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Antibiotic Use in Animals

Edited by Sara Savić

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Meet the editor

Sara Savić, PhD, DVM, is a researcher, working in a diagnostic laboratory within the Scientific Veterinary Institute "Novi Sad" from Novi Sad, Serbia. Her main work is based on the diagnostic procedures for zoonotic diseases and vector-borne zoonoses. Dr. Savić has completed her PhD degree on Diagnostics of Lyme disease in dogs and ticks, after which her interests and career went toward One Health issues. The significance of multidisciplinary, transdisciplinary, and interdisciplinary work has become most interesting during the past decade. Her expertise is in bacterial and parasitic vector-borne zoonoses, especially in blood parasites. Dr. Savić has published over 100 publications so far as a leading author or as a coauthor, in different scientific journals and proceedings from different scientific meetings.

Contents

Preface

This book is about the most known term in medicine "antibiotics," which have lately often been characterized as a cause for "more bad than good" during treatment. In this book, we have tried to show both sides of the story—how good antibiotics can be when used properly and how bad they can turn out if they are misused.

The book *Antibiotic Use in Animals* has everything said in the title. But please do not think that this book is only meant for the veterinarians. It is intended to be used also by medical doctors, animal owners, consumers of food of animal origin, etc. The book has five sections: "Introduction," "Use of Antibiotics in Animals," "Antibiotics and Nutrition," "Probiotics," and "Antimicrobial Resistance." Each of the sections discusses about one side of the antibiotic usage. Each group of authors has dedicated their work to one of the topics with key roles of antibiotics in the health of animals and public health in general.

The introduction section is about the current state of knowledge on antibiotics today. The section on "Use of Antibiotics in Animals" is about the choices we make and the alternatives we have, in order to control bacterial infections and diseases. This responsible use of antibiotics in animals is important in companion animals as much as in food-producing animals. In the section "Antibiotics and Nutrition," the authors deal with potential danger from constant everyday intake of hidden antibiotics in our food. Probiotics are the opposite side of antibiotics that are also produced by bacteria, and in this chapter, their influence is shown. Antimicrobial resistance (AMR) is the most intriguing topic at the moment among public health and One Health specialists. In the chapter dedicated to antimicrobial resistance it is shown how the resistance can be triggered or stimulated to appear.

This book is a work of scientists and researchers as a contribution to general knowledge and sophisticated expertise on the topic of antibiotic use. With this book, we hope to open new questions and deepen the research on roles of antibiotics in everyday life.

As the editor, I would like to dedicate this book to all my colleagues and researchers working hard on protecting public health.

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Introduction

Chapter 1

Introductory Chapter: Antibiotic Use in Animals Today

Sara Savić

Additional information is available at the end of the chapter

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1. Introduction

Use of antibiotics in everyday life has become an issue of different opinions and debates during the past couple of years. Due to the appearance of several occurrences, and as a consequence, of antibiotic usage, it became a topic of different studies. In the book "Antibiotic Use in Animals," we have tried to show and explain the different aspects of antibiotic use, different points of view, and a multidisciplinary and transdisciplinary approach to this topic.

According to Wikipedia—"antibiotic use in livestock is the use of antibiotics for any purpose in the husbandry of livestock, which includes treatment when ill (therapeutic), treatment of a batch of animals when at least one is diagnosed as ill (metaphylaxis, similar to the way bacterial meningitis is treated in children), and preventative treatment (prophylaxis) against disease" [1].

In the past, antibiotics were used as additions in animal feed and/or water for better growth of the animals or for higher feed efficiency. They were added in subtherapeutic doses. This opinion was eliminated in 2017, as a result of new FDA Veterinary Feed Directive. This practice has been banned in Europe since 2006 by the European Commission (Ban on antibiotics as growth promoters in animal feed).

In usage of antibiotics in animals, it is not only important to show the necessity of utilization, but also responsibility and moderation while handling antibiotics. There is no doubt that antibiotics have to be used in different cases of disease in animals. But during the past decades, antibiotics have been used sometimes irresponsibly and sometimes even abused. Some antibiotics "stopped working," so pharmaceutical industries had to search for new generations of antibiotics, which were again overused in practice—new antibiotics, over usage again, and after several decades, we have found ourselves in a closed circle with no way out, and then, a new term has appeared called—antimicrobial resistance. After a number of years of use of antibiotics, antimicrobial resistance has occurred and the way of handling and use of antibiotics had to change.

The topic of Antibiotic Use in Animals is of scientific nature, but it is also meant to bring the topic of antibiotic use to wider reading audience. The problem of antibiotic resistance and antibiotic overuse cannot be solved or tackled by a single book. The purpose of this book is to at least cut into the topic of antibiotic use in animals and antimicrobial resistance.

The significance of this topic is not in question, since there is a whole public debate going on for a while about the use of antibiotics in animals as well as in humans. There are reviews on antibiotic use through the history, like the one on Antibiotic Use in Food Animals: Perspective, Policy, and Potential, published in Public Health Reports [2]—where it is stated that "antibiotic use today plays a major role in the emerging public health crisis of antibiotic resistance." Massive antibiotic use in agriculture, leads to a topic of how antibiotic use in farm animals contributes to the overall problem of antibiotic resistance in humans and in animals. The mentioned review summarizes literature on the role of antibiotics in the development of resistance and its risk to human health with the search of multiple databases to identify major lines of argument supporting the role of agricultural antibiotic use in the development of resistance and to summarize existing regulatory and policy documents.

Antibiotic resistance became a public health crisis, and whole research teams are dedicated identifying the resistant strains and ways how to overcome the current situation with hospital acquired infections. Antibiotic resistance is a product of natural selection in bacteria, their survival abilities. Individual bacteria carry mutations that can lead to ineffectiveness of antibiotics.

The Federation of Companion Animal Veterinary Associations (FECAVA) is also interested in solving the problem about the antibiotic use and they have dedicated a meeting to European Antibiotic Awareness Day, which has been going on for the 10th time. FECAVA is also dedicated to fight antimicrobial resistance and to raise awareness of the public on responsible use of antibiotics. This organization has issued a chart named as "Advice on responsible use of antibiotics," which gives a detailed instruction how to handle the use of antibiotics and to support decision making and also the diseases and conditions when antibiotic use is recommended [3, 4].

Antibiotics are irreplaceable in some cases of illness, but not all. Some infectious diseases are caused by bacteria, but some are caused by viruses or other causative agents that do not respond to antibiotic therapy. Not even all bacterial infections demand antibiotic therapy. FECAVA appeals also to the animal owners, not only practitioners, that inappropriate use of antibiotics can even harm the animal and that the responsibility has to be globalized as responsibility of society!

There is no doubt that antibiotics are important, and that many infections, in animals or humans, cannot be treated with anything else. But with antimicrobial resistance raise, the treatment of these diseases may become a problem. Medical doctors and veterinarians have to work together on this issue, including the International Health Institutions. The diagnostic procedure in establishing the cause of the disease is essential. After identification of the cause of the disease, a decision can be made if the antibiotics are necessary. If antibiotic therapy is the solution to illness, the advice of medics/veterinarians has to be followed. If antibiotic treatment is to be applied, there are several rules that have to be followed during the treatment…the dosage of the antibiotic given per one intake cannot be changed at free will of the animal owner

or the patients themselves. Therapy must not be stopped earlier or prolonged at free will. The treatment of one animal cannot be shared with other animals in the same house holding or farm, at free will of the owner. just because they have similar symptoms to the owners eye. The left over medicaments should never be reused, they have to be safely disposed. The use of antibiotics with the aim of infection control has to be responsible on the intention of a medic/vet, and not on the intention of the patient/owner. Usage of antibiotics as a precaution is also not appropriate. Antibiotics should be used responsibly, only when necessary and only when there is no other choice.

The usage of antibiotics can be such in variety of cases and when used responsibly, it can only contribute to Public Health. In this book that variety is shown! The intention is for a wide audience to comprehend the subject—experts such as medical doctors and veterinarians, but also pet and animal owners and even wide population of potential patients which is almost everybody. The story of antibiotic use has to be presented as a positive one but in responsible hands and not as a villain.

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Section 2

Use of Antibiotics in Animals

Chapter 2

Necessary Usage of Antibiotics in Animals

Magdy Moheb El-Dein Saad and
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Abstract

Animals could become sick at any time of their lives, just like all people exposed. Many of the antibiotics administered to animals are identical to or closely drugs used in human. All animal species in general and food-producing animals, in particular, are commonly exposed to antibiotics to treat and prevent infectious diseases or to promote growth. Antibiotics would not be necessary if animals were raised differently under good veterinary and husbandry practices that were less crowded and more sanitary. The proper and responsible use of antibiotics in veterinary medicine mandate an active cooperation between all the interested parties involved in livestock production cycles. All parties are invited to act together to ensure the ultimate goals of maintaining the efficacy and safety of veterinary antibiotics and complying the established maximum residue limits (MRLs) of the products of animal origin intended for human consumption. Antibiotics as hazardous substances should be applied and directed during the different steps starting from prescription until ensuring the withdrawal period under the supervision of professionals and veterinarians. Practices indicated that there is a need to improve sensitivity testing services and facilities before prescribing the proper antibiotic.

Keywords: prophylaxis, metaphylaxis, curative, therapy, misuse, resistant bacteria, food-producing animals, drug prescription

1. Introduction

Since the veterinary antibiotic residues and occurrence of bacterial resistance problems had got the attention of scientists and public communities, many questions raised focusing on the main five logics of (5W + 1H) being: What are the alternatives and choices to avoid using veterinary antibiotics? Why we cannot stop usage of antibiotics in food-producing animals? When do veterinary antibiotics are necessary to use? Where do the herd producers get

antibiotics for animal use? Who is authorized to prescribe and regulate antibiotics for animal use? And how could antibiotics be effective without any negative effects on public health?

2. Choices and alternatives to control bacterial infections and diseases in companion and food-producing animals

Undoubtedly, animals could become sick at sometime of their lives, just like all people do. Food-producing animals involving large and small ruminants, poultry and aquaculture are often exposed to antibiotics to treat and prevent infectious diseases or to promote growth [1, 2]. Many of the antibiotics administered to animals are identical to or closely resemble drugs used in human [2, 3]. Also, other animal species like pets, dogs, horses and animals used for fur are commonly exposed to bacterial infections during their lives. These groups of animals when showed clinical signs, they could be treated separately [4]. As an example, when clinical symptoms of infections were reported or suspected in horses, dogs and other companion animals, they could be easily monitored with a possible quarantine. So, the risk of the infection spreading in veterinary hospitals and veterinary clinics could be at minimum. The same approach was followed when dogs showed clinical symptoms, they should not be kept with other animals [5, 6]. The issue is completely different when flocks or herds of food-producing animals are exposed to bacterial diseases or infections [7]. The responsibility of antibiotics in veterinary medicine is still the best choice to control and treat bacterial infections. Guidelines on the responsibility and prudent use of antibiotics in animal husbandry were issued by the United Nation Organization of UN-Office International des Epizooties [8] and confirmed by the European Union [6]. The aims of the guidelines are to maintain antibiotic efficacy, avoid dissemination of resistant bacteria and finally, avoid such bacteria to reach human food. Also, the guidelines are addressed to all the interested parties involved in animal husbandry; that is, the veterinary pharmaceutical industry, practitioners, breeders and farmers. The guidelines also exhibited the main role and responsibility of the competent authorities dealing with the production and marketing of veterinary antibiotics. The approved guidelines for the use of antibiotics in veterinary medicine had a set of recommendations and measures acting together to ensure the ultimate goals of: (a) comply with the mandatory standards recommended by the international organizations, (b) maintain the efficacy and safety of antibiotics, (c) prevent or at least reduce transformation of resistant bacteria to human, (d) fulfill and comply the established maximum residue limits (MRLs) of the applied antibiotics and finally, (e) ensure the safety of the products of animal origin intended for human consumption.

3. The prudent and responsible use of antibiotics

The technical committee of UN-Office International des Epizooties [8] recommended the following criteria for the proper and responsible use of antibiotics:

a. Antibiotics as hazardous substances should be applied and technically directed under the supervision of professionals and those have the required experience and skills.

b. Antibiotics usage should be applied within the good veterinary practices (GVP) and animal husbandry practices, considering diseases prevention practices like vaccination and improving husbandry conditions.

c. The usage of antibiotics should be limited to their approved and intended use.

d. Where appropriate, testing of isolates from food-producing animals during their production period to adjust therapy.

e. The proper and responsible use of antibiotics in veterinary medicine mandate an active cooperation between all the interested parties involving, administrative and scientific authorities, veterinary pharmaceutical industry, distributors and handlers, veterinary practitioners and livestock breeders and producers.

It is worthy to mention that prudent use of veterinary antibiotics is the most important part of the good veterinary practices (GVP). The first step is commonly dealing with antibiotic prescribing habits. Some practitioners take into account responsible use warnings when antibiotic sensitivity testing is performed. No doubt, those significant differences could be obtained, due to the frequency of sensitivity testing, practitioners skills, background as well as some interfering factors. Practices indicate that there is a need to improve sensitivity testing services and facilities aiming to offer rapid, accurate and cheaper testing before prescribing antibiotics [9]. As more antibiotics are discovered and applied to veterinary clinical use, there is a need to update codes of practices, conduct and ethics. Applying such codes will help to ensure maximizing therapeutic efficacy and minimizing the resistance of microorganisms. Veterinary antibiotics are not only widely used in many countries, if not in all countries to treat and protect animal health, but also they are incorporated into animal feed to improve growth and feed utilization. Regarding antibiotic feed additives, they are poorly absorbed in the gut, so the great portion of such antibiotic additives will take their way into animal secretions such as feces and urine leading to contamination of soil and environment [10–12]. So, the routine use of antibiotics as growth promoters is no longer recommended. Also, veterinarians, practitioners and livestock breeders should have enough knowledge about infectious diseases exposure pathways, fate and effects of veterinary antibiotics, besides the environmental cycle and occurrence.

4. Antibiotic usage in food-producing animals and aquaculture

4.1. Mass production of beef

Raising beef cattle needs relatively little intervention and use of antibiotics comparing with those managed in intensive and feedlots. But commonly beef calves, after weaning aged 6 months and more, are routinely shipped to mass production farms, then maintained in large groups and fed high energy rations. In most developing countries, feedlot animals are kept at high densities which lead to more morbidity, especially in newly received calves. During such production cycle, both pneumonia and diarrhea are the major threat to the herd life. Bovine respiratory diseases have occurred with many causal organisms. These organisms could

change during the progression of diseases [13]. Thus, calves are often treated with individual or grouping medication. Consequently, a variety of viral secondary infections contribute to the primary of pneumonia and diarrhea, but treating bacterial infection is still the right medication. Also, the major feedlot health problem of shipping fever complex of pneumonia is an important determinant of antibiotic use. Since beef cattle change ownership more than once during their life cycles, feedlot owners could not easily followed the good veterinary practices. So, the common approach based on the assumption that animals in the group are either susceptible or already carrying diseases [14]. This assumption is applied in USA, when 83% of feedlot cattle received antibiotics through feed or water [15]. The most commonly used oral antibiotics are tylosin, tetracyclines and florfenicols, which could act as prophylactic treatment against liver abscesses, diarrhea, foot rot and respiratory diseases, as well as acting as growth promoters at sub-therapeutic levels [16]. The relation between antibiotic use and resistance in beef cattle appeared wide conflicting results between calves treated with penicillin, streptomycin and tetracyclines and the resistant *Escherichia coli* isolated from their feces [17]. However, in mass production of beef, the fewer antibiotics are used comparing with the other categories of animal production [18].

4.2. Dairy production

As dairy industry is increasing, average herd size and average milk production per head had significantly increased. The common system in most dairy farms depends upon the separation of new-born calves from mothers within a day of birth. The new-born calves are housed separately to control infection and fed milk and/or milk replacers commonly contained tetracycline up to the weaning age 6–8 weeks. To treat or prevent the common diseases of pneumonia and diarrhea, the antibiotics of tetracycline, penicillin and sulfonamides may be administered orally or by injection. Dairy cows are commonly housed at higher densities with great metabolic stress. As milk production increases, parallelly related disease increased, which could negatively affect animal welfare and food quality [19]. Contrary to poultry and beef industry, antibiotics in dairy industry are used for therapeutic functions [20]. Antibiotics are very necessary to treat the common diseases of mastitis, lameness, respiratory diseases and gastrointestinal disorders [21, 22]. In most countries, especially the developing ones, intra-mammary use of antibiotics was frequent, with little if no information, about pharmacokinetics, efficacy and withdrawal period. So, antibiotics use in dairy cattle should be related to the production stage "lactation, dry period and heifers replacement". In lactating dairy cows, mastitis is the most common challenge of diseases. Mastitis caused by intra-mammary infections which could be categorized as clinical or sub-clinical based on clinical signs and some milk composition criteria [23]. To select an appropriate therapeutic protocol, it necessitates enough data about: clinical signs, milk composition and the results of sensitivity testing [24]. Some forms of chronic mastitis like those caused by *Staphylococcus aureus* are poorly respond to antibiotic therapy, but survey studies showed that cephapirin, pirlimycin and amoxicillin were the preferred therapy of clinical mastitis [25]. Because causative organisms of mastitis are classified as environmental, changes in management system could lead to increasing environmental mastitis. However, supportive care and good husbandry practices are recommended for resolution of clinical cases makes antibiotic therapy is not necessary [26]. It is well known

that high yielding dairy cows are more susceptible to infectious diseases, especially when transition period of lactation resumes [19]. Good husbandry practices including nutritional support, controlling environmental stress, applying dry cow therapy are the recommended successful criteria for dry period. Dry cow therapy products are available over-counter purchasing. Using teat sealants at dry off represent good option for the prevention of addition intra-mammary infections [27]. Dry cow therapy had positive effect on reducing incidence of mastitis early in lactation without an increase in intra-mammary infection at calving [28]. During the stage of heifers replacement, the selected heifers were fed either on milk replacers with added antibiotics, commonly tetracycline or neomycin, or received whole milk in their home dairy [29]. In general, the primary infections of respiratory diseases and diarrhea necessitate antibiotic therapy in replacement heifers. Diarrhea is the most common cause of mortality in pre-weaned calves commonly treated with ceftiofur, while the primary indication in weaned heifers is respiratory diseases often treated with florfenicols or tilmicosin [27].

4.3. Small ruminants

Sheep and goats are farmed for different products, that is, milk, meat and wool. Sheep and goats necessitate the use of antibiotics to treat the common diseases of mastitis, lameness, respiratory and gastrointestinal disorders [21, 22]. The licensed antibiotics for sheep and goats are very rare, so the use of such drugs depends upon the practitioners experience. Dosage estimation and withdrawal time had not adequately reported for sheep and goats, which increases the risk of residues in human food [30]. The use of antibiotics in small ruminants is relatively low, especially with meat sheep. For therapeutic use, penicillin and tetracyclines are the most common antibiotics, meanwhile tetracycline was more common in feed medication [31]. Usually, small ruminants mastitis is sub-clinical and does not necessarily reduce milk yield and often localized to one udder half. The main adverse effects are related to milk quality and increasing somatic cells counts (SCC) in the yield. It is well established that high SCC are positively related to increase antibiotic residues [32]. Because of the rare antibiotics labeled for small ruminants, mastitis is commonly treated with bovine intra-mammary products, which leads to many adverse effects due to the improper dosage and the estimated withdrawal period [33]. In small ruminants, both broad and narrow spectrum antibiotics can enhance animal performance [31].

4.4. Poultry

Poultry products including eggs and meat are very important sources of animal protein in most developed and developing counties. During the last five decades, broiler chicken production all over the world had increased significantly to meet the increased requirement of animal protein. In Egypt, as an example, poultry industry were grew fast to be highly integrated industry with fewer companies controlling most sources of birds, feed mills, farms, slaughter and processing facilities. Integration of such industry led to standardized management practices including drug treatment practices, especially those related to prevent and control of infection diseases. An intensive production system resulted the spread of pathogens including the zoonotic ones like *Salmonella*. Broiler rations usually contain a coccidiostat,

several of which are broader antibiotics of ionophores and sulfonamides. Besides, bacitracin, bambermycin, chlortetracycline, penicillin and virginiamycin are commonly used for growth promotion, feed efficiency in broilers, turkey and egg layers. Also, bacitracin and virginiamycin could be used to control intestinal infections caused by *Clostridium* sp. and/or as growth promoters [34]. Poultry industry necessitates the use of antibiotics at therapeutic and subtherapeutic doses. As gastrointestinal and respiratory diseases represent the common problems challenging poultry industry, coli-bacillosis, necrotic enteritis and *E. coli* are the most causative pathogenic organisms [35]. Survey studies exhibited the common therapeutic use of amoxicillin and tylosin, while lincosamides are used both preventive and curative [36]. Most poultry producers in the developing countries believe well on the use of antibiotics to prevent and curate challenging diseases. In these countries, the use of antibiotics in poultry industry applied without prescription, lacking the necessary information about withdrawal period and the adverse effects to human health and environment [37].

4.5. Fish aquaculture

Antibiotics are very essential additions in fish aquaculture. The ability of antibiotics to control fish diseases is influenced by four main factors: (1) the actual bacterial component, (2) the sensitivity and/or resistance of the bacterial strains to the chosen antibiotic, (3) the proper dosage and treatment intervals and (4) the other contributing stress factors. Antibiotics do not directly cure treated fish, but they are controlling the population growth of a bacteria in a fish which promoting their immune system to eliminate them [38]. Before antibiotics are prescribed to fish and aquaculture, sources of stress involved water quality and temperature, differences between aquaculture species, nutrition and means of antibiotics handling and transportation should be eliminated or at least minimized [39]. Special experience and skills are needed to identify the interfered factors which could be primary or secondary factors affecting bacterial infectious diseases. Such experience and skills besides the sensitivity testing could lead to more efficient treatments and less loss of fish.

5. Persistence development of bacterial resistance threaten human health and environment

Many reports exhibited the variable resistant response of bacterial pathogens [40, 41]. Resistant species of *Salmonella typhimurum* rapidly observed after exposure to certain antibiotics, while *Salmonella duplin* remains sensitive when treated with the same antibiotics. Similarly, *S. aureus* became resistant to penicillin very shortly after administration, meanwhile it needs about 20 years to be observed in *Streptococcus pneumonia* [42]. As bacterial resistance is affected by two main variables of antibiotic category and bacterial species. Resistance to fluoroquinolones in *Campylobacter* sp., apramycin in *E. coli* and *Salmonella* sp. were rapidly observed after drugs administration [43]. Meanwhile, resistance to ampicillin needs to develop more gradually [44]. Similarly, tetracycline and avoparcin persistence are widely affected by many interfered factors including the re-exposure withdrawn antibiotics [42, 45]. The medical impact of previous antibiotic usage in food-producing animals

on human health received little attention except for Salmonella and *E. coli*. ***E. coli*** may acquire resistance from the gut micro-flora of the food animal even if the antibiotic is used as a growth promoter [46]. When livestock flocks treated with sulfonamides, amino glycosides or tetracyclines, widespread resistance of bacteria was observed. While the corresponding resistances to other antibiotics like ampicillin and olaquindox is less widespread [44]. It is worthy to mention that multiple resistances to more than one class of antibiotics seemed to be common with animal strains of *E. coli*. ***Salmonella* sp**. showed wide variation between different isolates. The monitoring programs showed that *Salmonella typhimurium* isolates are more resistant to tetracyclines, sulfonamides and streptomycin comparing with the isolates of *Salmonella dublin* and *Salmonella enteritidis* [40]. ***Campylobacter* sp**. had often showed erythromycin resistance, in particular to the isolates of *E. coli* [2]. Enteric *Campylobacter* are rarely requiring treatments, but they showed more tetracycline resistance in human than poultry isolates. Also, isolates of *Campylobacter* obtained from pig exhibited more macroloide and streptomycin resistance comparing with human and poultry isolates [47]. *Enterococci* as *Campylobacter*, both are enteric bacteria in animals. Monitoring programs revealed that pigs and poultry fed avoparcin develop vancomycin resistant *Enterococci* in human. Resistance had been reported in Enterococcal strains obtained from animals to the macroloide lincosamide-streptogramin group including the common antibiotic of tylosin [48]. Resistant non-enteric bacteria were also commonly reported in respiratory tract pathogens in all livestocks and resistant *Staphylococci* from bovine mastitis and small animal infections [49].

6. Cautions and precautions before prescribing veterinary antibiotics

When it is necessary to use veterinary antibiotics to safeguard animal health, many precautions should be considered:

a. The prescription should be based on clinical diagnosis by qualified veterinarian and conducting sensitivity testing to choose the proper antibiotic [39].

b. In general, metaphylaxis antibiotics should never be used, when good veterinary and husbandry practices are available [50].

c. Metaphylaxis antibiotics, when it is necessary, should be prescribed on the basis of clinical findings about the progress of a disease in certain herd or flock [19].

d. It is much better to isolate and separate sick animals and treated them individually [6].

e. Livestock producers should keep files and records to register causes and nature of infections and the available antibiotic products to facilitate the right and correct decision [14].

f. Narrow-spectrum antibiotics could be the first choice, unless sensitivity testing exhibit that they could be ineffective. Contrary, the broad-spectrum antibiotics should be avoided [6].

g. In re-current infection cases, tracing the causal bacteria is recommended to determine why the disease is recurring and to facilitate the pathogenic microorganisms eradication [9].

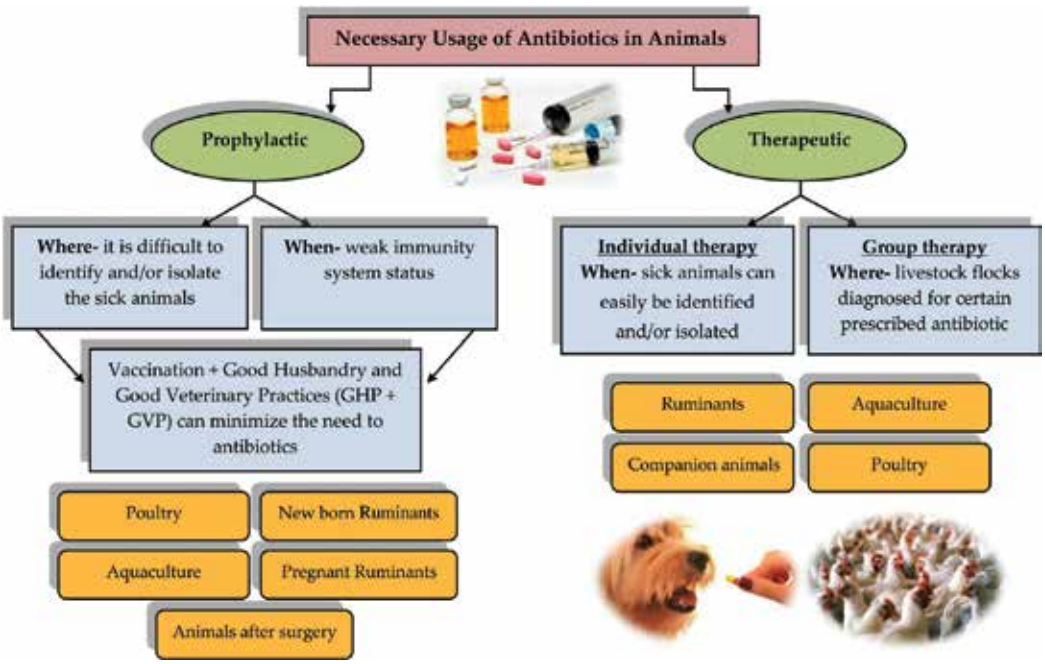

Figure 1. Cases necessitate the use of antibiotics in animals.

h. The need for antibiotic therapy should be reassessed on scientific basis to avoid unnecessary medications.

i. Antibiotics should be administered as the instructions of the prescriber and the manufacturer of the drug [13].

j. All alternative programs to control diseases like vaccination, good veterinary and husbandry practices should be applied together to minimize or reduce the need to veterinary antibiotics [7].

k. Advanced laboratories are recommended to perform rapid and accurate sensitivity testing and more advanced once are required to evaluate and control zoonotic and commensal microorganisms [9].

