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Microbial Biofilms

Importance and Applications

Edited by Dharumadurai Dhanasekaran and Nooruddin Thajuddin

MICROBIAL BIOFILMS - IMPORTANCE AND APPLICATIONS

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Notice

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Meet the editors

Dr. Dharumadurai Dhanasekaran is working as an Assistant Professor, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, India. He has experience in fields of actinobacteriology and mycology. His current research focus is on actinobacteria, microalgae, fungi and mushroom for animal and human health improvement. He has received the UGC-Raman Post doctoral Fellowship to visit USA, University of New Hampshire, Durham. He has deposited around 64 nucleotide sequences in GenBank, 5 bioactive compounds in Pubchem, published 89 research and review articles and books on Fungicides for Plant and Animal Diseases and Antimicrobial compounds: Synthetic and Natural compounds. He guided 7 Ph.D candidates and organized several national level symposia, conference and workshop programs. He received major research projects from Department of Biotechnology, University Grant Commission, Indian Council for Medical research, and International foundation for Science, Sweden. He is a life member in Mycological Society of India, National Academy of Biological Sciences and member in editorial boards in National, International Journals, Doctoral committee member and Board of study member in Microbiology. As per the reports of Indian Journal of Experimental Biology, 51, 2013, Dr. Dharumadurai Dhanasekaran is rated in second position among the top five institutions in the field of Actinobacteria research in India.

Dr. Nooruddin Thajuddin is working as Professor and Head, Department of Microbiology, School of Life Sciences and Dean, Faculty of Science, Engineering and Technology, Bharathidasan University, Tiruchirappalli, India. He has vast experience in microbial taxonomy, isolation, cultivation, harvesting, extraction of valuable products, and most importantly expertise in employing molecular tools in the identification and phylogeny of various microorganisms, bioremediation of effluents and bioenergy from Microalgae and Cyanobacteria. He had one-year post–doctoral training on molecular taxonomy and phylogeny of cyanobacteria at the Department of Biology, Rensselaer Polytechnic Institute, Troy, New York, USA, through Dept. of Biotechnology (Govt. of India) overseas fellowship. Recently Department of Biotechnology (Govt. of India) sanctioned to a major grant to Prof. N. Thajuddin for the Establishment of National Repository for Freshwater Microalgae & Cyanobacteria. He deposited around 515 nucleotide sequences in GenBank and developed barcodes for 6 indigenous fungi using ITS region. published 242 research and review articles and 3 books on microbiology related topics, developed Germplasm of cyanobacteria, microal-

gae, bacteria, actinobacteria and fungi in his laboratory, guided 30 Ph.D candidates, acted as doctoral committee member for 142 Ph.D. candidates and currently guiding 8 post doctoral and 8 doctoral candidates on various aspects of microbiology. He organized several national level symposia, workshops, refresher courses and DST-INSPIRE (Govt. of India) Programs. He received major research projects from DBT, DST, UGC and MoES at the tune of INR 4.48 corers. He is a life member of various academic bodies and member in editorial boards in national and international journals. He received Dr. G. S. Venkataraman Memorial Best Scientist Award of National Academy of Biological Sciences for the year 2014. He visited the United States of America, the United Kingdom, the Kingdom of Saudi Arabia, Republic of Korea, Malaysia, Honk Kong, Singapore and Germany to disseminate his expertise and to keep himself abreast of the advanced techniques in the field of Microbiology.

Contents

Foreword

Biofilms have a greater role to play in public health because of their involvement in various infectious diseases and a proper understanding of the biofilm processes would lead to find out novel and effective strategies for biofilm control and hence patient management improvement.

In this context, the book on "Microbial Biofilms: Importance and Applications" edited by Dr. D. Dhanasekaran and Dr. N. Thajuddin is an excellent contribution and a welcome addition to the research field of biofilm formation and development. Editors and contributing authors deserve appreciation for their laudable efforts. The book is presented in four major areas namely Biofilm fundamentals, Biofilm applications, Biofilm in health and diseases, and Biofilm control, embracing topics on grazing effects of ciliates on microcolony formation in bacterial biofilms, role of the biofilms in the waste water and rain water treatment, dental biofilm formation, biofilms in diabetic foot ulcers, biofilm interactions in epizootic shell disease of the American lobster, and natural compounds for new diagnostics and drugs against biofilm infections.

As a whole, this book depicts an excellent overview, importance, and applications of biofilms and it will be a very useful resource for the teachers, researchers, and industries, interested in furthering research in the field of biofilms. Researchers in the fields of clinical, food and water, and environmental microbiology have already begun to investigate microbiological processes from a biofilm perspective. It is heartening to note that considerable efforts have been taken to control biofilms through the application of antiquorum sensing compounds by various microorganisms. Hope these efforts will continue with more vigor, reaping fruits.

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Preface

Despite the discovery of microbial biofilms as far back as the seventeenth century, in recent years, scientists have increased their attention of these dense colonies of bacteria that produce extracellular polymeric substances that bind a community of different microorganisms together and anchor them to both living and inanimate surfaces. Biofilms are not only responsible for chronic bacterial infection account for more than 80% of all microbial infections of the human body, infection on medical devises, deterioration of water quality, and the contamination of food, but they also can promote remediation of contaminated groundwater and soils and they play an important natural role in cycling of nitrogen, sulfur, and metals. The protective nature of the biofilm structure makes the bacteria embedded within them remarkably difficult to treat with antimicrobials; biofilms are resistant to doses of antimicrobials 100- to 1000-fold over the minimum lethal dose for microbes outside of biofilms. Hence, understanding "the good and the bad" characteristics of biofilms under diverse conditions is of great interest.

In this book "Microbial Biofilms: Importance and applications", eminent scientists provide an up-to-date review of the present and future trends on biofilm-related research. This book is divided with four subdivisions as biofilm fundamentals, applications, health aspects, and their control. Moreover, this book also provides a comprehensive account on microbial interactions in biofilms, pyocyanin, and extracellular DNA in facilitating *Pseudomonas aeruginosa* biofilm formation, atomic force microscopic studies of biofilms, biofilms in beverage industry, *Staphylococcus* and Salmonella biofilms, wound biofilm and therapeutic strategies, oral microbiome in dental biofilm formation, antimicrobial strategy against mature oral biofilm, and novel models to manage biofilms on microtextured dental implant surfaces.

The book comprises a total of 21 chapters from valued contributions from world–leading experts in Australia, Bulgaria, Canada, China, Serbia, Germany, Italy, Japan, the United Kingdom, the Kingdom of Saudi Arabia, Republic of Korea, Mexico, Poland, Portugal, and Turkey. We are grateful to the experts who have provided state-of-the-art valued contributions of this book. This book may be used as a text or reference for everyone interested in biofilms and their applications. It is also highly recommended for environmental microbiologists, soil scientists, medical microbiologists, bioremediation experts, and microbiologists working in biocorrosion, biofouling, biodegradation, water microbiology, quorum sensing, and many other related areas. Scientists in academia, research laboratories and industry will also find it of interest.

We offer our special thanks and appreciation to Ms. Dajana Pemac, Publishing Process Manager for their encouragement and help in bringing out the book in the present form. We are also indebted to InTech publisher for their concern, efforts, and encouragement in the task

of publishing this volume. Appreciations are due to our research scholars for their sincere efforts and diligence towards this volume.

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Section 1

Biofilm Fundamentals

Chapter 1

Viewing Biofilms within the Larger Context of Bacterial Aggregations

Olena V. Moshynets and Andrew J. Spiers

Additional information is available at the end of the chapter

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Abstract

The 'Microbial Cities' vision of bacterial biofilms has dominated our understanding of the development and functioning of bacterial aggregations for the past 20 years, during which active sludge, clumps, colonies, flocs, mats, pellicles, rafts, slimes, zooglea, etc. have been largely forgotten or ignored. Although the medically inspired developmental model of human pathogen biofilms has merits including providing a rationale for the development of anti-biofilm therapeutics, it fails to provide links to other types of bacterial aggregation that are commonly found in a wide range of natural and man-made environments. Possibly as a result, applied and environmental microbiologists tend to avoid the term 'biofilm' and use others such as 'microbial mats' instead. Here we challenge the simplistic planktonic (independent and free-swimming bacteria)-biofilm (sessile and co-operative bacteria) dichotomy, and consider biofilms within the larger context of bacterial aggregations. By placing biofilms into context, which we see as a continuum of aggregations or communities with varying abiotic and biotic properties, fundamental physical, biological, and evolutionary ecological processes that effect community development and function can no longer be considered unique to biofilms, but may also be important in other aggregations that develop over time and change in nature depending on prevailing conditions. By doing this, we will be better able to distinguish those processes which govern bacterial colonisation and ecological success in a wider sense from those that are unique to particular environments and specialised strategies.

Keywords: Bacterial aggregations, Biofilms, Colonies, Communities, Planktonic and sessile bacteria

1. Introduction

Modern biofilm research often acknowledges the seminal reviews of Costerton et al. [1,2] in which a conceptual model of biofilm development and structure was first presented. In this model, biofilm development is described as a series of linked events, from the attachment of free-swimming planktonic bacteria to a submerged solid surface, the growth of microcolonies in simple conical structures, and subsequent maturation as larger mushroom-shaped structures which have been envisioned as 'Microbial Cities' (this appellation may derive from reviews entitled 'City of Microbes' and 'Microbial Metropolis' [3,4], but we are unsure). Equally important to this description was the dichotomous differentiation between independent free-swimming planktonic bacteria with the co-operative and co-ordinated communities of sessile bacteria forming biofilms, and the somewhat teleological suggestion that surface-attached communities allowed growth in harsh conditions which planktonic bacteria could not survive [2]. This view of complex bacterial behaviour and growth strategies was in contrast with the apparently contemporary idea that bacteria were unsophisticated organisms [5].

Since the publication of the Costerton et al. reviews, our understanding of biofilms has developed through the study of model bacteria as well as of natural communities forming multispecies biofilms (we direct the reader to the reviews cited in the following sections as a means of accessing recent biofilm research and current understanding). Model human pathogens forming biofilms important for virulence include *Escherichia coli* [6], *Pseudomonas aeruginosa* [7], *Salmonella enterica* [8], *Staphylococcus aureus* [9], *Vibrio cholera* [10], etc., though the archetype is probably *P. aeruginosa*, an opportunistic pathogen of the human respiratory tract and a key factor in cystic fibrosis patient mortality [11]. As a result of an understanding of pathogen biofilm formation, critical points in the developmental processes are now being scrutinised as possible targets for anti-biofilm therapeutics [7]. Biofilms are also recognised as having importance in a range of other natural and man-made environments, impacting on crop productivity, food technology, metal corrosion, veterinary medicine, etc. [12–15], and microbial mats, a term seemingly preferred by applied and environmental microbiologists, are found on rock surfaces, in caves, wetlands, sediments, salt marshes, lakes and seas, thermal springs, hypersaline ponds and lagoons, methane and petroleum seeps, oil wells, etc. [16–21]. Comparisons between pathogenic and environmental biofilm-forming bacteria highlighting commonalities suggest that biofilm developmental pathways or responses may not be unique to species or particular environments.

Investigations of biofilm-forming bacteria have revealed key sensory-regulatory pathways, including intercellular communication and intracellular regulation, required to control biofilm development by altering motility and attachment behaviour, physiology and metabolism, the production of extracellular polymeric substances (EPS) forming the matrix of biofilms, and dispersants required to release bacteria from mature structures [22–27]. The use of a variety of different experimental systems, including in vitro flow cells, microtitre plates, static microcosms, etc., as well as animal models [28–31], has also identified the impact of abiotic factors such as liquid flow and mass transport; O_2 and nutrient diffusion; surface physical-chemistry and topology; and biotic interactions between bacteria, surfaces, and matrix components; and

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so on, on biofilm formation and structure [23–26,32]. In addition, research focussed more on medical and environmental microbiology, rather than on biofilm formation per se [33–37], as well as on evolutionary ecology and social microbiology [11,38–41], are increasingly providing explanations for the role or function of biofilms in different environments and evidence of their ecological success.

Despite the obvious diversity in biofilm research as evidenced by publications in journals covering a wide range of disciplines including microbiology, microbial biotechnology, environmental science, and medical microbiology, our general understanding of biofilms is nonetheless dominated by a few human pathogens and the submerged solid-surface interface biofilms they produce in flow cells or microtitre plates [28,29,31] (here we refer to these as liquid-solid surface (L-S) interface biofilms to differentiate them from other types of biofilms or bacterial aggregations). Of these, the medically inspired developmental model of *P. aeruginosa*, often chosen to represent the 'Microbial Cities' vision, is perhaps the most persuasive, with bacteria growing in these structures almost exclusively compared to free-swimming planktonic bacteria. We are growing concerned that this vision is beginning to dominate biofilm research in a negative manner.

2. A continuum of aggregations

In this opinion piece, we challenge the simplistic planktonic-(sessile) biofilm dichotomy and advocate the inclusion of biofilms within the larger context of bacterial aggregations. We believe that by recognising biofilms within a continuum of aggregations or communities with varying properties, it will enable a more extensive investigation of bacterial colonisation, and in particular, allow us to distinguish those processes governing general colonisation and ecological success from those unique to particular environments and specialised strategies.

Costerton et al. [2] defined biofilms as 'matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces ... (and) includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media'. Although this definition is broad (i.e. sensu lato), there is a presumption by current researchers that biofilms are those structures formed on submerged solid surfaces (i.e. at the L-S interface) and that other structures associated with surfaces or interfaces are somehow different or inconsequential. We would suggest that L-S interface biofilms as observed in flow-cells and microtitre plates are a means to investigate biofilm formation independently of natural environments or context, as it is difficult or impossible to extrapolate from these simple in vitro systems to the more complex natural environments from which the bacteria of interest were first isolated [29–31]. We note that in some later reviews, the description of biofilms is extended with more examples. However, this has also lead to a more relaxed *(sensu amplo)* definition in which 'biofilm' is frequently used as a synonym of 'aggregation', even though the former is often defined by the latter (e.g. [42]). As a matter of etymology, 'aggregation' which originates in late Middle English (1150–1500 AD) should take precedence over 'biofilm' whose usage largely stems from the 1990s.

As an example of how the biofilm definition has been extended following the definition of Costerton et al. [1,2], we consider the inclusion of air-liquid (A-L) interface biofilms and agar plate-grown colonies as suggested by Branda et al. [42].

3. Biofilms at the air-liquid interface

Material, including bacteria, accumulates at the air-liquid interface of sea or fresh water to form surface films often subject to highly variable conditions [19]. A-L interface biofilms also form on the surface of static liquids in experimental microcosms, and are sometimes referred to as pellicles or floating biofilms. These are produced by a wide range of bacteria, including *Bacillus subtilis* [43], *Gluconacetobacter xylinus* (formerly known as *Acetobacter xylinum* and since reclassified as *Komagataeibacter xylinus*) [44], as well as numerous enteric bacteria and pseudomonads [44–46].

Although A-L interface biofilms may look superficially similar, the diversity of bacteria which form them would suggest that they vary in structure and other characteristics as well. We have been investigating this by comparing biofilms produced by environmental *Pseudomonas* spp. isolates, using relatively large static microcosms in 30-ml glass universal vials containing liquid growth medium [44,47,48]. These allow us to undertake combined biofilm assays which determine growth, attachment to the vial walls, and biofilm strength [49–51]. Using this approach, we have been able to quantitatively differentiate biofilms produced at the meniscus and A-L interface [50,52]. These include biofilms limited to the meniscus region, attached biofilms which extend across the A-L interface, and unattached 'floating' biofilms (as well as 'invisible' attached biofilms too thin or transparent to see by eye [52]). It is possible that the floaters and attached biofilms represent substantially different colonisation strategies, with the former recruiting planktonic cells directly from the liquid column to the A-L interface and growing from multiple loci, and the latter developing from sessile cells attaching in the meniscus region and subsequently growing out across the A-L interface [52].

Although floating biofilms have been reported in which buoyancy is the result of trapped CO_2 released by respiration (e.g. *G. xylinus* [44]), the two different A-L interface biofilms produced by our model environmental pseudomonad, *P. fluorescens* SBW25, known as the viscous mass and Wrinkly Spreader biofilms, are not buoyant per se and readily sink when disturbed [49,53,54]. It is likely that they are maintained at the A-L interface by hydrophobic cell surfaces, matrix components, and surfactant which pierce or weaken the A-L interface [53,54]. Interestingly, we have recently found that for the Wrinkly Spreader, a class of adaptive mutants of the wild-type strain which evolves in static microcosms, drip-fed glass bead columns, and soil [48,51,55], attached A-L interface biofilm growth can be seamlessly linked to swarming motility and colony growth using 'transitional microcosms' in which a layer of agar is set along the side of the vial, providing both liquid and dry agar surfaces for colonisation (C. Immoor, O. Moshynets , A. Spiers, Unpublished Observations).

In these transitional microcosms, we wonder whether there is more than just planktonic bacteria growing in the liquid column and a single, distinct structure colonising both the liquid and agar surfaces in these simple environments, as suggested by the simplistic planktonic-

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(sessile) biofilm dichotomy. Instead, we think it is likely that there are different types of aggregation including L-S interface biofilms attached to the vial walls at the meniscus, attached biofilms extending out across the liquid surface, confluent colonies growing on the agar surface, and microcolonies developing in the swarm-front as bacteria move up the agar surface and away from the liquid media. We are interested in identifying which abiotic and biotic factors drive growth at different points across the transitional zone, and understanding how these might alter behaviour and gene expression patterns to produce the structures we can observe within a few hours and over several days.

4. Colonies are not biofilms

Whilst we approve of the inclusion of A-L interface biofilms in the *sensu amplo* Costerton et al. [1,2] definition, we resist the suggestion that colonies grown on agar plates should be included too, despite the fact that investigations of colony morphology have been presented as biofilm research (e.g. [43,56–61] etc.). In contrast, we have investigated the colony morphologies of Wrinkly Spreader mini-*Tn* mutants on agar plates as a means to identify the genes required for biofilm formation; importantly, we also tested the mutants in static microcosms to determine the impact on biofilm formation to confirm the identity of these genes as important in biofilm formation [48].

Our objection to the inclusion of colonies as biofilms is based on a consideration of the O_2 and nutrient gradients established in these aggregations, as well as liquid flow (**Figure 1**). Rather than argue that these are insignificant differences, we would suggest that colonies, A-L and L-S interface biofilms would be better presented within a larger continuum of aggregations in which O_2 and nutrient gradients (and other chemicals including communication signals and waste, etc.), and liquid flow can be used to differentiate between types of aggregation. In this way, it now becomes reasonable to ask whether the parallel O_2 and nutrient gradients observed

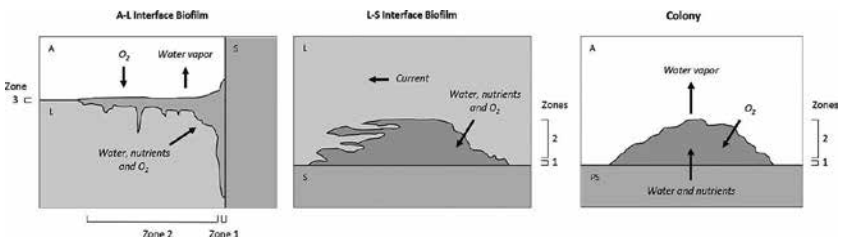

Figure 1. Bacterial aggregations include biofilms and colonies with significant similarities and interesting differences. Liquid-solid surface (L-S) interface biofilms (middle) are subject to physical stress and establish various chemical gradients. Similarly, air-liquid interface (A-L) biofilms (left) or colonies (right) also experience physical stress and establish gradients. In each type of aggregation, a layer of cells is attached to the solid surface (Zone 1) with distal regions are held in place by cell and matrix component interactions (Zone 2). In A-L interface biofilms, cells and matrix components may also break through the interface (Zone 3). However, nutrients are supplied by capillary flow (mass transport) from beneath colonies, whereas in A-L and L-S interface biofilms, they are transported or diffuse from the surrounding liquid. (A, air; L, liquid; PS, permeable or porous solid; S, solid.)

in L-S interface biofilms present a substantially different set of conditions for bacteria than the opposing gradients found in colonies. Similarly, it would be interesting to compare liquid flow within biofilms subject to external liquid currents, with the evaporation- and capillary-driven liquid flow within colonies subject to different drying regimes.

Notably, colonies show different growth patterns, have highly structured morphologies, are sometimes surrounded by EPS, and may show co-operative behaviour, so it is not unreasonable to consider that they are responding to abiotic and biotic conditions as do biofilms [6,42, 43]. In soil, water availability, often described by the matrix potential, is a significant factor restricting bacterial motility, the formation of aggregations, and colonisation through the pore network [62–64]. In such systems it is highly likely that contiguous bacterial populations colonise non-permeable solid surfaces covered or linked by thin films of water with slimes or swarms; permeable solids through which water is available with microcolonies and colonies; and partially and fully saturated pores with A-L and L-S interface biofilms, slimes, and planktonic bacteria.

5. Aggregations respond to different conditions

We would argue that bacteria colonising a range of environments should develop into different aggregations in response to local conditions and opportunities. These aggregations might appear to be superficially similar (e.g. A-L and L-S interface biofilms) or substantially different (cf. a colony), depending on bacterial responses and colonisation strategies aimed at maximising ecological success, as well as on our ability to recognise which abiotic and biotic factors have the greatest impact on the developing population.

Our observations of linked Wrinkly Spreader A-L interface biofilms and colonies in the transitional microcosms might suggest that these are very similar aggregations used to colonise two interfaces (the liquid and agar surfaces) which do not pose significantly different challenges to bacterial growth. However, competitive fitness assays in each environment suggests that Wrinkly Spreaders achieve substantially different levels of ecological success in colonising liquid and agar surfaces: they have a fitness advantage in static microcosms but are at a disadvantage in colonies compared to wild-type *P. fluorescens* SBW25 [65–68]. These fitness differences suggest that environmental conditions probably change across the transitional zone, with the initial Wrinkly Spreader population responding to these changes to colonise the A-L interface and agar surfaces in different manners.

We speculate that the fitness advantage of Wrinkly Spreaders is determined by the subtle trade-off in energy expenditure needed to produce A-L interface biofilms in static microcosms and the increased access to O_2 biofilm formation allows. The growth of *P. fluorescens* SBW25 is limited by O_2 and it drives the evolution of the Wrinkly Spreaders [69]. Relative small numbers of wild-type colonists rapidly generate an O_2 gradient through respiration, converting the homogeneous liquid column into a shallow upper zone having normal levels of O_2 and a deeper lower zone with rapidly diminishing O_2 levels (these colonists are in effect environmental engineers) [69]. As this population rapidly expands, random mutation results in

Wrinkly Spreader genotypes which are recruited to the A-L interface through the expression of attachment factor and cellulose which provides the main matrix component for the biofilm [49,53,68]. Those bacteria localised to the A-L interface have access to higher levels of O_2, compared with those lower down, and consequently grow faster [69]. Higher levels of O_2 might also induce a SOS response via reactive oxygen species (ROS) leading to the expression of an error-prone DNA polymerase, as in the case of *P. aeruginosa* PA01 [70], increasing mutation rates and the appearance of Wrinkly Spreader mutants.

As a result of access to higher levels of O_2, Wrinkly Spreaders have a fitness advantage over non-biofilm-forming competitors [69]. We speculate that in colonies where Wrinkly Spreaders still express attachment factor and cellulose, these components have no essential function and therefore pose a fitness cost to the growing population, explaining why the Wrinkly Spreaders are poorly adapted to growing on agar surfaces. Interestingly, improved O_2 access has also been suggested as an explanation for the wrinkled colonies produced by *P. aeruginosa* PA14. However, it appears that a more complex level of redox control regulating the use of different electron donors, including O_2 diffusing into the colony from above and redox-active phenazines produced by bacteria located at the base of the colony, may govern metabolism in *P. aeruginosa* PA14 and other bacteria producing wrinkled colonies including *B. subtilis* [57,71,72].

More generally, O_2 gradients determine the distribution of aerobic and anaerobic bacteria in a wide range of environments, including water columns, sediments and soils, where it effects growth, gene expression patterns and metabolism, and along with other competing electron acceptors, help define aerobic, micro-aerobic (transitional) and anaerobic niches [73–76]. It is therefore not surprising if O_2 gradients also played a central role in the development and function of a wider range of bacterial aggregations, and not just in biofilms and colonies.

6. Other terminology

As a slight digression, we present a non-exhaustive selection of vernacular and scientific terminology used to describe bacterial aggregations which includes active sludge, biofilms, clumps, colonies, flocs, mats, pellicles, rafts, slimes, zooglea, etc. (**Table 1**). We would expect that a more extensive review of the early microbiology literature, including French, German, and Russian publications, and of current microbiology, microbial biotechnology, environmental science, and medical microbiology publications, would result in more terms being identified. Although the first observations of biofilms (dental plaque) were made by van Leeuwenhoek (1683–1708) [34], microbial mats appeared much earlier, and are identified today as the fossilised remains of 3.5-billion-year-old stromatolites [77]. Arguably the first observation by a microbiologist was made by Pasteur (1864) [34], and by the end of the nineteenth-century, environmental microbiologists were investigating them as well (we list several early observations following Pasteur in **Table 2**). For example,Winogradsky (1895) [78] described jelly-like masses of bacteria as 'zooglea', whilst Egunov (1895) [79]) and Sorokina (1938) [80] more obviously referred to biofilms in the current sense, using terms that translate into bacterial 'plate' or 'plane', and 'film', respectively.

Abscesses, inclusion bodies and metastasis	A mass of bacteria growing within another structure, including sediments, soils, plant and animal tissues.
Active fluids	Formed by motile bacteria moving together at high density in a liquid.
Active sludge	A complex mixture or community of bacteria and other microorganisms produced and used in wastewater treatment.
Aggregates, blobs, clumps, colloids, lumps and masses	A mass of bacteria (and other microorganisms) having some sort of physical cohesion; these may have developed by growth, or they may be the result of physical mixing or disturbance.
Bacterial plates	A layer of bacteria formed in a water column at a certain depth depending on oxygen levels.
Biofilms	A mass of bacteria enclosed in a protective matrix of EPS and associated with a surface or interface; most often used to refer to liquid-solid surface (L-S) interface biofilms such as those developing in flow-cells and in microtitre plates.
Biolaminites, microbialites, stratifera and stromatolites	Living and fossilised microbial mats which may trap and bind sediments and/or cause mineral precipitation, sometimes intercalated by sediment laminae; sometimes referred to as microbial-induced sedimentary (MIS) structures.
Clusters	A zone of physiologically synchronised bacteria within a lager aggregation, or a small mass of bacteria such as a colony or floc.
Collapsed cakes	Formed by the collapse of clumps or flocs on membranes during filtration.
Colonies, macro- and micro-colonies	A mass of bacteria having some sort of physical cohesion and having developed by growth on a solid dry surface; micro-colonies are those associated with the biofilm development process on submerged solid surfaces, or small aggregations of bacteria not noticed as colonies unless observed with magnification on leaf surfaces, detritus, or agar plates.
Communities and consortia	A complex mixture of multiple bacterial species (or genotypes) and possibly other microorganisms in which biotic interactions define structure and function.
Crusts, dust particulates and aerosols	Microbial communities developing on the surface of soils and dessert sand; also the dried remnants of colonies etc.
Deposits and sediments	Mass or body of bacteria that accumulate on dry or submerged surfaces due to wind or water movement.
Desert varnish	A dark stain or coating covering rock surfaces and colonised by bacteria.
Filamentous structures and streamers	Long strands of material stretching out from the main mass of a biofilm subject to liquid flow.
Films, layers, planes, plates, volumes and zones	A thin layer or volume containing bacteria which may or may not be physically connected to one another, solid surfaces, or other interfaces, and occurring in liquids, porous or permeable solids.
Flocs and snow	Masses of bacteria formed by growth, self-association, hydrophobic interactions, or by attachment to suspended inert particles; flocs in sea water are referred to as snow.
Floaters	Biofilms at the air-liquid interface having no appreciable attachment to a solid surface; these may be localised at the liquid surface by buoyancy, penetration of the interface, or by hydrophobic surfaces.
Foams	Air-water emulsions containing high concentrations of bacteria and compounds such as polymers and surfactants, which may help stabilise the structure.
Granules	A mass of bacteria growing on small solid particles.

Jellies and zooglea (or zoogloea)	A slimy mass of bacteria encased in a gel-like material, often found floating in water or found on plant stems or leaf litter.
Laminae	Layers of microbial mat generations, or microbial mats overgrowing sediments.
Mats	Microbial communities, often with clear layering or stratification, found in streams, lake or sea beds; these may also contain, algae and plants, and trap small particles including sand and stones.
Meniscus growth	A mass of bacteria adhered to a solid surface in the meniscus region of static liquids (the air-liquid-solid surface (A-L-S) interface).
Microcenosis, microbial cenosis	Microbial communities formed in a particular niche or site.
Micro-zones and pelogens	Microbial communities growing in thin layers structurally segregated with different characteristics or activities in sediments or silt.
Pellicles	A thin film or gel-like coating surrounding of individual bacteria, as well as air-liquid (A-L) interface biofilms.
Phlegm balls	Flocs found in underground streams.
Plaque	Dental biofilms formed largely by anaerobic bacteria on the surfaces and cavities in teeth.
Rafts	Flat sections on the edges of colonies, often associated with twitching motility, or flat pieces of un-attached biofilm found at the air-liquid interface.
Remains and remnants	A mass of bacteria and cellular debris found at a site at which they probably did not develop or a portion of a larger mass which has been removed by physical disturbance, predation or decay.
Sediments	Bacteria from liquids no longer in suspension, or microbial communities developing in sediments or silt.
Slimes, glycocalyx and viscous liquids	Viscous liquids or regions of a larger volume of liquid containing high densities of bacteria and EPS.
Snottites and snoticles	Pendulous or dripping masses of bacteria developing on cave walls or at the bottom of stalactites, especially limestone cave speleothems.
Swarms	Bacteria showing a particular form of surface-associated motility, moving in high densities across moist or wet surfaces.

This is a non-exhaustive list where terms, meanings and usage varies between contexts, and grouped terms may not be synonymous.

Table 1. Vernacular and scientific terminology used to describe bacterial aggregations

1864 Pasteur describes slimy material called Mother of Vinegar [34].

1887 Winogradsky observed bacterial growth in ring-like structures in a liquid microcosm containing H_2S and covered with a glass slide to restrict O_2 diffusion [86].

1893 Beijerink observed bacteria growing in zones of enriched water microcosms, the positions of which could be altered depending on O_2 and H_2 levels. Described 'Bakterienniveau' or 'niveau' as an aggregation formed by motile bacteria [87].

1895 Winogradsky observes bacterial zoogleas on potato slices [78].

1895	Egunov observed bacterial plates forming in microcosms containing Black Sea sediments, the position of which depended on anaerobic conditions, O_2 and H_2S levels. Some of these bacteria were motile, and Egunov asked what forces drove them to form a stationary aggregation [79,88].
1900	Egunov describes bacterial attachment during plate formation, and his 'bioanisotropy' concept for environments (continuous matter exchange between an organism and its surroundings) [89].
1914	Isachenko describes 'pink water' caused by the aggregation of purple bacteria in sea water as well as bacteria forming cloud-like structures in liquid microcosms [36].
1933	Henrici observed that bacteria mostly grow on submerged surfaces, not in free flowing water [34].
1935	Zobell describes marine bacteria attachment to surfaces [34].
1938	Sorokina describes a bacterial film forming on a submerged slime surface in a liquid microcosm [80].

Table 2. Early observations of bacterial aggregations.

More recently, pendulous and dripping snottites have been described on cave walls and stalactites [81] and collapsed cakes are a problem in filtration [82]. More interesting, perhaps, are the reports that bacterial remains have been misidentified as dinosaur soft tissues [83] and desert varnishes are being used to train sensors for future planetary explorations [84]. Regrettably, we also note that a chance to create a more evocative science fiction term for biofilms on the International Space Station was missed [85].

We argue that such an extensive collection of terms used to describe bacterial aggregations should not be considered a plethora, but rather an indication that the diversity of aggregations we are aware of may reflect the multitude of ways bacteria to respond to differing ecological opportunities. However, we do not suggest that each of the terms are unique, as quite evidently different types of aggregations may be associated closely or more distantly from one another, depending on which abiotic or biotic factors are considered.

7. Key features linking aggregations

Bacteria interact with abiotic and biotic factors by responding to physical and chemical cues according to behavioural and adaptive strategies under constant selection to maximise fitness. When these cues and strategies are considered in the context of a larger continuum of aggregations, it is possible to identify key features that link aggregations within a larger continuum (**Table 3**). Here we briefly indicate how physical interactions, diffusion radii, and increasing genetic diversity might be used to compare different types of aggregations.

Chemical gradients	Controlling the behaviour of individuals and groups, defining zones of optimal and restricted growth, providing information about local conditions; of nutrients, O_2 and other electron acceptors and metabolites, chemosensory, regulatory and communication compounds, and waste.
Competition	Between genotypes or lineages for resources, drives adaptation, allows investment in public goods but also results in cheaters.
Complexity	Of abiotic and biotic interactions, of genotypes, metabolism, structures, etc.

Communication, co-operation and co-ordination	Linking individuals into a group through the exchange of communication signals and/or response to the same environmental signals, resulting in similar behaviours or activities, and the production of common goods such as EPS and other secreted products.
Developmental pathway	Guiding the behaviour individuals and groups through a series of defined stages and resulting in a specific type of aggregate; responding to abiotic and biotic factors including communication signals.
Environmental conditions and modification	Abiotic chemical and physical factors, biotic factors; niche, opportunities, substrates, resources, stress, variation and instability; local, large-scale and irreversible changes, depletion, contamination.
Gene expression	Controlling behaviour, communication, metabolism, the production of compounds required for the formation of aggregations, etc.
Genotypes and diversity	Aggregations may arise from a single individual or founder population of the same genotype, but diversity will develop over time with radiation and immigration; single or multi-species aggregations; cheaters, invaders and persisters.
Liquid flow	Around and within aggregations, effecting the development of the physical structure, deformation and breakage, boundary layers, mass transport and diffusion of molecules, as well as the movement of bacteria.
Mobility	Of individual bacteria, small groups and larger aggregations, over small and large-scale distances, across interfaces, surfaces and volumes, and through environments.
Physical interactions	With interfaces, surfaces, bacteria, EPS, etc., in terms of strength, elasticity or resilience, distances, duration and reversibility.
Resilience	To chemical and physical stress, external competition, predation, and in terms of physical structure.
Sensory zones	The ability of individuals to detect the presence of others using altered chemical gradients, metabolites and communication signals (diffusion radii), and the distance separating individuals or groups.
Stratification	From homogeneous mixtures to clustered or layered differences resulting from age, metabolism, diversity and function.
Structure and rheology	Aggregations having properties similar to Newtonian liquids, visco-elastic gels and solids.
Succession	The diversity and function of aggregations will develop over time and with changing environmental conditions.
Time	The time-scale for the development, presence or persistence of aggregations may vary considerably, effecting population size, radiation, succession, aggregation structure and stratification, and environmental impact.

Table 3. Features linking bacterial aggregations within a larger continuum.

Physical interactions involving bacterial surface coatings, appendages and matrix, solid surfaces and interfaces within A-L and L-S interface biofilms are known to be complex (**Figure 2**), but there is no reason to believe that all types of interactions are unique to these particular aggregations. We therefore propose that one feature of the continuum of aggregations could be expressed by a scale going from no interactions with solid surfaces or other interfaces, as in the case of a population of planktonic bacteria, through short-term, weak, or distant physical contacts with surfaces, interfaces, or material, to the complexity of interactions seen in A-L and L-S interface biofilms, and presumably in other aggregations such as flocs, granules, and snow.

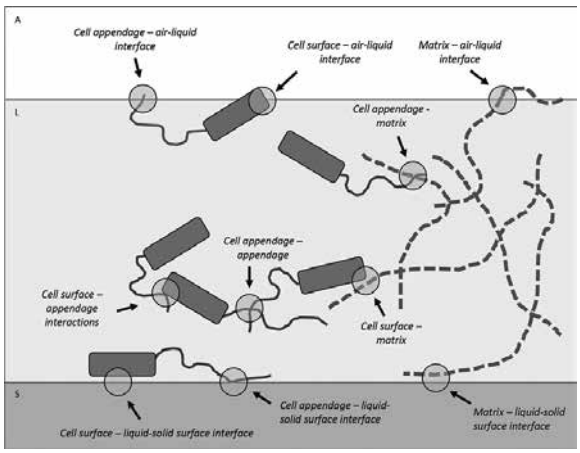

Figure 2. Bacterial aggregations are constructed by numerous and varied physical interactions. Bacterial cells within aggregations will interact with one another through close, intermediate, and long-range interactions involving surface coatings and extended appendages (e.g. flagella), matrix components, solid surfaces and interfaces (e.g. the air-liquid interface). (Key: A, air; L, liquid; S, solid; Matrix components or fibres are artistically depicted by dashed lines and bacteria as bacilli with a single flagella.)

Interactions with surfaces and interfaces also clearly limit the ability of an aggregation to develop, so the ability to expand across surfaces or to penetrate volumes, along with altered mass transport and diffusion characteristics, also present other scales with which to compare aggregations (**Figure 3**). In particular, diffusion radii will determine the ability of individual bacteria to detect the proximity of others and to respond competitively or with co-operation. Clearly, low-density planktonic and surface-attached bacteria may be beyond detection distances, but as bacterial numbers increase, their individual and collective impacts on local environmental conditions will lead to a situation where they are now within the same micro-environment, and similar conditions may result in coordinated changes in gene expression

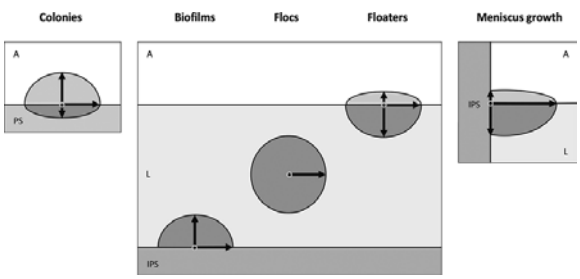

Figure 3. Bacterial aggregations develop at interfaces, within liquids, porous solids, and visco-elastic materials. Colonising bacteria may develop into communities restricted primarily by diffusion or liquid flow (mass transport) of chemicals and the ability to expand across interfaces or into spaces (radial arrows). Shown here (left to right) are colonies growing on and into a solid, biofilms growing on a solid submerged surface, flocs developing from a suspended particle, floaters forming at the air-liquid interface, and meniscus growth occurring at the air-liquid-solid surface interface. (Key : A, air; IPS, impermeable solid; L, liquid; PS, permeable or porous solid; S, solid; arrows indicate expansion radii and the opposite direction indicates diffusion gradients that limit growth.)

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patterns, behaviour, and metabolism. We propose that separation distance, scaled in terms of chemical gradients, will also be a useful means of comparing and differentiating bacterial aggregations.

Aggregations developing over significant periods of time will also gain diversity by radiation and immigration, leading to multispecies communities subject to ecological succession, driven by internal competitive and co-operative interactions and changing environmental conditions (**Figure 4**). We propose that both diversity in terms of genotype or species composition and community function could also be used to consider bacterial aggregations, and this perspective is complementary to understanding the physical interactions and the molecular biology underlying the development of these structures.

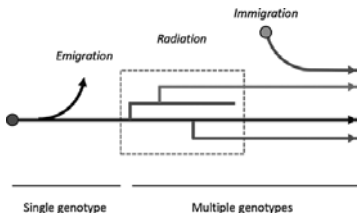

Figure 4. Bacterial aggregations gain diversity over time. An aggregation developing from a single genotype will gain diversity through radiation (mutation), immigration, and succession. Stochastic events and changing selective pressures will result in different genotypes within the community; some genotypes may become extinct and diversity may fall. (Key: Time progresses from left to right; colonising genotypes are shown as circles; mutation events as vertical lines; successful genotypes are indicated by arrows and an extinction event by a truncated line.)

By considering the ability of individual bacteria to respond to their local environments via different growth and colonisation strategies, the impact of abiotic conditions on individuals and the structures they create, and the longer-term development of the community, it is possible to speculate how altered environmental conditions and circumstance can lead to the cycling between different types of aggregation (**Figure 5**). The ability of bacteria to move between different types of aggregation with changing conditions or to exploit new opportunities will clearly differentiate those able to colonise a wide range of environments with those adapted to very specific niches.

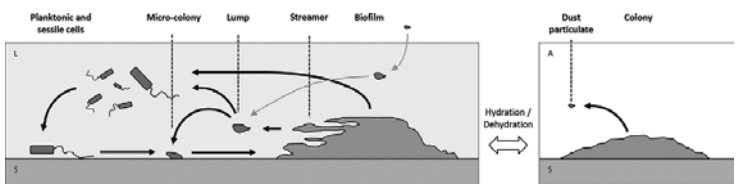

Figure 5. Bacterial aggregations change nature with altering environmental conditions. Although aggregations may develop and persist in one site, conditions may change resulting in an aggregation with a substantially different nature. The archetypal L-S interface biofilm developmental process is shown on the left. Biofilms may dry out to form slimes and colonies, and further drying might result in dust particulates which may rehydrate to form aggregations. (Key: A, air; L, liquid; S, solid; bacteria are artistically depicted as bacilli with a single flagella.)

8. Conclusion

In this opinion piece, we advocate the inclusion of biofilms within the larger context of a continuum of bacterial aggregations. We do this because we are growing concerned that the 'Microbial Cities' vision originating from the seminal reviews by Costerton et al. [1,2] is beginning to dominate biofilm research, and that such a narrow view limits our ability to better understand bacterial colonisation of a variety of different environments. In this continuum, we consider A-L and L-S interface biofilms to be biofilms, but argue that other aggregations such as colonies are significantly different and should not be referred to using this particular term. It is also possible that applied and environmental microbiologists prefer to refer to microbial mats rather than to biofilms, because the latter is too closely associated with experimental L-S interface biofilms produced in flow-cells or microtitre plates, and too far removed from the aggregations found in natural and other man-made environments.

We believe that the advantages of taking a wide view will allow us to distinguish those processes governing general colonisation through the formation of aggregations and ecological success, from those unique to particular environments and specialised strategies (as an apologia, we remind readers of the sensu lato definition of biofilms, secundum Costerton et al., which was inclusive of a number of different aggregations). By suggesting that biofilms are better considered as one of a variety of different aggregations, the simplistic planktonic-(sessile) biofilm dichotomy is also challenged, and perhaps the best reference or comparator for different aggregations will not always be logarithmic phase planktonic bacteria.

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Chapter 2

Role of Pyocyanin and Extracellular DNA in Facilitating *Pseudomonas aeruginosa* Biofilm Formation

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Additional information is available at the end of the chapter

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Abstract

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that is primarily responsible for infections related to cystic fibrosis (CF) airways, burn wounds, urinary tract infections, surgery-associated infections, and HIV-related illness. Pyocyanin and extracellular DNA (eDNA) are the major factors dictating the progression of biofilm formation and infection. Pyocyanin is a potent virulence factor causing cell death in infected CF patients and is associated with high mortality. eDNA is a key player in *P. aeruginosa* biofilm formation and is also responsible for the high viscosity of CF sputum that blocks the respiratory airway passages. In this chapter, we summarize our recent findings on the role of pyocyanin in facilitating *P. aeruginosa* biofilm formation. Pyocyanin promotes eDNA release in *P. aeruginosa* by inducing cell lysis mediated via hydrogen peroxide (H_2O_2) production. Pyocyanin intercalates with the nitrogenous bases of DNA and creates structural perturbation on the double-helix structure. Pyocyanin-eDNA binding significantly influences *P. aeruginosa* cell surface hydrophobicity and influences the physicochemical interactions facilitating bacterial cell-to-cell interaction (aggregation) and ultimately facilitates robust biofilm formation. A pyocyanin knockout (Δ*phzA-G*) mutant is shown to have significantly reduced eDNA release and biofilm formation in comparison to its wild-type. To this end, we discover that antioxidant glutathione directly binds to pyocyanin and modulates pyocyanin structure and function, thus inhibiting pyocyanin-eDNA binding and consequently hampering biofilm development.

Keywords: *Pseudomonas aeruginosa*, Pyocyanin, extracellular DNA, glutathione, biofilm

1. Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that readily forms biofilms and causes life-threatening infections, particularly in immunocompromised persons. *P. aeruginosa* is primarily responsible for infections related to airways in cystic fibrosis (CF) and bronchiectasis, burns wounds, urinary tract infections (UTIs), and nosocomial infections. For instance, *P. aeruginosa* infection and subsequent biofilm formation is implicated in 80% of CF deaths worldwide [1]. In CF patients, *P. aeruginosa* infection is responsible for lung tissue damage, impaired lung function, respiratory failure, and premature death [1, 2]. In spite of intensive antibiotic therapy against infections, the mean life expectancy of CF patients is short (~45 years) [1]. A recent study suggests that *P. aeruginosa* and its associated infections are more persistent and dominant in CF patients aged over 18 years (91%) than in patients less than 18 years (39%) [3]. In the case of burn injury infections, *P. aeruginosa* along with *Staphylococcus aureus* are recognized as principal pathogens responsible for serious complications in ~20.9% and 22.9% of patients, respectively [4]. Studies suggest that, in the United States, approximately 2.5 million patients with burn injury requires medical attention in which it is estimated more than 100,000 hospitalizations result every year, causing approximately 12,000 deaths from associated infections [5]. In hospital-acquired UTIs, *P. aeruginosa* is the third most common pathogen next to *Escherichia coli* and *Proteus mirabilis* [6]. In United States, UTI-associated hospital admission is estimated to be 300,000 patients every year, and urological disease costs more than 3.5 billion dollars annually in the United States alone [6].

Persistent *P. aeruginosa* infections that culminate in biofilm formation are attributed to a matrix of extracellular polymeric substances (EPS) in which bacterial cells are embedded [7, 8]. The EPS matrix of *P. aeruginosa* primarily consists of biomolecules, such as polysaccharides (alginate, lipopolysaccharides), proteins (protease, elastase), extracellular DNA (eDNA), metabolites (phenazines), exotoxin, and siderophores [6, 7]. These molecules present in the matrix play a significant role in the pathogenesis of *P. aeruginosa* infections. In addition, in many bacterial species, the matrix as a whole provides an inherent protection against both host immunity and traditional antibiotic therapy, which makes eradication extremely difficult [7, 9]. Previous studies acknowledge that the production of phenazines such as pyocyanin and the release of DNA from cells, providing freely available eDNA by *P. aeruginosa*, are the major factors dictating the formation of a biofilm and the persistent infection within the host [7, 8, 10, 11]. For instance, *P. aeruginosa*-infected CF and bronchitis sputum contains a significant amount of pyocyanin (up to 16 and 23 μg/ml, respectively) and eDNA (3–14 mg/ml) compared to none in non-CF patients [11, 12]. Pyocyanin is a highly versatile molecule recognized for its effect as a potent virulence factor, causing cell death in chronically infected CF patients and directly associated with decreased lung function and high mortality [11, 13]. eDNA is similarly a key factor in *P. aeruginosa* biofilm formation and protecting bacteria by inducing antibiotic resistance [8, 14, 15] and is also a contributing factor to the high viscosity of CF sputum that blocks the respiratory airway passages [12].

In this chapter, we focus our discussion on very recent findings that elucidate the essential role of pyocyanin in promoting eDNA production and interacting with eDNA to facilitate biofilm

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formation in *P. aeruginosa*. To this end, we highlight novel strategies targeting the interaction of pyocyanin with eDNA to prevent *P. aeruginosa* biofilm formation.

2. Pyocyanin production by *P. aeruginosa*

P. aeruginosa synthesizes a variety of phenazines; however, the most abundantly produced is pyocyanin [5-methyl-1(5H)-phenazinone]. Up to 95% of *P. aeruginosa* isolates preferentially produce pyocyanin [16], which at a neutral pH is a distinctive blue in color and turns red in acidic pH conditions. It is a small and highly diffusible nitrogen-containing aromatic compound with a multitude of biological activities [16, 17]. In *P. aeruginosa*, pyocyanin production involves a stepwise process, beginning with the synthesis of the primary quorum sensing (QS)

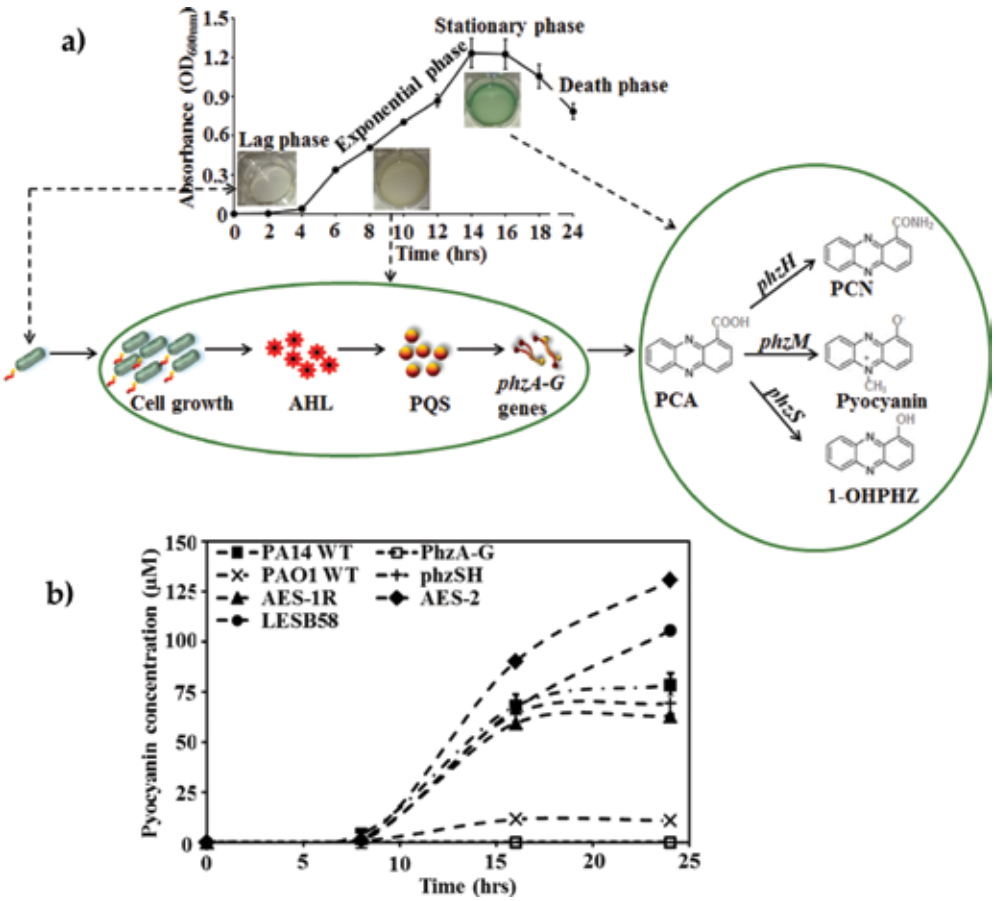

Figure 1. (a) Schematic of phenazine (pyocyanin) production by *P. aeruginosa*. Pyocyanin production is triggered at the early stationary phase through QS molecules (AHL and PQS), phenazine-producing genes (*phzA-G*), and finally by gene *phzM*. (b) Pyocyanin production by various *P. aeruginosa* clinical (AES-1, AES-2, and LESB58) and laboratory (PA14 wild-type, Δ*phzA-G*, PAO1 wild-type, and Δ*phzSH*) strains at different (0, 8, 16, and 24 h) time points. Error bars represent standard deviations from the mean (n=4).

molecule *N*-acyl-L-homoserine lactone (AHL) during the exponential growth phase followed by the secondary QS molecule *Pseudomonas* quinolone signaling (PQS) during the late exponential phase. PQS directly controls the expression of *phzA-G* operons resulting in the production of phenazine-1-carboxylic acid (PCA) from its precursor chorismic acid. PCA is then modified to produce three metabolites during the early stationary phase of which pyocyanin is the predominant product and is regulated by the *phzM* gene. The two other types of phenazine products are phenazine-1-carboxamide (PCN; encoded by *phzH*) and 1-hydroxyphenazine (1-OHPHZ; encoded by *phzS*) [16, 18] (**Figure 1a**).

Figure 1b shows pyocyanin production by various *P. aeruginosa* clinical (AES-1R, AES-2, and LESB58) and laboratory (PA14 wild-type, Δ*phzA-G*, PAO1 wild-type, and PAO1 *phzSH*) strains at different time points (0, 8, 16, and 24 h) grown under planktonic conditions in Luria broth (LB) at 37°C and 150 rpm (unpublished data). The cell-free supernatant of various bacterial strains was used to quantify pyocyanin by absorbance at 691 nm. A standard curve for pyocyanin was generated by addition of a known concentration (0–150 μM) of purified pyocyanin (Sigma-Aldrich, USA) to LB medium in a 1 ml cuvette, and absorbance was assayed at 691 nm (λ_{max} of pyocyanin) using Bio-Rad Smartspec 3000 (Bio-Rad Laboratories, Australia). This curve was used to calibrate pyocyanin concentration in bacterial supernatants. For most of the *P. aeruginosa* strains (AES-1R, AES-2, LESB58, PA14 wild-type, PAO1 wild-type, and PAO1 *phzSH*) used in this study, at initial growth stage (8 h postinoculation), the concentration of pyocyanin produced was less than 5 μM followed by a significant increase in pyocyanin production recorded at ≥16 h of growth and reaching between 75 and 100 μM pyocyanin for AES-1R, LESB58, PA14 wild-type, and PAO1 *phzSH* strains and ~140 μM pyocyanin for AES-2 strain after 24 h growth, whereas PAO1 wild-type produces less than (<) 15 μM pyocyanin. In contrast, the *phzSH* mutant of PAO1, which is deficient of *phzS* and *phzH* genes, was unable to convert PCA to PCN or 1-OHPHZ [16, 19] and consequently overproduced pyocyanin, whereas, after 24 h, phenazine-deficient mutant (Δ*phzA-G)* does not produce any pyocyanin.

3. Pyocyanin a virulence factor

Pyocyanin was formerly disregarded as a bacterial secondary metabolite but has recently been ascribed a variety of roles in microbial ecology, and importantly a relationship with the severity of *P. aeruginosa* infections [20]. Previous research has shown a correlation between pyocyanin concentration in CF sputum and severity of infection [20]. The role of pyocyanin has been intensively studied due to its electrochemical and redox activity. The diffusible nature of pyocyanin means that it can easily diffuse through the host cell membrane and undergo redox reactions with other molecules [2]. For instance, it accepts electrons from NADH and subsequently donates electrons to molecular oxygen to form reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [13]. The H_2O_2 generated via redox reactions has been identified as a potent inhibitor of other bacterial and fungal species found within the microbiome of the CF lung as well as significantly altering host cell functions [2, 21]. In CF patients, pyocyanin-mediated ROS oxidize host intracellular and extracellular reduced glutathione (GSH) to form glutathione disulfide or oxidized glutathione (GSSG; **Figure 2**) [13].

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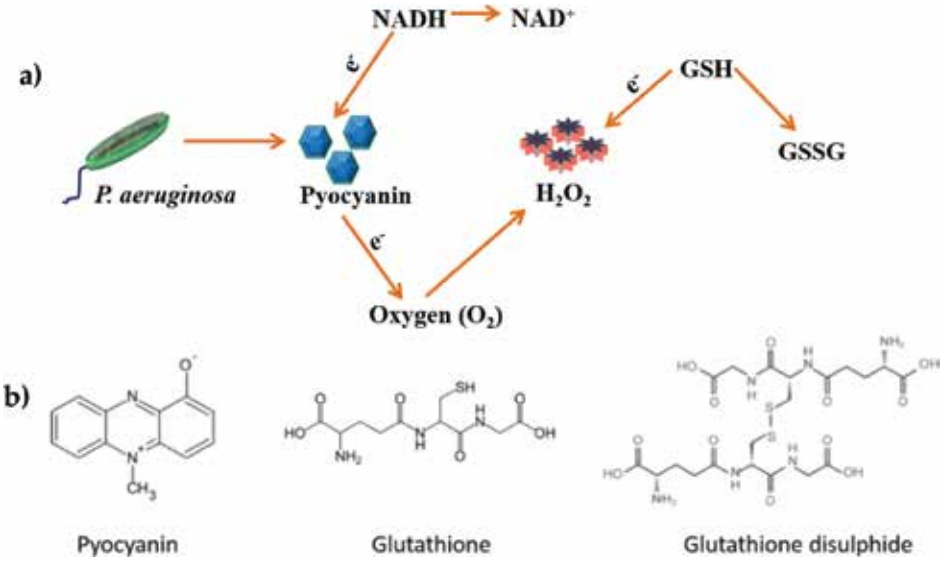

Figure 2. (a) Oxidation of GSH to GSSG by pyocyanin. *P. aeruginosa* produces pyocyanin, and pyocyanin accepts electrons (e^-) from NADH and donates these electrons to oxygen to generate H_2O_2. Glutathione also donates electrons to H_2O_2 forming GSSG. (b) Structure of pyocyanin, GSH, and GSSG.

Depleted GSH levels during the chronic stage of CF infection leads to widespread epithelial cell death and consequently lung damage, respiratory failure, and mortality [21, 22]. Pyocyanin also inhibits catalase activity in the airway epithelial cells and thus aggravates oxidative stress in lung epithelial cells [23].

4. Pyocyanin promotes eDNA release in *P. aeruginosa* via H_2O_2 generation

In bacteria, eDNA release is mediated by both lytic and nonlytic mechanisms. The lytic mechanism involves the controlled lysis of a small number of bacterial cells via the production of various QS-mediated cell lysing agents, such as lytic prophages, autolysin proteins, enzymes, and H_2O_2. In nonlytic mechanisms, eDNA release is through bacterial outer membrane blebs/vesicles that contain large amounts of DNA [24–26]. QS-dependent mechanisms involve the AHL and PQS systems, whereas QS-independent mechanisms operate via release through flagellum and type IV pili [27, 28]. PQS in *P. aeruginosa* PAO1, a virulent laboratory strain, triggers eDNA release in the early phase of planktonic culture through the induction of prophages [27]. In accordance, a mutant for QS synthesis in *pqsA* and a *pqsL* mutant that overproduces PQS show low and high amounts of eDNA release, respectively [27]. QS-independent mechanisms, which also include phage induction [28], are responsible for eDNA release only in well-established PAO1 biofilms (>10 days) and not in planktonic cultures.

A recent work in this field by Das and Manefield has shown that pyocyanin production in *P. aeruginosa* laboratory strains PAO1 and PA14 triggers a significant increase in eDNA release

[19]. Quantitative analysis of eDNA in PA14 wild-type (120 h grown in LB medium) cell-free supernatant showed ~20 to 25 μg eDNA/ml. In contrast, cell-free supernatant of pyocyanin-deficient mutant of PA14 (Δ*phzA-G*) produced 50% less eDNA. Interestingly, Δ*phzA-G* culture grown in the presence of exogenous addition of pyocyanin showed significant increase in eDNA release [19]. In another study, Steinberger and Holden recorded up to 220 and 500 mg eDNA/g of cellular DNA in 5-day-old biofilms of *P. aeruginosa* and *Pseudomonas putida*, respectively; however, their study did not compare the link between pyocyanin and eDNA production in *P. aeruginosa* and *P. putida* [29].

In general, phenazines such as pyocyanin induce H_2O_2 production and subsequently trigger cell death in host (mammalian) and competing fungal and bacterial cells are well documented. A recent study showed concrete evidence of *P. aeruginosa* strains producing the highest concentrations of pyocyanin generated the highest concentrations of H_2O_2 [19]. The production of H_2O_2 initiates ~7 to 14% increase in cell lysis in planktonic cultures of *P. aeruginosa* and consequently promotes eDNA release. **Figure 3** shows a schematic of pyocyanin-mediated eDNA release via H_2O_2 in *P. aeruginosa*. H_2O_2-mediated eDNA release is not limited to *P. aeruginosa* strains; for instance, in oral Gram-positive bacteria (*Streptococcus sanguinis*), pyruvate oxidase activity induces ~10% increase in cell death in its own population via H_2O_2 production and consequently facilitates eDNA release [30].

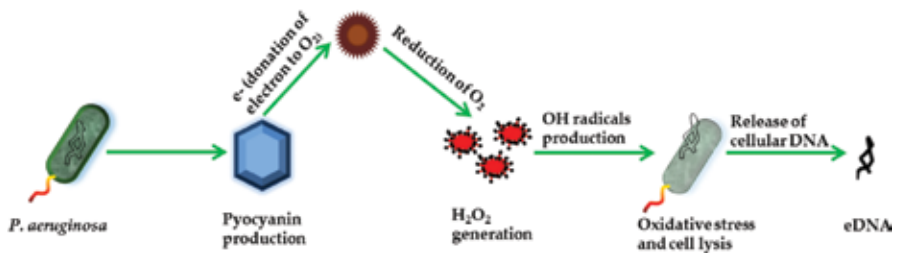

Figure 3. Schematic showing pyocyanin-mediated release of eDNA via H_2O_2 production.

By examining pyocyanin production in different strains of *P. aeruginosa* in relation to H_2O_2 generation, cell lysis, and eDNA concentration, a clear relationship emerged. The findings in this study demonstrate that pyocyanin is involved in eDNA release in growing *P. aeruginosa* planktonic cultures, raising interesting questions about its role in biofilm biology. Pyocyanin-mediated eDNA production, which likely happens as a consequence of cell lysis via H_2O_2 generation, could possibly assist *P. aeruginosa* biofilm formation in several ways, as eDNA has been shown to be an essential biomolecule that necessitates the establishment of *P. aeruginosa* biofilm [7, 8]. Previous studies suggest that the production of phenazines enhance bacterial adhesion, microcolony formation, and increased biomass in *P. aeruginosa* biofilms and current generations of *P. aeruginosa* PA14 in bioelectrochemical systems [31, 32]. From previous findings in concurrence with the current results, we hypothesized that the phenazine pyocyanin may promote biofilm formation in *P. aeruginosa* via eDNA release through H_2O_2-mediated cell lysis. However, the exact mechanism of how pyocyanin facilitates *P. aeruginosa* biofilm formation is yet to be elucidated.

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5. Pyocyanin binding to eDNA

Previous studies have demonstrated that eDNA is a key constituent in the construction and structural integrity of the biofilm matrix in many bacterial species, and the cleaving of eDNA by nuclease enzymes such as DNase I disintegrates the biofilm matrix, thereby increasing the susceptibility of bacterial cells within the biofilm matrix to antimicrobial agents such as detergents and antibiotics [33, 34]. eDNA acts as a scaffold for the whole biofilm by binding with other biomolecules such as peptides/enzymes/proteins and polysaccharides. For example, in *Streptococcus mutans*, an oral pathogen responsible for dental plaques, the competence stimulating peptide (CSP) and DNA-binding protein ComGB interact with eDNA, and this interaction is essential for the uptake of eDNA by *S. mutans*, which facilitates bacterial cell-to-cell interaction and biofilm formation [35]. In another example, HU and IHF (DNA-binding proteins) produced by human pathogenic bacterium *E. coli* strain U93 specifically bind to double-stranded DNA (dsDNA) in the EPS, and by binding with eDNA, such proteins generate specific structures within eDNA. The enzymatic digestion of these proteins makes eDNA lose its structural stability and thereby disrupts *E. coli* biofilms [36]. In *Listeria monocytogenes*, a food-borne pathogen, eDNA binding with *N*-acetylglucosamine, a type of peptidoglycan, has been shown to be an essential molecular interaction that initiates *L. monocytogenes* adhesion to surfaces [37]. To our knowledge, our discovery was the first to demonstrate that, apart from biopolymers such as proteins and polysaccharides, a secondary metabolite such as pyocyanin strongly binds with DNA to dictate the establishment of *P. aeruginosa* biofilm.

5.1. Mechanism of pyocyanin-DNA binding

The mechanism of pyocyanin-eDNA binding was elucidated using different types of spectroscopic techniques by Das et al. [10]. In this study, we used *P. aeruginosa* pyocyanin (Sigma-Aldrich, USA) and calf thymus DNA sodium salt (type I fibers; 42% GC content; Sigma-

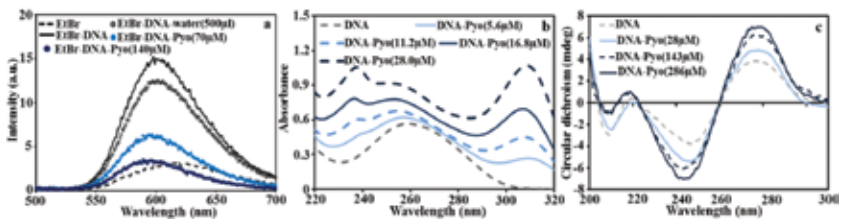

Figure 4. Mechanism of pyocyanin-DNA binding using spectroscopic techniques. (a) Fluorescence emission spectroscopy techniques show that pyocyanin displaces EtBr bound to dsDNA, indicating that pyocyanin is an intercalating agent. (b) UV-vis spectra of DNA with pyocyanin showed that hyperchromic (increase in absorbance intensity) and hypochromic (shift in wavelength of DNA peak from 259 to 253 nm) effects are indicative of the intercalation of pyocyanin between nitrogenous base pairs of DNA and exposure of nitrogenous base pairs due to the unwinding of the DNA helix. (c) CD spectra of DNA-pyocyanin mixtures confirm that DNA binds to pyocyanin. The increase in mdeg at 277 nm in DNA peak confirms that pyocyanin intercalates into the nitrogenous bases of DNA, whereas the shift in DNA peak at 247 nm to 244, 243, and 242 nm in the presence of 28, 143, and 286 μM pyocyanin, respectively, confirms that pyocyanin also binds to sugar-phosphate backbone of DNA.

Aldrich, USA), which has predominantly dsDNA (~90%) quantified using Qubit fluorescent dye assay and Qubit 2.0 Fluorometer (Invitrogen, USA) as mentioned previously.

Using fluorescence emission spectroscopy (Varian Cary Eclipse Fluorescence Spectrophotometer, USA), it was found that pyocyanin displaces ethidium bromide (EtBr) bound to dsDNA. All experiments were done in SHE buffer (2 mM HEPES, 10 μM EDTA, and 9.4 mM NaCl in Milli-Q water adjusted to pH 7 with NaOH). Light emission at 615 nm (λ_{ex}=480 nm) was quantified at room temperature in 1 ml quartz cuvette. The fluorescence emission spectra suggest that the addition of pyocyanin (70 or 140 μM) reduced the DNA (6 ng/μl)-EtBr (4 μM) peak maxima to that of an EtBr solution without DNA (**Figure 4a**). It is well known that EtBr is a classic intercalating agent that strongly binds to DNA via intercalation and the displacement of EtBr by pyocyanin suggests that pyocyanin can bind to DNA. However, the mechanism of pyocyanin-DNA binding was not immediately clear.

To determine the binding mechanism between pyocyanin and DNA, a Varian Cary 100 Bio UV-visible (UV-vis) spectrophotometer technique was used [10]. UV-vis spectroscopic scans from 200 to 800 nm were performed in 1 ml quartz cuvette on DNA, pyocyanin, and the DNA-pyocyanin complex in Milli-Q water. **Figure 4b** shows the UV-vis range spectra of DNA (50 ng/μl) in the presence of increasing concentrations of pyocyanin (5.6, 11.2, 16.8, or 28.0 μM). The spectra of DNA with pyocyanin showed a gradual increase in absorbance intensity of the DNA peak and a shift of the DNA peak from 259 to 253 nm with increasing pyocyanin concentrations. The observed hyperchromic (due to the increase in absorbance intensity) and hypochromic (due to the shift in wavelength of DNA peak) effects are indicative of the intercalation of pyocyanin between nitrogenous base pairs of DNA and exposure of nitrogenous base pairs due to the unwinding of the DNA helix [38].

The pyocyanin-DNA binding mechanism was further confirmed using a Chirascan circular dichroism (CD) spectrophotometer (Applied Photophysics, UK). The experiments were conducted using 1 mm path length quartz cuvette, and mixtures of dsDNA at 135 ng/μl with varying pyocyanin (0, 28, 143, and 286 μM) concentrations in 350 μl Milli-Q water were scanned from 200 to 320 nm after a 15-min static incubation at 25°C. The spectra of DNA-pyocyanin mixtures confirm that DNA binds to pyocyanin with statistically significant changes in peak intensity (P<0.05) at all four characteristic DNA peaks (209, 221, 247, and 277 nm) achieved with pyocyanin concentrations above 28 μM. The significant increase in mdeg at 277 nm in DNA peak is a clear confirmation that pyocyanin intercalates into the nitrogenous bases of DNA. Additionally, the shift in DNA peak at 247 nm to 244, 243, and 242 nm in the presence of 143 and 286 μM pyocyanin, respectively (**Figure 4c**) show that pyocyanin also binds to sugar-phosphate backbone of DNA and therein creates local perturbations in the DNA double-helix structure but, however, does not cause any significant transition in form (i.e. B-DNA to A or Z form).

5.2. Pyocyanin-eDNA binding influences *P. aeruginosa* cell surface hydrophobicity and physicochemical interactions

Pyocyanin is well known as an electron shuttle [18], and our recent investigation revealed that pyocyanin intercalates with DNA [10]. In line with this, previous studies revealed that eDNA

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promotes bacterial aggregation through acid-base interactions involving electron-donating and electron-accepting groups [39]. Contact angle measurements on various Gram-positive and Gram-negative bacterial cell surfaces revealed that eDNA significantly influences modulation in bacterial cell surface hydrophobicity [8, 39]. For instance, the cell surface of *Streptococcus epidermidis* 1457 wild-type is more hydrophobic in the presence of eDNA (average water contact angle of 85°), whereas *S. epidermidis* 1457 Δ*atlE* (eDNA-deficient mutant) and DNase I (DNA cleaving enzyme)-treated *S. epidermidis* 1457 wild-type showed significant reduction in the water contact angle (39–44°) [39]. Based on these findings, we hypothesized that pyocyanin is involved in facilitating eDNA binding to *P. aeruginosa* cells and thus possibly influences *P. aeruginosa* cell surface properties such as hydrophobicity and surface energies and consequently influences physicochemical interactions.

The hypothesis was tested by measuring contact angles of *P. aeruginosa* PA14 and PAO1 lawn growths prepared by the deposition of bacteria, from a planktonic suspension, onto a 0.2 μm pore diameter filter (nitrocellulose membrane filter; Millipore, USA) using negative pressure [39, 40]. Contact angles were measured with standard polar (water and formamide) and nonpolar (diiodomethane) liquids using goniometer (KSV model 200; KSV Instrumentation Pvt. Ltd., Finland) following the sessile drop technique [39]. To remove eDNA, *P. aeruginosa* strains were treated with DNase I before lawn preparation and its contact angle was measured as above. DNase I untreated *P. aeruginosa* PA14 and PAO1 wild-type showed significantly higher degree of water contact angle in comparison to untreated Δ*phzA-G*. After DNase I treatment only, the wild-type strains showed a significant decrease in the water contact angle and ultimately equivalent to the water contact angle of the Δ*phzA-G* mutant (**Figure 5a**) [40]. This result demonstrates that the interaction of pyocyanin with eDNA modulates cell surface hydrophobicity in *P. aeruginosa*.

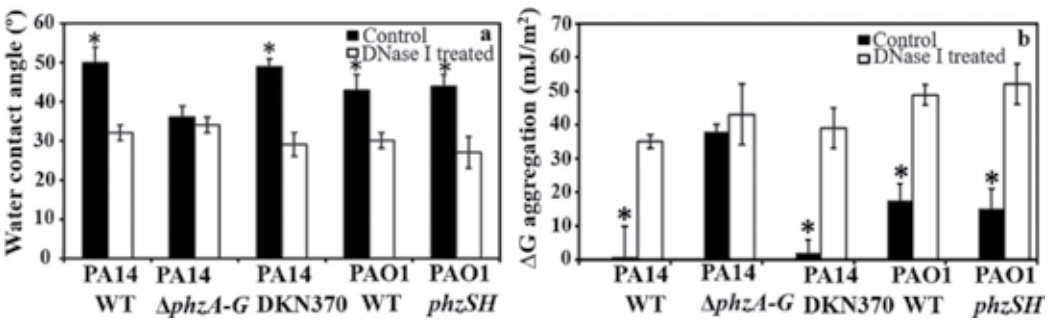

Figure 5. (a) Effect of DNase I and pyocyanin on *P. aeruginosa* cell surface hydrophobicity. The removal of eDNA by DNase I treatment shows a significant decrease in cell surface hydrophobicity (water contact angle) on all pyocyanin-producing strains, whereas pyocyanin-deficient strain (Δ*phzA-G*) showed no change in the water contact angle regardless of DNase I treatment. (b) Gibbs free energy of aggregation indicates that the removal of eDNA or the absence of pyocyanin significantly declines the efficiency of *P. aeruginosa* cell-to-cell aggregation. Error bars represent standard deviations from the mean (n=3). Asterisks indicate statistical significance (Student's t-test P<.05) in comparison to DNase I treatment. PA14 strain data are taken from Das et al. [40], whereas PAO1 contact angle and Gibbs free energy of aggregation results are unpublished.

The modulation in *P. aeruginosa* cell surface hydrophobicity has shown a direct impact on bacterial surface energy components as determined using theoretical surface thermodynamics (**Figure 5b**). Surface thermodynamics elucidated the physicochemical interactions that are responsible for bacterial cell-to-cell interactions at close approach. Physicochemical interactions are nonspecific interactions forces that derive from molecules that are present on the bacterial cell surface. In our study, pyocyanin and eDNA are the molecules that facilitate attractive physicochemical interactions such as short-range acid-base and long-range Lifshitz-van der Waals interactions. The removal of eDNA from *P. aeruginosa* wild-type cell surface or the absence of pyocyanin in Δ*phzA-G* mutant strain showed significant impact (i.e. nonattractive interaction, especially on the short-range acid-base interaction, which includes electron-donating and electron-accepting parameters). However, the long-range Lifshitz-van der Waals interactions remained unaffected between wild-type and Δ*phzA-G* regardless of DNase I treatment [40]. Another important parameter that commonly exists and drives bacterial interactions includes the presence of biopolymers (DNA, polysaccharides, and proteins) and cell appendages (pili, fimbriae) that extend hundreds of nanometers from the cell surface [41]. These biopolymers/appendages initiate hydrogen bonding by colliding with its neighboring cells and thereby help bacterial cells to overcome small physicochemical energy barrier and promote bacterial cell-to-cell interactions [7, 41].

5.3. Pyocyanin-eDNA binding is essential for *P. aeruginosa* biofilm formation

eDNA was previously acknowledged as a biofilm-promoting factor, whereas pyocyanin was mainly considered as a secondary metabolite essential for the persistence of *P. aeruginosa* cells in highly dense biofilm by enabling maintenance of a basal rate of respiration for energy harvesting and to maintain cytoplasmic redox homeostasis [18]. From our previous investigation, it was revealed that pyocyanin-eDNA binding influences essential physicochemical interactions that drive bacterial cell-to-cell interactions. Such findings prompted the hypotheses that pyocyanin-eDNA binding is essential for *P. aeruginosa* biofilm formation.

P. aeruginosa PA14 wild-type and Δ*phzA-G* mutant biofilm formation was investigated on a glass substratum using confocal laser scan microscopy (CLSM) technique complemented using ImageJ software to quantify biofilm characteristic properties (**Figure 6**). Biofilm growth, staining, imaging, and analysis were performed using the protocol described previously [10]. Comparative studies were undertaken between biofilms (grown for 24 h in LB medium, 37°C, 150 rpm) of wild-type (**Figure 6a** and **b**) and Δ*phzA-G* (**Figure 6c** and **d**) grown in the absence and presence of DNase I. Findings indicated that *P. aeruginosa* PA14 biofilm architecture, thickness, and biofilm biomass in wild-type biofilms grown in the presence of DNase I are similar in biofilms of a phenazine-deficient mutant strain (**Table 1**). The removal of eDNA reduced wild-type biofilm thickness and biomass by ~40% and 65%, respectively, whereas phenazine-deficient mutant also showed ~40% reduction in biofilm thickness and up to 80% reduction in biomass. This result clearly indicates that the influence of eDNA on developing a three-dimensional and structurally integrated biofilm is equivalent to the influence of pyocyanin on biofilm.

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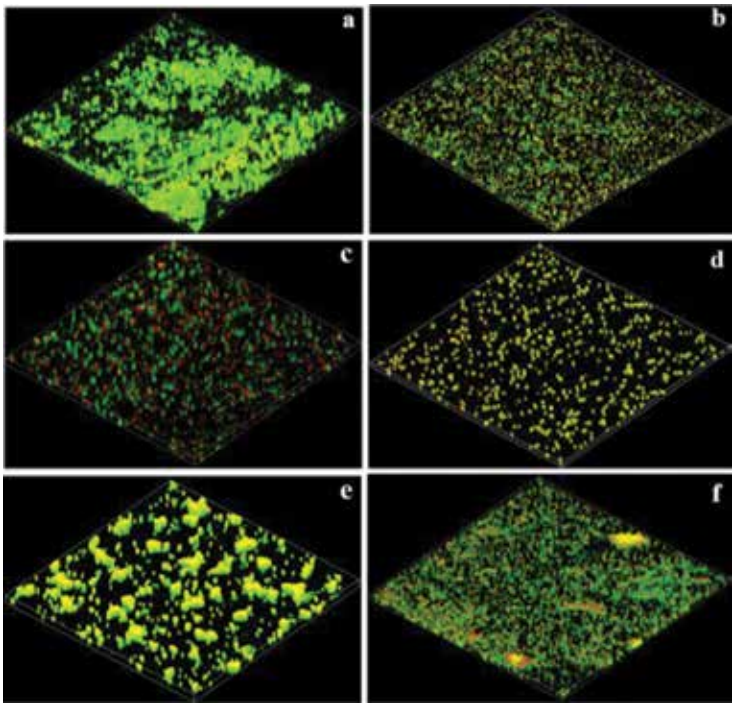

Figure 6. Confocal microscopy imaging of *P. aeruginosa* PA14 strains. (a) PA14 wild-type, (b) PA14 wild-type grown in the presence of DNase I (40 U), (c) Δ*phzA-G*, (d) Δ*phzA-G* in the presence of DNase I (40 U), (e) Δ*phzA-G* in the presence of exogenous pyocyanin (200 μM), and (f) Δ*phzA-G* in the presence of exogenous DNA (1 ng/μl) and exogenous pyocyanin (200 μM).

P. aeruginosa	Thickness (μm)	Biomass ($\mu m^3/\mu m^2$)
Wild-type	8.3±0.3	1.4±0.4
Wild-type+DNase I (40 U)	**5.2±1.5**	**0.5±0.1**
Δ*phzA-G*	**4.9±0.4**	**0.3±0.1**
Δ*phzA-G*+DNase I (40 U)	**3.9±0.5**	**0.3±0.3**
Δ*phzA-G*+pyocyanin (200 μM)	***7.2±1.0***	***0.6±0.2***
Δ*phzA-G*+pyocyanin (200 μM)+DNA (1 ng/μl)	***7.9±0.4***	***1.0±0.1***

Table 1. Quantification of *P. aeruginosa* (nonestablished) biofilm properties using ImageJ software. Significant differences were observed in biofilm thickness and biomass between control (PA14 wild-type) and wild-type grown in the presence of DNase I (40 U) and Δ*phzA-G* regardless of whether they were grown in the presence of DNase I. Mean ±standard deviations (n=3). Boldfaced data indicate that the differences were statistically significant (Student's t-test $P<0.05$) in comparison to control (wild-type), whereas boldfaced italicized data indicate that the thickness and biomass of Δ*phzA-G* grown in the presence of pyocyanin and the combination of pyocyanin+DNA is significantly (Student's t-test $P<0.05$) higher than the biofilm of Δ*phzA-G* alone (control).

A previous study by Ramos et al. also showed similar results with phenazine-deficient mutant but could not elucidate the mechanism behind the influence of phenazine on biofilm formation in *P. aeruginosa* [30]. This finding is compatible with the hypothesis that eDNA and pyocyanin act in concert to influence *P. aeruginosa* biofilm formation, with a direct interaction between pyocyanin and eDNA likely underlying the mechanism of enhanced biofilm integrity. The fact that biofilm formation in the phenazine-deficient mutant is partly restored by the addition of exogenous pyocyanin (**Figure 6e**), with the addition of both exogenous pyocyanin and DNA showed further enhancement in biofilms in comparison to the pyocyanin only treatment (**Figure 6f**). This suggests that this particular phenazine is partly, but not wholly, responsible for the phenomenon, and that interaction with eDNA is an essential factor to facilitate biofilm formation.

6. Glutathione disrupts *P. aeruginosa* biofilm formation

With the increased tolerance of bacteria to existing antibiotic therapies [1] and the necessity to use high doses of antibiotics with their related side effects [42, 43], there is an urgent public health priority to identify new methods and targets for the control of *P. aeruginosa* biofilms.

6.1. Glutathione interacts with pyocyanin and inhibits its binding with eDNA

GSH is a thiol tripeptide (γ-glutamylcysteinylglycine) found in all human cells and in some bacterial species. GSH is considered to be a master antioxidant and its primary functions include defense against ROS and free radicals and maintaining a healthy immune system [13]. In humans, intracellular GSH levels vary from 2 to 10 mM, whereas, in the extracellular lung lining fluid (ELF), levels range from 250 to 800 μM [44]. In contrast, intracellular GSH concentration in bacterial cell differs significantly from species to species [45–47]. For instance, in *E. coli*, GSH is an essential molecule and exists in the millimolar range [47], whereas, in *P. aeruginosa* PAO1, intracellular GSH concentration is reported in the nanomolar range (70 nM) [46]. However, in many Gram-positive bacteria such as *Bacillus cereus*, GSH is not found [45, 48].

Pyocyanin undergoes oxidation by donating electron to molecular oxygen to form superoxides and H_2O_2 [49]. GSH, being a thiol antioxidant, will donate electron/protons to pyocyanin directly through the -SH group from cysteine [50], thereby interfering in the pyocyanin oxidation process by inhibiting H_2O_2 generation [50]. The antioxidant property of GSH makes it a potential inhibitor of pyocyanin toxicity.

Our recent investigation using CD and UV-vis suggests that pyocyanin-GSH complex interferes with pyocyanin binding to DNA (**Figure 7a** and **b**) [10], whereas nuclear magnetic resonance (NMR; Bruker Avance 400 spectrometer) of the pyocyanin-GSH complex at various pyocyanin-GSH ratios clearly indicates that GSH (with at least fivefold higher concentration than pyocyanin) is required to modulate pyocyanin aromatic structure (**Figure 7c**; unpublished data). As discussed earlier, pyocyanin is a planar molecule that intercalates into the nitrogenous base of DNA. By instead conjugating with GSH, intercalation with DNA is restricted. However, it is interesting to observe that with the increases in GSH concentration, the

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inhibition of pyocyanin binding to DNA is increased; almost complete inhibition was observed at a molar ratio of pyocyanin/GSH of ~1:6. A similar observation was reported recently by Muller and Merrett [50] demonstrating that thiol concentration needs to be available in the millimolar range to neutralize pyocyanin toxicity. Their work also suggests that GSH forms a cell-impermanent conjugate with pyocyanin. This suggests that thiol antioxidants could be a potential clinical target against *P. aeruginosa* toxicity by preventing pyocyanin entry into host cells and prohibiting pyocyanin-mediated cell lysis.

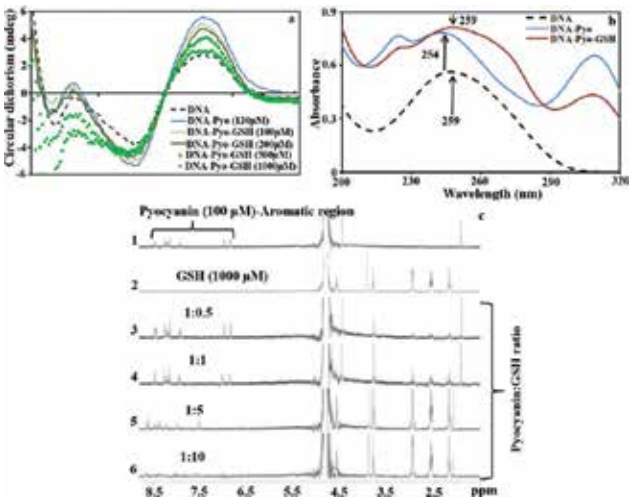

Figure 7. Inhibition of pyocyanin intercalation with DNA. (a) CD spectra of DNA-pyocyanin-GSH mixtures confirm that GSH inhibits pyocyanin intercalation with DNA; however, the inhibition is GSH concentration dependent. At above 1000 μM, GSH (which is ~1:9 ratio to pyocyanin concentration used in this study) successfully inhibits pyocyanin intercalation to DNA nitrogenous base (peak 277 nm) and binding to DNA backbone (peak at 244 nm). (b) UV-vis spectra of DNA with pyocyanin-GSH complex inhibit the shift of wavelength of DNA peak (i.e. retained DNA peak at 259 nm) indicative of the absence of hypochromic effect, which indicates no intercalation. (c) Proton NMR spectra of pyocyanin (100 μM)-GSH (50, 100, 500, or 1000 μM) mixtures indicated a considerable downfield shift of the pyocyanin aromatic peaks. However, this shift was observed only at higher GSH (i.e. at 500 and 1000 μM) concentration ratios (i.e. at pyocyanin/GSH 1:5 and 1:10), whereas, at low GSH (i.e. at 50 and 100 μM) concentrations, pyocyanin aromatic peaks remain stable.

6.2. Glutathione disrupts clinical strains of *P. aeruginosa* biofilms and facilitates bactericidal activity

Biophysical techniques confirmed that GSH inhibits pyocyanin-eDNA intercalation. This made us to hypothesize that GSH could disrupt biofilms, as pyocyanin and eDNA are the crucial factors that initiate biofilm formation. Our recent investigation confirmed that interrupting the pyocyanin-eDNA intercalation using GSH results in a significant disruption of the biofilms of pathogenic Australian epidemic strain (AES-1R and AES-1M isolated from a CF patient; unpublished data). **Figure 8** shows the effect of GSH, DNase I treatment (12 h, 37°C, 150 rpm) on preestablished biofilms grown in LB medium (24 h, 37°C, 150 rpm) imaged using CLSM.

An analysis of biofilm image using ImageJ software revealed that GSH or DNase I-treated AES-1R and AES-1M biofilm showed significant difference (Student's *t*-test $P>0.05$) of ~30 to 40% and 60 to 80% reduction in its biofilm thickness and total biofilm biomass, respectively (**Table 2**). This shows that eDNA and pyocyanin are vital molecules in the building of biofilms of clinical strains of *P. aeruginosa*, and by modulating pyocyanin structure and function, biofilm formation can be restrained while concomitantly reducing the toxicity of pyocyanin.

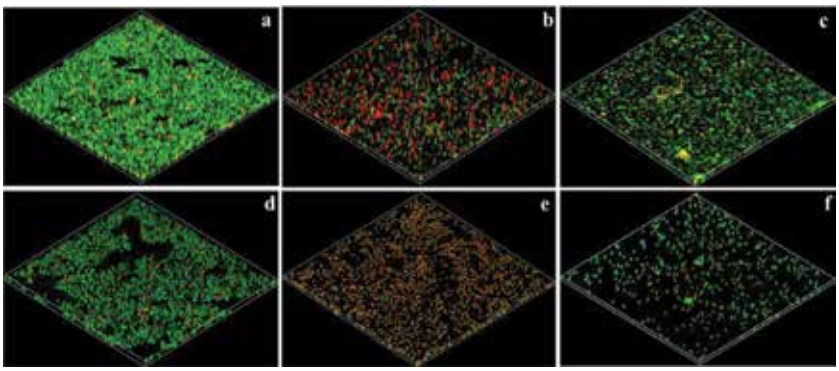

Figure 8. Confocal microscopy imaging of 24 h preestablished biofilms of *P. aeruginosa* clinical strains AES-1R and AES-1M. (a) AES-1R (control), (b) AES-1R treated with 2 mM GSH, (c) AES-1R treated with DNase I (40 U), (d) AES-1M (control), (e) AES-1M treated with 2 mM GSH, and (f) AES-1M treated with DNase I (40 U).

P. aeruginosa	Treatment	Thickness (μm)	Biomass ($\mu m^3/\mu m^2$)	Average Live/Dead (%)
AES-1R	—	8.6±1.1	1.8±0.4	83/17
AES-1R	GSH (2 mM)	**5.1±0.8**	**0.5±0.2**	37/**63**
AES-1R	DNase I (40 U)	**4.9±1.0**	**0.7±0.3**	84/16
AES-1M	—	7.4±1.4	1.1±0.3	79/21
AES-1M	GSH (2 mM)	**4.1±0.7**	0.7±0.4	12/**88**
AES-1M	DNase I (40 U)	**3.9±0.6**	0.2±0.1	93/7

Table 2. Quantification of *P. aeruginosa* (24 h preestablished) biofilm properties using ImageJ software. Significant differences were observed in biofilm thickness, biomass, and average percentage of live and dead biomass between control (AES-1R and AES-1M) and GSH (2 mM) or DNase I (40 U)-treated biofilms. Mean±standard deviations (n=3). Boldfaced data indicate that the differences were statistically significant (Student's *t*-test $P<0.05$) in comparison to control.

Biofilm image (**Figure 8**) and quantification of live (green) and dead (red) biofilm biomass (**Table 2**) clearly show significant increase in dead biomass when biofilm exposed to GSH treatment. GSH-mediated bactericidal activity in *P. aeruginosa* is proposed through the generation of H_2O_2 via the auto-oxidation of GSH (**Figure 8**). H_2O_2 generation by GSH was quantified using Amplex red H_2O_2 assay kit (Thermo Fisher Scientific, USA) as per manufacturer's instruction using multi-well plate reader (Perkin-Elmer, USA). About 11.5 μM H_2O_2

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was generated by 2 mM GSH after 4 h; this finding supports previous published work that suggested that GSH undergoes auto-oxidation to produce H_2O_2 [51–53]. In biological system of healthy individuals, the production of catalase and other chelating agents inhibits the oxidation stress generated by the auto-oxidation of GSH [52]; however, in infected patients, pyocyanin inhibits catalase activity in lung epithelial cells [23] and consequently inhibits the protection against oxidative stress generated by all kinds of endogenous and exogenous molecules.

7. Conclusion and perspective

Bacterial biofilms form a highly protective biological matrix that enables persisting populations of bacteria to survive in otherwise highly hostile environments. These matrices vary highly between species; however, they share a common structural element (eDNA). Within *P. aeruginosa*, eDNA is both necessary and sufficient for structured biofilm formation and maintenance. We have demonstrated that eDNA enhances intercellular aggregation by reducing the Gibbs free energy between cells while also enhancing hydrophobicity by increasing the water contact angle. Furthermore, these properties are enhanced by the presence of the virulence factor pyocyanin, which is able to directly bind to nitrogenous base pairs within dsDNA by mode of intercalation. Pyocyanin is uniquely produced by *P. aeruginosa* and as such confers a unique method by which *P. aeruginosa* is able to strengthen its biofilm.

Biofilm formation is associated with increased resistance to antibiotic therapies and persistence of bacterial colonization within the CF lung. Novel treatment strategies seek to act on molecules that are essential for bacterial persistence such as biofilm constituents. Biofilm disruption is associated with increased antibiotic susceptibility and the clearance of bacteria. We have shown that, by disrupting the biofilm association with thiol-based antioxidants, namely GSH, which directly binds and clears freely available pyocyanin, intercellular aggregation and overall biofilm structure are perturbed. This is enhanced by the activity of nucleases such as DNase I, which remove the underlying eDNA scaffold, resulting in a complete disruption of the biofilm structure by decreasing the water contact angle of bacterial cells and increasing the Gibbs free energy between cells.

Thus, the intercalation between pyocyanin and eDNA is both a unique and highly exploitable interaction in *P. aeruginosa* biofilms, and this interaction is necessary for any kind of structured biofilm architecture in *P. aeruginosa*. By exploring the disruption of this interaction, both GSH and DNase I have arisen as potential therapeutic candidates for the elimination of persistent infections of *P. aeruginosa* existing as a biofilm. This is particularly useful for long-term host-adapted strains of *P. aeruginosa*, such as those persisting in the CF lung, which typically develop a multidrug-resistant profile as the result of repeated antibiotic therapies.

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Chapter 3

Microbial Interactions in Biofilms: Impacts on Homeostasis and Pathogenesis

Yung-Hua Li and Xiao-Lin Tian

Additional information is available at the end of the chapter

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Abstract

Microbes in nature or in the human body are predominantly associated with surfaces and living in biofilms. Species diversity, high cell density and close proximity of cells are typical of life in biofilms, where organisms interact with each other and develop complex interactions that can be either competitive or cooperative. Competition between species is a well-recognized ecological force to drive microbial metabolism, diversity and evolution. However, it was not until recently that microbial cooperative activities are also recognized to play important roles in microbial physiology and ecology. Importantly, these microbial interactions in biofilms profoundly affect their overall function, biomass, diversity and pathogenesis. It is now known that every human body contains a personalized microbiome that is essential to maintain host health. Remarkably, the indigenous species in most microbial communities often maintain a relatively stable and harmless relationship with the hosts despite regular exposure to minor environmental perturbations and host defence factors. Such stability or homeostasis results from a dynamic balance of microbial–microbial and microbial–host interactions. Under some circumstances, however, the homeostasis may breakdown, predisposing a site to diseases. The evidence has accumulated that such biofilm or community-based diseases can be prevented or treated not only by targeting putative pathogens, but also by interfering with the processes that drive breakdown of the homeostasis in biofilms.

Keywords: biofilms, microbial interactions, microbial homeostasis, microbiome, pathogenesis, community-based diseases

1. Introduction

The human body is host to a wide variety of microbial life, termed as the human microflora or microbiota, or more recently microbiome [1, 2]. The human microbiome contains hundreds of

species and trillions of cells that are predominantly associated with surfaces as communities, such as dental plaque and biofilms on many mucosal surfaces of the human body [1–3]. Species diversity, high cell density and close proximity of microbial cells are typical of life in biofilms, where microbes interact with each other and develop complex social interactions that can be either competitive or cooperative among species [4, 5]. Even without physical contact, microorganisms living In the same community may secrete small diffusible signal molecules to interact with each other [6]. The human microbiome, including "core" microbiota shared by all individuals and "personalized" microbiota exclusive to the individuals, plays important roles in human health, such as breakdown of complex molecules in food, protection from exogenous pathogens and stimulation of healthy immune development [3]. One of the most striking aspects of these complex communities is their long-term stability in healthy individuals. The indigenous species in a community often maintain a relatively stable and harmless relationship with the host despite regular exposure to minor environmental perturbations and host-defense factors [7]. Such stability or homeostasis is considered critical for host health and wellbeing. Under some circumstances, however, such homeostasis may break down, leading to population shifts in a community and predisposing a site to diseases [8]. What determines such homeostasis in a community? What factors can change homeostasis and what are the mechanisms behind? How can these changes be detected and prevented? This chapter aims to briefly review the current advances relevant to these questions.

2. Social structure of microbial biofilms

Microorganisms in nature are predominantly associated with surfaces and live in multispecies biofilms, which account for over 99% of microbial life on this planet [9]. Similarly, the host-associated microbes largely reside in biofilm communities on the surfaces of human body, including nonshedding surfaces, such as teeth, and shedding surfaces, such as the mucosa of the mouth, upper respiratory tract, digestive tract and urogenital tracts, although large numbers of microbial cells may be washed or shed off from these surfaces by mechanical and biological movements [9–11]. Biofilm formation is a dynamic process that often results in a developmental biofilm life cycle [9, 10]. During the process of biofilm formation, some organisms are early colonizers that express biochemical components allowing them to effectively adhere to a surface [10]. Others are the later colonizers, which often contain components enabling them to adhere to the early colonizers, bringing metabolic and other competitive advantages into the community [9, 12]. Biofilms are spatially structured communities that often display a high degree of organization and their functions depend on complex webs of symbiotic interactions [11]. If viewing an intact biofilm under a microscope, then one will immediately find that microbes in biofilms do not randomly stick together, but rather form a well-organized community with numerous specialized configurations [10, 13]. One may also find that microbial cells in biofilms physically interact with each other and maintain intimate relationships [12]. Even without physical contact, microbes living in the same community may secrete small diffusible signal molecules to interact with each other [14]. For example, many bacteria are found to regulate diverse physiological processes through a

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mechanism called quorum sensing, in which bacteria secrete, detect and respond to small signal molecules for coordinated activities in a cell density-dependent manner [15]. During quorum sensing, bacterial cells cooperate to obtain group-specific benefits, such as signal molecules, extracellular polymers, exoenzymes, antibiotics and virulence factors [16–18]. Structural and physiological complexities of biofilms have led to the idea that microbes in biofilms frequently cooperate for social activities as groups, like multicellular organisms [19]. Indeed, microbiologists have discovered an unexpectedly high degree of multicellular behaviours that have led to the perception of biofilms as "cities" of microbes [20]. Through cooperation, microbes can impact their environments in many ways that are simply impossible for individual cells. Clearly, microbes in such "cities" can achieve strength by increasing their cell density and interactions or by collectively producing virulence factors required for the pathogenesis [17–20].

3. Microbial interactions in biofilms

Microbial biofilms are characterized by species diversity, high cell density and close cell-cell proximity [6, 9, 12]. This suggests that microbial cells in biofilms likely display intermicrobial interactions that contribute to the formation of a highly structured community, allowing cells to carry out metabolic activities that may enhance the overall function of the community [21]. The significance of intermicrobial interactions was first realized and thoroughly described for microorganisms residing in the oral cavity [10, 12]. Dental plaque is a well-recognized biofilm community characterized by its vast diversity (>700 species) and high cell density (1011 cells/g wet wt), which allow organisms to develop complex interactions [12]. Cooperative interactions among organisms in dental biofilms have been well studied, including bacterial co-aggregation and co-adhesion that facilitates bacterial colonization on saliva-coated teeth and effectuates temporal and spatial formation of highly organized biofilm architectures [10, 12]. Biofilm matrix also plays important roles in promoting bacterial adhesion, trapping nutrient molecules, forming microenvironments and protecting microbial cells from lethal challenges or antimicrobial agents [22, 23]. Cooperative metabolic interactions are even more common among microbial species, involving nutritional synergy or complementation enabling organisms to breakdown complex salivary components [6, 12]. Cross feeding is another type of cooperation in which microbes obtain available nutrients, allowing formation of food chains in the community [24]. For example, oral streptococci are well known by their ability to generate lactic acid from sugar fermentation, whereas some neighbouring species, for example *Veillonella* sp., are unable to ferment sugar but use lactic acid as a preferred carbon source to generate energy [25]. Many bacteria in biofilms also use quorum-sensing mechanisms to regulate biofilm development and other coordinated activities, including symbiosis, formation of spore or fruiting bodies, bacteriocin production, genetic competence, virulence and pathogenesis [14–18]. The processes controlled by quorum sensing are diverse and reflect the specific needs of particular communities. In many bacteria, quorum sensing represents a central mechanism to regulate cooperative activities, enabling bacteria to reap benefits that

would be unattainable to them as individual cells [6]. Clearly, cooperative interactions among species probably play important roles in biofilm development and metabolic activities.

However, microbes in most ecosystems often face major challenges of limited space and nutritional resources, which inevitably results in competition among species. To survive and pass their genes to the next generation, microbes have to cope with constant battles of resource competition [26]. The potential pool of microbial competitors is vast, and a wide range of mechanisms can be responsible for the emergency and radiation of dominant microbial populations. Microbial ecologists have long recognized two types of competition: exploitation competition that occurs indirectly through resource consumption and interference competition that causes a direct, antagonistic effect on competitors [5, 27, 28]. There is good evidence that both exploitative and interference competition are prevalent in biofilms, strongly influencing the homeostasis and outcome of natural selection of microbes in biofilms. Microbial competition for common resources is a typical exploitative competition and can be strong in many natural ecosystems [28]. However, microbes cannot be viewed as passive nutritional sinks, but rather have evolved numerous strategies to augment their acquisition of resources. Many microbial activities, such as motility, attachment, antibiotic production and secretion of extracellular polymers, can tip the competition balance, resulting in outcomes that may differ from those predicted in planktonic cultures (27). Particularly, biofilms often form gradients in nutrient concentrations, oxygen tension, pH and waste products due to the thickness [12]. These factors can significantly affect the outcomes of microbial competition and compositions in a biofilm community. Interestingly, despite high levels of competition among species, the majority of the resident organisms in a host-associated community can co-exist and maintain a relative stability in the community [8, 30]. This indicates that some regulatory mechanisms must exist and play critical roles in balancing microbial cooperative and competitive activities in microbial communities.

Based on recent community structure and dynamic studies using metagenomics and 16S pyrosequencing, microbial interactions can have three types of outcomes: a positive impact (win), a negative impact (loss) and no impact (neutral) on the microbial species involved [31]. The possible combinations of win (+), loss (−) and neutral (0) outcomes for two interacting partners allow classification of various interaction types. For example, different species of bacteria may cooperate to build a biofilm, which confers protection of the interacting members from antibiotics, a win–win (+/+) relationship known as mutualism. Other examples for cooperation are certain cases of cross feeding, in which two species exchange metabolic products to the benefit of both. In contrast, competition between two species is a classic loss–loss (−/−) relationship, which indicates that two species with similar niches exclude each other or competitive exclusion. In addition to typical cooperation or competition, predator–prey relationships and host–parasite relationships are considered to be win–loss (+/−) interactions, which are also common in natural and host-associated microbial communities [30]. For example, *Streptococcus mutans* in dental plaque can produce an array of bacteriocins that kill other related species in the community, a typical win–loss interaction (+/−) [32]. In most ecosystems, there are few cases of neutral or no (0) interaction among species in the same community. These microbial interactions are largely based on laboratory studies of pairwise

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species of microorganisms interested. Relatively few studies have been carried out to investigate microbial interactions and their impacts at community level until recently when genomics and metagenomics techniques are available to study communities [31]. However, detecting these various types of interactions in natural microbial communities is far from straight forward. Novel approaches to the investigation of community- or even ecosystem-wide networks may open a way towards global models of community and ecosystem dynamics. Ultimately, these studies will help to predict the outcome of community alterations and the effects of perturbations in complex microbial communities.

4. Roles of microbial interactions in maintaining the homeostasis in biofilms

It has been recognized that host-associated microbial communities are usually characterized by a remarkable stability among the component species, despite regular exposure to minor environmental perturbations and numerous host-defence factors [8, 33]. The ability of microbes to maintain the community stability is referred to as homeostasis (**Figure 1**). The homeostasis is believed to stem not from any indifference among the component species but rather results from a dynamic balance of microbial–microbial interactions and microbial–host interactions [8]. Interestingly, such stability in a microbial community is often associated with a healthy condition. However, despite our rapidly increasing knowledge of the composition of the human microbiome, we know relatively little about what determines the homeostasis in a microbial community and what mechanisms have been involved in maintaining the homeostasis. There are few *in vivo* studies on the relative significance of microbial interactions in maintaining microbial homeostasis. Most studies have characterized potential interactions *in vitro* with the assumption that they may operate similarly *in vivo*. It has been proposed that the tendency of a microbial community to maintain its homeostasis often increases with species diversity or with a greater biological complexity of the community [7, 8, 34]. This suggests that some regulatory mechanisms must operate to favour the development of species diversity and complexity of a microbial community. When the homeostasis is disturbed in a community, the self-regulatory mechanisms may come to work and restore the previous homeostasis status in the community. However, it is not always certain what regulatory mechanisms operate to maintain the homeostasis in a community. Recent studies have revealed that most stable microbial communities contain high levels of species diversity with complementing and seemingly redundant metabolic capabilities [35]. Microbial interactions in these communities can promote high species richness and bolster community stability during environmental perturbations. Clearly, species diversity within a microbial community is an important indicator of the homeostasis [7, 8]. The need for microbial diversity in health may suggest that every species can carry out a specific function that is required to maintain the homeostasis in a community.

Recent studies of microbial community dynamics show that although positive microbial interactions or feedbacks, such as cooperation and synergism, play important roles in increasing community productivity, the positive microbial interactions can come at costs to the

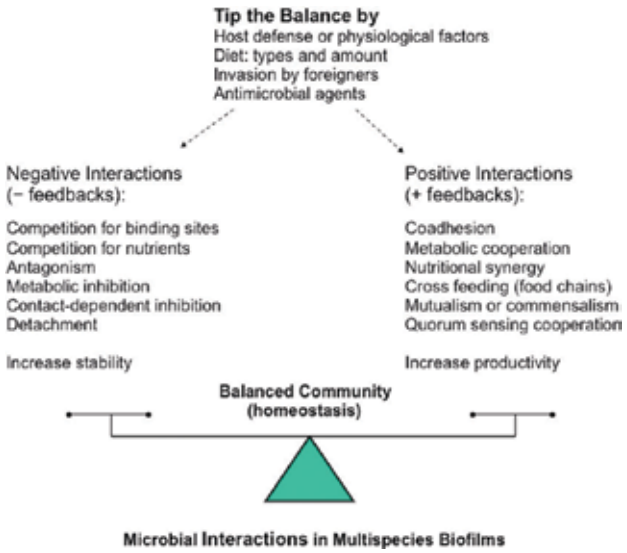

Figure 1. A schematic diagram describes microbial–microbial interactions and their roles in maintaining the homeostasis in a community. Microbial interactions include negative interactions (− feedbacks) such as competition and antagonism and positive interactions (+ feedbacks) such as cooperation, synergy and mutualism. Positive interactions likely increase the productivity of the community but potentially destabilize the community, while negative interactions often dampen cooperative activities but favour species diversity and community stability. These interactions form complex networks that finely balance the homeostasis of the community. However, a number of ecological factors can tip the balance of these microbial interactions, disturbing the stability of a community. Dashed arrows indicate the potential of these factors to tip the balance of the community.

community, so potentially destabilizing the community [7, 34]. Microbial cooperation is destabilizing the community because it introduces positive feedbacks, which can generate runaway effects. For example, when two species cooperate, an increase in the abundance of one species increases the abundance of the second, which in turn will increase the abundance of the first species and so on. If these increases are not sufficiently checked by other constraints, then this can lead to runaway increases in cooperating species that can cause the collapse of interacting populations and destabilization of the community [7]. In contrast, negative microbial interactions or feedbacks, such as competition and antagonism, are considered as major and essential mechanisms in maintaining the homeostasis in microbial communities. This means that adding species that primarily engage in competitive interactions to the community may counterintuitively help to stabilize the community by dampening positive feedbacks, stopping the community from cooperating its way to collapse [34]. Human and animal hosts may also suppress positive interactions or feedbacks between cooperating species in order to stabilize the community. Hosts could do this possibly by three mechanisms. First, the host immune response could be a stabilizing force. When certain species in a community rapidly increase in abundance, this could provoke a targeted host immune response, stopping positive feedbacks between cooperating species in their tracks. Second, the host could attempt to block cooperative interactions among species by spatially segregating them: when species grow in separate locations, their interactions will be weakened, thereby, preventing positive

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feedbacks. Third, the host could feed microorganisms to reduce cooperation among species by providing alternative carbon sources, so that these species no longer rely so strongly on their cooperative partners [34]. Analysis of mouse gut microbiome reveals that cooperative interactions are rare in the gut microbiome (only ~10% of pairwise interactions are mutually beneficial), possibly because of their destabilizing effect [7].

An additional unexplored factor that could drive community stability is natural selection on both microbiomes and hosts. The human microbiome is the product of long adaptive processes of constituent species, their interactions and host factors governing their growth [36]. Given the possibility of selection driving communities towards higher stability, it will be important to ascertain not only how species interactions affect stability on average, but also what characteristics of the most stable communities are, and whether they are achievable by evolution. Work on animal and plant communities has shown that factors that decrease community stability on average can also counterintuitively be over-represented in most stable communities [34]. These approaches will be critical in understanding evolutionary ecology of microbial communities, therefore, helping manipulation of component species in communities to promote stable microbiomes and health in hosts.

5. Potential factors disrupting microbial homeostasis: tipping the balance

Human microbiome research reveals that every human body contains a variety of microbial communities that consist of hundreds of microbial species important to human health [1–3]. The key to human health is an ecological-balanced microbiome that practices commensalism or mutualism within itself and with the host [7]. Microbial–microbial and microbial–host interactions play important roles in maintaining such a homeostasis in these microbial communities (**Figure 1**). Despite these interactions, however, the homeostasis in a microbial community can breakdown under certain circumstances, leading to population shifts and predisposing a site to diseases [8]. What factors can disrupt the homeostasis in such stable communities? Studies of various host-associated microbiomes, such as those in the oral cavity, gastrointestine and vagina, have provided some clues to the type of factors indicating homeostasis disruption in a community, including (1) a significant change in the relationship between a microbial community and the host; (2) acquisition of a virulence factor or pathogenic trait by a resident species in the community; (3) a sudden increase or decrease in relative abundance of one or more species in the community and (4) more recently, "keystone" species or pathogens that play key roles in the breakdown of host–microbial homeostasis leading to dysbiosis in a community and diseases, based on the keystone-pathogen hypothesis [3, 8, 33, 37].

The relationships between microbiome and its hosts during health are often mutually beneficial because the host is providing its microbial communities with an environment in which they can flourish and, in turn, keep their host healthy [34]. The presence of an immune or physiological disorder can tip the balance of a microbial community. As the immune defense system regulates microbial–host interactions, a compromised immune system often disrupts

the balance relationships between microbes and the host, resulting in the homeostasis breakdown and predisposing to disease. For example, immune-deficient or chemotherapy patients have an increased susceptibility to opportunistic infections [11]. Individuals with reduced saliva flow or dry mouth also have an increased susceptibility to dental caries, periodontitis or oral candidosis caused by once-normal resident microbes within the oral cavity [33]. Another example is that increase in female sex hormones can sometime have the capacity to disrupt microbial homeostasis in several ecosystems of the body, predisposing or enhancing opportunistic infections [8].

Acquisition of virulence factors or pathogenic traits via horizontal gene transfer between microbes in biofilms is a common mechanism to trigger population shifts by antibiotic-resistant species leading to the homeostasis breakdown in a community. For example, an antibiotic-resistant gene transfer within or between species may lead to dominance by these populations in the community, particularly when the community is exposed to a subinhibitory antibiotic stress condition [38].

A sudden increase or decrease in relative abundance of one or more species in a microbial community often indicates the homeostasis breakdown of the community [8, 33]. A common feature is a significant change in nutrient status, for example, introduction of an excess substrate such as sugar or a chemical compound that can disturb the ecosystem [8]. For example, frequent consumption of fermentable dietary carbohydrates in the oral cavity may favor the overgrowth of sugar-fermenting bacteria (**Figure 2**) such as *S. mutans* and *Lactobacillus* sp. in a dental biofilm [33]. Such carbohydrate metabolism from these bacteria generates large amounts of lactic acid that acidifies the local environment, resulting in selection of acid-resistant bacteria but elimination of acid-sensitive bacteria in the community. The dominance by a few acid-resistant species in the community indicates the breakdown of the homeostasis, predisposing the site to tooth decay [33]. In this case, the microbial community is often dominated by fewer species or reduced species diversity [8]. Clearly, frequent consumption of fermentable carbohydrates is a powerful determinant that disturbs the homeostasis in dental biofilms. Similarly, antimicrobial agents that kill bacteria are the best-characterized mechanisms resulting in homeostasis breakdown in many host-associated microbial communities [11]. Antibiotic treatment often causes a rapid reduction in sensitive species followed by an emergence of resistant organisms. This inevitably results in population shifts and the homeostasis breakdown in the communities. It is then not surprising that an infectious disease may occur due to the overgrowth of an antibiotic-resistant organism during an improper antibiotic therapy.

More recently, a novel hypothesis, called the "keystone-pathogen hypothesis", has been proposed to describe mechanisms underlying the breakdown of host–microbial homeostasis that precipitates dysbiosis (microbiota imbalance) of a community, leading to diseases [37]. The keystone-pathogen hypothesis holds that certain low-abundance microbial pathogens can orchestrate inflammatory disease by remodeling a normally benign or resident microbiota into a dysbiotic one in a community. Importantly, the keystone pathogens have the capacity of instigating inflammation and triggering dysbiosis even when they are present as quantitatively minor components in the community. Recent studies suggest that keystone pathogens play

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key roles in initiating periodontitis, chronic inflammatory bowel disease, colon cancer and obesity. For example, periodontitis is a biofilm-induced chronic inflammatory disease, which affects the tooth-supporting tissues or periodontium (**Figure 3**), and also increases patients' risk of developing atherosclerosis, diabetes and possibly rheumatoid arthritis [38, 39]. The tooth-associated dental plaque is required but not sufficient to induce periodontitis, because it is the host inflammatory response to this microbial challenge that ultimately can cause destruction of the periodontium. There has been significant progress in the quest to identify specific periodontal pathogens, including the identification of several candidates, mostly Gram-negative anaerobic bacteria that colonize subgingival tooth sites. Foremost among this group are three species that constitute the so-called "red complex", are frequently isolated together and are strongly associated with diseased sites in the mouth: *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* which are the keystone pathogens in subgingival dental biofilms [39]. Much research have been directed towards understanding the pathogenic mechanisms and virulence determinants of these three bacterial species. Dysbiotic microbial communities of these keystone pathogens are thought to exhibit synergistic virulence, whereby not only they can endure the host response but can also thrive by exploiting tissue destructive inflammation, which fuels a self-feeding cycle of escalating dysbiosis and inflammatory bone loss, ultimately leading to tooth loss and systemic complications [40].

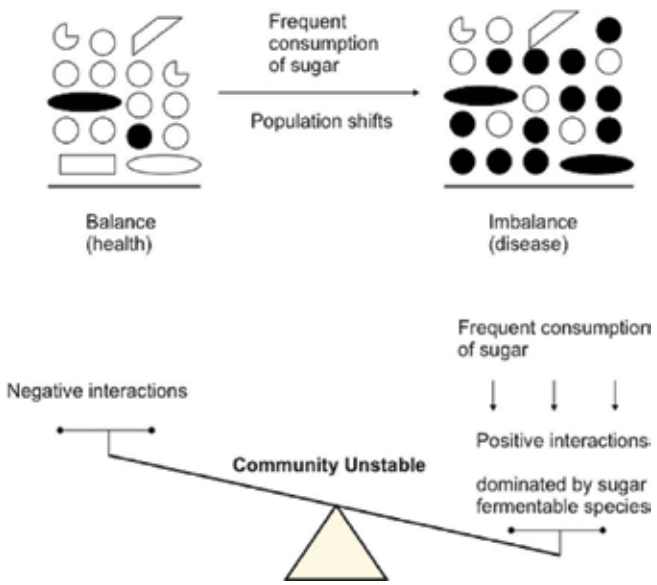

Figure 2. A schematic diagram describes an example of an ecological factor, frequent consumption of sugar (fermentable carbohydrates), to tip the balance of the community. In the human oral cavity, frequent consumption of dietary sugar is a powerful ecological factor that can cause population shifts and tip the balance of a dental biofilm community. Sugar favours the overgrowth of sugar-fermentable and acid-resistant bacteria such as *Streptococcus mutans* (black circles) and *Lactobacillus* sp. (black ovals) in dental biofilms. This will result in population shifts characterized by dominance of *S. mutans* and *Lactobacillus* sp., but reduction or elimination of acid-sensitive bacteria (blank shapes) in the community, leading to the homeostasis breakdown and predisposing the site to dental caries. In this case, fewer species remain in the imbalanced community.

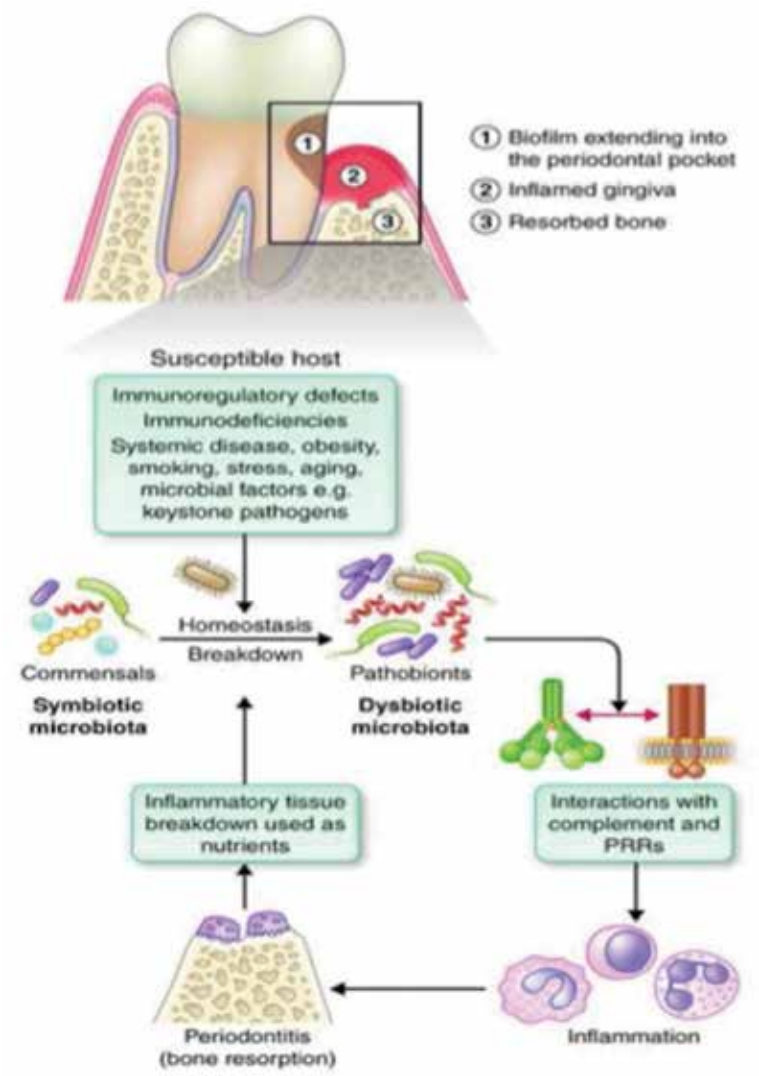

Figure 3. A proposed model describes the roles of pathobionts or keystone pathogens in the initiation and development of periodontitis. In healthy periodontium, a commensal microbe–host relationship is maintained because of a controlled inflammatory state. However, this balanced relationship or homeostasis can breakdown due to defects in the immunoinflammatory state or predisposing conditions or environmental factors, leading to the balance shift towards dysbiosis, a state in which former commensal organisms become proinflammatory pathobionts. In addition, the presence of keystone pathogens can similarly tip the balance toward dysbiosis even in hosts without apparent predisposing factors. The inflammation caused by the dysbiotic microbiota depends in great part on crosstalk signaling between complement and pattern recognition receptors (PRRs). This has two major interrelated effects: it causes inflammatory destruction of periodontal tissue, which in turn provides nutrients (destructed tissues) further promoting dysbiosis. This generates a self-perpetuating pathogenic cycle. It should be noted that host susceptibility might not simply be a determinant of the transition from a symbiotic to dysbiotic microbiota but it may underlie the predisposition of the host to develop inflammation sufficient to cause irreversible tissue damage.

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6. Implications in the pathogenesis of biofilm diseases

Traditional studies on infectious diseases have focused extensively on pathogenic microbes that directly damage tissues in hosts. It is increasingly recognized that direct attack is not the only way that microbes cause diseases. Evidence has accumulated that some commensal microbes living as the normal residents in a host can also induce diseases or contributes critically to disease development. These commensal microbes that can cause or promote diseases under certain conditions are often called opportunistic pathogens or "pathobionts" [40]. When some species become dominant in their relative abundance in a community, the relationships among the resident members in the community might become imbalanced called dysbiosis, which indicates the breakdown of the homeostasis in the community. The keystone pathogens identified from various ecosystems also play key roles in disturbing the microbial–host homeostasis, leading to dysbiosis, which can be the cause or the consequence of diseases and is largely dependent on microbial–host interactions in a microbial community. Recent studies reveal that factors that can disturb the microbial homeostasis likely result in the dominance by pathobionts in a community, predisposing a site to diseases [39, 40]. A common feature of these diseases is that they are often associated with multiple species of pathobionts, so these diseases are referred to as polymicrobial or community-based diseases [12, 42]. However, only certain species play major roles in driving a commensal community toward the pathogenic shift [41]. Despite multispecies features, a major challenge using antibiotics to treat these diseases is that wide-spectrum antibiotics may indiscriminately kill the resident organisms in the community, resulting in ecological disruption or other negative clinical consequences [43]. Current understanding of polymicrobial or community-based diseases has changed the strategies for diagnosis, prevention and treatment of these diseases.

