IntechOpen

Metagenomics for Gut Microbes

Edited by Ranjith N. Kumavath

METAGENOMICS FOR GUT MICROBES

Edited by **Ranjith N. Kumavath**

Metagenomics for Gut Microbes
http://dx.doi.org/10.5772/intechopen.68370
Edited by Ranjith N. Kumavath

Contributors

Takehiko Kenzaka, Katsuji Tani, Prudhvi Lal Bhukya, Renuka Nawadkar, Rodrigo Carvalho, Fillipe Luiz Rosa Do Carmo, Aline Vaz, Barbara Cordeiro, Sara Heloisa, Enrico Gimenez, Aristóteles Góes-Neto, Vasco Azevedo, Luis Goulart, Gwénaël Jan, Yves Le Loir, Wilhelm Boland, Beng Teh, Tilottama Mazumdar, Ranjith Kumavath, Madangchanok Imchen

Notice

First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Metagenomics for Gut Microbes
Edited by Ranjith N. Kumavath

p. cm.

Print ISBN 978-1-78923-110-6

Online ISBN 978-1-78923-111-3

eBook (PDF) ISBN 978-1-83881-312-3

Meet the editor

Dr.Ranjith Kumavath's Laboratory focuses on cutting-edge drug delivery systems to tackle the challenges with various types of cancers and infectious diseases. At present the lab is engaged in the discovery of bioactive compounds and peptides, like PMAP-18, SlpAs, carotenoid pigments, cardiac glycosides, etc., which are found in a host's immune system and membrane-action to understand the altered pathways. Gene expression mechanism of CYP19A1 in breast cancer by Rbn and Rdn for therapeutic biomarkers. Ranjith Kumavath's group is also working on the functional sequence-specific antibiotic resistance genes underlying the Two Component Signal Transduction TCST mechanisms to make sense of the deluge of transcriptomics and metagenomics data by identifying the specifics of sequence-encoded molecular functions. Ranjith Kumavath has published many research publications, e.g., *Nature Scientific Reports* and *System Biology*, among others.

Contents

Preface

Gut microbes play a vital role in regulating human health and conduct a multitude of physiological and biochemical reactions with metabolic entities that play a key role in nutrition exchange between mammalian hosts and their intestinal microorganisms. The intestinal ecosystem is shaped by interactions between intra- and interspecies communications, which elucidate its roles in human health. The use of culture-independent methods for studying host-associated microbial communities could prove invaluable in the expansion of our current knowledge. Next-generation sequence (NGS) targeting of the 16S rRNA gene allows comprehensive clarification of the sampled bacterial community and their associated movement with migratory humans and animals. The application of NGS will lead to a better understanding of the whole picture of bacterial communities in migratory birds. Narrowing down the target bacteria using NGS will enable us to identify unknown pathogens or reveal the potential migration status of known pathogens that have not been noticed so far due to methodological constraints.

This renaissance in host-associated microbial ecology is spurred by advances in metagenomics tools. As never before, these tools collect massive amounts of information through functional and phylogenetic profiling of microbial isolates and complete microbial communities. Metagenomics tools have a huge potential to describe the diversity of microbiomes in gut microflora and most importantly directly in infectious samples. With rapid improvement in genomic sequencing techniques, the overall metagenomics approach is valuable for the discovery of new viruses, novel genes, and new pathways, and the surveillance of pathogens, host–virus interaction, and functional studies. The leads obtained through this exercise may have great impact on early diagnosis and treatment.

A healthy human body functions in sync with a wide array of gut microbes collectively known as the human gut microbiome. By conducting community composition investigations in parallel with functional investigations (e.g., drug resistance), these methods will lead to an understanding of the mechanisms by which multidrug-resistant bacteria spread around the world, which are essential in our daily life such as in food metabolism. Various illnesses, including colon cancer, autism, obesity, and autoimmune diseases, have been linked to imbalanced gut microbiota. However, antibiotics are indispensable drugs, although administration of antibiotics in humans as well as in animals has shown to increase antibiotic resistance genes (ARGs) in the gut microbiome.

In addition, numerous studies have consistently shown that the gut microbiome is unique to each individual. Hence, depth of knowledge on the gut microbiota community and the factors responsible for the shaping and spreading of ARGs are essential. This would in turn enable the development of custom-tailored food and drugs in the future. Hence, the book will give more precise information on gut microbes, which are associated in host organisms.

Ranjith N. Kumavath
Department of Genomic Science
Central University of Kerala, India

Section 1

Antimicrobial Resistance

Chapter 1

Metagenomics of Antimicrobial Resistance in Gut Microbiome

Madangchanok Imchen and Ranjith Kumavath

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76214

Abstract

A healthy human body functions in sync with a wide array of gut microbes collectively known as human gut microbiome. They complement in a number of functions which are essential in our daily life such as in food metabolism. Various illnesses including colon cancer, autism, obesity, and autoimmune diseases have been linked to an imbalanced gut microbiota. Antibiotics are indispensable drug; however, the administration of antibiotics in humans as well as in animal farms has shown to increase antimicrobial resistance genes (ARGs) in gut microbiome. This is of serious concern since the commensals in gut microbiome could capture ARGs through horizontal gene transfer which in turn could cause postsurgical infections. In addition, numerous studies have consistently shown that the gut microbiome is unique to each individual. Hence, in-depth knowledge on the gut microbiota community and the factor responsible for shaping and spreading of ARGs is essential. This would in turn enable the development of custom-tailored personalized food and drugs in the future.

Keywords: metagenomics, gut microbiome, antimicrobial resistance genes (ARGs) and gut resistome

1. Introduction

1.1. The gut microbiome and its significance

The human gut microbiome, also known as "second genome" [1], hosts over 100 trillion microorganisms [2] collectively covering over 150 folds more unique genes than the host [3, 4]. Several projects such as the Human Microbiome Project, MyNewGut, and Meta-HIT have been initiated with the aim to understand the entirety and the functional potential of gut microbiome and to find possible strategies to benefit the host though the alteration of gut

microbiome [5]. The gut microbiome has been linked to various functions, some of which are discussed subsequently.

1.1.1. Gut microbiome is a necessary digestive "organ"

The gut microbiome is also considered as a "metabolically active organ" [6]. The distal human intestine is an anaerobic bioreactor consisting of numerous microbes having the ability to degrade and harvest nutrients which are otherwise inaccessible to the host [7]. In return, the host provides the raw materials and shelter to the microbiome. In this way, the host is relieved of various genotypic attributes which the microbiome complements. Studies have shown that the microbiome coevolved with us by having a mutualistic association [8]. It would seem that the microbiome might compete with the host for food and nutrients. However, conventional animals require 30% more calorie intake than the germfree counterparts in order to maintain the same body weight, implying that the microbiome actually aid in the host metabolism [9, 10].

1.1.2. Personalized gut microbiome

The gut microbiome, similar to fingerprint, has its own unique signature for every individual which is, however, very dynamic [11, 12]. The changes in the microbiota, also called dysbiosis, have also been associated to several health issues [13]. This has led to the possibility of personalized medicine and diet tailored uniquely for every individual depending on his/her unique microbiome [14].

1.1.3. The gut-microbiome-brain connection

Alterations in gut microbiota have also been linked to autism spectrum disorder (ASD) and gut-microbiome-brain connection. Maternal immune activation (MIA) mouse exhibits similar symptoms to ASD such as neurodevelopmental disorders, dysbiosis, alterations in gastrointestinal (GI), and serum metabolites [15–18]. Such MIA mouse when treated with *Bacteroides fragilis* improves intestinal permeability, tight junction proteins, and colon cytokines IL-6 which is required by the MIA offspring for the development of behavioral deficits [9, 19]. Precursor 4-ethylphenol (4EP) found in MIA mice have also been shown to increase anxiety-like behavior in naïve mice. 4EP is produced by several species of *Clostridium* which are also abundant in MIA mice. Treatment with *B. fragilis* resorts the 4EP level in MIA further supporting the role of microbiota in behavioral development [9].

1.2. Types of ARGs in gut microbiome resistome

The gut microbiome resistome can be broadly classified into intrinsic and mobile resistance genes [20]. As the name suggests, intrinsic resistance genes are non-mobile resistance genes which are inherited and render tolerance to a particular drug without prior exposure. Although less mobile, there are possibilities of intrinsic resistance genes getting captured into mobile genetic elements (MGEs). Such events would turn it into mobile-resistant genes. Hence, studying such intrinsic resistance would provide knowledge on the mechanism and the possible treatment to tackle in case of outbreaks [20]. On the other hand, mobile resistomes are the resistance genes which are encoded in the highly mobile mobile genetic elements (MGE).

Mobile genetic elements include plasmids, transposons, integrons, integrative conjugative elements, genomic islands, and phages [20–25]. Resistance genes can get accumulated into a particular segment of DNA forming a special genomic island encoding multiple antimicrobial resistance genes (ARGs) called resistance islands (RIs). For instance, *Acinetobacter baumannii* Resistance Island of 86 kb is the largest known RI harboring 45 ARGs [26]. Resistance genes encode for proteins that render the microbe resistance to various antibiotics (**Figure 1**).

1.3. Factors that shape and spread gut microbiome ARGs

It is essential to understand the factors that shape and spread ARGs in the gut microbiome since gut microbiota regulates the human body in a diverse way, many of which are yet to

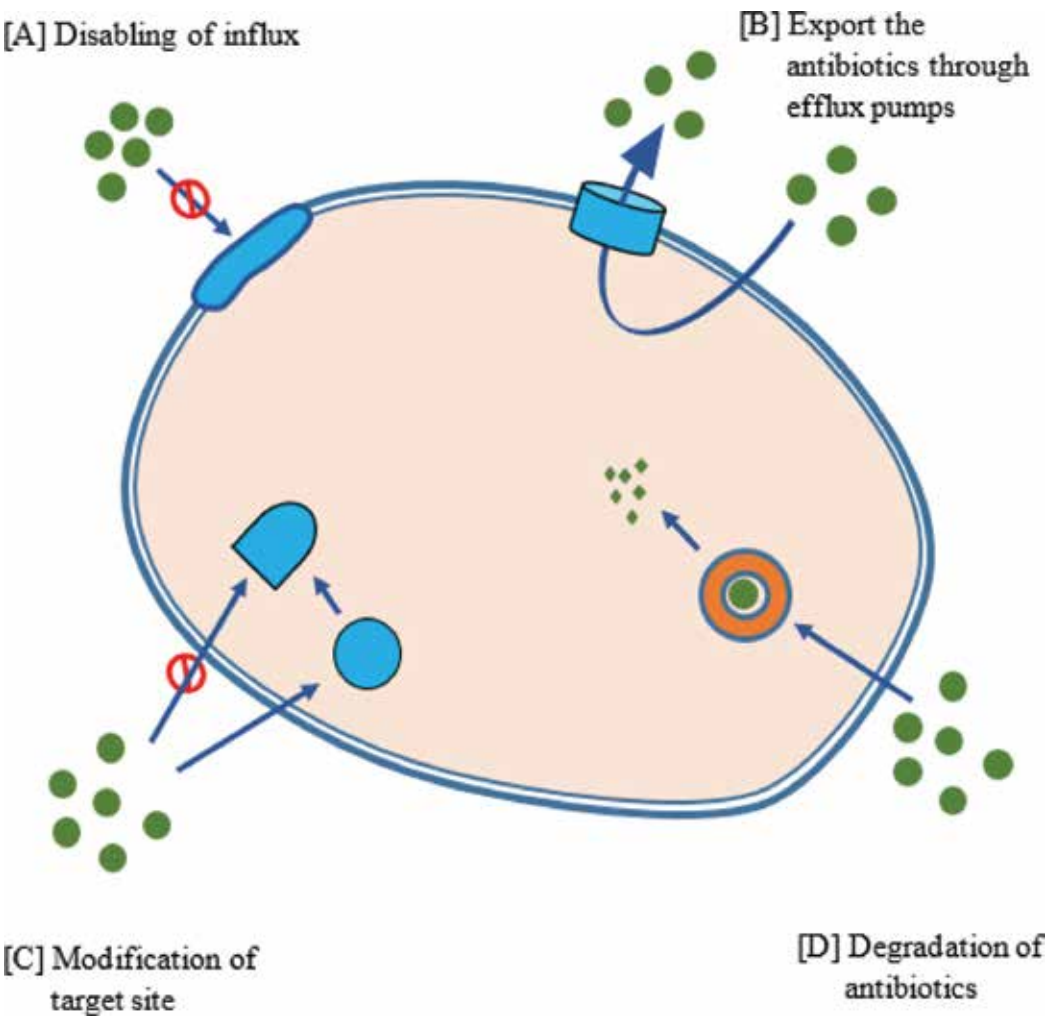

Figure 1. Antibiotic resistance mechanisms: They are broadly classified into four types: (A) the influx of the antibiotics is disabled into the cell, (B) the antibiotics that manage to get into the cell is pumped out by active efflux pumps, (C) the target site for antibiotic in the cell is modified so that the antibiotic cannot bind, and (D) the antibiotics that enters the cells are degraded by the cell machinery.

be known. It is indeed an important part of our body as discussed earlier which need special attention. However, the human gut microbiome is exposed to every food and drugs we consume. The microbiota is, therefore, reflected by the dynamic nature it faces. Cataloging ARGs in gut microbiome is essential in order to study and determine the source and the possible measure to tackle the problem.

1.3.1. Horizontal gene transfer through mobile genetic elements

Mobile genetic elements (MGEs) are transferred between microbes through horizontal gene transfer (HGT) involving conjugation, transduction, and transformation. Transformation is the capturing of naked DNA from the environment into the microbe. If the naked DNA has ARG encoded in it, the microbe taking up the naked DNA would gain resistance owning to the resistant gene encoded in the naked DNA. However, such events are found to be considerably rare in the mammalian gut [27]. Hence, comparatively, conjugation and transduction seem to have a higher impact in ARG horizontal gene transfer [28]. Conjugation involves the formation of mating bridge though which the ARGs are transferred from the donor to the recipient cell. Bacterial HGTs are more common among the same phylogenetic taxa [29]. ARG transfer was boosted between the commensal *Escherichia coli* and other pathogens during gut inflammation [30]. However, ARG transfer through conjugation was significantly reduced between *E. coli* strains in the healthy human gut since the intestinal epithelial cells produce a proteinaceous compound [28, 31] which could interfere with the conjugative process. In transduction, the ARG is encoded in the bacteriophages which get incorporated into the host once the bacteriophages invade a bacterium. It is postulated that transduction could be a major player in gut resistome [32] since the amount of phages and bacteria is equivalent in the intestinal tract [33, 34]. This is supported by the work of Goren et al. [35] showing that the phages isolated from antibiotic-treated mice when inoculated to aerobic microbiota culture showed higher ARG isolates when compared to culture which was treated with non-antibiotic-treated mice.

1.3.2. Gut resistome and antibiotic usage in farm animals

In the United States, nearly 80% of the antibiotics produced is used up in animal farm for treatment purposes [36]. As a result, the gut microbiome of farm animals is highly enriched in ARGs due to regular antibiotics treatment [37, 38]. ARGs enrichment up to 28,000 folds, including numerous unique ARGs, were detected in Chinese Swine farm [38] having efflux pumps, antibiotic deactivation, and cellular protection resistance mechanism. However, antibiotic-free organic pig guts were also found to harbor novel genes encoding resistance to the tetracyclines which were associated with putative mobile genetic elements [39]. Tetracycline resistance gene had the highest ratio of total ARGs according to a large-scale human gut microbiome analysis within the population from Denmark, Spain, and China. The study suggests the possibility of tetracycline resistance gene being transferred from animals since tetracyclines were highly used in animal farms [40, 41]. Subjects from country with comparatively tighter policies on antibiotic usages in humans and animals have considerably lesser ARG levels [42]. In addition, the antibiotic resistance genes revealed signature clustering of Chinese samples separate from other European countries thought single nucleotide polymorphisms (SNPs) analysis [41]. An independent study [43] on another population further supports this

idea of ARG signature. The country-wise signature patterns could be linked to different policies adapted in different countries [28].

1.3.3. Travelers and migratory birds spread ARGs

ARGs can also spread through traveling. In a study involving Swedish students exchange programs to India or Central Africa, the level of sulfonamide, trimethoprim, and beta-lactams were increased after the completion of the exchange programs [44]. The spread of ARGs can also be affected widely by migratory birds, which fly long distances [45].

1.3.4. Antibiotic therapy enriches ARGs

Gut microbiome is a reservoir of ARGs which can indirectly pass the ARGs into the environment. The application of antibiotics has been largely linked to increase in ARGs. Resistance to aminoglycosides was found to increase after admitting to intensive care unit (ICU) [46]. ARGs were also found to increase on patients after treatment with antibiotics [47]. Studies on large-scale human gut samples from 10 different countries have shown that the ARGs in gut microbiome are highly influenced by the antibiotic usage and food products [48] while other factors such as age, sex, body mass index (BMI), and health status did not show significant contribution to ARGs level. The administration of cephalosporin, cefprozil, increased *Lachnoclostridium bolteae* in 16 out of 18 participants, as revealed from a study by Raymond et al. [49]. It also increased opportunistic pathogen *Enterobacter cloacae* in those participants whose initial microbiome diversity was comparatively lower. The treatment also enriched ARGs which were undetectable before the treatment. The alternation in the microbiome was specific to each subject, however, in a specific and reproducible manner. The authors, Raymond et al. [49], hypothesized that the initial analysis of microbiome before the treatment of antibiotics could bypass adverse effects during and after the antibiotic treatments. Nonetheless, the reduction of ARGs was seen in some studies when combinatorial antibiotic treatment was administrated [28, 46, 47]. This could happen when the resistant microbe is susceptible to another antibiotic when given in combination [28]. The application of antibiotic treatment, in addition to alteration of gut microbiome, can also cause long-term persistence of the ARGs in the gut microbiota [50]. Hence, alternative approach to antibiotic therapy is of urgent need to avoid undesirable effects to the microbiota. Alternative therapies such as probiotic intervention, vaccination, and bacteriotherapy [51–54] have been developed. However, such alternative strategies are still at infancy stage; hence, focus on such strategies have to be encouraged.

1.4. Gut microbiome ARGs

Human gut microbiota is a home to numerous commensals, microbes that derive benefit from the host without causing harm. However, such commensals can acquire ARGs from microbes that are merely passing through the gut which can cause serious postsurgical infections [20]. In addition, disruption in the composition of gut microbiome in animal models has shown to cause non-communicable diseases (NCDs) such as colon cancer, autism, obesity, and autoimmune diseases [55, 56]. Salyers et al. [57] proposed the concept of ARGs in human gut microbiome. Since then, the technological advancement in high-throughput robotic screening

and next-generation-sequencing (NGS) technologies in the last decade has pushed the gut microbiota research into full swing [20].

1.4.1. The infants' gut resistome

The infant microbiota is highly dynamic and susceptible to antibiotics [58]. The disruption of microbiota at such stage could have significant ill effects throughout life by interfering with the metabolic and immune system [59]. The infant microbiota development is linked to various factors such as the host genetic makeup, nutrition, and environment [60–62]. The microbiota of a new born baby, even without antibiotic treatment, harbors a diverse resistance gene in their resistome [63, 64]. However, antibiotic treatments increase the abundance of pathogenic *Enterobacteriaceae* and lower healthy microbiota such as *Bifidobacteriaceae*, *Bacilli*, and *Lactobacillales* spp. [59, 65–67]. It is believed that the *Lactobacillus* and *Bifidobacterium* spp. are originated from maternal microbiome which is an essential component for the development of infant gut microbiome [62, 68]. The treatment of *L. acidophilus* and *Bifidobacterium* as probiotics in low birthweight infants increases the daily weight gain and recedes morbidity [69, 70], possibly by promoting the healthy gut microbiome and intestinal epithelial layer [58, 71]. The two modes of delivery, vaginally and C-section, can also distinctly affect an infant's microbiota in the first year after delivery. Vaginally delivered infants harbor comparatively higher resemblance to mother's microbiota [72]. Microbes such as *Bacteroides* and *Bifidobacterium* are less frequent in C-section-delivered infants; however, an increased frequency of bacteria is associated to oral and skin [73]. Studies have also found that the microbiota of a 2-month infant and their mother shares distinction in resistome which includes broad-spectrum beta-lactam antibiotics to be found only in the infants [74]. In fact, comparison between infant and their mother to an unrelated infant showed no significant difference [74]. It is proposed that the host genetic makeup and the environmental factors could play a role in the shaping resistome [74]. Infant microbiota shapes into an adult-like by increasing the alpha diversity while reducing the beta diversity which continues until the age of 3 [60]. Maturation of the infant microbiota is also driven by the feeding habit. The addition of solid food does not induce the maturation of microbiota significantly. However, cessation of breastfeeding enriches the gut microbiota to adult-like [72]. Infants with breastfeeding are enriched by *Bifidobacterium and Lactobacillus* even at the age of 1 while infants who no longer breastfeed are enriched with *Roseburia*, *Clostridium*, and *Anaerostipes,* which are prevalent in adults. Functionally, polysaccharide-degrading genes are enriched only after the cessation of breastfeeding [72]. The microbiota also acquires significant essential amino acids, irons, and vitamins genes after 4 months, which are essential for normal brain development [9, 75]. Functional metagenomics from healthy infants and children isolated three novel ARGs and also demonstrated that the ARG in gut resistome is significantly higher than previously estimated [64, 76].

1.4.2. The adult gut resistome

Large-scale metagenomic study of 252 fecal metagenomes samples identified 50 antibiotic classes [42]. Tetracycline resistance gene, tetQ, is the most abundant resistance gene in fecal samples of Chinese, Danish, and Spanish individuals. In fact, tetracycline resistance genes

were the most abundant genes in multiple studies [41, 42]. Although sufficient evidence for the diversity and abundance of ARGs have already been shown to light, the numbers could still be underestimated since during the annotation of metagenomic data, only those ARGs which have been identified and added into the database would yield a positive hit. This would exclude all the ARGs which have not yet been identified. For instance, 290 ARGs having an average similarity of only 69.5% against the GenBank were isolated using functional metagenomics of fecal samples from two healthy individuals [77].

2. Conclusion

Gut microbiome is an essential "organ" without which the host would be deprived of various benefits derived from the numerous gut microbes. The benefits range from food metabolism to the mental health of the host. Hence, it requires attention as much as any other organ in our body. Various studies have, however, noticed the dynamic nature in the compositing and diversity of the gut microbiome making it one of the most dynamic "organs" in us. In addition, the wide application of antibiotic treatment for human as well as animals has enriched the gut ARGs. Hence, strict polices has to be implemented in order to maintain a moderate antibiotics usage. In addition, the surge in ARGs is a clear indication that the research on antibiotic alternative is a necessity for the coming future.

Author details

Madangchanok Imchen and Ranjith Kumavath*

*Address all correspondence to: rnkumavath@gmail.com; rnkumavath@cukerala.ac.in

Department of Genomic Science, School of Biological Sciences, Central University of Kerala, Kasaragod, India

References

[1] Zmora N, Zeevi D, Korem T, Segal E, Elinav E. Taking it personally: Personalized utilization of the human microbiome in health and disease. Cell Host & Microbe. 2016; **19**(1):12-20

[2] Tsai F, Coyle WJ. The microbiome and obesity: Is obesity linked to our gut flora? Current Gastroenterology Reports. 2009;**11**(4):307-313

[3] Ghaisas S, Maher J, Kanthasamy A. Gut microbiome in health and disease: Linking the microbiome–gut–brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. Pharmacology & Therapeutics. 2016;**158**:52-62

[4] Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR. A human gut microbial gene catalog established by metagenomic sequencing. Nature. 2010;**464**(7285):59

[5] Roeselers G, Bouwman J, Levin E. The human gut microbiome, diet, and health:"post hoc non ergo propter hoc". Trends in Food Science & Technology. 2016;**57**:302-305

[6] Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annual Review of Nutrition. 2002;**22**(1):283-307

[7] Savage DC. Gastrointestinal microflora in mammalian nutrition. Annual Review of Nutrition. 1986;**6**(1):155-178

[8] Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;**307**(5717):1915-1920

[9] Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrosino JF, Patterson PH. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013; **155**(7):1451-1463

[10] Wostmann BS, Larkin C, Moriarty A, Bruckner-Kardoss E. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Laboratory Animal Science. 1983;**33**(1):46-50

[11] Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, Knights D, Gajer P, Ravel J, Fierer N, Gordon JI. Moving pictures of the human microbiome. Genome Biology. 2011;**12**(5):R50

[12] Franzosa EA, Huang K, Meadow JF, Gevers D, Lemon KP, Bohannan BJ, Huttenhower C. Identifying personal microbiomes using metagenomic codes. Proceedings of the National Academy of Sciences. 2015;**112**(22):E2930-E2938

[13] Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: An integrative view. Cell. 2012;**148**(6):1258-1270

[14] Schloissnig S, Arumugam M, Sunagawa S, Mitreva M, Tap J, Zhu A, Waller A, Mende DR, Kultima JR, Martin J, Kota K. Genomic variation landscape of the human gut microbiome. Nature. 2013;**493**(7430):45

[15] Boukthir S, Matoussi N, Belhadj A, Mammou S, Dlala SB, Helayem M, Rocchiccioli F, Bouzaidi S, Abdennebi M. Abnormal intestinal permeability in children with autism. La Tunisie medicale. 2010;**88**(9):685-686

[16] Malkova NV, Collin ZY, Hsiao EY, Moore MJ, Patterson PH. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. Brain, Behavior, and Immunity. 2012;**26**(4):607-616

[17] Shi L, Smith SE, Malkova N, Tse D, Su Y, Patterson PH. Activation of the maternal immune system alters cerebellar development in the offspring. Brain, Behavior, and Immunity. 2009;**23**(1):116-123

[18] Williams BL, Hornig M, Parekh T, Lipkin WI. Application of novel PCR-based methods for detection, quantitation, and phylogenetic characterization of Sutterella species in intestinal biopsy samples from children with autism and gastrointestinal disturbances. MBio. 2012;**3**(1):e00261-11

[19] Smith SE, Li J, Garbett K, Mirnics K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. Journal of Neuroscience. 2007;**27**(40): 10695-10702

[20] Hu Y, Gao GF, Zhu B. The antibiotic resistome: Gene flow in environments, animals and human beings. Frontiers of Medicine. 2017:1-8

[21] Bennett PM. Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. British Journal of Pharmacology. 2008;**153**(S1):S347-S357

[22] Dobrindt U, Hochhut B, Hentschel U, Hacker J. Genomic islands in pathogenic and environmental microorganisms. Nature Reviews. Microbiology. 2004;**2**(5):414

[23] Hu Y, Zhu Y, Ma Y, Liu F, Lu N, Yang X, Luan C, Yi Y, Zhu B. Genomic insights into intrinsic and acquired drug resistance mechanisms in Achromobacter xylosoxidans. Antimicrobial Agents and Chemotherapy. 2015;**59**(2):1152-1161

[24] Rice LB. Tn916 family conjugative transposons and dissemination of antimicrobial resistance determinants. Antimicrobial Agents and Chemotherapy. 1998;**42**(8):1871-1877

[25] Rowe-Magnus DA, Mazel D. The role of integrons in antibiotic resistance gene capture. *International Journal of Medical Microbiology*. 2002;**292**(2):115-125

[26] Fournier PE, Vallenet D, Barbe V, Audic S, Ogata H, Poirel L, Richet H, Robert C, Mangenot S, Abergel C, Nordmann P. Comparative genomics of multidrug resistance in Acinetobacter baumannii. PLoS Genetics. 2006;**2**(1):e7

[27] Nordgård L, Brusetti L, Raddadi N, Traavik T, Averhoff B, Nielsen KM. An investigation of horizontal transfer of feed introduced DNA to the aerobic microbiota of the gastrointestinal tract of rats. BMC Research Notes. 2012;**5**(1):170

[28] Van Schaik W. The human gut resistome. Philosophical Transactions of Royal Society B. 2015;**370**(1670):20140087

[29] Hu Y, Yang X, Li J, Lv N, Liu F, Wu J, Lin IY, Wu N, Weimer BC, Gao GF, Liu Y. The bacterial mobile resistome transfer network connecting the animal and human microbiomes. Applied and Environmental Microbiology. 2016;**82**(22):6672-6681

[30] Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, Barthel M, Westendorf AM, Krogfelt KA, Walker AW, Ackermann M. Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. Proceedings of the National Academy of Sciences. 2012;**109**(4):1269-1274

[31] Machado AMD, Sommer MO. Human intestinal cells modulate conjugational transfer of multidrug resistance plasmids between clinical Escherichia coli isolates. PLoS One. 2014;**9**(6):e100739

[32] Mills S, Shanahan F, Stanton C, Hill C, Coffey A, Ross RP. Movers and shakers: Influence of bacteriophages in shaping the mammalian gut microbiota. Gut Microbes. 2013;**4**(1):4-16

[33] Kim MS, Park EJ, Roh SW, Bae JW. Diversity and abundance of single-stranded DNA viruses in human feces. Applied and Environmental Microbiology. 2011;**77**(22):8062-8070

[34] Reyes A, Semenkovich NP, Whiteson K, Rohwer F, Gordon JI. Going viral: Next generation sequencing applied to human gut phage populations. Nature Reviews. Microbiology. 2012;**10**(9):607

[35] Goren MG, Carmeli Y, Schwaber MJ, Chmelnitsky I, Schechner V, Navon-Venezia S. Transfer of carbapenem-resistant plasmid from Klebsiella pneumoniae ST258 to Escherichia coli in patient. Emerging Infectious Diseases. 2010;**16**(6):1014

[36] Yap MNF. The double life of antibiotics. Missouri Medicine. 2013;110(4):320

[37] Cheng W, Chen H, Su C, Yan S. Abundance and persistence of antibiotic resistance genes in livestock farms: A comprehensive investigation in eastern China. Environment International. 2013;**61**:1-7

[38] Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proceedings of the National Academy of Sciences. 2013;**110**(9):3435-3440

[39] Kazimierczak KA, Scott KP, Kelly D, Aminov RI. Tetracycline resistome of the organic pig gut. Applied and Environmental Microbiology. 2009;**75**(6):1717-1722

[40] Hu Y, Yang X, Lu N, Zhu B. The abundance of antibiotic resistance genes in human guts has correlation to the consumption of antibiotics in animal. Gut Microbes. 2014; **5**(2):245-249

[41] Hu Y, Yang X, Qin J, Lu N, Cheng G, Wu N, Pan Y, Li J, Zhu L, Wang X, Meng Z. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nature Communications. 2013;**4**:2151

[42] Forslund K, Sunagawa S, Kultima JR, Mende DR, Arumugam M, Typas A, Bork P. Country-specific antibiotic use practices impact the human gut resistome. Genome Research. 2013;**23**(7):1163-1169

[43] Ghosh TS, Gupta SS, Nair GB, Mande SS. In silico analysis of antibiotic resistance genes in the gut microflora of individuals from diverse geographies and age-groups. PLoS One. 2013;**8**(12):e83823

[44] Bengtsson-Palme J, Angelin M, Huss M, Kjellqvist S, Kristiansson E, Palmgren H, Larsson DJ, Johansson A. The human gut microbiome as a transporter of antibiotic resistance genes between continents. Antimicrobial Agents and Chemotherapy. 2015; **59**(10):6551-6560

[45] Arnold KE, Williams NJ, Bennett M. Disperse abroad in the land': The role of wildlife in the dissemination of antimicrobial resistance. Biology Letters. 2016;**12**(8):20160137

[46] Buelow E,Gonzalez TB, Versluis D, Oostdijk EA, Ogilvie LA, van Mourik MS, Oosterink E, van Passel MW, Smidt H, D'andrea MM, de Been M. Effects of selective digestive decontamination (SDD) on the gut resistome. Journal of Antimicrobial Chemotherapy. 2014;**69**(8):2215-2223

[47] Pérez-Cobas AE, Artacho A, Knecht H, Ferrús ML, Friedrichs A, Ott SJ, Moya A, Latorre A, Gosalbes MJ. Differential effects of antibiotic therapy on the structure and function of human gut microbiota. PLoS One. 2013;**8**(11):e80201

[48] Forslund K, Sunagawa S, Coelho LP, Bork P. Metagenomic insights into the human gut resistome and the forces that shape it. BioEssays. 2014;**36**(3):316-329

[49] Raymond F, Ouameur AA, Déraspe M, Iqbal N, Gingras H, Dridi B, Leprohon P, Plante PL, Giroux R, Bérubé È, Frenette J. The initial state of the human gut microbiome determines its reshaping by antibiotics. The ISME Journal. 2016;**10**(3):707

[50] Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS One. 2010;**5**(3):e9836

[51] Imchen M, Kumavath R. Vaccination to Combat as an Approach to Reduce the Antibacterial Resistance..! International Journal of Vaccines and Vaccination. 2017;**4**(1): 00074. DOI: 10.15406/ijvv.2017.04.00074

[52] Kale-Pradhan PB, Jassaly HK, Wilhelm SM. Role of lactobacillus in the prevention of antibiotic-associated diarrhea: A meta-analysis. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 2010;**30**(2):119-126

[53] Khoruts A, Dicksved J, Jansson JK, Sadowsky MJ. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent Clostridium difficile-associated diarrhea. Journal of Clinical Gastroenterology. 2010;**44**(5):354-360