The common cases necessitate the usage of antibiotics in animals were summarized in **Figure 1**.

7. Economic considerations

It is well agreed and understood that agriculture is practiced to return profit to the farmers and livestock producers. So, two main economic considerations influence the selected programs adopted by livestock producers. The first limiting factor is to select and elect individuals or animal flocks capable to challenge diseases, and the second one is the response

of elected animals to enhance most production parameters. It is more economical to prevent diseases than treating them. The preventive efforts involve variety of practices including, proper nutrition, immunization, good veterinary and husbandry practices [4]. Prevention is needed for many diseases and it will be necessary to use prophylactic antibiotics during certain critical periods of animal life. Referring to the second consideration aiming to the response to enhancing most production parameters, livestock producer has the option to do or do not using antibiotics [39]. An economic evaluation of antibiotic usage in animal agriculture is calculated on the basis of return on such use which is not easy to quantify when used as prophylaxis. When antibiotics are used to treat diseases, it is easy to quantify the return, because the cost represents a portion of the necessary total expenses. In both cases, many factors should be considered, that is, costs of diagnosis, culture and sensitivity testing, confirmatory tests and the estimated loss of production based on the affected animals. Actually, the economic return on the use of antibiotics for prevention could be determined by direct comparison with similar farms not using antibiotics. Sub-therapeutic antibiotics are commonly administered to protect animal populations during the critical time when they are susceptible or expose to specific infections and hazards [9]. Thus, such approaches could be considered as economically cost effective, since they reduce the necessity of using higher therapeutic levels of antibiotics.

8. Risk and impact of veterinary antibiotics on human health and environment

Recently, there is a great concern between public in general and scientific communities, in particular, that exposure to pharmaceuticals including antibiotics may have negative effects on both human health and environment [3]. The adverse effects on human health may include the risk of chemical poisoning [51, 52], hypersensitivity reaction, especially with penicillin [53, 54], liver injuries [55], disruption in the normal intestinal flora [56] and occurrence of antibiotic resistance [54, 57]. The adverse effects of exposure to veterinary antibiotic residues in food is very difficult to trace, because bacterial resistance could take very long period of time. For example, the antibiotic chloramphenicol which used in human medicine to treat severe illness, when used as veterinary medicine for food-producing animals, dramatic effects were observed. Thus US-FDA banned the use of chloramphenicol in food-producing animals; even they approved the utility of the drug in treating systemic infections in cattle [9]. Also, because there is no threshold predicted for human aplastic anemia, chloramphenicol is completely banned for use in food-producing animals in many countries including Australia, Canada, EU and USA [7]. It is worthy to mention that chloramphenicol can be synthesized in soil which could be reached to feedstuffs consequently to the edible tissues of the animals. The Codex Committee on veterinary residues had recommended certain criteria for risk assessment of chloramphenicol or any antibiotic when used in food-producing animals [9]. Such criteria evaluate the potential of both short and long term of dietary exposure to antibiotic(s) residues on human health. However, misuse of antibiotics in animal husbandry and aquaculture could lead to the presence of residues in human food [57].

Transformation of antibiotic residues could easily reach soil, surface and ground water via many routes such as sub-surface flow, drain flow or leaching [11, 58]. The concentration of the transformed residues are affected by many factors including the chemical and physical properties of the antibiotic molecule, sorption behavior and persistence, exchange capacity of the soil matrix besides the various climate conditions [16]. Recent reports showed that sulfonamides and chloramphenicol could easily reach ground water, while fluoroquinolones could not leach. The reports added that in most areas of intensive livestock breeding, the source of contamination mainly attributed to irrigation with sewage [12, 16]. The uptake and accumulation of antibiotics into edible plants is commonly initiated when expose to contaminated soil with considerable concentrations of the drugs over time as confirmed by several studies [59–63]. However significant amounts of antibiotics and/or their degraded metabolites are introduced to agro-system via irrigation, fertilization with antibiotic-polluted manures, bio-solids, sludge, sediments and contaminated water [16, 64]. Accumulation and transport of antibiotics to edible plants poses high risk to crops, soil and water ecosystems [63, 65, 66], consequently, increasing risk to both human health and environment [3]. Applying and implementing good veterinary and husbandry practices are most urgent issues to control and reduce both the problems of antibiotic residue contamination and persistence of resistant bacteria.

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Chapter 3

Antibiotics in Chilean Aquaculture: A Review

Ivonne Lozano, Nelson F. Díaz, Susana Muñoz and Carlos Riquelme

Additional information is available at the end of the chapter

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Abstract

Aquaculture in Chile has been practiced since the 1920s; however, it was not until the 1990s that aquaculture became an important sector here. Important species in Chilean aquaculture include salmonids, algae, mollusks, and turbot. Salmonids are the dominant species in Chilean aquaculture for both harvest volume and export value, their production reaching greater than 800-thousand tons in 2015. However, this growth has been accompanied by an increase in disease presence, requiring greater drug use to control. This increase in drug use is an environmental and public health concern for the authorities, the salmon industry itself, and the destination markets. In this chapter, we review the literature on drug use, antibiotic resistance, regulatory framework, and alternatives, with focus on Chile.

Keywords: aquaculture, Salmon, antibiotics, food safety

1. Introduction: brief history of antibiotics use in Chilean aquaculture

Antibiotics have been used to treat animals since the 1940s, which was soon followed by the appearance of resistant bacteria [1, 2]. In 1969, the House of Lords in the United Kingdom published the "Swann report," highlighting the excessive use of antibiotics in animals and its potential risks to human and animal health. The Swan report suggested that antibiotic use should be restricted and regulated. The accumulated evidence from Europe and North America supports the notion that antibiotic use should be regulated and restricted to specific clinical situations [2].

In Chile, between 1973 and 1976, the first commercial fish farming of salmonids in the Region of the Lakes was consolidated and has grown ever since [3]. In the subsequent decades, four species of salmonids of commercial importance have been cultivated in

Chile: rainbow trout (*Oncorhynchus mykiss*), Chinook (*Oncorhynchus tshawytscha*), coho (*Oncorhynchus kisutch*), and Atlantic salmon (*Salmo salar*). In the period between 1987 and 2010, four diseases appeared in the salmon farming industry in Chile, coinciding with the beginning of antibiotic use by the industry in 1989. The diseases reported in these years were primarily due to the ectoparasite *Caligus* and bacterial kidney disease in 1987 [4–6]; the presence of *Piscirickettsia salmonis* in 1989 [4]; presenting later high mortalities due to IPN in 1997; and by the outbreaks of the infectious salmon anemia disease in the years 2002, 2007, and 2008 [5].

Sixteen antibiotics are used in animal treatments in Chile, compared to three in the United States (US) and four in Norway [7]. In the case of aquaculture in Chile, antibiotics have been mainly used in sea water Atlantic salmon farming, which accounted for 80% of the total use of antibiotics used for 2015, followed by 11% for coho salmon, 9% for rainbow trout, and 0% for Chinook salmon [8].

The consumption of antibiotics in the salmon industry in Chile has increased by 56% from 2005 to 2015, with a production increase for those years of 23.48%. The highest consumption was recorded in 2014, with a total use of 563.2 tons of antibiotics with 955,179 tons of salmonids produced. In 2016, there was a 30.66% decrease in antibiotic use compared to 2015, using a total of 382.5 tons of antibiotics to produce 727,812 tons of fish (**Table 1**) [8–11].

The most used antibiotics in the salmon farming industry in Chile are florfenicol and oxytetracycline. Florfenicol use has increased steadily since 2013 and accounted for 87% and 82.50% of the total antibiotics used in 2015 and 2016, respectively [8–11]. Florfenicol

Year	Antibiotics use annual quantity (ton)	Annual production of salmonids (ton)
2005	239.1	614.435
2006	343.8	647.302
2007	385.6	600.862
2008	325.6	630.647
2009	184.4	474.174
2010	142.2	466.857
2011	206.8	649.492
2012	337.9	836.949
2013	450.7	786.091
2014	563.2	955.179
2015	557.2	846.163
2016	382.5	727.812

Table 1. Historical consumption of antibiotics in salmon farming industry in Chile [8–11].

is mainly used in the seawater stage to control piscirickettsiosis (SRS) caused by the Gram-negative facultative intracellular pathogen *P. salmonis*. In salmonids, this epizootic disease has high mortality rates (78.9% for Atlantic salmon, 82.9% for rainbow trout, and 59.3% for coho salmon) [9].

In 2005, SRS was the most diagnosed pathology, accounting for 77.04% of the antimicrobials used in the year 2005 by the industry [9]. This trend has been maintained; 89.3% of all antibiotics used for the year 2016 have been for the control of SRS at the seawater stage followed by 6.8% for the control of renibacterosis [10].

2. Antibiotic resistance

2.1. Mechanisms of *P. salmonis* infection

In the last decade, there have been significant advances in the knowledge of *P. salmonis*, including aspects of its survival behaviors under stress conditions and genomic data. One of the first achievements has been the culture of this pathogen in the cell-free medium [12, 13]; previously, it had been necessary to develop and maintain cultures of fish cell lines. This progress has allowed the study of the physiology and behavior of this bacterium. An interesting recent finding is that *P. salmonis* can form biofilms. The development of *P. salmonis* biofilms occurs under stress conditions and salt concentrations similar to those of seawater. The biofilm matrix of *P. salmonis* is composed of exopolysaccharides and is disaggregated when treated with cellulases, which are relevant since biofilm formation might be a survival mechanism in the marine environment of this bacterium [14].

It is known that within biofilms, microorganisms are more resistant to the action of chemotherapeutics and have better survival rates under adverse conditions [15]. Recent findings have identified genes that have a role in the formation of *P. salmonis* biofilms such as the cheA gene [16]. This gene plays a key role in modulating the initiation of bacterial chemotaxis in other bacteria, such as *Pseudomonas pseudoalcaligenes* KF707 [17]. Using real-time PCR, it has been shown that cheA expression is increased during *P. salmonis* biofilm development. The results obtained in this research also suggest interaction between the formation of biofilms and the genes involved in the chemotaxis of this pathogen. Biofilm production has been reported as a potential mechanism of pathogenicity in several aquatic bacteria [18, 19]. It is very likely that the first contact with fish for the development of biofilm is produced by chemotactic responses. Chemotaxis to fish mucus has been previously reported as the first step in the development of pathogenic activity [20].

Some authors have suggested that *P. salmonis* infections begin when bacteria overgrow the skin barrier or gills [21]. In this regard, experimental infections were performed in juveniles of *Oncorhynchus mykiss* obtained from areas where the presence of SRS has never been reported, infecting them at six different entry sites. These authors found that the main entrance routes

are through skin and gills and that the oral route is not used to initiate *P. salmonis* infection of salmonids [22]. Later, this same research group performed experimental infections in coho salmon (*Oncorhynchus kisutch*). The results of cumulative mortality and survival analyses showed that the most effective entry portal was the skin, followed by intestinal intubation and finally by gill infection [23]. These findings show that *P. salmonis* can penetrate and then systemically invade the Coho salmon through the skin and mucous membranes, which appear intact at the macroscopic level, and that the skin is probably the most important site of entry of this bacterium into salmonids. These findings support the notion that biofilm formation initiates colonization of the fish, thereby activating other virulence factors such as proteases to initiate ulcerations and invasion of the organism. *P. salmonis* is not a motile bacteria and a chemotaxis process could not be activated toward the fish mucus as it happens in other fish pathogens [20]. Studies have shown that other nonmotile pathogenic bacteria can adhere to their host through their net electrostatic charges [24]. Also, nonmotile pathogens infect a host using proteases, and the genes encoding these proteases can be transmitted to nonprotease mutant strains [24].

Recent research carried out on coding and noncoding transcript during an *in vivo* infection process of Atlantic salmon with *P. salmonis* identified a common response associated with oxidation-reduction processes, endocytosis, and ion responses. In the different types of analyzed tissues, the clathrin protein, which plays a major role in the formation of coated vesicles, was significantly upregulated in infected individuals, suggesting the importance of clathrin-mediated endocytosis for the bacterial internalization. Moreover, several endocytosis receptors were repressed during the challenge [25].

2.2. Piscirickettsia salmonis resistance to antibiotics

As mentioned above, the use of oxytetracycline and florfenicol are mostly used to control *P. salmonis*. Florfenicol use almost doubled between 2013 and 2016, suggesting that the battle against this pathogen has been unsuccessful. The evolution of resistance of *P. salmonis* to antibiotics has been demonstrated. Recent isolates of *P. salmonis* (SLGO94 and SLGO95) present a higher level of resistance to antibiotics than earlier isolates (LF-89 and EM-90), suggestive of antibacterial resistance [26]. Subsequently, a large-scale study conducted to evaluate the susceptibility profiles for quinolones, florfenicol, and oxytetracycline from 292 field isolates obtained from different farm sites over a 5-year period revealed a high incidence of resistance to quinolones and early resistance to oxytetracyclines and florfenicol [27, 28].

P. salmonis genes encoding membrane-carrying proteins are upregulated in the presence of antibiotics [29]. The *P. salmonis* genome encodes efflux pumps that enable this bacterium to survive at critical concentrations of florfenicol [30]. Thus, despite the use of antibiotics, there are antibiotic-resistant (especially quinolone) bacteria in sediments near farming areas [31]. These bacteria carry plasmids that confer resistance to quinolones in marine bacteria [32]. **Figure 1** shows a proposed model for *P. salmonis* infection during the seawater stage in salmonids.

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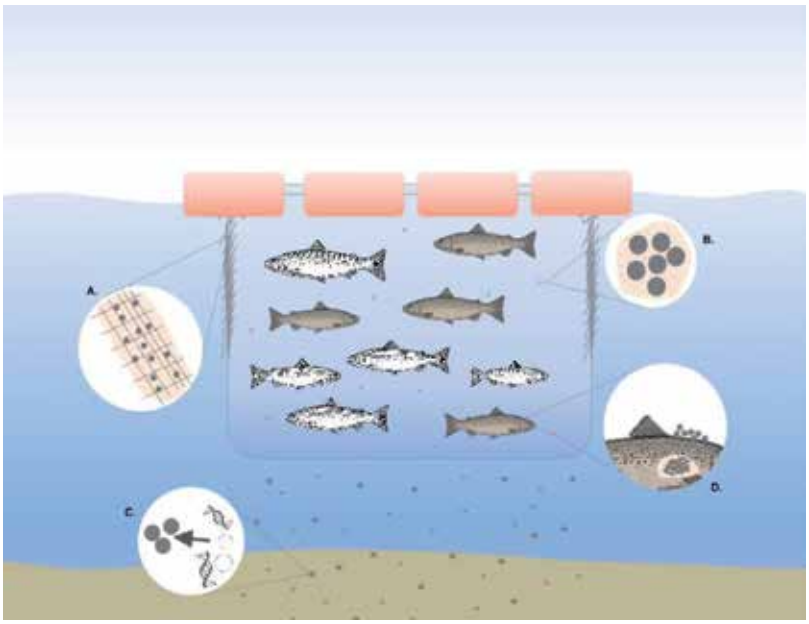

Figure 1. Model of *in situ* infection of *P. salmonis*. (A) Biofilms of *P. salmonis* in culture net; (B) biofilms in microaggregates; (C) transference of resistance genes in sediments; (D) colonization of *P. salmonis* on fish surface in captivity and onset of pathogenesis by contact with microaggregates or net. Elaborated by: Carlos Riquelme and Victor Sanchez.

3. Analysis of the regulatory framework

In Chile, in its Article 86, the Fisheries and Aquaculture law prohibits the preventive application of antimicrobials in a preventive way in aquaculture, as well as any use harmful to human health. Subsequently, there have also been the supreme decree N°319/2001, Regulation on Protection Measures for the Control and Eradication of High-Risk Diseases for Hydrobiological Species; the exempt resolution N°8228/2015 [33], the Manual of Good Practices in the Use of Antimicrobials and Antiparasitics in Chilean Salmon Farming [34]; the exempt resolution N° 5.125/ 2016, Manual on Food Safety and Certification [35]; and the Quality Assurance Program (PAC) for Fisheries and Factories Vessels [36]. This regulatory framework is jurisdiction of SENAPESCA (National Fisheries and Aquaculture Service) institution that is part of and depends on the Ministry of Economy, Development and Tourism.

According to the current legislation, only pharmaceuticals for exclusive veterinary use registered or authorized for application in hydrobiological species can be used [37]. The pharmaceutical products authorized by Servicio Agrícola Ganadero (SAG) are shown in **Table 2** [34]. The studies of effectiveness, adequate dosage, animal safety, and human food safety (toxicology) of these authorized pharmaceutical products are not available to the public or were not found.

Generic name	Trade name and registry no.	Presentation/route of administration	Registered by	Withdrawal period (degree days)	Dose mg/kg lw*/day	Tolerance level in muscle tissue (μg kg^{-1})
Oxolinic acid 80%	Reg. N°441	Powder/oral	FAV S.A.	450	20 per 10 days	100
	Litoflox Reg N°648	Powder/oral	Centrovet LTDA.	450	10–30 per 10 days	
	Bandrol Reg N°481	Powder/oral	Veterquímica S.A.	450	10 per 10 days	
Amoxicillin 50%	Amox-Feed Reg N°121	Powder/oral	Veterquímica S.A.	300	70.4 per 10 days	50
Erythromycin 50%	Vetromic Reg. N°1402-B	Powder/oral	Centrovet LTDA.	500	75–100 per 21 days	200
Erythromycin 80%	Eritofeed Reg. N°616-B	Powder/oral	Veterquímica S.A.	500	92.5 per 21 days	
	Vetromic Reg N°1803-B	Powder/oral	Centrovet LTDA	500	75–100 per 14–21 days	
Flumequine 10%	Flumepren Reg. N°79	Powder/oral	Centrovet LTDA	300	(–)	***500 ****600
Flumequine 50%	Reg. N°484	Powder/oral	FAV S.A.	300	12–25 per 10–12 days	
	Reg. N°646	Powder/oral	Centrovet LTDA	300	12–30 per 10 days	
Flumequine 80%	Reg. N°442	Powder/oral	FAV S.A.	300	20 per 10 days	
	Flox-Feed Reg. N°478	Powder/oral	Veterquímica S.A.	300	10 per 10 days	
	Flumepren Reg. N°645	Powder/oral	Centrovet LTDA.	600	12–30 per 10–15 days	
Florfenicol 50%	Florfenox Reg. N°1537	Powder/oral	Bayer S.A.	300	10 per 10 days	1000
	Veterin Reg. N°1556	Powder/oral	Centrovet LTDA.	300	10 per 10 days	
	Duflosan Reg. N°1769	Powder/oral	Veterquímica S.A.	300	10 per 10 days	
Florfenicol 50%	Duflosan L Reg. N°2264	Solution/oral	Veterquímica S.A.	100	10 per 10 days	1000
	Aquafen Reg. N°1193	Powder/oral	Intervet Chile LTDA.	200	10 per 10 days	
	Reg. N°1598	Powder/oral	FAV S.A.	300	10 per 10 days	

Generic name	Trade name and registry no.	Presentation/route of administration	Registered by	Withdrawal period (degree days)	Dose mg/kg lw*/day	Tolerance level in muscle tissue (μg kg^{-1})
Oxytetracycline hydrochloride 20%	Terrivet F200 Reg. N°2252	Suspension for injection	Veterquímica S.A.	1060	**20	200 (–) Tetracyclines
Oxytetracycline 50%	Terrivet Reg. N°149	Powder/oral	Veterquímica S.A.	600	75 per 15 days	
Oxytetracycline 80%	Terrivet Reg. N°485	Powder/oral	Veterquímica S.A.	600	75 per 15 days	
	Reg. N°1595	Powder/oral	FAV S.A.	600	55–82 per 10 days	
	Zanil Reg. N°1380	Powder/oral	Centrovet LTDA.	600	75 per 10 days	
Oxytetracycline 40%	Reg. N°309	Powder/oral	Laboratorio Veterinario Quimagro S.A.	600	13.57–20.75 per 10 days	

*lw = live weight.

**mg/kg/lw.

***Trout.

****Other salmonids.

Table 2. Antimicrobials for salmonids authorized by the Veterinary Medicines Registry (SAG) [34].

The Chilean authority supervises the use of pharmaceutical products in hydrobiological species, and in accordance with the provisions of the general and specific health programs, therapeutic treatments applied to populations of hydrobiological species should be prescribed by a veterinarian and the application of antimicrobials for prophylactic purposes is prohibited. Before the application of the antimicrobials, fish samples should be obtained for subsequent confirmation of the diagnosis by laboratory analysis [37]. Farming facilities should keep records of antimicrobial treatments performed and antimicrobial treatments should be reported monthly through the Aquaculture Inspection System (SIFA) [34].

Extralabel antimicrobials can be prescribed by a veterinarian when the health of an animal is at risk, there is danger of death, or there is suffering of the animal; or when one of the following is fulfilled: dosage, timing, duration of treatment or route of administration for a registered product does not obtain the expected response; the product is temporarily unavailable on the market; or there is no registered product to treat a diagnosed condition [34].

The Manual on Food Safety and Certification (resolution N° 5.125/ 2016) describes the norms and procedures that allow to guarantee the sanitary quality of the fishery and aquaculture products destined for international markets along the whole productive chain. Regarding

the procedures for the control of residues of pharmaceutical products, each farm facility must demonstrate (and issue a declaration of guarantee) that the concentrations of residues of pharmaceutical products in fish do not exceed the limits established by the Chilean authority [35].

The analysis must be carried out in authorized laboratories, according to the Methods of Analysis of Residues of Pharmaceutical Products and Contaminants for Export Fishery Products. If the maximum allowable limits are not met, the withdrawal period should be extended and a new sampling should be carried out [35].

The government of Chile also maintains a Program of Surveillance and Control of Piscirickettsiosis, in which monitoring system and the application of control measures are established for this disease [38]. Upon request, the Chilean authority issues fish farming centers a certificate stating that the fish are free of antimicrobial and/or antiparasitic treatments [39]. The Quality Assurance Program (PAC) is a voluntary certification program, based on the concept of hazard analysis and critical control point (HACCP), which applies only to fishing plants and factory vessels. This program, however, is mandatory for all companies that are authorized to export to the European Union and the United States. The Chilean authority must approve the quality assurance plan for the industry and supervise its subsequent operation [36]. The administrative procedures, work guides, and specific requirements of this program (PAC) are not publicly available.

The main Chilean salmon markets are the US and Japan. In the case of the US, the Food and Drug Administration (FDA) is in charge of regulating the use of antibiotics in fish, primarily through its regulation 21 Code of Federal Regulations (CFR) 123 "Procedures for the Safe and Sanitary Processing and Importing of Fish," which aims to ensure the safe and sanitary processing of fish and fishery products (seafood), including imported seafood [40]. The regulation mandates the application of HACCP principles to the processing of seafood as a preventive system of hazard control that can be used by processors to ensure the safety of their products to consumers. For the control of drugs for use in food of animal origin, direct medication or for addition to feed must be approved, conditionally approved, or index listed by the FDA (Federal Food, Drug, and Cosmetic Act Section 512) [41].

Under certain conditions authorized by FDA, unapproved new animal drugs may be used in conformance with the terms of an Investigational New Animal Drug (INAD) application (21 CFR 511) [42] and FDA's Center of Veterinary Medicine (CVM) guide 1240.3025. When a drug is approved by CVM, the condition of the approval is listed on its label or in the labeling (21 CFR 514.1) [43]; this condition specifies the species for which the drug is approved for use, indications for use, dosage regimen, and other limitations such as route of administration and withdrawal time. Labeled withdrawal times must be followed to ensure that no harmful drug residues are present in the edible tissue of the animal when harvested for human consumption; tolerances for some drug residues in the edible tissue have been established [44].

Relatively few drugs have been approved for aquaculture in the US (**Table 3**). This has led to the inappropriate use of unapproved drugs, general-purpose chemicals, or approved

Antibiotic	Dose	Approved for:	Route of administration	Tolerance level in muscle tissue	Withdrawal period
Terramycin 200 (oxytetracycline dehydrate)	2.5–3.75 g/100 lb. of fish/day Maximum dose = 8.33 mg/kg/day/10 days	Salmonids: For control of ulcer disease caused by *Haemophilus piscium*, furunculosis caused by *Aeromonas salmonicida*, bacterial hemorrhagic septicemia caused by *A. liquefaciens*, and pseudomonas disease, for control of mortality due to cold-water disease associated with *Flavobacterium psychrophilum*. Freshwater-reared *Oncorhynchus mykiss*: For control of mortality due to columnaris disease associated with *Flavobacterium columnare*	Administer in mixed ration for 10 days; do not liberate fish or slaughter fish for food for 21 days following the last administration of medicated feed.	2 ppm (As the sum of tetracycline residues)	21 days for disease control in salmonids 7 days for marking skeletal tissue in Pacific salmon
OxyMarine Oxytetracycline HCL soluble powder-343, TETROXY Aquatic	200–700 mg oxytetracycline/L of water for 2–6 h	For marking of skeletal tissues in finfish fry and fingerlings as an aid identification	Immersion	2 ppm (as the sum of tetracycline residues)	21CFR 556.500
Sulfadimethoxine/ormetoprim combination Romet-30		For control of furunculosis in salmonids (trout and salmon) caused by *Aeromonas salmonicida*	Medicated feed	0.1 ppm for each drug (21 CFR 556.490)	42 days (21 CFR 558.575)
Florfenicol Aquaflor	10 mg/kg fish per day for 10 consecutive days	For the control of mortality in freshwater-reared salmonids due to cold-water disease associated with *Flavobacterium psychrophilum*	Medicated feed	2 ppm in salmon muscle/skin	15 days

Table 3. FDA aquaculture approved drugs, route of administration, and tolerance levels [40, 44].

drugs in a manner that deviates from the labeled instructions [44]. Studies establishing the effectiveness, adequate dosage, animal safety, and human food safety (toxicology) of these approved drugs are available to the public [45].

In the case of Japan, fishery products are regulated by the Food Sanitation Act and the Food Safety Basic Act. The authorities involved with in the Food Sanitation Act are as follows: the Office of Import Food Safety; Inspection and Safety Division; Pharmaceutical and Food Safety Bureau; Ministry of Health, Welfare and Labor; and the Department of Food Safety. The purpose of the Food Sanitation Act is to prevent the occurrence of health hazard arising from human food so as to contribute to the protection of people health by conducting regulations and measures deemed necessary, from the view point of public health and for securing food safety [46].

The purpose of the Food Safety Basic Act is to promote comprehensive measures to secure food safety by laying down the basic principles of safety for food, defining the responsibility of the government, local authorities, and food-related businesses, clarifying the role of consumers, and establishing basic policies for developing measures. The authority concerned is the Consumer Affair Agency [46].

Antibiotic residue concentrations for edible products from food-producing animals are determined based on jurisdictional-specific regulations that result in the determination of a tolerance or maximum residue level (MRL) for specific drugs in a specific tissue for specific animal species and based on toxicological assessments. This index estimates the amount of substance in food that can be ingested over a lifetime by humans without significant risk to health [47]. There are notable differences among MRLs or tolerances set by the different agencies regarding the two antibiotics most used in Chilean salmon farming (**Table 4**).

Many methods have been developed for analysis of antibiotics in fish. HPLC and mass spectrometry (HPLC-MS/MS) is the most sensitive method for the detection of these antibiotics and is currently regarded as the tool of choice for analysis of antibiotic residues in

Antibiotic	Europe (μg kg^{-1}) MRL	[e]Chile (μg kg^{-1})	[f]USA tolerance (ppm)	[g]Japan (ppm)
Oxytetracycline	[a]100	200	[c]2	[d]0.2
Florfenicol	[b]1000	1000	1	0.2

[a]508/1999/EC.

[b]1322/2001/EC.

[c]As a sum of tetracycline residues [44].