7. Strategies for diagnosis, prevention and treatment of community-based diseases

7.1. Community-based assays of the microbiomes

It is now known that most biofilm diseases are associated with multiple species of microorganisms. These polymicrobial diseases such as dental caries, periodontitis, otitis media, cystic fibrosis lung infection, inflammatory bowel disease and other biofilm infections are clinically characterized by a chronic process with acute or subacute episodes [41–43]. The homeostasis breakdown leading to dysbiosis in a community is the key step for the initiation and development of these diseases [40]. Because alternations in the microbiota at a given site are potential biomarkers of disease activity, analyzing the microbiome at the early stages of diseases would allow clinicians to diagnose, predict and prevent potential risk, severity and outcomes of these diseases. In particular, identification of keystone pathogens could have substantial clinical benefits, as it may facilitate the development of targeted treatment by focusing on a limited number of pathobionts in biofilms. Since every human body contains a personalized micro-

biome, analyses of the microbiome will pave the way for more effective diagnosis, prevention and therapies, contributing to the development of personalized medicine.

For a long time, our understanding of microbial communities has been hampered by the intrinsic limitation of conventional culture-dependent techniques. Our views of the complexity and genetic diversity of microbial communities based on cultivation strategies are severely biased. Fortunately, a number of DNA-based assays or genomic approaches have been developed to help overcome such limitation, allowing us to obtain a clearer picture of microbial communities in terms of their structural complexity and genetic diversity. Since intermicrobial interactions in a community often create many new physiological functions that cannot be observed with individual species, community-based assays have emerged to analyze microbial compositions and associated physiology, which has greatly contributed to our understanding of the microbiomes and dysbiosis. Common strategies used to analyze microbial communities or the microbiomes include 16S rRNA gene (pyro)sequencing [44, 45], genomic or metagenomic approaches [46], checkerboard DNA–DNA hybridization [47], PCR-based denaturing gradient gel electrophoresis (DGGE) [48] or denaturing high performance liquid chromatography (DHPLC) analyses [49] and terminal restriction fragment length polymorphism (T-RFLP) analysis [50]. The application of these community-based techniques in the analysis of the human microbiomes has revealed astonishing diversities of largely uncultivated microorganisms present in human samples. These approaches have been expanded to many clinical samples collected from a broader patient pool with a diverse range of healthy conditions and diseases, promoting the discovery of many new species of the human microbiome.

7.2. Modulating community ecology to reduce potential risk of a disease

With our new understanding of microbial communities and their associated diseases, there is an increasing interest in approaches that modulate the ecology of microbial communities to achieve reduction or control of community-based diseases. These diseases may be prevented or treated not only by inhibiting the putative pathogens, but also by interfering with the factors disturbing the homeostasis in microbial communities. Among them, probiotic approach has been a popular method for modulating microbial ecology [51]. The probiotics refers to live microorganisms that can confer health benefits on the host when administered in adequate amounts [52]. In the past decades, there have been numerous exciting discoveries that reveal beneficial effects resulting from administering probiotics, ranging from direct inhibition of pathogenic microbes to improving host immune functions [53]. The rationale of using probiotics is based on the fact that probiotics can interfere with invasion by foreign pathogens or with pathogenic shifts by keystone pathogens in microbial communities. These may reduce the potential of a community to become a pathogenic one or dysbiosis [51–53].

Another strategy is to interfere with microbial cell–cell communication via quorum sensing in microbial communities, since quorum-sensing mechanisms play important roles in biofilm formation and cell density-dependent virulence [13–18]. In recent years, scientists actively search for natural and synthetic compounds that act as quorum-sensing inhibitors (QSIs) that can target bacterial quorum-sensing mechanisms and their controlled pathogenic activities [54–56]. It is believed that QSIs target bacterial cell–cell signaling and coordinated activities

required for infections, thereby, essentially disarming the bacteria and tipping the balance in favor of the host and allowing the immune system to clear the infectious pathogen [54]. QSI therapies that specifically block bacterial quorum sensing can make the pathogens become 'deaf', 'mute' or 'blind' rather than directly killing them. Therefore, QSI therapy may achieve the treatment but much less likely cause selective pressure to create resistant microbes [54–56].

For some community-based diseases, such as periodontitis and intestine inflammatory diseases, anti-inflammatory agents can be used to break the cycle of inflammation and tissue destruction, both of which promote the homeostasis breakdown or dysbiosis in a community [42, 43]. In particular, these agents combined with some antimicrobials that specifically target the keystone pathogens or pathobionts would provide much better therapy both by targeting the putative pathogens and by interfering with the processes that drive breakdown of the homeostasis in the community [43].

Other strategies in regulating microbial ecology to prevent homeostasis breakdown in some microbial communities include diet regulation such as sugar substitutes that reduce carbon source for bacterial fermentation, increasing flow of body fluids such as saliva, use of oxygenating or redox agents that reduce the growth of obligate anaerobes in a biofilm community, and use of nonantimicrobial agents such as fluoride, chelating agents such as EDTA, and metal ions that compromise some metabolic activities of certain microbes [8, 33]. For example, fluoride can inhibit enzyme activity required for bacterial metabolism, particularly under low pH, but shows little bacterial killing, thereby, not significantly affecting community ecology [8].

7.3. Targeted antimicrobial therapy to reduce pathobionts in a community

Currently, available antibiotics exhibit broad killing spectra with regard to bacterial genus and species. Indiscriminate killing of microbes by these conventional antibiotics may disrupt the ecological balance of the indigenous microflora, resulting in negative clinical consequences [51]. To circumvent the problem, a new class of such antimicrobials, called pheromone-guided antimicrobial peptides (PG-AMP), has been developed as potential alternatives [57, 58]. The rationale of using such antimicrobial agents is based on the addition of a targeting domain of a quorum-sensing signal pheromone from a target organism to the killing domain of a known antimicrobial peptide. Both domains are fused via a small linker to generate a fusion PG-AMP without detrimental change of their activities [58]. The targeting domain can guide such a fusion peptide to bind selectively to the target organism, leading to selective killing [57–59]. These narrow-spectrum antimicrobials can selectively target specific organisms with little effect on the other members of the community [51, 57–59]. Therefore, PG-AMPs have added an exciting opportunity to develop new antimicrobials that target keystone pathogens in a community-based disease. Recent studies explored the possibility of utilizing a pheromone produced by *S. mutans* as a targeting domain to mediate *S. mutans*-specific delivery of an antimicrobial peptide domain [57–59]. It is found that PG-AMPs constructed in this way are potent against *S. mutans* in animal dental caries model [60]. The PG-AMPs are capable of eliminating *S. mutans* from multispecies biofilms without affecting other noncariogenic species, indicating the potential of these molecules to be developed into targeted antimicrobial

agents. This proof-of-principle strategy suggests that it may be possible to develop PG-AMPs that specifically target other keystone pathogens and modulate microbial ecology in community-based diseases [58–60].

8. Concluding remarks

Research over the last 30 years has generated a substantial amount of knowledge on microbial biofilms. We have learned that microbes form highly diverse communities on surfaces of human body, which are increasingly recognized to have profound impacts on human health and diseases. It has been well established that microbes in such biofilm communities can develop complex social interactions and networks, which play important roles in modulating the community stability or homeostasis important to host health. Despite our rapidly increasing knowledge of the compositions of the human microbiome, we know little about what determines the stability of these communities. However, significant advance has been made to identify factors that affect microbial interactions, ecology and pathogenesis. Evidence shows that some biofilm diseases can be prevented or treated not only by targeting the putative pathogens, but also by interfering with the processes that drive the breakdown of the homeostasis in biofilms. Studies of the human microbiomes in health and disease will open a new avenue for the development of more effective diagnosis, prevention and treatment of community-based diseases, contributing to personalized medicine.

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Chapter 4

Adherence and Biofilm Production of *Streptococcus pyogenes*

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Additional information is available at the end of the chapter

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Abstract

Streptococcus pyogenes (group A streptococcus – GAS) can cause numerous human infections, varying from mild skin infections to life-threatening, e.g. necrotizing fasciitis. Adherence and biofilm production are important in streptoccocal pathogenesis. GAS adhesins are numerous and diverse, with the ability to bind to several different receptors at the same time, which leads to difficulties in their precise identification and classification. Biofilm production is one of the most probable explanation for therapeutic failure in the treatment of GAS infections. Most researchers agreed that biofilm formation is a trait of individual strains rather than a general serotype attribute. The aim of our study is to investigate differences in adherence to laminin and biofilm production between invasive and non-invasive isolates (NI) of GAS. In this study the correlation between adherence to laminin and invasiveness in GAS isolates is noticed. The strains isolated from GAS carriers and highly invasive (HI) GAS strains have excellent capacity for binding to laminin. When testing biofilm production, there was noticeable positive correlation between adherence and biofilm production among non-invasive isolates. Non-invasive isolates were stable biofilm productors. There was no correlation between adherence and biofilm production among invasive isolates. Invasive isolates were also unstable biofilm productors.

Keywords: *Streptococcus pyogenes*, Invasiveness, Adherence, biofilm production, hydrophobicity

1. Introduction

Streptococcus pyogenes (group A streptococcus – GAS) is one of the most frequent exclusively human pathogen. When speaking about human infections and clinical conditions, there are only

few bacteria like GAS showing so much different faces and such a wide spectra of different virulence factors. Even systematization of group A streptococcal diseases was not specified for a long time. Classification of GAS diseases has recently been successful, clarified, and systematized. Diseases caused by *S. pyogenes* are divided into pyogenic (superficial and invasive) and toxemic, with autoimmune complication such as post-infectious sequelae (acute rheumatic fever and acute post-streptococcal glomerulonephritis) [1]. Streptococcal carriage is a special clinical condition, during which, this strictly human pathogen became a not precisely opportunistic bacteria, but normal flora-like bacteria.

S. pyogenes is a successful human pathogen and is a cause of so many diseases by virtue of its numerous virulence factors, which in this high numbers are possessed only by few bacteria like *Staphylococcus aureus* and *Clostridium perfringens*. Therefore, it is quite incomprehensible that the bacteria with such numerous and different virulence factors is not expected to be the cause of life-threatening human diseases. Although it is so much discovered and known about GAS pathogenesis, there is still unknown why this bacteria so rare and unexpectedly activates his most powerful virulence factors such as toxins and hydrolytic enzymes, which are most active in necrotizing fasciitis and streptococcal toxic shock syndrome (STSS).

On the other hand, such invasive bacteria could become normal flora-like bacteria during pharyngeal or nasal carriage. Streptococcal carriage has been defined as the recovery of GAS from the nasopharynx or oropharynx in the absence of any evidence of acute infection [2]. Streptococcal carriers should not be treated with antibiotics, except in the cases of reappearance of disease or possible occurence of post-streptococcal sequelae. Genesis of streptococcal carriage was for a long time poorly understood. Nowadays, there are two theories which explain streptococcal carriage as the consequence of therapeutic failure happened after infection of strains capable to produce biofilm [3] or internalize into epithelial cells [4].

Considering that *S. pyogenes* is one of the few bacteria still sensitive to penicillin *in vitro*, and due to the development of new antibiotics, it would be expected that the incidence of streptococcal infections should decrease over time. But, epidemiological data suggest that percentage of streptococcal carriage has remained unchanged, and also the fact that frequency of the invasive GAS diseases have become more frequent, led to the establishment Strep-EURO study group for monitoring streptococcal invasive disease in 11 European countries. All these information and facts have led to actualizations of GAS, revelation of new virulence factors which contribute to new perspectives in the understanding of group A streptococcal pathogenesis.

2. Adherence and biofilm production of *Streptococcus pyogenes*

2.1. New insight into old problem of group A streptococcal adherence

Although sometimes not sufficiently emphasized, efficient adherence is the prime step in the pathogenesis of infective disease. Factors that influence adherence are diverse and can

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originate from environment such as the substrate on which the biofilm creates, initial bacterial layer coating the substrate, and characteristics of bacteria multiplying in this medium [5].

Adherence is complex process that includes several different steps. In the first step, bacteria have to overcome the repulsive forces which are consequence of negative charge of the bacterial superficial adhesins and substrate. Afterwards, in the second step positively attracting forces (such as covalent, ionic, van der Waals, hydrophobic) are established between bacterial adhesin and compatible receptor on the human cell. These attractive forces act on the small distance and only after bacterial surpassing of the repulsive electrostatic forces. Most of these interactions are low affinity bonds, but acting together they turn out to be strong and high affinity. Van der Waals forces play crucial role in protein-protein recognition, when complementary lock-and-key shapes are involved [6]. Hydrophobic side chains on the proteins could be connected to each other also using the low affinity hydrophobic forces. This is very plain and simple observation of adherence, and we should highlighted here that in the same or similar environmental conditions even closely related species in genus *Streptococcus* could demonstrate very diverse attractive forces [7].

Nowadays, there is proposition of two-step adherence of *S. pyogenes* [6]. In the first step, lipoteichoic acid (LTA) as amphipathic molecule, enables overcoming of the repulsive electrostatic forces between bacteria and substrate. In the second step, microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), such as M protein, fibronectin binding protein (SfbI), serum opacity factor (SOF), etc. adhere to specific receptor on the human cell. Besides of the MSCRAMMS, it is demonstrated that this second step of adherence could be provided by bacterial pili. The initial attachment is very dynamic process in that they demonstrate on-off kinetic effect and includes several chemical molecular interactions, such as hydrophobic, ionic and electrostatic forces. Second step of adherence probably involves more specific, complex and irreversible interactions with higher affinity between one or several different MSCRAMMS and human cells. Group A streptococcal ability to bind our cells with several different MSCRAMMS at the same time make difficulties in identification of streptococcal adhesins.

Bacterial adherence to human cells could be on the direct or indirect way. Direct way of adherence is displayed by binding of bacterial adhesion to specific receptor on the cell surface; e.g. capsular hyaluronic acid interacts with CD44 receptor on the surface of keratinocytes and induces reorganization of cytoskeletal actin and rupture of intercellular bridges enabling bacteria to penetrate the epithelium still staying extracellular and reaching deeper into the tisssue [8]. The other, indirect way of adherence is more common. Streptococcal adhesins first bind to proteins of extracellular matrix (ECM) such as fibrinogen, fibronectin, laminin, collagen as bridging molecules which than attach to cell membranes integrins [9].

2.1.1. Fibronectin binding proteins

Fibronectin (Fn) is a high-molecular weight glycoprotein that circulates free as a dimer in the soluble form in blood plasma or as a fibrillar form is assembled by cells as major component of the ECM. So far, fibronectin binding proteins are the best studied adhesins of *S. pyogenes* and currently 11 different such adhesins have been identified [10], divided in two types. First

type proteins are SfbI, PrtF2, SOF, SfbX, Fbp54, FbaA, and FbaB and they all contain Fn-binding repeats. Second type proteins are M1, Shr, Scl1, and GAPDH and they do not contain these repeats. It's estimated that 60% of initial attachment to epithelial cells is realized by streptococcal lipoteichoic acid, but afterwards MSCRAMMS, e.g. fibronectin binding proteins are the most important in the irreversible stage of adherence. Binding of these adhesins to Fn could result in irreversible attachment to the cell or biofilm production in tissue or bacterial internalization. Fibronectin acts as a bridge molecule for binding to $\beta 5\alpha 1$ integrins, with subsequently rearrangement of cytoskeletal actin and uptake of the invading bacteria [11].

Expression of Fn-binding proteins is regulated as response to the environmental conditions in which streptococci survive and multiply. Protein F/SfbI, which allows binding to epithelial cells of the dermis and Langerhans cells, show increased expression on bacterial surface with increasing pressure of oxygen, e.g. on the cell surface, thereby enabling a better adherence of the bacteria. When oxygen level is decreased, e.g. in deep tissue, expression of this protein is also diminished, allowing bacterial dissemination into deeper tissues [12]. SfbI expression could be diminished also by catalytic cleavage with serine protease streptococcal pyrogenic exotoxin B (SpeB) or by other bacterial surface proteases where infection occurs. Protein F2, detected in most SfbI negative- GAS strains, binds fibronectin with high affinity and is homologous to Fn-binding proteins of group C streptococci. Similarly as in protein F1/SfbI, F2 activity is also response to the environmental oxygen pressure [13]. Unlike these two proteins, M protein expression is enhanced in the deeper tissues with increased pressure of carbon dioxide, preventing phagocytosis and contributing to the dissemination of GAS [14].

2.1.2. Anchorless adhesins

Anchorless adhesins are attached to bacterial cell surface in the unknown mode, probably through hydrophobic interactions. Importance of these proteins is in their ability to separate from the cell surface, get away from the cell, detects environmental signals around streptococci and the information transferred back to *S. pyogenes* [6]. The anchorless adhesins are not grouped toghether because they are functionally and structurally diverse. Most of them have enyzmatic functions. For example, five anchorless adhesins are enyzmes in glycolytic pathway and they tipically are located in bacterial citosol: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, phosphoglycerate kinase, phosphoglycerate mutase, and triose phosphate isomerase [15]. GAPDH, also designed as SDH and Plr, could bind to several human proteins (plasmin/plasminogen, fibronectin, and fibrinogen), cytoskeletal actin and myosin, acting as an important colonization factor. These five anchorless adhesins operate together as a complex in generating ATP molecules. As anchorless adhesins, they could produce extracellular ATP, which is attached to $P2X_7$ receptors on epithelial and immune cells, inducing apoptosis of these cells. In this way, *S. pyogenes* establishes control on the behavior of human cells and facilitates further progression of the infection [16].

2.1.3. Laminin binding adhesins

Laminin is high-molecular weight ECM protein and one of the major components of the basal lamina, which is part of the basement membrane in human cells. Although laminin is widely

distributed in our body, only a few laminin binding proteins are identified in GAS so far. Currently, proteins nominated as streptococcal hemoprotein receptor (Shr), laminin binding protein (Lbp), and streptococcal pyrogenic exotoxin B (SpeB) are identified as laminin binding proteins for *S. pyogenes*.

SpeB is anchorless adhesin, with enzymatic function as cysteine protease. SpeB was first identified as exotoxin, but this protein can be attached to the bacterial surface as adhesin. SpeB is synthesized during early stationary phase in nutritious poor media [15]. This protein, like M protein, has multiple functions (adhesin, proinflammatory effect, and enzymatic function). Besides his function as laminin binding adhesin, this protein can bind to fibronectin and vitronectin, allowing streptococcal dissemination in deep tissue [17] and activates metalloproteases included in remodeling and degrading of ECM [15].

Shr is probably the protein attached to cell membrane, because it contains nor LPXTG either QVPTG repeats, that recognize housekeeping or accessory sortases, enzymes which incorporate proteins in the cell wall [18]. Its membrane position corresponds to the primary role of Shr protein in uptaking of the heme and binding to its transporter in the cytoplasmic membrane [19, 20]. In addition to its metabolic role, and by the virtue of surface position, it has been shown that this protein have the ability to bind laminin and fibronectin, participating in this way in adherence and acting as MSCRAMMS [21].

Lbp belongs to the group of metal-binding receptors with modified accessory proteins. Lbp scavenges environmental zinc and transports it to carriers of the cell membrane, to which it is attached [22]. Also, Lbp is laminin binding adhesin [23]. This protein is not identified in the oral streptococci, but is present in all surveyed so far M serotypes of GAS [23]. Lbp is very short, even shorter than the thickness of the cell wall, and because of its location in the cell membrane, it is likely to have greater importance in the metabolism of metal than in the adherence to laminin [22].

2.1.4. Our experience with group A streptococcal adherence

Considering that the adherence of streptococci is still insufficiently examined process and that the streptococcal adhesins are numerous and irregularly and inadequately identified, in our study isolates were divided in three groups according to invasiveness of the disease they caused. The aim of our study was to investigate differences in adherence to laminin between invasive and non-invasive isolates (NI) of GAS.

2.1.4.1. Material and methods

2.1.4.1.1. Bacterial strains

In total, 172 GAS isolates were included in the study. They were divided into three groups: (1) 100 non-invasive isolates (NI) obtained from GAS carriers; (2) 50 low invasive (LI) isolates obtained from patients with tonsillopharyngitis; and (3) 22 highly invasive (HI) recovered from blood of patients with sepsis and STSS. All the isolates are part of the national collection of GAS strains formed at the National Reference Laboratory for Streptococci, Institute of

Microbiology and Immunology, Faculty of Medicine, University of Belgrade. The NI and LI isolates were collected during 2012, while HI isolates had been collected over the last two decades.

2.1.4.1.2. Laminin coating of microtiter plates

We investigated adherence of GAS strains to uncoated and laminin-coated microtiter plates. Laminin coating of the polystyrene microtiter plates (Kartell, Italy) was performed by using 0.5 mg/ml laminin (Sigma Aldrich, USA), in accordance with the manufacturer's instructions. The plates were coated for 2 hours at 37°C with laminin previously diluted in Hanks balanced salt solution (Sigma Aldrich, USA) to achieve final concentration of 5 μg/ml, and afterwards were washed three times with Hanks balanced salt solution.

2.1.4.1.3. Capsule removal by hyaluronidase

Prior to adherence testing, all isolates were treated with bovine testicular hyaluronidase, type VI-S, (Sigma-Aldrich, USA) diluted in enzyme diluent (20 mM Sodium Phosphate, 77 mM Sodium Chloride, 0.01% Bovine Albumin, pH 7.0 at 37°C) in order to remove their capsules, as previously described [24].

2.1.4.1.4. Quantification of adherence to laminin by GAS strains

Quantification of adherence of GAS strains to uncoated and laminin-coated microtiter plates was based upon the protocol described by Stepanovic *et al.* [25]. The strains were incubated overnight in THY at 37°C, and then diluted in fresh THY medium to achieve final concentration of 10^6 CFU/ml. Aliquots of bacterial suspension (100 μL) were transferred to each well of the 96-well microtiter plate and incubated for 30 minutes at 37°C. The content of each well was then aspirated and wells were washed three times with sterile phosphate buffer solution (PBS). The plates were left overnight at room temperature for drying and air fixation. The plates were stained with 100 μL of 2% (w/v) crystal violet and, afterwards, the dye bound to the adherent cells was solubilized with 100 μL of 33% (v/v) glacial acetic acid. The negative control wells contained Todd Hewitt broth supplemented with 1% yeast extract (THY) broth only. *Staphylococcus epidermidis* ATCC 14990 was used as the positive control. The optical density (OD) of each well was measured at 570 nm using an automated microtiter plate reader. The cut-off optical density OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Strains with OD above ODc were considered adherent to microtiter plates. Strains were classified as follows:

$OD \leq ODc$ = non-adherent isolates, $ODc < OD \leq (2 \times ODc)$ = weakly adherent isolates (+), $(2 \times ODc) < OD \leq (4 \times ODc)$ = moderately adherent isolates (++) and $OD > (4 \times ODc)$ = strongly adherent isolates (+++). All analyses were performed in triplicate and repeated at least two times.

2.1.4.1.5. Statistical analysis

Student's *t*-test was used to measure the differences in adherence to uncoated and laminin-coated plates as well as the differences in adherence to laminin before and after the penicillin and erythromycin treatment within each group of GAS strains tested. ANOVA was used to determine the differences in adherence to laminin among different groups of GAS strains. Data analyses were done with the SPSS version 20. The differences were considered significant if $p < 0.05$, and highly significant if $p < 0.01$.

2.1.4.2. Results and discussion

To determine correlation between invasiveness of tested GAS strains and their ability to bind to laminin, we investigated adherence of NI, LI, and HI isolates to uncoated and laminin-coated microtiter plates. All isolates were treated with hyaluronidase in order to eliminate the interference of hyaluronic acid capsule on adherence. The proportions of NI, LI, and HI isolates that displayed adherence to uncoated microtiter plates were 98%, 71%, and 91%, respectively. In all adherent isolates the level of adherence was estimated as weak, but adherence of NI and HI isolates to uncoated plates was significantly higher than adherence displayed by isolates of the LI group ($p \leq 0.001$). All isolates tested displayed adherence to laminin-coated microtiter plates (**Table 1**).

	No (%) of weakly adherent isolates (+)	**No (%) of moderately adherent isolates (++)**	**No (%) of strongly adherent isolates (+++)**
NI group (total 100 isolates)	13 (13)	40 (40)	47 (47)
LI group (total 50 isolates)	13 (26)	27 (54)	10 (20)
HI group (total 22 isolates)	0	5 (23)	17 (77)

Table 1. Adherence to laminin of invasive and non-invasive GAS isolates.

The overall results showed significantly higher adherence ($F = 6.952$, $p \leq 0.001$) of GAS strains tested to laminin-coated microtiter plates than adherence to uncoated plates. This was noted in all three groups of strains, and the ratios of adherence to laminin-coated vs. uncoated plates were as follows: 1.86 in HI group ($t = 15.603$, $p \leq 0.001$), 1.36 in NI ($t = 19.730$, $p \leq 0.001$) and 1.24 in LI group ($t = 13.355$, $p \leq 0.001$). However, the level of adherence was different in different groups of strains, as shown in **Table 1**. Majority (54%) of LI isolates were moderately adherent, while most of NI (47%) and, in particular, HI isolates (77%) were strongly adherent. To our knowledge, this is the first study evaluating differences in adherence to laminin between invasive and non-invasive isolates of GAS, and, thus, there are no other previously reported results for direct comparison. Musumeci *et al*. [26] showed that the proportion of *S. pyogenes* strains carrying the *prtF2* gene, encoding internalization-associated fibronectin binding protein F2, was significantly higher among asymptomatic carriers than among children with pharyngitis. This suggests significant contribution of this adhesion to the ability of *S. pyo-*

genes to persist in the throat of asymptomatic carriers. Similarly, we established high adherence ability in isolates obtained from GAS carriers. As far as highly invasive isolates are concerned, positive correlation between invasiveness and adherence to laminin found in our study was also shown for group B streptococcus (GBS) [27]. The surface laminin-binding protein (Lmb) was significantly more expressed in invasive GBS strains isolated from cerebrospinal fluid of the neonates with meningitis than in non-invasive strains isolated from feces or vaginal swabs of colonized asymptomatic pregnant women [27].

In conclusion, this study showed correlation between adherence to laminin and invasiveness in GAS isolates. The strains isolated from GAS carriers and highly invasive GAS strains have excellent capacity for binding to laminin.div4close

2.2. Biofilm

The greatest importance of the effective bacterial adherence is in the attachment to host cells and the aggregation of bacteria, which then create a signal for the biofilm production. In a collective way of existence bacteria gain a protective matrix layer, which in planktonic lifestyle does not exist and is responsible for the most of mechanisms that bacteria avoid eradication from the infection site.

According to literature, *S. pyogenes* is also capable for biofilm production. Researchers have noticed GAS microcolonies in skin lesions of patients with impetigo [28], and after that the same was observed in experimental zebrafish skin infection [29]. Also, three-dimensional communities resembling biofilm were detected in pediatric tonsillar samples after tonsillectomy of patients with adenotonsillar hypertrophy, contributing to the theory that biofilm formation is one of the probable explanations for the GAS persistence and carriage [30]. Besides these *in vivo* experiments, several authors have been proven *S. pyogenes* biofilm production also *in vitro* in static or flow conditions, e.g. in polystyrene microtiter plates, plastic coverslips or flow chambers [31, 32].

Biofilm production is one of the most probable explanation for therapeutic failure in the treatment of infections with this bacteria, *in vitro* sensitive to the tested antibiotic. Several authors have noticed better biofilm production in non-invasive streptococcal strains compared to invasive strains [33, 34], and also in erythromycin-sensitive isolates compared to erythromycin-resistant isolates of *S. pyogenes* [35]. These results strongly indicate that biofilm production is protective mechanism enabling bacterial survival of antibiotic treatment and immune system reaction. Also, collective lifestyle allows *S. pyogenes* easier horizontal gene transfer by transformation, generating virulent clones and biofilm phenotype [36].

Although Baldassari *et al*. [35] have proven biofilm production in 90% of tested invasive and non-invasive isolates, still most researchers agreed that biofilm formation is a trait of individual strains rather than a general serotype attribute. GAS biofilm production is complex process influenced not only by environmental conditions such as ECM proteins, incubation temperature and medium, but also by many bacterial virulence factors such as capsule, SpeB, M protein, pili, etc. Manetti *et al.* [37] assumed that on this multifactorial process also affect antigenic variation of M protein and pili, in addition to a variety known and poorly surveyed

virulence factors, thus further complicating the already insufficiently elucidated process of biofilm assembling. Doern *et al*. [38] suggested significance of in the timely manner production of several enzymes, such as SpeB, which in addition to his role in adhesion, is also one of the crucial factors in biofilm formation. SpeB expression is very high during both planktonic and biofilm lifestyle of GAS, that is almost a unique and uncommon phenomenon, because genetic expression is very different during this two distinct bacterial life stages. In planktonic lifestyle SpeB is highly synthesized in the early stationary phase. In the early phase of collective lifestyle, in order to begin with biofilm formation, it is necessary to prevent SpeB production. In the later stages of biofilm dispersion, production of SpeB is extensive, leading to protein degradation in the biofilm matrix. SpeB expression and disintegration of matrix protein are activation signal for other secreted proteases and nucleases, like Sda1, which degrade DNA and proteins in biofilm, contributing to biofilm dispersal and dissemination of bacteria throughout the body.

Fibronectin-collagen-T antigen (FCT) classification is only partially managed to link biofilm production with certain FCT groups. In FCT region of *S. pyogenes* genome are placed genes encoding several virulence factors important for regulation of matrix production: fibronectin binding proteins F1 and F2 (prtF1, prtF2), pilus – associated proteins (Cpa, Fca, Fcb) and RofA/Nra regulator [39–42]. Koller *et al*. [43] have shown correlation between FCT type 1, 2, 5, 6, and 9 and homogenous biofilm production. FCT type 9 isolates were poor biofilm producers, while FCT type 3 and 4 isolates were unequally and irregular biofilm producers. Manetti *et al.* [44] demonstrated association between biofilm formation among FCT types 2, 3, 5, 6 and FCT subset 4 in acidic surroundings.

2.2.1. Our experience with biofilm production of Streptococcus pyogenes

Considering that biofilm production, like adherence, is still not sufficiently explained virulence factor, we supposed that dividing isolates according to invasiveness would be interested. Like in adherence experiments, we also divided isolates in three groups in order to show correlation between biofilm production and invasiveness of strains tested.

Our goal was to find out whether biofilm production as virulence factor is correlated with specific disease/clinical condition. Considering that adherence and hydrophobicity are in relationship with biofilm production, we also wanted to show possible association between them.

2.2.2. Material and methods

2.2.2.1. Determination of hydrophobicity

Hydrophobicity was measured by two different methods described previously by Rosenberg *et al.* [45] and by Lindhal *et al*. [46]. Microbial adhesion to hydrocarbons (MATH) test described by Rosenberg *et al*. [45] was measured with determination of percentage of bacteria adhered to xylene and hexadecane. The salt aggregation test (SAT) described by Lindhal *et al*. [46] is based on the principal of salting out of the surface proteins and bacterial precipitation with a

series of ammonium sulfate solutions with various molarities (0.008 M to 4 M). The highest molarity of solution giving the visible aggregation was scored as numerical value of bacterial surface hydrophobicity or SAT value.

2.2.2.2. Biofilm production

Biofilm production was determined by the same methodology [25] as adherence testing with modification in incubation period of 12, 24, and 48 hours. As in adherence testing, according to ODc isolates were designed as non-producers = OD < ODc, weak biofilm producers = ODc < OD ≤ (2 × ODc) (+), (2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producers (++) and OD > (4 × ODc) = strong biofilm producers (+++).

2.2.2.3. Statistical analysis

ANOVA was used to determine the differences in hydrophobicity and biofilm production among different groups of GAS strains. Correlation between adherence, hydrophobicity, and biofilm production was determined by Pearson test. Data analyses were done with the SPSS version 20. The differences were considered significant if $p < 0.05$, and highly significant if $p < 0.01$.

2.2.3. Results

2.2.3.1. Adherence

Adherence results are shown in Section 2.1.4.2.

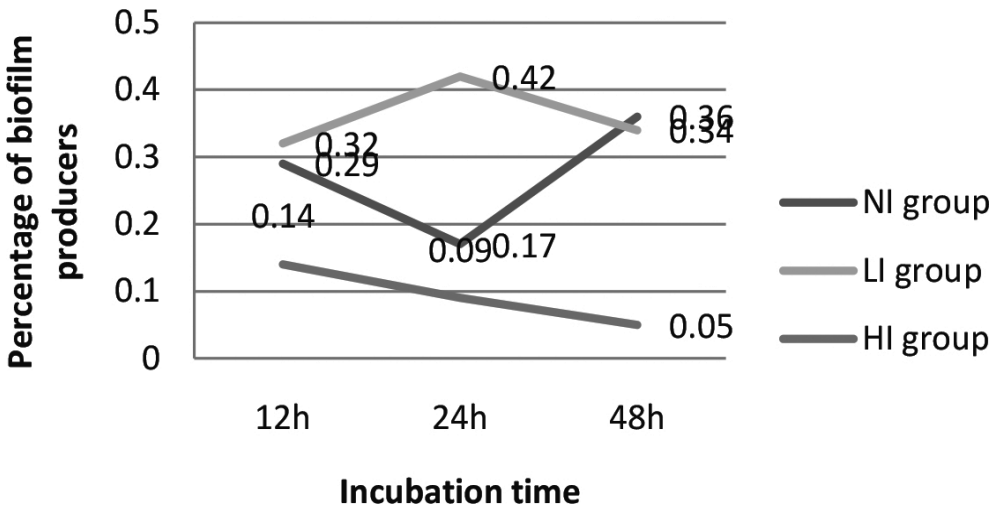
2.2.3.2. Hydrophobicity

Measurement of bacterial hydrophobicity was first performed by MATH test using hexadecane as hydrocarbon after removal of the capsule, which hinders superficial hydrophobic proteins. Adherence to hexadecane was very low and with no statistical difference between groups. After that we tested GAS adherence to xylene. In our assay adherence to xylene were 48.49, 22.78, and 36.09 for NI, LI, and HI group, respectively. It was noticed statistically significant difference between groups, particularly NI group isolates were more hydrophobic in relation to other two groups ($p < 0.001$, $p = 0.041$) and HI group isolates in relation to LI group ($p = 0.044$).

2.2.3.3. Biofilm production

When we did dynamic analysis of biofilm production during specified incubation periods (12–48 hours) all three groups have shown different pattern ($p = 0.040$). Although NI and LI groups started and finished at similar percentages, and as well as that NI group increased biofilm production, and LI group decreased during the time, there was no difference between groups

($p = 0.262$). HI group of isolates constantly were low biofilm producers, which was significantly different from two other groups ($p = 0.026$, $p = 0.001$), as it is shown in **Graph 1**.



Graph 1. Percentage of biofilm producers during tested periods.

2.2.3.4. Analysis of correlation between adherence, hydrophobicity and biofilm production

Adherence and hydrophobicity are very important in process of the biofilm formation. We used various measurement methods to establish possible connection between these three traits of bacteria.

When non-invasive group of isolates was analyzed, positive correlations were noticed between adherence and biofilm formation after 48 hours of incubation ($r = 0.205$, $p = 0.040$), and between biofilm production after 12 and 24 hours ($r = 0.166$, $p = 0.03$), and after 12 and 48 hours of incubation ($r = 0.255$, $p = 0.001$). These results indicated that isolates which adhered efficiently and establish biofilm after 12 hours will also be good biofilm producers after 24 and 48 hours of incubation. In this group of strains, negative correlation was noticed between hydrophobicity measured with xylene and biofilm production after 12-hours ($r = -0.236$, $p = 0.018$) and 24 hours of incubation ($r = -0.201$, $p = 0.045$), i.e. more hydrophobic strains were worse biofilm producers. In NI group of strains, early biofilm producers were stabile producers during entire examined period.

When low invasive group of strains was explored, no correlation was noticed between adherence, hydrophobicity, and biofilm production ($p > 0.05$). Positive correlation was noticed in biofilm production between 24 and 48 hours incubation ($r = 0.166$, $p = 0.03$). In LI group of strains, late biofilm producers were stabile producers.

When highly invasive group of isolates was studied, no correlation was noticed between adherence, hydrophobicity, and biofilm production ($p > 0.05$) or in biofilm production during different incubation periods ($p > 0.05$). Isolates of HI group were non-stable biofilm producers, i.e. they formed and rapidly disbanded their biofilm and matrix.

2.2.3. Discussion

In this study it is demonstrated correlation between adherence, hydrophobicity and biofilm production for non-invasive isolates, while for low and highly invasive isolates no correlation was noticed.

Group A streptococcal adherence is still unrevealed process depending on unspecific hydrophobic bonds and on specific protein-protein or protein-carbohydrate interactions. Hydrophobic interactions are weak non-covalent interactions between water and hydrophobe (non-polar low-water soluble molecules). Hydrophobic interactions are stronger than other weak intermolecular forces (van der Waals or Hydrogen bonds) and depend on several factors: temperature, number of carbon atoms on hydrophobe and shape of hydrophobe.

According to Rosenberg *et al.* [45] MATH test should be performed with phosphate buffer molarity higher than 150 mM, because only under these conditions hydrophobic bonds are stronger than electrostatic. We used PUM buffer containing phosphate, potassium, urea and magnesium with recommended 150 mM molarity and pH 7.1. Even in this recommended conditions, our isolates have not adhered to hexadecane, even after capsule removal, as suggested by Offek *et al*. [47]. According to experiment performed by Nagao and Benchetritt [48], we used xylene instead of hexadecane, afterwards our isolates adhered to this new hydrocarbon. Hexadecane is low-reactive saturated alkane hydrocarbon in contrast to xylene, which is more reactive non-saturated aromatic hydrocarbon. This structural modifications and differences in molecular polarization could be probable explanations for low ability of GAS strains to adhere to hexadecane. In this study it was noticed differences between groups in hydrophobicity measured with xylene, and it was also observed that groups which were more hydrophobic also better adhered to laminin, as expected.

Since *S. pyogenes* is not associated with indwelling device-infections, it was assumed that isolates tested will not at all or will have weak ability for adherence to uncoated microtiter plate. Because of *S. pyogenes* strains weak ability to adhere we also supposed that biofilm production will be at low level. Our assumption was approved in this study, especially for highly invasive isolates, which were low and unstable biofilm producers during tested periods of incubation. Similar results for invasive isolates were observed for *S. pneumoniae* [33, 34]. According to these results we could assume that for invasive isolates biofilm production is not crucial virulence factor.

Non-invasive isolates from streptococcal carriers have shown direct, positive relationship between adherence to uncoated microtiter plate and late stage biofilm production. These isolates were also the most stable biofilm producers during all three incubation intervals, confirming the latest theory that biofilm production could be possible explanation for phar-

yngeal carriage [3]. Marks *et al*. [36] supposed that biofilm producing bacteria down-regulate genes associated with dissemination and invasive disease to adapt to asymptomatic oropharyngeal colonization in mice and the tonsil and adenoid tissue are less toxic to epithelia cells and inducing less inflammation. Somewhat surprising result in this study is the negative correlation of the hydrophobicity with biofilm production in non-invasive isolates tested. This could be explained by the fact that GAS posses abundance of surface adhesins, most of them are inadequate identified and tested, but all of them could affect the hydrophobicity and subsequent formation of biofilm. The most hydrophobic streptococcal surface adhesins are M protein and LTA [49]. Therefore one possible explanation of our findings could be the quantification of LTA in cell envelops of tested isolates.

In our study, we did not find any relationship between different methods of adherence and hydrophobicity measurements for low and highly invasive isolates. Also, we showed that highly invasive isolates have been unstable biofilm producers, contributing to previous findings of other researchers that biofilm production is not crucial virulence factor for invasive strains.

According to literature, this was first work about determination of the relationship between adherence, hydrophobicity and biofilm production for *S. pyogenes*. Surprisingly when were compared adherence and biofilm production, no direct correlation was found for isolates of *S. epidermidis* [50] and *Acinetobacter baumannii* [51], indicating that adherence and biofilm production are not always and explicitly linked.

In conclusion, it is obvious that adherence and biofilm production are not phenotypic traits of all species, but rather individual characteristic of every strain. It is important to emphasize that our experiments have been conducted *in vitro*, so in order to define the role of these three tested virulence factors *in vivo*, complex interactions between various streptococcal adhesins with ECM proteins and host cells should be considered. Due to the numerous virulence factors GAS has excellent adherence capacity, which enables the occurrence of infections, usually not as serious as expected.

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Chapter 5

Grazing Effects of Ciliates on Microcolony Formation in Bacterial Biofilms

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Additional information is available at the end of the chapter

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Abstract

The attachment to surfaces and the subsequent formation of biofilms are a life strategy of bacteria offering several advantages for microorganisms, for example, a protection against toxins and antibiotics and profits due to synergistic effects in biofilm environment. Moreover, biofilm formation is thought to serve as grazing protection against predators. From pelagic systems it is known that feeding of bacterivorous protists may strongly influence the morphology, taxonomic composition and physiological status of bacterial communities and thus may be an important driving force for a change in bacterial growth and shift in morphology towards filaments and flocs. Bacteria in biofilms had to evolve several other defence strategies: production of extrapolymeric substances (EPS) or toxins, formation of specific growth forms with strong attachment, specific chemical surface properties and motility. In addition, bacteria can communicate via quorum sensing and react on grazing pressure. The results of the case study presented here showed that even microcolonies in bacterial biofilms are affected by the activity of grazers, though it may depend on the nutrient supply. Feedback effects due to remineralization of nutrients because of intensive grazing may stimulate biofilm growth and thereby enhancing grazing defence. Predator effects might be much more complex than they are currently believed to be.

Keywords: Bacterial biofilms, protozoan grazing, predator-prey interactions, defence mechanisms, colony formation

1. Defence mechanisms of biofilm bacteria—implications from plankton

Bacteria are an important food source for protozoans. The impact of protozoan grazing on bacterial communities can significantly affect bacterial biomass and may shape morphology

and taxonomic composition of bacterial communities. While this is well known for pelagic microbial communities [e.g. 1–4], bacterial communities in biofilms have mainly been viewed from a microbial perspective rather than the food web perspective [5]. However, biofilm studies have shown that bacterivorous organisms, such as protozoans, can effectively reduce the biovolume and morphology of bacterial biofilms, too [e.g. 6, 7]. Especially, amoebae may significantly influence biofilms [e.g. 8].

For pelagic habitats, a variety of defence mechanisms has been described: a widespread observation is a shift to larger cells in bacterial communities that are subject to strong protozoan grazing. In addition, several other defence strategies had been described as for example extreme reduction in cell size, certain motility patterns, specific surface properties of the bacteria, toxin production and the production of exopolymeric substances that surround bacteria (for a summary see [3]).

The change of size as response to protozoan grazing has been observed in several planktonic field studies (e.g. [9–11] as well as in laboratory experiments [12–16]). Pernthaler et al. [17] found a shift in size classes of bacteria as result of intensive protozoan grazing in oligomesotrophic lake plankton during spring and argued that small cells (<0.4 μm) and large cells (>2.4 μm) being the most resistant groups with reference to their size. Matz and Jürgens [18] found that bacteria > 0.5 and <0.1 μm^3 (the latter are called 'ultramicrobacteria') showed the highest survival rates. The shift to larger cell sizes and filaments and flocs has been reported from several laboratory studies (e.g. [15, 16, 18, 19]). These latter studies also pointed to the decrease in cell size as a potential strategy for bacteria to escape protozoan grazing. However, Boenigk et al. [20] could demonstrate that bacteria may even feed on this small prey size which implies that small-sized bacteria are not generally protected against grazing.

Furthermore, exopolymeric substances secreted by the bacteria may hinder bacterial predators from grazing. This has been found for example in a study on batch and continuous cultures of two pelagic bacterial species isolated from the field [21]. In this study, the extrapolymeric substances were shown to form an essential portion of flocs and microcolonies in suspensions at strong flagellate grazing. Grazing experiments with the flagellate *Ochronomas* and the bacterium *Pseudomonas* as prey by Matz et al. [22] revealed that thenon-mucoid-producing morph of *Pseudomonas* was severely affected and reduced in terms of abundance, whereas the primary mucoid-producing type survived due to the formation of inert suspended microcolonies stabilized by an extracellular matrix.

Moreover, it could be shown that bacteria might kill their prey by producing substances that are toxic for their potential predators. Matz et al. [23] found in a study where they analysed grazing of different common heterotrophic flagellates on violacein-producing bacterial strains, a rapid cell death of the flagellates after ingestion of these bacteria.

The production of toxins was shown to be induced by quorum sensing, which emphasizes that this kind of communication between bacterial cells plays an important role in the grazing defence.

Motility of bacteria has been identified as another defence mechanism [24]. Small bacteria, which increased in size under strong grazing pressure, were additionally much more motile.

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Increased swimming speed of bacteria enhanced the probability of capturing of bacteria by flagellates, but the ingestion rates dropped down with increasing swimming speed [25]. Increased motility clearly increased the survival rate of bacteria under protozoan predation.

The surface properties of bacteria have also been shown to influence the rate of their ingestion by protozoa. In two studies, it has been shown that gram-positive bacteria were grazed to a lower extant than gram-negative bacteria by flagellates and ciliates [26, 27].

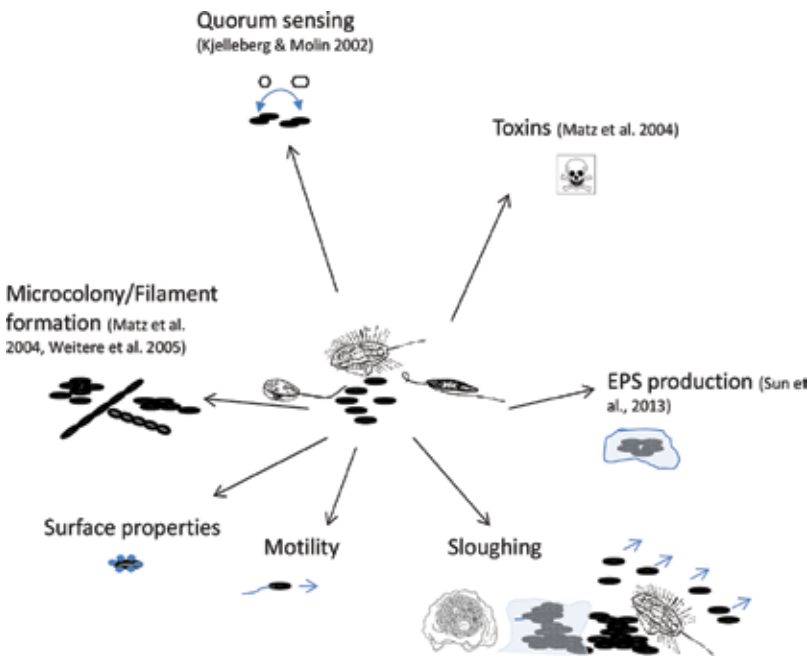

Figure 1. Defence mechanism of bacteria against grazing in biofilm communities.

Defence strategies of bacteria in biofilms are much less understood than those for the pelagial. We summarized the potential defence phenomena that could be derived from studies of planktonic communities (**Figure 1**). The importance of protozoans on biofilms has been reviewed by Arndt et al. [28] and Ackermann et al. [29]. In biofilms, a very common phenomenon is the increase in size due to the formation of microcolonies and filaments. Matz et al. [30] reported that a wild-type strain of *Pseudomonas aeruginosa* formed microcolonies if faced with protozoan grazing by the benthic flagellate *Rhynchomonas*. They showed that type-IV pili of bacteria creating the so-called 'twitching motiliy' (a certain way of movement over the substrate) are important for microcolony formation, as bacteria lacking these pili could form only a considerably lower number in microcolonies than the wild type. Weitere et al. [31] showed that grazing protection due to microcolony formation in bacterial biofilms is dependent on the protozoans' feeding mode. This was underlined by a consecutive study by Erken et al. [32] who analysed the influence of grazing of three gliding flagellates (*Neobodo*, *Rhynchomonas* and *Planomonas*) differing in feeding modes, that is, regarding the contactrates, handling

times and relative predation success of each species. A longer handling time, as found for *Planomonas*, was shown to result in a significantly higher success in ingestion rates. However, the lower ingestion rates of the other two species were compensated by higher contact rates. Microscopic observations revealed that heterotrophic flagellates contacted microcolonies, but in no case bacterial cells of these bacterial aggregations were ingested. Another study on stream biofilms indicated that various protozoans may differently affect microcolony formation [7]. The ciliate *Dexiostoma* did not change biofilm volume and porosity but stimulated the formation of larger microcolonies. In contrast to this, the heterotrophic flagellate *Spumella* and the ciliate *Chilodonella* did not stimulate microcolony formation; however, the biofilm volume was decreased 2.5–6.3-fold compared to ungrazed biofilms. Contrary to this, grazing of the raptorial feeding amoebae *Vannella* reduced microcolony size clearly. On the other hand, the porosity and the ratio of biofilm surface area to biofilm volume were 1.5–3.7 and 1.2–1.8 times higher under grazing pressure. This points to possible stimulating effects as grazing might improve the exchange of nutrients and gases in deeper biofilm layers and enhance microbial growth.

These examples clearly show that the formation of microcolonies may serve as defence strategy for protozoans. However, in contrast to the pelagial, for biofilms, the reduction in individual cell size seems not to play a role in biofilms.

The secretion of exopolymeric substances (EPS) is a typical characteristic of biofilms and is believed to be a clue for grazing defence, as it has been shown for pelagic bacteria. Weitere et al. [31] showed that alginate-mediated microcolony formation served as effective defence mechanism against grazing on *Pseudomonas aeruginosa* biofilms by flagellates of the following two different feeding types: the suspension feeding *Bodo saltans* and the surface feeding *Rhynchomonas nasut*a. In a parallel study with an alginate-overproducing mutant strain of *P. aeruginosa,* the bacteria built significantly larger microcolonies under grazing pressure of a surface-feeding flagellate (*Rhynchomonas nasuta*) compared to the wild-type strain [30]. Hence, the production of EPS might provide a sufficient grazing defence. Moreover, this production was shown to be quorum-sensing regulated, which underlines the importance of communication between bacteria for their defence against grazing. This is supported by Sun et al. [33], who emphasized the importance of EPS-production and quorum sensing. Biofilms with mutants of the pathogenic bacteria *Vibrio cholera,* which expressed less polysaccharides, were also less resistant against grazing. The same was true for mutants with a deficiency in quorum-sensing ability.

An additional defence factor is the production of inhibitors. Weitere [31] found flagellate growth to be affected in *Pseudomonas aeruginosa* biofilms in a late phase of biofilm development.

To summarize the knowledge of defence strategies of bacteria in biofilms, the most well-known phenomenon is the increase in size or the shift to a more grazing-resistant morphology by the formation of microcolonies and filaments. However, this mechanism depends on quorum-sensing-mediated communication among bacteria. The production of exopolymeric substances or toxic substances additionally may strongly affect protozoan predators or kill them, respectively.

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2. Bacteria defence from grazing in the course of biofilm aging

Within the process of maturing, bacterial biofilms have shown to undergo certain morphological changes (for an overview see e.g. [34]). From a scattered distribution of bacteria, this changes to clustered microcolonies and increases in height, followed by the establishment of mushroom-like structures. With an increase in height, an increase in the detachment of single bacteria or bacterial flocs into the pelagial occurs due to increasing shear stress in running waters affecting the biofilm thickness [5, 34]. This effect is called 'sloughing' and may also decrease the probability of being captured by protists (**Figure 2**). Ammendola et al. [35] found that *Serratia liquefaciens* exposed to certain surfaces formed elongated, highly motile swarm cells which were grazing-resistant provided their length exceeded 15 μm.

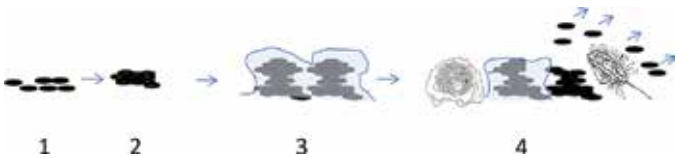

Figure 2. Different phases of biofilm development including bacterial settlement (1), aggregation (2), EPS formation (3) and sloughing (4).

The grazing pressure by protozoans changes with the ongoing process of biofilm maturation. Weitere et al. [31] showed that the early formation of microcolonies in *Pseudomonas aeruginosa* biofilms resulted in a grazing protection against early biofilm colonizers (e.g. the kinetoplastid flagellate *Bodo saltans*). In contrast to this, grazing by late biofilm colonizers such as the browsing ciliate *Tetrahymena pyriformis* or the amoeba *Acanthamoeba polyphaga* caused high losses of bacterial biomass. A different result was obtained by Chavez-Dozal et al. [36] for *Vibrio fischerii* biofilms. In late biofilms, the expression of antiprotozoan substances affected the late biofilm colonizers and grazers (*Tetrahymena pyriformis)*, whereas the flagellates *Rhynchomonas nasuta* and *Neobodo designis* were able to graze and show significant growth in early biofilms.

These studies suggest that the vulnerability of biofilms to grazing by protists may significantly change in the course of biofilm aging. The production of toxins and extrapolymeric substances may play an important role. Biofilm communities are complex systems and we are just at the beginning to understand the interactions occurring on biofilms.

The occurrence of macroinvertebrates on biofilms and their influence on the different trophic levels have to be considered. Ackermann et al. [29] showed that increases in macrofauna populations increased the surface and biovolume of biofilms in a river. Multifactorial field studies by Haglund and Hillebrand [37] found that the presence of grazers tended to increase bacterial biomass at ambient nutrient conditions but tended to decrease bacterial biomass under enrichment nutrient conditions. Remineralization of nutrients due to the feeding process of macroinvertebrates may play a significant role. And there may also be another indirect effect of metazoans by reducing bacterivorous protozoans [29].

3. Defence mechanisms of biofilm bacteria may change with substrate supply

From pelagic studies, it is known that the response of bacteria to grazing is dependent on the availability of nutrients. Matz and Jürgens [24] could demonstrate in their study on grazing of two flagellates (*Ochromonas* and *Spumella*) on a natural bacterial community that the nutrient quality decides how the bacterial community reacts. Small and motile bacteria dominated under carbon limitation, whereas large and elongated bacteria occurred if phosphorous was limited. On the other hand, Simek et al. [38] demonstrated that the portion of grazing resistant forms (flocs and filaments) increased when bacteria were exposed to protozoan grazing at limiting nutrient concentrations. However, up to now, this has not been analysed in detail for biofilm communities, but a comparable influence is likely.

As it has been pointed out in the first paragraph, bacterial biofilms may show microcolony formation as a defence mechanism against grazing by protozoans. Hence, we conducted an experiment with a bacterium, a variant of the genus *Acinetobacter*, which generally forms microcolonies during biofilm growth. We investigated whether this bacterium was affected by grazing of the ciliate *Tetrahymena pyriformis* under different nutrient supply for bacteria. We hypothesized that the microcolony-forming *Acinetobacter* sp. strain C6 would be resistant to grazing by *T. pyriformis* as long as microcolony formation is not affected due to limiting nutrients, and we assumed that less optimal substrate supply will weaken this defence mechanism. The experiments were run in flow chambers in a mineral medium [39] which was supplemented either with sodium benzoate or with citrate as a carbon source. Citrate is known to be a less optimal carbon source for the microcolony formation *Acinetobacter*[40, 41]. For the analysis of biofilms under the laser-scanning microscope, *Acinetobacter* was either tagged with green fluorescent protein or stained with propidium iodide.

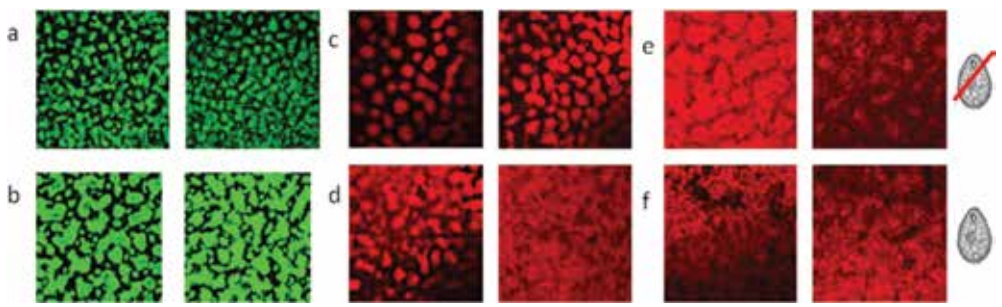

Figure 3. Structural changes of biofilms of *Acinetobacter* with and without grazing pressure by *Tetrahymena pyriformis* (confocal laser-scanning-microscope pictures in *x-y* direction; size: 230 µm × 230 µm; a and b: GFP-tagged bacteria, c–e: propidiumiodide stained bacteria). Biofilms are shown for day 4 and day 8. (a) Sodium benzoate as medium, high medium supply rate, no *Tetrahymena*. (b) Sodium benzoate as medium, high medium supply rate, *Tetrahymena* present. (c) Sodium benzoate as medium, low medium supply rate, no *Tetrahymena*. (d) Sodium benzoate as medium, low medium supply rate, *Tetrahymena* present. (e) Citrate as medium, high medium supply rate, no *Tetrahymena*. (f) Citrate as medium, high medium supply rate, *Tetrahymena* present.

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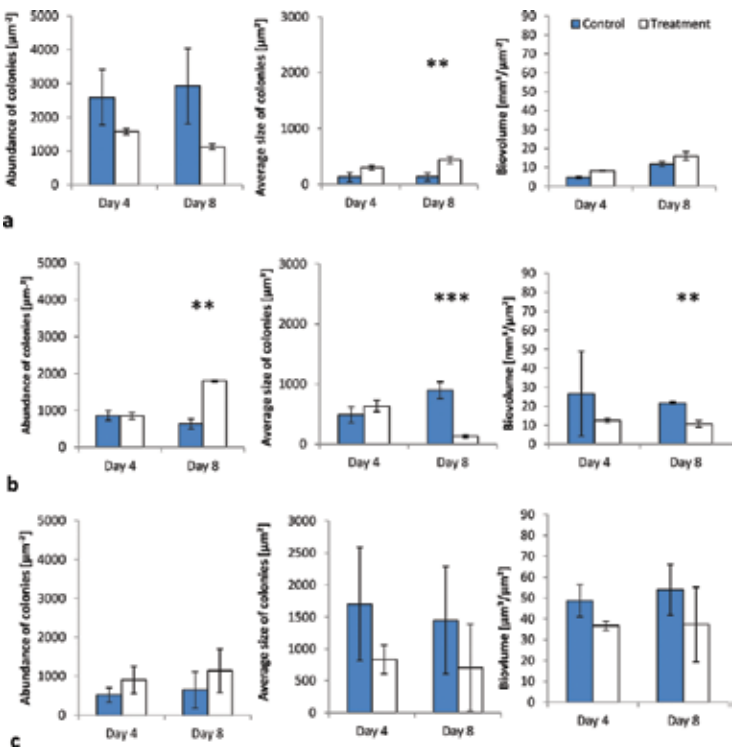

Figure 4. Quantitative analysis of biofilms (abundance of colonies, average size of colonies and biovolume) of *Acinetobacter* with and without grazing pressure by *Tetrahymena pyriformis* (average +/- standard deviation) after 4 and 8 days. Quantification was made with 3D for LSM (Zeiss, Germany) and Image J (NIH, Maryland, USA). (a) Sodium benzoate as medium, high-medium supply rate, (b) sodium benzoate as medium, low-medium supply rate, (c) citrate as medium, high-medium supply rate.

Grazing of *Tetrahymena* led to different microcolony formation of *Acinetobacter* biofilms depending on different growth conditions for bacteria and under grazing pressure of *Tetrahymena*. Under high supply rate of medium and by the use of an optimal carbon source (sodium benzoate as carbon source), round microcolonies dominated the biofilm, which were regularly distributed over the substrate (**Figure 3a**). In contrast, microcolonies showed a more irregular, elongated shape in the presence of the grazer (**Figure 3b**) and the size of the colonies was significantly larger (**Figure 4a**). Interestingly, the biovolume of the biofilm increased under grazing influence of *Tetrahymena* during the whole experiment which points to a stimulating effect of protzoan grazing to bacterial growth (**Figure 4a**). In contrast, biofilms grown with low medium supply (and sodium benzoate as carbon source) were affected by the presence of the protozoan grazer in terms of size and biovolume. The nongrazed biofilms showed similar, clearly visible round microcolonies as found with high medium supply (**Figure 3c**), being evenly distributed over the substrate during the whole experiment. In the presence of *Tetrahymena*, the microcolonies showed always an irregular shape (**Figure 3d**). Moreover, a significant decrease in microcolony size and biovolume in the course of the experiment was recorded (**Figure 4b**). The effect of weakening the potential to form microcolonies was even

more pronounced in the biofilms grown under high medium supply with citrate in comparison to the biofilms with low medium supply of sodium benzoate (**Figure 3e**). In the control treatments without ciliates, round microcolonies dominated the biofilm though with a more irregular, larger shape compared to the sodium benzoate treatments. In the grazed biofilms, microcolonies were severely affected and did not show any round shape, but irregular flocs and after 8 days, the microcolony formation nearly disappeared completely. The remaining microcolonies were smaller in size and had a lower biovolume (**Figure 4c**).

We hypothesized that the microcolony-forming *Acinetobacter* sp. strain C6 would be resistant to grazing by *T. pyriformis* as long as microcolony formation is not affected by substrate supply. However, we found that the presence of protozoans had a considerable impact on the structure of microcolonies of *Acinetobacter* sp. strain C6 in every treatment tested and thereby affect this defence mechanisms of bacteria regardless of the available nutrient source. Without *T. pyriformis* being present, we observed formation of round microcolonies in the *Acinetobacter* sc. strain C6 biofilm with sodium benzoate as carbon source. After introduction of *T. pyriformis* to this array at the beginning of the experiment, the shape of microcolonies was changed, as microcolony size increased and single microcolonies connected to each other. This enlargement of microcolonies could also be observed when *Tetrahymena* was added later (e.g. at day two of biofilm formation, data not shown). This morphological change of the shape of microcolonies probably serves as a further enhanced protection against protozoan grazing. This structure was also found in the study of Dopheide et al. [42], who examined the effect of *Tetrahymena* grazing on biofilms built by the bacterium *Serratia plymuthica.* This points to the fact that the browsing feeding mechanism of this protozoan may stimulate this kind of microcolony formation in biofilms, which is also underlined by the fact that the biovolume of the biofilm increased in the present study with sodium benzoate as nutrient source. Here, nutrient remineralization may be facilitated by the grazing activities of the protozoa. We compared the loss due to grazing with the bacterial production to check whether *Acinetobacter* may be able to grow fast enough to compensate feeding losses to predators. For this, we considered data of grazing of *Tetrahymena* (Tanasescu, pers. comm.) and used published carbon conversion factors for ciliates and bacteria [43, 44]. The calculations revealed that the mean growth rate of *Acinetobacter* of 0.4 pg C μm^{-3} day^{-1} can match the average demand of 0.29 pg C μm^{-3} day^{-1} for *T. pyriformis.* This supports the idea that grazing losses at least can be compensated by the growth of *Acinetobacter* sp. Due to sloppy feeding and excretion of nutrients, grazers release bacteria from nutrient limitation [45]. Movements of bacterivores within the biofilms (e.g. ciliates as *T. pyriformis*) may create free patches and ventilate the bacterial biofilm. Thus, bacteria at the base of the biofilm that might otherwise starve or become inactive might receive increased nutrient and oxygen supply. Additionally, substances produced by either grazers or bacteria (chemical cues or quorum-sensing signals) might have additional growth-stimulating effects [25, 46]. These feedback effects between grazers and bacteria might have had a significant influence on the observed structural and quantitative changes and thereby might result in an increased bacterial growth. The reduction in medium supply to the bacteria enhanced the competition for substrate between the biofilm bacteria. As a consequence, bacteria could not maintain the regular structure and distribution of microcolonies, and furthermore, the biovolume of the biofilm was reduced significantly under

grazing pressure. This supports our hypothesis that less optimal growth conditions in bacteria biofilms may affect the ability to defend against grazing by microcolony formation. If citrate as alternative carbon source was used, a high structural heterogeneity occurred in the presence of *Tetrahymena* for the whole course of the experiments. Such a high heterogeneity could also be seen with biofilms of *Pseudomonas aeruginosa*, if grown with citrate as carbon source [40]. The results of this study thereby showed that even microcolonies in bacterial biofilms are affected by the activity of grazers and that the interactions between biofilm bacteria and its predators might be much more complex than currently believed.

Biofilms might serve as grazing defence, though it may differ between different species and moreover depend on the nutrient supply. Additionally, feedback effects due to remineralization of nutrients as result of intensive grazing may stimulate biofilm growth and thereby enhance grazing defence.

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Chapter 6

Atomic Force Microscopy of Biofilms—Imaging, Interactions, and Mechanics

Sean A. James, Lydia C. Powell and Chris J. Wright

Additional information is available at the end of the chapter

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Abstract

Atomic force microscopy (AFM) has proven itself to be a powerful and diverse tool for the study of microbial systems on both single and multicellular scales including complex biofilms. This chapter will review how AFM and its derivatives have been used to unravel the nanoscale forces governing the structure and behavior of biofilms, thus providing unique insight into the control of microbial populations within clinical and industrial environments. Diversification of AFM-based technologies has allowed for the creation of a truly multiparametric platform, enabling the interrogation of all aspects of microbial systems. Advances in traditional AFM operation have allowed, for the first time, insight into the topographical landscape of both microbial cells and spores, which, when combined with high-speed AFM's ability to resolve the structure of surface macromolecules, have provided, with unparalleled detail, visualization of this complex environmental interface. The application of AFM force spectroscopies has enabled the analysis of many microbial nanomechanical properties including macromolecule folding pathways, receptor ligand binding events, microbial adhesion forces, biofilm mechanical properties, and antimicrobial/antibiofilm affectivities. Thus, AFM has offered an outstanding glimpse into the biofilm, how its inhabitants create and use this complex adaptive interface, and perhaps most importantly what can be done to control this.

Keywords: atomic force microscopy, imaging, force measurement, nanomechanical properties, adhesion

1. Introduction

Biofilms remain a primary concern in industrial and clinical fields. The tendency of planktonic cells to form these structures in moist environments and the resulting increase in resistance

to antimicrobials, in combination with an increasing frequency of innate antimicrobial resistance, demonstrates the continued need for novel biofilm control strategies and innovative methods to unravel the fundamental properties of biofilms. Atomic force microscopy (AFM) has proven to be a useful addition to the microscopy family providing imaging and force measurement capabilities that can interrogate the nanoscale properties of surfaces. Indeed, AFM has been used with great success to provide novel insight into the structure of biofilms and the interplay of interaction forces and mechanical properties that govern the behavior of biofilms and their response to chemical and physical attack as part of control strategies. AFM can be used to study whole biofilms or the influence of their component parts, from bacterial surface proteins to extracellular polysaccharides (EPSs) and individual cells. This chapter will first introduce the reader to the basic operation of the instrument relevant to the study of biofilms. The different capabilities of the instrument and their application to biofilm will be then reviewed with examples from the authors' laboratory.

2. AFM basic principles

AFM was first developed as part of the family of scanning probe microscopies in 1986 [1]. It was very quickly applied to the imaging of biological materials, including DNA, bacteria, viruses, and mammalian cells [2]. The components of atomic force microscope is shown in **Figure 1**. A very small, sharp tip held at the free end of a cantilever systematically scans a surface of interest to generate a topographical image. The tip is held in intimate contact with the surface, and its apex has a radius of curvature in the range of nanometers, which sets the image resolution. As the tip is systematically scanned across the surface, it encounters surface forces that cause the cantilever to be deflected. The deflection of the cantilever is monitored

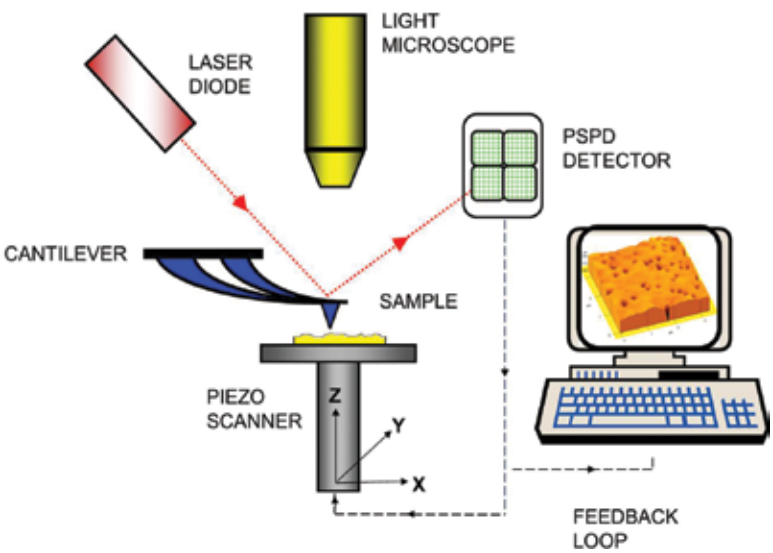

Figure 1. A schematic representation of the AFM instrument.

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by the displacement of a reflected laser beam and used to create a topographical image. In contact mode, the forces of the bent cantilever keep the tip in intimate contact with the surface.

When imaging a soft sample such as a bacterial cell surface or biofilm, the tapping mode or intermittent contact mode is used. The intermittent contact of this imaging mode reduces the degree of friction or drag on a sample compared with imaging in contact mode. To achieve the intermittent contact, a vibrating cantilever technique is used, and the changes in the vibrational parameters are monitored as the cantilever scans the surface. In response to changes in topography, the piezo-scanner moves up and down to maintain a constant vibration of the cantilever, and the feedback signal is used to produce the image data set. A further advantage of this imaging mode is that measurement of the phase angle between the free oscillation at the end of the cantilever and the imposed driving vibration provides a map of phase angle across a surface; this data, referred to as phase imaging, is captured simultaneously as the standard topographical data. This phase angle is often used to qualitatively distinguish between materials on the surfaces of heterogeneous samples as the phase angle change is a function of the mechanical properties of the surface and the area of contact between the AFM tip and the surface.

The advantages of tapping mode have meant that this is the most frequently used method when imaging soft biological samples. The authors have found tapping mode in combination with phase imaging extremely useful in identifying structures on the cells and within biofilm.

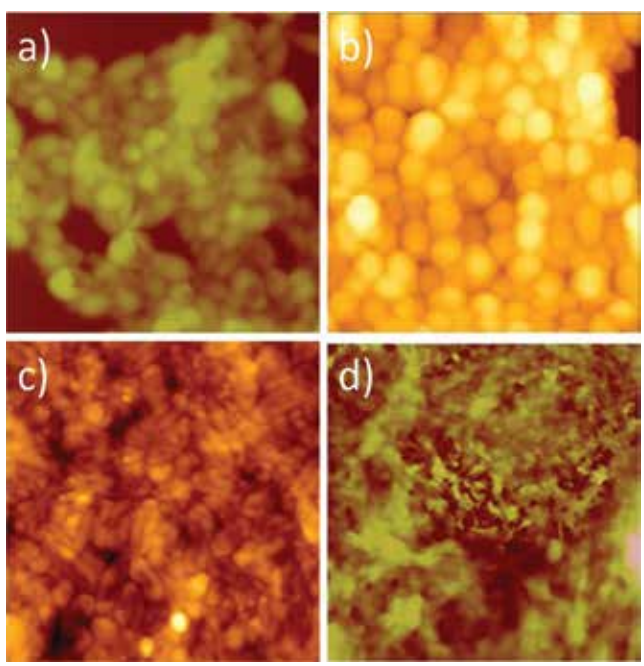

Figure 2. AFM tapping mode images of microbial biofilms: (**a**) *Candida tropicalis* (50 μm^2), (**b**) *Staphylococcus aureus* (10 μm^2), (**c**) *Pseudomonas aeruginosa* (10 μm^2), (**d**) mixed species biofilm at an industrially fouled reverse osmosis membrane (10 μm^2).

Figure 2 presents AFM tapping mode images of a range of microbial biofilms. When imaging biofilms, the mechanical robustness of a biofilm should be considered; it is simpler to image model biofilms with minimum components, which have been grown on adhesion-promoting substrates, compared to biofilms that have been sampled from natural or industrial settings that consist of multiple components (**Figure 2d**). As AFM imaging is a technique that relies on surface contact, the imaging of a hydrated diffuse biofilm is very difficult without fixation methods.

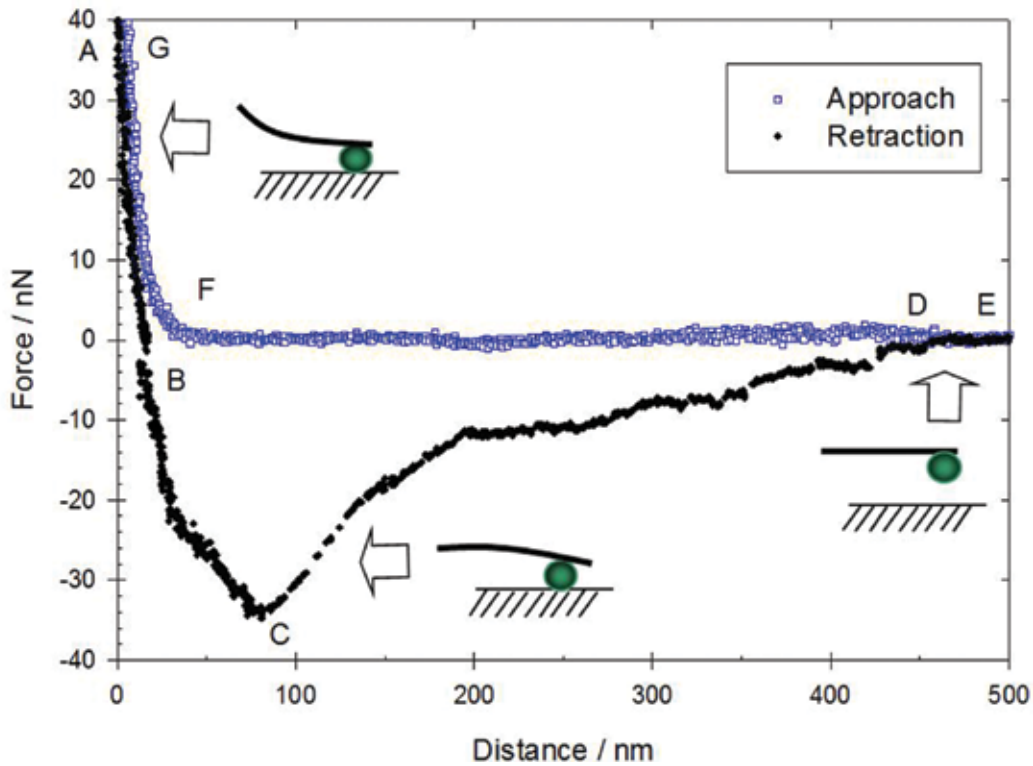

Figure 3. A typical force measurement between an AFM cell probe (*Saccharomyces cerevisiae*) and a surface in a process-relevant environment (10^{-2} M NaCl).

The AFM can measure the forces of interactions between surfaces, which have obvious implications in the study of biofilms. AFM has been added to the group of instruments that can be used to study microbial interactions involved in biofilm formation. Such instruments include flow chambers, micropipette aspiration, and centrifugation devices. However, AFM has the advantage of allowing the imaging and identification of points of interest on a surface prior to the measurement of the forces of interaction. AFM also allows the direct measurement of forces as opposed to techniques that estimate force from the application of shear to a cell

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population. In addition, surface forces are measured over very small contact areas, minimizing contamination problems. To generate a force–distance curve, the deflection of the cantilever is recorded as a function of tip-to-sample separation, as the piezo-scanner of the AFM brings the sample and tip together. The deflection of the cantilever is converted to a value of force using Hooke's law. Force–distance curves are characteristic of the system under study. For biofilms, they have features that reflect the chemical and physical properties of the surfaces that are interacting, including the substrate, the cells, EPS, and the AFM probe. **Figure 3** shows a typical force measurement between an AFM cell probe (*Saccharomyces cerevisiae*) and a surface in a process-relevant environment (10^{-2} M NaCl) [3]. The force is plotted as a function of separation distance and shows some key features for the characterization of the surfaces involved. At position D (referring to **Figure 3**), the cantilever and probe are moving independently of the surface, as the probe is brought into contact with the surface, until at position F it encounters physiochemical forces, which in this case are repulsive and likely to be dominated by electrostatic forces. The extension of the scanner continues to push the cell into contact (F–G) until a predefined loading force is reached, whereupon the movement is reversed and the probe is retracted away from the surface by the retraction of the piezo-scanner. At position C, the bending of the cantilever is inflected and the forces in the bent cantilever begin to rupture the adhesion between the cell and the surface. If this was an inorganic hard particle, a sudden break in contact would be observed. However, with the yeast cell with macromolecular tethers (and any deformable surface), a sequential breaking of contact is observed as the forces in the bent cantilever peel the cell from the surface, until at position E the cell probe is moving independently of the surface. The adhesion measurement is determined from the difference in force between positions C and D. Integration between the approach and diffraction curves gives an estimate of the energy of adhesion. The mechanical properties of the system can be determined from the contact region (F–G and A–C) and the adhesion component of the curve (C–D).

Operating the AFM as a nanoindenter allows the measurement of microbial cell and biofilm mechanical properties, which include elastic moduli and turgor pressure [2]. **Figure 4** shows how the indentation depth is measured by comparison between force curves measured at a reference hard surface and at the softer sample surface. The indentation depth can then be plotted as a function of applied force and compared with a theoretical framework to quantify sample mechanical properties. The most commonly used theoretical framework is based on the Hertz model, which describes the elastic deformation of two perfectly homogeneous smooth bodies touching under load. The geometry of the system is assumed to consist of an indenter with a parabolic shape and a sample that is of much greater thickness than the indentation depth. The Hertz model that describes force on the cantilever $F(\delta)$ as a function of indentation depth is:

$$F(\delta)_{\text{parabolic}} = \frac{4E\sqrt{R}}{3(1-\nu^2)}\delta^{3/2}$$

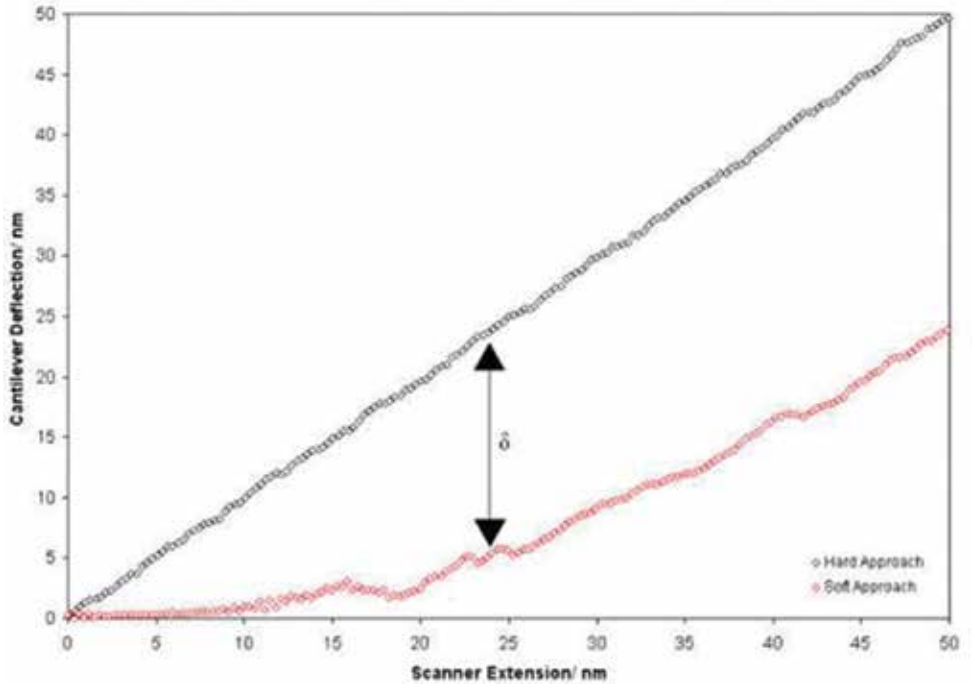

Figure 4. The measurement of indentation depth (δ) by comparison of the slope of the contact region of force curves at hard and soft surfaces.

where the tip is approximated with the radius R, the depth of indentation is denoted by δ, E is the Young's modulus of the sample surface, and ν is the Poisson ratio for the sample material (assumed to be a value of 0.5 for biological samples). Other theoretical frameworks have been used to interrogate AFM nanoindentation curves such as the JKR (Johnson, Kendall, Roberts) model. When choosing which model to use and interpreting the data, a number of considerations should be taken into account. The mechanical properties of microbial cells and biofilms will not be homogeneous across their surface and will be a convolution of whole cell compression as well as material close to the tip. In addition, nanoindentation is an invasive technique which applies a disruptive force to the surface. Repeated indentation at the same location on the cell or biofilm will disrupt the structure and its mechanical robustness rendering subsequent measurements invalid.

3. Imaging

Examination of microbial systems in native, aqueous environments is central to the validity of the data collected. However, AFM imaging in such environments is often difficult due to a number of factors. For instance, microbial cells are often attached to the surface via week Lifshitz-Van der Waals forces, and as a result are easily disrupted by the scanning of an AFM

cantilever, resulting in the destruction of the sample [4, 5]. Additionally, microbial cells are often motile with some recent papers suggesting that motility may even be the largest governing factor in the physiological imaging of microbes [6]. Consequently, immobilization of microbial cells prior to analysis has become imperative to the application of AFM in the imaging of microbial systems.

3.1. Cell immobilization for single-cell analysis

Immobilization of microbial cells has often proven to be the most problematic step in the imaging of microbial samples under aqueous conditions. The immobilization must be secure enough to withstand the lateral forces exerted by the tip during scanning, but benign enough to not force physiochemical, physiological, or nanomechanical changes in the sample. As a result, a number of different techniques have arisen; these protocols can be broadly divided into two categories: mechanical, whereby microbial cells are physically trapped within a porous media, and chemical, whereby chemical treatment of the substrate is used to facilitate binding.

Initial studies into the use of mechanical protocols to immobilize microbes utilized agar or membranes with pore diameters similar to the cell diameter of the organism to be captured [4, 7–9] (**Figure 5**). Later work expanded upon this through the use of more complex or functionalized surfaces such as lithographically patterned silica [5, 10–12]. Though, while mechanical entrapment offers immobilization secure enough to alleviate the destructive scanning of the cantilever, the immobilization is sporadic and unpredictable, reducing the reproducibility of

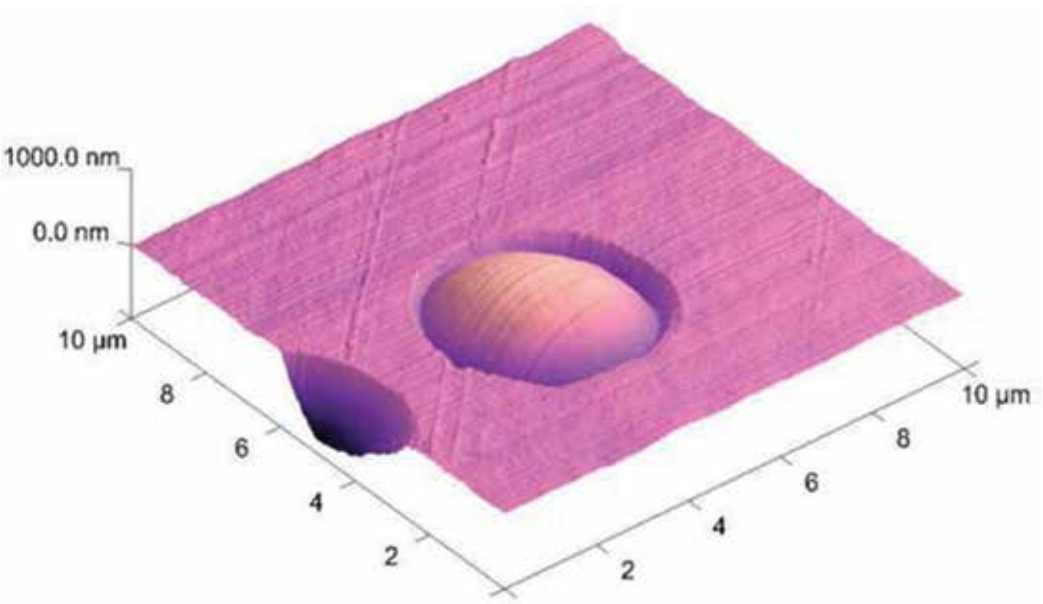

Figure 5. A yeast cell (*Saccharomyces cerevisiae*) trapped in a microfiltration membrane prior to AFM study.

the results. Recent work by Formosa et al. [13] developed a protocol in which selective tuning of polydimethylsiloxane (PDMS) stamps were used to immobilize spherical microorganisms of various sizes. The protocol requires the creation of a glass and chromium blank that holds the microstructure, from which the pattern is transferred to a silicon wafer by deep reactive ion etching. The dimensions of the silicon master can be varied with the group reporting dimensions of 1.5–6 μm wide, a pitch of 0.5 μm, and a depth of 1–4 μm, accommodating a variety of target cell sizes. A PDMS stamp is then cast from the silicon wafer master and cells deposited through the use of convective and capillary forces. Further work by the group has shown this immobilization technique to be an effective way to immobilize spherical cells, in this case *S. cerevisiae* and *Candida albicans,* and, spore of *Aspergillus fumigatus* with no effect on viability [14–17]. Additionally, the technique allows for the rectification of one of AFMs greatest flaws, analysis of multiple cells to achieve statistical significance. Previously, this has not been feasible using other immobilization techniques due to the relatively low rate and sporadic nature of deposition; thus, the development of a platform capable of producing arrays of uniform cells for multiparametric analysis will increase the reliability of AFM analysis. However, this technique is limited due to its inability to immobilize nonspherical organisms.

A number of chemical fixation methods for the immobilization of microbial cells have been used, including, poly-L-lysine, trimethoxysilyl-propyl-diethylenetriamine, mica, and carboxyl group cross-linking [18–22]. While these techniques offer a high level of immobilization, some cross-linking agents have been shown to negatively impact the nanocharacteristics and viability of the immobilized cells [23]. Despite this, some techniques, such as the use of photocatalytically active silicon, also offer a high level of cell orientation and organized immobilization not offered by conventional mechanical techniques, which, depending on application may be favorable over the associated reduction in viability [24]. Other recent advances also indicate that the addition of divalent cations, such as Mg^{2+} and Ca^{2+}, and glucose may provide optimal attachment without the associated reduction in viability. In one such study, Lonergan et al. [25] reported that *Escherichia coli* cells immobilized on poly-L-lysine in 0.01× PBS-S, with a rehabilitation period in minimal media were sufficiently immobilized to perform AFM analysis while maintaining membrane integrity.

3.2. Cell topography

Analysis of the topography of single cells has proven to be a powerful addition in the real-time visualization of cellular surface structures. However, the structural landscape of metabolically active cells exists in a constant state of flux; thus, the ability to image surface morphologies under physiological conditions is vital for characterization. Previous studies have utilized AFM to image a number of key microbial features. In 2010, negative mutants of cell wall polysaccharide (WPS^-) of *Lactococcus lactis* were shown by AFM imaging to exhibit a 25 nm corded like structure perpendicular to the long axis of the cell; further mutagenesis studies confirmed that these structures were not due to hydrolysis, and AFM chemical spectroscopy (imaging with a functionalized tip) using LysM confirmed that the bands consisted of peptidoglycan [26]. In a more recent study, an in-depth analysis of *Streptococcus agalactiae* (Group B *Streptococci*) peptidoglycan confirmed the presence of approximately 25 nm corded

structure running perpendicular to the long axis of the cell [27]. However, during this study the bands were found to periodically interlink to form a net-like structure. Imaging of other Group B *Streptococci* showed that this net-like structure, while exhibiting some variation in pore dimensions, remained constant. The group then imaged a number of cell wall deficient mutants in an attempt to identify structural abnormalities associated with other surface macromolecules; however, no significant alterations in the peptidoglycan net to suggest macromolecular anchoring were observed. Significant alterations in the solute concentration were found to alter the net-like structure with the group observing a near doubling (~25 to ~47 nm) of the peptidoglycan bands, suggesting that the net-like structure may influence adaptation of the cell to changes in turgor pressure. Similarly, the growth phase of the organism was found to have a significant effect on peptidoglycan structure; topographical images of a high proportion of Group B *Streptococci* grown to stationary were shown to exhibit a tendency to express a rough peptidoglycan layer as opposed to the previously described net-like structure. Upon further investigation, this roughness was shown to consist of highly ordered strands aligned in parallel with the divisional plane having a periodicity of approximately 4.5 nm; the group suggests that these may in fact be glycan strands; however, the structure and density of the strands prevented the researchers from coming to a clear conclusion.

As outlined above, in vitro AFM has been used to map the topography on cellular structures at a number of cell life stages, as in the work of Abscali et al. [28] who examined changes in the macromolecular structure of the cell wall of *Streptomyces coelicolor* during its life cycle from vegetative hyphae to spores. Yet, such studies merely offer a snapshot of cellular processes. Thus, several studies have aimed to image the dynamics of cellular processes. Germination of *Bacillus atrophaeus* has been successfully imaged; post exposure to a germination solution, the rodlets comprising the spore coat were shown to disassemble and form 2–3 nm etched pits [29]. The pits were subsequently shown to mature into highly orientated fissures perpendicular to the rodlet orientation, beneath which a highly ordered hexagonal structure was observed. The study continued to image the germinating spore through to the emergence of the germling cell, and the spore fissures were observed to form apertures of approximately 70 nm that dilated with germination. In vitro analysis of the germling confirmed the presence of vegetative cell wall structures prior to emergence, which were similar to those of mature vegetative cells.

3.3. Microbial cellular surface layers

Microbial membranes consist of a number of surface layers, the outermost of which, the S-layer, consists of a monomolecular layer composed of self-assembling single proteins, or glycoprotein monomers exhibiting oblique, square, or hexagonal symmetry. Due to its self-assembling nature and its role in many innate immunities associated with microbes, S-layers have become the focus of many AFM studies. Initial studies into S-layers successfully imaged PS2 monomers of *Corynebacterium glutamicum* and in the process highlighted the presence of a bilayer of hexagonally arranged monomers and a nanogrooved substrate; further work suggests that this substrate may be involved in the creation of the monolayer [30, 31].

In a recent study, the nanomechanical and structural properties of *Propionibacterium freudenreichii* surface layer protein A (SlpA) was characterized [32]. SlpA was found to consist of a hexagonal *p1* monomer with a high level of disorder; upon heating to 45°C, SlpA was found to maintain structural integrity post recrystallization. However, a marked reduction in the elasticity of the SlpA layer from 4.2 ± 0.9 MPa at 25°C to 1.8 ± 0.3 and 0.9 ± 0.1 MPa for 35 and 45°C, respectively, demonstrate that while topographically comparable, the nanomechnical properties of SlpA had altered. Additional work conducted by the group showed the SlpA exhibited the same, albeit less pronounced, behavior in response to alteration in pH. The topographical characteristics of SlpA were maintained to pH 3; however, a corresponding reduction in the elastic properties was observed: 5.7 ± 1.4 MPa and 5.5 ± 1.6 MPa at pH 6.7 and 5, respectively, followed by a reduction to 2.2 ± 0.3 MPa at pH 3. The group attributes this reduction in the elastic properties to be a result of a number of physiochemical interactions such as the reduction in pH below that of the theoretical pI of SlpA and protonation of the disordered regions.

3.4. High-speed AFM

While spatial resolution using AFM has remained high, the lack of high temporal resolution has limited the application of topographical studies of microbial systems. Optimal scan speed varies; however, the minimum is restricted to the order of approximately 30 s for an AFM image. This level of temporal resolution is sufficient for the imaging of relatively low fluctuating structures and processes, such as S-layers and cell division. The high-resolution imaging of surface macromolecules has remained elusive due to the limited speed of standard AFM imaging. However, the recent development of high-speed AFM (HS-AFM) has enabled the resolution of such structures primarily due to HS-AFMs to show exceptional temporal resolution (>100 ms) and significantly reduced scanning forces [33–35].

In one such series of studies, the dynamics of conformational changes of bacteriorhodopsin (bR) was successfully imaged in response to electrochemical radiation stimulation [33, 36, 37]. During initial studies, the group observed conformational changes in the form of a 0.69 ± 0.15 nm displacement of the center mass of the trimer structure when exposed to green light. Furthermore, the group was able to ascertain that these changes in the center mass were actually the result of the displacement of trimer monomers into close proximity with monomers of neighboring trimers via displacement of the E–F loop. Through combination of selective mutagenesis and HS-AFM, Yamashita et al. [33] were able to characterize the monomer association of bR trimers. During the study, five bR mutants were created: W10I, Y131I, W12I, F135I, and W12F, and HS-AFM used to image the structure of each trimer within the membrane. The study showed that W12I and F135I mutants were unable to form membrane-stable trimers, with only a small number of trimers assembling and quickly dissipating. Conversely, W10I, Y131I, and W12F were able to form a stable trimer structure, suggesting the presence of an aromatic residue at positions 12 and 135, which is essential to the formation of a stable trimer.

Further HS-AFM studies have been able to track the motion of membrane-bound macromolecules through three-dimensional space. In one such study, the rotational and translational

membrane dynamics of outer-membrane protein F (OmpF) were imaged to an optical resolution of approximately 750 Å [38].

While initial studies using HS-AFM revolved around its ability to resolve surface macromolecules, some focus has shifted to topographical analysis. In the first such study, the surface of *Magnetospirillum magneticum* was found in contradiction to initial models to consist of a very highly ordered series of nanometer-sized pores consistent with that of porin molecules [34]. Further work set out to ascertain if this was in fact a characteristic of all *Proteobacteria,* wherein Oestreicher et al. [39] imaged the surfaces of the *E. coli* and *Rhodobacter sphaeroides*. This was shown to be the case, and nanometer-sized pores of 8 and 6.6 nm were observed for *E. coli* and *R. sphaeroides*, respectively. Oestreicher et al. [39] concluded that due to the similarities in distribution and size when compared to *M. magneticum* (7 nm), and with the crystal structure size estimation of the outer membrane proteins of *E. coli* (OmpF and OmpC)—7.5 and 7.38 nm, respectively—that they must also be porins.

4. Force spectroscopy

AFM force measurement has been used extensively to study biological systems. In the past, AFM was limited to physics laboratories, and microbiologists focused on the benefits of AFM to imaging of single bacteria; bacterial studies were restricted to model surfaces, and the heterogeneity inherent to natural systems compromised quantification and discouraged the use of AFM force measurement. However, AFM technology has been disseminated to microbial laboratories that have the advantage of prior knowledge to guide AFM research strategies. In addition, the advent of improved data capture rates has permitted statistically viable AFM measurements to quantitatively characterize biological systems including biofilms. Modern AFM studies of biofilm orchestrate AFM imaging of microbial surfaces with force spectroscopy to unravel structure function relationships. The force-curves measured at surfaces have a number of components which can be used to characterize the mechanical and interaction properties of biofilms that are now discussed.

4.1. Microbial surface proteins

Surface macromolecules play an essential role in a number of physiological processes essential to the success of microbes including adhesion and existence within a biofilm; the activity of these molecules has been shown to be dependent on a number of environmental conditions [40–45]. Consequently, research into the nanomechanical and physiological properties of surface macromolecules has expanded over the last decade with the fundamentals of AFM tip-molecule binding forces in vitro having become well documented [46–48].

Several models have been described to interpret the nanomechanical properties of long-chain surface macromolecules. Typically, these models revolve around the use of the Worm-Like Chain (WLC) and Freely Jointed Chain (FJC) models, as these allow for the description of force-curve profiles and the definition of tether and binding partner interaction entropy, thus leading to contour length (L_0) definition [49, 50]. Defining L_0 offers a number of advantages, such as

the filtering of noise and predictions in the unfolding pathways of uncharacterized protein complexes [51, 52]. If the structure is unknown, L_0 allows collaboration of experimentally derived data to a theoretical value defined from the estimation of the sum of individual components fitted to a normal (Gaussian) distribution, therefore acting as a confirmation that the interaction is the one of interest, while offering a level of insight into the unbinding pathway. Studies conducted by Farrance et al. [53] expanded on traditional models, whereby a physical basis for the prediction of L_0 was described. The model, through the use of theoretically idealized tethering surfaces and the probability of two such chains meeting, is able to predict the distributions expected from experimentally derived data with a high level of agreement to existing studies.

4.2. Functional proteins at microbial surfaces

Microbial adhesion to biotic and abiotic surfaces is reliant on a number of macromolecular interaction including binding of small microbial peptides (SMPs), capsules, recognition proteins, fimbriae, and flagella. Single-molecule force spectroscopy (SMFS) has been used to characterize a number of microbial surface-bound receptors including antibiotic receptor ligand sites, fimbriae, flagella, and adhesins [54–56]. In an interesting example of the use of SMFS, the holdfast proteins of *Caulobacter crescentus* were characterized for adhesion to surfaces of varying polarities [57]. Holdfasts were allowed to adhere to each surface for an extended period of time greater than 16 h and imaged via AFM to determine the height and diameter; it was found that the holdfast height varied independently of the surface polarity; from 5 to 100 nm, however, the average height varied between 30.6 ± 2.4 nm and 21.5 ± 0.9 nm for mica and graphite, respectively. Holdfast foot diameter was also found to vary on both surfaces: 90.2 ± 2.7 nm for mica and 119.2 ± 4.1 nm for graphite; however, both showed large distributions in the data—30–280 nm and 45–450 nm, respectively. The group then proceeded to access the binding strength on holdfast-coated cantilevers to mica, graphite, clean glass, and 3-TMSM-treated glass, and the maximum adhesion force was measured—0.05, 0.08, 0.13, and 0.66 nm, respectively. Adhesion was concluded to be primarily a result of residence time and surface polarity.

4.3. Microbial mechanical properties

One of the distinct features of AFM over other SPMs is its ability to quantifiably resolve physiochemical properties of materials at the nanoscale. To date, AFM has been used to resolve the nanomechanical behaviours of a bacteria in a number of ways, from single-cell indentation studies to the characterization of molecular appendages such as pili and flagellum [58]. A number of techniques can be employed dependent on the type of nanomechanical measurement that is required, with most alterations involving functionalization of the cantilever. All nanomechanical studies revolve around the use of the force-curve analysis as detailed earlier in this review.

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5. Adhesion studies

Biofilm adhesion qualities have been measured through AFM in a number of ways. EPS has been confirmed as a major mechanism controlling biofilm adhesion [59–62]. As a result, a number of studies have been undertaken to assess the effect of growth conditions, chemical treatments, and novel antimicrobials on the production of EPS and the reduction in adhesion. Oh et al. [61] used AFM force spectroscopy to study the influence of nutrient concentrations on *E. coli* biofilm maturation. The adhesion of an AFM tip at the surface of the biofilm increased as biofilms matured, indicating a release and accumulation of extracellular polymeric substances over the cell surface after primary colonization. Nunez et al. [63] used AFM imaging and force measurement to study the action of *Bdellovibrio bacteriovorus* on *E. coli* biofilms. AFM characterized the change in *E. coli* cells, as they were attacked by the predatory bacterium with cells changing from rod-shaped to a round shape, with a shrunken texture and the visible coil of *B. bacteriovorus* growing inside. *Bdellovibrio bacteriovorus* was shown to prevent biofilm formation and destroy established biofilms. This work was extended by Volle et al. [64] who used force spectroscopy to observe that the spring constant of predated *E. coli* cells was three times softer than that of normal cells and that there was change in cell wall morphology on predation, as there was much larger adhesion forces between an AFM tip and predated cells. This important work demonstrates that dynamic events in living unfixed cells can be characterized and investigated using AFM. Rodriguez et al. [65] used AFM force measurements to study the formation of *Listeria monocytogenes* biofilms at stainless steel surfaces. They found that the adhesiveness of biofilms was not influenced by contact time, loading force, or relative humidity, but surface chemistry is important; force measurements using SiO_2 and polyethylene colloid probes showed that *L. monocytogenes* cells within a biofilm adhered more strongly to hydrophobic surfaces. The mechanical properties of the surface that biofilms form at are important determinants on the properties of the biofilm.

Oh et al. [66] studied the formation of *Pseudomonas aeruginosa* biofilms at a range of surfaces including steel, rubber, and polypropylene. Biofilms were treated with hot water, and all surfaces with and without biofilms were characterized using AFM. Force spectroscopy revealed that adhesion was greatest at the untreated biofilm surfaces and that the reduction of adhesion after hot water treatment indicated the removal of extracellular matrix from the biofilm.

6. Indentation studies

AFM has been implemented to analyze several mechanical properties of microbial cells, such as elasticity and hardness [56, 67, 68]. Typically, this is done using the Hertz model, wherein the indentation of a material by a nonadherent probe can be used to calculate the elastic modulus of the substrate. Volle et al. [69] measured cell spring constants and AFM tip adhesion on cells within the biofilms of *E. coli*, *Pseudomonas putida*, *Bacillus subtilis*, and *Micrococcus luteus*. Gram-positive bacteria were observed to have largest spring constants with all cells having values in the range 0.16 ± 0.01 to 0.41 ± 0.01 N/m. These workers also demonstrated that the mechanical properties of chemically fixed cells are significantly different. Fang et al. [60] also

used AFM force spectroscopy to quantify tip-cell adhesion and surface elasticity of sulfate-reducing bacteria (SRB) biofilms. To achieve this, they used a force volume technique to map forces across the biofilm surface. Greater adhesion was measured at the cell–cell and cell–substratum interfaces; this was compared to a smaller and constant force at the bacterial cell surfaces and argued to be due to the accumulation of EPS at the interfaces. Another interesting study conducted by Longo et al. [70] demonstrated that AFM can be used to characterize the variations in nanomechanical properties across a single cell membrane. In the study, nanoindentation was performed across the surface of an immobilized *E. coli* cell, and it was found that there was a variation in the Young's modulus of the cell membrane. Upon further analysis, this heterogeneity was attributed to the presence of submembranous structures, hinting at the possibility that AFM may be capable of resolving the organization of such structures.

As confidence in the technique grew, focus of nanoindentation studies shifted from single cells to biofilms. However, use of the classic Hertz model to interpret the viscoelastic properties of biofilms, until recently, remained problematic [71, 72]. In a recent example of one such study, the elastic moduli of *P. aeruginosa* was found to be heterogeneous in nature, varying between approximately 40 and 45 kPa [73]. SEM and AFM topographical studies of the same sample showed variations in packing density of the cells throughout the biofilm, offering possible insight into the cause of the variation in mechanical properties. However, these variations may also be the result of underlying physiological structures such as nutrient channels. Finite element analysis performed by the group showed that the variation may be a result of the combined effect of the EPS and cell orientation.

There have been further studies into the nanomechanical properties of biofilms that have focused on the effect of growth conditions and novel antimicrobials on the nanostructure of biofilms. One such study showed that increasing concentrations of $CaCl_2$ resulted in not only an increase in EPS production but also alterations in EPS structure of *Pseudomonas fluoroscenes* biofilms [74]. Consequently, a reduction in stiffness and increase in both viscosity and adhesive forces were observed. In another study, AFM was used to assess the changes in the nanomechanical properties of *P. aeruginosa* and *Acinetobacter baumannii* biofilms after treatment with OligoG. During the study, OligoG was found to significantly lower Young's moduli and increase the surface roughness (R_a) when compared to untreated biofilms [75]. However, this study highlights one of the main challenges facing the characterization of biofilms via AFM: continuity of sample preparation. In the aforementioned study, the biofilms were dried prior to analysis, while others made use of hydrated samples. While both techniques remain valid, interstudy comparisons will remain difficult until a level of interstudy continuity is achieved.

7. Single-cell force spectroscopy

Single-cell force spectroscopy (SCFS) has become an essential tool in unravelling the forces involved in intermicrobial, host–microbe, and substrate–microbe binding. This is of particu-

lar importance in the field of biofilm formation as the forces governing such interactions are pertinent in the initiation of a biofilm. The research was pioneered by Bowen et al. [76] who first constructed a cell probe to measure the adhesion of *S. cerevisiae* cells at surfaces (**Figure 6**). The author then moved this on to look at the adhesion of fungal and bacterial spores [77, 78]. Protocols for the construction of cell probes have varied in the method of cantilevers functionalization: electrostatic compounds; poly(ethyleneimine) (PEI), poly-L-lysine, or hydrophobic substances, and the use of glue, chemical fixation, and bio-inspired wet adhesives have all been used, and in the type of probe that was created: single versus multicellular [3, 79–85]. While all methods succeeded in the creation of a cellular functionalized tip and the acquisition of adhesive force-curve data, the results and validity of the techniques varied.

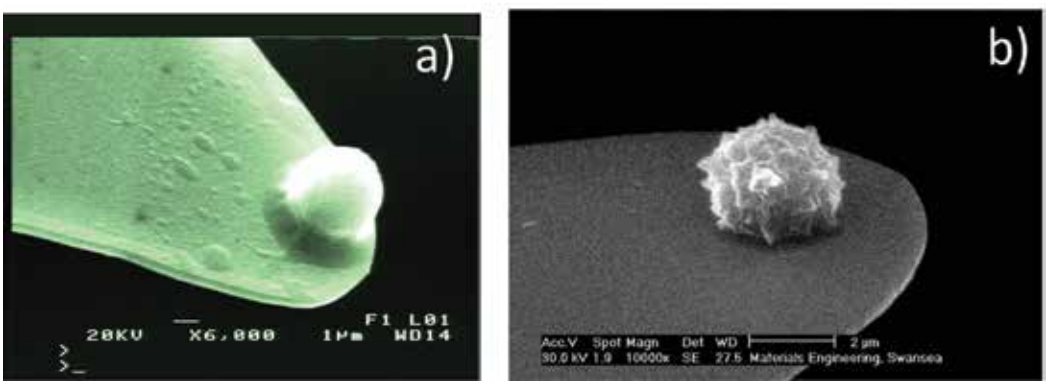

Figure 6. Scanning electron images of AFM probes used in single cell force spectroscopy (SCFS). (**a**) *Saccharomyces cerevisiae* and (**b**) *Aspergillus niger*.

Recently, a method for the direct immobilization of single microbial cells was developed [86]. A colloidal probe was attached to the tip of a cantilever and coated in polydopamine, and a single microbial cell was then attached to the colloid particle. Fluorescence microscopy validated the viability and orientation of the microbial cell, and force-curve analysis was performed across a number of surfaces and a number of probes to ensure reproducibility of results. The technique was shown to offer a high level of cell orientation; thus, a high level of control of the surface area, ensuring reproducibility of results and enabling statistical analysis of force curves. The group went on to create cellular probes functionalized with *Lactococcus plantarum, C. albicans*, and *Staphylococcus epidermidis* to prove the versatility of the technique [84].

Studies using SCFS have characterized a number of microbial binding structures, such as bacterial pili, to show how these structures influence microbial adhesion. During one such study, the nanomechanical binding of *P. aeruginosa* type IV pili to a hydrophobic substrate was examined. During the study, type IV pili were shown to have the same constant force plateaus associated with a nanospring-like mechanism; this may be explained by the fracturing of internal amino acid bonds and the unravelling of the three-dimensional structure to resist the increase in mechanical force. This model is consistent with the previous interpretations of

Gram-negative pili structure [87–89]. In a similar study, strains of *Lactococcus lactis* were immobilized onto polyethyleneimine (PEI)-coated cantilever, and adhesion to a pig gastric mucin-coated substrate was characterized [90]. In the study, long-range adhesion was found to be predominantly the result of pili-mediated binding, while surface adhesion was primarily mediated by mucus-binding adhesins.

The implementation of SCFS has not been limited to the characterization of microbial binding to surfaces. While uncommon, the use of SCFS to characterize microbial aggregation and the formation of heterogeneous biofilms has grown as a field in recent years. One such interaction to be studied is the common co-colonization of *S. epidermidis* and *C. albicans*; a recent study attempting to characterize such an interaction showed that despite the complex nature, SCFM is able to offer a window of insight into the adhesion forces at work [91]. During the study, the group was able to establish that *S. epidermidis* adhesion was strongly influenced by the life stage of *C. albicans* and primarily mediated by the binding of long-range macromolecules.

SCFS techniques have been used to study the mechanisms of biofilm control agents. Chaw et al. [92] measured the adhesion between *S. epidermidis*-coated AFM tips and a substrate before and after addition of silver ions (50 ppb) to the liquid medium. For both *S. epidermis* strains studied, the adhesion decreased and was argued to demonstrate how the biofilm matrix is destabilized in the presence of silver ions.

8. Conclusion

AFM has provided researchers with the tools necessary to unravel the intimate, complex, and traditionally illusive processes governing the formation and resilience of biofilms. AFM has provided the platform necessary for the application of classical engineering techniques, such as indentation analysis in the exploration of microbial nanomechanics with unprecedented resolution. Nanoindentation studies have elucidated the heterogeneity of the microbial membrane landscape. Studies utilizing nanoindentation have provided evidence of the variation in Young's moduli of both single cells and biofilms, while also hinting at the possible application of the technique in the visualization of the assembly of submembranous structures. AFM studies have also demonstrated the importance of such measurements in the evaluation of novel antimicrobial and other therapeutics.

Through the use of functionalized cantilevers, SMFS has revolutionized our understanding of microbial cell surface topography and nanomechanical properties. Tips functionalized with ligands or with alterations in hydrophobicity have been used to not only map the receptor landscape at the macroscale, but also to visualize the structure of individual membrane-bound protein complexes. AFM quantification of the nanoscale forces of adhesion has offered unparalleled insight into the forces governing microbial adhesion, a crucial event in biofilm formation, and how these individual forces may be manipulated to promote dissolution. The formation of cellular probes has been a mainstay of microbial-based AFM, and this continues with the recent development of protocols for the immobilization of a singular, highly orientated bacterial cells.

In conclusion, microbiology and the study of biofilms is no longer a microscience. The elucidation of microbial behavior at the nanoscale has now become an essential avenue of research in the understanding of the complex interplay of the microbial world, and AFM has proved itself to be an essential tool in this endeavor. The increase in sensitivity and analytical power, as well as ingenuity shown by researchers in the creation of more imaginative probes will ensure that unique insights into biofilms through AFM will continue.

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Section 2

Biofilm Applications

Chapter 7

Role of the Biofilms in Wastewater Treatment

Shama Sehar and Iffat Naz

Additional information is available at the end of the chapter

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Abstract

Biological wastewater treatment systems play an important role in improving water quality and human health. This chapter thus briefly discusses different biological methods, specially biofilm technologies, the development of biofilms on different filter media, factors affecting their development as well as their structure and function. It also tackles various conventional and modern molecular techniques for detailed exploration of the composition, diversity and dynamics of biofilms. These data are crucial to improve the performance, robustness and stability of biofilm-based wastewater treatment technologies.

Keywords: biofilm, wastewater treatment, biofilm technologies, molecular methods, biofilter media

1. Introduction

Water is a basic necessity, but its availability for human use is hardly about 1%. Current global water crises are due to a rapid increase in population, climatic variation, environmental pollution, urbanization, industrialization and contamination of existing water reservoirs. The quality of freshwater in rivers and streams is affected because much of the wastage is discharged without prior treatment from industries, municipal sewers and agricultural areas. The quality of groundwater is declining due to unprocessed sewage containing domestic waste along with human and animal excretion products, leading to worldwide deaths and other environmental factors, including biodiversity reduction and an increasing number of water-related infections, among others. According to WHO, approximately 30% of all diseases and 40% of deaths throughout the world are due to polluted water [1].

Wastewater is a broad term comprising effluents or discharge from household seepage, agriculture, industries and storm water [2]. The organic material present in wastewater includes detergents, pesticides, fats and oils. In addition, many types of microorganisms, including bacteria, viruses, protozoa and helminths, can be present in wastewater. Basic nutrients (nitrogen, phosphorous and ammonia, etc.) as well as metals and inorganic materials (mercury, lead, cadmium, nickel and hydrogen sulfide, etc.) are also present in wastewater. By keeping the hazardous effects of wastewater and its usage for daily lives, wastewater treatment plants have become a focal path in securing our future water supply.

2. Types of biological wastewater treatment systems

There are a number of wastewater treatment processes based on the physical and chemical removal of contaminants. These processes offer varying degrees of effectiveness in addition to presenting environmental and economic disadvantages. However, biological wastewater treatment technologies have been gaining much attention in recent years. They offer low operational costs, provide easy handling and have comparatively less harmful effects on the corresponding environment. On the basis of structural configuration of biomass, biological wastewater treatment processes can be divided into two basic configurations: dispersed growth system and attached growth system.

2.1. Dispersed growth system

In dispersed/suspended growth systems, biomass grows in suspended or dispersed form in liquid medium without any attachment to the surface (**Figure 1**).

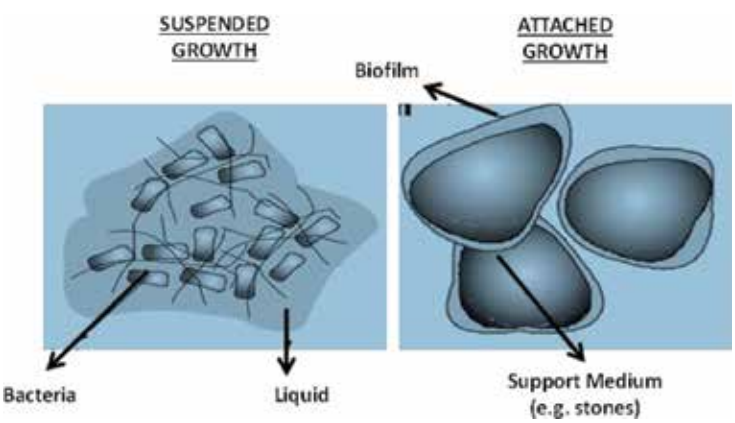

Figure 1. Typical examples of biomass growth [3].

Microorganisms in biomass absorb organic matter and nutrients in their vicinity, which allows them to grow and reproduce to form microcolonies. These microcolonies settle as sludge, which is then either removed or treated in a sludge treatment process or reused in the process

by being resuspended. In dispersed growth systems, the density of dispersed biomass is close to the sewage and moves in the same direction and velocity thereof. Thus, biomass is exposed to the same fraction of liquid for a larger interval with less substrate concentration in the neighboring cell, leading to low bacterial activity and substrate removal rate. The hydraulic retention time (average time water molecules stay in the system) has to be greater than the doubling time of microorganisms (time required to generate new cells) to increase bacterial activity and population size. Bacteria can easily be "washed out" of the system if the hydraulic retention time is shorter than the bacterial doubling time [4]. This is the main hurdle in sizing biological reactors, as reactor volume and retention time are directly related to each other. Some of the commonly used dispersed growth systems are described in the following subsections.

2.1.1. Activated sludge technology

Activated sludge systems comprise a multichamber reactor unit in which aerobic microorganisms are used to degrade organic components of wastewater to produce a high-quality effluent. Constant supply of oxygen is required to maintain aerobic conditions in an aeration tank. Besides aerobic bacteria, anaerobic and/or nitrifying bacteria along with higher organisms can be present. These microorganisms oxidize the organic carbon present in wastewater to produce carbon dioxide, water and new cells that form small clusters or flocs during the aeration and mixing process. After aeration, the mixture is transferred to a secondary clarifier for settling of floc particles and the effluent moves on for further treatment or discharge. The sludge is then recycled back to the aeration tank, where the process is repeated. A schematic of the entire process is shown in **Figure 2**. Activated sludge technology is most commonly used

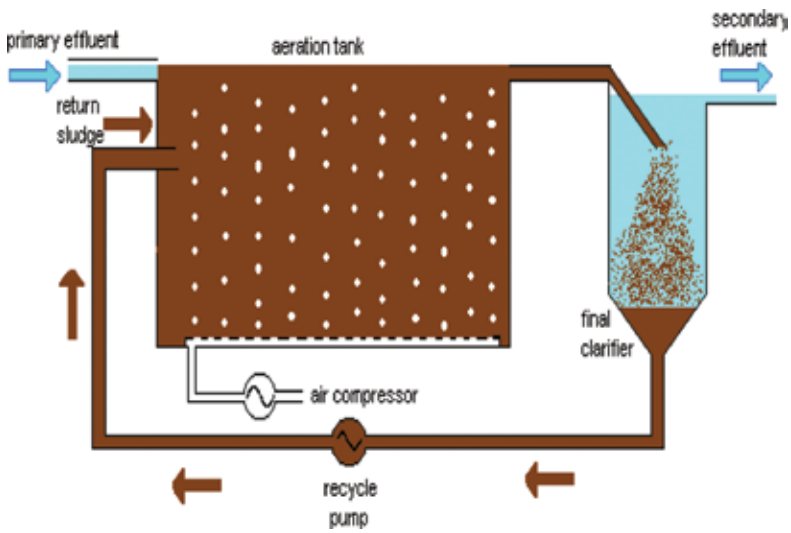

Figure 2. Schematic of a typical activated sludge system [6].

in industrialized countries for the removal of biological solids by sedimentation. Poor settling of these solid pollutants can lead to increased solid treatment costs, increased effluent solid concentrations, decreased disinfection efficiencies, washout/low biomass concentration and increased risks to downstream ecosystems and public health [5].

2.1.2. Extended aeration system

The extended aeration system is one of the modifications of the activated sludge process. It is a complete mixed system that provides biological treatment for the removal of biodegradable organic waste under aerobic conditions. Air may be supplied by mechanical or diffused aeration means. The raw sewage directly flows into the aerobic digestion chamber where all the solids are digested by aerobic bacteria. This is possible because the sewage is aerated for a minimum of 24 h, giving vastly increased time for almost complete digestion of all solids. Since there is complete stabilization in the aeration tank, there is no need for a separate sludge digester. Furthermore, there is no need for a primary settling tank as organic solids are allowed to settle in the aeration tank due to their long detention time. The major advantages of extended aeration include ease of construction as well as operation, high oxygen transfer efficiency, absence of odor, less sludge yield and exceptional mixing energy from a controlled aeration chain environment. However, extended aeration plants do not achieve denitrification and phosphorus removal without additional unit processes.

2.2. Attached growth system

In attached growth systems, the biomass grows attached to a support medium to create a biofilm. Attachment to the support medium is influenced by composition of the media used, cell-cell interactions and the presence of polymer molecules on the surface [7]. The support medium can be immersed in the liquid medium or receive continuous or intermittent discharges. The support medium can be of any nature, such as solid natural (rocks, stones, gravels, sand and soil), artificial (rubber, plastic) or agglomerates of the biomass itself (granules). These biofilms grow on support media by feeding off the organic matter and nutrients in the wastewater that flows over them. In attached growth systems, there is a difference in the density gradient of the support medium together with biomass and the density of the liquid inside the reactor that allows the velocity gradient between the liquid and the external layer of biofilm. Therefore, bacterial cells being continually exposed to new substrates tend to increase their activity. Some of the commonly used attached growth systems are described in the following subsections.

2.2.1. Trickling filters

Wastewater treatment through trickling filters (TFs) is among the oldest and most well characterized treatment technologies. TFs generally comprise a vessel packed with inert media (rocks, coke, lava, slag, gravel, polyurethane foam, ceramic, sphagnum peat moss or plastic media). The distribution system is used to sprinkle wastewater over filter media, and the wastewater trickles through the filter media supporting biomass under the influence of gravitational force. A biological slime layer grows on the media, and treatment is provided by

the microbes that absorb dissolved organic matter for their growth and reproduction as the wastewater cascades randomly through the voids between the media [8]. A schematic of the entire process is shown in **Figure 3**. TFs are suitable for small- to medium-sized communities with a high filter loading rate and marked by their ease of operation, self-cleaning capacity and efficient removal of ammonia. However, additional treatment may be needed for the effluent to meet strict discharge standards as it generates large amounts of sludge and a relatively high incidence of clogging [9].

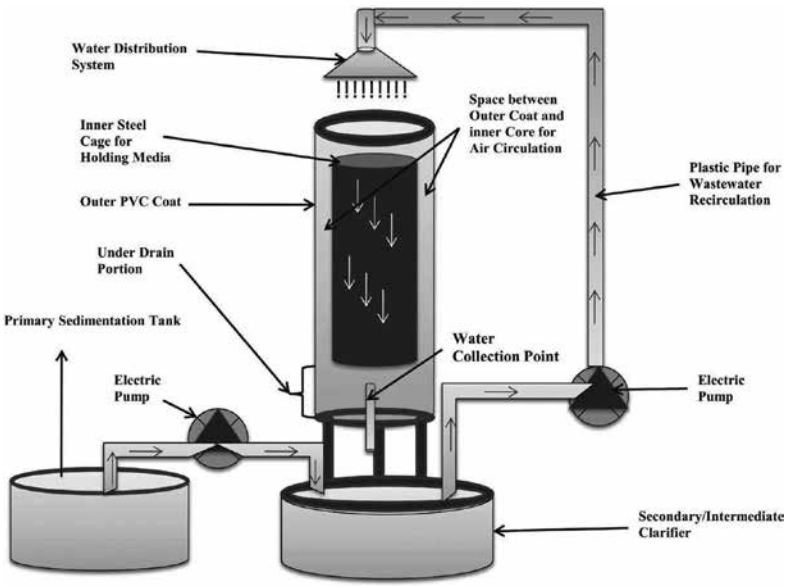

Figure 3. Schematic of a typical trickling filter system [10].