[54] Lee CR, Cho IH, Jeong BC, Lee SH. Strategies to minimize antibiotic resistance. International Journal of Environmental Research and Public Health. 2013;**10**(9):4274-4305

[55] Deehan EC, Walter J. The fiber gap and the disappearing gut microbiome: Implications for human nutrition. Trends in Endocrinology & Metabolism. 2016;**27**(5):239-242

[56] Logan AC, Jacka FN, Prescott SL. Immune-microbiota interactions: Dysbiosis as a global health issue. Current Allergy and Asthma Reports. 2016;**16**(2):13

[57] Salyers AA, Gupta A, Wang Y. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology. 2004;**12**(9):412-416

[58] Gibson MK, Crofts TS, Dantas G. Antibiotics and the developing infant gut microbiota and resistome. Current Opinion in Microbiology. 2015;**27**:51-56

[59] Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, Kim SG, Li H, Gao Z, Mahana D, Rodriguez JGZ. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell. 2014;**158**(4):705-721

[60] Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC. Human gut microbiome viewed across age and geography. Nature. 2012;**486**(7402):222

[61] La Rosa PS, Warner BB, Zhou Y, Weinstock GM, Sodergren E, Hall-Moore CM, Stevens HJ, Bennett WE, Shaikh N, Linneman LA, Hoffmann JA. Patterned progression of bacterial populations in the premature infant gut. Proceedings of the National Academy of Sciences. 2014;**111**(34):12522-12527

[62] Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biology. 2007;**5**(7):e177

[63] Alicea-Serrano AM, Contreras M, Magris M, Hidalgo G, Dominguez-Bello MG. Tetracycline resistance genes acquired at birth. Archives of Microbiology. 2013;**195**(6):447-451

[64] Moore AM, Patel S, Forsberg KJ, Wang B, Bentley G, Razia Y, Qin X, Tarr PI, Dantas G. Pediatric fecal microbiota harbor diverse and novel antibiotic resistance genes. PLoS One. 2013;**8**(11):e78822

[65] Arboleya S, Sánchez B, Milani C, Duranti S, Solís G, Fernández N, Clara G, Ventura M, Margolles A, Gueimonde M. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. The Journal of Pediatrics. 2015;**166**(3):538-544

[66] Bennet R, Eriksson M, Nord CE, ZetterstrÖm R. Fecal bacterial microflora of newborn infants during intensive care management and treatment with five antibiotic regimens. The Pediatric Infectious Disease Journal. 1986;**5**(5):533-539

[67] Tanaka S, Kobayashi T, Songjinda P, Tateyama A, Tsubouchi M, Kiyohara C, Shirakawa T, Sonomoto K, Nakayama J. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. FEMS Immunology & Medical Microbiology. 2009;**56**(1):80-87

[68] Makino H, Kushiro A, Ishikawa E, Kubota H, Gawad A, Sakai T, Oishi K, Martin R, Ben-Amor K, Knol J, Tanaka R. Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota. PLoS One. 2013;**8**(11):e78331

[69] Abrahamsson TR, Rautava S, Moore AM, Neu J, Sherman PM. The time for a confirmative necrotizing enterocolitis probiotics prevention trial in the extremely low birth weight infant in North America is now! The Journal of Pediatrics. 2014;**165**(2):389-394

[70] Härtel C, Pagel J, Rupp J, Bendiks M, Guthmann F, Rieger-Fackeldey E, Heckmann M, Franz A, Schiffmann JH, Zimmermann B, Hepping N. Prophylactic use of lactobacillus acidophilus/Bifidobacterium infantis probiotics and outcome in very low birth weight infants. The Journal of Pediatrics. 2014;**165**(2):285-289

[71] Jones RM, Luo L, Ardita CS, Richardson AN, Kwon YM, Mercante JW, Alam A, Gates CL, Wu H, Swanson PA, Lambeth JD. Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. The EMBO Journal. 2013;**32**(23):3017-3028

[72] Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, Li Y, Xia Y, Xie H, Zhong H, Khan MT. Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host & Microbe. 2015;**17**(5):690-703

[73] Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences. 2010;**107**(26):11971-11975

[74] Moore AM, Ahmadi S, Patel S, Gibson MK, Wang B, Ndao IM, Deych E, Shannon W, Tarr PI, Warner BB, Dantas G. Gut resistome development in healthy twin pairs in the first year of life. Microbiome. 2015;**3**(1):27

[75] Lozoff B, Brittenham GM, Wolf AW, McClish DK, Kuhnert PM, Jimenez E, Jimenez R, Mora LA, Gomez I, Krauskoph D. Iron deficiency anemia and iron therapy effects on infant developmental test performance. Pediatrics. 1987;**79**(6):981-995

[76] Wang WL, Xu SY, Ren ZG, Tao L, Jiang JW, Zheng SS. Application of metagenomics in the human gut microbiome. World journal of gastroenterology: WJG. 2015;**21**(3):803

[77] Sommer MO, Dantas G, Church GM. Functional characterization of the antibiotic resistance reservoir in the human microflora. Science. 2009;**325**(5944):1128-1131

Section 2

Metagenomic Applications

Chapter 2

Potential Applications and Challenges of Metagenomics in Human Viral Infections

Prudhvi Lal Bhukya and Renuka Nawadkar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75023

Abstract

Complex association of human host and pathogenic viruses makes a necessity to understand the overall host and virus interaction network. Identification of virus population and its systematic classification will help in understanding the viral association with the disease outcome. Metagenomics is a recently developing approach for the detection of pathogens in the samples with precise interpretation in a short period of time. Metagenomic approaches have been employed for studying the predominance or spread of the virus within a particular locality and nature of virus during infection. Metagenomics is basically a collective approach of lab-based techniques and in-silico methods for identification of pathogenic viruses without culturing them in specific aseptic conditions. Lack of unique conserved genes in viruses has made metagenomics study difficult in this juncture. Other challenges in the field of metagenomics are like cellular DNA contamination, free environmental DNA contamination and continuous evolution of viruses. Recent studies have shed light on the advancement of this field in virus identification and characterization however still needs further investigations to overcome the challenges. Current chapter focuses on the application and challenges faced in metagenomic analysis of human viral infections.

Keywords: metagenomics, viral metagenomics, gastrointestinal infections, applications of metagenomics

1. Viruses

In Latin, the term virus means toxin, virus are obligate intracellular parasites with RNA or DNA as a genetic material. They vary in size from ~20 nm to ~1.5 μm and simple machinery. Viruses cant able to replicate themselves as they are intracellular parasites and require susceptible host

for their propagation. Extracellular viral particles are noninfectious in nature. They can infect a wide range of hosts including plants, bacteria, fungi, algae, protozoa, vertebrate or non-vertebrate animals. In nature, around 1×10^{31} number of different viruses are present. The number itself suggests the diversity of viruses in nature. They play a very important role such as an increase in diversity via horizontal gene transfer in hosts, and nutrient recycling [1]. Report from Hooda et al. showed the abundance of viruses in nature is around 1000 times more than observed via cell culture dependent technique [1, 2]. This suggests the large pool of viruses is still unknown, only around 219 pathogenic viruses have been yet identified [2, 3].

2. Role in pathogenesis

Human viruses: More than 200 viruses are known to infect humans and number is increasing with time, but the diversity of viruses suggests a huge number of viruses still unknown. In humans, yellow fever virus was the first pathogenic virus discovered in 1901. 1900 was the era of human virus discovery and most of the common pathogenic viruses studied during this time. In current scenario, two out of three infection causing organisms are viruses [4] and known to cause a variety of disease ranging from normal acute infections such as common cold, flu, and gastroenteritis to deadly diseases such as Hantavirus pulmonary syndrome (Huntavirus), AIDS (HIV) ebolavirus disease (ebolavirus). Recent outbreaks of viruses show the emergence of previously known viruses with modified virulence properties.

3. Human gut and viral infection

For decades human gut-associated pathogenic viruses are known for many gastrointestinal diseases as gastroenteritis. Following are the main group of viruses has been identified. Rotavirus, adenovirus (serotype 40 and 41), astrovirus, calicivirus, norovirus, torovirus, herpesviruses, coxsackieviruses, human papillomaviruses [3], Norwalk-like viruses, coronaviruses, picornaviruses, Sapporo-like viruses [4, 5]. They infect epithelial cell linings, mucosal linings of the stomach and small intestine, a specific portion of epithelium in the intestine. Depending upon the infection type observed, different samples are used for detection of the infectious agent. In general, feces sample used for general microbiological examination during gut-associated infection [6, 7]. Apart from feces, gastric biopsy, gastric juice, saliva [8, 9] duodenal fluid, cotton swabs [5] are collected. These samples are very essential for diagnosis as they directly contain the pathogen.

4. Methods for diagnosis and virus identification

4.1. Traditional methods

Since viruses are extracellular inert particles they need to be propagated into on susceptible host or host cells for their growth. Initially, viruses were cultured in vitro with the help

of embryonated eggs or laboratory animals. Discovery of tissue culture technique in the 1900s provides an indispensable tool for in vitro virus culture. Tissue culture technique has been then recognized as a "gold standards" for virus discovery. Major advantages of using tissue culture technique for virus identification are an amplification of viruses, characterization of the virus, functional studies, drug targeting, and genome extraction. Due to authentic results and sensitivity of the technique, tissue culture-based techniques are still in use for virus discovery, as well as immune responses study, altered gene expression and characterization of viruses. Successful use of tissue culture technique in virus identification depends on crucial steps involved such as collection of a sample from high titer area of the body, immediate transport of sample, sample processing and selection of appropriate cell line [10]. The major defects of traditional method for virus identification are difficulties in identification of susceptible cell line, time-consuming and laborious in nature [10]. Moreover, culture-based virus identification is further succeeded with the evolution of new scientific techniques and modification in existing techniques. Shell vials with centrifugation, PRE-CFE stain technique, immune-based techniques, e.g., ELISA, agglutination, precipitation, flocculation, microscopy-based techniques, reduced the time of virus identification but compromising sensitivity.

4.2. Molecular methods

Gradually field of virology shifted their particles toward molecular biology methods. Together, traditional culture-based methods and molecular biology techniques are used hand in hand for studying virus associated samples [11]. Broadly molecular biology methods are of two types: sequence dependent and sequence independent. Both the methods have proven its usefulness; many viruses have been identified using these techniques.

1. **Sequence-dependent method**: These techniques are most sensitive molecular biology techniques; it can amplify selective DNA from mixed samples [12]. Since the time of discovery of PCR, it has opened the door for many other variations of PCR for multiple gene modulations. The basic backbone of molecular biology PCR is, it has been used in several approaches such as for sequencing of known viruses depending on similarly in sequence in DNA or consensus sequence of previously known viruses, RFLP and diagnostic purposes [13–16]. Another technique, microarray introduced in 1995, it is used mainly for gene expression studies, used in gene profiling, usually in infected samples. Two methods have been used for discovery of new viruses, taxa, gammaretroviruses and xenotropic murine leukemia virus, SARS-CoV are few best examples [17]. The subsequent studies were unable to reproduce the earlier results [6, 7].

2. **Sequence-independent method:** This approach is independent of prior knowledge of virus genome sequence. Sequence subtractive hybridization and representational difference analysis were methods used for detection of gene expression studies and comparison of genome sequence repetitively [18]. Use of these methods was helpful for detection of human herpes simplex virus type 8 (HHV-8) [9, 19], GBV-A, GBV-B virus [20, 21], Tonovirus and norovirus [22].

Another independent approach is (SISPA) sequence-independent single-primer amplification circumvents used for detection of the unknown viral sequence by ligation of linker oligonucleotide sequence [23]. Further, it can be used for molecular cloning of viral genome for subsequent characterization. This method has been used successfully for the discovery of well-known Hepatitis E virus [10, 24] Parvovirus 2 and 3 [24] and Norwalk virus [11]. As viruses are devoid of consensus sequences, generally culture-based traditional and molecular biology-sequence-dependent and sequence independent technique are useful for the study of limited samples with limited output. Most of the viruses remain unidentified due to this reason.

Compared to above techniques metagenomics is the less biased approach. Any type of virus with either RNA or DNA as a genome, cultivable or uncultivable or novel viruses can be quickly detected. The word metagenomics denotes "transcendent" and "ome" is the all or every in Greek collectively means all genomic content. Metagenomics is the study of genetic material with the help of advanced genomic research technique's and computational tools, directly from the environmental sample. Metagenomics approach bypasses the need for classical biochemical laboratory techniques for microbial analysis. With the help of metagenomics, one can investigate all types of genomic contents of a variety of organisms. This technique provided an indispensable tool for identification of nonculturable species of microbes. It is also used for investigation of known and culturable organisms with great accuracy. Another advantage to use this tool is it bypasses the need to isolate and culture individual species manually and the thereby it reduces the time required to study while providing more information. Initial metagenomics analysis of samples directly from raw environmental samples subsequently provides a necessary foundation for further lab-based analysis (**Table 1**). Metagenomics has been used for a variety of purposes, in diverse areas from the time of its discovery in 2002 when for the first time this approach was used in the virology field [12, 52].

4.3. Process of metagenomics

Metagenomics tool is a successful tool for surveillance in different environmental conditions such as freshwater, soil, marine water and gut of different organisms (**Table 1**) Recent advances in sequencing technology improved the speed of novel virus discovery and surveillance of environment [13, 53]. In 2000s, increase in literature related to metagenomics use in virome study and increase in a number of virus database show the ease of process. Recently government organization takes active participation in conducting surveillance programs [14, 15, 54, 55].

Basically, there are three main steps involved in metagenomics analysis of sample as follows:

1. Sample preparation
2. Sequencing
3. Bioinformatics analysis

Year of study	Sample type	Method of sequencing	Virus detected	Reference
2002	Sea water	Sanger's		[12]
2003	Feces	Sanger's		[16]
2004	Marine sediments	Sanger's		[17]
2005	Blood	Sanger's	Novel anellovirus	[25]
2005	Plasma	SISPA	Novel parvoviruses	[18]
2005	Nasopharyngeal aspirates	Sanger's	Novel bocavirus	[26]
2006	Seawater	Sanger's	Novel RNA viruses	[27]
2006	Feces	Sanger's	Plant RNA viruses	[28]
2007	Honey bees	454 NGS	Israeli acute paralysis virus	[29]
2007	Faces, urine, blood	rolling circle amplification (RCA) technique	Novel polyomavirus	[30]
2007	Soil	Sanger's	Soil metagenomics overview	[31]
2007	Virioplankton	Sanger's	Virioplankton metagenome	[32]
2008	Feces	Sanger's	Study of diversity viruses in growing infants	[33]
2008	Feces	Sanger's	Novel picobirnavirus, picornavirus, norovirus and anellovirus, picornavirus, norovirus, picobirnavirus	[34]
2008	Turkey feces	454NGS	Novel bornavirus	[35]
2008	Hotspring water	Sanger's	Novel viruses in hot springs	[36]
2008	Bush kuru rat	454NGS	Novel arenavirus	[37]
2008	Insect pool, skunk brain, human feces, sewer effluent	454NGS	Orthoreovirus and orbirus	[38]
2008	SISPA	Novel paralysis virus		[39]
2009	plasma, liver biopsy	454NGS	Novel LUJO virus	[40]
2009	grapevine	454NGS	Novel marafivirus	[41]
2009	plant	454NGS	Novel cucumovirus	[41]
2009	potable, reclaimed water	454NGS	Several animal and plant viruses	[42]
2009	Sea lion lungs	Sanger's	Novel California sea lion anellovirus	[43]
2009	Sea turtle swabs/tissues	454NGS	Novel sea turtle fibropapilloma virus	[43]
2009	Ant	Sanger's	*Solenopsis invicta* virus	[44]
2009	Feces	454NGS	Klassevirus	[45]
2009	Plant	Sanger's	Sweet potatoes badnavirus and mastrevirus	[46]
2010	Brain	454NGS	Astrovirus	[45]
2010	Feces	454NGS	Novel chimpanzee associated circular virus	[47]
2010	Mosquitoes	454NGS	Novel mycovirus	[48]

Year of study	Sample type	Method of sequencing	Virus detected	Reference
2011	Plasma	454NGS	Novel simian hemorrhagic fever virus	[49]
2011	Feces	454NGS	Many novel species in pig: astrovirus, bocavirus	[50]
2011	Liver, pancreas, intestine biopsy	454NGS	Novel turkey hepatitis virus	[51]

Table 1. Viruses discovered with metagenomics approach.

1. **Sample preparation and processing:** Since in metagenomics any type of sample can be analyzed with some pretreatment (or enrichment methods). However, for analysis of gut-associated virome collection of the different sample is done from different parts of the human gastrointestinal region. For accurate results, sample collection, proper handling, transportation, stage of the sample is very crucial. There are many standard protocols available for collection of different samples to laboratory and its storage techniques [37]. Different protocols are used for fluid sample and for tissue samples. The tissue sample is generally homogenized in autoclaved saline and collected supernatant filtered through 0.8, 0.45 and 0.2 μm filters, this serial filtration procedure is used to separate larger particles and bacteria from viruses. See **Figure 1**.

   There are different types of sample processing methods used earlier for extraction of viral genomic material [16, 56–58]. Based on studies done by many groups [56, 58–60], a framework designed by Shah et al. in 2014. A comparative analysis of three widely used sample processing methods for gut-associated RNA virome was done. The second processing method used in the separation of virus partials and DNA preparation gave good results. In that method, PEG treatment and ultracentrifugation steps are spatially separated by sonication step in PBS buffer to remove remnants of PEG. In this method based on bioinformatics

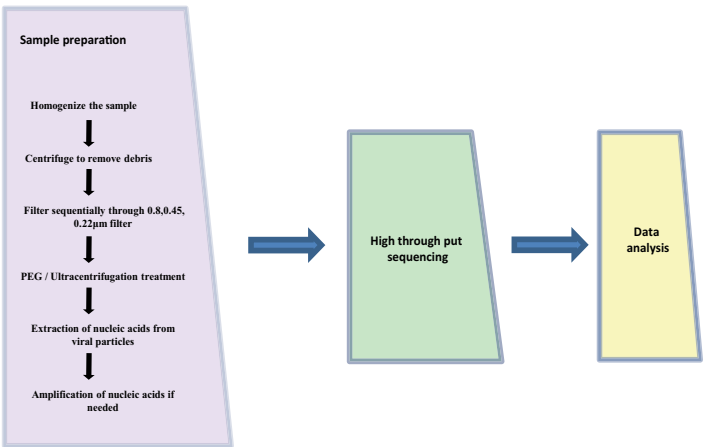

Figure 1. Overview of general procedure of metagenomics.

tools, like riboPicker tool version and blast of viral RNA sequence showed more number of virus domains present in the sample which were processed via the second method, while other methods showed more cellular noise [19].

2. **Sequencing:** The rate of metagenomics study was slow during Sanger sequencing when around 2005 other methods are yet to be evolved, Sangers sequencing was in use. Many studies in this period showed abundant diversity in viruses, analysis of human clinical samples also showed plenty of diversity, while speed of viral genome sequencing is increased several times during pyrosequencing. New viral communities of human and animals have been identified during this period. Some important discoveries are as follows: Astrovirus [21], Rhabdovirus [22], Coronavirus [23], Picornavirus [24], gammapaillomavirus [61]. This technology becomes popular in short time because of low cost, a high number of reads. This technology is also used for sequencing of the clinical sample from tissue fluids and tissue samples [11].

   Ion Torrent: This is pH-based sequencing method with few steps are similar to pyrosequencing technology. Ion Torrent technology gives very rapid runs so it was very useful for targeted deletion of viral sequences from clinical samples such as HIV, HCV, polyomavirus, influenza virus, etc. This method was not a good choice for virologists for identification of new viruses because of low output.

   Illumina: This technology is a high-throughput platform with low-cost rate of virus identification; many viruses from clinical samples have been identified using this technique.

   Pacific bioscience sequencing and nanopore sequencing: These sequencing methods were not popular for metagenomics study because of high error rate [52].

3. **Bioinformatics analysis:** Bioinformatics analysis of raw sequence data generated from high-throughput sequencer is a critical step in novel virus discovery and even in diagnostics. There many ready to use pipelines available for analysis of raw data. VIP, VirFinder, Vipie, METAVIR, PHACCS, VIROME, HP Viewer, Fast virome Explorer, EzMAP, Vanator, viruspy and Viral_genome_annotator are few commonly used pipelines for viral metagenomics analysis. Typical workflow of viral metagenomics includes the following steps. Next-generation sequencing (NGS) data obtained is first subjected to trimming for removal of low-quality sequences and adaptor sequences, (Refer **Figure 2**). Second the trimmed data is subjected for removal of host (humans or bacteria) related sequences and third, these sequences are aligned to reference viral genomes for advance functional characteristics such as novel virus identification, viral taxonomy, identification of viral proteins and phylogenic analysis.

   Challenges involved in metagenomics: For analysis of sequencing data of viral genome through high throughput, sequencing machine needs standard computational tools, software with a high accuracy of data analysis. This needs high-cost involvement with technical expertise. Few high-quality tools available for sequence data analysis such as Diamond [53], UBLAST [52] and Kaiju [54] have increased the speed of metagenomics study. Still, there is a need for technical improvement for rapid and accurate data analysis. The second challenge involved in data analysis of metagenomics sequencing is an assembly of the genome from thousands of small fragments. Assemblers used for the assembly of single genome sets during early times of sequencing study are outdated or non-useful for

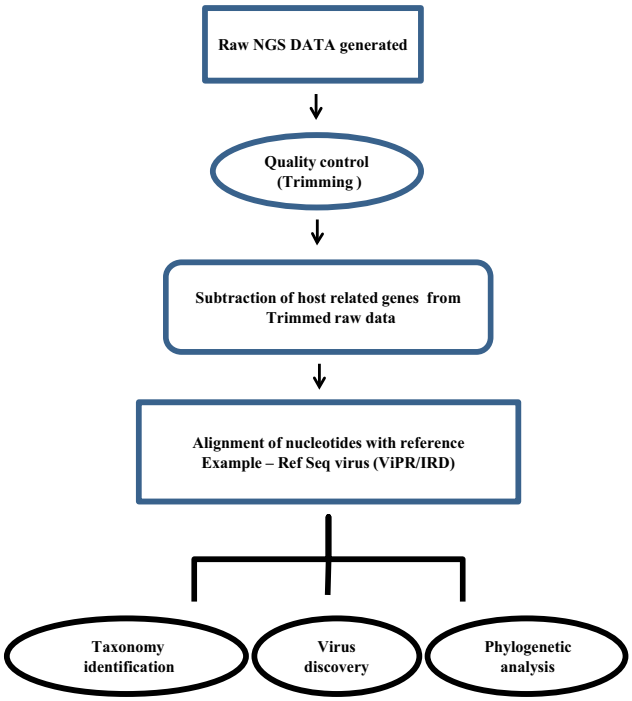

Figure 2. Workflow of metagenomics data analysis.

metagenomics; they create chimeric genomes which misinterpret the genome sequence. Now a days for such studies MetAMOS [55], Meta Velvet [62], MetaSPADes [57] assemblers are available. Still assembly process requires manual editing to sort out genomic chimera generation [15]. Another challenge of virologists for data analysis is reference database deposited which sometimes may cause confusion or problems. If reference database is misinterpreted it will give a wrong interpretation of results. If reference database is high, it decreases the speed as a large number of sequence alignments are required to test data. Sequence data interpretation is a last and very decisive step for metagenomics. Still, we lack clear knowledge about the link between the diversity of virus in the environment and during outbreaks, our surveillance is merely based on a biased collection of only clinical samples and their study. This limits our knowledge about disease spread [63]. Prediction of future outbreaks and limiting the spread of disease needs proper study, development of strong tools [15] Therefore further extensive studies should be encouraged for obtaining maximum and precise knowledge of environmental and gut-associated virome.

4.4. Applications in gut-associated virome analysis

1. **Epidemic and endemic surveillance**: Several reports of unknown pathogenic virus outbreaks in history suggest the need for comprehensive study of virus-host interaction during disease and disease-causing viruses is a big threat to the human population. Well, known examples of zoonotic virus transmission are Nipah virus from fruit bats [58] and Ebola virus from bushmeat [60]. This creates a need for continuous surveillance of diseases in the community. David et al. in 2017 [15] gave a comprehensive explanation about disease outbreak and its diagnosis with

the help of surveillance pyramid. The surveillance pyramid explains during disease spread in the community only a few diagnosed cases are reported, the individuals carrying symptoms of the disease and the carriers of the disease are not reported. This phenomenon creates biasedness in sampling. Therefore metagenomics study has been proved a useful tool for constant surveillance of gastrointestinal tract pathogenic virome community. As well as some endemic viral diseases, which causes common gastrointestinal health concerns in community, e.g., astrovirus, calicivirus, norovirus, and torovirus [64], herpesviruses, hepatitis E virus, epstein bar virus, coxsackieviruses, and surveillance with the metagenomics study is useful.

2. **Discovery of new viruses and classification**: Metagenomics is a powerful tool for identification of novel organism(s). Screening of different gut samples can be useful to study novel gut-associated viruses. Initially with the sequence-based studies of Markel cell carcinoma new *human papillomavirus* has been identified. Markel cell carcinoma is human skin tissue carcinoma, where virus DNA found to be integrated into tumor tissue [65]. Subsequent studies have revealed the diversity of gut-associated viruses in different animals which help in the study of past zoonotic occurred in history. Human-rodent's interaction is well known due to civilization in forest areas or due to the domestication of animals this is leading cause of zoonotic outbreaks. Knowledge of outbreaks in past and monitoring of the present status of the spread of known pathogenic viruses and closely associated pathogenic human viruses provides a base to predict future outbreaks. This approach is also useful to limit the epidemiology of recurrent outbreaks with the study of disease-prone viruses and characterization of unknown viruses. Phan et al. in 2011 extensively studied fecal sample from wild rodents in Virginia and they characterized viruses belonging to mammalian virus families, many new viral families, two new genera were identified. Two viruses closely related to *Aichivirus*, an associated with acute gastroenteritis worldwide, were characterized through the study [66].

   Turkey meat is very popular in the USA and its production is an important part of US economy. One study conducted in California in March 2011on turkey which was suffering from turkey viral hepatitis. Pyrosequencing of RNA, extracted from liver revealed the presence of novel picornaviruses named as *turkey hepatitis virus* [51]. Another study on cattle's suffering from the unknown disease in Germany and Netherlands affected milk production. Metagenomics study discovered the new virus, *Schmallenberg virus*, from infected cow sample [67]. Identification and characterization of such viruses will help in facing problems which have a negative impact on countries economic status. Similar to domestic animals, wild-type animals can also act as a reservoir of novel pathogens. Two novel simian hemorrhagic fever viruses diverse from original simian hemorrhagic fever virus were identified from African green monkeys. *Simian hemorrhagic fever virus* has not yet found to infect human but clinical indices comparable with human *Ebola* and *Marburg viruses*. This analogy makes it in the suspect list of emerging viruses [49].

3. **Diagnostic Metagenomics** is a potent method that allows broad analysis of relative genetic variation among viruses and can be used for the study of host-pathogen interactions. This is also more popular because it can be used for uncultivable organisms as well. The recently rising approach is to use metagenomics during epidemics and outbreaks, with a given large number of samples in a lesser time. In *hepatitis C virus* (HCV) infection, identification of infection is a challenging task due to lack of apparent symptoms and lack of easy laboratory tests for differentiation of acute and chronic phase of the disease. Available

molecular methods for virus diagnostic purpose are tedious, time-consuming and costly. A recent report from Escobar-Gutierrez et al. described the use of next-generation sequencing (NGS) method in the diagnosis of HCV infection. NGS allows cost-effective analysis of a large number of samples in detail. The study showed low-frequency mutations, genetic variation [68]. Genetic shift and re-assortment viruses are a leading cause of the emergence of a new strain of viruses, especially in RNA viruses. Well a known example is influenza virus, many pandemics and deaths in history. The recent H1N1 virus is a combination of swine, human and avian genomic segments of RNA [69]. The best approach of metagenomics study in 2009 H1N1 pandemic is the use of metagenomics for characterization and detail study of the virus, followed by manufacture of microarray-based virochip for rapid detection and differential screening from seasonal virus [70].

4. **Evolution of host-virus interaction:** Evolution of RNA viruses is comparatively fast process than DNA viruses. Study of evolution is necessary to understand the source of new variance, spread and keep a check on epidemic initiating variant. In emerging RNA virus, *norovirus* causative agent of gastroenteritis inter-host, intra-host, and transmission of the new variant has been studied. Usually, it is a self-limiting acute disease but in immune-compromised individuals and in newborns it may cause morbidity and mortality. No vaccine or drugs are available for treatment. A report from Bull et al. hypothesized based on metagenomics study that, *norovirus* has multiple mechanisms of evolution. Chronic hosts are a major reservoir of new variants while acute patients generally possess a single variant. NGS approach for use assists in comprehensive study of viral population dynamics [71]. Characterization of cardiovirus genus originally believed to possess two genera, metagenomics study has revealed five new genera with full characterization. Cardioviruses are the causative agent of enteric diseases in mice with multiple symptoms. In humans, it causes encephalitis-like condition and diarrhea in children's [72]. Metagenomics based studies help in designing future approach with these new genotypes and associated diseases.

5. Conclusion

The metagenomics studies have a huge potential to describe about diversity of microbiome in gut microflora and most importantly directly in infectious samples. Among all pathogens viruses are the ones, who cause severe illness to mankind. With rapid improvement in the genomic sequencing techniques, the overall metagenomics approach is very valuable for discovery of new viruses, novel genes, surveillance of pathogens, discover new pathway, host virus interaction, functional studies. The leads obtained through this exercise may have great impact on early diagnosis and treatment. While metagenomic studies also experience limitations and challenges, which need to overcome in near future to obtain a precise results. Unified genomic extraction techniques and development of improved analysis modules may suffice the needs of metagenomics in future.

Conflict of interest

Authors declare no conflict of interest.