[d]Calculated as oxytetracycline.

[e][34].

[f][44].

[g][48].

Table 4. Antibiotics used in Chilean salmon farming and their maximum residue limits in salmonids (MRLs).

animal-derived food [47, 49], having a limit of detection (LOD) in fish of 10.3 ng/l [50] and a limit of quantification of 20 ng/l for tetracyclines [51].

4. Alternative antibiotic treatment in salmon farming

Fish are considered as the earliest class of vertebrates to have both innate and adaptive immunity, though the latter defense mechanism is not as elaborate as in higher vertebrates. Unlike in mammals, the alternative complement pathway in teleosts is relatively high and can mediate the lysis of target erythrocytes from several species. These features, along with their potential to function at varying temperatures, suggest that the complement system is a powerful defense mechanism in fish [52–54], and they are in constant interaction with their surroundings and therefore could easily encounter potential pathogens. In the wild, fish can protect themselves using innate defense mechanisms (either constitutive or responsive) [52].

The various alternatives to the use of antibiotics can be classified according to the action toward the pathogen or host. Pathogen-directed strategies include inhibitors of growth and virulence genes, antibacterial compounds, and the phage therapy. Host-directed strategies include the improvement of health, stress prevention, stimulation of the defense system, and selective breeding for disease resistance [55].

One of the first lines of defense against bacterial infection is the withholding of nutrients, termed nutritional immunity. The most significant form of nutritional immunity is the sequestration of iron [56]. Recent studies have detected a relationship between iron transporter glycoproteins and *Salmo salar* susceptibility to pathogens [57, 58]. In salmonids, an iron transporter glycoprotein has been identified as a vaccine enhancer [59]. In vertebrates, it has been shown that iron transporter glycoproteins exert antibiofilm therapeutic [60, 61] and antimicrobial activity by binding to iron, thereby preventing its use by bacteria [61–64] and thus causing alterations in the bacterial wall and, ultimately, death. Because of its cationic nature, this glycoprotein binds to the lipopolysaccharides of Gram-negative bacteria, thereby attenuating those proinflammatory processes induced by bacterial lipopolysaccharides [63]. Among alternative sources of bioactive compounds, ingredients or products derived from marine algae show great potential for use in aquaculture [65]. Rainbow trout–supplemented diets with phytopharmaceutical of herbal and macroalgal origin have improved resistance against *P. salmonis* [66].

Several bacteriophages have been isolated against the following pathogenic bacteria, *Edwardsiella tarda, Edwardsiella ictaluri, Lactococcus garvieae, Pseudomonas plecoglossicida, Streptococcus iniae, Flavobacterium columnare, Flavobacterium psychrophilum, Aeromonas salmonicida, Aeromonas hydrophila, Vibrio anguillarum, Vibrio harveyi, and Vibrio parahaemolyticus*, and their potential to be used as a therapeutic agent has been studied by several researchers [67]. In salmonids, *Flavobacterium psychrophilum* phages have shown protection

against bacterial cold water disease *in in vitro* conditions. Each phage isolate rarely infected *F. psychrophilum* strains other than the strain used for its enrichment and isolation. Some bacteriophages decrease mortality from intraperitoneal injection of their host strain when added together with the bacteria at a ratio of 10 plaque-forming units per colony-forming unit [68].

Promising results have been obtained in laboratory studies. However, high concentrations of bacteriophages in seawater can induce bacterial genetic variation. This occurs through mutation and bacteriophage-mediated horizontal transmission of genetic material between different bacteria mediated by bacteriophages [5, 69]. The use of phages can also influence bacterial community dynamics and ecosystem biogeochemistry. These influences differ depending on whether phages establish lytic, chronic, or lysogenic infections. The impacts of lysogeny are well studied at the cellular level, but ecosystem-level consequences remain underexplored [70].

Probiotics have been credited for producing improved nutrition, health benefits, reduced disease incidence, improving growth, health status, immunity, feed conversion, microbial balance, and water quality, as well as food production in an environmental-friendly way [71–73]. Probiotics in aquaculture can be live or dead preparations, including cellular/extracellular components of the microorganism(s), administered either as a feed supplement or to the rearing water. Probiotics can be used to control a range of bacterial pathogens in various fish species [69]. For example, rainbow trout (*Oncorhynchus mykiss*) were protected against *Aeromonas salmonicida* and *Yersinia ruckeri* when administered with dietary *Carnobacterium maltaromaticum* and *C. divergens* [72]. The efficacy of *Carnobacterium* sp. at reducing diseases caused by *A. salmonicida*, *V. ordalii*, and *Y. ruckeri* in salmonids has also been demonstrated [72]. However, there is no solid knowledge regarding the potential of probiotic against *P. salmonis*.

The Food and Agriculture Organization of the United Nations (FAO) has now highlighted the use of probiotics in aquaculture as a means of improving the quality of the aquatic environment [72]. However, concerns have been voiced about the possible acquisition of antibiotic resistance and virulence genes via horizontal gene transfer, which might lead to safety problems if using live probiotics in an open aquatic environment. Probiotics can also affect host tissue and result in severe cell damage. To avoid this, probiotic strains must be recognized as safe for the cellular integrity of the host [72].

In the aquaculture industry, vaccination strategies include traditional inactivated and attenuated vaccines, as well as next-generation vaccines comprising recombinant, subunit, vectored, genetically engineered, DNA and peptide vaccines, reverse vaccinology, plant-based edible vaccines, and nanovaccines [74]. Current vaccination protocols for *P. salmonis* include whole cell, inactivated and adjuvant vaccines for injection (primary immunization), followed by oral boost (where the timing of boost delivery is determined by measuring circulating antibody levels against the pathogen). Live vaccines and DNA vaccine studies have been unsuccessful under laboratory conditions. There are more than 25 different vaccines against SRS that are available in the Chilean market. These vaccines confer good short-term protection against disease and mortality but are inefficient at conferring long-term protection, or the duration of protection is insufficient to protect the fish throughout their economic life [75–77].

5. Conclusions

Veterinarians in charge of the salmon industry in Chile have used large quantities of antibiotics relative to its production volumes. In the years of highest production, an average of 600 g ton^{-1} produced was used. The antibiotics used by this industry are florfenicol and oxytetracycline for the control of *P. salmonis* at the seawater stage; studies have demonstrated the resistance of this pathogen to quinolones, oxytetracycline, and florfenicol, as well as their mechanisms of resistance.

There are 12 different types of generic and 25 branded antimicrobials authorized for use in salmonids in Chile, with no specifications related to pathogens or diseases. This is in contrast to the US situation, where the FDA has approved just four antibiotics for specific uses and against certain pathogens.

The studies of the effectiveness, adequate dosage, animal safety, and human food safety (toxicology) of the authorized pharmaceutical products, as established by the Chilean authority, are not available to the public or were not found. This was also the case for the administrative procedures, work guides, and specific requirements of the Quality Assurance Program (PAC), whereas the effectiveness and toxicology studies of the FDA-approved antibiotics are freely available online. This absence of Chilean regulation and antibiotic data is concerning. To avoid chemical hazards and ensure food safety, we propose that a mandatory legal framework based on international regulations is needed in Chilean aquaculture. Antimicrobial treatment is required for an efficient production of animal products; however, antibiotics should never be used as a substitute for proper nutrition and hygiene management.

The alternatives to the use of antibiotics in Chilean salmon farming, such as the use of nutritional immunity, phytopharmaceuticals, probiotics, antimicrobial peptides, and selective breeding for disease resistance, require advanced research with *in vivo* studies. Although several vaccines have been authorized, this remains an inefficient strategy for the control of pathogens in aquaculture.

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Chapter 4

Antibiotic-Treated SPF Mice as a Gnotobiotic Model

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Abstract

Decontamination of specific pathogen-free (SPF) mice of BALB/c line was accomplished by administration of amoxicillin *per os* potentiated with potassium clavulanate at a dose of 387.11 mg/kg body weight and ciprofloxacin administered s.c. at a dose of 18.87 mg/kg body weight every 12 h for 5 days. This resulted in a decreased viability of microorganisms in feces and the cecal content of mice and decreased counts of cultivable microorganisms in the feces, which by day 3 of study declined below the recovery level and to the reduction of animal microbiota to two detected cultivable species, namely *Escherichia coli* (GenBank KX086704) and *Enterococcus* sp. (GenBank KX086705). Convalescence of decontaminated animals under gnotobiotic conditions for 10 days prevented restoration of species diversity of mice microbiota and sufficed to return the metabolic, hematological and morphological values to the physiological range. It also restored the fermentative activity of the intestine to the level similar to that observed before antibiotic treatment. Animals subjected to this procedure can be used in further studies. As a result, we created a mouse gnoto model with reduced and controlled microbiota without alteration of the overall health status of the respective animals.

Keywords: amoxicillin-clavulanic acid, ciprofloxacin, mice, gnotobiotic, decontamination

1. Introduction

Autochthonous microbiota in the gastrointestinal tract (GIT) of mammals are a complex, dynamic, spatially and density diverse community of non-pathogenic micro-organisms. They are a metabolically active entity [1], playing an important role in affecting morphology of the intestine and thus also in its maturation and development, in forming a key barrier

against pathogenic bacteria, affecting the immune system through modulation and providing essential products of its metabolism to the host. Accumulating evidence reveals that the gut microbiota plays a major role in promoting health, as a result of which it is often referred to as the "forgotten organ" [2, 3]. These microbiota are key factors in maintaining homeostasis, with functions affecting virtually every organ in the body, such as the regulation of bone mass [4], brain development and behavior [5–7], hepatic function [8], and aspects of adipose tissues [9] and the cardiovascular system [10].

In the several past decades, many animal models were used in the studies of dynamically and ecologically diverse community of micro-organisms in gastrointestinal tract (GIT). These micro-organisms are exactly those that help us to understand better the biological complexity of processes underlying their symbiotic relationships with the host. Extensive use of rodents in experiments is related to the fact that these animals can adjust easily to new conditions, multiply quickly, exhibit low nutritional needs and have low requirements on their environment [11]. Like human beings, conventional rodents harbor trillions of bacteria and viruses [12]. The uniformity of microbiota assumed previously in the controlled populations of inbred laboratory animals may not be so high. Some variations may be caused by differences in rearing and handling of animals, and others may result from factors that have not been identified as yet and may affect composition of the microbiota within populations and individuals over time. This should be taken into account when designing experiments involving laboratory animals and interpreting results of such experiments [13]. Despite the fact that only few studies were dealing with systematic comparison of microbiota of highly hygienically standardized mice to those kept in less strict environment, there is sufficient background that allows one to assume limited species complexity in highly microbiologically standardized animals [14, 15]. With increasing use of such rodents, it is reasonable to expect that microbiota of limited diversity alters the known responses of rodents within experimental settings [16]. Using a simplified approach, laboratory animals can be divided to conventional laboratory animals, i.e. those harboring various proportions of other live organisms, and gnotobiotic laboratory animals with accurately defined microbiota. The term germ-free (GF) (axenic) refers to an animal demonstrably free from microbes, including bacteria, viruses, fungi, protozoa, and parasites, throughout its lifetime [17, 18]. GF animals selectively colonized with one or more bacterial species are referred to as gnotobiotic [19, 20]. This term is derived from the Greek "gnotos", meaning known, and "bios" which means life [17, 21]. Gnotobiotic animals offer a wide range of advantages compared to other animal models when studying the physiology of the digestive tract. This involves particularly the study of mutual interaction of natural microflora and pathogens in the digestive tract and the mechanisms of probiotic effects of microorganisms [22]. Germ-free animal models have been used to explore host-microbiota interactions in entire fields, including lipid metabolism [9], cardiology [10], neurogastroenterology [5, 6, 23, 24], reproductive biology [25, 26], and bone homeostasis [4].

An alternative is a temporary gut sterilization, which may involve absolute or selective elimination of microflora [27, 28]. Some researchers [29, 30] described procedures based on oral administration of antibiotics that allowed them to achieve complete elimination of bacterial

flora of rats' digestive tract and to maintain its bacteria free status. In other studies, various cocktails of antibiotics sufficed to completely or selectively sterilize the gastrointestinal tracts of mice and rats [31–34]. Administration of oral antibiotic for the purpose of gut sterilization facilitated physiological studies of the nutritionally important relationship between the intestinal microflora and the host. However, when carrying such studies one must consider the extreme variability of such gut flora and thus expect considerable variations of the efficacy of antibiotics in gut sterilization between and within species. Therefore, it is necessary to test effectiveness of any antibiotic cocktail before its implementation [27]. Since the microflora of laboratory specific pathogen-free (SPF) mice is partially controlled and these animals do not come into contact with antimicrobial substances, they are the most suitable model for decontamination [35]. Due to the frequent testing, these animals do not serve as a reservoir of multiresistant or nosocomial micro-organisms [16]. By using antibiotics for decontamination of these animals, one can reduce considerably the number and species diversity of their microbiota.

Our study focused on obtaining an animal model with reduced and controlled microflora ensuring at the same time good health of these model animals.

2. Material and method

2.1. Isolator technology

The experiment was carried out in three germ free isolators (Velaz s.r.o., Prague, Czech Republic) using a gnototechnology described previously by Gancarčíková et al. [22]. A routine microbiological control of isolators was performed throughout the experimental study. Microbiological swabs were taken from gnotobiotic isolator walls, surface of animals and from their rectum. They were inoculated onto TSA agar (tryptic soy agar) with 5% ram's blood (BBL, Microbiology Systems, Cockeysville, USA).

2.2. Animals, housing and diet

The experiment was carried out on 66 specific pathogen-free (SPF) BALB/c female mice, (4 weeks old), obtained from Velaz s.r.o. (Prague, Czech Republic). All experimental procedures were approved by the Ethics Commission of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia. The experimental protocol No. 1177/14–221 was approved by the State Veterinary and Food Administration of the Slovak Republic and the animals were handled and sacrificed in humane manner in compliance with the guidelines established by the relevant commission. All applicable institutional, national and international regulations for the care and use of experimental animals were observed. The conventional SPF mice were transported by air in special transport containers to the experimental facilities of the Laboratory of Gnotobiology, University of Veterinary Medicine and Pharmacy (UVMP) in Košice. After a thorough surface disinfection of the containers with peracetic acid, these were transferred to gnotobiotic isolators (Velaz s.r.o., Prague, Czech Republic). After subsequent

venting of peracetic acid vapors, the mice were transferred to three breeding polypropylene cages, 7–9 mice per cage. The following groups were formed: negative control C (n = 7); decontaminated/antibiotic- treated group DC (n = 9); decontaminated/antibiotic- treated and convalesced group DC + R (n = 8). All animals were fed *ad libitum* complex mixed feed for mice in system, a barrier feeding system ST-1 (Velaz s.r.o., Prague, Czech Republic), and had unlimited access to water kept in glass bottles. The diet contained (kg diet) crude protein 24%, crude fat 3.4%, crude fiber 4.4%, ash 6.8%, calcium 11 g, sodium 1.8 g, phosphorus 7.2 g, copper 20 mg and selenium 0.38 mg (vitamin A 28000 IU, vitamin D 2200 IU, vitamin E 100 mg). The mice were kept at temperatures maintained between 20 and 24°C, at relative humidity of 45–65%, under a 12-h light/dark regimen. Lignocel 3-4S (Velaz s.r.o., Prague, Czech Republic) bedding intended for barrier breeding system was used.

2.3. Antibiotic treatment of SPF mice

The experimental mice were administered amoxicillin and clavulanate potassium (Amoksiklav 2 × 457 mg/5 mL, Sandoz Pharmaceuticals, Ljubljana, Slovenia) perorally at a dose of 387.11 mg/kg body weight (0.2 mL of dilution) every 12 h during the first 5 days of the experiment.

Ciprofloxacin (Ciloxan 1 × 5 mL/15 mg, Alcon Cusí S.A., Barcelona, Spain) was administered subcutaneously at a dose of 18.87 mg/kg body weight (0.1 mL of dilution) every 12 h during the first 5 days of the experiment.

2.4. Sampling procedures

Health of the animals and consistency of feces were observed and recorded daily. Fresh fecal samples were collected on days 0, 1, 2, 3, 5 and 15 of the study. Blood samples for hematological and biochemical analysis were collected from anesthetized animals using retro-orbital technique. Anesthesia was induced with sodium pentobarbital at a dose of 86 mg/kg body weight. The mice were euthanized by cervical dislocation at the end of the study for the purpose of sample collection. During dissection, weight of internal organs (heart, liver, spleen, kidneys and lungs) was recorded and samples of feces, caecum and *lobus caudatus hepatis* from the liver were collected. Samples for microbiological examination, determination of percentage survivability by fluorescence-activated cell sorting (FACS) and fluorescence microscopy examination by visualization method with viability fluorescent quick test on a polycarbonate filter (VFQTOPF) were processed immediately, while samples for determination of production of organic acids were stored at −20°C until analysis. Samples from *lobus caudatus hepatis* intended for histological analysis were fixed in 4% solution of paraformaldehyde until analysis.

2.5. Microbiological analysis

2.5.1. Bacterial enumeration

For microbiological analysis, samples of feces and caecum were collected individually from each animal. The samples (1 g) were homogenized Stomacher Lab Blender 80 (Seward

Medical Limited, London, UK) with 9 mL of a sterile anaerobic diluent (0.4 g $NaHCO_3$, 0.05 g), L-cysteine-HCl, 1 mL resazurin (0.1%), 7.5 mL mineral solution I (0.6% K_2HPO_4), 7.5 mL mineral solution II (1.2% NaCl, 1.2% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, 0.12% $CaCl_2$, 0.25% $MgSO_4$) and 84 mL distilled water (pH 6.8). A series of 10-fold dilutions (10^{-1} to 10^{-9}) were made under a CO_2 atmosphere. From appropriate dilutions, 0.1 mL aliquots were spread onto Trypticase soy blood agar (Oxoid Unipath, Ltd., Basingstoke, UK) with 10% sheep blood for total aerobes, Schaedler agar (BBL Microbiology systems, Cockeysville, USA) with 1% vitamin K1 - hemin solution for total anaerobes, and Man-Rogosa-Sharpe agar (MRS, Merck, Darmstadt, Germany) for lactic acid bacteria. Incubation of the inoculated media for anaerobic and lactic acid bacteria was carried out at 37°C for 3 days under anaerobic conditions (Gas Pak Plus, BBL). Plates for the enumeration of aerobic bacteria were incubated for 24 h at 37°C. Numbers of colony-forming units (CFU) were expressed as log CFU per gram of sample. The results were presented as arithmetical means ± standard deviation (SD).

2.5.2. Viability of microorganisms on fluorescence-activated cell sorting visualized with viability fluorescent quick test on a polycarbonate filter (VFQTOPF)

The samples of feces and cecal contents were diluted 1:100 in PBS (37°C; MP Biomedicals, France) and filtered through 70 μm and subsequently through 45 μm cell strainers (BD Falcon, NJ, USA). The prepared suspensions were stained with carboxyfluorescein diacetate (cFDA; Sigma) in final concentration of 25 μM and with propidium iodide (PI; Sigma) in final concentration of 45 μM at 37°C for 20 min. Flow cytometric analysis was performed employing a BD FACSCanto™ flow cytometer (Becton Dickinson Biosciences, USA) and BD FACS Diva™ software. The percentages of live and dead bacteria were evaluated based on presence of carboxyfluorescein (cF) (metabolized form of cFDA) detectable only in live bacteria, measured in FL-1 channel (530/30 nm) and the intensity of fluorescence was measured in FL-3 channel (695/40 nm) for propidium iodide (PI) which enters only damaged or dead bacteria [36]. Simultaneously, samples stained with cFDA and PI were analyzed by epifluorescence microscopy. Vacuum filtered samples were fixed on polycarbonate filters (Merck Millipore, Billerica, USA) and stained also with DAPI solution (1 mg of 4′,6-diamidino-2-phenylindole/mL). The filters were placed on microscopic slides and mounted with Vectashield Medium (Vector Laboratories, Peterborough, UK). The slides were examined under a Carl Zeiss Axio Observer Z1 epifluorescence microscope using filter sets 38HE, 64HE and Set 49 for detection of cF, PI and DAPI, respectively. Microphotography analysis was performed using Axio Vision Rel 4.8 software.

2.5.3. Determination of the minimum inhibitory concentration of antibiotics

The minimum inhibitory concentrations (MICs) of antibiotics against the tested strains were determined by Etest® strips for ciprofloxacin (AB bioMérieux, Marcy l'Étoile, France) and M.I.C. evaluator strips for amoxicillin and clavulanate potassium (Thermo Fisher Scientific, Basingstoke, UK). The results were read in accordance with the manufacturers' protocol, which is essentially identical for both strip products.

2.5.4. Phenotypical identification

Phenotypical identification of *Escherichia coli* was performed by means of a diagnostic kit ENTEROtest 24 N (Erba Lachema s. r. o., Brno, Czech Republic).

2.5.5. DNA identification

After microbiological cultivation on blood agar, DNAzol direct (Molecular Research Center Inc., Cincinnati, USA) was used to isolate DNA from bacterial colonies. The PCR reaction was performed with the help of primers 27F (5-AGAGTTTGATCMTGGCTCAG-3 and 1492R (5-CGGYTACCTTGTTACGACTT-3). The amplification protocol for PCR reaction was: 5-min at 94°C, 1 min at 94°C, 1 min at 55°C and 3 min at 72°C and a final at 72°C 10-min (TProfesional Basic, Biometra GmbH, Göttingen, Germany). PCR products were separated by electrophoresis on 0.7% agarose gel with the help of TAE buffer. The PCR amplicons were stained with GelRed™ (Biotium Inc., Hayward, USA) and visualized after the separation under UV light. Purification of PCR products was carried out by means of a kit NucleoSpin® Gel and PCR Clean-Up Kit (Mancherey-Nagel GmbH & Co. KG, Düren, Germany). The amplicons were submitted for sequence analysis to *E. coli* s.r.o. (Bratislava, Slovakia) and sequenced in both directions by either 27F or 1492R primers. The sequences were then analyzed by BLAST (compared with sequences available in the GenBank) and after the alignment and assembly processing by means of Genious 6.1.6 software they were submitted to the GenBank. The resultant sequences were published under GenBank accession numbers KX086704 and KX086705.

2.6. Blood and serum analysis

Hematological analysis was carried out using a BC-2008 VET automatic analyzer (Mindray, Shenzhen, China). An automated biochemical analyzer Ellipse (AMS, Rome, Italy) and standard kits (Dialab, Prague, Czech Republic) were used to determine concentrations of the following biochemical parameters: glucose; triglycerides; cholesterol; HDL-cholesterol; LDL-cholesterol; total protein; urea; albumin; creatinine; activities of enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Total activity of lactate dehydrogenase (CLDH) was determined spectrophotometrically (Alizé, Lisabio, France) and its isoenzymes (LDH-1: LDH-5) were determined by an electrophoretic method (Hydrasys, Lisses, France).

2.7. Short chain fatty acids (SCFAs) analysis

The produced organic acids were determined by isotachophoresis as described by Gancarčíková et al. [22]. After the collection, 0.5 g of feces and caecum contents were dissolved in 25 mL deionized water and 30 μL aliquots were used for analysis of short-chain fatty acids (SCFAs). The measurements were done on an Isotachophoretic analyzer ZKI 01 (Radioecological Institute, Košice, Slovakia). A leading electrolyte of the following composition was used in the pre-separatory capillary: 10^{-2} mol/L HCl + 2.2. 10^{-2} mol/L

ε-aminocaproic acid + 0.1% methylhydroxyethylcellulosic acid, pH = 4.3. A solution of 5. 10^{-3} mol/L caproic acid + 2. 10^{-2} mol/L histidine was used as a finishing electrolyte. This electrolytic system worked at 150 μA in the pre-separatory and at 40 μA in the analytic capillary.

2.8. Histology of the liver and kidneys

Liver samples from *lobus caudatus hepatis* and kidneys of mice were fixed in 4% paraformaldehyde in PBS (pH 7.2) (Amresco LLC, Solon, USA) for 72 h, washed for 5 h and paraffin blocks were prepared according to the standard procedure. Some paraffin sections (7 μm thick) were stained with Harrison's hematoxylin and eosin, and the tissue was mounted in Histochoice mounting fluid (Amresco LLC, Solon, USA). Tissue sections were examined using a light microscope (Olympus BX 51, Czech Republic) and Digital Analysis Imaging system "Analysis Docu" (Soft Imaging Systems 3.0, Prague, Czech Republic). A part of sections of livers and kidneys were used for fluorescent detection of late apoptosis seen as fragmented nuclei (blue color) and simultaneously for localization of neutral lipids (red color). Rehydrated sections were firstly stained with solution of Nile red (Sigma-Aldrich, USA) prepared in 75% glycerol in PBS at the concentration of 2 μg/mL for 2 h at 8°C. Following the washing step in PBS, sections were incubated with nuclei - staining solution prepared from Hoechst 33,342 (5 μg/mL) in PBS (Sigma-Aldrich, USA) for 2 h at 8°C. Then the washed slides were covered with mounting fluid (90% glycerol in PBS, 2.5% DABCO (Sigma-Aldrich, USA) and examined using fluorescent microscope Carl Zeiss Axio Observer Z1) and analyzed with Axio Vision Rel 4.8 software (Carl Zeiss Jena, Germany).

2.9. Statistics

Statistical evaluation of the results was performed using Statistic software GraphPad Prism 3.0 for Windows (GraphPad Software, San Diego, USA). One-way analysis of variance (ANOVA) was used, followed by a multiple comparison Tukey's test. Significance of differences between the groups of mice was tested using analysis of variance and unpaired Student's *t*-test. The significance level was set to $P < 0.05$. Most of the results are expressed as means ± SD (standard deviation).

3. Results

3.1. Clinical examination of animals

Laboratory SPF BALB/c mice were subjected to complex clinical examination during quarantine and at the end of the experiment. During experiment, all changes in clinical status were observed and recorded twice daily (8.00 and 15.00 h). The regular observation of overall health manifested by uptake of food, agility of animals and consistency of feces allowed us

to detect changes in consistency of feces from solid to pasty on day 3 of the experiment in 10 out of 17 animals treated with antibiotics. All SPF BALB/c mice were agile and their intake of food was unchanged.

3.2. Total body weight and relative weight of internal organs

On day 5 of the experiment, the total body weight of animals from experimental group DC (**Table 1**) was insignificantly lower by 0.23 g in comparison to negative control (C). Examination of internal organs showed a significant decrease in relative weight of the liver ($P < 0.05$) and spleen ($P < 0.01$) in decontaminated group (DC) in comparison with control group C. On day 15 of the experiment, group DC + R showed the highest relative weights of the heart, liver and spleen, approaching the weights of these organs in group C on day 5 of the experiment.

3.3. Hematology parameters

Total counts of leukocytes (WBC) and lymphocytes (Ly) in all investigated groups (**Table 2**) were in physiological ranges. However, the decontaminated group (DC) showed insignificantly lower counts of WBC (by 1.73 G/L) and lymphocytes (by 1.65 G/L) in comparison with control group C.

On day 5 of the experiment, group DC showed a significant increase ($P < 0.05$) in counts of monocytes (Mo), and their percentage value (Mo%) was also significantly increased ($P < 0.05$) in comparison with group not treated with antibiotics (C). Administration of antibiotics (ATB) affected also the number of granulocytes (Gran, Gran%). While the number of granulocytes (Gran) exceeded the physiological limit, it was only insignificantly higher compared to the control C (by 1.34 G/L). However, in case of percentage proportion of granulocytes (Gran%) the difference was significant ($P < 0.05$).