2.2.2. Rotating biological contactor (RBC) system

The rotating biological contactor (RBC) is an efficient attached growth system that purifies wastewater from different industries, namely food and beverage, refinery and petrochemical, pulp and paper industries. In addition, it is efficient in purifying municipal wastewater, landfill leachate and lagoon effluent. The system consists of biomass media, usually plastic (polyethylene, polyvinyl chloride [PVC] and expanded polystyrene), that are partially immersed in wastewater. As it slowly rotates, it lifts a film of wastewater into the air. The wastewater trickles down across the media and absorbs oxygen from the air provided by the rotating action. A living biomass (biofilm) attached to the discs assimilates the organic materials and nutrients in the wastewater. Any excess biomass that sloughs off the discs by shearing forces exerted with disc rotation and gravitational force is then removed from clear water through a conventional clarification process. A schematic of the entire process is shown in **Figure 4**. The RBC system has an edge over suspended growth systems in terms of reduced life cycle costs, less sludge production, less space requirement, ease of operation and high

process stability with load variations as well as high effluent quality with regard to both biological oxygen demand (BOD) and nutrients. However, RBC system optimization and adaptability under different environmental conditions and influent characteristics still pose challenges for the efficient design and use of this technology.

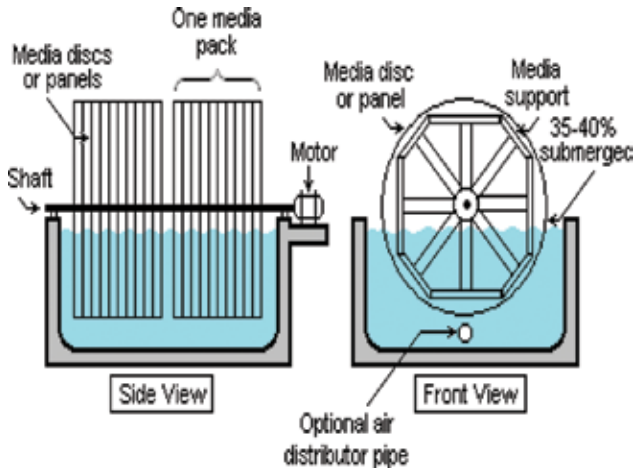

Figure 4. Schematic of a typical rotating biological contactor (RBC) [11].

2.2.3. Constructed wetland system

Constructed wetlands (CWs) are engineered attached growth or fixed film systems comprising beds loaded with inadequately sapped graded medium (sand, soil, gravel, etc.) and planted with suitable vegetation and their microbial inhabitants to treat contaminants in surface water, groundwater or waste streams. CWs generally may be categorized into two major groups: surface flow and subsurface flow. In the case of surface flow, the water runs over the surface, while for subsurface flow, it runs beneath the surface to overcome the issues of odor. In surface flow, the bacteria and substrate contact angle with water is lower than that in subsurface flow, resulting in the much enhanced treatment efficiency of subsurface flow systems [12]. Further subsurface flow systems are categorized into horizontal and vertical subsurface flow wetlands depending on the flow path. All these systems are efficient in removing contaminants and pathogens from wastewater; however, the evaporation rate of CWs in general is much higher than that of ponds or lagoons, thus posing a low potential for irrigation. The configuration of hybrid CWs (combination of vertical and horizontal flows) is considered to be an appropriate choice that has minimum water loss to overcome this flaw (**Figure 5**). Hence, the discharge of nitrified and partly denitrified effluents is possible with lower total N contents [13].

Generally, water purification in constructed wetlands involves a series of physical, chemical and biological processes, such as adsorption, filtration, sedimentation, chemical precipitation, microbial activities and macrophyte uptake. Various factors contribute to the removal

efficiency of CWs, including hydraulic retention time, temperature, macrophytes, composition of substrate or fill media and microorganisms [14]. In CWs, the role of macrophytes is very important for the removal of nutrients from wastewater, and they also speed up the purification process by increasing the chemical and biological reactions in the rhizosphere. CWs require low operational and maintenance costs, less energy consumption and a reduced amount of sludge, and they are environmentally friendly [15].

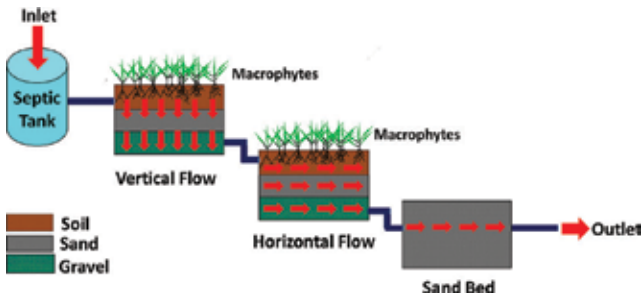

Figure 5. Schematic of a constructed wetland system for wastewater treatment [14].

2.2.4. Membrane bioreactors (MBRs)

Membrane bioreactors (MBRs), which are used for municipal/industrial wastewater treatment, are a combination of a suspended growth treatment method with membrane filtration equipped with low-pressure microfiltration (MF) or ultrafiltration (UF) membranes. A membrane is simply a two-dimensional material used to separate components of fluids usually on the basis of their relative size or electrical charge. MBRs are generally categorized into the following: (i) vacuum or gravity-driven systems, immersed and normally employing hollow fiber or flat sheet membranes installed in bioreactors or a subsequent membrane tank and (ii) pressure-driven systems or pipe cartridge systems located external to the bioreactor. A schematic of MBRs is depicted in **Figure 6**.

An MBR system is often composed of 10 or 11 subsystems and includes fine screening, the membrane zone and, in most cases, some type of post-disinfection process. The initial step in a biological process occurs in membrane zones where microbes are used to degrade pollutants that are then filtered by a series of submerged membranes. The individual membranes are housed in units known as modules, cassettes or racks, and a combined series of these modules is referred to as a working membrane unit. Air is introduced through integral diffusers to continually scour membrane surfaces during filtration, facilitate mixing and, in some cases, contribute oxygen to the biological process. The major advantage of MBRs is that they allow high concentrations of mixed liquor suspended solids (MLSSs) with low sludge production, increased removal efficiencies of BOD and COD, water reclamation, reduced footprints and no further polishing requirement for disinfection/clarification. However, membrane surface fouling is a major obstacle to the wide application of MBRs. Additionally,

membrane channel clogging and process complexity are the main cause of increased capital as well as running costs of the entire system [16].

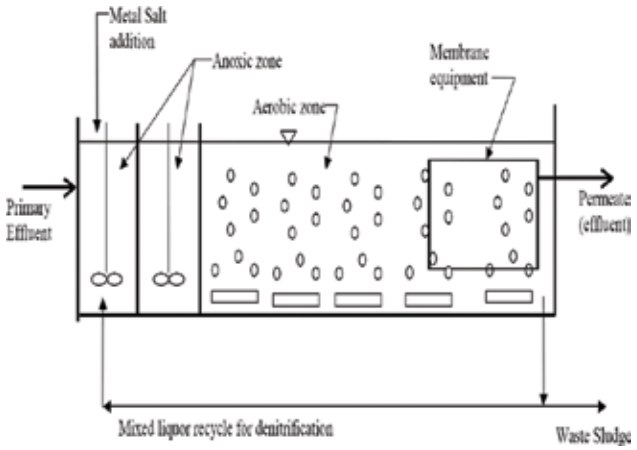

Figure 6. Typical schematic for a membrane bioreactor [17].

3. Biofilm development: structure and function

An assemblage of microbial cells enclosed in a matrix of bacterial self-generated extracellular polymeric substances (EPSs) irreversibly associated with a surface is termed a *biofilm*. Generally, the development of biofilms is composed of five main stages (**Figure 7**): (1) initial attachment of planktonic microorganisms with the exposure of a surface to an aqueous

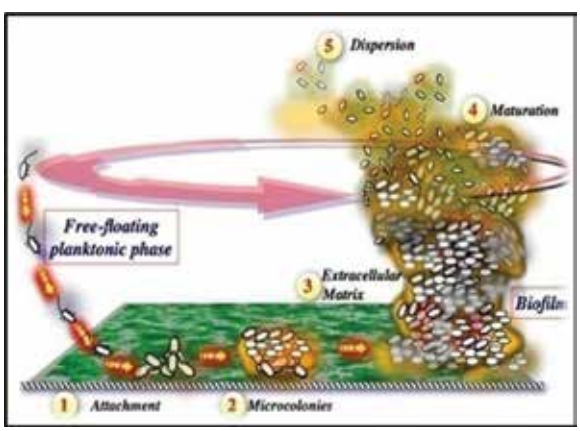

Figure 7. Stages of biofilm development [20]: (1) initial attachment; (2) irreversible attachment; (3) replication; (4) maturation and (5) dispersion.

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medium; (2) irreversible adhesion upon the production of microorganism-mediated EPSs as polyhydroxyl groups in EPSs colonize bacteria to the surface via hydrogen bonding [18]; (3) formation of monolayer microcolonies on the fixed surface due to replication of early colonizers; (4) maturation of biofilm into a three-dimensional arrangement by attaching debris from the adjacent environment and by employing new planktonic bacteria and (5) dispersion or expansion by active and passive processes in which sessile, matrix-encased biofilm cells convert to freely swimming planktonic bacteria through quorum sensing (QS) or a cell-to-cell signaling mechanism [19].

3.1. Factors effecting biofilm formation

The following subsections discuss the factors that help in promoting the process of biofilm formation.

3.1.1. Effects of nutrients, pH and temperature

Biofilm formation varies under diverse nutrient conditions ranging from high to almost non-detectable. However, they are more abundant and dense in a nutrient-rich environment as it promotes the transition of bacterial cells from planktonic to biofilm state, while depletion of these nutrients causes detachment of biofilm cells from surfaces. There are different means by which bacterial biofilms obtain nutrients: (i) concentrating trace organics on surfaces through extracellular polymer, (ii) using the waste products from secondary colonizers and (iii) pooling the biochemical resources with the help of different enzymes to break down food supplies.

Any change in pH greatly affects the growth and development of bacterial and biofilm formation as it can overwhelm different mechanisms and have negative or killing effects on the microorganisms. In response to internal or external changes in pH, bacteria quickly adjust the activity and synthesis of proteins that are associated with different cellular processes. However, some of the cellular processes, including excretion of exopolymeric substances or polysaccharides, do not adapt to pH variations so easily. The optimum pH for polysaccharide production varies among different species, but for the majority of bacteria, it is around 7 [21]. Microbial activities are very sensitive to change in temperature. Optimum temperature results in healthy growth of bacterial populations, whereas a slight variation may reduce bacterial growth efficiency. The reason for this is a reduction in bacterial enzyme reaction rates. For many bacteria found in cooling water systems, the optimum temperature for maximum growth is about 40°C [22].

3.1.2. Surface topography

Surface topography greatly influences the ability of bacteria to adhere to a surface. During the initial steps of colonization, surface roughness at nanoscale and microscale levels enhances the adhesion of bacteria to substrates by providing more surface area for cell attachment. Surface roughness reduces the shear force on bacterial cells and communities present in flowing liquids at high flow rates, such as water pipes in industrial plants. A material surface exposed in an aqueous medium will inevitably become conditioned or coated by polymers from the medium,

and the resulting chemical modification will affect the rate and extent of microbial attachment. Moreover, other factors such as charge, hydrophobicity and elasticity are also influential in microbial attachment [23].

3.1.3. Velocity, turbulence and hydrodynamics

The area from the surface where no turbulent flow is experienced is known as the boundary layer. Within this area, the flow velocity has been shown to be insufficient to remove biofilms. The area outside this layer is characterized by high levels of turbulent flow and has an influence on the attachment of cells to the surface. The size of the boundary layer is dependent on the flow velocity of water. At high velocities, the boundary layer decreases in size and the cells are exposed to a high turbulence level. Hydrodynamic conditions can influence the formation, structure, EPS production, thickness, mass and metabolic activities of biofilms [24].

3.1.4. Gene regulation and quorum sensing (QS)

Studies have shown that up-regulation and down-regulation of a number of genes are involved in the initial attachment of cells with the substratum. Approximately 22% of genes were up-regulated and 16% were down-regulated in the biofilm formation of *Pseudomonas aeruginosa* [25]. In addition, *algD*, *algU*, *rpoS* and genes controlling polyphosphokinase synthesis were also up-regulated in the biofilm formation of *P. aeruginosa* [23]. Biofilms of *Staphylococcus aureus* were up-regulated for genes encoding enzymes involved in glycolysis or fermentation, such as phosphoglycerate mutase, triphosphate and alcohol dehydrogenase [26]. Cell-to-cell signaling, also termed *QS*, has recently been proven to play a significant role in cell attachment and detachment from biofilms. Growth and development of biofilms on different surfaces are mediated by a density-dependent chemical signal released by bacterial cells densely packed with an EPS matrix. QS makes use of a transcriptional activator protein that acts in concert with small autoinducers (AIs) signaling molecules to stimulate expression of target genes, resulting in changes in chemical behavior. After accumulation of sufficient AIs, this form of intercellular communication serves to coordinate gene expression, morphological differentiation and the development responses of bacterial cells [27].

3.1.5. Production of extracellular polymeric substances (EPSs)

Extracellular polymeric substances (EPSs) are a complex mixture of high-molecular-weight polymer (M_w = 10,000) excreted by microorganisms, products from lysis and hydrolysis as well as adsorbed organic matters from wastewater. Generally, EPSs have been shown to be a rich matrix of polymers, including polysaccharides, proteins, glycoproteins, DNA oligomers, phospholipids and humic acids [28]. EPSs are also highly hydrated because they can incorporate large amounts of water into their structure by hydrogen bonding. EPSs are typically reported to aid in the formation of a gel-like network that keeps bacteria together in biofilms due to bridging with multivalent cations and hydrophobic interactions. In addition, EPSs also cause the adherence of biofilms to surfaces, flocculation and granulation, protect bacteria against noxious environmental conditions and enable bacteria to capture nutrients from the

surroundings [29]. Different biofilms produce different amounts of EPSs, and the amount of EPSs increases with the age of biofilms [30].

3.1.6. Extracellular DNA (eDNA)

Extracellular DNA (eDNA) has been reported to be a major constituent of various single and multispecies biofilms. eDNA or naked DNA is a central part of bacterial self-produced extracellular polymeric substances (EPSs) and has similarity to chromosomal DNA in its primary sequence [31]. Its role is very important in various stages of biofilm formation, such as initial bacterial adhesion, aggregation and microcolony formation that favors wastewater treatment. eDNA also helps strengthen biofilms, provides protection to biofilms from physical stress, antibiotics and detergents as well as serves as an excellent source of nutrients for biofilm growth [32]. In addition, eDNA can be utilized in engineering of biofilms for beneficial purposes, such as remediation of environmental pollutants and electricity or fuel production in bioelectrochemical systems or bioreactors.

3.1.7. Divalent cations

Divalent cations such as Ca^{2+} are abundant in terrestrial and aquatic environments; therefore, calcium may be one of the factors that bacteria sense during biofilm-associated growth. Recent studies showed that eDNA chelates divalent cations that help in the modification of bacterial cell surface properties and thus favor resistance of biofilms to detergents and antimicrobial agents [33]. Divalent cations, such as those of calcium, play a critical role in the initial attachment of microbial aggregates of activated sludge flocs, anaerobic sludge granules and biofilms by bridging negatively charged sites on extracellular polymers [34]. Recent studies have shown that the thickness of a biofilm can be enhanced by introducing more divalent cations, as a result of which the biofilm becomes denser and mechanically more stable [35]. Calcium has been found to not only act as a cofactor for certain proteins but also act in cell signaling, biofilm virulence, cellular and extracellular product formation and alginate regulation [36].

4. Biofilm in wastewater treatment

Biofilm system is a well-developed technology in which solid media are added to suspended growth reactors to provide attachment surfaces for biofilms, so as to increase the microbial concentration as well as rates of contaminant degradation biofilms to take advantage of a number of removal mechanisms, including biodegradation, bioaccumulation, biosorption and biomineralization [8]. The microbial communities in the biofilm break down different nutrients, such as phosphorous and nitrogen-containing compounds, carbonaceous materials as well as trapped pathogens from the wastewater. Once pollutants are removed, treated water of a biofilter is either released to the environment or used for agriculture and other recreational purposes. Removal of the pollutants from wastewater by biofilm on the filter media is schematically represented in **Figure 8**.

Wastewater treatment with biofilm systems has several advantages, including operational flexibility, low space requirements, reduced hydraulic retention time, resilience to changes in the environment, increased biomass residence time, high active biomass concentration, enhanced ability to degrade recalcitrant compounds as well as a slower microbial growth rate, resulting in lower sludge production.

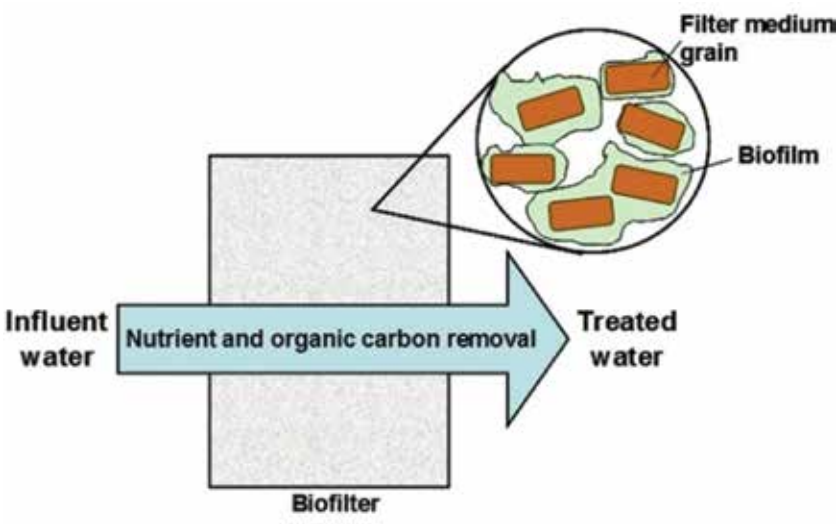

Figure 8. Removal of the pollutants from wastewater by biofilm on the filter media [37].

5. Biofilm development on different filter media

Packing or filter medium is the basic unit of attached growth wastewater treating technologies. It provides a surface for the growth of the biofilm. The filter medium needs to be durable, insoluble and resistant to chemicals. Its selection is based on size, porosity, density as well as resistance to erosion and chemicals [38]. The ideal medium provides a high specific surface area, low cost and porosity high enough to avoid clogging and promote ventilation. The surface area and geometry of the support materials affect the hydrodynamic conditions in the reactor and thus affect biofilm formation, which in turn affects wastewater treatment [39, 40]. Presently, different synthetic and natural materials have been employed. Various researchers have used polystyrene [41], polypropylene [42], tire-derived rubber [43] and pebbles [44, 45] as bio-filter media in fixed biofilm reactors for wastewater treatment. The chemical composition of the filter media is very critical, with respect to its compatibility with the developing biofilms; its elemental composition should be evaluated. For the detection and quantification of the elements in a filter medium, different spectroscopic techniques can be applied, such as X-ray photoelectron spectroscopy (XPS) and energy-dispersive X-ray spectroscopy (EDS or EDX or XEDS). XPS is a surface chemical analysis technique used to

analyze the surface chemistry of a material. It measures the elemental composition at the parts per thousand range, empirical formulas, chemical state and electronic state of the elements that exist within a material [46]. On the other hand, EDS is a useful technique applied for the elemental analysis/chemical characterization of filter media [47].

6. Biofilm community characterization approaches

The following subsections discuss various biofilm community characterization approaches.

6.1. Traditional methods

6.1.1. Determination of biofilm weight (wet weight and dry weight)

Biofilm weight can be determined in terms of dry weight and wet weight by using a digital weighing balance. The wet weight of the biofilm is measured after soft rinsing with distilled water. However, the dry weight of the biofilm is estimated by allowing it to dry under aseptic conditions in laminar flow until the attainment of the constant weight of polypropylene and polystyrene filter media [41, 42]. On the other hand, natural filter media, such as rock, granite or stone media, should be dried in the oven at 60°C to constant weight [44]. The weight of the biofilm is then calculated from the difference between the weight of medium with biofilm and that of medium without biofilm.

6.1.2. Determination of the biofilm optical density (OD)

The biofilm is also measured by the OD method. The filter media supporting biofilms are first rinsed with sterilized water to ensure the removal of any material on their surface. The biofilm is then removed from the filter media in 0.9% saline by sonication for 15 min. Finally, the spectrophotometric absorbance of dissolved biofilms is recorded at 550 nm wavelength (OD_{550}) using saline as blank [41, 42, 44].

6.1.3. Determination of heterotrophic plate count (HPC)

The HPC concentration (HPC/mL) of biofilms on filter media is determined by the conventional serial dilution method. The biofilm dissolved in 0.9% saline is serially diluted (up to 10^{-5}) and then spread on the selective growth media plates and incubated at 37°C for a specific period (24–48 h). The microbial growth appearing on specific media is enumerated in terms of HPC/mL (pathogen indicators). Pure cultures from these plates are further identified by colony morphology as well as microscopic and biochemical tests.

6.1.4. Microscopic analysis of biofilms

Non-invasive microscopic techniques provide a more accurate way of visualizing biofilms without disturbing their structure. The traditional microscopic techniques involve light microscopy (LM) and electron microscopy (SM), used for imaging analysis of biofilm sam-

ples. However, scanning electron microscopy (SEM) is a well-established fundamental technique to examine the morphology of bacteria and the topography of the material surface, and it is even capable of demonstrating the relation of biofilms to surfaces. On the other hand, other new advanced techniques have been established, including laser scanning microscopy (LSM), confocal laser scanning microscopy (CLSM), magnetic resonance imaging (MRI) and scanning transmission X-ray microscopy (STXM). These new techniques allow *in situ* analysis of the structure, composition, processes and dynamics of microbial communities. These techniques represent powerful tools for the examination of mixed microbial communities, those usually in the form of aggregates and biofilms [48].

6.1.5. Determination of biofilm activity

The metabolic activity of the microorganisms constituting biofilms can be estimated by considering the rate of the conversion of the specific substrate after inoculation with the seed of the biomass. For example, the physiological activity of *Nitrosomonas* spp. can be determined by measuring the strength of the nitrites (NO_2-N) formed in the growth medium from the known concentration of $(NH_4)_2SO_4$ after a specific period [49]. Similarly, the removal of carbonaceous (COD and BOD) and nitrogenous (NH_4—N) pollutants by biofilms can be estimated.

6.2. Advanced methods

6.2.1. Clone library technique

Cloning and sequencing of the 16S rRNA gene have been extensively and successfully employed for the study of microbial biofilms since the beginning of the 1990s, and this technique is still most widely used [50]. The cloning methodology for studying a biofilm community involves (1) extraction of the nucleic acid from the biofilm sample; (2) amplification of the 16S rRNA gene by polymerase chain reaction (PCR), usually using universal primers for bacteria or archaea, for obtaining a mixture of rDNA copies of the microorganisms; (3) cloning of the PCR products into an appropriately high number of copies of plasmid and then transformation of competent *Escherichia coli* cells with this vector; (4) selection of the transformed clones on the basis of an indicator contained in the plasmid; (5) extraction of the plasmid DNA from the colons; (6) creating a cone library by sequencing of the cloned gene and finally (7) identification and affiliation of the isolated cloned sequence with the aid of phylogenetic software and various dedicated computer programs (ARB, Seqlab, PAUP, PHYLIP).

These illustrate that the clone library method allows complete 16S rRNA sequencing and identification with very precise taxonomic studies of both cultured and non-cultured microorganisms in biofilms, design of primers for PCR and probes for fluorescence *in situ* hybridization (FISH) [51]. However, cloning is a time-consuming method, impractical for a high sample throughput and non-quantitative; in addition, extraction of a DNA pool from a microbial community can be difficult and the PCR steps are also biased. Furthermore, this technique needs specialized personnel and equipment [52]. In general, cloning and rRNA gene

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library construction have been applied in combination with other advanced techniques in wastewater treatment for the exploration of biofilm communities.

6.2.2. Microbial fingerprinting methods

Microbial fingerprinting methods provide the overall profile of a biofilm community by making a distinction between microorganisms and groups of microorganisms on the basis of their distinctive characteristics of a universal component/section of a biomolecule, such as phospholipids, DNA or RNA [52, 53]. These methods include phospholipid fatty acid analysis (PLFA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP).

6.2.2.1. Phospholipid ester-linked fatty acid analysis (PLFA)

Phospholipids are a structural component of all cell membranes, but their type and proportion are distinctive to different microorganisms and break down rapidly upon cell death. Thus, the mass of PLFAs in a biofilm sample is directly proportional to viable biomass. Some groups of organisms have unique or "signature" types of PLFA [54]. PLFA analysis of the biofilm involves (1) extraction of phospholipids from the biofilm sample; (2) separation by gas chromatography with flame ionization detection; and, if required, (3) confirmation and identification by mass spectroscopy. It is not a good choice as a stand-alone method and can be combined with stable isotope probing (SIP). The SIP technique includes (1) incorporation of the stable isotope label (typically ^{13}C) into biomass, (2) incubation of microorganisms to metabolize for a specific time, (3) extraction of biomolecules from the incubated biofilm sample, (4) quantification of the extracted biomolecules by ^{13}C-PLFA using GC/IRMS and separation of unlabeled nucleic acids by density gradient ultracentrifugation and (5) identification of the genes/microorganisms by PCR or fingerprinting or sequencing.

6.2.2.2. Denaturing gradient gel electrophoresis (DGGE)

DGGE is a nucleic acid-based technique employed to generate a genetic fingerprint of a complex microbial community [51]. It encompasses the following steps: (1) extraction of the DNA or RNA from the biofilm sample; (2) amplification of the extracted nucleic acids by PCR, generating a multitude of copies of a variable region within a target gene usually with universal primers to give a mixture of DNA fragments, all of the same length and each representing a species present in the original sample; (3) separation of the DNA mixture by denaturant gradient electrophoresis on an acrylamide gel with an increasing urea/formamide gradient, with the DNA molecules migrating toward the positive pole and halting on the gel upon reaching their corresponding denaturant force (T_m), depending on the DNA sequence, with every band on the gel corresponding to a different microorganism in the sample; (4) visualization of these bands and (5) sequence identification by excision of the individual "bands" from the gel and its comparison with the 16S rDNA database for the phylogenetic affiliation of the microorganism.

DGGE is the fastest and most economical way of comparing large numbers of samples without culturing on expensive media, isolations and analysis, and it permits rapid/simple monitoring of the spatial-temporal distribution of microbial populations by only considering band. However, depending on the nature of the sample, extraction and amplification of representative genomic DNA can be difficult. The DNA copy number, proportional to the abundance of a particular microorganism, can be very different after amplification by PCR, and thus the intensity of the bands on a DGGE gel is not quantitative. Furthermore, the sequences of the bands obtained from a gel correspond to short DNA fragments (200–600 bp), and so phylogenetic relations are less reliably established and short sequences are less useful for designing new specific primers (for PCR) and probes (for FISH).

6.2.2.3. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a nucleic acid-based method and provides the profile of a microbial community, which is used to identify specific microbial populations [55]. It has four steps: (1) total community DNA or RNA extraction from a sample; (2) PCR amplification with a fluorescent PCR primer to make multiple copies of a target gene; (3) enzymatic digestion of the PCR products with restriction enzymes to cut the DNA molecule at known sequences, indicative of a specific microorganism and finally (4) fragment identification by electrophoretically separating the amplified gene sequences of different sizes.

Furthermore, it is also possible to sequence and identify the generated sequences by comparison with a sequence database. The strength of the fluorescent signal yields additional information regarding the abundance of the different species, similar to the band intensity in the patterns of a DGGE gel. T-RFLP offers more sensitivity than DGGE, and it may detect the lower number sequences in a sample and is commercially available. However, sometimes, the heterogeneous size of fragments makes phylogenetic analysis less confident [56].

6.2.3. Fluorescence in situ hybridization (FISH)

For FISH, the most commonly used target molecules are 16S rRNA, 18S rRNA, 23S rRNA and mRNA. FISH is an excellent method for the identification, localization, visualization and quantification of non-cultured microorganisms in their microcosm. The specificity of the florescent probe enables detection/identification on any desired taxonomic level, from domain down to a resolution suitable for differentiating between individual species [57].

FISH is carried out in a few steps: (1) the specimen is fixed by precipitating agents (ethanol or methanol), cross-linking agents (aldehydes) or a mixture depending on the target organism and the type of sample; (2) the sample is prepared, with the process including specific pretreatment steps. For better attachment of specimens to glass slides, their surfaces should be treated with coating (gelatin, poly-L lysine) or silanising agents; (3) hybridization is directly carried out on the fixed sample by addition of a mixture of salts, formamide, detergents and fluorescent probe in a dark humid chamber, usually at temperatures between 37°C and 50°C. Its time varies between 30 min and several hours; (4) slides are rinsed with distilled water to remove unbound probe, dried and mounted and (5) visualization and documentation of

results are carried out with a conventional epifluorescence microscope for multicolor FISH. However, a charged coupled device (CCD) camera and appropriate image analysis software can be used for the digitalization/manipulation of images, enumeration of microorganisms and measurement of the activity of single cells in biofilms by quantification of their rRNA content.

On the other hand, CLSM is used with FISH analysis for thick samples with a high background (sludge flocs, biofilms) and for obtaining three-dimensional images. Different software packages are also available.

FISH is an easy and fast technique, and, if required, probes are available for direct visualization and quantification of microorganisms. This technique is apt for routine analyses, highly trained/specialized personnel are not necessary, and only basic knowledge of microscopy and laboratory experience is required. However, prior knowledge of the microbial habitat/environment conditions and the target microorganisms to be detected is necessary. The rRNA sequence for a particular microorganism to be detected and quantified must also be known [51].

FISH is a widely applied technique and can be combined with other techniques to increase its sensitivity and upgrade it to overcome some of its pitfalls. FISH-based methods have revolutionized investigations into the morphology and microbial composition of biofilms and enable bacteria to be mapped [58]. These methods include FISH-MAR (FISH with micro-autoradiography, CARD-FISH (FISH with catalyzed reporter deposition), Clone-FISH (FISH preceded by generating the expression of the 16S or 18S rRNA targeted gene), CLASI (combinatorial labeling and spectral imaging with FISH), DOPE-FISH (double labeling of oligonucleotide probes with FISH), RING-FISH (recognition of individual genes with FISH), DVC-FISH (FISH with direct viable count) and RCA-FISH (FISH with rolling circle amplification). In the FISH-BrdU method, identification of the microbes is carried out by using 5-bromo-2′-deoxyuridine (BrdU) without any need for paraformaldehyde for cell fixation or formamide for DNA denaturation. In a technique called Spike-FISH, quantification based on an internal standard (*E. coli*) is introduced by spiking the biofilm samples with known amounts of *E. coli* cells. In RAMAN-FISH, Raman microspectroscopy is combined with FISH. NanoSIMS is based on the visualization of oligonucleotide probe-conferred hybridization signals in single microbial cells and isotopic measurement using high-resolution ion microprobes [58].

6.2.4. DNA microarray technology

DNA microarray technology detects hundreds or even thousands of DNA sequences simultaneously and rapidly [59]. It involves (1) extraction of DNA from the sample, (2) amplification by PCR, (3) direct hybridization of the amplified PCR products from total DNA to known molecular probes attached on the microarrays and (4) scoring of positive signals using CLSM after hybridization of the fluorescently labeled PCR amplicons to the probes. Generally, the hybridization signal intensity on microarrays is directly proportional to the abundance of the target organism. The main pitfalls of this technique are cross-hybridization and that it is not useful in identifying and detecting novel prokaryotic taxa. Moreover, if the genus does not

have a corresponding probe on the microarray, then the biological significance of a genus could be totally missed. The application of this technique is comparatively less in the study of wastewater treating biofilms.

6.2.5. Next-generation sequencing (NGS) technology

NGS, such as pyrosequencing, is a novel DNA sequencing technology developed at the Royal Institute of Technology (KTH) based on the sequencing-by-synthesis principle [60] and on the detection of released pyrophosphate (PPi) during DNA synthesis [61]. This technology transforms microbial ecology, explores deeper layers of microbial communities and is vital in presenting an unbiased view of the composition and diversity of communities [62]. NGS platforms such as Roche/454, Illumina/Solexa, Life/APG and HeliScope/Helicos BioSciences are much faster and less expensive than the first-generation Sanger sequencing technology [63].

The steps in pyrosequencing techniques include the following:(1) extraction of the DNA from the biofilm samples; (2) quantification and detection of the purity of the extracted DNA using a NanoDrop spectrophotometer; (3) amplification of the sample 16S rRNA gene by using universal PCR primers (28F and 519R) and incorporation of different barcodes between the 454 adaptor and the forward primer, with the duplicate PCR products pooled and purified using the QIAquick Gel Extraction Kit; (4) use of the purified PCR products for pyrosequencing and then ligation of short adaptors onto both ends for the segregation of the sequences; (5) attachment of the modified products to DNA capture beads, followed by emulsion-based clonal amplification, with the beads set into the wells of a PicoTiterPlate device, with appropriate chemicals, four enzymes (DNA polymerase, ATP sulfurylase, luciferase, apyrase), adenosine 5′-phosphosulfate (APS) and luciferin, and then inserted into the Genome Sequencer according to the manufacturer's directions to record programs; (6) preprocessing of all partial 16S rRNA gene sequences using the pyrosequencing pipeline at the Ribosomal Database Project (RDP) to trim barcodes, remove primers from the partial ribotags and discard low-quality and short (<250 bp long) sequences; (7) denoising and assemblage of the sequences into clusters using the precluster command, thus generating the FASTA file data sets (*.fna and *.qual files) and (8) further analysis of these sequences through MOTHUR, with MOTHUR analysis pipeline and R-Scripts used to start sequencing the taxonomy and analyze the data

The technique of pyrosequencing has the potential advantages of accuracy, flexibility, parallel processing and easy automation. It has no need for labeled primers, labeled nucleotides and gel electrophoresis. It has been successful for both confirmatory sequencing and *de novo* sequencing [61].

7. Conclusions

Of the different wastewater treatment technologies, biofilm-based systems have potential advantages. For better designing of these biofilm wastewater treatment systems, knowledge

about the composition of filter media and developing biofilms is highly necessary. The composition and quantification of different elements in filter media can be determined using spectroscopic techniques, such as X-ray photoelectron spectroscopy (XPS) and energy-dispersive X-ray spectroscopy (EDS). The traditional methods used for the study of biofilms include analyses of their gravimetric weight spectroscopic absorbance, substrate utilization activity and viable plate count as well as microscopic techniques. Complete biofilm community profiling is carried out by advanced techniques such as microbial sequencing, clone library generation, genetic fingerprinting, DNA microarray, denaturant gradient electrophoresis (DGGE) and next-generation sequencing (NGS), on the basis of their availability, to increase the performance, stability and robustness of biofilm reactors.

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Chapter 8

Role of Biofilm in Rainwater Tank

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Additional information is available at the end of the chapter

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Abstract

In order to establish the role of biofilm in rainwater tank, it was investigated the phylogenetic distribution of the bacteria present in an operating rainwater tank. Most of the bacteria were closely related to fresh water, soil, and biofilm bacteria found in natural environments. The high proportion of proteobacteria indicates the generally clean oligotrophic nature of the tank water. To better understand the environmental conditions in rainwater tanks and the development of biofilms therein, the changes in biofilm cells and the bacterial community were investigated during biofilm development. We confirmed that the biofilm development process takes place in three stages: an initial stage characterized by the colonization of different populations, an intermediate stage characterized by a limited number of dominant populations utilizing similar resources, and a late/mature stage characterized by mature biofilms of a complex spatial structure. It was investigated microbial behaviour after inoculation of the bacterium, *Pseudomonas aeruginosa*, in pilot and full-scale rainwater tanks with different surface-to-volume (S/V) ratios. Ninety-nine percentage of the inoculated *P. aeruginosa* had been removed from the water phase. The faster removal rate in pilot and full-scale tank was due to its higher S/V ratio. From the results, several recommendations for tank design and management were suggested.

Keywords: bacterial composition, bacterial community, biofilm, biofilm development, CLSM, DGGE, microbial quality, *P. aeruginosa*, rainwater tank, surface-to-volume ratio

1. Introduction

Almost about one billion people in developing country suffer from water problem. Accordingly, rainwater harvesting is becoming now one of the major alternatives to tackle water scarcity and spreading to not only in developing country but also urban and remote rural communities

in developed country in the world. Rainwater management system has an advantages such as simple technology, low cost and low-energy consuming, but rainwater use is limited by uncertainty about rainwater quality, and especially its microbial quality [1, 2].

Krampitz and Holländer [1] concluded that tank cleaning was contra-productive and Deutsches Institut für Normung (DIN; in English, the German Institute for Standardization) recommend that people do not clean the rainwater tank <10 years. About 13% of all Australian households use rainwater tanks as a source of drinking water [3]. This study was motivated by those questions why the water quality was poorer after cleaning tank and why they are safe in spite of using untreated rainwater.

In the case of roof-harvested water, contamination could mainly occur on the roof collection system or in the storage facility [4]. The contaminant input is limited only from catchment area, and its management is very important for water quality in rainwater tank. Most of the contaminants come into the rainwater tank which is removed by sedimentation [5] and sludge generated thereby is able to lower the water quality by resuspension. Application of simple design such as sludge drain, calm inlet, intermediate wall, and baffle can control the contaminant in rainwater tank to the certain level [6].

Biofilm is one of the factors influencing the rainwater quality in tank. Many researches showed that presence of biofilm includes negative effects, such as biofouling in filter and biocorrosion and biocontamination in drinking water distribution networks, but also positive effects such as biofilm reactors for the degradation or production of chemical substances in wastewater treatment process [7–9]. It has been suggested that biofilm may have a function of self-cleaning of the tank and regulation of the microbial quality in rainwater [1, 2]. Although biofilm might have a positive impact on stored rainwater quality, only few studies investigated bacterial composition and distribution, its development and role in this particular environment. Through the research on these characteristics of biofilm in rainwater tank, it is possibly suggested a better information to improve the rainwater system in management and design perspectives

In this chapter, to establish the role of biofilm in rainwater tank, (1) it was investigated the kinds of bacteria that inhabit rainwater tanks, (2) the changes in the biofilm cells and the bacterial community during biofilm development, (3) the microbiological characteristics of rainwater in two tanks with different S/V ratios to identify how the internal design features of storage tanks affect the microbial quality of rainwater, and then (4) suggested design and maintenance guideline for rainwater tank.

2. Method and materials

2.1. Study sites

This study was carried out at Seoul National University in Seoul, Korea (**Figure 1**). In order to investigate the microbial community and how biofilms are developed and in operating

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rainwater tank, the choice of sites for sample collection was made mainly with regard to the availability of the rainwater facility.

The system 1 was built in November 2003 and consists of a 200 m^3 concrete storage tank located underground and a 2098 m^2 roof catchment area. The harvested rainwater supplies to the toilets of 167 households and a garden [10]. In this system, the study about microbial community was performed.

The system 2, which was constructed in October 2005, comprises a 250 m^3 main storage tank, a 27 m^3 smaller extra tank, and a 4 m^3 supply tank located underground. The catchment area is a concrete roof surface with a total area of 2828 and 824 m^2 terrace. Rainwater collected from the roof of one part of the building (960 m^2) flows into the main tank and that from the roof of another part of the building (1868 m^2) and the aforementioned terrace flows into the extra tank. When the water in the extra tank reaches 1.2 m in depth, it is pumped into the main tank. About 1000 full-time staff and students occupy the building, and the amount of water used each day to flush the toilet is approximately 60–90 m^3. In this system, study about biofilm development process in rainwater tank was performed.

System 3 and system 4 were installed at Buddle-gol, Seoul National University in October 2007, which collects rainwater from the valley of Mount Gwanak. Tank 3 was designed a concrete square with a storage volume of 20 m^3. Tank 4 was assembled from polypropylene units with 95% pore space and has a storage volume of 20 m^3. In these systems, study about microbial behaviour by full-scale spike test described was performed.

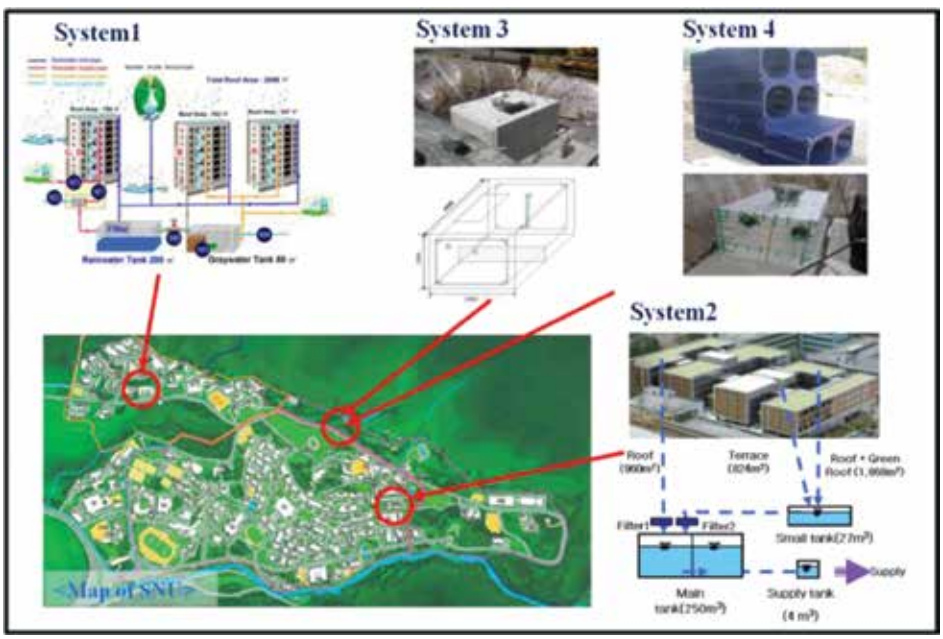

Figure 1. Schematic diagram of the four study sites in SNU campus.

2.2. Sampling sketches

2.2.1. Sampling and sample preparation for PCR-DGGE

In the system 1, the rainwater from the roof flows through a filter (VF6 type with a mesh size of 0.65 mm and a capacity of 70.5 L/s) at first, and then enters the main tank through a calm inlet. Inside the tank, the W × L × H ratio changes from 7.4 × 15.4 × 2 to 3.7 × 30.8 × 2 due to the installation of a baffle.

The sampling points were indicated in **Figure 2**. Rainwater of 1.5 L was sampled at a depth of 50 cm in the tank and was carried directly to the lab in a sterile water bottle. Biofilm was collected from 0.04 m^2 of the wall surface in the tank and placed in a sterile tube containing 20 ml of distilled water.

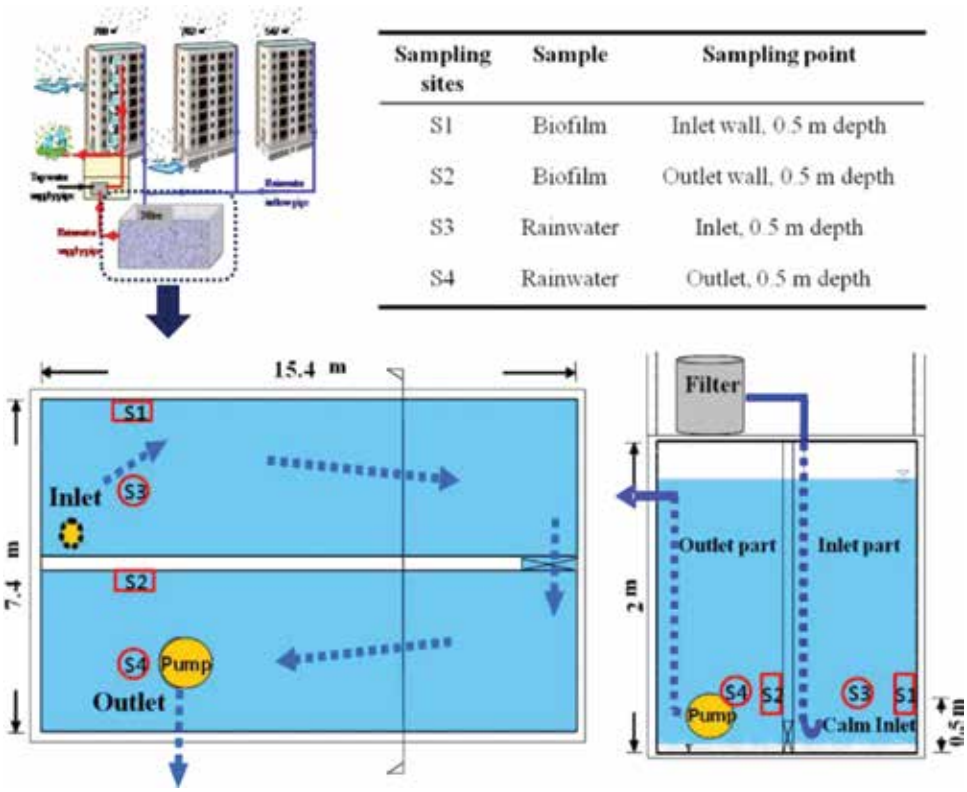

Figure 2. Schematic diagram and description of the sampling points in system 1.

Water samples in system 3 and system 4 were collected for physicochemical monitoring and after spiking test at a depth of 1.3 m from the bottom, around the point of supply in each tank (**Figure 3**). Three replicate samples were taken on four different occasions between May and August 2010.

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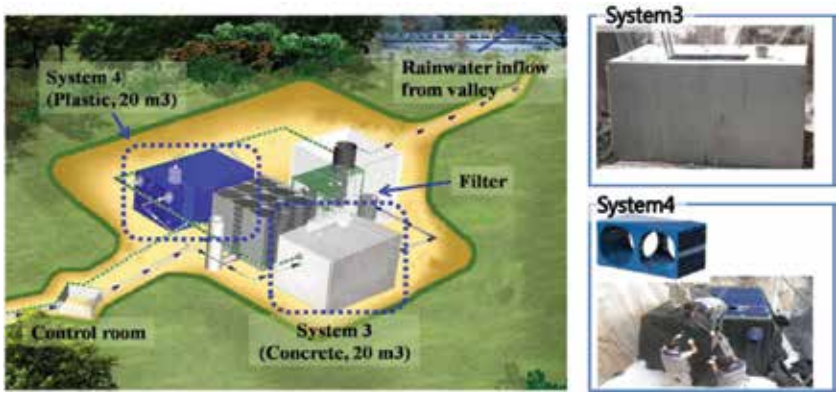


Figure 3. Schematic diagram of system 3 and system 4 used in full-scale experiments.

2.2.2. Coupon preparation and sampling

2.2.2.1. Characteristics of biofilm development on the surface

To study the biofilm formation on the surface, 3 × 8 × 0.5 cm acrylic coupons were prepared (**Figure 4**). These coupons were immersed at the inlet and outlet of the system 2 tied to an acrylic support placed in the middle of the tank at a depth of 2 m from the bottom (**Figure 5**).

Figure 5 shows the plans and cross-sectional views of the tank, and sampling points in system 2. To minimize the influence of flow velocity during the experiment period, no rainwater was supplied, but overflow was permitted.

To evaluate biofilm growth, several tests were carried out with quantify the biofilm development on the surface, following the schedule presented in **Table 1**.

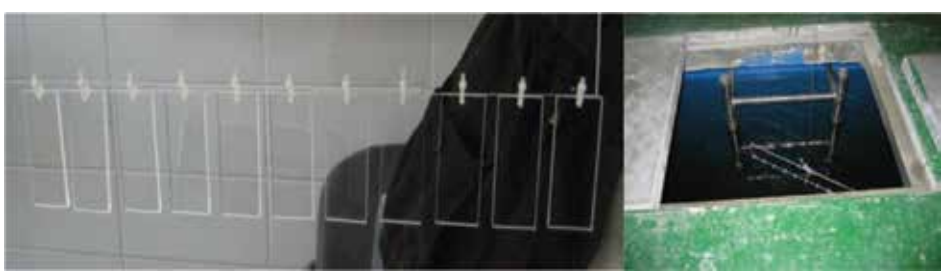


Figure 4. Picture for coupon prepared and set up in the tank.

Water samples were collected from the inlet and outlet portions of the tank once a month in two 1 L sterile screw-cap containers. The samples were taken at a depth of 2 m from the bottom of the tank to collect data on the coupons' environment. The samples were transported to the laboratory and analysed within 30 min of collection.

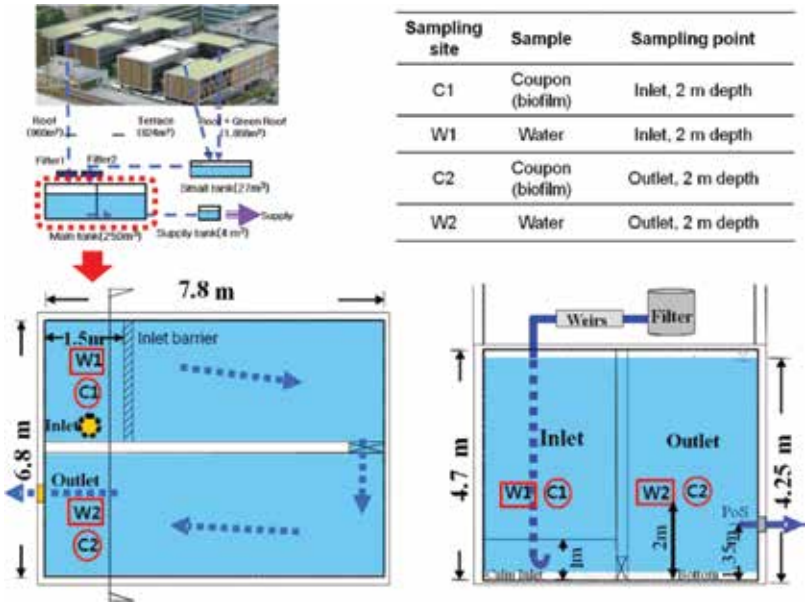


Figure 5. Schematic diagram and description of the sampling points in system 2.

Weeks (after immersion in the tank)	0	1	4	5	8	9	12	15	16
Coupon samples for HPC		✔	✔			✔		✔	
Coupon samples for CLSM		✔		✔		✔		✔	
Coupon samples for PCR-DGGE		✔		✔		✔		✔	
Water samples for pH, turbidity, EC, DO, Temp., SS, TN, TP, TOC, HPC	✔		✔		✔		✔		✔
Water samples for PCR-DGGE								✔	

CLSM, confocal laser scanning microscopy; EC, electric conductivity; HPC, heterotrophic plate count; SS: suspended solids.

Table 1. Experimental schedule for coupons and water sampling in system 2.

2.3. Physicochemical characteristics

The various physicochemical parameters of the rainwater, such as temperature (Sension 1, Hatch, Japan), pH (Sension 1, Hatch, Japan), dissolved oxygen (DO) (ProODO, YSI, USA), electric conductivity (EC) (Sension 378, Hatch, Japan), turbidity (2100P, Hatch, Japan), suspended solid (SS) total nitrogen (TN) (HS-TN-L kit, Humas, Korea), total phosphate (TP) (HS-TP-L kit, Humas, Korea), and total organic carbon (TOC) (V CPH kit, Shimadzu, Japan) were measured.

2.4. Enumeration of bacteria

The heterotrophic bacteria were quantified using the conventional microbiological culture method. Faecal coliform tests were carried out through membrane filtration procedure, Standard method 9222D (APHA, 1998).

2.5. PCR-DGGE analysis

2.5.1. DNA extraction

The sample of rainwater and the detached biofilm sample in the PBS were separately passed through a filter, and genomic DNA was isolated with a water RNA/DNA purification kit (Norgen, Canada) according to the manufacturer's instructions.

2.5.2. Polymerase chain reaction (PCR)

The EUB 341F-GC and PRUN518R primer pair, comprising universal primers specific to bacteria, was used [11] for PCR, which was performed with a thermal cycler (GeneAmp PCR System 9700, Perkin Elmer). The PCR conditions are described in **Table 2**.

Primer set	Operation temperature and thermal cycler time (Temp., Time)					Cycles
	Initial denaturation	Denaturation	Annealing	Elongation	Final extension	
EUB 341F-GC, PRUN518R	94°C,	94°C	55°C	72°C	72°C	33
EUB 341F, PRUN518R	15 min	45 s	45 s	45 s	7 min	

Table 2. Reaction conditions for the PCR.

2.5.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed using a D-Code system (Bio-Rad, USA). The 8% polyacrylamide gel contained a series of denaturant concentrations ranging from 30 to 60% (formamide and urea). The gels were run at 70 V for 11 h in a 1 × TAE buffer at 60°C. After electrophoresis, the gels were stained with ethidium bromide in a 1 × TAE buffer for 15 min and then destained in DDW(Deionized distiled water) for 20 min. The DGGE gels were visualized with a UV transilluminator (302 nm) mounted with a digital camera to capture photographs of them.

2.5.4. Re-amplification of the DGGE bands and sequencing

The DNA bands on the DGGE gels were excised under UV transillumination using sterile scalpels and then soaked overnight in 50 μL of sterile DDW at 4°C. Two μL of DNA solution was used for re-amplification with the same primer pair without a GC clamp. The reaction conditions for the PCR were the same as those described in **Table 2**. The PCR products were purified using a kit (AccuPrep PCR purification kit, Bioneer, Korea) and then sequenced using EUB341F (for bacteria) and F984 (for actinomycetes) in an automatic DNA sequencer (ABI

Prism 3730 XL DNA Analyzer, PE Applied Biosystems). The DGGE band sequences were compared with 16S rDNA sequences obtained through a BLAST search from the database of the DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/top-e.html).

2.6. CLSM analysis

To observe the thickness of the biofilm via CLSM, two coupons from each part of the tank were sampled in sterile Petri dishes, and a BacLight Live/Dead bacterial viability kit (L-7012, Molecular Probes, USA) was employed to stain the live and dead cells. Photographs of two random locations on each coupon were taken with a Carl Zeiss LSM 510 microscope. The CLSM images were analysed with an Image Structure Analyzer (ISA) [12].

2.7. Spike test

2.7.1. Pilot-batch tanks

To investigate the behaviour of microbial populations in spike tests in pilot-scale batch tanks with different S/V ratios, 200-liter (L) polyethylene (PE) tanks were filled with 100 L of rainwater. The S/V ratios were set to 10 and 50 m^{-1} by installing acrylic plates (50 × 20 × 0.2 cm) (**Figure 6**). To ensure that a sufficient amount of biofilm attached to the tank walls before

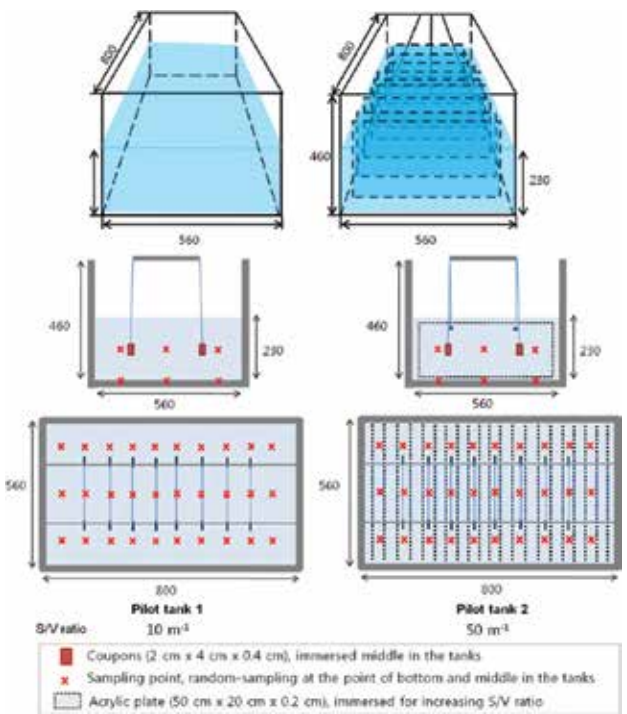


Figure 6. Schematic diagram for the pilot-scale batch experiments.

the spike test, the tanks were filled with 100 L of rainwater and stored for 4 weeks. Ten litres of rainwater per day were then replaced, and the retention time was controlled at 10 days. The water was stored in the dark at room temperature (20°C).

2.7.2. Full-scale tanks

Two full-scale rainwater tanks of system 3 and system 4 were employed to investigate the behaviour of the microbial populations in spike tests carried out with different S/V ratios (**Figure 3**). The S/V ratio was 2 m^{-1} in system 3 and 15 m^{-1} in system 4. The retention time was 10 days. The difference of tank material between two tanks was assumed to be negligible because material for biofilm formation primarily affects in the initial steps [13] and the two rainwater tanks used in this study had been in operation for 3 years.

2.7.3. Bacteria preparation and inoculation

Pseudomonas aeruginosa (KCTC #1636), a ubiquitous environmental bacterium that forms biofilms on wet surfaces such as those of rocks and soil, was used in the spike tests. The *P. aeruginosa* were grown to an exponential phase (OD600 = 1.2, containing approximately 5×10^7 CFU/mL) in Luria-Bertani (LB) broths and washed twice with phosphate-buffered saline (PBS) (centrifuge at 8000 rpm, 4°C for 10 min). *P. aeruginosa* was put into the tanks at a final concentration in rainwater of about 5×10^5 CFU/mL for the pilot tests and 1.3×10^4 CFU/mL for the full-scale tests.

2.7.4. Sampling

Fifty microlitres of water samples were taken in duplicate from the bottom and middle sections of the two pilot tanks, and coupons were tested randomly every day for 8 days (**Figure 6**). In the full-scale test, 1 L rainwater samples were taken in duplicate from the two tanks every day for 10 days. The pH value in both pilot tanks was 7.1 ± 0.1; the DO was 7.9 ± 0.5 mg/L in Pilot Tank 1 and 6.7 ± 0.4 mg/L in Pilot Tank 2.

3. Results and discussions

3.1. Composition and distribution of bacteria in an operating rainwater harvesting tank

3.1.1. Physicochemical conditions in rainwater tanks create a distinct microbial habitat

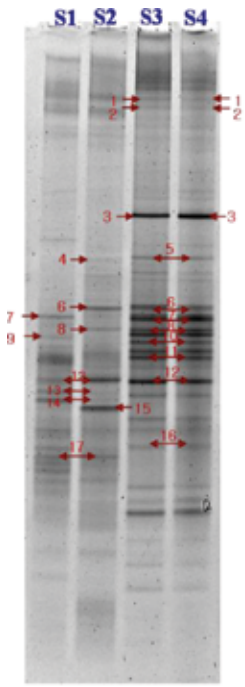
The turbidity, EC, SS and VSS were lower at the outlet than at inlet, and the DO was slightly lower at the inlet than at the outlet. The TN and total phosphorous were 4.9 ± 0.4 and 0.08 ± 0.04 mg/L at the inlet but decreased at the outlet to 4.4 ± 0.2 and 0.05 ± 0.01 mg/L. The COD was 1.9 ± 1.12 and 0.9 ± 0.01 mg/L, and the TOC was 0.78 ± 0.03 and 0.26 ± 0.15 mg/L at the inlet and outlet, respectively. The values of the parameters were better at the outlet than at the inlet.

Because the rainwater tank under study is installed underground, the lack of sunlight and the average water temperature of as low as 19°C led to the absence of photosynthetic microbes

such as algae. The nutrient input depended on rainfall. Rainwater tanks indicated an oligotrophic environment, as the concentration of dissolved organic matter in these habitats is commonly <10 mg/L [14]. The inflow and outflow of rainwater in such tanks change according to the precipitation and rainwater usage. Thus, rainwater tanks constitute a unique habitat for microbes.

3.1.2. Bacterial composition and distribution

The bacterial composition in the rainwater and biofilm samples showed different tendencies. Seventeen species were identified from the selected DGGE bands (**Figure 7**). According to the standard phylogenetic classification of prokaryotes, the species belonged to 13 genera, 10 families, 8 orders, 5 classes and 3 phyla. Proteobacteria accounted for 88% of the species identified, with the remainder being Bacteroidetes and Firmicutes.



Species	Similarity (%)	Band No.	Accession number
Proteobacteria			
Limnohabitans planktonicus	96	1	FM165535
Limnohabitans curvus	96	2	AJ938026
Limnohabitans sp	100	3	AJ938027
Ralstonia insidiosa	100	4	FJ772078
Aquaspirillum sp.	95	5	AF321032
Novosphingobium resinovorum	97	6	X94103
Sphingopyxis sp.	99	7	FJ889532
Sphingomonas sp.	97	8	AB288316
Blastochloris sulfoviridis	96	9	F654663
Rubrivivax gelatinosus	98	10	F655912
Sphingobium yanoikuyae	97	12	GQ214009
Sphingomonas sp.	100	14	Z23157
Sphingobium sp.	99	15	FJ966197
Rhodobacter gluconicum	97	16	AB077986
Beijerinckiaceae bacterium	98	17	FM252034
Bacteroidetes			
Roseivirga ehrenbergii	91	11	AY739663
Firmicutes			
Bacillus sp.	98	13	EF489025

Figure 7. DGGE profiles at each sampling point and closest identified phylogenetic relatives found in the DGGE bands.

The DGGE profiles showed a clear difference between the planktonic bacterial community and the community in the biofilm (**Figure 7**). The bacterial composition tended to differ across the biofilm samples, but was similar across the rainwater samples. *Rubrivivax gelatinosus*, *Roseivirga ehrenbergii*, *Limnohabitans* sp., *Aquaspirillum* sp. and *Rhodobacter gluconicum* were identified only in the rainwater, whereas *Sphingomonas* sp., *Sphingobium* sp., *Ralstonia insidiosa*, *Blastochloris sulfoviridis*, *Bacillus* sp. and *Beijerinckiaceae bacterium* were found in the biofilm. Some species,

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such as *Sphingopyxis* sp., *Sphingomonas* sp., *Novosphingobium resinovorum* and *Sphingobium yanoikuyae* were found in the both rainwater and biofilm samples.

The bacterial composition in the biofilm differed according to the location. *Sphingopyxis* sp. (Band No. 7) and *Blastochloris sulfoviridis* (Band No. 9) were detected in the inlet samples, whereas *Ralstonia insidiosa*(Band No. 4), *Novosphingobium resinovorum* (Band No. 6), *Sphingomonas* sp. (Band No. 8), *Sphingobium* sp. (Band No. 15) were found only in the outlet samples. *Sphingobium yanoikuyae* (Band No. 12), *Bacillus* sp. (Band No. 13), *Sphingomonas* sp. (Band No. 14), and *Beijerinckiaceae bacterium* (Band No. 17) were detected in both locations. Similar bacterial composition indicated at the inlet and outlet rainwater samples.

The samples contained mostly nonpathogenic proteobacteria. Many of the bacteria identified were closely related to fresh water, soil and biofilm bacteria found in natural environments [15–20]. Eighty-eight percentage of the identified bacteria were proteobacteria. It have been reported that proteobacteria are consistently more abundant at pristine sites, whereas Firmicutes and Actionobacteria are dominant at polluted sites [21]. Though estimates were made in terms of detection ratio only in this study and the species were not quantified, the results still indicate the clean oligotrophic nature of the tank water.

The bacterial composition in the biofilm was different from that in the rainwater. It is known that biofilm formation provides an advantage for bacteria that exist in oligotrophic environments [22]. Some of the species identified in the biofilm in this study, such as *Bacillus* sp., *Sphingomonas* sp. and *Sphingobium* sp., have been demonstrated to degrade certain contaminants and to act as bio-control agents [17, 18, 23]. These species may be relatively sensitive to nutrients in oligotrophic conditions and thus tend to develop a biofilm to survive. Therefore, in oligotrophic rainwater tanks, microbial species possibly remain constant in rainwater tank through biofilm formation.

3.1.3. Self-purification possibility of rainwater tanks and implications for rainwater quality

Bacterial communities in nature play a key role in the production and degradation of organic matter and many types of environmental contamination, and the cycling of nitrogen, sulphur, and many metals [24]. In addition, the sorptive capacity of biofilm for dissolved organic matter and metals has been widely demonstrated in sewage and marine systems [25, 26]. Thus, biofilm formation in rainwater tanks seems not only to promote the survival of bacteria, but also serves as a natural filter by removing contaminants and bacteria from rainwater.

3.2. Characteristics of biofilm development in rainwater tank

3.2.1. Physicochemical and microbial conditions in rainwater

The temperature of the stored rainwater ranged from 16 to 22°C, and the pH was around 7. At the inlet and outlet of the tank, the turbidity was 2.9 ± 1.6 and 2.1 ± 1.0 NTUs, respectively; the SS count was 3.2 ± 1.8 and 1.3 ± 0.8 mg/L, respectively; the TOC was 1.56 ± 0.54 and 0.91 ± 0.97 mg/L, respectively; and the TP count was 0.07 ± 0.04 and 0.04 ± 0.01 mg/L, respectively. Thus,

the particle and nutrient parameters of the rainwater, namely turbidity, SS, TOC, and TP, at the outlet of the tank were slightly lower than those at the inlet.

Figure 8 shows the number of viable cells at the inlet and outlet of the tank during the experimental period. The difference between the two sites was significant ($P < 0.05$): the number of viable cells at the inlet was triple that at the outlet (3×10^5versus 1×10^5 CFU/mL, respectively).

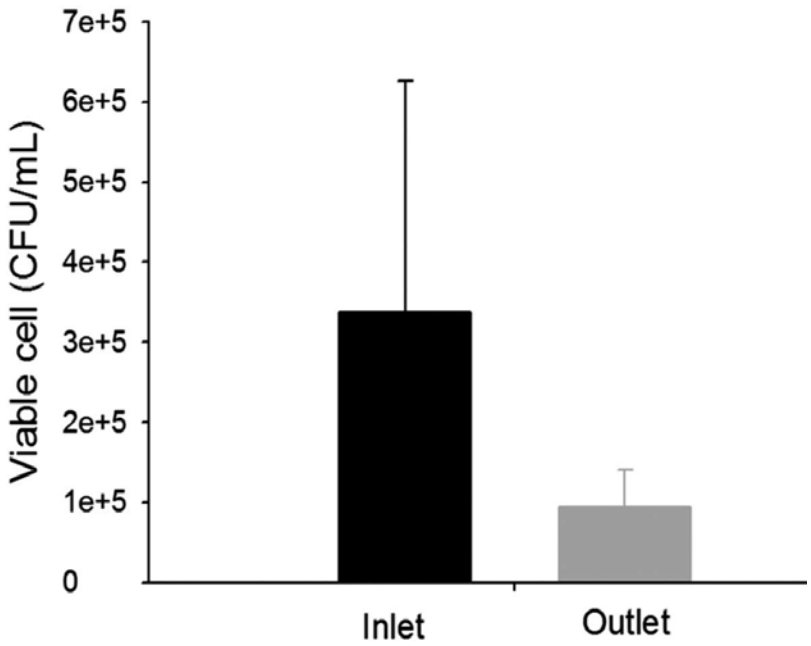

Figure 8. Comparison of the viable cell quantities at the tank inlet and outlet. The difference between them is statistically significant (Student's t-test; $P < 0.05$, $n = 12$).

The rainwater tank used in this study was designed with an internal wall in the inlet section and a baffle in the middle to improve sediment efficiency. Ryu [6] reported that such design factors as inlet barrier and baffles can affect the removal of the particles that come into a rainwater tank. Hence, the slight differences in the physicochemical characteristics identified at the tank inlet and outlet in this study appear to be due to these design factors. In addition, the physicochemical conditions appear to influence the microbes in the water, as can be seen in the different microbial numbers.

3.2.2. Comparing cell dynamics on coupon: cell number

To compare the biofilm development at the tank inlet and outlet, we also investigated the number of viable cells on the coupons immersed at each site (**Figure 9**). The two sites exhibited a similar number of cells until the fourth week. At week 9, however, the number at the inlet was 2.5 times higher than that at the outlet (2.7×10^5 and 1.1×10^5 CFU/cm^2, respectively),

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suggesting there was greater biofilm growth at the former. After 15 weeks, the corresponding figures were 3.6 CFU/cm^2 at the inlet and 3.0 CFU/cm^2 at the outlet, and the difference in cell numbers between the two sites had been reduced to 1.2 times.

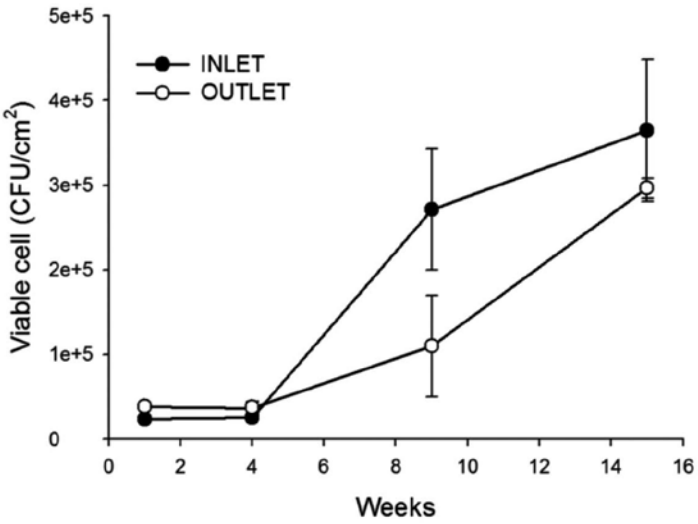

Figure 9. Number of viable cells on the coupons immersed at the tank inlet and outlet for 15 weeks.

Mature biofilm development may take anywhere from several hours to several weeks, depending on the system [27]. Biofilm formation is one possible survival strategy for bacteria, and one of the advantages of bacterial adherence is the greater availability of nutrients attached to the surface [28]. Geesey et al. [29] reported high rates of biofilm development in oligotrophic environments. In the current study, biofilm formation was observed on the coupons after 1 week of immersion. Considering the oligotrophic nature of rainwater tanks [2], it appears that biofilms develop within 1 week in this environment.

In this study, cell number in outlet site would be smaller than that in inlet part because of attachment to the existing biofilms on the wall in inlet part and sedimentation with small particles. The difference in biofilm formation between inlet and outlet part would be results of nutrient concentration and planktonic cell number in rain water flowing from inlet part. The influence of flow velocity and the substratum effect was most likely excluded in this study because, during the experiment period, a coupon of identical material was placed in the middle of the tank and to minimize the effect of water flow, the water flow was controlled without supply by overflowing.

3.2.3. Biofilm thickness

The CLSM images of the biofilm thickness on the coupons exhibited similar viable cell patterns (**Figure 10**). At the tank inlet, the thickness was 4.5 ± 0.1 μm at the end of the first week, increasing to 48.4 ± 1.3 μm at week 9 and then decreasing to 25.0 ± 2.8 μm at week 15.

Apilanez et al. [30] demonstrated that once biofilm has attained a certain weight, which can be related to a certain thickness, detachment occurs. The development of greater biofilm thickness can thus lead to earlier, and a great extent of detachment. Several processes can lead to detachment: erosion or shearing, and sloughing and abrasion. Donlan [31] wrote that when biofilm increases in thickness, its rate of erosion also increases. The detached biofilm possibly settles at the bottom of the tank, but it also provides a way for cells to migrate and colonize a less populated area.

At the tank outlet, in contrast, a biofilm thickness of 5.7 ± 0.7 μm was seen at the end of the first week and that thickness continued to increase until it reached 29.5 ± 2.0 μm at week 15. No detachment phase was observed at the outlet in this study. It seems that because the nutrient concentration is lower at the outlet, biofilm development is slower at that site.

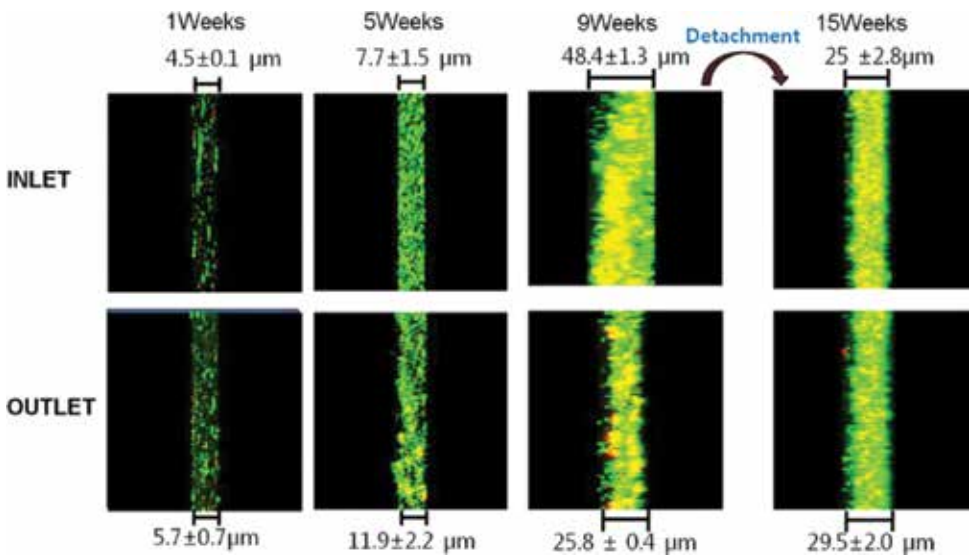

Figure 10. Comparison of biofilm thickness obtained by CSLM (average ± standard deviation, n = 4).

3.2.4. Dynamics in bacterial community

Two sets of biofilms displayed changes in their DGGE banding patterns and number of bands as they developed (**Figures 11** and **12**). Differences were apparent between the inlet and outlet samples, both in the individual samples of a specific age and in the overall pattern of bacterial community development. The biofilm at the inlet exhibited a greater number of bands in the earliest sample (1 week), displaying a decrease by the fourth week and then increasing again. At the outlet, there were also a greater number of bands after 1 week, a decrease by the ninth week, and then a slight increase. The band patterns appeared similar between the initial two stages (weeks 1 and 4) and later two stages (weeks 9 and 15), and seemed to simplify over time as the biofilm developed.

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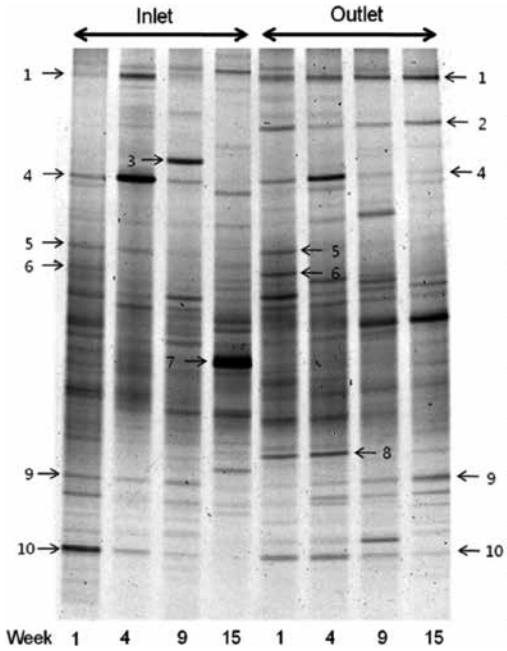

Band No.	Closest species	Similarity (%)	Accession No.
1	*Methylophilus methylotrophus*	94	GQ411499_1
2	*Methylophilus sp.*	95	AY436794_1
3	*Arcicella rosea*	98	GU368373_1
4	*Methylophilus sp.*	98	AY436794_1
5	*Methylotenera mobilis*	94	DQ287786
6	*Microbacterium pumilum*	99	AB234027
7	*Acidovorax delafieldii*	98	GU195154
8	*Bacillus sp.*	93	DQ305318
9	*Methylocella palustris*	95	Y17144
10	*Nitrospira sp.*	97	AF035813

Figure 11. DGGE profiles at each sampling time point and the closest phylogenetic relatives found in the DGGE bands.

Following a high number of bands at the initial stage, reductions occurred later, possibly arising from the competitive dominance of a few populations. The biofilms at the inlet and outlet demonstrated a reduction in the number of bands after the first sample date. The populations that were initially detected may still have been present in later biofilms, but the rapid growth of other populations made them more difficult to detect. As biofilm matures, the number of available microhabitats may increase (for example, from the formation of an anaerobic pocket within the biofilm), thereby supporting a greater number of bacterial populations.

Despite the overall differences in banding patterns, a number of bands appeared at the same position in the DGGE gels in almost all of the samples, including *Methylophilus methylotrophus* (Band No. 1), *Methylocella palustris* (Band No. 9) and *Nitrospira* sp. (Band No. 10), although their intensity differed. *Methylophilus* sp. (Band No. 2) was found only at the outlet site at all stages, whereas *Methylotenera mobilis* (Band No. 5), *Microbacterium pumilum* (Band No. 6) and *Bacillus* sp. (Band No. 8) were identified in the earlier samples (week 1 and/or week 4). Some bands, such as *Nitrospira* sp. (Band No. 10), faded over time.

In the initial stage of biofilm formation, free-swimming bacteria attach to the surface through hydrophobic and electrostatic interactions and through the use of flagella [32]. In this study, for example, both *Methylotenera mobilis* (Band No. 5) and *Microbacterium pumilum* (Band No. 6) were detected in the earlier samples. The former is mobile by means of a single flagellum [33], and the latter is non-motile [34]. Initial colonization on the surface may not be entirely random,

in that certain bacterial species may have greater colonization aptitude than others, such as greater mobility.

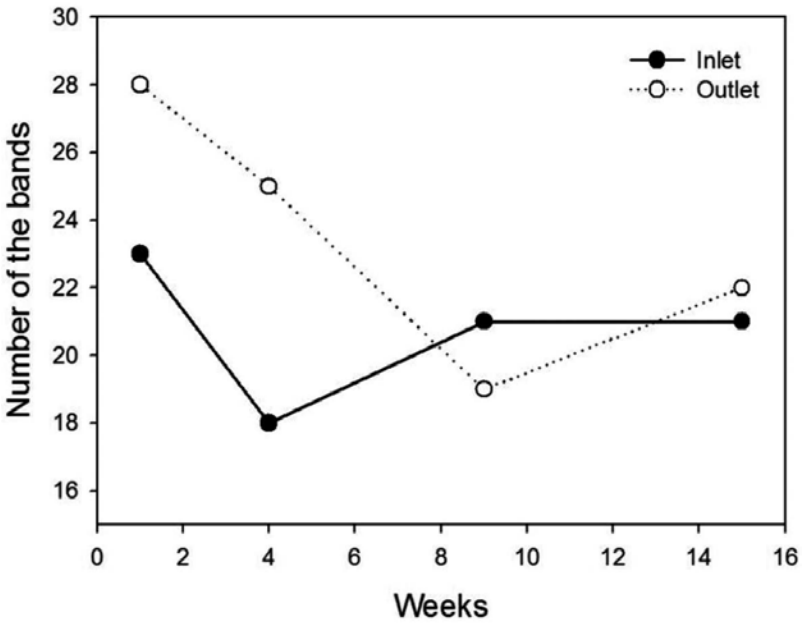

Figure 12. Changes in the number of DGGE bands in the biofilm during the experimental period.

Rank-abundance distributions provide insights into both richness and evenness. In this study, the rank-abundance plots displayed a trend towards a geometric distribution (**Figure 13**), and linear regression was performed to examine changes in the pattern of evenness during biofilm development (**Table 3**). Lower slope values indicate greater evenness, and higher values indicate greater dominance by certain populations. At the tank inlet, the slope value was −0.113 at the end of the first week. It then increased steeply after 4 weeks, decreased after the ninth week, and then increased again. At the outlet, in contrast, the slope value increased sharply at the 9-week sample and then exhibited a decrease at the last sample.

The pattern of biofilm development seems to follow three major stages. Jackson et al. [35] suggested an initial stage characterized by the colonization of different populations, an intermediate stage characterized by a limited number of dominant populations utilizing similar resources, and a late or mature stage characterized by mature biofilm of a complex spatial structure that facilitates greater diversity through increased variation in habitat and available resources. However, in the current study, this characterization appeared to apply only up until the mature stage, after which detachment occurred. Following detachment, biofilm development appeared to return to the intermediate and/or mature stage, and the process was then repeated. In this study, this pattern/cycle was confirmed at the tank inlet site, where biofilm development was more rapid throughout the experimental period than at the

outlet site, most likely due to the difference in nutrient concentrations. As the biofilm development process was faster at the tank inlet, more sludge from the detachment was also seen at this site.

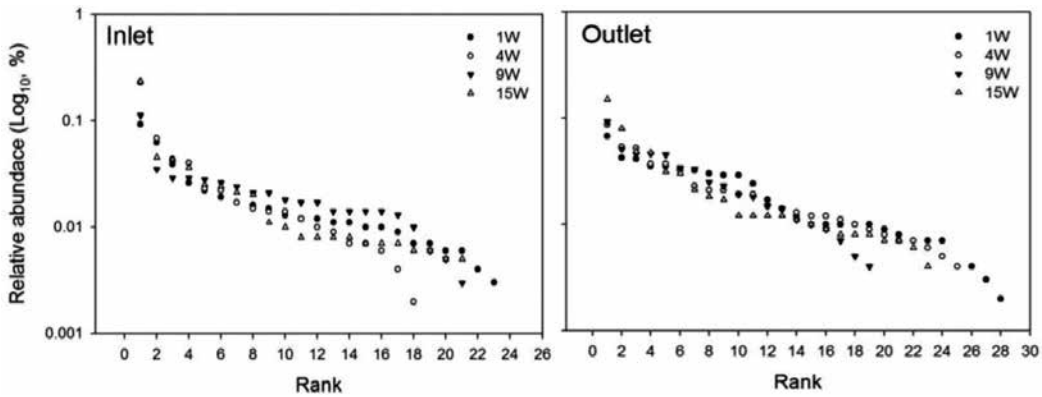

Figure 13. Rank-abundance distributions of bacteria in different periods of biofilm development.

Sample	Inlet		Outlet	
	Slope	r^2	Slope	r^2
1 week	−0.113	0.90	−0.106	0.96
4 weeks	−0.194	0.89	−0.105	0.97
9 weeks	−0.111	0.84	−0.150	0.98
15 weeks	−0.141	0.81	−0.123	0.88

Table 3. Regression statistics for rank-abundance distributions of bacteria in the biofilm at different sites and stages of development ($P < 0.05$; n varies by sample).

3.3. The effect of biofilms on microbial quality in rainwater tanks

3.3.1. P. aeruginosa removal in water

The persistence of the *P. aeruginosa* cells inoculated into the pilot tanks resulted from the interaction between the cell growth and death rates and from that among the attachment, detachment, and sedimentation processes. The total number of inoculated cells in the water samples and on the bottoms and walls of the tanks decreased in both tanks (**Figure 14**). Cell death contributed more to the observed cell decline than did growth in the tanks due to low-nutrient conditions.

Figure 15 shows the removal rate of *P. aeruginosa* from the water of the two pilot tanks. Ninety-nine percent of the inoculated *P. aeruginosa* was removed after 4 days in Pilot Tank 2 and after 5 days in Pilot Tank 1. The faster removal rate in Pilot Tank 2 was due to its higher S/V ratio.

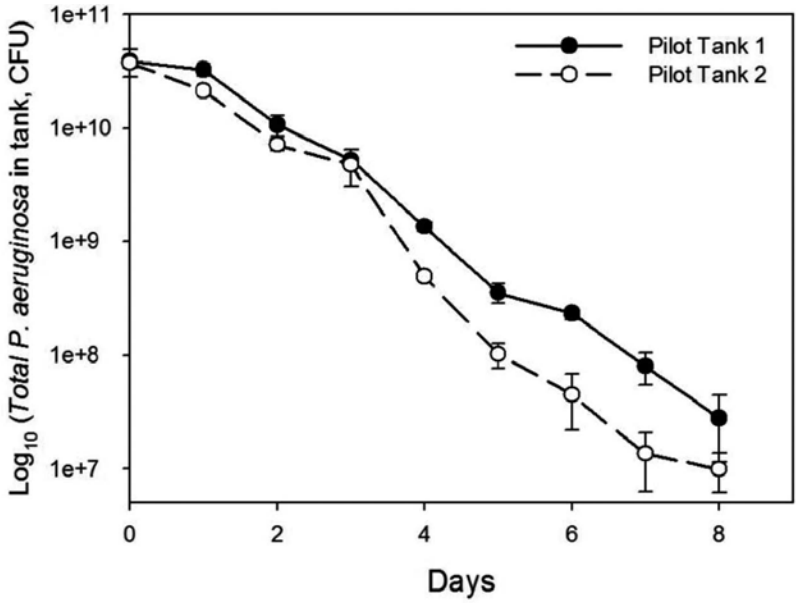

Figure 14. Total amount of *Pseudomonas aeruginosa* inoculated in pilot-scale tanks. . [Student's t-test; $P < 0.05$ except Day 0 ($P = 0.55$) and 3 ($P = 0.15$)].

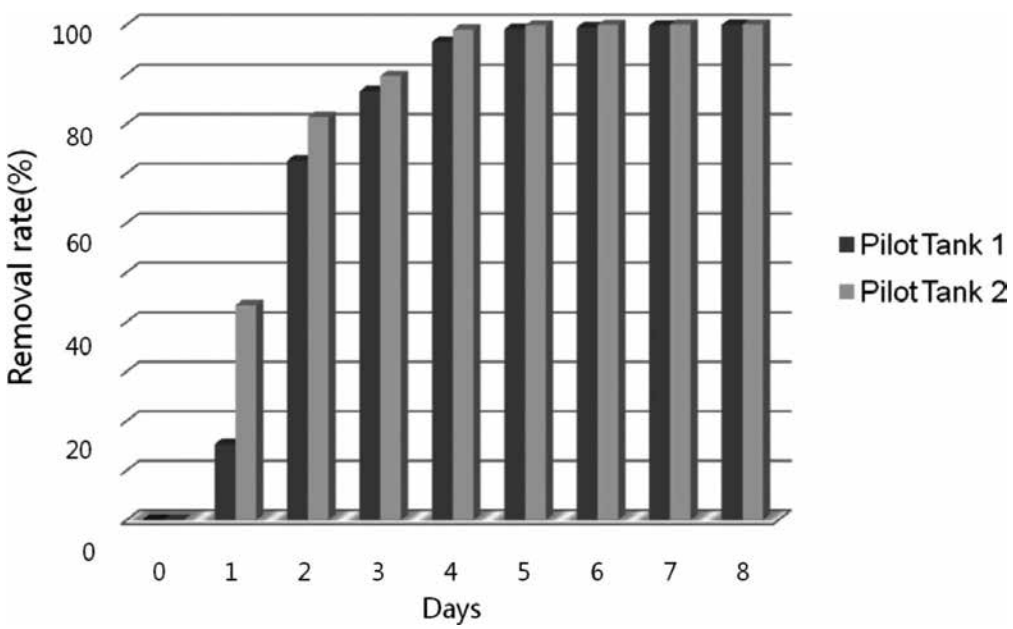

Figure 15. Removal rate of *Pseudomonas aeruginosa* inoculated in the water of the two pilot tanks.

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3.3.2. Microbial behaviour of P. aeruginosa put into the rainwater tanks

The number of *P. aeruginosa* in the water decreased by 3–4 log units, indicating that the death, attachment and sedimentation processes dominated the overall dynamics (**Figure 16A**). The removal rate of *P. aeruginosa* in the water phase was −0.57 $\log_{10}$ cells ml^{-1} day^{-1} ($r^2 = 0.93$) in Pilot Tank 1 and −0.74 $\log_{10}$ cells ml^{-1} day^{-1} ($r^2 = 0.98$) in Pilot Tank 2. A faster removal rate was shown in the tanks with higher S/V ratios.

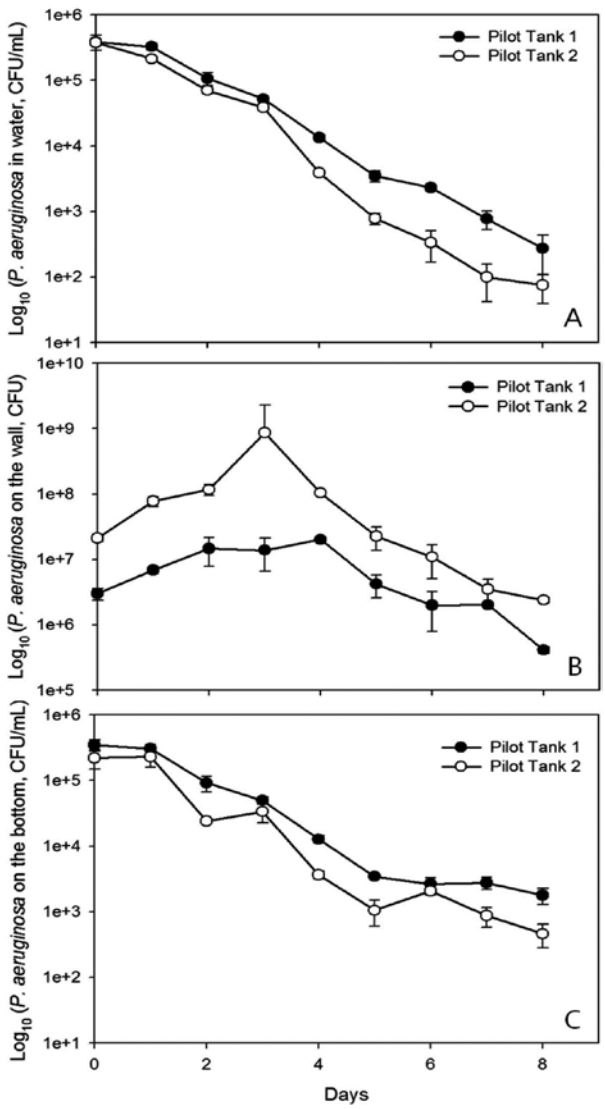

Figure 16. Behavior of Pseudomonas aeruginosa inoculated in (A) the water and on the (B) wall and (C) bottom of the8 pilot-scale tanks. [Student's t-test; (A) $P < 0.05$ except Day 0 ($P = 0.51$); (B) $P < 0.1$ except Day 0 ($P = 0.17$) and 1 ($P = 0.14$); (C) $P < 0.01$ except Day 7 ($P = 0.10$)].

The number of attached *P. aeruginosa* cells increased over 4 days in Pilot Tank 1 and over 3 days in Pilot Tank 2 (**Figure 16B**). Their attachment to the biofilm on the wall was initially dominant, and more bacteria were attached in Pilot Tank 2 because of the higher S/V ratio.

After 4 days, the number of attached *P. aeruginosa* cells declined by 2–3 log units, indicating that the death or detachment processes were the dominant bacterial dynamics on the wall (**Figure 16B**). Established biofilms developed from indigenous river water bacteria have been shown to reduce the persistence of introduced *E. coli* and other enteric pathogens [36]. Banning et al. [37] showed that, under certain conditions, the presence of mixed-populated biofilms may limit the survival potential of enteric bacteria pathogens introduced into groundwater. In addition, biofilm dynamics changes and pathogen persistence are affected by increasing in nutrient levels. It was reported that a significant decrease in the survival rate of the *Campylobacter jejuni* strain in heterogeneous tap-water biofilms following the addition of serine, a carbon source favoured by *C. jejuni*, and a concurrent increase in the number of indigenous biofilm microflora [38]. These studies demonstrate that, under certain conditions, biofilms represent sites of intensified competition for limited nutrients. Therefore, for the biofilms in oligotrophic rainwater tanks, a decrease in *P. aeruginosa* cells may result from the nutrients competition with indigenous microbial communities.

Inoculated *P. aeruginosa* were found on the bottom in tanks and decayed over time (**Figure 16C**). More bacteria observed at the bottom of Tank 1, which had a lower S/V ratio, and more bacteria observed on the wall in Tank 2. The number of *P. aeruginosa* increased slightly on days 3 and 6 in Tank 2, probably due to detachment from the wall rather than bacterial regrowth, as this effect was not observed in Tank 1.

3.3.3. Microbial behaviour of P. aeruginosa put into the full-scale tanks

The number of *P. aeruginosa* in the water decreased by 1.5 log units in Tank 1 and by 2 log units in Tank 2 (**Figure 17**). The removal rate was −0.604 $\log_{10}$ cells ml^{-1} day^{-1} ($r^2 = 0.99$) in Tank 1 and −0.854 $\log_{10}$ cells ml^{-1} day^{-1} ($r^2 = 0.98$) in Tank 2. In line with the results of the pilot test, a faster removal rate was shown in Tank 2 due to its higher S/V ratio. Thus, it can be concluded that increasing the S/V ratio in rainwater tanks to a certain level is possibly effective to remove bacteria from rainwater.

In this study, the removal rates of *P. aeruginosa* were determined by calculating the slope and correlation coefficient (r^2) of the linear regression of the log-transformed cell concentration data according to the first-order decay equation. Crane and Moore [39] reviewed a variety of modified models of first-order decay kinetics and concluded that the simplest model is the most advantageous. As noted, the findings of the current study suggest that increasing the S/V ratio in rainwater tanks is an effective way of improving their microbial quality. Accordingly, additional research aimed at identifying which range of S/V ratios is most effective in improving such quality may benefit from modifying the first-order kinetics. The resulting information would help in the development of appropriate guidelines for the design of rainwater tanks.

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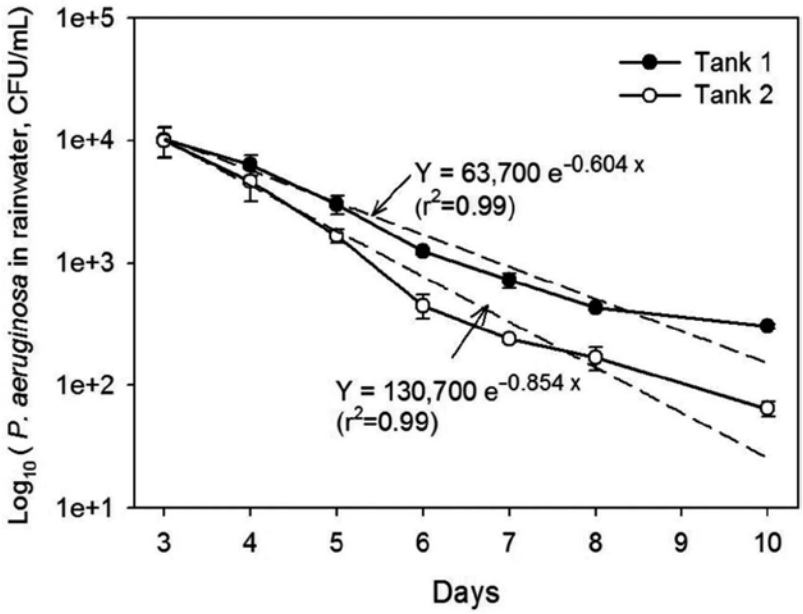

Figure 17. Number of *Pseudomonas aeruginosa* inoculated in the rainwater of the full-scale tanks. [Student's t-test; $P < 0.1$ except Day 0 ($P = 0.67$)].

3.3.4. Biofilm's role in rainwater tank

It has been suggested that rainwater tanks are unique ecosystems that support functional ecosystems comprising complex communities of environmental bacteria [2]. This study showed that a wider surface area for biofilm formation led to a higher removal rate of *P. aeruginosa* in rainwater. When opportunistic pathogens such as *P. aeruginosa* introduced to rainwater tanks with limited nutrient conditions, it seem to be removed due to their attachment to biofilms and die both naturally because of competition with indigenous microbial communities for nutrients.

4. Suggestion of a design and maintenance guideline for rainwater system

From this study, it was suggested the expected role of biofilms for improving water quality in rainwater tank. Contaminants including microorganisms in rainwater are possibly attached on biofilm, and the biofilms are grown by nutrient degradation and additional attachment. Then, followed sloughing and sedimentation processes, the rainwater quality seemed be sustained by certain level.

In addition, four recommendations were suggested for design and maintenance of RWH system as followed description.

1. There seems to be a unique microbial ecosystem which is able to control the microbial quality to the certain level by themselves. Thus, it is recommended that avoid mixing with chlorinated tap water which might disturb the microbial ecosystem in rainwater tank. When rainwater and tap water connected system designs, it is advisable to arrange separate supply tank without direct connection of tap water to the main storage tank.
2. Design of baffle and inlet barrier is recommended because they not only induce the sedimentation of inflow particle but also control bacterial quality in rainwater by increasing the surface for biofilm development.
3. Increasing the S/V ratio bacterial quality is possibly controlled by inducing more biofilm development. Therefore, it is recommended to consider the parameter of S/V ratio when rainwater tank is designed.
4. Frequent cleaning and/or disinfection of rainwater tank inside seems to be counterproductive because biofilm developed in rainwater tank improve the bacterial quality in rainwater tank by adhesion of bacteria in rainwater.

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Chapter 9

Biofilms in Beverage Industry

Dorota Kregiel and Hubert Antolak

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62940

Abstract

Over the years, numerous studies have been conducted into the possible links between biofilms in beverage industry and health safety. Consumers trust that the soft drinks they buy are safe and their quality is guaranteed. This chapter provides an overview of available scientific knowledge and cites numerous studies on various aspects of biofilms in drinking water technology and soft drinks industry and their implications for health safety. Particular attention is given to *Proteobacteria*, including two different genera: *Aeromonas*, which represents *Gammaproteobacteria*, and *Asaia*, a member of *Alphaproteobacteria*.

Keywords: biofilms, water, soft drinks, *Aeromonas* , *Asaia*

1. Drinking water systems

In water systems, both natural and industrial dominate *Proteobacteria*. This is the main group (phylum) of Gram-negative bacteria, taxonomically very diverse, consisting of more than 200 genera. Its membership includes both pathogenic bacteria of the genera *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and many other types of free-living or symbiotic, motile or nonmotile, chemoautotrophic or heterotrophic bacteria from outstanding aerobes to obligatory anaerobes.

Although bacteria are physiologically and morphologically diverse, they constitute a coherent set of six main classes: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Zetaproteobacteria*. Taxonomy of the group is determined primarily on the basis of ribosomal RNA sequences [1]. Species belonging to the classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* are very heterogeneous in their physiological characteristics. Each of the three classes includes aerobes and anaerobes, photosynthetic and nonphotosynthetic cells. They are distributed in both terrestrial and aquatic environments in very high abundance.

In natural systems, freshwater or potable water distribution networks, *Betaproteobacteria* dominate (87–99%), while *Alphaproteobacteria* are in marine waters [2]. *Proteobacteria* predominated in biofilms present in drinking water distribution systems, but the compositions of the dominant proteobacterial classes and genera and their proportions varied among biofilm samples [3]. The majority of strains isolated from biofilms in water distribution networks is *Alpha-* or *Gammaproteobacteria* [4]. Except *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Nitrospirae*, and *Cyanobacteria* are usually the major components of biofilm bacterial community.

One of the common features of *Proteobacteria* is the ability of biofilm formation and/or aggregation and formation of the so-called "flocs". An important component of such structures, in addition to microbial cells, is water – it represents about 97%. Besides water, the biofilm or flocs matrix are extracellular polymeric substances (EPSs). The bacterial cells in biofilms are embedded in a heteropolymeric matrix containing humic substances, glycoproteins, polysaccharides, and nucleic acids [5].

A first step in the successional development of biofilms is the coating of uncolonized surfaces with many particles, organic or inorganic (conditioning film), which enhances attachment of initial colonizing bacteria. Anything that may be present within the bulk fluid can through gravitational force or movement of flow settle onto a surface and become part of a conditioning layer. Surface charge, potential, and tensions can be altered favorably by the interactions between the conditioning layer and the surface. Factors such as available energy, surface functionality, bacterial orientation, temperature and pressure conditions are local environmental variables which contribute to bacterial adhesion. Physical forces associated to bacterial adhesion include the van der Waals' forces, steric interactions, and electrostatic (double layer) interactions, collectively known as the DVLO (Derjaguin, Verwey, Landau, and Overbeek) forces [6]. An extended DVLO theory takes into consideration hydrophobic/hydrophilic and osmotic interactions.

In real time, a number of the reversibly adsorbed cells remain immobilized and become irreversibly adsorbed. The physical appendages of bacteria (flagella, fimbriae, and pili) overcome the physical repulsive forces of the electrical double layer. Some evidence has shown that microbial adhesion strongly depends on the hydrophobic–hydrophilic properties of interacting surfaces. The first colonizers grow in surface-attached microcolonies and produce EPS. After an initial lag phase, a rapid increase in population is observed, which is described as the exponential growth phase. As the microcolonies develop, additional species, the so-called secondary colonizers, are recruited through coaggregation and nonspecific aggregation interactions, increasing the biofilm biomass and species complexity [7].

Simultaneously, expression of a number of genes for the production of cell surface proteins and excretion products increases. Surface proteins (porins) such as Opr C and Opr E allow the transport of extracellular products into the cell and excretion materials out of the cell, e.g., polysaccharides. EPS molecules impart mechanical stability and are pivotal to biofilm adhesion and cohesion, and evasion from harsh dynamic environmental conditions. The differences in gene expression of planktonic and sessile cells were identified, and as many as 57 biofilm-associated proteins were not found in the planktonic profile [6].

Physicochemical nature of such consortia implies differentiation of the physiological condition of individuals forming them [8]. Creating consortium is an effective adaptation strategy, including cell protection against adverse environmental factors; increased nutrient availability; increased binding of water molecules, thereby reducing the risk of dehydration; and increased ability to transfer DNA.

Microbial consortia exhibit altered phenotypic characteristics compared to planktonic cells, particularly with respect to growth and gene expression. All these factors increase the survival of cells forming biofilms. As a result, the inactivation of bacterial cells by conventional methods such as the use of antibiotics and disinfectants is often ineffective [9]. Especially this exopolymer matrix confers resistant properties to the whole system via the limitation of the effectiveness of disinfection by consuming the oxidants used, such as chlorine and chloramines [10].

At high cell concentration, a series of cell signaling mechanisms are employed by the biofilm, and this is collectively termed *quorum sensing*. *Quorum sensing* describes a process where a number of autoinducers (chemical and peptide signals in high concentrations, e.g., homoserine lactones) are used to stimulate genetic expression of both mechanical and enzymatic processes. In mature biofilms, enzymes are produced by the community itself which breakdown polysaccharides holding the biofilm together, actively releasing surface bacteria for colonization of fresh substrates. For example, alginate lyase produced by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, N-acetyl-heparosan lyase by *Escherichia coli*, and hyaluronidase by *Streptococcus equi* are used in the breakdown of the biofilm matrix [6].

Biofilms are polymicrobial communities, therefore the potential for cell coaggregation plays an integral role in spatiotemporal biofilm development and the moderation of biofilm composition. Coaggregation is mediated by the interaction between specific macromolecules on the cell surface of one species and cognate macromolecules expressed on the cell surface of the partner species. Microbial cells may also come into contact through hydrophobic interactions or electrostatic forces, but these last associations are relatively weak. Coaggregation-mediating proteins are referred as adhesins. Coaggregation may occur between lectin-like protein adhesins and their polysaccharide receptors or by protein–protein (adhesin–adhesin) interactions. These interactions may be unimodal, but in some cases are bimodal, involving two different interacting pairs of macromolecules [7].

Cell aggregation, as well as biofilm formation may have both intrageneric and intergeneric character [11]. Consortia are very changeable and their components depend on the environmental conditions. The study conducted by Rickard et al. [12] revealed that intergeneric and intraspecies coaggregation between water bacteria are common phenomena, and expression of coaggregation is dependent on cells being in the optimum physiological state for coaggregation, which usually occurs in stationary phase. Therefore, it is possible that since cells grow very slowly in nutrient-limited biofilms, these biofilms would provide suitable conditions for expression of coaggregation.

Different materials such as cast iron galvanized steel, stainless steel, copper, and polyethylene are used to manufacture water distribution pipes. It is worth noting that these materials favor biofilm formation in the water distribution systems. The presence of biofilms in drinking

water distribution pipes usually leads to a number of undesirable effects on the quality of water that is supplied to consumers. For example, the development of biofilms in copper pipes facilitates cuprosolvency which increases the release of copper into the distribution system. What's more, increased carbon influences the growth of heterotrophic plate count bacteria which are also involved in the corrosion of copper [13]. Silhan et al. [14] showed that among drinking water pipe materials such as galvanized steel, cross-linked polyethylene, copper pipes, and medium-density polyethylene, the most dense biofilm of *E. coli* was formed on the steel surface.

Molecular analysis of microbial communities by Yu et al. [15] indicated the presence of *Alpha-* and *Betaproteobacteria*, *Actinobacteria*, and *Bacteroidetes* in biofilms on the pipe materials. Moreover, the DGGE profile of bacterial 16S rDNA fragments showed significant differences among different surfaces, suggesting that the pipe materials affect not only biofilm formation potential but also microbial diversity.

The development of biofilms inside water distribution pipes facilitates the propagation of mixed microbial populations and is considered the main source of planktonic bacteria in water supply systems. Among the heterotrophic bacteria in drinking water systems, the pathogenic bacteria or at least opportunistic pathogens often appear. Enteropathogenic *E. coli* or other members of *Enterobacteriaceae* may appear in water supply systems due to contamination as a result of flooding, water supply failure, or insufficient disinfection. Other opportunistic bacteria such as *P. aeruginosa*, *Burkholderia* spp., *Stenotrophomonas maltophilia*, and *Legionella* spp. were quite often detected [16]. They increase the health risks associated with the consumption of water [13].

In the last decade, a group of new, potentially dangerous pathogens forming biofilms were classified as *Aeromonas* spp. rods from class *Gammaproteobacteria* [17, 18] (**Figure 1**). The experimental data and clinical and epidemiological evidence show that *Aeromonas* spp. may be an etiological factor of bacterial gastroenteritis in children and people with reduced immunity.

Bacteria *Aeromonas* spp. are capable not only of survival, but also propagation in water at temperatures up to 10°C and show a greater ability to utilize different carbon compounds than other Gram-negative bacteria.

According to Sautour et al. [19], the genus *Aeromonas* shows the ability to use not only carbohydrates, amino acids, and carboxylic acids, but also fatty acids and saturated hydrocarbons. Growth of these bacteria in an aqueous medium follows in the presence of even a small amount of biodegradable dissolved organic carbon compounds.

It was noted that there was an intense increase in the number of heterotrophic bacteria in the summer months. The results obtained by Craveiro et al. [20] demonstrated that *Aeromonas* spp. strains were able to form biofilm at both room and refrigeration temperatures. The chlorine-based disinfectant demonstrated to be efficient in removing preformed biofilm, but both were unsuccessful in preventing biofilm formation, highlighting the importance of adequate cleaning and disinfection procedures, with emphasis on food processing surfaces.

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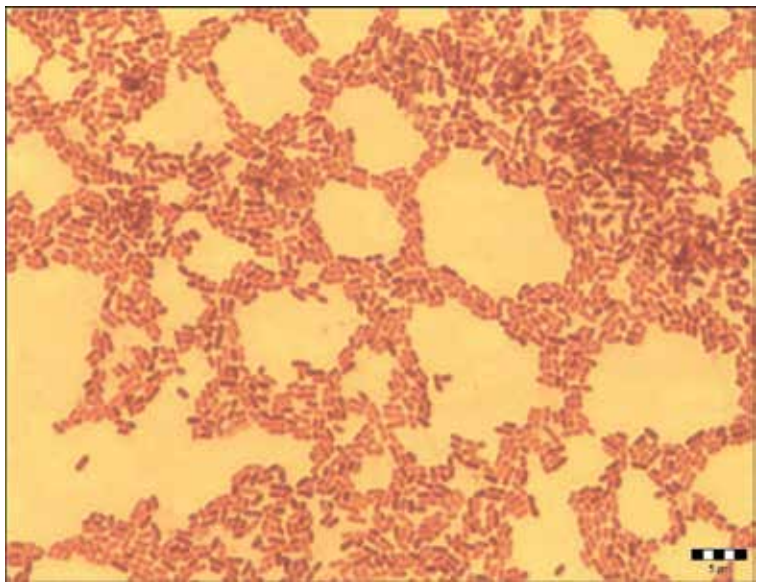

Figure 1. Gram-negative rods of *Aeromonas hydrophila*.

The vast majority of bacteria isolated from biofilms belonged to *Aeromonas hydrophila*. They showed the major virulence factors such as surface polysaccharides (capsule, lipopolysaccharide, and glucan), S-layers, iron-binding systems, exotoxins and extracellular enzymes, secretion systems, fimbriae, and other nonfilamentous adhesins, motility, and flagella [21, 22]. Despite the demonstration of the enterotoxic potential of some *Aeromonas* spp. strains, there is still a debate on its consideration as an etiological agent, as there were no big epidemical outbreaks described and no adequate animal model is available to reproduce the gastroenteritis caused by *Aeromonas*.

In clinical and environmental isolates of *Aeromonas* species, two distinct types of fimbriae have been found based on their morphology: short, rigid fimbriae (0,6–2 μm) that can be found in high numbers on the bacterial cell and long, wavy fimbriae (4–7 nm) found in smaller numbers. The short fimbriae are able to cause autoaggregation, and large ones considered hemagglutinins. Amino-acid sequence analysis indicates that they correspond to type IV pili, known as important structures for adhesion to epithelial cells and involved in biofilm formation. Some of them exhibit highest homology with the type IV pili of *Pseudomonas* and *Neisseria* species [22].

In studies conducted by Kregiel et al., *A. hydrophila* isolated from water distribution system, adhered to different abiotic surfaces such as glass, polystyrene, polyvinyl chloride, and gumosil, commonly used as packaging and installation materials [23–25]. After 3 weeks in an aqueous environment with a small amount of organic matter, bacteria formed numerous microcolonies surrounded extracellular mucilaginous substance (**Figure 2**). The results of microscopic examination demonstrated the strong adhesion properties of *A. hydrophila* and they were confirmed by luminometric measurements.

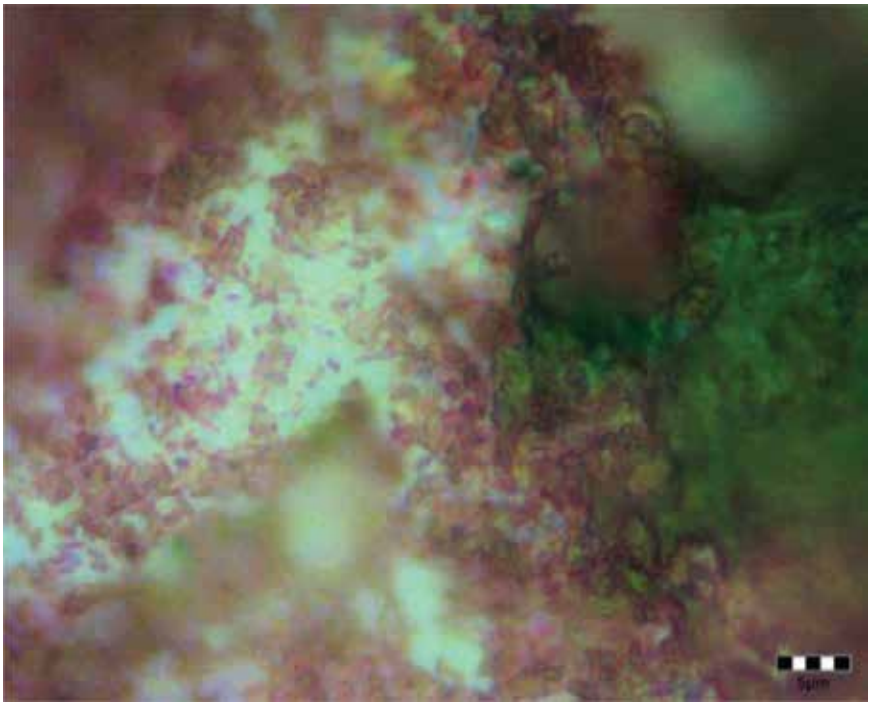

Figure 2. Biofilm of *Aeromonas hydrophila* on a glass surface.

The studies have found that both due to the strong adhesive properties of *A. hydrophila*, and the possibility of the virulence factors determining its pathogenicity, it should be considered the inclusion of *Aeromonas* rods for routine microbiological water analysis, especially for monitoring water or beverage distribution systems.

2. Soft drinks

When a change in the chemical nature of a fluid occurred, there is usually a qualitative shift created microbial consortia [8]. While the succession is a well-known process in classical ecology, in the case of biofilms or cell aggregates it is not fully understood. Despite many researches, the full knowledge on the formation of microbial consortia is still lacking. However, succession processes seem to be rather stochastic (reproduction and death) [26]. During growth of consortia, competition for resources makes that weak individuals are eliminated, and stronger competitors become dominant. Finally, in the mature consortia, cells are becoming more diverse by individual differences and "internal recycling."

Environmental factors may also shape the succession in microbial consortia. Changes in pH, the presence of carbon sources in the form of saccharides, and other additional substances cause significant qualitative changes in biofilms [27].

For example, the flavored drinking water samples with sucrose and natural fruit flavors showing signs of turbidity and the characteristic "flocs" formed by heterotrophic bacteria [28]. The developed specific methods allowed for the isolation of bacteria belonging to the *Asaia* spp. – a new, previously unknown in Poland, microbial contamination of mineral water and flavored beverages. Isolated bacteria were Gram-negative, aerobic rods with dimensions of 0.4–1.0 × 08–2.5 μm. These bacteria formed characteristic small (1–3 mm in diameter), pale pink or pink colonies in agar plates. The isolates were identified based on 16S rRNA gene sequences. It is worth noting that the same morphotypes and genotypes were isolated from fruit concentrates, which were previously used for production of flavored waters.

Asaia sp. was established in 2000 as the fifth genera of acetic acid bacteria of the class *Alphaproteobacteria*. Bacteria *Asaia* sp. were first isolated from the orchid tree flower (*Bauhinia purpurea*) and flowers of *Pueraria* (*Plumbago*), growing in tropical climates. Currently, Genera *Asaia* contains eight species named as: *As. bogorensis, As. siamensis, As. krungthhepensis, As. lannensis, As. spathodeae, As. astilbis, As. platycodi* and *As. prunellae.*. It is distinguished from other types of acetic acid bacteria not only by genetic features, but also by biochemical properties. The optimum pH and temperature of these bacteria are 5.5 and 30°C, respectively. Nevertheless, the strains belonging to *Asaia* sp. isolated from environments in tropical Indonesia, Thailand, and Japan have optimum growth even at 37°C [29].

Asaia spp. belongs to the risk 1 group, which means that it is a group of saprophytic microorganisms without causing diseases in humans. However, according to the literature, these bacteria can cause opportunistic infections when they get into the bloodstream of a man with weakened immune systems. Several cases of bacteremia caused by *Asaia* spp. were documented, especially in chronically ill adults and pediatric patients with cardiomyopathy or cancer. The first documented case of bacteremia caused by *As. bogorensis* was reported in a young patient with a history of intravenous-drug abuse. *As. bogorensis* was identified by sequencing the 16S rRNA gene. The isolate was resistant to almost all antibiotics routinely tested for Gram-negative rods, but was susceptible to gentamicin and doxycycline [30]. One of the last reports describes transient bacteremia due to *As. lannensis* in a patient with a psychiatric disorder and compulsive self-injection of different substances. Only restriction fragment length polymorphism of PCR-amplified 16S rRNA gene allowed for proper identification of isolate. The strain was also highly resistant to most antibiotics [31].

Asaia spp. show strong ability to aggregate and form characteristic "flocs" and to create biofilms on selected surfaces commonly used in the food industry: glass, polyethylene terephthalate, and polypropylene [32] (**Figure 3**).

It was found that the hydrophobicity of the cells decreased with increasing the age of the population. The higher hydrophobicity of young cells stimulates the process of aggregation and formation of flocs. The studies proved that the adhesive abilities of *As. lannensis* depend on the carbon source, nutrient availability, and physicochemical properties of abiotic surface. The strongest adhesion properties were characterized by cells in the minimal medium with sucrose.

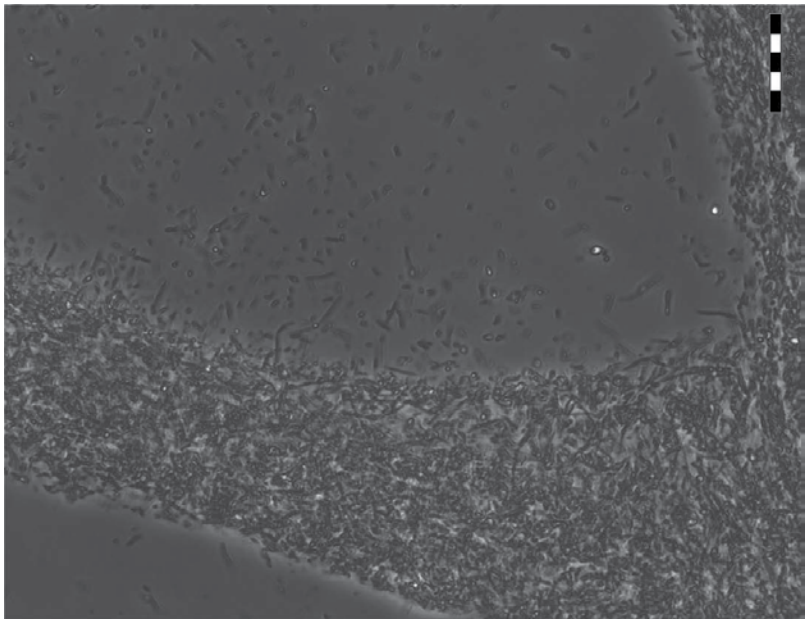

Figure 3. "Flocs" formed by cells of *Asaia* spp.

Definitely, the level of cell adhesion decreased in media that is rich in nutrients. Biofilm creation in a specific medium which was the commercial mineral flavored water had a dynamic character [32].

It is difficult to determine the origin of the contamination of soft drinks with the *Asaia* spp. However, the fruits and fruit concentrates are regarded as the source of the contamination [28, 33]. Most strains were isolated from the reclaimed fruit beverages and flavored mineral waters. This spoilage often occurs in the acid products preserved by the benzoate, sorbate, and dimethyldicarbonate. Horsakova et al. [34] found that these bacteria occur in the processing equipment in the form of biofilm, which is persistent and hardly removable by the common sanitation. The isolated bacteria *Asaia* spp. exhibit the polysaccharide encapsulation. The presence of preservatives is almost no effect on the *Asaia* spp. growth. The minimum inhibition concentration for sorbic and benzoic acid under the conditions of the model fruit drink (pH 3.45; Rf 10 Brix) were between 250 and 500 mg/l, while the concentration 250 mg/l is used for the stabilization of similar fruit beverage production.

The resistance of *Asaia* spp. to common preservatives limits the available possibilities to prevent spoilage of similar drinks. Additionally, the contamination of the technological equipment always brings the serious problem. The common sanitation procedures used in the beverage production may be insufficient to eliminate the very rigid biofilm, which is formed by *Asaia* spp. in the equipment. According to Horsakova et al. [34], the reliable elimination of such biofilm may require more forcing condition (e.g., hot sodium hydroxide and detergent and enzyme solutions) and in any hardly accessible points (pipe bends, branches, connections, and valves) mechanical treatment is the only possibility.

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3. New antiadhesion strategy: organosilanes

It is known that it is best to prevent than to fight against biofilm formed on the internal surface of a distribution system. For drinking waters and soft drinks, reduction or elimination of the formation of cell consortia can be obtained only by changing the physicochemical properties of abiotic surfaces or bioactive properties of consumption waters.

Compounds of the biocidal and/or antiadhesive properties applied in potable water systems have to inhibit effectively the growth of microorganisms without releasing toxic compounds with low molecular weight into aquatic environment. Such compounds may be organosilanes containing at least one bond between the carbon and silicon atom Si–CH_3. A carbon–silicon bond is very durable, and the presence of an alkyl group causes a change in surface tension. Additionally, organosilanes can contain other functional groups with antimicrobial properties, for example, methoxy, ethoxy, amino, methacrylic, and sulfide [35].

Organofunctional silanes are hybrid compounds that combine the functionality of a reactive organic group and the inorganic functionality of an alkyl silicate in a single molecule. This special property means they can be used as 'molecular bridges' between organic substrates and inorganic materials (**Figure 4**).

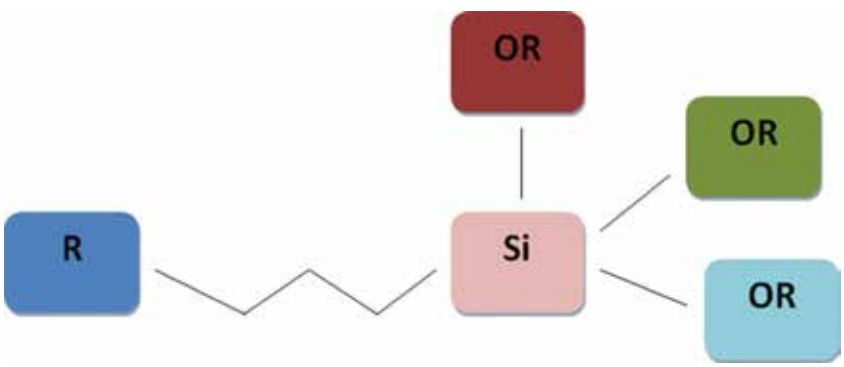

Figure 4. Model structure of organosilanes.

These compounds are relatively environmentally friendly, improve adhesion, and provide better protection against corrosion. Surfaces on which they can be used include metal, plastic, glass, rubber, ceramic, porcelain, marble, cement, granite, tile, silica, sand, appliances that have been enameled, polyester, polyurethane, polyacrylic, resins that are melamine or phenolic, siliceous, polycarbonate and wood, as well as painted surfaces.