Author details

Prudhvi Lal Bhukya[1]* and Renuka Nawadkar[2]

*Address all correspondence to: saiprudhvi21@gmail.com

1 Department of Biotechnology, Krishna University, Machilipatnam, Andhra Pradesh, India

2 Yashwantrao Chavan College of Science, Karad, India

References

[1] Hobbie JE, Daley RJ, Jasper S. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Applied and Environmental Microbiology. 1977;**33**:1225-1228

[2] Woolhouse M, Scott F, Hudson Z, Howey R, Chase-Topping M. Human viruses: Discovery and emergence. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences. 2012;**367**:2864-2871

[3] Salim AF, Phillips AD, Farthing MJ. Pathogenesis of gut virus infection. Baillière's Clinical Gastroenterology. 1990;**4**:593-607

[4] Brogden KA, Guthmiller JM. Polymicrobial Diseases. Washington, D.C.: ASM Press; 2002

[5] Rene E, Verdon R. Upper gastrointestinal tract infections in AIDS. AIDS GIT group. Baillière's Clinical Gastroenterology. 1990;**4**:339-359

[6] Solonenko SA, Sullivan MB. Preparation of metagenomic libraries from naturally occurring marine viruses. Methods in Enzymology. 2013;**531**:143-165

[7] Draghici S, Khatri P, Eklund AC, Szallasi Z. Reliability and reproducibility issues in DNA microarray measurements. Trends in Genetics. 2006;**22**:101-109

[8] Balsalobre-Arenas L, Alarcon-Cavero T. Rapid diagnosis of gastrointestinal tract infections due to parasites, viruses, and bacteria. Enfermedades Infecciosas y Microbiología Clínica. 2017;**35**:367-376

[9] Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science. 1994;**266**:1865-1869

[10] Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, et al. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature. 2010;**466**:334-338

[11] Quan PL, Firth C, Conte JM, Williams SH, Zambrana-Torrelio CM, Anthony SJ, et al. Bats are a major natural reservoir for hepaciviruses and pegiviruses. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**:8194-8199

[12] Breitbart M, Salamon P, Andresen B, Mahaffy JM, Segall AM, Mead D, et al. Genomic analysis of uncultured marine viral communities. Proceedings of the National Academy of Sciences of the United States of America. 2002;**99**:14250-14255

[13] Ansorge WJ. Next-generation DNA sequencing techniques. New Biotechnology. 2009;**25**: 195-203

[14] Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. Nature. 2012;**486**:207-214

[15] Nieuwenhuijse DF, Koopmans MP. Metagenomic sequencing for surveillance of food- and waterborne viral diseases. Frontiers in Microbiology. 2017;**8**:230

[16] Breitbart M, Hewson I, Felts B, Mahaffy JM, Nulton J, Salamon P, et al. Metagenomic analyses of an uncultured viral community from human feces. Journal of Bacteriology. 2003;**185**:6220-6223

[17] Breitbart M, Wegley L, Leeds S, Schoenfeld T, Rohwer F. Phage community dynamics in hot springs. Applied and Environmental Microbiology. 2004;**70**:1633-1640

[18] Jones MS, Kapoor A, Lukashov VV, Simmonds P, Hecht F, Delwart E. New DNA viruses identified in patients with acute viral infection syndrome. Journal of Virology. 2005;**79**:8230-8236

[19] Shah JD, Baller J, Zhang Y, Silverstein K, Xing Z, Cardona CJ. Comparison of tissue sample processing methods for harvesting the viral metagenome and a snapshot of the RNA viral community in a Turkey gut. Journal of Virological Methods. 2014;**209**:15-24

[20] Simons JN, Pilot-Matias TJ, Leary TP, Dawson GJ, Desai SM, Schlauder GG, et al. Identification of two flavivirus-like genomes in the GB hepatitis agent. Proceedings of the National Academy of Sciences of the United States of America. 1995;**92**:3401-3405

[21] Quan PL, Wagner TA, Briese T, Torgerson TR, Hornig M, Tashmukhamedova A, et al. Astrovirus encephalitis in boy with X-linked agammaglobulinemia. Emerging Infectious Diseases. 2010;**16**:918-925

[22] Grard G, Fair JN, Lee D, Slikas E, Steffen I, Muyembe JJ, et al. A novel rhabdovirus associated with acute hemorrhagic fever in Central Africa. PLoS Pathogens. 2012;**8**:e1002924

[23] Honkavuori KS, Briese T, Krauss S, Sanchez MD, Jain K, Hutchison SK, et al. Novel coronavirus and astrovirus in Delaware Bay shorebirds. PLoS One. 2014;**9**:e93395

[24] Boros A, Nemes C, Pankovics P, Kapusinszky B, Delwart E, Reuter G. Identification and complete genome characterization of a novel picornavirus in Turkey (Meleagris gallopavo). The Journal of General Virology. 2012;**93**:2171-2182

[25] Breitbart M, Rohwer F. Method for discovering novel DNA viruses in blood using viral particle selection and shotgun sequencing. BioTechniques. 2005;**39**:729-736

[26] Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**:12891-12896

[27] Culley AI, Lang AS, Suttle CA. Metagenomic analysis of coastal RNA virus communities. Science. 2006;**312**:1795-1798

[28] Zhang T, Breitbart M, Lee WH, Run JQ, Wei CL, Soh SW, et al. RNA viral community in human feces: Prevalence of plant pathogenic viruses. PLoS Biology. 2006;**e3**:4

[29] Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, et al. A metagenomic survey of microbes in honey bee colony collapse disorder. Science. 2007;**318**:283-287

[30] Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, et al. Identification of a third human polyomavirus. Journal of Virology. 2007;**81**:4130-4136

[31] Fierer N, Breitbart M, Nulton J, Salamon P, Lozupone C, Jones R, et al. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Applied and Environmental Microbiology. 2007;**73**:7059-7066

[32] Bench SR, Hanson TE, Williamson KE, Ghosh D, Radosovich M, Wang K, et al. Metagenomic characterization of Chesapeake Bay virioplankton. Applied and Environmental Microbiology. 2007;**73**:7629-7641

[33] Breitbart M, Haynes M, Kelley S, Angly F, Edwards RA, Felts B, et al. Viral diversity and dynamics in an infant gut. Research in Microbiology. 2008;**159**:367-373

[34] Finkbeiner SR, Allred AF, Tarr PI, Klein EJ, Kirkwood CD, Wang D. Metagenomic analysis of human diarrhea: Viral detection and discovery. PLoS Pathogens. 2008;**4**:e1000011

[35] Honkavuori KS, Shivaprasad HL, Williams BL, Quan PL, Hornig M, Street C, et al. Novel Borna virus in psittacine birds with proventricular dilatation disease. Emerging Infectious Diseases. 2008;**14**:1883-1886

[36] Schoenfeld T, Patterson M, Richardson PM, Wommack KE, Young M, Mead D. Assembly of viral metagenomes from yellowstone hot springs. Applied and Environmental Microbiology. 2008;**74**:4164-4174

[37] Palacios G, Druce J, Du L, Tran T, Birch C, Briese T, et al. A new arenavirus in a cluster of fatal transplant-associated diseases. The New England Journal of Medicine. 2008;**358**:991-998

[38] Victoria JG, Kapoor A, Dupuis K, Schnurr DP, Delwart EL. Rapid identification of known and new RNA viruses from animal tissues. PLoS Pathogens. 2008;**4**:e1000163

[39] Djikeng A, Halpin R, Kuzmickas R, Depasse J, Feldblyum J, Sengamalay N, et al. Viral genome sequencing by random priming methods. BMC Genomics. 2008;**9**:5

[40] Briese T, Paweska JT, McMullan LK, Hutchison SK, Street C, Palacios G, et al. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from southern Africa. PLoS Pathogens. 2009;**5**:e1000455

[41] Al Rwahnih M, Daubert S, Golino D, Rowhani A. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology. 2009;**387**:395-401

[42] Rosario K, Nilsson C, Lim YW, Ruan Y, Breitbart M. Metagenomic analysis of viruses in reclaimed water. Environmental Microbiology. 2009;**11**:2806-2820

[43] Ng TF, Suedmeyer WK, Wheeler E, Gulland F, Breitbart M. Novel anellovirus discovered from a mortality event of captive California Sea lions. The Journal of General Virology. 2009;**90**:1256-1261

[44] Valles SM, Hashimoto Y. Isolation and characterization of *Solenopsis invicta* virus 3, a new positive-strand RNA virus infecting the red imported fire ant, *Solenopsis invicta*. Virology. 2009;**388**:354-361

[45] Greninger AL, Runckel C, Chiu CY, Haggerty T, Parsonnet J, Ganem D, et al. The complete genome of klassevirus - a novel picornavirus in pediatric stool. Virology Journal. 2009;**6**:82

[46] Kreuze JF, Perez A, Untiveros M, Quispe D, Fuentes S, Barker I, et al. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: A generic method for diagnosis, discovery and sequencing of viruses. Virology. 2009;**388**:1-7

[47] Blinkova O, Victoria J, Li Y, Keele BF, Sanz C, Ndjango JB, et al. Novel circular DNA viruses in stool samples of wild-living chimpanzees. The Journal of General Virology. 2010;**91**:74-86

[48] Bishop-Lilly KA, Turell MJ, Willner KM, Butani A, Nolan NM, Lentz SM, et al. Arbovirus detection in insect vectors by rapid, high-throughput pyrosequencing. PLoS Neglected Tropical Diseases. 2010;**4**:e878

[49] Lauck M, Hyeroba D, Tumukunde A, Weny G, Lank SM, Chapman CA, et al. Novel, divergent simian hemorrhagic fever viruses in a wild Ugandan red colobus monkey discovered using direct pyrosequencing. PLoS One. 2011;**6**:e19056

[50] Shan T, Li L, Simmonds P, Wang C, Moeser A, Delwart E. The fecal virome of pigs on a high-density farm. Journal of Virology. 2011;**85**:11697-11708

[51] Honkavuori KS, Shivaprasad HL, Briese T, Street C, Hirschberg DL, Hutchison SK, et al. Novel picornavirus in Turkey poults with hepatitis, California, USA. Emerging Infectious Diseases. 2011;**17**:480-487

[52] Kumar A, Murthy S, Kapoor A. Evolution of selective-sequencing approaches for virus discovery and virome analysis. Virus Research. 2017;**239**:172-179

[53] Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nature Methods. 2015;**12**:59-60

[54] Menzel P, Ng KL, Krogh A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nature Communications. 2016;**7**:11257

[55] Treangen TJ, Koren S, Sommer DD, Liu B, Astrovskaya I, Ondov B, et al. MetAMOS: A modular and open source metagenomic assembly and analysis pipeline. Genome Biology. 2013;**14**:R2

[56] Thurber RV, Haynes M, Breitbart M, Wegley L, Rohwer F. Laboratory procedures to generate viral metagenomes. Nature Protocols. 2009;**4**:470-483

[57] Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: A new versatile metagenomic assembler. Genome Research. 2017;**27**:824-834

[58] Yob JM, Field H, Rashdi AM, Morrissy C, van der Heide B, Rota P, et al. Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia. Emerging Infectious Diseases. 2001;**7**:439-441

[59] Hurwitz BL, Deng L, Poulos BT, Sullivan MB. Evaluation of methods to concentrate and purify ocean virus communities through comparative, replicated metagenomics. Environmental Microbiology. 2013;**15**:1428-1440

[60] Mann E, Streng S, Bergeron J, Kircher A. A review of the role of food and the food system in the transmission and spread of Ebolavirus. PLoS Neglected Tropical Diseases. 2015;**9**:e0004160

[61] Phan TG, Vo NP, Aronen M, Jartti L, Jartti T, Delwart E. Novel human gammapapillomavirus species in a nasal swab. Genome Announcements. 2013;**1**:e0002213

[62] Afiahayati SK, Sakakibara Y. MetaVelvet-SL: An extension of the velvet assembler to a de novo metagenomic assembler utilizing supervised learning. DNA Research. 2015;**22**:69-77

[63] La Rosa G, Libera SD, Iaconelli M, Ciccaglione AR, Bruni R, Taffon S, et al. Surveillance of hepatitis a virus in urban sewages and comparison with cases notified in the course of an outbreak, Italy 2013. BMC Infectious Diseases. 2014;**14**:419

[64] Tran A, Talmud D, Lejeune B, Jovenin N, Renois F, Payan C, et al. Prevalence of rotavirus, adenovirus, norovirus, and astrovirus infections and coinfections among hospitalized children in northern France. Journal of Clinical Microbiology. 2010;**48**:1943-1946

[65] Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;**319**:1096-1100

[66] Phan TG, Kapusinszky B, Wang C, Rose RK, Lipton HL, Delwart EL. The fecal viral flora of wild rodents. PLoS Pathogens. 2011;**7**:e1002218

[67] Hoffmann B, Scheuch M, Hoper D, Jungblut R, Holsteg M, Schirrmeier H, et al. Novel orthobunyavirus in cattle, Europe, 2011. Emerging Infectious Diseases. 2012;**18**:469-472

[68] Escobar-Gutierrez A, Vazquez-Pichardo M, Cruz-Rivera M, Rivera-Osorio P, Carpio-Pedroza JC, Ruiz-Pacheco JA, et al. Identification of hepatitis C virus transmission using a next-generation sequencing approach. Journal of Clinical Microbiology. 2012;**50**:1461-1463

[69] Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 a(H1N1) influenza viruses circulating in humans. Science. 2009;**325**:197-201

[70] Greninger AL, Chen EC, Sittler T, Scheinerman A, Roubinian N, Yu G, et al. A metagenomic analysis of pandemic influenza a (2009 H1N1) infection in patients from North America. PLoS One. 2010;**5**:e13381

[71] Bull RA, Eden JS, Luciani F, McElroy K, Rawlinson WD, White PA. Contribution of intra- and interhost dynamics to norovirus evolution. Journal of Virology. 2012;**86**:3219-3229

[72] Blinkova O, Kapoor A, Victoria J, Jones M, Wolfe N, Naeem A, et al. Cardioviruses are genetically diverse and cause common enteric infections in south Asian children. Journal of Virology. 2009;**83**:4631-4641

Chapter 3

Public Health Implications of Intestinal Microbiota in Migratory Birds

Takehiko Kenzaka and Katsuji Tani

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72456

Abstract

Understanding the transboundary movement of microorganisms is a significant public health issue. It is possible that large amounts of various bacteria existing on the earth's surface are spreading across borders through migratory birds, but their identities and rates of migration have yet to be elucidated. Although modern bacteriology has advanced based on culture technology, many environmental bacteria may be in a "viable but nonculturable" state. To date, various novel culture-independent detection methods have been developed, including next-generation sequencing (NGS) technology that enables high-throughput sequencing and in-depth gene analysis independent of culture. By using NGS to comprehensively analyze the intestinal microbiota of migratory birds, research on bacterial and viral communities traveling over long distances has entered a new era, providing a new insight for the analysis of the livestock industry, agriculture, and human health risks. Here, we describe the current state and future outcomes of studying intestinal microbiota associated with migratory birds.

Keywords: migratory bird, avian, wild bird, gut microbiota, intestinal microbiota

1. Introduction

Understanding the transboundary movement of microorganisms is a significant issue regarding health, sanitation, and ecological conservation. Birds are susceptible to many microbial diseases that are common to humans and domestic animals [1]. The unprecedented spread of West Nile virus in North America in 1999 has raised concerns over the transport of pathogenic viruses by migratory birds [2]. Recent sporadic outbreaks of avian influenza have caused masses of avian deaths, and it is strongly suspected that the source of infection in East Asia is the feces of migratory birds. In 2014, studies revealed that the influenza virus of the Antarctic penguin had spread to migratory birds via the fecal-oral route and was highly likely to have infected horses on continents more than a few thousand kilometers away [3].

Waterfowls such as ducks, geese, coots, and cormorants can play a role in the environmental dissemination of *Giardia* cysts and *Cryptosporidium* oocysts [4]. Migratory birds are also thought to be one of the mechanisms responsible for the wide geographic distribution of various human pathogenic protozoans.

Organized and periodic surveillance methods of bacterial pathogens are not as efficient as those for the highly pathogenic avian influenza. Avian cholera, caused by *Pasteurella multocida*, occurs sporadically in various parts of the world, including North America and Europe, where migratory birds are also implicated as a vector [5]. Many human enteric pathogenic bacteria have been isolated from wild birds [6]. Additionally, studies reported that antibiotic-resistant bacteria travel long distances through migratory birds [7]. Therefore, migratory birds carry a wide range of viruses, bacteria, protozoa, fungi, and other microorganisms that may be transmitted to humans [8].

Attempts have been made to clarify the microbiota contained within migratory birds' feces using new culture-independent genetic-based methods such as next-generation sequencing (NGS). Research on the spreading of bacterial populations over the vast distances has led to the elucidation of the roles of migratory birds regarding human health risks. Further studies of the spatial and temporal distribution of pathogenic bacteria in wild birds will enhance the assessment of their roles, thereby enabling the prediction of potential outbreaks based on migratory patterns. NGS is useful for the understanding of bacterial diversity and the discovery of novel bacteria [9]. This review considers the potential role of wild birds in the transmission of intestinal microbiota and our current knowledge of microbiota associated with migratory birds using NGS technologies.

2. Intestinal microbiota in wild birds

The majority of information regarding enteropathogens in wild birds has been ascertained by applying traditional microbiological techniques [10–12]. Research on the fecal flora of wild birds in a few well-studied species has focused on the prevalence of enteropathogenic bacteria that are most likely zoonotic. Representative species that are often found in diseased wild birds include *Salmonella enterica* serotypes *Typhimurium* and *Enteritidis*, *Escherichia coli*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Clostridium botulinum*, and *Listeria monocytogenes*. The carrier birds are often diverse species, including house sparrows, brown-headed cowbirds, white-throated sparrows, tufted ducks, crows, pheasants, pigeons, hook-bills, finches, free-living flamingos, quails, pheasants, red grouse, and waterfowl [1, 6, 8].

Many classical methods such as microscopy, culture, or serology have been used to detect and isolate pathogenic protozoa or fungi from excreta of migratory birds. *Candida albicans* and *Candida tropicalis*, which can become pathogenic in immunocompromised individuals, have been isolated from excreta of migratory gulls and geese [13]. *Toxoplasma gondii* has been isolated from many migratory species including ducks and raptors. *Eimeria* spp., which cause severe intestinal coccidiosis, are distributed by geese and diving ducks. These infections are mainly caused by oral ingestion of oocysts excreted from feces. The oocysts of *Cryptosporidium baileyi*, an intracellular enteric coccidian parasite that can cause gastrointestinal and respiratory

tract disorders in birds, have been found in excreta of migratory gulls and Canada geese. Thus, waterfowls can disseminate intestinal protozoan parasites in the environment [4].

Representative virus species that often cause fecal-oral disease transmission in wild birds include *Influenza A virus* (found in species of ducks, geese, gulls, terns, shearwaters, guillemots, shorebirds, and passerines), Newcastle disease virus (found in many species of free-living birds), anatid herpesvirus 1 (found in many species of anseriform such as ducks, geese, and swans), and aviadenovirus (found in migratory anseriform species) [6, 8].

As vectors of enteropathogens, wild birds have been associated with global outbreaks of water-borne and food-borne diseases. For example, outbreaks of infectious diseases have been attributed to fecal water supply contamination by wild birds [14]. Birds were strongly suspected as a source of enteropathogens for livestock when infected birds were found foraging on the livestock feed, and domestic cats have been known to contract enteropathogens by feeding on infected dead birds [15]. The incidence of infectious diseases in humans has also been linked to the handling of dead wild birds [16].

3. Methodology

Traditionally, studies on microbial community composition have been based on culture-dependent methods [17]. When classifying and identifying bacteria, a combination of simple methods has helped to distinguish bacteria based on morphology, dyeability, and biochemical properties. Since automated devices have been developed, it is possible to assure the quality of microbial-based data in pharmaceutical fields and diagnostics fields as standards. When in need of more specific classification, sequence decoding for a specific gene or DNA fingerprinting is carried out. When determining bacterial species, it is a standard practice to analyze the 16S rRNA gene.

However, traditional culture methods underestimate bacterial populations because many environmental bacteria cannot yet to be cultivated by conventional laboratory techniques [18, 19]. Traditional microbiological methods of detection, enumeration, and identification using culture methods are often time-consuming and labor-intensive. These practical considerations often limit the extent to which microbiological tests are routinely applied to community analysis.

Various approaches have been developed to identify microorganisms in natural samples without the requirement for laboratory cultivation [20–23]. Since the latter half of the 1990s, direct sequencing approaches for 16S ribosomal RNA gene amplicon have been popularized. In these methodologies, DNA is extracted directly from the sample without culturing the bacteria. Target DNA is amplified by polymerase chain reaction (PCR) using a universal primer set targeting a conserved region of the 16S rRNA gene or a genus-specific primer, and then sequencing follows to identify the bacterial community members. Because the amplicons are mixed molecules derived from numerous complex bacterial species, PCR products should be separated using denaturing gradient gel electrophoresis or a clone library method in combination [24, 25]. With these methods, the number of bacterial clones that can be screened at one time is limited from several tens to thousands and proves to be labor-intensive.

In this decade, a comprehensive analysis of gene sequences using next-generation sequencing (NGS) has spread rapidly [26, 27]. The NGS is a powerful technology capable of concurrently determining nucleotide sequences for tens of millions to hundreds of millions of fragmented DNA strands. Originally, NGS was used for high-throughput sequencing of a single biological genome, but now it is possible to perform high-speed processing, allowing multiple samples to be sequenced simultaneously. Therefore, a wide variety of applications have been proposed for NGS. The price of NGS contract analysis service also has declined greatly in the past few years, making it easier to use so that it is now more useful for research on genetic diseases, clinical diagnoses, relationships between human intestinal flora and diseases, analyses of environmental bacterial community composition and succession in both time and space, and searches for useful microorganisms in various environments. Metagenomic methods provided by NGS technology have facilitated a remarkable expansion of our knowledge regarding uncultured bacteria [28].

A more recent detection method, quantitative real-time PCR, is known for its excellent accuracy and sensitivity when detecting known zoonotic pathogens [29]. On the other hand, it is difficult to identify target pathogens that are not previously known with this method, and often too many samples must be handled simultaneously for it to be convenient. A comprehensive analysis by NGS enables us to comprehend a whole picture of the bacterial community contained in a sample, so it is possible to carry out further analysis with specific pathogenic bacteria based on the taxonomic information obtained by NGS.

4. Variable region of the 16S rRNA gene

The 16S rRNA gene sequence was first used in 1985 for phylogenetic analysis [30]. Because it contains both highly conserved regions for primer design and hypervariable regions to identify phylogenetic characteristics of microorganisms, the 16S rRNA gene sequence became the most widely used marker gene for profiling bacterial communities [31]. Full-length 16S rRNA gene sequences consist of nine hypervariable regions that are separated by nine highly conserved regions [32]. Limited by sequencing technology, the 16S rRNA gene sequences used in most studies are partial sequences. Therefore, the selection of proper primers is critical for studying bacterial phylogeny in various environments [32].

Recent studies utilizing high-throughput technology also have demonstrated that the use of suboptimal primer pairs results in the uneven amplification of certain species, causing either an under- or overestimation of some species in a microbial community [32, 33]. Integrated bioinformatics tools were used to evaluate the phylogenetic sensitivity of the hypervariable regions compared with the corresponding full-length sequences. Results showed that using a combination of V4–V6 regions represented the optimal subregions for bacterial phylogenetic studies of new phyla [34].

5. Flyway

Bird migration is the regular seasonal journey undertaken by many species of birds. Bird movements occur as a response to changes in food availability, habitat, or weather. Approximately 1800

of the world's 10,000 bird species are long-distance migrants [35]. The bar-tailed godwit, *Limosa lapponica*, undertakes one of the avian world's most extraordinary migratory journeys. Recent research revealed that some individuals had made nonstop flights over 11,000 km, the longest continuous journey that has ever been recorded for a bird [36].

Many species migrate along broadly similar, well-established routes, known as flyways. Recent research has identified nine such pathways: the East Atlantic, the Mediterranean/Black Sea, the West Asia/East Africa, the Central Asia, the East Asia/Australasia, and four flyways in the Americas [37]. The most common pattern involves flying north in the spring to breed in the temperate or Arctic summer and then returning south in the fall to warmer wintering grounds. Migration is often annual and is linked closely with the cyclic pattern of the seasons. Migration is most evident among birds, which have a highly efficient means for traveling swiftly over long distances.

The East Asia/Australasia flyway extends from Siberia and North America to the southern limits of Australia and New Zealand. It encompasses large parts of East Asia, all of Southeast Asia, and includes eastern India and the Andaman and Nicobar Islands. The scale of avian movements along the flyway is awesome, with over 50 million migratory birds using the route annually [38]. Bacterial community compositions in migratory birds from the East Asia/Australasia flyway are described in the section below.

6. Bacterial community composition in migratory birds

A comprehensive analysis of the bacterial community structure in migratory birds using culture-independent methods is introduced below.

6.1. Confirmation of avian host

For field samples, it is important to confirm that the specimens are derived from the desired avian host. Mitochondrial DNA (mtDNA) sequences from avian hosts are ideal for this purpose because they provide phylogenetic information and a high copy number in host cells. Kenzaka et al. [39] amplified avian host DNAs by PCR with primers L5216 (5′-ACTCTTRTT-TAAGGCTTTGAAGGC-3′) and H6313 (5′-GGCCCATACCCCGRAAATG-3′) targeting the NADH dehydrogenase subunit 2 (ND2) gene and determined the sequences to confirm the avian host feces [40]. The mtDNA sequences from a variety of avian species are available in DNA database (e.g., GenBank).

6.2. Eurasian wigeon

The Eurasian wigeon (*Mareca penelope* or *Anas penelope*) breeds in the northernmost areas of Europe and Asia. The size of the wigeon is approximately 50 cm in length (**Figure 1a**). The global population is estimated approximately 2.8–3.3 million individuals [41]. The species is strongly migratory, undertaking significant cold weather movements of varying magnitudes. It leaves its breeding grounds in late summer to arrive in its wintering grounds across Europe and Asia in October and November. It lives primarily in lakes, rivers, and along coastlines and

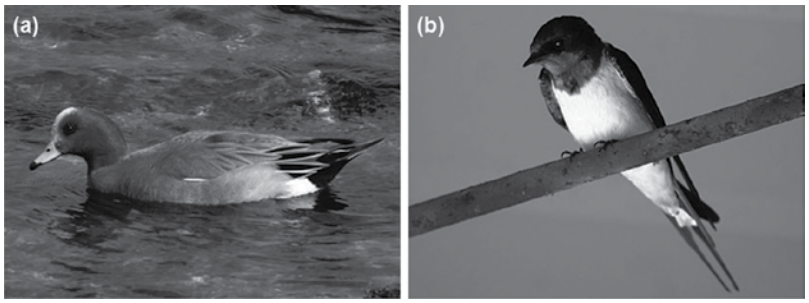

Figure 1. Photographs of (a) Eurasian wigeon and (b) barn swallow.

prefers a location near water plants and land plants that it can eat. The number of observed individuals in Japan has been about 180,000 per year.

Kenzaka et al. collected fresh feces from the Eurasian wigeon that had fallen on plant surfaces along the southern coast of Lake Biwa (Japan) [39]. From this research, most fecal sample bacterial communities were dominated by the phyla *Firmicutes* (51.7%) and *Proteobacteria* (45.1%), composing an average of about 97% (**Figure 2a**). At the family level, on average, *Enterobacteriaceae* composition was 37.6%, *Bacillaceae* was 21.5%, *Paenibacillaceae* was 16.5%, *Clostridiaceae* was 7.5%, and *Pseudomonadaceae* was 6.3% (**Figure 2b**). Although there were individual differences, these families were the dominant groups in all samples collected.

Detected genera that have been reported in association with human pathogenicity are shown in **Table 1**. The genera *Pantoea*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Clostridium*, *Escherichia/Shigella*, *Helicobacter*, and *Serratia* were found at a rate of more than 0.1% of total sequences. On the other hand, the genus *Campylobacter*, which is present in various birds and known as causative bacteria of food poisoning [42], was detected but composed less than 0.1% of the bacterial community in 60% of the samples. Compositions for both the genus *Listeria*, a zoonotic infectious pathogen-causing listeriosis [43], and the genus *Pasteurella*, a pathogen of poultry cholera [5], were less than 0.1% in all of the samples.

6.3. Barn swallow

The barn swallow (*Hirundo rustica*) has a total length of about 17 cm, and its weight is about 18 g (**Figure 1b**). The global population is estimated more than 190 million individuals approximately. This species breeds in a wide range of climates and over a wide range of altitudes, preferring open country like farmland and near water and buildings that provide nesting sites. The barn swallow is primarily a rural species in Europe and North America, while in North Africa and Asia, it often breeds in towns and cities [44]. Many swallows migrate to Japan from Southeast Asia (i.e., Philippines, Malaysia, Indonesia, etc.) and breed near human-living environments, such as private houses and the eaves. Swallows mainly feed on insects. After breeding, they gather at river beds and reed borders, forming group roosts of 1000–10,000 of individuals, and then return to Southeast Asia in autumn. The number of observed individuals in Japan is estimated at several hundred thousand birds per year.

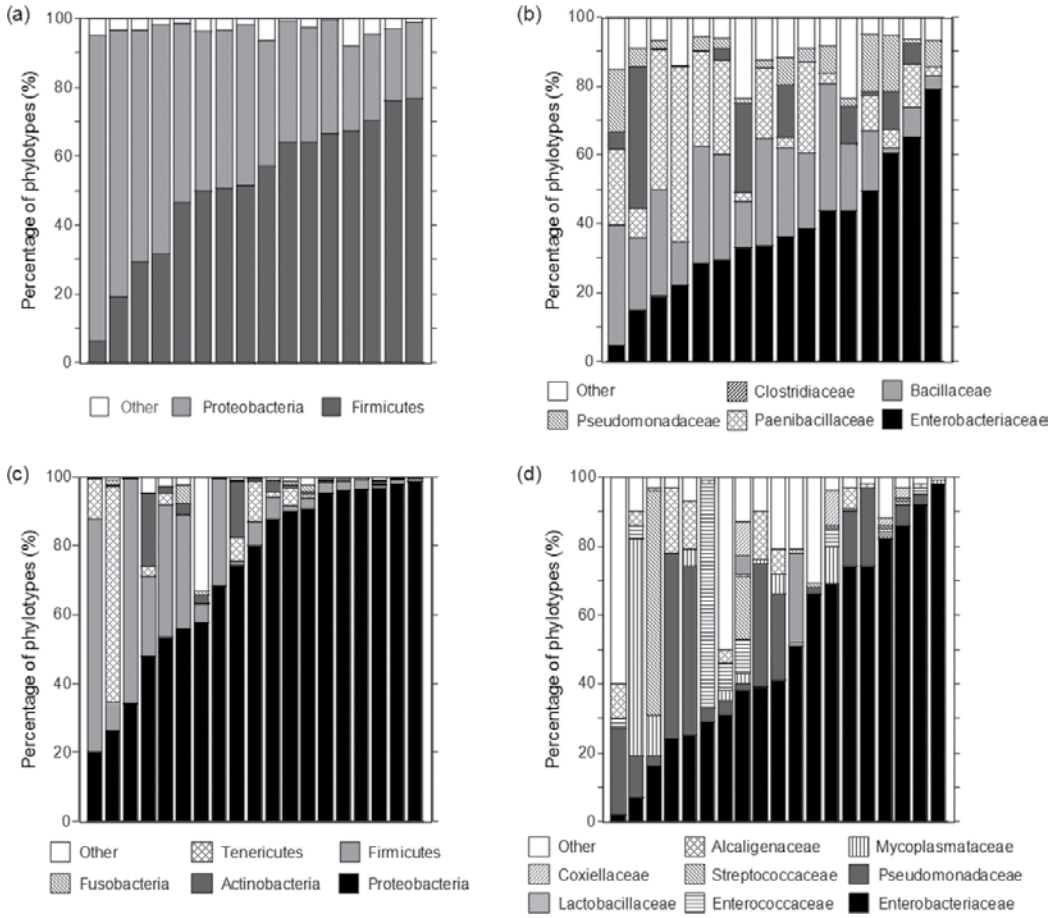

Figure 2. Relative proportions of bacterial phylotypes in individual fecal samples of barn swallow shown at the (a) phylum level and (b) family level and of Eurasian wigeon at the (c) phylum level and (d) family level.

In Osaka Prefecture (Japan), Kenzaka et al. collected specimens of fresh swallow feces from under a mating pair's nest, made at the edge of a private house or artificial building [45]. **Figure 2c** shows the results of the bacterial community composition analysis (at the phylum level) found in swallow feces. Most fecal samples were dominated by *Proteobacteria* (72.1%), *Firmicutes* (15.9%), and *Tenericutes* (5.7%), composing on average about 94% of the bacterial community. Moreover, the proportion of *Bacteroidetes*, which is a human gut-dominant bacterial phylum, was about 0.4%. On the family level, *Enterobacteriaceae* composition was about 53.3% on average, *Pseudomonadaceae* was 13.6%, *Mycoplasmataceae* was 5.5%, *Enterococcaceae* was 4.8%, *Streptococcaceae* was 4.6%, *Alcaligenaceae* was 4.3%, *Lactobacillaceae* was 1.7%, and *Coxiellaceae* was ~1.3% (**Figure 2d**). Although there were individual differences, any of these bacterial groups dominated more than 10% in all samples.

Table 1 shows the major genera with high abundance, namely, *Pseudomonas* spp., *Escherichia/Shigella* spp., *Enterobacter* spp., *Yersinia* spp., *Mycoplasma* spp., *Enterococcus* spp., *Achromobacter* spp., *Fusobacterium* spp., and *Serratia* spp. All of these genera include species that are reported as

Genus	Relative proportion (%)[a]	
	Eurasian wigeon	**Barn swallow**
Pseudomonas spp.	33.2	<0.1
Escherichia/Shigella spp.	21.1	<0.1
Enterobacter spp.	16.5	<0.1
Yersinia spp.	6.1	17.7
Mycoplasma spp.	5.7	<0.1
Enterococcus spp.	3.1	13.4
Achromobacter spp.	0.4	<0.1
Fusobacterium spp.	0.1	0.2
Serratia spp.	<0.1	11.2
Pantoea spp.	<0.1	9.9
Bacillus spp.	<0.1	9.2
Paenibacillus spp.	<0.1	7.2
Clostridium spp.	<0.1	4.8
Helicobacter spp.	<0.1	0.8

[a]>0.1% of total OTUs.

Table 1. Relative proportion of OTUs belonged to representative genus in feces samples determined by 16S metagenomics sequencing.

pathogenic to humans. The genus *Campylobacter* was detected in some samples but at a rate of <0.1% in only 40% of the samples. The genera *Pasteurella* and *Listeria* composed of <0.1% in all samples. Also, *Bacteroides* spp., *Bifidobacterium* spp., and *Prevotella* spp., which are all commonly dominant in the human intestine [46, 47], comprised <0.1% in more than 90% of samples.

6.4. Bar-headed goose

Wang et al. examined metagenomic profiling of gut microbial communities in both wild and artificially reared bar-headed geese in China [48]. The bar-headed goose (*Anser indicus*) breeds in the high plateaus of Central Asia in colonies of thousands near mountain lakes and winters in South Central Tibet and India. This species has been reported as migrating south from Tibet, Kazakhstan, Mongolia, and Russia, crossing the Himalayas [49].

The authors found that *Firmicutes*, *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes* were the four most abundant phyla in the gut of bar-headed geese. In wild bar-headed geese, the predominant phylum was the *Firmicutes*, with an average relative abundance of 83.2%. The second most predominant bacterial lineage, constituting 11.8%, was identified as phylum *Proteobacteria*, followed by *Actinobacteria* and *Bacteroidetes*, accounting for 2.5 and 0.9%, respectively, of the relative abundance.

At the genus level, the sequences from the wild samples represented 106 genera. Four major genera (*Streptococcus*, *Lactococcus*, *Bacillus*, and *Enterococcus*) belonged to phylum *Firmicutes*,

the genus *Pseudomona*s belonged to phylum *Proteobacteria*, and *Arthrobacter* belonged to *Actinobacteria*. Wang et al. compared the bacterial compositions between wild and artificially reared populations of bar-headed geese [48]. They found that *Bacteroidetes* was significantly more abundant in the artificially reared population compared to the wild population.

They also reported on functional profiling and found that artificially reared bar-headed geese had more genes related to carbohydrate transport and metabolism, energy metabolism and coenzyme transport, and metabolism, in general.