The changes in red blood components recorded in group DC after decontaminated with antibiotics (ATB) resembled those observed in white blood components in this group (**Table 2**). Increased counts exceeding the physiological range, although insignificantly different, were observed for erythrocyte counts (RBC), level of hemoglobin (HGB) and hematocrit

Group	The organ dimensions (g/kg)						Body weight (g)
	Heart	Liver	Spleen	Right kidney	Left kidney	Lungs	
C	5.49 ± 0.28	53.87 ± 2.6	4.40 ± 0.30	7.19 ± 0.34	7.16 ± 0.24	8.27 ± 0.67	16.13 ± 0.34
DC	5.07 ± 0.21	47.41 ± 0.68 ^{*C}	2.82 ± 0.13 ^{**C}	7.31 ± 0.30	7.33 ± 0.33	7.40 ± 0.33	15.90 ± 0.36
DC + R	5.96 ± 0.21	5.70 ± 1.49	4.71 ± 0.31	7.29 ± 0.29	7.16 ± 0.31	8.23 ± 0.27	17.33 ± 0.65

The results are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1. Body weight (g) and the organ dimensions (g/kg) of the BALB/c mice in control C (n = 7), treated with ATB for 5 days (DC group, n = 9) and then after 10 days without antibiotic treatment (DC + R group, n = 8).

Group	C	DC	DC + R	Ref BALB/c
WBC (G/L)	7.76 + 1.55	8.80 + 1.92	6.03 + 0.98	5.69–9.87
Ly (G/L)	6.08 + 1.31	5.18 + 1.09	4.43 + 0.65	3.60–7.29
Mo (G/L)	0.14 + 0.04	0.74 + 0.30^{*C}	0.15 + 0.04^{*DC}	0.34–0.70
Gran (G/L)	1.54 + 0.35	2.88 + 0.70	1.45 + 0.34	0.74 – 1.78
Ly (%)	77.64 ± 2.83	60.10 ± 5.06^{**C}	74.67 ± 1.98^{**DC}	55.06–73.44
Mo (%)	1.94 ± 0.20	7.62 ± 2.13^{**C}	2.75 ± 0.30^{**DC}	3.75–7.26
Gran (%)	20.42 ± 2.70	32.28 ± 3.34^{*C}	22.58 ± 1.77^{*DC}	10.46–18.94
RBC (T/L)	9.06 ± 1.17	10.77 ± 0.39	9.44 ± 1.16	8.16–9.98
HGB (g/L)	156.4 ± 19.85	189.00 ± 7.96	145.90 ± 13.52	124–154
HCT (%)	51.50 ± 7.01	61.00 ± 2.42	47.37 ± 4.20	43.50 – 55.4
MCV (fL)	56.60 ± 0.69	56.66 ± 0.45	55.88 ± 0.42	50.80 – 55.60
MCH (pg)	17.26 ± 0.28	17.46 ± 0.15	15.85 ± 0.96	13–15.5
MCHC (g/L)	306.4 ± 6.74	309.4 ± 1.03	297.5 ± 12.19	239–280
RDW (%)	14.78 ± 0.50	13.62 ± 0.43	13.76 ± 0.24	16.9–19.1

WBC white blood cells, *Ly* lymphocytes, *Mo* monocytes, *Gran* granulocytes, *RBC* red blood cells, *HGB* hemoglobin, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *RDW* red blood cell distribution width, *Ref* reference range [91]. The results are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 2. Hematology parameters of the BALB/c mice in control C (n = 7), treated with ATB for 5 days (DC group, n = 9) and then after 10 days without antibiotic treatment (DC + R group, n = 8).

(HCT) in comparison with control (C). *On day 10* after termination of treatment with antibiotics, we recorded an insignificant decrease in counts of both leukocytes and lymphocytes in group DC + R. This group showed a significant reduction in counts of Mo, Gran% ($P < 0.05$) as well as in Mo% ($P < 0.01$) in comparison with group DC. A decreasing trend in the observed parameters in group DC + R following convalescence of animals and return of their levels to the physiological range was observed not only for the white components but also for red ones, represented by decrease in RBC, HGB and HCT. Mean cell volume (MCV) of erythrocytes, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were moderately increased in all groups and red blood cell distribution width (RDW) was moderately decreased in comparison to physiological range, but there were no significant differences between the groups.

3.4. Biochemical parameters

3.4.1. Nitrogen profile

Nitrogen profile (**Table 3**) represented by concentration of total proteins (TP) and albumin showed significant differences between groups DC and C on day 5 of the experiment. Despite

Group	C	DC	DC + R	Ref BALB/c
Total protein (g/L)	70.20 ± 1.65	89.98 ± 0.90^{**C}	66.60 + 5.78^{**DC}	60.8–73.0
Urea (mmol/L)	6.66 ± 0.63	6.68 ± 0.08	5.87 ± 0.16	5.70–7.14
Albumin (g/L)	33.98 ± 0.48	37.48 ± 0.57^{**C}	31.57 ± 0.82^{***DC}	31.0–37.0
Creatinine (μmol/L)	27.50 ± 0.96	24.50 ± 0.96	30.00 ± 0.58^{**DC}	up to 33.59
Glucose (mmol/L)	8.03 ± 0.17	6.35 ± 0.06^{**C}	8.03 ± 0.47^{**DC}	4.72–10.71
Triglyceride (mmol/L)	2.59 ± 0.05	2.26 ± 2.09^{*C}	2.96 ± 0.06$^{*C, ***DC}$	up to 3.42
Cholesterol (mmol/L)	2.62 ± 0.09	3.41 ± 0.03^{***C}	3.22 ± 0.16^{*C}	2.09–3.65
HDL cholesterol (mmol/L)	1.77 ± 0.06	1.76 ± 0.02	1.79 ± 0.02	up to 1.78
LDL cholesterol (mmol/L)	0.38 ± 0.01	0.75 ± 0.02^{***C}	0.57 ± 0.01^{***C,DC}	up to 0.38
DC	up to 0.38			
AST (μkat/L)	3.27 ± 0.18	3.64 ± 0.10	3.13 ± 1.57	2.67–3.05
ALT (μkat/L)	2.50 ± 0.24	3.26 ± 0.55	8.20 ± 1.63^{**C,DC}	0.68–2.89
ALP (μkat/L)	6.47 ± 0.30	5.48 ± 0.48	5.96 ± 0.45	1.83–6.23
LDH-Total (μkat/L)	58.4 ± 2.9	78.98 ± 9.81	64.83 ± 12.3	
LDH-1				
% z LDH-T	2.9 ± 1.1	1.55 ± 0.13	2.0 ± 0.15	
(μkat/L)	1.73 ± 0.73	1.22 ± 0.16	1.27 ± 0.18	
LDH-2				
% z LDH-T	2.6 ± 0.1	2.38 ± 0.15	3.0 ± 0.15	
(μkat/L)	1.53 ± 0.14	1.85 ± 0.16	1.98 ± 0.43	
LDH-3				
% z LDH-T	16.75 ± 3.15	14.35 ± 2.54	21.2 ± 1.25	
(μkat/L)	9.88 ± 2.33	10.98 ± 1.53	13.57 ± 2.16	
LDH-4				
% z LDH-T	9.25 ± 0.55	8.53 ± 0.24	10.97 ± 1.52	
(μkat/L)	5.39 ± 0.06	6.7 ± 0.74	7.44 ± 2.38	
LDH-5				
% z LDH-T	68.5 ± 3.8	73.2 ± 2.7	62.83 ± 0.8	
(μkat/L)	39.9 ± 0.24	58.24 ± 8.7	40.58 ± 7.4	

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *LDH-T* lactate dehydrogenase total, *Ref* reference range [91]. The results are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 3. Biochemical parameters in blood serum of the BALB/c mice in control C (n = 7), treated with ATB for 5 days (DC group, n = 9) and then after 10 days without antibiotic treatment (DC + R group, n = 8).

decreased exogenous intake of feed by animals of group DC, this group exhibited significantly higher concentration of both TP and albumin ($P < 0.01$). Their levels exceeded the upper physiological limit due to hemoconcentration and dehydration of the organism. While administration of antibiotics to mice of group DC did not affect significantly the level of urea in comparison with group C, serum creatinine in decontaminated mice decreased by 11%. *On day 10* after termination of treatment with antibiotics, group DC + R showed return of concentrations of TP and albumin back to the physiological range with significantly lower levels of TP ($P < 0.01$) and albumin ($P < 0.001$) in comparison with those recorded in group DC on day 5 of the experiment (**Table 3**). While on day 10 after termination of treatment with antibiotics the level of urea in group DC + R decreased, concentration of creatinine significantly increased ($P < 0.01$). After 10-day convalescence, all investigated parameters of nitrogen profile were in physiological range.

3.4.2. Energy and lipid profile

On day 5 of the experiment (**Table 3**), animals from decontaminated group DC showed significantly lower levels of glucose ($P < 0.01$) and triglycerides ($P < 0.05$) in comparison with group C, indicating reduced intake of feed, however, concentration of total cholesterol, which was in physiological range, was significantly higher ($P < 0.001$) in this group and indicated moderate irritation of intestinal mucosa. While the level of HDL-cholesterol was about the same in both investigated groups (C, DC), LDL-cholesterol was significantly higher ($P < 0.001$) in group DC and exceeded the physiological range. *On day 10* after termination of treatment with antibiotics, group DC + R showed an opposite trend in concentration of investigated parameters of energy and lipid profile of mice in comparison with group DC (**Table 3**). After 10-day convalescence, a significant increase in glucose ($P < 0.01$) and triglycerides ($P < 0.001$) was observed in group DC + R in comparison with group DC. At the same time, we recorded in this group a significant decrease ($P < 0.001$) in LDL-cholesterol; however, its concentration exceeded the physiological limit determined for mice of BALB/c line.

3.4.3. Enzymatic profile

While on days 5 and 15 of the experiment none of the investigated groups showed increased activity of enzyme ALP (**Table 3**), activities of enzymes AST and ALT were insignificantly increased in group DC in comparison with group C. ALT is a liver-specific enzyme and its increased activity indicates irritation or damage to the liver. Its increase is associated with damage to membrane of liver cells, even at the absence of their necrosis, and the enzyme is excreted at both reversible and irreversible damage to liver parenchyma. Increased activity up to 3-fold the reference level is considered a moderate increase. After *10-day* convalescence without treatment with antibiotics, an insignificantly lower activity of non-specific hepatic enzyme AST and significantly higher ($P < 0.01$) activity of enzyme ALT was observed in group DC + R in comparison with group C and decontaminated group DC, indicating irritation of the liver. In this case, ALT was released, however, without damage to hepatocytes. There was no alteration of AST, the activity of which was increased only slightly and thus the coefficient of hepatocyte damage was not decisive.

3.4.4. Activity of LDH-total and isoenzymes

LDH-T is a multi-organ cytosol enzyme that exists as 5 isoenzymes. It is released to circulation already at slight tissue damage. Observation of specific activity of total LDH (**Table 3**) and its isoenzymes in the serum of mice of the investigated groups (C, DC a DC + R) showed no significant differences.

On day 5 of the experiment, we observed an insignificant increase in activity of total LDH in decontaminated group DC, which was by 20.5 μkat/L higher in comparison with control group C. Determination of relative proportions of individual isoenzymes in decontaminated group DC revealed that besides increase in LDH-1, specific activities of isoenzymes LDH-(2, 3 and 4) were also increased; however, as far as their percentage proportion of total LDH was concerned, we observed a decrease in specific activities of all isoenzymes LDH-(1-4) in favor of increased activity of isoenzyme LDH-5, indicating irritation of hepatic tissue. The greatest although insignificant decrease in specific activity was observed in isoenzyme LDH-3, found in pulmonary parenchyma. Its activity was lower by 2.4% in comparison with the period without treatment with antibiotics. The activity of isoenzyme LDH-1, known as a heart enzyme, was lower by 1.35%, and activities of isoenzymes LDH-4, found in the kidneys and pancreas, and LDH-2, primarily associated with the reticuloendothelial system, were decreased by 0.72% and 0.22%, respectively. The most pronounced although insignificant increase in specific activity was observed in isoenzyme LDH-5, found in liver parenchyma and striated muscles. Its activity was higher by 18.34 μkat/L and percentage proportion of total LDH higher by 4.7% in comparison with control group C.

An insignificant decrease in total LDH was observed again in group DC + R after convalescence period. The activity of this enzyme was lower by 14.15 μkat/L in comparison with group DC. *On day 10* following the termination of treatment with antibiotics, group DC + R showed most pronounced but insignificant changes in activities of isoenzymes LDH-5, LDH-3 and LDH-4. While the activities of isoenzymes LDH-3,4 after convalescence (DC + R) showed an increase by 6.85% (LDH-3) and 2.44% (LDH-4) of total LDH, an opposite trend was observed for LDH-5. The isoenzyme associated with liver parenchyma and striated muscles (LDH-5) showed an insignificant decrease in specific activity down to the level determined before treatment with antibiotics (40.58 ± 7.4 μkat/L), which indicated reparation of hepatic tissue.

3.5. Microbiological parameters

3.5.1. Determination of counts of cultivable microorganisms in mice feces

Before the application of antibiotics (ATB), the plate counts (**Figure 1**) of microorganisms in feces in all groups of SPF mice (C, DC, DC + R) ranged between 8.15 and 9.19 log10 CFU/mL. Determination of plate counts 24 h after the antibiotic treatment showed a significant decrease by 4 logs (4.58 ± 0.31 log10 CFU/mL) after aerobic cultivation and by 3–4 logs

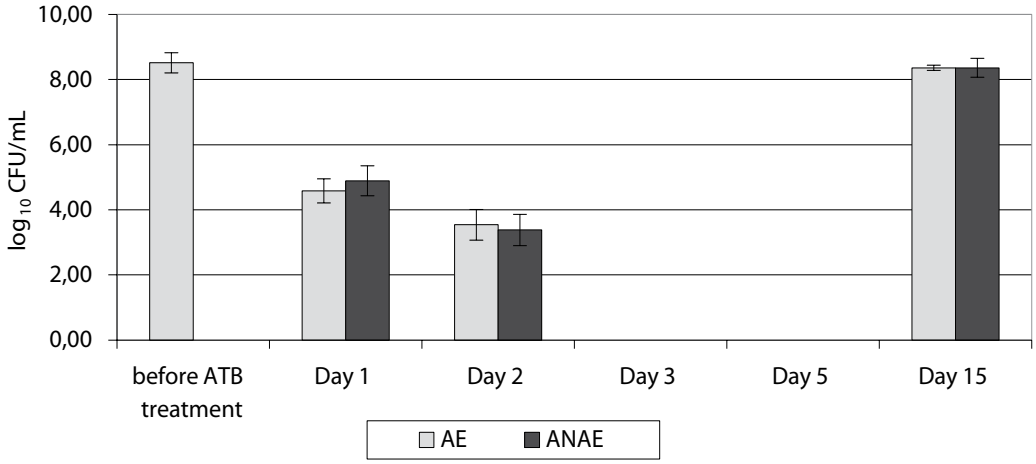

Figure 1. Plate counts of microorganisms in feces samples determined by cultivation on TSA agar. *AE* aerobic conditions, *ANAE* anaerobic conditions. The results are expressed as the means log 10 CFU/mL ± SEM.

(4.89 ± 0.46 log10 CFU/mL) after anaerobic cultivation when compared with the initial counts determined before antibiotic treatment (8.51 ± 0.31 log10 CFU/mL). Cultivation at 48 h from the beginning of antibiotic treatment revealed less pronounced decrease in plate counts of cultivable microorganisms. The counts were reduced by 1 log under aerobic conditions (3.54 ± 0.47 log10 CFU/mL) and by 1–2 logs when cultivated anaerobically (3.38 ± 0.48 log10 CFU/mL), in comparison with the counts determined at 24 h after the antibiotic treatment. The following investigations on days 3 and 5 of cultivation revealed absence of cultivable microorganisms in the feces (**Figure 1**). Determination of plate counts on day 10 after termination of antibiotic treatment showed recurrence of cultivable microorganisms in feces after both aerobic cultivation (8.36 ± 0.08 log10 CFU/mL) and anaerobic cultivation (8.36 ± 0.29 log10 CFU/mL).

3.5.2. Survivability of microorganisms in samples of feces and caecum content determined by FACS, visualized by means of VFQTOPF

Survivability of microorganisms in mice feces (BD FACS Canto flow cytometer, BD, USA) decreased significantly ($P < 0.01$) between days 1 (35.03 ± 2.43%) and 2 (28.33 ± 0.43%) of antibiotic treatment. The survival rates before the treatment reached 60.58 ± 5.28% (**Figure 2**). Survival rate of bacteria in the caecum on day 5 of treatment (**Figure 3**) was significantly lower ($P < 0.001$) in DC group (28.10 ± 1.56%) in comparison with control group C (76.77 ± 1.56%). Survival rate of microorganisms in the caecum of mice from group DC + R (kept in gnotobiotic isolators with microbiologically controlled environment) reached 75.47 ± 0.38% on day 10 after termination of antibiotic treatment. The viability fluorescent quick test on a polycarbonate filter (VFQTOPF) was also employed to detect survivability of microorganisms (**Figures 2** and **3**). It allowed visualization on the basis of color as the live bacteria stained green and dead bacteria turned red.

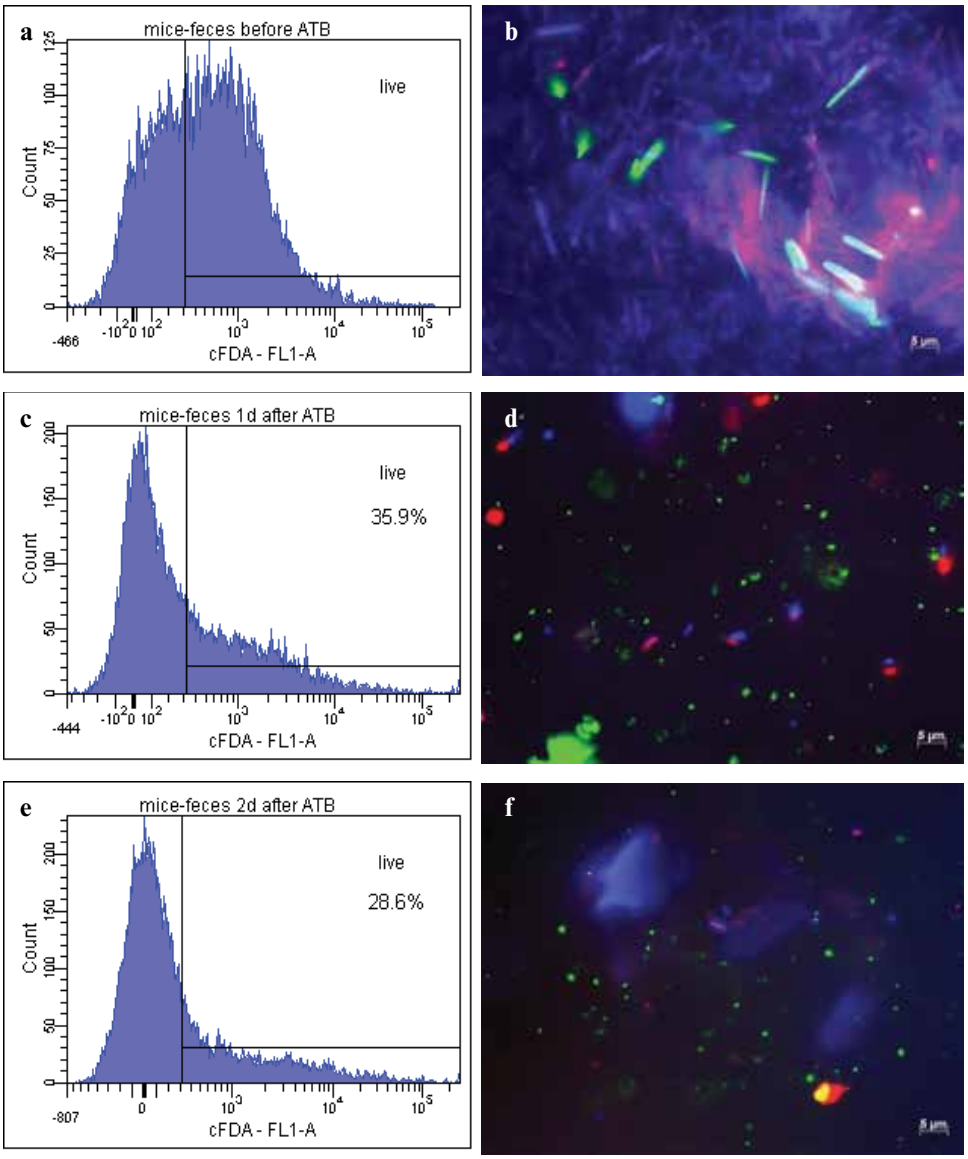

Figure 2. Viability of microorganisms in feces determined by FACS and visualized with VFQTOPF. Live bacteria are *green*. Dead bacteria are *red*. Barely active non-dead are *blue*. **a, b** Mice feces before antibiotic treatment. FACS analysis (**a**) and VFQTOPF visualization (**b**). **c, d** Mice feces on day 1 of the study. FACS analysis (**c**) and VFQTOPF visualization (**d**). **e, f** Mice feces analyzed on day 2. FACS analysis (**e**) and VFQTOPF visualization (**f**).

3.5.3. Cultivable bacteria detected in the study

At day 10 after termination of antibiotic treatment, the microbiota was reduced to two cultivable species. They were differentiated and identified on the basis of morphological, biochemical and genetic differences. The first species isolated from DC + R group was a Gram-negative

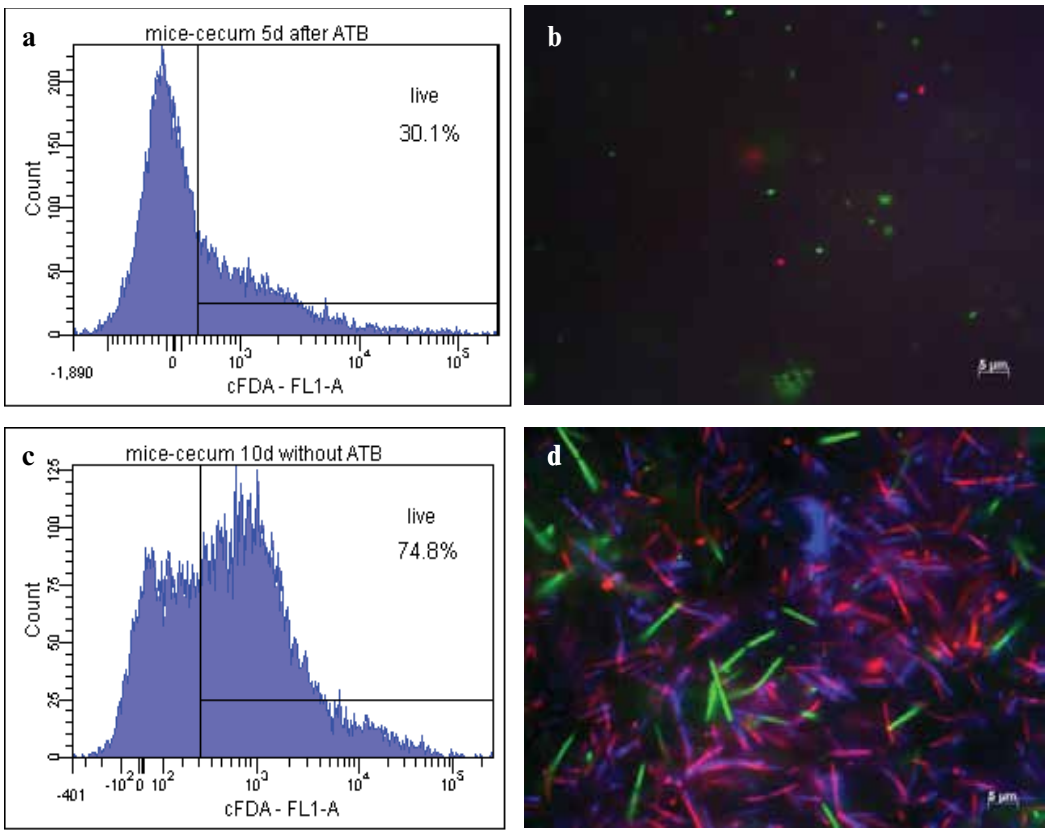

Figure 3. Viability of microorganisms in the caecum determined by FACS and visualized with VFQTOPF. Live bacteria are *green*. Dead bacteria are *red*. Barely active non-dead are *blue*. **a, b** Mice contents of caecum on day 5 of the study. FACS analysis (**a**) and VFQTOPF visualization (**b**). **c, d** Mice contents of caecum 10 days without ATB. FACS analysis (**c**) and VFQTOPF visualization (**d**).

rod-shaped bacterium. Determination of biochemical properties of this bacterium by means commercial ENTEROtest 24 N (Erba Lachema s.r.o., Brno, Czech Republic) showed that this involved species *E. coli* with accuracy ranging between 90.52 and 99.85%. Results of analysis of the DNA section corresponding to the 16S rRNA of bacteria by BLAST analysis and comparison of DNA templates showed that the best match was with *E. coli* (GenBank KU254762.1) species (**Figure 4**). The MIC determined by Etest® strips for ciprofloxacin (AB bioMérieux, Marcy l'Étoile, France) was 0.064 mg/L.

The second species isolated from DC + R group was a Gram-positive coccus. By analyzing the DNA section corresponding to 16S rRNA of bacteria by BLAST analysis and comparing it with DNA templates, the best match obtained indicated *Enterococcus* sp. (GenBank KT630829.1) (**Figure 5**). Determination of the MIC carried out by M.I.C. Evaluator strips for amoxicillin and potassium clavulanate (Thermo Fisher Scientific, Basingstoke, UK) showed that the MIC was equal to 0.25 mg/L.

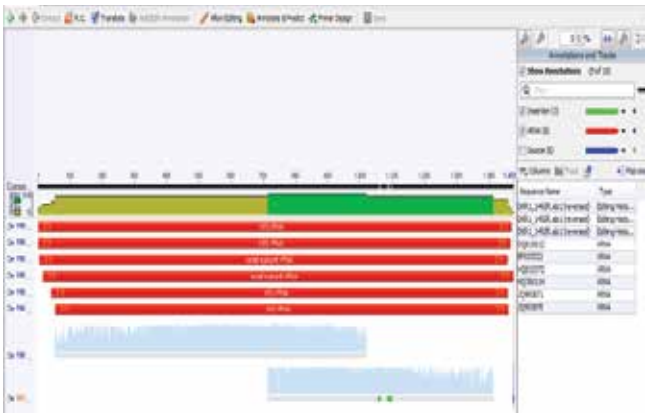

Figure 4. Assembly of 16S rRNA sequences identified as *Escherichia coli* (GenBank: KU254762.1).

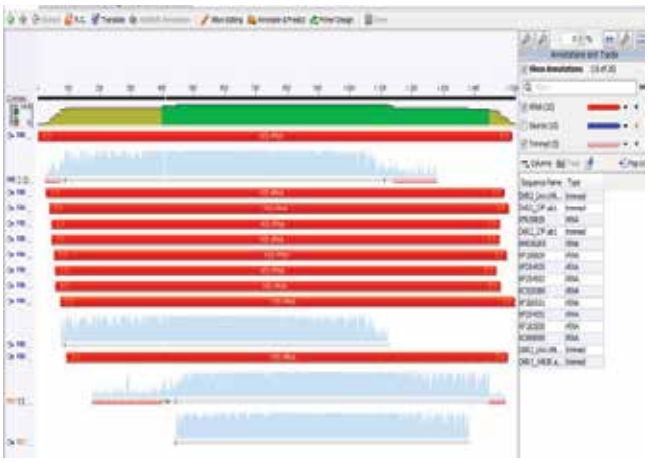

Figure 5. Assembly of 16S rRNA sequences identified as *Enterococcus* sp. (GenBank: KT630829.1).