The growth of many microorganisms can be reduced on surfaces treated with alkylsilanes. In general, the reactivity of hydroxylated surfaces with organofunctional silanes decreases in the following order: Si–NR_2 > Si–Cl > Si–NH–Si > Si–O_2CCH_3 > Si–OCH_3 > Si–OCH_2CH_3. The methoxy and ethoxysilanes are the most widely used organofunctional silanes for surface modification. The methoxysilanes are capable of reacting with substrates under dry, protic

conditions, while the less reactive ethoxysilanes require catalysis. The low toxicity of ethanol, a byproduct of the reaction, favors the use of ethoxysilanes in many commercial applications [35].

One of the most established and successful uses of the application of organosilanes is prevention against biofilm formation. The use of the proper quaternary amine-based organosilane can provide durable antimicrobial protection against a wide variety of microorganisms [36].

Adhesion abilities of *A. hydrophila* to the glass surface modified by coating with four different organosilanes with active functional groups were described by Kregiel [23]. The presence of active functional groups had an impact on a significant reduction in the surface tension of the test surfaces due to reduced participation polar forces – one of the components of surface forces. Among the modifiers, organosilanes containing methoxy groups and quaternary ammonium salts showed the best antiadhesive and antibacterial properties. Organosilanes were stable in an aqueous environment. Interesting results from the modification of the surface of the glass gave impulsion to extend the study on modification of plastic materials commonly used as pipe materials in water systems [24, 25]. The modified PVC surfaces were made by silane coupling on the native material. Modifications of silicone elastomer were carried by cocrosslinking organosilane with silicone. Almost all of the modified surfaces were characterized by antimicrobial and antiadhesive features. Among the modifications, especially polydimethylsiloxane with a quaternary ammonium salt and a methoxy group in the silicone elastomer showed the greatest antiadhesive and antibacterial properties against *A. hydrophila*.

4. New antiadhesion strategy: proanthocyanidins

Scientific studies showed that natural compounds from different fruits have potential health benefits against cancer, aging and neurological diseases, inflammation, diabetes, and bacterial infections. For example, cranberry juice was recognized for benefits of maintenance of a healthy urinary tract. Cranberry is a term derived from the contraction of "crane berry." This name is derived from the nickname of the bilberry flower, and the sand crane, a bird that often feeds on the berries of this plant. The cranberry is part of the *Ericaceae* family and naturally grows in acidic swamps full of peat moss in humid forests [37].

Bacterial adhesion is accomplished by the binding of lectins exposed on the cell surfaces of pili and fimbriae to complementary carbohydrates on the host tissues. Pili are small filaments that can be either mannose-resistant or mannose-sensitive. The mannose-sensitive pili, called type 1 pili, permit bacterial adhesion to the urothelium. The fimbriae (p-fimbriae) are inhibited by fructose, present in cranberries. The more virulent strains of *E. coli*, isolated from patients with urinary tract infections, have other types of these structures that bind to glycosphingolipids of the lipid double membrane of renal cells, which precedes renal parenchymal invasion.

The current hypothesis is that cranberries work principally by preventing the adhesion of type 1 and p-fimbriae *E. coli* strains to the urothelium. Without adhesion, the bacteria cannot infect the mucosal surface. In vitro, this adhesion is mediated by two components of cranber-

ries: fructose, which inhibits the adherence of type 1 fimbriae, and proanthocyanidins, which inhibits the adherence of p-fimbriae. The binding of the proteinaceous bacterial fimbrial tips to mucosal surfaces on the uroepithelium occurs as a specific receptor-ligand association favored by hydrophobic interactions. This possible mechanism is that the cranberry compounds, acting as receptor analogs, competitively inhibit the adhesion of *E. coli* to host cells by binding to the fimbrial tips. Another mechanism of cranberry activity is the in vitro reduction in the expression of p-fimbriae in *E. coli* by changing the conformation of surface molecules [38]. Zafriri et al. were the first to postulate that compounds in cranberry could affect p and type 1 fimbriae of *E. coli* [39]. In 1998, Howell et al. [40] identified specific proanthocyanidin compounds in cranberry responsible to antiadhesive properties.

Proanthocyanidins are one of many plant phenols, which are aromatic secondary metabolites found in the plant kingdom. They are mainly found in *Vaccinium* berries such as cranberries and blueberries. They are dimers or oligomers of catechin and epicatechin and their gallic acid esters. Proanthocyanidins are in the first place very strong antioxidants. Studies have shown that proanthocyanidins act as anticancer and antiallergic agents, and that they improve heart health. These flavonoids have several potential clinical effects, including antiatherosclerotic, anti-inflammatory, antitumor, antithrombogenic, antiosteoporotic, and antiviral. Some of these effects, such as antitumor, are still up for discussion, and the role of flavonoids in different effects is not fully known.

They are also known as olgoflavanoids, and consist of monomer flavan-3-ol units. When linked through either C4 to C8 or C4 to C6 bonds, the linkages are called B-linked. When the linkages were through a C2 and C7 compound, they are called A type [41]. While B-linked proanthocyanidins can be found in different fruit products including apple juice, purple grape juice, green tea, and dark chocolate, A-linked ones are found in cranberries and it is a linkage with unique antiadhesion properties associated with them [42] (**Figure 5**).

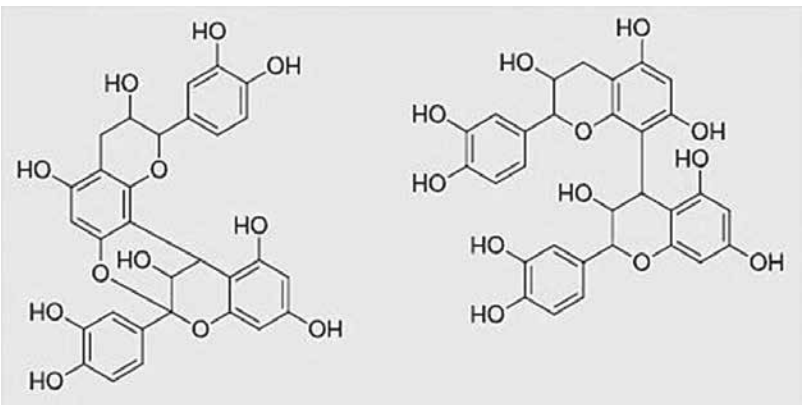

Figure 5. Proanthocyanidins: type A (left) and type B (right).

The antiadhesive properties of cranberry were demonstrated against different microorganisms: *E. coli*, *Proteus mirabilis*, or *Helicobacter pylori*, responsible for urinary tract infections and

gastritis, as well as other pathogenic Gram-negative and Gram-positive bacteria: *P. aeruginosa, Staphylococcus aureus*, or *Listeria monocytogenes* [43–45].

It was also noted that the adhesion of *Asaia* spp. cells in the presence of cranberry juice was much lower, especially for the packaging material – polystyrene [37]. In the presence of 10% cranberry juice, attachment of bacterial cells was three times lower. The obtained results suggested that compounds of cranberry inhibit both biofilm formation and coaggregation of microbial cells. This fact would help to utilize antioxidant-rich cranberry juice as a natural antiadhesive protectant and microbiological stability enhancing agent for functional soft drinks.

5. Conclusion

Problems related to microbial contamination in the beverage industry have been studied for more than a century. However, most of the knowledge acquired over the years relates to single-cells, but today it is generally accepted that microorganisms grow and survive in organized communities where their physiology is very different. This paper has given an overview of the most widely used research on the controlled attachment of specific bacteria present in drinking water or soft drinks. Both surfaces modified by organosilanes and cranberry juice supplementation are the latest developments in this area. Particularly, cranberry juice and cranberry extracts may be investigated as a natural solution for food industry by creating an additional barrier to inhibit the growth of spoilage bacteria and providing additional health benefits.

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Section 3

Biofilm in Health and Diseases

Chapter 10

Staphylococcal Biofilms: Pathogenicity, Mechanism and Regulation of Biofilm Formation by Quorum-Sensing System and Antibiotic Resistance Mechanisms of Biofilm-Embedded Microorganisms

Sahra Kırmusaoğlu

Additional information is available at the end of the chapter

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Abstract

Staphylococcal infections are reported to cause very important problems in hospitalized and immunocompressed patients worldwide due to their tough and irresponsive treatment by antibiotics. Biofilm-embedded bacteria that gain resistance to immune defense and antibiotics by antibiotic degrading enzymes, efflux pumps, and certain gene products of which expression are changed by the quorum sensing cause chronic and recurrent infections such as indwelling device–associated infections. Biofilm-embedded sessile community has heterogeneous cells that have wide range of different responds to each antimicrobials. *Staphylococcus epidermidis* (*S. epidermidis*) and *Staphylococcus aureus* (*S. aureus*) that are mostly known pathogenic strains can induce gene expression of biofilm that has an important role in the pathogenesis of staphylococcal infections and causes bacterial attachment and colonization on biotic such as tissues or abiotic surfaces such as prosthetic surfaces that may act as a substrate for microbial adhesion when microorganisms exposed to stress conditions. This expressed and matured biofilm causes bacterial spread to whole body, consequently, spread of infection in to whole body. It is hard to treat biofilm infections, and new agents are being researched to prevent formation and dissemination of biofilm. Defining the virulence and the role of biofilm of *S. epidermidis* and *S. aureus* in chronic and recurrent infections such as indwelling device–associated infections, the mechanism and the global regulation of biofilm production by quorum-sensing system, inactivation of biofilm formation, and the resistance patterns of biofilm-embedded microorganism against antimicrobials are important.

Keywords: staphylococcal biofilm, mechanism and regulation of biofilm formation, quorum-sensing system, antimicrobial resistance of biofilm, *Staphylococcus aureus*, *Staphylococcus epidermidis*, pathogenicity

1. Introduction

Staphylococcus epidermidis (S. epidermidis) and *Staphylococcus aureus* (S. aureus) are the most common causes of indwelling device–associated infections, and nosocomial and community acquired infections can produce biofilm as a virulence factor [1]. The biofilm infections such as *S. epidermidis* and *S. aureus* infections are important problems in hospitalized and immunocompressed patients worldwide due to their tough and irresponsive treatment by antibiotics. Biofilm-producing bacteria resist to immune defense, antibiotics, and many antimicrobial agents. Biofilm-embedded bacteria gain antibiotic resistance by antibiotic-degrading enzymes, efflux pumps, and certain gene products of which expression are changed by the quorum sensing [2, 3]. Biofilm-embedded sessile community has heterogeneous cells that have wide range of different responds to each antimicrobials [2]. So, every antibiotic has a different effect against different metabolically active cells that are present in the different layers of biofilm and persister cells that are evolved to survive in biofilm. It is hard to treat biofilm infections that are generally recurrent infections and of which treatments are tough and irresponsive [3].

Staphylococci that construct the human skin flora can contaminate indwelling devices. By this way, they are inserted to human by contaminated indwelling devices. When microorganisms exposed to stress conditions, gene expression of biofilm is induced as a stress response. The biofilm that is a slime-like glycocalyx causes bacteria to survive in the stress conditions. Staphylococci adhere, colonize, and infect biotic surfaces such as tissue or abiotic surfaces such as prosthetic surfaces that may act as a substrate for microbial adhesion and causes bacterial spread to whole body by forming biofilm that is a slime-like glycocalyx [1, 4, 5]. The virulence and the role of biofilm of *S. epidermidis* and *S. aureus* in chronic and recurrent infections such as indwelling device–associated infections, the mechanism, and the global regulation of biofilm production by quorum-sensing system, especially *agr*-quorum-sensing system, inactivation of biofilm formation, and the resistance patterns of biofilm-embedded microorganism against antimicrobials are discussed in this chapter.

2. The biofilm, virulence, and Staphylococcus

2.1. The pathogenesis of Staphylococcus biofilm

The biofilm has an important role in the pathogenesis of staphylococcal infections. The biofilm causes bacteria to survive in the stress conditions such as UV damage, metal toxicity, anaero-

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bic conditions, acid exposure, salinity, pH gradients, desiccation, bacteriophages, and amoebae and to resist antibiotics, antimicrobials, and host immune defense [5–8]. The main pathogen of implant infections is staphylococci that cause 80% of all prosthetic infections [9]. The biofilm of bacteria causes chronic infections such as indwelling device–related infections, chronic wound infections, chronic urinary tract infections (UTI), cystic fibrosis pneumonia, chronic otitis media (OM), chronic rhinosinusitis, periodontitis, and recurrent tonsillitis [10]. The biofilm infections are the main important problems in hospitalized and immunocompressed patients in worldwide due to their tough and irresponsive treatment by antibiotics. In biofilm, bacteria are not distrupted completely by antibiotics even high doses of antibiotics used *in vivo* [3, 11, 12]. Infected device can expose the patient to a higher risk of mortality. Orthopedic surgery and trauma indwelling device-related infections that make treatment difficult by antibiotics [13] cause removal of implant out of the body to eradicate biofilm and overcome biofilm-related infections [14] and may cause functional loss of the infected limb [15, 16].

2.2. Staphylococcal biofilms as a virulence factor

The biofilm that anchored to abiotic or biotic surfaces is a slime-like glycocalyx in which sessile community of microorganisms embedded. This extracellular polymeric substance that is constituted by matrix of polysaccharide, teichoic acids, extracellular DNA (eDNA), and staphylococcal proteins is produced by biofilm producing microorganisms [4, 17, 18]. Polysaccharide intracellular adhesin (PIA) is a specific polysaccharide in glycocalyx composed of β-1,6–linked N-acetylglucosamine residues (80–85%) and non-N-acetylated D-glucosaminyl residues that are an anionic fraction and contain phosphate and ester-linked succinate (15–20%) [18]. Although PIA is a main mechanism of biofilm formation in *S. aureus* and *S. epidermidis*, surface proteins are the other alternative mechanism of biofilm formation. Extracellular matrix has large water-filled channels, accumulates antibiotic-degrading enzymes such as β-lactamases [19], and plays a role in the adaptive resistance mechanisms due to eDNA constituent [20] (**Figure 3**).

2.3. Mechanisms of biofilm formation

Bacterial biofilm formation is a complex and multifactorial process. The biofilm formation process consists of adherence/adhesion/attachment, aggregation/maturation/accumulation, and detachment/dispersal phase. The last step is the dispersal of mature biofilm-embedded bacteria out of the biofilm [21] (**Figure 1**).

2.3.1. Attachment (adhesion or adherence) phase

When conditions favor biofilm formation, biofilm formation that begins with the adherence of the bacteria to a surface that act as a substrate for microbial adhesion continues with the aggregation formed by cell–cell adhesion [22] (**Figure 1**).

Staphylococcal adherence to an abiotic surface of indwelling prosthetic device depends on physico-chemical structure of medical device and surface components of Staphylococci such as wall teichoic acid (WTA) [23], lipoteichoic acid (LTA) [23], accumulation-associated

protein (Aap) [24], autolysins AtlA [25] and AtlE [26]. The staphylococcal adherence to a biotic surfaces such as host cells and plasma protein-coated prosthetic surface is mediated by cell wall-anchored (CWA) proteins such as the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* and fibrinogen-/fibronectin-binding proteins FnBPA and FnBPB and clumping factors A and B of *S. aureus* [27].

Several microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that are able to bind to human matrix proteins such as fibronectin and fibrinogen and colonize are expressed in *S. epidermidis* and *S. aureus* at the first step [28]. Adherence of bacteria to an extracellular matrix component, fibronectin, fibrinogen, and plasma clot is mediated by expressed surface adhesins such as Bap coded by bap gene [29], surface protein G (SasG) [22], fibronectin-binding proteins (FnbA and FnbB) of *S. aureus* [30], and the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* [27]. Adherence of *S. aureus* to collagenous tissues and cartilage is mediated by collagen-binding protein, Cna. Some antibodies can block bacterial attachment to these tissues by blocking Cna. Adherence of *S. aureus* to fibrinogen in the presence of fibronectin is mediated by clumping factor A and B (ClfA, ClfB) that are effective in foreign body and wound infections. Also, plasma-sensitive surface protein (Pls) participates in the attachment to fibrinogen and fibronectin. Protein A that is present in cell wall and encoded by *spa* gene in *S. aureus* impair opsonization and phagocytosis by binding to Fc domain of immunoglobulin G (IgG) in the wrong orientation. Endovascular diseases are emerged by *S. aureus* as a result of the binding of protein A to von Willebrand factor in damaged endothelium [31].

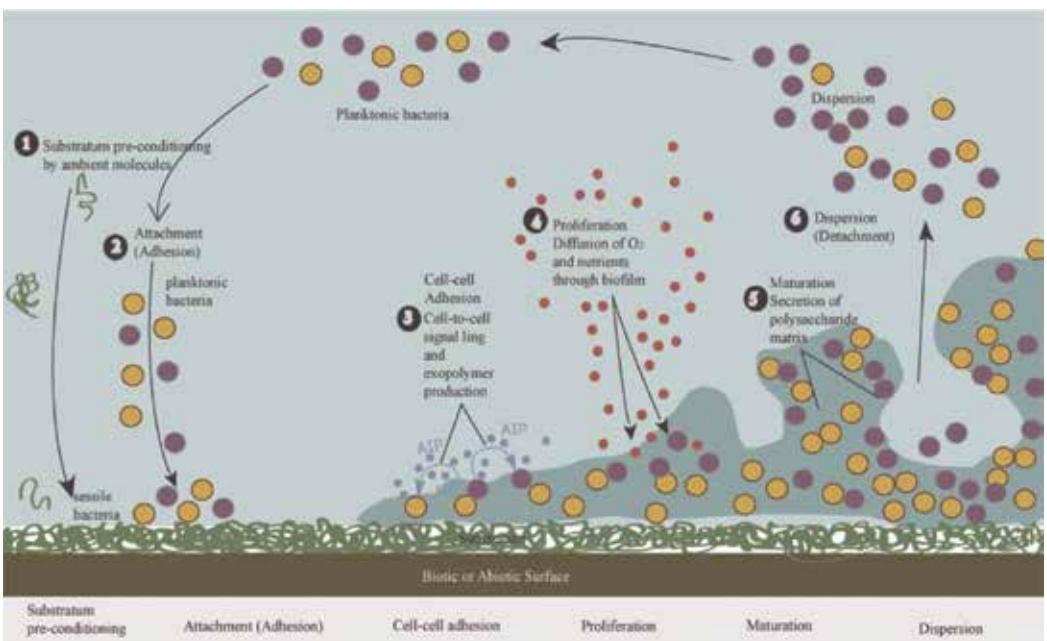

Figure 1. The stages of biofilm formation.

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2.3.2. Accumulation (aggregation or maturation) phase

After adherence of staphylococcus to biotic and abiotic surfaces, exopolysaccharide (EPS) such as PIA or PNAG that are produced by *ica* operon (*ica*-dependent form) starts to be produced, extracellular matrix (ECM) is constructed by PIA/PNAG, extracellular DNA (eDNA), and surface proteins [cell wall-anchored (CWA) proteins] in *ica*-independent form, and bacterial colonies become mature [2, 27]. The cell wall-anchored (CWA) proteins not only provide bacterial adherence but also provide intercellular adhesion, biofilm accumulation, and maturation [27]. Aggregation that is mediated by the synthesis of either polysaccharide intercellular adhesion/poly-N-acetylglucosamine (PIA/PNAG) [30, 32] is formed in cell clusters till multi-layer-structured biofilms formed. Several staphylococcal surface proteins that mediate primary attachment of bacteria such as clumping factors A and B, fibrinogen-/fibronectin-binding proteins FnbA and FnbB of *S. aureus* or the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* that are cell wall-anchored proteins (CWA) also promote intercellular adhesion and construct the aggregation of bacteria in *ica*-independent biofilm formation rather than PIA [33] (**Figure 1**).

In the initial cell-surface interaction of motile bacteria, adherence of motile cell to surface is facilitated by flagella of motile cell. After adherence motile species that undergo cellular differentiation in biofilm lose their motility by paralyzing their flagella and become nonmotile [34]. Klausen et al. [35] revealed that wild-type strain and isogenic flagellar mutant of *Pseudomonas aeroginosa* both forms biofilms which have structural differences.

2.3.3. The detachment (or dispersal) phase

In the detachment stage, sessile cells turn into planktonic state that can spread and colonize other surfaces and form biofilm on these infected regions [2] (**Figure 1**). Detachment of microorganisms from biofilm can be caused by bacteria themselves, such as enzymatic degradation of the biofilm matrix such as dissolution of adhesins by proteases, nucleases, and a group of small amphiphilic α-helical peptides, known as phenol-soluble modulins (PSMs) functioning as surfactants [27], and quorum sensing or by external forces such as fluid shear forces, corrosion, and human intervention [36] (**Figure 2**). During detachment of motile microorganism rather than staphylococcus, cells express genes that are for motility such as transcription of pilus and ribosomal proteins and are almost seen in planktonic cells [37].

2.4. Types of biofilm formation

2.4.1. PIA-dependent biofilm formation

Positively charged PIA provides intercellular attachment via binding to bacteria of which surface is negatively charged [27]. All *S. aureus* strains contain *icaADBC* gene of which product is PIA constructs biofilm formation [31]. *Ica* locus have been identified in many staphylococcus species like *S. aureus* and *S. epidermidis* but except *S. haemolyticus* and *S. saphrophyticus* [9]. *ica* is regulated by stress conditions, such as anaerobic conditions, extreme temperature, osmolarity, ethanol, and antibiotics. icaA, icaD, icaC, and icaB are the genes of *ica*ADBC locus.

icaA and icaD contribute to exopolysaccharide synthesis and encode N-acetylglucosaminyl transferase as a transmembrane enzyme to synthesize poly-N-acetylglucosamine polymer. While poly-N-acetylglucosamine polymer is translocated to cell surface of bacteria by *ica*D gene, the polymer is fixed to the outer surface of bacteria by deacylation of poly-N-acetylglucosamine polymer by the product of *ica*B gene [9]. Regulator gene *icaR* that is located upstream of the *icaADBC* operon encodes a transcriptional repressor in both *S. epidermidis* and *S. aureus* and *icaADBC* genes are upregulated in response to anaerobic growth such as inside of biofilm. Under anaerobic conditions, PIA is induced by SrrAB (the staphylococcal respiratory response regulator) that binds to upstream of the *icaADBC* operon. Insertion sequence (IS256) can regulate *ica* by reversible inactivation in *S. epidermidis* and some strains of *S. aureus*. TcaR (transcriptional regulator of the teicoplanin-associated locus) and IcaR are repressors of ica operon transcription and repress PIA expression. While deletion of *icaR* gene increases *ica* gene expression, PIA production, deletion of *tcaR* gene had no effect against *ica* gene, PIA production. Transcription of IcaR is repressed by Rbf that is a protein regulator of biofilm formation and leads expression of *ica* gene, PIA production, whereas transcription of IcaR is induced by Spx that is a global regulator of stress response genes and regulates biofilm formation negatively [18].

2.4.2. PIA-independent biofilm formation

Biofilms not only can be constructed by *ica* gene of which product is PIA, but also constructed by *ica*-independent (PIA-independent) form. Biofilm is generated not only by PIA that is a main component of biofilm production but also by a number of proteins. When *icaADBC* is deleted, PIA is not produced but the biofilm formation so, virulence is not affected. In this case, biofilm formation can be constructed rather than PIA. In the catheter infection, biofilm formation of clinical isolates of *S. aureus* of which *ica* cluster is mutated is not reduced [18]. Fitzpatrick et al. revealed that biofilm formation of the *icaADBC* operon-deleted MRSA mutants was not affected, whereas biofilm formation of the *icaADBC* operon-deleted MSSA mutants was impaired. This study showed that ica-independent biofilm formation is strain specific [38].

PIA-independent biofilms were constructed by accumulation-associated proteins (Aap) of *S. epidermidis*, biofilm-associated protein (Bap) that is a surface protein of *S. epidermidis* and *S. aureus* and Bap-related proteins of *S. aureus* [18]. Other surface proteins that involve in the PIA-independent biofilm formation are SasG, SasC, protein A, fibronectin-binding proteins FnBPA and FnBPB, cell wall-anchored (CWA) proteins including clumping factors A and B, autolysins AtlA and AtlE or wall teichoic acid (WTA), the fibrinogen-binding protein SdrG/Fbe, lipoteichoic acids (LTA) of *S. aureus* and the fibrinogen-binding protein SdrG/Fbe of *S. epidermidis* [27].

Scientists determined that medical MRSA isolates produce protein-dependent biofilm such as FnBP- and Aap-dependent biofilms in animal models that have indwelling device–associated infection. O'Neill et al. [30] and McCourt et al. [39] revealed that biofilms of certain isolates of HA-MRSA from CC8 and CC22 and CA-MRSA from USA300 lineage (CC8) were FnBPs-dependent.

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Autolysin Atl that is a wall-anchored protein of *S. aureus* and causes initial attachment of *S. aureus* to surfaces can be cleaved into amidase and glucosaminidase that cause cell lysis, eDNA release, and cell accumulation. Then, biofilm maturation of FnBP-dependent biofilm phenotype is constructed by FnBPs [25].

In biofilm production of *S. aureus*, cell-cell interactions are facilitated by α-toxin that is a haemolytic toxin. Nevertheless, the mechanism of integral role of α-toxin has not been known clearly. β-toxin that is a sphingomyelinase and causes hemolysis and lyse lymphocytes plays a stimulative role in the biofilm production of *S. aureus* by covalently cross-linking to itself in the occurrence of DNA in matrix of staphylococcal biofilms [40].

S. aureus biofilms can be stabilized by amyloid fibrils that are formed by aggregated PSM on the surface of bacteria and aggregated signal peptide AgrD [41].

2.5. The global regulation of biofilm formation

2.5.1. The regulation of Staphylococcal biofilm by agr-quorum-sensing system

Biofilm production is provided by the equilibrium between the productions of amyloid fibrils and phenol soluble modulins (PSMs) that are extracellular polymeric substances and their catabolism by enzymes such as nucleases and proteases that are expressed by agr-QS regulator system that use two-component system signal transduction system (TCS). The control of planktonic and sessile bacteria and the biofilm expression is regulated by coordinated mechanisms [41] (**Figure 2**).

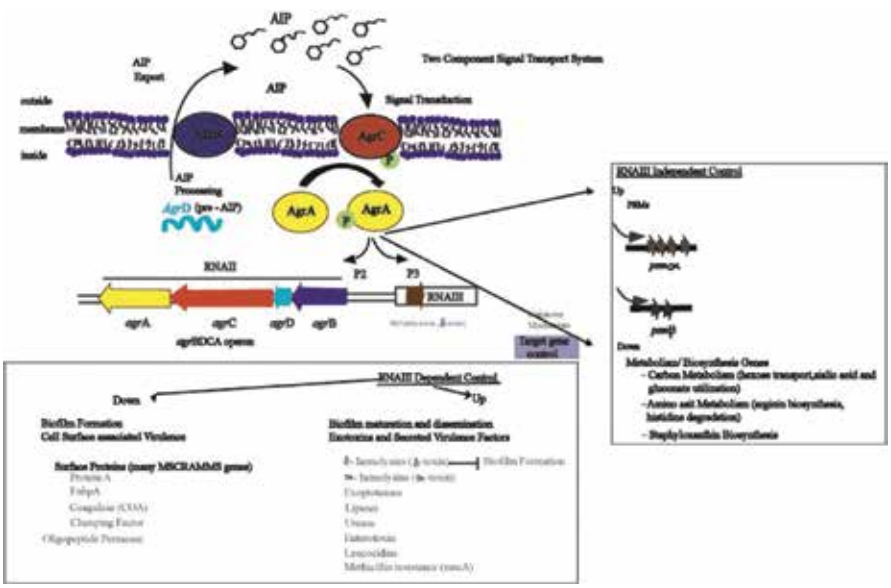

Figure 2. The regulation of biofilm formation by agr-quorum-sensing system.

The biofilm formation of staphylococci is fully expressed *in vivo*, whereas the biofilm formation of staphylococci is not fully expressed all the time *in vitro* unless nutrient supplementations are added to growth media and is provided. Increased amount of biofilm formation due to fully expression occurs in stress conditions such as starvation, thermal stress, heat shock, salt, certain antibiotics, iron limitation, subinhibitory concentrations of ethanol, accumulations of metabolites, oxidative stress, low pH, and changes in osmolarity *in vitro*. Bacteria sense stimuli from the environment and bacterial density and then respond to stimuli by upregulating expression of biofilm formation, virulence factors production such as toxins, etc. [9].

Staphylococcus use quorum-sensing systems (QS) for intercellular communication and biofilm formation. Accessory gene regulator (Agr) system regulates cell density-dependent gene expression using two-component signal transduction system [42]. Agr and LuxS systems that are required for autoinducer peptide (AIP) production as a pheromone are quorum-sensing systems in staphylococci [43]. Bacteria sense pheromones as stimuli that are released by the density of bacteria belonging to the same group and express biofilm formation [9]. AIP production starts in exponential phase of bacterial growth [44]. There are four proteins that are sensor histidine protein kinase AgrC, DNA-binding response regulator AgrA, AgrD that is a prepheromone, and AgrB that exports and modifies AgrD, present in this system. The signal is transported to bacteria by binding of AIP to AgrC. When AIP binds to AgrC, DNA-binding regulator AgrA is activated by His-dependent phosphorylation of AgrC [42]. By the binding of activated DNA-binding regulator AgrA to P2 and P3 promoters in *agr* operon (*agr*BDCA), RNAII and RNAIII are transcripted, respectively [44]. The *agr*BDCA operon codes RNAII transcript that encodes AgrB, D, C, A from *agrB*, *D*, C, *A* genes as a components of agr system, and RNAIII transcript that include *hld* gene encodes the δ-hemolysin (termed δ-toxin or δ-PSM) [42]. RNAIII regulates the expression of agr-governed virulence factors such as CWA proteins as a surface proteins and exotoxins at transcriptional and translational level. Independently of RNAIII (RNAIII independent control), AgrA also directly regulates the expression of α-PSMs and β-PSMs by binding to their promoters in *psm* operon in *S. aureus* and involves in the downregulation of genes contribute carbohydrate and amino acid metabolism [44] (**Figure 2**).

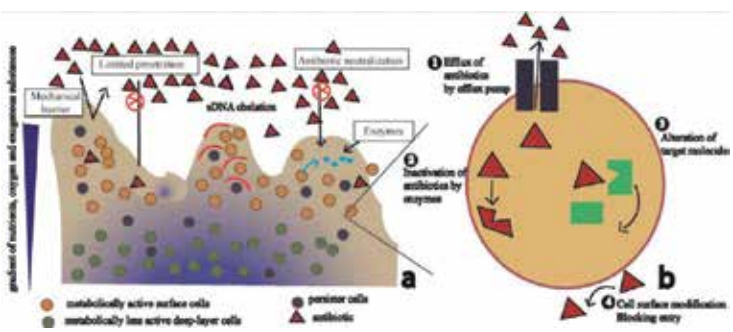

Figure 3. The biofilm-embedded bacteria. (a) The heterogeneous sessile community of biofilm. (b) Antibiotic resistance mechanisms of biofilm-embedded bacteria.

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The regulation mechanisms of RNAIII for target genes can be at transcriptional and translational level, and its regulation can be direct or indirect. Fourteen stem-loop and two long helices construct structure of RNAIII. Each domain regulates the expression of each target gene. Translation of α-hemolysin (*hla*) upregulated by hairpin loop H2 and H3. In contrast to this, the repression of early expressed virulence genes of *S. aureus* such as coagulase, protein A, and the repressor of toxins (Rot) is comprised by hairpin H13, H14, and H7 of RNAIII. Hairpins such as H7, H13, and H14 that are complementary to Shine-Dalgarno sequences (SD) of target mRNA act as an antisense RNA and inhibit initiation of translation and cause RNAaseIII-mediated degradation of target mRNA [45] (**Figure 4**).

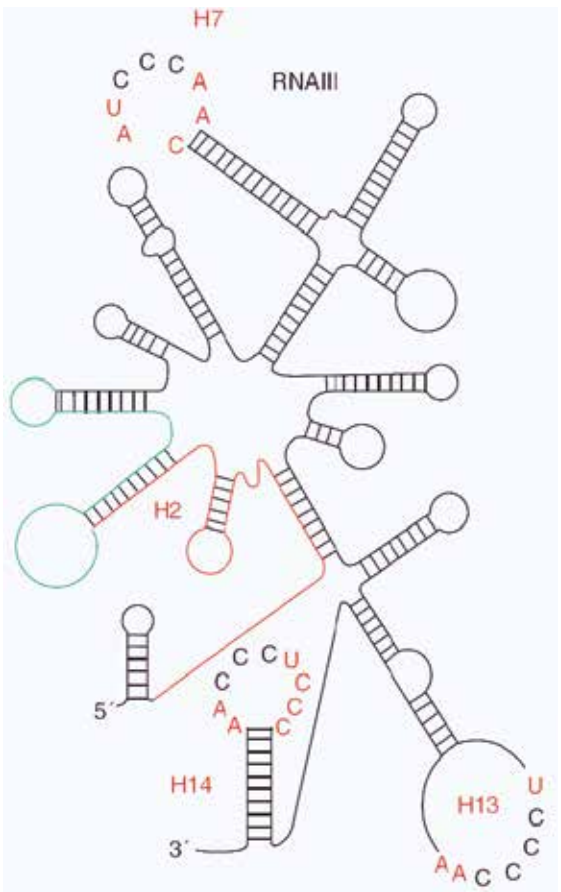

Figure 4. The structure of RNAIII [44]

Staphylococcal virulence factors are expressed with accessory gene regulator (agr) system in response to cell density [9]. During the beginning of the biofilm-related staphylococcal infection, adhesion factors (surface proteins) such as MSCRAMMs are upregulated. After initial attachment and colonization had been happened, during early stationary growth phase

of bacteria, toxins and other acute virulence factors such as degradative exoenzymes (such as δ-hemolysin, lipases and proteases that disperse bacteria) are upregulated and non-aggressive colonization surface proteins such as MSCRAMMs are downregulated by *agr*-QS regulator system [1, 46]. Adherence is reduced by downregulated genes of CWA, due to surface proteins are no longer needed after colonization, by the way initial biofilm formation is decreased indirectly [5]. Expression of staphylococcal toxins such as enterotoxin B, toxic shock syndrome toxin-1, exfoliative toxins, fibrinolysin, α, β, γ, and δ hemolysins, other phenol-soluble modulins (PSMs), leucocidin, capsular polysaccharide (type 5 and 8), serine protease, and DNase is increased (upregulated), and expression of surface proteins and biofilm formation is decreased (downregulated) by *agr* of *S. aureus* and *S. epidermidis* [9, 44]. Infection is dispersed to other surfaces by the detachment of biofilm that is caused by the upregulation of the expression of PSMs that have an important role in acute infection [1]. In chronic biofilm-associated infection of *S. aureus* high amount of QS or *psm* gene mutants are present, by the way, mutants favor compact biofilm development and biofilm/infection cannot be dispersed to other surfaces [46, 47].

The production of PIA/PNAG, PIA/PNAG-degrading enzymes, and matrix components of staphylococcal biofilm is not regulated by QS [44, 46].

Phenol-soluble modulins (PSMs) are surfactant-like staphylococcal peptides and are controlled by *agr* locus function in biofilm maturation, biofilm structuring/destructuring, dispersal, and dissemination by distruption of non-covalent interactions between biofilm matrix molecules. PSMs have a role in the pathogenesis of *S. aureus* and *S. epidermidis* biofilm-associated infections [9, 21, 46]. In contrast to soluble PSMs, PSMs that are aggregated form amyloid fibrils that contribute to stability of the biofilm [27, 41]. *S. aureus* and *S. epidermidis* catheter-related infections can be controlled by PSM surfactant-mediated QS control of biofilms for biofilm maturation and dissemination [48, 49]. The biofilm maturation is not only caused by PSM surfactants but also enzymatic degradation of biofilm matrix components by proteases and nucleases [46]. But Beenken et al. [50] revealed that nuclease did not disperse *S. aureus in vitro*. Hochbaum et al. [51] revealed that D-amino acids trigger biofilm dispersal of *S. aureus*.

Agr (AIPs) of each strain belongs to different *agr* classes of which biofilm-forming capacities and syndromes are different. Four main classes of AIPs (Agr) are present in *S. aureus* and *S. epidermidis*. *S. aureus* strains of which *agr* classes are *agr* II and *agr* III are high and medium biofilm formers due to having defective and inactive *agr*, respectively. Non-defective and active *agr* is present in *agr* I and *agr* IV strains that are weak biofilm producers [52]. *agr* IV *S. aureus* strains are more associated with exfoliative syndromes. *agr* I *S. aureus* strains are isolated from endocarditis and superficial infections. *agr* II and *agr* III *S. aureus* strains are isolated from endocarditis and nasal colonization, respectively [53]. Mortality due to *agr* II–caused infections is higher than *agr* I–caused infections [54]. The prevalences of *agr* I type among the *S. epidermidis* clinical isolates and *S. epidermidis* localized in skin flora are approximately 89% and 52%, respectively [55]. The sequences of AIPs that belong to *agr* I, II, III, and IV classes in *S. aureus* and *S. epidermidis* are YSTCDFTM, GVNACSSLF, YINCDFLL, YSTCYFTM, YNPCASYL, DSVCASYF, YNPCSNYL, YNPCANYL, respectively [55, 56].

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To control biofilm-associated staphylococcal infections, production of virulence factors and antibiotic resistance, QS can be disrupted by inhibition of signal production, degrading signals, and suppressing synthase and receptors [9].

2.5.2. The regulation of Staphylococcal biofilm by other than Agr

2.5.2.1. sarA

Two-component regulator gene locus encoded by arlRS is regulated by *agr* and *sarA* loci. *sar*A and *agr* have opposite functions in staphylococcal global regulation. When enough quorum population is present, at the beginning of attachment phase *sar*A is upregulated. During the initial stages, SarA enhances expression of PIA, adhesions, and EPS, by the way, induces attachment and early biofilm formation. SarA also represses nuclease and protease synthesis. After attachment of bacteria, agr system works and virulence factors that cause dispersal, nucleases and proteases and PSMs are produced [18].

2.5.2.2. sigB

The *sigB* operon of which product is σ^B in *S. aureus* upregulates ica transcription, and the factors for early stages of biofilm formation including FnbpA, clumbing factor, and coagulase and downregulates factors that are efficient in dispersal and in passing to planktonic state such as β-hemolysin, enterotoxin B, serine protease (SplA), cysteine protease (SplB), the metalloprotease Aur, staphopain, and leukotoxin D [18].

2.5.2.3. ArIRS

The biofilm formation of *S. epidermidis* [57] and *S. aureus* [58] can be also regulated by ArIRS that uses TCS. The biofilm formation of *S. epidermidis* is regulated by ArIRS in ica-dependent manner, whereas in *S. aureus*, this is ica-independent manner [59]. ArlRS also plays a role in the modulation of bacterial autolysis, as a result of eDNA release that participates in biofilm matrix [9].

2.5.2.4. lytSR

LytSR operon that is the other TCS of *S. aureus* plays a role in the activity of murein hydrolase that is an autolysin and distrupt structural components of the bacterial cell wall, consequently, autolysis. *Lrg*/*cid* operon that is a target of this system regulates lysis of cell during biofilm formation [60]. The regulator LytR that is effected by stimuli bound LytS sensor histidine kinase protein activates transcription of genes under its control. The regulator LytR upregulates the expression of *lrgA* and *lrgB* genes [61]. Encoded LrgA by *lrgA* is an antiholin and inhibits the extracellular activity of murein hydrolases, whereas *cidA* gene encodes holin protein that effects the activity of murein hydrolase, consequently, cell lysis and release of eDNA that participate in biofilm matrix [9].

2.5.3. Inactivation of ica by sequences

2.5.3.1. IS256

Although *S. epidermidis* strains are *ica* positive, they cannot produce biofilm due to IS256 insertion sequence that is inserted within the *ica* operon. Ziebuhr et al.[62] revealed that if bacterial genomic DNA contained IS256, IS256 was not seen within *ica* locus. They also revealed that although *S. epidermidis* strains that caused indwelling device–associated infection was *ica* positive and the insertion of IS256 is not seen within *ica* locus, strains did not produce biofilm ("off switch") [62]. These results showed that IS256 is not a natural occurring global regulator mechanism of biofilm production. The similar results were gained for *S. aureus*. IS256 that was inserted within *ica*C gene of *S. aureus* strain prevented biofilm formation by inactivating *ica*C gene [63].

2.5.3.2. Tetranucleotide tandem repeat

*ica*C inactivation caused by the expansion or contraction of tetranucleotide tandem repeat inhibits PIA/PNAG formation in *S. aureus* [64]. The reading frame of *ica*C is shifted by tetranucleotide tandem repeat ("ttta"), and this contributes premature stop of IcaC protein, consequently, inhibited PIA/PNAG production ("off switch"). Mutated *ica*C is preferred for the indwelling device-associated infections due to off switching of PIA/PNAG production.

2.6. Treatment of biofilm

To provide protection against *S. aureus* and *S. epidermidis* biofilm-associated infections vaccine that causes production of antibodies against PNAG and PSM peptides can be used. Researchers had revealed that mutant *S. aureus* of which icaB is over-expressed and produces high amount of surface associated PNAG was more opsonized by antibodies and undergoes to phagocytosis. But immune response is ineffective antibodies produced against PIA/PNAG of vaccine bind secreted PIA/PNAG of bacteria rather than surface-associated PIA/PNAG of bacteria [65]. Conjugate vaccine that contains *S. aureus* PNAG and clumping factor A can accelerate immune response [66]. Bacterial dispersal from indwelling medical devices can be prevented by antibodies against PSM peptides [48]. Brady et al. [67] had treated chronic osteomyelitis with a combination of antibiotic and quadrivalent vaccine that contains four antigens, which are glucosaminidase, an ABC transporter lipoprotein, a conserved hypothetical protein, and a conserved lipoprotein. By this way, Brady et al. [67] had reduced biofilm formation of *S. aureus* on infected tibias.

Kaplan et al. [68] and Whitchurch et al. [69] concluded that DNase I in human serum can degrade eDNA in biofilm matrix, by the way bacterial biofilms are degreased.

Nitric oxide (NO) that is a product of anaerobic respiration can cause dispersal of microorganism from mature biofilm by stimulation of c-di-GMP phosphodiesterases activity [70]. c-di-GMP biosynthesis inhibitors can be an alternative treatment for preventing biofilm formation and mature biofilm dispersal. The combinations of dispersin B (EPS-degrading

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enzymes) and disinfectants such as triclosan with antibiotics that are used in the treatment of wound and skin infections provides synergistic removal of biofilms [71].

3. The mechanisms of antibiotic resistance in biofilm-embedded microorganism

Biofilm-embedded bacteria are more resistant to antimicrobial agents than planktonic bacteria. It is difficult to eradicate biofilm, and this causes serious clinical problem [72].

Antibiotic resistance (tolerance) that is caused by biofilm and permit bacteria to survive is a physiological state by which mutational changes not caused [73]. Impermeability of peptidoglycan by efflux pumps, antibiotic-degrading enzymes, the charge of polymers [73], and certain gene products that are produced in biofilms [3] are the other antibiotic resistance mechanisms of bacteria rather than the biofilm [3]. Biofilm can gain higher antibiotic tolerance by antibiotic degrading enzymes such as beta-lactamases, efflux pumps, and certain gene products of which expression are changed by the quorum sensing as a stress response [3, 74]. Biofilms resist to beta-lactam antibiotics by beta-lactamases. Beta-lactamases that are produced by bacteria play a key factor in the biofilm caused resistance to beta-lactam antibiotics [3].

3.1. The heterogeneous sessile community and the physiology of biofilm

Biofilm-embedded sessile community has heterogeneous cells that are in the different growth states. Bacterial growth rate is reduced by stress conditions such as nutrient and oxygen limitation at the lower parts of the biofilm, and low metabolic activity. Low metabolically active cells (slow growing cells) are seen at the deeper parts of the biofilm, whereas high metabolically active cells (rapid growing cells) are seen at the surfaces of the biofilm. These heterogeneous cells that consist of low and high metabolically active cells have wide range of different responds to each antimicrobial. Antibiotic penetration through the biofilm is reduced by reduced bacterial growth rate. The biofilm-related resistance mechanisms such as oxygen limitation and low metabolic activity, reduced antibiotic penetration through the biofilm, and gaining genetic adaptations such as increased changes in the genes of the DNA repair systems play a key factor in the biofilm tolerance to antibiotics [3]. But some antibiotics such as colistin are just effective against slow-growing cells seen at the deeper parts of the biofilm not against rapid growing cells that acquired adaptive resistance by upregulation of the LPS-modification (arn) operon [75]. Persister cell population that is present in the biofilms of *S. epidermidis* can withstand to inhibitory concentrations of antibiotics [76] (**Figure 3**).

3.2. Nutrient limitation

Some researchers demonstrated that nutrient limitation-related antibiotic resistance is not due to the reduced growth rate of microorganism, but rather to the activation of regulated stress responses. Nutrient limitation-related antibiotic resistance is controlled by complex regulato-

ry pathways [77]. During starvation, the activation of the stringent response participates in antibiotic resistance such as fluoroquinolone resistance in *E. coli* biofilms [23]. Also, some researchers demonstrated that certain efflux pumps in *P. aeruginosa* are upregulated in the low-oxygen conditions [78] (**Figure 3**).

3.3. Biofilm matrix

Usually, the decreased antibiotic penetration through the biofilm is caused by antibiotics that may bind to the structural contents of biofilm matrix [3] rather than reduced diffusion of antibiotics through the biofilm matrix [10] (**Figure 3**).

3.4. Agr expression

Antibiotic susceptibility of biofilm-embedded bacteria decreases according to the planktonic state. The virulence of *agr* defective strains is lesser than the wild type. Expression of *agr* that imposes a fitness cost on *S. aureus* effects drug resistance of staphylococcal biofilm. It has been revealed that RNAIII production (provides fitness cost of bacteria) of *agr*-positive bacteria is induced by subletal doses of ciprofloxacin, mupirocin, and rifampin [79]. The adaptability of *S. aureus* to antibiotics involves the *agr* locus. *S. aureus* resists to drugs by adapting to antibiotics with *agr* locus. Ciprofloxacin, mupirocin, and rifampin are more effective against *agr*-defective bacteria. These antibiotics just must be used in *agr*-deficient mutants or *agr*-negative *S. aureus* when designing antimicrobial chemotherapy. *agr*-defective strains are isolated frequently in hospital-acquired *S. aureus* (HA-*S. aureus*) infections. Due to broad antibiotic usage in hospitals, the prevalence of agr-defective strains among hospital-acquired *S. aureus* infections is high and ranges between 15% and 60% [80].

Agr expression of biofilm producer staphylococcus has also been associated with the drug resistance of some antibiotics. It has been also revealed that the effect of rifampin against *agr*-defective *S. aureus* mutants was increased, whereas the effect of oxacilline unchanged [79]. *agr* negative or *agr* dysfunction strains have a fitness advantage over *agr* positive strains in the presence of some antibiotics such as vancomycin. Vancomycin susceptibility is reduced in VISA (vancomycin-intermediate *S. aureus*) due to the thickening of cell wall that is the result of the combination of cell wall biosynthesis activation and decreased autolytic activity. *agr* mutations have been correlated with the rise of VISA. *agr* defects that reduce autolysis decrease susceptibility of vancomycin of VISA [81].

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Chapter 11

Staphylococcus Biofilms

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Additional information is available at the end of the chapter

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Abstract

The majority of staphylococci produce biofilm on medical devices, which is the main mechanism to infect humans. Staphylococcal biofilms attach to abiotic or biotic surfaces, forming aggregates and protecting themselves against the immune system and the antimicrobial compounds of the host. Few studies on biofilm formation mechanism in *Staphylococcus epidermidis* and other coagulase-negative staphylococci (CNS) have been performed; however, there is a great interest in studying and controlling biofilm formation of this genus. This chapter exhibits the state of the art on biofilm formation in *S. epidermidis* and other staphylococcal species. The main goal of this chapter is to recognize the importance of biofilm formation in *Staphylococcus*. The participating molecules in staphylococcal biofilm formation are described. Currently, biofilm producer strains of *Staphylococcus* and mainly CNS have been frequently isolated at hospitals, causing significant economic losses. This chapter includes promising solutions in order to prevent medical device-associated infections, as the development of medical devices possessing anti-biofilm materials or surfaces that act against the adhesion or viability of the microorganisms.

Keywords: Biofilm, *Staphylococcus epidermidis*, *Staphylococcus aureus*, medical devices, anti-biofilm

Introduction

I. *Staphylococcus* biofilms

During the last years, the study of biofilms has become relevant due to their significance on many microbiology areas. In the health field, biofilms have been of great relevance because many pathogenic and non-pathogenic bacteria can produce biofilm as a part of its virulence

mechanism and protection against the host. A biofilm is considered a complex microbial community (or communities) attached to a defined surface and embedded within a cell matrix. Regarding the surface, biofilms may be formed on a wide variety of chemical or biological surfaces. Regarding bacteria of the *Staphylococcus* genus, biofilm is the main virulence mechanism of the coagulase-negative staphylococci (CNS) species. Biofilm formation in staphylococci is carried out in at least three stages: i) bacterial attachment to a defined surface, a process termed primary attachment; ii) assembly of these originating bacteria into a small cluster, also known as microcolony or cellular accumulation; and iii) biofilm growth and disassembly (also known as detachment or dispersal) mediated by a mechanical process or by active metabolites produced by the biofilm-embedded bacteria.

I.1. Medical and epidemiological relevance of staphylococci biofilms

Staphylococci are commensal bacteria inhabiting the human skin and mucus. However, they have been identified as infection-causing agents associated to biofilms. Animal models of biofilm-associated infections using staphylococci have allowed to determine the importance of their biofilms as a virulence mechanism. Therefore, staphylococci, particularly *Staphylococcus epidermidis*, are currently the most studied microorganisms regarding their biofilm formation capacity. Nosocomial Infections Surveillance System recognizes that *Staphylococcus aureus* and CNS (e.g., *S. epidermidis* and most of the remaining staphylococci species) are the most frequent nosocomial pathogens isolated from patients at the intensive care unit. Epidemiological data show that CNS are the third most common infective agent causing native valve infective endocarditis (NVIE), and they occupy the first place in prosthetic valve infective endocarditis (PVIE), demonstrating their importance for these two clinical entities.

Regarding *S. epidermidis*, it is an inhabitant of the human skin microbiota. *S. epidermidis* is an opportunist pathogen that causes disease only in patients subjected to predisposing factors. This includes patients with particular features such as premature newborns, inborn immunological impairments, or concomitant medical conditions, for example, human immunodeficiency virus (HIV) infection, immunosuppression after solid organ or bone marrow transplants, and chemotherapy-related neutropenia. Epidemiological data point out *S. epidermidis* as the most commonly isolated microorganism from foreign materials-related infections such as infected prosthetic joints, central venous catheters (CVC), cerebrospinal fluid shunts, intracardiac devices, artificial heart valves, and vascular grafts. Regarding prosthetic joints infections, *S. epidermidis* is the main infective agent of prosthetic joint implants. In UK, CNS and *S. epidermidis* are isolated from a 36% of total hip and 49% of total knee arthroplasty infections [1]. In an additional study on infected total hip and knee arthroplasties, it is pointed out that nearly 70% of the CNS isolates were identified as *S. epidermidis* [2].

I.2. Experimental models to study biofilm formation

The clinical relevance of biofilm formation on foreign materials has been demonstrated using cell culture models, a *Caenorhabditis elegans* infection model, and animal models of device infections, for example, CVC or prosthetic device infection models. The first study on the importance of biofilm formation in vivo using animal models and genetically distinct *S.*

epidermidis isolates with both positive- and negative-biofilm phenotypes failed to show evidence that could demonstrate that biofilm-forming isolates are more virulent in comparing with those possessing a biofilm-negative phenotype. Nevertheless, compelling results were obtained on subsequent studies using genetically defined strains and comparing the wild-type strain with its respective isogenic mutant strain. Using a mouse model of subcutaneous catheter infection and a rat model of venous catheter infection, the polysaccharide intercellular adhesion (PIA)-producing *S. epidermidis* 1457 strain was more virulent than its isogenic counterpart, the biofilm-negative 1457-M10 strain [3]. In a different model of CVC infection, the *icaRADBC*-expressing *S. epidermidis* strain and its *icaRADBC*-negative isogenic mutant displayed the same result [4]. An infection model of *Caenorhabditis elegans* was used in order to study the biofilm-positive phenotype of the *S. epidermidis* 9142 strain, in comparison to the *icaA* mutant, resulting in a higher virulence of the wild type than the mutant [5]. Recently, using a catheter infection model, *icaADBC* inactivation apparently had no effect on colonization, whereas *aap* inactivation completely abolished *S. epidermidis* ability to establish the infection [6]. One explanation for the null pathogenicity of the mutant strain regarding biofilm formation is the lack of protection against the innate immune system. Experiments conducted with cell culture showed that the biofilm-positive 1457 strain was less susceptible to antimicrobial peptides (AMPs) and to phagocytosis performed by polymorphonuclear granulocytes (PMNs) compared to the biofilm-negative 1457-M10 isogenic strain [7]. Physiological status is also important, when *S. epidermidis* 1457 grown on biofilm conditions was less susceptible to phagocytes than it was grown on planktonic conditions [8].

PIA-dependent biofilm formation also interferes with the host's complement activation. Biofilm-positive wild-type bacteria pre-opsonized with normal human serum are more resistant to complement-mediated elimination than the corresponding biofilm-negative isogenic bacteria [8]. It has been also shown that *S. epidermidis* biofilm formation interferes with the phagocytosis process and macrophage activation. This biofilm-forming phenotype may contribute to the chronic persistence of *S. epidermidis* in inflammatory conditions.

Conversely, *S. epidermidis* produces a set of pro-inflammatory peptides termed phenol-soluble modulins (PSMs), which are produced in a tightly regulated manner by the accessory gene regulator (*agr*) system. It has been demonstrated that PSM δ is able to lyse neutrophils, supporting the concept that these peptides are relevant for *S. epidermidis* pathogenesis. However, PSM δ is expressed at low levels by the biofilm-producing *S. epidermidis* 1457 strain, grown either in biofilm conditions or in planktonic conditions [9].

II. Mechanisms and molecules participating in staphylococci biofilm formation

In this chapter, we will divide the study of the biofilm formation process in three phases. During primary attachment, bacteria adhere to the biotic or abiotic surface in order to colonize it, whereas on the accumulation phase, bacteria build a tridimensional multi-cell and multilayer array. Then, staphylococci are able to disassemble biofilm structure in order to release those cells capable to colonize other sites on the surface. *S. epidermidis* and *S. aureus* biofilm models have been the most studied among staphylococci and the overall biofilm formation process is very similar. In this chapter, we will address *S. epidermidis* biofilm as the base model.

II.1. Participating molecules on the biofilm primary attachment phase

An essential step performed during the primary attachment stage is the tight binding of bacteria to the foreign material (medical device). This bacterial tight binding leads to a successful establishment of a medical device-associated infection. Regarding *S. epidermidis*, it has been found that cell wall proteins are the main elements of such interactions and this is similar for *S. aureus*. Genetic evidence has allowed establishing that bacterial binding to unmodified polystyrene (non-biotic surface) is conveyed by the *S. epidermidis* AtlE autolysin protein [10]. AtlE is a 115 KDa protein that belongs to the bacterial peptidoglycan (PGN)-hydrolases group that plays an important role in bacterial cell wall degradation. The protein is composed by an N-terminal signal peptide, a propeptide, a catalytic domain possessing N-acetylmuramyl-L-alanine amidase activity, three repeated sequences (R1-3), and one C-terminal catalytic domain, possessing N-acetylglucosaminidase activity. In addition to its role during cell wall turnover, AtlE is also important for unmodified polystyrene binding. This function was demonstrated by the *S. epidermidis* O-47 strain harboring a mutation caused by the *atlE*::Tn*917* transposon, which has an impaired ability to adhere to the polystyrene surface [10]. The binding mechanism of AtlE to the polystyrene surface is unclear; however, it is thought that the first event is AtlE recruitment on the bacterial cell wall through the R1-3 domain and those domains possessing enzyme activity. Based on AtlE expression and functional activation studies, it has been suggested that this protein leads to significant changes in cell wall hydrophobicity contributing to the primary attachment process [11]. Another assigned function for AtlE is its autolysin activity in order to cleave the cells wall and thus releasing extracellular DNA (eDNA), which is a common component in staphylococci biofilm [12]. *S. aureus* autolysin also shares this function at this biofilm formation phase.

The interaction between *S. epidermidis* and an artificial unmodified surface (polystyrene) is mediated by non-specific interactions without the participation of a receptor-specific ligand. On surfaces coated with the host's extracellular matrix (ECM), both *S. epidermidis* and *S. aureus* express cell surface proteins leading to a specific interaction with the components of this ECM of the host. Proteins exhibiting ECM-binding activity are important in order to initiate the infection of medical devices because once foreign materials are introduced inside the body, they are covered by ECM materials (e.g., fibronectin; fibrinogen; vitronectin; collagen). It has been described that *S. epidermidis* AtlE can adhere to vitronectin-covered surfaces, whereas the GehD lipase is involved in interactions with collagen [13]. In addition to these proteins, both *S. epidermidis* and *S. aureus* express proteins possessing a specific function for their interaction with ECM. These belong to the serine-aspartate repeat (Sdr) protein group and they are members of the microbial surface components family that recognize adhesive matrix molecules (MSCRAMM) [14]. In *S. epidermidis*, three Sdr proteins referred to as SdrF, SdrG, and SdrH have been identified [14]. SdrG (also known as Fbe) is a protein containing the LPXTG motif that is covalently bound to the bacterial cell wall surface and specifically recognizes fibrinogen and thus *S. epidermidis* cells expressing SdrG adhere to fibrinogen-covered surfaces [15]. The gene coding for SdG/Fbe is found on *S. epidermidis* clinical isolates. The SdrG protein contains four distinct regions: an N-terminal export motif sequence, the A region containing the fibrinogen-binding activity, the B region with unknown function, and the R

region containing the serine-aspartate repeat sequence. SdrG specifically binds to a 14-amino acid-long peptide sequence on the N-terminal of fibrinogen's beta chain. SdrF, display a similar organization to SdrG and it specifically binds to collagen I [16]. So far, a specific function has not been assigned to the A region of SdrF. However, it has been demonstrated that its B region is sufficient to interact with collagen I and apparently, this binding occurs through the alpha1 and alpha2 chains of type I collagen [16]. Using a *Lactococcus lactis* heterologous expression system and a murine infection model, it has been established that SdrF may contribute to cardiac assist device driveline infections [17]. SdrF also participates in binding to unmodified Dacron surfaces covering drivelines. Anti-SdrF antibodies inhibited 50% of *S. epidermidis* 9491 binding to collagen using an in vivo model [17], indicating that additional collagen-binding factors may participate.

eDNA function during *S. epidermidis* and *S. aureus* biofilm formation has been established as another crucial component for cell attachment to a surface. Some studies confirm that eDNA is a structural component of the biofilm's matrix in both species. Independent studies have demonstrated that eDNA is released through increased cell lysis. In *S. epidermidis*, autolysis is carried out mainly by the autolysin activity of AtlE. A role for eDNA in *S. epidermidis* 1457 was evidenced during primary attachment through the addition of DNase I that results in inhibition of bacterial binding to a glass surface. In spite of the fact that eDNA participates in the primary attachment phase, a function during intercellular attachment phase (accumulation phase) has been ascribed [18]. During the surface colonization phase by *S. aureus* under flow conditions, eDNA is crucial during the transition between primary attachment and accumulation phases [19]. This points out that eDNA plays an important role in early stages of staphylococci biofilm formation.

II.2. Participating molecules during the biofilm accumulation phase

The main component during the accumulation phase is the expression of molecules possessing intercellular (cell-cell) adhesion properties leading to cell aggregation and to subsequent biofilm development having a multi-cell and multi-layer tridimensional structure. Based on the early electron microscopy studies, it has been shown that *S. epidermidis* are embedded within an amorphous matrix. Afterwards, the studies were focused on the biochemical analysis of the matrix components. These efforts resulted in the discovery of the PIA polymer, a component participating in great proportion on intercellular adhesion.

The PIA structure was first described in biofilm-forming *S. epidermidis* 1457 and RP62A strains. Through biochemical analysis, the existence of the structurally related polysaccharide I (>80%) and polysaccharide II (<20%) was determined and separated based on their different ionic properties. Using chemical analysis and NMR spectrometry, it has been demonstrated that polysaccharide I is a lineal homoglycan consisting of beta-1,6-linked 2-amino-2-deoxy-D-glucopyranosil residues. Approximately 80–85% of them are N-acetylated (GlcNAc) and the rest are not N-acetylated, this polymer has an overall negative charge. PIA's polysaccharide II has a low proportion of N-acetylated 2-amino-2-deoxy-D-glucopyranosyl residues and it is modified with succinate residues linked by ester bonds, which confers it with anionic features [20]. The synthesis of an actively functional PIA molecule requires the expression of

all four *icaADBC* genes [21] constituting the *ica* operon. The *icaR* gene confines the repression of the *ica* operon expression. The synthesis process has been studied in detail using *S. carnosus* recombinant strains expressing different combinations of the *icaADBC* genes and using UDP-GlcNAc as the sugar donor [21]. The IcaA protein belongs to the glycosyltransferase family 2. It is an integral membrane protein consisting of 412 amino acids and four transmembrane domains. This protein performs the synthesis of beta-1,6-linked GlcNAc oligosaccharide composed by up to 20 GlcNAc units. The IcaD protein is required for IcaA full activity in vitro. IcaD is a membrane protein of 101 amino acids possessing two putative membrane space domains and it is thought that it may be a chaperone guiding IcaA protein folding and its membrane insertion and it also may act as a link between the IcaA and IcaC proteins [21]. Essential to PIA synthesis is the presence of the IcaB, an integral membrane protein of 355 amino acids possessing 10 predicted transmembrane domains, which may be involved in the externalization and elongation of the nascent polysaccharide [21]. IcaB is a member of the polysaccharide deacetylase family that includes chitin deacetylases or the chitooligosaccharide deacetylase NodB of *Rhizobium melioti*. IcaB in its mature form is a secreted protein consisting of 259 amino acids with a predicted signal sequence, which is responsible for PIA N-deacetylation and it is crucial for PIA activity during biofilm formation and for *S. epidermidis* virulence [22]. A strain harboring an *icaB* deletion mutation, in which the *icaB* gene has been eliminated, produces a weakly retained PIA at the cell's surface, as it does not contain N-deacetylated GlcNAc [22].

Conversely, the first observation made through biochemical analysis on biofilm matrix extracts indicated the presence of oligosaccharide, proteins, and nucleic acids. The specific proteins that comprise a biofilm have been identified and characterized; one of them is the biofilm-associated protein Bap [23]. The Bap is rarely found in invasive *S. epidermidis* biofilms from human infections [23] and it similarly occurs for *S. aureus*. Another protein found in biofilms is SesC, which has been proposed as playing an important role during biofilm formation [24]. SesC is a surface protein of 68 KDa containing the *S. epidermidis* LPXTG motif and it is related to the *S. aureus* clumping factor A (ClfA). SesC protein is strongly expressed in biofilm conditions in contrast to planktonic conditions by the *S. epidermidis* 1457 strain [24]. An anti-SesC antibody inhibited biofilm formation in vitro in several *S. epidermidis* isolates. All 105 *S. epidermidis* isolates collected from nose swabs of infection possessed the *sesC* gene in their genomes [24]. Actively or passively immunized animals using SesC as antigen displayed a decreased biofilm formation using the in vivo CVC infection model [25]. Nevertheless, a specific role of SesC during the intercellular adhesion of the accumulation phase of the biofilm remains to be demonstrated.

II.3. Multifunction proteins during the biofilm accumulation phase

Protein factors contributing to the accumulation phase of staphylococci biofilm have features of multifunctional proteins. In *S. aureus*, it has been found that fibronectin-binding proteins (FnBPs) (FnBPA and FnBPB), ClfA protein and *S. aureus* surface protein (SasG), may be considered multifunctional proteins as they do not have an exclusive role in either of the biofilm phases: primary attachment or accumulation [26]. FnBPs are constituted by an N-

terminal end, the A domain, and within the C-terminal, the LPXTG-anchoring domain separated by tandem repeats, which are involved in binding to fibronectin. Both, FnBPA and FnBPB are involved in the biofilm's accumulation phase in isolates from hospitals and under flow conditions. This concept of multifunctional proteins with important roles during some of the biofilm formation and surface colonization phases is also applied to *S. epidermidis* with its respective proteins participating in the accumulation phase: the accumulation-associated protein (Aap) and the extracellular matrix-binding protein (Embp).

The Embp protein and its *S. aureus* orthologue, designated as Ebh, were simultaneously pinpointed during identification studies of protein factors [27]. In a clinically relevant *S. epidermidis* isolates collection, the *embp* gene was detected in 90% of all strains [2]. Furthermore, studies conducted in vivo indicated the presence of anti-Embp antibodies in patients with prosthetic joint infections by *S. epidermidis*, suggesting that the Embp protein is expressed and it has a role during the infection. Surprisingly, when using a bacterial cell model in flow conditions, it was observed that addition of anti-Embp antibodies to the system inhibited biofilm formation by the *S. epidermidis* 1457 strain [28]. This result leads to propose the Embp protein as a potential candidate for preventive strategies against biofilm formation. Experimental evidences suggest that Embp has a defined role during the primary attachment phase. This proposal is supported by the fact that Embp overexpression had no effect on bacterial attachment on an unmodified polystyrene surface, although it had a negative effect on bacterial binding to fibronectin-covered surfaces [29]. Additionally, it was observed that the Embp-fibronectin interaction is necessary for the biofilm's accumulation phase on plastic surfaces [29].

The Aap protein is covalently bound to the cell wall and consists of an A domain and a B domain. The A domain has 584 amino acids and includes an export signal at its N-terminal, 16 amino acid repeats and a globular region of 212 amino acids with alpha-helical and beta-sheet contents. This 212-amino acid-long region is highly conserved between Aap and its *S. aureus* orthologue, SasG. Through bioinformatics analysis, it has been shown that this domain possess lectin-type activity [6]. The B domain consists in a variable number of repeats of 128 amino acids, the G5 subdomains [2]. The number of G5 subdomains in the B domain is different among the *S. epidermidis* strains, for example, the RP62A reference strain possesses 13 G5 subdomains, whereas the *S. epidermidis* 1457 strain has only seven [6]. This fact has been also observed in clinic isolates with clonal genotypes subsequently recovered from ongoing infections on devices in patients [2]. This observation leads to the hypothesis stating that the number of G5 subdomains of the Aap B domain may represent a mechanism contributing to the immune evasion of *S. epidermidis* mediated by the modification of the major epitopes on the cell's surface [2]. Aap is detected on the bacterial cell wall and its retention mechanism is through anchoring of its C-terminal by a covalent bond with the cell wall PGN [30]. A more detailed analysis by confocal microscopy showed that Aap is strictly localized at the bacterial cell surface, whereas minimal amounts of Aap are released within the biofilm matrix [30]. This result is supported by electron microscopy observations in which Aap appears as elongated fibers of 120 nm projecting outwards from the cell wall and in form of tufts [31].

The importance of Aap for *S. epidermidis* biofilm formation was recognized during studies in which the expression of the B domain does not modify the primary adherence properties, although it is very important to cellular aggregation, indicating that Aap is a protein that participates in the intercellular adhesion [32]. Similarly, the importance of the B domain for intercellular adhesion was also described for SasG in *S. aureus* [33]. Another fact that evidences the properties of Aap in intercellular adhesion is the ability of the B domain to undergo homodimerization in the presence of Zn [34]. The proposed mechanism of intercellular adhesion through Aap is that the protein must undergo proteolytic processing in order to remove the A domain [32–33]. Thus, Aap proteolytic processing does not normally occur under in vitro growth conditions [32].

Although the intercellular adhesion property of Aap was recognized, currently there is evidence supporting its significant role in the primary attachment phase as well. The binding of *S. epidermidis* NCTC 11047 strain expressing Aap to squamous epithelial cells was partially inhibited by the addition of the recombinant A domain of Aap [35]. In a clinical isolate, the binding of *S. epidermidis* CSF41498 strain expressing a non-processed Aap (thus containing the A domain) to polystyrene was completely impaired by the addition of an anti-A domain antiserum, whereas an anti-B domain antiserum did not affect its adhesion ability [36]. Thus, a new bifunctional role of Aap during the biofilm formation is suggested: its participation on the primary attachment phase through its A domain and also its participation on the accumulation phase through its B domain [32, 35–36].

II.4. Molecular mechanisms for mature biofilm disassembly

A primary biofilm disassembly mechanism used by *S. aureus* and *S. epidermidis* is the production of extracellular enzymes or surfactants that degrade or solubilize the adhesive components of the biofilm matrix. Because this matrix cover bacterial cells within the biofilm colony, its degradation results in cell detachment from the colony and its release toward the environment. The products of the matrix-degrading genes, which are implicated on the dispersion of the staphylococcal biofilm, include proteases, DNases, and surfactants.

The *agr* system is a putative regulator controlling the production of the enzymes degrading the biofilm matrix. The *agr* is controlled by a cyclic autoinducing peptide (AIP) that is synthesized and secreted within the environment. When the AIP concentration reaches a critical threshold level, it activates a two-component signal transduction cascade leading to the production of secretory virulence factors [37]. The extracellular proteins induced by the *agr* system are multiple proteases and pore-forming toxins termed PSMs. *S. aureus* does not form a biofilm when the *agr* system is highly active and its reactivation within the mature biofilm results in its disassembly [38]. Furthermore, the *agr* system of *S. aureus* is more active in cells detached from the biofilm [39]; the same phenomenon was observed in *S. epidermidis* [9], contributing evidence showing that induction of the *agr* system results in biofilm disassembly.

Extracellular protease production has been implicated on the disassembly of the mature biofilm. In *S. aureus*, the mutation of the protease-coding genes results in a significant increase of biofilm formation under flow conditions and the disassembly decrease when the *agr* system

is inactivated [38]. Additionally, protease inhibitors have a promoter role during biofilm formation in *S. aureus* under conditions that normally accelerate its disassembly [38]. Similarly, mutations leading to extracellular protease overexpression, such as those on the *sarA* and *sigB* genes, enhance a planktonic growth in *S. aureus* [40]. This leads to a concept of an inverse correlation between protease expression and biofilm formation.

S. aureus secretes a potent DNAse, also known as thermonuclease or micrococcal nuclease, which has been implicated on cell detachment from the biofilm. *S. aureus* mature biofilm is disintegrated by the exogenous addition of DNAse I or restriction enzymes [41]. It has been shown that a nuclease-mutant *S. aureus* strain exhibited an increase of biofilm formation under flow conditions regarding to the wild-type strain [41]. These findings suggest that nucleases may function as endogenous mediators for biofilm disassembly in this species.

PSMs are peptides possessing surfactant features, which are produced by both *S. aureus* and *S. epidermidis*, and they are capable of contributing for mature biofilm disassembly. PSMs are regulated by the *agr* system and their amphiphilic alpha-helix structure confers it with a surfactant-type property. PSMs promote both disassembly of the mature biofilm of *S. epidermidis* in vitro and dissemination from colonized catheters on a mouse model of device-related infection [9]. Additionally, antibodies against PSMs inhibit bacterial dispersal from the implanted catheter, indicating that the disassembly manipulation strategy may prevent the spreading of the infection.

III. Regulation of biofilm formation in staphylococci

Biofilms are a lifestyle adopted by a wide variety of microorganisms that requires the consumption of an enormous amount of energy. Thus, it is expected that biofilm growth may be controlled by more regulatory mechanisms regarding planktonic growth. Some of the factors that impact on biofilm formation are mentioned in the following sections.

III.1. Regulation of the factors participating on the primary attachment phase

The *agr* is a quorum-sensing system in staphylococci that regulates the expression of adhesion molecules, thus it participates in the primary attachment phase. These adhesion molecules, such as the MSCRAMMs, display an increased expression when cell density is low, a situation favoring the onset of the infection by staphylococci. Once the colonization has concluded, the increased activity of the *agr* quorum-sensing system represses the expression of unnecessary colonization factors. Among these, the MSCRAMMs are included, which are negatively regulated by *agr* in *S. aureus* [42]. In *S. epidermidis*, the knowledge regarding colonization factors and their regulation is more limited. Results obtained by transcriptional profiling and the assessment of MSCRAMM expression [43], suggest that some of the latter do not follow the classic notion of regulation mediated by the *agr* system.

III.2. Regulation of PIA synthesis

The regulation of PIA expression is probably the best-studied system among those regulation systems involved in staphylococci biofilm formation. Anaerobiosis significantly increas-

es PIA expression [44]. This constitutes an important finding for biofilm physiology, as the oxygen concentration would restrict biofilm formation at the oxygen-loaded arterial bloodstream. In an already established biofilm, PIA expression would be higher at the most deep biofilm sections because oxygen concentration significantly decreases. Conversely, it has been found that sub-inhibitory concentrations of specific antibiotics increase the transcription of the *ica* operon in *S. epidermidis* [45].

Some overall regulators of *S. aureus* or *S. epidermidis* participate in the *ica* operon transcription regulation or PIA expression, such as the SarA DNA-binding protein and the alternative sigma factor SigB that increase the expression of the *ica* operon, whereas the luxS quorum-sensing system represses the expression of this operon [46]. Contrastingly, the *agr* system does not regulate PIA. The exact mechanism explaining the influence of SarA and SigB on the *ica* operon transcription is complex. Briefly, the SigB regulator represses the *icaR* gene transcription, as its protein product, IcaR, in turn, represses the transcription of the *icaADBC* operon [47]. Besides, SarA regulates positively the *icaA* gene, independently from IcaR [48].

III.3. Regulation of the PSMs expression

It has been discussed that the *agr* quorum-sensing system represses the expression of surface proteins after the primary attachment. The major *agr* control relies on the expression of the PSMs. The expression of the *agr* system within a biofilm is limited to its periphery, in which the *agr* regulator controls cell detachment from the biofilm by regulating the increased expression of the PSM effector molecules [49]. The staphylococcal PSM δ is a major effector molecule for cell detachment from the biofilm and it is tightly controlled by the *agr* system of *S. aureus* [50]. In *S. epidermidis*, the PSM β is the most important.

III.4. Biofilm regulation against host's defenses and antibiotics

One of the advantages possessed by bacteria in the biofilm state is high resistance toward antibiotics and the host innate defense, such as AMPs and the phagocytosis performed by neutrophils. However, the molecular basis of this phenomenon has been recently investigated. Two of the main mechanisms contributing to biofilm resistance are: (1) keeping antibacterial substances from reaching their target, for example, by limited diffusion or repulsion and (2) biofilm's specific physiology that limits the efficiency of antibiotics, mainly those targeting active cells, and it may include specific subpopulations of resistant cells ("persistent").

Limited antibiotic diffusion provided by the biofilm is mainly due to the nature of the biofilm matrix. However, this limited diffusion is the resistance mechanism toward some antibiotics, such as ciprofloxacin in *P. aeruginosa* [51], whereas some others (e.g., rifampicin and vancomycin) are able to cleave the exopolysaccharide envelope of *S. epidermidis* [52]. Interestingly, PIA has the ability to protect the cells within the biofilm from both cationic or anionic AMPs, as it possess an overall positive or negative charge, and thus PIA interacts or repels molecules depending on its charge. Similarly, the poly-gamma-glutamic acid (PGA) exopolymer, of *S. epidermidis* and a number of CNS species, contributes to the resistance toward AMPs of either charge.

Phagocytosis, mainly performed by neutrophils, is a major mechanism by which the innate immune system eliminates microorganisms invading the human body. Staphylococci in a biofilm are not readily subjected to phagocytosis by neutrophils. The responsible elements for this constraint are the PIA exopolysaccharide and the PGA exopolymer, and therefore they contribute to biofilm resistance toward the host's innate defense mechanisms.

IV. Therapeutic strategies against biofilm formation in medical devices

Medical devices are widely used for diagnostic and therapeutic treatment in most medical specialties. Infection risk is a frequent complication linked to the permanent use of medical devices such as orthopedic or heart prostheses, vascular catheters, urinary catheters, and endotracheal tubes. A promising solution in order to prevent medical device-associated infections is to develop devices possessing materials or surfaces that act against microorganism adhesion or their viability. The first strategy was the use of biocides in coatings. A number of clinical assays have been conducted producing conflicting results. Some authors suggest that the extended use of biocide on the coating may lead to an increase of microbial resistance toward the microbiocide agent. The other strategy consists in the development of materials impeding bacterial adhesion.

IV.1. Biological strategies for biofilm treatment

The chemical diversity of the biofilm matrix, including protein material, eDNA, and polysaccharides, is susceptible to degradation by a wide variety of exogenously added enzymes. Some research groups have observed that proteinase K and trypsin may disperse the mature biofilm of *S. aureus* and *S. epidermidis* [53]. Bovine DNase addition has also been successful for dispersing the mature biofilm of *S. aureus* [54]. Similarly, the enzymes able to degrade PNAG cleave biofilms containing this polysaccharide as their primary component. An enzyme called dispersin B (DspB) inhibits biofilm formation and promotes its disassembly in several *S. epidermidis* and *S. aureus* strains having PNAG as the main component of their respective biofilm matrices [54]. Finally, the treatment with lysostaphin was effective in a catheter mouse model of *S. aureus* biofilm [55], suggesting that it may be a general therapy against staphylococcal biofilm infections.

A current topic is the development of an antimicrobial coating interfering with quorum-sensing mechanisms. This has been observed for halogenated furanones synthesized by the red algae *Delisea pulchra* possessing anti-adhesive properties against a wide range of bacteria [56].

IV.2. Anti-adhesive chemical strategies

IV.2.1. Hydrophobicity and surface charge

Bacterial adhesion depends on hydrophobocity of the cell and material constituting the surface. The self-autoassembled monolayers (SAMs) can modulate the exposure of their different residues on a surface and they are used in bacterial adhesion studies as models of surfaces

with chemically controlled properties. SAMs with hydrophilic residues (OH, NH_2) tend to decrease bacterial adhesion when compared to those with hydrophobic surfaces containing methyl groups (CH_3) [57]. Some hydrophilic linings, such as hydrogels or medical devices with chemically modified surfaces have been developed in order to restrict biofilm development. Some clinical studies have reported that urinary catheters lined with heparin can reduce *Proteus mirabilis* biofilm [58]. However, it has been observed that heparin may stimulate biofilm formation by *S. aureus* [59]. This demonstrates that initial adhesion is not always sufficient to avoid biofilm development. Treatment with plasma can also create hydrophilic residues at the surface of medical devices producing antimicrobial activity.

IV.2.2. Steric barriers

The chemical modifications of the surface may also consist on grafting long-chain polymers in order to form brush-type structures on it. The density of the chains provides a steric barrier that repels bacterial adhesion. The most widely studied polymers are derivatives of polyethylene oxides. In fact, residues of SAMs with ethylene glycol (4EG and 3EG) have lower bacterial adhesion in comparison to hydrophilic surfaces [57]. Polymers with ester residues (CHO_2-) or cyclic hydrocarbons (C4H-, C6H-) exhibit less bacterial attachment strength than materials containing ethylglycol or hydroxyl group fragments [60].

IV.2.3. Anti-adhesive strategies based on topographic modifications of the surface

In the theories of bacterial adhesion, the appearance of the surface of the material was not considered. The relief of a surface depends on the scale, that is, for bacterial adhesion, the submicron scale is used. The reliefs are divided into: i) areas with irregular or random traits defined as rough; ii) areas with organized features, often made by an engineering process, defined by the term surface topography.

One study showed that adherence of *S. epidermidis* was similar on titanium surfaces, both rough and smooth [61]. SEM observations showed that this strain tends to adhere to grooves and depressions possessing dimensions similar to that of bacteria [62]. Regarding surface topography, it has been found that a surface constituted by titanium nanotubes is more hydrophilic than a conventional titanium surface. These properties have a biomedical application in orthopedics by decreasing bacterial adhesion [63]. Additionally, nanotubes could be filled with biocides in order to enhance its activity against biofilm. Superhydrophobic surfaces are being developed with nano or micro-features in order to create bacteria-free medical devices.

IV.2.4. The influence of nanofeature physical structure on bacterial response

Nanofeatures may adopt different shapes: nanotubes, notches, channels or grooves, holes or pillars. There are few studies regarding the relationship between nanofeatures and bacterial adhesion. Ercan et al. compared *S. aureus* and *S. epidermidis* adhesion on commercial titanium surfaces with nanotubes of 20–80 nm of diameter. A decreased bacterial adhesion was observed for larger diameters (60 and 80 nm) [64]. However, the study conducted by Yu et al. produced opposite results: staphylococci adhesion increased proportionally to nanotube

diameter (30, 70, and 120 nm) [65]. The nanofeature's array is also important for bacterial adhesion and it may form large patterns having several effects on microbial adhesion. For instance, the crest-shaped array (2 μm wide, 3 μm spacing, and different lengths) in polydimethyl siloxane elastomer was bioinspired from the shark's skin. This elastomer structure exhibited no signs of *S. aureus* biofilm formation after 14 days, unlike the smooth surface, which allowed the formation of a mature biofilm [66].

As mentioned above, the nanoscale level, size, and bacterial shape regarding nanofeature dimensions play a significant role. Bacterial features (adhesion, surface charge) are also important to the adhesion process. The surface of a nanostructure must be tested with several bacterial strains, as they exhibit different adhesion behaviors. For instance, a titanium surface nanostructured by femtosecond laser ablation and mimicking the superhydrophobic surface of the lotus leafs was not colonized after 18 hours by *P. aeruginosa*, whereas *S. aureus* adhesion was stronger when compared to the smooth surface [67]. This result suggests that some nanostructure surfaces may not be appropriate for medical applications in which the adhesion properties of the microorganisms are unknown.

Conversely, silver nanoparticles (AgNPs) are gaining interest for biomedical applications because of their features having a higher surface/mass ratio and a potent antibacterial activity. These AgNPs may be applied as monolayers at the surface of biomaterials. A study on glass surfaces modified with AgNPs was carried out and it was found that they possess a great stability in aqueous media, an extended Ag^+ release without AgNPs detachment and a strong anti-biofilm activity against *S. epidermidis* RP62A [68]. These AgNP-coated surfaces could be applied on a great variety of biomaterials. Nevertheless, it is important to conduct more studies to verify the anti-biofilm capacity with clinical isolates of different staphylococci species.

5. Conclusions

Staphylococcus biofilms is a virulence factor widely distributed in this genus, currently there are many studies about this subject; however, there are still questions to be answered about the process of biofilm formation. Some molecules involved in biofilm formation are recognized; nevertheless, the interaction between them is unknown, such as the formation of the structural network of the biofilm, the assembly and disassembly process, and the mechanisms of intrinsic and extrinsic regulation during these events. Many molecular pieces remain to be resolved, which allow us to fully understand the construction of a biofilm. Furthermore, it is evident that a strain of *Staphylococcus* can form different types of biolfilm (PIA-dependent or dependent protein), suggesting that staphylococcal biofilm is dynamic and adaptable to growth conditions. In fact, biofilm dynamics can be interpreted as a mechanism of resistance to environmental variations. The use of medical devices covered with anti-biofilm materials represents an alternative strategy but it is not decisive. The picture is complicated by the biological and physical characteristics of the different types of biofilm, the high genetic diversity into the genus, and the lack of comprehensive knowledge on biofilm formation properties, which leads to a complex and complicated scenario that prevents a successful anti-

biofilm treatment. By understanding the processes of biofilm, it could control its formation and have biofilm-free medical devices.

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Chapter 12

Biofilm Formation of *Salmonella*

Daxin Peng

Additional information is available at the end of the chapter

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Abstract

Salmonella spp. may form biofilm, and bacteria in biofilm are more resistant to drug, chemical, physical and mechanical stresses, and host immune system. The progress on biofilm research will be helpful for the development of new tools and strategies to prevent biofilm-related disease and decontaminate biofilm-derived *Salmonella* in food production. In this review, we present a comprehensive overview of biofilm formation in *Salmonella*, included that (1) the component of *Salmonella* biofilm, (2) the detection methods for biofilm, (3) the identification of biofilm-formation-associated genes, (4) the regulation mechanism of biofilm formation, and (5) virulence or resistance of *Salmonella* in biofilm.

Keywords: *Salmonella*, biofilm, component, mechanism, gene, pathogenicity, drug resistance

1. Introduction

Salmonella enteric is an intracellular gram-negative pathogen that infects various hosts, which is classified into more than 2500 serovars [1]. Many serovars, such as those most commonly associated with human infections, including *Salmonella enteritidis, Salmonella typhimurium*, have a broad host range [2]. In contrast, other serovars, such as *Salmonella typhi, Salmonella paratyphi, Salmonella gallinarum, Salmonella choleraesuis, Salmonella abortusovis*, and *Salmonella dublin*, have restricted host ranges and are associated primarily with one or a few hosts [3]. *Salmonella* can cause disease in domestic animals, ranging in severity of asymptom, diarrhea and enteritis to systemic syndrome, and result in a huge economic loss in pig and poultry industry. Salmonellosis is also a growing public health concern in both the developed and developing countries, since nontyphoidal *Salmonella* disease, a major cause of diarrheal disease globally, is estimated to cause 93 million enteric infections and 155,000 diarrheal deaths each year [4]. The

illnesses and outbreaks are most commonly attributed to exposure to contaminated food, and the eggs, broiler chickens, and pigs are among the top sources [5]. *Salmonella* often exist not only as planktonic cells but also as sessile, multicellular forms such as biofilms attached to surfaces. Biofilm formation is important for the spread of *Salmonella* because bacteria in the biofilm are resistant to disinfectants and chemical, physical, and mechanical stresses [6–8]. The biofilm formation is also contributed to *Salmonella* virulence, since bacteria in the biofilm are more resistant to antibiotics and host immune system, resulting in a chronic infection and the development of *Salmonella* carrier state [9, 10]. In our review, we present a comprehensive overview of biofilm formation in *Salmonella*.

2. The component of *Salmonella* biofilm

The biofilm formation is a multistep developmental process that always has several distinguishable steps: (a) attachment to the carrier surface, reversible, (b) irreversible attachment, binding to the surface with the participation of adhesions or exopolysaccharides, (c) the development of microcolonies, a distinct mushroom shape, (d) the maturation of biofilm architecture [11, 12], (e) under favorable conditions, the synthesis of martrix compounds decreases and the matrix is enzymatically cleaved, leading to biofilm dispersion [13]. In natural environments, *Salmonella* forms biofilms on plant [14], abiotic surfaces, including plastics, metal and glass [15–17], meat and meat-processing environments [18, 19]. In addition, *Salmonella* can colonize gallstones under laboratory conditions [20], and the *Salmonella* biofilm can be directly visualized by confocal micrographs of extracellular matrix on the surface of human cholesterol gallstones [21]. They can also form biofilms on chicken intestinal epithelium [22] or HEp-2 cells that are suspended in once-flow-through continuous culture conditions [23].

The extracellular matrix of *Salmonella* biofilm is majorly composed of curli (amyloid fimbriae), cellulose [24, 25], biofilm-associated protein (Bap) [26], O-antigen capsule [14, 27], extracellular DNA [28, 29]. The expression pattern of the biofilm is serovar specific and correlates with contact surface [30]. Curli were first discovered in the late 1980s on *Escherichia coli* strains that caused bovine mastitis, and they are mainly involved in adhesion to surfaces, cell aggregation and biofilm formation. Curli also mediate host cell adhesion and invasion, and they are potent inducers of the host inflammatory response [12]. The curli protein is encoded by the divergently transcribed *csgBAC* (*agfBAC*) and *csgDEFG* (*agfDEFG*) operons [31, 32]. The *csgBAC* operon encodes the major structural subunit, CsgA, and the surface-exposed nucleator protein CsgB. A third gene, *csgC*, is in the *csgBAC* operon, but no transcript for *csgC* has been detected in curli biogenesis [32]. The other study shows that both CsgC and CsgE facilitate extracellular thin aggregative fimbriae synthesis in *Salmonella enteritidis* [33]. The *csgDEFG* operon encodes accessory proteins required for curli assembly. The *csgD* gene encodes a transcriptional regulator belonging to the LuxR family, CsgD, for active transcription of *csgBAC* promoter [24]. Although Giaouris et al. [34] found that CsgF was expressed in biofilm growth when compared with planktonic and biofilm cells of *Salmonella enteritidis* on stainless steel surface, the function of *csgF* and *csgG* genes has not been re-

solved in *Salmonella*. Cellulose is a polysaccharide composed of β(1 → 4)-linked D-glucose units [35], which is an important exopolysaccharide normally synthesized in the *Salmonella* biofilms. The production of cellulose and curli by *Salmonella* leads to a matrix of tightly packed cells covered in a hydrophobic network. The operons, *bcsABZD* and *bcsEFG*, are required for cellulose biosynthesis [36]. Cellulose biosynthesis is positively regulated by CsgD, which stimulates the transcription of AdrA that harbours a cytoplasmic GGDEF domain. AdrA activates cellulose production on the post-transcriptional level either by direct interaction with *bcs* operons or indirect interaction with bis-3′-5′-cyclic dimeric guanosine monophosphate (c-di-GMP) [25, 37, 38]. BapA, a large cell-surface protein required for biofilm formation, is encoded by *bapA* gene and secreted through a type-I protein secretion system (*bapBCD* operons) situated downstream of the *bapA* gene. The expression of *bapA* is coordinated with that of genes encoding curli fimbriae and cellulose, through the action of *csgD* [26, 39]. The *bapA* gene is also highly conserved in *Salmonella* [40]. *Salmonella* produces an O-antigen capsule coregulated with the fimbria- and cellulose-associated extracellular matrix. The operons yihU-yshA and yihVW are responsible for capsule assembly and translocation [41] and regulated by CsgD. Although the O-antigen capsule do not appear to be important for multicellular behavior, they play an important role in attachment and environmental persistence [14]. However, the O-antigen capsule is required for biofilm formation of *Salmonella typhimurium* and *Salmonella Typhi* on cholesterol gallstones, and the operons are regulated in a *csgD*-independent manner [42]. Extracellular DNA is shown to be a matrix component of *Salmonella* biofilms cultivated in flow chambers and on glass surfaces [28]. However, the presence of extracellular DNA plays an inhibitive and destabilizing effect during biofilm development of *Salmonella* on abiotic surfaces [29].

3. The detection methods for biofilm

3.1. Quantification of biofilm formation

Biofilm formation of *Salmonella* can be quantitated by microplate-based crystal violet staining [43]. Briefly, the overnight broth cultures of bacterium are diluted 1:100 in the diluted tryptic soy broth (TSB). One hundred μl of bacterial suspension is added into 96-well U-bottomed polystyrene microtiter plates. Plates are incubated at 28°C for 24 h under static conditions. Then, non-adherent bacteria are removed and the wells are washed gently three times with 200 μl of distilled water. One hundred μl of 0.4% crystal violet (v/v) is added into each well and stained for 20 min. After discard of staining liquid, all loosely adhering bacteria and dye are gently washed off with distilled water for three times. The dye bound to the adherent cells is solubilized with 100 μl of anhydrous ethanol per well. The optical density (OD) is measured at 590 nm, and OD value of biofilm-formation strain is significantly higher than that of negative control. It provides more reproducible results with an addition of a fixtion step (80°C for 30 min) prior crystal violet staining [19]. Combined with resazurin assay, the number of metabolically active cells is able to be evaluated [44]. With wheat germ agglutinin-Alexa Fluor 488 conjugate, which selectively binds to N-acetylglucosamine residues in

biofilms, the spectrofluorometric assay provides a more sensitive method for quantification and characterization of bacterial biofilms [45].

3.2. Biofilm formation in glass tube

The overnight cultures of bacteria are diluted 1:100 in the diluted TSB. Two milliliters of each bacterial suspension are added into borosilicate glass tubes and incubated at 28°C for 48 h. Then, the liquid is decanted and the tubes are washed gently three times with distilled water. Two ml of 0.4% crystal violet (v/v) are added into each tube and stained at room temperature for 20 min. The stained biofilm is observed at the liquid–air interface on the glass test tube walls or at the bottom of the tube [46]. The glass tubes may also be incubated at 37°C at 200 rpm by using an orbital shaker, and biofilm is observed at interphase without staining [47].

3.3. Congo red/carbol fuchsin staining

The overnight culture (1:100 diluted in TSB) is inoculated into 3 ml of fresh TSB in a 6-well plate containing sterile polystyrene coverslip (20 × 20 mm). After incubation at 28°C for 24 or 48 h without agitation, the coverslips are removed carefully, treated with cetylpyridinium chloride (10 mM) for 30 s, rinsed with distilled water and air dried for 20–30 min. After fixation by gentle heating, the coverslips are stained with a mixture of saturated aqueous Congo red solution and 10% Tween-80 (2:1, V/V) for 30 min and rinsed with distilled water. After staining with 10% (v/v) Ziehl carbol fuchsin for 6 min and rinsing in distilled water, the coverslips are air dried and mounted on slides [48]. Under a light microscope, bacterial cells on slides show purple staining, while the exopolysaccharides of biofilm show pink staining [46].

3.4. Field emission scanning electron microscopy

The coverslips with cultured bacteria are fixed in 3% glutaraldehyde in 0.1 M phosphate-buffered saline at 4°C for 2 h. The samples are then dehydrated with increasing concentrations of ethanol (50, 70, 80, 90, and 100%) followed by isoamyl acetate (100%), each for 15 min. The samples are critical point dried for 5 h, coated with gold palladium alloy, and observed under a field emission scanning electron microscope [49]. The biofilm-formation strain exhibits increased clusters of bacteria cells with curli fimbriae and has meshwork-like structures surrounding the cell surfaces.

3.5. Congo red and calcofluor plates

LB agar plate without salt supplemented with 40 mg/L Congo red and 20 mg/L brilliant blue is used to determine the Congo red-binding property of the colonies. LB agar plate supplemented with 200 mg/L calcofluor (fluorescent brightener) is used to determine the cellulose production by comparing the fluorescence of the test strains under UV light [49]. Biofilm of *Salmonella* is mainly composed of curli and cellulose, and *Salmonella* strains were grouped into distinct morphotypes according to Congo red binding: (a) red, dry, and rough indicating curli and cellulose production (RDAR), (b) brown, dry, and rough, indicating a lack of cellulose synthesis (BDAR), (c) pink, dry, and rough, indicating a defect in curli expression (PDAR), (d)

smooth, brown, and mucoid, indicating a lack of cellulose synthesis but overproduced capsular polysaccharide (SBAM), and (e) smooth and white, indicating a lack of both curli and cellulose production (SAW) [19, 31, 50].

3.6. Confocal laser microscopy

Bacteria cultured on coverslipes, dish, or microplate are stained by 0.1 M phosphate-buffered saline (pH 7.2) containing SYTO 9 and propidium iodide. After 10 min incubation in the dark at room temperature, stained samples are examined using a confocal scanning laser microscopy. Fluorochromes are excited using an argon laser source at 488 nm. Images are collected in two channels, 490–515 and 620–640 nm, corresponding to the emission maxima for SYTO 9 and propidium iodide, respectively. Optical sections approximately 1 μm in height are collected starting from below the focal plane to upward through the entire biofilm. The biofilm cells are clearly observed in a multilayer community [20, 51].

4. Identification of biofilm-formation–associated genes

The most common biofilm-formation–associated genes are the genes encode adhesins. The best characterized of the *Salmonella* fimbriae is type-1 fimbriae. This fimbrial type is encoded by the fim gene cluster and is assembled by the chaperone–usher system [52]. The fimA gene encodes the major structural subunit, while the fimH gene encodes the adhesin protein that is located at the tip of the assembled fimbrial structure and mediates binding to the receptor. The FimH adhesin is involved in biofilm formation on HEp-2 tissue culture cells, murine intestinal epithelium, and chicken intestinal epithelium [22, 23]. The long polar fimbriae (Lpf) are encoded by the *lpfABCDE* genes and have been implicated in the colonization of the murine intestinal mucosa [53, 54]. Plasmid-encoded fimbriae (Pef) are encoded on the 90-kb *Salmonella* virulence plasmid and are majorly encoded by *pefBCD*, orf5, and orf6 genes. Both Lpf and Pef contribute to the early steps of biofilm formation [55]. *Salmonella enteritidis* produce a variety of potentially adherent fimbrial types including SEF14 (SefA), SEF17 (CsgA), SEF18 (SefD), and SEF21 (type I, FimA), the role of each fimbrial in biofilm formation is different. The SEF17 encoded by *csgA* gene stabilize cell–cell interactions during biofilm formation, while SEF21 fimbriae may involve cell surface adherence [56]. SadA is trimeric autotransporter adhesin of *Salmonella typhimurium*, the expression of SadA resulted in cell aggregation, biofilm formation, and increased adhesion to human intestinal Caco-2 epithelial cells [57]. *Salmonella* may persist on post-harvest lettuce during cold storage, the genes *stfC, bcsA, misL*, and *yidR*, encoding a fimbrial outer membrane usher, a cellulose synthase catalytic subunit, an adhesin of the autotransporter family expressed from the *Salmonella* pathogenicity island-3, and a putative ATP-/GTP-binding protein, respectively, have a role in persistence of the pathogen. The *bcsA, misL*, and *yidR* knockout mutants are impaired in attachment and biofilm formation, suggesting that these functions are required for biofilm formation [58].

Salmonella flagella are not required for the formation of the multicellular morphotype on plates. However, the global behavior of the bacterial community on air–liquid, surface–liquid, or cell–

liquid interfaces is changed in the absence of flagella. In a mutant lacking flagella and thin aggregative fimbriae, the contribution of the latter to the multicellular morphotype is dominant [59]. Biofilm formation of an flgK mutant in meat and poultry broths and their attachment on surfaces of stainless steel and glass are significantly reduced compared with that of the wild-type strain, suggest that expression of flagella could be involved in biofilm formation and attachment of *Salmonella* on contact surfaces [60]. The presence of the flagellar filament enhances binding and biofilm formation in the presence of bile, while flagellar motility and expression of type-1 fimbriae were unimportant in biofilm formation on cholesterol gallstones [61].

Pathogenicity islands accommodate large clusters of genes that contribute to a particular virulence phenotype. *Salmonella* possess at least seven *salmonella* pathogenicity islands (SPIs). Among these, SPI1 is primarily required for bacterial motility and invasion of host cells. *Salmonella typhimurium* cultures containing cloned SPI-1 display an adherent biofilm and cell clumps in the media. This phenotype was associated with hyper-expression of SPI-1 type-III secretion functions. Surprisingly, mutations in genes essential for known bacterial biofilm pathways *(bcsA, csgBA, bapA*) did not affect the biofilms formation, indicating that this phenomenon is independent of established biofilm mechanisms [62]. *Salmonella* biofilm cells exposed to superheated steam show decreased transcription of flagella and SPI-1 genes, respectively, whereas increased transcription of SPI-2 genes, important for bacterial survival and replication inside host cells, is detected [63]. In contrast, when compared biofilms of *Salmonella typhimurium* with planktonic cells, the most highly downregulated genes in the biofilm are located on SPI-2 and that a functional SPI2 secretion system regulator (*ssrA*) is required for *Salmonella typhimurium* biofilm formation. Genes involved in tryptophan (*trp*) biosynthesis and transport are upregulated in the biofilm. Deletion of *trpE* results in decreased bacterial attachment and biofilm formation, indicating that aromatic amino acids make an important contribution to biofilm formation [64]. The *aro* mutants of *Salmonella* are frequently used as live vaccines for the oral vaccination of domestic animals, and they are unable to synthesize chorismate, which is a key intermediate in the synthesis of aromatic amino acids. The *aro* mutants exhibit a decreased production of cellulose, N-acetyl-D-glucosamine, or N-acetylneuraminic acid-containing capsular polysaccharide and fimbriae, which explains their inability to form biofilms [65].

Lipopolysaccharide (LPS) synthesis also involves the biofilm formation of *Salmonella*. Two Tn5 insertion mutations in genes that are involved in *ddhC* and *waaG* result in diminished expression of colony rugosity. Both mutants have impaired biofilm formation when grown in rich medium with low osmolarity, they constitutively form larger amounts of biofilms when the growth medium was supplemented with either glucose or a combination of glucose and NaCl [49]. The *rfbA* gene also involve in lipopolysaccharide biosynthesis. Biofilm formation by the *rfbA* mutant in meat and poultry broths and their attachment on surfaces of stainless steel and glass is significantly reduced [60]. Using transposon mutagenesis, the genes *metE, ompR, rpoS, rfaG, rfaJ, rfaK, rfaP, rfbH, rhlE, spiA*, and *steB* are found to be associated with biofilm formation of *Salmonella enteritidis* [66, 67]. When eight mutants with knockout of genes *ompR, rpoS, rfaG, rfbH, rhlE, metE, spiA*, or *steB* from the *Salmonella pullorum* are constructed. Only the

ompR mutant showed a complete loss of production of curli and biofilm formation. The other mutants showed a modified production of curli and cellulose with less effect related to biofilm formation [68]. Therefore, an integral LPS, at both the O-antigen and core polysaccharide levels, are important in the modulation of curli protein and cellulose production, as well as in biofilm formation.

5. Regulation mechanism of biofilm formation

Biofilm formation is majorly regulated by CsgD protein, a regulator belonging to the LuxR family [69]. CsgD has an N-terminal receiver domain with a conserved aspartate (D59) as a putative target site for phosphorylation and a C-terminal LuxR-like helix-turn-helix DNA binding motif. The unphosphorylated CsgD directly binds the *csgBA* and *adrA* promoter regions to activate transcription [70]. Multiple factors bind to the promoter sequence of *csgD* and regulate its transcription, such as OmpR, RpoS, RpoE, integration host factor (IHF), histone-like nucleoid structuring protein (H-NS), and MlrA. OmpR is one of first discovered to be required for *csgD* transcription [71]. Six binding sites (D1–D6) for OmpR are identified in *csgD* promoter regions. Binding of OmpR-P to D2 centered immediately upstream of D1 is proposed to repress promoter activity. IHF competes with OmpR-P for binding at its upstream site IHF1, which overlaps with D3–D6 and thereby activate the transcription of *csgD* [72]. The mutant of *ompR* in *Salmonella enteritidis* and *Salmonella pullorum* has inability to produce cellulose, curli, and biofilm [68, 73]. RpoS, encodes an alternative sigma factor of RNA polymerase, is critical for bacterial endurance under the most-stressful conditions, including stationary-phase entrance and host adaptation. RpoS is required for transcriptional activation of the *csgD* promoter in *Salmonella typhimurium* strains that rdar morphotype are normally expressed at low temperature [31]. However, in two *Salmonella typhimurium* strains, spontaneous mutants are found forming rdar colonies independent of temperature, the regulation of *csgD* is independent of *rpoS* [71]. Partially independent of *rpoS* for regulation of *csgD* is observed in *Salmonella enteritidis*. The *rpoS* mutant in *Salmonella pullorum* also shows similar biofilm forming ability as the wild-type strain [68], suggests that another sigma factor may recognize the *csgD* promoter. RpoE is an another regulator in the expression of thin aggregative fimbriae in *Salmonella* [74], since the *rpoE* deletion mutant shows significantly reduced amounts of *csgD* expression and modulated biofilm formation. Compared the expression of six different Sigma factors during biofilm formation in a *rpoS*-independent biofilm-formation strain, the expression of *rpoE* gene was the highest, and the *rpoE* mutant could not produce biofilm [75]. Therefore, RpoE acts as a regulator for *csgD* expression. IHF is a histone-like heterodimeric protein composed of two homologous subunits. IHF interacts with a define DNA sequence that has a supportive A-tract upstream of the consensus sequence by binding to the minor groove of the DNA. The *ihf* mutants show altered and reduced biofilm morphotypes on Congo Red agar plates [72]. H-NS prefers to bind AT-rich sites in the intergenic *csgBAC* and *csgDEFG* regions and causes moderate activation of *csgD* promoter. The inactivation of *hns* gene result in reduced expression of the rdar morphotype on agar plate [72]. MlrA (MerR-like regulator) acts directly or indirectly on the *csgD* promoter, the *mlrA* mu-

tants of *Salmonella typhimurium* no longer produce curli or rugose colony morphology. However, inactivation of *mlrA* did not affect curli production and aggregative morphology in an upregulated curli producing *Salmonella typhimurium* derivative containing a temperature- and RpoS-independent *csgD* promoter region. Therefore, MlrA acts as a positive regulator of RpoS-dependent curli and extracellular matrix production by *Salmonella typhimurium* [76].

c-di-GMP is recognized as a ubiquitous bacterial second messenger and a key regulator in bacterial transition from a motile and planktonic to a sessile and biofilm lifestyle. High intracellular c-di-GMP levels promote extracellular matrix production and subsequent biofilm formation and repress motility, whereas low intracellular c-di-GMP levels suppress matrix production and promote single-cell motility [77]. The synthesis/degradation of c-di-GMP depends on diguanylate cyclase/phosphodiesterase enzymatic activities. The cyclase activity, which converts two molecules of GTP to c-di-GMP, is encoded in the GGDEF protein domain, while phosphodiesterase activity, which hydrolyzes c-di-GMP to linear 5′-pGpG or two GMP molecules, is encoded in the EAL and HD-GYP domains. For example, Adar, containing a GGDEF domain, encodes diguanylate cyclase synthesizing c-di-GMP, is required for cellulose production and biofilm formation. In another seven GGDEF family (GcpA-G), only GcpA and GcpE are critical for biofilm formation [37]. The EAL domain protein STM4264, STM3611, and the GGDEF-EAL domain protein STM1703 play a determinative role in the expression level of multicellular behavior of *Salmonella typhimurium* [78, 79]. In contradiction, the EAL-like protein STM1697, neither degrade nor bind c-di-GMP, promotes biofilm formation and *CsgD* expression through interaction with proteins that regulate flagella function [80]. High intracellular amounts of c-di-GMP in *Salmonella typhimurium* inhibited invasion and abolished induction of a pro-inflammatory immune response in the colonic epithelial cell line HT-29. Inhibition of the invasion and IL-8 induction phenotype by c-di-GMP requires the major biofilm activator CsgD and/or BcsA. Therefore, c-di-GMP signaling is at least equally important in the regulation of *Salmonella*–host interaction as in the regulation of biofilm formation at ambient temperature [81].

CsgD synthesis is also regulated at the post-transcriptional level by sRNA. sRNAs have emerged as a diverse group of trans- or cis-encoded regulatory molecules of approximately 50–250 nt in size. The RNA chaperone Hfq protects sRNAs form degradation and facilitates their binding to the target mRNAs. All these sRNA may negatively regulate *csgD* gene expression by binding to the overlapping 5′-region of the transcript, masking the ribosome binding site, resulting in the inhibition of translation or the degradation of mRNA [82]. In *Escherichia coli*, sRNAs, OmrA/B, McaS, RprA, and GcvB are identified, which downregulate *CsgD* translation [83]. In *E. coli* and *Salmonella*, RydC's 5′-domain interacts with *csgD* mRNA translation initiation signals to prevent initiation, stimulation of RydC expression reduces biofilm formation by impairing curli synthesis [84]. Surprisingly, two Hfq-dependent sRNAs (ArcZ and SdsR) are responsible for positively regulation of rdar morphotype expression in *Salmonella typhimurium* [85]. *Salmonella* biofilm development depends on the phosphorylation status of RcsB. The unphosphorylated RcsB is essential to activate the expression of the biofilm matrix compounds. The inhibition of biofilm development by phosphorylated RcsB is

due to the repression of *CsgD* expression, through a mechanism dependent on the accumulation of the sRNA RprA [86].

Many gram-negative bacteria utilize N-acyl-L-homoserine lactones (AHLs) to bind to transcriptional regulators leading to activation or repression of target genes. *Salmonella* do not synthesize AHLs but do contain the AHL receptor, SdiA. The *Salmonella sdiA* gene regulates the *rck* gene, which mediates its adhesion and invasion of epithelial cells and the resistance of the organism to complement [87]. The *rck* gene is located on the virulence plasmid of pRST98, AHLs increase *rck* expression in pRST98-carrying strains, thereby enhancing bacterial adherence, serum resistance, and bacterial biofilm formation [88].

6. Virulence or resistance for biofilm

Biofilm formation may involve in the virulence of *Salmonella. Salmonella enteritidis* stains isolated from either the environment, dairy products, or infected patients are divided into two groups on the basis of their virulence (50% lethal dose) in chickens infected intraperitoneally. Only the virulent strains produce aggregates and formed visible filaments attached to the glass tube [47]. Further study confirms that the virulence of the biofilm-producing strain in infected chickens increases proportionally to the amount of stored glycogen, suggesting a possible role of the glycogen depot in the virulence of *Salmonella enteritidis* [89]. When tested for infection in Caco-2 cells and HEp-2 cells, the more virulent strains of *Salmonella enteritidis*, which are biofilm producers in adherence test medium, are able to disrupt monolayers. In contrast, the low-virulence strains of *Salmonella enteritidis*, which do not produce biofilms in adherence test medium, have no effect on the same cells. The high-virulence *Salmonella enteritidis* strains incubated under optimum biofilm-forming conditions may release a soluble factor, which enables the disruption of the integrity of Caco-2 monolayers [90]. The relationship between biofilm-forming ability and the pathogenicity is also evaluated in *Salmonella pullorum*. Although the virulence of *Salmonella pullorum* strains is independent of their ability of biofilm formation, prior growth as a biofilm for a biofilm producer of *Salmonella pullorum* leads to enhanced virulence in chickens, suggested that biofilm formation may be one of important virulence factor for *Salmonella pullorum* infection [46].

The *csgBAC* operon is required for curli biosynthesis in *Salmonella*. The *csgA* mutation is not reduced in ability to attach or colonize alfalfa sprouts, whereas the *csgB* mutation is reduced. Thus, *csgB* alone can play a role in attachment of *Salmonella* to plant tissue [91]. Competitive infection experiments in mice shows that *csgA* mutant cells outcompeted rdar-positive wild-type cells, indicating that aggregation via the rdar morphotype is not a virulence adaptation in *Salmonella typhimurium*. Furthermore, in vivo imaging experiments show that thin aggregative fimbriae genes are not expressed during infection but are expressed once *Salmonella* was passed out of the mice into the feces [92]. However, *Salmonella typhimurium* strains isolated from water buffalo calves affected by lethal gastroenteritis are tested in vivo in a mouse model of mixed infection. The most pathogenic strain is characterized by a high number of virulence factors and the presence of the locus *csgA*, coding for a thin aggregative fimbria [93].

The *bcsABZC* and *bcsEFG* operons are required for cellulose biosynthesis in *Salmonella*. Bacterial adherence and invasion assays of eukaryotic cells and in vivo virulence studies of cellulose-deficient mutants of *bcsC* and *bcsE* genes indicate that the production of cellulose is not involved in the virulence of *Salmonella enteritidis*. However, cellulose-deficient mutants are more sensitive to chlorine treatments, suggesting that cellulose production and biofilm formation may be an important factor for the survival of *S. enteritidis* on surface environments [36]. *Salmonella typhimurium* makes cellulose when inside macrophages. An attenuated mutant lacking the *mgtC* gene exhibits increased cellulose levels due to increased expression of the cellulose synthase gene *bcsA* and of cyclic diguanylate, the allosteric activator of the BcsA protein. Inactivation of *bcsA* restore wild-type virulence to the *Salmonella mgtC* mutant, indicating that *Salmonella* promotes virulence by repressing cellulose production [94].

BapA, a large cell-surface protein, is required for biofilm formation by *Salmonella*. Studies on the contribution of BapA to *Salmonella enteritidis* pathogenesis reveal that orally inoculated animals with a *bapA*-deficient strain survived longer than those inoculated with the wild-type strain. Also, a *bapA* mutant strain showed a significantly lower colonization rate at the intestinal cell barrier and consequently a decreased efficiency for organ invasion compared with the wild-type strain [26]. Osmoregulated periplasmic glucans (OPGs) are major periplasmic constituents of Gram-negative bacteria. An *opgGH* mutant strain in *Salmonella typhimurium*, which is defective in OPG biosynthesis, severely impaires biofilm formation. The opgGH mutant strain poorly colonizes mouse organs when introduced orally along with the wild-type strain [95].

Besides, the constitutional components of biofilm, there are many regulation proteins involved in both biofilm formation and virulence. An *ompR* mutant of *Salmonella enteritidis* has no ability to produce cellulose, curli, and biofilm and shows similar adherence percentage to and invasion percentage of epithelial cells as wild-type strain. Intraperitoneal challenge of bacteria in BALB/c mice reveals that the *ompR* mutant strain is significant attenuated [73]. A *spiA* gene mutant shows reduced biofilm formation and significantly decreased curli production, and reduced intracellular proliferation of macrophages during the biofilm phase. In addition, the *spiA* mutant was attenuated in a mouse model in both the exponential growth and biofilm phases [67]. Deletion of genes *ompR* and *spiA* in *Salmonell pullorum* strains contribute to attenuation of virulence in 1-day-old chickens [68]. DksA is a conserved gram-negative regulator that binds directly to the RNA polymerase secondary channel. In *Salmonella typhimurium*, expression of the *dksA* gene is induced during the logarithmic phase and DksA plays an important role in motility and biofilm formation. DksA positively regulates the *Salmonella* pathogenicity island 1 and motility-chemotaxis genes and is necessary for *Salmonella typhimurium* invasion of human epithelial cells and uptake by macrophages. The *dksA* gene is induced at the midcecum during the early stage of the infection and required for gastrointestinal colonization and systemic infection in a colitis mouse model [96].

Salmonella in biofilm is resistant to antibiotic. One of key mechanisms of antibiotic resistance is efflux. There are five families of multidrug resistance (MDR) efflux pumps, in which the AcrAB–TolC efflux system is the best characterized MDR system. Ten mutants of *Salmonella typhimurium* lacking MDR efflux systems, such as *tolC, acrB, acrD, acrEF, mdtABC, mdsABC,*

emrAB, *mdfA*, *mdtK*, and *macAB* are compromised in their ability to form biofilms. The mutants expressed significantly less *csgB* or *csgD* than wild type, indicating that loss of all multidrug resistance efflux pumps of *Salmonella typhimurium* results in impaired ability to form a biofilm [97]. Further study confirms that mutants of *Salmonella typhimurium* that lack TolC or AcrB, but surprisingly not AcrA, are compromised in their ability to form biofilms. The biofilm defect results from transcriptional repression of curli biosynthesis genes and consequent inhibition of production of curli. Therefore, the inhibition of efflux is a promising antibiofilm strategy [98]. However, recent studies offer contradictory findings about the role of multidrug efflux pumps in bacterial biofilm development. When no selective pressure is applied, *Salmonella typhimurium* is able to produce biofilms even when the AcrAB efflux pumps are inactivated. Upon exposure to chloramphenicol, the formation of biofilms on solid surfaces as well as the production of curli are either reduced or delayed more significantly in both AcrA and AcrAB mutants, implying that the use of efflux pump inhibitors to prevent biofilm formation is not a general solution and that combined treatments might be more efficient [99]. Triclosan is a potent biocide that is included in a diverse range of products. *Salmonella* biofilm-derived cells are more resistant to Triclosan. Within biofilms, triclosan upregulate the transcription of *acrAB, marA, bcsA*, and *bcsE* genes. Thus, *Salmonella* within biofilms could experience reduced influx, increased efflux and enhanced exopolysaccharides production. The data suggest that tolerance of *Salmonella* towards triclosan in the biofilm is attributed to low diffusion through the extracellular matrix, while changes of gene expression might provide further resistance to triclosan and to other antimicrobials [100].

In summary, *Salmonella* biofilm formation is major controlled by CsgD regulatory network and regulated by multiple transcriptional factors, c-di-GMP, and sRNAs. More and more genes are found to be associated with both biofilm formation and virulence. Dissection of their function and relationship will helpful for development of new tools and strategies to prevent biofilm-related disease and decontaminate biofilm-derived *Salmonella* in food production.

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Chapter 13

Bacterial Biofilms in Diabetic Foot Ulcers: Potential Alternative Therapeutics

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Additional information is available at the end of the chapter

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Abstract

Diabetes *mellitus* is a major health problem that affects approximately 171 million people globally. One of its most severe complications is the development of diabetic foot ulcers (DFU). Ischemic and neurophatic lesions are of major importance for DFU onset; however, it is the infection by multidrug-resistant and biofilm-producing microorganisms, along with local microenvironmental conditions unfavorable to antibiotics action that ultimately cause infection chronicity and lower limbs amputation. Novel therapeutic protocols for DFU management are extremely urgent. Bacteriophages, probiotics and antimicrobial peptides (AMP) have recently been proposed as alternatives to currently available antibiotics. Bacteriophages are viruses that specifically infect and multiply within bacterial cells. Their ability to diffuse through polymeric matrixes makes them particularly efficient to eradicate biofilm-based bacteria. Promising results were also observed with probiotic therapy. Probiotics are well-characterized strains with the ability to compete with pathogenic microorganisms and modulate the host immune response. AMP are molecules produced by living organisms as part of their innate immune response. Unlike conventional antibiotics, AMP also act as immunomodulators and resistance to AMP was rarely observed, supporting their potential as therapeutic agents. These innovative therapeutic strategies may in the future substitute or complement antibiotherapy, ultimately contributing for the decrease in multidrug-resistant bacteria dissemination.

Keywords: antimicrobial peptides, antimicrobial resistance, bacteriophages, biofilm, diabetic foot ulcer, probiotics

1. Introduction

Diabetes *mellitus* is a serious health problem in rapid expansion worldwide. It is estimated that there are 171 million diabetic patients worldwide and this number is expected to double by the year 2030 [1]. Diabetic foot ulcers (DFU) are one of the most frequent complications of diabetes, resulting from a complex interaction of factors, namely ischemia and neuropathy [2].

Neuropathy, which is characterized by modifications in sensitive and autonomic functions, causes ulceration due to trauma or excessive pressure in a deformed foot without protective sensibility. Autonomic neuropathy causes dryness of the skin by decreasing sweating, and therefore the vulnerability of the skin to break down increases. Once the protective layer of skin is damaged, deep tissues are exposed to bacterial colonization [3].

Diabetes-associated ischemia is caused by peripheral arterial disease. Poor arterial inflow decreases blood supply to ulcer area and is associated with reduced oxygenation, nutrition, and ulcer healing [3].

These ulcers are frequently colonized by pathogenic bacteria and infection is facilitated by immunological deficits related to diabetes [4], rapidly progressing to deeper tissues, increasing the presence of necrotic tissue, rendering amputation inevitable [5]. In fact, diabetic patients frequently require minor or major amputations of the lower limbs (15-27%) [2], which not only contribute dramatically to high morbidity among diabetic patients, but is also associated with severe clinical depression and increased mortality rates [6].

Although ischemic and neuropathic changes have the initial role in DFU pathophysiology, in the majority of cases it is the infection by multidrug-resistant microorganisms and the unfavorable microenvironmental conditions to the action of antibiotics that leads to amputation [5].

Diabetes-associated foot ulcer infections are predominantly polymicrobial and several bacterial genera can be part of the DFU microbiota, namely *Staphylococcus, Pseudomonas, Streptococcus, Enterococcus, Corynebacterium, Acinetobacter, Prevotella, Porphyromonas*, and members of the family *Enterobacteriaceae*. The predominant Gram-positive and Gram-negative species present in DFU are *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively [7–9].

There is, to date, little understanding of the ecology of such chronic infections, but bacterial biofilms seem to play a major role [10]. These are ubiquitous and complex structures consisting of an interactive community of polymicrobial cells embedded in a self-produced extracellular matrix of hydrated polymeric substances, such as proteins, polysaccharides, nucleic acids and others, irreversibly attached to the biological surface of the ulcer. These characteristics make them recalcitrant to the action of most antibiotics and also resistant to the innate immune system [11].

administration of biofilm-based infections generally requires local surgical procedures as well as antibiotic administration. However, in infected DFU, because of deficient vascularization, antibiotics frequently reach the local ulcer microenvironment only at subtherapeutic concen-

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trations [5]. Even when topically applied, antibiotics rarely reach bacteria that reside within mature biofilms at therapeutic concentrations [12].

Biofilm formation is a major mechanism of adaptation that is able to protect bacteria from antibiotics, due to several physiological traits. Firstly, biofilm spatial structure provides a protective coat against antimicrobial compounds. Secondly, in most cases, biofilms are polymicrobial, formed by complex mixtures of different species. It was proposed that, in such biofilms, the chemical interactions that occur between polymeric substances produced by different bacterial strains might lead to a more viscous matrix, impairing the contact between the bacterial cell wall and the antibiotic. Lastly, the production of degradative enzymes by different pathogenic species can act synergistically against antimicrobial compounds. These biofilm features are responsible for a reduced diffusion of the antibiotic within the biofilm matrix [13, 14].