6.5. Shorebirds

Ryu et al. examined intestinal microbiota of migrating shorebirds in Delaware Bay (Delaware, United States) on Atlantic flyway using a 16S rRNA clone library [50]. The authors collected the pellets from ruddy turnstones, red knots, and semipalmated sandpipers, which use the Atlantic flyway. The flyway route generally follows the Atlantic Coast of North America and the Appalachian Mountains.

The ruddy turnstone (*Arenaria interpres*) is a small wading bird. The global population is estimated approximately 460,000–730,000 individuals [51]. The ruddy turnstone breeds in northern latitudes around the sea. A subspecies occurs in Northern Alaska and in Arctic Canada, Greenland, Northern Europe, and Northern Russia. In the America, the species winters on coastlines from Washington and Massachusetts southward to the southern tip of South America. The red knot (*Calidris canutus*) is a medium-sized shorebird. The global population is estimated approximately 891,000–979,000 individuals [52]. The species has an extremely large range, breeding from Alaska across the Arctic to Greenland and Northern Russia. It winters on the Atlantic and Pacific coasts of North and South America, as well as Northwestern Europe. The semipalmated sandpiper (*Calidris pusilla*) is a very small shorebird. The global population was estimated at about 2 million individuals in 2006 [52]. It is a common breeder in the Arctic and subarctic, from Far Eastern Siberia east across Alaska and Northern Canada to Baffin Island and Labrador. In the non-breeding season, the species uses coastal estuarine habitats, wintering on the Pacific coast from Mexico to Peru and on the Atlantic coast from the Yucatan and the West Indies south to central Argentina. At one particular staging site in Delaware Bay, thousands of these shorebirds aggregate every spring to refuel for their migration to the Canadian Arctic.

Of about 4000 16S rRNA clone sequences analyzed from these shorebirds, the bacterial community was mostly composed of *Bacilli* (63.5%), *Fusobacterium* (12.7%), *Epsilonproteobacteria* (6.5%), and *Clostridia* (5.8%). The high abundance of *Firmicutes* in shorebird excreta was consistent with other avian studies. At the genus level, three main genera, *Bacillus* spp., *Catellicoccus* spp., and *Lysinibacillus* spp., constituted about 60% of the total sequences. The relatively low abundance of phylum *Bacteroidetes* and genus *Bacteroides* in shorebird excreta also was consistent with other avian studies. Analysis of epsilonproteobacterium-specific 23S rRNA gene clone libraries showed that sequences were dominated by *Campylobacter* (82.3%) or *Helicobacter* (17.7%) spp. In particular, *Campylobacter jejuni*, *C. coli*, and *C. lari* are known to be pathogenic species causing human gastroenteritis worldwide. *C. lari* constituted about 30% of the total *Epsilonproteobacteria* clones, but the pathogenic species of *C. jejuni* and *C. coli* were not detected in the feces of the three shorebird species.

6.6. Bacterial community composition in migratory and nonmigratory birds

Application of NGS for analyzing the intestinal flora of various animals, including humans, is rapidly increasing. In studies on nonmigratory birds, such as chickens, turkeys, ducks, and penguins, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* are reported to be high at the phylum level in all birds [53–55]. In particular, *Firmicutes* was present in almost all bird samples, while *Proteobacteria* and *Bacteroidetes* were present in about 90% of samples. It has been reported that *Tenericutes* was present in about 60% of samples. In the swallow samples, it was characteristic that *Proteobacteria* occupied a high percentage of the community, 50% or more, but the proportions of phylum *Bacteroidetes*, represented by genera *Bacteroides*, *Bifidobacterium*, and *Prevotella*, which are widely present in human intestines, were low.

In the case of the Eurasian wigeon, it was characteristic that the proportions of *Bacteroidetes*, *Actinobacteria*, and *Tenericutes* were lower, which is different from other birds. Also, genera *Bacteroides* and *Bifidobacterium*, which were widely present in human intestine, were low although the genera which belonged to *Firmicutes* and *Proteobacteria* were high.

Figure 3 shows the relative proportions of bacterial phylotypes in intestinal microbial communities of the Eurasian wigeon, the barn swallow, other birds, and mammals registered in DNA database GenBank. **Figure 4** shows the results of principal component analysis comparing the similarities between the intestinal microbial communities of the migratory birds with other birds and

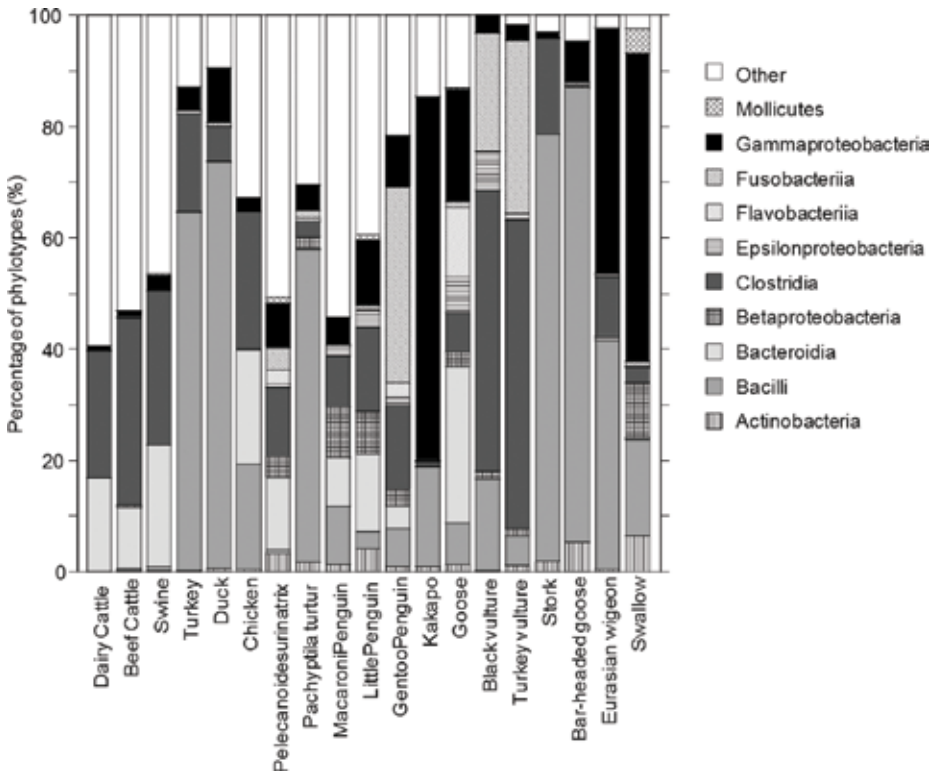

Figure 3. Relative proportions of bacterial phylotypes shown at the class level in gut samples of migratory birds and others.

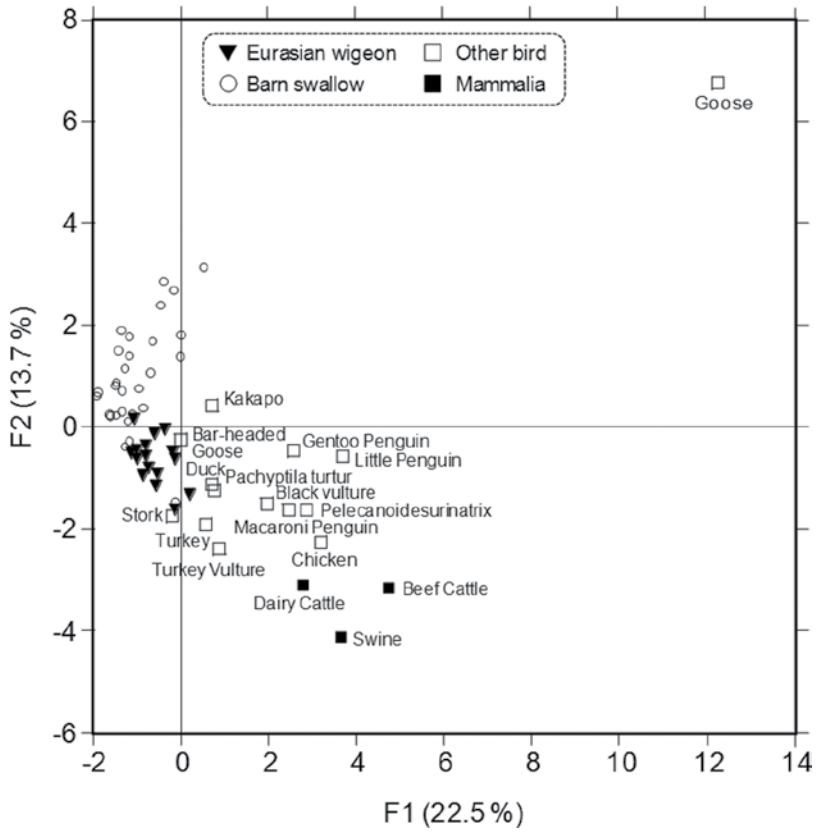

Figure 4. Principal component analysis of class abundance data from migratory birds and others.

mammals registered. It is highly likely that migratory birds may eat different foods from individual to individual, so differences across individuals are large compared to poultry; however, compared with other organisms (□, ■ in **Figure 4**), individual intestinal microbiota from the Eurasian wigeon (▼) and the swallow (○) were relatively similar. In particular, intestinal bacterial composition was found to be greatly different from mammals, such as swine, beef cattle, and dairy cattle (■). It seems that each intestinal bacterial community is formed by the food consumed, whether it is an insect meal, an herbivorous meal, an omnivorous meal, a carnivorous meal, and so on.

7. Protozoa and fungi in migratory birds

For comprehensive analysis by NGS of eukaryotic parasite, 18S rRNA gene, 28S rRNA gene, or cytochrome c oxidase I (COX1) gene on mitochondrial DNA has been used. The universal primers can amplify species across a broad variety of taxa, making them a time- and cost-effective alternative to group-specific primers. Using multiple markers may provide a broader taxonomic resolution of biological communities including diet. The diversity of sequences that can be detected by universal primers is often compromised by high concentrations of DNA templates of some groups. Moreover, up to 90% of the sequences obtained from NGS can be

less-degraded host DNA [56]. If the DNA within the sample contains a small number of interesting sequences in relatively high concentrations of non-interesting sequences, less sequences are often not amplified. In this case, the use of annealing inhibiting primers which overlap with the 3′ end of one of the universal primers is effective [57]. The inclusion of primers to block host DNA amplification can increase the number of nonhost sequences significantly.

As fungi contained in the intestinal tract of seabirds, *Blastocladiomycota*, *Chytridiomycota*, *Entomophthoromycotina*, *Ascomycota*, *Mucoromycotina*, and *Basidiomycota* have been detected [58, 59]. *Nebela* spp., Alveolata, Stramenopila, Rhizaria, Amoebozoa, Excavata, Choanoflagellatea, Glaucophyta, Cryptophyceae, Chlorophyceae, Trebouxiophyceae, Ulvophyceae, Prasinophyceae, and Mamiellophyceae have been detected as protozoa contained in the intestinal tract of seabirds.

8. Conclusion and future perspectives

The use of culture-independent methods for studying bird-associated microbial communities could prove invaluable in the expansion of our current knowledge. NGS targeting the 16S rRNA gene allows comprehensive clarification of the sampled bacterial communities and their associated movement with migratory birds. This methodology also is clarifying the details of bacterial communities, which are moving long distances with migratory birds. Since the 16S rRNA gene differs from 1 to 16 in the number of copies per cell depending on genus [60], the relative proportion obtained by NGS does not necessarily agree with the ratio of actual community composition, but the dominant populations can be ascertained. Applications of NGS will lead to a better understanding of the whole picture of the bacterial communities in migratory birds. Narrowing down the target bacteria using NGS will enable us to identify unknown pathogens or reveal the potential migration status of known pathogens that have not been noticed thus far due to methodological constraints.

The dynamics of individual pathogenic bacteria and drug-resistant bacteria need to be examined in detail in the future. By conducting community composition investigations in parallel with functional investigations (e.g., drug resistance), these methods will lead to an understanding of the mechanisms by which multidrug-resistant bacteria spread around the world.

Addressing the current implications of birds as potential vectors of pathogenic bacteria is of great interest. Analysis of the indigenous bacterial flora of migratory birds may highlight the importance of human hygiene and the environmental significance of microbial transfer associated with natural avian migratory patterns. When wild birds are vectors of disease, it is important to identify the true source of the infectious organisms. NGS, being a culture-independent method, will facilitate further understanding of the complexities and interactions of the genera inherently present in the avian gut and of those acquired from the environment.

Acknowledgements

This work was supported by the JSPS Grant-in-Aid for Scientific Research (C) (15K00571) and Grant-in-Aid for Scientific Research on Innovative Areas (15H05946). We thank Takashi Fujimitsu, Kenji Kataoka, and Yuina Ishimoto for technical assistance during this work.

Author details

Takehiko Kenzaka* and Katsuji Tani

*Address all correspondence to: kenzat@osaka-ohtani.ac.jp

Faculty of Pharmacy, Osaka Ohtani University, Osaka, Japan

References

[1] Benskin CM, Wilson K, Jones K, Hartley IR. Bacterial pathogens in wild birds: A review of the frequency and effects of infection. Biological Reviews of the Cambridge Philosophical Society. 2009;**84**(3):349-373. DOI: 10.1111/j.1469-185X.2008.00076.x

[2] Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, Garmendia AE, Van Kruiningen HJ. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science. 1999;**286**(5448):2331-2333. DOI: 10.1126/science.286.5448.2331

[3] Hurt AC, Vijaykrishna D, Butler J, Baas C, Maurer-Stroh S, Silva-de-la-Fuente MC, Medina-Vogel G, Olsen B, Kelso A, Barr IG, González-Acuña D. Detection of evolutionarily distinct avian influenza a viruses in Antarctica. MBio. 2014;**5**(3):e01098-e010914. DOI: 10.1128/mBio.01098-14

[4] Plutzer J, Tomor B. The role of aquatic birds in the environmental dissemination of human pathogenic *Giardia duodenalis* cysts and *Cryptosporidium oocysts* in Hungary. Parasitology International. 2009;**58**(3):227-231. DOI: 10.1016/j.parint.2009.05.004

[5] Petersen KD, Christensen JP, Permin A, Bisgaard M. Virulence of *Pasteurella multocida* subsp. multocida isolated from outbreaks of fowl cholera in wild birds for domestic poultry and game birds. Avian Pathology. 2001;**30**(1):27-31. DOI: 10.1080/03079450020023168

[6] Hubálek Z. An annotated checklist of pathogenic microorganisms associated with migratory birds. Journal of Wildlife Diseases. 2004;**40**(4):639-659. DOI: 10.7589/0090-3558-40.4.639

[7] Bonnedahl J, Järhult JD. Antibiotic resistance in wild birds. Upsala Journal of Medical Sciences. 2014;**119**(2):113-116. DOI: 10.3109/03009734.2014.905663

[8] Abulreesh HH, Goulder R, Scott GW. Wild birds and human pathogens in the context of ringing and migration. Ringing & Migration. 2007;**23**(4):193-200. DOI: 10.1080/03078698.2007.9674363

[9] Novais RC, Thorstenson YR. The evolution of pyrosequencing(R) for microbiology: From genes to genomes. Journal of Microbiological Methods. 2010;**86**(1):1-7. DOI: 10.1016/j.mimet.2011.04.006

[10] Brittingham MC, Temple SA, Duncan RM. A survey of the prevalence of selected bacteria in wild birds. Journal of Wildlife Diseases. 1988;**24**(2):299-307. DOI: 10.7589/0090-3558-24.2.299

[11] Kapperud G, Rosef O. Avian wildlife reservoir of *Campylobacter fetus* subsp. jejuni, *Yersinia* spp., and *Salmonella* spp. in Norway. Applied and Environmental Microbiology. 1983;**45** (2):375-380

[12] Waldenström J, Broman T, Carlsson I, Hasselquist D, Achterberg RP, Wagenaar JA, Olsen B. Prevalence of *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter coli* in different ecological guilds and taxa of migrating birds. Applied and Environmental Microbiology. 2002;**68**(12):5911-5917. DOI: 10.1128/AEM.68.12.5911-5917.2002

[13] Buck JD. Isolation of *Candida albicans* and halophilic *Vibrio* spp. from aquatic birds in Connecticut and Florida. Applied and Environmental Microbiology. 1990;**56**(3):826-828

[14] Varslot M, Resell J, Fostad IG. Water-borne campylobacter infection–probably caused by pink-footed geese. Two outbreaks in Nord-Trøndelag, Stjørtdal in 1994 and Verdal in 1995. Tidsskrift for den Norske Lægeforening. 1996;**116**(28):3366-3369

[15] Alley MR, Connolly JH, Fenwick SG, Mackereth GF, Leyland MJ, Rogers LE, Haycock M, Nicol C, Reed CE. An epidemic of salmonellosis caused by *Salmonella* Typhimurium DT160 in wild birds and humans in New Zealand. New Zealand Veterinary Journal. 2002;**50**(5):170-176. DOI: 10.1080/00480169.2002.36306

[16] Thornley CN, Simmons GC, Callaghan ML, Nicol CM, Baker MG, Gilmore KS, Garrett NK. First incursion of *Salmonella enterica* serotype typhimurium DT160 into New Zealand. Emerging Infectious Diseases. 2003;**9**(4):493-495. DOI: 10.3201/eid0904.020439

[17] HS X, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microbial Ecology. 1982;**8**(4):313-323. DOI: 10.1007/BF02010671

[18] Yamaguchi N, Kenzaka T, Nasu M. Rapid in situ enumeration of physiologically active bacteria in river waters using fluorescent probes. Microbes and Environments. 1997;**12** (1):1-8. DOI: 10.1264/jsme2.12.1

[19] Kenzaka T, Yamaguchi N, Utrarachkij F, Suthienkul O, Nasu M. Rapid identification and enumeration of antibiotic resistant bacteria in urban canals by microcolony-fluorescence in situ hybridization. Journal of Health Science. 2006;**52**(6):703-710. DOI: 10.1248/jhs.52.703

[20] Kenzaka T, Yamaguchi N, Tani K, Nasu M. rRNA-targeted fluorescent in situ hybridization analysis of bacterial community structure in river water. Microbiology. 1998;**144** (8):2085-2093. DOI: 10.1099/00221287-144-8-2085

[21] Kenzaka T, Utrarachkij F, Suthienkul O, Nasu M. Rapid monitoring of *Escherichia coli* in Southeast Asian urban canals by fluorescent-bacteriophage assay. Journal of Health Science. 2006;**52**(6):666-671. DOI: 10.1248/jhs.52.666

[22] Kenzaka T, Ishidoshiro A, Yamaguchi N, Tani K, Nasu M. rRNA sequence-based scanning electron microscopic detection of bacteria. Applied and Environmental Microbiology. 2005;**71**(9):5523-5531. DOI: 10.1128/AEM.71.9.5523-5531.2005

[23] Kenzaka T, Tani K, Nasu M. High-frequency phage-mediated gene transfer in freshwater environments determined at single-cell level. The ISME Journal. 2010;**4**(5):648-659. DOI: 10.1038/ismej.2009.145

[24] Iwamoto T, Tani K, Nakamura K, Suzuki Y, Kitagawa M, Eguchi M, Nasu M. Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiology Ecology. 2000;**32**(2):129-141. DOI: 10.1111/j.1574-6941.2000.tb00707.x

[25] Kawai M, Matsutera E, Kanda H, Yamaguchi N, Tani K, Nasu M. 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology. 2002;**68**(2):699-704. DOI: 10.1128/AEM.68.2.699-704.2002

[26] Mardis E, Next-generation DNA. Sequencing method. Annual Review of Genomics and Human Genetics. 2008;**9**:387-402. DOI: 10.1146/annurev.genom.9.081307.16435

[27] Morozova O, Marra MA. Applications of next-generation sequencing technologies in functional genomics. Genomics. 2008;**92**(5):255-264. DOI: 10.1016/j.ygeno.2008.07.001

[28] Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, Perry T, Kao D, Mason AL, Madsen KL, Wong GK. Characterization of the gut microbiome using 16S or shotgun metagenomics. Frontiers in Microbiology. 2016;**7**:459. DOI: 10.3389/fmicb.2016.00459

[29] Pabinger S, Rödiger S, Kriegner A, Vierlinger K, Weinhäusel A. A survey of tools for the analysis of quantitative PCR (qPCR) data. Biomolecular Detection and Quantification. 2014;**1**(1):23-33. DOI: 10.1016/j.bdq.2014.08.002

[30] Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences of the United States of America. 1985;**82**(20):6955-6959. DOI: 10.1073/pnas.82.20.6955

[31] Tringe SG, Hugenholtz P. A renaissance for the pioneering 16S rRNA gene. Current Opinion in Microbiology. 2008;**11**(5):442-446. DOI: 10.1016/j.mib.2008.09.011

[32] Baker GC, Smith JJ, Cowan DA. Review and re-analysis of domain-specific 16S primers. Journal of Microbiological Methods. 2003;**55**(3):541-555. DOI: 10.1016/j.mimet.2003.08.009

[33] Wang Y, Qian PY. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One. 2009;**4**(10):e7401. DOI: 10.1371/journal.pone.0007401

[34] Yang B, Wang Y, Qian PY. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinformatics. 2016;**17**:135. DOI: 10.1186/s12859-016-0992-y

[35] Sekercioglu CH. Conservation ecology: Area trumps mobility in fragment bird extinctions. Current Biology. 2007;**17**(8):R283-R286. DOI: 10.1016/j.cub.2007.02.019

[36] Hedenström A. Extreme endurance migration: What is the limit to non-stop flight? PLoS Biology. 2010;**8**(5):e1000362. DOI: 10.1371/journal.pbio.1000362

[37] Kirby JS, Stattersfield AJ, Butchart SHM, Evans MI, Grimmett RFA, Jones VR, O'Sullivan J, Tucker GM, Newton I. Key conservation issues for migratory land- and waterbird species on the world's major flyways. Bird Conservation International. 2008;**18**(S1):S49-S73. DOI: 10.1017/S0959270908000439

[38] Yong DL, Liu Y, Low BW, Espanola CP, Choi CY, Kawakami K. Migratory songbirds in the East Asian-Australasian flyway: A review from a conservation perspective. Bird Conservation International. 2015;**25**(1):1-37. DOI: 10.1017/S0959270914000276

[39] Kenzaka T, Fujimitsu T, Kataoka K, Tani K. Intestinal microbiota in migrating Eurasian wigeon around Lake Biwa. Journal of Antibacterial and Antifungal Agents. 2018 (in press)

[40] Sorenson MD, Ast JC, Dimcheff DE, Yuri T, Mindell DP. Primers for a PCR-based approach to mitochondrial genome sequencing in birds and other vertebrates. Molecular Phylogenetics and Evolution. 1999;**12**(2):105-114. DOI: 10.1006/mpev.1998.0602

[41] BirdLife International. *Mareca penelope* (amended version published in 2016) the IUCN red list of threatened species 2017: E.T22680157A111892532. 2017. DOI: 10.2305/IUCN.UK.2017-1.RLTS.T22680157A111892532.en

[42] Keller JI, Shriver WG, Waldenström J, Griekspoor P, Olsen B. Prevalence of *Campylobacter* in wild birds of the mid-Atlantic region, USA. Journal of Wildlife Diseases. 2011;**47**(3):750-754. DOI: 10.7589/0090-3558-47.3.750

[43] Hellström S, Kiviniemi K, Autio T, Korkeala H. *Listeria monocytogenes* is common in wild birds in Helsinki region and genotypes are frequently similar with those found along the food chain. Journal of Applied Microbiology. 2008;**104**(3):883-888. DOI: 10.1111/j.1365-2672.2007.03604.x

[44] BirdLife International. *Hirundo rustica*. The IUCN red list of threatened species 2016: E. T22712252A87461332. 2016. DOI: 10.2305/IUCN.UK.2016-3.RLTS.T22712252A87461332.en

[45] Kenzaka T, Kataoka K, Fujimitsu T, Tani K. Intestinal microbiota in migrating barn swallows around Osaka. Yakugaku Zasshi. 2018 in press

[46] Engels C, Ruscheweyh HJ, Beerenwinkel N, Lacroix C, Schwab C. The common gut microbe *Eubacterium hallii* also contributes to intestinal propionate formation. Frontiers in Microbiology. 2016;**7**:713. DOI: 10.3389/fmicb.2016.00713

[47] Gorvitovskaia A, Holmes SP, Huse SM. Interpreting *Prevotella* and *Bacteroides* as biomarkers of diet and lifestyle. Microbiome. 2016;**4**:15. DOI: 10.1186/s40168-016-0160-7

[48] Wang W, Zheng S, Sharshov K, Sun H, Yang F, Wang X, Li L, Xiao Z. Metagenomic profiling of gut microbial communities in both wild and artificially reared bar-headed goose (*Anser indicus*). Microbiology. 2017;**6**(2):e00429. DOI: 10.1002/mbo3.429

[49] BirdLife International. *Anser indicus*. The IUCN red list of threatened species 2016: E. T22679893A92834171. 2016. DOI: 10.2305/IUCN.UK.2016-3.RLTS.T22679893A92834171.en

[50] Ryu H, Grond K, Verheijen B, Elk M, Buehler DM, Santo Domingo JW. Intestinal microbiota and species diversity of *Campylobacter* and *Helicobacter* spp. in migrating shorebirds in Delaware Bay. Microbiome. 2014;**80**(6):1838-1847. DOI: 10.1128/AEM.03793-13

[51] BirdLife International. *Arenaria interpres*. The IUCN red list of threatened species 2016: E. T22693336A86589171. 2016. DOI: 10.2305/IUCN.UK.2016-3.RLTS.T22693336A86589171.en

[52] BirdLife International. *Calidris canutus*. (amended version published in 2016) the IUCN red list of threatened species 2017: E.T22693363A111379432. 2017. DOI: 10.2305/IUCN.UK.2017-1.RLTS.T22693363A111379432.en

[53] Dewar ML, Arnould JP, Dann P, Trathan P, Groscolas R, Smith S. Interspecific variations in the gastrointestinal microbiota in penguins. Microbiology. 2013;**28**(1):195-204. DOI: 10.1002/mbo3.66

[54] Waite DW, Taylor MW. Exploring the avian gut microbiota: Current trends and future directions. Frontiers in Microbiology. 2015;**6**:673. DOI: 10.3389/fmicb.2015.00673

[55] Barbosa A, Balagué V, Valera F, Martínez A, Benzal J, Motas M, Diaz JI, Mira A, Pedrós-Alió C. Age-related differences in the gastrointestinal microbiota of chinstrap penguins (*Pygoscelis antarctica*). PLoS One. 2016;**11**(4):e0153215. DOI: 10.1371/journal.pone.0153215

[56] Shehzad W, Riaz T, Nawaz MA, Miquel C, Poillot C, Shah SA, Pompanon F, Coissac E, Taberlet P. Carnivore diet analysis based on next-generation sequencing: Application to the leopard cat (*Prionailurus bengalensis*) in Pakistan. Molecular Ecology. 2012;**21**(8):1951-1965. DOI: 10.1111/j.1365-294X.2011.05424.x

[57] Vestheim H, Jarman SN. Blocking primers to enhance PC amplification of rare sequences in mixed samples – A case study on prey DNA in Antarctic krill stomachs. Frontiers in Zoology. 2008;**5**:12. DOI: 10.1186/1742-9994-5-12

[58] McInnes JC, Alderman R, Deagle BE, Lea M-A, Raymond B, Jarman SN. Optimised scat collection protocols for dietary DNA metabarcoding in vertebrates. Methods in Ecology and Evolution. 2017;**8**(2):192-202. DOI: 10.1111/2041-210X.12677

[59] Bowser AK, Diamond AW, Addison JA. From puffins to plankton: A DNA-based analysis of a seabird food chain in the northern gulf of Maine. PLoS One. 2013;**8**(12):e83152. DOI: 10.1371/journal.pone.0083152

[60] Stoddard SF, Smith BJ, Hein R, Roller BRK, Schmidt TM. rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucleic Acids Research. 2015;**43**:D593-D598. DOI: 10.1093/nar/gku1201

Section 3

Infectious Diseases

Chapter 4

Metagenomic Approaches for Investigating the Role of the Microbiome in Gut Health and Inflammatory Diseases

Rodrigo Carvalho, Fillipe Carmo, Sara Heloisa, Barbara Cordeiro, Aline Vaz, Enrico Gimenez, Luis Goulart, Aristóteles Góes-Neto, Yves Le Loir, Gwénaël Jan and Vasco Azevedo

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72031

Abstract

The human gut microbiota makes fundamental contributions to host metabolism and immune system. Therefore, perturbations in its composition, a process known as dysbiosis, have an important role in the development of several chronicle diseases, mainly intestinal inflammatory disorders. Culture-independent molecular methods are allowing scientific community to uncover substantive findings, thus giving a more detailed description of the human intestinal microbiota. This chapter presents a review on current metagenomic approaches, based on next-generation sequencing technologies, for investigating bacterial taxonomic classification and predictive function associated with the human gut in health and disease. In this context, we describe recent studies that have been trying to elucidate important alterations in microbiome composition across individuals according to delivery mode, aging, diet and medication that might be linked to susceptibility to immune-mediated diseases. A description of the main bacterial taxa and genes acting in dysbiosis during inflammation, focusing on chronic inflammatory bowel diseases and colorectal cancer, is also explored in this chapter.

Keywords: gut microbiota, metagenomics, dysbiosis, inflammatory bowel diseases, colorectal cancer

1. Introduction

Microbiota is considered as an essential organ of humans and other animals, which carry out many functions that host cells cannot. A major portion of these mutualistic microbes is found

in the human gut, where they occupy niches that make contributions to nutrient processing, pathogen colonization resistance and mucosal immune system development [1]. The intestinal microbiome is formed by hundreds of different bacterial species colonizing mucosal surfaces. Its compositional structure differs across human populations according to geographic regions in the world method of delivery at childbirth, breast or bottle feeding, age, diet and medications [2]. Actually, the role of one individual microbiota is composed by the repertoire of expressed genes, known as metagenome. Impressively, it is estimated that humans possess 10 million extra genes from intestinal bacteria [3]. Significant perturbation of the gut microbiota can lead to a dysbiosis state, which compromise important functions in host immunity and raise susceptibility to immune-mediated diseases [4]. Therefore, there has been great interest in identifying the metagenomic content of the gut microbiota which can be used to treat or prevent diseases. In this context, extensive endeavor are being carried out to elucidate the gut ecosystem and molecular mechanisms underlying the pathogenesis of several intestinal disorders [5]. Culture-independent methods, in particular next-generation sequencing technologies, have prompted a huge breakthrough in our knowledge regarding the microbial communities colonizing the human body and their functional beneficence to host health [6].

2. Metagenomic sequencing approaches for investigating intestinal microbiomes

2.1. Bacterial identification by 16S rRNA gene sequencing

New generation sequencing technologies are capable of processing high amount of DNA in a relative short time using 16S ribosomal (16S rRNA) genetic information. Several high throughput platforms such as 454 Roche GS FLX, Applied Biosystems SOLiD System, Illumina HiSeq and MiSeq System and Ion Torrent Personal Genome Machine (PGM) have been used for this kind of metagenomic approach [7, 8]. The molecular-based taxonomic investigation for bacteria employs direct sequencing of PCR-amplified small sequences of 16S rRNA gene from extracted DNA, generally using universal primers annealing conserved nucleotides to amplify one or more fragments of variable regions. As a few numbers of base pairs can change in a very short period of evolutionary time, amplicons around 300 bp are frequently enough for taxonomic assignment [9]. The sequences at a pre-defined level of identity stand for grouped clusters of similar sequencing reads, known as Operational Taxonomic Unit (OTU), which corresponds to a group of very similar 16S sequences. Reference databases (GreenGenes, myRDP, NCBI) are used to classify OTUs providing identification of taxonomy, relative frequencies and diversity of community composition in samples obtained from the certain ecosystem [10, 11]. This approach allows identification of new species and investigation of low-abundance bacteria and even uncultivated gut microbial communities from a single analysis. In addition, these technologies are faster and more accurate compared to classical identification methods (cloning and culture) [12]. However, this approach has some limitations regarding information about the microbiome function, mainly because several species of bacteria have not been characterized yet and secondly due to a great variability found among individuals, it is expected that the microbiota function present high redundancy, in which different species may occupy the same niche in the gut [13].

2.2. Shotgun sequencing for predictive functional analyses

The whole metagenome sequencing can be performed using a shotgun approach. In such genomic survey strategy, multiple continuous overlapping sequences (contigs), which are assembled from fragmented sequences and obtained from total purified genomic DNA, are used for identifying genes through alignment with bacterial reference genomes and databases (KEGG, SEED and NCBI) [6]. Shotgun approach is quite versatile, in which the samples can be submitted to various methods, including nebulization, endonucleases, or sonication for random fragmentation of DNA, sequencing a subsequent contig assembly and annotation. Furthermore, advanced computational methods applying different algorithms are frequently being developed for more accurate assembly and annotation of genes, thus allowing functional characterization in complex environments like the human gut [14, 15]. This method also provides identification of variants and polymorphisms and gives a more comprehensive understanding on the functional information of microorganism communities, for example, by reconstructing metabolic pathways *in silico* [12]. A major limitation of this strategy is that metagenomic sequencing of multiple individuals is extremely expensive in comparison with 16S rRNA sequencing and generation of a large amount of data demands intense computational analysis, most of time to be performed by bioinformatics specialists [16].