3.6. Production of SCFAs in feces and caecum

Production of organic acids (**Figure 6**) in the *caecum* of decontaminated DC group resulted in very low concentrations of these acids in comparison with control group C and group after convalescence (DC + R). The highest concentrations did not exceed the level of 27 mmol/L. *On day 5* of the experiment, examination of the caecum of decon taminated group DC showed a decrease in concentration of all investigated acids (**Figure 6**) with the exception of succinic acid in comparison with group not treated with antibiotics (C). The most pronounced decrease was observed in production of acetic and acetoace-tic acids. The decrease in production and resulting concentrations of both acetic and acetoacetic acid was significant (26.97 ± 3.58 mmol/L, $P < 0.01$ and 12.69 ± 1.48 mmol/L,

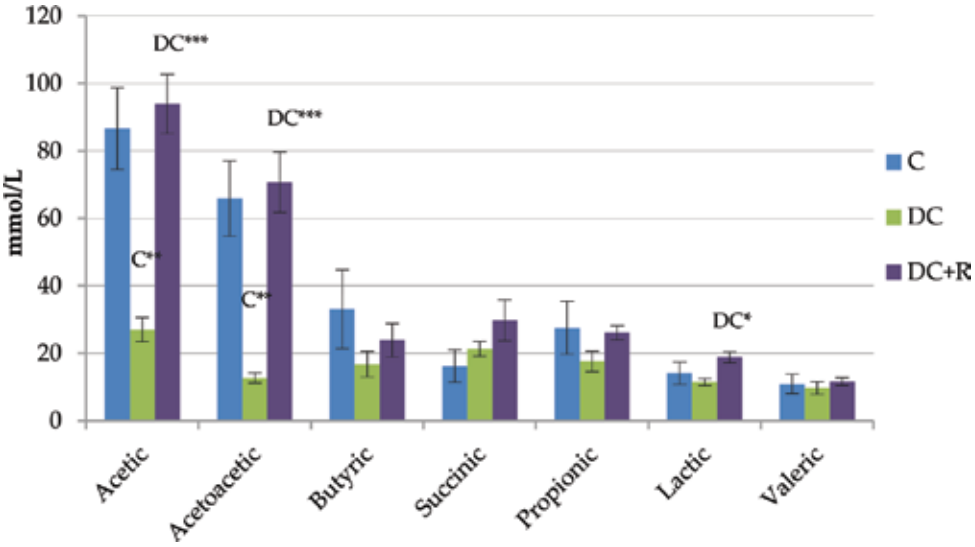

Figure 6. The caecum concentration of organic acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic treatment (DC + R group). The results are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

$P < 0.01$, respectively) in comparison with concentrations of these acids in control group C (86.65 ± 12.11 and 65.87 ± 11.20 mmol/L, respectively). After termination of treatment with antibiotics (ATB) and *10-day convalescence* period (DC + R), the concentrations of organic acids in cecal contents of mice (**Figure 6**) were higher with the exception of butyric and propionic acids in comparison with both DC group and control group C on day 5 of the experiment. A significant increase in concentration of acids ($P < 0.001$) after convalescence in comparison with 5-day period of treatment with ATB (DC) was recorded for acetic acid and acetoacetic acid (93.90 ± 8.76 and 70.69 ± 8.96 mmol/L, resp.) and in production of lactic acid (18.78 ± 1.66 mmol/L; $P < 0.05$).

Within the 5-day decontamination period, examination of *feces* of mice from group DC (**Figure 7**) showed the most pronounced significant decrease in concentration of *acetic acid* ($P < 0.01$) and *lactic acid* ($P < 0.05$) at 24 h after onset of treatment with ATB. The dynamics of concentration of acetic acid in group DC showed a similar course in the following days of decontamination (days 2–5) with concentrations varying around 40 mmol/L. The differences on days 2 and 5 of treatment with ATB were significant at levels $P < 0.01$ and $P < 0.001$, respectively, in comparison with concentrations before the treatment. In the same period, concentrations of lactic acid in group DC showed a gradual decrease with significant differences on day 2 ($P < 0.01$) and 5 ($P < 0.001$) of treatment, in comparison with concentrations before antibiotic treatment. The dynamics of concentrations of acetic and lactic acids (**Figure 7**) in group DC + R in the above period resembled that observed in group DC but the decrease in concentrations of acids at 24 h after onset of treatment with Antibiotics

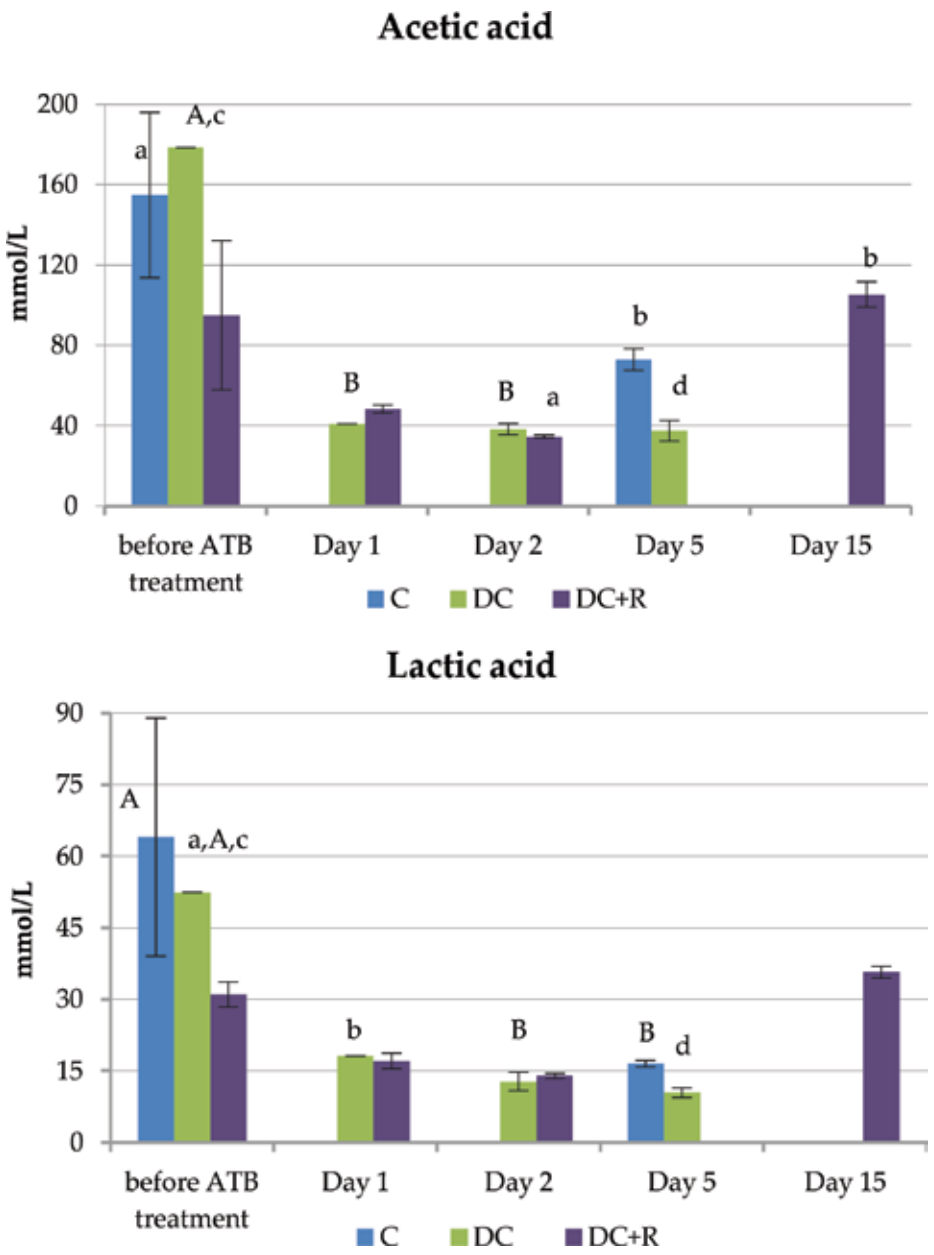

Figure 7. The fecal concentration of acetic and lactic acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic (ATB) treatment (DC + R group). The results are expressed as the mean ± SD. $^{a,b}P < 0.05$, $^{A,B}P < 0.01$, $^{c,d}P < 0.001$ (statistical differences within groups).

(ATB) was less pronounced and insignificant. By day 2 of decontamination, both acids reached similar levels as those recorded in group DC (acetic acid 34.52 ± 0.79 mmol/L; lactic acid 3.96 ± 0.50 mmol/L). Concentrations of both acids lactic and acetic in group DC + R returned back to the level observed before treatment only after termination of treatment

with ATB and 10-day convalescence period. The increase in acetic acid was significant ($P < 0.05$; 105.4 ± 6.27 mmol/L) in comparison with day 2 of treatment with ATB.

Despite the fact that control mice (C) were not treated with ATB, they showed a significant decrease in concentrations of acetic and lactic acid ($P < 0.05$; $P < 0.01$) in comparison the level before treatment with ATB, probably as a result of their keeping in gnotobiotic (germ-free) environment and feeding with sterile food and water.

More pronounced although insignificant decrease in *propionic acid* (**Figure 8**) was recorded after 24-h treatment with ATB in feces of mice of both decontaminated groups (DC, DC + R). The level of propionic acid decreased from 54.97 ± 0.01 to 10.2 ± 0.01 mmol/L in group DC and from 60.94 ± 29.34 to 20.47 ± 1.68 mmol/L in group DC + R. In the following period (days 2 and 5 of treatment with ATB), the concentration of this acid in both decontaminated groups was very low and did not exceed 20 mmol/L in group DC and 24 mmol/L in group DC + R. The proportion of propionic acid (**Figure 8**) in feces of mice from group DC + R after convalescence period was insignificantly different (34.99 ± 5.92 mmol/L), and reached only 57.4% of the level determined before antibiotic treatment (60.94 ± 29.34 mmol/L).

Although the concentration of *succinic acid* (**Figure 8**) declined gradually in both decontaminated groups during the period of treatment (days 1–5), it was relatively high particularly at 24 h after treatment with ATB when it reached 34.97 ± 0.01 mmol/L in group DC and 31.14 ± 7.99 mmol/L in group DC + R. On day 5 of the experiment, we recorded in feces of control group C similar decreasing tendency of concentration of succinic acid as that observed for lactic, acetic and propionic acids. The level of succinic acid was significantly lower ($P < 0.05$) in comparison with that observed before the treatment with ATB. In group DC + R after convalescence, we recorded an insignificant increase in succinic acid to the level of 40.70 ± 3.46 mmol/L, which slightly exceeded its concentration from the period before decontamination (34.45 ± 9.13 mmol/L).

While the concentrations of acetic, lactic, succinic and propionic acids in groups DC and DC + R showed a decreasing tendency in the decontamination period (days 2–5) the concentrations of *acetoacetic acid* (**Figure 8**) exhibited an opposite trend. After 24 h of treatment with ATB, group DC showed an insignificant increase in acetoacetic acid from 71.95 ± 0.009 to 122.2 ± 0.01 mmol/L. In the same period, the second decontaminated group DC + R showed an opposite trend, i.e. insignificant decrease in the concentration of acetoacetic acid from 106.0 ± 9.04 to 79.67 ± 0.35 mmol/L. In the following period, concentration of acetoacetic acid decreased significantly in group DC ($P < 0.05$), however, its concentrations were still relatively high and reached the level of 54.73 ± 11.04 mmol/L by day 2 and 72.89 ± 12.50 mmol/L by day 5 of the treatment. Even more pronounced although insignificant increase was observed in group DC + R where concentration of acetoacetic acid reached 120.0 ± 20.04 mmol/L by day 2 of the experiment. High concentration of this acid persisted up to the convalescence period when it reached similar level (99.86 ± 7.106 mmol/ L) as that before treatment with ATB (106.0 ± 9.04 mmol/L).

Concentrations of *butyric acid* (**Figure 9**) in group DC were relatively even up to day 2 of the experiment, ranging from 30.34 to 36.98 mmol/L. By day 5 of the study, they decreased insignificantly down to 22.89 ± 1.51 mmol/L. Except for day 1 of treatment, group DC + R

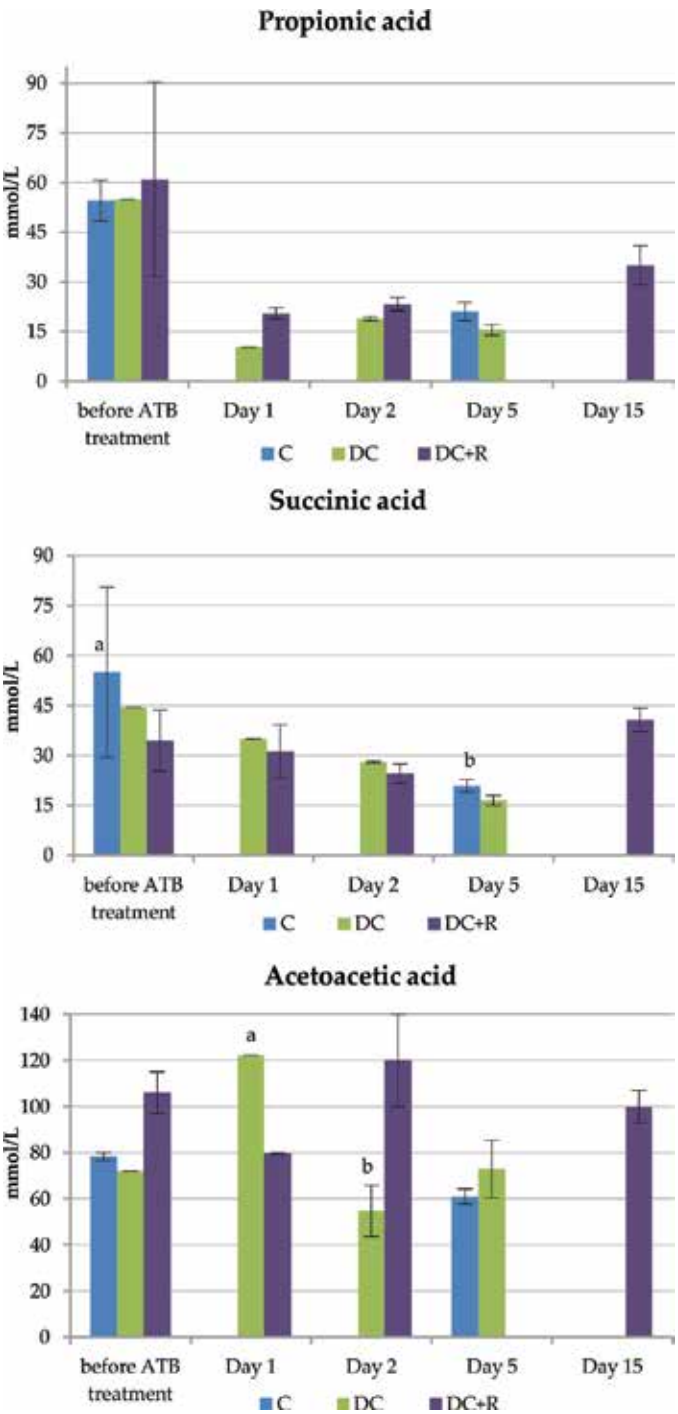

Figure 8. The fecal concentration of propionic, succinic and acetoacetic acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic (ATB) treatment (DC + R group). The results are expressed as the mean ± SD. $^{a,b}P < 0.05$ (statistical differences within groups).

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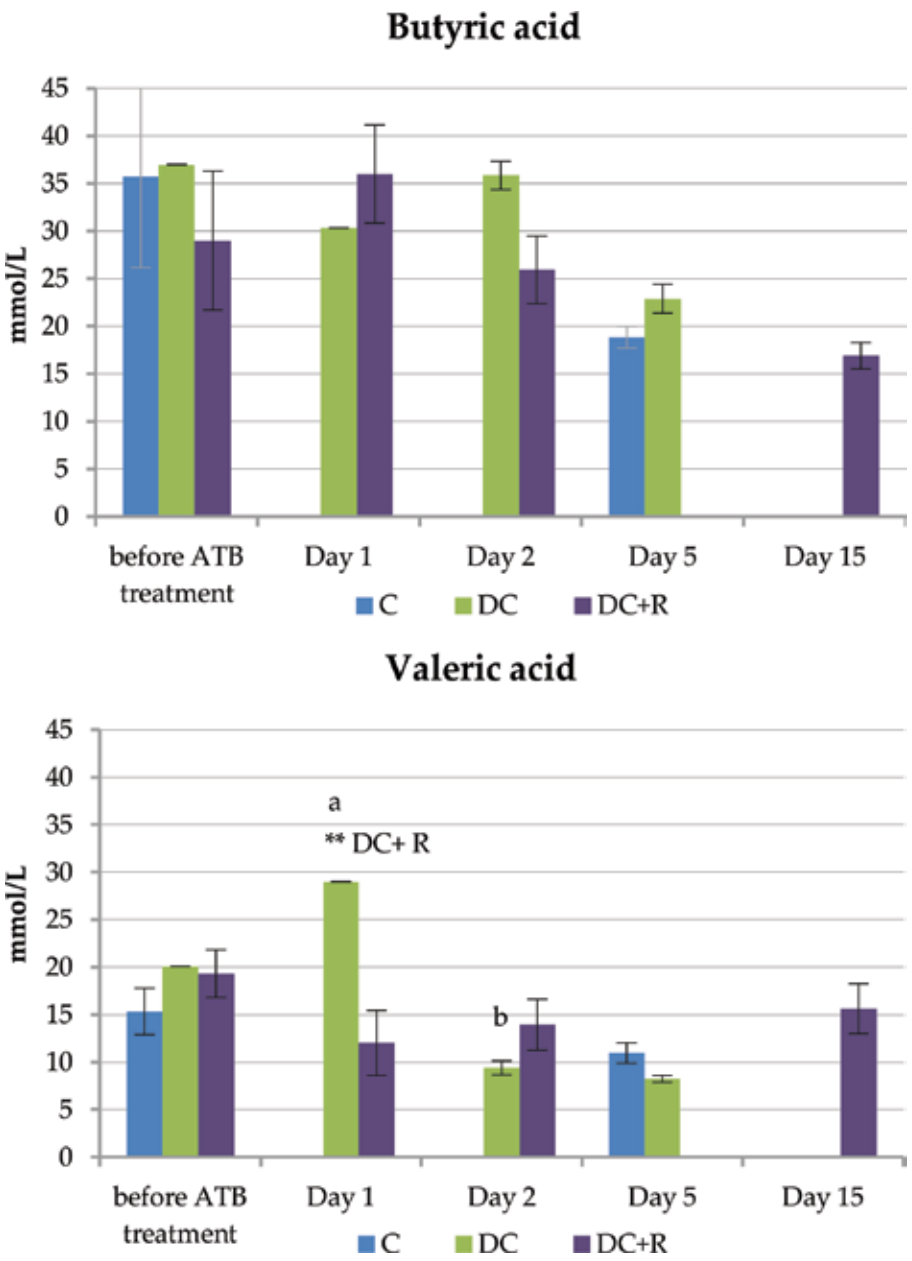

Figure 9. The fecal concentration of butyric and valeric acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic (ATB) treatment (DC + R group). The results are expressed as the mean ± SD. $^{a,b}P < 0.05$ (statistical differences within groups). $^{**}P < 0.01$ (statistical differences between groups).

showed an insignificant increase in butyric acid, which persisted up to the end of the experiment. Concentration of this acid on day 15 of the study reached 58.5% of production recorded in the period before treatment with ATB.

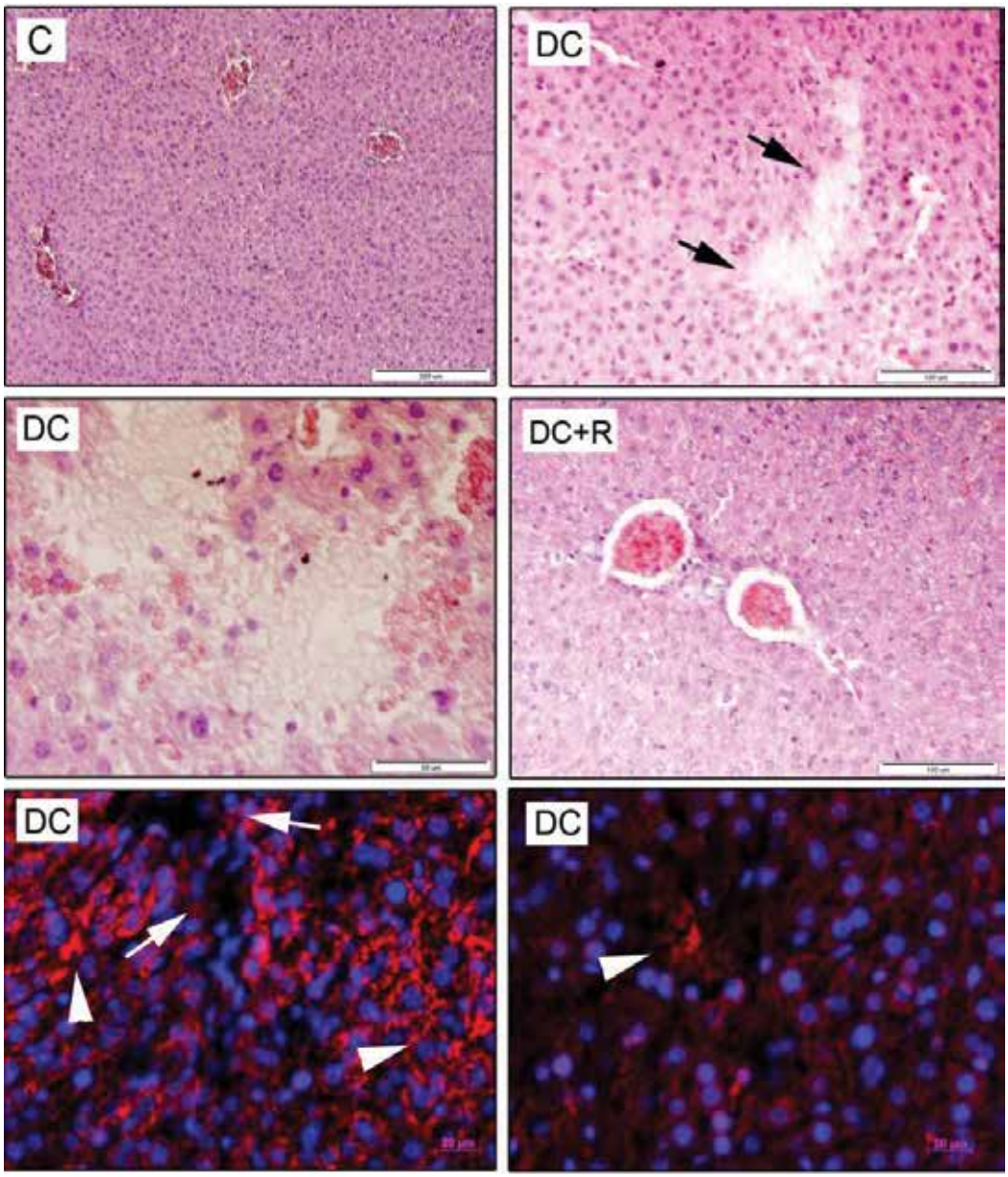

Figure 10. Representative microphotographs of the liver sections from control untreated mice (C), mice treated with antibiotics (group DC) and antibiotics-treated group after a period of recovery (DC + R). Upper panel formed of four images was prepared with light microscope on paraffin sections stained with hematoxylin/eosin. Lower panel formed of two images was prepared with fluorescent microscope on paraffin sections stained with Nile red (lipids showed in red) and Hoechst 33342 stains (nuclei showed in blue). In the livers from DC group, the sporadic occurrence of lesions (arrows) with advanced vacuolization containing a few, usually necrotic, hepatocytes and disrupted sinusoids (arrowheads) was observed. In this group, fluorescent stains demonstrated the presence of lipids droplets in some hepatocytes (arrowheads) and in the lesions (arrows) as well as the absence of fragmented apoptotic nuclei of hepatocytes and other cells. The representative microphotograph of the liver from DC + R group showed normal tissue morphology without any histopathological changes.

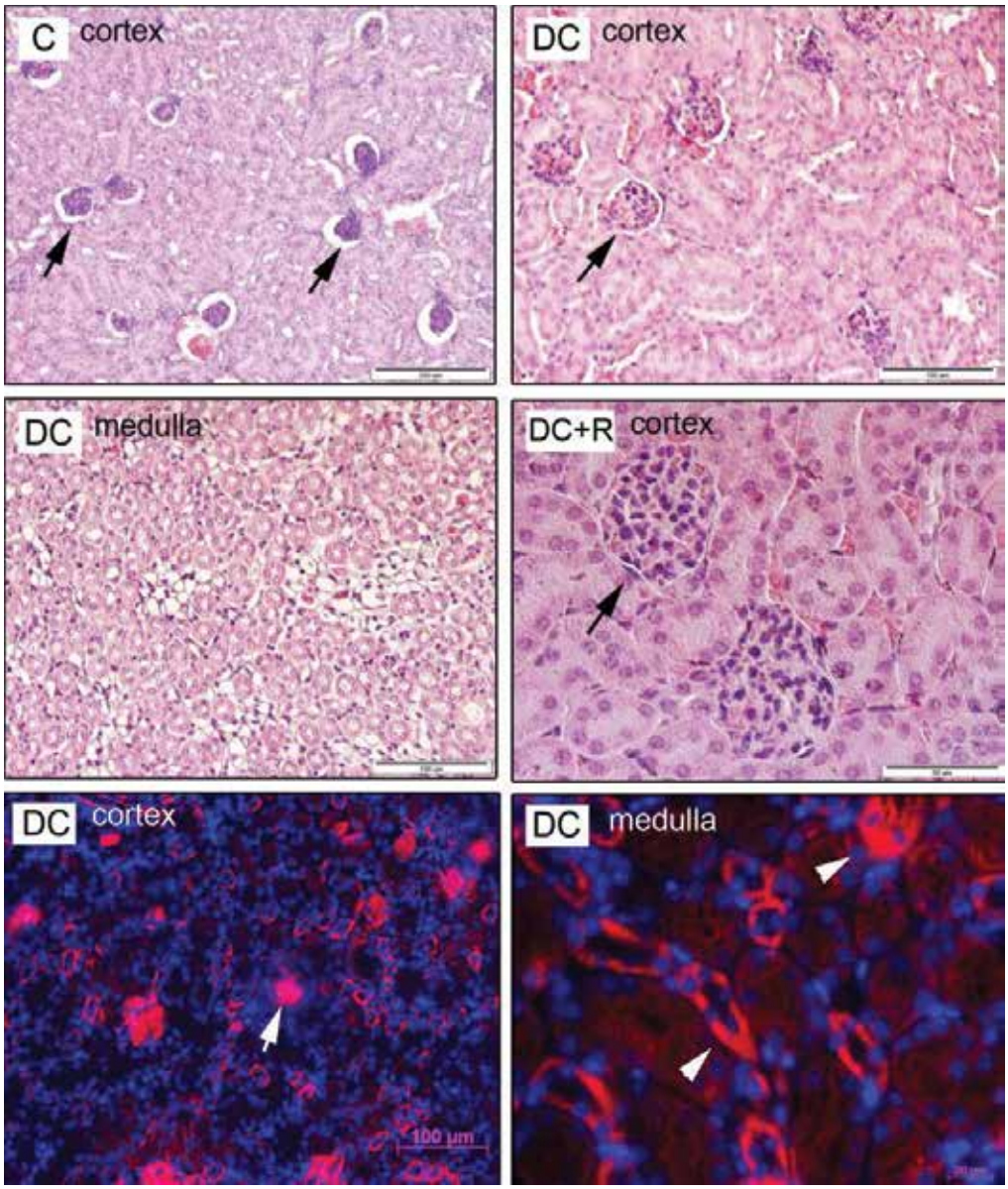

Figure 11. Representative microphotographs of the kidney sections from control untreated mice (C), mice treated with antibiotics (group DC) and antibiotics-treated group after a period of recovery (DC + R). Upper panel formed of four images was prepared with light microscope on paraffin sections stained with hematoxylin/eosin. Lower panel formed of two images was prepared with fluorescent microscope on paraffin sections stained with Nile red (lipids showed in red) and Hoechst 33342 stains (nuclei showed in blue). Normal morphology of the cortex of kidney from control group (C) showing multiple renal corpuscles consisting of the glomerulus and the surrounding capsule (arrows). In DC group, the overall morphology of cortex, appearance of these Bowman's capsules as well as morphology of central medullar part did not show any pathological alterations or damage to cells. A representative image of kidney´s cortex from DC + R group showed normal morphology. Images of DC group showing positive signal for neutral lipids droplets in the cortex (left, arrows) and in some of renal cells in medulla of kidneys (right, arrowheads). No apoptotic process in kidney cells was seen in either of examined groups.