In addition, patients suffering from DFU face the emergence and dissemination of antibiotic resistant bacteria, which is not a recent biological phenomenon. Seventy years ago, after the discovery of penicillin and the beginning of the antibiotic era, Alexander Fleming noticed the emergence of bacterial strains resistant to penicillin. Indeed, resistance began to appear in target microorganisms, including *S. aureus* isolates from hospitals, a few years after the introduction of penicillin into medical practice [15]. Fleming described the occurrence of antibiotic resistance and warned the scientific and medical community of this phenomenon in his Nobel Prize lecture in 1945 [16].

Several causes can explain the emergence and dissemination of antibiotic resistance. Firstly, the overuse and, most importantly, the misuse of antibiotics in different but interconnected areas, like human and veterinary medicine, agriculture and animal production. Secondly, the effects of antibiotic compounds in the environment are not yet completely described and understood. Finally, antibiotic compounds are stable and static chemical substances that are used to fight living and evolving bacterial cells [17]. Microorganisms, namely bacteria, are ubiquitous and interact with all other living beings. Considering that nature is a highly complex system supported by extremely dynamic interactions and exchanges between all its elements, the emergence and evolution of bacterial populations able to resist against antibiotic substances is not surprising. In fact, over the last decades, microbiologists have demonstrated the influence that antibiotics exert upon bacterial populations. Previously seen as miracle drugs, capable of virtually eradicating all species of bacteria, antibiotics are now seen as substances with limited antimicrobial capacity and multifaceted proprieties. These compounds have the ability to induce or inhibit different bacterial responses and to influence bacterial virulence and survival strategies [18, 19].

As mentioned above, biofilm formation is a well-known virulence factor of some bacterial strains that, along with many other advantages, confers them a protective layer against adverse elements. Recently, it was demonstrated that some antibiotics are able to induce this adaptative strategy. In 2005, when Hoffman *et al*. [18] were testing the efficacy of aminoglycosides, a widely exploited antibacterial therapeutic agent, against biofilm-forming bacteria, they observed an unexpected bacterial response. Aminoglycosides not only did not eliminate the *P. aeruginosa* strain used in the study, but also stimulated their ability to form biofilm. In fact,

they demonstrated that aminoglycosides interact with the *P. aeruginosa* aminoglycoside response regulator gene, *arr*, which encodes for an inner-membrane phosphodiesterase essential to the regulation of cyclic di-guanosine monophosphate levels, which represents a bacterial second messenger that regulates cell surface adherence [18]. Later on, Kaplan *et al*. [19] also reported that in *Escherichia coli*, not only sub inhibitory antibiotic concentrations but also disinfectants such as chlorhexidine are responsible for the induction of biofilm formation. From their work, one can conclude that, for some bacterial strains, biofilm formation can be a specific defensive reaction to the presence of antibiotics.

Despite all the evidences showing that biofilms provide advantages to microorganisms, namely enhanced resistance towards environmental stresses including the presence of antimicrobial compounds, many antibiotics that are currently in use were developed, tested, and regulated using *in vitro* tests against planktonic bacteria.

It is known that microbial cells growing within a biofilm are physiologically distinct from planktonic cells of the same strain. The overall resistance level in biofilms is distinct from the one observed at a cellular level [20]. As a consequence, the antimicrobial concentration required to inhibit biofilms can be up to hundreds or even a thousand times higher than the corresponding concentration necessary to eliminate free-living bacterial cells [21]. Such phenomena cannot be overlooked in the development of novel strategies to combat infectious diseases.

Taking into account that biofilm formation is a threatening characteristic of the microbiome that colonizes diabetic foot wounds, it is not unexpected that in the past few decades a major problem in treating DFU infections has been the increasing rate of colonisation by antibiotic resistant pathogens. This is the case of methicillin-resistant *S. aureus* (MRSA), and to a lesser degree, glycopeptide-intermediate *S. aureus*, vancomycin-resistant enterococci, extended-spectrum β-lactamase- or carbapenamase–producing gram-negative bacilli, and highly resistant strains of *P. aeruginosa*. In fact, the infection by polymicrobial communities of multidrug-resistant bacteria is an important cause of DFU healing impediment [7, 22–27].

The rates of isolation of these multidrug-resistant pathogens vary widely among geographical area and treatment center. However, the increasing incidence of multidrug-resistant microorganisms together with the incapacity of antibiotics to act on resistant and biofilm-producing bacteria at therapeutical concentrations emphasizes the importance of developing new treatment strategies to effectively eradicate these infections.

Considering that biofilms were only described by the scientific community by the end of the twentieth century, it is comprehensible that research on biofilms is still an expanding area [28]. The lack of understanding of the mechanisms behind the biofilm mode of life has impaired the development of antimicrobial compounds that specifically operate on biofilm polymicrobial communities [28]. However, in recent years, the increased failure in infectious diseases therapeutic protocols and the dissemination of antibiotic resistance has demonstrated the importance of developing such substances and several novel therapeutic strategies, namely bacteriophages, probiotics and antimicrobial peptides (AMP), are recently been explored and proposed as potential alternatives to eradicate bacterial biofilms in DFU.

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2. Bacteriophages

Bacteriophages were discovered almost a century ago by two independent microbiologists, Twork in 1915 in the United Kingdom and D'Herelle in 1917 in France. D'Herelle named these bacteria-eating entities as bacteriophages and explored them as antibacterial agents [29, 30].

Bacteriophages are bacteria-specific viruses that infect and multiply within bacterial cells. In contrast to lysogenic bacteriophages, the replication of lytic bacteriophages and release of the newly formed virus particles always involves lysis of the host bacterial cell. Bacteriophage therapy is the use of lytic bacteriophages to reduce or eliminate pathogenic bacteria [31].

Lytic bacteriophages seem to be efficient therapeutical agents in biofilm microenvironment due to several particular characteristics: specificity and efficiency in lysing pathogenic bacteria; absence of pathogenicity to man and animals; efficiency over bacteria organized in polymeric matrixes, namely biofilms; action in microaerophilic environments with high bacterial load; and rapid and economical accessible production capability [32, 33].

Bacteriophage therapy has become a broadly relevant technology for veterinary, agricultural and food microbiological applications; however, the treatment of human infections with bacteriophage-based protocols attracts the greatest interest [34].

Bacteriophages are viruses that specifically infect prokaryotic bacterial cells. In fact, the prokaryotic biochemical machinery that enables the interaction between bacteriophages and bacterial cells has particular characteristics that are not present in eukaryotic cells. For instance, the outer membrane receptors of bacterial cells, with which bacteriophage capsid coat or molecular appendages first connect with the purpose of being anchored on the bacterial cell wall, as well as the polymerases required for the bacteriophage genome replication, are specific of prokaryotic bacterial cells and are structurally and functionally different from those presented by eukaryotic cells [31]. For that reason, bacteriophages can only directly interact and infect bacterial cells, and not eukaryotic cells. The bacterio-specificity features allow classifying bacteriophages as 'safe' for use in eukaryotic organisms, namely plants and animals, including humans.

The use of bacteriophages as antibacterial agents for suppurative infections began shortly after their discovery, with Bruynoghe's and Maisin's application for treating *S. aureus* skin infections [35]. However, following the discovery and general application of antibiotics, interest in the therapeutic uses of bacteriophages waned. Recently, the increase in antibiotic-resistant bacterial strains has reinvigorated enthusiasm about these bacteria-specific viruses [36]. This interest is particularly true in cases in which bacteriophages can be applied topically, as is the case of DFU.

Recently, a topically delivered bacteriophage suspension was tested for its antimicrobial activity and wound healing capability against ulcers chronically infected with *S. aureus, P. aeruginosa* and *Acinetobacter baumannii*. In this study, conducted by Mendes *et al*. in 2013 [37], the bacteriophage suspension was applied in debrided infected cutaneous wounds and microbiologic, histological and planimetric parameters were evaluated. It was shown that the

bacteriophage treatment successfully decreased bacterial colony counts and improved wound healing, as indicated by smaller epithelial and dermal gaps. The bacteriophage therapy protocol developed was proven to be an effective methodology in the treatment of two animal models of Diabetes *mellitus*, rodents and porcines [37].

The same bacteriophage suspension also demonstrated *in vitro* activity against both planktonic cells and established biofilms. Using metabolic activity as a measure of cell viability, it was observed that bacteriophage treatment significantly increased cell impairment within biofilms. Moreover, bacteriophage exposure repeated every four hours caused a further decrease in cell activity [9].

There is still much to unravel regarding bacteriophage therapy. For instance, not all phages are suitable for clinical application. More information is required, namely detailed studies of potentially useful phages with respect to their interaction with target bacteria and their genetic content.

Nonetheless, despite the paucity of experimental data regarding bacteriophage therapy in DFU, a consensus appears to have emerged on the feasibility of this potential alternative to treat biofilm-infected DFU.

3. Probiotics

The increasing global antimicrobial drug resistance problem led to an urge in researching alternatives to drug therapies, making the concept of bacteriotherapy more interesting and pertinent than ever. Bacteriotherapy is a promising alternative approach to fight infections by employing harmless bacteria to displace pathogenic microorganisms [38].

The concept of 'probiotic' arose in 1907 from a hypothesis proposed by Noble Prize-winning Ilya Mechnikov. At the turn of the twentieth century, Mechnikov noticed that peasant populations in Bulgaria had increased average life spans in comparison with wealthier European populations [39]. He also observed that yogurt and other fermented milk products were a substantial part of their diets and described the beneficial effects of the 'Bulgarian *bacillus'* present in those foods [40, 41]. These healthy bacteria, later classified *Lactobacillus bulgaricus*, helped digestion, impaired the putrefactive effects of gastrointestinal metabolism, and contributed to the improvement of the immune system [41].

Mechnikov was not the only one to notice the health benefits of lactic acid bacteria. A few years before, in 1899, another important discovery was made at the Pasteur Institute in Paris. Henri Tissier demonstrated that children suffering from diarrhea had a low number of bacteria characterized by a peculiar Y-shaped morphology. On the other hand, these "bifid" bacteria were abundant in the gut flora of healthy breast-fed infants. Moreover, Tissier demonstrated that the administration of these Y-shaped bacteria, later classified *Bifidobacterium*, to patients with diarrhea allowed them to re-establish a healthy intestinal microbiome [42].

The definition of probiotic as well as their characteristics have evolved in the last century and nowadays probiotics are defined By the Food and Agriculture Organization and the World

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Health Organization as: 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [43]. Probiotics are either a single strain or a mixture of commensal microorganisms with the ability to outcompete pathogenic bacteria through several mechanisms of action. The two most common are direct modification of the microbial populations and modulation of host immune system [43].

Direct modification of the microbiome includes competition with pathogenic bacteria for adhesion to epithelial receptor, production of antimicrobial substances like acids, hydrogen peroxide and bacteriocins, and inhibition of toxic substances produced by pathogens. Immunomodulation includes strengthening of host immune response, promotion of anti-inflammatory action, and enhancement of the wound healing process by stimulating the accumulation of inflammatory cells like lymphocytes, macrophages and polymorphonuclear cells in the site of wound [44].

As one would expect, not all commensal bacteria are suitable to be used as a probiotic. The screening and selection of probiotics includes a rigorous evaluation of the probiotic candidate strain in order to determine whether it fulfills all the required criteria.

Firstly, it is important to assess its safety. An evaluation that includes strain identification and typing, antimicrobial resistance profiling, and determination of virulence and pathogenic properties, including metabolic activities associated with toxic compounds production, is mandatory [45]. Secondly, it is relevant to determine its technological potential. It is essential for a probiotic strain to be genetically stable and bacteriophage-resistant. In addition, it must present viability during processing and storage and be adequate for large-scale production [46]. Thirdly, it is required to establish its physiological properties. To survive the host inner environment, which is rather complex and hostile, a probiotic strain must possess specific characteristics such as gastric acid and bile tolerance and mucosal surface adhesion stability [47]. Lastly, the functional properties must be evaluated. Validated and documented health effects are mandatory, namely antagonistic activity towards pathogens, immunomodulatory activity, and anticarcinogenic properties. Some probiotic strains are also able to interfere with the host cholesterol and lactose metabolism, preventing damages by its metabolites [48].

Probiotics have already been exploited for prevention as well as treatment of a number of health disorders including irritable bowel syndrome, hypersensitivity such as food allergies, hypercholesterolemia, renal failure, gastritis and gut infection, parasitic infections, urogenital infections, colorectal cancer, and dental disorders [49, 50]. Since the putative probiotic mechanisms of action should be the same in the peripheral wounds as they are in other parts of the body, these can be considered as a potential DFU treatment alternative.

Lactic acid bacteria (LAB), in particular *Lactobacillus* and *Bifidobacterium* species, have been extensively used as probiotic strains. The genus *Lactobacillus* is formed by ubiquitous and usually harmless bacteria. In animals, including humans, they are present in the gastrointestinal and genitourinary tracts where they act as health promoters [51]. The genus *Bifidobacterium* includes anaerobic bacteria that produce acetic and lactic acid without release of carbon dioxide. *Bifidobacterium* is the third most abundant genus in the complex microbiome of the human intestinal tract where it exerts beneficial functions of paramount importance [52].

However, other species of bacteria, and even some fungi, also present probiotic properties, such as *Enterococcus faecium, Bacillus cereus, E. coli* strain Nissle, *Propionibacterium freudenreichii, Propionibacterium acnes* and the yeasts *Saccharomyces cerevisae* and *Saccharomyces boulardii* [53–55].

LAB commonly produce antimicrobial substances with effect against gastric and intestinal pathogens and compete for cell surface and mucin binding sites [56]. Recent studies have demonstrated the efficacy of LAB-based therapy for DFU infections control. A study on effectiveness of bacteriotherapy using *Lactobacillus plantarum* on infected chronic DFU demonstrated that topical application of this bacterial culture induced debridement, granulation tissue formation and total healing in half of the diabetic patients treated [57, 58]. *Lactobacillus fermentum* also showed promising applications in treating DFU infections. When co-incubated *in vitro* with *S. aureus and P. aeruginosa, L. fermentum* reduced the cytotoxicity and biofilm formation ability of several pathogenic strains [59].

Additional studies have suggested that *Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus acidophilus* and *Lactococcus lactis* are also promising probiotics with the ability to naturally eliminate pathogenic microorganisms, including MRSA clinical isolates [60].

In the last years, probiotics have been widely studied and all these recent data point out the beneficial effects of probiotics to human and animal health. Naturally, no probiotic strain will provide all the proposed benefits. However, one can no longer ignore the emergence of probiotics as a novel approach to fight multidrug-resistant and biofilm-producing bacteria commonly present in DFU.

4. Antimicrobial peptides

Antimicrobial peptides are major components of the host innate immune system that act as endogenous antibiotics [61, 62]. These multifunctional molecules are produced by living organisms from all kingdoms, including bacteria, fungi, plants, insects and vertebrates, as part of their defense strategy against pathogens. Most AMP act as the first defense barrier against dissemination of a wide spectrum of microorganisms, such as bacteria, fungi, viruses and protozoan parasites [62].

In addition to their antimicrobial activity, AMP serve as modulators of the immune system and even show antitoxic activity, since they neutralize bacterial toxins, including lipopolysaccharide lipid A [63, 64]. Some AMP are also able to prevent biofilm formation and act on preformed biofilms [65].

The majority of AMP are polypeptides with ten to forty amino acid residues; however, some can have up to a hundred. AMP are amphipathic molecules, with two regions in their structure, a polar or hydrophilic region and a nonpolar or hydrophobic region. Due to the presence of multiple lysine, arginine, and histidine residues, the polar region of AMP is cationically

charged. On the other hand, hydrophobicity derives from the abundant presence of hydrophobic amino acids, such as tryptophan, tyrosine and phenylalanine [66, 67].

The distinctive physical-chemical properties of AMP are what confers them their potential as antimicrobial compounds. It has been generally accepted that AMP exert their bactericidal activity through electrostatic interactions with the negatively charged bacterial cytoplasmic membrane, followed by permeabilization of the membrane, which causes cell lysis. Membrane permeabilization can occur through pore formation in the lipid membrane, membrane dissolution, narrowing of the membrane bilayer or lipid-peptide domain formation [68]. The AMP amphipathic structure, namely their cationic and hydrophobic regions, interacts with the negatively charged phospholipids present in the surface of the microorganisms' cytoplasmic membranes. Bacterial membranes are rich in lipids such as phosphatidilglycerol and cardiolipin, whereas host cells have eukaryotic membranes that are rich in phosphatidylcholine, cholesterol, and sphingomyelin [69].

It is the difference in the lipids that constitute the membranes of bacteria and host cells that allows AMP to selectively target the microbial cells over mammal cells and confers them the criterion of safety to be use in eukaryotic organisms, including humans.

Additionally to their role as membrane disruptors, several studies have also suggested alternative targets for AMP. In fact, it was proven that some AMP are able to translocate into the cytoplasm of pathogens and attack intracellular targets. This way, AMP impair essential bacterial metabolic processes, including nucleic acids synthesis and cell wall assembly [70–72]. AMP can present multiple and simultaneous mechanisms of action, including both membrane permeabilization and intracellular effects. This property is probably the reason why they present antimicrobial activity against such a wide range of pathogens.

Regarding their immunological functions, AMP are also known as host-defense peptides [73–76]. By interacting with a variety of host cell receptors, AMP promote the recruitment of leukocytes to the site of infection through direct chemotactic activity and stimulation of chemokine production by leukocytes, epithelial cells, and other cell types [77, 78]. Finally, some AMP also play a role in angiogenesis and wound healing [79, 80].

The production of AMP is not limited to multicellular organisms; bacteria can also synthesize AMP that are active against other bacteria. These AMP of bacterial origin include non-ribosomally synthesized peptides such as gramicidins, and ribosomally synthesized peptides such as bacteriocins, and have been used for years [81, 82]. Gramicidin S is a cyclic decapeptide produced by *Bacillus aneurinolyticus* and has been used as a topical antimicrobial agent against Gram-positive bacteria since 1946 [83]. Nisin is a bacteriocin produced by *L. lactis* that acts primarily against Gram-positive bacteria and has been used safely as a food preservative for over 50 years [84].

Several studies have analyzed the *in vitro* activity of different AMP against DFU clinical isolates. In 2013, Okuda *et al*. [85] evaluated the antimicrobial activity and mode of action of three bacteriocins, nisin A, lacticin Q, and nukacin ISK-1, against a clinically isolated and biofilm-producing MRSA strain. Nukacin ISK-1, produced by *Staphylococcus warneri*, presented only bacteriostatic effects. However, both nisin A and lacticin Q, produced by *L. lactis*,

showed bactericidal efficacy against planktonic and biofilm cells [85]. Synthetic cationic antimicrobial peptides, namely NP101 and NP108, also showed *in vitro* activity against bacterial species commonly associated with DFU infections, such as *S. aureus* and *P. aeruginosa*, as demonstrated by O'Driscoll *et al*. [86] in 2013. These results suggest that bacteriocins that act on biofilm-producer cells are highly suitable for the treatment of DFU infections.

However, there are some limitations in the use of AMP as a clinical alternative for Antibiotics, in spite of the fact that bacteria resistance to AMP is rare, in opposition to what is observed towards classic antibiotics [87]. This characteristic of AMP is likely to be related to the ionic interaction between the positively charged AMP and the negatively charged bacteria membrane. Since these interactions are not dependent of specific protein binding sites, in order to develop resistance to AMP, bacteria would have to change the basic structure, namely the lipid bilayer, of its cytoplasmic membrane [88]. Moreover, attachment of the AMP with the bacterial membrane and consequent cell lysis happens in such a short period of time, rendering the possibility to develop AMP resistance quite scarce [89]. However, there are reports of distinct species of bacteria, which present resistance towards AMP. The mechanisms of resistance include degradation of AMP through secretion of proteases; removal of AMP from their site of action via efflux pumps; production of inhibitors that bind to AMP and prevent them from reaching their target; and modulation of AMP gene expression [90–92].

Another obstacle to the successful implementation of AMP as an alternative to conventional antibiotics is the production costs. AMP discovery and development is time consuming, reaching up to 10 years, and can cost millions of euros or dollars. In fact, production costs are estimated to be approximately 50-400 American dollars per gram of amino acid [93].

Even so, AMP are still a promising alternative to antibiotics. A possible solution to reduce costs associated with AMP production is the reduction of the peptide size, maintaining its antimicrobial activity [94]. Moreover, AMP exhibit physiological and functional advantages over other molecules that make them so attractive to be used in clinical practice. For instance, physiological concentrations of AMP *in vivo* are much lower than the minimal inhibitory concentrations required for its antimicrobial activity *in vitro* [95]. In fact, AMP are antimicrobial agents with a broad-spectrum activity displayed at micromolar concentrations, usually in the 1-50 μg/ml range [96]. A plausible justification for this fact may be the synergistic effect that some AMP possess, which enhances their antimicrobial activity *in vivo* [97].

For all these reasons, the development of AMP-based therapies to eliminate microbial pathogens, such as those present in DFU infections, is extremely promising and deserves further exploration.

5. Conclusive remarks

The severity of diabetic foot infections and the economic burden associated with its prevention, treatment and control have compelled scientists and clinicians to invest substantial time and effort in not only understanding how these mechanisms work, but also how they can interfere with them.

As mentioned before, a major factor responsible for healing impediment of DFU are infections by multidrug-resistant or biofilm-producing bacteria. Dissemination of these strains, coupled with disinvestment in new antibiotics development, calls for increasing research to find new approaches to prevent and control these pathogens. In this chapter, the potentialities of bacteriophage viruses, probiotic strains and antimicrobial peptides as novel strategies for management of DFU, were reviewed. Several studies, conducted by independent research teams, have demonstrated promising results, both *in vitro* as *in vivo*, regarding their competence to eradicate the pathogenic microorganisms present in DFU. However, further investigation is required so that in the future, these strategies could be applied in clinical practice alongside with conventional therapeutics.

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Chapter 14

Wound Biofilm and Therapeutic Strategies

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Additional information is available at the end of the chapter

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Abstract

Since the turn of the millennium, an evolving body of scientific and clinical evidence indicates that biofilm is implicitly linked to delayed wound healing and infection. Currently, wound anti-biofilm strategies rely on non-specific wound bed preparation techniques involving physical debridement and cleansing, and innovative technologies designed to specifically manage biofilm have only just begun to emerge. The first output of anti-biofilm research and product development in wound care show great promise for patients, clinicians and healthcare institutions. The aim of this chapter is to address the current clinical biofilm problem, describe existing and emerging strategies to combat wound biofilm and review the available evidence.

Keywords: wound, anti-biofilm, dressing, healing, infection

1. Introduction: the clinical problem

Fossil evidence of microorganisms existing as surface-attached microcolonies dates back 3.4 billion years [1], establishing biofilm as one of the oldest life-forms on earth. The scientific study of surface-attached microorganisms dates back to the seventeenth century [2], but it is only in recent decades that their relevance has been appreciated in both natural and pathogenic ecosystems [2, 3]. Although the term 'biofilm' has been used to describe surface-attached, matrix-encased microbial communities in industrial and environmental applications since the 1930s, it was not until 1985 that Bill Costerton introduced the term into medical microbiology [2]. The importance of biofilm in chronic infections is now widely accepted and there has been an exponential rise in related medical publications since 1975, reaching a number of 3251 in 2013 alone [2].

The refractory nature of many infections has been largely attributed, in recent decades, to the continuing rise in antibiotic resistance, but the involvement of biofilm in microbial tolerance to antimicrobial agents and immune cells is increasingly recognised. The combined effect of biofilm tolerance and antibiotic resistance are the two most important microbial defence strategies, and in combination present a significant risk to public health. In 1999, Costerton *et al.* [4] reported bacterial biofilm as being a common cause of persistent infections that include conditions such as periodontal disease, otitis media, cystic fibrosis, pneumonia and device related infections. Although the authors also considered necrotising fasciitis and osteomyelitis as biofilm infections, wound infections in more general terms were not considered.

Any wound that is not healing and that has not followed a normal wound healing trajectory is likely to involve biofilm. Healthy skin is an effective microbial barrier; therefore dermal tissues are intrinsically sterile. However, the surface of the skin is heavily colonised. Damage to or removal of the epidermal barrier layer will inevitably lead to wound contamination and opportunistic microbial colonisation. The innate human immune system can usually counter this invasion but, if the initial contamination event is overwhelming (such as a severe traumatic wound), or contamination is repetitive (for example for a faecally incontinent subject suffering from a sacral pressure ulcer), or if the casualty has a weakened immune system (as a result of age, disease, malnutrition, obesity, smoking, etc.) then biofilm may become established.

The earliest indirect indication of wound bacteria existing in biofilm form involved the detection of extracellular polysaccharide capsules surrounding the cells of both aerobic and anaerobic wound pathogens, using light and scanning electron microscopy [5]. Capsule production is a key component of a biofilm mode of life that can protect bacterial communities from the host immune system [6]. The earliest scientific research into wound biofilm was reported by Serralta *et al.* in 2001 [7]. In this *in vivo* study, both planktonic and biofilm bacterial lifestyles were observed, with biofilm bacteria (*Pseudomonas aeruginosa*) exceeding planktonic bacteria by approximately 100-fold. Whereas the planktonic *P. aeruginosa* could be removed by vigorous flushing, adherent biofilm *P. aeruginosa* could only be removed by forceful scrubbing with a detergent agent.

The development and evolution of wound biofilm from contamination to a pathogenic state was proposed in 2004, and the point at which an evolving biofilm begins to interfere with wound healing and increase the risk of infection has largely replaced the previously-used term 'critical colonisation' [8]. In 2008, a hypothesis (that was considered novel at the time) relating to why chronic wounds fail to heal was reported [9]. Based on their previous experiences with chronic *P. aeruginosa* infections in patients suffering from cystic fibrosis, Bjarnsholt *et al*. [9] proposed that the inability of polymorphonuclear leukocytes and antibiotic treatment to eliminate *P. aeruginosa* biofilm was the cause of recalcitrance in chronic wounds. Subsequent clinical studies using microscopy and molecular analytical techniques demonstrated that biofilm existed in a majority of non-healing chronic wounds [10, 11]. Since 2008, an increasing number of studies have demonstrated the presence of biofilm in wounds of varied aetiology [10–21] as indicated in **Table 1**.

Since the turn of the millennium, wound biofilm has been proposed, investigated and confirmed, as a factor in chronic wound pathogenesis. From initial evidence of their

existence [7], role in wound healing [8], and the simultaneous and pioneering confirmation of their clinical existence in 2008 [10, 11], a large body of scientific and clinical evidence now suggests that biofilm is inextricably linked to wound infection and delayed healing [7, 22–37] (**Table 2**).

Wound types	No.	Methods	Observations	Ref.
Chronic (mixed)	50	Light microscopy, scanning electron microscopy (SEM)	30 chronic wounds observed to contain biofilm (60%)	10
Chronic (mixed)	22	Confocal laser scanning microscopy (CLSM)	13 chronic wounds observed to contain biofilm (59%)	11
Chronic (mixed)	9	Fluorescence microscopy, CLSM	*P. aeruginosa* observed deeper in wound than *S. aureus*	12
Chronic (mixed)	10	Fluorescence microscopy, CLSM	*P. aeruginosa* elicited greater inflammation than *S. aureus*	13
Chronic	1	Fluorescence microscopy	Both samples contained biofilm	9
Mixed aetiologies	15	Fluorescence microscopy	7 wounds observed to contain biofilm (47%)	14
Full-thickness burns	11	Light & transmission electron microscopy, SEM	Only ulcerated and escharotomy sites contained biofilm	15
Diabetic foot ulcers (DFU)	2	CLSM	Both samples contained biofilm	16
Acute	16	Light microscopy, SEM	1 acute wound contained biofilm (6%)	10
DFU	4	Light & fluorescence microscopy, environmental SEM	Microcolonies associated with biofilm observed in all wounds	17
Surgical sternal	6	Light & fluorescence microscopy, CLSM, SEM	3D biofilm aggregates observed in all 6 infected wounds	18
Venous leg ulcers (VLU)	45	Transmission electron microscopy	Biofilm matrices of polysaccharides, proteins & DNA observed	19
Malignant	32	Fluorescence microscopy	Biofilm observed in 35% of wounds	20
Mixed & non-wound	113	Biofilm-forming capacity of isolates by culture & SEM	Significantly more biofilm formed by wound isolates than others	21

Table 1. Key scientific evidence for the presence of biofilm in human wounds.

Model	Biofilm species	Observations	Ref.
Porcine acute	*S. aureus (S.a)*	Challenge with antimicrobial agents confirmed the recalcitrance of biofilm bacteria	7
Porcine acute	*S.a*	Polymorphonucleocytes observed on the surface of, but not within, biofilm	22
Porcine partial thickness	MRSA, *P. aeruginosa (P.a)*	Interactions between MRSA and *P.a* were observed, delaying healing due to suppression of epithelialisation and expression of	23

Model	Biofilm species	Observations	Ref.
		virulence factors	
Murine burn	*P.a*	Microscopic biofilm observed that was not readily removed by rinsing with saline	24
Murine diabetic chronic	*P.a*	Biofilm-colonised wounds highly inflamed; 8 weeks for biofilm-colonised wounds to heal, 4 weeks for controls	25
Murine diabetic chronic	*P.a*	Biofilm significantly delayed wound healing, even in diabetic mice treated with insulin	26
Murine infected surgical	*P.a*	Biofilm highly-tolerant to antibiotics & sodium hypochlorite once established over several days	27
Murine chronically-infected surgical	*S.a*, *P.a*, *E. faecalis*, *F. magna*	Polymicrobial biofilm maintained for 12 days, & delayed healing more than *P.a* biofilm, by wound closure	28
Murine splinted	*S.a*/*S. epidermidis*	Biofilms significantly delayed epithelialisation; inhibition of biofilm restored normal healing	29
Rabbit ear	*S.a*	Biofilm and active infection significantly delayed healing; biofilm-colonised wounds expressed significantly lower levels of inflammatory cytokines than infected wounds	30
Rabbit ear	*P.a*	Biofilm significantly delayed healing; debridement, lavage and silver sulphadiazine in combination were more effective at restoring healing than individual treatments	31
Rabbit ischaemic ear	*K. pneumonia (K.p)*, *S.a*, *P.a*	*K.p* biofilm was least virulent, *P.a* biofilm most virulent, measured by healing inhibition and inflammation; extracellular polymeric substances (EPS)-deficient *P.a* did not delay healing	32
Rabbit ear	*S.a*, *P.a*	2-species biofilm elicited significantly elevated inflammatory response & impaired epithelialisation & granulation tissue formation compared to single-species	33
Rabbit ear	*P.a*	Dressing designed specifically to manage biofilm gave significant reductions in biofilm count & significantly improved wound healing (granulation & epithelialisation)	34
Murine	Natural skin microflora	Biofilm developed over time in chronic wounds (similar to humans); reducing oxidative stress increased their susceptibility to antibiotics & dismantled biofilm	35
Rabbit ear	*K.p*, *P.a*	Wounds showed increased inflammation and delayed healing with *P.a* biofilm infection as determined by of wound healing cells transcriptome analysis	36
Porcine burn	*P.a*, *A. baumannii*	Biofilm-infected wounds, tolerant to silver dressings, eventually closed, but skin barrier function compromised	37
Diabetic mouse	*S.a*	Diabetic wounds had significantly more biofilm & less neutrophil activity, thus poorer healing than wild type	38

Table 2. Evidence that biofilm delays wound healing from porcine, murine and rabbit ear models.

In our laboratory, we have used microscopy techniques to better understand the structure and development of wound biofilm. Planktonic cultures of *P. aeruginosa* were grown on track-

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etched membrane cell culture inserts in culture wells for up to 48 hours, and biofilm growth was examined at various stages of development by light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Biofilm was shown to form within 6 hours, and becoming established after 24–48 hours (**Figure 1**). This favourably compares with previous work reported [39] in which mature *P. aeruginosa* biofilm formed within 5 hours of initial inoculation. Stages of biofilm observed *in vitro* may correlate with our understanding of wound biofilm development and its link to chronicity and infection (**Figure 1**).

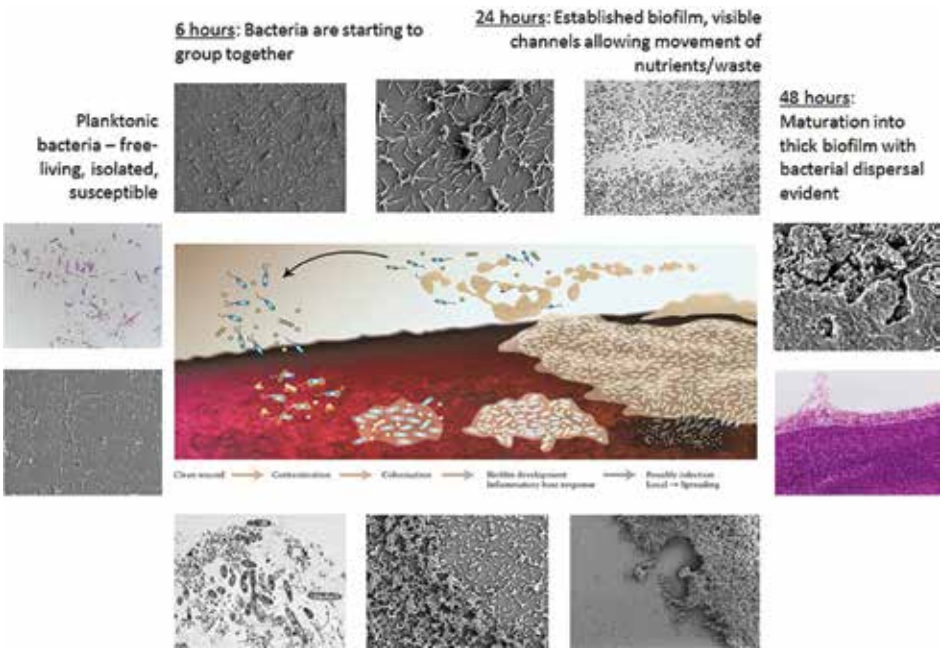

Figure 1. Evolution of *P. aeruginosa* biofilm over 48 hours, with evidence of microcolony formation, maturation and dispersal.

Numerous *in vivo* models have been reported in recent years, and these have been critically reviewed and compared [40]. The rabbit ear model developed by Tom Mustoe's team in Illinois, USA is perhaps the most developed and validated of the models [30], has demonstrated clear links between wound biofilm and healing, and has also been used to compare the effectiveness of anti-biofilm strategies [31, 34, 41–43].

Scientific, clinical and animal evidence strongly suggests that biofilm delays wound healing [44], and efforts are underway to understand and develop ways to visualise and control biofilm to aid clinical practice [44, 46]. It has been shown that by targeting and suppressing biofilm

healing can be improved, so the onus is now on wound care product developers and manufacturers to offer technologies with anti-biofilm effectiveness.

2. Therapeutic anti-biofilm strategies

With only relatively recent recognition of the existence of biofilm in wounds and consequent role it plays in delayed healing and chronicity [8, 10, 11], the development of effective therapeutic treatments and strategies to date has been very limited. However, this late recognition does mean that wound care researchers can benefit from the knowledge gained in other industries and in related healthcare areas such as dentistry and indwelling medical devices. Here treatment strategy options are well developed and broadly similar. Although the intention to prevent, remove and kill bacterial biofilm is the same, there is a significant challenge in selecting wound treatments that have an appropriate balance of safety versus efficacy. There are also challenges in simultaneously addressing the other clinical needs of the wound as, unlike inert medical devices or tooth enamel, the surface of a wound, particularly a chronic wound, can be acutely sensitive and fragile.

Wound biofilm is generally initially attached to the wound bed which is a dynamic mixture of viable and non-viable (slough) tissues. Exudate permeates through this underlying tissue

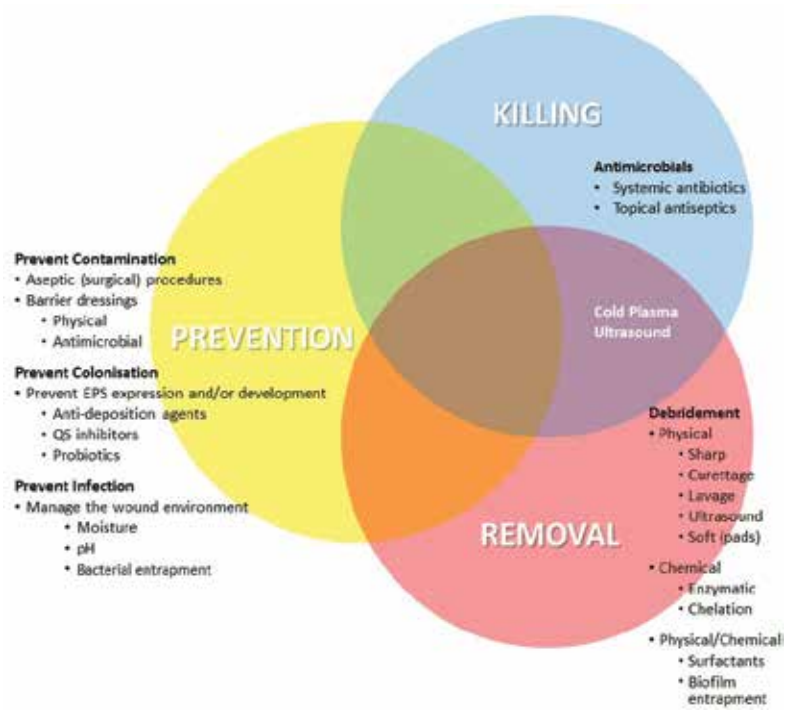

Figure 2. Anti-biofilm strategies for wound care—prevention, removal, killing.

providing moisture and nutrients to developing biofilm. Treatment and prevention of wound infection usually consists of systemic antibiotics and/or topical antiseptics. The polymicrobial nature of wound bioburden [5] and difficulty in identification of species present means that antibiotic selection and coverage is imperfect. Chronic wounds are often poorly perfused with blood (a causative factor) therefore delivery of systemic antibiotics, at a sustained therapeutic concentration, for the period necessary to diffuse into a biofilm and take effect, is an additional challenge [47]. Topical antiseptics are preferred because of their broad spectrum of activity, but they suffer from a lack of selectivity towards bacteria and therefore can be toxic to host tissues. General reaction with organic matter and continual dilution and removal with wound exudate mean that an antimicrobial product needs to be continuously instilled [48] or formulated for slow release and physical retention within the wound or dosed at high concentration. Clinical evidence suggests that none of the above existing systemic or topical treatments are particularly effective against wound biofilm. Therefore, different strategies are required.

It is convenient to consider these strategies in broad groups aligned with the clinical intent as mentioned above—to prevent, to remove, to kill biofilm-associated organisms (**Figure 2**)—and many treatments will involve combinations of these with the best being a combination of all three. We will also discuss the anti-biofilm effectiveness of existing methods together with associated devices, and how some of these are providing therapeutic advances in the emerging 'biofilm era' of wound care.

2.1. Prevention of biofilm

Microbial contamination of breaches in skin integrity is inevitable, unless the wound is created under aseptic surgical conditions, and wounds that are not successfully managed can become chronic and at risk of infection. The ideal situation is to prevent bacteria entering the wound in the first instance. The risk of wound contamination post-surgery can be addressed by applying effective microbial barrier dressings. In the 1990s *DuoDERM*® hydrocolloid adhesive dressings were shown to reduce the rate of infection (compared to traditional gauze dressings) [49] and these dressings were later shown to provide a physical barrier to both bacteria and viruses [50]. More recently, combined physical and antimicrobial barrier dressings have been developed to minimise the risk of post-operative infection [51].

Although barrier dressings have an important role to play in minimising infection, it is most likely that any open wound will become contaminated to some extent (chronic wounds will become significantly more contaminated than most surgical wounds). Consequently, an important infection control strategy at this point is to prevent microbial attachment to the wound tissue. This might be achieved by chemical or biological treatment of the wound surface. Examples of the former are lactoferrin and xylitol. Lactoferrin [52, 53] is a protein that is believed to inhibit the effectiveness of bacterial adhesins by its ability to sequester iron from acidic environments, particularly for Gram-negative bacteria [54]. It has proven useful in the preservation of meat but in a living system with a functioning circulating system supplying an excess of iron and buffering to neutral pH there will be challenges in maintaining efficacy. If derived from non-human sources there is also the possibility that lactoferrin may be

identified by the immune system as a threat and illicit an inflammatory response. Xylitol is a naturally occurring sugar that binds to the surface of Gram-positive bacteria preventing adhesion [53], inhibiting glycocalyces (exopolymeric substances) and disrupting cell wall growth [54]. But xylitol faces the same challenge as lactoferrin in that it is a freely soluble substance and will be difficult to maintain at an effective concentration in an exuding wound. Gallium is also mooted in this space as Ga^{3+} is similar to Fe^{3+} in size but does not undergo the same redox reactions ($Fe^{3+} \longleftrightarrow Fe^{2+}$) and therefore interferes with bacterial attachment and proliferation [52].

If microorganisms gain the opportunity to attach to wound tissue and acclimatise to the environment, then subsequent colonisation will lead to the development and maturation of a predominantly biofilm population that is protected from the immediate hostilities within the wound environment (**Figure 1**). Two potential biological approaches for controlling biofilm development are quorum sensing (QS) inhibitors and probiotics. Quorum sensing is an active field of research with over 100 bacterial species identified as having the ability to communicate by release of small signalling molecules [55]. At a critical concentration, microbial communication between cells triggers a change in gene expression which results in a change in behaviour. QS is involved at all stages in the biofilm life cycle (initial attachment, EPS expression, proliferation, maturation and dispersal) and is implicated in biofilm virulence. In practical terms, this minimum concentration dependence translates into a minimum threshold bacterial population density. However, it must be borne in mind that wound biofilms are polymicrobial [56–58] and although approximately 50% of all known QS bacterial species have the 'universal' autoinducer 2 (AI-2) [55], QS signal molecules vary between species and strains. Therefore, a universal inhibitor for wound biofilm formation seems unlikely in the near future. Probiotics [59, 60] offer the interesting possibility of prophylactically colonising the wound with non-pathogenic bacteria. *Lactobacillus* species have been shown to successfully outcompete and inhibit the pathogenic activity of *P. aeruginosa* and *S. aureus* possibly through QS inhibiting effects [59]. However, there are significant challenges, particularly for chronic wounds, in how to selectively provide conditions for the survival and growth of probiotic microorganisms in an already contaminated and inflammatory wound.

Finally, often overlooked is the management of the wound environment itself—establishing the best conditions in which the body's immune system can function and/or creating conditions which reduce bacterial proliferation and biofilm development. The optimal moisture balance in the wound bed is reported to be 100% humidity with no free liquid [61], and it has recently been suggested that poor exudate control is likely to encourage the development of biofilm [62]. Chronic, non-healing wounds are often characterised by a high pH (7.15–8.9) and healing wounds tend to have a lower pH [63]. The increased production of *S. aureus* EPS with increasing pH has been reported [64], but other examples describe the opposite, so the relationship between biofilm and pH appears to be complex [65, 66].

2.2. Removal of biofilm

By the time wounds are presented to a wound care specialist, the majority of non-healing traumatic and chronic wounds are likely to be biofilm impeded. There is a long history of

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removing non-viable and necrotic host tissue from wounds (debridement) in order to encourage the inflammatory, granulation and epithelialisation processes of wound closure. Surgical debridement techniques can range from aggressive surgical or sharp removal of tissue to less invasive techniques such as curettage and lavage. This practice is likely to have coincidentally been removing biofilm with beneficial effect. With increasing familiarity with the appearance of biofilm or more likely, the symptomatic signs of its presence, clinicians are seeking methods to physically remove biofilm from wounds. In recent years, debridement devices utilising a number of very different technologies have emerged.

Sharp debridement is the most radical approach and requires expertise [67]. Excision of devitalised host tissue (i.e. necrosis) or infected/biofilm tissue via scalpel or other surgical instrument until the exposed tissue is bleeding would certainly be expected to remove a majority of any biofilm residing in the wound, but the deleterious effects on healing tissues need to be balanced with the need to remove unhealthy tissue. However, sharp debridement has proven successful and advocates such as Wolcott have developed protocols where regular sharp debridement has provided a 'healing window' during which improved effectiveness of concurrent antimicrobial treatment has been observed [68]. Hurlow has also reported the atraumatic removal of biofilm above a non-healing surgical wound with exposed tendon using curettage and antimicrobial cleansers [69, 70] (**Table 3**).

A number of other devices that can be used for wound debridement are now commercially available. Examples include devices that emit energy in the form of water jets (lavage), ultrasound and cold plasma (**Table 3**). High pressure lavage using hand-held devices [67] has been assessed in several laboratory and clinical investigations, and there is evidence that removal of unwanted tissue (which may include biofilm) using this method encourages wound progression [71]. Ultrasonic wound debridement has proven effective in clinical cases [72], and scientific studies support the ability of ultrasound to disrupt biofilm and encourage healing *in vivo* [42], as well as potentiate the effects of antiseptics via its anti-biofilm action *in vitro* [73]. Finally, encouraging *in vitro* anti-biofilm effectiveness of cold atmospheric pressure plasma technology suggests that the reactive oxygen species produced in precise beams by these devices may disrupt and kill biofilm while sparing host tissues [74].

Unfortunately, in many clinical institutions the skills, training and equipment for the use of advanced debridement techniques or devices may not be available. Under these circumstances simple cleansing, enhanced with 'soft debridement' using engineered textiles, may be helpful. Recently, debridement pads or wipes have emerged which aim to gently brush and lift away wound debris. A polyester filament pad has generated encouraging clinical effectiveness data [75, 76] and cost-saving estimates [77]. In addition to disrupting and lifting surface-associated wound debris (which is likely to include biofilm), these soft debridement devices are simple and safe to use, gentle on patients and relatively low-cost, compared to most other debridement techniques and devices discussed in **Table 3**.

More thorough biofilm removal may be achieved by degrading the structure of the EPS such that it flows away from the wound or can be more readily irrigated or absorbed by absorbent dressings. General proteolytic enzymes have been used for many decades to remove slough and necrotic tissue, but, as EPS is not primarily comprised of extracellular proteins for its

structural integrity, these are ineffective. This fact has been utilised by an aid to detect the presence of wound biofilm [45]. Alternative enzymatic candidates that are effective against polysaccharides have been identified and reviewed [78, 79], and include: α-amylase (mammalian), polysaccharide depolymerase (bacteriophage), alginate lyase (bacterial) and glycoside hydrolase (DspB) (bacterial). Generally, the kinetics of enzyme reactions are known to be sensitive to pH, for example Dispersin B, despite demonstrating some activity *in vitro* [80], has optimal activity at pH 5, and as proteins, enzymes will be vulnerable to the high concentrations of proteolytic enzymes often associated with chronic wounds. Hence, careful formulation of any enzyme based anti-biofilm treatment would be required. Sun *et al*. also point out that the current high cost of industrial enzyme production makes the application of these enzymes relatively expensive [79].

Debridement method	**Evidence for effectiveness against biofilm**	**Ref.**
Curettage	(Clinical) Gentle scraping of suspected biofilm (in combination with other antimicrobials) improved healing in case studies	69,70
Lavage/water jets	(Clinical) Indirect anti-biofilm evidence; debridement with *Versajet II* (Smith & Nephew) removed unwanted tissue & encourages healing	71
Ultrasound	(*In vitro*) Anti-biofilm action demonstrated in agar biofilm model using *Ultrasonic-Assisted Wound (UAW)* device (Soring)	73
	(*In vivo*) Leporine ear model showed *MIST* (Celleration) reduced *P. aeruginosa* biofilm & inflammation, & improved healing parameters	42
	(Clinical) Indirect anti-biofilm evidence; case study evidence that *UAW* can effectively debride unwanted tissue	72
Cold plasma	(*In vitro*) Biofilm significantly reduced using *Coblation* (Smith & Nephew) compared to lavage or curettage in porcine explant model	74
Soft debridement pads	(Clinical) Indirect anti-biofilm evidence in case studies where sloughy wounds were well managed using *Debrisoft* (Lohmann & Rauscher)	75
	(Clinical) Debridement was classed as effective in 94% of patients, removing debris and slough, in a 57-patient study using *Debrisoft*	76

Table 3. Biofilm removal using debridement methods and devices.

Structural degradation of biofilm EPS can also be achieved chemically. Divalent cations such as calcium and magnesium are known to be involved in cross-linking polysaccharides within EPS and manganese and iron are involved in bacterial metabolism and cell wall structure [81]. Competition for these ions or their removal (chelation) will therefore affect biofilm formation and strength. Metal chelating agents are a diverse set of compounds but biocompatibility and safety considerations restrict those that can be considered for wound care to ethylenediamine tetra acetic acid (EDTA) and its homologues and polyanionic compounds such as phosphates and citrate. The most widely discussed of these as an anti-biofilm agent and the one with the greatest affinity for calcium and magnesium cations is EDTA. The literature primarily focuses on the tetra-sodium salt form but only at high pH (>pH 10) [81], which is incompatible with wound management practices. Lower pH forms of EDTA, such as

the di-sodium salt, are effective but all anionic chelating agents are pH-sensitive. A water-soluble gel formulation that contains 0.1% EDTA, acetic acid, citric acid and carbopol has demonstrated anti-biofilm effectiveness against *P. aeruginosa* and *S. epidermidis in vitro* [82].

Empirical experience in other industries such as food, laundry, personal washing and dental products [83] has shown the utility of surfactants as anti-biofilm agents to facilitate penetration of combination agents through biofilm EPS [84], leading to detachment from surfaces and prevention from re-deposition by micelle formation. An anti-biofilm gel comprising a surfactant and calcium chelator has shown *in vitro* and *in vivo* anti-biofilm activity. Although not yet commercially available, use of this gel has shown clinical superiority to standard wound care, as well as apparent synergy with standard care [85].

2.3. Killing of biofilm microorganisms

The efficacy of existing antimicrobial therapies in wound care has almost exclusively been based on their activity against susceptible planktonic bacteria. Whilst associated devices may be useful in controlling this bacterial phenotype by reducing the risk of contamination and dispersal, their effectiveness against biofilm is unproven. Indeed, the prevalence and recurrence of chronic wounds suggests that most antimicrobial therapies are ineffective.

Considering the selective and specific action of antibiotics, the polymicrobial nature of wound bioburden and the increasing threat of multi-drug resistant organisms (MDROs), the effectiveness of antibiotics in chronic wound care is questionable. However, utilising state of the art molecular microbiological techniques, personalised cocktails of topically-applied antibiotics yielded better results than patients receiving systemic antibiotics prescribed using the same diagnostic techniques who, in turn, yielded better outcomes than a standard-of-care group treated upon data from standard culture techniques [86]. Unfortunately, this level of diagnostic sophistication is not within the reach of most health care systems, and therefore we must await further technological advancements so that it becomes generally affordable.

The majority of wound treatments do not have the benefit of sophisticated microbiological analysis; therefore any antimicrobial therapy administered must have broad-spectrum activity. Choice then becomes restricted to antiseptics which can only be applied topically. Antiseptics are chemically reactive species that are largely non-selective in their action, therefore potential cytotoxicity (local toxicity to skin cells) and systemic toxicity must be taken into account. Toxicity is generally managed by limiting the concentration and time of exposure to the antimicrobial agent. Therefore, antimicrobial cleansing at dressing change may involve slightly higher concentrations of antiseptic than an antimicrobial dressing which may stay *in situ* for several days. However, given that the minimum biofilm eradication concentration (MBEC) for antiseptics in wound relevant bacteria is often between 10–1000 times greater than its minimum inhibitory concentration (MIC) for planktonic bacteria (the value upon which most products are formulated) [87], the balance of safety versus efficacy of antiseptic agents is challenging. Topical wound antiseptic treatments typically involve 0.5 to 12% for silver in dressings (depending on the form of silver), approximately 1% for molecular iodine in dressings (or 10% as povidone iodine), 2 to 4% for chlorhexidine gluconate in cleansers and

0.1% for polyhexamethylene biguanide (PHMB) in cleansers. Increasing the concentration of the antiseptic component to be effective against biofilm may not be possible, practical or safe.

Clinical experience and safety reviews have limited the number of usable antiseptic substances. Currently, silver is the most widely-used topical antiseptic agent, primarily due to its good safety versus efficacy balance [88]. Silver is the most studied topical antiseptic [52, 53], and ionic silver—the antimicrobial active form—has a particularly high affinity for sulphur atoms, binding irreversibly to thiol groups. Ionic silver also binds to nitrogen atoms in amines and oxygen atoms in carboxylates, although less strongly. These three interactions lead to very efficient denaturing of peptides, proteins and enzymes—all of which are essential to bacterial structure and metabolism. However, carboxylate functional groups are also found within the polysaccharide in EPS. Therefore, although ionic silver may be inactivated by EPS and other organic matter within the biofilm, there is a theoretical basis for it to have some biofilm disruptive effects. Evidence for this effect was a reduction in EPS mechanical strength of an *S. epidermidis* biofilm after the application of dilute silver ion solution [89] (**Table 4**). Similar observations have been made for a silver-containing carboxymethylcellulose dressing [90, 91], and it was reported wound dressings with hydrophobic base material impregnated with silver had sustained anti-biofilm activity [92] (**Table 4**).

Molecular iodine has proven too toxic for direct application but, by complexation with a carrier molecule and careful formulation, acceptable slow release products have been developed. Although the mode of action of molecular iodine is not fully understood [52], studies suggest that in common with silver, sulphur atoms are a reaction target resulting in protein denaturing and subsequent changes to cell wall structure [93]. Iodine will react with unsaturated fats and lipids and organic matter within the wound, and is known to be trapped by polysaccharides. There is limited evidence that molecular iodine has anti-biofilm properties, aside from in simple *in vitro* models [94], but *ex vivo* studies of a formulated cadexomer iodine product suggest that sustained release may result in biofilm penetration [95] (**Table 4**).

Evidence for the anti-biofilm effects of the cationic, nitrogen containing, surfactant-like antibacterials—chlorhexidine (CHG), PHMB and octenidine—in wound care is limited. CHG has been shown to have limited effect against some biofilms *in vitro* but to be ineffective against others, and the theoretical electrostatic effect on bacterial cell walls as the antimicrobial mechanism is believed to be negated by biofilm, so the observed effects are unexplained [96]. PHMB is similar in structure to CHG and is proposed to accumulate within biofilm by electrostatic interactions [97], i.e. cationic PHMB binds to the anionic polysaccharide of EPS [98]—initially, at least, this will have an inactivating effect on the antimicrobial action. Available anti-biofilm data focus on formulated products [99–104], so laboratory and clinical results cannot be attributed solely to PHMB (**Table 4**). Octenidine has been tested *in vitro* against *S. aureus* biofilm, and above a critical concentration bioburden reduction rate was seen to increase, but biofilm was possibly removed to surfactancy rather than specific anti-biofilm activity [105].

The next most popular traditional antiseptic substances are the molecular halogens and related oxidising compounds. Chlorine itself is too toxic to be used and hypochlorite-based bleaches are considered too cytotoxic for general wound care. Hypochlorous acid (HOCl) and chlor-

ine dioxide (ClO_2) are under consideration [52] as potent, fast-acting cleansing solutions, and some early anti-biofilm effectiveness has been observed *in vitro* or inferred clinically for various HOCl formulations [104, 106–108] (**Table 4**).

Antimicrobial agent	Evidence for effectiveness of formulated product against biofilm	Ref.
Ionic silver	(*In vitro*) Reduction in *S. epidermidis* biofilm EPS mechanical strength after application of dilute (50 ppb [ng/ml]) silver solution	89
	(*In vitro*) Anti-biofilm activity of silver Hydrofiber (*AQUACEL Ag*; ConvaTec) shown over 48 hours using range of biofilm models	90,91
	(*In vitro*) Sustained anti-biofilm activity evident for at least 7 days, independent of the microbial strain	92
Molecular iodine	(*In vitro*) Povidone iodine dressing (*Inadine*; Systagenix) demonstrated greater anti-biofilm activity than silver dressings	94
	(*In vitro*) Cadexomer iodine dressing (*Iodoflex*; Smith & Nephew) demonstrated anti-biofilm activity in *P. aeruginosa ex vivo* model	95
PHMB with alkylamidopropyl betaine solution /gel	(*In vitro*) Biofilm matrix on human dermal cell line was disrupted, releasing bacteria for killing, by *Prontosan* (B. Braun)	104
	(*In vivo*) Anti-biofilm effectiveness of *Prontosan* was significantly more effective than inactive controls in porcine dermal wounds	100
	(Clinical) Signs of biofilm & infection reduced, healing progression observed in 124-patient study using *Prontosan* gel	101
Hypochlorous acid	(*In vitro*) 0.01% HOCl killing of CDC reactor-grown *P. aeruginosa* biofilm by live-dead staining with confocal microscopy	106
	(*In vitro*) Biofilm matrix on human dermal cell line was disrupted, releasing bacteria for killing, using a concentrated HOCl solution	104
	(Clinical) Signs of infection reduced & progress toward healing in a 31-patient study using *Vashe* (PuriCore)	107
	(Clinical) Infection contolled & wounds healed in 14 osteomyelitis patients using *Dermacyn* (Oculus)	108

Table 4. Examples of existing topical antimicrobial products with some anti-biofilm activity.

2.4. State of the art today

2.4.1. Multi-modal strategies

Perhaps the most straightforward way for wound care clinicians to implement more effective biofilm management strategies today is to consider how dental care has embraced multiple strategies to manage dental plaque biofilm. By using combinations of debridement (brushing, flossing), surfactants with antimicrobials (toothpaste), and antimicrobial rinses (mouthwash), most consumers manage biofilm effectively on a daily basis to maintain oral hygiene, and prevent conditions such as dental caries and periodontitis.

Biofilm-based wound care (BBWC) is an emerging and evidently effective way of combining multiple modes of wound treatment to improve the health of chronic and infected wounds [68, 85, 109]. Practised initially by the pioneering wound care physician, Randall Wolcott in Texas, a first assumption of BBWC is made that most (if not all) chronic or infected wounds contain biofilm. A further assumption is that one mode of treatment may not suffice, therefore the use of combinations of vigorous debridement, cleansers or gels, topical antimicrobial or anti-biofilm agents, and wound dressings, is required. Wolcott *et al.* [85, 109–111] have shown how BBWC can result in significantly improved outcomes compared to standard wound care in several large patient cohorts. A number of case studies reported by Hurlow and Bowler [69, 70] have also described how protocols of care designed to target biofilm result in improved wound outcomes. Combining lactoferrin and xylitol (see Section 2.1) in a hydrogel in conjunction with a silver wound dressing demonstrated good efficacy against biofilms [53].

We also firmly believe that the multi-modal approach is the most effective way of rapidly improving wound health in chronic wounds that are likely compromised by biofilm or infection. A key component in such protocols of care is undoubtedly efficacious wound dressings which can provide effective, sustained and safe antimicrobial and anti-biofilm action. Although the focus here is on therapeutic approaches towards wound biofilm, biofilm cannot be considered in isolation. Other challenging wound conditions must be considered alongside biofilm—exudate must be managed, infection must be controlled, the wound must be protected, and pain must be considered—to provide outcomes that can improve quality of life.

Most established antimicrobial dressings are very efficient at managing planktonic bacteria, thereby limiting initial contamination and spread of infection. However, they all suffer the same challenge in the treatment of biofilm in that the antimicrobial agent must penetrate the EPS in order to reach the target bacteria and, when they do so, they largely rely on metabolism to draw them into the bacterial cell for them to act. EPS can restrict the movement of antimicrobial agents by binding them and increasing the likelihood of reaction with other organic matter. If the agent is able to reach the target bacterial cell it must do so in a concentration sufficient to be cidal for the sessile (biofilm) phenotype. Therefore, it is clear that universally successful antimicrobial therapy using a topical antiseptic agent can only be achieved by a sustained application or release in combination with some form of EPS (biofilm) disruption.

2.4.2. An anti-biofilm wound dressing

In 2009, the authors of this chapter undertook a substantial research project to design a wound dressing specifically to manage biofilm. The starting point was taken as an existing antimicrobial dressing, *AQUACEL® Ag*. This dressing has a well-documented clinical history for patient acceptance, safety, management of exudate and reducing the risk of infection [112–115]. *In vitro* studies have demonstrated this product to be effective against a broad spectrum of pathogenic bacteria in their planktonic form, including pathogenic multi-drug-resistant species and clinical wound isolates that have shown high levels of antiseptic tolerance

in vivo [116–121]. In addition, this dressing has shown some *in vitro* anti-biofilm activity in simple models [90, 91]. Using the MBEC method [122] the ability of combinations of anti-biofilm agents, surfactants and ionic silver to eliminate mature *P. aeruginosa* biofilm after 30 minutes contact was investigated. Component concentrations were varied, as was pH, and in all over 60000 tests were performed. Very few combinations proved to be beneficial, but a number of strong synergistic effects were identified, in particular between ionic silver, quaternary ammonium surfactants and metal-chelating agents (especially EDTA), all at a slightly acidic pH [123]. These synergistic components (termed *Ag+ Technology*) were incorporated into the dressing and subjected to extensive safety testing. We believe that the resultant dressing, *AQUACEL® Ag+ Extra™*, is the first commercially available wound dressing specifically designed to manage biofilm.

In the laboratory *AQUACEL Ag+ Extra* dressings have demonstrated effectiveness against biofilm microorganisms in sophisticated *in vitro* wound models. Here, thick biofilms of multi-drug-resistant *S. aureus* or *P. aeruginosa* were grown on cotton gauze substrates, and placed on to a simulated wound bed of nutrient agar, within model peri-wound skin [124] (**Table 5**). Further studies using isothermal microcalorimetry demonstrated how neither the standard silver dressing alone (*AQUACEL Ag*) nor silver nitrate solution showed a marked anti-biofilm activity, while the *AQUACEL Ag+ Extra* dressing eradicated the *S. aureus* biofilm *in vitro* [125]. Interestingly, in this study the anti-biofilm agents alone, without silver, were also shown to be ineffective unless combined with silver, demonstrating the synergistic nature of this anti-biofilm formulation (**Table 5**).

The efficacy of this combination of ionic silver, metal chelator and surfactant has also been demonstrated in an FDA-recognised *in vivo* model of wound healing [30]. Here, the controlled formation of *P. aeruginosa* biofilm and polymicrobial biofilm (*P. aeruginosa* and *S. aureus*) in an acute wound of defined size, and its subsequent treatment, was assessed over time by measuring parameters such as viable biofilm counts, granulation tissue formation and epithelialisation. The anti-biofilm dressing was found to be significantly superior to a PHMB-containing dressing in improving these wound parameters [34] (**Table 5**).

Most encouraging is the early clinical performance data emerging for this new anti-biofilm technology. Harding *et al.* [126] demonstrated the safety and effectiveness of this dressing containing *Ag+ Technology* in a 42-patient study in VLU patients. In particular, the authors highlighted a subset of 10 clinically-infected wounds (where biofilm was assumed to be a problem) that responded in a more dramatic fashion (**Table 5**). European and Canadian clinical evaluations summarised 113 cases which were selected on the basis of being difficult-to-heal wounds, with suspected involvement of infection or biofilm. Following an average of 4.1 weeks of use of the new dressing in otherwise standard wound care protocols, an average wound closure of 73% was achieved, with 17% of wounds healing completely [127] (**Table 5**). More detailed individual case studies from these evaluations have also been presented [128]. In more recent UK-based evaluations of *AQUACEL Ag+ EXTRA*, a 29-case evaluation reported reductions in all described signs of clinical infection, including a reduction in suspected biofilm from 76 to 45%. This was accompanied by an average wound closure of 62%, with 34% of

wounds fully healing after an average of 5.4 weeks of dressing use [129] (**Table 5**). Finally, a 112-patient post-market surveillance study further demonstrated the safety and effectiveness of this dressing, shifting stagnant or deteriorating wounds, that had previously been managed with a large variety of standard antimicrobial products, onto healing trajectories [130] (**Table 5**).

Study type	Summary of results	Ref.
In vitro	Assessed quantitatively by viable counts & confocal scanning laser microscopy, *AQUACEL Ag+ Extra* gave 6 $\log_{10}$ kill of *P. aeruginosa* and CA-MRSA biofilm after 4 & 5 days; standard *AQUACEL Ag* dressing did not fully eradicate either biofilm	124
	Dressing & silver nitrate+EDTA+BC eradicated *S. aureus* biofilm; silver CMC dressing, CMC dressing, EDTA+BC, & silver nitrate alone did not eradicate biofilm; demonstrating synergy of silver with metal chelator & surfactant	125
In vivo	The new dressing technology gave 2 $\log_{10}$ reductions in *P. aeruginosa* or polymicrobial biofilm after 4 & 6 days compared to PHMB gauze & CMC dressings; granulation tissue formation & epithelialisation significantly better after new dressing	34
Clinical study	An acceptable safety profile was demonstrated; after 4 weeks of the new dressing then 4 weeks CMC 12% of wounds healed, 76% showed improvement; mean ulcer size reduction 55%; subset of 10 infected wounds reduced in area by 70%	126
Clinical evaluation	The new dressing resulted in an average wound closure of 73% after average of 4.1 weeks of use in 113 cases; 17% of wounds healed completely	127
	62% average wound closure after 5.4 weeks of *AQUACEL Ag+ Extra* dressing use; 34% of wounds healed completely; exudate & signs of suspected biofilm & infection reduced in 29-case evaluation	129
	Safety & effectiveness demonstrated in 112-case evaluation; suspected biofilm coverage of wound reduced; 13 of wounds healed completely, 65% improved after 3.9 weeks of *AQUACEL Ag+ Extra* dressing use	130

Table 5. Evidence for a dressing designed specifically to manage exudate, infection and biofilm.

3. Conclusions and future perspectives

Biofilm is increasingly accepted as an integral component of wound recalcitrance and infection, and is likely a key reason for the frequent failure of antibiotics and antiseptics in wound healing. Strategies for combating wound biofilm are currently limited and non-specific physical debridement techniques—from physical removal with absorbent dressings, pads and wipes, to sharp and surgical tissue removal—remain the most effective approach. Despite the limited available anti-biofilm wound strategies, efforts are in progress to develop durable medical devices and wound dressings that combine anti-biofilm and antimicrobial activity. To-date and to our knowledge, only one dressing has been designed to combat biofilm (**Figure 3**), and there is a growing body of evidence demonstrating the exceptional clinical

effectiveness of this dressing (*AQUACEL Ag+ Extra*) [126–130]. It is likely that future efforts will continue to investigate combination technologies that will disrupt biofilm to enhance the antimicrobial efficacy of antiseptics and antibiotics. Certainly, eradication of wound biofilm is critical to promotion of healing and hence improving the lives of patients debilitated by wound recalcitrance.

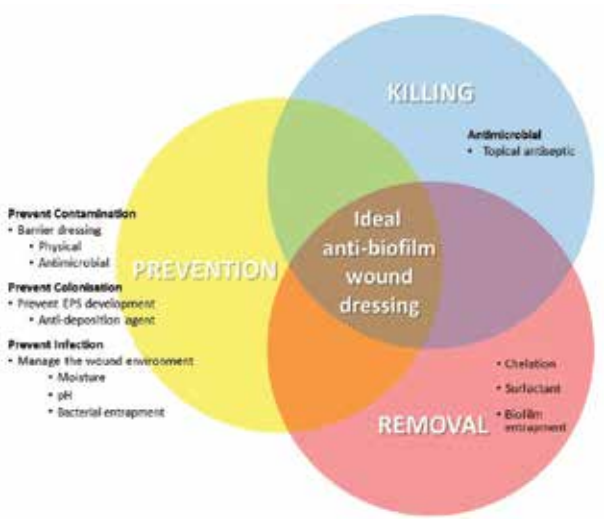

Figure 3. The ideal anti-biofilm wound dressing—prevention, removal, killing.

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Chapter 15

Interactions and Mechanisms of Respiratory Tract Biofilms Involving *Streptococcus Pneumoniae* and Nontypeable *Haemophilus Influenzae*

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Additional information is available at the end of the chapter

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Abstract

The pathology associated with human respiratory tract bacterial agents that exist as opportunistic commensals in the nasopharynx cause infections. This is particularly true for the middle ear disease otitis media (OM) and exacerbations of chronic obstructive pulmonary disease (COPD). *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) are a commonly recurrent combination and the formation of bacterial biofilms by these pathogens in the bronchial airway or middle ear contributes significantly to the chronic nature of these diseases. While S. *pneumoniae* and NTHi have been extensively studied in mono-culture, our knowledge about how they exist together, either in their free-living (planktonic) form or as a biofilm, or indeed the implication of co-infection is still limited. Several key elements are believed to contribute or are induced: (1) a set of sugar metabolic pathways; (2) surface structures in *S. pneumoniae* and NTHi when they are able to co-exist equally; (3) epithelial cell contact that dramatically increases the rate of biofilm formation; (4) chemical modifications of NTHi surface structures involved in host cell interactions; and (5) transcription factors that regulate particular surface molecules and the switch to a biofilm state. There appears to be multiple mechanisms involved and that these are active under specific conditions.

Keywords: biofilm metabolism, multispecies biofilm, *Streptococcus pneumoniae*, *Haemophilus influenzae*, metal ions in biofilm

1. Introduction

Human respiratory tract bacterial infections, like otitis media (OM) and exacerbations of chronic obstructive pulmonary disease (COPD), are caused by bacterial agents that exist as opportunistic commensals in the nasopharynx. *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) are a commonly recurrent combination. The formation of bacterial biofilms in the bronchial airway or middle ear contributes significantly to the chronic nature of these diseases. Biofilms are very difficult to remove by either the host's natural processes or antibiotic therapies, making them an important element within the vicious cycle of the infection exacerbations.

S. pneumoniae and NTHi are extensively studied individually, yet our knowledge about how they exist together, either in their free-living (planktonic) form or as a biofilm, or indeed the implication of co-infection is still limited in contrast to a single species. Studies have shown that in mono- and co-culture planktonic states and in biofilm development several key elements contribute or are induced. These include: (1) a set of sugar metabolic pathways employed especially by *S. pneumoniae* in co-culture when it dominates; (2) surface structures in *S. pneumoniae* and NTHi when they are able to co-exist equally; (3) epithelial cell contact that dramatically increases metabolic process associated with biofilm formation; (4) chemical modifications of NTHi surface structures that have a direct role in the interaction with host epithelial cells; and (5) certain transcription factors that have an integral role in the regulation of particular surface molecules and the switch to a biofilm state. There appears to be multiple mechanisms involved and that these are active under specific conditions.

2. Co-existence within the multispecies biofilm as a mode of bacterial resistance and persistence

In most environmental situations that bacteria exist, they are within communities and in a biofilm. By definition therefore, in nature these are multi-species biofilms. It is surprising, therefore, that the vast amount of knowledge that exists on bacterial biofilm formation and function is from mono-species studies. How the individual species function within an environment, the physical and chemical nature of their biofilm and its eventual impact on the environment (this is particularly true of bacterial persistence within an anatomical *niche*) will be different when as a mono-culture compared to multi-species culture. *H. influenzae* is a commensal bacterial species that inhabits the nasopharynx of healthy humans, and it is accepted knowledge that its asymptomatic nasopharyngeal carriage is in the range of up to 80% [1]. However, *H. influenzae* is not the only species to colonise the nasopharyngaeal *niche*; the other bacterial species within this *niche* include *S. pneumoniae*, *Staphylococcus aureus,* and *Moraxella catarrhalis*. Most commonly the species known to co-colonise the nasopharyngaeal *niche* with *H. influenzae* is the Gram-positive species *S. pneumoniae*. Asymptomatic nasopharyngaeal carriage for *S. pneumoniae* has been documented to be at least 20% [2].

Both *H. influenzae* and *S. pneumoniae* are able to transit from this site of their commensal lifestyle to other anatomical *niches* and thereby cause various diseases. This includes the subsequent

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infection of the bronchi to cause bronchitis [3, 4], the lungs to cause pneumonia [5], the middle ear to cause OM [6], the blood to cause septicaemia, and across the blood–brain barrier to cause meningitis [7]. An increasing number of clinical, diagnostic or epidemiological studies with a focus either on bacterial carriage or the microbiota within an infection have co-located *S. pneumoniae* and *H. influenzae* together [8]. Further to this, in many diseases there are other bacteria present – as mentioned, in the middle ear of OM patients there is *S. aureus* and *M. catarrhalis*, but then in different parts of the respiratory tract, these and other microorganisms are known to co-exist (whole genome sequencing of the bacterial population in patients with cystic fibrosis has shown the presence of a diverse range of bacteria including *S. pneumoniae* and NTHi but also *Pseudomonas aeruginosa)* [9]. In the case of OM, to some degree, there is evidence that at least infection with *S. pneumoniae* alone represents a different clinical and epidemiological case than compared to *S. pneumoniae* together with NTHi [10]. There seems to be a distinction also based on strains; specifically *S. pneumoniae* serotype variations effecting their colonisation and interaction with NTHi. Also, there are non-encapsulated *S. pneumoniae* strains that obviously have a different molecular pathogenesis but also cause OM, and have been shown to co-exist with NTHi [11].

Upon entry to their new *niche H. influenzae* and *S. pneumoniae* require systems that permit their adaptation to the specific physical and chemical properties that exist in the lung, middle ear, blood or cerebrospinal fluid. These include oxygen levels, pH, nutrient availability, the presence of toxic reactive chemicals (reactive oxygen and nitrogen species), and immune factors such as antimicrobial compounds. Given the likely inhospitable nature of migration from nasopharyngaeal *niche*, it seems necessary that there are eventual benefits from this switch in lifestyle. However, the specific molecular factors and signals that cause the transit from the commensal colonisation of the nasopharynx to, for instance, an invasion of sterile sites of the respiratory tract is not well known. There are clearly host factors such as the anatomy of the eustachian tubes [12], and then age and immune competence [13]. Within either their original commensal site or the further migrated locations (in particular the middle ear and the lung), it is known that *H. influenzae* and *S. pneumoniae* have an ability to persist for prolonged periods of time. In the first instance this requires the bacterial cells to attach and remain present. This process includes expression of appropriate adhesins and the ability to evade the host immune response. For both these bacterial species, an essential factor in colonisation and then their survival and persistence is their formation of a biofilm. In the case of the lung and middle ear, this is now known to be as a multi-species biofilm.

3. The nature of bacterial persistence and resistance within a biofilm

A biofilm is a bacterial lifestyle in which the cells reside adhered to a substratum and to each other and are encased in a self-produced extracellular polymeric substance (EPS) matrix [14, 15]. An important feature of bacterial biofilms is their persistent nature and their insensitivity to immune mediators and clinically used antimicrobial agents [16]. These features can be explained both by the changed physiology of the biofilm-resident bacterial cells themselves and by the physical properties of the EPS matrix components. The presence of an EPS matrix

provides protection and biofilm persistence by physically limiting the diffusion of antimicrobial compounds into the biofilm [17]. Additionally, within the biofilm the bacteria have altered gene expression profiles as compared to their planktonic state [18–20]. This switch in gene expression has global effects on cellular functions. This includes changes not only in the surface structures that are expressed for adhesion and cell-cell interactions, but also in the metabolic and biosynthetic pathways and the systems for maintaining intracellular conditions such as pH and redox balance [21]. There is a reduced metabolic activity; a reduction in energy production, cell division, protein synthesis, and other molecular pathways. These changes create a cellular state with an increased recalcitrance to a broad range of antimicrobial agents [22], at the very least they have reduced or no targets for many antibiotics (DNA replication, protein synthesis, and cell wall biosynthesis). The resistance provided by a biofilm state to the bacterial cells is against effectors of both the innate and acquired immunity as well as antibiotics. The nature of the biofilm (its chemical composition and physical properties), the process of its initiation and formation, and the eventual maturation (the structure), will impact the function and stability of the biofilm. This will be different for a mono-culture compared to a multi-species biofilm.

In summary, a model for different stages of biofilm development by *H. influenzae*/*S. pneumoniae* is well demonstrated [23]. **Figure 1** shows this model in the development of the mixed-species biofilm. Stage 1 involves adaptation and adhesion, where the bacteria recognise the physical and chemical conditions of their new environment (such as the oxygen level, nutrients, and pH) and indeed the biological conditions (the immune mediators that are present and the host cells and their receptors). Specific bacterial adhesins bind to cognate host cell receptors. These surface exposed adhesins structures are expressed with the particular function for attachment to host cells. For *H. influenzae*, these include the type IV pili, lipooligosaccharide (LOS) decorated to form sialyated LOS or phosphorylcholine LOS (discussed later), outer membrane proteins (OMP P5, P6, Hap, HMW1, HMW2), and extracellular DNA (eDNA). For *S. pneumoniae,* there is also a role for eDNA including a range of surface structures and proteins such as capsule, Pht, CbpA, PsrP. Stage 2 is the recognition and response to the interspecies stresses. This involves the expression of systems designed for survival in the presence of the stresses generated by the other species such as; chemical stresses, pH, and immune-mediated stress. *S. pneumoniae* growth generates acidic by-products that lowers the local pH. It is also well known that *S. pneumoniae* can produce H_2O_2 either naturally or perhaps by induction, and this has been argued to be a factor in *S. pneumoniae* out-competing *H. influenzae* (although there is evidence that this not the case) [24]. In addition, *S. pneumoniae* produces an extracellular enzyme (NanA) that desialyates the sialic acid decorated *H. influenzae* LOS, thereby reducing its ability for adhesion. At the same time, *H. influenzae* stimulates certain host immune factors that specifically induce opsonophagocytosis of *S. pneumoniae*, although capsule-specific strains will survive this process [25]. These events through Stage 2 will remove the sensitive strains of both species from the *niche* such that in Stage 3 there is now co-operation between the strains that have survived and is ready to form a multi-species biofilm. This co-operation is complex and still poorly understood but does include quorum sensing (QS; as discussed in the next section of this chapter), co-aggregation and adhesion, formation of an EPS matrix, and subsequently biofilm formation. Stage 4 is the

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maturation of the biofilm permitting its persistence and, through an extension of the EPS matrix, the resistance to exogenous antimicrobial compounds. Host neutrophil extracellular traps (NETs) seem to be incorporated specifically into the EPS matrix and further to this, there is non-specific binding of host immune factors to EPS matrix components such as to the eDNA, further protecting the bacteria that exist within the biofilm. The role of eDNA and the association of NETs in the integrity and structure of biofilm is discussed in the next section of this chapter.

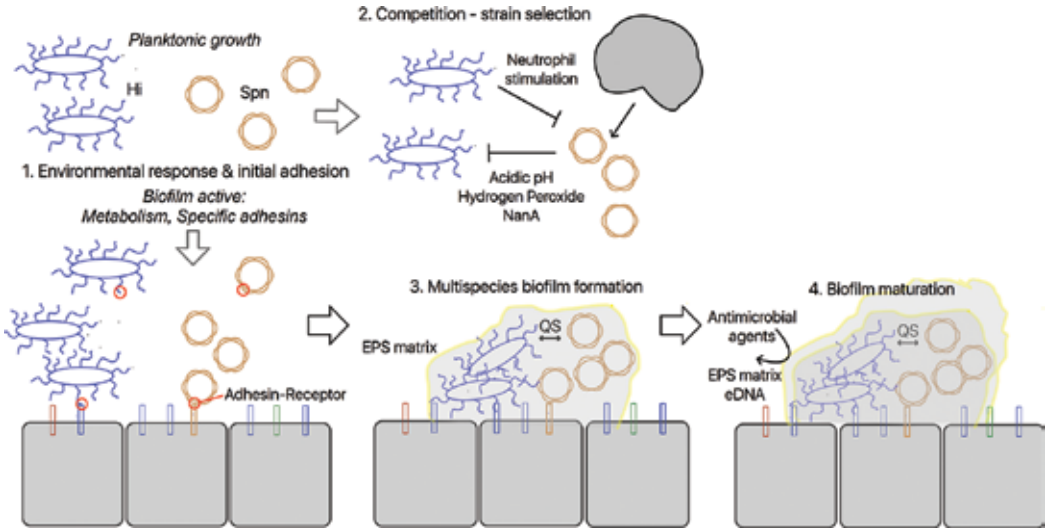

Figure 1. A model for the development of the *H. influenzae-S. pneumoniae* multispecies biofilm. Based on the available literature, there can be identified discrete stages for *H. influenzae* (Hi) and *S. pneumoniae* (Spn) response to the host environment and their survival together and biofilm formation. *Stage 1 – Environmental response and initial adhesion*: the bacteria respond to stresses in the host-pathogen environment by switching from a free-living lifestyle (planktonic) to a biofilm active form (a change in cellular metabolism and surface structures). This includes cell-cell interactions and specifically the binding of bacterial adhesins to host cell receptors. *Stage 2 – Competition and strain selection*: As Spn grows it lowers the local pH and generates hydrogen peroxide, both of which are bactericidal to Hi. The SpnNanA enzyme desialyates the Hi lipooligosachcaride (LOS) reducing its capacity to attach to host cells. The Hi is known to stimulate neutrophils and opsonophagocytosis of Spn. The strains that do survive can then co-operate. *Stage 3 – Multispecies biofilm:* there is signalling between the bacterial cells by quorum sensing (QS) through AI-2/AI-3 such that the bacteria recognise the multispecies environment. There is development of the extracellular polymeric substance (EPS) matrix made up of components from both bacterial species (type IV pili, eDNA, LOS and protein), providing co-operative adhesion and stability of the biofilm structure. *Stage 4 – Biofilm maturation*: the EPS develops providing further protection to the bacterial cells from antibiotics and host phagocytic cells. There is also incorporation of host immune factors, such as NET structures into the EPS.

In very particular disease situations, it is apparent that the biofilm formation is a key virulence factor. For *S. pneumoniae* and the NTHi, they are clearly present together in middle ear tissues of recurrent OM (ROM) and chronic OM (COM) patients [6] and in sputum samples from COPD [26]. The mono-species biofilm formation of both bacterial species has been well described [27, 28], and although both are known to co-exist in planktonic and biofilm states, the understanding of the nature of the interplay between these pathogens and the effect of co-

infection on the disease is only just starting to emerge [23]. The persistence of these species within a biofilm provides a vast array of phenotypes that allow for both the bacterial adaptation to a host anatomical *niche* and for the persistence of these species within a *niche* for a prolonged period. The switch to a biofilm state is characterised by global changes to their surface structures, physiology (energy production), metabolic processes, and stress response (discussed in a later section of this chapter).

4. Antibiotic resistance within a biofilm

Within a biofilm, bacteria display added resistance to host defences and antibiotic therapies; biofilms are 1000x more resistant to antibiotics than the planktonic state. An unusual stress response by NTHi that employs nickel [Ni (II)] ion uptake seemingly as a signalling process that links the cell's stress response to the cell physiology and the composition of its surface structures has also been identified [29].

Fluorescence ***i****n* ***s****itu***h**ybridization (FISH) techniques have shown that both *S. pneumoniae* and *H. influenzae* are present in middle ear tissues excised from chronic OM patients [6]. The bacterial aetiological agents in OM are *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis* [6]. All these bacteria have also been located in tissue samples within a biofilm. While OM is commonly treated with antibiotics [30] or tympanostomy tube placement [31], COM or ROM forms of OM are often unresponsive to these treatments. The antibiotic treatment is complicated by the presence of multiple species of bacteria as well as the biofilm lifestyle of these bacteria. This has been shown in various studies [8, 32]. Firstly by the induction of a polymicrobial biofilm in the presence of each other and then specifically the antibiotic resistance provided to the oto-pathogenic bacteria within a polymicrobial biofilm [32]. This can be in a directed role (through signalling pathways, see later) or passively. In addition to this antibiotic resistance, there is some thought that the tympanostomy tube insertion has little impact on removing the bacteria or even on the biofilms formed; it is believed that it could promote biofilm formation and particularly in a polymicrobial situation [33].

The formation of bacterial biofilm during COM largely explains the difficulty in treating COM with antibiotics, as well as the resistance to tympanostomy tube placement, as both pathogens are able to re-establish the biofilm on the tympanostomy tube. However, while it has been established that both species are capable of forming a multi-species biofilm, the physical or molecular interactions between *H. influenzae* and *S. pneumoniae in vivo* or furthermore, when within the biofilm, have not been well defined. Research findings relating to the outcomes of this interaction are conflicting, as recently reviewed [23]. There are studies showing that the interaction between *H. influenzae* and *S. pneumoniae* is of a synergistic nature, whereby the formation of a multi-species biofilm would benefit both species, and protect them from host antimicrobials, shear forces, and antimicrobial agents. It was shown that a β-lactamase producing strain of *H. influenzae* could protect *S. pneumoniae* from β-lactam treatment [34]. This same study showed that the formation of a multi-species biofilm with a bla^- strain of *H. influenzae* also had a protective effect on both the biofilm resident *S.*

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pneumoniae and the biofilm resident *H. influenzae* bla$^-$cells, which alone were susceptible to beta-lactamase treatment. A synergistic interaction was also shown, where *H. influenzae* and *S. pneumoniae* reached higher cell densities in co-culture than in mono-culture, and were able to modulate each other's gene expression in the biofilm [35]. Associated with this has been the demonstration that *H. influenzae* inhibits autolysis and fratricide of *S. pneumoniae*, and thereby *H. influenzae* improves the biofilm formation by *S. pneumoniae*, although this effect was only observed at later stages of culturing, and suggested that bacteria in co-culture biofilms may have altered biofilm formation processes, as previous study showed that in mono-cultures of *S. pneumoniae* autolysis promoted biofilm formation [36].

As is obvious from this array of findings, the nature of the interactions between these species remains unclear. It is likely, that these interactions are dependent on a multitude of specific host, genomic, and environmental factors, and that the discrepancy observed between studies is a result of the variation of one or more of these parameters. In addition, most studies have investigated the role of *H. influenzae*/*S. pneumoniae* interactions from the perspective of biofilm formation. Our recent study analysed global gene expression patterns in the *H. influenzae*/*S. pneumoniae* co-culture situation. This revealed the potential for either synergistic or antagonistic interactions between *H. influenzae* and *S. pneumoniae*, which is largely dependent on the growth dynamics and environmental conditions. We have shown that both species undergo vast changes in their transcriptional profile in response to the growth environment, and further influenced by the presence of the other species, and we thereby proposed that these environmental parameters and transcriptional patterns determine the synergistic or antagonistic nature of the *H. influenzae*/*S. pneumoniae* interactions [24]. Indeed under conditions of neutral or lower pH, the presence of different strains of *S. pneumoniae* induces *H. influenzae* into a **V**iable **B**ut **N**on-**C**ulturable (VBNC) state [24]. For other bacterial species, this VBNC state was differentiated from the dead state by several observations; firstly, VBNC cells have an intact membrane, in contrast to dead cells, they are metabolically active and continue respiration, VBNC cells continue gene transcription and mRNA production, and were shown to have continued uptake and incorporation of amino acids into proteins [37, 38]. Given these characteristics, the induction of *H. influenzae* cells under specific conditions into the VBNC state by *S. pneumoniae* does not preclude it from a multi-species biofilm. This switch in cell type during co-culture highlights the complex nature of the impact of the bacteria being together than being in mono-culture.

5. Signalling and sensing mechanisms associated with biofilm formation

Most acute respiratory infections are often dominated by one organism, however, chronic bacterial infections mostly encompass mixed species microbial communities. In the natural environment, bacteria mostly coexist or compete with various microbial species, therefore, it is important to understand the impact of co-infections on persistent infections. The nasopharyngeal commensals such as *H. influenzae*, *S. pneumoniae*, *S. aureus*, and *M. catarrhalis* are linked with many respiratory tract infections, with several virulence factors of these microbes involved and recognised in biofilm formation. This highlights the need to understand the

complex interactions between these microbes and how they influence each other to form biofilms that contribute to persistent and chronic infections.

The matrix of microbial biofilm is usually composed of biopolymers that include polysaccharides, protein, and extracellular DNA (eDNA), referred to as the EPS. It is well established that bacteria use a signalling network for cell-to-cell communication, known as QS, to carry out co-ordinated activities including migration to a suitable environment, nutrient acquisition, and biofilm formation with the release of various signal molecules or autoinducers (AI) [39]. Such mechanisms have been identified in both *S. pneumoniae* and NTHi. Although different systems are used by different bacteria, the principles of QS such as: AI signal molecules are often undetected when bacteria are in low density, but commonly detected at high density; availability of receptors for AI are usually cytoplasmic or membrane bound; and their detection is critical for any co-ordinated gene expression and/or repression to be carried out by the bacteria [40, 41]. N-acyl homoserine lactones (AHL's) are the most studied class of AI signal molecule and are commonly involved in the QS by gram-negative bacteria. The enzymes involved in the synthesis of autoinducer *N*-3-(oxo-hexanoyl)-homoserine lactone (3OC6HSL) AHL's are; LuxI and LuxR-type synthases, and substrate S-adenosylmethionine. The AHL's then traverse across the bacterial membranes through efflux pumps to bind to their respective regulators and initiate their activity. LuxR, a receptor for 3OC6HSL, and a well recognised transcriptional activator of the luciferase *luxICDABE* operon that activates its expression [42]. In contrast, in gram-positive bacteria, the modified oligopeptides or autoinducing peptides (AIPs) are mediated by specialized transporters that act as autoinducers in the QS systems. The AIP's bind to the bacterial membrane bound two-component histidine kinase receptors, which further activates the cytoplasmic regulator that transcribes the genes associated with QS [43]. A recent review on AI-2 mediated signalling in bacteria has compiled different functions that are regulated by AI-2 including biofilm formation, antibiotic susceptibility, virulence factor production, motility, in both gram-positive and gram-negative bacteria [44]. Some of the noted examples of AIPs based QS include; ComD/ComE in *S. pneumoniae*, AgrC/AgrA in *S. aureus*, and ComA/ComP in *Bacillus subtilis* [42]. The manipulation of the identified QS systems in both gram-positive and gram-negative bacteria must be addressed further to develop newer biotechnological therapies towards treating chronic and persistent infections.

6. Quorum sensing mechanisms and signalling in *H. influenzae* biofilm formation

NTHi biofilm formation is well recognised due to bacterial aggregation involving various bacterial components such as lipooligosaccharide, proteins, extracellular DNA (eDNA), and host material derived from inflammation [45]. QS for NTHi was first suggested because of the presence of *luxS* gene in *H. influenzae* Rd genome, with *luxS* gene known to be involved in the production of AI-2 [46]. The most studied, identified QS systems in NTHi are the LuxS/RbsB and QseB/QseC systems.

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6.1. LuxS/RbsB system

The role of *luxS* gene in NTHi biofilm formation has been extensively studied in both *in vivo* and *in vitro*, with the mutants lacking this gene forming biofilm, although with decreased biofilm thickness and biomass, that was further shown to be due to decreased phosphorylcholine incorporation into the LOS structure of the NTHi [47–49]. A certain *in vivo* study recently demonstrated the involvement of RbsB protein, a known periplasmic binding protein in mediating the uptake of AI-2 signals in NTHi [50]. Similar to the *luxS* mutants, the *rbsB* mutants also produced biofilms with reduced thickness and biomass, which were reflective of the decreased phosphorylcholine levels in the LOS of NTHi. These observations strongly indicate that QS clearly contributes to the establishment of a chronic infection.

6.2. QseB/QseC system

This two-component signalling system in NTHi was first described in enterohemorrhagic *Escherichia coli* and shown to regulate expression of virulence genes in a QS system independent of AI-2 [51]. A certain study involving the NTHi mutants lacking the *qseC* gene showed decreased biofilm production and was AI-2 independent, indicating that there could be other alternative signalling molecules affecting NTHi biofilm formation [52]. Although much progress has been done in understanding and identifying the QS system involved, not much is known about the nature of the QS signal molecules secreted by NTHi, or how does AI-2 affect the gene expression that could further alter the bacterial phenotype to produce biofilm is yet to be determined.

6.3. Role of extracellular DNA in NTHi biofilms

eDNA has been implicated as a major structural component of NTHi biofilms facilitating survival and replication of NTHi within a biofilm [53]. The association of NTHi pili and eDNA in biofilms, and its involvement in increasing bacterial adherence and biofilm formation is also well recognised [53, 54]. Recently, the protein responsible for providing the stabilisation of eDNA within the NTHi biofilm was identified as DNABII that binds to the eDNA and offers stabilization to the biofilm structure [55]. In addition to the bacterial eDNA, host eDNA also facilitates NTHi biofilm formation. The human neutrophils through making the NETs entrap the pathogens with the help of their genomic DNA [56]. The presence of these NETs had been demonstrated in various studies [53, 55, 57] but their role in pathogenesis is still unclear. A recent review has described the diverse mechanisms by which both gram-positive and gram-negative bacteria release eDNA, how eDNA and extracellular polymer matrix of a biofilm interact with each other, and the chemical behavior of eDNA and these interactions are responsible for the integrity and structure of biofilm development [58]. eDNA is often supplied by both host and a pathogen, and is linked to bacterial biofilms, QS, structural maintenance of biofilm, and offers a protective environment to pathogens residing inside, and further contributes to chronic and persistent infections. This prompts the need for developing therapeutics to target disruption of the extracellular matrix. A recent study has provided with a promising result to show an effective way involving human β-defensin to remove the eDNA

from the extra cellular polymer matrix, alter the NTHi biofilm formation, and effectively kill the NTHi residing within the biofilm [59].

6.4. Quorum sensing mechanisms and signalling in *S. pneumoniae* biofilm formation

In *S. pneumoniae* and in most of the gram-positive bacteria, QS often involves recognition of secreted peptides through the two-component regulatory systems. Over the last 40 years, the main QS systems in *S. pneumoniae* that have been identified and deciphered in detail are: LuxS/Autoinducer 2 (AI-2), the ComABCDE, and the BlpABCSRH systems [60]. Recently, there has been a growing interest in deciphering QS signalling or bacterial cross-talk between different strains of the same species. It has been suggested that bacteria belonging to the same pherotype are able to recognise peptides secreted by the same group but not the ones secreted by the other members. These pherotypes were previously identified for different QS systems including Agr in *S. aureus*, ComCDE in *S. pneumoniae*, ComQXPA in *B. subtilis*, and PapR/PlcR in *B. cereus* [61–63]. A recent study identified a QS mechanism in *Streptococci* genus that belongs to the Rgg family and involves a short hydrophobic peptide (SHP) that acts as a pheromone [64]. The functionality of the SHP/Rgg cell-cell communication mechanism in three different *Streptococci* species was demonstrated and cross-talk between strains was observed. More recently, an *in vitro* study demonstrated the involvement of a secreted peptide pheromone, competence-stimulating peptide (CSP) in influencing and development of *S. pneumoniae* biofilm [65].

In the recent years, an alternative group of QS peptides have been identified which are secreted by bacteria upon interaction with an oligotransporter and a cytoplasmic receptor protein, and initiate the process of QS [66, 67]. One of such peptides is the Phr signalling peptides of the *Bacillus species* that regulate different functions such as; sporulation, genetic competence, virulence gene expression, biofilm formation, and transfer of genetic elements [68]. The role of pneumococcal oligopeptide permease (Opp) (homologous to the *phr* peptides in the *Bacillus species*) in colonisation and virulence is well known [69, 70]. A recent study has identified TprA/PhrA signalling system to mediate QS in various strains of the pneumococci and its involvement in regulating the QS system in media containing galactose, which is one of the main energy sources required by the pneumococci during nasopharyngeal colonisation [68]. As biofilms are associated with colonisation, further studies are warranted to investigate the involvement of TprA/PhrA signalling system in biofilm formation, if any.

6.5. ComABCDE pathway

ComABCDE pathway is one of the most studied QS system regulated by the CSP, encoded by the *comC* gene and exported by the ATP-dependent ComAB transporter. In this system, the membrane-bound histidine kinase receptor, ComD, recognises the CSP which further leads to autophosphorylation of the histidine kinase, involving a transfer of a phosphate group from ComD to ComE [60]. Although the biological role of the Com QS in colonisation, bacterial carriage, and disease by *S. pneumoniae* is not yet fully known, there are certain studies that has demonstrated genetic transference to be more efficient in competent *Streptococci* biofilm cells

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in comparison with planktonic cells [71], and induction of competence a well recognised link between the switch from planktonic to biofilm form [72]. A certain study demonstrated competent cells releasing pneumolysin from the neighbouring non-competent cells by a cell-lysis mechanism, suggesting an indirect relationship between competence and virulence [73]. Further studies are warranted to understand how this mechanism relates to pneumococcal infections. Various pathogenic bacteria including *Ps. aeruginosa, H. influenzae, S. pnuemoniae* form biofilms on different substrates including tissues and human epithelia. Moreover, *S. pneumoniae* and *H. influenzae* upon interaction with human airway epithelial cells have been shown to produce more biofilm in comparison with no contact of epithelial cells [74, 75]. However, the production, regulation, and the mechanism by which enhanced pneumococcal biofilms are formed upon host-microbial interactions are not fully elucidated. Recently, an *in vitro* study has described a mechanism involved in the regulation of biofilm autolysis, and studies involving mutant strains lacking the *comC* and *luxS* showed that early pneumococcal biofilm on human cells are regulated by both Com and LuxS/AI-2 QS system [76]. In other *Streptococcus species*, such *S. gordonii* and *S. mutans*, there are reports about the involvement of ComCDE QS system in regulating both competence development and biofilm formation [77, 78].

6.6. BlpABCSRH pathway

This pathway is also one of the well characterised QS system in *S. pneumoniae*. The pathway consists of a secretion apparatus (BlpAB), a two-component regulatory system (BlpSRH), and an ABC transporter (BlpA) [79]. Being similar to the Com pathway, it is suggested that both pathways could converge at a common site where the response regulators of both pathways bind to the same motif, and activate the transcription of the same target gene [79]. In this QS system, a peptide pheromone encoded by *blpC* gene regulates the production of class II bacteriocins and their immunity proteins [80]. As bacteriocins are known to inhibit growth of competing bacteria, leading to intense microbial competition, it could be important to further elucidate how these complex regulatory networks operate during the course of an infection.

6.7. LuxS/AI-2

Autoinducer-2 (AI-2) is one of the most common QS signal in both gram-positive and gram-negative bacteria synthesised by S-ribosyl homocysteine lyase (LuxS) [81]. LuxS converts S-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), which further cyclises to active AI-2 [82]. Although its involvement in the biofilm development and virulence in several bacterial species is widely recognised [83, 84], the regulation and mechanism of LuxS has not been clear until now. It is believed that LuxS-controlled QS system might be only a part of the regulatory network that controls competence and LytA-dependent autolysis. Recently, the role of LuxS in controlling *S. pneumoniae* biofilms was first demonstrated using *luxS* mutants that failed to form early biofilms [85], and overexpression of *luxS* gene resulted in hyper-biofilm-forming phenotype [36]. Another study involving human respiratory cells showed that both the LuxS/AI-2 and Com QS systems as the main regula-

tors in the process of biofilm production [76]. Another report confirmed the down regulation of pneumolysin in mutants lacking the *luxS* gene but not in a *comC* knockout mutant, suggesting that pneumolysin is predominantly regulated by the LuxS/AI-2 system [86]. Future studies involving molecular interactions between Com and LuxS/AI-2 QS systems could provide new research directions in further elucidating gene expressions during early biofilm formation, or how mature biofilms are formed upon activation of either of these systems.

7. Quorum sensing mechanisms and signalling in mixed species biofilm formation

Although AI-2 signalling has been vastly studied under monospecies experimental set-ups, the polymicrobial nature of any microbial biofilm cannot be underestimated. There are certain co-culture studies that have demonstrated how AI-2 signalling by *E. coli* induced production of haemagglutinin protease, which facilitates detachment of *Vibrio cholera* from the intestinal mucosa [87]. This indicates how the presence of *E. coli* influences the dynamics and spread of cholera disease. Similar approaches have also been adopted to investigate interspecies signalling amongst nasopharyngeal microflora such as *M. catarrhalis* and *H. influenzae*, and demonstrated that biofilm formation by *M. catarrhalis* is promoted by the AI-2 signalling by *H. influenzae* [88]. Both *H. influenzae* and *M. catarrhalis* are well recognised causative pathogens of many respiratory infections including OM, and chronic OM is often associated with multi-species biofilm formation and antibiotic resistance [89]. Although *M. catarrhalis* lacks LuxS/AI-2 QS system, there are certain studies that have demonstrated increased biofilm production, antibiotic resistance by *M. catarrhalis* in the presence of *H. influenzae* [88]. Our own studies have found that nasal co-colonisation of *M. catarrhalis*, *H. influenzae,* and *S. pneumoniae* resulted in increased colonisation load and incidence of OM in mice [90], increased bacterial adherence to epithelial cells *in vitro*. The complex microbe-host interactions during biofilm production in our study suggested the importance of understanding why certain strains and serotypes differentially influence biofilm formation, in which the epithelial cell contact was a key contributor to increased biofilm formation [75]. **Figure 2** shows the possible mechanisms that could be involved in a multi-species biofilm. It shows how mixed bacterial species could behave differently and produce more biofilm upon interaction with host epithelial cell contact in comparison with no cell contact on abiotic surfaces. A recent review has collated the extensive work on *H. influenzae* and *S. pneumoniae* multi-species biofilm including; co-existence within the biofilm to reflect their persistence, changes in gene expression and physiology, how they adapt to environmental conditions, and molecular factors involved in bacterial cross-talk [23]. Another study showed how co-existence of *M. catarrhalis* and *S. pneumoniae* within a biofilm confers antibiotic resistance and bacterial persistence, and facilitates increased *M. catarrhalis* biofilm production which is not dependent on AI-2 signalling [32]. These observations highlight the importance of interspecies AI-2 signalling and the resilient nature of multi-species biofilm, and how do they impact on bacterial persistence and virulence.

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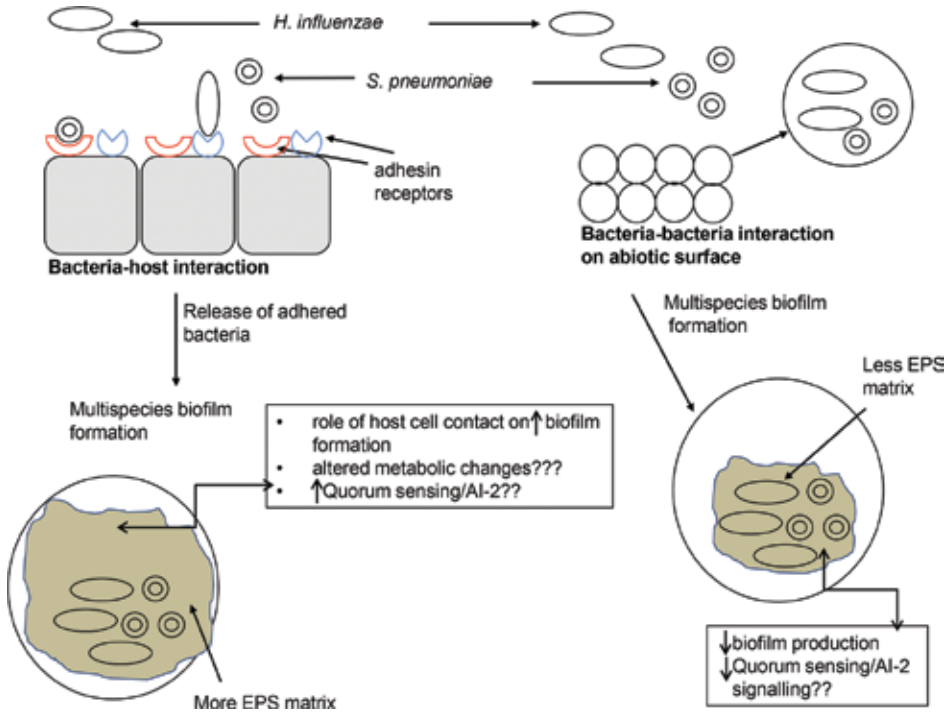

Figure 2. Possible mechanisms involved in multispecies biofilm formation. Mixed bacterial species behave differently and produce more biofilm upon interaction with host epithelial cell contact in comparison with no cell contact on abiotic surfaces. The figure shows increased biofilm production [more extracellular polymeric substance (EPS) matrix] by *H. influenzae-S. pneumoniae* in a multispecies environment upon contact with human epithelial cells (left panel). The right panel shows a decreased production of biofilm when grown on an abiotic surface (tissue-culture polystyrene plate). The potential role of host cell contact, possibility of any altered metabolic changes, or increased quorum sensing (QS)/autoinducer-2 (AI-2) signalling, upon host-cell interactions could hold key to further elucidate the mechanisms involved in increasing multispecies biofilm formation.

8. Future directions in QS signalling research

Despite the progress made in QS system and signalling pathways, there are several challenges ahead to better understand how these networks function. The challenges include; deciphering the messages obtained from the chemical properties, different sensing mechanisms and integration with other QS pathways, environmental factors, and cellular metabolism. A modern approach that involves developing a chemical probe to identify novel AI-2 receptors [91] along with the availability of genetic screening and bioinformatics could be a promising tool to further elucidate the role of different signalling systems in individual organisms. Therapeutic approaches to combat horizontal gene transfer by bacteria, multidrug resistance, or to target induction of bacterial community behaviours could be helpful in answering control of bacterial communities within multi-species biofilm that presents a major problem in chronic disease including cystic fibrosis or OM. Another approach could be the use

of quorum quenching or cause interference in AI-2 based signalling by developing antagonistic analogue molecules [92]. Recently, nanotechnology has provided some promising results with the manipulation of the AI-2 signalling on certain subpopulation of targeted bacteria [93].

9. The host-pathogen environment and role of metal ions in bacterial cells

The exact nature of the properties of an environment will influence if a resident bacterial species can grow freely in an active, planktonic lifestyle or whether the environmental properties represent non-optimal or stressed conditions and therefore act as a trigger for the bacteria to switch to a biofilm lifestyle. These properties include the chemical and physical properties affecting growth; the pH, oxygen levels, nutrient levels, temperature, osmotic pressure, redox state, water availability, and the presence of toxic compounds such as reactive chemicals. In host-pathogen environment the presence, absence, or changes in the levels of these properties can become a stress for the bacteria. Many host cells either intrinsically or by induction as a response to the bacterial being present, generate toxic levels of reactive oxygen or nitrogen species (ROS and RNS respectively). The immune response from cells such as macrophages (and other cells) stimulates the production of the ROS superoxide and hydrogen peroxide as part of their anti-microbial processes. They are also known to generate nitric oxide (NO) and other RNS as a response to infection. It is appreciated that there are differences in this range of physical and chemical properties between anatomical *niches*. It has been well established in numerous bacterial species that the presence of hydrogen peroxide and NO stimulate biofilm formation [94, 95]. In the context of bacteria such as *S. pneumoniae* and NTHi, when acting as a commensal of the nasopharynx and when persisting in *niches* such as the lung and middle ear, and perhaps more relevant than the short-lived immune generated toxic chemicals (ROS and RNS), are the intrinsic features of the environment (the oxygen levels, the pH nutrients levels, and the availability of essential micro-nutrients such as the metal ions, iron, copper, manganese, and nickel).

It is not understood how measures of variation observed in the secretions taken from the middle ear and lungs of patients relate to conditions that favour biofilm formation and persistence of bacteria. The pH in serous (7.92), mucous (8.55), and serous/mucous (8.33) middle ear fluids varies slightly across a weak alkaline range [96]; sputum from COPD patients was lower in pH from those with more significant disease, and this was shown to be associated with increased cytokine levels [97]. The contribution to defined growth conditions for the co-existence of *S. pneumoniae* and NTHi have been important determinants for understanding the mechanisms and factors important at different growth phases in planktonic and biofilm states. Studies ultimately found that at a higher pH, NTHi survived in co-culture with *S. pneumoniae* and as part of the competitive microbial environment in batch cultures, *S. pneumoniae* drives a decrease in pH that continues below pH 5.75, at which point NTHi is unable to grow. Such *in vitro* observations do not appear to be a representative of the host environment, therefore studies using flow cell chambers have provided a more realistic model system, showing that even under low pH condition, the NTHi can survive [24].

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The environmental concentration of transition metal ions can have significant influence on the survival of the bacteria and its lifestyle. For many metals, even though essentially they can quickly become toxic. Obviously, there is therefore a necessary tight regulation of their homeostasis; under metal starvation, there is an up-regulation of uptake systems, but as the metal concentration exceeds the cellular requirement there is activation of efflux systems [98]. In addition to some metal ions, their toxicity is closely linked to other environmental factors (or stresses) that the cells also required to respond – for instance iron toxicity is associated with oxidative stress (through Fenton Chemistry) and therefore iron homeostasis is regulated in conjunction with oxidative stress responses. Nickel levels and nickel function (such as binding to proteins) is affected by pH. Copper also is linked to pH, as the concentration increases there is an increase in acidity, and for copper there is also a link to Fenton chemistry and oxidative stress. Other metals seem to have an anti-oxidant role for the cell (such as zinc and manganese). The homeostasis of a metal is therefore affected by cellular requirement as well as other environmental factors that are affecting the metal toxicity or function. The influence of metal ions on bacteria within the environment is complex. It is further the subject of the metal bioavailability and cellular requirements. It is known that the correct access to metals influences cell lifestyle – this can be direct (where their role is either as a co-factor for a biomolecule or through directly within transcriptional pathways) or indirectly (acting as signalling system for stresses). As suggested previously, the response of cellular networks to the environmental level of a metal ion, the metabolic and physiological (energy generation) cell systems, and cell surface structures, will vary under different growth and environmental conditions.

Metal ion uptake is known to be important for bacterial survival within the host. Iron uptake systems are proven virulence factors for many pathogens [99, 100] and the control of zinc, copper, and manganese levels within the host environment has been shown to be important for bacterial survival and virulence. There have been different studies associating the *niche*-specific metal ion concentration and subsequent expression and role of particular surface structures [101]. A key example is the struggle between host and bacteria for iron. Iron is central for many pathways required in growth and survival, indeed for humans as well as for the bacteria, but iron chemistry also links it tightly to oxidative stress. Alternatively, an inability to acquire iron simply in itself can become a stress for the bacteria and induce the bacteria into a biofilm lifestyle. Iron acquisition and homeostasis is considered a virulence factor for many pathogens. In addition to the stress, some iron acquisition pathways have surface structures and of these iron-acquiring systems they have a dual role in adhesion and initiation of biofilm formation. In the context of multi-species biofilms, there is very little known of the role of transition metals. For Pneumococcus alone, iron sensing and iron transport is complicated, overlapping with other transition metal systems. The regulation is predominantly through RitR and transport is via PiaABCD, PitADBC, and PiuCDA [101]. Disruption of these pathways is shown to affect nasopharyngeal carriage and adhesion (indeed using OM model assays; these different reports have been well reviewed) [101]. Iron has been suggested through different studies to act as a signal for numerous processes in Pneumococcus, although the fine balance required for iron levels seems to be highlighted by its positive and negative effects on biofilm formation. Certainly, iron-limited conditions altered the protein expression of a

number of surface structures (such as PsaA) and therefore affected biofilm formation [102]. Other work linked iron levels to LuxS regulatory controlled processes, increasing levels of iron actually enhanced biofilm formation and other processes [36]. For NTHi, there is also a correlation to iron acquisition pathways, iron regulatory pathways, and biofilm formation. Iron uptake is up-regulated as NTHi migrates to the middle ear, a *niche* that it is known to exist within a biofilm. The central iron-responsive transcription factor (ferric uptake regulator, Fur) is required for long term survival of NTHi *in vivo* [103], and by regulating many genes it controls biofilm initiation and maturation. More directly, culturing NTHi sequentially through iron-replete and iron-depleted conditions revealed iron restriction induces biofilm formation [104]. These studies also used an experimental OM model and observed survival in the middle ear and the biofilms formed in the middle ear of bacterial cells taken from iron-rich and iron depleted cultures. The iron-depleted cultures survived longer and interestingly showed a changed architecture in their biofilm [104]. While this work revealed significant outcomes and raised intriguing questions with regards to the cell biology, it did clearly show that iron levels play an essential role in NTHi biofilm formation in the middle ear.

Other transition metal ions variously have vital role for bacterial survival. Some of these roles are as co-factors for important enzymes and then simply for growth, while other functions includes in stress response. In the case of *S. pneumoniae* these functions for transition metals have been intensively studied and this has been well reviewed [101]. In pneumococcal pathogenesis, transition metal ions such as iron, zinc, copper, and manganese are critical for its survival within a host, although in differing degrees. Although the exact function is not always clear and could be direct or indirect such as; the signalling through global transcriptional pathways or in competition with other metals for specific binding sites in biomolecules [101]. There are a series of surface proteins in *S. pneumoniae* that function in metal ion uptake (or even in efflux) but are concurrently essential in adhesion and at least the first stages of biofilm formation on epithelial cells. These structures include the choline binding protein PcpA, the serine protease PrtA, and the manganese uptake system PsaBCA. PsaR regulates all these and this is at least in response to environmental and cellular manganese levels. While the transport proteins for copper seem to be up-regulated during infection, their role in virulence is not known [105]. Although the copper export proteins (CopA certainly) do seem to be involved at various stages during pathogenesis and certainly are linked to pneumococcal survival in the nasopharynx and lung [106]. Likewise, with other transition metals, there seems to be a central role in survival as perhaps shown in infection and systems animal model studies. These however have not always been directly associated with an exact process within pathogenesis or directly in biofilm formation [101].

The local concentration of zinc has a significant role in the pathogenesis of *S. pneumoniae* and this includes in its biofilm formation within the host [107]. There are a number of zinc uptake and efflux systems that have been studied in pneumococcus (AdcABC and AdcAII regulated by AdcR, Pht proteins and CzcD). Metal limiting conditions can result in a growth limitation. Maintaining cellular zinc (and manganese) levels is important to controlling the redox balance and defending against oxidative stress. AdcAII has been shown itself to have a direct role in pathogenesis; while mutants lacking either *adcA* or *adcAII* increased invasive

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infection of human lung epithelial cells, it was *adcAII* alone that was required for attachment and colonization on the nasopharynx, presumably through biofilm formation [108]. The Pht proteins bind zinc and facilitate zinc uptake, have been shown to be essential in attachment of *S. pneumoniae* to respiratory epithelial cells [109]. The *pht* genes along with *adc* operon are regulated by AdcR in response to zinc but the PsaR regulator is now also known to be zinc-responsive (in addition to manganese). Further to the complex nature of the transcriptional response to environmental metal ion levels, both AdcR and PsaR have been shown to additionally respond to cellular concentrations of nickel. In PsaR, the nickel competes against manganese's binding and has an opposite effect to manganese for PsaR function on its regulon [110]. AdcR also independently responds to nickel – exogenous nickel levels having a direct role in regulating the Pht proteins and affecting the AdcR control of the *adc* operon [111]. Nickel has clearly been shown for NTHi to directly have a role in cell's lifestyle. The maintenance of intracellular nickel has a role in the nature of the cell surface, the surface charge and hydrophobicity, and the outer membrane protein and LOS composition, and this is independent of nickel binding proteins [112]. Further to this, it was shown that this nickel-induced effect on the bacterial cell also translated to a loss in type IV pili-mediated twitching motility in NTHi [29]. The importance of nickel uptake for the growth of NTHi is well known, and when limited, the bacteria makes the switch to a biofilm state [112]. This was correlated to a control of intracellular pH levels. However, it was shown not simply to be pH stress that was influencing NTHi survival or biofilm formation when in co-culture with *S. pneumoniae*, but growth dynamics [24].

The exact nature of the environmental transition metal composition therefore impacts on both NTHi and *S. pneumoniae* lifestyle and their ability to attach to host cells and initiate biofilm formation. There is little analysis of the consequential impact of metal ions in NTHi/*S. pneumoniae* co-culture and biofilm formation and much of our discussion has therefore focussed on mono-culture studies.

10. Bacterial metabolic pathways and mechanisms contributing to the biofilm production

Several studies have investigated gene and/or expression to identify the unique metabolic changes associated with transition form planktonic form to biofilm for *S. pneumoniae*. Yadav and co-workers [113] identified the exclusive up-regulation of genes involved in the mevalonate pathway, pyruvate metabolism, carbohydrate metabolism, galactose metabolic process, cell wall biosynthesis, translation, and purine and pyrimidine nucleotide metabolic pathways in biofilm formation, and suggested that these were also important to the growth and survival of bacteria in biofilms. In addition, changes to related genes suggested that the cells in biofilms may be under stress conditions that result in changes in the protein synthesis required to adapt to a new environment. Protein profiles have been compared between log-phase planktonic *S. pneumoniae* serotype 14 and 1-day and 7-day biofilm cultures using iTRAQ (isobaric tagging for relative and absolute quantification) [114]. This study by Allan and co-workers identified 244 proteins of which >80% were differentially expressed during

biofilm development. Their results indicated that metabolic regulation appears to play a central role in the adaptation from the planktonic to biofilm phenotype. Their study found that 47% of proteins were down-regulated during biofilm development (day 1) and 16% were up-regulated compared to the bacteria in log-phase. As the biofilm matured, approximately 24% of the proteins expressed during biofilm development returned to expression levels similar to the planktonic state and a further 16% were up-regulated. In general, up-regulation was observed with proteins associated with pyruvate, amino acid, and carbohydrate metabolism and there was a down-regulation in glycolysis and some other metabolic proteins. By day 7 of the biofilm, the most noticeable difference was the increase in some proteins that were associated with protein biosynthesis/alteration or degradation and cell division. Changes in metabolism potentially serve two purposes, firstly changing the bacterial phenotype so as to adapt to the different lifestyle and secondly, the need to utilise alternative metabolic pathways for survival.

The results of these studies suggest that *S. pneumoniae* uses a range of carbohydrates during biofilm formation. The biofilm also has a changing oxygen environment and increases in pyruvate metabolism, particularly lactate dehydrogenase that indicates an adaptation to this. The down-regulation of many virulence proteins generally associated with infection, persistence, and its ability to compete suggest a significant shift in its need to protect and respond to threats from the external environment. This is accompanied by the down-regulation in NADH oxidase that acts as an oxygen sensor and improving glucose catabolism. At the same time, processes important to carbohydrate selection and capsule production were increased, as was pyruvate oxidase, important to *S. pneumoniae* aggregate formation.

Comparison of NTHi biofilm to planktonic form in one study has shown that 127 proteins are significantly differentially expressed [115]. Of particular note was the major down-regulation in proteins involved in purine, pyrimidine, nucleoside, and nucleotide processes; protein synthesis; and energy metabolism. Up-regulation was detected for proteins involved in the cell envelope, DNA metabolism, transcription, and metabolism of phospholipids and fatty acids. Similar to the conclusions drawn from the metabolic changes to *S. pneumoniae*, NTHi appears to enter a state of decreased energy metabolism and protein biosynthesis at the same time adjusting its metabolism to the changes in the aerobic environment and energy derived from carbohydrate metabolism. Another study found that one of the triggers for biofilm formation was exposure to sub-inhibitory concentrations of beta-lactam antibiotics [116]. While very similar gene expression changes were found as reported by Post *et al.*, including an increase in biofilm biomass and decreased protein production, the concomitant up-regulation of the genes involved in glycogen production was proposed to be associated with an ability for the bacteria to be sustained as they become metabolically inactive. This aligns with recent work reported by Kidd where in mixed *S. pneumoniae* and NTHi biofilm, *S. pneumoniae* is able to convert NTHi to a non-culturable state [29].

It appears that similar changes in metabolic processes might occur as bacteria transition from the planktonic state, through early biofilm development to the mature biofilm (**Table 1**). As yet, very little is known about the metabolic changes that enable mixed biofilm formation, particularly associated with the shift in the processes associated with interspecies competi-

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tion and mechanisms of cooperation. Additionally, the role of the human mucosal surface and respiratory tract environment on metabolic changes have not yet been investigated.

Planktonic	**Biofilm development**	**Mature biofilm**
Up-regulation		
Stress response	Cell wall organisation	Some enzymes involved in biosynthesis/alteration or degradation and cell division
Virulence	Amino acid, pyruvate, pyrimidine processes	Transport
Bacteriocin prod/secretion	Glycolysis and some other metabolic proteins	Amino acid metabolism
Rapid metabolism glucose		
Specific carbohydrate metabolism		
Down-regulation		
	Translation	Many metabolic processes changed during biofilm development return to normal levels of expression
	Pyruvate processes	
	Some amino acid processes	
	Cell division	
	Monosaccharide metabolism	
	DNA replication	
	Purine metabolism	

Table 1. Summary of key metabolic processes altered during biofilm formation and maturation.

11. Conclusion

The biofilm is the dominant factor in persistence; being recalcitrant to antibiotic and host antimicrobial processes. Understanding the mechanisms that contribute to this persistence will help to design the next generation therapeutics. Many key questions are still unresolved. Identifying the genes involved in enabling bacterial co-existence, particularly in the transition to a biofilm state, may provide new targets for preventing the transition to a state of chronic, persistent colonisation. Understanding the specific cell-to-cell factors affecting the signalling/sensing mechanisms that could alter bacterial cell-surface and the host characteristics that play a role might enable us to identify individuals likely to be susceptible to chronic disease situations. Our knowledge is still limited about the differences in the general charac-

teristics, biofilm architecture, and signalling mechanisms associated with single and co-species biofilms.