2.3. Metagenomic consortium

Massive increase in the amount of data from human gut microbiota and identification of genes or families of genes submitted in the databases has prompted the creation of consortia such as the Human Microbiome Project in healthy individuals, which led to establishment of a reference microbial genome database according to results of 16S profiling of 242 healthy adults from the United States [17]. The European milestone Metagenomics of the Human Intestinal Tract (MetaHIT) intended to identify potential links between the association of gut microbiome with obesity and inflammatory bowel disease (IBD) from 540 Gb of DNA from stool samples of 124 healthy or sick individuals [18]. Moreover, about 1000 bacterial species were found and each individual in this study is estimated to contain at least 160 species, and in addition, 18 species of bacteria were common to 124 individuals [19]. Nevertheless, complementary approaches to metagenomic studies as well as integrative analysis are required to understand the complex and intrinsic interactions with gut microbiota and hosts, like metatranscriptomics, metaproteomics for studying the functional aspects of the microbiota and metabolomics [20, 21].

3. The gut microbiome in health

3.1. Nutritional and metabolic functions

Gut microbiota is essential for the host digestion and nutrition, as they can produce unique nutrients from indigestible substrates [22]. For instance, many polysaccharides, which are found in vegetables from our diet, such as cellulose, xylans, resistant starch and inulin, are digested by certain species that colonize the intestines. Metagenomic studies revealed that the capacity to digest xyloglucans found in onion and lettuce is a specific trait of *Bacteroides* sp. [23]. Through

a mutualistic relationship, the digestion of non-digestible compounds by microbiota may yield energy for microbial growth and end products such as short-chain fatty acids (SCFAs). SCFAs, mainly acetate, propionate and butyrate, are an important energy source for the colonic epithelium and as a key factor for regulating pro-inflammatory immune responses in the gut. Acetate and propionate are important for the liver and peripheral tissues as well, because they act as substrates for metabolic functions such as gluconeogenesis and lipogenesis [5]. The capacity of gut microbiomes metabolic activity, when regarding SCFAs production, may depend on how much complex carbohydrates are ingested through our diet and on the composition of the gut microbiota as well [24].

A study using a model of gnotobiotic mice demonstrated that fermentation of dietary fructans increases when animals colonized with *Bacteroides thetaiotaomicron* are co-colonized with *Methanobrevibacter smithii*. The first species produces more acetate and formate, whereas the second uses formate for methanogenesis, illustrating the importance of interaction between the microbiota organisms to promote nutrient fermentation and absorption and consequently metabolic functions [25, 26].

The ingestion of prebiotics such as inulin can promote expansion of *Faecalibacterium prausnitzii* and *Bifidobacterium* sp. in humans. Both species are important for metabolic functions and immune response regulation in the intestinal mucosa as they produce butyrate and folate, respectively. Folate can be synthesized by the large bowel microbiota and is essential for the synthesis of precursors of nucleic acids, contribute to epigenetic effects, and amino acid metabolism. For example, *B. bifidum* and *B. longum* produce folate in high concentrations [27, 28].

Vitamin B12 is synthesized by different bacteria, for example, *Propionibacterium freudenreichii* and *Lactobacillus reuteri* [29]. Vitamin B12 and its corrinoid precursors play an important role in the gut microbiota as 80% of human intestinal microbes express transporters to capture corrinoids and use vitamin B12 as a cofactor for metabolic pathways. For example, *B. thetaiotaomicron* encodes three vitamin B12 acquisition systems. Folate and vitamin B12 also serve as regulators of gene expression in human gut bacteria and might control genomic interactions between the microbiota and host [30].

3.2. Immune system regulation and resistance against opportunistic pathogens colonization

The mucosal immune system is responsible for maintaining gut homeostasis as it must remain vigilant against pathogen infections while limiting over inflammatory responses against the majority of commensal organisms that comprise the microbiota. Symbiotic bacteria play an important role in preventing inflammatory diseases, however in dysbiosis, opportunistic pathogens may stimulate a local immune response resulting a tissue damage. Therefore, the microbiota has the potential to exert both pro-inflammatory and anti-inflammatory responses, meaning that the balance in the composition of gut microbiome may be intrinsically involved in the proper function of the immune system [31, 32]. Many bacterial species from the gut microbiota such as *Lactobacillus* sp. and *Bifidobacterium* sp. have the ability to activate molecular

mechanism on several cell types constituting the epithelial barrier function, such as specialized epithelial cells like goblet cells and paneth cells [33, 34]. Goblet cells are the main cells involved in mucus production, which is considered the first line of host defense against pathogens. Current studies reveal that several commensal microbes can stimulate Muc2 expression, a major component of the intestinal mucus [35].

Enterocytes, such as paneth cells, which reside at the base of the intestinal crypts, are specialized in producing antimicrobial peptides preventing overstimulation of the immune system by keeping commensal bacteria 50 μm apart from the small intestinal epithelial surface [35]. Studies suggest that expression of these antimicrobial peptides, including RegIIIγ and defensins, is driven by the microbiota. Certain components of the microbiota, including *B. thetaiotaomicron*, confer resistance to *Candida albicans* by promoting the expression of H1F-1α, a transcriptional regulator that induces the expression of the antimicrobial peptide LL-37, with anti-Candida activity [36].

Microbiota induction of the adaptive responses of the immune system, including B cells and T cells, plays a central role in the defense against intestinal pathogens and regulation of inflammation in the gut. Certain intestinal bacterial species play a major role in the differentiation of T cells into different subsets, including T helper cells Th1s, Th2s, Th17s and T regulatory cells (Tregs) [35]. The development and functionality of Tregs in the gut depend on the presence of specific commensal microbes. Administration of a pool containing bacterial strains from the Clostridia clusters IV, XIVa and XVIII has been shown to restore the population of Tregs to those observed in conventional mice. One mechanism by which Clostridia species, including *F. prausnitzii*, may enhance Treg differentiation is through production of SCFA [37]. Similarly, vitamin A metabolite retinoic acid, whose production has been shown to be partly controlled by the microbiota, also supports anti-inflammatory function [38].

Production of IgA by B cells is a key factor for the host to control infections on mucosal surfaces, including the gastrointestinal tract. Several studies have demonstrated that the intestinal microbiota influences B-cell development and antibody production. The gut microbiota affects IgA class-switch recombination in either T-cell–independent or dependent pathways. T-cell–independent produces IgA with low affinity but directed towards the microbiota. Bacteria that colonize the epithelial surface of the intestinal mucosa, such as *Mucispirillum*, *Clostridium scindens* and *Akkermansia muciniphila* which are segmented filamentous bacteria, can activate t-cell-dependent pathway and regulation of mucosal IgA responses [39, 40].

The microbiota can also confer resistance to pathogens by directly inhibiting them, without the involvement of the gut immune system. Certain commensal strains can produce and secrete small molecules with bacteriostatic or bactericidal activity, such as bacteriocins or microcins produced by Gram-positive and Gram-negative species, respectively. SCFA can also influence the expression of virulence factors. For example, butyrate and propionate can downregulate the expression of pathogenicity island 1 (SP1) genes in *Salmonella typhimurium*, which is crucial for this bacterium to invade intestinal epithelial cells. A major mechanism by

which the microbiota inhibits intestinal colonization by bacterial pathogens is through nutrient competition [35].

3.3. Microbiome composition in human ontogeny

The gut microbiome assumes different characteristics regarding diversity, structure and functional gene repertoires over human lifetime (**Figure 1**). From the compositional perspective, microbiota becomes more and more complex with time, along with periods of ecological stability and fluctuation due to new environmental expositions, until it reaches a dynamic equilibrium in adulthood [13].

3.3.1. Birth

It is commonly accepted by scientific community that our first exposition to microbes may occur at birth delivery. However, recent metagenomics studies reveal that non-harmful bacteria may colonize the placenta, amniotic fluid and other fetal components from healthy term pregnancies [41]. The bacterial taxa identified in the fetal environment present low abundance but are diverse, including many species associated with the oral and intestinal microbiomes, such as *Fusobacterium* spp. and *Bacteroides* spp. Moreover, similar bacterial communities can be found in meconium, suggesting that the colonization of the gut may start with intrauterine resident bacteria [42]. Nevertheless, the elucidation of the probable mechanism in which those bacteria translocate to the gut of the fetus is required to sustain the hypothesis of prenatal colonization. Regarding the gut microbiome just after birth, it has significantly lower diversity and higher variability among individuals compared to the microbiome in adulthood [24]. The dominant bacteria at phylum level in the neonatal gut include Firmicutes, Proteobacteria and Actinobacteria with lower levels of Bacteroidetes, a dominant phylum in the adult gut microbiome [43].

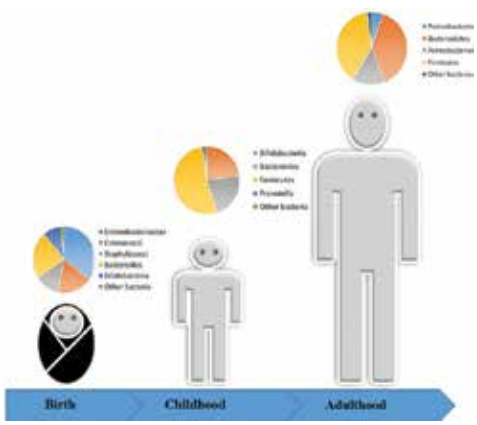

Figure 1. Intestinal microbiota in birth, childhood and adulthood. Birth delivery starts the colonization of the gut, thought the caesarian mode can alter the microbiome composition in newborns. In childhood, gut microbiota shows increased diversity due to introduction of solid foods. In adulthood, the gut microbiota achieves stability but several lifestyle factors may influence the microbiome composition, such as diet, physical activities, alcohol, smoking, drugs and hygiene.

3.3.1.1. Mode of delivery

The modes of delivery significantly influence the neonatal microbiome [44]. In vaginally delivered infants, the gut is primarily colonized by bifidobacteria as well as lactobacilli and enterobacteria. When compared to children delivered in natural terms, the microbiomes of caesarian-delivered individuals show lower diversity and lower abundance of these bacterial taxa [45]. Moreover, infants delivered by cesarean section have more *Escherichia coli* as well as *Clostridium difficile* [46]. A recent study has been trying to evaluate whether cesarean-delivered infants exposure to maternal vaginal fluids are able to colonize them with the natural microbiota that is supposed to be acquired during passage through the birth canal. However, these findings revealed that such inoculation provides minimal effect on the bacterial community of the infants, which presented fewer species and lower levels of *Lactobacillus* spp. and *Bacteroides* spp. comparing with samples originally obtained from vaginally delivered infants [47]. It is important to reiterate that despite of discrepancies between studies in the literature, most of them show that mode of delivery may select certain bacterial groups over other types.

3.3.2. Childhood

Previously, based on classical microbiological studies, it was thought that the human gut microbiome would achieve a stable status during the first 4 years of life. However, contemporary studies using metagenomic approaches have shown a different composition in childhood regarding the human gut microbiota when compared to adulthood [48, 49]. In American children cohorts, a study demonstrated that they present higher frequencies of Firmicutes, Proteobacteria and Actinobacteria and less Bacteroidetes [50]. Moreover, teenager's microbiomes seem to be enriched in butyrate-producing bacteria such as *Alistipes* spp., *B. vulgatus* and *B. xylanisolvens* and *Roseburia* spp., *Faecalibacterium* spp., *Ruminococcus* spp., that seems to play anti-inflammatory roles [51]. Also, on a nutritional function perspective, microbiomes of children contain more vitamin B12-producing bacterial species than adults [52].

Although many functional features of the microbiome of children remain to be profoundly investigated, in general, it is known that childhood microbiome represents a dynamic ecosystem, which probably has an impact on health later in life. In this context, several environmental exposures are supposed to be associated with differences found among individuals. These factors include drugs exposure, contact with domestic animals, hygiene, geography and diet [52]. Metagenomic studies suggest that diet has a determinant role for driving the development of childhood microbiome as it needs to adapt to different conditions of nutrients availability [53].

3.3.2.1. Breast feeding versus bottle feeding

In the first years of life, the human gut microbiome is richer in genes involved in digestion of oligosaccharides found in breast milk, while in later childhood, due to the ingestion of solid foods, the gut metagenome is richer in genes involved in the digestion of polysaccharides and vitamin biosynthesis. Therefore, different microbe exposure in modes of feeding might significantly influence microbiome composition and function [52, 53]. Breast-fed children

show an increase in Actinobacteria and a decrease in Firmicutes and Proteobacteria, whereas bottle-fed ones exhibit more abundance of potential pathogens such as *E. coli* and *C. difficile* [42, 54]. Interestingly, breast milk contains many compounds that might affect the microbiome composition of the infant gut in a positive way, such as immunoglobulin, prebiotic oligosaccharides and diverse maternal milk microbiota species that continually colonize the infant gut [55]. The majority of the studies show that the composition of the gut microbiota in breast-fed infants is enriched in aerobic organisms compared to formula-fed ones, which present higher prevalence of anaerobic and facultative anaerobic organisms such as *Bacteroides* sp. Certain *Bacteroides* sp. strains are able to digest milk oligosaccharides, suggesting a potential competitive relationship between *Bifidobacterium* sp. and *Bacteroides* sp. in breast-fed infants. Metagenomic findings have been important for providing new advances regarding translational researches such as the development of infant formulas that are more similar in composition and function to breast milk. In this context, Oligosaccharide-enriched formulas have been developed, favoring the colonization of infants gut by greater numbers of bifidobacteria [43].

3.3.3. Adulthood

Healthy adult humans may harbor more than 1000 species of bacteria belonging to different bacterial phyla with Bacteroidetes and Firmicutes being the dominant ones [56]. The microbiota of adults can achieve the highest diversity regarding human ontogeny development. There is also considerable variation in the intestinal environment, compared to other sites of the gut, and among healthy individuals. The proportion of each phylum apparently varies according to geographical distribution [2, 57]. For instance, some studies have shown that Firmicutes are more prevalent in adults in rural communities, whereas adults in industrialized societies seem to present higher levels of Bacteroidetes [58]. A higher Firmicutes to Bacteroidetes ratio has been mostly associated with metabolism function and body weight gain, although further investigation is required to shed light on the species-associated role in healthy individuals cohort across different geographical location to understand their influence in leanness/obesity [24]. In general, the gut microbiome in adulthood remains relatively stable through adulthood, except following perturbations such as pathogen infections, antibiotic drugs or drastic dietary shifts [13]. However, as we age, the gut microbiome is enriched in more traits associated with inflammation and metabolic dysfunction. A reduction in *Bacteroides* spp., *Prevotella* spp. and *F. prausnitzii* and an increase in Enterobacteriaceae have been associated with an overall decrease in the quality of life in old age [51]. In general, the gut microbiome in the elder adults exhibits a higher ratio of Firmicutes to Bacteroidetes when compared to young adults and reduction in symbiotic microbes such as Bifidobacteria and Bacteroides [59].

4. Dysbiosis in the pathogenesis of inflammatory bowel diseases

As described above in this chapter, substantial changes in microbiota may sometimes lead to dysfunction resulting in a dysbiosis state. This process has an important role in diseases involving inflammatory responses in the gut, for example, in inflammatory bowel diseases

http://dx.doi.org/10.5772/intechopen.72031

(IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC). Moreover, excessive oxidative long-term activity of IBD is associated with colorectal cancer (CRC) (**Figure 2**) [60]. IBDs have been considered as global health problem [61], affecting around 3.1 million people in the USA [62, 63]. CD and UC are chronic diseases characterized by periods of relapse and remission. Clinical symptoms of both diseases are similar to each other; however, UC activity is restricted to the colon while CD may affect any part of the gut, more frequently in the ileum and proximal colon [64].

It has been reported that patients with IBD have a reduced diversity of gut microbiota, characterized by the depletion of commensal species belonging to the phylum Firmicutes and Bacteroidetes [65, 66]. Metagenomic studies have pointed out a decreasing in short-chain fatty acids (SCFAs) metabolism genes such as butyrate, which play an important role in the maturation of regulatory T cells, in the gut microbiota of IBD patients [67]. Moreover, it was observed that patients have a reduction in a number of many SCFA-producing species from Clostridia groups, remarkably *F. prausnitzii* [68, 69]. Microbiota patterns such as the reduction in *F. prausnitzii* have been consistently associated with CD patients, during period of active disease or remission, from different geographical regions including Europe and South Asia and may thus serve as a reliable clinical marker [70, 71]. The reduction in species from *Lactobacillus* and *Bifidobacterium* genus has an important role in IBD pathogenesis as well, as these bacteria can downregulate the expression of key proinflammatory cytokines and chemokines in the gut [70]. In the other hand, opportunistic commensals, such as *Mycobacterium avium* sub. *paratuberculosis*, *C. difficile*, *Ruminococcus gnavus* and enterobacteria, are increased [19]. Commensal Sulfate-reducing bacteria are considered as a key factor in the initiation and maintenance of IBD as these bacteria reduce disulfide bonds of the mucus barrier, thereby allowing exposure of the host cells to pathogenic bacteria and toxins [72]. Therefore, these

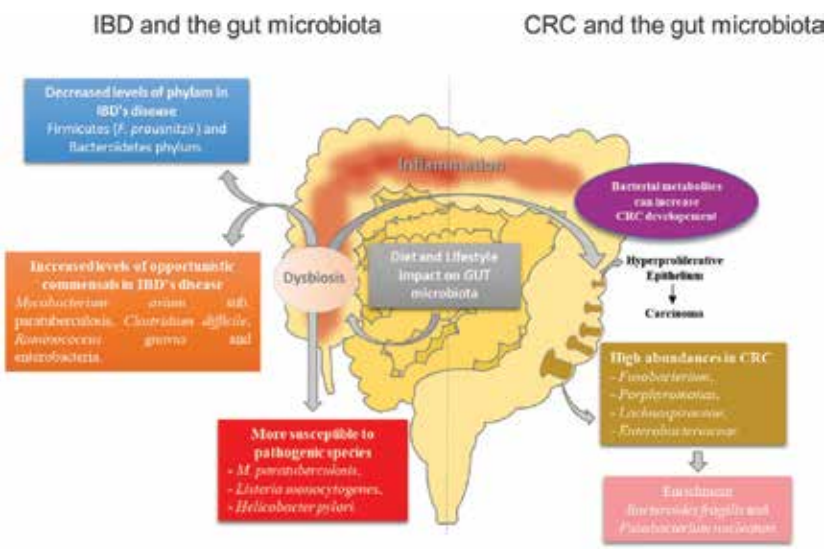

Figure 2. Dysbiosis in pathogenesis of IBD and CRC. Increased levels of metabolites produced by opportunistic commensals of the gut microbiome modulates the effects of inflammation process and cancer tumorigenesis.

patients develop a certain predisposition for the colonization of facultative pathogens, such as invasive *E. coli*, that express several virulence factors involved in adhesion and invasion of the epithelial barrier [73, 74]. Moreover, colonization by pathogenic species, such as *M. paratuberculosis*, *Listeria monocytogenes* or *Helicobacter* species, can worsen the symptoms of IBD as these bacteria can activate proinflammatory signaling cascades into the host [75].

Besides bacteria, gut microbiome fungi and virus may also be involved in IBD. Metagenomic studies demonstrated that inflamed guts present high frequency of certain bacteriophages [76, 77]. In regard of fungi, few studies in mice show some yeast species that can inhibit the over growth of certain opportunistic commensals and affect the gut homeostasis. However, in humans, much research is required to elucidate the role of fungi and virus microbiome in the gut [78].

Currently, there is much discussion about the use of fecal transplant for transference of microbiota from healthy individuals to IBD patients as an alternative therapeutic strategy. Promising outcomes have been achieved against *C. difficile* infection in clinical trials, which is often found in the gut of IBD patients [79]. However, in UC clinical trials, the fecal transplant efficacy is controversial [80, 81]. Regarding CD clinical trials, children cohorts have shown better results with fecal microbiota transplantation than adult cohorts [82, 83].

4.1. Colorectal cancer

CRC is the most common form of gastrointestinal tract cancer, globally the third leading cause of cancer, and is associated with significant mortality affecting both men and women with 1.4 million people diagnosed annually [60, 84].

Different from other kinds of cancer affecting the large bowel which are caused by point mutations in several genes that control cell proliferation, survival, differentiation and migration [60], CRC is mostly attributed to environmental factors and lifestyle such as high fat diet, alcohol, red meat, smoking, obesity, or lack of physical activity [56, 85]. There are several studies showing a connection between inflammatory processes and carcinogenesis, although the contribution of immunological mechanisms and inflammation to malignancy of CRC, it is not fully elucidated [86–88]. Immune cells, cytokines and other mediators of the immune system that are directly influenced by dysbiosis play an important role in the stages of tumorigenesis in the colon, including onset, promotion, progression, and metastasis [86].

Several studies have been trying to identify components of the microbiota that play pivotal roles in inflammation process and in the progression of colorectal cancer by creating microenvironments that favor tumorigenesis development (**Table 1**). Metagenomic analysis of microbiome provides insights of interactions and contributes to understand how the bacterial species can be related to CRC development, as it has been observed that bacterial populations present in fecal samples are distinct from not only tissue biopsies but also between inter-individual microbial communities, even in samples with the same subtypes of cancer. Usually fecal samples and biopsies are collected for 16S RNA gene sequencing generating results about diversity and abundance of the species making part of the gut microbial community [89, 90].

Main findings	Disease	Sampling description	Ref
Microbiomes of IBD subjects fluctuate more than those of healthy individuals	IBD	Fecal samples from 109 patients with IBD (CD, n = 49; UC, n = 60)	[100]
UC and CD have distinct microbiomes. *F. prausnitzii* and *E. coli* were found decreased and increased, respectively, in CD. These species among others could be used as microbiome markers to discriminate CD and UC	IBD	2045 non-IBD and IBD fecal samples from four countries (Spain, Belgium, the UK and Germany)	[101]
Microbiome colonizing the mucosa is different between inflamed subjects with CD and UC. At phylum level, Bacteroidetes is more frequent in CD while Proteobacteria and Firmicutes were more frequently observed in UC. At genus level, *Faecalibacterium*, *Bacteroides* and *Pseudomonas* were significantly different between the inflamed CD and UC	IBD	Analysis of the microbiota composition of ileum, cecum, mid-colon and rectum samples from 166 individuals	[102]
The role of the metabolites produced by the microbiota in dysbiosis was correlated to IBD. Dysbiosis characterized by changes in Firmicutes and Proteobacteria phyla. Decreased levels of *Roseburia* and *Faecalibacterium* were found in CD and UC	IBD	Microbiota of intestinal biopsies and stool samples from 231 IBD and healthy	[103]
Metabolites and fecal microbiome can be useful to discriminate between healthy subjects and patients with IBD. At genus level, *Escherichia*, *Faecalibacterium*, *Streptococcus*, *Sutterella* and *Veillonella* were increased, while level of *Bacteroides*, *Flavobacterium* and *Oscillospira* decreased in IBD group	IBD	Microbiota and the metabolites in stool of 183 subjects (UC—82, CD—50 and 51 healthy controls)	[104]
Mucosa-associated dysbiosis was identified in IBD patients. CD and UC may be distinguishable from the mucosa-associated microbial community structure. CD patients have increased levels of *Escherichia*, *Ruminococcus*, *Cetobacterium*, *Actinobacillus* and *Enterococcus* comparing to controls and UC subjects, and a significant decrease in *Faecalibacterium*, *Coprococcus*, *Prevotella* and *Roseburia* as well	IBD	174 mucus samples from 43 UC subjects, 26 CD subjects and 14 non-IBD controls	[105]
No consistent overrepresentation of potential pathogenic bacteria in CRC tissue Increased abundance of Coriobacteridae, *Roseburia*, *Fusobacterium* and *Faecalibacterium* Decreased abundance of Enterobacteriaceae (*Citrobacter*, *Shigella*, *Cronobacter*, *Kluyvera*, *Serratia* and *Salmonella* spp.)	CRC	Resections for primary colon adenocarcinoma of 6 patients	[106]
The gut microbiome could be used as a biomarker for CRC Adenoma: increased abundances of Ruminococcaceae, *Clostridium*, *Pseudomonas* and Porphyromonadaceae. Lower abundances of *Bacteroides*, Lachnospiraceae, Clostridiales and *Clostridium* Carcinoma: increased abundances of *Fusobacterium*, *Porphyromonas*, Lachnospiraceae and Enterobacteriaceae. Lower abundances of *Bacteroides*, Lachnospiraceae and Clostridiales	CRC	Analysis of fecal samples from healthy individuals, adenoma and carcinoma patients (30 subject for each clinical group)	[107]
F. nucleatum is prevalent in cases of proximal colon cancer. Amount of *F. nucleatum* increases linearly along the bowel subsites from rectum to cecum	CRC	1102 samples provided from database of colorectal carcinoma cases	[108]

Main findings	Disease	Sampling description	Ref
F. nucleatum, *B. fragilis* and *F. prausnitzii* could be identified as useful prognostic biomarkers for CRC *B. fragilis* and *F. prausnitzii* correlated with patient's survival in CRC *F. nucleatum* presented higher abundance in non-survival group	CRC	Tissues samples from 108 patients in stages I–IV of CRC with different prognosis	[109]
CRC microbiome is stage-specific and appears to evolve with disease progression. Enrichment of organisms including *Bacteroides fragilis*, *Fusobacterium nucleatum* and under-representation of *Bacteroides vulgatus*, *Bacteroides uniformis* and *Faecalibacterium prausnitzii* Enrichment of oral pathobionts in poor prognosis tumors and cancers: *Parvimonas micra*, *Porphyromonas gingivalis* and *Prevotella* spp.	CRC	Tissue was sampled from 158 CRC patients, 24 adenoma patients and 14 normal colon controls	[110]
Multiple fusobacteria members did not correlate with CRC. Enriched *F. nucleatum*, *F. necrophorum*, *Leptotrichia trevisanii*, *B. fragilis*, *Parvimonas micra*, *Peptostreptococcus stomatis* and *Gemella morbillorum* Low levels of *F. varium* and *Cetobacterium somerae* in CRC	CRC	16S rRNA amplicon sequence raw datasets from 12 studies	[111]

Table 1. Recent metagenomic studies in clinical investigation of intestinal inflammatory diseases.

Considering CRC microenvironment and the functionality of intestinal microbiome, it has a high importance as a risk factor or can be directly associated to CRC. Chronic inflammatory processes driven by dysbiosis can affect all stages of tumor development by compounds that can damage DNA, for example, reactive oxygen species (ROS) promoting CRC development [91]. During an inflammatory response, this microenvironment generates ROS and reactive nitrogen intermediates (RNIs) that lead to deleterious DNA promoting carcinogenesis damage or activating pivotal signaling pathways for adenoma formation and growth [86]. In a study investigating CRC in mouse model of tumorigenesis, it was demonstrated that long-term inflammation-mediated breakdown of protective intestinal barriers promotes the production of some inflammatory cytokines such as IL-17 and IL-23 that lead to tumor growth by facilitating bacterial translocation and consequently microbial products that trigger tumorigenesis resulting in adenoma invasion [92]. Blooms of enterobacteria also seem to play a role in CRC as indicated by metagenomic studies of luminal microbiota of inflamed Il10−/− mice reveal that *E. coli* can promote cancer activity modulating tumor development once host inflammation has been established [87, 93].

In a study with fecal samples from 74 patients with CRC, the microbiome was enriched in *Fusobacterium nucleatum* and *Peptostreptococcus stomatis*. Moreover, co-occurrence of these taxa with the other two, *Parvimonas micra* and *Solobacterium moorei,* was found. Interestingly, *P. micra* and the Gram-negative *F. nucleatum* can induce inflammatory responses by binding to lipopolysaccharides from Gram-negative bacteria. Furthermore, *F. nucleatum* was shown to increase intestinal tumorigenesis through recruitment of infiltrating immune cells and through activation of β-catenin signaling [90]. In another study, in which the role of *F. nucleatum* in CRC was investigated by metagenomic analyses of 511 colorectal carcinomas from Japanese patients, it was identified a significant increase in the occurrence of the bacteria [89].

An investigation in 34 patients with four CRC subtypes showed enrichment of Fusobacteria and Bacteroidetes, and decreased levels of Firmicutes and Proteobacteria, including *Porphyromonas gingivalis*, *P. micra*, *P. stomatis* and *F. nucleatum*, create a particular condition by recruiting T cells resembling the immunological aspects in specific colorectal tumors [94]. Prorok-Hamon et al. (2014) have shown, by PCR screening, that from 281 *E. coli* isolates from IBD patients, CRC patients presented increased levels of 21 colonic mucosa-associated *E. coli* strains, with pathogenic traits, including M-cell translocation, angiogenesis and genotoxicity properties [95].

Some works using metagenomics and metabolomics integrative analyses found that enriched levels of Fusobacteria and Proteobacteria create a specific mucosal metabolic microenvironment that was associated with CRC pathogenesis through a large number of chemical and molecular signaling pathways [96–98]. Although fecal microbiome and metabolome may positively correlate with CRC conditions, the role of such interactions is still poorly understood in the pathogenesis of the disease [96].

Helicobacter pylori which well established in the development of stomach cancer since 1994 being classified as carcinogenic by the International Agency for Research on Cancer has also been pointed out as risk factor for CRC, as the infection leads to initial inflammatory response by stimulating IL-1β production, and consequently causing epithelium injury such as metaplasia [85]. In some studies, there was an indication of increased risk of colorectal adenomas by the presence of *H. pylori* due to hypergastrinemia; however, there is a controversy in other metagenomic studies that did not found correlation or even did not identify the presence of the pathogen in the analyzed samples [99].

5. Concluding remarks

These recent metagenomic studies reiterate that microbiome intrinsic factors from particular communities and lifestyle are extremely important and should be considered for future development of novel therapies for IBD. Despite of all efforts to accurately unravel the microbiome role in the gut and its relationship with gut inflammation through metagenomic sequencing approaches, it seems that more complex mechanism might be involved in dysbiosis linked to chronic inflammatory diseases, such as IBD and CRC. In this context, further advances in the area are required to achieve a more precise definition of gut microbiome role, which could allow investments for the search of more effective therapies.