The concentration of *valeric acid* in group DC (**Figure 9**) on day 1 of treatment with antibiotics (ATB) showed a similar increase as that observed for acetoacetic acid. The difference compared to group DC + R was significant ($P < 0.01$). Subsequently, a significant decrease ($P < 0.05$) in concentration of butyric acid was observed by day 2 of the experiment in comparison with day 1 of treatment with ATB. Similar increased concentrations of this acid persisted by day 5 after treatment with ATB. The group that convalesced after treatment (DC + R) showed an insignificant decrease in production of valeric acid after 24 h of treatment with ATB. In the subsequent period, the level of this acid did not exceed 16 mmol/L.

3.7. Histological examinations of livers and kidneys

The liver and kidney cells are highly sensitive to harmful effects of xenobiotics including antibiotics; therefore, we examined histomorphology of the livers and kidneys from control mice without treatment (C), from treated mice (DC) and from treated group of mice after a period of recovery (DC + R). Light microscopy revealed that liver sections from control mice showed normal liver architecture and hepatocytes were arranged in rows radiating out from central veins (**Figure 10**). In the livers from DC group, we observed sporadic occurrence of lesions (arrows) with advanced vacuolization containing a few, usually necrotic, hepatocytes and disrupted sinusoids (arrowheads). Such altered or loose liver parenchyma indicated an early metabolic injury to the cells. In this group, fluorescent staining specific for neutral lipids demonstrated the presence of lipids droplets in some hepatocytes (arrowheads) and in these lesions (arrows). However, we did not find the fragmented nuclei of hepatocytes and other cells indicating that treatment did not elicit apoptosis. The representative microphotograph of the liver from DC + R group showed normal tissue morphology without any histopathological changes.

The representative **Figure 11 (C)** of paraffin section after hematoxylin/eosin staining of cortex from untreated group demonstrates multiple renal corpuscles consisting of the glomerulus and the capsule around it (arrows). In DC group, the overall morphology of cortex and appearance of these Bowman's capsules did not show any pathological alterations or damage to cells. Central medullar part of kidneys from DC group had the same morphology as was observed on sections from control mice (not shown). A representative image of kidney's cortex from DC + R group (**Figure 11**) showed normal morphology. Using the fluorescent double staining methods we demonstrated the positive signal for neutral lipids droplets in the cortex (left, arrows) and in some of renal cells in medulla of kidneys (right, arrowheads) in all groups. No apoptotic process in kidney cells was seen in either of examined groups.

4. Discussion

Animal gut microbiota is a complex community of trillions of microbes colonizing the digestive tract of animals. This extensive community, comprising as many as 10^{12} colony-forming

units/mL in the colon, affects physiology of the gastrointestinal tract, the function of distant organs and susceptibility of animals to diseases [37]. Despite the enormous bacterial load carried by the gastrointestinal tract and the sheer variety of species present, an exquisite balance is maintained at almost all times. The combination of an efficient, self-repairing barrier, abundant mucus secretion, continuous luminal flow of contents and a vigorous yet finely regulated immune system is capable of keeping a massive foreign population contained within the limits of the mucosa [34]. This delicate equilibrium represents a well-balanced opposition of considerable forces. Alteration of this equilibrium is pivotal in the development of diseases of gastrointestinal tract.

Laboratory animals such as germ-free (GF) rodents have proved important for studying the effects of microbial mono- and poly-colonizations on host phenotype [38–40] and in the search for a mechanistic understanding of microbe-mediated changes in several disease models [41–45]. An alternative is temporary gut sterilization, which may involve absolute or selective elimination of microflora [27, 28]. The first studies devoted to decontamination of the digestive tract by ATB investigated successfulness of such decontamination and removal of microorganisms from the animal digestive tract. Results indicate that decontamination of mice [46], monkeys [47], dogs [48], Syrian hamsters [49] and pigs [50] with oral antibiotics is feasible. However, these studies did not investigate the effect of ATB on animal health. In human medicine, the beginnings of decontamination of digestive tract were related to prevention of septicemia in patients with granulopenia [51], in studies of burns therapy [52], acute pancreatitis [53], and later in acute stroke [54], critically ill patients [55] or esophageal resection [56] and prevention of acute graft-versus-host disease following allogeneic bone marrow transplantation [57]. Selective antibiotic treatment resulting in decontamination of the digestive tract was capable of preventing severe infections and reducing mortality rate in patients in the critical stages of diseases. Concern about development of bacterial resistance associated with the use of such decontamination and the absence of its influence on mortality, have not been confirmed [58]. The aim of SDD (Selective Digestive Decontamination) is to prevent or eradicate, if present, the oropharyngeal and intestinal abnormal carriage of potentially pathogenic microorganisms, such as Gram-negative aerobic microorganisms, methicillin-sensitive *Staphylococcus aureus* and yeasts [58, 59].

Various antibiotic cocktails have been shown to completely or selectively sterilize the gastrointestinal tracts of mice and rats [31, 32, 60]. Our study was aimed at decontamination of BALB/c SPF mice in a way that would not have adverse effect on their health. Similar to Johnson et al. [27], we strived to develop a non-invasive, relatively simple and inexpensive method of decontamination of the gut, testing for the sterility and maintaining controlled microbiota in model animals suitable for further experiments. In the study by Johnson et al. [27] animals were decontaminated and sterile environment in their gastrointestinal tract was maintained by enrofloxacin in Baytril 10% (Bayer, Germany) without barrier maintenance or using a laminar box. In other studies, the decontamination of gastrointestinal tract was carried out using ampicillin [61–63], bacitracin and neomycin [39], meropenem

[64, 65] and vancomycin [66] added to the drinking water. On the basis of our previous results [67], mice in our study were decontaminated with amoxicillin administered *per os*, potentiated with potassium clavulanate at a dose of 387.11 mg/kg in the form of preparation Amoksiklav (Sandoz, Slovenia) and subcutaneously administered ciprofloxacin at a dose of 18.87 mg/kg as a preparation Ciloxan (Alcon, Spain), while keeping the animals in strictly defined environment of gnotobiotic isolators. The administered doses were considerably lower than lethal doses (LD_{50}) of the selected ATB to mice. In mice the LD_{50} of amoxicillin potentiated with clavulanic acid was found to be 4526 mg/kg and of ciprofloxacin 5000 mg/kg. This means that the dosage of ATBs used in our study were lower 11.7-fold with amoxicillin and 265-fold with ciprofloxacin than the respective LD_{50} doses. In the case of ciprofloxacin, such a low dose was selected due to subcutaneous route of its administration and high nephrotoxicity associated with this ATB, which, however, was not manifested at the low dosage used in our study. While in our study we used a combination of *per os* and subcutaneous administration of the ATBs, the other studies used intragastric gavage [68–70], administration and withdrawal of antibiotics in drinking water [33, 71–73], or administration in food and water provided ad libitum [27]. The study by Donskey et al. [74] was also based on subcutaneous administration of ciprofloxacin.

Some research studies were conducted dealing with the comparison of antibiotic decontamination carried out on the basis of cultivation and studies based on commonly used antibiotic combinations. They included the clinical study E.O.R.T.C. [75], which investigated the effect of ATB selected on the basis of cultivation and compared it with the effect of combination of neomycin, cephaloridine, polymyxin (B or E) and nystatin or amphotericin B in granulocytopenic patients. Comparisons indicated good effectiveness of both methods and the differences were insignificant. However, it is worth mentioning that only non-absorbable ATB were used in the E.O.R.T.C. [75] study. In our study, we used the ATB selected on the basis of cultivation, as recommended by Johnson et al. [27] with the aim to eliminate the ATB with marked adverse impacts on animal health.

The length of antibiotic administration in the available studies differed. In our study, we administered ATB for 5 days. This was based on preliminary examinations and procedures carried out at our institution that showed null cultivation recovery of bacteria from feces on day 3 of antibiotic administration. Van der Waaij et al. [50] arrived to similar conclusions while the length of administration of ATB in other studies varied as follows: 4 days [27, 76], 7 days [71], 14 days [68], 21 days [77] or 28 days [73, 78–80]. While in our study the DC + R group of animals was kept under gnotobiotic conditions for 10 days following the antibiotic administration, in other studies, the mice convalescence period lasted from 14 days [71, 77] up to 5 weeks [68].

Following the 10-day convalescence period, the cultivable colonies obtained from feces and caecum content of SPF mice were tested biochemically and subjected to 16S ribosomal DNA (rDNA) sequencing that allowed us to identify *E. coli* and *Enterococcus* species. Puhl et al. [77] administered ATB for 21 days and after 14-day convalescence were able to identify by

sequencing only limited number of *Clostridium*-like and *Bacteroides* species. Ubeda et al. [71] detected an increased bacterial density 2 weeks after cessation of 7-day antibiotic treatment with ampicillin, vancomycin or combination of metronidazole, neomycin and vancomycin (MNV). They observed decreased frequencies of microbiota native to the *Bacteroidetes* phylum and the *Lactobacillaceae* family and increased frequencies of bacteria associated with the *Clostridium* and *Enterococcus* genera and *Enterobacteriaceae* family. In the study by Ubeda et al. [71], the effect of antibiotics on microbial density was investigated by quantitative PCR (qPCR) of bacterial 16S rRNA genes. The results showed that all three tested antibiotic regimens caused a decrease in the number of 16S rDNA copies in the ileum by a factor of approximately 100, whereas consistent reduction of bacterial density in the caecum was achieved only by ampicillin. Tenfold reduction in the quantity of 16S genes was observed in four of six mice treated with vancomycin and three of six mice that were administered MNV. Similar results were obtained by [81] by analyzing samples of feces after using a combination of four antibiotics (vancomycin, neomycin, metronidazole and amphotericin). By day 13, as many as 86% of the mice subjected to antibiotic treatment exhibited successful depletion of their cultivable aerobic and anaerobic fecal microbiota. The corresponding fraction determined on day 24 was 74%. Thus, a minimum of 100-fold reduction of cultivable aerobic Gram-negative rods and 106-fold reduction of cultivable aerobic Gram-positive cocci as well as cultivable anaerobic fecal bacteria was detected in depleted mice (1 CFU/mg feces). The authors observed significantly reduced copy number of 16S rRNA genes in feces of all mice subjected to the depletion protocol: all samples collected from mice treated with antibiotics displayed similar level of bacterial DNA that was, on average, more than 400-fold lower in comparison with untreated mice. In the study by Ge et al. [73], after 4 weeks of antibiotic treatment (ampicillin, neomycin sulfate, metronidazole, and vancomycin), the average number of operational taxonomic units (OTU) of mice decreased significantly from 383.4 ± 23.4 to 74.9 ± 3.1 ($P < 0.01$). The antibiotics resulted in changes in the composition of commensal bacteria examined by 16S rRNA analysis. At phylum level, only Proteobacteria accounted for more than 0.5% of all the microbiota in antibiotic-treated mice. Our study showed that a decrease in plate counts of microorganisms occurred 24 h after the antibiotic treatment. By this, time the bacterial counts decreased by 4 logs under aerobic conditions and by 3–4 logs at anaerobic cultivation. In the study by Ubeda et al. [71] bacterial density in the caecum increased after antibiotic cessation. This was in an agreement with the results of our study. Ubeda et al. [71] observed that the *Enterobacteriaceae* operational taxonomic units that predominated in antibiotic-treated mice were also present in the ileum wall of some of the untreated mice. This observation suggests that the ileum wall may be the source of the *Enterobacteriaceae* that had increased after antibiotic treatment. Stecher et al. [82] reported that the recovery of the normal microbiota, as measured at the phylum level, occurred 5 days after termination of treatment with streptomycin. Yuan et al. [68] demonstrated that neonatal amoxicillin treatment affected significantly the biodiversity of the murine intestinal *Lactobacillus* community and the impact was long lasting. In agreement with previous studies [71, 83], it seems reasonable to assume that some bacterial populations do not recover after antibiotic withdrawal.

The ciprofloxacin MIC against *E. coli* (GenBank KX086704), determined in our study by Etest® strips, was 0.064 mg/L. According to EUCAST [84], the MIC breakpoint for *E. coli* is ≤1.0 mg/L; therefore, we can assume absence of ciprofloxacin-resistant bacteria. This resembles observations of Bergan et al. [85] who studied the pharmacokinetics of ciprofloxacin in 12 volunteers, given 500 mg of ciprofloxacin orally twice a day for 5-days. In their study, counts of enterobacteria and enterococci in feces decreased markedly, whereas no marked changes were observed in anaerobic flora (anaerobic cocci, fusobacteria and bacteroids). Fourteen days after termination of drug treatment, the salivary and fecal microbiota returned to normal. The amoxicillin and potassium clavulanate MIC against our second cultured strain, specifically *Enterococcus* sp. (GenBank KX086705), determined by M.I.C. Evaluator strips was 0.25 mg/L. According to EUCAST breakpoint table for bacteria [84], the MIC breakpoint of amoxicillin-clavulanic acid for *Enterococcus* spp. is ≤2.0 mg/L. Although the bacteria recovered in our study were not resistant to the relevant antibiotics, they may have been inactivated as reported by Van der Waaij and Nord [86]. The ATB effective doses may be reduced to various degrees by enzymatic activity, or non-enzymatically by intestinal contents. Such reduction may be dependent on individual differences in microbiota and pharmacokinetic properties of the respective antibiotics [86].

Flow cytometry results obtained in our study showed a decrease in viability of microorganisms in feces. The differences were significant ($P < 0.01$) between days 1 (36.03%) and 2 (28.33%) following the antibiotic treatment and survival rates before the treatment (60.58%). Very similar method based on BacLight™ Live/Dead Viability Kit was used by Johnson et al. [27]. The authors investigated antibiotic inactivation by determination of bacterial viability in feces employing fluorescence staining of samples. Before antibiotic treatment, the mean proportion of live bacteria found in the feces of one mouse (expressed as a percentage of the total bacterial cells present) was 13.86%. By antibiotic treatment, this proportion was reduced to 0.17%. The corresponding values for the second mouse reached 13.37% before and 0.15% after the treatment. In both animals, the treatment with Baytril caused a significant reduction in viability of bacterial cells in feces to less than 1% of the originally determined values ($P < 0.05$ and $P < 0.05$).

Short chain fatty acids are the principal metabolites of intestinal fermentation and their concentrations in the digestive tract reflect the level of this fermentation. The most pronounced decrease in production of organic acids, particularly acetic, lactic and propionic acids in feces of both decontaminated groups (DC, DC + R), was recorded as soon as 24 h after starting with administration of ATB, which correlates with decreased plate counts of microorganisms in these groups by 4 logs after aerobic cultivation and 3–4 logs after anaerobic cultivation. Also during the following days of administration of ATB (days 2–5), low level of intestinal fermentation was detected in the feces of decontaminated mice. Eleven days lasting antibiotic treatment (ampicillin, bacitracin, meropenem, neomycin and vancomycin) caused marked changes in colon microbiota and gut dysbiosis was reflected in changed concentrations of several metabolites in the colon luminal contents [87]. The depletion of the SCFAs acetate, n-butyrate and propionate, the products of microbial fermentation of dietary fiber, agreed with results presented in other studies [88–90]. In our

study, we observed high concentrations of acetoacetic and butyric acids, the products of biodegradation of lipid tissue. Keto compounds that formed at physiological state of passive degradation of lipid stores became a substitute source of energy for normal functioning of the organism at the time of energy starvation. However, by day 15 of the study, the intestinal fermentation activity was restored in group DC + R and production of organic acids returned to the level before the treatment with ATB. An interesting observation was that concentration of organic acids decreased also in the feces of control group of mice C that was not treated with ATB. This decrease could be explained by the fact that these animals were fed sterile commercial feed, supplied sterile water in bottles and sterile bedding was replaced every day.

The macroscopic picture of all decontaminated mice was typical of germ-free animals, such as megacaecum condition and a significant decrease in relative weight of the liver ($P < 0.05$) and spleen ($P < 0.01$) in decontaminated group DC in comparison with control group C. Due to complete or partial absence of microbiota, we can find some typical morphological peculiarities in the digestion tract of germ-free and gnotobiotic animals. Distinctive features of germ-free rodents are considerably thinner intestinal mucosa and enormously enlarged caecum [20] the weight of which may be 10-fold greater than the physiological one. These morphological properties of the small intestine of germ-free animals are a consequence of the absence of both immunological stimuli induced by digested bacterial antigens and the potentiating influence of bacteria that affect the level of extrusion of cells from the tip of the villi.

The potential effect of antibiotic decontamination of mice on overall health of treated animals was investigated only in small number of relevant studies. Our study showed only a slight change in the blood picture of mice from DC group in comparison with group C. Moreover, after the convalescence, all parameters determined in group DC + R returned to the physiological range [91]. The mice from DC group showed increased levels of Mo ($P < 0.05$), as well as percentage proportion of Mo % ($P < 0.01$) and Gran % ($P < 0.05$). This may be associated with intense metabolic load on the liver during the ATB breakdown. Simultaneously, the levels of RBC, HGB and HCT were increased in DC group, possibly due to reduced water intake by decontaminated animals. As the liver is the main organ involved in detoxification of various xenobiotics introduced from the external environment, it plays the principal role also in the breakdown of ATB. For this reason, we selected this organ as a reliable indicator of overall health of the tested SPF mice. Our determinations focused on the activity of hepatic enzymes and histology of the liver parenchyma. We observed an increase in the activity of enzymes AST and ALT in DC group. Results obtained after 10-day convalescence period showed a significantly higher ($P < 0.01$) activity of ALT enzyme in this group exceeding twice the upper physiological limit [91]. Although no necrosis or reversible and irreversible damage to the liver parenchyma was observed, the increased level of ALT was associated with disturbances of liver cell membranes. An increase in ALT up to 3-fold the reference value [91] is referred to as moderate and higher than this level as marked. The decontaminated group of animals (DC) exhibited also increase in specific liver enzyme LDH-5. This enzyme catalyzes reversible conversion of lactate to pyruvate and is found in

the circulation already at minimum tissue damage. The above results correspond to histological findings in group DC, which showed structural alterations (presence of necrotizing hepatocytes, vacuolization and damage to sinusoids, multinuclear cells and lipid infiltration) in comparison with group C. However, after the convalescence period, the activity of isoenzyme LDH-5 returned to the level recorded before treatment with ATB. In our study, the activity of ALP enzyme showed no augmentation in any of the investigated groups. Marked elevation of serum ALP levels is characteristic for cholestatic hepatotoxicity [92]. Substances known to lead to this type of injury include amoxicillin/clavulanate and chlorpromazine. Cholestatic hepatotoxicity rarely progresses to the stage of chronic damage to the liver and gradual destruction of intrahepatic biliary tract [92]. In our study, we used amoxicillin and potassium clavulanate but no signs of development of cholestatic hepatotoxicity were not observed. Instead, mice from DC group showed signs of fatty liver, a reversible condition also known as fatty liver disease (FLD). Impaired metabolism of fatty acids results in accumulation of triglycerides that form nonmembrane-bound vacuoles in cells. These vacuoles may displace the nucleus from its usual location [93]. By day 10 of convalescence, the majority of hematological and biochemical parameters in group DC + R returned back to the physiological range. With respect to biochemical parameters, we observed an increased activity of enzyme ALT, hyperbilirubinemia and increased level of LDL-cholesterol indicating irritation of hepatic cells, however, the structure of liver tissue showed no marked changes. An interesting observation was that of isoenzyme LDH-3 in group convalesced for 10 days (DC + R) was increased by 20% in comparison with decontaminated group DC. As this is a pulmonary isoenzyme, its increased activity is associated with damage to pulmonary parenchyma. During the experiment, the mice from group DC + R were kept 3-fold longer in a gnotobiotic isolator with active ventilation and ventilation was probably the main cause of the change.

Animals obtained under this protocol can be used in our further studies such as nutritionally important relationship between the intestinal microflora and the host, interactions between microorganisms in the gut or modulation of metabolic and physiological parameters of host with selected probiotics.

In conclusion, decontamination of SPF BALB/c mice with combination of per oral administration of amoxicillin and clavulanate potassium and subcutaneous administration of ciprofloxacin every 12 h during 5 days reduced viability of microorganisms in feces and caecum content and resulted in absence of cultivable microorganisms in feces. After 10-day convalescence of antibiotic-treated SPF mice under gnotobiotic conditions the diversity of gut microbiota of mice was not recovered as it was reduced to only two detectable cultivable species, specifically to *E. coli* (GenBank KX086704) and *Enterococcus* sp. (GenBank KX086705), that returned to metabolic and morphological values within the physiological range. Finally, a mouse gnoto-model with reduced and controlled microflora was created without evident alteration of the overall health status. The animals obtained under this protocol can be used in further studies dealing with nutritionally important relationship between the intestinal microflora and the host, interactions between microorganisms in the gut, or modulation of metabolic and physiological parameters of the host using selected probiotics.

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Chapter 5

Influence of Selected Per Orally Administered ATB on Microflora of GIT in Experimental Animals

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Additional information is available at the end of the chapter

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Abstract

Composition of gastrointestinal (GIT) microbiota differs in individual parts of GIT. Only 40% of GIT bacteria are cultivable. Fluorescence-in-situ-hybridization (FISH) can detect non-cultivable bacteria. Perorally administered antibiotics (ATB) affect the composition of microbiota in GIT. The absorbed ATB, namely penicillins, tetracyclines, macrolides or fluorochinolons, have different influence in comparison with poorly absorbed oral ATB, such as aminoglycosides, aminocoumarines or polypeptides. This effect is due to retention of high concentration of non-absorbed ATB during passage through GIT and their longer influence on bacteria living in different parts of GIT. Study methods were based on scientific literature review from PubMed, Elsevier databases and Slovak scientific publications. We searched for publications between years 1980 and 2016, with keywords: ATB, influence, microbiota, FISH. The literature review focuses on peroral administration of ATB to humans and animals and its potential effect on composition of GIT microbiota. The relevant studies showed that per orally administered ATB produced many important changes in microbiota of GIT. FISH method was more frequently used for screening the normal composition of microbiota than for studying the effects of ATB although there were some studies dealing also with this issue.

Keywords: peroral ATB, effect, microbiota, GIT, FISH

1. Introduction

Although the use of antibiotics administered antibiotics (ATB) is nowadays often necessary, there is still a number of issues that arise from their abuse. It is known, that excessive use of ATB has a negative impact on physiological composition of intestinal microbiota,

especially when they are administered *per os*. This is due to increase in gastrointestinal (GIT) diseases. To understand the impact of ATB on GIT microbiota it is necessary to know the correct composition of the GIT microbiota and changes induced by various ATB in this convocation. The most common pattern for tracking changes in the microflora is faeces. However, there is little knowledge on microbiological changes in various parts of GIT. Experimental animals, both conventional and gnotobiotic, were used in relevant studies. However, they were fed a different type of food in addition to a number of anatomical and physiological differences. Therefore, for many scientists this issue still remains a great mystery. Also, until the development of sensitive molecular methods, conventional culture methods were used to track these changes. However, since 40–90% of the intestinal bacteria are not cultivable, scientists looked for and tested more sensitive and accurate methods for the detection and quantification of microorganisms [1]. For example, developed were methods based on PCR-DGGE, real-time PCR, and others. However, even these methods have shortcomings that require an amplification process which may introduce an untargeted error. The fluorescent-in-situ-hybridization (FISH) method is independent of the amplification and is sufficiently sensitive to trap even non-cultivable microorganisms. So far scientists have used a number of FISH to determine the physiological composition of microbiota of GIT, either animal or human. In addition, the new development allows one to monitor potential changes under the impact of substances added to the diet in both experimental animals and clinical patients. The aim of this study was to summarise the findings on the impact of ATB on composition of intestinal microbiota by means of FISH method using available sources and compare them with previously published knowledge in this area. The importance of this study consists in finding out whether it is possible to track by this method the changes in GIT microbiota produced by ATB and thus contribute to the body of knowledge in this area.

Recently, the increasing resistance of bacterial agents to ATBs, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) or *Pseudomonas*, stressed the importance of the development of novel ATB derivatives [2]. New classes of antibiotics are urgently needed to treat nosocomial infections. The risk of increasing ATB resistance also increases due to the increased use of broad-spectrum ATBs in unprofessional human and veterinary clinical practice, without detecting the bacterial origin of the disease and its sensitivity to ATBs. ATB residues in food of animal origin from countries not complying with the 2006 EU Directive have an impact on the increased risk of spreading antibiotic resistance. Development of ATB-resistant strains can be prevented by using correct therapeutic dose of ATB and completing the prescribed course of treatment. Properly balanced intestinal microflora prevents the development of resistant microbial strains. Normal microbiota acts as a barrier against the colonisation of potentially pathogenic microorganisms and against the excessive growth of opportunistic microorganisms already present. Administration of ATBs either therapeutically or as a prophylactic measure disturbs the ecological balance between the host and the normal microbiota. The clinically most common symptoms of intestinal microbiota disruption are diarrhoea and fungal infections that usually resolve after the treatment has ended [3]. It is difficult to assess the long-term consequences of microbial symbiosis disorders in the intestine. In addition to

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changes in intestinal microbiota, many chronic diseases such as asthma and atopic diseases are associated with the use of ATB in childhood [4].

2. Antibiotics

In 1928, Alexander Fleming discovered that the growth of *Penicillium notatum* suppressed the growth of staphylococci, and then, as this phenomenon was studied, it was found that the cause was an exoproduct of a mould called penicillin that was released into the cultivation medium. In 1938, Howard Florey and Ernst Chain began experimenting with penicillin mould. By 1941, a sufficiently purified form of penicillin was obtained and by early 1942, American pharmaceutical companies were mass producing penicillin for distribution to Allied soldiers during the Second World War [5]. Since the first effects of ATB have been discovered, other substances with ATB properties have appeared and many have found a wide range of applications in medicine for the treatment of infections caused by bacteria, pathogenic fungi, mycoplasmas, rickets, chlamydia and some other agents [6]. Attempts to influence GIT microbiota with ATB date back to the very beginning of their use. The impact of ATB was observed in clinical practice as well as during preoperative patient preparation. With regard to animal production, it raised interest particularly for economic reasons, as it was shown that ATBs accelerate the growth and weight gain in mice, dogs, but also in pigs and calves. Experiments on germ-free chickens revealed that the nutritional effect of ATB is mainly related to suppression of some subclinical infections [7].

Antibiotics are substances of organic origin produced by bacteria and moulds, possibly from higher plants or animal tissues, and can be prepared synthetically or semi-synthetically [8]. Their name was derived from the phenotype of Pasteur, which was described by Pasteur in the 1960s.

According to their biological effect on microorganisms they are divided to two groups, one with bacteriostatic action and another one with bactericidal effect. Bacteriostatic ATBs arrest multiplication of bacteria so the bacteria are not killed and natural dying of quiescent bacterial cells is not affected. Bactericidal effect of ATBs results in death of bacterial cells. The bactericidal effect during the first 4 hours of action of ATBs is of specific importance. If at least 99% of bacteria is killed within this time we can speak about clinically relevant bactericidal action.