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Chapter 16

The Role of Human Oral Microbiome in Dental Biofilm Formation

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Additional information is available at the end of the chapter

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Abstract

Each surface of the human body, which stays in contact with the external environment, is covered by a layer of microorganisms. This layer—the human microbiome—is characterized by a high diversity of species and huge number of cells. Its name was proposed by Joshua Lederberg at the turn of the twentieth and twenty-first centuries and was originally referred to as a group of microorganisms colonizing a certain habitat. Currently, the term also defines a set of genomes of all organisms inhabiting a particular niche. Since the human microbiota affects many aspects of human health, it has become the subject of different studies. The use of sequencing methods enabled to obtain genetic material derived directly from the human environment with simultaneous explanation of mutual relationships between microorganisms inhabiting different ecological niches of human organism (i.e., commensal, symbiotic, and pathogenic microorganisms). It is hard to determine the amount of microbiota inhabiting human oral cavity because microbiota represents distinct anatomically limited ecological niches; for example, microbiota of tongue surface, cheek, teeth, palate, gingiva, and periodontal pocket. Apart from anatomical structure, other factors determine different composition of particular oral cavity microbiota. These factors are various qualities of saliva—a natural protective barrier ensuring maintenance of healthy condition of the oral cavity—and habits of diet and hygiene. Generally, bacteria are passively transported by flowing saliva toward teeth surfaces. In turn, the pioneering microorganisms initiating changes in the environment of oral cavity through the production and secretion of products of their metabolism induce mutual microbiota–biofilm interactions. The formation of biofilm of the plaque is a complex and rapidly evolving process in which several stages of development can be distinguished arbitrarily: (i) reversible binding of bacteria to solid surfaces, (ii) production of exopolysaccharide matrix, (iii) irreversible binding to the surface, (iv) maturation of biofilm structure, (v) disintegration and dispersion of an organized structure, and (vi) the formation of new habitats. An oral microbiome

analysis depending on the genotypic characteristics of the host, as well as its metabolic phenotype, will allow us to understand all these factors which are responsible for maintaining host-microbiota homeostasis. The formation of genetic maps (including host, as well as microbiota) of such environments and the detection of biofactors indicating the predisposition for a given disease may contribute to the development of new diagnostic methods in reference to individual persons, and thus individualized therapy.

Keywords: microbiome, pathogenicity, biofilm, antimicrobial peptides, dental plague

1. Introduction

1.1. Formation of oral cavity microbiome

Each of the human body surfaces, which are in contact with the external environment, is covered with a layer of microorganisms. This layer, called the human microbiome, is characterized by high species diversity and cell number. The term proposed by Joshua Lederberg at the turn of the twentieth and twenty-first centuries originally defined a group of microorganisms living in a certain habitat. Currently, it also defines a set of genomes of all organisms inhabiting a particular niche.

The human microbiome became the subject of many studies since it affects many aspects of human health. The use of sequencing methods enabled the correct identification of bacteria on the basis of obtained genetic material sourced directly from the human environment. This allowed explaining the mutual relationships between microorganisms inhabiting different ecological niches of the human body (i.e., commensal, symbiotic, and pathogenic microorganisms). In addition, particular attention is paid to the ability of microflora to modulate the expression of host genes. This phenomenon is a part of the cross-talk process.[1]

The oral cavity is one of the most numerous in terms of bacterial species diversity microbiome of the human organism [1,2]. Microbiome of the human oral cavity consists of difficult-to-determine number of microbiota representing anatomically limited distinct ecological niches, e.g., microbiota of the surface of the tongue, cheek, teeth, palate, gums, gingival pocket, etc. Except for the anatomical structure, the factors determining the variable composition of particular microbiota of the oral cavity are as follows: variable quality of saliva, being a natural protective barrier ensuring the maintenance of proper condition of the oral cavity, and also habits of diet and hygiene.

Environment of the oral cavity is subject to constant transformation depending on the age, appearance of first teeth, their extractions, carious lesions, dentures, fillings, edentulous and transitional changes that may be induced by diet, variable flow of saliva, and prolonged use

[1] Multidirectional network of connections that enables signal transfer and communication of bacteria with bacteria, bacteria with host, and host with bacteria, thus creating a comprehensive interactive ecosystem determining a wide variety of biological processes, including health or disease, between the host and their indigenous bacterial flora, leading to the molecular dialogue with the host cells.

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of antibiotics [3,4]. Environmental conditions such as temperature, salinity, availability of oxygen, nutrients, variable conditions of pH, and redox potential may affect the ecosystem and contribute to changes in species composition of biofilms present in every place [5]. The formation of oral cavity microbiota begins at the moment of birth through the contact between the surface of the newborn's skin and mucous membrane of its mouth with mother's vaginal microbiota. In the case of birth by cesarean section, microflora is transferred from the mother's skin to the surface of the skin and mucous membranes of the newborn.

Immediately after the birth (<5 min), the species of bacteria covering most of the body surface of the newborn (oral cavity, nasopharynx, skin, and intestines) are very similar to each other [6]. Children born vaginally have the microbiome of the oral cavity similar to the mother's vaginal microbiome, with the predominance of the following bacterial species: *Lactobacillus, Prevotella, Corynebacterium, Staphylococcus, Streptococcus, Enterococcus, Bacteroides*, and *Sneathia* spp., while children born via cesarean section have a flora similar to the one present on the mother's skin, with the dominance of the following bacterial species: *Staphylococcus, Corynebacterium, Propionibacterium* spp. belonging to the phylum such as *Actinobacteria, Firmicutes, Proteobacteria*, and *Bacteroidetes* (**Figure 1**) [7].

At the time of birth and in the following hours, newborn's oral cavity is massively exposed to microorganisms coming from the external world (breathing, breastfeeding, and contact with parents and medical staff). The process of permanent colonization of the oral cavity begins in the postnatal period. The so-called pioneering microorganisms determining the composition of the oral microbiome are established within 24 hours of birth. The most common colonizers of the oral cavity at this stage are gram-positive cocci, including *Streptococcus* (inter alia *S. sanguinis, S. mitis* and *S. oralis, S. salivarius*) and *Staphylococcus* genus (**Figure 1**) [8,9].

Pioneering microorganisms initiate the changes in the environment through the production and secretion of their metabolism products, which often enhances the growth of other species. For example, *Streptococcus salivarius* present in the oral cavity of the newborns, having the ability to adhere to epithelial cells and to produce extracellular polymers from sucrose, promotes the growth of other bacterial species including *Actinomyces* spp., which may adhere to the so-formed structure [8]. The increase in diversity of the formed complex produces a more stable structure of the oral cavity microenvironment.

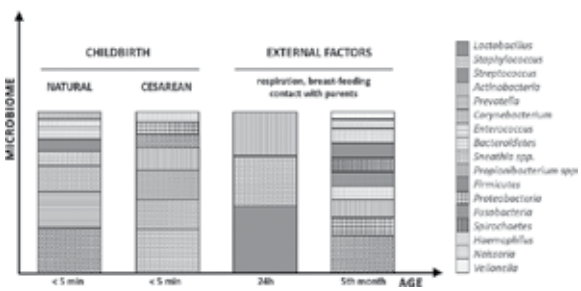

Figure 1. Human oral microbiome diversity during the age.

During the first two months of the child's life, bacteria colonize only the surfaces of mucous membranes, and further species of microorganisms, which determine the changes within oral microbiome, occur with the eruption of deciduous teeth [10]. As the child grows, the oral microbiome is subject to evolution. In about fifth month of life, the microflora of infant's oral cavity shows a clear resemblance to the oral microflora of the mother due to environmental conditions that occur in the first months of life, in particular, through feeding, contact with other adults and children, contact with pets, hygiene, eating habits, etc. [11]. The microflora of the newborn's oral cavity is then composed mainly of bacteria, including those of the following six phyla: *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria*, and *Spirochaetes*, among which the most widespread genera include *Streptococcus, Haemophilus, Neisseria*, and *Veillonella* (**Figure 1**) [11]. Interestingly, some of these microorganisms, such as *S. mitis* and *S. oralis*, produce proteases against immunoglobulin (IgA), which specifically degrade the secretion of salivary IgA. Because of this feature, the microorganisms are able to survive in an environment rich in IgA, which is excreted with mother's milk [12]. Although infants during that period have lower number of microorganisms in the oral cavity than their parents, their species composition is richer [11].

The formation of a new ecological niche in the oral cavity environment is observed during the eruption of teeth, with the occurrence of new adhesion surfaces. It was believed that the colonization of *Streptococcus* spp. cariogenic strains, such as *S. mutans*, starts just at this stage at the moment of the appearance of hard surfaces. Caufield et al. have defined this moment as a so-called "window of infectivity" [13]. However, recent research overthrew the above view showing the presence of this species in edentulous children, which suggests that the soft tissues can act as a reservoir for pathogenic microorganisms such as, e.g., *S. mutans* [6]. This fact highlights the importance of oral hygiene habits in a child, even before the eruption of teeth.

At the age of three, oral microbiome seems to be complete, but its maturation process continues until adulthood [14]. The bacterial flora of the oral cavity in the child varies during the development of teeth: deciduous, mixed or permanent. The oral cavity microflora in children with deciduous teeth in relation to the other groups demonstrates a higher incidence of bacteria belonging to the following families: *Pseudomonadaceae* (*Pseudomonas* genus) *Moraxellaceae* (genera *Acinetobacter Moraxella* and *Enhydrobacter*), *Enterobacteriaceae* and *Pasteurellaceae* (*Aggregatibacter* genus). During the replacement of deciduous teeth on the permanent ones, population of bacteria belonging to the family *Veillonellaceae* (genera *Veillonella* and *Selenomonas*) and *Prevotella* genus is subject to an increase, whereas bacteria of the family *Carnobacteriaceae* (*Granulicatella* genus) decrease [14].

The population of oral cavity microorganisms in children aged 3–12 years is composed of the species of bacteria inhabiting different ecological niches, e.g., surface of the cheeks, gums, tongue, etc. One cannot talk about the microflora of teeth focusing only on the selected anatomical areas associated with certain bacterial species. Also the number of pathogenic species occupying specific ecological niches is not synonymous with the development of caries [15–18].

This is proved by the observations [19] that showed there was no presence of *S. mutans* in 10% of the examined children and adolescents (aged from 2 to 21 years) with caries. Also the

contribution of other bacterial species-such as *Lactobacillus, Veillonella, Bifidobacterium, Propionibacterium*, acidic species independent of *Streptococcus mutans* (*S. gordonii, S. oralis, S. mitis*, and *S. anginosus* [18,20], *Actinomyces*, and *Atopobium*—in the development and progression of caries is suggested, which proves the influence of mixed bacterial flora on the development of dental caries. In the case of white spots the contribution of *S. mutans* is higher than in the control, but still quite low at a level up to 10% of the whole microflora [21]. *Streptococcus* (except *S. mutans*) and *Actinomyces* species are considered as the main reason of damage to the enamel. In the absence of *S. mutans* and *Lactobacillus*, initial enamel demineralization may be induced by the early colonizers, i.e., *S. sanguinis, S. mitis*, and *S. oralis* [18].

On the contrary, in case of the appearance of dentin lesion, the contribution of *S. mutans* represents about 30% of the total microflora, which indicates that this species is associated with advanced stage of caries. However, *S. mutans* was less common in caries progression, where *Lactobacillus, Bifidobacterium*, and *Prevotella* species dominated [6,18,22] . The studies evaluating the composition of microflora associated with early childhood caries show the share of bacteria of the following genera: *Streptococcus, Veillonella, Actinomyces, Propionibacterium, Granulicatella, Leptotrichia, Thiomonas, Bifidobacterium* and *Atopobium*, suggesting the lack of pathogenic species. The virulence of cariogenic bacterial population is correlated with the phenotype adopted for specified environment related to the acidic potential of bacteria that can induce the changes in the environment leading to the development of dental caries.

Under physiological conditions, oral cavity of children has a higher proportion of bacteria belonging to the following phylum: *Firmicutes* (genera *Streptococcus, Veillonella, Lactobacillus*, and *Granulicatella*) and *Actinobacteria* (genera *Rothia* and *Actinomyces*) and a smaller percentage of bacteria from phylum *Bacteroidetes* (genera *Bacteroidales* and *Prevotella*), *Fusobacteria* (*Fusobacterium* genus), *Spirochaetes*, and TM7 strain, compared to adults [14]. Interestingly, the share of cariogenic bacteria is also subject to an increase as the child grows. This change concerns the decrease in aerobic bacterial populations or facultative gram-positive cocci for the favor of relatively anaerobic gram-negative bacteria [17, 23].

Adolescence is a stage of major hormonal changes that accompany the enrichment of oral environment with a variety of nutrients, leading to the growth of certain groups of microorganisms, including gram-negative anaerobic bacteria and spirochete. This change in bacterial flora of oral cavity may be associated with an increased incidence and severity of gingivitis during the maturation [24].

The formation of a full oral microbiome takes several years, and in the case of certain surfaces, e.g., intestines, even longer. In the case of microbiota of oral mucosa, this process can be considered as complete after the eruption of permanent teeth.

Microorganisms colonizing the oral cavity live not only in the form of single cells (in the form of plankton), but are also capable of forming the clusters immersed in a mucilaginous extracellular matrix (ECM). In some diseases, the ability of bacteria to create a multidimensional, complex structure called "biofilm" plays a very important role. This structure adheres strongly to the surface so that the microbiome cells, particularly during the new biofilm formation, communicate with the cells of epithelium or mucous membranes by contact with

their receptors sending various signaling substances [25,26]. In this way, a network of relationships is created, which results in both control of functioning of mucosal epithelial and immune cells present in the epithelium under the influence of microbial cells of the microbiome, as well as the control of microorganism populations and their metabolism by the cells of the host's organism[2]. Only the presence and integrity of the microbiome are able to ensure the proper functioning of the cells of mucous membrane and skin surface, as well as protection against infections [2].

2. Definition of pathogenicity and determinants of microorganism's pathogenicity

An increasing number of opportunistic bacterial infections [27] have been reported in the past year. Microorganisms, which were previously considered as saprophytes incapable of causing human diseases, have now become an etiological factor in even serious conditions such as heart failure [28]. These microorganisms are referred to as "newly occurring pathogens" or "microorganisms on the newly acquired pathogenicity," which are generally called the emerging pathogens. These particular species develop pathogenicity due to evolutionary changes progressing slowly, as microorganisms adapt to new habitats, including the ecosystem, which is the human body.

Bacteria belong to *Prokaryota* and, thus, do not have complex genome repair and regulation systems; therefore they undergo changes at a much faster rate compared to the higher structured fungi (*Eukaryota*). In contrast to fungi, horizontal gene transfer does not occur in bacteria; this means that in practice the feature acquired by one strain can be transferred to other strains [29]. It is possible that an increased number of opportunistic infections recorded might be due to evolutionary adaptation of pathogenic strains to environmental changes [30,31].

Aggressiveness of current medicine creates an increasing number of the gate infections. The use of antibiotics and other antimicrobial agents with a wide spectrum of action interferes with human microbiota ontogenesis. Elimination of one species involves an increase in the number of surviving species populations. Consequently proliferation mainly concerns opportunistic microorganisms that are often resistant to the applied chemotherapeutic agents.

The presence of a high content of sugar in the diet, particularly in beverages, facilitates the formation of dental plaques by microorganisms inhabiting the oral cavity. Demineralization acquired due to excessive acidity or constant contact of enamel with dietary sugars favors the infections. Patients from the so-called window of infectivity (between 19 and 31 months of age), patients during the eruption of teeth, patients with hypercalcemia, patients with different types of malocclusions subject to treatment, patients with disturbed functioning of the immune

[2] Host; organism, the parasite lives at the expense of (including parasitic bacteria). Parasite relationship with the host may be permanent or temporary.

system, patients treated for systemic diseases and mentally handicapped, as well as patients with immunosuppression constitute the group to be at risk of opportunistic infections.

The features that determine the ability of a microorganism to cause disease, but which themselves are not required for their survival, are referred to as the determinants of pathogenicity [32]. Henderson et al. defined the determinants of pathogenicity as components of pathogens that are important in causing an infection in the host organism; they may include the factors vitally important for the microorganisms. A classic example may be the adhesins present on the bacterial cell surface that facilitate adhesion to receptors on the host cells surface, thus facilitating the colonization of bacterial species such as endotoxin lipopolysaccharides and gram-negative bacteria, which have a resistance towards the bactericidal activity of the complement and phagocytosis. On the other hand, the activity of heat-labile enterotoxin, for example, *Escherichia coli*, results in the activation of adenylate cyclase, increased intracellular cAMP concentration, and activation of protein kinase that phosphorylates cell proteins and is involved in ions transport. Increased cAMP concentration directly disturbs the function of the sodium potassium pump, which results in an excessive secretion of water and potassium ions from the cell and inhibition of the reabsorption of sodium ions and water. An excessive amount of water that is not absorbed is accumulated in the intestine, which increases intestinal passage and leads to the development of secretory diarrhea and tissues dehydration [33].

However, these definitions do not reflect the role of host's susceptibility to infection, indicating that only the features of the pathogen are causing disease. Consequently, only those organisms are microbe.

New definition of pathogenicity and pathogenicity determinants was created [34]. Pathogenicity of a specified microorganism is expressed as a range of damages that are caused by the microorganism itself and by the immune system as a response to the pathogen. They distinguished six classes of pathogens and are as follows.

Class I: This class of microorganisms damages only hosts with a weak immune response (with reduced host's immunity) and are commonly referred to as opportunistic or commensal; they do not cause damages to organisms with an undisturbed immune response; for example, damages due to *Pneumocystis carinii* are associated with the host response and those that are due to *Pseudallescheria boydii* are mainly associated with the growth of pathogens in host tissues (**Figure 2**).

Class II: This class of microorganisms causes damages both to people with impaired and normal immune responses. In the case of patients with weak immunity, damages are more severe and more frequent (**Figure 2**). Infections due to organisms such as *Candida albicans* and *Cryptococcus neoformans* are more often observed in individuals with weakened immune system and severe infections due to *Streptococcus pneumoniae* are usually associated with extreme age groups. This class also includes pathogens secreting toxins. For example, the toxins of *Corynebacterium diphtheriae* can cause damage immediately before the immune system responds, so the damage is regardless of the immune status of the patient.

Class III: This class of microorganisms causes damages to hosts both with impaired and normal immune responses. In both cases, the form of infection is similar (**Figure 2**), which distin-

guishes that class from class II. For example in patients with impaired immunity, *Histoplasma capsulatum* causes infections that are associated with high mortality rate due to the proliferation of the cells to different organs; in patients with strong immune response, mediastinum fibrosis results from chronic inflammation.

Class IV: This class of microorganisms causes damages, especially in extreme cases—impaired immunity or in cases of immune system overactivity (**Figure 2**). This is a relatively small group of pathogens that causes symptomatic infections only in people with impaired immunity or in those with prolonged immune response. For example, *Aspergillus fumigatus* causes aspergillosis in patients with neutropenia or bronchial aspergillosis in patients chronically exposed to antigen.

Class V: This class of pathogens causes damages in any condition of the immune system, but mostly acute in the case of host's immune system overactivity (**Figure 2**). For example, infections of *Salmonella* and *Campylobacter sp*. do not usually cause any permanent changes, but in the case of increased immune response, they may develop into Reiter's syndrome, manifested by intestinal inflammation.

Class VI: This class of microorganisms causes damages only in the conditions of strong immune response of the host. This is a theoretical class, not reported so far (**Figure 2**); it includes inter alia a group of diseases of unknown etiology, for example, Crohn's disease or Whipple's disease. *Helicobacter pylori* is the closest to meet this criterion. The infection caused by this species is asymptomatic in most patients; however, in some of them, it leads to the development of ulcers and, consequently, to carcinogenesis. Damages are associated with factors dependent both on the pathogen and on the host.

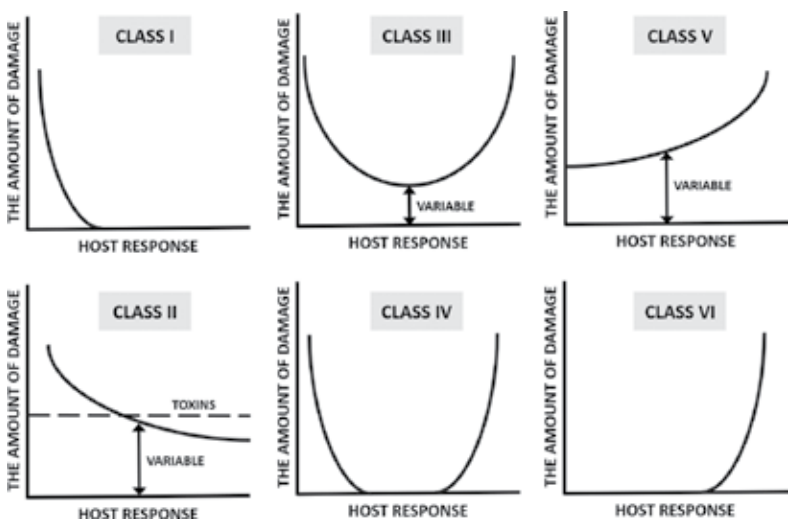

Figure 2. Pathogenicity presented as a function of the amount of damage and immune response of the host. Figure based on the study by Casadevall and Pirofski [34].

The state of host's immune defense is the main factor determining the course and cure of infection [34].

The determination of pathogenicity determinants for class I microorganisms based on the classification presented above poses some interpretation problem, since it seems that the key role in these infections is played by the condition of the host. Human is a complex ecosystem, in which bacteria–immune system homeostasis is observed in the physiological state. Bacteremia develops when this system is disturbed, usually through immunodeficiency and more rarely by the overexpression of bacteria pathogenicity determinants. However, most of the oral bacteria are rarely pathogens. So that even low-virulence pathogens have a minimum set of features that determine their pathogenicity and enables them to penetrate and proliferate in a host's body.

Writing about biofilm—pathogenicity determinant of *Streptococcus* genus—Kreikemeyer relates it with the discovery of long thread-like structures resembling pilus, which are located on the bacterial surface [35]. In *S. pyogenes* presented pilus are responsible for bacterial adherence and microcolonies creation on the surface of host cells, as well as aggregation itself, especially under the influence of saliva. These construction are involved in the interaction with pathogen and host; they are also causing bacteria clustering in the biofilm [36]. Pathogenicity of *S. mutans* is primarily associated with the ability to adhere to host cells and biofilm formation.

The bacteria in the biofilm resemble other organisms, for which the clustering is an evolutionary adaptation for survival. Bees and ants die if they are not in their "community;" similarly birds migrating for the winter organize themselves together to travel, and even most living mammals, starting from mice to *Homo sapiens*, are social organisms.

The complexity of relationships between microorganism and host, as well as differentiated expression of the features that determine the microorganism's pathogenicity, makes the pathogenicity an unpredictable phenomenon, since even a complete knowledge of hosts and microorganisms does not allow identifying all the possible interactions between them.

Due to certain specific characteristics of the host and microorganisms, all kinds of infectious diseases are often increasingly observed. Opportunities to travel around the world in a very short time not only contribute to the transfer of microorganisms in environments non-inhabited by them but also extend their pathogenicity [37].

3. Biofilm formation

The most common diseases of the oral cavity, in which an important causative role is played by biofilm formed by microorganisms on the surface of teeth and gums, are dental caries and periodontitis. One of the main etiological factors of these diseases is *Streptococcus mutans* [38–40].

3.1. Stages of biofilm formation

Biofilm formation is a multi-stage and very complicated process. There is a need for a number of relevant factors and conditions that must exist in the oral cavity, in order to assure the proper course of the whole process. However, five characteristic phases can be distinguished in it.

1. The initial phase of the microorganism adherence to a solid surface. *S. mutans* adheres to the tooth enamel and other materials such as tooth root or dental implant [41] using two mechanisms: sucrose dependent (based on the activity of glycosyltransferases and glucan-binding proteins) (**Figure 3**) and the sucrose independent (using interactions between adhesion particles of microorganisms and saliva agglutinins) (**Figure 3**) [42, 43].

2. Irreversible connection of bacteria with the surface, constituting the start of exopolysaccharide (EPS) matrix formation (EPS exopolysaccharide, which is the open architecture of nutritional channels, spaces, and other properties, including heterogeneity of the environment (pH and oxygen gradients, co-adhesion) that forms the protection from the host defense factors and desiccation (**Figure 3**) [44].

3. Biofilm maturation, when the matrix is still being developed and another bacterial species join the biofilm [45]. The bacteria synthesize extracellular polymers (soluble and insoluble glucans, fructans, and heteropolymers), which are constituents of the plaque matrix (**Figure 3**). The presence of matrix is a feature of all the biofilms; however, it is much more than the chemical scaffold retaining the biofilm shape. The matrix is biologically active, retaining water, nutrients, and enzymes inside the biofilm structure.

4. Bacterial succession associated with the shift of initial dominance of the species of *Streptococcus* genus in the direction of the predominance of *Actinomyces* and other gram-positive cocci. Newly occurring species of bacteria adhere to the previously attached pioneering species. The presence of one microorganism creates the ecological niches for other microorganisms, which facilitates their survival in the new favorable conditions. In 2003, this phenomenon, referred to as co-aggregation, was proposed by Rickard et al. [46]. An example of this phenomenon is the elimination of lactate by biofilm-forming streptococci, which becomes a source of carbon for the growth of *Candida albicans*. This, in turn, reduces the availability of oxygen to the level preferred by streptococci, thereby stimulating the growth of facultative anaerobic bacteria.

5. The formation of a mature biofilm is associated with growth-rate reduction of particular bacteria. The doubling of the bacteria amount occurs in 1–2 h in the initial stage of mature biofilm while in 1–3-day dental plate, it last 12–15 hours. A three-dimensional biofilm structure is formed. At this stage, the interactions between microorganisms (antagonism and synergism), i.e., their mutual influence on each other and microorganism–host interactions associated with the host's immune system plays the most important role not only in the formation of mature biofilm structure, but also in the disconnection of bacterial species from such formed structure, occupying subsequent ecological niches within the oral cavity microbiome [47–49]. Bacteria can "feel" the adverse changes in the environmental conditions and induce the genes related to active detachment. An example is

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Prevotella loescheii, which produces proteases hydrolyzing own adhesins related to fimbriae, responsible for co-aggregation with *S. mitis*.

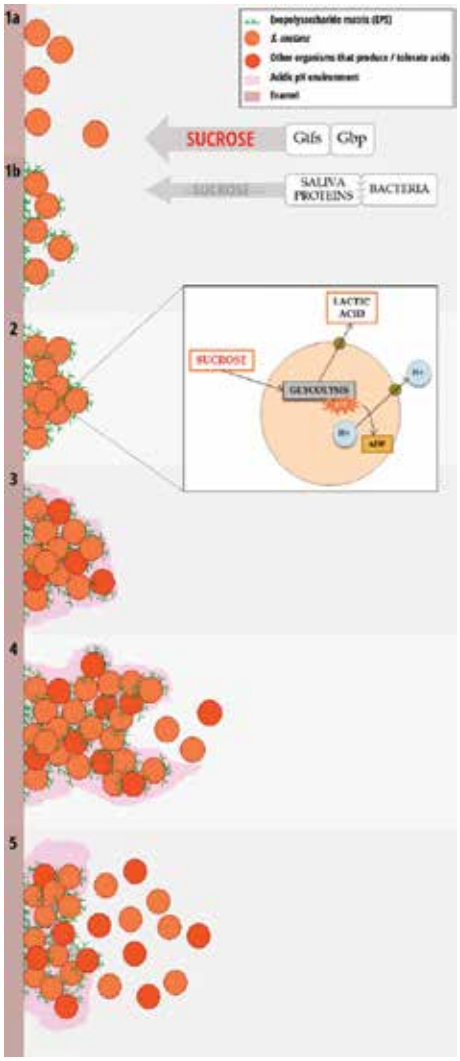

Figure 3. Stages of bacterial biofilm formation.

Mature biofilm structure is thus the result of the balance between the adhesion, growth, and removal of microorganisms. The development of plague as the biomass lasts up to the moment of reaching a critical size, i.e., when the shear forces limit further expansion; however, the structural development and reorganization may be continued. A classic example of a mature biofilm structure is dental calculus, which is mineralized dental plaque. It contains intracellular and extracellular deposits of minerals that grow around each bacterium and mineralized spaces coalesce and form a hard lodgment coated then with a layer of new bacteria. The

calculus is observed both supragingival and subgingival, where it acts as an additional retention site for the accumulation of plaque causing gingivitis (which is related to the presence of, inter alia, above-mentioned *Prevotella loescheii*).

It is believed that in stages 4 and 5 of the biofilm formation (**Figure 3**), it is impossible to inhibit further formation of its structure. This is due to the interactions occurring between particular species accumulated in its structure and signals from the external environment (derived from microorganisms of oral cavity microbiome). At this stage, one can talk about hyperadditive synergism, during which the combination of the effects of particular species forming biofilm activity is observed.

Keeping in mind the general scheme shown in **Figure 3**, important aspects for the biofilm formation such as biocompatibility during microorganism adhesion [50], nutritional conditions [51] as well as hydrodynamic conditions [52], the type of surface (smooth, rough, and their combinations) [53, 54], and many other unexplained and undiscovered factors should be remembered.

3.2. Factors affecting biofilm formation

3.2.1. Saliva and bacterial surface adhesins

The process of biofilm formation begins with the coating of tooth surface by acquired enamel pellicle (AEP) [44, 55]. The membrane is formed from the components of salivary origin (such as proline-rich proteins, amylase, lysozyme, histatin, peroxidase, mucin 2) and bacterial components (e.g., FtF GtF, lipoteichoic acid), specifically adsorbed on the surface of tooth enamel [55, 56]. AEP constitutes the basis for biofilm formation by microorganisms that colonize the oral cavity [56]. Single cells of *S. mutans* or their aggregates connect to the membranes by two independent mechanisms: sucrose dependent and sucrose independent [38, 39, 44].

The mechanism of adhesion (sucrose independent) involves an interaction between particles of *S. mutans'* adherence and AEP, which constitutes a diffusion barrier that protects the teeth against direct activity of organic acids [56]. Agglutinins are involved in the adhesion and aggregation by the interplay with adhesin P1 placed in bacterial cell wall [38–40, 57–59].

Salivary agglutinins play an equally important role in the process of biofilm formation as surface bacteria adhesins. The effect of salivary agglutinins on *S. mutans'* ability to form the biofilm was investigated *in vitro* [38, 40]. In addition to the strain of wild type (WT), the scientists also used strains of *S. mutans* with mutated *spaP* gene, encoding protein P1, which allowed examination of the differences and interactions observed during the biofilm formation [60, 61].

The salivary proteins of high significance in the biofilm formation also include salivary proteins binding inorganic ions, mainly calcium. About 33% calcium pool in the dental plaque is in free form, 17% is present in the bonds with phosphate ions and other organic anions. The remaining 50% is associated with specific proteins. The changes in protein profile may lead to calcium deficiency in dental plaque, and thus lead to the development of caries. Proline-rich

proteins, statherines, and histatins (present in AEP); cysteine-containing phosphoproteins (in dental plaque); and low molecular peptides in saliva may play an important role as calcium ion binding sites. Furthermore, proline-rich proteins and statherines inhibit the precipitation of calcium phosphates. Thus, calcium-binding proteins play a significant role in the remineralization of tooth enamel, constituting these ions' reservoir [62]. Reduced amount of salivary proteins binding inorganic ions may consequently lead to an advantage of demineralization processes over remineralization processes and the development of caries disease.

3.2.2. Presence of sucrose

The discovery that the pH of the dental plaque is subject to a decrease after sucrose consumption and then returns to its original value [63] initiated intensive study on oral cavity microorganisms. They were the subject of research not only due to their importance in the health care, but also for the recognition of the number of interspecies interactions and the desire to know the behavior of microorganisms occupying a common ecological niche [45].

Sucrose-dependent way of dental plaque formation are the glucosyltransferases (Gtfs B, C, D) produced by *S. mutans* in combination with glucan-binding proteins (Gbp) [39, 55, 64].

Gtfs are responsible for the formation of glucans from sucrose. The synthesized glucans provide the possibility of bacterial adhesion to the tooth enamel and microorganisms to each other. This process allows forming microcolonies, which favors biofilm formation [55, 64].

In conditions *in vivo*, Gtfs adsorb very fast with salivary film (sHA). The highest connection to sHA is demonstrated by GtfC, which is a hydrophilic compound. However, it has a hydrophobic domain, which is responsible for the affinity to plaque. It enables overlapping of the interactions with saliva proteins contained in the salivary film such as lysozyme or α-amylase. GtfB is glucosyltransferase, which in *S. mutans* is primarily responsible for interactions with other bacteria. It is responsible for the formation of highly differentiated microcolonies comprising the biofilm structure. Its activity is significantly increased in the presence of glucose [64].

The ability of GtfD to bind glucans is due to five of 65-five amino acids sequences repeated at the C-end of the chain. Glucosyltransferases binding other bacteria, even if they are not synthesizing their own glycosyltransferases [55, 65, 66]. Among the numerous polymers forming the plague (α-(1→6)-, α-(1→4)-, and α-(1→3)-glucans; β-(2→6)-fructans), a decisive role in etiopathology of tooth caries is played by α-(1→3)-glucan. This polymer constitutes 1.3–1.4% of plate dry matter and exhibits several unique features that allow it to form a skeleton (matrix), i.e., it easily adsorbs to saliva or pellicle-coated enamel, promotes mutual sticking of bacteria (aggregation), and greatly improves the consistency of plaque. α-(1→3)-glucans are insoluble in water and have a fiber structure, which means that they are not dissolved and washed out by fluids of oral cavity. Moreover, α-(1→3)-glucans are neither subject to the activity of enzymes nor to those present in the oral cavity or produced by inhabiting its microorganisms, which ensures the stability and durability of the plaque [67].

Another component of sucrose-dependent mechanism is Gbps mediating in bacteria binding with glucans. There are four known types of that protein: GbpA, -B, -C, and—D [39, 55, 64].

GbpC protein (and possibly GbpB) is associated with a cell wall of bacteria, and therefore is a specific receptor for glucans. All four types of the proteins play a role in microorganism adhesion and biofilm formation, but the protein GbpD seems to be the key one [55]. The studies using strains of *S. mutans* GS5 (*gbpB* gene deletion) and UACA2 (strain expressing an antisense RNA for gbpB gene) demonstrated, in turn, that the absence or mutation of GbpB encoding gene results in a change in cell shape and its slow growth [64]. This prevents normal development of the biofilm, which, instead of being a diverse, compact formation, becomes a creation formed of irregular cell clusters surrounded by a matrix of untypical structure.

Thus, sucrose plays a dual role in the formation of biofilm pathogenicity, provides a source for organic acid production by acidic species in dental plaque, and serves as a substrate for the production of extracellular polysaccharides (EPS) [48].

3.2.3. Mechanisms of low pH tolerance

The primary feature distinguishing cariogenic bacteria such as *Streptococcus mutans* and *Lactobacilli* is their ability to tolerate low pH of the environment, which results from the maintenance of intracellular pH homeostasis.

S. mutans species tolerance to low pH (acid tolerance response, ATR) is associated with a number of mechanisms regulating its cell physiology involving, inter alia, the changes in the synthesis and activity of cellular proteins and fatty acids [42, 68, 69]. One of the main mechanisms enabling *S. mutans* bacteria to survive at low pH is based on the increase in ATPase activity, mainly F_1F_0-ATPase, proton pump, which removes H^+ ions outside the cell, thus contributing to the maintenance of intracellular pH at a level of about 7.5 [69–71]. This mechanism involves the active removal of H^+ ions from the cell through mentioned ATPase. This process requires energy input, which is obtained from the conversion of L-malate to L-lactate (during malolactic fermentation, MLF). In addition to ATP, CO_2 is also produced during this process, which additionally reduces the cytoplasm acidity, affecting the regulation of intracellular pH [70,71].

In the case of a prolonged period of starvation, during which the adequate nutrients such as sugars are not provided to bacteria, ATPase begins to act as ATP synthase, providing the cell with energy required for survival, and which the microorganism is not able to obtain from the food [72]. The neutralization of acidic products of metabolism in the cells additionally requires CO_2, and ammonia resulting from the conversion of urea or arginine by urease or arginine deiminase system and agmatine deiminase system (AgDS) [70]. The result are products like ammonia and carbamoyl *putrescine*, which is subsequently converted to putrescine and CO_2. These reactions are further stage comprising the maintenance of intracellular homeostasis in the conditions of the external environment decreased pH.

Another process affecting low pH tolerance is the change in stress response proteins synthesis. The main products are present in the cell just after 30 minutes from the pH change to a lower value [73]. A large part of them demonstrate an increased glycolytic activity, which allows microorganisms to preserve the metabolic ability under new conditions [69, 70]. Another concept of *S. mutans* tolerance to low pH suggests a role of the cell membrane in that

process [70, 71, 74]. The expression of *fabM* gene responsible for the synthesis of monounsaturated fatty acids is subject to the changes in an environment of acidic pH, which increases their share in cell membrane structure. The changes in the membrane lipid profile results in a decrease in its permeability, which allows for more efficient maintenance of H^+ ions out of the bacterial cell [74].

An additional mechanism supporting ATR is the biofilm formation, which focuses in its structure the clusters of microorganisms of different species, additionally protecting them with EPS matrix. As demonstrated by Welin-Neilands and Svensäter [69], the cells of *S. mutans* in the planktonic phase are seventy thousand times more sensitive to low pH compared to the biofilm-forming cells of that microorganism. This feature is explained by an increased tolerance to the low pH of the cells adhering to the surface. Microorganisms grouped in this way, mutually affecting their metabolism, weaken the activity of produced glycolytic enzymes, which results in a decrease in the production of acidic substances that lower the pH of the environment [69]. A large role in the biofilm resistance to acidic conditions in the oral cavity is played by glucans as discussed below, which slow down the diffusion of hydrogen ions into the biofilm [75].

Other microorganisms resist the low pH of the environment by an increase in the gene expression associated with the production of bases, e.g., *S. salivarius* activates urease enzyme which degrades urea into ammonia, while *S. sanguinis* system of arginine *deiminases* causing arginine decomposition to ornithine, carbon dioxide, and ammonia at lower pH (4.0) than that enables the growth and may cause glycolysis (pH 5,2).

Streptococcus mutans can also use branched-chain amino acid (BCCA) biosynthesis as one of the ways to deal with cytoplasm acidification [42, 70]. In this mechanism, *S. mutans* redirects pyruvate formed in glycolysis for the pathway of biosynthesis of amino acids belonging to the group of BCAA. This reduces the amount of substrate for organic acid production, mainly lactic acid. The initial reaction in the biosynthesis of valine and leucine amino acids is *acetylacetonate* formation by condensation of two pyruvate molecules. α-keto acid is produced during further reactions, which finally is converted by aminotransferase to the BCAA. In addition, NH^{3+} ion is secreted during isoleucine biosynthesis, also belonging to BCAA, whose binding with H^+ causes the formation of ammonia and alkalization of cytoplasm [76]. Santiago et al. (2012) obtained an increase in expression *ilvE* gene encoding a BCAA aminotransferase (ILvE) in *S. mutans* in response to environment acidification. In addition, they demonstrated that the activity of membrane ATPase is subject to a decrease in microorganisms lacking genes for ILvE, which is one of the main mechanisms to maintain pH homeostasis in the bacterial cell [76, 77].

Another important mechanism protecting *Streptococcus mutans* against the harmful effect of low pH is the permeability of the cell membrane. During the adaptation to an acidic extracellular pH, *S. mutans* can change fatty acid composition in the cell membrane to prevent the passive penetration of H^+ ions into the cell. In turn, the amount of monounsaturated fatty acids and long-chain fatty acids is subject to an increase, which reduces membrane permeability [42, 68, 70].

The change in the activity of numerous cellular proteins is observed during the adaptation to stress conditions. The study conducted by Gong et al. demonstrated that during the adaptation to environment acidification (pH = 5.5) [68], *S. mutans* alters the expression of about 14% of their genes, most of which are genes whose expression is subject to an increase (e.g., encoding subunits of membrane proton pump; two-component regulatory systems: *comD, comE, ciaH, ciaR*, and others; transcription factors; protein transporters, e.g., P-type ATPase responsible for inorganic ion transport). A significant part of genes of an increased expression is the genes that protein products exhibit glycolytic activity, which protects microorganisms against a decline in metabolic ability under new conditions [70].

3.2.4. Carbohydrate metabolism

In oral cavity conditions, cariogenic bacteria *S. mutans* are characterized by altered metabolism compared to the cells of physiological flora. It is based on glycolysis, which also occurs in presence of oxygen (a phenomenon known as the Warburg effect). The low concentration of oxygen (<2%), i.e., hypoxia inside the biofilm, increases an expression of genes encoding glycolytic enzymes and inhibits the oxidative phosphorylation.

Carbohydrate metabolism is one of the key metabolic pathways that subject to changes during mixed biofilm development. It was demonstrated that the amount of synthesized glycolytic enzymes, including glucokinase, aldolase, phosphoglycerate mutase, and pyruvate kinase, is subject to an increase [77]. Therefore, *S. mutans* is more resistant compared to other streptococci to glycolytic enzyme inactivation in a response to the lowered pH of the environment. This may be due to an increased amount of ATP produced during glycolysis, and consequently, increased proton removal by H^+-ATPase [78, 79]. Glycolytic enzymes are sensitive to low pH, which results in a decrease in their activity when the pH inside the cell is lowered. The optimum pH for glycolysis process is neutral pH (pH = 7), an increase in the activity of glycolytic enzymes during ATR can therefore be explained by the need to overcome the inhibitory effect of environment acidification in relation to the metabolic activity [77]. Furthermore, when the bacterial cells adapt to the new conditions, the optimum pH for the glycolysis is reduced and the process rate is increased [42]. This means that *S. mutans* may continue its metabolism, even when the external pH falls below 4, which for most microorganisms do not have ATR mechanisms causing stop their metabolism [42].

The glycolytic activity increases in the newly formed biofilm (a few hours), which is caused by an increased activity of the processes needed to adapt to new environmental conditions, requiring energy inputs in the form of ATP. However, the glycolytic activity of enzymes decreases with dental plaque biofilm aging. The probable cause is the desire to avoid further acidification of the environment, when biofilm becomes thicker. Reduced rate of glycolysis process, combined with decreased rate of cell growth in the biofilm, can make the structure more stable and resistant to external factors [78].

Glycolysis provides not only ATP, but also intermediate products involved in anabolic processes [77]. An example would be an increased activity of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase. This enzyme bypasses the stage of first ATP molecule formation, in order to provide the reduced form of nicotinamide adenine dinucleotide

phosphate (NADPH), which is necessary in the reduction processes. This is a significant reaction since *S. mutans* has neither the enzymes of oxidative pentose pathway portion nor transhydrogenase, which would generate NADPH [77]. In addition, the expression of other enzymes, transketolase, increases in the conditions of environment acidification. This enzyme redirects the two intermediate products of glycolysis, glyceraldehyde 3-phosphate and fructose 6-phosphate, to the non-oxidative part of the pentose pathway, and consequently, for the pathway of nucleotide biosynthesis needed for DNA synthesis [77].

Of the three enzymes involved in the final conversion of 3-phosphoglycerate to pyruvate, two of them demonstrate higher expression in pH=5 than at a neutral pH. The first of them, phosphoglycerate mutase, exhibits more than twofold increase in expression, while the activity of pyruvate kinase increases nearly threefold [77]. Pyruvate kinase, the last of the enzymes involved in glycolysis, is considered as an enzyme conditioning the rate of the whole process since it is activated by glucose-6-phosphate, a substrate of glycolysis in *S. mutans*.

Len et al. demonstrated nearly 93% reduction in the activity of the enolase, an enzyme that converts 2-phosphoglycerate into phosphoenolpyruvate (PEP) [77]. PEP is a precursor of pyruvate, which is involved in carbohydrate transport inside the bacterial cell in phosphoenolpyruvate-sugar phosphotransferase system (PEP-PTS), where it plays a role of phosphate donor [80]. In the conditions of reduced pH, the amount of PEP produced is subject to a decrease as a consequence of reduced enolase activity. However, the amount of pyruvate produced by pyruvate kinase increases concurrently, which may indicate the conversion of 2-phosphoglycerate to pyruvate without phosphoenolpyruvate accumulation [77]. In addition, PEP is also an inhibitor of lactate dehydrogenase, an enzyme that converts pyruvate to lactic acid. Therefore, a decrease in PEP amount results in an increased production of lactic acid at low pH, which was confirmed in the studies [69, 77].

Increased expression of some glycolytic enzymes, e.g., glyceraldehyde 3-phosphate dehydrogenase, enolase, phosphoglycerate kinase, or aldolase, can be explained by their dual function, which is related to adhesion to the substrate [81]. These enzymes are found on the bacterial cell surface of many species of *Streptococcus* genus [69]. The study conducted by Ge et al. revealed the presence of enolase on the surface of *S. mutans*, and the ability of that enzyme to bind to salivary mucin MG2, as well as plasma plasminogen [81]. Thus, enolase acts not only as a glycolytic enzyme, but also as the surface adhesin facilitating cell adhesion to the pellicle acquired on the surface of the tooth. Double function of enolase may explain the results demonstrated almost a twofold increase in the expression of the genes for enolase during the initiation of bacterial biofilm formation. Due to the presence of surface enzymes on the bacterial cells, *S. mutans* is considered, next to the *streptococcus* of *viridans* group, as one of the etiological factors of bacterial endocarditis or bacteremia [82, 83]. Also rare cases of recurrent bacteremia in women with Sjogren's syndrome were noted [84]. In addition to *endocarditis* and bacteremia, *S. mutans* induced sepsis [85], and it was also the etiologic factor of other systemic diseases [86]. Also, a rare case of retroperitoneal abscess caused by *S. mutans* was described [87].

3.2.5. Nitrogen metabolism

Metabolism of nitrogen compounds is related to an activity of many endopeptidases and exopeptidases. *S. sanguinis, S. gordonii*, and some *lactobacilli* species release arginine from peptides decomposing it using arginine *deiminase* to ammonia, ornithine, and carbon dioxide with the release of energy. Urea present in saliva at a relatively high concentration (200 mg/L) is decomposed by certain bacteria having enzyme urease (e.g., *S. salivarius, A. naeslundi*) to carbon dioxide and ammonia. This example is proof of the existence of interspecies interactions associated with the ability of some bacterial species to the reduction of the environment acidity through the production of ammonia, creating more favorable conditions for the survival of bacteria sensitive to low pH. Such ability is demonstrated by microorganisms such as *Fusobacterium nucleatum* and *Prevotella intermedia*, which, through the production of ammonia during glutamate and aspartate fermentation, enable the growth of sensitive species such as, e.g., *Porphyromonas gingivalis* [88].

3.2.6. Oxygen metabolism

Expression of *S. mutans* pathogenicity determinants and biofilm formation in specific conditions of the oral cavity can be modulated, inter alia, by the environment and the presence of oxygen [39].

The culture of *S. mutans* strains under aerobic conditions produces 80% reduction in bacterial ability to biofilm formation [38]. The availability of oxygen is the causative agent of the changes in the bacterial cell surface composition, as well as modifications that occur in autolysin production and specific system of signal transduction VicRK. The production of autolysin AtlA is conditional by an expression of gene SMu0629 demonstrating oxidoreductase activity. An increased expression of this gene can be observed under aerobic conditions, and therefore the overproduction of autolysin AtlA, which inhibits biofilm formation. Transducer of kinase VicK constitutes a system regulating the expression and activity of autolysin AtlA. The study on the strains mutated for the gene SMu0629 and *vicK* was conducted in order to confirm the changes in autolysin affected by oxygen. Strains with the removal of the above-mentioned genes adapted better to the aerobic conditions and showed a greater ability to biofilm formation compared to the parent strain UA159 [89].

Multi-species biofilm formation is conditioned by physiological relationships favoring the connection of microorganisms with different environmental requirements or different type of metabolism. An example of such relationship is oxygen absorption by the early colonizers such as *Neisseria* genus, providing thus favorable conditions for the growth of the absolute anaerobic bacteria like *Porphyromonas gingivalis* [78]. Another example of interspecies cooperation in biofilms is an increase in expression of gene *amyB* for α-amylase in *Streptococcus gordonii* caused by the co-aggregation with *Veillonella atypica* species. Decomposing carbohydrates, such as α-amylase, contribute to the formation of acidic end products of *S. gordonii* metabolism, mainly lactic acid. The resulting acid then becomes a source of carbon for *V. atypica*, bacteria, which itself cannot ferment carbohydrates [90].

This example proves that the existence of the relationship between oral cavity bacterial microbiome, inter alia, absolute anaerobes, which occur at different locations of the oral cavity, and, as evidenced by the studies, can survive oxygen exposure due to the interactions with the bacterial biofilm species, metabolizing oxygen and having appropriate enzymes removing toxic products of free radical reactions.

3.2.7. Production of intracellular polysaccharides

Since bacteria found in the oral cavity are subject to continuous "feeding" and "hunger" cycles with respect to dietary sugar supply, the resident microflora develops, as a consequence, a mechanism for carbohydrate storage. They help avoid the lethal effects of excessive amounts of intermediate products of intracellular glycolysis and provide a source of carbon and energy during the "starvation" period. Intracellular polysaccharide (IPS) repositories are created and are a source of energy during starvation; they can promote tooth decay, by extending the time in which the teeth are exposed to organic acids [48, 91]. IPS are high molecular polymers containing α-1,4 and α-1,6-glycosidic bonds in their molecules, which are readily metabolized in the absence of other carbohydrate sources.

It was proved that the strains of *S. mutans*, which have an increased repository of these polysaccharides, are considered to be more cariogenic than other strains of the microorganisms colonizing the dental plaque. The studies demonstrated that organic acid production by *S. mutans* from intracellular substrates resulted in an increased and prolonged drop in environment pH, leading to the acceleration of demineralization processes [62].

3.2.8. Production of extracellular polysaccharides (EPS)

EPS promote biochemical and physiological changes in bacterial biofilm matrix. EPS allow adhesion and clustering of microorganisms accumulated in the biofilm structure, and they provide structural integrity and stability and increase the biofilm biomass, matrix acidity, as well as reduce the concentration of inorganic ion. With an access to sugars, especially sucrose, EPS are produced in a continuous manner, which, as a consequence, leads to the formation of bacterial microcolonies and then three-dimensional structures [48]. Polysaccharide EPS matrix provides the protection for bacterial microcolonies present in the biofilm against adverse external factors, including bactericides or host's defense mechanisms. It also provides the protection for all kinds of microorganisms contained in the biofilm, creating environmental niches in which they can communicate with each other, adapt to the conditions, and even change the surrounding microenvironment. The matrix of EPS biofilm may also affect the availability of nutrients, metabolites, or signaling substances [92]. Moreover, EPS increases the mass and porosity of biofilm matrix, allowing more nutrients to diffuse into the surface of the teeth. Therefore, the deeper layers of biofilm are exposed to acidic pH activity, increasing thus the risk of caries [62]. In addition, the polysaccharide matrix is a place of accumulation of organic acids produced from carbohydrates fermented by acid-forming bacteria, including streptococci from *mutans* group and bacteria of *Lactobacillus* genus [55]. It constitutes an impermeable barrier for the buffering activity of saliva, which causes the formation of acid niches, mainly inside the biofilm structure [48]. Due to a limited diffusion to and from biofilm,

it is possible to collect the nutrients or metal ions inside it that are necessary for microorganism survival and to reduce the penetration of external bactericidal substances, and also antibiotics [55]. The composition and structure of biofilm matrix may change over time, and this may be due to both effects of bacterial populations and microenvironment factors. An example can be the presence of extracellular DNA (eDNA), which enhances the adhesion of *S. mutans* to biofilm surface. The eDNA is a component of the (ECM) and is formed from chromosomal DNA during the processes such as autolysis or active secretion [93]. It is incorporated in the matrix, affecting the biofilm architecture and development. It was demonstrated in the studies that eDNA is involved in several stages of biofilm formation, including the initial bacterial adhesion, aggregation, and mechanical stabilization of biofilm structure. eDNA is also involved in genetic competence, the process in which genetic information can be transferred from one bacterial species to another related one. This is a kind of horizontal gene transfer, inter alia, encoding virulence factors or genes of resistance to bactericidal agents [94].

3.2.9. Proteases

Also other features of *S. mutans*, such as production of proteases, which can decompose the proteins derived from the host, are not without significance [95]. Some examples include collagenases, proteins degrading collagen in the dentin and teeth cement, which may in turn lead to caries of the root surface of the tooth [96], and proteases degrading the components involved in host's immune response, e.g., proteases IgA1, which degrade secretory immunoglobulins A1 (sIgA1) present in the saliva. Secretory antibodies IgA1 are the first line of host's defense against bacterial agents, preventing their adhesion and colonization on tooth surfaces. These antibodies block surface adhesins AGI/II [97–99]. In the conditions prevailing in the oral cavity, the expression of pathogenicity determinants, in particular species of bacteria, both those forming oral cavity microbiome and those contained in the biofilm, is affected by both the environment in which the biofilm grows and the influence of accompanying bacterial flora.

3.3. Mixed biofilm

The interactions observed between microorganisms of oral cavity microbiome is another major factor affecting the development of the biofilm [39, 100]. The interactions that occur between the microorganisms can result in both acceleration and inhibition of this process. This way, the pathogenicity of *S. mutans* depends not only on the environmental conditions of the oral cavity, but also on the composition of its bacterial flora.

In a response to rapidly changing environmental conditions of the biofilm, such as pH, the content of EPS, or the amount of available nutrient substrates, bacteria had to develop molecular adaptation pathways that would ensure their survival and optimal metabolism. The interactions that occur between the microorganisms can result in both acceleration and inhibition of this process. Microorganisms can change the expression of their genes and communicate with each other in response to the density of their distribution. "*Quorum sensing*", i.e., density signaling, "overcrowding sense", is based on the interactions between chemical molecules, autoinducers and their receptors. In the case when the concentration of autoinduc-

er in the local environment reaches the threshold concentration, it causes an induction of corresponding genes expression, concerning, inter alia, the division and differentiation of cells, virulence (e.g., bacteriocins), responsible for the production of enzymes (e.g., glycosyltransferases). Communication can occur between the cells of the same or different species. This system differs in gram-positive and gram-negative bacteria. Autoinducers of gram-positive bacteria are peptide molecules, whereas in gram-negative bacteria, these functions are played by acylated homoserine lactone (HSL) [101].

Quorum sensing (QS) in *S. mutans* is mediated by a two-component regulatory system (TCS) ComDE. *ComDE* genes are included in the operon *comCDE*, which together with operon *comAB* play a significant role in the biofilm formation, bacteriocin production, response to stress conditions, e.g., low pH of the environment, and in the so-called genetic competence. This process involves the ability of bacteria to the heterologous DNA uptake and is quite often found in the biofilm environment [102]. Competence stimulating peptide (CPS) is an autoinducer in QS system in *S. mutans*, and its protein precursor is the product of gene *comC*. CPS is a signaling molecule and is secreted outside the cells of the bacteria by ABC transporter, *comAB* gene product. Once a sufficient threshold concentration in the biofilm environment is reached, CPS mediator combines with the bacterial cell surface receptor ComD, which is a membrane histidine kinase. After joining to ligands, the receptor is subject to autophosphorylation with the participation of ATP. Then, the signal is transmitted to the response regulator (ComE), which after conformation change affects the transcription of genes *comAB, comCDE*, and *comX*. ComX is an alternative sigma subunit of RNA polymerase that recognizes other promoter sequences and determines the level of expression of the genes associated with the genetic competence and response of the increase in the density of the microorganism population [103–104]. Competences stimulating peptide (CSP) is involved in the regulation of bacterial biofilm formation, which was demonstrated constructing the mutants lacking genes *comC* and *comDE*. Strains of *S. mutans* devoid of gene *comC* formed the biofilm with altered structure compared to the wild strains, while the biofilm of mutants of genes *comDE* had changed the structure and significantly decreased biomass. It was also demonstrated that the presence of CPS increases the expression of genes for glycosyltransferases (*gtfB*/*C*/*D*), fructosyltransferases (*ftf*), and glucan-binding protein B, i.e., the factors involved in the biofilm formation of *S. mutans* [78, 105]. Interestingly, other studies demonstrated that an excess of CPS may contribute to the death of bacterial cells. This might be a way of a selective control of *S. mutans* survival in mixed biofilms and its virulence control [104,105], but on the contrary, bacterial cell death leads to the release of chromosomal DNA into the biofilm matrix and provides the nutrients, which increases the ability of bacteria to survive in the biofilm [42]. Zhang et al. pointed out the role of CPS in the late stages of the biofilm formation, i.e., its maturation without affecting the growth and division of bacterial cells [104].

An important signaling molecule participating in interspecies communication is autoinducer AI-2—affecting the expression of *gtfB* and *gtfC* [39, 55]. It is produced by both gram-positive and gram-negative bacteria. It is formed during the spontaneous conversion of 4,5-dihydroxy-2,3-pentanedione, whose biosynthesis is catalyzed by LuxS protein. LuxS protein, the product of *luxS* gene, is present in many species of bacteria observed in biofilms (dental

plaque), including *S. mutans, S. gordonii, S. oralis, L. casei*, and *P. gingivalis*. LuxS enzyme plays a dual function, except AI-2synthesis, it also participates in the conversion of toxic S-adenosyl-L-homocysteine to homocysteine [106]. AI-2 regulates, inter alia, the biofilm formation, tolerance of oxidative stress or stress induced by environment acidification, and the expression of virulence factors in response to the increase in bacterial density in the local environment, e.g., the production of bacteriocins [39, 106–108].

Bacteria present in mixed biofilm can not only interfere with each other on the changes in gene expression, but they may also provide each other with plasmids, e.g., antibiotic resistance genes [78]. Among the *S. mutans* species, inter alia, erythromycin or kanamycin resistance gene (gene *aphA3*) can be transferred, which causes the changes in above bacterial phenotype in the direction of multidrug resistance phenotype, impeding an effective targeted therapy [82, 107]. An example of such plasmids may be a new transposon vector called "pMN100," containing, among others, selective kanamycin resistance gene, an aminoglycoside antibiotic [82]. As the multi-species biofilm develops, *S. mutans* increases the expression of genes related to the synthesis, alterations, and adhesion of EPS, particularly genes for surface enzymes, glycosyltransferases. These changes can be caused by the presence of other bacterial species in the biofilm [109, 49]. There is also an increase in the activity of enzymes decomposing extracellular glucans, mainly dextranases. Dextranase, hydrolyzing α-1,6-glycosidic bonds during the synthesis of glucans, leads to an increase in the ratio between α-1,3 and α-1,6 bonds, resulting in an increase in insoluble matrix components. Due to its activity, this enzyme also provides the primers for insoluble glucan production. Moreover, the degradation of soluble glucans and fructans (via fructanase) provides the substrates for organic acid production, thus leading to acidification of biofilm microenvironment [48, 110].

Also, an expression of factors involved in response to stress related to a decrease in environment pH is increased in mixed biofilms. This starts a complex mechanism of ATR, comprising many processes, whose aim is to maintain the pH at an appropriate level, inter alia, the activity of proton pumps is subject to an increase, e.g., F1F0-ATPase, whose task is to remove H^+ ions outside the cell, an increase also involves fatty acid biosynthesis, BCAA metabolism, and other processes that were mentioned in the section concerning factors of *S. mutans* pathogenicity (Section 2.3) [48, 109].

Also a decrease in the expression of proteins aimed in repairing DNA was observed in a mixed biofilm, which may indicate a decreased DNA susceptibility to damage, which is caused, e.g., by chemical agents or radiation [111]. In turn, the share of proteins involved in the synthesis is increased, inter alia, ribosomal proteins, including factors affecting translation initiation and elongation (e.g., aminoacyl-tRNA synthetase) or factors involved in the translation elongation, e.g., factors Tu and G [77], proteins involved in protein folding and secretion, e.g., chaperone protein DnaK, whose task is to prevent erroneous folding of proteins and their aggregation [42, 77]. Also the number of proteins involved in biosynthesis of amino acids and fatty acids is subject to an increase, e.g., BCAA aminotransferase [76], or fabM, involved in the production of monounsaturated fatty acids [42]. The change in membrane lipid profile results in a decrease in its permeability, which allows more efficient maintenance of H^+ ions outside the bacterial cell [68, 74].

Bacteria in the biofilm compete with each other due to limited space for growth and the amount of nutrient substrates. Many species evolved their own mechanisms assuring their survival, e.g., the production of bacteriocins, specific or non-specific proteins involved in inter-bacterial interactions [88]. The analysis of the interactions between the physiological flora and cariogenic factors of dental caries was possible due to the studies using double cultures. The control group for double cultures in the study was single cultures of each bacterial species. The results of the experiments are summarized in **Table 1**.

Double culture	Biofilm growth and formation	Expression of *S. mutans* pathogenicity factors	References
S. mutans + *S. gordonii*	Decrease in the growth rate and biomass	Decrease in expression of genes involved in the transport and metabolism of carbo hydrates (e.g., sucrose phosphotransferase), biosyntheses, and cell division (e.g., sucrose-6-phosphate hydrolase)	[118]
		Inactivation of the competence stimulating peptide (CSP), and in a consequence, a decrease in the amount of produced bacteriocins	[113]
S. mutans + *S. salivarius*	Decreased biomass in the biofilm	Inactivation of CSP, a decline in the ability to produce the biofilm	[114]
S. mutans + *V. parvula*	Insignificant effect on the growth rate	Decrease in the expression of genes of malate transport and metabolism (malolactic fermentation, MLF)	[118]
	Increase in formed biofilm thickness	Spatial biofilm change, resulting in an increased resistance to antibacterial agents	[115]
S. mutans + *Actinomyces naeslundii*	Increase in the biofilm thickness	No effect on the amount of produced bacteriocins	[113]
Actinomyces naeslundii	biofilm thickness		
S. mutans + *S. mitis*	Increase in the biofilm thickness	A larger decrease in the extracellular pH	[116, 117]

Table 1. Results obtained in the experiments using double cultures [112–117] with modification.

Some contradictory relations based on antagonism are observed between the streptococci of oral cavity (**Table 1**). Species from the group of "mitis", i.e., an important group considered

as non-cariogenic, have the ability to suppress the growth and development of the cariogenic biofilm with the participation of *S. mutans* species and also cause a decrease in the expression of virulence factors in this microorganism; therefore, the species such as *S. gordonii, S. oralis, S. mitis*, and *S. sanguinis* are considered by many researchers as the species promoting the health of oral cavity [71, 93, 118]. Epidemiological studies demonstrated a reverse correlation between these two groups of streptococci: the high number of the species from "mitis" is accompanied by a low percentage of microorganisms from "mutans" group [112]. Competition between these two groups of microorganisms is caused by mutual competition for nutrients and a place to live in the biofilm environment [71, 88]. Microorganisms from "mitis" group produce significant quantities of hydrogen peroxide (H_2O_2), which inhibits protein glycolysis and synthesis in *S. mutans* and also causes oxidative damage to DNA and proteins, leading to impaired metabolic processes [119]. Glycolytic enzyme, which is most sensitive to the toxic effects of H_2O_2, is glyceraldehyde 3-phosphate dehydrogenase, as well as glucose transport system dependent on the phosphoenolpyruvate (PEP-PTS) [88]. Hydrogen peroxide in *S. sanguinis* and *S. gordonii* species is formed in the reaction catalyzed by pyruvate oxidase, in which, next to H_2O_2, also and CO_2 and *acetyl phosphate* are formed from pyruvate [88, 118]. In turn, H_2O_2 production in *S. oligofermentas* species is mediated by two additional enzymes, lactate oxidase, which converts lactate to pyruvate, and L-amino acid oxidase, which catalyzes the oxidative deamination of L-amino acids [71, 118].

Another example of antagonism between these two groups of streptococci is the ability to inactivate the CSP, which is part of *QS* mechanism in *S. mutans*. This mechanism mediates, inter alia, the capability for the biofilm formation and bacteriocin production by *S. mutans* in response to increasing bacterial density in the biofilm. *S. gordonii* produces specific serine proteases encoded by gene *sgc*, which are able to inactivate CSP. Wang et al. [113] demonstrated that bacteria lacking gene *sgc* did not cause CSP inactivation and did not inhibit biofilm formation by *S. mutans*. Also Tamura et al. [114] proved the inhibitory effect of *S. salivarius* on the biofilm formation by *S. mutans*, which depends on CSP inactivation. In *S. mutans*, CSP regulates an expression of gene *glrA* responsible for the morphology of the newly formed biofilm. Causing CSP inactivation, *S. salivarius* causes, as a consequence, a decrease in the expression of gene *glrA* and the inhibition of proper biofilm formation. *S. sanguinis* species can also reduce the amount of mutacins produced by *S. mutans* and show faster growth, thus often gaining an advantage over its competitor. *S. mutans* grow more slowly in mixed biofilm than in the single-species one [39, 88].

S. mutans has its own mechanisms enabling it to compete with other species in the microenvironment of the biofilm. It produces higher amounts of organic acid during carbohydrate metabolism, which causes a decrease in the growth rate of other streptococci [71, 88]. *Streptococci* from the group of non-mutans have the ability to survive and adapt in low pH conditions; however, in contrast to *S. mutans*, they are unable to proliferate under conditions of considerable acidity. Therefore, an increase in the count of bacteria from the group of mutans, with concurrent reduction in the number of bacteria from the group of mitis, is observed in the case of frequent drop in pH that occurs in the oral cavity [120]. In addition, *S. mutans* produces special bacteriocins, called mutacins, which inhibit the growth of other

bacterial species inhabiting the same ecological niches. Bacteriocins are proteins of an antagonistic effect toward closely related species. *S. mutans* produces several different mutacins, which belong to the following two groups: lantibiotics and non-lantibiotic bacteriocins. Mutacin IV, belonging to non-lantibiotic bacteriocins, demonstrates an activity against streptococci of the groups of *mitis* and *salivarius* observed in the biofilm of dental plaque [121, 122]. Mutacins not only cause the inhibition of the growth of microorganisms present in the biofilm, but they can also participate in the mechanism of genetic competence. *S. mutans* can obtain DNA from closely related species, including *S. gordonii*. High level of mutacin IV causes the death of neighboring microorganisms, which, as a consequence, causes the release of DNA into the local environment, which can then be incorporated into the genome of *S. mutans* [123].

Under *in vitro* culture conditions, the result of the above interactions between these two groups of streptococci depends on which one of the first will inhabit particular environmental niche. However, under *in vivo* conditions, both groups can coexist together. This is caused, inter alia, by the presence of other bacterial species in the biofilm [39, 112]. As demonstrated by Liu et al. [112], the growth and formation of biofilm by *S. mutans* was significantly inhibited during the double culture with *S. gordonii*. However, after the introduction of third species into the culture, i.e., *V. parvula*, the growth rate was comparable to that observed in a single biofilm of *S. mutans*. This suggests that *V. parvula*, belonging to the early colonizers in the dental plaque, can affect the mutual competition between *S. mutans* and streptococci from *mitis* group. Kara et al. [115] have also demonstrated that in the double culture with *V. parvula, S. mutans* exhibits higher resistance to bactericidal agents, e.g., chlorhexidine, increasing thereby its ability to survive in such formed structure.

Another example of a protective effect of one species to another is the ability of *Actinomyces naeslundii* microorganism to neutralize toxic hydrogen peroxide, protecting other microorganisms from the damaging effect of this metabolite [107]. Relationships between bacteria living in the biofilm of dental plaque are complex and subject to dynamic changes with the changes occurring in the biofilm environment. Interspecies interactions play a significant role in the formation, growth, and maturation and stabilization of the biofilm. In the future, a better understanding of these mechanisms may allow to improve the fight with dental biofilm.

3.4. Modification of already existing biofilm

A number of substances demonstrating an effective antibacterial activity and inhibiting the development of biofilm are known today, e.g., chlorhexidine, delmopinol, and phenolic compounds. Unfortunately, most of them cause side effects such as vomiting, diarrhea, addiction, and discoloration of the teeth. Therefore, there is still a search for alternative substances demonstrating antibacterial activity that would be safe for the users [124].

For years, the primary role in the fight against tooth decay is played by calcium phosphate having remineralization properties [125, 126]. A special calcium-phosphate resin was formed very quickly, whose aim was the gradual release of large amounts of these elements in sites requiring reconstruction. Later, this compound gained the form of nanoparticle composite (nanoparticles of amorphous calcium phosphate, NACP), which also found a wide application in dentistry as its predecessor. The benefits of nanoparticle composite are as follows: better

mechanical opportunities; increase in ions release in acidic environment, rapid neutralization and increase of low pH to a safe value (pH=6) [125, 126].

NACP was enriched with compounds of quaternary ammonium salts (QAS) that in addition to the remineralization properties would have antibacterial properties. Special application was found for quaternary ammonium dimethacrylate (QADM). The compound has two active antibacterial domains at its ends, which enhance the desired properties, and, in addition, easily mixes with other dental media [125, 126]. This modification created a completely new composite, demonstrating a strong antibacterial activity. Its application results in lowered viability of microorganisms, both in the planktonic form and in biofilms (including *Streptococcus* spp. and *Lactobacillus* spp.), decreased acidity, and depletion of pathogens' metabolic activity [125, 126]. The mechanism of that feature is connected with QAS compounds amphiphilicity, which allows entering the reactions interfering cell membrane functions. This process is connected with lipid part of membrane and also affects indirectly in taking part in the transport of substances enzymes activity. QAS influence on lipid membrane alter the bacterial cell metabolism activity. Lethal properties of QAS toward a broad spectrum of microorganisms were also used in antibacterial nanoemulsions. In this case, the salt used was cetylpyridinium chloride (CPC) [127]. An antimicrobial nanoemulsion is a dispersing substance, i.e., water and a lipid substance containing surfactant that forms nano-droplets of the emulsion. It is not toxic to humans or animals; however, it exhibits an antibacterial, antifungal, and antiviral properties. The antibacterial properties of the emulsion result from nano-droplets' effect on bacterial cell membrane destabilizing its lipid integration [127]. CPC is capable of inhibiting bacterial fructosyltransferases, which play an important role in the biofilm formation by microorganisms in the oral cavity. Due to this feature, CPC plays a role of the antimicrobial substance (affecting microorganism cell membrane) inhibiting the development of biofilm. An increase in nanoemulsion's efficiency enriching them with CPC resulted in the extension of their use in products for oral hygiene such as toothpastes, mouthwashes, and dental materials, e.g., varnishes and dental fillings [127].

Among QAS, 12-methacryloyloxydodecylpyridinium bromide (MDPB) also found its application in the fight against tooth decay [128, 129]. MDPB properties and the possibility of its use attracted the attention of a team of scientists [129]. In their experiments, the authors studied the effect of MDPB on the bacterial flora of the oral cavity, interactions with dental materials, and the possibility of synergistic action of that compound with silver nanoparticles (NAg). MDPB is a monomer demonstrating antibacterial properties with respect to aerobic and anaerobic bacteria isolated from caries lesions (i.e., *Actinomyces, S. mutans*), as well as antifungal activity (e.g., *Candida albicans*) [129]. Hardened by the polymerization of binding layer of the filling, it remains active in its against bacteria, concurrently not affecting adversely either the human cells or the binding capacity of the filling material [129].

Silver compounds became the aim of studies conducted by Cheng et al. (2013), who drew the attention to their use combined with QADM. Silver is known for its lethal activity against a wide range of bacteria, viruses, and fungi. Its antibacterial properties are because of the ability of disintegration of microorganism cell membrane, penetration through the membrane, and organelles destruction. Another mechanism causes the bacterial enzymes inactivation

(inhibits capabilities of bacterial DNA replication). All these pathways increase silver compounds efficacy. The other advantages of these compounds are low toxicity, long-term antibacterial effect (gradual silver release). In the fight against microorganisms significant is lower bacterial resistance to silver compounds than to antibiotics [128–130]. Therefore, many mechanisms and properties that make up the activity of substances containing silver particles cause the inability of formation of the systems of full protection against their effects by microorganisms. An additional difficulty is an activity of silver compounds as catalysts, not as substances incorporating into chemical reactions [131]. Silver nanoparticles have a large active surface, and therefore they represent only 0.05 to 0.1% wt. of the filling in the dental composites. This amount provides both effective antibacterial activity and no effect on color and mechanical properties of the filling [128].

The study of both teams demonstrated that the combination of NAg with MDPB and Nag with QADM give much better results than the use of these substances alone. It can be noticed as shown in **Table 2** that the combination of silver nanoparticles with MDPB and QADM does not affect the mechanical properties of dental material and is not toxic to the cells of the human body. The big advantage of such combinations is an increased ability for inhibiting the growth of microorganisms, as well as a significant reduction in their metabolic activity and vitality.

Feature	Control	MDPB + NAg	QADM + NAg
Zone of bacterial growth inhibition	1 mm	Tenfold higher	Eightfold higher
Strength of dental material binding	30–32 MPa	no changes	no changes
Metabolic activity of bacteria determined by measuring the absorbance of the result of enzymatic MTT[a] reduction at wave length of 540 nm	0.5 A_{540}/cm^2	0.05 A_{540}/cm^2	No data
Viability of bacteria determined by the ratio of the number of live cells to dead ones on the basis of fluorescence microscope image	High viability	Considerably reduced with respect to the control	Considerably reduced with respect to the control
CFU (*colony-forming unit*, the number of microorganisms in examined material) after the application of antibacterial agents	23×10^6 for MDPB control 2.5×10^6 for QADM control	0.5×10^6	0.9×10^4

[a]*MTT – tetrazolium dye.*

Table 2. An effect of MDPB+NAg and QADM+NAg on the selected features; [128, 129] with modification.

Silver also found a combination with fluoride forming silver diamine fluoride (SDF) demonstrating antibacterial properties, which in turn are the result of metal component and remineralization properties due to the presence of fluoride [132]. It was demonstrated that SDF can

be used as an element in the prevention of caries [133], as well as a substance inhibiting the development of the disease in children, reducing the number of cariogenic microorganisms in oral cavity and corrective action in the sites of tooth enamel demineralization [132]. As described above, the study on the compound also demonstrated the sensitivity of *S. mutans* and *Actinomyces naeslundii* to its activity [134]. However, there are the studies verifying SDS effect on two-species bacterial biofilm formed by *Streptococcus mutans* and *Lactobacillus acidophilus*. Oral biofilm is not a single species structure, so such studies better illustrate the activity of oral cavity biofilm compound [132]. This is confirmed by the ratio of live to dead cells, which for the control was 0.02 while for the sample containing SDF was 6.74. These studies, in addition to the confirmation of antibacterial SDF activity on *S. mutans* and *L. acidophilus*, also proved that the substance slows down the process of enamel demineralization and protects collagen against destruction. This dual activity of SDF can contribute to widen its use in the products for oral cavity hygiene, as well as clinical success in the fight against dental caries.

There is still a search for antibacterial substances that may be included in the formulations for oral cavity hygiene and would not exert adverse effects. One of such substances is chitosan—a polysaccharide formed as a result of N-acetylation of chitin [135]. Both of these compounds are present in the world of plants, fungi, and animals and demonstrate the natural antibacterial and antifungal properties. However, a limitation of chitosan use in formulations for oral cavity hygiene is its insolubility in water since the compound is soluble only in acids. Attempts aimed to modify that the property, as a result of Millard reaction or sugar modification, caused the formation of chitosan soluble in water with unchanged antibacterial properties. The studies [135] demonstrated that such modified chitosan exhibits the highest antimicrobial activity with respect to microorganism isolated from the oral cavity in an environment of pH ranges between 5 (for e.g. *Staphylococcus aureus, Streptococcus mutans)* and 8 (for e.g. *Staphylococcus saprophyticus*). The optimum temperature in which the antimicrobial activity remained at a level of 50–96% in relation to the above bacterial species for chitosan activity is 37 °C. The minimum concentration of chitosan inactivate *K. pneumoniae, L. brevis* and *S. saprophyticus* was 400 μg/ml, while other species need 500 μg/ml. It was also found that only 5s is sufficient for chitosan to exhibit antimicrobial activity with respect to the microorganisms of the above species at a level of 99.6% and 20s caused that this level was 99.9%. It was proved therefore that the water-soluble chitosan demonstrates comparable efficacy in the fight against dental caries like the commonly used antibacterial substances used in the fluids of the oral cavity care. A very strong argument for the use of chitosan in such formulations is also its much lower toxicity than that of the conventionally used alcohols, chlorhexidine, and cetylpyridinium chloride [135], which are commonly used in compositions for oral cavity disinfection in upper respiratory tract diseases, e.g., Tantum Verde.

Another safe, potential solution for humans for the problem of dental plaque is antimicrobial peptides (AMPS). Currently, the information from more than 2600 characterized antimicrobial peptides is available. An ever-increasing number of antimicrobial peptides allowed to create a database gathering all available and characterized antimicrobial peptides (http://aps.unmc.edu/AP/main.php/). The vast number and variety of AMPs exclude a general

discussion of this issue. AMPS are alternative to the conventional antibiotic therapy. The works on dozens of antimicrobial peptides that can be used for a more targeted approach associated with selective control or elimination of the strains that cause tooth decay in children have been conducted currently. In addition, their features cause that they are much safer for the youngest users. A number of features of these substances, such as a broad antibacterial spectrum, stability, low toxicity, lack of staining on the teeth, odorless nature, suggest that these compounds should be studied further. Their potential use in the oral therapy should be discussed [124,136].

Within the limitations of the existing data, presented overview of AMPs has been elaborated based on the following features of selected peptides: documented antimicrobial activity with respect to cariogenic microorganisms, broad spectrum of antibacterial activity (gram-positive bacteria and other microorganisms that colonize oral cavity), profitable structural characteristics (i.e., size and conformation), durability (resistance to proteases or other salivary protein activity), and low cytotoxicity (only those peptides for which the data were available) [137,138]. The first large group consists of eukaryotic antimicrobial peptides, which include beta defensins of a structure of beta harmonica stabilized by the presence of two or three disulfide bridges, linear peptides that adopt an amphipathic alpha helical structure when associated with the cell membrane lipids, which include *magainins*, histatins; rope peptides modified by the presence of cysteine binding bridges, such as cathelicidins, peptides with loop structure; and peptides with cyclized peptide chain. One can include those with cationic residues, like histidine, arginine or lysine [137].

A range of histatin derivatives, like its derivative PAC113, demonstrates an increased activity against *S. mutans* and *S. sobrinus* [139]. PAC113 found practical application of for the treatment of dental caries. The results of the second phase of the clinical trials using the above discussed derivative of PAC113, in which the children are given lotions and jellies, confirm that the PAC113 is safe and effectively inhibits the growth of fungi and cariogenic bacteria in accordance with its intended purpose, which makes it an ideal candidate for further clinical trials with the indication for caries prevention [137]. A high therapeutic potential was demonstrated for the derivative of histatin 5, called Dhvar1-5, used as a scaffold for the design of analogues of histatin 5. Due to the fact that histatin 5 is a poorly amphipathic peptide, its derivatives Dhvar 1 and 2 are designed to improve amphipathic properties of alpha-helical conformation by the change of histidine to lysine or glutamic acid with lysine at the hydrophobic end. This way, derivatives Dhvar 1 and 2 at higher ionic strength demonstrate lethal concentrations LC50 against *S. mutans* in the range between 15.6 and 16.3 μg/ml, compared to the concentration of 200 μg/ml for the natural form of histatin 5 [140]. Competitive derivatives Dhvar 3 and 4, in which glutamic acid replaced lysine, have lower lethal concentrations LC50 against *S. mutans* at a level of 0.7 and 7.6 μg/ml. Moreover, the analysis of protein proteolysis during *S. mutans* incubation with the examined derivatives showed no proteolysis fragments, suggesting that the derivatives Dhvar 3 and 4 are resistant to cleavage by the proteases excreted by *S. mutans*. Also in the case of Dhvar 4, the number of strains of *S. mutans, S. sanguinis*, and *Actinomyces naeslundii* in a multi-species bacterial biofilm is decreased by more than 90% (100 μg/ml peptide after 30 min). Such a strong antibacterial activity of the dis-

cussed histatin derivatives, in particular Dhvar4, constitutes a promising tool against caries [137, 141].

Another large group is bacteriocins, which owes their name to the organism from which they originate. Based on the structure and composition, they are classified into three groups. Most bacteriocins exhibit a cationic character, but there are neutral and anionic bacteriocins also, which have strong antibacterial properties [142]. Class I bacteriocins include the so-called lantibiotics, thermostable, polycyclic peptides having a molecular weight of less than 5 kDa, which contain untypical amino acids in their structure: lanthionine, 3-*methylanthionine*, dehydroalanin, *dehydrobutyric* acid. Best known bacteriocin is nisin (derived from *Lactococcus lactis*) [143]. The main object of interest in the case of nisin is its use in food industry, and also as an antibiotic and a probiotic in a number of infectious diseases. Also there are a large number of patent applications that present the use of nisin as a component of chewing gum with an emphasis on its anticariogenic effects. The studies also prove that the activity of nisin is comparable to the fluorine ions that are used in oral formulations. However, despite the existing scientific evidences, there is a lack of commercial application of nisin against dental caries or other diseases of the oral cavity. The MIC values for nisin at neutral pH with respect to *S. mutans* and *S. sobrinus* are in the range between 625–1250 and 1250–2500 μg/ml, respectively, and its stronger activity is demonstrated against *Lactobacillus* (39–1250 μg/ml). Furthermore, research on the activity of nisin in the saliva suggests that the needed concentrations for the growth inhibition of cariogenic bacteria are lower (0.1–100 μg/ml). In addition, it was found that the presence of dental caries may enhance the antibacterial activity of nisin at acidic pH, assuring its effective mechanism. More importantly, antibacterial activity against bacteria that cause dental caries is maintained particularly at low pH at the site of cariogenic bacterial action. There are some trials using nisin, by its dilution with saliva [137, 143]. Another quite well-known bacteriocin is poly-lysine, originally isolated from *Streptomyces albulus*, sold by Japan mainly for food applications. Like nisin, it exhibits a relatively broad spectrum of antimicrobial activity and is also stable over a wide pH range and is highly soluble in water. The reported MIC values against *S. mutans* for poly-L-lysine are 20 μg/ml. Moreover, poly-lysine and nisin exert a synergistic effect with respect to *S. mutans* in partial inhibition. Complete inhibition of the growth of *S. mutans* is observed in the case of combination of 10 and 50 or 5 and 200 μg/ml [144]. Both nisin and poly-lysine seem to have a common mechanism of action on the same species of oral cavity microorganisms, and further research on the application of the above-described peptides may explain the degree of specificity of the synergistic action. The use two or more active substances in combination, considerably better cope with many obstacles, such as salivary proteases. Thus such complexes can provide better effectiveness and reduce the likelihood of resistance development. Commercialization is preferred in this case since the application of lower concentrations of the peptides in the combination will cause their lower cost. Combination therapy using poly-lysine and nisin may have significant anti-caries potential, hence the specificity of above peptides action [137]. The group of bacteriocins also includes mutacins, as the name implies, it is derived from *S. mutans*, proposed as anticariogenic agents [145].

The production of large, active enzymes on an industrial scale can be expensive, and this seems to be reflected in the high cost of the product. On the contrary, antimicrobial peptides are extensively studied and seem to have a great possibility of purposeful treatment of dental caries. The oral cavity, due to the availability, is appropriate for oral therapy involving these antimicrobial peptides. One of these proteins is α-helical protein, chrysoidine-1—amphipathic. Chrysoidine-1 has a hydrophilic domain at the C-end of polypeptide chain. Electrostatic interactions of chrysoidine-1 with negatively charged phospholipids of bacterial cell membranes cause tight adhesion to microorganisms. It demonstrates a wide demonstrating a broad spectrum of activity against both gram-negative and gram-positive bacteria. The hydrophilic domain penetrates the bacterial membrane structure, forming the numerous pores in it. Changes in the cell shape were first observed by Wang et al. [124] during their studies using chrysoidine-1 at the concentrations of 2–4 mg/ml (depending on bacterial species). Thus, chrysoidine-1 exhibits a strong destructive effect with respect to the lipid components of cell membrane of microorganisms such as *S. mutans, S. sanguinis, S. sobrinus, S. gordonii, Actinomyces* spp. and *Lactobacillus* spp., and therefore is able to induce its disintegration and bacterial lysis. This contributes to the reduced viability of these pathogens, both in the planktonic form and grouped in the biofilm. In relation to *S. mutans*, chrysoidine-1 demonstrates lethal activity after 30 min using the concentration eightfold higher than the minimal inhibitory concentration (MIC). For comparison, *E. faecalis* is killed after 5 min and *L. fermentis* after 60 min using only fourfold MIC. *A. viscosus* proved to be more resistant than *S. mutans*. Its eradication required the use of eightfold MIC for 60 min. [124].

Biofilm structure microorganisms demonstrate about 1000 times higher resistance compared to planktonic phase. Therefore, AMPs could be used most effectively in the early stages of biofilm formation. This would prevent its development and colonization of the oral cavity by another pathogen. In the phase of biofilm maturation, AMPs would only slow down the process of cariogenesis [124].

The problem of infectious diseases inevitably involves a growing number of microorganisms resistant to increasingly broader spectrum of antibiotics (multidrug resistance). Therefore, the attention of scientists is increasingly focused on antimicrobial, antifungal, and antiviral substances of natural origin. Such properties are demonstrated by many metabolites of plant origin, such as polyphenols, alkaloids, tannins, terpenoids, steroids, and flavonoids [146,147]. Despite the proved antibacterial activity, these compounds so far are the only additives in formulations intended for oral cavity hygiene.

Most of the above-presented substances found their application in dentistry and are included in the composites and resins for dental fillings, whose properties allow the reduction of biofilm formation on them and reduce the likelihood of repeated episodes of dental caries in the same place. They were introduced to the treatment, since the commonly used methods for developing of tooth occupied by caries usually do not allow for the removal of bacteria from the hard tissues of the tooth. In such situations, it is suggested to use the antimicrobial agents on already developed dentin. These are usually the fillings releasing ions [148] in the form of nanoparticle of dicalcium phosphate anhydrous (DPCA) or tetracalcium phosphate (TTCP),

often enriched with fluorine [148], or modifications of such filling, e.g., with an addition of QAS and silver nanoparticles [125, 126, 128, 129].

Iodine compounds (potassium iodine or povidone iodine PVP-I) also reduce the incidence of dental caries. Free iodine ions are the active element of PVP-I complex, which consists of iodine and polyvinylpyrrolidone. Iodine, slowly released from the complex, is able to penetrate bacterial membranes and get into the cytosol, where it inactivates the key proteins in cell metabolism, fatty acids, and nucleotides [149]. No side effects such as discoloration of the teeth, tongue, or change of taste were reported [151]. Moreover, the slow release of iodine minimizes its toxic effect for human cells [150], and its short-term use does not damage even irritated oral cavity mucosa. Hosaka et al. [151] examined the antibacterial activity of PVP-I on two microorganisms associated with the diseases. They demonstrated that PVP-I at a concentration of 7% obtains an activity that allows to kill 100% microorganisms of *P. gingivalis* species after 3 min of activity, while the same effectiveness with respect to *F. nucleatum* was already produced by 5% PVP-I after 30 s. The results obtained for two-species biofilm formed by the examined microorganisms demonstrated that it is two-hundred times more resistant to the action of the examined substance compared to single-species cultures. Despite the activity of 5% PVP-I already allowing for the killing of biofilm-forming bacteria, unfortunately, it was demonstrated that concentrations of PVP-I used in common mouthwash fluids (0.23–0.47%) are not sufficient for a significant decrease of the biofilm-forming ability of *P. gingivalis* and *F. nucleatum* in a time of 30 s–1 min, which was defined as an average mouthwash time [151]. It can thus be concluded on the basis of these reports that mixed bacterial biofilm will be much more resistant to PVP-I compared to planktonic cells.

In children with early childhood caries antibacterial activity of PVP-I was also verified for strains of *S. mutans*. It was concluded that the application of 10% PVP-I in healthy and cariously damaged teeth every three months for one year significantly reduced the number of these microorganisms in the oral cavity compared to baseline levels [152]. Lower number of cariogenic bacteria may therefore help to reduce the likelihood of caries levels in children. In the research conducted on a group of 127 children it was demonstrated, that combined treatment with 10%PVP-I and 5% fluoride varnish reduces the incidence of new caries lesions by 31% in relation to the application of the varnish alone [133]. An increasing range of substances that protect from the onset of caries, and concurrently not causing side effects, becomes the hope of creating a formulation for oral cavity hygiene allowing for an effective fight with this disease and complete protection of children from the development of the first changes in deciduous teeth.

4. Dental plague

Biofilms present in the oral cavity are three-dimensional structures consisting of the bacteria strains anchored to a solid surface, such as tooth enamel, root cement, or dental implant and are embedded in exopolysaccharides matrix [44]. One of the best known and most commonly reported types of biofilm in the human body is a dental plaque [153]. This is an example of

supragingival plaque, unlike subgingival plague, involved in the pathogenesis of periodontal diseases or gingivitis [154].

More than 1000 different species of bacteria forming plaque have been indentified so far, and more than half of them failed to grow using the classical microbiology methods [19, 71, 103, 155, 156]. In recent studies using advanced methods of identification with molecular biology techniques, a number of species identified in dental plaque biofilm increased to about 1500 species [71], and some researchers estimate that this number may be as high as 10,000 [118]. The studies conducted using molecular biology methods demonstrated five dominant phylum of bacteria in dental plaque: *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria*. They constitute 80–95% of the oral cavity microflora (**Table 3**) [93, 118].

Molecular biology technique	**Bacterial microflora compositions (the most often isolated species)**	**References**
16S rRNA pyrosequencing and 16S cDNA analysis	**Firmicutes:***Streptococcus* **Bacteroidetes:***Prevotella, Capnocytophaga* **Fusobacteria***: Leptotrichia*, *Fusobacterium* **Actinobacteria**: *Actinomyces, Corynebacterium* **Proteobacteria:***Camphylobacter*	[157]
16S rRNA pyrosequencing and 16S cDNA analysis	**Firmicutes**: *Streptococcus ,Gemella, Paenibacillus, Veillonella* **Actinobacteria**:, *Actinomyces, Rothia, Angustibacter, Kineococcus* **Proteobacteria**: *Neisseria, Kingella, Alysella* **Bacteroidetes:***Capnocytophaga* **Fusobacteria***: Fusobacterium*	[158]
16S rRNA pyrosequencing and 16S rDNA analysis	**Firmicutes:***Streptococcus, Veillonella, Lactobacillus* **Proteobacteria:***Neisseria, Kingella, Derxia* **Actinobacteria:***Actinomyces, Corynebacterium* **Bacteroidetes:***Prevotella, Capnocytophaga* **Fusobacteria:***Leptotrichia*	[159]
16S rRNA pyrosequencing and 16S rRNA hybridisation (Human Oral Microbe Identification Microarray, HOMIM)	**Firmicutes:***Streptococcus, Veilonella, Granulicatella, Gemella, Abiotrophia, Selenomonas* **Proteobacteria:***Neiserria, Camphylobacter* **Bacteroidetes:***Capnocytophaga* **Fusobacteria:***Fusobacterium*	[71, 93]
16S rRNA of V5–V6 region pyrosequencing and 16S cDNA analysis	**Firmicutes:***Streptococcus, Veillonella, Granulicatella* **Proteobacteria:***Neisseria, Haemophilus* **Actinobacteria:***Actinomyces, Corynebacterium, Rothia* **Bacteroidetes:***Capnocytophaga, Prevotella, Porphyromonas* **Fusobacteria:***Fusobacterium*	[160]

Molecular biology technique	Bacterial microflora compositions (the most often isolated species)	References
16S rRNA pyrosequencing and 16S cDNA analysis	**Firmicutes:***Streptococcus, Veillonella* **Actinobacteria:***Corynebacterium, Actinomyces, Rothia* **Fusobacteria:***Fusobacterium, Leptotrichia* **Bacteroidetes:***Prevotella, Capnocytophaga* **Proteobacteria:***Neisseria, Haemophilus, Camphylobacter*	[161]

Table 3. Composition of dental plague microflora.

In the studies conducted using the latest methods of molecular biology, the largest group of microorganisms are the bacteria belonging to the *Firmicutes* phylum. The second place is occupied by bacteria belonging to the *Proteobacteria* phylum, further *Actinobacteria, Bacteroidetes*, and *Fusobacteria*. Many studies confirmed the dominant share of *Streptococcus* genus, which constitutes more than half of all bacteria in dental plaque biofilms [71, 93, 118, 155, 157, 158]. Another position in this ranking is occupied by bacteria of the genus *Veilonella, Granulicatella, Fusobacterium, Neisseria, Actinomyces, Capnocythophaga, Kingella, Camphylobacter, Gemella, Haemophilus*, and *Prevotella*.

Plaque-forming microorganisms can be divided into the so-called early and late colonizers, forming early and late biofilm, respectively. The species of the early colonizers include streptococci, as well as bacteria of the genus *Veillonella, Haemophilus, Propionibacterium, Capnocytophaga, Prevotella, Eikenella*, or *Actinomyces* [107]. Late colonizers are microorganisms of the genera *Eubacterium, Treponema*, and *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum*, and *Prevotella intermedia* [107]. As the biofilm matures, the domination of bacteria is transferred from one group to another. Streptococci give way to genera such as *Actinomyces, Fusobacterium*, or *Veillonella* [107]. The entire flora of dental plaque was considered for many years as the etiological factor of caries formation (nonspecific plague theory), or only specific microorganisms were considered as pathogenic (specific plague theory). Currently, the main hypothesis of dental caries formation is the so-called ecological plaque hypothesis [162]. The ecological plaque hypothesis combines elements of nonspecific plaque theory and specific plaque theory of caries formation. This theory assumes that changes occurring in the oral environment initiate the changes in the balance of bacterial flora, resulting in the development of more cariogenic species, and are responsible for enamel demineralization over the remineralization. Carbohydrates from the diet are considered to be the most important factor responsible for biochemical and physiological changes observed in the biofilm. Consumption of sugar causes a rapid pH decrease in the dental plaque. In addition, frequent consumption of carbohydrates increases the percentage of streptococcus from Mutans group, and bacteria from of the *Lactobacillus* genus, as well as reduces the number of streptococci from mitis group, including *S. sanguinis*. This is caused by a decrease in the pH of oral cavity, which is accompanied by carbohydrates metabolism and organic acid production (**Figure 4**). Additionally, frequent consumption of sucrose promotes tooth decay not only through the acidification of the environment but also by the production

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of extracellular exopolysaccharides (EPS) forming a biofilm matrix (**Figure 5**). All these processes contribute to an increased risk of dental caries development [62].

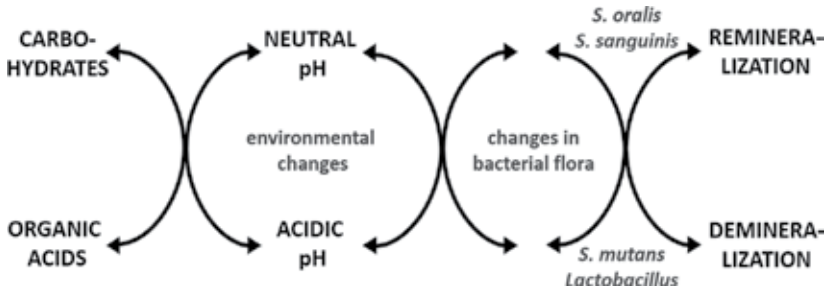

Figure 4. The ecological plaque hypothesis—effect of fermentable carbohydrates on caries development (based on Paes Leme et al. [62]).

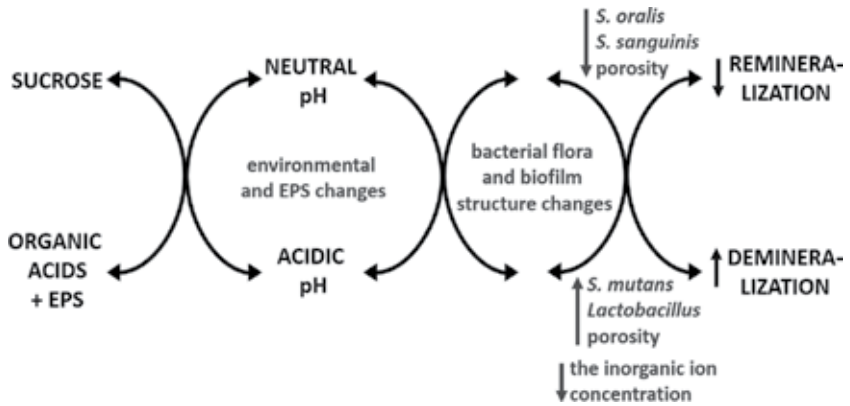

Figure 5. Sucrose effect on caries development (based on Paes Leme et al. [62]).

The species involved in the cariogenic process include *Streptococcus sobrinus, Lactobacillus acidophilus, Actinomyces viscosus*, and *S. mutans* species which is the most known and widespread 129]. However, studies indicate that the induction of carious lesions on the dental plaque can occur even in the absence of *S. mutans* [163]. The factors initiating the formation of the above lesions are acidity and acidophilic bacteria other than *S. mutans* [164, 165]. Except the species mentioned above, some authors have also classified *Actinomyces naeslundii, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia*, and *Porphyromonas gingivalis* as caries-forming species [166], as well as bacteria of the genera *Bifidobacterium* or *Selenomonas* [71].

The six types of bacteria, that is, *Streptococcus, Veillonella, Actinomyces, Granulicatella, Leptotrichia*, and *Thiomonas* demonstrate a large differentiation between the dental plaques with active caries and caries without lesions [15]. Studies showed that three types of bacteria, including *Streptococcus, Granulicatella*, and *Actinomyces*, occurred predominantly in patients with severe

forms of caries. In control (free of caries) noted predominant numbers of the *Aestuariimicrobium* genus [167]. Microbiome of bacteria associated with the formation of dental caries is much more differentiated than previously believed [168].

The microflora of oral cavity demonstrates specificity depending on the stages of caries progression [169]. The study conducted by Gomar-Vercher et al. in 2014 on saliva samples of children with caries showed that the dominant caries-forming species found were bacteria classified to genera *Porphyromonas* and *Prevotella* [169]. Using pyrosequencing techniques, attention was paid to species variability on the dental plaque depending on the activity of caries process—in the case of intact plaque, in the presence of the so-called white spots, in the case of damage to the dentin. Diversity of dental plaque microflora of healthy individuals exhibits a much more homogeneity compared to the plague of individuals with caries. Thirteen bacteria of genera *Capnocytophaga, Fusobacterium, Porphyromonas, Abiotrophia, Comamonas, Tannerella, Eikenella, Paludibacter, Treponema, Actinobaculum, Stenotrophomonas, Aestuariimicrobium*, and *Peptococcus* were associated with healthy teeth. Eight bacteria of genera *Cryptobacterium, Lactobacillus, Olsenella, Megasphaera, Scardovia, Shuttleworthia, Cryptobacterium*, and *Streptococcus* increased significantly in dentine damage, while *Actinomyces* and *Corynebacterium* were present predominantly in the so-called "white spots" and *Flavobacterium, Neisseria, Bergeyella*, and *Derxia* were numerous with intact dental plaque of individuals with caries [159]. In case of damaged dentin, the study by Obata et al. reported a relatively high percentage of bacteria of the genus *Atopobium, Prevotella*, or *Propionibacterium* along with *Streptococcus* or *Actinomyces* [170].

However, taking into account the complexity of microorganism–microorganism interactions and the number of microorganisms, which is essential for the formation of biofilm and creation of specific conditions for caries development, it can be concluded that particular species of bacteria acts as the forefront of cariogenic factors [71, 164]. An increasing attention is paid to the study of relations between the course of biofilm formation and its maturation, development of resistance to increased spectrum antibiotics, analysis of microorganisms genome, and mechanisms of action of specific bacterial proteins [171–173]. Considerable focus is also on the structure of biofilm and its relationship with the species forming the microbiota of human oral cavity.

5. Role of oral microbiome in the formation of dental plaque

The knowledge of the microbial diversity in human oral cavity has recently been improved and was subjected to a considerable transformation in the last two decades. Understanding biological characteristics and functions of individual bacterial species colonizing oral cavity, which are subject to dynamic changes, has become the fundamental objective of studies on human microbiome. The function of the oral microbiome can be determined on the basis of characteristics of microbes isolated *in vitro*. Currently, no efficient referential method exists for the evaluation of the actual presence of bacteria in the oral cavity. The culture methods carry their limitations related to the lack of ensuring all the necessary growth conditions for each of

the microbiota species under *in vitro* conditions. Both the interpretation of culture results and molecular tests pose numerous problems, in particular, due to the fact that the mere presence of an occasional isolation or determination of bacterial DNA does not mean that a given species colonizes the oral cavity and its individual anatomical areas—it can occur there temporarily. Bacteria in the oral cavity can (which we discussed in subchapter) occur accidentally as a result of age-dependent transformation, coming through primary teeth, their extractions, carious lesions, dentures, restorations, edentulism, and also temporary changes, which can be induced by diet, variable saliva flow, or lasting antibiotic treatment. Environmental conditions, such as temperature, salinity, availability of oxygen and nutrients, variable pH conditions, and redox, may have an effect on the ecosystem and contribute to the modification of the species composition of biofilms found on every location.

This is corroborated by taxonomic studies comparing bacterial species composition of the oral microbiome with species forming the dental plaque, and in these studies, more attention was paid to the occurrence of species diversity, however, with a very similar metabolic activity characteristic of individual representatives of the oral microbiome and the dental plaque [155]. The presented data suggest that modifications in the taxon proportions occur between the oral microbiome and biofilm. However, it is important to note that these constitute changes caused by the functional abilities of certain taxonomic groups (induced by their metabolic activity, i.e., the use of carbohydrates and tolerance to low pH). Populations of microorganisms exhibit similar stability, which enables us to gather the basic knowledge on the behavior of the abovementioned microbes under *in vivo* conditions.

As a result of the evaluation of phylogenetic relationship of bacteria found in saliva samples of healthy persons and bacteria forming the biofilm model imitating the dental plaque *in vivo*, attention was paid to the occurrence of affinities between species, in particular, within the *Streptococcus* genus and to a lesser degree in *Veillonella* (**Figure 6**) [2, 4, 155, 174]. In the case of the *Streptococcus* genus, the most dominant units within the human oral taxa (HOT) were *S. vestibularis, S. salivarius, S. mitis, S. parasanguinis*, and different undiscovered *Streptococcus* spp. The strains of *S. vestibularis* constituted approximately 40% of the total biofilm microbes, whereas *S. salivarius* (HOT-755) and various other undiscovered *Streptococcus* sp. (HOT-C65) each occupied 10%. *S. vestibularis* is the representative of physiological flora of the oral cavity, and its presence is uncommonly associated with the disease, except for few cases of infectious endocarditis, neonatal sepsis, bacteraemia, and in patients with cancer [175]. Furthermore, a few studies demonstrated that *S. vestibularis* may be the etiological factor of caries in rats, compared to other *Streptococcus* species (e.g., *S. salivarius*), which are associated with the development of the disease much more commonly [176]. *S. salivarius*, which has extensive functions from being highly cariogenic [177] to the protective function against caries development (i.e., through hydrolysis of urea to ammonia [178], influences the health state of the human oral cavity. The strains of *S. parasanguinis* (HOT-721 and HOT-711) constituted a considerable participation (10%) in the presented *in vitro* biofilm model. The role of *S. parasanguinis* in the health of oral cavity is poorly known. Due to the ability of this microorganism for carbohydrate fermentation to lactate and other organic acids, *S. parasanguinis* is considered to be an organism with moderate tolerance to low pH and a low cariogenic

species [179,180]. The previous studies suggested that these bacteria were significantly related to both caries in small children and the physiological flora of the oral cavity [181, 182]. This fact is not confirmed by recent study Krzyściak et al.'s [183] in which, apart from a detailed description of *S. mutans*, constituting 24% of natural physiological flora of children without symptoms of the caries disease and 77% of cariogenic flora in children with early childhood caries (ECC), presence of *S. parasanguinis* constituted only 2%.

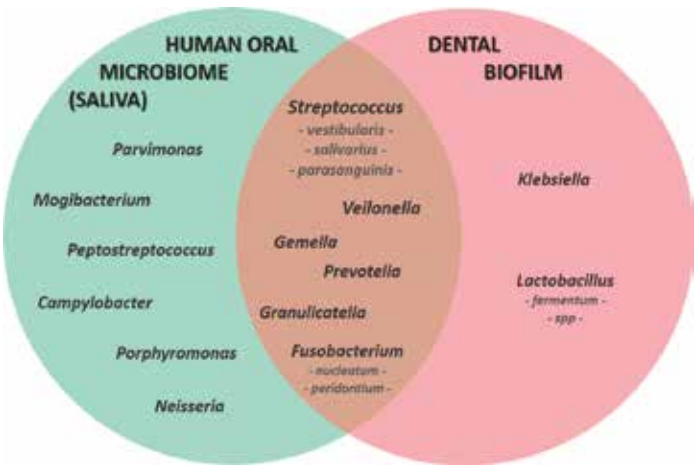

Figure 6. Results of phylogenetic relationship evaluation of bacteria found in saliva samples (oral microbiome) of healthy persons and bacteria forming the biofilm model imitating *in vivo* dental plaque.

The presence of *S. mutans* bacteria onto the teeth surfaces is usually related to poor hygiene and diet. These bacteria dwell in the oral cavity, occupying hard tissues; but they also colonize soft tissues from which they can be easily transferred to the oral cavity, in particular, with shared use of personal care products or during kissing. It appears also that maintaining the oral cavity in the state of lowered moisture (frequent snacking) may favor colonization and the resulting infection. It is possible that irrigation itself, without the use of disinfectants, may also constitute the source of colonization [157, 184].

The presence of the *Veillonella* genus in the presented biofilm model and saliva samples creates new opportunities related to the role of this genus in the interspecific relations between the oral microbiome and caries development. In earlier studies, the role of *Veillonella* remained unexplained, and all the available laboratory tests demonstrated that the growth effects of microbes of the *Veillonella* genus can be mixed [185, 186]. It is also suggested that the presence of the *Veillonella* genus can be used for forecasting the possibility of caries development in children without symptoms of the disease due to its close relationship with *S. mutans*, which was identified in all samples of the above study (HOT-686) [187].

On the contrary, the lactic acid bacteria of the *Lactobacillus* genus were identified in the few samples obtained from the supragingival area of healthy persons. The representatives of this genus are considered to be late colonizers [2]; they commonly occur in advanced caries (low

pH) [22]. Two *Lactobacillus* species (*L. fermentum* HOT-608 and *Lactobacillus* sp. HOT-A89) were identified in the presented model with a relatively low growth ($\overline{X}$ =0.8%). This means that the opportunity to alter the view on the participation of this genus is only as normal physiological flora exhibiting an increased possibility to cause the disease as a result of the movement of this genus from the physiological flora to the disease conditions [155, 188].

Interestingly, *Fusobacterium* genus, which appears to play a less significant role in the oral microbiome in children, similar to the TM7 strain [14], constituted approximately 10% of the total diversity of microbial genes forming the presented biofilm model. Similar to the majority of other microbes, microorganisms belonging to the *Fusobacterium* genus are known not only as the normal physiological flora of the oral cavity, but also as the flora responsible for causing the diseases of oral cavity under special conditions [189]. Their ability to grow in different environments can be explained by their universal metabolism, which enables them to obtain energy from fermentation of simple sugars, simple and branch-chained amino acids [189].

Many bacteria of the oral microbiome, including species forming the dental plaque, are opportunistic species (until recently considered to be non-pathogenic), and due to this fact, their appearance in sites normally unavailable to them in the case of habitat disturbance (disturbance of the immune system functioning of the host) may initiate the development of infection. This is corroborated by the results described above, indicating an increased frequency of the occurrence of the bacteria of physiological flora in the dental plaque, which are exposed not only to the changes in the oxidation-reduction potential, local pH, but also in the response to diet and all types of environmental conditions constituting the source or nourishment for bacteria. In children, studies on the oral microbiome independent of cultures demonstrated that apart from the DNA of *Streptococcus, Veillonella, Lactobacillus* and *Granulicatella, Rothia* and *Actinomyces*, the most common type of DNA belonged to *Bacteroidales* and *Prevotella*, and also *Fusobacterium, Spirochaetes*, and the TM7 strain [14]. *Streptococcus*, and to a lesser degree *Veillonella*, also dominated in the studies evaluating phylogenetic relationships of bacteria forming the dental plaque with the oral microbiome species [155, 190].

6. Conclusion

As it was mentioned earlier, only approximately 40% of the oral microbiome bacteria are cultured *in vitro*. Due to this fact, studies on the function of non-cultured bacteria should be carried out using other methods, and thus looking for such a solution that enables understanding their properties without the need for *in vitro* isolation. Such possibilities are provided by metagenomics, which studies sequences of DNA obtained directly from the environment of the oral cavity, treated as one common gene set [191]. An oral microbiome analysis depending on the genotypic characteristics of the host, as well as its metabolic phenotype, will allow us to understand all these factors which responsible for maintaining host- microbiota homeostasis. The formation of genetic maps (including host, as well as microbiota) of such environments and the detection of biofactors indicating the predisposition for a given disease may contribute to the development of new diagnostic methods in reference to individual

persons, and thus individualized therapy. So far, numerous active bacterial factors and molecular mechanisms of their interactions with the host have been discovered. However, the problem of the presence of healthy oral microbiome bacteria and relating teeth colonization with a potential infection development requires further study.

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Chapter 17

Surface Biofilm Interactions in Epizootic Shell Disease of the American Lobster (*Homarus americanus*)

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Additional information is available at the end of the chapter

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Abstract

Epizootic shell disease (ESD) is a persistent malady that affects American lobsters (*Homarus americanus*) in the southern extent of the commercial fishery. Emerging at the turn of the 21st century, ESD presented as bacterial ulcerations on the carapace of affected lobsters. The research presented here examined the bacterial community of the lobster carapace and represented the first such attempt to characterize the lobster surface microbiome. Culture-independent techniques, such as amplicon length heterogeneity and pyrosequencing, yielded sequence data of hypervariable regions of the genes for ribosomal RNA that upon comparison revealed the likely identities of the taxa present on the lobster carapace. Although some researchers have identified a novel chitinolytic bacterium of the genus *Aquimarina* (*A. homaria*) as consistently appearing on lobsters with shell disease, this research found no evidence of a correlation of this species with the disease. Instead, analysis revealed that the genus *Aquimarina* was ubiquitous and correlated only weakly with the diseased state. The data suggest that this disease is not caused by a single pathogen but by a state of dysbiosis where normally occurring microflora emerge as potential opportunistic pathogens when there is some apparent environmental stressor that alters the interaction of the surface biofilm of the lobster.

Keywords: epizootic shell disease, lobster microbiome, next-generation sequencing, multitag pyrosequencing, amplicon length heterogeneity

1. Introduction

Since the end of the 20th century, American lobsters (*Homarus americanus*) have suffered from a host of problems that have led to morbidity and mortality. Some appear to be environmen-

tal, such as increased bottom temperatures during the summer season, and general effects of eutrophication [1]. Others are possibly the result of intoxication from anthropogenic substances [2]. Emergent pathogens, such as paramoebiasis, have significantly reduced commercially important populations in western Long Island Sound and elsewhere [1]. What makes this situation especially problematic is that the observed decline in lobster health and viability may have causes that can be linked to a convergence of environmental stressors and pathogenic microorganisms.

One such challenge to lobster health that is reducing the quality, and possibly the quantity, of commercial landings is epizootic shell disease (ESD), a condition that is manifesting itself in geographically isolated portions of the commercial lobster fishing grounds [3].

Documentation of diseases that cause lesions and ulcerations in the carapace of lobsters can be found at least as early as 1937 [4]. Initial reports of shell disease in other crustaceans are contemporary with Sindermann [5]. In many cases, the pathology appeared to be linked to degraded environments, such as the proximity to pollution sources, for example, the municipal waste dump site in an offshore canyon used by New York City where lobsters and crabs presented with shell disease [6, 7]. Another common place to find crustacean shell diseases is in commercial impoundment facilities [5]. In this situation, chitinolytic bacteria appeared to be eroding the carapace faster than it could be replaced by molting, leading to a pitting of the cuticle. However, there has only been one report of an infectious process after the lobster's carapace was abraded and swabbed with *Vibrio* sp. [8]. Waddy et al. described the integument during the intermolt phase as consisting of four layers, beginning externally: the epicuticle, the exocuticle, the endocuticle, and the membranous layer [9]. Beneath these four layers are the epidermis, the basement membrane, and the connective tissue. The transport to the epicuticle of extracellular substances, presumably with protective properties, from the epidermis occurs through pore canals that transverse the exoskeleton. All but the membranous layer is calcified either with regularly organized calcite crystals or with amorphous calcium carbonate and all but the epicuticle contains the acetyl-aminated polysaccharide, chitin.

In the epicuticle, calcification consists of spherulitic calcite surrounded by a lipid-protein matrix; in the exocuticle and endocuticle, calcite crystals are dispersed throughout chitin-protein fibers that are referred to as lamellae. In addition, the exocuticle contains trabeculae that are composed of apatite (calcium phosphate) that resembles spongy bone tissue in vertebrates [10]. The endocuticle is the most calcified layer of the exoskeleton [11].

According to Smolowitz et al. [3], ESD is identifiable by erosions of the carapace that have a unique histology. The presence of lesions on the carapace of an infected lobster is the most visible sign of the disease. Histological examination revealed bacteria as the most common organisms in the lesions, whereas some protist constituents were found in more advanced cases. The lesions were grouped into three categories based on the depth of the bacterial incursion, presuming that depth reflects a progressive erosion of the cuticle.

Category 1 is the least severe erosion, with shallow lesions extending into the epicuticle and exocuticle. The margins of the lesions often exhibited evidence of melanization, but inflam-

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mation in the underlying connective tissue or other evidence of an immune response is rarely observed at this stage. Bacteria are found in the leading edges of the lesion and in the crystalline chitin lattice.

Category 2 lesions are moderately deep, penetrating the calcified endocuticle. The crystalline lattice structure of the chitin takes on a "pillar-like" appearance as the bacteria degrade the protein structure between the lattice crystals. The endocuticle exhibits melanization, especially in the vertical areas of bacterial incursion. Evidence of immune response to the infection includes inflammation of the underlying cuticular epithelium and "moderate numbers" of hemocytes in the tissues. Secondary invasion by small protists occurs during this stage. These organisms are apparently responsible for the degradation of the crystal lattice structure of the chitin. In some category 2 lesions, an "inflammatory cuticle" forms between the uncalcified endocuticle and the cuticular epithelium. The latter has some areas of hyperplasia and hypertrophy.

Deeper erosions into the uncalcified endocuticle are characterized as category 3 lesions. At this stage, the overlying structures of the carapace are absent, and the exposed areas are melanized. The cuticular epithelium is hyperplastic and hypertrophic and exhibits an intense inflammatory response accompanied by greater hemocyte infiltration. The underlying connective tissue also exhibited signs of inflammation and immune response. The most extreme types of category 3 lesions had no more tissue than the inflammatory cuticle overlying the cuticular epithelium. In some cases, the lesions progressed to ulcerations, which were characterized by a complete absence of cuticular tissue and cuticular epithelium. Degranulated hemocytes developed a multilayered pseudomembrane to cover the connective tissue. The outer layer of the pseudomembrane was necrotic and melanized.

Certain groups of bacteria have been found as common constituents on the carapaces of moribund lobsters, and overall populations of bacteria are higher than on the carapaces of healthy lobsters [11]. Using a combination of culture-dependent techniques, polymerase chain reaction (PCR), and denaturing gradient gel electrophoresis, the group of Chisosterdov isolated three novel bacteria that were ubiquitous in ESD lesions: *Aquimarina homaria* I32.4, *Thalassobius* sp. 131.1, and *Pseudoalteromonas gracilis* ISA7.3 [11]. Quinn et al. [12] were successful at initiating lesions that are histologically similar to ESD in captive lobsters using these three bacteria. The abrasion of the carapace was necessary for lesions to form. Researchers were also able to isolate *A. homaria* from lesions in "diet-induced" shell disease. These lobsters were fed strictly herring and were deprived of sources of astaxanthan, rendering them nearly colorless [13].

The characterization of microbial communities using terminal restriction fragment length polymorphism revealed that, although no grossly significant difference in communities on healthy lobsters was present compared to diseased lobsters, there were some minor differences [14]. More anaerobic bacteria and greater numbers of α- and β-proteobacteria were found in the lesions. Bell et al. used lobsters from eastern and western Long Island Sound. Lobsters from a coastal Maine site were used as reference specimens. This study also elucidated the activities of four bacterial ectohydrolases on shell samples from healthy and diseased specimens and found that, whereas chitinase activity was high in all samples, cellulase and

proteinase activities were significantly higher in diseased specimens. Lipase activity was higher in Long Island Sound lobsters compared to those from Maine but was apparently similar between healthy and diseased lobsters. They concluded that chitin degradation was not as important to the progression of the disease as the degradation of lipids and protein in the carapace. Bell et al. [14] interpreted the spatial difference in lipase activity in bacteria to indicate that the degradation of the lipid moieties in the epicuticle may be an important initial step in disease progression and hypothesized that it should correlate positively with geographic areas of ESD prevalence.

The works of Smolowitz et al. [3] and Bell et al. [14] make clear that ESD differs from earlier types of shell disease. ESD is ostensibly bacterial in origin, but other research indicates that the bacterial infection may only be a proximate cause.

Shields et al. [15] found that some idiopathic conditions were more prevalent in lobsters from Rhode Island compared to lobsters from Maine. Rhode Island lobsters presented with vibriosis, hepatopancreatitis, and eye lesions at a significantly higher rate than did Maine lobsters. When Rhode Island lobsters with and without ESD were compared, these and other idiopathic conditions were present but did not occur more frequently in lobsters with the disease.

Laufer et al. [16] found that ESD lobsters had higher levels of alkylphenols than did unaffected lobsters and that bottom sediments had higher than normal levels of these compounds in areas where diseased lobsters resided. Using gas chromatography-mass spectrometry, Laufer et al. identified four alkylphenol species: 2-*t*-butyl-4-(dimethylbenzyl) phenol, 2,6-bis-(*t*-butyl)-4-(dimethylbenzyl) phenol, 2,4-bis-(dimethylbenzyl) phenol, and 2,4-bis-(dimethylbenzyl)-6-*t*-butyl phenol. The second compound, 2,6-bis-(*t*-butyl)-4-(dimethylbenzyl) phenol, was developed but never used by Monsanto Corporation as a mosquito larvicide. Biggers and Laufer [17] have identified alkylphenols as endocrine disrupters having juvenile hormone activity. This hormone, also referred to as methyl farnesoate in crustaceans, is important to the metamorphosis of larvae [18]. Laufer et al. [19] confirmed that alkylphenols do inhibit larval development in lobsters. In adult lobsters, however, these compounds displace tyrosine molecules and delay the hardening of the shell by preventing the tyrosine cross-linking of proteins and chitin [19]. Kunkel et al. [10] found that, in lobsters with ESD, the exocuticular apatite layer was greatly reduced or absent. Another group of researchers found that several measurements of immune response in lobsters, including phenoloxidase activity and antimicrobial activity of the hemolymph, phagocytic activity, and reactive oxygen species production, was depressed in lobsters from eastern Long Island Sound (one of the prime ESD areas) compared to lobsters from western Long Island Sound and Maine [20].

Tarrant et al. found that lobsters with ESD had gene expression patterns that were consistent with exposure to xenobiotics. Specifically, they found an elevated expression of ecdysteroid receptors and elevated expression of CYP450 (a cytochrome *P*450 moiety that is positively correlated with increased ecdysteroid levels). In addition, decreased arginine kinase expression in thoracic muscles was observed in lobsters presenting with ESD. Arginine kinase is analogous to creatine kinase in vertebrates [21]. These findings suggest that lobsters from Rhode Island waters that were sampled for these studies were subjected to atypical environmental stress factors.

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In response to the ESD crisis, a group of scientists, fisheries managers, and lobstermen formed the New England Lobster Health Initiative. It was this ad hoc committee that ultimately received funding that was awarded to several university and institutional researchers, including the award that funded the study reported here. The findings of each group were published as a special edition of the *Journal of Shellfish Research* [22]. Included in that edition was an article that summarizes some of what is contained herein [23].

A systems approach to ESD was employed in this study to elucidate the surface biofilms of lobsters in three states of health: Healthy, Diseased, and Healthy-on-Diseased (areas of the carapace on a lobster presenting with ESD that do not yet have the characteristic lesions). Using culture-independent molecular techniques coupled with multivariate statistical and network analysis models, this study surveyed the biofilms of the carapaces of lobsters from the geographic area that is most affected by ESD.

This study represents the first survey of the surface microbiome of the American lobster. Although the sequencing techniques can provide a comprehensive overview of bacteria in a sample, this study cannot be regarded as a global assessment of the lobster microbiome. The lobsters surveyed here are from a one geographic location and represent a statistically small sample of lobsters. Elucidating the complete lobster microbiome should make use of lobsters from as much of the natural range as possible and should include a statistically large sample of specimens. Bell et al. [14], for example, have demonstrated that there is a biochemical activity in the lobster surface microbiome that varies geographically.

This survey does, however, offer a glimpse into the subtle microbiome differences between lobsters that present with ESD ("Diseased") and those that do not ("Healthy"). In addition, a third class, "Healthy-on-Diseased," has been defined as a region of the shell of a lobster with ESD that does not present with lesions. This third class may provide some insight into the disease progression.