Author details

Rodrigo Carvalho[1]*, Fillipe Carmo[1], Sara Heloisa[1], Barbara Cordeiro[1], Aline Vaz[1], Enrico Gimenez[1], Luis Goulart[2], Aristóteles Góes-Neto[1], Yves Le Loir[3], Gwénaël Jan[3] and Vasco Azevedo[1]

*Address all correspondence to: rodrigodoc2@gmail.com

1 Federal University of Minas Gerais (UFMG-ICB), Belo Horizonte, MG, Brazil

2 Federal University of Uberlandia, Uberlandia (UFU-IGB), MG, Brazil

3 INRA-Agrocampus Ouest UMR 1253 STLO, Rennes, France

References

[1] Messer JS, Liechty ER, Vogel OA, Chang EB. Evolutionary and ecological forces that shape the bacterial communities of the human gut. Mucosal Immunology. 2017;**10**:567-579. DOI: 10.1038/mi.2016.138

[2] Rook G, Bäckhed F, Levin BR, McFall-Ngai MJ, McLean AR. Evolution, human-microbe interactions, and life history plasticity. The Lancet. 2017;**390**:521-530. DOI: 10.1016/S0140-6736(17)30566-4

[3] Burcelin R. When gut fermentation controls satiety: A PYY story. Molecular Metabolism. 2016;**6**:10-11. DOI: 10.1016/j.molmet.2016.11.005

[4] Lin L, Zhang J. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. BMC Immunology. 2017;**18**:2. DOI: 10.1186/s12865-016-0187-3

[5] Boulangé CL, Neves AL, Chilloux J, Nicholson JK, Dumas M-E. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Medicine. 2016;**8**:42. DOI: 10.1186/s13073-016-0303-2

[6] Nagarajan N, Pop M. Sequence assembly demystified. Nature Reviews. Genetics. 2013;**14**:157-167. DOI: 10.1038/nrg3367

[7] Shendure J, Ji H. Next-generation DNA sequencing. Nature Biotechnology. 2008;**26**:1135-1145. DOI: 10.1038/nbt1486

[8] Fuller CW, Middendorf LR, Benner SA, Church GM, Harris T, Huang X, et al. The challenges of sequencing by synthesis. Nature Biotechnology. 2009;**27**:1013-1023. DOI: 10.1038/nbt.1585

[9] Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM, et al. The ribosomal database project (RDP-II): Introducing myRDP space and quality controlled public data. Nucleic Acids Research. 2007;**35**:D169-D172. DOI: 10.1093/nar/gkl889

[10] Handelsman J. Metagenomics: Application of genomics to uncultured microorganisms. Microbiology and Molecular Biology Reviews (MMBR). 2004;**68**:669-685. DOI: 10.1128/MMBR.68.4.669-685.2004

[11] Lepage P, Leclerc MC, Joossens M, Mondot S, Blottière HM, Raes J, et al. A metagenomic insight into our gut's microbiome. Gut. 2013;**62**:146-158. DOI: 10.1136/gutjnl-2011-301805

[12] Tringe SG, von Mering C, Kobayashi A, Salamov AA, Chen K, Chang HW, et al. Comparative metagenomics of microbial communities. Science. 2005;**308**:554-557. DOI: 10.1126/science.1107851

[13] Moya A, Ferrer M. Functional redundancy-induced stability of gut microbiota subjected to disturbance. Trends in Microbiology. 2016;**24**:402-413. DOI: 10.1016/j.tim.2016.02.002

[14] Kuczynski J, Lauber CL, Walters WA, Parfrey LW, Clemente JC, Gevers D, et al. Experimental and analytical tools for studying the human microbiome. Nature Reviews. Genetics. 2011;**13**:47-58. DOI: 10.1038/nrg3129

[15] Thomas T, Gilbert J, Meyer F. Metagenomics—A guide from sampling to data analysis. Microbial Informatics and Experimentation. 2012;**2**:3. DOI: 10.1186/2042-5783-2-3

[16] Fraher MH, O'Toole PW, Quigley EMM. Techniques used to characterize the gut microbiota: A guide for the clinician. Nature Reviews. Gastroenterology & Hepatology. 2012;**9**:312-322. DOI: 10.1038/nrgastro.2012.44

[17] Morgan XC, Huttenhower C. Chapter 12: Human microbiome analysis. PLoS Computational Biology. 2012;**8**:e1002808. DOI: 10.1371/journal.pcbi.1002808

[18] Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, et al. An integrated catalog of reference genes in the human gut microbiome. Nature Biotechnology. 2014;**32**:834-841. DOI: 10.1038/nbt.2942

[19] Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalog established by metagenomic sequencing. Nature. 2010;**464**:59-65. DOI: 10.1038/nature08821

[20] Lee PY, Chin S-F, Neoh H, Jamal R. Metaproteomic analysis of human gut microbiota: Where are we heading? Journal of Biomedical Science. 2017;**24**:36. DOI: 10.1186/s12929-017-0342-z

[21] Petriz BA, Franco OL. Metaproteomics as a complementary approach to gut microbiota in health and disease. Frontiers in Chemistry. 2017;**5**:4. DOI: 10.3389/fchem.2017.00004

[22] Tremaroli V, Bäckhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012;**489**:242-249. DOI: 10.1038/nature11552

[23] Larsbrink J, Rogers TE, Hemsworth GR, McKee LS, Tauzin AS, Spadiut O, et al. A discrete genetic locus confers xyloglucan metabolism in select human gut Bacteroidetes. Nature. 2014;**506**:498-502. DOI: 10.1038/nature12907

[24] Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. Nutrients. 2014;**7**:17-44. DOI: 10.3390/nu7010017

[25] Samuel BS, Gordon JI. A humanized gnotobiotic mouse model of host–archaeal–bacterial mutualism. Proceedings of the National Academy of Sciences of the United States of America. 2006;**103**:10011-10016. DOI: 10.1073/pnas.0602187103

[26] Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M. De los Reyes-Gavilán CG, Salazar N. Intestinal short chain fatty acids and their link with diet and human health. Frontiers in Microbiology. 2016;**7**. DOI: 10.3389/fmicb.2016.00185

[27] Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P. Effect of inulin on the human gut microbiota: Stimulation of Bifidobacterium adolescentis and *Faecalibacterium prausnitzii*. The British Journal of Nutrition. 2009;**101**:541-550. DOI: 10.1017/S00071 14508019880

[28] Zhao Y, Yu Y-B. Intestinal microbiota and chronic constipation. SpringerPlus. 2016;**5**. DOI: 10.1186/s40064-016-2821-1

[29] LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: A gut microbiota perspective. Current Opinion in Biotechnology. 2013;**24**:160-168. DOI: 10.1016/j.copbio.2012.08.005

[30] Biesalski HK. Nutrition meets the microbiome: Micronutrients and the microbiota. Annals of the New York Academy of Sciences. 2016;**1372**:53-64. DOI: 10.1111/nyas.13145

[31] Sherwin E, Sandhu KV, Dinan TG, Cryan JF. May the force be with you: The light and dark sides of the microbiota–gut–brain Axis in neuropsychiatry. CNS Drugs. 2016; **30**:1019-1041. DOI: 10.1007/s40263-016-0370-3

[32] Zmora N, Bashiardes S, Levy M, Elinav E. The role of the immune system in metabolic health and disease. Cell Metabolism. 2017;**25**:506-521. DOI: 10.1016/j.cmet.2017.02.006

[33] Carmo FLR, Rabah H, Cordeiro BF, Silva SHD, Jan G, Azevedo VA, et al. Applications of Probiotic Bacteria and Dairy Foods in Health. Current Research in Microbiology. 1ed. Wilmington: Open Access eBooks. 2017;**1**:1-33

[34] Carvalho RDDO, do Carmo FLR, de Oliveira Junior A, Langella P, Chatel J-M, Bermúdez-Humarán LG, et al. Use of wild type or recombinant lactic acid bacteria as an alternative treatment for gastrointestinal inflammatory diseases: A focus on inflammatory bowel diseases and mucositis. Frontiers in Microbiology. 2017;**8**. DOI: 10.3389/fmicb.2017.00800

[35] Ubeda C, Djukovic A, Isaac S. Roles of the intestinal microbiota in pathogen protection. Clinical & Translational Immunology. 2017;**6**:e128. DOI: 10.1038/cti.2017.2

[36] Fan D, Coughlin LA, Neubauer MM, Kim J, Kim MS, Zhan X, et al. Activation of HIF-1α and LL-37 by commensal bacteria inhibits *Candida albicans* colonization. Nature Medicine. 2015;**21**:808-814. DOI: 10.1038/nm.3871

[37] Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013;**500**:232-236. DOI: 10.1038/nature12331

[38] Hand TW, Vujkovic-Cvijin I, Ridaura VK, Belkaid Y. Linking the microbiota, chronic disease, and the immune system. Trends in Endocrinology and Metabolism. 2016;**27**:831-843. DOI: 10.1016/j.tem.2016.08.003

[39] Sommer F, Bäckhed F. The gut microbiota—Masters of host development and physiology. Nature Reviews. Microbiology. 2013;**11**:227-238. DOI: 10.1038/nrmicro2974

[40] Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;**535**:75-84. DOI: 10.1038/nature18848

[41] Cao B, Stout MJ, Lee I, Mysorekar IU. Placental microbiome and its role in preterm birth. NeoReviews. 2014;**15**:e537-e545. DOI: 10.1542/neo.15-12-e537

[42] Arrieta M-C, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: Health and disease. Frontiers in Immunology. 2014;**5**. DOI: 10.3389/fimmu.2014.00427

[43] Gritz EC, Bhandari V. The human neonatal gut microbiome: A brief review. Frontiers in Pediatrics. 2015;**3**. DOI: 10.3389/fped.2015.00017

[44] Mueller NT, Bakacs E, Combellick J, Grigoryan Z, Dominguez-Bello MG. The infant microbiome development: Mom matters. Trends in Molecular Medicine. 2015;**21**:109-117. DOI: 10.1016/j.molmed.2014.12.002

[45] Neu J, Rushing J. Cesarean versus vaginal delivery: Long term infant outcomes and the hygiene hypothesis. Clinics in Perinatology. 2011;**38**:321-331. DOI: 10.1016/j.clp.2011.03.008

[46] Yang I, Corwin EJ, Brennan PA, Jordan S, Murphy JR, Dunlop A. The infant microbiome: Implications for infant health and neurocognitive development. Nursing Research. 2016;**65**:76-88. DOI: 10.1097/NNR.0000000000000133

[47] Dominguez-Bello MG, De Jesus-Laboy KM, Shen N, Cox LM, Amir A, Gonzalez A, et al. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. Nature Medicine. 2016;**22**(3):250. DOI: 10.1038/nm.4039

[48] Houghteling PD, Walker WA. Why is initial bacterial colonization of the intestine important to the infant's and child's health? Journal of Pediatric Gastroenterology and Nutrition. 2015;**60**:294-307. DOI: 10.1097/MPG.0000000000000597

[49] Rodríguez JM, Murphy K, Stanton C, Ross RP, Kober OI, Juge N, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. Microbial Ecology in Health and Disease. 2015;**26**. DOI: 10.3402/mehd.v26.26050

[50] Cheng J, Ringel-Kulka T, Heikamp-de Jong I, Ringel Y, Carroll I, de Vos WM, et al. Discordant temporal development of bacterial phyla and the emergence of core in the fecal microbiota of young children. The ISME Journal. 2016;**10**:1002-1014. DOI: 10.1038/ismej.2015.177

[51] Hollister EB, Riehle K, Luna RA, Weidler EM, Rubio-Gonzales M, Mistretta T-A, et al. Structure and function of the healthy pre-adolescent pediatric gut microbiome. Microbiome. 2015;**3**. DOI: 10.1186/s40168-015-0101-x

[52] Greenhalgh K, Meyer KM, Aagaard KM, Wilmes P. The human gut microbiome in health: Establishment and resilience of microbiota over a lifetime. Environmental Microbiology. 2016;**18**:2103-2116. DOI: 10.1111/1462-2920.13318

[53] Thompson AL, Monteagudo-Mera A, Cadenas MB, Lampl ML, Azcarate-Peril MA. Milk- and solid-feeding practices and daycare attendance are associated with differences in bacterial diversity, predominant communities, and metabolic and immune function of the infant gut microbiome. Frontiers in Cellular and Infection Microbiology. 2015;**5**:1-13. DOI: 10.3389/fcimb.2015.00003

[54] Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World Journal of Gastroenterology (WJG). 2015;**21**:8787-8803. DOI: 10.3748/wjg.v21.i29.8787

[55] Laforest-Lapointe I, Arrieta M-C. Patterns of early-life gut microbial colonization during human immune development: An ecological perspective. Frontiers in Immunology. 2017;**8**:1-13. DOI: 10.3389/fimmu.2017.00788

[56] Shreiner AB, Kao JY, Young VB. The gut microbiome in health and in disease. Current Opinion in Gastroenterology. 2015;**31**:69-75. DOI: 10.1097/MOG.0000000000000139

[57] Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. Genome Medicine. 2016;**8**:1-11. DOI: 10.1186/s13073-016-0307-y

[58] De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences of the United States of America. 2010;**107**:14691-14696. DOI: 10.1073/pnas.1005963107

[59] Saraswati S, Sitaraman R. Aging and the human gut microbiota—From correlation to causality. Frontiers in Microbiology. 2015;**5**:1-4. DOI: 10.3389/fmicb.2014.00764

[60] Coleman OI, Nunes T. Role of the microbiota in colorectal cancer: Updates on microbial associations and therapeutic implications. BioResearch Open Access. 2016;**5**:279-288. DOI: 10.1089/biores.2016.0028

[61] Gismera CS, Aladrén BS. Inflammatory bowel diseases: A disease (s) of modern times? Is incidence still increasing? World Journal of Gastroenterology. 2008;**14**:5491-5498

[62] Dahlhamer JM, Zammitti EP, Ward BW, Wheaton AG, Croft JB. Prevalence of inflammatory bowel disease among adults aged ≥18 years—United States, 2015. MMWR. Morbidity and Mortality Weekly Report. 2016;**65**:1166-1169. DOI: 10.15585/mmwr.mm6542a3

[63] Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012;**142**:46-54.e42; quiz e30. DOI: 10.1053/j.gastro.2011.10.001

[64] Tontini GE, Vecchi M, Pastorelli L, Neurath MF, Neumann H. Differential diagnosis in inflammatory bowel disease colitis: State of the art and future perspectives. World Journal of Gastroenterology (WJG). 2015;**21**:21-46. DOI: 10.3748/wjg.v21.i1.21

[65] Walker AW, Sanderson JD, Churcher C, Parkes GC, Hudspith BN, Rayment N, et al. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. BMC Microbiology. 2011;**11**:7. DOI: 10.1186/1471-2180-11-7

[66] DeGruttola AK, Low D, Mizoguchi A, Mizoguchi E. Current understanding of Dysbiosis in disease in human and animal models. Inflammatory Bowel Diseases. 2016;**22**:1137-1150. DOI: 10.1097/MIB.0000000000000750

[67] Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013;**341**:569-573. DOI: 10.1126/science.1241165

[68] Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux J-J, et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn's disease patients. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**:16731-16736. DOI: 10.1073/pnas.0804812105

[69] Takahashi K, Nishida A, Fujimoto T, Fujii M, Shioya M, Imaeda H, et al. Reduced abundance of butyrate-producing bacteria species in the Fecal microbial Community in Crohn's disease. Digestion. 2016;**93**:59-65. DOI: 10.1159/000441768

[70] Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. Nature Reviews. Gastroenterology & Hepatology. 2012;**9**:599-608. DOI: 10.1038/nrgastro.2012.152

[71] Rehman A, Rausch P, Wang J, Skieceviciene J, Kiudelis G, Bhagalia K, et al. Geographical patterns of the standing and active human gut microbiome in health and IBD. Gut. 2016;**65**:238-248. DOI: 10.1136/gutjnl-2014-308341

[72] Ijssennagger N, van der Meer R, van Mil SWC. Sulfide as a mucus barrier-breaker in inflammatory bowel disease? Trends in Molecular Medicine. 2016;**22**:190-199. DOI: 10.1016/j.molmed.2016.01.002

[73] Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, et al. Bacterial flagellin is a dominant antigen in Crohn's disease. The Journal of Clinical Investigation. 2004;**113**:1296-1306. DOI: 10.1172/JCI20295

[74] Subramanian S, Roberts CL, Hart CA, Martin HM, Edwards SW, Rhodes JM, et al. Replication of colonic Crohn's disease mucosal Escherichia Coli isolates within macrophages and their susceptibility to antibiotics. Antimicrobial Agents and Chemotherapy. 2008;**52**:427-434. DOI: 10.1128/AAC.00375-07

[75] Lebeer S, Vanderleyden J, De Keersmaecker SCJ. Host interactions of probiotic bacterial surface molecules: Comparison with commensals and pathogens. Nature Reviews. Microbiology. 2010;**8**:171-184. DOI: 10.1038/nrmicro2297

[76] Breitbart M, Hewson I, Felts B, Mahaffy JM, Nulton J, Salamon P, et al. Metagenomic analyses of an uncultured viral community from human Feces. Journal of Bacteriology. 2003;**185**:6220-6223. DOI: 10.1128/JB.185.20.6220-6223.2003

[77] Minot S, Sinha R, Chen J, Li H, Keilbaugh SA, GD W, et al. The human gut virome: Inter-individual variation and dynamic response to diet. Genome Research. 2011;**21**:1616-1625. DOI: 10.1101/gr.122705.111

[78] Ni J, GD W, Albenberg L, Tomov VT. Gut microbiota and IBD: Causation or correlation? Nature Reviews. Gastroenterology & Hepatology. 2017;**14**:573-584. DOI: 10.1038/nrgastro.2017.88

[79] Gough E, Shaikh H, Manges AR. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America. 2011;**53**:994-1002. DOI: 10.1093/cid/cir632

[80] Angelberger S, Reinisch W, Makristathis A, Lichtenberger C, Dejaco C, Papay P, et al. Temporal bacterial community dynamics vary among ulcerative colitis patients after fecal microbiota transplantation. The American Journal of Gastroenterology. 2013;**108**:1620-1630. DOI: 10.1038/ajg.2013.257

[81] Suskind DL, Brittnacher MJ, Wahbeh G, Shaffer ML, Hayden HS, Qin X, et al. Fecal microbial transplant effect on clinical outcomes and fecal microbiome in active Crohn's disease. Inflammatory Bowel Diseases. 2015;**21**:556-563. DOI: 10.1097/MIB.0000000000000307

[82] Suskind DL, Singh N, Nielson H, Wahbeh G. Fecal microbial transplant via nasogastric tube for active pediatric ulcerative colitis. Journal of Pediatric Gastroenterology and Nutrition. 2015;**60**:27-29. DOI: 10.1097/MPG.0000000000000544

[83] Cui B, Feng Q, Wang H, Wang M, Peng Z, Li P, et al. Fecal microbiota transplantation through mid-gut for refractory Crohn's disease: Safety, feasibility, and efficacy trial results. Journal of Gastroenterology and Hepatology. 2015;**30**:51-58. DOI: 10.1111/jgh.12727

[84] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA: A Cancer Journal for Clinicians. 2015;**65**:87-108. DOI: 10.3322/caac.21262

[85] Sun J, Kato I. Gut microbiota, inflammation and colorectal cancer. Genes & Diseases. 2016;**3**:130-143. DOI: 10.1016/j.gendis.2016.03.004

[86] Grivennikov SI. Inflammation and colorectal cancer: Colitis-associated neoplasia. Seminars in Immunopathology. 2013;**35**:229-244. DOI: 10.1007/s00281-012-0352-6

[87] Arthur JC, Gharaibeh RZ, Mühlbauer M, Perez-Chanona E, Uronis JM, McCafferty J, et al. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. Nature Communications. 2014;**5**:4724. DOI: 10.1038/ncomms5724

[88] Robles AI, Traverso G, Zhang M, Roberts NJ, Khan MA, Joseph C, et al. Whole-exome sequencing analyses of inflammatory bowel disease-associated colorectal cancers. Gastroenterology. 2016;**150**:931-943. DOI: 10.1053/j.gastro.2015.12.036

[89] Nosho K, Sukawa Y, Adachi Y, Ito M, Mitsuhashi K, Kurihara H, et al. Association of Fusobacterium nucleatum with immunity and molecular alterations in colorectal cancer. World Journal of Gastroenterology. 2016;**22**:557-566. DOI: 10.3748/wjg.v22.i2.557

[90] Yu J, Feng Q, Wong SH, Zhang D, Liang QY, Qin Y, et al. Metagenomic analysis of faecal microbiome as a tool towards targeted non-invasive biomarkers for colorectal cancer. Gut. 2017;**66**:70-78. DOI: 10.1136/gutjnl-2015-309800

[91] Meira LB, Bugni JM, Green SL, Lee C-W, Pang B, Borenshtein D, et al. DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. The Journal of Clinical Investigation. 2008;**118**:2516-2525. DOI: 10.1172/JCI35073

[92] Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. Nature. 2012;**491**:254-258. DOI: 10.1038/nature11465

[93] Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews. Immunology. 2009;**9**:313-323. DOI: 10.1038/nri2515

[94] Purcell RV, Visnovska M, Biggs PJ, Schmeier S, Frizelle FA. Distinct gut microbiome patterns associate with consensus molecular subtypes of colorectal cancer. Scientific Reports. 2017;**7**:11590. DOI: 10.1038/s41598-017-11237-6

[95] Prorok-Hamon M, Friswell MK, Alswied A, Roberts CL, Song F, Flanagan PK, et al. Colonic mucosa-associated diffusely adherent afaC+ *Escherichia coli* expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer. Gut. 2014;**63**:761-770. DOI: 10.1136/gutjnl-2013-304739

[96] Jiménez B, Mirnezami R, Kinross J, Cloarec O, Keun HC, Holmes E, et al. 1H HR-MAS NMR spectroscopy of tumor-induced local metabolic "field-effects" enables colorectal cancer staging and prognostication. Journal of Proteome Research. 2013;**12**:959-968. DOI: 10.1021/pr3010106

[97] Veselkov KA, Mirnezami R, Strittmatter N, Goldin RD, Kinross J, Speller AVM, et al. Chemo-informatic strategy for imaging mass spectrometry-based hyperspectral profiling of lipid signatures in colorectal cancer. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**:1216-1221. DOI: 10.1073/pnas.1310524111

[98] Kinross J, Mirnezami R, Alexander J, Brown R, Scott A, Galea D, et al. A prospective analysis of mucosal microbiome-metabonome interactions in colorectal cancer using a combined MAS 1HNMR and metataxonomic strategy. Scientific Reports. 2017;**7**:8979. DOI: 10.1038/s41598-017-08150-3

[99] Wu Q, Yang Z-P, Xu P, Gao L-C, Fan D-M. Association between *Helicobacter pylori* infection and the risk of colorectal neoplasia: A systematic review and meta-analysis. Colorectal Disease: The Official Journal of the Association of Coloproctology of Great Britain and Ireland. 2013;**15**:e352-e364. DOI: 10.1111/codi.12284

[100] Halfvarson J, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nature Microbiology. 2017;**2**:17004. DOI: 10.1038/nmicrobiol.2017.4

[101] Pascal V, Pozuelo M, Borruel N, Casellas F, Campos D, Santiago A, et al. A microbial signature for Crohn's disease. Gut. 2017;**66**:813-822. DOI: 10.1136/gutjnl-2016-313235

[102] Forbes JD, Van Domselaar G, Bernstein CN. Microbiome survey of the inflamed and noninflamed gut at different compartments within the gastrointestinal tract of inflammatory bowel disease patients. Inflammatory Bowel Diseases. 2016;**22**:817-825. DOI: 10.1097/MIB.0000000000000684

[103] Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biology. 2012;**13**:R79. DOI: 10.1186/gb-2012-13-9-r79

[104] Santoru ML, Piras C, Murgia A, Palmas V, Camboni T, Liggi S, et al. Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. Scientific Reports. 2017;**7**:1-14. DOI: 10.1038/s41598-017-10034-5

[105] Nishino K, Nishida A, Inoue R, Kawada Y, Ohno M, Sakai S, et al. Analysis of endoscopic brush samples identified mucosa-associated dysbiosis in inflammatory bowel disease. Journal of Gastroenterology. 2017. DOI: 10.1007/s00535-017-1384-4

[106] Marchesi JR, Dutilh BE, Hall N, Peters WHM, Roelofs R, Boleij A, et al. Towards the human colorectal cancer microbiome. PLoS One. 2011;**6**:e20447. DOI: 10.1371/journal.pone.0020447

[107] Zackular JP, Rogers MAM, Ruffin MT, Schloss PD. The human gut microbiome as a screening tool for colorectal cancer. Cancer Prevention Research (Philadelphia, PA). 2014;**7**:1112-1121. DOI: 10.1158/1940-6207.CAPR-14-0129

[108] Mima K, Cao Y, Chan AT, Qian ZR, Nowak JA, Masugi Y, et al. Fusobacterium nucleatum in colorectal carcinoma tissue according to tumor location. Clinical and Translational Gastroenterology. 2016;**7**:e200. DOI: 10.1038/ctg.2016.53

[109] Wei Z, Cao S, Liu S, Yao Z, Sun T, Li Y, et al. Could gut microbiota serve as prognostic biomarker associated with colorectal cancer patients' survival? A pilot study on relevant mechanism. Oncotarget. 2016;**7**:46158-46172. DOI: 10.18632/oncotarget.10064

[110] Alexander J, Perdones-Montero A, Scott A, Poynter L, Atkinson S, Soucek P, et al. OC-061 a prospective multi-national study of the colorectal cancer mucosal microbiome reveals specific taxonomic changes indicative of disease stage and prognosis. Gut. 2017;**66**:A32. DOI: 10.1136/gutjnl-2017-314472.61

[111] White JR, Drewes J, Sears CL. Abstract 844: High-resolution microbiome profiling and meta-analysis yields insight into microbial consortia associated with colorectal cancer. Cancer Research. 2016;**76**:844-844. DOI: 10.1158/1538-7445.AM2016-844

Chapter 5

The Microbiome of *Spodoptera littoralis*: Development, Control and Adaptation to the Insect Host

Tilottama Mazumdar, Beng-Soon Teh and Wilhelm Boland

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72180

Abstract

The symbiotic microbial consortium in the gut of *Spodoptera littoralis* shows dramatic, but reproducible changes in line with the development of the insect from the egg via six larval instars to the pupa. Since the food is kept constant during development, factors from the insect host and certain microbial symbionts are assumed to control the composition of the microbiome. A GFP-tagged *Enterococcus mundtii*, one of the major players of the consortium, easily integrates into the microbiome and can be monitored in all gut segments at all developmental stages. The reporter organism can be recovered from the gut using a preparative flow cytometry allowing subsequent RNA extraction for transcriptomic analyses. The transcriptomic profile from the fluorescent *Enterococcus* cells provides information on the adaptation of the reporter organism to the local gut conditions. The concept of using a fluorescent reporter organism that can be recovered at any time from any area of the intestinal tract will allow a holistic analysis of adaptation strategies used by the microbes to adapt to the insect gut. In combination with the analysis of transcript patterns from the gut membranes, a first insight into the molecular interaction between the insect host and the microbiome can be expected.

Keywords: *Enterococcus mundtii*, *Spodoptera littoralis*, gut microbiome, transcriptomics, flow cytometry

1. Introduction

The development of a gut in multicellular organisms is an evolutionary achievement of the highest order. The gut allows the host to exploit the metabolic and catabolic abilities of a multitude of microbial inhabitants to degrade and digest recalcitrant and complex organic matter.

The symbionts may also be involved in the detoxification of poisonous metabolites in food [1, 2]. The membranes of the intestinum carefully separate the bacterial symbionts from the host organism and prevent infection by invasive and deleterious members of the microbiome. Accordingly, the gut membrane is a complex structure that allows the exchange of nutrients with both high- and low-molecular weight (signaling) compounds and, on the other, blocks the entry of microbes and many of their macromolecular components [3]. The flux of nutrients and even more complex metabolites across the membrane is controlled by transport proteins expressed in the gut membrane [4, 5]. The microbiome also defends against parasites or pathogens [6–8]. The diverse functions provided by the microbial partners are vital for the insect's survival, especially in adverse ecological niches.

Although almost all organisms rely on core microbiomes [9], in many cases the gut community changes according to the insect's developmental stage. In early instars of *Spodoptera littoralis,* several *Enterococcus* spp. dominate, whereas in late instars, Clostridia contribute significantly (ca. 50%) to the microbial population [10]. A core community, consisting of Enterococci, Lactobacilli and Clostridia was revealed in the insect larvae. These bacteria were always present in the digestive tract at a relatively high frequency; although developmental stage and diet have a great impact on shaping bacterial communities, clearly the insect gut selects for particular bacterial phylotypes. Enterococci are also prominent in the gut of insects such as *Drosophila*, ground beetles and desert locusts [11, 12]. The strong dependence of the gut community on the developmental stage of the insect host suggests that unknown low- and high-molecular weight factors control symbiotic interactions among the partners. For example, in *Drosophila melanogaster*, the immune system not only plays a central role in preventing pathogen infection, but also controls the resident bacterial population. The intestinal homeobox gene *Caudal* regulates the resident gut microbial community by repressing the antimicrobial peptide genes that are dependent on the nuclear factor kappa B. Silencing the *Caudal* gene by RNAi resulted in the overexpression of antimicrobial peptides, which in turn reduced the microbial population in the gut [13].

To monitor such developmentally controlled changes in the microbiome of *S. littoralis,* a fluorescent member of the gut symbionts—in particular, the dominant *Enterococcus mundtii*—appeared to be an ideal reporting organism. A GFP-labeled *E. mundtii* [14] would easily integrate into the gut community and survive adverse conditions embedded in the community of enterococci, bacteria which are largely resistant to environmental stresses, such as antibiotic exposure, disinfection, desiccation and starvation [15]. As the transgenic *E. mundtii* are fluorescent, their presence can be monitored in all gut areas of the larvae and at developmental stages such as the pupa and the adult. Moreover, the reporter organisms can be easily recovered from the gut and used for transcriptomic analyses. By comparing transcriptomes from adjacent gut tissue and from the microbes, a "dialog" between the insect host and the symbiotic bacteria could be unraveled. This concept is generally applicable and can be used to holistically analyze host microbial interactions. The protocol of the approach based on the use of a fluorescent reporter organism—for example, using GFP-tagged *E. mundtii*—is described in this chapter.

2. Fluorescent reporters and their applications for *in vivo* imaging in microbiomes

The green fluorescent protein (GFP) isolated from the jellyfish, *Aequorea victoria*, is widely used as a reporter for studying gene expression [16], and the localization and structure of living cells [17]. The GFP has a major excitation peak at about 395 nm and an emission peak at about 508 nm. The GFP contains 238 amino acids with a molecular weight of 26.9 kDa. It emits green fluorescence when exposed to light in the blue to ultraviolet range [18]. The GFP requires only oxygen as a cofactor for chromophore formation, which gives it an advantage over other reporter proteins [19]. It is sensitive and non-toxic, and does not affect cell growth [20, 21]. In addition, the GFP is stable at temperatures below 65°C and pH 6–11 [22]. Since the GFP was discovered, many mutants have been developed with modification in spectral and folding properties, or enhanced fluorescence intensity [23–26]. The choice of a GFP variant depends on several factors, such as pH, environmental temperature, toxicity, multimerization and photostability [26]. The first *gfp* gene was cloned in 1992 [27], and 2 years later, the gene was successfully expressed in both eukaryotes and prokaryotes [20]. Apart from GFP, many variants of red fluorescent proteins, such as mCherry and tdTomato have been developed based on DsRed originally isolated from *Discosoma* sp. [28]. Since then, over 40 coral fluorescent proteins with different colors, from cyan to chromo-red, have been described [29].

The reporter proteins provide important tools with which to monitor gene expression from within the cells in real time and in the *in vivo* environment, such as the gastrointestinal tract. For a gene to be selected as a reporter, it must be able to easily detect signals secreted by the expressed reporter gene in the cells [30]. The *lux* gene derived from bacteria, and luciferase from the firefly and click beetle (*luc*), are two other common reporter genes used in bioluminescence imaging. Fluorescence imaging is commonly associated with the use of green and red fluorescence proteins [31, 32]. Rats and mice are popular model organisms which study the proliferation and colonization of lactic acid bacteria (LAB) [33, 34]. LAB has been tagged with green fluorescent protein (GFP) and mCherry to study their colonization of the intestinal tract of chickens, mice and zebrafish [35–39].

2.1. Construction of a GFP fluorescent system for *E. mundtii*

LAB is widely used as probiotics due to the benefits they bring to human and animal health by balancing the gut microbiome and by eliminating pathogenic microorganisms through the production of antimicrobial peptides [40, 41]. Due to the importance of LAB in many applications, it is essential to study how they survive and colonize by monitoring their metabolic activities *in vivo* through the development of fluorescent reporter microorganisms. It is important that the reporter gene in the fluorescent bacteria is stably expressed [42].

Plasmids are present in most of the members of LAB, including *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Streptococcus*, *Pediococcus, Leuconostoc*, etc. Plasmids found in LAB

vary in size (0.87 kb to more than 250 kb), copy number (1 or more per cell) and gene content [43–46].

Enterococci harbor plasmids that are resistant to a wide range of antibiotics, including erythromycin, tetracycline, gentamicin and vancomycin [47–50]. Some of these plasmids encode bacteriocins [51–53], virulence factors [54, 55], toxins [56] and sex pheromones [57]. Plasmids replicate via rolling circle replication (RCR) and theta replication [58]. Theta-replicating plasmids can carry large DNA fragments and are more stable than RCR plasmids [59]. The enterococci plasmid pAMβ1 replicates via theta mode. In the early 1990s, shuttle vectors in the pTRK family of high and low copy number carrying the origin of replication of pAMβ1 for LAB and p15A for *E. coli* were developed [60]. The plasmids carrying the replicon pAMβ1 isolated from *Enterococcus faecalis* [61] have been reported to replicate in Gram-positive bacteria [62].

The choice of a reliable expression vector depends on several factors, such as the mode of replication, copy number and stability [63]. The expression vector used in this study is derived from pTRKH3 plasmid with a broad host range. pTRKH3 is a shuttle vector for *E. coli*, *Enterococcus*, *Lactococcus*, *Streptococcus* and *Lactobacillus* [60]. The vector has a copy number (30–40) in *E. coli*, and a somewhat higher copy number (45–85) in *Lactococcus* and *Streptococcus* species [64]. It carries a gene for erythromycin resistance, which is expressed in *E. coli* and LAB. In this chapter, we report the expression of mutated *gfp* (mgfp5) on a pTRKH3 plasmid controlled by a strong constitutive promoter, erythromycin ribosomal methylase (*ermB*) [61], in *E. mundtii* (**Figure 1A**). The lactate dehydrogenase (*ldh*) promoter from *Lactobacillus acidophilus* [65] has also been used to control the expression of GFP. In contrast, the use of a surface-layer (*slp*) promoter from *L. acidophilus* [66] was not able to induce the expression of GFP [14]. Accordingly, the selection of an appropriate promoter to achieve a high level of GFP expression is crucial. Nisin-inducible promoters have been used for heterologous gene expression in lactobacilli [67, 68]. Nisin that can be degraded within the intestinal environment is a drawback of this inducible expression system [69, 70].

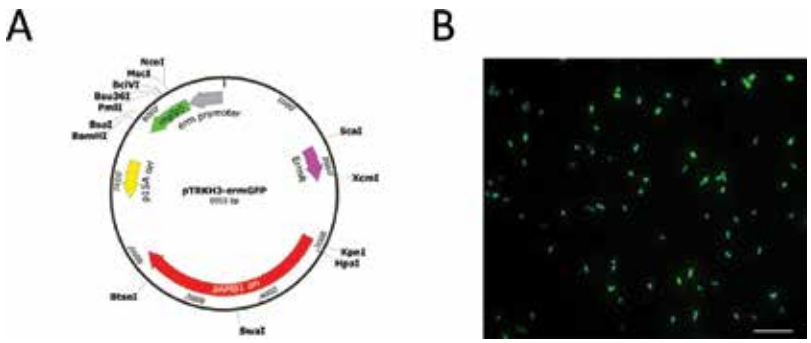

Figure 1. Construction of GFP-tagged *E. mundtii* by electroporation. (A) Plasmid map of pTRKH3 harboring the *mgfp5* gene regulated by an erythromycin ribosomal methylase (*ermB*) promoter. The plasmid is an *E. coli*-LAB shuttle vector with p15A and pAMβ1 as the origins of replication. (B) The *ermB* promoter was used to increase the expression of GFP from *E. mundtii* grown in Todd-Hewitt Bouillon (THB) broth culture. Scale bar: 10 μm [14].