ATBs are divided into 5 groups according to the mechanism of action:

1. Inhibition of cell wall synthesis (bactericidal effect), (typical of penicillins, vancomycin, cycloserin)
2. Effect of cell wall function (bactericidal effect), (typical of polymyxins)
3. Inhibition of protein synthesis (bacteriostatic and bactericidal effect) (chloramphenicol, tetracyclines, aminoglycosides, macrolide ATBs)

4. Inhibition of nucleic acid synthesis (bactericidal effect), (griziofulvin, rifampicin)
5. Interference in the intermediary metabolism of bacteria (sulfonamides)

2.1. Oral antibiotics

Not all ATBs can be administered orally, but ATBs capable of influencing GIT microbiota must be available in the form suitable for oral administration. The most commonly used orally administered ATBs include: penicillins, cephalosporins, tetracyclines, polypeptide ATBs, aminoglycosides, macrolides, Lincosamide ATB, ansamycin ATB, diterpenes, aminocoumarin ATBs, steroid antibiotics, sulfonamides and quinolones. Among these, we include the following representatives:

1. **Penicillins**:

   (A) **Phenoxypenicillins**: Phenoxymethylpenicillin—Penicillin V, Penamecillin, Penetacillin, Benetaminpenicillin, Phenticillin, Propicillin, Phenbenicillin, Klometocillin

   (B) **Wide spectrum of penicillins**:

   1. **Aminopenicillins**: Ampicillin, Bakampicillin, Pivampicillin, Talampicillin, Amoxicillin, Epicillin, Cyclaclin
   2. **Carboxypenicillins:** Carbenicillin Esters: Indanyl Carbenicillin, Carfecili
   3. **Amidopenicillins:** Mecilinam esters: Bakmecilinam, Pivmecilinam
   4. **Isoxazolylpenicillins:** Oxacillin, Dicloxacillin, Kloxacillin, Flucloxacillin, Pirazocillin
2. **Cephalosporins**: Cefalexin, Cefadroxil, Cefixim, Metacyclin, Tiacycline
3. **Amphenicols**: Chloramphenicol, Tiamfenicol, Florfenicol
4. **Tetracyclines**: Chlortetracycline, Oxytetracycline, Tetracycline, Doxycycline, Minocycline,
5. **Polypeptide antibiotics**: Polymyxins: Polymyxin B
6. **Aminoglycosides**: Streptomycin, Neomycin, Kanamycin, Apramycin, Gentamicin, Tobramycin and Aminocyclitols: Spectinomycin
7. **Macrolides**: Erythromycin, Spiramycin, Tylozine, Oleandromycin, Troleandromycin, Josamycin, Tilmicosine, Clarithromycin, Roxithromycin and Azalides: Azithromycin
8. **Linkozamide antibiotics**: Linkomycin, Klindymycin
9. **Ansamycin antibiotics**: Rifampicins: Rifampicin, Rifaximin, Rifabutin, Rifapentin
10. **Diterpenes**: Tiamulin, Valnemulin
11. **Aminocoumarin antibiotics**: Novobiocin
12. **Antibiotics with steroid structure**: Fusidic acid

According to some authors, other peroral drugs with antibacterial activity are considered antibiotics:

13. Other antimicrobials

1. Nitroimidazole derivatives: Metronidazole, Tinidazole Nimorazole
2. Sulfonamides:

   Short-acting: Sulfathiazole, Sulfacetamide, Sulfisoxazole.

   Medium-effective sulphonamides: Sulfadimidine, Sulfadiazine, Sulfamerazine, Sulfamethoxazole + Trimetoprim = Kotrixomazole, Sulfachloropyridazine.

   Long-term effective: Sulfamethoxypyridazine, Sulfadoxine, Sulfadimetoxin Enteric-acting sulfonamides: Phthalylsulfathiazole, Succinylsulfathiazole, Sulfachinoxaline, Sulfaclozine
3. Quinolones: Nalidixic acid, Flumequin, Enrofloxacin, Difloxacin, Ciprofloxacin, Marbofloxacin, Norfloxacin, Sarafloxacin, Pefloxacin, Ofloxacin, Ibafloxacin, Orbifloxacin

3. Materials and methods

Search method: we searched the PubMed, and Elsevier databases and Slovak scientific literature for the studies dealing with the effect of ATBs on GIT composition. We searched for publications in the period from 1980 to 2016 using keywords related to ATB, Influence, microbiota, FISH. A literature review was produced aimed to identify association between peroral administration of ATB to humans or animals and its effect on composition of normal microbiota in GIT.

4. Influence of ATB on GIT microflora

Administration of ATBs can seriously disturb the balance of the intestinal microbiota in terms of multiplication of bacteria and development of resistant microorganisms. This can lead to infections and to the transfer of resistance factors between bacteria [9]. According to the majority of authors, the effect of ATB on nutrition is mediated by intestinal microbiota. Antibiotics are divided according to their effect on GI microbiota to ATBs capable of absorption across the intestinal wall and to those that cannot be absorbed at all or only in very small amounts. The lower the bioavailability of ATB the more it remains in the colon and thus the risk of suppression of intestinal microflora increases. If ATBs are absorbable (e.g. tetracycline, penicillin, chloramphenicol, etc.), their concentration is lower in the GIT endpoints. In contrast, ATBs incapable of absorption (e.g. streptomycin, polymyxin, neomycin, etc.) may have a strong toxic effect on the microbiota throughout the GIT. The effect of ATB is generally dependent on the dose, the active substance, the duration of administration and other factors. The search results clearly demonstrated that the effect of ATB on the GIT microbiota is as follows:

1. Breach of microbial balance (in GIT, urinary tract, reproduction tract, etc.).
2. Vitamin K hypovitaminosis as result of long-term use ATB (especially p.o.)

3. Resistance of resistant strains, superinfection: *Candida*, *Staphylococcus*, *Pseudomonas*, *Clostridium difficile* and others.
4. Evidence of rapid bacteriolysis, particularly Gram-negative bacteria (endotoxin release)

4.1. Testing of ATB effect on animals

The studies of the effect of ATB on microbiological-clinical microbiota date back more than 50 years ago [10, 11]. The effect on microbiota was investigated with regard to the weight gains of conventional experimental animals. Studies on germ-free animals (without GIT microbes) showed weight gains related to ATB [11]. It is still an up-to-date topic as indicated by recent studies [12]. To demonstrate the presence of bacteria and changes in their numbers, whether under the influence of antibiotics and other substances, conventional cultivation methods are still used. However, these methods have recently been supplemented by more sensitive molecular methods. One of the methods used for quantification of bacterial population is the fluorescent-in-situ-hybridization method (FISH). These methods can be used to accurately identify and quantify the species representation of microorganisms [13]. While radioactive labelling was previously used in the FISH methodology, today we use fluorochrome-labelled probes [14]. The probes serve to specifically bind to that part of the target sequence that exhibits a high degree of sequence complementarity. The probes consist mostly of 15–30 nucleotides and are covalently labelled with a fluorescent dye at the 5 'end—fluorescein, tetramethylrodamine, Texas red, carbocyanine. Up to now, several probes have been standardised, which are currently used to quantify the major intestinal bacteria (**Table 1**). For example, a probe called (S-G-Lab-0158-a-A-20) or abbreviated Lab158 is designed to detect the presence of *Lactobacillus* spp./*Enterococcus* spp. in the monitored samples. It is an oligonucleotide with the sequence 5'X-GGT AAT AGC A (T/C) C TGT TTC-3 'wherein X is fluorochrome [16]. This method is particularly useful in the study of the effect of probiotics, which are often required to identify probiotic bacteria of the commensal microflora [17]. Recently it was reported that the simultaneous use of ATB and supportive probiotic therapy, which can help to restore intestinal microbiota, can also expand the antibiotic resistance of bacterial intestinal bacteria [18, 19].

4.2. Changes in GIT miroflora after ATB treatment in laboratory animals by FISH methods

In addition to scientific papers dealing with the impact of antibiotics on the microflora of GIT by means of conventional culture methods, studies using FISH method were also published focusing mainly on quantification of bacterial representatives in samples of various origin. This later led to the use of this method also for the purpose of monitoring the effect of ATB on the GIT microflora not only in humans [20] but also in experimental animals that were used to determine changes in composition of microbiota. For example, using of FISH for research of effect of amoxicillin potentiated by clavulanic acid on human faecal microflora in germ-free mice [21]. To provide more clear overview, the sources obtained by search were divided on the basis of their ability to absorb across the GIT.

Short name	Full name	Target microorganism	Sequences (5′ - 3′)
Sal 303	L-S-Sal-1717-a-A-18	*Salmonella* spp.	AATCACTTCACCTACGTG
Bif164	S-G-Bif-0164-a-A-18	*Bifidobacterium* spp., *Parascardovia denticolens*	CATCCGGCATTACCACCC
Lab158	S-G-Lab-0158-a-A-20	*Lactobacillus*, *Weissella* spp.; *Lactococcus lactis*; *Vagococcus*, *Enterococcus*, *Melisococcus*, *Tetragenococcus*, *Catellicoccus*, *Pediococcus* a *Paralactobacillus* spp.	GGTATTAGCAYCTGTTTCCA
Bac303	S-Bacto-0303-a-A-17	*Bacteroides sensu stricto*, *Prevotella* spp., *Parabacteroides*; *Barnesiella viscericola* a *Odoribacter splanchnicus*	CCAATGTGGGGGACCTT
Chis150	S-Chis-0150-a-A-23	*Clostridium tyrobutyricum*; *Adhaeribacter aquaticus*, *Flexibacter canadensis*, *Flexibacteriaceae*; *Propionibacteriaceae*	TTATGCGGTATTAATCTYCCTTT
Rbro730	S-Rbro-730-a-A-18	*Ruminococcus bromii-like*; *Clostridium sporosphaeroides* a *Clostridium leptum*	TAAAGCCCAGYAGGCCGC
Rfla729	S-Rfla-729-a-A-18	*Ruminococcus albus* a *Ruminococcus flavefaciens*	AAAGCCCAGTAAGCCGCC
Ato291	S-Ato-0291-a-A-17	*Atopobium*, *Colinsella*, *Olsenella Eggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium Mycoplasma elephantis*	GGTCGGTCTCTCAACCC
Erec482	S-Erec-0482-a-A-19	*Clostridium saccharolyticum*, *Syntrophococcus sucromutans*, *Bacteroides galacturonicus Bacteroides xylanolyticus Lachnospira pectinschiza*	GCTTCTTAGTCARGTACCG

Source: http://onlinelibrary.wiley.com/doi/10.1111/j.1574-6941.2008.00610.x/pdf
Table processed by the author from the original Table [15].

Table 1. Probes for FISH analysis used to detect bacterial populations in samples from *in vitro* fermentation.

4.2.1. Absorbable ATBs

4.2.1.1. Penicillin ATBs: aminopenicillins

4.2.1.1.1. Amoxicillin

The results of studies dealing with the effect of amoxicillin on the microbiota indicate that *per os* administration caused a significant decrease in the number of total faecal bacteria by almost 30%, as determined by the universal Eub338 probe. Major microbiota populations such as *Fusobacterium*,

Eubacterium and *Atopobium* were affected by amoxicillin. There was observed also a percentage increase in *Bacteroides* and *Bifidobacterium*. The results also showed that not all evaluated populations were affected by the ATB. The greatest change was observed in *E. coli* counts, which increased significantly during ATB administration [3, 22]. By using FISH, the effect of amoxicillin potentiated by clavulanic acid on human faecal microbiota in germ-free mice was observed [21]. In this study, amoxicillin with clavulanic acid was administered orally for 7 days and the results were compared with the control group of mice not treated with ATB. Molecular analysis of digestive microbiota was performed in a 2-week experiment using FISH in combination with flow cytometry (FC) using specific 16S rRNA target probes for *Bacteroides-Porphyromonas-Prevotella*, *Clostridium coccoides-Eubacterium rectale*, *Clostridium histolyticum*, *Faecalibacterium prausnitzii*, *Enterobacteriaceae*, *Lactobacillus*, *Enterococcus*, and *Bifidobacterium*. *Clostridium coccoides-Eubacterium rectale* and *Bacteroides-Porphyromonas-Prevotella*, which represented the dominant flora, were found to be the most abundant bacterial groups. The *Clostridium coccoides* group was stable in control mice (from 40.7 ± 1.6% to 45.6 ± 2.8%) but significantly decreased in the treated mice on the second day of treatment and remained at a low level throughout the ATB treatment (3.9 ± 0.8%). At the end of ATB administration, the levels increased (17.7 ± 4.7%) and by day 14 reached 36 ± 1.8%. The *Bacteroides-Porphyromonas-Prevotella* group in control mice persisted at 35.9 ± 4.3%, whereas in treated mice it increased from 1 to 6 days when it reached 58.5 ± 0.4.5%. From day 9, the level decreased to 38.6 ± 5.7% until it reached the same level as in control mice at the end of the experiment [21]. This animal model allowed the authors to conclude that amoxicillin potentiated by clavulanic acid disrupts the balance of the dominant anaerobic microflora and that the *Clostridium coccoides* group is very susceptible to amoxicillin potentiated by clavulanic acid. No *Enterobacteriaceae* bacteria were detected in control mice, on the other hand their number increased and they were detectable in the treated mice from day 2 of administration of ATB. From day 8, their counts decreased and from day 11 until the end of the experiment they were no more detectable. *Faecalibacterium prausnitzii* and *Clostridium histolyticum* were present in 1.3 ± 2.1% and 0.4 ± 0.4% of control mice [21]. No bacteria were detected in the treated mice during administration of ATB, i.e. these bacterial groups were sensitive to amoxicillin-clavulanic acid. From day 1 to day 14 after administration of ATB, the counts of these groups of bacteria were similar to those in control mice. The probes for *Bifidobacterium*, *Lactobacillus* and *Enterococcus* did not detect any signals in either treated or control mice [21]. During 7 days of *per os* treatment with amoxicillin potentiated with clavulanic acid, the effect of *Saccharomyces boulardii* yeasts on the composition of intestinal microbiota in mice associated with human microbiota was also investigated. The predominant groups of bacteria were quantified by FISH in combination with flow cytometry. Probes for *Eubacteria*, *Bacteroides-Porphyromonas-Prevotella*, *Clostridium coccoides-Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Clostridium histolyticum*, *Lactobacillus-Enterococcus*, *Enterobacteriaceae* and *Bifidobacterium* species have been used. The observed mice were divided into two groups of mice, the first group received yeast and the second did not. In the second group the level of *Enterobacteriaceae* and *Bacteroides* increased but the numbers of *C. coccoides-E rectale* dropped dramatically. After ATB treatment, the original intestinal flora was restored more rapidly for *C. coccoides-E. rectale* and *Bacteroides-Porphyromonas-Prevotella* in *S. boulardii* mice versus control mice ($p < 0.05$) [21]. The effect of other beta lactam ATBs on the microbiota, in particular of imipenem, was also observed using the FISH method (Dubourg et al., [23]). The susceptibility

of *Akkermansia muciniphila* with respect to the effect of imipenem was also studied. In this case, the FISH method utilised a specific protozoan 5 '[Alexa488/546] GCTGCCACCCGTAGGTGT for *Verrucomicrobium*, which confirmed the presence of the bacterium. EUB338 '[Alexa488/546] 5-GCTGCCTCCCGTAGGAGT-3 [23] was also used. Stool samples with *Akkermansia muciniphila* were susceptible to imipenem.

4.2.1.2. Lincosamide ATBs

4.2.1.2.1. Clindamycin

Clindamycin was used in the study dealing with development of vancomycin-resistant enterococci (VRE) because this ATB inhibits anaerobes in the intestine without the reduction of facultative Gram-negative bacilli and VRE [24]. It has been shown that clindamycin causes VRE growth in mice and colonised patients [25]. In this study, a mouse model was used to test the hypothesis that the anaerobic microflora in the large intestine inhibits development of vancomycin-resistant enterococci. Anaerobic growth of VRE was assessed in the caecal content and cervical mucus of mice receiving subcutaneous clindamycin and in negative control administered saline solution. Following orogastric inoculation of VRE-*Enterococcus Faecium* C68, the mice were sacrificed and tested. To confirm that some Gram-positive cocci visualised in this experiment using light microscopy, a specific commercially available kit for the detection of *E. faecium* by fluorescence in situ hybridization (Microscreen) was used with *E. faecium*. In saline treated mice, no *E. faecium* was detected by in situ hybridization. In contrast, the presence of *E. faecium* was confirmed in clindamycin-treated mice [25].

4.2.1.3. Fluorochinolons

4.2.1.3.1. Ciprofloxacin

In a study investigating the role of intestinal bacteria in the pathogenesis of chronic, immuno-mediated inflammation of the intestine, ciprofloxacin has been shown to affect the inflammation of the intestine but not the inflammation of the colon. This has confirmed the selective effect of ciprofloxacin in the gut. Experimental pathogen free (SPF) mice were used. Furthermore, mice lacking the gene encoding interleukin 10 (IL10) producing colitis have been used. However, this does not occur in germ-free mice. Germ-free, IL-10 deficient mice were colonised by SPF bacteria, and narrowed and broad-spectrum ATBs were observed to influence the development and development of intestinal inflammation in IL10 deficient mice. ATBs were administered to mice orally, either preventively prior to colonisation of SPF with bacteria or therapeutically. Quantitative bacterial analysis using the FISH method used parts of the blind and the colon [26]. BAC303 for *Bacteroides*/*Prevotella*, *E. coli* specific EC1531 and other Enterobacteriaceae, Lab158 for the detection of lactobacilli and enterococci were used for FISH detection. By the FISH method, ciprofloxacin was found to reduce total aerobic bacteria in both the colon and caecum. *E. coli* was not detectable and the number of luminal enterococci was reduced. Reduction of lactobacilli was also confirmed [26].

4.2.1.4. Other antimicrobial substances: imidazole derivatives

4.2.1.4.1. Metronidazole

A study [26] on germ-free, IL10 deficient mice that were colonised by SPF bacteria (no specific pathogens) and were monitored for the effect of a particular narrow spectrum ATB metronidazole on the development of inflammation of the intestine showed a selective effect of metronidazole in the large intestine. The effect of this ATB on inflammation of the cervix was not confirmed. BAC303 for *Bacteroides/Prevotella*, *E. coli* specific EC1531 and other *Enterobacteriaceae*, Lab158 for the detection of lactobacilli and enterococci were used for FISH detection. Metronidazole is selectively effective against anaerobic bacteria, including predominantly *Bacteroides*. FISH revealed that administration of metronidazole reduced the number of *Bacteroides* species to a detectable level. Also, the amount of luminal *E. coli* was significantly reduced. FISH analysis showed that metronidazole had no significant effect on intestinal lactobacilli. Enterococci were confirmed, in particular *E. faecalis*. The study [23] confirmed that *Akkermansia muciniphila* were resistant to metronidazole.

4.2.1.5. Tetracycline ATBs

4.2.1.5.1. Tetracycline

ATBs such as tetracycline have the ability to interfere with bacterial populations in the gut. If the formation of a microbial barrier against pathogens and potential pathogens is impaired, it can lead to the proliferation of undesirable microorganisms such as *Candida albicans*. In *in vitro* studies, growth of *C. albicans* was observed in growth media in the presence of tetracycline, with a significant increase in *C. albicans*. The potency of the probiotic culture of *Lactobacillus plantarum* LPK, which was added to the *in vitro* fermentation system, was also tested to determine whether this organism had any effect on the Candida population. Although *C. albicans* was not completely removed in the presence of this bacterium, its numbers were significantly reduced. This study showed that the use of probiotics, in particular *Lactobacillus plantarum*, had a positive effect on the reduction of undesirable *C. albicans*, the number of which was increased by tetracycline administration. It also pointed out that normal intestinal microflora can itself develop a 'natural' resistance to *C. albicans* (Payne et al., [27]). In the future, it would be necessary to use a probe detecting the presence of *C. albicans* to quantify this bacterium when studying the effect of tetracycline on GIT microbiota. For this purpose, oligonucleotide 020 (5 'CCCCCTTTCCTAAACCAATCCGGA 3') can be used [28].

4.2.1.5.2. Doxycycline

One of the few studies that dealt with the effect of doxycycline on microbiota using the FISH method was a study aimed at monitoring its effect on *Akkermansia muciniphila*. For the FISH method, a specific probe 5'[Alexa488/546] GCTGCCACCCGTAGGTGT for Verrucomicrobium was used to confirm the presence of the bacterium. Also, EUB338 '[Alexa488/546] 5-GCTGCCTCCCGTAGGAGT-3 [22] was used. In the stool specimen with *Akkermansia muciniphila*, the sensitivity of this bacterium to doxycycline was confirmed.

4.2.2. Not resorbing ATB

4.2.2.1. Aminoglycoside ATB

4.2.2.1.1. Streptomycin

In streptomycin-treated conventional mice most of the facultatively aerobic Gram-negative rods, amounting to about 0.1 to 1% of microbiota, were eliminated by streptomycin treatment [29]. Multiple model experiments were used to study the effect of streptomycin on microbiota of mice. To detect the presence and quantify *E. coli* strains in streptomycin-treated mice, the authors used ribosomal probe ES 1531 specific for *E. coli* 23S rRNA and *E. coli* BJ4 reference strain that was detected in stool samples [29]. Also, the adhesion properties of *E. coli* to colonic mucosa were studied in streptomycin-treated mice and reduced numbers of *E. coli* were detected [30]. Sekirov 2008 used for the study of the effect of streptomycin on the intestinal microbiota the EUB338 mouse probe for all bacteria (*Eubacteriaceae*) with the sequence (5 '[TxRd]-GCT GCC TCC CGT AGT AGG-3'), *Cytophaga-Flavobacterium-Bacteroides* CFB286 '[Fluorescein]-TCC TCT CAG AAC TAC CCC-3') and for the Gammaproteobacteria probe GAM42a (5 'fluorescein-GCC TTC CCA CAT CGT TT-3'). Sekirov investigated the ability to produce *Salmonella* infection after ATB treatment [31]. He demonstrated that after the administration of streptomycin, the equilibrium of the microbial community of the intestine changes, giving the possibility of infection with Salmonella. He also found that increasing doses of streptomycin resulted in a gradual increase in the strains of *Firmicutes* and *Cytophaga-Flavobacterium-Bacteroides* (CFB). At the genus level, the numbers of lactobacilli and enterococci/group D streptococci decreased significantly. Gradually, the number of *Firmicutes* and other bacteria was reduced. Sekirov, however, concluded that ATB treatment changes the composition of intestinal microbiota depending on dose and type of ATB, but does not significantly change the total number of gut microbiota [31]. After 8 days of per oral administration, the use of a combination of streptomycin and penicillin caused a significant reduction in all bacterial counts measured by FISH and intestinal content analysis (Swann et al. [32]). Although almost every ATB treatment induces an increase in pathogenic colonisation, the development of enterocolitis was particularly observed after the use of streptomycin or vancomycin (Ferreira et al. [33]). In the current research, three types of mouse models were used to study the interaction between the host and the given bacterium: gnotobiotic, conventional and streptomycin-treated. Studies have shown that mice pre-treated with ATBs (e.g. streptomycin) have a higher chance of competitive growth of intestinal pathogens in the intestine, although the mechanism is poorly elucidated [34]. Streptomycin-treated mice are the best model for studying the growth and survival of extraneous microorganisms in the intestine without causing pathogenesis [35].

4.2.2.2. Macrolide ATBs

4.2.2.2.1. Erythromycin

Using a FISH method, a study was conducted that investigated the resistance of the *Campylobacter* strain to macrolide ATB erythromycin. This strain is the most common cause of inflammation

of the intestines (enteric). Because its resistance to quinolones rises, macrolides are currently the drug of first choice. In humans, the resistance of *Campylobacter* to macrolides is about 5%, but in some animals it is up to 80%. Probes for the detection of macrolide resistance in *H. pylori* were used [36, 37]. The theoretical applicability of these probes for *Campylobacter* was assessed by controlling previous publications [38, 39]. FISH may also be useful for detecting macrolide resistance in other bacteria, e.g. mycobacteria or haemophiliacs. However, for this purpose, probes must be adapted to different sequences accompanying the mutation point [40].

4.2.2.2.2. Clarithromycin

The effect of clarithromycin as the most commonly used ATB for the treatment and eradication of *Helicobacter pylori* was studied using the FISH method [36]. In this study, FISH methods were used to demonstrate the presence of *H. pylori* and to identify the 23S rRNA spot mutation responsible for macrolide resistance directly from a biopsy specimen. All oligonucleotide probes used in this study were previously described and evaluated [41]. Briefly, the HPY-1 (5′-CACACCTGACTGACTATCCCG-3′) probe targeting 16S rRNA was used to identify *H. pylori*, while the ClaR1 (A2143G) (5′-CGGGGTCTTCCCGTCTT-3′), ClaR2 (5′-CGGGGTCTCTCCGTCTT-3′) and ClaR3 (A2143C) (5′-CGGGGTCTTGCCGTCTT-3′) were used to detect the 23S rRNA spot mutation responsible for the resistance of the bacterium to clarithromycin. A ClaWT probe (5′-CGGGGTCTTTCCGTCTT-3′) was also used to identify *H. pylori* strains sensitive to clarithromycin that were not detected either by ClaR1, ClaR2 or ClaR3. Similar studies have also been addressed [42].

4.2.2.3. Glycopeptide ATBs

4.2.2.3.1. Vancomycin

In mice, the effect of vancomycin on GIT microbiota differs significantly from that of streptomycin [31]. Low doses of vancomycin reduce bacterial counts of *Firmicutes* and *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) strains and cause a small increase in the class of *Gammaproteobacteria*. Higher doses of vancomycin already cause an increase in thecounts of *Gammaproteobacteria*, to nearly 50% of the total microflora, while the counts of CFB remain reduced. The genera *Lactobacillus-Enterococcus*, group D streptococci, are affected by the overgrowth of the *Enterobacteriaceae* and cultivated aerobic bacteria. ATB treatment alone does not cause significant changes in the total number of microbes, although vancomycin administration has a much greater effect on GIT microbiotas than streptomycin [31]. In the study by [43], the broad-spectrum vancomycin-imipenem combination was shown to be effective in mice, both in the cecum and in the colon. Despite the significant decrease in *E. coli* and *E. faecalis*, the total aerobic microflora was not reduced after administration of vancomycin with imipenem. However, the amount of total anaerobic bacteria was significantly reduced. Lactobacilli were eliminated after administration of the vancomycin-imipenem combination. Using FISH, it has also been found that by administering this combination, many *Bacteroides* species have been reduced below a detectable level [43]. Also, vancomycin resistance was investigated in *Akkermansia muciniphila* [23].

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Section 3

Antibiotics and Nutrition

Chapter 6

Nutrition: From the First Medicine to the First Poison

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Additional information is available at the end of the chapter

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Abstract

Severe adverse reactions of the organism to environmental elements have been dizzily rising in humans and pets over the last 50 years. Such reactions can be expulsive (vomit, diarrhea, dandruff, and abundant secretion or excretion) or driven by an inflammatory process (which has been considered as healing process) in charge to destroy every toxic introduced into the body. Thus, it is clear that if a contaminated food is assumed daily, the inflammatory process becomes inevitably chronic. Most common inflammatory processes of dogs and cats originate from this condition, which we observed to be frequently caused by well-defined contaminants: toxic residues of oxytetracycline (OTC). In fact, once everything containing in this compound is eliminated, all inflammatory processes tend to rapidly and spontaneously regress. Here, we reviewed and discussed the problem related to the amount of pharmacological and chemical substances, which are used to increase the production of fruits, vegetables, intensive farming-derived meat and fish, milk, eggs, and grain. Such substances can persist within the products in variable amount and, gradually or rapidly (often in a few hours), poison the organism causing reactions such as allergies, anaphylactic shocks (not so frequent), autoimmune diseases (fortunately not so frequent but continuously increasing), and inflammatory processes, the most common reaction. In this context, nutrition, as a daily and frequent habit, should be taken seriously into account; given that wild animals do not seem to have the same pathologic reactions, there are no doubts that many foods deriving from intensive farming have become a poison rather than a remedy.

Keywords: food intolerances, food as carrier of chemical and/or pharmaceutical residues, oxytetracycline, increase of excretions and secretions, inflammatory processes, adverse food reactions

1. Introduction

Pathologic reactions of the organism to environmental elements sharply rose in humans and pets in the last 50 years. In this context, nutrition, as a daily and frequent habit, should

seriously be taken into account. Moreover, given that wild animals do not have the same pathologic reactions, there are no doubts that food has become a poison rather than a remedy. Here, there are few simple reflections.