As described previously, Quinn et al. [12] were successful in initiating lesions similar to ESD with three bacteria that were isolated from ESD lesions. Some of the infections were initiated with only one bacterium (*A. homaria*), whereas other lesions were initiated as "coinfections" with *Thalassobius* sp. and *P. gracilis*. The removal of the epicuticle by abrasion was necessary to initiate the infection. These authors acknowledge that ESD lesions present with many more bacteria and that ESD appears to be a polymicrobial disease.

The polymicrobial nature of ESD, and the desire to illuminate something of its etiology, is the focus of this research. To that end, culture-independent techniques were employed to elucidate the bacterial community, and multivariate statistics were used to interpret the results. Specifically, length heterogeneity-PCR (LH-PCR) was used to survey a typical lobster. This provided an overview of the distribution of bacteria on different regions of the lobster carapace. LH-PCR data do not provide sequence information but identify PCR amplicons by size (basepair length) that indicate different taxa. The number of representative fragments found in the cephalothorax region of the carapace, combined with the knowledge that this region had the highest incidence of ESD lesions, led to the decision to focus the next phase of molecular interrogation on the cephalothorax. This phase used multitag pyrosequencing (MTPS) to

generate raw sequence data of each PCR amplicon, which could be identified by comparing these sequences to known sequences in a standard database.

Discriminant analysis (DA) was chosen as a means of determining if there is a statistically significant difference between the bacterial communities found on the three classes of data (Healthy, Diseased, and Healthy-on-Diseased). In addition, DA assigns each variable (bacterial taxon) with coefficients that indicate its contribution to the discriminant function. Correlational network analysis, which uses techniques similar to social network analysis to create a visual image of correlations between the bacterial taxa, was employed to identify which taxa are positively and negatively correlated in each of the three states examined. Using correlational difference analysis to compare the states, it may be possible to more clearly identify the changes in bacterial population on the lobster carapace that are correlated with a decline in health.

We approached the problem using the following hypothesis:

H: The bacterial communities on lobsters with ESD are significantly different in quality and quantity than unaffected lobsters.

HA: The bacterial communities on the carapaces of healthy and shell-diseased lobsters are similar.

2. Materials and methods

2.1. LH-PCR

We initially performed a survey of the microbiome on normal lobsters using a culture-independent method to characterize the taxa composition of the biofilm on various locations of the lobster shell. Characterizing a microbial population using molecular techniques is advantageous over culture-dependent techniques in that the latter relies on the growth of bacteria on artificial media usually at a controlled temperature. Many bacteria have particular nutritional and environmental conditions that render them difficult to grow in laboratory cultures and would not be present in cultures taken from the environment. Existence in culture medium, as discussed in the previous chapter, requires that the bacteria assume a planktonic phase rather than embedding in a biofilm, which may be an abnormal state for most species [24]. These molecular techniques are sometimes referred to as "culture independent" in that they identify bacteria from a sample without first attempting to grow them under artificial conditions. Identifying bacteria by amplifying and sequencing the entire genome of each individual would be impractical and inordinately time-consuming. Several techniques exist that enable the identification of bacteria by taking advantage of variable regions within genes that are ubiquitous in all prokaryotes. This research used the first two variable regions of the genes encoding for the small (16S) subunit of ribosomal RNA (rRNA) to discriminate between different operational taxonomic units (OTUs) that are surrogates for taxa identification. There are nine such variable regions within this gene.

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The technique employed in this research is called LH-PCR [25]. The variability, or heterogeneity, of the basepair lengths of the variable regions can be used to define the OTUs that represent individual taxa, but the technique does not assign a taxon name to them.

An LH-PCR survey of the diversity of the bacteria in the shell microbiome of a store-bought lobster was initially performed to determine the compositional patterns throughout the surface of the lobster. In this procedure, bacterial DNA was extracted from various regions of the lobster carapace (claw, abdomen, cephalothorax, and telson). The carapace samples were dissolved in EDTA and proteinase K to recover all microorganisms from the surface and subsurface, and DNA was extracted using the FastDNA Spin Kit (MP Biomedicals, Solon, OH). PCR was employed to amplify the bacterial genes for the first two hypervariable regions (V1 and V2) of the 16S rRNA subunit using universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 355R (5'-GCT GCC TCC CGT AGG AGT-3'; Invitrogen Corporation, Carlsbad, CA) [26].

The LH-PCR products were diluted according to their intensity on agarose gel electrophoresis and mixed with ILS-600 size standards (Promega, Madison, WI) and HiDi Formamide (Applied Biosystems, Foster City, CA). The diluted samples were then separated on an ABI 3130xl fluorescent capillary sequencer (Applied Biosystems) and processed using the Genemapper™ software package (Applied Biosystems). Normalized peak areas were calculated using a custom PERL script, and OTUs constituting less than 1% of the total community from each sample were eliminated from the analysis to remove the variable low abundance components within the communities.

2.2. MTPS

The technique described above employed a culture-independent technique that is capable of resolving amplified fragments of DNA by size (OTUs). This can be useful in making observations on gross changes of microbial communities over time, such as in a site that is contaminated by petroleum or some other organic material. In this case, LH-PCR was used to demonstrate that the biofilm on the cephalothorax was the best representative sample of the shell microbiome. The next phase of interrogation employed MTPS [27] to further elucidate the nature of the microbial community. The OTUs from the lobster samples were sequenced using next-generation sequencing technology, and the sequence reads were matched as closely as possible to known bacterial taxa. This provided us with data that could be used in a variety of ways to demonstrate the correlation between bacterial taxa and various states of health in the lobster.

Cuticle samples (0.5 cm^2) were harvested as part of the "100 Lobsters" Project [28] and in a manner described therein. The lobsters were collected from within Narragansett Bay, Rhode Island. A total of 55 lobsters had ESD, whereas 47 were categorized as apparently healthy (i.e., the lobsters did not have visible ESD lesions). The samples were shipped in plastic vials on dry ice and stored at -80°C until processed. Three types of carapace samples were taken: those from lesions of diseased lobsters (Diseased), those from lesion-free areas of diseased lobsters (Healthy-on-Diseased), and those from apparently healthy lobsters (Healthy). The carapace samples were dissolved in EDTA and proteinase K to recover all microorganisms from the

surface and subsurface, and DNA was extracted using the FastDNA Spin Kit (MP Biomedicals).

PCR was employed to amplify the bacterial genes for two hypervariable regions of the rRNA subunit using modified primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 355R (5'-GCT GCC TCC CGT AGG AGT-3'; Invitrogen Corporation). The forward primer listed above was modified with a "barcode" sequence that was unique to each DNA sample extracted from individual lobsters, allowing us to track the various bacterial sequence reads to their specific sample origin (i.e., the lobster and carapace region from which the bacteria were sampled). MTPS was employed to characterize the microbiome from a subset of the carapace samples that were used in the LH-PCR analysis. We generated a set of 96 fusion primers that contained the emulsion PCR linkers (454 Life Sciences) on the 27F and 355R universal 16S rRNA primers along with different eight-base "barcodes" on the 27F primer. Each lobster DNA sample was then amplified with a unique set of tagged 16S rRNA primers, pooled, subjected to emulsion PCR, and pyrosequenced using a GS-FLX pyrosequencer as per manufacturer's instructions (Roche, Branchburg, NJ). Data from the pooled sample were "deconvoluted" by sorting the sequences into sample bins based on the barcodes using custom PERL scripts. This technique allows the rapid sequencing of multiple samples at one time, yielding thousands of sequence reads per sample. The sequence reads were identified using the Bayesian analysis from the Ribosomal Database Project [29]. We used a custom PERL script to normalize the abundances of the taxa in a sample based on the total reads in that sample.

The sequences were aligned that were identified as *Aquimarina* spp. using the RDP 10 analysis [29] with reference sequences available in GenBank and constructed a neighbor joining tree for the genus. The clades in the tree were labeled by the major species present therein. Dr. Andrei Chisosterdov, a colleague from Louisiana State University and a participant in the New England Lobster Health Initiative, supplied an rRNA sequence for *A. homaria*. None of the bacteria from our samples that were identified as members of the genus *Aquimarina* were greater than 95% similar to Dr. Chisosterdov's reference sequence, indicating that we identified similar examples of the same genus.

The software program Quantitative Insights into Microbial Ecology (QIIME) [30] was used to compare the microbial communities on the three types of carapace samples and to construct a UniFrac neighbor joining tree that graphically displays the similarities in microbiome structure between each lobster sample in the study. The technique was validated using jackknifing to estimate the dispersion of the data [31]. According to Lozupone and Knight [32], the UniFrac metric "measures the phylogenetic distance between sets of taxa in a phylogenetic tree as a fraction of the branch length that leads to descendents from either one environment or the other, but not both." In the process, UniFrac was able to depict the evolutionary differences between environments. In this application, UniFrac was used to compare phylogenetic differences between bacterial communities and provide a graphic representation of the differences. Each terminal node, therefore, represents a unique phylogenetic tree of that sample. The distance between the nodes is the distance between these trees.

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2.3. DA

DA, sometimes referred to as discriminant function analysis, is a multivariate statistical method that identifies variables of different cases that are useful in the discrimination of those cases into different classes. The analysis of the data results in the construction of a linear equation in which the variables are factors of both unstandardized and standardized coefficients. The products of these variable coefficient pairs are summed to produce a score for each case, and that score determines the membership of that case in a specific class [33]. The summation of the unstandardized coefficients includes a constant. Standardizing the coefficients eliminates the constant. These coefficients are partial coefficients that compare the relative importance of the independent variables [34]. We define the cases as individual lobsters in the study and the variables as the bacterial taxa that were identified by MTPS.

In addition to producing unstandardized and standardized linear coefficients that can be used to discriminate classes, DA produces full coefficients that are referred to as structure coefficients. These are measurements of the importance of the independent variables and the discriminant function itself [34, 35]. Structure coefficients are a more definitive coefficient for determining relative importance of the variables [35]. In this research, the structure coefficients are useful in providing us with a ranking of the variables, which reflects their relative importance to ESD.

The abundance data for the 170 variables (taxa) from the 102 lobster samples were analyzed by DA with PASW version 18 (IBM, Chicago, IL). In the first analysis, we compared the taxa on the cuticle from Diseased versus Healthy animals, the second analysis compared the taxa on the cuticle from Diseased animals versus Healthy-on-Diseased animals, and the third analysis compared the taxa on the cuticle from Healthy animals versus Healthy-on-Diseased animals.

2.4. Correlational network analysis

The computations described above are useful in identifying variables (bacterial taxa) that are likely candidates for determining the class of each sample, in this case, the state of health of the lobster being sampled. DA, however, does not account for the effects of one variable upon another. The members of a microbial community interact not only with their host or source of food but also with each other [36].

Network analysis is a statistical-graphical technique that has wide applications in fields as diverse as the social sciences and epidemiology and has gained some popularity among people working in "homeland security." Arguably the most common of these types of applications is social network analysis, which can be defined as a "study of human relationships by means of graph theory" [37].

Microbes occupying a biofilm are exposed to the biochemical output of their neighboring microorganisms. Some of these molecules are metabolic wastes of one microbe but serve as nutritive material for another, which can be viewed as metabolic cooperativity [36]. Members of a community also secrete antimicrobial agents in an effort to reduce competition, and still

other bacteria engage in quorum sensing, the detection of chemical signals as a means of monitoring population density [36]. It would be incorrect to equate human interactions with those of microorganisms, but it is arguably valid to recognize that biofilms exhibit a social structure that is mediated via biochemical signaling. Therefore, network analysis has the potential to elucidate microbial interactions within a biofilm. Within this study, the chemical interaction between various members of the biofilm was not directly investigated. Instead, we calculated the statistical correlations between bacterial taxa as a means of inferring their interactions and relationships.

The pyrosequencing abundance data were analyzed using Spearman rank correlation using a custom R module [38]. Spearman rank correlation is a nonparametric statistical function that allows the comparison of nonlinear data [36]. All possible correlation coefficients between variables are calculated, and correlations between variables are ranked in an output table. The parameters for the ranking were set as follows: $p \leq 0.01$, four minimum pairs per variable (taxa) and five minimum nonzero pairs.

The correlation tables were generated by the R module into the networking software Cytoscape [39] version 2.8.2 to construct correlational network diagrams of the three states of health of the lobster microbiome: Diseased, Healthy, and Healthy-on-Diseased. In addition, the data were used to construct correlation difference networks that plot edges between features whose correlations have significantly changed between the two states being compared. One can interpret these correlation difference networks as a depiction of what correlations were altered between the two states. The author constructed two such maps: Healthy versus Diseased and Healthy-on-Diseased versus Diseased.

3. Results

3.1. LH-PCR

Initially, the lobster carapace was surveyed to determine the bacterial community distribution on various regions of the carapace. **Figure 1** is a histogram of the normalized abundance of OTUs that were present on the various regions of the carapace (pink and red: cephalothorax, yellow: claws, green: abdomen, and tail: blue). The LH-PCR profiles revealed that, with a few exceptions, the OTUs present on the cephalothorax are representative of the microbiome present on other regions, such as the claw and the abdomen. Of the 29 OTUs identified, 22 are present on the cephalothorax, 17 are present on the claws, 17 are present on the tail (telson), and 13 are present on the abdomen. Using the Shannon index of diversity [40], the cephalothorax region had a index of 2.81 and an eveness score of 0.85. The claw region had an index value of 2.80 and a eveness score of 0.833. The tail had an index value of 2.37 and an eveness score of 0.75. The abdomen had an index value of 1.95 and a eveness score of 0.61.

The peaks in the fingerprints are described as OTUs, because this method alone does not identify the bacteria. In fact, a peak may contain more than one species, and strains of the same species may show up in different peaks. The normalized OTU abundance for each sample was

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plotted as a stacked histogram to demonstrate the distribution of the microbiome on the lobster carapace.

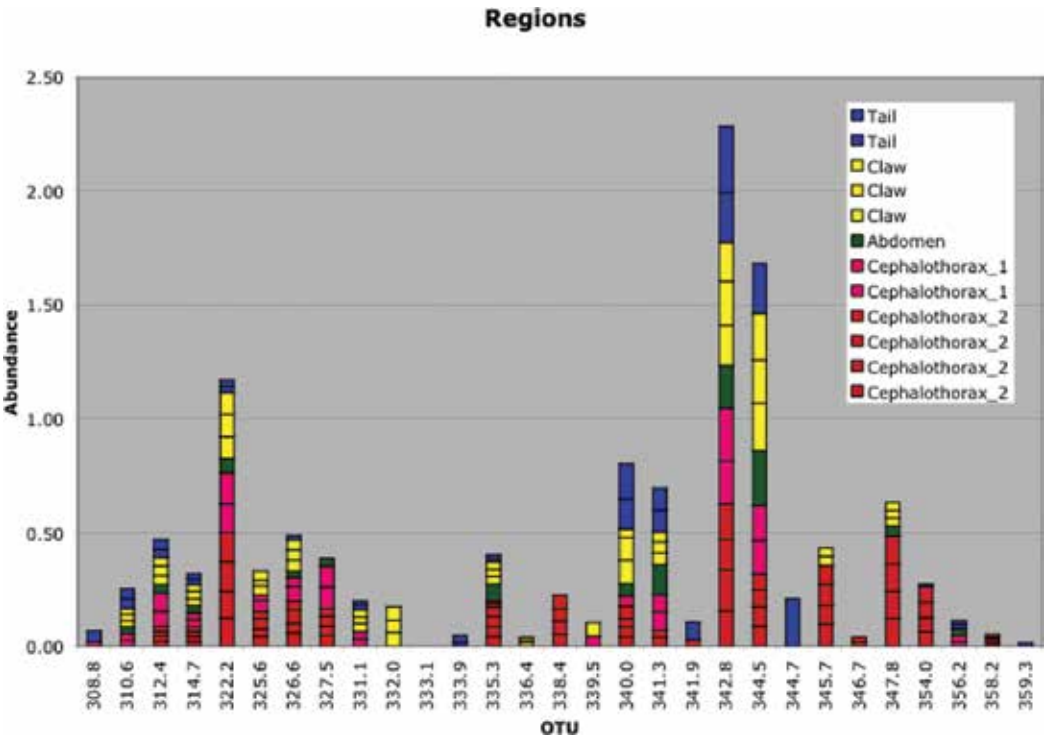

Figure 1. Replicate LH-PCR fingerprint analysis of various regions of the carapace. The normalized OTU abundance for each sample was plotted as a stacked histogram to demonstrate the distribution of the microbiome on the lobster carapace. Multiple bars of the same color represent OTU abundances from several samples of the same region of the shell. Therefore, OTU abundances from multiple samples may add up to greater than 100%.

3.2. MTPS

MTPS was performed on 102 lobster samples and the resulting sequence reads were sorted based on their tags or barcodes. The analysis yielded 212,019 reads with an average of 1594 reads per sample. We identified 170 bacteria present on the cuticle of lobsters from Narragansett Bay, Rhode Island, having culled taxa that were less than 1% of the total community under the *a priori* assumption that rare taxa will not contribute to the disease process. Of these 170 bacteria, 167 were identified to the level of genus, 1 was identified to the level of family, and 2 were identified as OTUs whose complete identities are unknown.

Figure 2 is a histogram of the average abundance of the taxa found in each sample class (i.e., Diseased, Healthy-on-Diseased, and Healthy) rank ordered by the average abundance of the Disease class (red bars). The entire histogram for all 170 taxa is depicted in the insert. The most abundant taxa found in the carapace microbiome include the genera *Jannaschia*, *Aquimarina*,

Cardiobacterium, *Thalassobius*, and *Loktanella* and the suborder *Micrococcineae*. However, essentially all genera are found in all disease classes (i.e., Healthy, Healthy-on-Diseased, and Diseased).

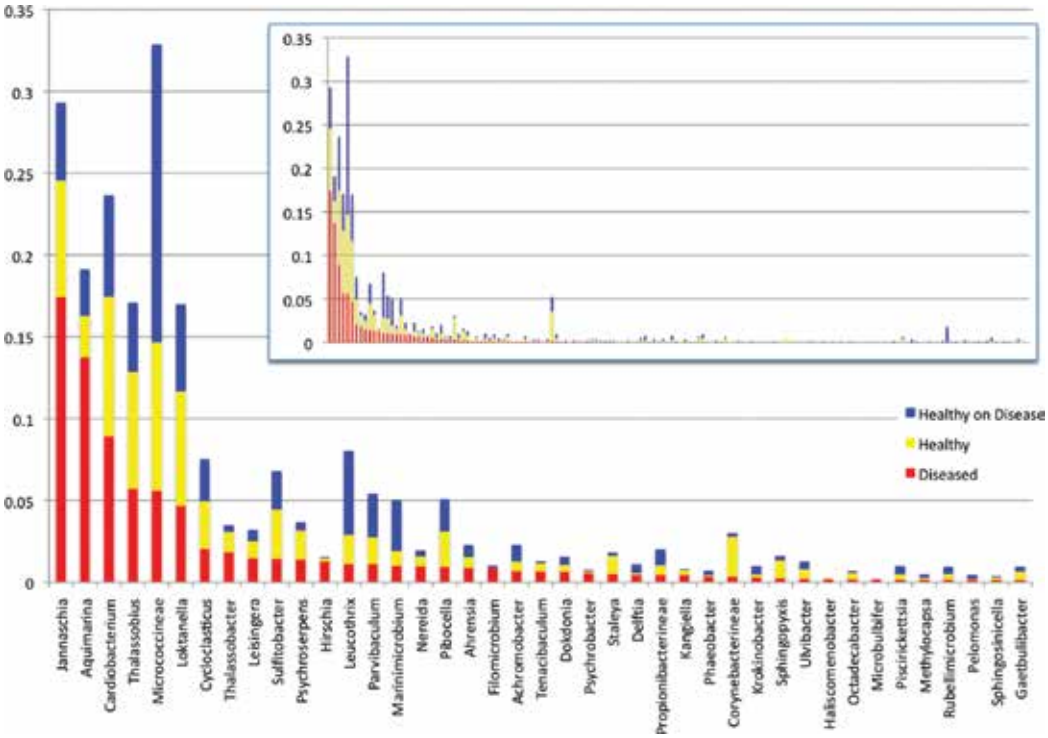

Figure 2. MTPS analysis of the cephalothorax microbiome. The normalized abundances of the most prominent taxa found on the cephalothorax of 102 lobsters is plotted as a histogram, rank ordered by those taxa in the disease state. The histogram of all 170 taxa is plotted in the insert.

Figure 3 is a neighbor joining tree that displays all of the members of the genus *Aquimarina* that were found in GenBank along with all the *Aquimarina* sequences found in these lobster samples. They are labeled according to their sample origin, and clades were labeled by the most abundant identified species in that clade. This tree is included here to illustrate the diversity of the genus and to demonstrate its ubiquity in samples of all three sampling classes of this study. There are three clades in that represent known *Aquimarina* species: *A. intermedia*, *A. mulleri*, and *A. laterculi*. We see an additional two clades that represent previously identified *Aquimarina* species. In addition, we can see that there are at least three clades of previously unidentified *Aquimarina* species (unlabeled clades). There are representatives of Healthy, Healthy-on-Diseased, and Diseased samples in all clades.

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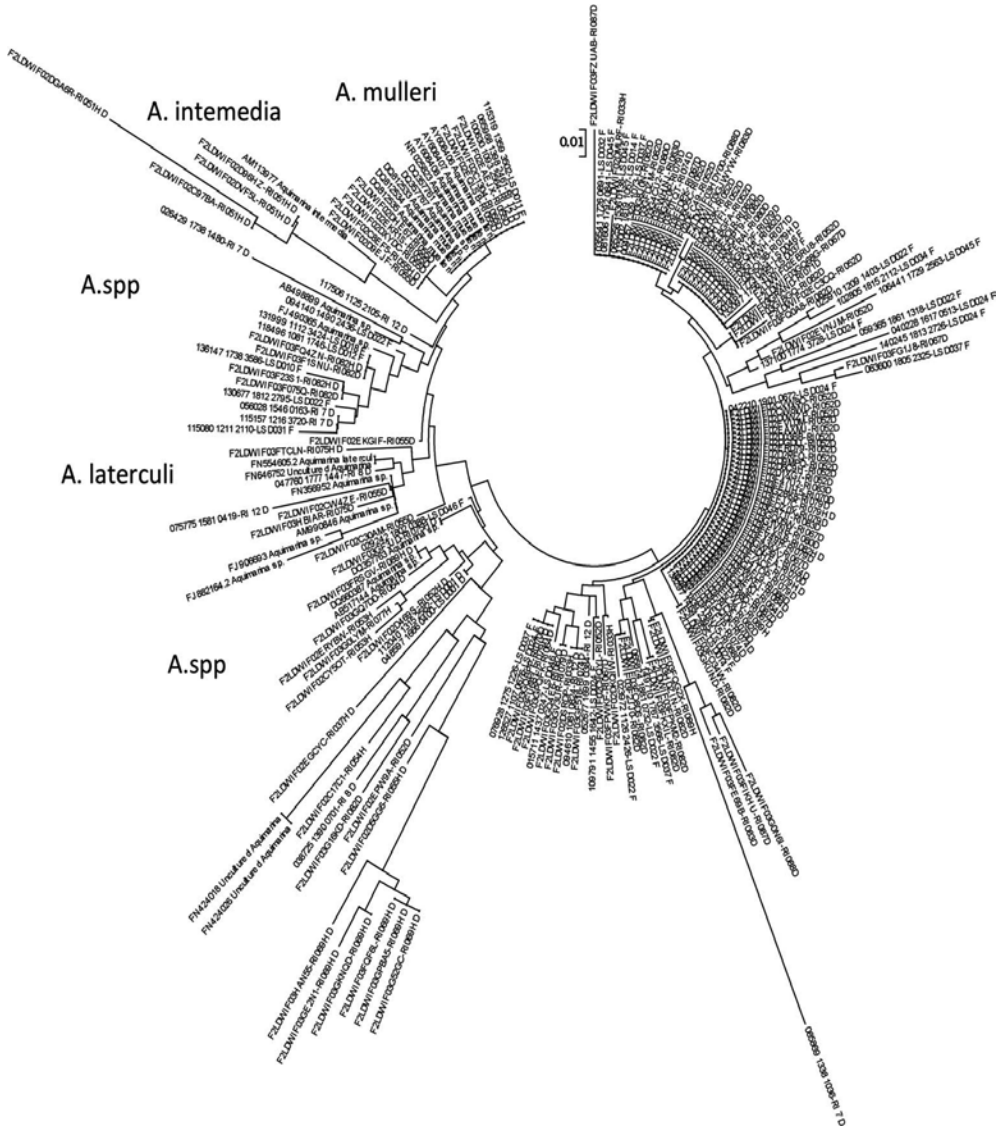

Figure 3. Neighbor joining tree of *Aquimarina* spp. identified in the lobster microbiome.

The weighted UniFrac tree is displayed in **Figure 4**. Although a cursory inspection of the figure may lead one to conclude that there is indeed a clustering of the three disease classes (Diseased, Healthy, and Healthy-on-Diseased), significance tests reveal that the differences between the three classes are not significant (Diseased vs. Healthy-on-Diseased: p=0.45, Diseased vs. Healthy: p=1.0, and Healthy-on-Diseased vs. Healthy: p=0.85). This demonstrates that, although there is some clustering, there is no clear separation between the three disease classes (Diseased, Healthy, and Healthy-on-Diseased), that is, they are more or less equally distributed throughout the neighbor joining tree.

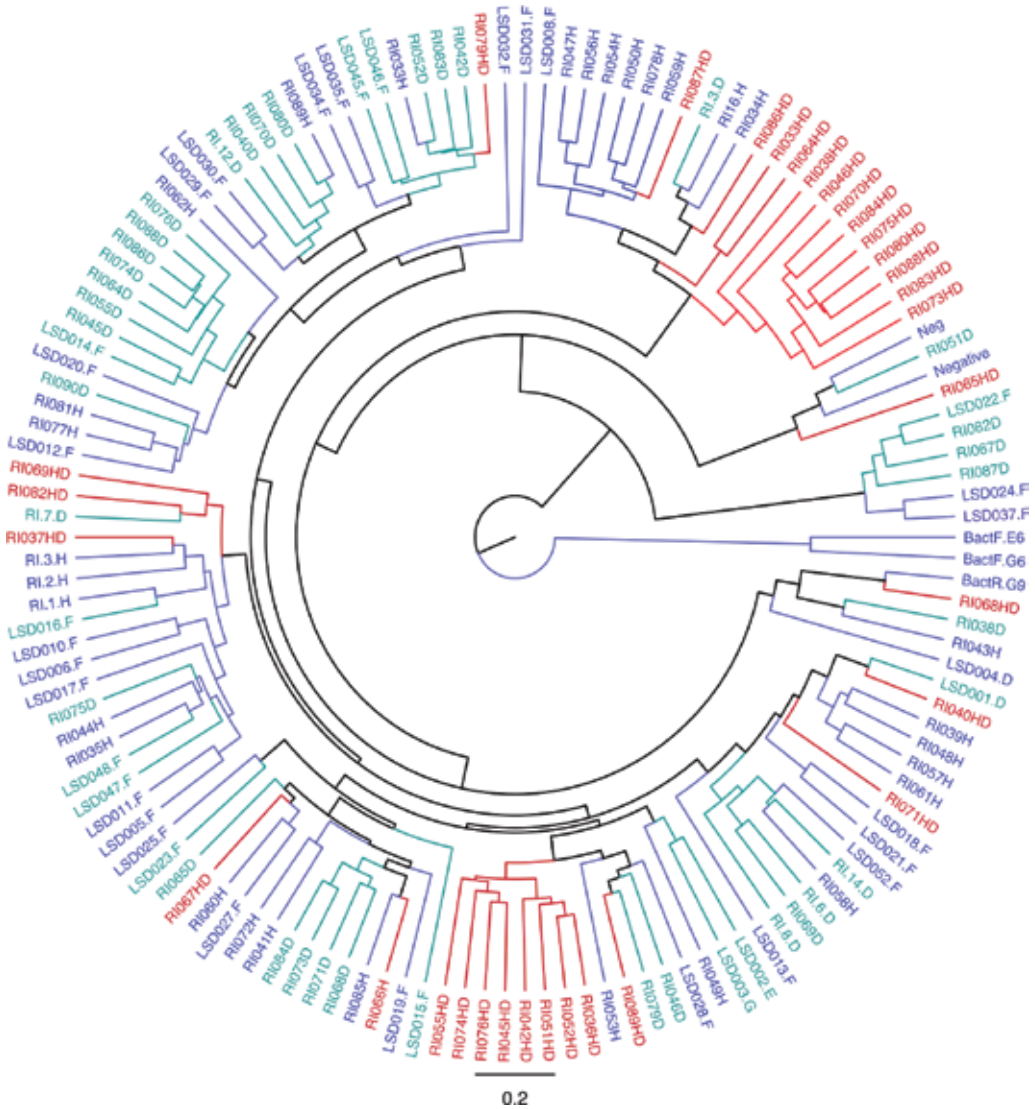

Figure 4. Weighted UniFrac tree. The disease classes are color coded. Healthy samples are blue (n = 47), diseased samples are green (n = 55), and healthy-on-diseased samples are red (n = 33).

Figure 5 is a principal coordinate analysis (PCO) of the normalized abundances of the genera identified in each disease class using a Bray-Curtis distance metric. The PCO performs an Eigen analysis that clusters the data based on the variance of all features (genera) of the data matrix [33]. One can see a clustering of the Diseased samples (red dots) that is somewhat distinct from the Healthy (yellow) and Healthy-on-Diseased (blue) samples.

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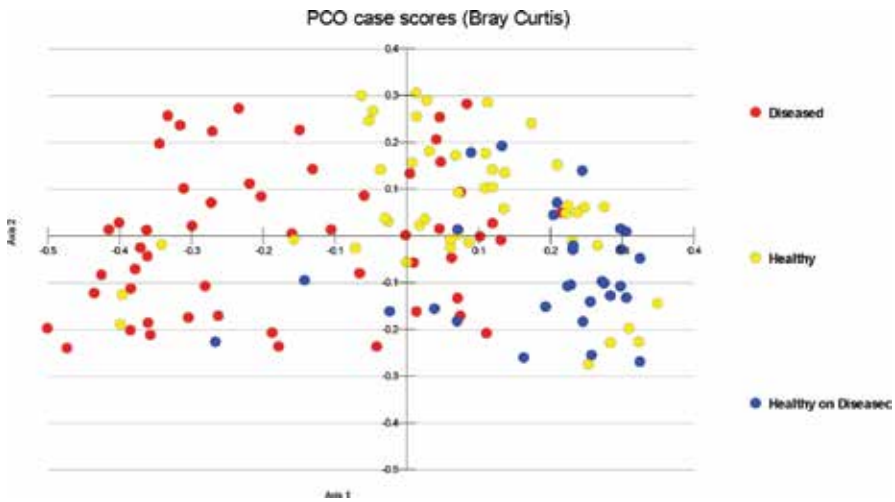

Figure 5. PCO of the normalized abundances of the genera from each disease class. The Healthy samples are yellow dots (n = 47), the Diseased samples are red dots (n = 55), and the Healthy-on-Diseased samples are blue dots (n = 33).

3.3. DA

The DA for the first comparison, the bacterial taxa on cuticle from Diseased versus apparently Healthy lobsters, included a tolerance test that eliminated 112 variables because they lacked variance between groups and thus did not significantly contribute to the discriminant function. Thus, the analysis between the two groups relied on 58 variables. The functions at the group centroids were 1.463 for disease class 1 (Diseased) and -1.1712 for disease class 2 (Healthy). The group centroids are a central measure of a set of multivariate data similar to the mean in univariate analyses. These scores demonstrate that the centroids were well separated. Such a separation between centroids demonstrates that the function is able to discriminate one class from another. The canonical correlation coefficient (R_c^2=0.848) is equivalent to the coefficient of determination (r^2) in univariate statistical analyses [35]. Wilks' λ (=0.281) is indicative of the contribution of the independent variable to the discriminant function. The lower the value of Wilks' λ is, the greater is the contribution. When Wilks' λ approaches 1, then the group means are nearly the same, and the contribution of the independent variable to the discriminant function approaches zero. The χ^2 test (χ^2=91.287; p=0.002; df=56) also indicates that the function is discriminating between classes [34]. In the second analysis (Diseased vs. Healthy-on-Diseased), the functions at group centroids were 2.060 for disease class 1 (Diseased) and -3.541 for disease class 3 (Healthy-on-Diseased). Again, these scores demonstrate that the function was discriminating between the classes (R_c^2=0.939; Wilks' λ=0.118; χ^2=131.382; p=0.000; df=47). In the third analysis (Healthy vs. Healthy-on-Diseased), the functions at group centroids were 2.439 for disease class 2 and -3.582 for disease class 3 (R_c^2=0.948; Wilks' λ=0.100; χ^2=122.991; p=0.000; df=47).

The classification results (confusion matrix) of all three analyses are displayed in **Table 1**. The confusion matrix is run as a test of the ability of the discriminant function to predict the

membership of the cases accurately. In the first analysis (Diseased vs. Healthy), the function predicted 94.1% of the original grouped cases correctly. The second analysis (Diseased vs. Healthy-on-Diseased) predicted 98.9% and the third analysis (Healthy vs. Healthy-on-Diseased) predicted 100% of original grouped cases correctly. This is further evidence that the respective functions for each of these comparisons are reliably discriminating between cases.

Classification results: Diseased vs. Healthy					
		Disease class	Predicted group membership		
			Diseased	Healthy	Total
Original	Count	Diseased	52	3	55
		Healthy	3	44	47
	%	Diseased	94.5	5.5	100.0
		Healthy	6.4	93.6	100.0
Diseased vs. Healthy-on-Diseased					
		Disease class	Predicted group membership		Total
			Diseased	Healthy-on-Diseased	
Original	Count	Diseased	54	1	55
		Healthy-on-Diseased	0	32	32
	%	Diseased	98.2	1.8	100.0
		Healthy-on-Diseased	0	100.0	100.0
Healthy vs. Healthy-on-Diseased					
		Disease class	Predicted group membership		Total
			Healthy	Healthy-on-Diseased	
Original	Count	Healthy	47	0	47
		Healthy-on-Diseased	0	32	32
	%	Healthy	100.0	0	100.0
		Healthy-on-Diseased	0	100.0	100.0

Table 1. DA confusion matrices.

Table 2 displays the standardized canonical discriminant coefficients for all three classes. These coefficients are semipartial coefficients that compare the relative importance of the variable to the independent variable. They are analogous to the β weights of regression analysis [34]. In this table, the variables with positive correlation coefficients are listed in descending order, whereas those with negative coefficients are listed in ascending order. This is done to reflect that the magnitude conveys the quantity of the correlation, and the sign indicates the quality (positive or negative). An inspection of this table reveals that only a small

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number of taxa have a number higher than 1, indicating that their correlation with the disease is not strong.

Diseased vs. Healthy		**Diseased vs. Healthy-on-Diseased**		**Healthy vs. Healthy-on-Diseased**	
Taxon	**Coefficient**	**Taxon**	**Coefficient**	**Taxon**	**Coefficient**
Anaerococcus	2.769	*Anaerococcus*	1.601	*Leucothrix*	1.082
Fluoribacter	2.613	*Leucothrix*	0.805	*Fluviicola*	1.064
Alkalilimnicola	2.031	*Jannaschia*	0.786	*Burkholderia*	1.045
Crenothrix	1.745	*Corynebacterineae*	0.748	*Corynebacterineae*	1
Burkholderia	0.831	*Cellulophaga*	0.747	*Turicibacter*	0.784
Pasteuriaceae Incertae Sedis	0.691	*Filomicrobium*	0.609	*Jannaschia*	0.708
Cellulophaga	0.472	*Aquimarina*	0.552	*Cycloclasticus*	0.705
Haliscomenobacter	0.433	*Crenothrix*	0.493	Carnobacteriaceae_1	0.647
Fluviicola	0.378	*Cycloclasticus*	0.456	Alishewane	0.63
Ahrensia	0.361	Glycomycineae	0.447	*Branhamella*	0.612
Jannaschia	0.359	*Hyphomicrobium*	0.413	*Erythrobacter*	0.607
Devosia	0.349	*Alkalilimnicola*	0.297	*Frankineae*	0.597
Kangiella	0.335	*Geothermobacter*	0.297	*Frateuria*	0.479
Frateuria	0.324	*Achromobacter*	0.244	*Fluoribacter*	0.443
Hyphomicrobium	0.296	*Leisingera*	0.213	*Lactococcus*	0.429
Hirschia	0.253	*Hirschia*	0.186	*Leisingera*	0.423
Aquimarina	0.249	*Haliscomenobacter*	0.114	*Erythromicrobium*	0.347
Glycomycineae	0.192	Caldilineacea	0.113	Flexithrix	0.344
Hydrogenovibrio	0.166	*Brumimicrobium*	0.106	Glycomycineae	0.293
Lacinutrix	0.164	*Achromatium*	0.103	*Algibacter*	0.24
Branhamella	0.159	Flexithrix	0.099	*Cardiobacterium*	0.229
Aminomonas	0.155	*Geopsychrobacter*	0.021	*Aquimarina*	0.19
Brumimicrobium	0.149	*Erythrobacter*	0.015	*Kaistia*	0.157
Oceanibulbus	0.122	*Agrobacterium*	0.012	*Hydrogenovibrio*	0.097
Kaistia	0.11	*Delftia*	-1.584	*Chrysiogenes*	0.037
Caldilineacea	0.101	*Dokdonia*	-0.684	*Haliscomenobacter*	-1.52
Algibacter	0.087	*Erythromicrobium*	-0.559	*Oceanibulbus*	-0.78
Leisingera	0.087	*Kangiella*	-0.474	*Abiotrophia*	-0.73
Colwellia	0.078	*Ahrensia*	-0.423	Acidimicrobineae	-0.578

Diseased vs. Healthy		Diseased vs. Healthy-on-Diseased		Healthy vs. Healthy-on-Diseased	
Taxon	**Coefficient**	**Taxon**	**Coefficient**	**Taxon**	**Coefficient**
Kordiimonas	0.052	Carnobacteriaceae_1	-0.395	*Kangiella*	-0.531
Geopsychrobacter	0.024	Pasteuriaceae Incertae Sedis	-0.392	*Hoeflea*	-0.505
Agrobacterium	0.011	*Chrysiogenes*	-0.384	*Devosia*	-0.448
Turicibacter	0.01	*Devosia*	-0.371	*Dokdonia*	-0.348
Delftia	-3.464	*Oceanibulbus*	-0.308	*Ahrensia*	-0.341
Flexithrix	-2.637	*Algibacter*	-0.301	*Cellulophaga*	-0.257
Chromatium	-1.586	*Kaistia*	-0.255	*Hirschia*	-0.246
Hoeflea	-1.11	*Gaetbulibacter*	-0.247	*Achromobacter*	-0.235
Chrysiogenes	-1.072	*Frankineae*	-0.23	*Crenothrix*	-0.228
Gaetbulibacter	-1.015	*Kordiimonas*	-0.207	Pasteuriaceae Incertae Sedis	-0.212
Erythrobacter	-0.867	Acidimicrobineae	-0.176	*Delftia*	-0.16
Frankineae	-0.78	*Burkholderia*	-0.176	*Alkalilimnicola*	-0.144
Geothermobacter	-0.771	*Lactococcus*	-0.162	*Hyphomicrobium*	-0.124
Dokdonia	-0.452	*Fluviicola*	-0.078	*Kordiimonas*	-0.082
Carnobacteriaceae_1	-0.429	*Fluoribacter*	-0.059	*Geothermobacter*	-0.051
Corynebacterineae	-0.378	*Lacinutrix*	-0.028	*Gaetbulibacter*	-0.031
Leucothrix	-0.267	*Cardiobacterium*	-0.018	*Lacinutrix*	-0.013
Cycloclasticus	-0.194			*Aminomonas*	-0.008
Acidimicrobineae	-0.191				
Filomicrobium	-0.161				
Lactococcus	-0.15				
Erythromicrobium	-0.147				
Cardiobacterium	-0.143				
Alishewane	-0.119				
Achromobacter	-0.01				

Table 2. Standardized canonical discriminant coefficients for all three classes.

Table 3 displays the structure coefficient table. The structure coefficients are full coefficients, meaning that they are pooled as within-groups correlations between the independent variable and the standardized canonical discriminant coefficients [34]. According to Klecka [35], the structure coefficient is the authoritative coefficient for determining the importance of the independent variable to the discriminant function. As with the standardized canonical

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discriminant coefficient, the absolute value represents the quantity and the sign indicates quality of the correlation. In the case of structure coefficients, however, the largest absolute is 1.0. If a taxon had a correlation close to +1, then it could be identified as a cause of the disease. An examination of the table reveals that *Aquimarina* spp. have a structure coefficient of 0.268, indicating that it has a weak correlation with the function. *Aquimarina* ranks second in the structure coefficient table to the genus *Jannaschia*, which has a structure coefficient of 0.325. This table has been arranged to display a descending order of importance, with the positive coefficients listed first. In the case of the negative coefficients, they appear to be listed in ascending order because the absolute value is indicative of a stronger correlation. These weak correlations indicate that there is no one pathogen that correlates definitively with the disease.

Diseased vs. Healthy		**Diseased vs. Healthy-on-Diseased**		**Healthy vs. Healthy-on-Diseased**	
Taxon	**Coefficient**	**Taxon**	**Coefficient**	**Taxon**	**Coefficient**
Jannaschia	0.325	*Jannaschia*	0.315	*Jannaschia*	0.248
Aquimarina	0.268	*Aquimarina*	0.156	*Leisingera*	0.127
Hirschia	0.173	*Cycloclasticus*	0.125	*Corynebacterineae*	0.113
Oceanicola	0.152	*Hirschia*	0.112	*Frankineae*	0.112
Methylosarcina	0.148	*Corynebacterineae*	0.076	*Leucothrix*	0.094
Schineria	0.148	*Filomicrobium*	0.073	*Cycloclasticus*	0.09
Shigella	0.143	*Cardiobacterium*	0.066	*Hirschia*	0.063
Tenacibaculum	0.142	*Leisingera*	0.059	*Kaistia*	0.062
Terasakiella	0.141	*Leucothrix*	0.055	*Aminomonas*	0.057
Filomicrobium	0.139	*Haliscomenobacter*	0.051	*Crenothrix*	0.057
Thalassobacter	0.125	*Kaistia*	0.043	*Hoeflea*	0.054
Microbulbifer	0.105	Glycomycineae	0.043	*Erythrobacter*	0.052
Photobacterium	0.104	*Burkholderia*	0.043	*Cardiobacterium*	0.048
Lacinutrix	0.099	*Frankineae*	0.04	Glycomycineae	0.045
Streptococcus	0.092	*Brumimicrobium*	0.03	*Hydrogenovibrio*	0.045
Woodsholea	0.089	*Geopsychrobacter*	0.03	*Frateuria*	0.044
Vibrio	0.073	*Agrobacterium*	0.03	*Fluviicola*	0.044
Haliscomenobacter	0.065	*Fluviicola*	0.03	*Haliscomenobacter*	0.044
Ruegeria	0.063	*Achromatium*	0.03	*Lactococcus*	0.036
Psychrobacter	0.06	Caldilineacea	0.03	*Chrysiogenes*	0.035
Roseovarius	0.058	Flexithrix	0.03	*Chromatium*	0.031
Shinella	0.058	*Alkalilimnicola*	0.03	*Branhamella*	0.031
Fluoribacter	0.058	*Chromatium*	0.03	*Alkalilimnicola*	0.031
Ralstonia	0.058	*Colwellia*	0.03	Flexithrix	0.031

Diseased vs. Healthy		Diseased vs. Healthy-on-Diseased		Healthy vs. Healthy-on-Diseased	
Taxon	**Coefficient**	**Taxon**	**Coefficient**	**Taxon**	**Coefficient**
Carnobacteriaceae_2	0.058	*Anaerococcus*	0.03	Alishewane	0.031
Curvibacter	0.058	*Hoeflea*	0.03	*Burkholderia*	0.031
Schlegelella	0.058	*Delftia*	0.025	*Turicibacter*	0.031
Diaphorobacter	0.058	*Hyphomicrobium*	0.025	Carnobacteriaceae_1	0.019
Pseudomonas	0.058	*Ahrensia*	0.023	*Ahrensia*	0.013
Asticcacaulis	0.058	*Lactococcus*	0.021	Acidimicrobineae	0.011
Stenotrophomonas	0.058	*Lacinutrix*	0.019	*Delftia*	0.007
Staphylococcus	0.058	*Crenothrix*	0.015	*Algibacter*	0.004
Colwellia	0.058	*Dokdonia*	0.001	*Aquimarina*	-0.002
Methylobacterium	0.058	*Achromobacter*	-0.274	*Abiotrophia*	-0.004
Hyphomicrobium	0.058	*Cellulophaga*	-0.191	*Dokdonia*	--0.016
Achromatium	0.058	*Chrysiogenes*	-0.131	*Achromobacter*	-0.018
Geopsychrobacter	0.058	*Abiotrophia*	-0.116	*Cellulophaga*	-0.046
Brumimicrobium	0.058	*Fluoribacter*	-0.115	*Hyphomicrobium*	-0.046
Spirochaeta	0.058	*Erythrobacter*	-0.109	*Lacinutrix*	-0.046
Caldilineacea	0.058	*Algibacter*	-0.091	*Erythromicrobium*	-0.063
Agrobacterium	0.058	*Hydrogenovibrio*	-0.079	*Fluoribacter*	-0.066
Cellulophaga	0.058	Carnobacteriaceae_1	-0.068	*Gaetbulibacter*	-0.069
Anaerococcus	0.058	Acidimicrobineae	-0.052	*Geothermobacter*	-0.072
Roseobacter	0.049	*Erythromicrobium*	-0.052	*Oceanibulbus*	-0.09
Nereida	-0.263	*Geothermobacter*	-0.046	*Kordiimonas*	-0.103
Glycomycineae	-0.236	*Oceanibulbus*	-0.042	*Devosia*	-0.118
Silicibacter	-0.206	*Kordiimonas*	-0.037	Pasteuriaceae Incertae Sedis	-0.169
Stappia	-0.179	*Gaetbulibacter*	-0.015	*Kangiella*	-0.258
Cycloclasticus	-0.177	*Devosia*	-0.013		
Sulfitobacter	-0.175	Pasteuriaceae Incertae Sedis	-0.013		
Phaeobacter	-0.173	*Kangiella*	-0.004		
Cloacibacterium	-0.172				
Erythromicrobium	-0.165				
Salinibacter	-0.153				
Carnobacteriaceae_1	-0.15				

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Diseased vs. Healthy		Diseased vs. Healthy-on-Diseased		Healthy vs. Healthy-on-Diseased	
Taxon	**Coefficient**	**Taxon**	**Coefficient**	**Taxon**	**Coefficient**
Branhamella	-0.143				
Turicibacter	-0.141				
Leadbetterella	-0.132				
Geothermobacter	-0.124				
Alishewane	-0.12				
Hyphomonas	-0.115				
Nannocystaceae	-0.113				
Comamonas	-0.11				
Algibacter	-0.11				
Piscirickettsia	-0.11				
Fluviicola	-0.101				
Propionibacterineae	-0.099				
Chrysiogenes	-0.099				
Gp4	-0.096				
Crenothrix	-0.095				
Leucothrix	-0.095				
Methylocapsa	-0.094				
Krokinobacter	-0.092				
Frateuria	-0.09				
Zobellia	-0.088				
Sphingomonas	-0.088				
Rubellimicrobium	-0.086				
Kaistia	-0.083				
Maribacter	-0.081				
Gaetbulibacter	-0.078				
Hoeflea	-0.068				
Hydrogenovibrio	-0.068				
Acidimicrobineae	-0.068				
Saccharophagus	-0.068				
Meganema	-0.068				
Aminomonas	-0.068				
Nitrospira	-0.068				
Thiorhodospira	-0.068				

Diseased vs. Healthy		Diseased vs. Healthy-on-Diseased		Healthy vs. Healthy-on-Diseased	
Taxon	**Coefficient**	**Taxon**	**Coefficient**	**Taxon**	**Coefficient**
Thioclava	-0.068				
Rhodomicrobium	-0.068				
Sphingopyxis	-0.068				
Pibocella	-0.068				
Winogradskyella	-0.068				
Rhodobaca	-0.063				
Nitrosospira	-0.063				
Microvirga	-0.06				
Erythrobacter	-0.06				
Corynebacterineae	-0.059				
Frankineae	-0.051				
Micrococcineae	-0.05				

Table 3. Structure coefficient table.

For the second comparison of the bacterial taxa on the cuticle of Diseased lobsters versus Healthy cuticle on Diseased lobsters, the functions at the group centroids were well separated with a value of 2.060 for disease class 1 (Diseased) and -3.541 for disease class 3 (Healthy-on-Diseased; R_c^2=0.939; Wilks' λ=0.118; χ^2=131.382; p<0.001; df=47). As in the first comparison, these data indicate that the function is discriminating between the classes.

Jannaschia spp. appears to have roughly the same structure coefficient as in the previous analysis (0.315). *Aquimarina* spp. has a structure coefficient of 0.156.

Although these are the taxa with the highest positive structure coefficients, they are considerably less than 1. This indicates that, although they do discriminate for the disease compared to Healthy-on-Diseased samples, they do so weakly. *Aquimarina* spp. discriminated more weakly between Diseased and Healthy-on-Diseased classes than between Diseased and Healthy classes. This might indicate that, as a Healthy lobster converts to a Diseased lobster, the abundance of *Aquimarina* spp. increases. There would be more of these bacteria present on the unblemished surface of a lobster with ESD compared to Healthy lobsters but still less than in the lesions.

For the third comparison of the bacterial taxa on the cuticle of Healthy lobsters versus Healthy cuticle on Diseased lobsters, the discriminant functions at the group centroids demonstrated a good separation with values of 2.439 for disease class 2 (Healthy) and -3.582 for disease class 3 (Healthy-on-Diseased; R_c^2= 0.948; Wilks' λ=0.100; χ^2=122.991; p<0.001; df=47). In this analysis, *Aquimarina* spp. have a structure coefficient of -0.002, which indicates that it correlates weakly and negatively in discriminating between carapace from lobsters that show no

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signs of the disease and carapace samples without lesions that are taken from lobsters that have the disease.

3.4. Correlational network analysis

Figure 6 is the network diagram of bacteria from the lobsters identified as Diseased. The edges (connecting links) between the nodes (taxa) are either red (negative correlation) or blue (positive). The width of the edge corresponds to the magnitude of the correlation coefficient. An inspection of the map reveals that the genera *Aquimarina* and *Jannaschia* are negatively correlated with several other taxa. *Jannaschia* is positively correlated with *Thalassobacter* and *Thalassobius* and, by extension, is negatively correlated with *Cardiobacterium*. *Jannaschia* is also negatively correlated with *Uvibacter*, *Pibocella*, and *Leucothrix*. *Aquimarina* is also negatively correlated with *Leucothrix* as well as with *Loktanella* and *Micrococcineae*.

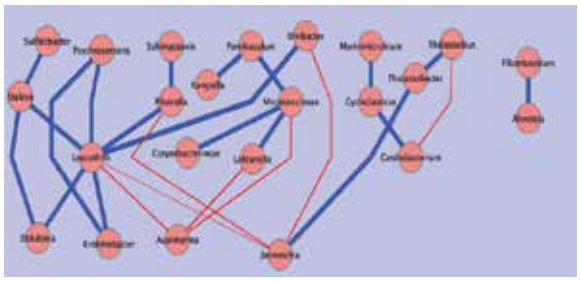

Figure 6. Correlational network map of the Diseased microbiome.

Figure 7 is the network diagram of bacteria from the lobsters identified as Healthy. In this diagram, *Corynebacterineae* appear to occupy a central point that connects most of the nodes and is negatively correlated with *Cycloclasticus* and *Cardiobacterium* but positively correlated with *Maribacter*, *Propionibacterineae*, *Frankineae*, and *Micrococcineae*. *Cardiobacterium* also appears to occupy a hub-like position and is negatively correlated with *Micrococcineae* and positively correlated with *Cycloclasticus* and *Psychroserpens*. There is a secondary cluster that is disconnected from the larger one. In this disconnected cluster, *Ahrensia* occupies a central position between *Erythrobacter*, *Sphingocinella*, and *Parvibaculum*. *Erythrobacter* and *Parvibaculum* are also linked to form a clique with *Ahrensia*, and *Erythrobacter* is also linked to *Sphingopyxis*, which in turn is linked to *Rubellimicrobium*. All of these taxa are positively correlated with one another. There are also three unconnected dyads. *Leucothrix* is associated with *Crenothrix*, *Thalassobacter* is associated with *Nereida*, and *Ulvibacter* is associated with *Marinimicrobium*. The pairs in all three dyads are positively correlated with one another.

Figure 8 represents the Healthy-on-Diseased microbiome correlations. In this map, there are five unconnected clusters. The largest consists of four taxa in which all are positively correlated. The connection is an unbranched chain with *Micrococcineae*, *Marinimicrobium*, *Algibacter*, and *Cycloclasticus* in sequence. There are two triads that do not form a complete clique. *Ulvibacter*, *Rubellimicrobium*, and *Ahrensia* are all positively correlated. In the second triad,

Cardiobacterium is negatively correlated with *Leucothrix*, which is positively correlated with *Nitratireductor*. There are two dyads in which all taxa are positively correlated: *Thalassobius* and *Loktanella* form one and *Krokinobacter* and *Piscrikettsia* form the second.

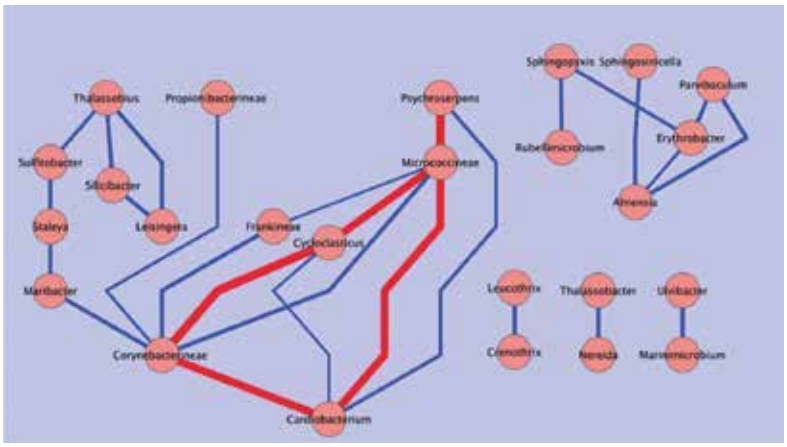

Figure 7. Correlational network map of the Healthy microbiome.

Comparing the three maps, the average clustering coefficient of the Diseased microbiome is 0.289, the Healthy microbiome has an average clustering coefficient of 0.337, and the Healthy-on-Diseased microbiome has an average clustering coefficient of 0.0. Clustering coefficients are the ratios of the actual number of connections in comparison to the total possible connec-

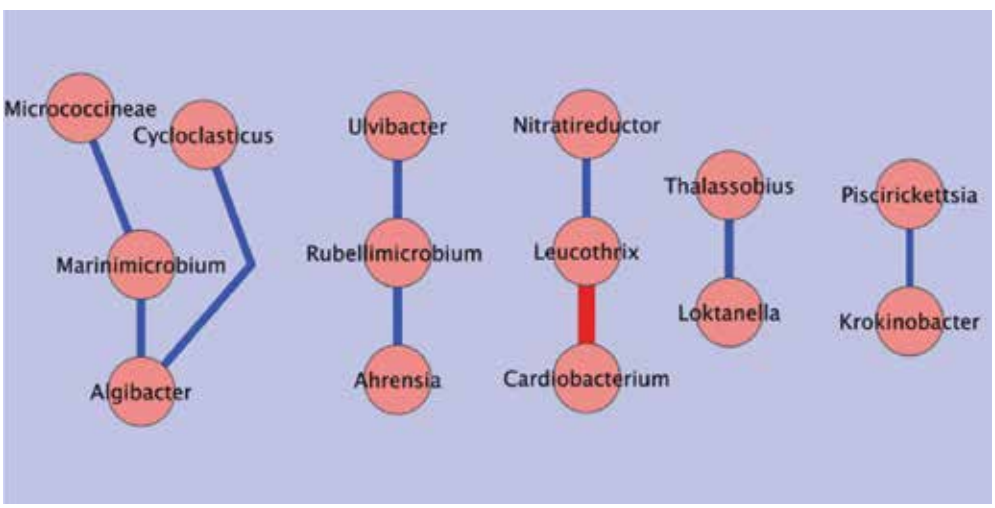

Figure 8. Correlational network map of the Healthy-on-Diseased microbiome.

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tions and range from 0 to 1 [41]. In other words, the clustering coefficient is the probability that one node is connected to another.

Figure 9 is a correlational difference network diagram that compares Healthy and Diseased microbiomes. This map displays taxa whose correlation coefficient changes significantly from Healthy to Diseased class. They are color coded to reflect the nature of the change. Red edge color indicates that the taxa correlate positively with the Healthy state and negatively with the Diseased state. Blue indicates that they correlate with both states positively but have a larger positive correlation coefficient in the Healthy state. Green indicates a negative correlation with both states but a larger negative correlation coefficient with the Healthy state.

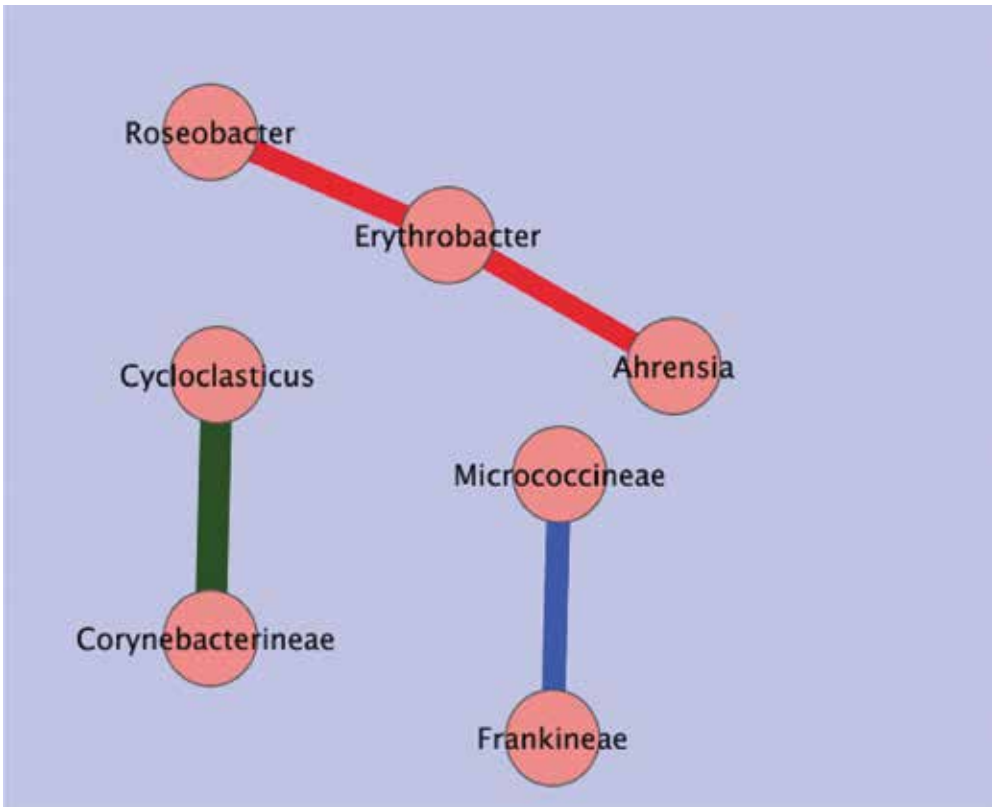

Figure 9. Correlational difference map of the Healthy versus Diseased microbiomes.

The network consists of three disconnected groups of taxa. *Ahrensia*, *Erythrobacter*, and *Roseobacter* are connected in one group. *Erythrobacter* bridges this triad, and they all are positively correlated to the Healthy state and negatively correlated to the Diseased state. *Frankineae* and *Micrococcineae* form a dyad that has a higher positive correlation with the

Healthy state than the Diseased state. *Cycloclasticus* and *Corynebacterineae* form the second dyad that correlates negatively with both states but more negatively with the Healthy state. Neither *Aquimarina* nor *Jannaschia* appears in this correlation difference network, suggesting that the observed correlation between these two taxa in the Diseased state (**Figure 6**) was not statistically different from the Healthy state. Correlational difference maps illustrate differences in the microbial community when comparing one state (disease class) to another. If there is no significant difference in the correlations between taxa when one state is compared to the other, then those taxa will not be visible on the diagram.

Figure 10 is a correlational difference diagram that compares Healthy-on-Diseased to Diseased microbiomes. The color coding is the same as **Figure 9**.

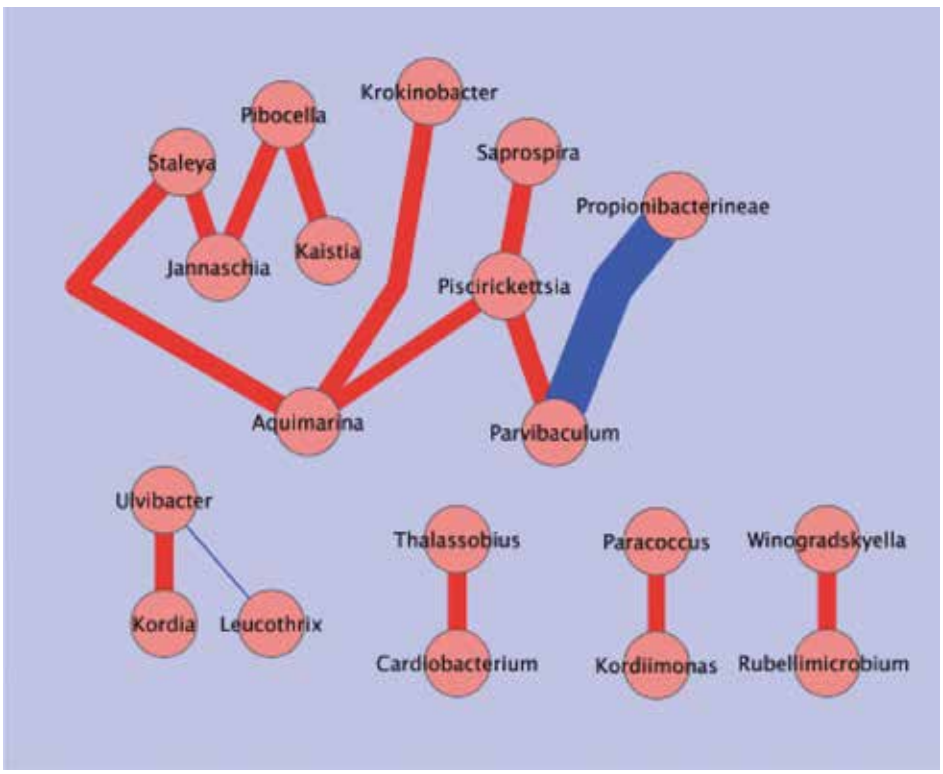

Figure 10. Correlational difference map of the Healthy-on-Diseased versus Diseased microbiomes.

This network is more complex, with a large group consisting of 10 taxa, 3 dyads, and 1 triad, suggesting that a number of potentially pathogenic processes or interactions are different in the Healthy-on-Diseased biofilm that do not occur in the Healthy biofilm. Within the large group, *Aquimarina* is connected to three taxa: *Staleya*, *Krokinobacter*, and *Piscirickettsia*. Following the first branch, *Staleya* is connected to *Jannaschia*, which is connected to *Pibocella*

and *Kaistia* in a linear fashion. *Krokinobacter* is a terminal end of its branch, and *Piscirickettsia* connects to two taxa: *Saprospira* and *Parvibaculum*. To this point, the edge color of this group is red, indicating that all are positively correlated with the Healthy-on-Diseased state and negatively with the Diseased state. *Parvibaculum* connects to *Propionibacterineae* with a blue-colored edge, indicating that this edge-node combination correlates positively more positively with Healthy-on-Diseased than with Diseased.

The triad, similar to the triad in the Healthy versus Diseased correlational difference map, forms an incomplete clique [37], with *Ulvibacter* bridging between *Kordia* and *Leucothrix*. Between *Ulvibacter* and *Kordia*, the red edge color reveals that they correlate positively with the Healthy-on-Diseased state but negatively with the Diseased state. The second edge of the triad, however, between *Ulvibacter* and *Leucothrix*, is colored blue. The three dyads have red edge colors. They consist of the pairs *Thassobius* and *Cardiobacerium*, *Paracoccus* and *Kordimonas*, and *Windgradskyella* and *Rubellimicrobium*.

4. Discussion and conclusion

Smolowitz et al. [3] found that, although more advanced cases of ESD presented with extensive lesions throughout the dorsal surface, most cases were restricted to the cephalothorax.

The bacterial diversity as measured by the Shannon index appears to be roughly similar on all regions, with the claw and cephalothorax regions having the most similar indices (see **Figure 1**). Smolowitz et al.'s observations that ESD lesions were more prevalent on the upper cephalothorax, however, influenced the decision that a further molecular study would be focused on this region of the carapace. Furthermore, as the taxa distribution is relatively similar in all regions, we would be unlikely to miss key taxa by focusing on the cephalothorax.

As mentioned previously, the inset histogram (**Figure 2**) represents 170 taxa that were identified and at a normalized abundance greater than 1%. The most abundant taxa include the genera *Jannaschia*, *Aquimarina*, *Cardiobacterium*, *Thalassobius*, and *Loktanella* and the suborder *Micrococcineae*.

Jannaschia are characterized as rod-shaped, nonmotile, Gram-negative, catalase-positive, strict aerobes that do not reduce nitrate to nitrite and found to use a wide variety of carbon sources but not chitin. Their growth appears to be restricted to seawater and cannot be cultured in the absence of seasalts [42].

Aquimarina are described as rod-shaped bacteria that possess gliding motility. They are Gram-negative, catalase-positive, strict aerobes and are capable of degrading chitin. They are also known to produce various flexirubin and carotenoid pigments. Similar to *Jannaschia*, *Aquimarina* appear to be dependent on seawater to grow [43].

Cardiobacterium are described exclusively in the literature as human pathogens implicated in endocarditis and peritonitis. They are also found as normal constituents in human nasal and oral cavity microflora. They are catalase-negative, Gram-negative pleomorphic bacilli. They

are facultative anaerobes that ferment carbohydrates but do not reduce nitrate to nitrite. Although implicated in serious infections, members of this genus are not exceptionally virulent (a large inoculate is necessary to initiate an infection) [44].

Thalassobius are Gram-negative, strict aerobes that do not produce pigments and tend to favor organic acids as a carbon and energy source rather than carbohydrates. Some members of the genus exhibit motility; others do not [45].

Loktanella are Gram-negative, strict aerobes that do not use carbohydrates but can degrade urea, Tween 80, citrate, and aesculin [46].

The suborder *Micrococcineae* includes 15 families that collectively include more than 90 genera as well as 4 genera that have not been classified as members of a family. This group is a metabolically diverse, Gram-positive taxon [47]. What this specific OTU represents is unclear from these data.

These taxa do conform, however, to what has been observed about ESD. There is some, but not extensive, degradation of chitin. In these six genera, only one genus (*Aquimarina*) is observed to be chitinovorous. As mentioned above, there is high lipase activity in lesions [14], and lipids appear to be the preferential carbon and energy source of *Loktanella*. There are generalists in the group, such as *Jannaschia*, and there are those that use organic acids, including amino acids (*Thalassobius*). The presence of a fermenter, such as *Cardiobacterium*, could indicate that anaerobic conditions develop concomitantly with the progression of the lesions.

Bell et al. [14] found that ectohydrolase activity differed within the biofilms of lobsters from the geographic areas with high ESD incidence compared to lobsters inhabiting areas of little to no ESD. This could indicate that there is a shift in metabolic activity of the bacteria that are present or could indicate a shift in population.

The neighbor joining tree (**Figure 3**) of *Aquimarina* species reveals that the genus is present in all samples. There is no emergent pattern of members of the genus that correlates with either state of health. These data suggest that members of the genus *Aquimarina* are ubiquitous but do not eliminate them as opportunists if there is an underlying susceptibility in the lobster. This supports the dysbiosis model of ESD. A discrete pathogenic microorganism does not cause the disease; rather, the bacterial infection is the result of a change in the biofilm, either in community structure or in metabolic activity, which could have a different underlying cause. Laufer et al. [16], as explained in Section 1, found that lobsters with ESD had higher tissue concentrations of alkylphenols than did lobsters that did not present with the disease. Other studies of the same lobsters used in this study also found a higher incidence of idiopathic ailments such as hepatopancreatitis and ocular lesions in Rhode Island lobsters compared to their Maine counterparts [15], and other researchers found differences in bacterial ectohydrolase activity on the surface of lobsters from these distinct regions [14]. This suggests that there may be environmental conditions present in Rhode Island waters that are causing the native lobsters to be more susceptible to disease.

Researchers have implicated a novel species (*A. homaria*) in the initiation of a laboratory-induced form of shell disease [12]. The author's investigation found no evidence of this specific

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species in any of the samples, although we did find related species. Furthermore, the induced shell disease studied here does not conform to the histological profile that has been described for ESD [12].

An inspection of the UniFrac data that are depicted by the tree (**Figure 4**) reveals that, although there is some clustering, the lobsters identified as Healthy and Diseased show enough similarity that they exist as neighbors on all of the major branches of the tree. There is no statistically significant difference between the three classes as demonstrated by the enumeration of their *p* values. Again, this supports the hypothesis that this disease is not caused by a discrete pathogen.

The PCO reveals much the same pattern; although there are identifiable regions, there is an overlap between the classes.

Our original simple hypothesis is that there is a significant difference between the bacterial communities in the Healthy and Disease states, indicating that this difference is associated with the etiology of the disease. The null hypothesis asserts that the bacterial communities are similar on the Healthy and Diseased lobsters. The phylogenetic metrics employed to compare the microbiomes of subjects in this study indicate that there is no significant difference between the three classes, which leads us to accept the null hypothesis and reject the original hypothesis. However, these data suggest that, although there may not be a gross difference between the microbiomes of Healthy and Diseased lobsters, there is a subtle shift that requires further investigation and the definition of a more complex hypothesis. A major avenue of inquiry would be to determine whether this shift is one of the metabolic activities among essentially the same bacteria, if there is an emergence of some members of the population that displace other extant members, or if there are new recruits to the community from outside the biofilm. Wahl et al. [48] suggested the use of emergent techniques, such as desorption electrospray ionization-mass spectrometry, as a means of surveying the surface of the biofilm to elucidate the metabolic compounds that are present in biofilms. Coupled with confocal microscopy techniques, as suggested by Costerton [24], researchers could begin to develop a spatial representation of the lobster surface microbiome that would include both the identity of the bacterial cells and the metabolic activity that was occurring in their vicinity.

From the perspective of disease etiology, this kind of shift may point to a cause other than bacterial. If the same microflora, albeit in different abundances, are found on lobsters that have the disease as well as on those that are apparently healthy, then perhaps this dysbiosis represents a sign of some kind of disorder that is caused by something other than bacteria. Laufer et al. [16] established a correlation between lobsters with ESD and high tissue concentrations of alkylphenols. As noted earlier, these compounds retard the cross-linking of tyrosine moieties in the carapace, which makes shell hardening take longer. Tarrant et al. [21] found evidence to suggest that gene expression in lobsters affected by ESD might indicate increased exposure to xenobiotics. Homerding et al. [20] found that lobsters living in the geographic area with a high incidence of ESD presented with reduced immunocompetence. This evidence supports an assertion that ESD is not primarily a bacterial disease but that bacterial lesions are a manifestation of a systemic dysfunction.

Kunkel et al. [10] discovered that lobsters with ESD had a loss of calcification in and around the disease lesions, particularly in the trabecular structures that are composed of apatite. One question that needs to be investigated further is whether the decalcification is related to the presence of alkylphenols. Could exposure of the underlying structure to the environment lead to decalcification? Alternatively, could acidic metabolic products of bacterial action produce the same results?

To use an analogy, suppose there is a house whose roof is defective. Imagine the nails were weak and could not hold the protective components of the roof in place, similar to the protein-chitin structures in the lobster carapace in which the tyrosine cross-linking was not complete. Rainwater would leak into the underlying structure, analogous to seawater getting into the trabecular apatite of the lobster carapace. In both cases, this would physically weaken the underlying structure. In both cases, normal microflora would have access to parts of the structure from which they would normally be excluded and may start to use these energy and carbon sources. This would further weaken and disfigure the structure and increase the likelihood of a breach in the outer protective structure. In this analogy, it is not the microflora that causes the problem in the roof. Rather, the rot that would ensue from its invasion is the result of the ineffectiveness of barriers whose function is to keep these organisms out of the underlying structure. In the case of ESD, perhaps the bacterial lesions are not the cause of the disease but merely the inevitable result of structural weaknesses in the lobster carapace. Additionally, the trabecular apatite and the other calcium moieties present in the lobster cuticle may have antimicrobial properties themselves [10]. Their physical dissolution could be the result of the intrusion of acidified seawater, as there is now evidence that ocean acidification is affecting organisms that produce calcareous shells [49]. The absence calcium moieties from the site of lesions could represent an additional reduction in the ability to exclude microbiota from the exoskeleton.

Although there have been other studies of biofilms related to crustaceans [50, 51], this represents the first survey of the surface microbiome of the lobster that uses high-throughput, culture-independent molecular techniques. As mentioned above, it is by no means an exhaustive analysis of the lobster microbiome, as the subjects of this study are geographically restricted and are not a large enough size to yield a global generalization. It is comprehensive enough, however, to demonstrate that, although there is no gross difference between the microbiomes of lobsters with and without ESD in Rhode Island samples, there may be a subtle shift in the microbial population that correlates with dysfunction. As mentioned previously, Bell et al.'s work indicates a variance in the metabolic activity of bacteria in eastern Long Island Sound, an area of high ESD occurrence, compared to those found on the surface of lobsters in regions that have little to no ESD incidence [14]. This shift may be more closely related to a shift in activity rather than of population.

Considering the first DA analysis (Diseased vs. Healthy), the canonical correlation coefficient (R_c^2) value of 0.848 indicates a high correlation of the discriminant function and the groups [34]. The second and third analyses have lower values. This indicates that the 58 bacteria that were retained after the initial tolerance test are useful in discriminating between disease classes. Presumably, the 112 bacteria that were rejected because their abundan-

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ces are similar in both classes are not contributing to the disease state. The structure matrix indicates that the genus *Aquimarina* is weakly correlated with the Diseased state. Its structure coefficient is second only to the genus *Jannaschia*.

This analysis supports the affirmative hypothesis that there is a difference between the microbial taxa in shell-diseased lobsters compared to healthy lobsters. The study identifies 58 bacteria that are significantly different between the two classes and rejects 112 that do not contribute to discrimination between them. The relatively low correlation coefficients for these 58 taxa, however, do indicate that they are present on all three classes but in slightly different abundances.

This analysis also provides additional evidence that ESD in the American lobster correlates with dysbiosis rather than the presence of a single overt pathogen. In a disease caused by a single pathogen, one would expect a structure coefficient of one of the variables to approach 1. An inspection of the structure coefficients reveals that none of the bacterial taxa have a coefficient greater than 0.325.

The role of *Aquimarina* spp. in the disease lesions is unclear. In the DA that compared the bacteria on cuticle from Diseased animals to Healthy cuticle from Diseased animals, *Aquimarina* spp. had a smaller structure coefficient (0.156) than that on Diseased versus apparently Healthy animals (0.268). One possible interpretation is that, if *Aquimarina* spp. were present at the initiation of the disease, then it should be strongly negatively correlated with the Diseased cuticle bacteria. Instead, the genus correlates weakly and positively, indicating that it discriminates the Diseased state more strongly than the pre-Diseased state. Moreover, in the analysis of taxa from Healthy lobsters versus Healthy cuticle on Diseased lobsters, the structure coefficient of *Aquimarina* spp. was negative and the lowest value of all the structure coefficients in the structure coefficient table. This indicates a weakly negative correlation. If this bacterial taxon was definitive in the initiation of the disease, a more likely scenario would be that it would correlate strongly with the unaffected surface of a Diseased lobster and would therefore have a more negative coefficient compared to the surface of Healthy lobsters.

This analysis supports the observations that were articulated above. The weak correlations of some of the taxa with the three states of health reinforce the assertion that, although there are differences in the abundances of these taxa when comparing these three states, none of them are exclusive to one or the other state. The weak correlations also demonstrate that this is not a disease caused by a discrete pathogen, which would have a much stronger correlation. The works of the author's colleagues within the New England Lobster Health Initiative strongly suggest that there are underlying causes that render some lobsters, particularly within the waters of eastern Long Island Sound and Rhode Island, susceptible to an opportunistic infection [10, 14–16, 21]. Exposure to abnormally high levels of endocrine-disrupting compounds such as alkylphenols appears to fit the profile of intervening agents, which is described by Laufer et al. and Tarrant et al.; not only are they capable of eliciting a response consistent with an endocrine-disrupting compound, but they also appear to retard proper shell development. Hitherto, there is a correlation, but no direct evidence, linking alkylphenol exposure to ESD. Obtaining such evidence would require an experiment in which a group of lobsters was exposed to alkylphenols, whereas a control group would be kept in an alkylphe-

nol-free environment. As discussed previously, however, captive lobsters are more susceptible to impoundment shell diseases [5]. Designing an experiment that would mitigate such confounding results is likely to prove challenging.

The abundance tables, as well as the DA computations reported in the previous chapter, reveal that *Aquimarina* and *Jannaschia* are more abundant (and correlate positively in DA) when the Diseased and Healthy states are compared. An inspection of the correlation network of the Diseased microbiome indicates that these two genera are negatively correlated with several of the other taxa present, indicating that, although these two are more abundant in the Diseased state, most of the others are reduced in abundance, with the exception of *Thalassobacter* and *Thalassobius*, which are positively correlated with *Jannaschia*. The average clustering coefficient for the Diseased microbiome was 0.289. For the Healthy microbiome, the coefficient was 0.337. Both of these are well below 1; therefore, neither network demonstrates a great deal of connectivity or interaction between members of the community. With a difference in the coefficients of less than 0.05, the difference between the two is negligible. In the Healthy-on-Diseased microbiome, the average clustering coefficient is 0.0, indicating that the associations between the taxa are essentially random.

The correlational difference networks may shed some light on the role of *Aquimarina* and, for that matter, *Jannaschia*. Neither can be found in the correlational difference network map that compared Healthy and Diseased lobsters. In the comparison of Healthy-on-Diseased to Diseased, however, both appear as taxa that correlate positively with the Healthy-on-Diseased state but negatively with the Diseased state. This may indicate that both are early opportunists in the lesion formation and there is a shift in their function when they move to the Diseased state.

The only data used in these correlational networks were those derived from MTPS of sample lobsters from the "100 Lobsters" Project [28]. As described in Section 1, samples from these lobsters were used in other investigations, and the data obtained have been recorded [14, 16, 20]. Integrating these data by means of correlational network analysis with alkylphenol concentrations in the tissues and ectohydrolases on the surface of the same lobster, for example, may produce a more definitive correlation between ESD and alkylphenols and may allow researchers to correlate which bacterial taxa are involved in what metabolic activity.

When comparing the data obtained using previous methods, a slightly different picture emerges. Taxa such as *Aquimarina* and *Jannaschia* appear to correlate with ESD, albeit weakly. In the DA analysis, they serve as reliable factors in discriminating cases of the disease. They appear, however, to have somewhat weaker interactions with other constituents of the biofilm as demonstrated by these correlational diagrams.

As stated above, the hypotheses addressed are as follows:

H: The bacterial communities on lobsters with ESD are significantly different in quality and quantity than unaffected lobsters.

HA: The bacterial communities on the carapaces of healthy and shell-diseased lobsters are similar.

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There are no major shifts in the microbiome between the Healthy and Diseased states, so we accept the alternative hypothesis and reject the original simple hypothesis. However, there are minor differences in the microbiomes of Healthy and Diseased lobsters. Furthermore, the microbiomes of lesion-free carapace on Diseased lobsters exhibited different microbiome compositions. However, the microbiomes of all three classes of the Diseased state that were identified here were similar enough that they occupied the same branches on a weighted UniFrac tree, and the difference between the microbiomes was determined to be statistically insignificant. Although there appears from these data to be a subtle difference between the microbiomes of samples from these three sample classes, the difference is not enough to fully support the original simple hypothesis. We must move forward with a more complicated hypothesis where environmental factors play a major role in the etiology of the disease. As such, we can start to define ESD as a complex environmental disease.

Arguably one of the most important advances in health and disease prevention has been the recognition that multiple pathogens are the cause of many diseases and it is now well accepted that these "polymicrobial disease" are in fact quite common [52]. As the body of knowledge has increased and we have extended our limits of detection and sequencing throughput, a growing number of diseases and syndromes have emerged that have been shown to be polymicrobial in nature. ESD is an example of one such polymicrobial disease.

This research has employed culture-independent techniques coupled with multivariate statistical treatment of the resultant data that present a shift in the lobster microbiome that correlates with the disease. This phenomenon is defined here as a dysbiosis. Although this research has elucidated this subtle shift in the microbiome of the lobster, it has not addressed the etiology of the disease. Indeed, it is not clear that the bacterial manifestations of this disease are anything more than a proximal cause. Researchers have successfully induced a condition that resembles ESD in captive lobsters but under extraordinary conditions. The evidence of involvement of alkylphenol contamination, for example, is circumstantial at this point. A direct evidence might be obtained through controlled experimentation as long as the confounding effects of captivity can eliminated.

The bulk method of extracting bacterial DNA from the lobster samples is itself confounding to the process of understanding the disease. Removing the biofilm and extracting the microbiomic DNA in bulk fashion precludes the interrogation of the spatial aspect of the biofilm. If microbes are cooperatively harvesting material and energy from a site, understanding their positional relationship may be useful. In addition, the nature of bulk extraction is that a relatively large amount of microbiota is captured. Such an approach can obscure members of the microbial community whose importance to the ecological function is disproportional to their relative abundance. It may be that minority members of the community, when observed in their spatial context within the biofilm, play a pivotal role in metabolic function. In the research described here, these bacteria may have been discarded due to insufficient abundance. *In situ* techniques, such as the use of laser capture microdissection on prepared sections of ESD lesions, coupled with DNA extraction of captured microbes, might shed some light on the spatial arrangements of the biofilm. In addition, visualization of intact biofilm colonies using confocal microscopy [24] combined with *in situ* hybridization techniques could

round out the picture in a way that would be the best of systems biology. In addition, the emergent field of metabolomics, the study of the net metabolic effects of epibiotic communities, would shed light on the changes in metabolic activity of a dysbiotic shift [48]. It is possible that what is described here is not a change in microbial organisms but merely a change in metabolic output of the same actors.

As noted previously, this research did not subdivide the disease lesions based on severity. Doing so would have yielded a more complete picture of the disease process and may have provided us with more insight into the roles of *Aquimarina* and *Jannaschia* and other taxa in the colonization of the lesions. A larger sample size, with more geographic diversity, would also be advantageous to increasing statistical confidence.

In response to the ESD crisis, a group of scientists, fisheries managers, and lobstermen formed the New England Lobster Health Initiative. It was this ad hoc committee that ultimately received funding that was awarded to several university and institutional researchers, including the award that funded what is reported here. The findings of each group were published as a special edition of the *Journal of Shellfish Research* [22]. Included in that edition was an article that summarizes some of what is contained herein [23]. A synthesis of all the research is summarized in the final article of the journal [53]. This research contributed a broad molecular-based survey that helped integrate studies such as those that elucidated immune response [20], potentially pathogenic microorganisms [12], gene expression [21], and xenobiotics [16]. Although the integration of these data is far from complete, there is sufficient evidence to suggest that ESD may represent a dysbiotic shift whose etiology could be alkylphenol intoxication.

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Section 4

Biofilm Control

Chapter 18

Adverse Influences of Antimicrobial Strategy against Mature Oral Biofilm

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Additional information is available at the end of the chapter

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Abstract

Antimicrobial measures, such as topical antiseptics and local drug delivery, have proven effective as complements to mechanical control. However, recent investigations have reported some adverse influences of antimicrobial strategy.

One possible negative reaction is that residual structure may serve as a scaffold for redevelopment of biofilm. It is reported that no or little biofilm structure was removed when oral biofilms were treated with chemical compounds and that the secondary adhesion was promoted in the presence of residual structure.

Second, residual structure may also act as pathogens. It is well known that various microbial components in the biofilm can play a role in disease pathogenesis, even if the microorganisms in the biofilm are completely killed.

Third, low-dose antibiotics may promote bacterial biofilm formation. The short-time exposure of chemical agents will cause gradient of concentration inside biofilm. In this case, the cells in deeper area may be exposed to subminimal inhibitory concentrations (sub-MICs) of antimicrobial agents. Recent studies have demonstrated that a variety of antibiotics or antimicrobial agents at sub-MIC levels can induce biofilm formation *in vitro*, interfering with bacterial biofilm virulence expression.

This chapter reviews studies demonstrating adverse influences of antimicrobial strategy against mature oral biofilm.

Keywords: oral biofilm, antimicrobial agent, residual structure, sub-MIC, stress response

1. Introduction

Mechanical approach by procedures such as self-performed oral hygiene, scaling and root planning (SRP), or periodontal surgery is fundamental in the control of mature oral biofilms [1]. Chemical approaches such as topical antiseptics, local drug delivery, and systemic antibiotics are used with the expectation of producing an adjunctive effect [2–5]. In fact, it has been demonstrated that adjunctive antimicrobials improve clinical parameters, including plaque index, gingival inflammation, and probing pocket depth [3, 5–7]. It has also been reported that antiplaque biocides do not cause the microbial resistance and alterations of microbial flora [8].

However, recent investigations have demonstrated that antimicrobial compounds do not work as intended [9–12. Especially in short-time exposure, the antimicrobials failed to penetrate into deeper area inside biofilm. Wakamatsu et al. have reported the penetration kinetics of mouthrinses into *in vitro Streptococcus mutans* biofilms by direct time-lapse microscopic analysis. The antimicrobial penetration was critically restricted within 30 s of exposure; the average penetration velocity was ranging from 4.2 to 30.1 μm/min [13]. This phenomenon can be explained by retarded penetration due to degradation and/or modification by the biofilm matrix. Extracellular polymeric substance (EPS) produced by microorganisms make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix that reduces antimicrobial penetration [14,15]. Representative four models of how these polymer strands might interact are shown in **Figure 1** [16]. Panel A is the alginate paradigm. Calcium forms a complex with negatively charged polymer strands. Panel B shows tight adhesion of a negatively charged polymer and a positively charged polymer. Panel C indicates an insoluble polymer. Polymer complex formation is probably driven by hydrogen bonding or hydrophobic interactions. Panel D indicates that bacteria have surface receptors

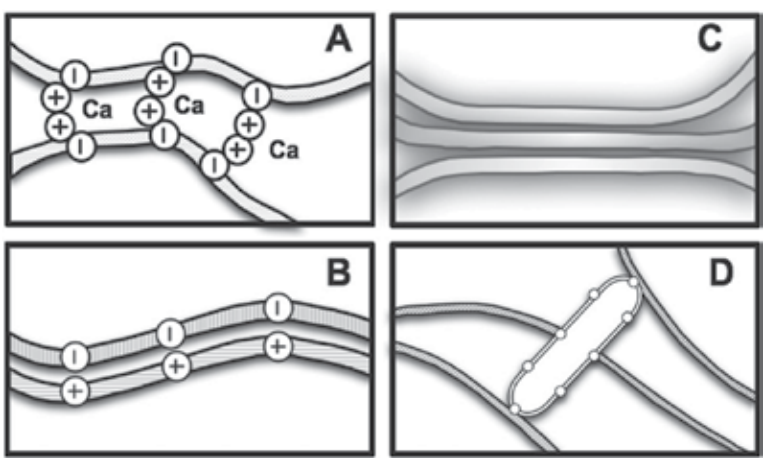

Figure 1. Conceptual models of matrix cohesion. (A) Alginate paradigm. Calcium is cross-linked between alginate. (B) Adhesion of a negatively charged polymer and positively charged polymer. (C) Hydrogen bonding or hydrophobic interaction. (D) Bacteria are partially cross-linked to the matrix. Reproduced from Takenaka et al. [16] with permission.

that bind to the EPS strands and partially cross-link them to the matrix. Diffusion limitation arises readily in these polymer strands because the fluid flow is reduced and the diffusion distance is increased in the biofilm mode of growth [17]. On the other hand, prolonged antimicrobial stress causes the biofilms facilitating the spread of antibiotic resistance by promoting horizontal gene transfer [18]. The existence of tolerant or dormant cells is critical factor in chronic infection [19, 20] (**Figure 2**).

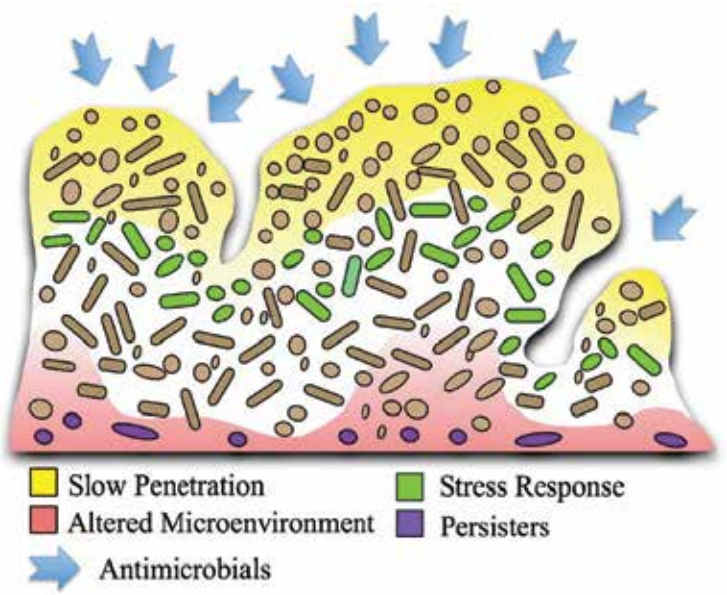

Figure 2. Mechanisms of biofilm tolerance. Antimicrobial penetration is retarded in the presence of EPS (yellow). The some microorganisms in the biofilm change activity in response to antimicrobial stress (green). The microenvironment in deeper area is altered to resist eradicating (pink). Persister cells are present in higher concentration in biofilm (violet). This image was modified from CBE Image Library by the Center for Biofilm Engineering at Montana State University.

This chapter is focusing to the studies demonstrating adverse influences of antimicrobial strategy against mature oral biofilm.

2. Adverse influences of antimicrobial strategy

2.1. Residual structure

Recent investigations have demonstrated that chemical disinfection for oral biofilm may leave intact biofilm structures. We performed a direct time-lapse microscopic observation throughout continuous exposure of commercial mouthrinses to an oral biofilm model [10]. Consequently, no removal of biomass was observed in control, ethanol (EtOH), 0.12% chlorhexidine gluconate (CHG), and Biotene, which contains lysozyme, lactoferrin, lactoperoxidase, glucose

oxidase, and potassium thiocyanate, even after 20 min exposure. Treatments with CHG and EtOH resulted in only a slight contraction of the biofilm (**Figure 3**).

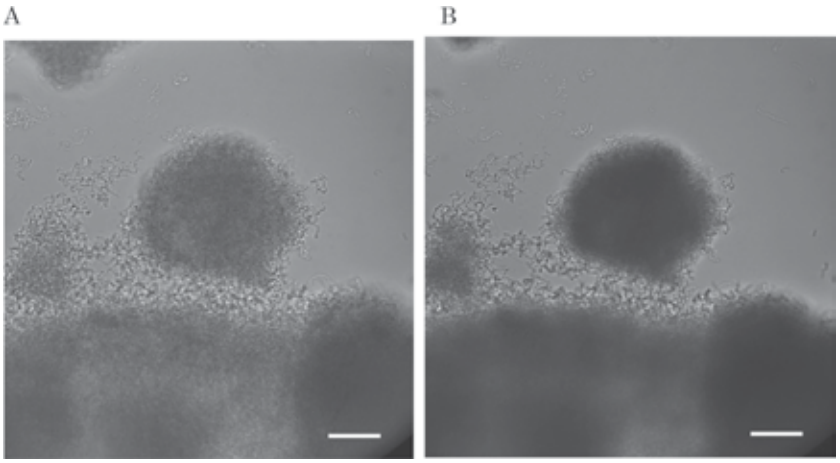

Figure 3. Transmission images of biofilm cluster before (A) and after (B) 0.12% chlorhexidine (CHG) treatment. The biofilm was exposed to CHG continuously inside glass capillary biofilm reactor for 20 min. Scale bar, 30 μm. Reproduced from Takenaka et al. [10] with permission.

Davison et al. investigated the dynamic antimicrobial action of chlorine, a quaternary ammonium compound, glutaraldehyde, and nisin within biofilm cell clusters of *Staphylococcus epidermidis* using time-lapse confocal scanning laser microscopy [21]. Chlorine among these chemicals was the only antimicrobial agent that caused any biofilm removal. Yamaguchi et al. showed that treatment of *Porphyromonas gingivalis* biofilms with CHG for 5 min does not degrade their external structure, or reduce the volumes of protein and carbohydrate constituents [22]. A summary of representative experiments demonstrating that chemical approach failed to detach the biofilm structure is shown in **Table 1**.

Bacterium	Experimental design	Incubation time	Antimicrobial agent	Exposure time	Judgment	Reference
Multispecies (*Streptococcus oralis*, *Streptococcus gordonii*, *Actinomycesnaeslundii*)	Flow-cell	20h	11.6% EtOH 0.12% CHG Biotene	20 min	Microscopic observation (transmission image)	[10]
Multispecies (*Streptococcus oralis*, *Streptococcus gordonii*, *Actinomycesnaeslundii*)	Flow-cell	20h	40% EtOH 0.1% SLS 0.03% TRN 0.12% CHG 0.05% CPC 0.005% nisin	60 min	Microscopic observation (transmission image)	[12]

Bacterium	Experimental design	Incubation time	Antimicrobial agent	Exposure time	Judgment	Reference
Streptococcus mutans	Glass-based dish	24h	0.12% CHG EO CPC IPMP	5 min	Microscopic observation (transmission image)	[13]
Staphylococcus epidermidis	Flow-cell	24h	0.14mM QAC 0.5mM Glutaraldehyde 14.9μM nisin	60 min	Microscopic observation (transmission image)	[21]
Porphyromonasgingivalis	Chambered coverglass	24h	0.05 to 0.2% CHG	5min	Microscopic observation (transmission image), Quantitative analysis of protein and carbohydrate composition	[22]

EtOH: ethanol; CHG: chlorhexidinegluconate; SLS: sodium lauryl sulfate; TRN: triclosan; CPC; cetylpyridinium chloride; IPMP: isopropyl methyl phenol; QAC: quaternary ammonium compound

Table 1. A summary of representative experiments demonstrating that chemical approach failed to detach the biofilm structure.

In contrast, there are some reports that the biofilm structure has been successfully degraded by repeated exposures of mouthrinse [23–25]. Although it is likely that biofilm reduction may be enhanced by repeated pulse of a mouthrinse, this approach may not always be effective. Pratten and Wilson have reported that anaerobic counts in dental plaque biofilm returned to pretreatment levels with altered bacterial composition after 4 days, despite the continuous pulsing of CHG [26].

Summarizing the above, these results suggest that chemical approach such as the mouthrinse, especially without repeated use, may not be sufficient to eradicate oral biofilm structure. Residual structure may cause adverse effects in oral environment, even if the microorganisms in the biofilm are completely killed.

2.1.1. Antigen and host inflammatory reaction

As the remaining biofilm matrix contains carbohydrates, proteins, polysaccharide, lipids, and nucleic acid [27], dead bacteria and biofilm components could work as antigens and induce inflammatory reactions.

For example, *Actinobacillus actinomycetemcomitans*, *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola* have been implicated in the development of various forms of periodonti-

tis. An extensive review of the literature revealed that lipopolysaccharide or outer membrane lipids, polysaccharide, fimbriae and outer membrane, and secreted proteins are antigens of all four bacteria that may play a role in disease pathogenesis [28].

In addition, even if the microorganisms in the biofilm are completely eradicated, various microbial components in the biofilm could play a role in disease pathogenesis. Augustin et al. reported that injection of dead components of *Enterococcus faecalis* into rats following mechanical aortic damage by a catheter produced endocarditic vegetation enriched with polymorphonuclear cells [29]. Bacterial components have also been attracted considerable attention as an adjuvant. It has been reported that injection of structural components of the outer surface membrane led a variety of immunopotentiative actions following the activation of phagocytes and leukocytes [30–32].

2.1.2. Calculus formation

The remaining dental biofilm structure will absorb calcium and phosphate from saliva for the formation of supragingival calculus and from crevicular fluid for the formation of subgingival calculus. Calculus formation begins with the deposition of kinetically favored precursor phases of calcium phosphate, octacalcium phosphate, and dicalcium phosphate dihydrate, which are gradually hydrolyzed and transformed into less soluble hydroxyapatite and whitlockite mineral phases [33].

The calculus surface may not in itself induce inflammation in the adjacent periodontal tissue [34, 35]. Jepsen et al. stated that periodontal healing occurs even in the presence of calculus as long as the bacteria is removed or disinfected [34]. For example, it has been reported that autoclaved calculus does not cause pronounced inflammation or abscess formation in connective tissues [36]. Listgarten et al. have demonstrated that a normal epithelial attachment can be formed on its structure when microorganisms on calculus surface were completely disinfected with CHG [37]. Johnson et al. investigated the clinical outcomes of treatment with locally delivered controlled-release doxycycline (DH) or SRP in adult periodontitis patients. Treatment with either DH or SRP resulted in significant statistical and clinical improvements in clinical attachment levels, pocket depth, and bleeding on probing. These clinical outcomes were equivalent regardless of the extent of subgingival calculus present at baseline, suggesting that positive clinical change depend on altering the subgingival biofilm rather than the removal of calculus [38].

However, calculus is known to be a plaque retention factor as well as a reservoir for toxic bacterial products and antigens. Histological section of a human tooth root showed that calculus is covered with viable bacterial plaque [34]. Nichols et al. reported that the dihydroceramide lipids produced by *P. gingivalis* were found in subgingival calculus [39]. Hence, the presence of calculus will be a secondary etiological factor.

2.1.3. Scaffold for secondary bacterial adhesion

Recent investigations revealed that residual structure would promote a secondary bacterial adhesion and biofilm redevelopment [22, 40]. Yamaguchi et al. compared the volume of *P.*

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gingivalis adherent with the residual biofilm developed in saliva-coated well following a CHG treatment for 5 min using a confocal laser microscopy [22]. The volume of *P. gingivalis* adhering to the residual structure was greater than that in saliva-coated wells. This result indicates that the residual biofilm could serve as a scaffold for the secondary biofilm formation. Outer membrane vesicles produced by *P. gingivalis* promote autoaggregation and coaggregation of another bacterial species [41, 42]. In addition, they also enhance the attachment to and invasion of epithelial cells by *T. forsythia* [43].

Our research group has demonstrated that residual structure of *S. mutans* biofilm following complete disinfection favors secondary bacterial adhesion and biofilm redevelopment [40]. At first, *S. mutans* biofilm generated on a resin-composite disc in a rotating disc reactor was disinfected completely with 70% isopropyl alcohol, and returned to the reactor. The same bacterial strains in the logarithmic phase were then flowed into the reactor for 4 h. The amount of secondary adhered cells on the remaining structure was compared with that on a disc without structure using confocal laser scanning microscopic (CLSM) analysis and quantitative analysis. Three-dimensional reconstruction revealed that viable bacteria appear to get caught to upstream edges of disinfected biofilm structure (**Figure 4**). The cryosectioned sample demonstrated stratified patterns of viable cells beside the structure. Mean viable count adhered on the structure was significantly higher than that on plane surface. This result showed that

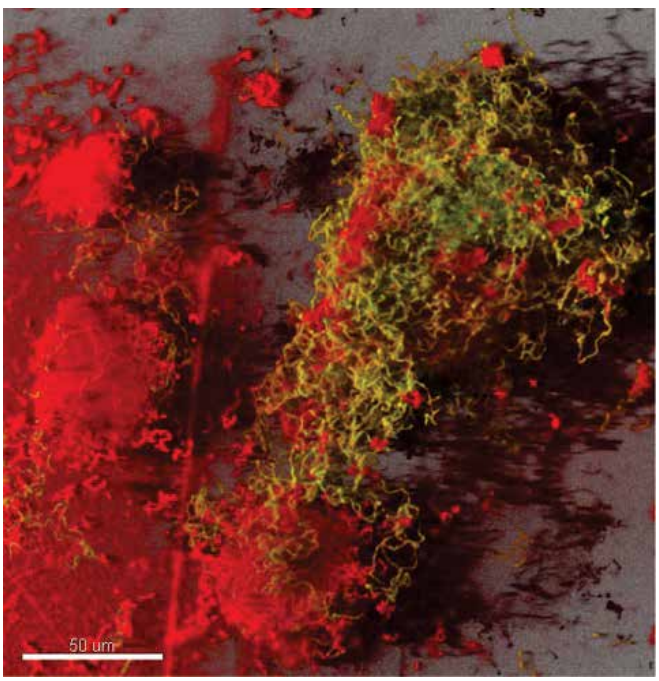

Figure 4. Three-dimensional reconstructed images of 4-h secondary biofilm (green) on disinfected 72-h biofilm structure (red). Fresh planktonic *S. mutans* cells flowed into the completely disinfected 72-h biofilm structure for 4 h. Viable bacteria were stained green by calcein fluorescence and appeared to get caught in upstream edges of disinfected biofilm structure.

the residual structure following antimicrobial disinfection promoted bacterial secondary adhesion and biofilm formation.

The mechanism of *S. mutans* adhesion on the residual structure can be explained by cell-cell aggregation and glucan-dependent aggregation. The cell surface protein antigen c (PAc) of *S. mutans* is known to correlate with cellular hydrophobicity, sucrose-independent adhesion to tooth surface and self-aggregation between cells [44, 45]. The glucan-dependent aggregation is mediated by glucosyltransferase enzymes and glucan-binding proteins [46]. Glucan-binding protein C, which is a cell-wall anchoring protein and a cell surface glucan receptor, plays an important role in sucrose-dependent adhesion by binding to soluble glucan synthesized by glucosyltranseferase D [47, 48].

Thus, since a numerous and diverse range of microorganisms reside in our intraoral environment, the residual biofilm will contribute to biofilm redevelopment.

2.2. Antimicrobials-induced biofilm formation

Numerous studies have shown that subminimum inhibitory concentrations (sub-MICs) of various antibiotics and chemicals can inhibit biofilm formation. A representative example is the macrolide antibiotics. Although *Pseudomonas aeruginosa* that contributes to progress respiratory infection is resistant to azithromycin, low-dose azithromycin has been shown to inhibit protein synthesis [49] and improve clinical symptom [50, 51]. Sub-MIC concentrations of azithromycin have also been shown to inhibit quorum sensing and alginate production [52, 53].

In the field of dentistry, it has also been reported that sub-MICs of antimicrobial agents or compounds can inhibit bacterial attachment [54, 56, 57], biofilm formation [54, 55, 57, 58], and downregulate virulence genes [54, 56, 59, 60]. Moon et al. reported N-acetyl cysteine (NAC) that is an antioxidant possessing anti-inflammatory activities, showed a significant decrease of *Prevotella intermedia* biofilm formation in the presence of sub-MIC [55]. NAC was demonstrated to present the expression of LPS-induced inflammatory mediators in phagocytic cells and gingival fibroblasts during the inflammatory process. Lee and Tan showed that treatment of *E. faecalis* with 1/2 sub-MIC of (–)-epigallocatechin-3-gallate (EGCG) significantly inhibited the expression of virulence genes related to collagen adhesion, cytolysins activator, gelatinase, and serine protease compared with the untreated control [60].

In contrast to the inhibitory effects of sub-MIC antimicrobials against biofilm formation, recent studies have shown that some antibiotics at sub-MIC can significantly induce biofilm formation in a variety of bacterial species such as *S. epidermidis, Staphylococcus aureus, Staphylococcus lugdunensis, Escherichia coli*, and *P. aeruginosa* [61]. Kaplan et al. demonstrated that sub-MIC of four different β-lactam antibiotics significantly induce biofilm formation in some strains of *S. aureus* [62]. The amount of biofilm induction was 10-fold in maximum and sub-MIC β-lactamantibiotics induce autolysin-dependent extracellular DNA release. However, the pattern of biofilm induction was strain and antibiotic dependent, indicating that biofilm formation by sub-MICs of antimicrobial agents do not always occur in all the strains of the same species.

This phenomenon may have clinical relevance because bacteria are exposed to sub-MIC of antibiotics at the beginning and end of a dosing regimen [63]. In addition, antimicrobials are retarded to diffuse within the biofilm matrix [14, 15]. In such cases, the bacteria in deeper areas are exposed to antimicrobials at sub-MICs.

As for oral biofilm, there are a few studies reported that sub-MICs of antimicrobial agents upregulate the genes related to EPS production and induce biofilm formation. Dong et al. evaluated the expression of genes related to *S. mutans* biofilm formation following treatment with 1/2 MIC of CHG, tea polyphenols, and sodium fluoride (NaF) [64]. The results showed that expression of *gtfB, gtfC, luxS, comD*, and *comE* was significantly upregulated after treatment with each antimicrobial agent in planktonic cells. Similarly, *gtfB, luxS, comD*, and *comE* were also upregulated in biofilm. Morphological observation using a FE-SEM and CLSM revealed that the biofilms of *S. mutans* treated with sub-MICs of NaF or CHG became denser, containing more EPS and fewer water channels. However, tea polyphenols appear to not promote *S. mutans* biofilm formation, as evidenced by SEM and CLSM images. Little EPS was produced on the surface of teeth after *S. mutans* was treated with a sub-MIC of tea polyphenols, although the expressions of gtfB and gtfC genes were upregulated. The inconsistency of these results can be explained by that sub-MICs of tea polyphenols may prevent from bacterial adhesion to the surface of teeth in the presence of fluid shear force. Because the gene analysis was performed using a 24-well plate under a static condition, whereas the biofilm formation for morphological analysis was prepared under a controlled flow. It has been reported that tea polyphenols could decrease the adherence of *S. mutans* to glass surface [65, 66].

Bedran et al. investigated the effect of triclosan at sub-MICs on *S. mutans* biofilm formation, adherence to oral epithelial cells and expression of several genes involved in adherence and biofilm formation [67]. The authors reported that biofilm formation increased six-fold in the presence of 1/4 MIC of triclosan. Growth of *S. mutans* in the presence of triclosan at sub-MICs also increased its capacity to adhere to a monolayer of gingival epithelial cells. Furthermore, the expression of *comD, gtfC*, and *LuxS* was significantly upregulated in the presence of 1/2 and 1/4 MIC, although the expression of *atlA* and *gtfB* was less pronounced.

Even in limited works with regard to oral biofilms, it is likely that short-time exposure of antimicrobial agents in oral cavity sometimes cause adverse influences because the survived microorganisms after exposure to the agents will alter gene expressions in a positive and negative way.

3. Conclusion

Although chemical agents provide some benefits in terms of controlling oral biofilms, they have the limitation of leaving biofilm structures that may induce adverse reactions such as biofilm regrowth. Furthermore, sub-MICs of certain antimicrobial agents might induce biofilm formation and upregulate pathogenic genes. Future strategies for the control of oral biofilms may therefore shift to the degradation and/or detachment of biofilm matrix.

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Chapter 19

Modulation of Biofilm Growth by Sub-Inhibitory Amounts of Antibacterial Substances

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Additional information is available at the end of the chapter

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Abstract

It is generally accepted that bacteria in biofilm are more resistant to antibacterials than their planktonic counterparts. For numerous antibiotics, it has been shown that minimal inhibitory concentrations (MICs) for bacteria grown in broth are much lower than the minimal biofilm inhibition concentrations. While sub-inhibitory concentrations, that is, amounts of antibacterials below the MIC, do not either influence or suppress to some extent or other the bacterial growth in liquid media, these same amounts of drugs, natural substances, etc., may have diverse effects on bacterial biofilms, ranging from suppression to stimulation of the sessile growth and varying with regard to the bacterial species and strains. This is a source of additional risks for both biofilm infection of host tissues and contamination indwelling devices. When considering the data for biofilm modulation, differences in experimental protocols should be taken into account, as well as the strain-specific mechanisms of biofilm formation.

Keywords: biofilm, sub-MIC, antibiotics, bacteriocins, antimicrobial peptides, plant metabolites

1. Introduction

While the development of antibiotics during the twentieth century resulted in remarkable advances in the fight against infectious microorganisms, it was unfortunately paralleled with the highly increasing risks for the development of antibiotic resistance. These risks are a consequence of the extensive use of antibacterial preparations in both human medicine and agriculture. Resistance has become a threat to human and animal health worldwide, and it

necessitates the development of key measures. Among these, the identification of critical points of control, the development of surveillance measures, and the prevention of environmental contamination are in focus [1].

In the aquatic and terrestrial environments, the contaminated sites (wastewater systems, pharmaceutical factories effluents, animal husbandry facilities, etc.) are characterized by the presence of subtherapeutic concentrations of antibiotics [1–3]. Thus, bacteria present in the environment are often subjected to drug amounts lower than the minimal inhibitory concentrations (MICs) [4]. Antimicrobial sub-MICs are encountered in the human body as well, during treatment, which can occur irregularly at intervals at the site of infection [5] or in cases of low-dose antibiotic prophylaxis [6]. When microorganisms grow in the presence of sub-MICs, the antibiotics can potentially alter the physicochemical characteristics of microbial cells, their functions, and the expression of some virulence genes [7]. While sub-MICs generally do not interfere with bacterial growth dynamics, the microorganisms are subjected to stress. As a way to counter stress, microbes would often form biofilms both in external environments and on indwelling medical devices [3, 8, 9].

It is noteworthy that, when MICs or sub-MICs are considered, this concerns values obtained with bacteria grown in liquid media, that is, as plankton. Such will be the use of the term also in the present review. In biofilms, the inhibitory doses exceed 10 to even 1000 times these of plankton [1, 9, 10]. Interestingly, when plankton and bacteria dispersed from biofilm have been examined, they were shown to have similar antibiotic susceptibility [11]. Hence, increased resistance is likely associated with characteristics that are a consequence of the structure of the sessile microbial communities. They themselves represent heterologous microenvironments in which gradients of physical and chemical parameters exist [3]. The advantages of these structured bacterial communities comprise limited antibiotic diffusion, enhanced transmission of resistance genes, expression of efflux pumps, drug adsorption by extracellular matrix, as well as the presence of metabolically inactive persister cells [12].

Provided the growing concern about the wide spread and the role of environments containing subinhibitory amounts of antibacterials, the present review will focus on the interplay of sub-MICs with biofilm growth and/or detachment. In a previous review, the antibiotic-induced biofilm formation has been discussed [13]. However, the sub-MIC of antibiotics, but also other antibacterials (e.g. antibacterial peptides, natural and synthetic substances, etc.), dependent on the combination drug-bacterial strain or species, may have diverse effects on biofilm, from suppression through no effect to promotion. This determined the aim of the present review: to summarize current data and concepts about the modulation of biofilm growth by sub-MICs of antibacterial substances.

2. Sub-MIC of antibiotics and biofilms

While it was initially believed that antibiotics in nature have the role for fighting against competitors, and that therefore also sub-MICs would reduce virulence, recent evidence reveals a more complicated picture, showing the capacity of some antibiotics at low dose to act as chemical signals to modulate metabolic processes [14] or regulate gene (including virulence gene) expression [15].

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The idea on the effects of antibiotic sub-MICs on biofilms is getting more and more complicated with the accumulation of experimental data. This puts forward the question of methodology. The conventional approaches to antibiotic sensitivity do not apply to biofilm-grown bacteria [9]. Due to the potentially very high intrinsic biofilm resistance, the focus has mainly been put on their prevention [16]. Probably for this reason, most results have been obtained while applying the drug during the sessile growth, with only a few studies testing the agent's effects on pre-formed biofilms [17–19]. The routinely applied methodology is to test biofilm biomass on 96-well plates by the crystal violet assay, with only a few other studies that explore cell viability as well, for example, the viable cell counts [20] or live-dead staining for fluorescence microscopy.

We have summarized the available experimental data on the action of sub-MICs of antibiotics on biofilms in **Table 1–5**. We could find no strict pattern with regard to the effects of the separate groups of antibiotics. All groups were shown to influence some biofilms positively, and others, negatively. An important observation is the bacterial species and strain specificity of the response to the sub-MICs. Thus, sub-MICs of ampicillin increased biofilm growth of *Staphylococcus saprophyticus* [6], reduced it in *Escherichia coli* K-12 [21], and had no effect on *E. coli* UTI8 and *Mycobacterium avium* [6, 22] (**Table 1**). Sub-MICs of ciprofloxacin promoted biofilms of *S. saprophyticus* [6] and *E. coli* UTI8 [6], but reduction was registered in *Streptococcus suis*, *Salmonella enterica* serovar Typhimurium clinical strains, *Stenotrophomonas maltophilia*, *Streptococcus pyogenes*, *Staphylococcus epidermidis*, and *Proteus mirabilis* [17, 18, 20, 23–25] (**Table 2**). Diverse effects have been illustrated for sub-MICs of erythromycin on biofilms of *S. suis*, *Corynebacterium diphtheriae*, and *S. epidermidis* [25–27] (**Table 3**); for gentamycin on *S. enterica* serovar Typhimurium, *S. saprophyticus*, *E. coli*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (**Table 4**); and for tetracyclin on *E. coli*, *Staphylococcus lugdunensis*, *M. avium*, *P. aeruginosa*, and *S. epidermidis* [9, 15, 21, 22, 28] (**Table 5**).

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
		Penicillins		
Dicloxacillin	1/2 MIC	*S. epidermidis* strains 9142, IE186, and M187	32–60% BF inhibition	[29]
	8 μg/ml	*S. epidermidis* M187 *S. haemolyticus* M176	BF biomass reduction; decreased synthesis of the EPS, poly-*N*-acetyl-glucosamine	[16]
Penicillin	1/16–1/2 MIC	*S. suis* NJ-3	Dose-dependent BF suppression	[25]
	1/8 MIC	*C. diphtheriae* subsp. mitis strains	No effect	[26]
Methicillin	1/3–1/8 MIC	*S. aureus* Newman	Denser BF formed by the strain and its small-colony variants	[30]
Nafcillin	0.0625–0.5 MIC	*S. lugdunensis*—15 isolates from BF-related infections (endocarditis, prosthetic joint infections, etc.)	Increase in 93% of the tested strains, no effect in 7%	[9]
	1/3–1/8 MIC	*S. aureus* Newman	No effect on BF	[30]
		Cephalosporins		
Cefazolin	1/2 MIC	*S. epidermidis* strains 9142, IE186, and M187	32–55% BF inhibition	[29]
	0.0625–0.5 MIC	*S. lugdunensis*—15 isolates from BF-related infections (endocarditis, prosthetic joint infections, etc.)	Increase in 13% of the tested strains, no effect in 80%, and decrease in 7%	[9]
	0.5 MIC	*S. epidermidis* strains SE5 and RP62A	BF decrease	[31]

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
Cefalotin	1/3–1/8 MIC	*S. aureus* Newman	Three- to fourfold denser BF formed by the strain and its small-colony variants	[30]
Cefoperazone	1/3–1/8 MIC	*S. aureus* Newman	No effect on BF	[30]
Cefotaxime	1/2–1/16 MIC	*Salmonella enterica* serovar Typhimurium clinical isolates 75 strains	At 1/2 MIC—significantly increased production of BF and EPS	[5]
Ampicillin	0.005–500 μg/ml	*E. coli* MG1655 wt and MG1655 (pBR322)	BF reduction	[21]
	0.1–1.1 μg/ml	*S. saprophyticus* 15305	Sub-MIC (0.3–0.7 μg/ml) stimulate BF formation	[6]
	1/2–1/1024 MIC	*E. coli* UTI89	BF stimulation at MIC, no effect of sub-MIC	[6]
	10 μg/l	*M. avium* strains 104; 101, A5; 3362-33 and 3362-34)	No effect on BF	[22]
		Carbapenems		
Imipenem	2–4 μg/ml	*P. aeruginosa* PA01	BF induction, changes in BF morphology, upregulation of *ampC* and genes for alginate biosynthesis	[32]
	0.03 and 0.125 μg/ml	73 isolates *A. baumannii*	BF stimulation	[33]
Ceftazidime	1/2–1/8 MIC	5 clinical isolates strains *S. maltophilia*	BF inhibition and reduction of viable cell counts	[20]
	0.125–1.0 MIC	5 strains *P. mirabilis*	BF inhibition	[24]
	2; 8; 32 mg/l	6 clinical isolates of *P. aeruginosa*	Synergistic effect with polymorphonuclears against developed 48 h BF	[34]

Abbreviations: BF, biofilm; EPS, exopolysaccharide; MIC, minimal inhibitory concentration.

Table 1. Effects of sub-MIC of β-lactam antibiotics on biofilms.

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
Ciprofloxacin	1/16–1/2 MIC	*S. suis* NJ-3	Dose-dependent BF suppression	[25]
	1/2–1/16MIC	*Salmonella enterica* serovar *Typhimurium* clinical isolates 75 strains	Inhibition of BF formation and EPS synthesis	[5]
	0.1–1.1 μg/ml	*S. saprophyticus* 15305	BF stimulation by sub-MIC (0.4–0.9 μg/ml)	[6]
	1/2–1/1024 MIC	*E. coli* UTI89	Statistically significant BF increase and upregulation of BF-associated genes at 1/4 MIC	[6]
	1/2–1/8MIC	5 clinical isolates *S. maltophilia*	BF inhibition and reduction of viable cell counts	[20]
	1/2–1/64 MIC	*S. pyogenes* isolates (M56; st38;M89; M65; M100; M74)	Dose-dependent BF inhibition	[23]
	0.5 × MIC	*E. coli* strains (8; 9; 10; 31 ;1583)	Reduction of BF formation and survival of the BF bacteria	[18]
	1/2–1/8 MIC	*Staphylococcus epidermidis*—20 clinical isolates	Reduces BF growth and pre-formed BF	[17]
	0.125–1.0 MIC	5 strains *P. mirabilis*	BF inhibition	[24]
	2; 8; 32 mg/l MIC	*P. aeruginosa*—6 clinical isolates	Synergistic effect with polymorphonuclears against developed 48-h BF	[32]

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
	0.001–100 mg/l	*P. aeruginosa*	BF reduction	[15]
Norfloxacin	1–10,000 mg/l	*S. aureus* ATCC 25923	BF stimulation by 1 mg/l	[8]
	1–10,000 mg/l	*P. aeruginosa* NNRL-B3509	BF stimulation by 1 mg/l	[8]
	1/2–1/8 MIC	*S. maltophilia*—5 clinical isolates strains	BF inhibition and reduction of viable cell counts	[20]
	1/2–1/64 MIC	*S. pyogenes* isolates (M56; st38;M89; M65; M100; M74)	Dose-dependent BF inhibition	[23]
	1/2–1/8 MIC	*Staphylococcus epidermidis*—20 clinical isolates	Suppression of BF growth and reduction of pre-formed BF	[17]
	1/16–1/2 MIC	*S. suis* NJ-3	Dose-dependent biofilm suppression	[25]
Ofloxacin	1/2–1/8MIC	*S. maltophilia*—5 clinical isolates strains	BF inhibition and reduction of viable cell counts	[20]
	1/2–1/64 MIC	*S. pyogenes* isolates (M56; st38;M89; M65; M100; M74)	Dose-dependent BF inhibition	[23]
	0.5 × MIC	*S. epidermidis* strains (SE5; RP62A)	No effect on BF formation	[31]
	1/2–1/8 MIC	*Staphylococcus epidermidis*—20 clinical isolates	Suppression of BF growth and reduction of pre-formed BF	[17]
	1/2–1/8 MIC	*S. maltophilia*—5 clinical isolates strains	Biofilm inhibition and reduction of viable cell counts	[20]
Levofloxacin	1/2–1/64 MIC	*S. pyogenes* isolates (M56; st38; M89; M65; M100; M74)	Dose-dependent BF inhibition	[23]
	0.0625– 0.5 MIC	*S. lugdunensis*—15 isolates from biofilm-related infections (endocarditis, prosthetic joint infections, etc.)	Increase in 20% of the tested strains, in 47% no effect, and in 40% decrease	[9]
Moxifloxacin	1 μg/l	*M. avium* strains (104; 101; A5; 3362-33; 3362-34)	No effect on BF	[22]
	1/2–1/8 MIC	*S. maltophilia*—5 clinical isolates strains	BF inhibition and reduction of viable cell counts	[20]
	0.03–0.06 MIC	*S. maltophilia* strains Sm 132 and Sm 144	Decrease in adhesion and BF formation	[7]
	2; 10; 50; 100 x MIC	*S. aureus*—6 strains of coagulase negative	No effect on BF	[35]
	1 μg/ml	*M. avium* strains (101, 104, 109, and A5)	BF inhibition	[36]
	1/2–1/8MIC	*S. maltophilia* —5 clinical isolates strains	BF inhibition and reduction of viable cell counts	[20]
Grepafloxacin	1/2–1/8MIC	*S. maltophilia*—5 clinical isolates strains	BF inhibition and reduction of viable cell counts	[20]
Pefloxacin	1/2–1/8 MIC	*S. epidermidis*—20 clinical isolates	Reduces BF growth and pre-formed BF	[17]

Table 2. Effects of sub-MIC of fluoroquinolones on biofilms.