Therefore, the use of constitutive or native-based promoters would be favorable, as these promoters could ensure the constant production of the target protein, especially in the gut environment. Several studies using homologous promoters have been reported to achieve efficient gene expression [71, 72], as the transcriptional signal induced by native promoters is recognized by the host bacteria. Bacteria with the *gfp* gene cloned downstream of a native constitutive promoter express GFP efficiently in broth culture (**Figure 1B**).

2.2. Transformation of *E. mundtii* KD251 using electroporation

Several methods have been used to introduce exogenous DNA into microbial cells; these include chemical treatment, electroporation, the use of a biolistic gun, ultrasound, polyethylene glycol, microwave and hydrogel [73]. Of all the methods, electroporation most efficiently transforms a broad array of microorganisms [74] by introducing foreign DNA-like plasmid into bacteria. Electroporation is one of the transformation techniques for rapid introduction of foreign DNA-like plasmid into bacteria. The method uses an electric pulse that forms pores on the bacterial cell walls so that DNA can pass into the cell. In recent years, numerous lactic acid bacteria have been transformed using electroporation [75]. The success rate of electro-transformation depends on the cell wall becoming sufficiently permeable to allow DNA to enter. In some cases, to improve a cell's electro-transformation efficiency, the cell wall is pre-treated with chemicals such as lysozyme [76, 77], threonine [78, 79], penicillin G [80], ethanol [81] and glycine [82, 83]. These weaken the cell walls only for certain bacteria species. It has been shown that the efficiency of electro-transformation of *Lactococcus lactis* was affected by several parameters, such as the cell's growth phase and density, the medium, the plasmid concentration and the strength of the electrical field [84].

The choice of method in the preparation of the competent cells is important for a successful transformation. Although competent *E. coli* cells have reportedly been prepared with ice-cold calcium chloride [85], the transformation achieved with this method is less successful than that achieved using the electroporation method [86]. The electrocompetent cells, the equipment and the washing buffers all have to be prepared at cold temperatures [87, 88].

In this chapter, we report the use of a conventional method to transform *E. mundtii* based on the modified protocol of *Escherichia coli* [89]. The electrocompetent cells and electroporation protocol for *E. mundtii* have been published [14]. Briefly, the bacterial cells were grown to the exponential phase and then washed with ice-cold water for two rounds to remove salts from the growth medium. Glycerol at a final concentration of 10% was added to the bacterial suspension so that the cells could be preserved and stored frozen. A concentration of plasmids between 0.15 and 0.2 μg worked fine for us. The competent cells were mixed with the plasmid DNA and then transferred to a 0.2 cm plastic cuvette for electroporation at a pulse of 1.8 kV, 600 Ω parallel resistance and 10 μF capacitance. The pulsed cells were recovered with fresh broth medium, and the cell suspension was incubated at 37°C for 2 hours before plating on plates containing antibiotic erythromycin. After 2 days, the bacterial transformants were screened for the plasmid-containing *gfp* gene.

2.3. Colonization of GFP-tagged bacterium in the gut of *S. littoralis*

The fluorescent reporter *E. mundtii* has been integrated into the gut microbiome across all developmental stages of *S. littoralis* [14], indicating its symbiotic relationship with the insect host. Microorganisms have the ability to face environmental stresses, particularly those within the gastrointestinal environment. Constructing the fluorescent reporter *E. mundtii*, we explored the mechanisms these bacteria use to adapt to stress; we recovered the reporter bacteria from the gut of *S. littoralis* using the state-of-the-art technology of flow cytometry. The dominance and persistence of *E. mundtii* in the gut motivates us to look deeper into their gene expression system. Therefore, it is important to unravel the mechanisms used by microorganisms living within the gastrointestinal environment. Construction of the fluorescent reporter *E. mundtii* is one of the strategies to find out those mechanisms, since it has been possible to recover the reporter bacteria from the gut of the very same insects using the *state-of-the-art* technology of flow cytometry. Fluorescence-activated cell sorting (FACS) enabled us to pick out the GFP-tagged reporter *E. mundtii* from a mixture of insect and other bacterial cells.

3. Fluorescence-activated cell sorting

Flow cytometry separates cells based on their intrinsic physical and chemical characteristics, integrating electronics, fluidics and optics. The sample, from which the cells of interest are to be sorted, is passed through a flow cell. The sheath fluid escorts the cells down the channel, where they encounter a laser beam. Light beams of specific frequencies and wavelength are emitted. Detectors measure the forward scatter (FSC) and the side scatter (SSC) based on cell size and granularity. FSC and SSC are unique for every particle. A combination of the two can differentiate among cell types within a cohort of cells. This way, the qualitative and quantitative data of a particular kind of cell can be assessed.

Fluorescence-activated cell sorting is an application of flow cytometry. The cells of interest are fluorescently tagged and sorted by the machine. Here the GFP-tagged fluorescent *E. mundtii* is isolated from a mixture containing insect gut homogenate with other bacteria. The solution is delivered to the flow channel and carried by the sheath fluid. The pressure from the compressor, which is adjustable, forces the solution through a laser beam using hydrodynamic focusing. Then monochromatic beams of high intensity interrogate cells one at a time. Depending on the excitation wavelength of the fluorophore, the laser wavelength is chosen. The scatters are then recorded. The forward scatter (FSC), which refers to light that is refracted by the cell and continues in the same direction, tells us about the size of the cell. In contrast, the side scatter (SSC), which refers to light that is refracted by the cells and travels at right angles to the excitation axis, tells us about the fluorescence and granularity of the cells. The more granular a cell, the more scattered light it produces. Furthermore, each cell enclosed in a droplet is assigned a charge, depending on the extent of the cell's deflection [90]. After passing through an electrical field, the cells are deflected to the collection tubes and the uncharged droplets are directed to the waste. The detector system consists of

a set of photo multiplier tubes that have specific filters to select for certain wavelengths of the beam and are set at the excitation range to view GFP.

Once the larvae are fed with the fluorescent *E. mundtii*, the number of larvae that survive can be determined and eventually recovered for further studies. The *E. mundtii* cells are sorted and their transcriptomes can be studied. This technology has made it possible to focus on a single cell or cells of interest, to study their function or their physiological state.

4. RNA extraction

The GFP-tagged *E. mundtii* are sorted by the flow cytometer and collected in a RNA-protective reagent (RNAlater®). The role of such reagents is twofold: first, they preserve the integrity of RNA, which has a very short half-life, for a few minutes. We need the RNA to be intact and of good quality in order to process it for sequencing. Second, addition of protective reagents minimizes subsequent changes from being introduced when the cells are handled. As soon as the cells are collected in a Falcon tube filled with the protective reagent (RNA Protect or RNA Later), the reagent percolates into the cells and prevents an alteration in the gene expression [91]. Additionally, the entire process is maintained at 4°C, as all metabolic activities slow down at low temperatures. The Falcon tube is centrifuged at a high speed to pellet down the cells, and care is taken not to disturb it while draining the supernatant.

RNA is very sensitive to exogenous and endogenous RNases. The entire extraction procedure is done in an area free of RNase. Moreover, RNase inhibitors are used to clean all equipment, ranging from gloves to microcentrifuge tubes to get rid of RNase. *E. mundtii* is a Gram-positive bacterium with a cell wall containing a thick layer of peptidoglycan and lipoteichoic acid, followed by a single lipid membrane. The cell wall is anchored to the membrane by diacylglycerols. To release the nucleic acid from the cell, it has to be detached from its peptidoglycan-containing cell wall and membrane. Lysozyme is a glycoside hydrolase that hydrolyzes the 1,4-beta linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues of the peptidoglycan. Additionally, guanidium thiocyanate, beta-mercaptoethanol and a detergent called dithiothreitol help in cell lysis and deproteinization. Proteinase K frees the RNA from the bound proteins and endogenous RNase.

Following lysis, the RNA is separated by density gradient centrifugation using phenol, chloroform and isoamyl alcohol, and further precipitation with ethanol. The RNeasy® Mini Kit (Qiagen) based on silica-matrix RNA extraction was used in our work. Several studies have reported on extraction of high quality bacterial RNA using this kit [92–94]. Thus, RNA is obtained from the cells of the sorted *E. mundtii* [95].

The extraction of total RNA from the low number of bacterial cells that remain after sorting by the flow cytometer is challenging. The concentration of RNA was as low as a few picograms to 50 ng. The minimum threshold quantity for a successful RNA library preparation is 100 ng. This is too low an amount to proceed with RNA sequencing. Hence, the total RNA must be amplified before we can use it further.

4.1. Amplification of RNA

Amplification of RNA is required if the aim is to create an effective transcriptomic profile from a very low starting quantity of RNA. MessageAmp II aRNA amplification kit (Ambion) was used for amplification [96–99]. The principle is based on *in vitro* transcription. The steps are as follows:

4.1.1. Polyadenylation of RNA

Bacterial RNA is devoid of a poly (A) tail. The *E. coli* poly (A) polymerase enables a poly (A) tail to be added at the ends of RNA. This stretch is required for cDNA synthesis.

4.1.2. Synthesis of first-strand cDNA

Primers against the poly (A) stretch are used to synthesize the first strand of cDNA by reverse transcription. The primers are anchored with a bacteriophage promoter sequence: T7 oligo(dT) sequence, T3 or SP6. dNTPs are added to the reaction mix.

4.1.3. Synthesis of second-strand cDNA

RNaseH is used to degrade the RNA from the RNA-cDNA pair; DNA polymerase is required to synthesize the second strand of cDNA. The result is a double-stranded cDNA fragment with a T7 promoter sequence.

4.1.4. Purification of cDNA

cDNA is cleaned by removing the fragmented RNA, enzymes and salts, all of which could hinder *in vitro* transcription.

4.1.5. In Vitro transcription

Multiple copies of antisense RNA are generated using DNA-dependent RNA polymerase. Linear amplification is employed for this. Depending on the bacteriophage promoter sequence attached to the cDNA, a polymerase is selected. Promoter-specific dNTPs are added to the reaction mix. 37°C is optimum for this reaction. The reaction time depends on the extent to which one wants to amplify the RNA.

4.1.6. Purification of amplified RNA

The residual enzymes, salts and unincorporated dNTPs must be removed from the final product [100–104].

At this point, the RNA has been amplified several fold: 1–2 ug.

RNA amplification procedures have a drawback. When the concentration of RNA is brought to a point at which successful transcriptomic profiling is possible, certain biases are unavoidably introduced.

Certain amplified transcripts may be misunderstood as duplicates and *vice versa*, which could give a false positive read [105]. In PCR-based amplification procedures, duplicates that can arise from sample handling may have features such as fragmentation, sequencing depth or library complexity; unfortunately, these cannot be distinguished from PCR duplicates. Removing duplicates does not improve the accuracy of quantification or the power; rather, makes it worse [106]. The *Taq* polymerases used for the PCR-based approach are more prone to introduce errors than the RNA polymerases for *in vitro* transcription. Thus, *in vitro* transcription is favored over PCR-based amplification [105], although premature transcription termination can occur in low complexity sequences [107]. Nevertheless, *in vitro* transcription is an efficient method to follow when the starting quantity is limited [107].

5. Transcriptomics

At this point, we have enough RNA to get a transcriptomic profiling of the bacterial cells done. The transcriptome is the entire set of genes expressed in a type of cell at a particular time point and/or condition. This is in contrast to a genome, which refers to the full complement of genes in a cell-type. Not all genes are constitutively induced. Information about transcripts, or genes expressed, may shed light on the developmental or physiological state of the cell. It also talks about other species of RNA, small RNAs and non-coding RNAs, novel transcripts, the transcriptional start sites, splicing regions, post-transcriptional modifications, and 3′ and 5′ ends. Another purpose of transcriptomic profiling is to quantify the expressed genes. One can judge the extent of regulation of a particular gene in the given conditions. As compared to one situation, when cells behave differently in another, one can now say which genes are differentially regulated to bring about the same.

In this chapter, our aim has been to investigate the survival and adaptation strategies of *E. mundtii* living inside the gut of *S. littoralis* as compared to in the laboratory. This unraveling has been done by cataloging the genes of *E. mundtii* which are differently regulated and which make it as one of the dominant bacterial species in the gut.

5.1. RNASeq

Transcriptome sequencing has improved dramatically over the past few years, starting with EST-based Sanger sequencing. The early method was mainly useful with the most abundant transcripts, whereas subsequent next-generation sequencing has been successfully carried out on all transcripts with sensitivity and accuracy even allowing the identification of low expressed genes. The situation has ameliorated with the advent of deep sequencing, which can increase the average number of times a nucleotide is sequenced. The deeper the sequencing is, the better the probability of detecting the less abundant transcripts. Next-generation sequencing has several hierarchies of its own. These days, RNA-seq is more widely used than the microarrays. The former gives us a base-pair level of resolution. Whereas microarrays can be used only when the reference genome sequence is available, RNA-seq can build the transcriptome *de novo*. Also, background noise is taken better care of in the case of RNA-seq. These

days, sequencing is not confined to a larger number of cells. It is possible to obtain resolution up to a single cell. Naturally, the amount of RNA obtained from one single cell is in picograms and must be processed as discussed above. Among all the increased sensitivity of next-generation technologies, so far, Illumina allows us to start with the smallest amount of RNA.

The fragmented and adapter-ligated cDNA is allowed to flow through a flow cell of the sequencer, which has oligonucleotides that complement the adapter sequences embedded in them. After hybridization, the oligonucleotides prime the polymerization process with the provided dNTPs and DNA polymerase. Each of the dNTPs is tagged with a fluorophore. As the nucleotide is incorporated, the resulting fluorescence is detected. With the addition of each nucleotide, the fluorophore is released, regenerating the 3′ hydroxyl group for the next nucleotide to join. This way, the fluorescent intensity is recorded and converted into nucleotide identity using an algorithm.

The amplified RNA from the fluorescent *E. mundtii* cells sorted by flow cytometry went through deep sequencing (Hiseq) to detect as many genes as possible to tell us the story of their adaptation to the gut environment of *S. littoralis* (**Figure 2**).

The complications arising from several different forms of RNA, alternate splicing, removal of introns, that is, the ones that are profound in eukaryotes are not required to be considered in the case bacteria. Although, there are several regulatory and non-coding RNAs in bacteria, but this particular case dictates one to follow a rather straightforward approach of unraveling the upregulated and downregulated transcripts only.

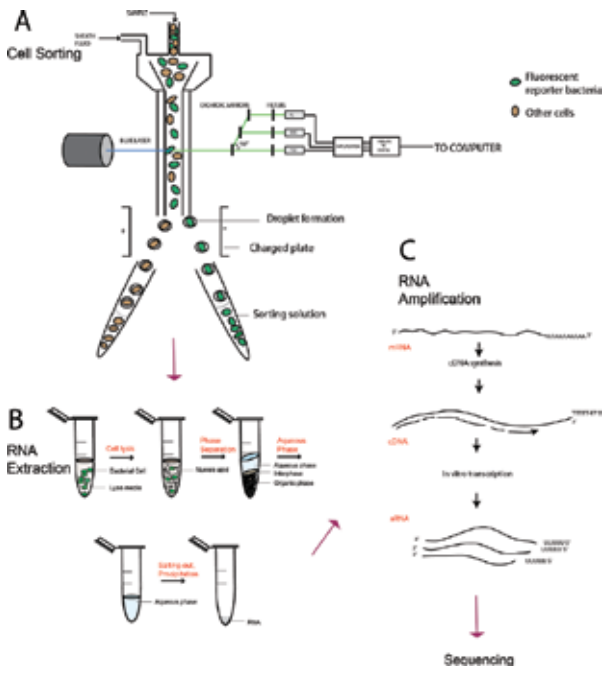

Figure 2. Overview of the workflow for bacterial RNA-seq. (A) Flow cytometry to sort fluorescent bacteria from gut homogenates. (B) Extraction of total bacterial RNA. (C) Amplification of the total RNA by *in vitro* amplification (unpublished).

5.2. Adaptation and survival strategies of *E. mundtii* in the gut of the insect

The GFP-tagged *E. mundtii* was fed to the *S. littoralis* larvae at early instars. The bacterial reporter was able to colonize the gut at various stages of the insect's life cycle, as seen in the fluorescent microscopic images (**Figure 4**).

The production of antimicrobial substances from insects or their resident symbionts is a survival strategy to keep pathogens at bay. The dominant gut bacterium *E. mundtii* has been shown to produce an antimicrobial peptide called mundticin KS, which is a stable class IIa bacteriocin. It establishes a chemical barrier, which prevents colonization by competitors [108]. If allowed to persist, the early colonizers of the *S. littoralis* gut, *Enterococcus faecalis* and *Enterococcus casseliflavus*, could be potential pathogens for the insects. Successful antimicrobial activities against them have been shown in the presence of *E. mundtii* [108].

The larvae were allowed to grow until the fifth instar, at which stag the guts were homogenized to retrieve the fluorescent *E. mundtii* by flow cytometry. The RNA of these sorted bacteria was used to probe their differential behavior inside the gut. RNA sequencing and analysis of differential gene expression were performed later.

Numerous genes are differentially regulated in the *E. mundtii* obtained from the gut, when compared to the *E. mundtii* grown in bacterial culture under lab conditions (**Table 1**, **Figure 3**). Reactive oxygen species, such as superoxide radicals, hydrogen peroxide or hydroxyl radicals, from metabolic activities may cause oxidative stress and damage macromolecules. To survive the stress, resident bacteria have to come up with means to fight it. Superoxide dismutase and catalase are effective enzymes, over-produced by *E. mundtii* when inside the gut, as compared to the broth culture.

Gene/protein	Pathway	Function
Superoxide dismutase (*SOD*)	Oxidative stress management	Quenching reactive oxidation species by partial reduction of O_2^-
Catalase	Oxidative stress management	Quenching reactive oxidation species, converting hydrogen peroxide to water and oxygen
LPxTG-motif cell wall anchor domain protein	Cell surface adhesion	Signal peptide cleaved by sortase for cell surface adhesion
WxL domain surface cell wall-binding protein	Cell surface adhesion	Cell surface adhesion and adaptation
Accessory gene regulator (*Agr*)	Two-component system	Virulence factor
General stress protein	Adaptation	Various stress management
Universal stress protein	Adaptation	Adaptation to diverse stress sources
Ferric (Fe^{+3}) ABC superfamily ATP binding cassette transporter (*fetC*)	Iron transport	Iron transporter permease
Phosphotransferase systems	Sugar transport	Regulates carbohydrate metabolism in diverse sources and adaptation

Table 1. Upregulation of genes and pathways in *E. mundtii* living in the gut of *S. littoralis*.

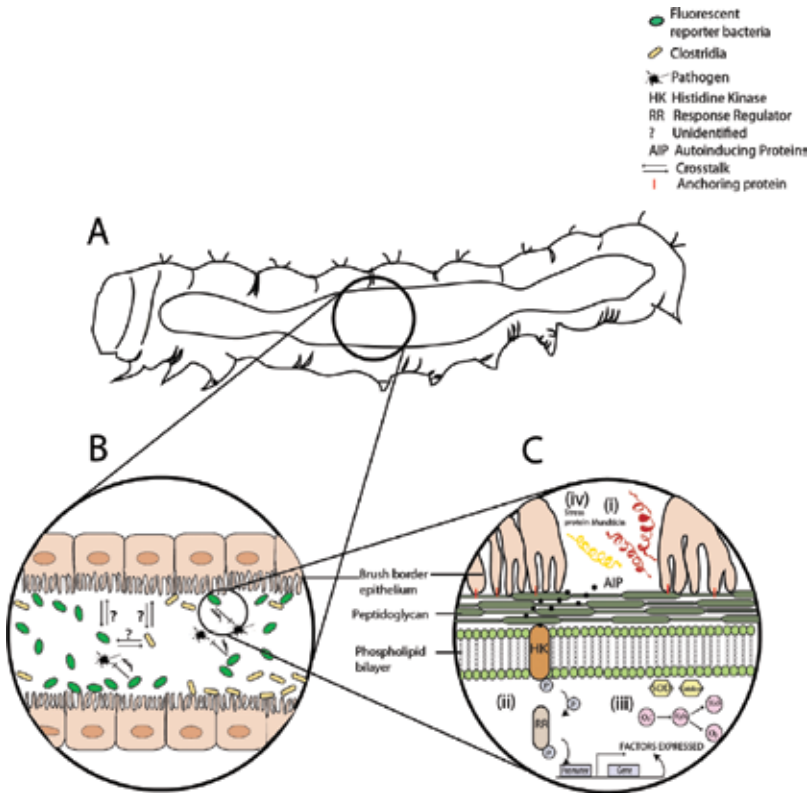

Figure 3. The gut microbiome of *S. littoralis* was dominated by *E. mundtii* and *Clostridia* sp. (A) Overview of the gut structure of fifth-instar larva of *S. littoralis*. (B) Illustration from within the gut space, which harbors major symbionts *E. mundtii*, *Clostridia* sp. and other bacteria. Bacteria adhere to the mucus layer of insect gut epithelium. Unknown interactions occur between microbe-microbe and host-microbe. (C) Illustration of some major expressed pathways *E. mundtii* used for survival in the gut. (i) Secretion of mundticin, an antimicrobial peptide, keeps pathogens at bay and helps the *E. mundtii* dominate the colonization process. (ii) A two-component system involving the accessory gene regulator (*agr*) system, which directs a histidine kinase to phosphorylate the response regulator, leads to the activation of transcription factors required for adaption. (iii) The induction of superoxide dismutase and catalase to manage oxidative stress leads to the conversion of superoxide radicals to water and oxygen. (iv) General or universal stress proteins help to overcome different kinds of stresses, such as oxygen starvation, heat or oxidative stress (unpublished).

Adhesion to the host gut epithelial surface is another key to successful colonization. Endosymbionts employ certain proteins (motifs and domains) for this purpose. These are mostly surface proteins associated with the cell wall and employing certain motifs, which act as the signal peptide for attaching to the cell wall. For example, the motif called LPXTG is a sorting peptide. The endopeptidase sortase cleaves it at the site between threonine and glycine residues, and links the peptide covalently to the peptidoglycan of the cell wall [109]. There is up-regulation in the genes encoding this motif and also in the sortase enzymes, indicating attachment of *E. mundtii* to the insect gut wall and biofilm formation. The up-regulation of the WxL domain hints at the increased colonization of the bacteria by their adherence to the gut epithelium. The WxL domain proteins are also crucial for adapting to varying environmental conditions [110].

The ability to adapt to variable living conditions is very much attributed to "two-component systems." These systems form a class of signal-transduction mechanisms that are induced when the insect senses stress in the environment. The main players in the system are auto-inducing proteins (AIPs), histidine protein kinases (HPKs) and response regulators. AIPs, which interact with the HPKs, are produced in response to stress. The signal is relayed to the response regulators. This cascade ultimately produces certain factors or proteins that aid *E. mundtii* to survive in the stressful environment [111]. Accordingly, the agr family of genes was found upregulated in *E. mundtii* living in the insect gut.

Quorum sensing is a phenomenon where the bacterial cells interact and communicate with one another for survival. AIPs are also key players for quorum sensing. In addition, also several quorum-sensing strategies are two-component systems. AIPs accumulate in response to increases in bacterial cell density; these increases are followed by a signaling cascade and lead to cooperative gene expression by the bacteria [112].

Stress proteins are adaptive factors that are induced when living conditions become stressful. There exist general and universal stress proteins. General stress proteins help bacteria deal with oxidative stress, heat stress, salt stress or oxygen limitation [113]. Universal stress proteins are induced in response to temperature fluctuations, heat or oxidative stress and hypoxia. Both of these protein classes were upregulated in *E. mundtii* in response to the insect gut's living conditions [114].

The type of sugar transport system expressed by bacteria depends on the types of carbon sources available. Phosphotransferase systems form a class of sugar transporters that sense the sugar source available in the environment and allow the respective transporters for fructose, glucose, mannose or cellobiose to act on it. Using energy from phosphoenolpyruvate, the transport system utilizes a cascade of cytoplasmic protein components with an accompanying phosphorylation of each component [115]. These transporters are generally sugar specific and because they help bacteria to survive in presence of complex carbohydrate conditions, they are said to help in their adaptation. Several of these PTS systems are upregulated by *E. mundtii* living in the gut of *S. littoralis*.

6. Discussion

Lactic acid bacteria are important in the production of fermented foods, such as dairy products. LAB is potential probiotics that provide benefits to human health [116]. Modified LAB could also be used as live vaccines or vaccine delivery systems [117]. It has been shown that the genetically modified *L. lactis* can survive and colonize the digestive tract of humans [118] and gnotobiotic mice [119]. In this chapter, we report the use of GFP to tag *E. mundtii* to monitor the bacteria's survival and activities in the intestinal tract of cotton leafworm, *S. littoralis*.

It has been shown that spatial and temporal distribution of fluorescent *E. mundtii* was observed across all developmental stages (**Figure 4**), as well as in the foregut, midgut and hindgut of *S. littoralis*. Data from the colony forming units (CFUs) show that the midgut houses the most

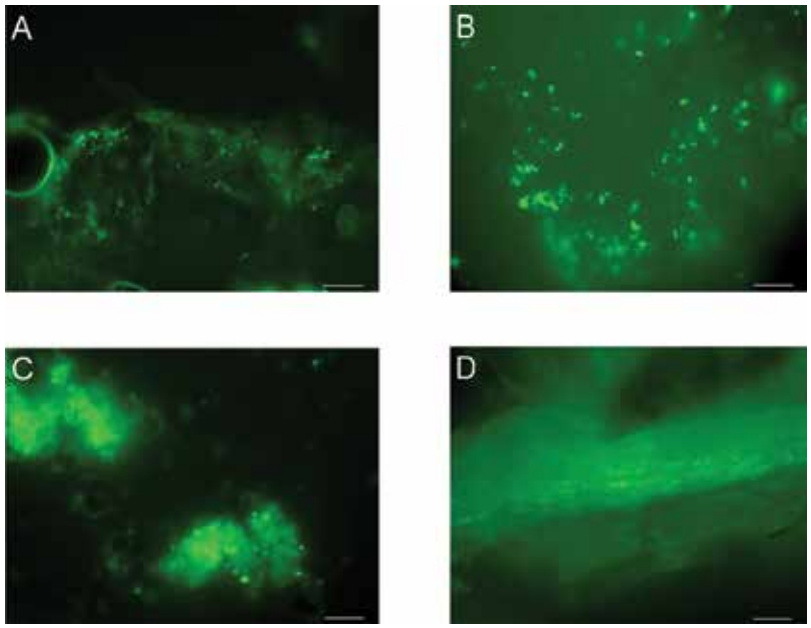

Figure 4. Photo showing the localization of fluorescent *E. mundtii* in the intestinal tract of *S. littoralis* at different life stages. (A) Bacterial cells accumulate on the peritrophic matrix separated between gut lumen and epithelium of fourth-instar larvae. (B) Bacteria cluster in the gut of fifth-instar larvae. (C) Fluorescent bacteria are visibly colonizing the tissue of pupae, although no gut tissue has been formed. (D) Vertical transmission of symbiont is evident as fluorescent *E. mundtii* survive first-generation and colonize second-generation first-instar progeny. Scale bars: 10–20 μm [14].

abundant bacterial counts, followed by the hindgut and foregut. Interestingly, the fluorescent *E. mundtii* were also detected in the eggs of *S. littoralis* [14], supporting a direct symbiont transmission from one generation to another. Other studies have shown that fluorescent bacteria were transmitted from the gut to the eggs in *Tribolium castaneum* [120]. The symbiotic *E. mundtii* was transmitted to the second-generation progeny, suggesting that the bacteria co-evolve with the insect host (**Figure 4D**). In addition, the fluorescent bacteria were detected in fecal samples of the larvae, indicating they had traveled successfully along the intestinal tract of *S. littoralis* (data not shown). The details of how a bacterial symbiont is transmitted from one generation to the next remain to be clarified. The symbiont that co-evolves with a host has a great chance to secure vertical transmission, for example, a symbiotic relationship exists between the aphid and its endosymbiont *Buchnera aphidicola*. It has been shown that the GFP-tagged *Asaia* strain is vertically transmitted from the mother to the offspring in *Anopheles stephensi* [121]. Bacterial symbionts can be horizontally transferred via "egg smearing," a phenomenon that involves a female stinkbug covering the surface of its eggs with symbiotic bacteria during oviposition. The newly hatched juveniles acquire the symbionts by ingesting the egg case [122].

Several factors, including the pH, redox potential, oxygen availability, and the nutrient and immune systems, can shape the microbial composition of the gut of insects [123]. Furthermore, constant change in gut contents due to molting and metamorphosis can affect the colonization of microorganisms. Many insects have an intestinal pH in the range of 6–8, and some

lepidopteran larvae have an even higher pH (11–12) in their midguts [124, 125]. The hindgut harbors high bacterial diversity and density in several insects, such as cockroaches, crickets and termites [126–128].

Microorganisms that live in the hindgut benefit from the metabolites and ions transported from the malpighian tubules into the hindgut. The hindgut, which stores nitrogenous and food waste, may contain nutrients for insect gut bacteria [123]. The hindgut is involved in water resorption [129]. The microbiota in the ileum of the hindgut of scarab beetles metabolizes plant polysaccharides into components that can be used by the insect [130]. In contrast to the hindgut, the midgut is an unfavorable environment for microorganisms. Many antimicrobial peptides [131] and digestive enzymes (lysozymes) [132] are secreted by the midgut epithelium cells of *D. melanogaster*. The peritrophic matrix secreted by midgut epithelial cells tends to accumulate digestive enzymes and to serve as a barrier to separate food particles, toxins and microorganisms [133]. The high alkaline pH in the gut of lepidopteran insects could kill many microorganisms. However, alkaline conditions favor the dominance of Firmicutes-related bacteria in the midgut of the beetle *Pachnoda ephippiata* [134]. Both culture-dependent and culture-independent methods have detected the presence of *Enterococcus* in the alkaline midgut of the gypsy moth larva [135].

The mechanisms of bacterial colonization in specific regions of the gut are not well understood. The gut of *S. littoralis* does not possess specialized structures called bacteriomes that contain endosymbionts, such as are found in aphids, whiteflies and other insects. How *S. littoralis* houses *E. mundtii* remains unknown, as no compartmentalized structures exist to protect the bacterium; for example, the gut of the pupae has been strongly reduced. Several mosquito species, especially newly emerged adults, that undergo metamorphosis eliminate their gut bacteria [136]. The host organism selects its own microorganisms as it depends on these for growth and development. As an example, see the case of the honeybee, whose bacterial symbionts were unable to survive in the gut of bumble bees [137].

Only a few of the important survival strategies of *E. mundtii* have been mentioned. There are several other pathways that are meant for their adaptation to the differential living conditions inside the gut. We anticipate that further RNA sequencing will help explain some of the other mechanisms that help the bacteria to survive in the gut.

E. mundtii is clearly a successful and a major symbiont in the gut of *S. littoralis.* The method that we have developed here can be used to investigate an indigenous bacterial species within the whole community. With further improvements and modifications, this kind of reporter system may be useful in many other species-specific interaction studies.

7. Future prospects

The survival strategies of *E. mundtii* in the gut of *S. littoralis* have been unveiled, yet the mechanisms employed by host insect to control the bacterium remain poorly understood. Transcriptomic analyses of the reporter organism indicated already a pattern of relevant

enzymes allowing the microbes to adapt to the harsh conditions of the insect gut. The studies can be extended to the very special conditions in the pupae where fluorescent bacteria could be observed. Thus, the concept of using a fluorescent reporter organism that can be recovered at any time from any area of the intestinal tract will allow a holistic analysis of adaptation strategies used by the microbes to adapt to the different developmental stages of the insect, as well as to study the impact of food-ingested plant toxins. In combination with the analysis of transcript patterns from the gut membranes, a first insight into the molecular interaction between the insect host and the microbiome can be expected. In conjunction with CRISPR/CAS9-created specific knock downs of defined metabolic capacities of the insect, detailed questions concerning the molecular dialog between the insect host and the microbial consortium can be answered.

Author details

Tilottama Mazumdar, Beng-Soon Teh and Wilhelm Boland*

*Address all correspondence to: boland@ice.mpg.de

Max Planck Institute for Chemical Ecology, Hans-Knöll-Straße, Jena, Germany

References

[1] Dowd PF. *In situ* production of hydrolytic detoxifying enzymes by symbiotic yeasts in the cigarette beetle (Coleoptera: Anobiidae). Journal of Economic Entomology. 1989;**82**:396-400. DOI: 10.1093/jee/82.2.396

[2] Genta FA, Dillon RJ, Terra WR, Ferreira C. Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. Journal of Insect Physiology. 2006;**52**(6):593-601. DOI: 10.1016/j.jinsphys.2006.02.007

[3] Terra WR. The origin and functions of the insect peritrophic membrane and peritrophic gel. Archives of Insect Biochemistry and Physiology. 2001;**47**(2):47-61. DOI: 10.1002/arch.1036

[4] Dale C, Moran NA. Molecular interactions between bacterial symbionts and their hosts. Cell. 2006;**126**(3):453-465. DOI: 10.1016/j.cell.2006.07.014

[5] Dillon RJ, Dillon VM. The gut bacteria of insects: Nonpathogenic interactions. Annual Review of Entomology. 2004;**49**:71-92. DOI: 10.1146/annurev.ento.49.061802.123416

[6] Currie CR, Scott JA, Summerbell RC, Malloch D. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature. 1999;**398**:701-704. DOI: 10.1038/19519

[7] Kroiss J, Kaltenpoth M, Schneider B, Schwinger MG, Hertweck C, Maddula RK, et al. Symbiotic Streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nature Chemical Biology. 2010;**6**(4):261-263. DOI: 10.1038/nchembio.331

[8] Oliver KM, Russell JA, Moran NA, Hunter MS. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**(4):1803-1807. DOI: 10.1073/pnas.0335320100

[9] Shade A, Handelsman J. Beyond the Venn diagram: The hunt for a core microbiome. Environmental Microbiology. 2012;**14**(1):4-12. DOI: 10.1111/j.1462-2920.2011.02585.x

[10] Tang X, Freitak D, Vogel H, Ping L, Shao Y, Cordero EA, et al. Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. PLoS One. 2012;**7**(7):e36978. DOI: 10.1371/journal.pone.0036978

[11] Cox CR, Gilmore MS. Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. Infection and Immunity. 2007;**75**(4):1565-1576. DOI: 10.1128/IAI.01496-06

[12] Lehman RM, Lundgren JG, Petzke LM. Bacterial communities associated with the digestive tract of the predatory ground beetle, *Poecilus chalcites*, and their modification by laboratory rearing and antibiotic treatment. Microbial Ecology. 2009;**57**(2):349-358. DOI: 10.1007/s00248-008-9415-6

[13] Ryu JH, Kim SH, Lee HY, Bai JY, Nam YD, Bae JW, et al. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science. 2008;**319**(5864):777-782. DOI: 10.1126/science.1149357

[14] Teh BS, Apel J, Shao Y, Boland W. Colonization of the intestinal tract of the polyphagous pest *Spodoptera littoralis* with the GFP-tagged indigenous gut bacterium *Enterococcus mundtii*. Frontiers in Microbiology. 2016;**7**:928. DOI: 10.3389/fmicb.2016.00928

[15] Lebreton F, Manson AL, Saavedra JT, Straub TJ, Earl AM, Gilmore MS. Tracing the enterococci from paleozoic origins to the hospital. Cell. 2017;**169**(5):849-861 e13. DOI: 10.1016/j.cell.2017.04.027

[16] Phillips GJ. Green fluorescent protein – A bright idea for the study of bacterial protein localization. FEMS Microbiology Letters. 2001;**204**(1):9-18. DOI: 10.1111/j.1574-6968.2001.tb10854.x

[17] Margolin W. Green fluorescent protein as a reporter for macromolecular localization in bacterial cells. Methods. 2000;**20**(1):62-72. DOI: 10.1006/meth.1999.0906

[18] Tsien RY. The green fluorescent protein. Annual Review of Biochemistry. 1998;**67**:509-544. DOI: 10.1146/annurev.biochem.67.1.509

[19] Stepanenko OV, Verkhusha VV, Kuznetsova IM, Uversky VN, Turoverov KK. Fluorescent proteins as biomarkers and biosensors: Throwing color lights on molecular and

cellular processes. Current Protein & Peptide Science. 2008;**9**(4):338-369. DOI: 10.2174/138920308785132668

[20] Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science. 1994;**263**(5148):802-805

[21] Valdivia RH, Hromockyj AE, Monack D, Ramakrishnan L, Falkow S. Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. Gene. 1996;**173**(1 Spec No.):47-52. DOI: 10.1016/0378-1119(95)00706-7

[22] Bokman SH, Ward WW. Renaturation of *Aequorea* gree-fluorescent protein. Biochemical and Biophysical Research Communications. 1981;**101**(4):1372-1380. DOI: 10.1016/0006-291X(81)91599-0

[23] Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene. 1996;**173**(1 Spec No):33-38. DOI: 10.1016/0378-1119(95)00685-0

[24] Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. Engineering and characterization of a superfolder green fluorescent protein. Nature Biotechnology. 2006;**24**(1):79-88. DOI: 10.1038/nbt1172

[25] Scholz O, Thiel A, Hillen W, Niederweis M. Quantitative analysis of gene expression with an improved green fluorescent protein. p6. European Journal of Biochemistry. 2000;**267**(6):1565-1570

[26] Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. Nature Methods. 2005;**2**(12):905-909. DOI: 10.1038/nmeth819

[27] Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene. 1992;**111**(2):229-233. DOI: 10.1016/0378-1119(92)90691-H

[28] Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nature Biotechnology. 2004;**22**(12):1567-1572. DOI: 10.1038/nbt1037

[29] Alieva NO, Konzen KA, Field SF, Meleshkevitch EA, Hunt ME, Beltran-Ramirez V, et al. Diversity and evolution of coral fluorescent proteins. PLoS One. 2008;**3**(7):e2680. DOI: 10.1371/journal.pone.0002680

[30] Koo J, Kim Y, Kim J, Yeom M, Lee IC, Nam HG. A GUS/luciferase fusion reporter for plant gene trapping and for assay of promoter activity with luciferin-dependent control of the reporter protein stability. Plant & Cell Physiology. 2007;**48**(8):1121-1131. DOI: 10.1093/pcp/pcm081

[31] Pinheiro LB, Gibbs MD, Vesey G, Smith JJ, Bergquist PL. Fluorescent reference strains of bacteria by chromosomal integration of a modified green fluorescent protein gene. Applied Microbiology and Biotechnology. 2008;**77**(6):1287-1295. DOI: 10.1007/s00253-007-1253-9

[32] Rhee KJ, Cheng H, Harris A, Morin C, Kaper JB, Hecht G. Determination of spatial and temporal colonization of enteropathogenic *E. coli* and enterohemorrhagic *E. coli* in mice using bioluminescent *in vivo* imaging. Gut Microbes. 2011;**2**(1):34-41. DOI: 10.4161/gmic.2.1.14882

[33] Botes M, van Reenen CA, Dicks LM. Evaluation of *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 as probiotics by using a gastro-intestinal model with infant milk formulations as substrate. International Journal of Food Microbiology. 2008;**128**(2):362-370. DOI: 10.1016/j.ijfoodmicro.2008.09.016

[34] Duangjitcharoen Y, Kantachote D, Ongsakul M, Poosaran N, Chaiyasut C. Potential use of probiotic *Lactobacillus plantarum* SS2 isolated from fermented plant beverage: Safety assessment and persistence in the murine gastrointestinal tract. World Journal of Microbiology and Biotechnology. 2009;**25**(2):315-321. DOI: 10.1007/s11274-008-9894-0

[35] Drouault S, Corthier G, Ehrlich SD, Renault P. Survival, physiology, and lysis of *Lactococcus lactis* in the digestive tract. Applied and Environmental Microbiology. 1999;**65**(11):4881-4886

[36] Wang Y, Wang J, Dai W. Use of GFP to trace the colonization of *Lactococcus lactis* WH-C1 in the gastrointestinal tract of mice. Journal of Microbiological Methods. 2011;**86**(3):390-392

[37] Geoffroy MC, Guyard C, Quatannens B, Pavan S, Lange M, Mercenier A. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Applied and Environmental Microbiology. 2000;**66**(1):383-391

[38] Russo P, Iturria I, Mohedano ML, Caggianiello G, Rainieri S, Fiocco D, et al. Zebrafish gut colonization by mCherry-labelled lactic acid bacteria. Applied Microbiology and Biotechnology. 2015;**99**(8):3479-3490. DOI: 10.1007/s00253-014-6351-x

[39] QH Y, Dong SM, Zhu WY, Yang Q. Use of green fluorescent protein to monitor *Lactobacillus* in the gastro-intestinal tract of chicken. FEMS Microbiology Letters. 2007;**275**(2):207-213. DOI: 10.1590/S1517-838246320140556

[40] Foulquie Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of enterococci in food and health. International Journal of Food Microbiology. 2006;**106**(1):1-24. DOI: 10.1016/j.ijfoodmicro.2005.06.026

[41] Bhardwaj A, Gupta H, Kapila S, Kaur G, Vij S, Malik RK. Safety assessment and evaluation of probiotic potential of bacteriocinogenic *Enterococcus faecium* KH 24 strain under *in vitro* and *in vivo* conditions. International Journal of Food Microbiology. 2010;**141**(3):156-164. DOI: 10.1016/j.ijfoodmicro.2010.05.001

[42] Andreu N, Zelmer A, Wiles S. Noninvasive biophotonic imaging for studies of infectious disease. FEMS Microbiology Reviews. 2011;**35**(2):360-394. DOI: 10.1111/j.1574-6976.2010.00252.x

[43] Zhang WY, Zhang HP. Genomics of lactic acid bacteria. In: Zhang, H. P, Cai YM, editors. Lactic Acid Bacteria-Fundamentals and Practice. 1st ed.. New York, NY: Springer Publishing Inc.; 2014. pp. 235-238. DOI: 10.1007/978-94-017-8841-0

[44] Wang TT, Lee BH. Plasmids in *Lactobacillus*. Critical Reviews in Biotechnology. 1997; **17**(3):227-272. DOI: 10.3109/07388559709146615

[45] Schroeter J, Klaenhammer T. Genomics of lactic acid bacteria. FEMS Microbiology Letters. 2009;**292**(1):1-6. DOI: 10.1111/j.1574-6968.2008.01442.x

[46] Mills S, McAuliffe OE, Coffey A, Fitzgerald GF, Ross RP. Plasmids of lactococci – Genetic accessories or genetic necessities? FEMS Microbiology Reviews. 2006;**30**(2):243-273. DOI: 10.1111/j.1574-6976.2005.00011.x

[47] Clewell DB, Yagi Y, Dunny GM, Schultz SK. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: Identification of a plasmid determining erythromycin resistance. Journal of Bacteriology. 1974;**117**(1):283-289

[48] Francia MV, Clewell DB. Amplification of the tetracycline resistance determinant of pAMalpha1 in *Enterococcus faecalis* requires a site-specific recombination event involving relaxase. Journal of Bacteriology. 2002;**184**(18):5187-5193. DOI: 10.1128/JB.184.18.5187-5193.2002

[49] Tanimoto K, Ike Y. Complete nucleotide sequencing and analysis of the 65-kb highly conjugative *Enterococcus faecium* plasmid pMG1: Identification of the transfer-related region and the minimum region required for replication. FEMS Microbiology Letters. 2008;**288**(2):186-195. DOI: 10.1111/j.1574-6968.2008.01342.x

[50] Flannagan SE, Chow JW, Donabedian SM, Brown WJ, Perri MB, Zervos MJ, et al. Plasmid content of a vancomycin-resistant *Enterococcus faecalis* isolate from a patient also colonized by *Staphylococcus aureus* with a VanA phenotype. Antimicrobial Agents and Chemotherapy. 2003;**47**(12):3954-3959. DOI: 10.1128/AAC.47.12.3954-3959.2003

[51] Balla E, Dicks LM. Molecular analysis of the gene cluster involved in the production and secretion of enterocins 1071A and 1071B and of the genes responsible for the replication and transfer of plasmid pEF1071. International Journal of Food Microbiology. 2005;**99**(1):33-45. DOI: 10.1016/j.ijfoodmicro.2004.08.008

[52] Ruiz-Barba JL, Floriano B, Maldonado-Barragan A, Jimenez-Diaz R. Molecular analysis of the 21-kb bacteriocin-encoding plasmid pEF1 from *Enterococcus faecium* 6T1a. Plasmid. 2007;**57**(2):175-181. DOI: 10.1016/j.plasmid.2006.06.003

[53] Criado R, Diep DB, Aakra A, Gutierrez J, Nes IF, Hernandez PE, et al. Complete sequence of the enterocin Q-encoding plasmid pCIZ2 from the multiple bacteriocin producer *Enterococcus faecium* L50 and genetic characterization of enterocin Q production and immunity. Applied and Environmental Microbiology. 2006;**72**(10):6653-6666. DOI: 10.1128/AEM.00859-06

[54] De Boever EH, Clewell DB, Fraser CM. *Enterococcus faecalis* conjugative plasmid pAM373: Complete nucleotide sequence and genetic analyses of sex pheromone response. Molecular Microbiology. 2000;**37**(6):1327-1341. DOI: 10.1046/j.1365-2958.2000.02072.x

[55] Paulsen IT, Banerjei L, Myers GS, Nelson KE, Seshadri R, Read TD, et al. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science. 2003;**299**(5615):2071-2074. DOI: 10.1126/science.1080613

[56] Brede DA, Snipen LG, Ussery DW, Nederbragt AJ, Nes IF. Complete genome sequence of the commensal *Enterococcus faecalis* 62, isolated from a healthy Norwegian infant. Journal of Bacteriology. 2011;**193**(9):2377-2378. DOI: 10.1128/JB.00183-11

[57] Hirt H, Manias DA, Bryan EM, Klein JR, Marklund JK, Staddon JH, et al. Characterization of the pheromone response of the *Enterococcus faecalis* conjugative plasmid pCF10: Complete sequence and comparative analysis of the transcriptional and phenotypic responses of pCF10-containing cells to pheromone induction. Journal of Bacteriology. 2005;**187**(3):1044-1054. DOI: 10.1128/JB.187.3.1044-1054.2005

[58] Janniere L, Gruss A, Ehrlich SD. Plasmids. In: Sonenshein AL, Hoch J, Losick R, editors. *Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics. Washington, DC: American Society for Microbiology; 1993. pp. 625-644

[59] Kiewiet R, Kok J, Seegers JF, Venema G, Bron S. The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. Applied and Environmental Microbiology. 1993;**59**(2):358-364

[60] O'Sullivan DJ, Klaenhammer TR. High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. Gene. 1993;**137**(2):227-231. DOI: 10.1016/0378-1119(93)90011-Q

[61] Swinfield TJ, Oultram JD, Thompson DE, Brehm JK, Minton NP. Physical characterisation of the replication region of the *Streptococcus faecalis* plasmid pAM beta 1. Gene. 1990;**87**(1):79-90

[62] Brantl S, Behnke D. Characterization of the minimal origin required for replication of the streptococcal plasmid pIP501 in *Bacillus subtilis*. Molecular Microbiology. 1992;**6**(23):3501-3510

[63] Shareck J, Choi Y, Lee B, Miguez CB. Cloning vectors based on cryptic plasmids isolated from lactic acid bacteria: Their characteristics and potential applications in biotechnology. Critical Reviews in Biotechnology. 2004;**24**(4):155-208. DOI: 10.1080/07388550490904288

[64] Lizier M, Sarra PG, Cauda R, Lucchini F. Comparison of expression vectors in *Lactobacillus reuteri* strains. FEMS Microbiology Letters. 2010;**308**(1):8-15. DOI: 10.1111/j.1574-6968.2010.01978.x

[65] Kim SF, Baek SJ, Pack MY. Cloning and nucleotide sequence of the *Lactobacillus casei* lactate dehydrogenase gene. Applied and Environmental Microbiology. 1991;**57**(8):2413-2417

[66] Boot HJ, Pouwels PH. Expression, secretion and antigenic variation of bacterial S-layer proteins. Molecular Microbiology. 1996;**21**(6):1117-1123. DOI: 10.1046/j.1365-2958.1996.711442.x

[67] Sheehan VM, Sleator RD, Fitzgerald GF, Hill C. Heterologous expression of BetL, a betaine uptake system, enhances the stress tolerance of *Lactobacillus salivarius* UCC118. Applied and Environmental Microbiology. 2006;**72**(3):2170-2177. DOI: 10.1128/AEM.72.3.2170-2177.2006

[68] CM W, Chung TC. Green fluorescent protein is a reliable reporter for screening signal peptides functional in *Lactobacillus reuteri*. Journal of Microbiological Methods. 2006;**67**(1):181-186. DOI: 10.1016/j.mimet.2006.03.009

[69] Bernbom N, Licht TR, Brogren CH, Jelle B, Johansen AH, Badiola I, et al. Effects of *Lactococcus lactis* on composition of intestinal microbiota: Role of nisin. Applied and Environmental Microbiology. 2006;**72**(1):239-244. DOI: 10.1128/AEM.72.1.239-244.2006

[70] Reunanen J, Saris PE. Survival of nisin activity in intestinal environment. Biotechnology Letters. 2009;**31**(8):1229-1232. DOI: 10.1007/s10529-009-9995-3

[71] Chouayekh H, Serror P, Boudebbouze S, Maguin E. Highly efficient production of the staphylococcal nuclease reporter in *Lactobacillus bulgaricus* governed by the promoter of the *hlbA* gene. FEMS Microbiology Letters. 2009;**293**(2):232-239. DOI: 10.1111/j.1574-6968.2009.01522.x

[72] Fang F, Flynn S, Li Y, Claesson MJ, van Pijkeren JP, Collins JK et al. Characterization of endogenous plasmids from *Lactobacillus salivarius* UCC118. Applied and Environmental Microbiology 2008;**74**(10):3216-3228. DOI: 10.1128/AEM.02631-07

[73] Singh M, Yadav A, Ma X, Amoah E, Plasmid DNA. Transformation in *Escherichia Coli*. Effect of heat shock temperature, duration, and cold incubation of CaCl2 treated cells. International Journal of Biotechnology & Biochemistry. 2010;**6**:561-568

[74] Miller JF, Dower WJ, Tompkins LS. High-voltage electroporation of bacteria: Genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proceedings of the National Academy of Sciences of the United States of America. 1988;**85**(3):856-860

[75] Rixon J, Warner PJ. Background, relevant genetic techniques and terms. In: Wood BJB, Warner PJ, editors. Genetics of Lactic Acid Bacteria. New York, NY: Kluwer Academic/Plenum Publishers; 2003. pp. 1-24. DOI: 10.1007/978-1-4615-0191-6

[76] Powell IB, Achen MG, Hillier AJ, Davidson BEA. Simple and rapid method for genetic transformation of lactic streptococci by electroporation. Applied and Environmental Microbiology. 1988;**54**(3):655-660

[77] Rodriguez MC, Alegre MT, Mesas JM. Optimization of technical conditions for the transformation of *Pediococcus acidilactici* P60 by electroporation. Plasmid. 2007;**58**(1):44-50. DOI: 10.1016/j.plasmid.2006.12.005

[78] van der Lelie D, Bron S, Venema G, Oskam L. Similarity of minus origins of replication and flanking open reading frames of plasmids pUB110, pTB913 and pMV158. Nucleic Acids Research. 1989;**17**(18):7283-7294

[79] Dornan S, Collins MA. High efficiency electroporation of *Lactococcus lactis* subsp. *lactis* LM0230 with plasmid pGB301. Letters in Applied Microbiology. 1990;**11**(2):62-64. DOI: 10.1111/j.1472-765X.1990.tb01275.x

[80] Wei MQ, Rush CM, Norman JM, Hafner LM, Epping RJ, Timms P. An improved method for the transformation of *Lactobacillus* strains using electroporation. Journal of Microbiological Methods. 1995;**21**:97-109. DOI: 10.1016/0167-7012(94)00038-9

[81] Assad-Garcia JS, Bonnin-Jusserand M, Garmyn D, Guzzo J, Alexandre H, Grandvalet C. An improved protocol for electroporation of *Oenococcus oeni* ATCC BAA-1163 using ethanol as immediate membrane fluidizing agent. Letters in Applied Microbiology. 2008;**47**(4):333-338

[82] Holo H, Nes IF. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Applied and Environmental Microbiology. 1989;**55**(12):3119-3123

[83] Thompson K, Collins MA. Improvement in electroporation efficiency for *Lactobacillus plantarum* by the inclusion of high concentrations of glycine in the growth medium. Journal of Microbiological Methods. 1996;**26**(1-2):73-79. DOI: 10.1016/0167-7012(96)00845-7

[84] McIntyre DA, Harlander SK. Genetic transformation of intact *Lactococcus lactis* subsp. *lactis* by high-voltage electroporation. Applied and Environmental Microbiology. 1989; **55**(3):604-610

[85] Hanahan D, Jessee J, Bloom FR. Plasmid transformation of *Escherichia coli* and other bacteria. Methods in Enzymology. 1991;**204**:63-113. DOI: 10.1016/0076-6879(91)04006-A

[86] Borralho T, Chang Y, Jain P, Lalani M, Parghi K. Effect of electroporation versus hanahan protocols on the transformation of *Escherichia coli* HB101 with chromosomal DNA from *Escherichia coli* HB101, *Escherichia coli* B23, and *Bacillus subtilis* WB746 and the plasmid p328.5, including an analysis of competent *Escherichia coli* HB101 cellular freeze tolerance. Journal of Experimental Microbiology and Immunology. 2002;**2**:194-200

[87] Aune TE, Aachmann FL. Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. Applied Microbiology and Biotechnology. 2010;**85**(5):1301-1313. DOI: 10.1007/s00253-009-2349-1

[88] Yoshida N, Sato M. Plasmid uptake by bacteria: A comparison of methods and efficiencies. Applied Microbiology and Biotechnology. 2009;**83**(5):791-798. DOI: 10.1007/s00253-009-2042-4

[89] Dower WJ, Miller JF, Ragsdale CW. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Research. 1988;**16**(13):6127-6145. DOI: 10.1093/nar/16.13.6127

[90] Muller S, Nebe-von-Caron G. Functional single-cell analyses: Flow cytometry and cell sorting of microbial populations and communities. FEMS Microbiology Reviews. 2010; **34**(4):554-587. DOI: 10.1111/j.1574-6976.2010.00214.x

[91] Uhlenhaut C, Kracht M. Viral infectivity is maintained by an RNA protection buffer. Journal of Virological Methods. 2005;**128**(1-2):189-191. DOI: 10.1016/j.jviromet.2005.05.002

[92] García-Nogales P, Serrano A, Secchi S, Gutiérrez S, Arís A. Comparison of commercially-available RNA extraction methods for effective bacterial RNA isolation from milk spiked samples. Electronic Journal of Biotechnology 2010;13:19-20. DOI: 10.2225/vol13-issue5-fulltext-10

[93] Beltrame CO, Cortes MF, Bandeira PT, Figueiredo AM. Optimization of the RNeasy mini kit to obtain high-quality total RNA from sessile cells of *Staphylococcus aureus*. Brazilian Journal of Medical and Biological Research = Revista brasileira de pesquisas medicas e biologicas. 2015;**48**(12):1071-1076. DOI: 10.1590/1414-431X20154734

[94] Dotsch A, Eckweiler D, Schniederjans M, Zimmermann A, Jensen V, Scharfe M, et al. The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing. PLoS One. 2012;**7**(2):e31092. DOI: 10.1371/journal.pone.0031092

[95] Heptinstall J, Total RNA. Isolation from bacteria. In: Rapley R, editor. The Nucleic Acid Protocols Handbook. Totowa, NJ: Humana Press; 2000. pp. 47-52. DOI: 10.1385/1-59259-038-1:47

[96] Stewart FJ, Ottesen EA, DeLong EF. Development and quantitative analyses of a universal rRNA-subtraction protocol for microbial metatranscriptomics. The ISME Journal. 2010;**4**(7):896-907. DOI: 10.1038/ismej.2010.18

[97] Yost S, Duran-Pinedo AE, Teles R, Krishnan K, Frias-Lopez J. Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. Genome Medicine. 2015;**7**(1):27. DOI: 10.1186/s13073-015-0153-3

[98] Kukutla P, Steritz M, Xu J. Depletion of ribosomal RNA for mosquito gut metagenomic RNA-seq. Journal of Visualized Experiments (JoVE). 2013;**74**. DOI: 10.3791/50093

[99] Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW, et al. Microbial community gene expression in ocean surface waters. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**(10):3805-3810. DOI: 10.1073/pnas.0708897105

[100] Feldman AL, Costouros NG, Wang E, Qian M, Marincola FM, Alexander HR, et al. Advantages of mRNA amplification for microarray analysis. BioTechniques. 2002;**33**(4):906-912 14

[101] Kacharmina JE, Crino PB, Eberwine J. Preparation of cDNA from single cells and subcellular regions. Methods in Enzymology. 1999;**303**:3-18

[102] Pabon C, Modrusan Z, Ruvolo MV, Coleman IM, Daniel S, Yue H, et al. Optimized T7 amplification system for microarray analysis. BioTechniques. 2001;**31**(4):874-879

[103] Polacek DC, Passerini AG, Shi C, Francesco NM, Manduchi E, Grant GR, et al. Fidelity and enhanced sensitivity of differential transcription profiles following linear amplification of nanogram amounts of endothelial mRNA. Physiological Genomics. 2003;**13**(2):147-156. DOI: 10.1152/physiolgenomics.00173.2002

[104] Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH, Amplified RNA. Synthesized from limited quantities of heterogeneous cDNA. Proceedings of the National Academy of Sciences of the United States of America. 1990; **87**(5):1663-1667

[105] Degrelle SA, Hennequet-Antier C, Chiapello H, Piot-Kaminski K, Piumi F, Robin S, et al. Amplification biases: Possible differences among deviating gene expressions. BMC Genomics. 2008;**9**:46. DOI: 10.1186/1471-2164-9-46

[106] Parekh S, Ziegenhain C, Vieth B, Enard W, Hellmann I. The impact of amplification on differential expression analyses by RNA-seq. Scientific Reports. 2016;**6**:25533. DOI: 10.1038/srep25533

[107] Hoeijmakers WA, Bartfai R, Francoijs KJ, Stunnenberg HG. Linear amplification for deep sequencing. Nature Protocols. 2011;**6**(7):1026-1036. DOI: 10.1038/nprot.2011.345

[108] Shao Y, Chen B, Sun C, Ishida K, Hertweck C, Boland W. Symbiont-derived antimicrobials contribute to the control of the lepidopteran gut microbiota. Cell Chemical Biology. 2017;**24**(1):66-75. DOI: 10.1016/j.chembiol.2016.11.015

[109] Roche FM, Massey R, Peacock SJ, Day NP, Visai L, Speziale P, et al. Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. Microbiology. 2003;**149**(Pt 3):643-654. DOI: 10.1099/mic.0.25996-0

[110] Brinster S, Furlan S, Serror P. C-terminal WxL domain mediates cell wall binding in *Enterococcus faecalis* and other gram-positive bacteria. Journal of Bacteriology. 2007;**189**(4): 1244-1253. DOI: 10.1128/JB.00773-06

[111] Alm E, Huang K, Arkin A. The evolution of two-component systems in bacteria reveals different strategies for niche adaptation. PLoS Computational Biology. 2006;**2**(11):e143. DOI: 10.1371/journal.pcbi.0020143

[112] Rutherford ST, Bassler BL. Bacterial quorum sensing: Its role in virulence and possibilities for its control. Cold Spring Harbor Perspectives in Medicine. 2012;**2**(11). DOI: 10.1101/cshperspect.a012427

[113] Volker U, Engelmann S, Maul B, Riethdorf S, Volker A, Schmid R, et al. Analysis of the induction of general stress proteins of *Bacillus subtilis*. Microbiology. 1994;**140**(Pt 4):741-752. DOI: 10.1099/00221287-140-4-741

[114] O'Connor A, McClean S. The role of universal stress proteins in bacterial infections. Current Medicinal Chemistry. 2017. DOI: 10.2174/0929867324666170124145543

[115] Kotrba P, Inui M, Yukawa H. Bacterial phosphotransferase system (PTS) in carbohydrate uptake and control of carbon metabolism. Journal of Bioscience and Bioengineering. 2001;**92**(6):502-517. DOI: 10.1016/S1389-1723(01)80308-X

[116] Gasson MJ. Progress and potential in the biotechnology of lactic acid bacteria. FEMS Microbiology Reviews. 1993;**12**:3-19. DOI: 10.1111/j.1574-6976.1993.tb00010.x

[117] Wells JM, Wilson PW, Norton PM, Gasson MJ, Le Page RW. *Lactococcus lactis*: High-level expression of tetanus toxin fragment C and protection against lethal challenge. Molecular Microbiology. 1993;**8**(6):1155-1162. DOI: 10.1111/j.1365-2958.1993.tb01660.x

[118] Klijn N, Weerkamp AH, de Vos WM. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Applied and Environmental Microbiology. 1995;**61**(7):2771-2774

[119] Gruzza M, Duval-Iflah Y, Ducluzeau R. Colonization of the digestive tract of germ-free mice by genetically engineered strains of *Lactococcus lactis*: Study of recombinant DNA stability. Microbial Releases: Viruses, Bacteria, Fungi. 1992;**1**(3):165-171

[120] Knorr E, Schmidtberg H, Arslan D, Bingsohn L, Vilcinskas A. Translocation of bacteria from the gut to the eggs triggers maternal transgenerational immune priming in *Tribolium castaneum*. Biology Letters. 2015;**11**(12). DOI: 10.1098/rsbl.2015.0885

[121] Favia G, Ricci I, Damiani C, Raddadi N, Crotti E, Marzorati M, et al. Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**(21):9047-9051. DOI: 10.1073/pnas.0610451104

[122] Funkhouser LJ, Bordenstein SR. Mom knows best: The universality of maternal microbial transmission. PLoS Biology. 2013;**11**(8):e1001631. DOI: 10.1371/journal.pbio.1001631

[123] Engel P, Moran NA. The gut microbiota of insects – diversity in structure and function. FEMS Microbiology Reviews. 2013;**37**(5):699-735. DOI: 10.1111/1574-6976.12025

[124] Wieczorek H, Beyenbach KW, Huss M, Vitavska O. Vacuolar-type proton pumps in insect epithelia. The Journal of Experimental Biology. 2009;**212**(Pt 11):1611-1619. DOI: 10.1242/jeb.030007

[125] Funke M, Buchler R, Mahobia V, Schneeberg A, Ramm M, Boland W. Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. Chembiochem: A European Journal of Chemical Biology. 2008;**9**(12):1953-1959. DOI: 10.1002/cbic.200700781

[126] Cruden DL, Markovetz AJ. Microbial ecology of the cockroach gut. Annual Review of Microbiology. 1987;**41**:617-643. DOI: 10.1146/annurev.mi.41.100187.003153

[127] Santo Domingo JW, Kaufman MG, Klug MJ, Tiedje JM. Characterization of the cricket hindgut microbiota with fluorescently labeled rRNA-targeted oligonucleotide probes. Applied and Environmental Microbiology. 1998;**64**(2):752-755

[128] Bauer S, Tholen A, Overmann J, Brune A. Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood- and soil-feeding termites by molecular and culture-dependent techniques. Archives of Microbiology. 2000;**173**(2):126-137. DOI: 10.1007/s002039900120

[129] Chapman RF, Simpson SJ, Douglas AE. The Insects: Structure and Function. 5th ed. Cambridge: Cambridge University Press; 2013

[130] Huang SW, Zhang HY, Marshall S, Jackson TA. The scarab gut: A potential bioreactor for bio-fuel production. Insect Science. 2010;**17**:175-183. DOI: 10.1111/j.1744-7917.2010.01320.x

[131] Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. Annual Review of Immunology. 2007;**25**:697-743. DOI: 10.1146/annurev.immunol.25.022106.141615

[132] Shanbhag S, Tripathi S. Epithelial ultrastructure and cellular mechanisms of acid and base transport in the *Drosophila* midgut. The Journal of Experimental Biology. 2009;**212**(Pt 11):1731-1744. DOI: 10.1242/jeb.029306

[133] Shao L, Devenport M, Jacobs-Lorena M. The peritrophic matrix of hematophagous insects. Archives of Insect Biochemistry and Physiology. 2001;**47**(2):119-125. DOI: 10.1002/arch.1042

[134] Egert M, Wagner B, Lemke T, Brune A, Friedrich MW. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Applied and Environmental Microbiology. 2003;**69**(11):6659-6668. DOI: 10.1128/AEM.69.11.6659-6668.2003

[135] Broderick NA, Raffa KF, Goodman RM, Handelsman J. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Applied and Environmental Microbiology. 2004;**70**(1):293-300. DOI: 10.1128/AEM.70.1.293-300.2004

[136] Moll RM, Romoser WS, Modrzakowski MC, Moncayo AC, Lerdthusnee K. Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. Journal of Medical Entomology. 2001;**38**(1):29-32. DOI: 10.1603/0022-2585-38.1.29

[137] Kwong WK, Engel P, Koch H, Moran NA. Genomics and host specialization of honey bee and bumble bee gut symbionts. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**(31):11509-11514. DOI: 10.1073/pnas.1405838111

Edited by Ranjith N. Kumavath

This book focuses on metagenomics for gut microbiomes. The host is deprived of various benefits derived from the numerous gut microbes for food metabolism and health. However, polysaccharides such as cellulose, xylans, resistant starch, and inulin that are found in vegetables in our diet are digested by certain species that colonize the intestines. In contrast, metagenomic studies that reiterate microbiome intrinsic factors from particular communities and lifestyles and are extremely important for bacterial communities traveling over a long distance have entered a new era. Predominantly to understand the behavior of organisms and their action in a host, next-generation sequencing will provide a new insight into analyzing the livestock industry, agriculture, and human health risks and will consider for future development novel therapies for various diseases through identification of advanced tools. Hence, the book will give more precise information on the role of gut microbiomes in the host.

Published in London, UK

IntechOpen

ISBN 978-1-83881-312-3