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
Erythromycin	1/16–1/2 MIC	*S. suis* NJ-3	Dose-dependent BF suppression	[25]
	1/8 MIC	*C. diphtheriae* subsp. mitis strains	BF increase	[26]

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
	0.25 MIC	*S. epidermidis*—96 clinical isolates	BF inhibition in 4 strains; BF enhancement in 20, other strains—unaffected	[27]
Azithromycin	1/2–1/16 MIC	*S. suis* NJ-3	BF inhibition by 1/4 and 1/2 MIC	[25]
	2.5–10 mg/ml	*S. aureus* strains (B1 487; B1 493; B1 412; B 391; B1 468; B1 483; B1 379; B1 472)	BF reduction	[37]
	0.125 μg/ml	*H. influenzae* NTHi2019	Decreased BF formation, reduction of established BF	[19]
	8 μg/m	*M. avium* strains (101, 104, 109, and A5)	BF inhibition	[36]
	sub-MICs	*P. aeruginosa*—35 clinical isolates	Dose-dependent BF reduction	[38]
Clarithromycin	1 μg/ml	*M. avium* strains (101, 104, 109, and A5)	BF inhibition	[36]
	MIC b/n 50–550 mg/ml	*P. aeruginosa*	Sub-MIC result in altered structure and architecture of BF	[39]

Table 3. Effects of sub-MIC of macrolides on biofilms.

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
Gentamycin	1/2–1/16	*Salmonella enterica* serovar *Typhimurium* clinical isolates 75 strains	Inhibition of BF formation and EPS synthesis	[25]
	0.1–1.1 μg/ml	*S. saprophyticus* 15305	Statistically significant BF increase by 0.6—0.7 μg/ml	[6]
	1/2–1/1024 MIC	*E. coli* UTI89	Statistically significant BF increase by 1/32 MIC	[6]
	8 μg/ml	*H. influenzae* NTHi2019	No effect on BF	[19]
	From sub-MIC up to 100× MIC	*S. aureus* strains (RN6390 ATCC 25923)	BF increase	[11]
	0.1–1.5 μg/ml MIC	*P. aeruginosa* PAK and isogenic mutants	BF increase	[40]
Streptomycin	0.5–2 μg/ml	*M. avium* strains (104; 101; A5; 3362-33; 3362-34; 8G12; 5G4; 6H9)	BF increase, induction of BF-associated genes	[22]
Tobramycin	0.05–2 μg/ml	*P. aeruginosa* PAK and isogenic mutants	BF increase	[40]
	0.3; 0.5; 1.0 μg/ml	*Xylella fastidiosa* ATCC 700964 and 3 isogenic mutants	BF reduction	[41]
	0.001–100 μg/l	*P. aeruginosa*	BF reduction	[15]
Amikacin	0.5× MIC	*E. coli* strains (8; 9; 10; 31; 1583)	Reduction of BF formation and survival of the BF-bacteria	[18]
	2; 8; 32 mg/l	*P. aeruginosa*—6 clinical isolates	Synergistic effect with polymorphonuclears for developed 48-h BF	[32]

Antibiotics	Amount	Bacteria/strains	Effect on biofilm	Ref.
Kanamycin	10–110 μg/ml	*P. aeruginosa* PAK and isogenic mutants	BF increase	[40]

Table 4. Effects of sub-MIC of aminoglycosides on biofilms.

Antibiotics	Amount	Bacteria/ strains	Effect on biofilm	Ref.
Streptogramins Quinupristin-dalfopristin	0.5 μg/ml	*S. epidermidis* strains 567 and *S. epidermidis* 561	Enhancement of *ica*ADBC operon expression and EPS synthesis	[28]
	0.0625–0.5 MIC	*S. lugdunensis*—15 isolates from biofilm-related infections (endocarditis, prosthetic joint infections, etc.)	Increase in 20% of the tested strains; in 47% no effect and in 33% decrease	[9]
Glycopeptides Vancomycin	1/2 MIC	*S. epidermidis* strains 9142, IE186 and M187	8–24% BF inhibition	[29]
	0.0625–0.5 MIC	*S. lugdunensis*—15 isolates from biofilm-related infections (endocarditis, prosthetic joint infections, etc.)	Increase in 27% of the tested strains; in 47% no effect and in 27% decrease	[9]
	0.5× MIC	*S. epidermidis* strains SE5 and RP62A	Decrease in BF	[31]
Tetracyclins Tetracyclin	0.005–500 μg/ml	*E. coli* MG1655 wt and MG1655 (pBR322)	Significant BF increase in the presence of (pBR322)	[21]
	0.0625–0.5 MIC	*S. lugdunensis*—15 isolates from biofilm-related infections (endocarditis, prosthetic joint infections, etc.)	Increase 7% of tested strains; decrease 93%	[9]
	0.5–2 μg/ml	*M. avium* strains (104; 101; A5; 3362-33; 3362-34)	BF increase	[22]
	0.01–100 mg/l	*P. aeruginosa*	BF reduction	[15]
	0.5 μg/ml	*S. epidermidis* strains 567 and *S. epidermidis* 561	Enhancement of *ica*ADBC operon expression and EPS synthesis	[28]
DHFR inhibitors Trimethroprim-sulfamethoxazole	0.0625–0.5 MIC	*S. lugdunensis*—15 isolates from biofilm-related infections (endocarditis, prosthetic joint infections, etc.)	Increase in 20% of the tested strains, in 40% no effect, and in 40% decrease	[9]
	1/2–1/8 MIC	5 clinical isolates strains *S. maltophilia*	BF inhibition and reduction of viable cell counts	[20]
Oxazolidonones Linezolid	0.0625–0.5 MIC	*S. lugdunensis*—15 isolates from biofilm-related infections (endocarditis, prosthetic joint infections, etc.)	In 80% no effect, in 20% decrease	[9]

Table 5. Effects of sub-MIC of streptogramins, glycopeptides, tetracyclins, dihydrofolate reductase (DHFR) inhibitors and oxazolidonones on biofilms.

Antibiotics with identical mechanisms of antibacterial action, for example, gentamicin and erythromycin, may have different effects on biofilm [19, 29]. In addition, the sub-MICs of a given antibiotic may have diverse effects on different strains of one species of microorganism. For example, such is the case with the effects of cefazolin and levofloxacin on 15 isolates of *S. lugdunensis* [9] (**Table 1**), the effects of erythromycin on 69 clinical isolates of *S. epidermidis* [27] (**Table 3**), of vancomycin on 3 strains of *S. epidermidis* and 15 isolates of *S. lugdunensis* [9, 30] (**Table 5**), and of trimethoprim-sulfamethoxazole on 15 isolates of *S. lugdunensis* [9] (**Table 5**). Obviously, individual strains use different response mechanisms to oppose the action of sub-MICs [9].

Sub-MICs of antibiotics have the potential to affect the structure of individual bacterial cells. Changes of morphology have been registered in several studies. For instance, sub-MIC of

penicillin induced filamentation of cells of *C. diphtheriae*, while erythromycin reduced cell size of this microorganism [26]. The sub-MIC of the drug combination piperacillin/tazobactam induced the occurrence of filamentous forms in *P. aeruginosa*, while subinhibitory amounts of imipenem resulted in the formation of roundish forms (coccobacilli) of this bacterium [43]. Sub-MICs of ciprofloxacin caused the occurrence of filamentous cells when applied to *E. coli* isolated from urinary tract infections. This was accompanied by alterations in the morphology of the outer membrane cardiolipin domains of the strain [44]. The cell-surface physicochemical characteristics of the bacteria would also be affected. Several studies focus on cell-surface hydrophobicity. For example, sub-MICs of penicillin and streptomycin which enhanced biofilm formation in *C. diphtheriae* also rendered the cell surface more hydrophobic [26]. The combination piperacillin/tazobactam applied as sub-MIC that suppressed biofilm growth also reduced cell-surface hydrophobicity of *P. aeruginosa* [45]. While these examples appear to show a likely positive correlation between cell-surface hydrophobicity (which is also related with cell adhesion) and the effects on biofilm growth, this may not be the rule throughout. Thus, sub-MICs of moxifloxacin that reduced biofilm growth had no influence on the cell-surface hydrophobicity of *S. maltophilia* [7]. Changes in zeta potential [8], flagellum-mediated swimming [45] and type IV fimbria-related twitching motility [39, 45] have been registered as well. When examined, the overall morphology of the biofilm, its thickness, substratum coverage, and roughness would change as well [16, 39].

The extracellular biofilm matrix is an important component of these structured microbial consortia. It has both structural and protective functions. In the interplay with the antibiotics, its barrier role against drug penetration should be underlined [46]. For the time being, available publications show a strict correlation between the effects of sub-MICs on the biofilm and on the extracellular matrix components. More data are available on the extracellular polysaccharide (EPS). In cases of biofilm biomass reduction (e.g. by gentamicin and ciprofloxacin on *S*. Typhimurium, by fluoroquinolones on *P. aeruginosa*, and by dicloxacillin on *S. epidermidis*) this was accompanied by reduced release of EPS [5, 16, 47]. In cases of biofilm biomass increase (by sub-MIC of erythromycin on *S. epidermidis*, of cefotaxime on *S. enterica* serovar Typhimurium, and of azithromycin on representatives of several bacterial genera), this coincided with EPS increase [5, 48, 49]. While less studied, such correlation might also characterize another component of the extracellular matrix, the extracellular DNA. It was registered with increased amounts in *S. epidermidis* biofilms treated with sub-MICs of vancomycin [50, 51].

Sub-MICs of antibiotics can interact with the bacterial-host interactions. Together with their capacity to affect phenotypes, they can influence bacterial sensitivity to oxidative stress [45], suppress host proinflammatory responses [6], and cooperate with host polymorphonuclear leucocytes to destroy biofilms [34].

There is evidence that in nature, antibiotics at non-inhibitory concentrations can have the role of signalling molecules that can interfere with quorum sensing [4, 42]. It was shown that sub-MICs of antibiotics influence quorum-sensing-related phenotypes of *Chromobacterium violaceum*, like the production of the pigment violacein, of acyl-homoserine lactones, and of chitinase [52]. Sub-MICs of tobramycin inhibited the Rhl/R system of *P. aeruginosa* thus

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reducing the production of C4-homoserine lactone [53]. Azithromycin also antagonized quorum sensing in *P. aeruginosa* [4]. Cephalotin and cephalotaxime suppressed the *agr* quorum-sensing system in *S. aureus* [54].

Sub-MICs of antibiotics can interact with bacterial regulation mechanisms and gene expression. Transcriptomic studies indicated that the expression of approximately 5% of bacterial promoters may be affected [13]. Genes related with antibiotic resistance should be mentioned in the first place. There was a correlation between the transcription of the *ermC* gene and the biofilm formation of erythromycin-treated *S. epidermidis* [27]. In *P. aeruginosa*, among the 34 genes influenced by imipenem, the most strongly induced gene was *ampC* coding for chromosomal β-lactamase [54]. Genes related with the synthesis of EPS or bacterial capsules may be affected, like the genes from the *ica*ADBC operon in *S. epidermidis* [28, 49]. Other genes related with adhesion and biofilm development that should be mentioned are *comD*, *gtfC*, *luxS*, *gtfB*, and *atlA* of *Streptococcus mutans* upregulated by sub-MIC of triclosan [55] and *guaB2* and *gtf* in *M. avium*, upregulated under the action of sub-MICs of streptomycin and tetracycline [22]. The biofilm growth and detachment have been related with the intracellular levels of the second messenger cyclic-di-GMP [40]. The *eal* gene participating in the pathways for its synthesis was upregulated by sub-MIC of tobramycin [41]. Definitely, the effects of sub-MICs on gene expression are not confined to these related to biofilm, many other genes can be influenced as well [40].

3. Antibiofilm bacterial metabolites

The capacity of released metabolites of bacterial species and strains to modulate biofilm growth of other bacteria is continuously in focus because of the potential for the isolation of novel biofilm modulating substances. As an initial screening step, the action of cell-free supernatants (CFSs) is tested. The activities may vary from stimulation [56, 57] to suppression [58, 59]. Noteworthy, the effects of CFSs on bacterial growth in liquid media do not predict the effects on sessile growth. Thus, subinhibitory amounts of 10^{-2} diluted CFSs from two bacteriocinogenic strains of *Lactobacillus plantarum* slowed down the growth of laboratory and uropathogenic strains of *E. coli*, but stimulated significantly the biofilm development [59]. The active substances in CFSs may be proteins/peptides, carbohydrates, low molecular weight metabolites, etc. [see comments by 57] and for some of them the nature, structure, and mode of action have been explored. However, we shall restrict our review to molecules which have inhibitory activity on bacterial growth, and for which there is data on the effects of subinhibitory amounts on biofilms.

Bacteriocins are proteins/peptides produced by prokaryotes which are active against other bacterial species or strains. For example, colicins are produced by some strains of *E. coli*. One of them, colicin M, is a phosphatase that hydrolyses the peptidoglycan lipid II intermediate, thus interfering with peptidoglycan synthesis and causing cell lysis. In subinhibitory amounts, it upregulated in *P. aeruginosa* PAO1 the *ydeH* gene related with the synthesis of cyclic-di-GMP, as well as several biofilm-related genes, *ycfJ*, *rprA*, *omrA*, and *omrB*. However, no biofilm

stimulation was confirmed by the crystal violet assay [60]. RIP is an RNAIII-inhibiting heptapeptide originally isolated from CFS of *Staphylococcus xylosus.* It inhibits staphylococcal pathogenesis. In sub-MICs, it suppressed biofilm formation by interfering with the quorum-sensing mechanisms [61]. Nisin is a polycyclic antibacterial peptide produced by *Lactococcus lactis*. At growth inhibitory concentrations, it suppressed sessile growth of *S. aureus*; however, sub-MICs had no effect on biofilm [62].

Mupirocin is an antibacterial substance of the monoxycarbolic acid class that was originally isolated from *Pseudomonas fluorescens*. At sub-MIC, it can reduce both biofilm formation and glycocalyx production by *P. aeruginosa* [63]. Phenyl lactic acid is a metabolite of *Lactobacillus* probiotic strains. At subinhibitory amounts, it attenuated the virulence and pathogenicity of *P. aeruginosa* and *S. aureus*, including biofilm formation, by interacting with quorum sensing [64]. The antifungal and antibacterial molecule, 2,4-diacetylphloroglucinol, was isolated from the CFS of *Pseudomonas protegens*. At subinhibitory amounts, it reduced pellicle and biofilm formation, and sporulation of *B. subtilis* [65].

4. Antimicrobial peptides and biofilm modulation

The host-bacterial interactions are also explored with the aim of identifying of novel molecules that would help overcoming the bacterial resistance mechanisms and combating infections. One important group of substances is that of the antibacterial peptides, an important part of the innate immune system.

Colistin is a cationic antimicrobial peptide which is gaining importance in the fight against *P. aeruginosa* cystic fibrosis infections. Subinhibitory concentrations altered the expression of 30 genes of the bacterium. Genes related with quorum sensing, lipopolysaccharides (LPSs) modifications, quinolone biosynthesis, and biofilm formation were upregulated while genes involved with motility and osmotolerance were downregulated. However, biofilm biomass remained unaffected [66].

The major human host defence peptide LL-37 is found in mucosal surfaces, the granules of phagocytes, as well as in bodily fluids. At very low concentrations, far below those that kill or inhibit the growth of *P. aeruginosa*, LL-37 prevented the *in vitro* biofilm formation [67]. It interfered with biofilm growth in at least three ways: by reduction of initial attachment, promotion of twitching motility, and downregulation of key components of the Las and Rhl quorum-sensing systems [67].

The synthetic antimicrobial peptide 1018, derived from the bovine neutrophil defence peptide bactenecin, has recently been identified as biofilm inhibitory compound. While not reflecting on bacterial growth, it could prevent the biofilm growth of *P. aeruginosa*, *E. coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *S. enterica*, *Burkholderia cenocepacia*, and methicillin-resistant *S. aureus*. This co-related with degradation of ppGpp [68]. In addition, peptide 1018 acted in synergism with conventional antibiotics, like ceftazidime, ciprofloxacin, imipenem, or tobramycin, to both prevent development and disperse existing biofilms [69].

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Invertebrate antibacterial responses are also explored. Thus, thanatin is an insect antimicrobial peptide on the basis of which a shorter synthetic derivative, R-thanatin, was synthesized. When applied in sub-MIC amounts to *S. epidermidis* (including methicillin-resistant staphylococcus epidermidis, MRSE), *Staphylococcus haemolyticus*, and *Staphylococcus hominis*, it inhibited biofilm formation. Parallelly, MRSE underwent serious morphological alterations like swelling and abnormal divisions [70].

5. Subinhibitory amounts of plant substances

The application of plants for treatment of illness dates back to the very early moments of mankind history, and has laid the basis of modern phytotherapy. The studies on the antibacterial activities of medicinal plant products have a long tradition, more recently expanded to biofilm research. Studies include tests on essential oils and plant extracts, partially purified enriched fractions, as well as isolated pure substances. Also, plant products may be used as a basis for chemical modifications aiming improved antibiofilm activity.

Among the plant products, essential oils are most popular for their wide use in ethnomedicine. Some of them that have antibacterial action proved successful against bacterial biofilms as well. Among the essential oils that at subinhibitory amounts could suppress sessile growth are, for example, these from *Satureja hortensis* L. (active against *Prevotella nigrescens* biofilm) [71], from *Thymus vulgaris* (active against *P. aeruginosa* and *E. coli* biofilms) [72], and from *Mentha piperita* (active against biofilms of *P. aeruginosa* and *A. hydrophylla*) [73]. In addition, peppermint oil suppressed EPS production [73].

Methanol and aqueous branch extracts of five *Juniperus* sp. were examined for their activities against two *S. aureus* strains. The extracts had minimal activity on planktonic growth of *S. aureus* ATCC 3538P but suppressed biofilm formation, while the other strain, *S. aureus* 810, was not affected in either mode of growth [74]. The extract from *Leonurus cardiaca* L. suppressed the adherence of *S. aureus* to both abiotic surfaces and surfaces covered with fibrinogen, fibronectin, or collagen [75]. Sub-MICs (1/2 to 1/32 MIC) of extracts from *Boesenbergia pandurata* (Roxb.) Schltr. and *Eleutherine americana* Merr. significantly prevented biofilm formation. Together with this, the extract from *E. americana* also suppressed quorum sensing in *C. violaceum* test system [76].

In a study on 14 fractions from plant extracts, the total extract and the phenyl propanoid-containing fraction from *Rhodiola rosea*, and the total extract and the sesquiterpene lactone-containing fraction (Am2) from *Arnica montana*, were shown to have no antibacterial effects. However, they suppressed the biofilm growth in *E. coli* urinary tract infection isolates. These same extracts had the opposite effect—biofilm stimulation, on a multidrug-resistant *E. coli* strain isolated from asymptomatic bacteriuria [77]. Noteworthy, the sesquiterpene lactone-containing fraction Am2 also suppressed the quorum-sensing-controlled bioluminescence in *Vibrio harveyi* bioreporter strains (ATCC1116 and ATCC1117) [78, 79].

Carvacol is an antimicrobial monotherpenic phenol with antibacterial potential that is present in many essential oils. In subinhibitory doses, it suppressed sessile growth of a number of

Gram-positive and Gram-negative bacteria [80, 81]. Polyphenols from muscadine grapes with antioxidant and antibacterial activity, at 0.5 MIC, inhibited biofilm growth of *S. aureus* [82]. Similar was the effect of the essential oil components eugenol and citral on *S. aureus* and *Listeria monocytogenes* [83], epigallocatechin-3-gallate from green tea on *S. maltophilia* [84], ursolic acid, genistein, cranberry extract, *p*-hydroxybenzoic acid, and resveratol on *S. aureus* [75, 85]. Menthol, together with biofilm suppression, was shown to inhibit both the *las*- and *pqs*-related quorum sensing [73].

Fenchone is a substance that is present in many essential oils. It had neither antibacterial nor antibiofilm effects on a panel of Gram-positive and Gram-negative strains. This molecule was used to synthesize its chemical derivatives. While the substitutions did not improve the antibacterial properties against *E. coli* ATCC 25922 and six *E. coli* K-12 strains, some of the derivatives showed biofilm modulation potential [86]. Chalcones are a group of flavonoids with antibacterial potential, found in many plants. Synthetic chalcones are applied as well. The effects of sub-MICs of three newly synthesized chalcones on methicillin-resistant *S. aureus* were examined. Both biofilm formation and adherence to human fibronectin were reduced, as well as the release of EPS [87].

6. Other compounds with biofilm-modulating potential

Other substances have also proved a good anti-biofilm potential when applied in sub-MICs. For example, sodium ascorbate, together with suppressing *P. aeruginosa* virulence factors (elastase, protease and haemolysin activities, pyocyanin production, and quorum sensing) also reduced biofilm formation [88]. Biofilm growth was inhibited by subinhibitory amounts of thiourea derivatives [89], thiazolinediones [90], and certain anthraquinones [91]. Organic complexes of metals are also elaborated as antibacterial and/or antibiofilm substances. Newly synthesized dimethylguanin-copper complexes [92], the organo-tellurium compound AS101 [93], and bismuth thiols [94] have shown anti-biofilm activity at sub-MICs. The latter substances are considered as possible coating agents for indwelling devices. For prevention of medical devices from bacterial contamination, other substances may prove useful as coating material, like ovotransferrin, protamine sulfate, ethylenediaminetetraacetic acid (EDTA) [95], cerium nitrate, chitosan, hamamelitannin [96], polyvinyl pyrrilidine [97], etc.

As an opposite effect, the biocides used in food processing facilities, trisodium phosphate, sodium nitrite, and sodium hypochlorite, when applied in sub-MICs, enhanced the capacity of *E. coli* to form biofilms. This was accompanied by a reduction of the antibiotic susceptibility [98].

7. Some final considerations

Presently, there is growing concern about the relationship between the rise of widespread antibiotic resistance and the role of environments containing subinhibitory amounts of

antibacterials [1]. As a major risk for human health, biofilm communities provide the bacteria with prerequisites for rapid resistance development [99]. Among the other more direct risks that biofilms may cause to human health, should be mentioned the possibility for enhanced colonization of indwelling medical devices in the presence of subinhibitory amounts of antibacterial substances, and the contamination of surfaces in medical or food-processing environments. Depending on the aim of a given antibiofilm strategy, different effects may be in the focus. Disinfection of outer surfaces in hospitals and in food industry requires that the used agents have the capacity to detach established biofilms. On the opposite, if biofilms on indwelling devices are concerned, once established, their detachment is hazardous. It may be accompanied with dissemination of the bacteria to other sites in the human body, and there are risks of sepsis [69]. Therefore, the development of medical materials should be directed to biofilm prevention. However, when the effects of a given substance are estimated, the biology of the biofilm as a whole is better to be addressed, starting from the attachment and establishment of the sessile community, and going as far as its detachment. The methodologies used by the predominant amount of the present-day studies search for the effects of sub-MICs by applying the tested agents during biofilm growth. It can be recommended that in the future a more standardized methodology is applied which includes as well tests for dispersion of established biofilms and for microbial vitality. The present review showed several critical points in the effects of sub-MICs of antibacterial substances as biofilm modulators. Among these, the strain- and species-specific responses of the bacteria in their biofilm development, the expression of virulence factors and quorum sensing should necessarily be taken into account when novel antibacterials are tested.

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Chapter 20

Novel Models to Manage Biofilms on Microtextured Dental Implant Surfaces

Carol Tran and Laurence J. Walsh

Additional information is available at the end of the chapter

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Abstract

Dental implants are used extensively to replace missing teeth. To enhance their integration with the bones of the jaws, the surfaces of titanium dental implants are modified to make them hydrophilic, high energy, and microtextured. These same features make biofilm development occur readily upon exposure to the saliva. The presence of mature biofilms on dental implant surfaces drives local inflammatory responses in the adjacent soft and hard tissues (peri-implantitis), which leads to pathological loss of bone and the formation of a saucer shaped bone defects. This chapter examines the unique challenges posed by biofilms formed on highly complex dental implant surfaces, which are difficult to access for cleaning, and easily damaged by conventional cleaning approaches. We explore how biofilms can be removed from implant surfaces using a variety of novel methods, without causing surface damage or other undesirable modifications, and show how different laboratory and clinical models can be used to assess the performance of both conventional and novel methods of biofilm removal.

Keywords: biofilms, dental implants, surface characterization, debridement, lasers, particle beams

1. Introduction

Dental implants have been used for over 40 years to replace missing teeth, with good clinical success when placed in most sites in the jaws [1]. They are inserted into specially prepared channels in the bone, and once fully integrated into the bone, can be restored with a ceramic crown to replace a single missing tooth (**Figure 1**). Typically, dental implants are fabricated from commercially pure titanium (Ti), or titanium alloys which include small amounts of vanadium and aluminum. Dental implants made from ceramic materials such as sapphire and

zirconium oxide exist but are not in common use. The use of Ti is favored over other materials because it is biocompatible when inserted into direct contact with bone, resistant to corrosion, lightweight, and durable.

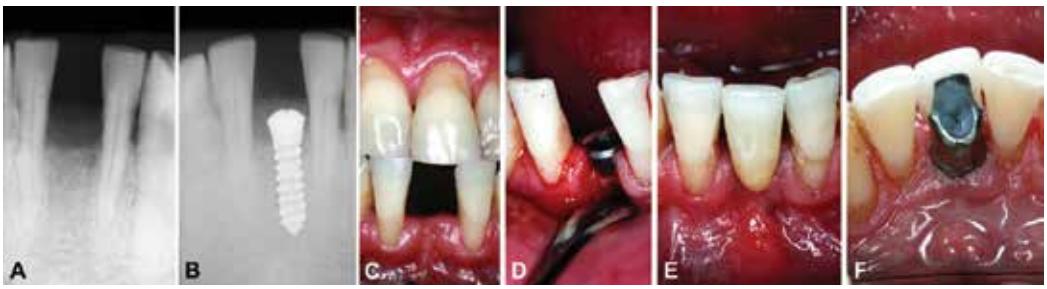

Figure 1. An overview of dental implant placement. (A) Radiograph of the site of a missing tooth showing adequate bone levels. (B) Post-operative radiograph of the same area after placement of a titanium dental implant. (C) Pre-operative clinical view of the site of the missing lower incisor tooth. (D) Immediately after placing the implant into a site prepared in the bone. The collar of the implant can be seen. The soft tissues are being displaced from the bone by the metal instrument. (E) Implant supported crown in place. (F) Mirror view showing the attachment to the implant which supports the crown.

2. The complexities of implant surfaces

While early dental implants had simple threaded forms and plain surfaces which were unmodified after milling, almost all modern dental implants have surface features which increase the surface area and surface energy, enhancing the adhesion of blood, matrix proteins, and human cells. Altering the surface of a Ti implant to increase its roughness does not compromise its biocompatibility but enhances the total area available for integration with bone [2, 3]. The surfaces of most modern dental implants are microtextured, to support and enhance osseointegration [4].

A range of methods have been used to achieve modification of the milled surface. Treatments such as titanium plasma-spraying, grit-blasting, acid-etching, and anodization create a favorable roughened, high-energy surface, which aid in the process of osseointegration [4]. The roughness of most current implant surfaces created using these methods ranges from 0.5 to 2 μm [3]. Newer surface modifications involving treatment using sulfuric acid and hydrogen peroxide can create nanoscale roughness, while technologies such as micro-arc oxidation can create nanostructured bioactive titanium oxide layers to enhance cell attachment and adhesion onto the dental implant surface. Examples of typical implant surfaces are shown in **Figure 2**. These patterns are superimposed onto a variety of different types of thread patterns (**Figure 2 A , B**). Regions which have only been milled, such as the uppermost collar region, show typical lathe marks on the surface (**Figure 2 C , J**), whereas the thread regions have microtextured surfaces.

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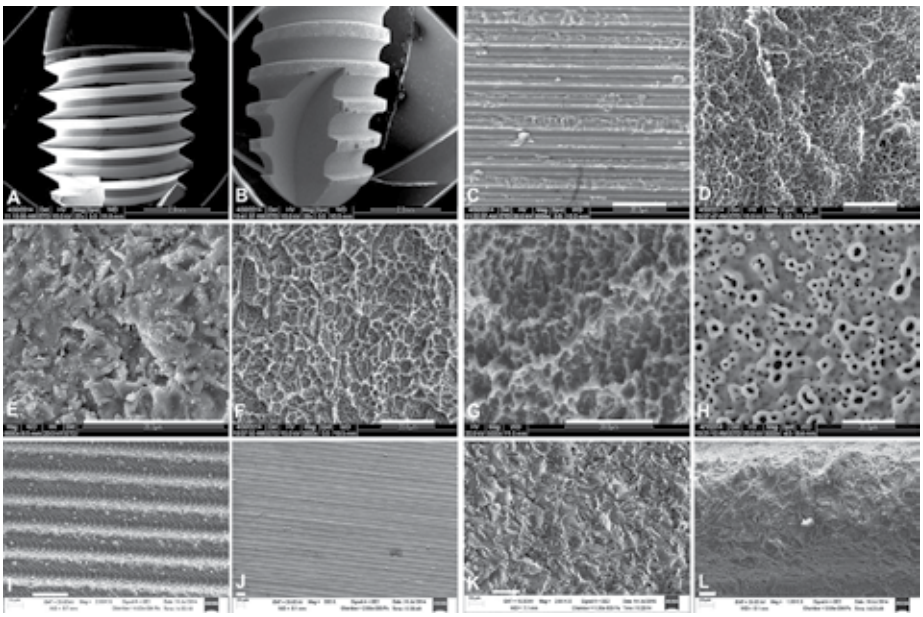

Figure 2. Scanning electron microscope images of dental implants. (A) The collar (upper) and thread regions of a 3i Biomet implant. (B) The lower end of an Ankylos implant. Note the difference in thread patterns compared to (A). (C – L) Implant surfaces of various implant brands. White scale bars represent 20 microns. (C) Non-textured surface showing lathe cutting marks (3i Biomet). (D) MIS, (E) Neoss, (F) Ankylos, (G) MID, (H) Nobel Biocare, (I) Biohorizons, (J) Southern (non-textured surface), (K) Southern ITC, (L) Southern (collar region).

3. Biofilm development on implant surfaces

The roughened implant surface which assists in achieving integration of the implant with the bones of the jaws provides an exceptionally favorable microenvironment for biofilm formation, when the surface comes into contact with saliva [2]. In vivo studies have shown that the extent of bacterial colonization of roughened Ti surfaces is greater than that of smooth surfaces [5]. Moreover, the extent of bacterial adhesion has been shown to correlate directly with the extent of surface roughness [5]. Several authors have shown that methods which increase surface roughness resulted in enhanced attachment of bacteria [6, 7]. Biofilms then develop quickly and mature rapidly, nourished by nutrients from the host, both through saliva and through gingival crevicular fluid and blood. The latter is found as a consequence of the development of inflammatory reactions in the adjacent host tissues and contributes to the growth of Gram-negative species, which utilize iron and porphyrins in their normal metabolic pathways.

Bacteria from the oral cavity readily adhere to the surfaces of dental implants, and mature biofilms develop over several days. Such biofilms can be seen on the surfaces of implants removed because of clinical failure from peri-implant inflammation, and are identical to those

which can be developed under laboratory conditions using human saliva as the sole inoculum (**Figure 3**).

Once a biofilm has become established on an implant surface, conventional methods of debridement are not effective for its removal [8]. An implant surface which is positioned below the position of the oral soft tissues cannot be reached with the bristles of a conventional toothbrush, as these only penetrate 0.5 mm into crevices around teeth and dental implants. Likewise, products used in the mouth such as mouthwashes only penetrate to a similar extent.

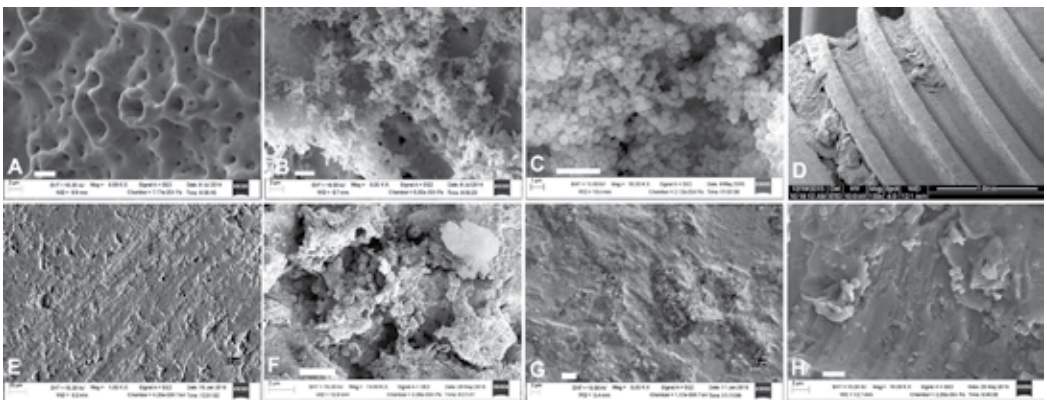

Figure 3. Rapid development of bacterial biofilm on dental implant surfaces. Scale bars in parts (A–C) and (E –G) represent 2 microns. (A) Pristine surface of a new Nobel Biocare implant. (B) The same surface after 4 days incubation in brain heart infusion broth inoculated with human saliva. (C) Four days biofilm on the surface of a Southern implant from a human saliva inoculum, which shows similar characteristics to image (B). (D) Biofilm between the threads of an implant which was removed from the mouth because of peri-implantitis, which had led to bone loss and eventual failure of the implant. (E) Damage to an abraded titanium surface (Southern implant) caused by an ultrasonic scaler with a metal tip used for 60 s to remove biofilm. Compare to **Figure 2K** which is the undamaged surface at the same magnification. (F–H) High power views showing bacteria still present and surface damage (flattening of irregularities) on different types of micro-roughened titanium surface after using an ultrasonic scaler.

4. Biofilm-induced peri-implant diseases

Peri-implant inflammatory diseases caused by biofilm accumulation on implant surfaces may segregated into two forms: peri-implant mucositis (PIM) and peri-implantitis (PI). These differ in the extent to which the inflammatory reaction of the host immune response extends to involve the bone surrounding the implant [9–11]. In PIM, inflammation is confined to the soft tissues surrounding the dental implant, and there is no progressive loss of supporting bone over time [10]. In peri-implantitis (PI), the biofilm-induced inflammatory process causes both changes in the soft tissues as well as progressive loss of the supporting bone [12]. Both PIM and PI are relatively common. The most recent systematic review conducted in 2015 estimated that the prevalence to be in the range of 19–65% for both PIM and PI [13]. This is consistent with recent longitudinal studies conducted in communities, where dental implant treatments are very commonly performed as the standard of care for single missing teeth [14].

At both the microbial and immunohistological levels, there are numerous similarities between PIM and gingivitis, and likewise between PI and periodontitis. The microbial flora is dominated by Gram-negative species, with smaller numbers of streptococci and other Gram positive bacteria. The profile is similar to biofilms, which develop on implant surface sunder laboratory conditions (**Table 1**). Key pathogens which have been implicated in PI include anaerobic Gram-negative rods, motile organisms, and spirochetes which numerically are present at low levels in these biofilms [15, 16]. A point of difference between biofilm formation on implant surfaces versus teeth is that Staphylococci (particularly *Staphylococcus aureus*) and yeasts can be found in biofilms on implant surfaces, whereas these rarely occur in biofilms associated with teeth [16, 17].

Neisseria and other Gram -negative species	79%
Streptococci	7.2%
Bacilli	4.6%
Veillonella	3.5%
Gemella	1.3%
Porphyromonas	0.5%
Actinomyces	0.3%
Peptostreptococci	0.1%
Fusobacteria	0.1%

Major groupings in a typical 96 h multispecies biofilm grown on Southern dental implant surfaces from a human salivary inoculum, showing major groups according to next-gen sequencing analysis.

Table 1. Dominant organisms in the taxonomic analysis of biofilm on dental implants.

Dental implants are increasingly utilized in the restoration of partially dentate or fully edentulous patients. This raises issues of pathogens being transferred from sites with periodontitis to the surfaces of implants as a vector for infection of implant surfaces [18]. Demand for dental implants for single-tooth replacement has been driven by their lower biological cost than conventional dental bridges, and better long-term outcomes, since implants, unlike teeth, are not affected by dental caries and its complications. When failures with implant treatment occur, these may be classified as being either early or late, reflecting surgical or mechanical factors in the former, and biological factors in the latter [19]. PI accounts for most of the late failures, since the biofilm-induced inflammatory reaction causes extensive cratering of the bone, making the implant unsuitable for supporting a crown or other prosthesis [20].

The seminal work of Lang et al. [21] documented how inflammation in the implant-mucosal unit (i.e., PIM) can, in susceptible patients when biofilms are allowed to accumulate for prolonged periods of time, progress from PIM to PI, with accompanying loss of circumferential bone. This conversion is not merely an expansion in volume of the host immune response to the biofilm but represents fundamental changes in the composition of the biofilm

(such as emergence of different pathogens in a cyclical pattern) and accompanying shifts in the composition of immune cells present in the tissues and their behavior, particularly their production of inflammatory cytokines and mediators which alter host tissues, such as proteases. These add to the effects of proteases of bacterial origin, especially those produced by *Porphyromonas* species. As expected, an effective treatment would address the fundamental driving factors within the host response to the biofilm which accumulates on the surfaces of implants by removing the microorganisms and their products [22]. Reducing the biofilm volume and changing its composition should then reduce the intensity of the inflammatory response and alter its character so that the destruction of tissue no longer outweighs formation of tissue [23].

As biofilms develop on implant surfaces, the appearance of key pathogens such as Porphyromonas species is a relatively early event. As seen in **Table 1**, such organisms can be present in saliva and can reach significant levels in the biofilms which form on dental implant surfaces after a period of several days. Quirynen et al. [24] followed the colonization of newly placed implants by bacteria, took samples of the microbiota, and examined these using checkerboard DNA–DNA hybridization, cultural techniques, and by real-time polymerase chain reaction (RT–PCR). They found that bacterial species associated with periodontitis can be detected in peri-implant pockets as early as 2 weeks after implant placement.

The risk factors for peri-implant diseases are strikingly similar to the known predisposing and modifying factors for periodontal diseases. Various prospective and retrospective analyses have shown that the systemic health of the host (e.g., type II diabetes mellitus) [25], genetic traits [26], environmental factors (e.g., smoking) [27, 28], a past history of periodontitis [28], poor compliance with mechanical cleaning recommendations [27], and infrequent dental maintenance visits [29] is major risk factors for the development of peri-implantitis.

Adding to this, there are significant effects of the brand of the implant used, which reflects the different surface topography on which the biofilm will form [29–32], as illustrated in **Figure 2**. For implants with PI, on average around 30% of the bone surrounding, the implant had been lost. Several studies have reported that over periods ranging from 1 to 20 years, the prevalence of bone loss can vary from 27.8 to 47% of patients [30–32]. There is a clear message from such studies that if PI which is left untreated is a strong predictor of future implant loss.

5. The complexities of biofilm removal and implant debridement

While different protocols for professional care of dental implants have been suggested, it is unclear at present which is the most effective [33]. Traditional dental treatment modalities, such as the removal of biofilms using scaling instruments originally designed for debriding the roots of teeth to remove such deposits, cannot be applied in exactly the same way to threaded implant surfaces [8]. The implant surface structure has far more areas which are protected, and much of the surface is inaccessible to conventional professional instruments. Conventional dental therapies such as and scaling instruments and ultrasonic scalers been shown to have minimal effectiveness for removing biofilm and eradicating pathogens from implant surfaces through mechanical means [34].

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Although there are many studies of peri-implantitis treatments, including randomized controlled clinical trials, the latest Cochrane systematic review conducted in 2012 concluded that based on current evidence, no particular treatment can be established as a gold standard approach for the treatment of peri-implantitis [33].

In dental clinical practice, chairside methods to assess the levels of key pathogens present in biofilms on the surfaces of implants or teeth do not exist, and sampling followed by genetic analysis is too expensive for routine use. Therefore, the approach taken follows a nonspecific approach, namely the removal of all biofilm from the surface, regardless of the pathogenicity of bacterial species growing within it. Since the treatment of peri-implantitis aims to also achieve re-osseointegration of the implant surface with bone, it is necessary to remove not only all viable bacteria but all traces of bacterial products such as endotoxins, in order to maximize the likelihood of success.

Treatment of peri-implantitis needs to be implemented as early as the problem is diagnosed, since the likelihood of implant failure due to PI is reduced significantly when the condition is detected early so that treatment can be instituted [35]. Such treatment involves decontamination of the implant surface, as well as surgical augmentation of the associated bony defects [36]. The desired goal of achieving re-osseointegration of the implant after decontamination, despite the use of guided bone regeneration (GBR) with or without bone grafting, is regarded as either difficult or impossible to achieve [37]. The reasons include the challenges of biofilm removal from the surface of the implant, alterations of the implant surface caused by the cleaning procedure used.

6. Methods which have attempted to clean implant surfaces

Implant surfaces are notoriously difficult to clean [38, 39]. The difficulty in cleaning the surfaces of titanium dental implants lies in the complex topography of the implant surface, as is readily apparent at high magnifications such as those used in **Figure 2**. Most implants have threads at the macro-level (e.g., **Figure 2 A , B**), which impede with the action of hand scalers and ultrasonic scalers, so that they only touch the outer parts of the threads but do not reach areas between the threads. On the microscopic level, the highly roughened surfaces mean that there is a large surface area. The microscopic roughness of the surface is a major obstacle for the removal of bacteria and their products [38], as these types of surfaces defy effective debridement by mechanical means alone [39].

The various conventional methods that have been examined for biofilm removal from implant surfaces include ultrasonic scalers fitted with various types of tips, hand periodontal curettes with steel, titanium, plastic or Teflon® tips, abrasive and polishing rubber cups and brushes, and particle beam (air powder abrasion) devices [40]. Most or all of these are found in a modern dental office. Ultrasonic scalers used with metallic tips and stainless steel hand scalers damage and scratch the surfaces of titanium implants [40–42], and for this reason, their use is contraindicated [41]. Furthermore, plastic hand scalers leave residual scaler material on implant surfaces during use [43, 44]. Examples of typical damage to surfaces from ultrasonic

scalers are shown in **Figure 3** (panels E–H), which also show residual bacteria and biofilm matrix which persist on the surface despite intense professional cleaning. No conventional cleaning method will remove all traces of biofilm from microscopically rough titanium surfaces, and the careless application of stainless steel instruments will damage the surface and encourage further biofilm growth.

Some implant designs use a smooth collar near the attachment point for the overlying crown, which is designed to be easier to clean by toothbrushing. Hand and ultrasonic scalers can readily damage this smooth surface, with the resultant scratches promoting the growth of biofilms in the supragingival areas. As this matures, it can track down the protected areas of the grooves and scratches created by dental instruments to penetrate into the subgingival environment, where it can then become established on the threads, leading to peri-implantitis. For this reason, plastic curettes and rubber polishing cups are recommended for the removal of plaque from smooth implant collars, rather than metal instruments of any type [40, 42, 45].

Within the group of conventional instruments, particle beam or air-powder abrasive methods have been shown to provide the most effective cleaning option to date [46]. The range of available particles for such devices includes aluminum oxide, calcium carbonate, sodium bicarbonate, and glycine. Several manufacturers have fabricated tip designed to apply the particle beam into subgingival implant surfaces; however, the pattern of the threads causes many regions on the surface to be protected. It must be recognized that air-powder abrasion causes undesirable microscopic alterations of titanium implant surfaces, and so are not ideal [47].

6.1. Cavitation-based approaches

Ultrasonic scalers have been used in dental practice for the removal of dental biofilms on the root surfaces of teeth [48, 49]. Modern ultrasonic scalers fall into two main categories: piezoelectric and magneto-strictive devices. A part of their cleaning action is through vibrational energy, which shatters any calcified hard deposits. Only the tip of the ultrasonic is considered active; thereby, effective debridement is limited by how much contact the tip has with the surface area of the tooth [48]. Traditional ultrasonic inserts are made from stainless steel, and these damage implant surfaces through a mechanical vibrating contact action. Typical patterns of surface damage are shown in **Figure 3**.

Ultrasonic scalers also create cavitation, with the resultant shock waves from explosions and implosions disrupting bacterial cell walls. The accompanying stream of irrigant water both cools the tip and introduces air and thereby oxygen to the area. The movement of fluid can help remove endotoxins [49]. A number of manufacturers have released the so-called "implant safe" ultrasonic tip inserts for use in both implant maintenance and for the treatment of peri-implantitis. These tips are usually made of carbon fiber, titanium, Teflon®, graphite, or plastic. A number of studies have demonstrated that ultrasonic tips designed for implant maintenance do not cause significant damage at the macroscopic level [50–52]. Paradoxically, some investigators have proposed the use of instruments that deliberately flatten the microscopically rough implant surface to reduce its roughness and thereby area

available for the attachment of bacteria [53, 54]. This is certainly a compromise since the original goal has always been to remove biofilm without causing any surface modifications.

In terms of clinical outcomes, Karring et al. treated 11 patients diagnosed with PI and cleaned the implants either with an ultrasonic scaler or plastic hand scalers. They found no clinically relevant difference in the outcomes obtained [55]. No instrumentation applied by the dentist can resolve peri-implantitis (or periodontitis) if the oral hygiene of the patient remains poor, and bleeding scores remain high, which indicates persisting biofilms and persistent inflammation. Despite the advent of new ultrasonic scaler inserts made of titanium, plastic, or graphite, the general consensus in the literature is that ultrasonic scalers have the same fundamental limitations as hand instruments in that cannot access the undercuts of the implant found between the threads [56]. Their zealous use causes surface alternations [52, 57]. Moreover, the treated surface is not yet biologically compatible, since biofilm and endotoxins remain [58].

6.2. Particle beam systems for the removal of biofilm

Particle beam (air polishing /air abrasion) units have been marketed for the treatment of the roots of teeth affected by periodontal disease, because of their ability to disrupt biofilms [59], while causing little damage to the roots of teeth or the adjacent oral soft tissues [60]. They are well suited for repeated use at the same site, in contrast to hand instruments which when used repeatedly on the same tooth can cause significant removal of tooth structure from the root surface [60, 61].

The principle behind these particle beam devices is that steady flow of compressed air accelerates abrasive particles, which then impact on the tooth surface and fracture or abrade away deposits, including biofilms and external stains [62]. Ideally, the powder used should not damage the target and preferably would also exert some modest antibacterial actions [60]. A number of manufacturers now produce particle beam devices and powders for different periodontal applications. The tip designs vary according to the mode of clinical application (supragingival or subgingival tip) since these require different angulations for applying the particle beam at the appropriate working distance from the surface being cleaned. The powders available vary in particle size, shape, composition, and density [62], and include sodium bicarbonate, calcium carbonate, bioactive glass, pumice, and glycine [61]. The choice of powder type and the application method used both influence the effectiveness of biofilm removal and the potential for tissue harm [60]. Logically one would want to avoid powders which are harder than grade 4 or 5 titanium, as these could damage the surface and roughen it even more, enhancing the problems caused by the attachment and growth of bacteria [63]. This is exactly the same issue as discussed earlier for stainless steel instruments such as ultrasonic scalers and hand scalers, which will damage titanium implant surfaces [64].

The first study of implant surface debridement using a particle beam approach was undertaken by Barnes et al. [65], who used four different implant systems and exposed samples on the bench to particle beams for 0.5 s up to 10 s. Using scanning electron microscopy (SEM), no major differences between the surfaces were found. Since that time, numerous studies have examined the effects of particle beams on implant surfaces. Most in vitro studies and narra-

tive reviews based on these have concluded that particle beams are a safe treatment for decontaminating a titanium implant surface without causing major modifications to the surface [46, 52, 66]. The extent of surface damage is influenced strongly by the choice of abrasive powder [46], with sodium bicarbonate and aluminum oxide (alumina) powders being more likely to damage the implant surface than glycine [46]. There has been emerging support in the most recent literature for the use of glycine powder as the particle material of choice, due to it exerting bacteriostatic actions when used at a 10% concentration [67], having a low risk of air emphysema [68], and causing less damage to implant surfaces than sodium bicarbonate.

6.3. Laser-based methods

As discussed above, the complete removal of biofilm from titanium implants has proved elusive to date. Traditional dental instruments used to debride root surfaces have proved particularly ineffective [69]. Lasers have been suggested as an alternative means of decontaminating dental implants [39, 70], with some studies using Er:YAG lasers showing nearly complete removal bacteria and debris from titanium surfaces [36, 38]. The logic behind using lasers relates to the various photothermal bactericidal effects of lasers as well as their ability to create photomechanical effects such as cavitation when used in a way that generates cavitation in water [71]. The three-dimensional effects created by the scatter of laser energy, when combined with the shear forces generated by cavitation from a static laser tip would seem to be a very promising approach. The scatter of laser energy from a microscopically rough surface would enhance the extent of photothermal disinfection achieved, so that under certain conditions laser treatment could render the implant surface not only decontaminated but also sterile. This stands in marked contrast to the effects of hand or ultrasonic scalers or particle beam devices, none of which can produce a sterile surface [72]. Likewise, decontamination and detoxification of a titanium implant surface cannot be achieved with hand curettes alone [36]. In contrast, with a laser, the ability to decontaminate the implant is limited primarily by the degree of access that the laser energy has to affected implant surfaces. The choice of system used to deliver laser light then becomes an important consideration, with aspects such as the physical size and light distribution properties of the sapphire tips, glass, and non-glass optic fibers, or hollow waveguides used to deliver laser light having an effect [73].

The biocompatibility of a laser-treated surface must also be considered. Guided bone regeneration or bone grafting may be used to treat peri-implant bone loss; however, these surgical techniques both require a meticulously clean implant surface in order to achieve a good outcome [74]. Romanos et al. [75] established that cell attachment and morphology after laser irradiation is equal to that of sterile implant surfaces. Kreisler et al. [47] examined the biocompatibility of contaminated implant surfaces after treatment with either a particle beam device or the Er:YAG laser. The lowest cell growth and proliferation was seen for contaminated Ti surfaces, while cell growth was significantly greater on sterile (new), air powder-treated, and Er:YAG laser-cleaned surfaces.

Infrared lasers can exert powerful photothermal effects which can inactivate or destroy bacteria. The highly water absorbing far infrared energy from a carbon dioxide laser has potential application for the destruction of bacteria. Deppe et al. [36] found that the carbon

dioxide laser when used for disinfection gave faster initial healing than conventional methods. Nevertheless, the long-term outcomes were not significantly different, particularly when bone levels were compared after 4 years. The authors of this study also pointed out that the shape of the defect could have prevented the perpendicular delivery of laser energy and that optimal therapy with this laser when used for disinfection would require changes to the delivery system of the laser to make laterally emitting or side firing. They also noted that bleeding from the surgical site during the procedure would have reduced the amount of laser energy reaching the implant surface, and this attenuation by water absorption may have put the actual levels of energy reaching the implant surface well below those required for sterilization.

As well as the Er:YAG and carbon dioxide lasers already mentioned, other lasers have been found to be of benefit in the treatment of peri-implantitis. Bach et al. [89] found that near infrared diode laser irradiation reduced the rate of recurrence of peri-implantitis to only 7%, most likely because of the disinfecting action of this laser. Likewise, several wavelengths of laser light have been shown to impede the progression of bone resorption in peri-implantitis treatment regimens [77–80].

6.4. Guided Er: YAG and Er,Cr:YSGG lasers for implant surface decontamination

Sterilization and cleaning of implant surfaces by infrared lasers has been demonstrated in several reports [39, 70, 81], and surface decontamination has been reported for both CO_2 and Er:YAG lasers. Bone has been found to reattach to implants after infrared laser irradiation in a peri-implantitis models in dogs, suggesting that laser treatment leaves a biocompatible surface [78].

A key issue is that while laser irradiation can rapidly reduce the bacterial load on an implant surface, it may not be able to render the surface sterile in all circumstances, depending on the geometry of how the laser light interacts with the biofilm on the implant surface. The ability of laser irradiation to reduce bacterial viability is influenced by the implant surface roughness. Kreisler et al. [74] found greater bacterial killing for laser energy delivered at right angles to the surface for microbial deposits on smooth surfaces, and lower effectiveness for those on rough surfaces. They also showed that intensity (power density) strongly influences the disinfecting action.

In order to optimize the effect of laser energy, it is important to achieve a side-firing effect so that laser light applied using a fiber which is parallel to the long axis of the implant is directed onto the implant surface at an optimal angle. Simplistically, one could consider this angle 90 ° to the surface; however, the presence of micro- or nano- roughness on surfaces means that a spread of angles should be even more effective. Depending on the light wavelengths used, such the optical fibers used to deliver energy to the side of a dental implant may be plain glass, glass which has been modified with fluoride, germanium, or other dopants to enhance infrared light transmission, or rare earth element compounds such as germanium or gallium oxides. The latter are used with middle infrared lasers (Er:YAG and Er,Cr:YSGG).

Fibers with plain 90 ° ends (from a right angle cleave of the fiber) emit light with a typical divergence of 18°–20° . Cone-shaped, periscope, and other specialized applicators have been developed for the ends of optical fibers, to make them have enhanced side-firing actions [73, 76]. An alternative approach is to modify the end of the fiber itself, through various physical processes such as acid etching and particle abrasion [73]. Using such methods, it is possible to create radial-firing tips with cone-shaped ends to provide a broader pattern of light collection and emission than a right angle cleaved end. The most interesting modifications to the surfaces of glass and ceramic optical fibers involve the combination of various processes including tube etching, particle abrasion, and further etching, which creates unique surface architectures known as the "honeycomb" surface, to increase transmission and collection of visible red and infrared light [73, 76]. Various modifications of the parameters used for this technique are required for doped glass fibers (e.g., a longer primary etch stage for fluoride-doped glass), or for fibers containing germanium.

The applications of such honeycomb surfaces include broad lateral dispersion of visible red light as well as near and middle infrared light, for photodynamic and photothermal disinfection of subgingival areas and confined spaces, including biofilms present inside the root canals of teeth. This type of optical fiber technology also reduces thermal stress in adjacent hard and soft tissues [77]. It can also be used for fluorescence detection of biofilms on complex surfaces, including those which are only several cell layers thick, and of free-floating planktonic bacteria [78–81]. There is potential application for the automated detection and removal of biofilms from implant surfaces [82–84]. The value of laser fluorescence systems for detecting subgingival deposits on the roots of teeth is well established, even for those which have become calcified to become subgingival dental calculus [85]. The debriding action to remove biofilms then comes from the ability of the laser to generate cavitation in a water irrigant or water-based fluid. Various optical fiber modifications can enhance dramatically fluid agitation for cleaning complex surfaces and spaces, which are difficult to access [86, 87].

6.5. Laser-induced damage to implant surfaces

An important issue to consider with lasers is whether irradiation causes adverse changes to the implant surface [70]. One would expect that higher peak powers would cause greater alterations, and this has been shown for CO_2 lasers, which can cause undesirable implant surface alterations when used in the super-pulsed mode (when there are very high peak powers), but less damage occurs when the same laser is used in continuous wave mode [82]. Likewise, the Er:YAG laser, which normally operates in free running pulsed mode, can cause damage to titanium surfaces when used at very high peak power settings [45, 88]. Such areas have a melted volcanic appearance, which contrasts with the adjacent surface (**Figure 4**). For this reason, laser parameters such as peak power must be kept below the point where melting or surface ablation of titanium occurs, and water flow rates must be sufficient to minimize effects of plasma formation.

A further issue when using a powerful laser is the possibility of adverse thermal effects on bone. If the laser energy is absorbed strongly into titanium, not only can the surface be damaged, but the heat generated can be transferred to the adjacent bone [70, 71]. For safe

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clinical use, the temperature elevation which occurs in the peri-implant bone as a result of laser irradiation should be <10° C, since bone temperatures of 47° C or above may result in bone necrosis [83]. Using fibers and tips, which emit laser energy in a side-firing manner, lowers the total irradiance of the bone, while still achieving even irradiation along the length of the exposed threads fiber. Low average powers will also preserve the morphological and chemical characteristics, which provide titanium with its excellent biocompatibility.

In summary, the concept of using lasers to treat implant surfaces holds considerable promise, yet certain technical issues remain to be addressed, including controlling the laser effect (for example, through fluorescence feedback), achieving the correct geometry for delivery of laser energy (such as using side-firing fiber tips), and controlling undesirable thermal effects on the titanium surface and on the adjacent supporting bone [73–75]. The laser-treated surfaces have high biocompatibility, and this is reflected in the clinical studies that have been undertaken to date and produced promising results [89–95].

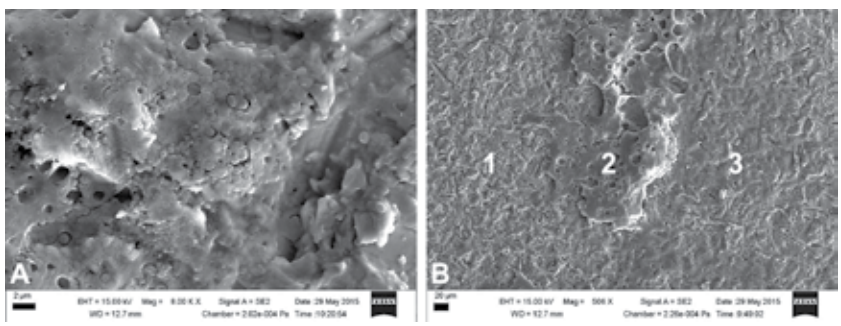

Figure 4. (A) Laser debridement. Biofilm growing on micro-rough abraded titanium surface from a saliva inoculum after 4 days, prior to laser treatment. The scale bar represents 2 microns. Individual bacteria are embedded into a dense matrix. (B) The surface after application of 120 mJ Er:YAG laser pulses with a fine mist water spray. The original abraded surface can now be seen (1), as well as a large central area where the titanium surface has been melted by laser pulses (2), and regions with remaining biofilm which have not yet been treated (3).

7. Laboratory models for assessing biofilm removal from implant surfaces

One of the most informative ways to assess how well a particular method can clean biofilm from an implant surface is to use a physical material which replicates the adhesive nature of biofilm, covers the surface at the microscopic level, and requires a similar process for its removal. The model which fulfils these three requirements involves the application of permanent marker ink of a certain type. The ink model was first described by Sahrmann et al. [96] . In our laboratory, when the same model is used, an abutment is attached to each implant so it can be handled without touching the surface, and the implants are dip-coated in a cyan blue indelible ink (Sharpie Fine Point Permanent Marker, Sanford L.P., Illinois, USA). This ink forms a uniform, visually detectable biofilm-like layer over the implant surface and penetrates well to cover fully the regions between the threads. The implants are inspected

under a light microscope to confirm an even distribution of ink over the implant surface. Each implant is subsequently mounted in an acrylic resin block (Sawbones, Pacific Research Laboratories, Washington USA) prepared with a 6- mm-deep, circumscribed saucer-shaped defect at 60° to simulate the bony defects found in sites of peri-implantitis environment. Implants are fixed into the Sawbones by screwing them in with two revolutions, to the desired position, typically so the third thread of the implant is level with the base of the intra-bony defect. After applying particular treatments, ink removal is then assessed by analysis of the area of ink remaining [96, 97]. We have developed a special system to photograph the ink distribution across the surface, which involves digitally stitching images from macro-photography so that the sharply in focus regions are combined into one image which shows the entire surface. This is suitable for quantitative analyses of the area of ink removed (**Figure 5**). At the microscopic level, the ink be identified using SEM in backscatter mode as its low atomic number signal appears dark which contrasts well with the higher atomic number signal from the underlying titanium.

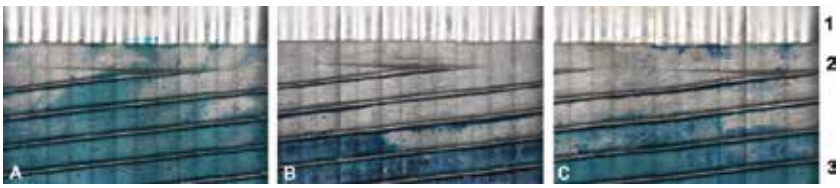

Figure 5. Ink model of biofilm removal from implant surfaces using blue ink placed onto 10- mm-long 4 mm-diameter Southern implants (ITC 410) fixed into Sawbone with peri-implant defects. Zone 1 represents the implant upper collar, and Zone 2 represents the area, where the ink has been removed and the underlying grey implant surface can be seen, and Zone 3 is the ink, which has remained in the deeper regions of the defect. Three different treatments have been applied in an attempt to clean the surface. (A) Ultrasonic scaler for 15 s. (B) Ultrasonic scaler for 120 s. (C) Particle beam device with glycine powder for 30 s. Note the persistence of dye on the areas which are shadowed by the threads, while the adjacent easily accessible areas have little dye. No treatment has reached to the base of the defect.

7.1. Mixed biofilm models

Biofilms which grow on implant surfaces contain multiple species, which are derived from the approximately 700 species of bacteria which are found in the saliva. These bacteria form a complex multispecies microorganism community in the biofilm, along with fungi such as *Candida albicans*. Using single species models in the laboratory cannot replicate the complexity or the biofilms which form in the clinical situation. Some laboratory studies have developed a mature anaerobic biofilms from multiple strains of known primary, secondary, and tertiary colonizers enriched in a high protein broth [98–100]; however, a major limitation in such studies is that the biofilms have been grown on flat hydroxyapatite (HAP) discs.

To ensure that a biofilm is established with features more like those found in vivo, we have developed complex multispecies biofilms on titanium disks with surface micro-roughness, and on dental implants under laboratory conditions (**Figures 3 B, C,** and **4A**). For this purpose, we have used human stimulated saliva to inoculate a broth of brain heart infusion (BHI) medium enriched with 5% defibrinated sheep or horse blood and 1 mg/mL menadione, which

is then kept under anaerobic conditions (0% O_2, 20% CO_2, and 80% N_2) at 37°C. This medium is rich in protein and hemoglobin in order to encourage the growth of facultative and obligate anaerobes. The saliva is collected from healthy adult subjects who have refrained from toothbrushing and other oral hygiene practices for 12 h prior to the collection of stimulated saliva, collected whilst chewing on sterile paraffin wax for 5 min. The incubation times of 72–96 h which we have used in these studies are the same as those used by Sánchez et al. [100] in their studies of the growth of pure species using the same BHI growth medium . Their work showed that by 12 h the early colonizers had adhered, the intermediate colonizers appeared at 24 h, the late colonizers were found after 48 h, and the biofilm reached a steady state between 72 and 92 h after initiation. Therefore, this model using BHI supports the development of a biofilm that is similar in composition and structure to a subgingival biofilm in vivo.

A key aspect of the process of biofilm formation is the deposition of a glycoprotein pellicle layer by the adsorption of salivary glycoproteins onto the pristine titanium surface before it is placed into the broth. Surfaces of titanium discs are abraded with alumina particle beams and then steam sterilized before being placed into the collected saliva for 5 min, to allow a pellicle layer to form. The same process is undertaken for titanium implants. The discs or implants are then placed into the BHI broth and incubated under anaerobic conditions. The resulting biofilms on the discs and implants can then be treated with various methods, and the extent of remaining biofilm assessed using vital staining with confocal microscopy, or scanning electron microscopy. For the latter, an appropriate fixation regimen involves 24 h in 10% neutral buffered formalin solution, followed by rinsing in 0.1 M cacodylate buffer solution for 30 min, and then post-fixing in osmium tetroxide for 1 h. The fixed samples can then be dehydrated with graded ethanol solutions (50–100%), dried, and placed onto aluminum stubs using conductive carbon tabs, and sputter coated with a 10- nm-gold layer, prior to being viewed using secondary electron emission or backscatter modes under high vacuum conditions.

7.2. In situ models

We have also developed an in situ model of biofilm formation on implants, using a specialized removable oral appliance [101]. The rationale behind this work is that past studies of implant biofilms have been laboratory based and have used only single species biofilms of oral bacteria. They have little or no direct relevance to clinical patient care. It was desirable to have a reproducible in situ model with naturally formed complex biofilms of mixed species, which should form under low oxygen conditions in an environment which is partially protected from the washing action of saliva, but able to access nutrients from the saliva. There should be contact with normal host protective mechanisms such as the gingival crevicular fluid produced around the gingivae. To meet these objectives, a removable appliance was designed which uses a removable dental bleaching tray as its base. This appliance carries an implant on its side, which is located within a tube and held against the oral soft tissues beside the gingival crevice (**Figure 6**). Using this model, we have generated realistic biofilms on dental implants in 48 h and then used these to test the effectiveness of various debridement methods. Other groups have likewise developed methods for developing dental plaque on implants using in

situ appliances [102–104], and this will likely be a productive approach for future studies. A particular advantage of our own system is that it can use both a flat titanium disc and an actual commercial titanium implant, whereas other models use flat titanium discs. While flat surfaces are easier to both clean and analyze, they lack features such as threads which make them hard to clean.

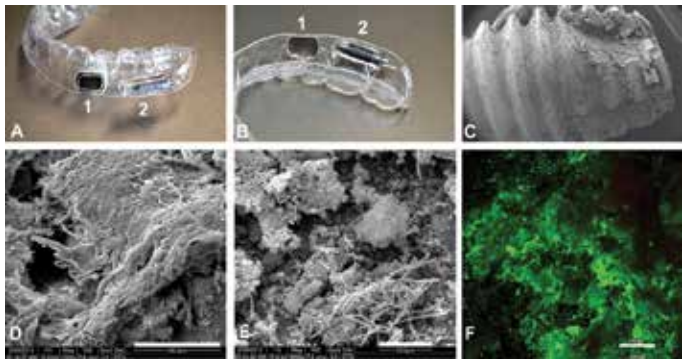

Figure 6. Removable dental appliance for the development of biofilms on dental implants. (A , B) The appliance design showing the flat titanium disc (1) and the titanium dental implant (2) mounted into a vacuum-formed removable appliance. (C–E) Low, medium, and high power SEM views of a 48 h biofilm on the implant in the in situ model. Scale bars in panels (D) and (E) are 100 and 10 microns, respectively. (F) Vital staining of biofilm grown on a flat titanium surface, using confocal microscopy.

8. Conclusions

The complex surface properties of titanium dental implants which give them excellent biocompatibility also facilitate the attachment of bacteria and the development of biofilms. The macroscale and microscale topographies of threaded implants make these difficult to clean with conventional dental instruments. Technologies such as particle beams and pulsed lasers appear promising in terms of better biofilm removal from surfaces. The development of various clinical and laboratory models for dental implant biofilms allows the systematic comparison of different approaches to biofilm removal.

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Chapter 21

Compounds from Natural Sources for New Diagnostics and Drugs against Biofilm Infections

Laura Selan, Marco Artini and Rosanna Papa

Additional information is available at the end of the chapter

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Abstract

Biofilm infections represent a new medical challenge that drives towards the discovery of new diagnostics and new drugs specifically designed for this purpose. All living organisms offer a huge source of compounds which represent the biochemical substrate of the biological competition on the Earth and can be used to this aim. We describe an innovative diagnostic tool to early diagnose medical device infections sustained by Staphylococci; then we list new compounds that modulate bacterial phenotype and reduce virulence without affecting bacterial viability so as to avoid the emergence of genetic resistances. These compounds are all derived from natural sources: prokaryotes, plants, and human body. From prokaryotes we studied new compounds extracted from different environmental bacterial species, including Antarctic species growing in extreme environments. We describe also the anti-biofilm properties of extracts obtained from plants well known since centuries in folk medicine. The humoral immune response is the source of the last anti-biofilm compound: transferrin (Tf), a protein derived from human plasma involved in inflammation and natural immunity. All these compounds can be used as scaffolds for the design of new drugs active on the sessile form of pathogens prevalent in human biofilm infections.

Keywords: human, diseases, infection, therapy, diagnosis

1. Introduction

Since 1929, the discovery of penicillin radically changed the history of human infection diseases, opening the path to the discovery of a wide array of new antibacterial compounds. After a few decades, the appearance of genotypic resistances undermined the dream of definitive human victory on infectious diseases. In the 1970s, some unexplainable cases of drug resistance sustained

by strains prone to conventional antibiotics were attributed to biofilm. At the beginning, sessile phenotype has been related only to medical devices infections, but as long as knowledge on biofilm behavior increased, the majority of chronic human infections have been attributed to sessile phenotype. It has been demonstrated that sessile bacteria resist to antibiotics due to a variety of causes including, a reduced penetration of drugs in the deep layers of biofilm, a favored diffusion of resistance genes, a drift of bacterial metabolism towards anaerobiosis causing a reduced cellular division, and a dramatic reduction of susceptibility to drugs. Efforts have been devoted to restore sensitivity of sessile bacteria to antibiotics and to identify new compounds to treat biofilm. In this chapter, we describe our efforts in this field. Since the early 1990s, our lab dedicated many efforts to identify new diagnostics and new therapeutical strategies to counteract biofilm infections in humans. We shall first describe a diagnostic tool based on enzyme-linked immunosorbent assay (ELISA) technique to early identify Staphylococcal colonization of medical devices, then we shall list an array of different compounds active on bacterial biofilms.

2. An ELISA assays for early diagnosis of biofilm infections

Specimen culturing is the gold standard for diagnosing bacterial infections, but growing bacteria from a biofilm is not reliable. Other testing modalities, such as polymerase chain reaction Polymerase chain reaction (PCR) and serology assays are nonspecific for biofilm infections and include the risk of contamination during sampling. In recent years, many attempts have been performed to create new for early, noninvasive diagnosis. A main feature of infections on implanted medical devices is the absence or paucity of local signs and general symptoms of infection/inflammation. The first signs are due to dysfunction of the device itself (loosening of orthopedic prostheses, cardiac valve regurgitation) and device-related damage to the surrounding tissues. Prompt detection of biofilm infection in the initial asymptomatic phases can allow earlier medical/surgical treatment. Highly virulent organisms (e.g., *Staphylococcus aureus* and gram-negative bacilli) induce early appearance of symptoms. Less virulent bacteria (e.g., coagulase-negative staphylococci) usually induce low-grade, indolent infections that remain clinically silent for years. Laboratory tests suggesting inflammation and possible infection by a bacterial pathogen (i.e., C-reactive protein, Complete blood counts, erythrocyte sedimentation rate) do not demonstrate the presence of bacteria and can mislead. An interesting alternative is based on the use of immunodiagnostics detecting antibodies directed against antigens that are specific for the sessile form of bacterial species, especially those that are traditionally considered saprophytes. Since staphylococci are highly prevalent in biofilm infections on medical devices, we developed a simple and reliable immunodiagnostic assay devoted to the diagnosis of staphylococcal biofilm infections [1] based on enzyme-linked immunosorbent assay (ELISA), a simple, rapid, and repeatable technology, that do not require removal of the implanted device. Our ELISA test allows to detect antigen-bound immunoglobulins with peroxidase-conjugated antibodies against human immunoglobulin G (IgG) or immunoglobulin M (IgM), and expresses antibody titers as units of optical density. The assay was tested in patients with late-onset infections involving synthetic vascular grafts

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(LO-SVGI). Antibodies (IgG and IgM) against staphylococcal slime polysaccharide antigens (SSPA) were titrated in sera collected from 38 patients with active LO-SVGIs caused by different staphylococcal species (group A). For control purposes, assays were performed on sera from 10 patients with active LO-SVGIs caused by bacteria other than staphylococcus (group B); 16 healthy patients with histories of staphylococcal LO-SVGI that had been eradicated 6–72 months earlier by means of graft replacement (group C); 17 healthy patients with synthetic vascular grafts and no evidence of current or past graft infections (group D); and 58 healthy subjects with no implanted medical devices or prostheses of any type (group E). Infections were classified as late onset only when first manifestations occurred 2 years or more after implantation of the vascular graft. All infections (ongoing and past) were microbiologically confirmed based on cultures of the explanted graft. The results of this study are summarized in **Table 1** and **Figure 1**.

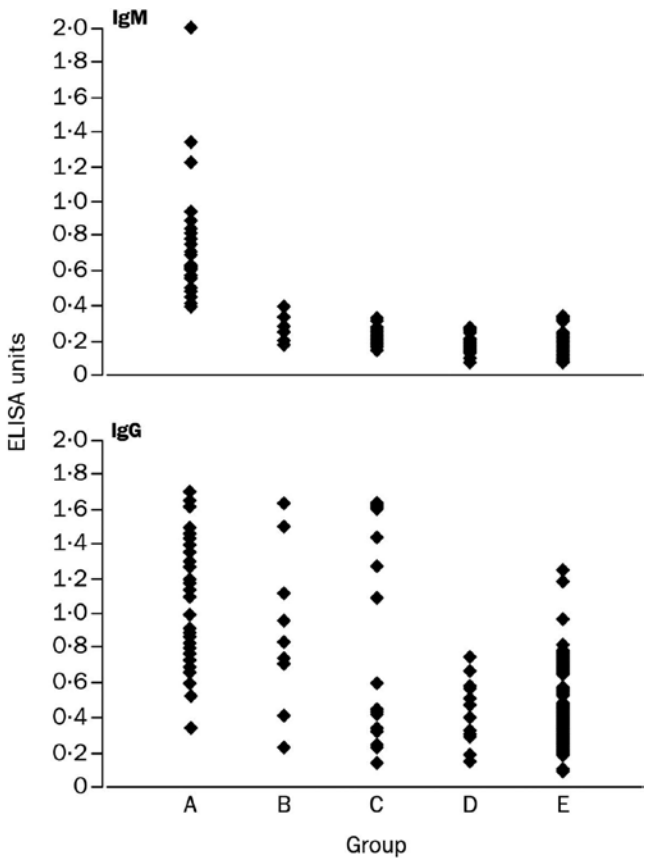

Figure 1. IgM and IgG antibody titres against SSPA. Group A = individuals with staphylococcal LO-SVGI; group B = individuals with non-staphylococcal LO-SVGI; group C = individuals with previous history of staphylococcal LO-SVGI, with successful graft replacement; group D = individuals with synthetic vascular graft implanted 14–78 months before study entry and no previous history of graft infections; group E = individuals free of prosthetic devices.

	Group					
	A (n = 38)	**B (n = 10)**	**C (n = 16)**	**D (n = 17)**	**E (n = 58)**	**B+C+D+E (n = 101)**
IgM titre (mean, SD) (EU)	0.69 (0.37)	0.28 (0.07)	0.26 (0.06)	0.18 (0.05)	0.17 (0.06)	0.19 (0.08)
IgG titre (mean, SD) (EU)	1.13 (0.36)	0.86 (0.46)	0.82 (0.57)	0.46 (0.27)	0.55 (0.26)	0.60 (0.37)
Positive tests for an IgM titre (number, %) (EU)						
≥0.35	38 (100%)	2 (20%)	0	0	0	2 (2%)
≥0.40	37 (97%)	0	0	0	0	0

Group A = patients with an ongoing LO-SVGI caused by *Staphylococcus epidermidis* (n = 6), *S. aureus* (n = 2), coagulase-negative staphylococci other than *S. epidermidis* (n = 24), and mixed infection by one more staphylococcal species and *enterococci, Pseudomonas aeruginosa*, or *Escherichia coli* (n = 6); group B = patients with LO-SVGI caused by bacteria other than staphylococcus—*P. aeruginosa* (n = 2), mixed infections by gram-negative bacilli (*Pseudomonas* spp. or Enterobacteriaceae; n = 5), mixed infection by *Enterococcus* spp and *Enterobacter cloacae* (n = 1), mixed fungal-bacterial infections (*Candida albicans* and *Enterococcus* spp. or *P. aeruginosa*) (n = 2); group C = healthy patients with a history of staphylococcal LO-SVGI, followed by successful graft replacement; group D = healthy patients with a synthetic vascular graft, with no previous history of graft infections; group E = patients with no prosthetic device.

Table 1. Comparison of titres of IgM and IgG antibodies against staphylococcal slime polysaccharide antigens (SSPA) (ELISA units [EU]) in sera from patients with an ongoing staphylococcal late-onset infection of synthetic vascular graft (LO-SVGI) and in controls.

The highest titers of IgG antibody to SSPA were noted in individuals with ongoing staphylococcal LO-SVGIs (group A). However, high titers were also seen in the control groups, which precluded the use of IgG titers for diagnostic purposes. In contrast, titers of IgM antibodies against SSPA were higher in the group A patients. There was virtually no overlap between the titers of these patients and those of controls. IgM antibody of 0.4 ELISA units (EU) or more indicated ongoing staphylococcal LO-SVGIs with detection rates of 97% and no false positives. When a cutoff of 0.35 ELISA units was used, the detection rate increased to 100%, but the false-positive rate also rose to 2%. The substantial difference observed between patients with ongoing versus previous staphylococcal LO-SVGIs (group A vs. group C) suggested that levels of IgM antibody against SSPA decrease rapidly after successful graft substitution. Recurrence of graft infection was associated with the return of elevated IgM antibody titers. We concluded that SSPA ELISA positivity can be used as a marker of active staphylococcal graft infections. Anti-SSPA ELISA was also tested for the diagnosis of orthopedic joint prosthetic infections (DOJP-Is). To this aim, we compared the titers of IgM antibodies against SSPA in the sera of 90 subjects [2]. Studied population included 29 subjects with ongoing staphylococcal DOJP-Is (group A), 34 subjects with orthopedic joint prostheses implanted at least 1 year previously without infection (group B), and 27 subjects not previously operated for orthopedic implants, attending the hospital for noninfectious diseases (group C). All subjects in group A underwent surgical removal of the infected prosthesis, and staphylococcal infection had been microbiologically confirmed by intraoperative cultures. For orthopedic applications, we adopted a cutoff value of 0.35 EU. The main results, summarized in **Table 2**, show that high anti-SSPA IgM levels may provide for noninvasive detection of the immune response elicited by biofilm colonization on artificial orthopedic implants. We did

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not evaluate IgG titers, because they were not associated with current infection both in the previous study on vascular grafts and in a preliminary analysis on five cases and eight controls. According to these results, we can affirm that anti-SSPA ELISA assay is effective in detecting antibodies in DOJP-Is caused by different staphylococcal species.

Group (no. of samples)	Mean (SD) IgM titre (EU)	% (no.) of positive tests for an IgM titre:	
		≥0.35 EU	≥0.40 EU
Prosthesis infection[a] (29)	0.72 (0.55)	89.7 (26)	69.0 (20)
Prosthesis, no infection[a] (34)	0.21 (0.09)[b]	8.8 (3)	5.9 (2)
No Prosthesis, no infection (27)	0.20 (0.05)[b]	0 (0)	0 (0)
All controls (61)	0.21 (0.07)[b]	4.9 (3)	3.3 (2)

[a] Prosthesis infection is defined by subjects with an ongoing prosthetic infection caused by *Staphylococcus epidermidis* (n = 15), *Staphylococcus aureus* (n = 8), coagulase-negative staphylococci other than *S. epidermidis* (n = 2), and mixed infection by one or more staphylococcal species plus enterococci, *Pseudomonas aeruginosa*, or *Escherichia coli* (n = 4).
[b] $P<0.001$ versus infected subjects (Kruskal-Wallis test).

Table 2. Comparison of titers of IgM antibodies against SSPA, expressed as ELISA units, in sera from subjects with an ongoing staphylococcal late-onset infection of orthopedic prostheses and in controls.

Periodic testing for IgM antibodies against SSPA could prove useful in the follow-up of patients with implanted vascular and orthopedic devices. The SSPA ELISA displays a strong advantage over other available methods used to diagnose biofilm-related infections, in fact it is versatile because it can detect antibodies in biofilm infections caused by different staphylococcal species. The higher diagnostic value of the IgM titers depends on the choice of the antigen: a mixture of purified polysaccharide antigens extracted from the biofilm matrix. Polysaccharide antigens are known to elicit a thymus-independent humoral response based exclusively on IgM production. This response is maintained as long as the antigenic stimulus is present, and there is no shift to IgG production. IgMs elicited by antigens of this type are synthesized by a particular subpopulation of B lymphocytes (B1): blood IgM + IgD + CD27 + cells that correspond to splenic marginal zone B cells. Because of the absence of thymic involvement and IgG production, these responses are regarded as expressions of innate immunity. The peculiar behavior of the immune response to polysaccharide antigens represents a diagnostic advantage since it can be used at any time to evaluate the possibility of device infection, even during the post-replacement follow-up. The interest in the development of alternative anti-infective approaches for the prevention and treatment of staphylococcal infections has increased in recent years [3–5]. But our group has been working on the search of new compounds active as anti-biofilm drugs since the early 1990s [6].

2.1. New compounds from prokaryotes for the therapy of biofilm infections.

Our first attempt, for the search of new anti-biofilm drugs, was based on the clinical observation that the administration of proteolytic enzymes can enhance therapeutic outcomes in the treatment of contact lens and endo-ocular prosthetic devices infections. The hypothesis was

that proteolytic treatment could damage the proteic structure of biofilm matrix reverting bacteria to a condition of susceptibility to antibiotics. In our first study [6] four different proteases were tested on 10 bacterial strains (5 *Staphylococcus epidermidis* and 5 *Pseudomonas aeruginosa*) with ofloxacin (dilution range, 200–0.1 mcg/ml): clostridiopeptidase A, fibrinolysin, streptokinase, and serratiopeptidase. Experiments were been performed in both planktonic and sessile form, the last one based on the colonization of polystyrene beads maintained in constant flow culture. Results showed a strong anti-biofilm activity of serratiopeptidase as demonstrated by bioluminescence count of adherent bacterial cells. A further confirmation was obtained by scanning electron microscope (SEM) image of the surface of polystyrene beads incubated for 5 days in a culture of *P. aeruginosa* containing ofloxacin alone (**Figure 2**) or ofloxacin and serratiopeptidase (**Figure 3**).

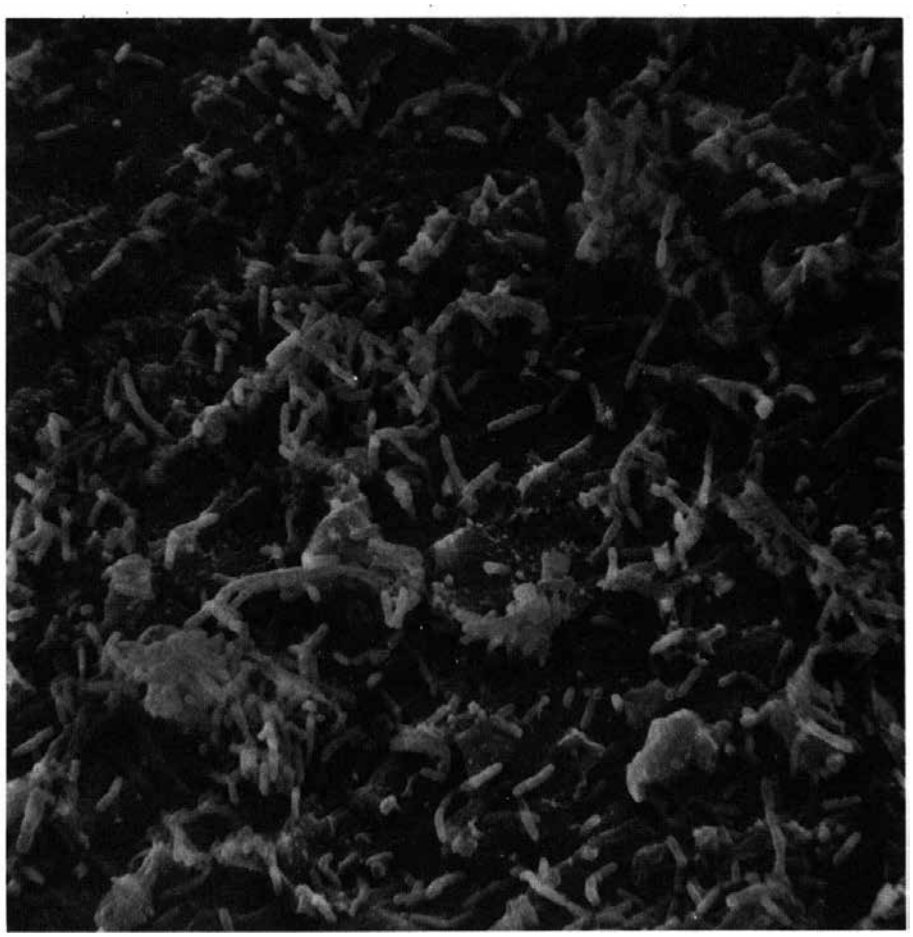

Figure 2. SEM image of a polystyrene bead incubated for 5 days at 37°C in a culture of *P. aeruginosa* in TSB with ofloxacin at a concentration equal to MIC under planktonic growth condition (0.78 mcg/ml) at a flow rate of 120 ml/h. Magnification × 1550.

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Figure 3. SEM image of a polystyrene bead incubated for 5 days at 37°C in a culture of *P. aeruginosa* in TSB with ofloxacin at a concentration equal to MIC under planktonic growth condition (0.78 mcg/ml) and serratiopeptidase at a concentration of 10 U/ml at a flow rate of 120 ml/h. Magnification × 2220.

Serratiopeptidase is a metalloprotease (containing zinc) cloned from *S. marcescens*, showing a strong proteolytic activity and widely used in therapy for decades for its anti-inflammatory properties and for its ability to enhance the penetration of antibiotics in the site of infection. We further studied serratiopeptidase (SPEP) trying to understand its mechanism of action on a molecular level. To this aim, our group examined the effect of two families of proteases on *S. aureus* and *S. epidermidis* strains. In particular, we used three serine proteases (proteinase-K, PK; trypsin, TRY; chymotrypsin, CHY) and two metalloproteases (SPEP; carboxypeptidase-A, CpA) in biofilm formation assays and in human cell invasion processes (invasion only for *S. aureus*) [4]. The study was intended also at obtaining a broader knowledge on the possible use of proteases as anti-adhesive molecules whose use could be proposed in combination therapy with antibiotics. SPEP seems the most promising molecule to be developed as a novel anti-virulence tool. Its action selectively affects a discrete number of proteins clearly involved in fundamental mechanisms associated with bacterial virulence, such as adhesion, invasion, and biofilm formation and would thus hinder staphylococcal virulence properties. Adhesion of bacterial cells and formation of biofilm are finely tuned in staphylococci by an interplay of adhesins including sialoprotein binding proteins (SdrC), fibrinogen binding proteins (FnBP-A/B, Embp, and ClfA), biofilm-associated protein (Aap and Bap), extracellular matrix binding proteins (SasG), autolysins (Alt and AtlE), proteins involved in polysaccharide intracellular adhesin (PIA) synthesis (IcaADBC), and others [7]. In order to ascertain the presence of genes that code for proteins that modulate adhesion and biofilm formation the studied strains were studied by PCR.

We found no relationship between bacterial gene profile and proteases activity. Cell surface protein samples from treated and untreated cultures of all staphylococcal strains were

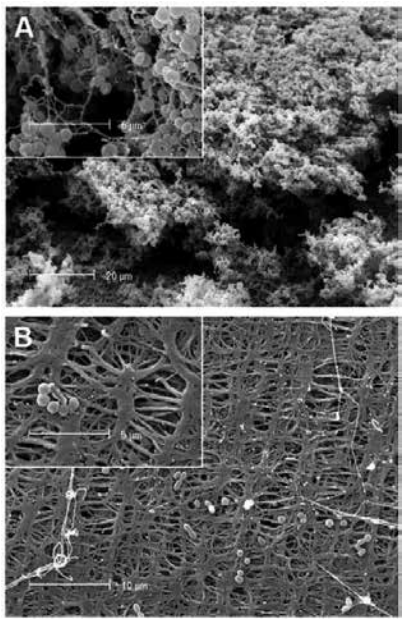

Figure 4. Scanning electron microscopy analysis of *S. aureus* 6538P grown overnight in BHI in presence of a PFTE filter as a substrate for sessile growth in the absence (A), or in the presence (B) of SPEP. In the magnification box of panel A, the extracellular matrix is visible and biofilm-embedded bacteria cover the filter surface. In panel B, the PTFE filter background is visible and only single cells are present.

simultaneously analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The ability of each protease to interfere with *S. aureus* capacity to adhere and invade human cells was tested by antibiotic protection assay on HeLa cell line. We found that only SPEP and CpA had a comparable action on all tested strains; on the contrary all tested serine proteases showed different behavior on each bacterial strain suggesting a nonspecific and indiscriminate effect of these proteases. For this reason, we focused on SPEP and CpA action on surface proteins. SPEP treatment resulted in an effective and broad-spectrum reduction of biofilm formation and its action appears to be more selective, sequence-specific [8, 9] and proportional to biofilm production. Antibiotic protection assay performed on HeLa cells showed that SPEP treatment strongly impaired *S. aureus* invasion efficiency. This is similar to what found by our group for *L. monocytogenes* [10], thus confirming the broad spectrum of this protease also against virulence properties different from biofilm formation. The action of CpA results in an increase of biofilm accumulation, while the action of SPEP impairs surface adhesins/autolysins and probably impairs also the adhesive moieties of the altruistic suicidal cells. The altruistic suicide mechanism was found to be responsible for the lysis of a bacterial subfraction in *S. aureus* biofilm [11]. SPEP and CpA are able to act both on surface adhesins and on the lysed cellular debris derived from the suicidal subpopulation. It is important to underline that SPEP neither

influences bacterial viability when used at the concentrations adopted in this work and at higher concentrations, nor displays a cytotoxic effect on eukaryotic cell lines. Based on these results, confirmed by the images obtained in SEM [12] (**Figure 4**), we focused on the molecular aspects of SPEP action on *S. aureus*, a bacterial pathogen often associated with nosocomial and community-acquired infections, capable to express a multiplicity of virulence factors secreted or associated to bacterial cell surface; these factors include bacterial products that mediate adhesion to the surface of host cells and to damaged tissues. It has been ascertained that in *S. aureus* surface proteins play a fundamental role in virulence properties, including biofilm formation.

A successful strategy to hinder bacterial infection should not affect processes essential for bacterial survival in order to avoid the rapid appearance of escape mutants. A smarter approach should target the main virulence factors of *S. aureus* and avoid interferences on the viability of bacteria.

	Biofilm formation	
	Control	SPEP-treated
6538P	1.64 ± 0.15	0.086 ± 0.015
25923	1.68 ± 0.23	0.63 ± 0.12
12598	0.35 ± 0.05	0.21 ± 0.03
BAA1556	2.15 ± 0.08	0.47 ± 0.04

Based on the 590 nm OD absorbance produced by *S. aureus* strains. Data represent the mean ± SD of three independent experiments.

Table 3. Effect of SPEP treatment on staphylococcal biofilm formation.

Two steps lead to biofilm formation are adherence to a surface by bacterial cells and progressive growth of cell clusters in multilayers. The study of the factors that gather cells into a biofilm has evidenced the existence of strains producing either polysaccharide intercellular adhesion/poly-N-acetylglucosamine (PIA/PNAG) or a protein-dependent biofilm, even if in staphylococci the best-known biofilm mechanism depends on the production of PIA/PNAG, an extracellular polysaccharide adhesin [13, 14]. It has been shown that, besides their best-known role in the eukaryotic invasion process, fibronectin-binding proteins (FnBPs) play a relevant role in biofilm-associated foreign-body infections. Some proteins are multifunctional factors involved in metabolic pathways, in adhesion to extracellular matrix and invasion of host cells, such serine-aspartate repeat-containing protein D (SdrD), Elongation factor-Tu (EF-Tu), Elongation factor-G (EF-G), Atl, SsA2, and second immunoglobulin-binding protein (Sbi). In order to understand the molecular mechanism of SPEP action on *S. aureus* we confirmed its action on biofilm growth and studied the proteomic patterns of treated and untreated bacterial cells. Experiments were performed on four *S. aureus* strains: ATCC 6538P (DSMZ 346), reference strain for antimicrobial testing; ATCC 25923 (DSMZ 1104); ATCC 12598 (DSMZ 20372); ATCC BAA1556 (FPR3757 strain) is an USA 300 strain [15]. The biofilm-forming ability

was tested by quantitative assay. They showed different capabilities to form biofilm: three strains were strong biofilm producers with biofilm amount higher than 1.6 OD at 590 nm, while ATCC 12598 is a medium biofilm producer. Biofilm quantification was performed in Static biofilm assay according to Christensen [16] and in dynamic condition (BioFlux 2000 microfluidics system) which allows the acquisition of microscopic images over time. Results of SPEP effect on biofilm formation of four *S. aureus* strains are summarized in **Table 3**. We found also that SPEP is also extremely effective in the dispersal of *S. aureus* preformed biofilm suggesting that SPEP affects also mature biofilm.

Experiments in BioFlux system, performed on MSSA 6538P and MRSA BAA1556, showed that SPEP clearly impairs biofilm formation as already seen in static system. The SPEP action on the proteome of tested strains was assessed on surface proteins extracted according to the method of Tabouret [17]. Extracted proteins were separated on SDS-PAGE. Many specific protein bands detected in the untreated *S. aureus* protein profiles either disappeared or drastically diminished in intensity after SPEP incubation. This effect was clearly visible for all bacterial strains analyzed, and peptide mixtures obtained by in situ digestion were analyzed by MALDI-TOF Mass Spectrometry; when necessary, peptide mixtures were analyzed by LCMS/MS using a 4000Q-Trap coupled to an 1100 nano HPLC system. Analysis of the results showed the disappearance of specific proteins after SPEP treatment, including some surface proteins that mediate adhesion and invasion in eukaryotic cells, such as SsA2, SdrD, Atl, and Sbi, homolog to SpA. Notably, treatment with serratiopeptidase influenced the expression of cytoplasmic proteins involved in carbohydrate metabolism (in particular, proteins of the glycolytic pathway) and in energy production. EF-G, EF-Tu, and the dihydrolipoyl transacetylase, an enzyme component of the multienzyme pyruvate dehydrogenase complex [18], are further examples of factors participating in energetic metabolic pathways that are also involved in adhesion and invasion in eukaryotic cells. Investigation of the surface subproteome of *Listeria monocytogenes* studied previously, revealed a remarkably high number of proteins with a function in the cytoplasmic compartment. Many reports suggest that anchorless proteins of the bacterial surface promote bacterial adhesion and invasion of eukaryotic cells [19]. Proteins may perform different functions that depend on other proteins they can transiently associate with. As a consequence, proteins involved in bacterial metabolism are not only involved in energy production, playing an alternative role on bacterial cell surface, but also they may facilitate efficient invasion of eukaryotic cells. Some proteins of the adhesion family, alkyltransferase-like (ATL) protein, Sbi, Elongation Factor-Tu, Elongation Factor-G, and Serine-aspartate repeat-containing protein D, deserve particular attention. Atl disappeared after SPEP treatment, confirming that SPEP modulates adhesins and autolysins in *S. aureus*. Recently, a novel mechanism involved in staphylococcal internalization by host cells, which is mediated by the major autolysin/adhesins Atl in *S. aureus* has been described [20]. A microbial surface components recognizing adhesive matrix molecule (MSCRAMMS) member acting as a homologous to SpA is Sbi; this multifunctional protein binds host complement components Factor H and C3, IgG and b2-glycoprotein I and hinders innate immune recognition. Sbi inhibits both complement activation and lyses of rabbit erythrocytes mediated by the alternative pathway of human serum [21]. SdrD, a MSCRAMM family surface protein, plays an important role in *S. aureus* adhesion and patho-

genesis. The crystal structure of the domains of this protein has been elucidated and the ligand-binding site of SdrD was characterized. EF-Tu and EF-G, belonging to the so-called 'moonlight' proteins, perform different functions unrelated to splice variants, gene fusions, and generation of proteolytic fragments. Elongation Factor-Tu is considered as a main factor associated to cell wall in most bacterial species; among them *S. aureus* [18], *M. leprae* [23], *L. johnsonii* [22], M. pneumoniae, where it mediates fibronectin binding together with the pyruvate dehydrogenase E1 subunit [24]. In *L. monocytogenes*, EF-Tu was identified together with EF-G. Recently, EF-Tu has been identified as a surface protein possessing the characteristics of an adhesion factor and showing the capacity to induce a proinflammatory response. We used a methicillin-sensible *S. aureus* strain (6538P) and a methicillin-resistant strain (BAA1556) to assess serratiopeptidase influence on their ability to adhere and invade eukaryotic cells. Adhesion and invasion efficiency after serratiopeptidase treatment is shown in **Table 4**. The test was performed according to the antibiotic protection assay. According to our experimental data serratiopeptidase does not affect adhesion efficiency of 6538P while, as regards BAA1556, the adhesion efficiency was partially upset. About the invasion, expressed as the percent of the adhered bacteria which invaded HeLa cells, our data showed that about 50% of the BAA1556 adhering to HeLa cells invaded them. Even if methicillin-sensible strains are scarcely invasive, SPEP induced a 200-fold reduction of its invasion efficiency; furthermore, methicillin-resistant strain (BAA1556) showed a drastic reduction of invasion efficiency (3000-fold) after treatment with serratiopeptidase.

	Untreated		**SPEP-treated**	
	Adhesion [a]	**Invasion** [b]	**Adhesion** [a]	**Invasion** [b]
6538P	2.75 ± 0.45	0.7 × 10–2 ± 0.1 × 10–2	2.80 ± 1.80	0.3 × 10–4 ± 0.0 × 10–4
BAA1556	4.94 ± 0.97	2.5 ± 0.79	2.82 ± 1.46	0.7 × 10–3 ± 0.1 × 10–3

[a] Adhesion is expressed as the percentage of the initial inoculum of bacteria that adhered to HeLa cells 1 h post-infection at 37°C.
[b] Invasion efficiency is expressed as the percentage of adhered bacteria that were gentamicin-resistant 1 h post-infection. Data represent the mean ± SD of three independent experiments.

Table 4. Adhesion and invasion capabilities of SPEP-treated and untreated *S. aureus*.

It is not yet clear if serratiopeptidase action is mediated by its proteolytic function or to the activation of specific signal transduction pathways that regulate expression of proteins.

In the last years, our group started working also on compounds obtained from marine bacteria. Marine bacteria from Antarctica represent an untapped reservoir of biodiversity and produce several compounds which may be of potential biotechnological interest; culture supernatants derived from most of them have been shown to exhibit anti-biofilm activity against both gram-positive and gram-negative bacteria, including *Acinetobacter*, *S. aureus*, *Salmonella typhimurium*, *Shigella sonnei*, *Listeria monocytogenes*, and several *Bacillus* species [25].

Marine bacteria belonging to the genus *Pseudoalteromonas* produce compounds of biotechnological interest, including anti-biofilm molecules [26]. Starting from these considerations, we tested on different staphylococcal strains the anti-biofilm activity of cell-free supernatant of Antarctic marine bacterium *P. haloplanktis* TAC125 grown in planktonic and sessile form [27, 28]. Our results show that *S. epidermidis* biofilm growth is inhibited only by *P. haloplanktis* TAC125 supernatant grown in static condition. A possible explanation for this result can be found in the peculiar biofilm microenvironment that lead to production of specific metabolites or polymers due to metabolic rewiring of sessile bacteria [29] that could inhibit growth of other microorganisms. In accordance with this hypothesis, many anti-biofilm compounds were identified from cultured biofilms [25]. It is interesting to note that previously characterized anti-biofilm compounds have often a broad-spectrum biofilm inhibition activity [25, 26, 30] while the *P. haloplanktis* TAC125 anti-biofilm molecule seemed to be species-specific. *P. haloplanktis* TAC125 supernatant was, in fact, inactive against biofilm of *S. aureus* and *P. aeruginosa*. The majority of the anti-biofilm compounds described in literature so far also exert an antibacterial activity. *P. haloplanktis* TAC125 supernatant does not show antibacterial activity against planktonic forms, while its action is directed against sessile forms possibly mediated by mechanisms other than growth inhibition. Only few natural molecules display this mode of action. Three hypothetical modes of action could be proposed. (i) anti-biofilm compounds could be surfactants acting on physical characteristics of biotic and abiotic surfaces; (ii) they could act by competitive inhibition of multivalent carbohydrate-protein interactions [31]. Thus, the anti-biofilm compound might block lectins or sugar-binding proteins present on the surface of bacteria, or block tip adhesins of fimbriae and pili [32]; (iii) *P. haloplanktis* TAC125 anti-biofilm compound might act as a signaling molecule that modulates the gene expression of recipient bacteria [33]. Indeed, bacterial communication is one of the regulatory mechanisms suggested to be involved in biofilm formation [34]. The results of the initial attachment assay indicated that the cultured supernatant-inhibited biofilm formation by contrasting the initial attachment of bacterial cells to the surface. *P. haloplanktis* TAC125 anti-biofilm compound affects mature biofilm but its action requires a long time. This observation suggests a mechanism of action mediated by a signaling system that down-regulates adhesive properties of biofilm matrix and bacterial cell surface rather than by surfactant activity that, on the contrary, would rapidly carry on its action. Different systems have been described so far for bacterial communication. A well-characterized system (acting for intra- and interspecies communication) is based on the autoinducer-2 (AI-2) produced by *luxS* gene, as a signaling molecule [35]. *LuxS* has also been identified in *S. epidermidis* [36], but *P. haloplanktis* TAC125 genome analysis revealed that the Antarctic bacterium is devoid of *luxS* gene [37] suggesting the anti-biofilm activity could be due to a not identified signaling molecule. The anti-biofilm effects of *P. haloplanktis* exoproducts could be due to a novel molecule or the synergistic actions of different molecules. The *P. haloplanktis* TAC125 anti-biofilm molecule was active against several *S. epidermidis* strains, among others it was effective on the clinical isolate O-47 which is a naturally occurring *agr* mutant [33], but it was inactive on *S. epidermidis* XX-17 *ica* mutant [38].

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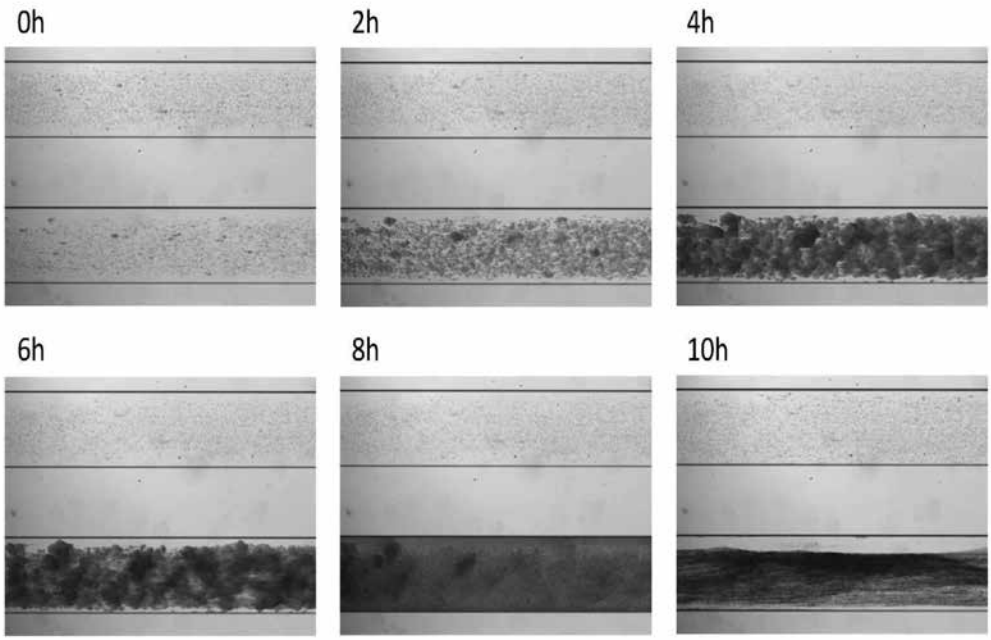

Figure 5. Biofilm formation of *S. epidermidis* O-47 in a BioFlux system. Each image contains two channels: top channel was SN-treated sample and bottom channel was the control one. Bright-field microscopic images were collected at 1-min intervals. The images presented were taken from the complete set of 720 images (see supplementary video bioflux for a video compilation of these images) taken at 40 × magnification.

The efficacy of *P. haloplanktis* supernatant was also confirmed on *S. epidermidis* biofilm formation in dynamic condition using BioFlux system (**Figure 5**). Our results demonstrated that in dynamic condition *P. haloplanktis* TAC125 anti-biofilm was effective against *S. epidermidis* biofilm formation. The use of *P. haloplanktis* TAC125 anti-biofilm molecule/s in combination therapy with antibiotics during persistent infection by staphylococci could be proposed. Furthermore, we evaluated the anti-biofilm activity of supernatants derived from cultures of several cold-adapted bacteria belonging to *Pseudoalteromonas, Psychrobacter*, and *Psychromonas* genera. Supernatants were obtained from bacterial cultures made both in sessile and planktonic conditions. The potential anti-biofilm activity was tested on bacterial cultures of *P. aeruginosa* PAO1, three different strains of *S. aureus*, and three different strains of *S. epidermidis* species [39]. In these species, the matrix composition, the molecules involved in attachment, development, detachment phases, and the regulation of quorum-sensing systems are different [40, 41]. Several cold-adapted bacteria produce molecules that are able to interfere with *S. aureus* biofilm formation. These molecules seem to be proteinaceous. On the contrary, only few Polar strains produce anti-biofilm molecules active on *S. epidermidis* O-47 and RP62A biofilms, and none were able to interfere with *S. epidermidis* XX-17 biofilm formation. It is important to underline that XX-17 strain produces a biofilm characterized by a polysaccharide ica-independent poorly characterized so far. Moreover, the six staphylococcal strains considered here were previously investigated to assess the presence of genes coding for

various proteins involved in adhesion and biofilm formation [4]. In *P. aeruginosa* the biofilm matrix is totally different, because the bacterium produces three exopolysaccharides, the glucose-rich Pel polysaccharide [42], the mannose-rich Psl polysaccharide [42], and alginate [43]. We used *P. aeruginosa* the reference strain PAO1 since the biofilm characterization of this strain was previously reported [44]. The reported differences in biofilm features of the three pathogens could explain the different ability of cold-adapted bacteria supernatants to impair their biofilm formation. It is interesting to note that, in all reported cases, the supernatants proved to be non-biocidal and specifically directed against biofilm. All studied Polar strains were able to produce anti-biofilm molecules against *P. aeruginosa* biofilm. Furthermore in almost all cases, the anti-biofilm molecules seemed to have the same chemical-physical features (were not heat-labile and seem to have a non-protein nature). These results could suggest that the molecule responsible for the anti-biofilm activity is the same for all cold-adapted strains, in particular could be polysaccharides or a small molecule acting as quorum sensing inhibitor. The ability of cold-adapted marine bacteria to produce several anti-biofilm molecules could suggest that the capacity to prevent the biofilm and colonization by bacterial competitors is a selective advantage in this extreme environment.

2.2. New compounds from Eukaryotes for the therapy of biofilm infections.

2.2.1. Compounds from plants.

Considering that plants have already yielded compounds with inhibiting activities against gram-positive bacteria [45], and that the use of medicinal and herbal remedies to treat infectious diseases is common in many countries [46], we have also attempted the discovery of new leads from plants. We explored several plant extracts searching for specific antibacterial activity from fractionated pools [47]. We considered extracts from *Krameria, Aesculus hippocastanum*, and *Chelidonium majus* plants; these plants have proved to possess a plethora of active principles in diverse pathologies. From active fractions, we purified and identified antimicrobial compounds. We identified and purified a dihydroxybenzofuran (DHBF) derivative that could be used as a possible active molecule from fractions of compounds extracted from the *Krameria lappacea* (Dombey) Burdet (para or Brazilian rhatany) and tested in microbiological assays. Our results demonstrated that extracts obtained from *C. majus* are the most active in a screening study [48], it has been shown that crude extracts *C. majus* exhibited antibacterial activity against *S. aureus* ATCC 25923 [49]. From the preliminary microbiological assays probing fractions of compounds extracted from *Aesculus hippocastanum* and *C. majus*, we identified and successively isolated the proanthocyanidin (proAc) from the former plant and the cheliritrin (CH) and the sanguinarin (SA) alkaloids from the latter plant to study their antimicrobial activity. All compounds are described in **Figure 6**. We tested bacteriostatic and bactericidal action on the planktonic form and quantified the efficacy on inhibition of biofilm formation and growth on *S. aureus* and *S. epidermidis*. We also presented proteomic evidence of the alteration of bacterial surface proteome. Both DHBF and SA had a similar marked bactericidal effect. Interestingly, both DHBF and SA were able to inhibit biofilm accumulation in *S. aureus* at concentrations between 1.4 to six-fold lower than those corresponding to MIC/MBC, **Figure 7** and **Table 5**. The inhibition data were interpreted using a

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Hill-type equation [50], **Figure 7**, where EC50 (the effective concentration at which 50% inhibition of biofilm formation is observed) is obtained with a fitting procedure. Experiments performed with sub-MIC concentration demonstrate that studied compounds had not antimicrobial activity at sub-MIC concentration. Therefore, we speculated that these compounds can inhibit biofilm formation but do not kill bacterial cells in the planktonic form. Their action should be rather based on the prevention of *S. aureus* transition to the sessile phenotype. On mature biofilm both DHBF and SA were not effective. Both SA and DHBF exhibited an inhibitory activity of de novo biofilm formation in *S. epidermidis* at a concentration about two-fold lower than the MIC and MBC range. SA also showed an inhibitory activity on the mature biofilm, DHBF showed no such activity at all. CH and proAc did not possess bactericidal activity. On the contrary, planktonic growth of both *S. aureus* and *S. epidermidis* was inhibited by CH (MIC 16–32 μM), while proAc did not show bacteriostatic activity (**Table 5**). However, biofilm growth of *S. aureus* and *S. epidermidis* was inhibited by both compounds. The proAc seemed to be the best inhibitor performing similarly on both strains, CH inhibited biofilm formation better in *S. epidermidis* than in *S. aureus*. While CH and proAc were good inhibitors of the de novo biofilm formation they displayed no meaningful inhibitory activity on the mature biofilm.

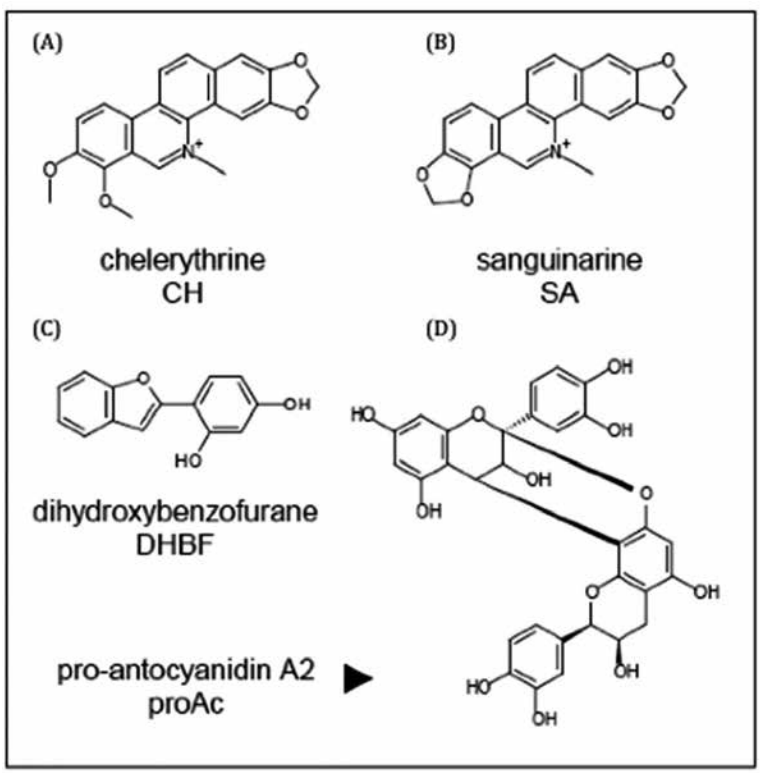

Figure 6. Structural formulas of the inhibitors used in this study, the positive charges in CH and SA are neutralized by chloride ion.

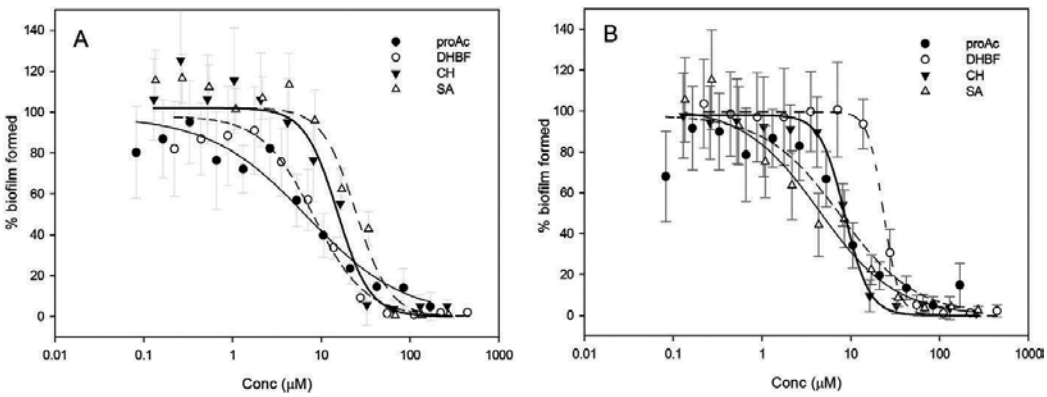

Figure 7. Inhibitory action of proAc, DHBF, CH, and SA, exerted on the ex-novo biofilm formation on *S. aureus* 6538P (panel A) and on *S. epidermidis* RP62A (panel B). Standard deviations from four replicates are represented as error bars in dark gray. The fitting curves shown on the curves were obtained with a single-step inhibition model and fitted with the equation "f={%biofilm formation / [1 + (C/EC50)^slope]}"; where C is the actual concentration of the inhibitor.

MW	Compound	*S. aureus* 6538P				*S. epidermidis* RP62A			
		M.I.C. [μM] [a]	M.B.C. [μM] [a]	EC50 (μM) [b]	slope	M.I.C. [μM] [a]	M.B.C. [μM] [a]	EC50 (μM)	slope
383.8	**CH**	16.3; 32.6	>260	15.2 ± 2.3	2.4 ± 0.7	16.3; 32.6	>260	8.6 ± 0.4	3.1 ± 0.5
367.8	**SA**	34.0; 68.0	34.0; 68.0	24.5 ± 3.6	2.3 ± 0.7	8.5; 17.0	34.0; 68.0	4.4 ± 1.3	1.0 ± 0.2
226.2	**DHBF**	55.3; 110.6	55.3; 110.6	8.2 ± 1.2	1.5 ± 0.3	55.3; 110.6	55.3; 110.6	23.5 ± 0.6	4.9 ± 0.6
592.6	**proAC**	>168.7	>168.7	6.9 ± 2.4	0.8 ± 0.2	>168.7	>168.7	7.6 ± 2.7	1.0 ± 0.3

[a] MIC and MBC were determined using CLSI guidelines.
[b] EC50 is referred to biofilm formation.

Table 5. Antibacterial activity of natural compounds.

According to our proteomics data, we observed that SA, CH, and proAc down-regulate proteins involved in *S. aureus* pathways. Notably the vast majority of down-regulated cell surface proteins were cytoplasmic. This suggests that SA, CH, and proAc can enter in the bacterial cells and possibly affect intracellular processes. In conclusion, we showed that two of these compounds possessed interesting potential to become active principles of new drugs. In particular, both proAc and CH were molecules which fulfill the requirements for inhibition of de novo biofilm formation without bactericidal activity.

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2.2.2. Compounds from human body.

Studies performed on various fish species and in swine which led to the identification of innate immuno factors important to be selected for resistance to gram-negative infections, have pointed the attention on Tf as a candidate gene for disease resistance [51, 52]. Therefore, Tf is considered as a relevant safeguard for human body, facing infections sustained by bacteria. Tf is a glycoprotein; its molecular structure shows the presence of two lobes, each binding one iron III ion. Apo-Tf is the denomination of its iron-depleted form; holo-Tf is the iron-loaded form; both forms are collectively named Tfs. Tf has been shown to exert both a bacteriostatic and bactericidal effect in vitro on a variety of microbial pathogens [53, 54]. The antimicrobial activity of transferrin is conventionally related to the iron-depleted form but some studies demonstrated that the mechanism of its antibacterial activity could not be referred only to iron deprivation [55, 56].

We investigated the effect of human apo-Tf and holo-Tf on biofilm formation by *S. aureus* (CA-MRSA USA300 type (ST8-IV) and ATCC 6538P strain) and *S. epidermidis* (A clinical isolate and ATCC 35984 strain). Our aim was to determine whether Tfs were able to interfere with microbial adherence of *S. aureus* and *S. epidermidis* to abiotic surfaces and to eukaryotic host cell [47]. A strong reduction in biofilm formation with both Tfs was obtained albeit at very different concentrations. In particular, the reduction in biofilm formation was higher with apo-Tf rather than obtained with holo-Tf. The *S. aureus* biofilm formation was 50% inhibited at about 50 μg/ml and 65 μg/ml, respectively for 6538P and USA300. The *S. epidermidis* biofilm was inhibited at higher concentrations, namely at 95 μg/ml and 250 μg/ml for RP62A and O-47, respectively. The holo-Tf also inhibited biofilm formation in all the strains, however at very high concentrations. A 50% reduction of biofilm formation by *S. aureus* strains (6538P and USA300) was obtained with concentrations of holo-Tf, respectively of 550 μg/ml and 600 μg/ml. *S. epidermidis* is a good biofilm former hence higher concentrations of holo-Tf are necessary to obtain a 50% reduction (2.0 mg/ml for RP62A and 2.5 mg/ml for O-47). As regards, the activity of holo-Tf on bacterial adhesion on eukaryotic cells our results show a 30% reduction for 6538P adhesion and an increase of adhesion for USA300. Tfs inhibited invasion, although with different efficacy. Similarly to the interference with adhesion process, holo-Tf exerts a stronger inhibition on bacterial invasion than Apo-Tf. Our result demonstrates that Tfs can be proposed as anti-infective compounds in *S. aureus* infections thank to their capability to reduce virulence of bacterial strains depending on adhesion, biofilm formation, and invasion. Our data suggest that holo-Tf plays a major role, as demonstrated on *S. aureus* pathogen strain CA-MRSA USA300 type (ST8-IV) responsible for severe community-associated staphylococcal disease, especially in the USA and in Europe.

3. Conclusions

More than four billion years ago, bacteria appeared on the earth and rapidly evolved in different species which spread and colonized nearly all ecological niches. Since that time prokaryotes and then eukaryotes (plants and animals, unicellular and multicellular) struggle

to maintain a perpetual condition of equilibrium between cohabitants. This equilibrium is obtained by the selection of organisms that can coexist and by the elimination of those who exert a negative influence on others. From the point of view of bacteria, this means that a bacterial cell has to defend its position in a niche; in fact in a prokaryotic community, all cells communicate by means of small molecules in order to allow only the presence of commensals, while multicellular organisms distinguish and select saprophytes from pathogens in order to allow the residency of the first ones and to counteract or even kill the last ones. In this struggle for survival, all living organisms including prokaryotes and all eukaryotes (plants and animals) produce compounds that counteract undesired bacteria by killing them or by modulating their virulence. Molecules that modulate bacterial virulence can be considered as a huge source of molecules to be studied as lead compounds for the development of new antibacterial drugs. We gave some examples derived from our experience in this field, by the description of compounds obtained from prokaryotes, from plants and from humans that can interfere with bacterial phenotype in order to reduce virulence. They represent thus promising scaffolds to use for further development of antibacterial drugs, which may overcome the insurgence of resistance.

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In the book Microbial Biofilms: Importance and applications, eminent scientists provide an up-to-date review of the present and future trends on biofilm-related research. This book is divided with four subdivisions as biofilm fundamentals, applications, health aspects, and their control. Moreover, this book also provides a comprehensive account on microbial interactions in biofilms, pyocyanin, and extracellular DNA in facilitating Pseudomonas aeruginosa biofilm formation, atomic force microscopic studies of biofilms, and biofilms in beverage industry. The book comprises a total of 21 chapters from valued contributions from world leading experts in Australia, Bulgaria, Canada, China, Serbia, Germany, Italy, Japan, the United Kingdom, the Kingdom of Saudi Arabia, Republic of Korea, Mexico, Poland, Portugal, and Turkey. This book may be used as a text or reference for everyone interested in biofilms and their applications. It is also highly recommended for environmental microbiologists, soil scientists, medical microbiologists, bioremediation experts, and microbiologists working in biocorrosion, biofouling, biodegradation, water microbiology, quorum sensing, and many other related areas. Scientists in academia, research laboratories, and industry will also find it of interest.

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