To preserve pet's oral health cavity, the use of a toothbrush and toothpaste is quite frequently necessary recommended in veterinary clinical practice [1]. It is the same for the ear and body cleaning where the weekly use of an ear cleaner and a dandruff shampoo is even more used [2].

The final reason of all these precautions is the owner desire to have "normal" dogs, with an oral cavity free of plaque, a tartar, and a clean fur, as normally occurs in wild environment without any care.

The only real difference is that wild animals eat naturally available foods, while domesticated ones, and also their owners, are daily bombarded by high amounts of pharmacological and chemical substances, which are used to increase the production of food (fruits, vegetables, meat, milk, eggs, or grain).

Such substances can remain within the food and, gradually or rapidly, poison the organism causing two kinds of reactions: one very negative and abnormal, represented by allergies, anaphylactic shocks (not so frequent) [3], and autoimmune diseases (unfortunately even more frequent and dramatic) [4, 5], and one "positive," represented by food intolerances [6].

The reason why we defined "positive" food intolerances is in the definition, which unequivocally identifies foods as normal, and the reactions to these as abnormal. On the contrary, foods result as abnormal because they systematically contain chemical and pharmacological residues and can cause symptoms related to damage induction and to the defense reactions triggered by the host. Such reactions can be expulsive (vomit, diarrhea, dandruff, and abundant secretion or excretion) or driven by an inflammatory process (which should be considered the healing process) in charge of destroying toxins introduced into the body.

Thus, it is clear that if a contaminated food is consumed daily, the inflammatory process becomes inevitably chronic.

Most common inflammatory processes of dogs and cats, but also humans, originate from this condition and it has been hypothesized and partially demonstrated that can be caused by a well-defined contaminant derived by the intensive farming: the oxytetracycline (OTC) [7–9].

2. Oxytetracycline

OTC, a widely and legally used antibiotic for intensive farming still used worldwide, can unfortunately reach the food chain supply (pet and human food) and then become the enhancer of inflammatory processes [7, 8].

We have also observed that its toxicity is exerted once bound to the bone of intensive farming-treated animals, e.g. chickens and turkeys [10]. Also, fat is considered an OTC deposit, but its role is less prominent.

It is not a case that chicken, at different concentrations, is the most used raw material in pet food [11].

It is important to point out that in vitro experiments showed a cytotoxicity of OTC even at lower concentrations with respect to minimal residual limits [7, 10, 12].

Both OTC (in its liquid form at 20%) and bone meal with OTC induced a significant release of interferon (IFN)-γ from human peripheral blood lymphocytes [7] and DNA damage features, such as the activation of ataxia-telangiectasia-mutated (ATM) protein and p53 oncogene, the phosphorylated histone H2AX (γH2AX), the modifications of histone H3 methylation of lysine K4 in the chromatin, and an increased expression of type 1 superoxide dismutase (SOD1) [13].

Moreover, current regulatory authorities do not contemplate the evaluation of bone because considered not eatable, while widely present in most of meat meals [10].

Providing a well-balanced food without the overdose of raw materials and toxic compounds able to promote inflammatory processes allows the organism to reach homeostasis, thus removing the inflammatory process. The restoration time is almost immediate depending on symptoms.

Therefore, we claim that the immune system is absolutely involved in the inflammatory and deregulatory process induced by OTC rather than in the allergic one [10, 14].

2.1. Antibiotic resistance

Already in 2014, the Food and Drug Administration (FDA) has published three papers aimed at reducing the use of antibiotics in animal nutrition [15]. According to experts, the habit of adding to the antibiotic feed used in humans has also led to an increase in bacteria resistant to their action, endangering human health seriously [16]. Hence, there is a need for conscious use of these drugs, which should be limited to cases where it is necessary to cure animals and not to increase their weight or make them more resistant to disease. Eighteen of these antibiotics, approved decades ago, have been judged by new FDA studies as being at high risk for humans because their use in feed promotes the development of resistant bacteria that can be transmitted to humans through the food chain. For the other 12 antibiotics, the producers had submitted safety records that would today be considered insufficient to obtain authorization. Nine are still used in herds. Among them, the tetracyclines are still heavily used. Europe's antibiotic resistance data provided by the EARS-Net Surveillance Network show a rather uncomfortable panorama: the resistance of the two types of bacteria under surveillance, *Escherichia coli* and *Klebsiella pneumoniae*, has increased considerably during the last 4 years. For this reason it is important to be alert because antibiotic resistance has become one of the major public health problems that threaten the health of European citizens. Antibiotic resistance causes difficulty or inability to effectively treat some bacterial infections, with increased hospitalization times, healthcare costs, and mortality. European data confirm the increase in resistance to the two types of bacteria under surveillance: *E. coli* and *K. pneumoniae* [17]. These two species are responsible for urinary infections, sepsis, and other nosocomial

infections. The World Health Organization (WHO) describes a rather reassuring scenario regarding the antibiotic resistance phenomenon: it is a serious threat to public health, which could lead to post-antibiotic age in the next few years, in which simple surgery, common infections, and minor injuries will be able to kill again. Only in our country, every year, they die from 5000 to 7000 people because of the antibiotic resistance with an annual cost of more than 100 million euros. The problem has long been known and has to do with intensive breeding. The overcrowding conditions of farms and stables of companies that have adopted an industrial production model make animal health precarious: excessive density and contiguity between garments make developing pathologies more likely. And, to prevent them from spreading throughout the game, the massive use of antimicrobial drugs is being resorted to. Many breeders argue that there is no preventive and default use and that pharmacological intervention only occurs in the presence of illness. But, even if only one animal has pathologic symptoms, it becomes necessary to subject all the garments to pharmacological treatment to avoid the risk of contagion. This systematic use of drugs has favored the prevalence of treatment-resistant bacteria, with serious risks also for people's health as some families of these pathogens, as described above, may also attack humans, who would find themselves without effective antibiotics. Basically, if antibiotics lose effect because the bacteria learn to overcome them, when they really do, they do not work anymore.

2.2. Allergies and intolerances: new elements for a differential diagnosis

Pharmacological intolerances are defined as pseudoallergic reactions due to their clinical similarities with IgE-mediated allergies. Food intolerances and their symptomatic manifestation are always dose dependent, and this helps in distinguishing them from real allergies, where symptoms are related to the intake of even small quantities of the responsible food [18, 19].

Until fairly recently, it was widely accepted that 90% of the adverse food reactions were allergies. We retain that 90% of the adverse food reactions are intolerances. Who is right? We believe that the answer can be given by the privation diet approach that, by means of a rapid disappearance of symptoms (within a few days for intolerances), can clarify the allergic or intolerance origin of suspected symptomatology. It is well known that food allergies are frequently caused by immune system food reactions (IgE release), with related symptomatology, that try to counteract the allergen within food culminating with an histamine release which in turn triggers the inflammatory process [20]. In fact, in most of "supposed" food allergies, the symptomatology is related to the administration of incorrect diets with an overdose of raw materials and toxic compounds, specifically OTC, frequently present within pet food [21].

2.3. Undefined food intolerances

"Mere" food intolerances are ascribed to the incessant consumption of some foods, are not IgE-mediated, and are characterized by a delayed onset once starting the accused food intake [22]. Related symptoms concern the gastroenteric apparatus (diarrhea, constipation, IBD, and eczema) [23]. Many foods are able to induce intolerances, but recent studies revealed the unexpected and toxic role of the OTC and its residues as one of the main triggering factors. This antibiotic, apparently harmless to chickens, turkeys, pigs, and cows,

becomes unexpectedly toxic once bound to the bone, promoting apoptosis and inflammatory processes related to the increased pro-inflammatory cytokine release. The overall result is the onset of several immune-mediated pathologies in dogs and cats [10, 14, 24]. It is worth noting that a rapid disappearance of clinical symptoms (otitis, conjunctivitis, keratitis, gingivitis, stomatitis, dermatitis, hot spot, pyoderma, gastritis, enterocolitis, colitis, enteritis, nephritis, cystitis, pancreatitis, and other inflammatory processes) with a privation diet without oxytetracycline and without therapy is a clear demonstration of our researches. Thus, OTC toxicity is exerted through apoptosis induction and interferon-γ release [7, 10]. We just listed that there are many inflammatory and reactive processes induced by OTC (generally those related to the intolerances), which can involve each organ. So far, the most effective tool, with respect to other unreliable commercially available tests, is the privation diet, which allows us to easily distinguish between intolerance and allergy. Moreover, such tests provide qualitative but not quantitative results. Thus, it is of relevance to distinguish, among food intolerances, between pharmacological and the so called "undefined" intolerances [25]. We can now refer these latter to OTC adverse reactions (OAR), which are characterized by physicochemical reactions without allergic reaction, but with immune-mediated inflammatory process implication; response times to privation diet between 3 and 10 days; localized itch on the neck and lumbar region and ear (less frequently in both ears), chin, neck, armpits, croup, thighs, volar carpus, and hot spot lesions; sensitization times of few hours; fundamental apparatus involvement, also with inflammatory, phenomena, and/or increase in excretions and secretions; and rapidly and strongly dose-related reaction. Reactions to such molecule can be accompanied by an increase in secretions and excretions, which should be considered as natural mechanisms of toxic expulsion.

2.4. Food residue syndrome in dogs and cats

By "food residue syndrome" (FRS), we mean the sequence of symptoms that develops in a sensitive subject following to the ingestion of foods that contain pharmacologically active molecules. These symptoms affect especially the gastrointestinal system, the skin, and the eyes, but the reaction could appear everywhere (mouth, pancreas, bladder, kidney, behavior, immune, and reproductive system):

- **Miliary dermatitis (cats and dogs)**: it has no precise cause. Several causes are just supposed, from flea allergy (that was recognized as the most frequent and most convenient cause, though the advent of new flea repellent with total efficiency did not alter the incidence of the disorder, thus contradicting this origin) to fungal, parasitic, and bacterial infections (all these elements are effects and nearly never the cause of the disorder). Atopic dermatitis is called into question too. It is another disease whose origin is often impossible to identify. The whole neck area is affected, and local hair loss may occur. The skin can be slightly thickened and wrinkled. The possible dermatitis will be characterized by thin dandruff. It is possible to observe the characteristic pannicular wave movement (looking back as if someone had stung it with a nail), annoyed licking (without the typical relaxation of normal cleaning activities), and biting of the concerned part; these are all symptoms that cannot be attributed to simple itching, but seem to show paraesthetic phenomena and possible neurologic reactions [21].

- **Granuloma (cats and dogs)**: possible onset of very itchy granulomas, with scratching injuries. The most frequent location is between the corner of the eye and the mouth of the cat, even though it can appear as a line on the distal part of the thigh or the foot.
- **Chin pyoderma (cats)**: it is relatively frequent in cats due to the presence of tetracycline residue in pet food. It always localizes near the chin. It appears with black scabs that strongly adhere to the skin. Chin pyoderma is associated with itching, which causes the partial detachment of the scabs, with bleeding and pus.
- **Repeated fasting vomiting (especially cats)**: the most characteristic manifestation of the food residue syndrome is fasting vomiting. It can occur at night or early in the morning, and it is very unpleasant, as the cat systematically identifies the fabric that is more similar to the ground or the grass and inevitably chooses carpets, rugs, and sofas, where it regurgitates yellowish stomach acids that leave permanent stains.
- **Malabsorption disorders (cats and dogs)**: these manifest through belching, borborigmo, feces of variable volume, consistence, color and smell, flatulence, and up to chronic or recurrent diarrhea. The clinical picture is usually characterized by the absence of high temperature and general signs. Dogs and cats can live rather well with the pathology, with no particular signs of dehydration even in lack of parenteral rehydration [26].
- **Forms of colitis (cats and dogs)**: these can be even very severe and characterized by vomiting, blood vomiting too, colic, pain, and profuse diarrhea, often hemorrhagic diarrhea, and can develop in the second part of the digestive tract. As it is known, there are no efficient treatments for chronic colitis (IBD), and symptomatic treatments often are nearly ineffective. On the contrary, a specific diet we developed for such disorder can be very effective.
- **Halitosis (cats and dogs)**: FRS causes several reactions in the oral cavity, from halitosis to dental plaque hyperproduction, tartar development, gingivitis and even to more serious forms of stomatitis [27].
- **Chronic interstitial nephritis (cats and dogs)**: although at present the FRS-related disorders cannot be distinguished from those with a different origin.
- **Idiopathic cystitis (cats and dogs)**: it is often labeled that way because it does not have an identified cause. It often arises from FRS [28].
- **Constant lachrymation (cats and dogs)**: numerous cats and small dogs, especially the breeds with brachygnathia, suffer from constant lachrymation, with secretions that are from transparent to brown or reddish. Even the consistency of secretions can vary from liquid to dense, with the accumulation of eye discharge that tends to dry. Very often, fair-haired cats have a real colored strip from the corner of their eyes.
- **Conjunctivitis (cats and dogs)**: these are characterized by the fact of being unilateral (just like paw-licking and ear infections). The manifestation is not dissimilar to the typical cases of conjunctivitis.

- **Keratitis (cats, but especially dogs)**: there is a growing awareness that keratitis in cats, that are not particularly frequent but are often autoimmune, can be attributed to FRS. It is now certain that *Keratoconjunctivitis sicca* in dogs is worsened and in some cases is caused by OTC only. Published studies on more than 50 chronic subjects clearly show: with numerous before and after pictures: the partial or total regression of the disease thanks to an elimination diet supplemented with immune-modulating herbal extracts [29]. In a large number of these cases, the regression occurs even without any drug. Even in this case, the manifestations are typical and unilateral.
- **Behavioral disturbances (cats and dogs)**: home cats apparently are less affected than dogs by the presence of the pollutant. In any case, similar to dogs, behavioral unbalance concerns all anxiety-related disorders and certain forms of exasperated or unjustified aggression. This could be the case of unjustified aggressive assaults to the members of one's family. The most common manifestations in dogs are aggression attacks, marking, anxiety, diffidence, irregular biorhythm, reactivity, activation, irritability, alertness, paw licking, environmental exploration, and attention requirement [30, 31].

2.5. Conclusions

The only effective treatment for FRS consists of the definitive elimination from the diet of all the foods that could contain the harmful pharmacological residues such as OTC, even in the smallest quantity. The various inflammatory phenomena that could affect different organs spontaneously regress, because they are the expression of the body's defense mechanisms. The dietary pattern that leads to the best results is based on residue-free foods and on the total elimination of all can have bones and fat from industrial farming. The food which proved to be the best both during the trial period and throughout the following maintenance diet is sea-caught fish: a very small number of dogs showed adverse reactions while eating fish constantly, even for a very long period. In cats, however, it is quite a common fish allergy.

Other foods that constantly proved to be free from this harmful residue are pasta (with the exception of egg pasta); rice; all fresh, frozen, and deep-frozen vegetables; all fruits; organic food meat of strictly wild animals; sheep meat that does not come from industrial farming; and vegetable fats.

Summer relapses are observed in those subjects who do not continue the prescribed privation diet in winter too, when there is a spontaneous, partial, or total regression of itching and skin disorders. You can likely assume that these improvements are connected with the seasonal disappearance of allergens that in spring and summer contribute to develop the disorder in question.

So, we reiterate that, in order to assist to a definitive remission of the FRS symptoms, the right diet must be constantly followed. It is extremely important to underline that, in the first phase, since each time you bend the rules, the effects last on average 4–5 days, two tidbits per week are enough to undermine all the efforts. In our experience most of the failures result from the owner's difficulty in following strictly the privation diet in the first 5–10 days. The success rate is objectively very high.

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Section 4

Probiotics

Chapter 7

Probiotic Bacteria as an Healthy Alternative for Fish Aquaculture

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Additional information is available at the end of the chapter

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Abstract

One of the problems of the aquaculture industry is the presence of pathogenic microorganisms whose proliferation is enhanced when the healthy quality of the culture systems do not meet comply with physical-chemical-biological parameters. In order to improve these problems, less aggressive alternatives to the environment have been sought. This is why probiotic bacteria are proposed as an alternative to the same systems where they will be applied, since they generate greater interest in not presenting a threat to the ecosystem, favor survival, improve the immune system of organisms and have antibacterial properties against pathogenic bacteria. This chapter reviews current research related to the search for marine probiotics for application in the aquaculture industry. Additionally, we deliver results from our work related to the research and application of probiotics. The reported studies demonstrate the positive effects of marine bacteria for their aquaculture application. The evidences found in our work allow us to conclude that larval survival is favored by the application of probiotics in the use of vectors such as rotifers, artemia and biofilms. However, depending on the species of interest, it is necessary to study the market for the biotechnological application of probiotics, to evaluate the feasibility of its production on a larger scale and its commercial feasibility.

Keywords: probiotics, pathogens, fishes, aquaculture, *Seriola lalandi*

1. Introduction

In recent years, the use of antibiotics in aquaculture has been reduced due to the diverse environmental problems that it generates in the ecosystems, as for example, the selection of bacterial strains resistant to antibiotics. The incorporation of antibiotics to the culture species, besides eliminating the pathogenic microbiota, also eliminates bacteria that are

beneficial for the same organism. Consequently, the accumulation of these chemicals in the organisms is not safe for human being who is the final consumer. The tendency today is to consume 100% natural foods, in search of a healthier and longer life. Likewise, the care of the environment over time has been regulated in different areas, privileging initiatives that have an environmental vision as a way to promote the care of the planet. In this area, the application of probiotics in fish culture mainly of commercial interest has been investigated for several decades. In this chapter, a bibliographical review of the recent probiotic studies in fish culture and the main results obtained from work on the use of probiotics in *Seriola lalandi* culture are made.

2. Updated definition of probiotics in aquaculture

The word probiotic was first introduced by [1] to describe "substances secreted by one microorganism that stimulate the growth of another." The name probiotic comes from the Greek "pro bios," which means "for life" [2]. Arora & Baldi [3] indicate that to date, there is no legal definition for the term probiotic. However, these authors define it as viable microorganisms with beneficial effect on the host. Akhter et al. indicate that probiotics are microorganisms that are administered orally in a sufficient amount to alter the microbiota (by implantation or colonization) of the specific host and lead to benefits for the host's health [4]. On the other hand, Banerjee et al. define probiotics as living microorganisms that confer beneficial effects to the host (improves immunity, helps digestion, protects against pathogens, improves water quality, and promotes growth and reproduction), and can be used as an alternative to antibiotics [5] (**Figure 1**). Probiotics include Gram-positive, Gram-negative bacteria, and many other organisms such as yeasts, bacteriophage, and single-celled algae [6]. In the field of aquaculture, the concept of probiotic should be

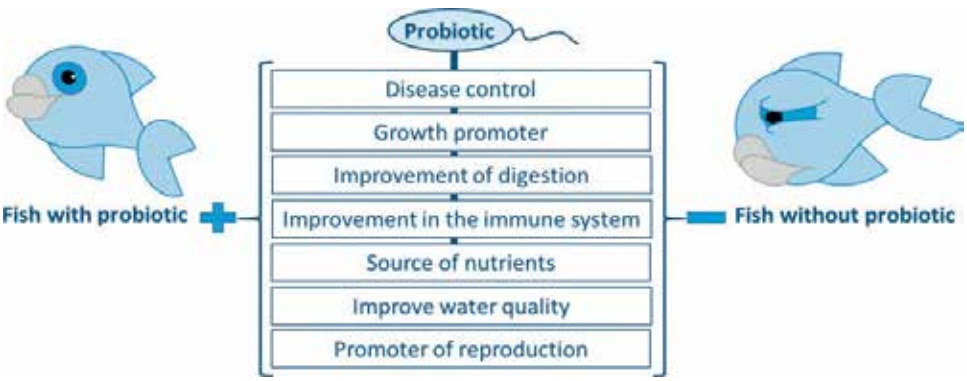

Figure 1. The benefits generated by the dominance of probiotics in confined systems are related to: control in water quality; disease control; promotion of growth; improvement in digestion (enzymes); improvement in immune system; and source of nutrients, among others.

defined taking into account other influencing factors that differentiate it from terrestrial probiotics. For example, Verschuere et al. extend this definition as "a living microbial complement that has a beneficial effect in the host by modifying the microbial community associated with the host or environment, ensuring a better utilization of the feed or improving the nutritional value, improving the host's response against a disease, or by improving the quality of its environment" [7].

Das et al. suggest that probiotics are a new tool in disease control and improved water quality in the aquaculture industry. Currently, probiotics have become fashionable in the worldwide market as a dietary supplement [8]. The interest in its consumption is related to be within the category of functional/natural foods. Rapid consumer awareness is due to the currently proven therapeutic benefits of probiotics. The benefits associated with probiotics are related to nutrient contribution, to promote survival, to improve the host immune system [4], and to promote growth and/or antibacterial properties against pathogenic bacteria [9]. In addition, probiotics isolated from the same systems where they will be applied, generate greater interest by not presenting a threat to the surrounding ecosystem.

The aquaculture industry is one of the fastest growing food producing sectors in the world, as well as of significant economic importance, expectations of development estimate that much of the food of marine origin and of sweet water in the future will be provided by aquaculture. However, closed crops have threatened industry because of the proliferation of pathogens that until recently were controlled with the addition of antibiotics. The development of bacteria resistant to antibiotics means an enormous risk of transmission from the environment to the human (Pandiyan et al 2013). The development of bacteria resistant to antibiotics means an enormous risk of transmission from the environment to the human [10]. In addition, the use of antibiotics does not discriminate and equally eliminates the beneficial microbiota in the gastrointestinal system of the organisms of interest, as well as, it accumulates in organisms affecting to man as a final consumer [8]. Because of these problems, a global trend has been created that has led to the search for healthy alternatives with the environment to control the pathogens that cause diseases of commercial interest.

The definition of probiotics has evolved over the years, integrating new terms that are related to the new investigations regarding its application in situ. However, the magnitude of the benefit of probiotics will depend on: the concentration of the probiotic; the use of one or a mixture of probiotics of different species; the species and sanitary quality of the host; the stage of development of the host receiving the probiotic supplement (larva, juvenile and/or adult); and the physical-chemical-biological conditions of the environment. Finally, there are many interactions involved that also define the success or failure of probiotic application in culture systems. For this reason, it is fundamental to standardize the protocols, independent for each host species to be treated, since, the success of a probiotic in a specific host, does not guarantee the same beneficial result in another species of host.

3. Influence of diet and water quality on the health fish

Water quality is one of the criteria associated with outbreaks of fish diseases in crops. Therefore, it is essential to maintain water quality parameters that allow the production of disease-free fish [11]. Improving water quality, avoiding the accumulation of organic, nitrogen, ammonia, and nitrite waste are constant concerns in aquaculture crops. High concentrations of these compounds can be extremely damaging and cause massive mortalities [8]. In nature, these toxic substances are transformed into safer forms by the oxidizing bacteria of ammonia (ammonia to nitrite) and oxidizing bacteria of nitrites (nitrite to nitrate) [12].

It has been argued that probiotic bacteria can be used as ecological biocontrol or bioremediation agent for the sustainable development of aquaculture [13–15]. Among the benefits attributed to probiotics are: decreased algae growth, decreased organic load, increased nutrient concentration, increased beneficial bacterial population, inhibition of potential pathogens, and increased concentration of dissolved oxygen [15]. Studies have shown that bacteria of the genus *Bacillus* have been considered as probiotics in water treatment because they have the particularity of converting organic matter into CO_2 [16]. Laloo et al. [17] verified that three isolates of the genus *Bacillus* decreased nitrite, nitrate, and ammonium concentrations in ornamental fish water. This same phenomenon was also observed by Kim et al. [18] with the species *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus licheniformis*, whose effects attributed it to mechanisms such as bioaccumulation, bioassimilation, and nitrification. In addition, it has been proven that the addition of probiotic bacteria reduces the load of pollutants such as heavy metals (Pb, Cd, Hg, Ni, etc.) [19]. Also, the use of *Bacillus* spp. can reduce the incidence of Vibriosis in water [16]. Other probiotic candidates such as *Nitrosomonas* sp. and *Nitrobacter* sp. have been shown to be beneficial in decreasing the pathogenic load in culture ponds [20]. Likewise, the species *Rhodopseudomonas palustris*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Saccharomyces cerevisiae* have been attributed to probiotic potential in the maintenance of water quality [21].

The application of probiotics for fish culture requires rigorous measures that determine its effectiveness. One of them is related to the abiotic (physical-chemical) or biotic (biological) factors that will stimulate the proliferation and dominance of the probiotic only if the conditions of its surroundings are favorable for this one. The application of probiotic can be done directly to the culture water or mixed with the inoculum of "green water," which is the entrance of microalgae in high concentrations, commonly used in fish culture for food consumption in the initial phase of the larval culture (2 days after hatching). Another pathway of probiotic entry in same fish culture is through live feed that fish receive as rotifers (up to approximately day 19 after hatching) (**Figure 2**), and then the addition of *Artemia* (until about day 25 of culture after of hatching). Another route of entry is through the skin of the fish where probiotics can colonize the surface layer of the skin and then enter through it. Consequently, probiotics after inoculum in culture systems can be found in water, sediment, and organisms of culture (**Figure 3**).

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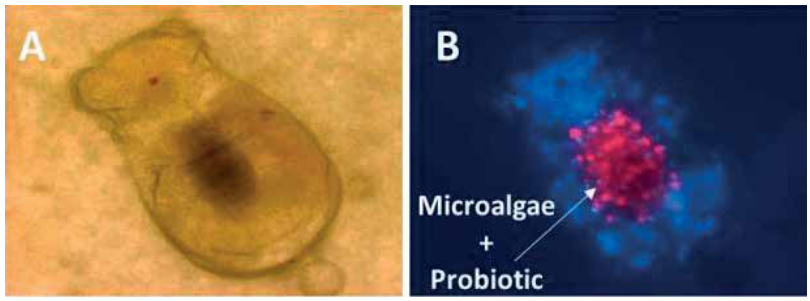

Figure 2. (A) Rotifers (*Brachionus rotundiformis*) in clear field 40×. (B) Rotifers (*B. rotundiformis*) fed with microalgae supplemented with probiotic bacteria stained with DAPI (4′, 6-diamino-2-phenylindole) and visualized by 40× epifluorescence microscopy.

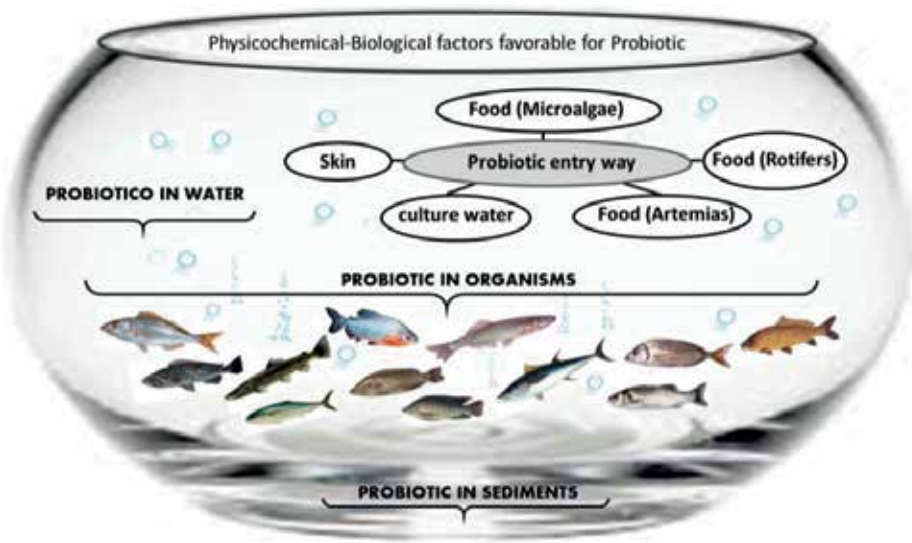

Figure 3. Probiotics in a confined system can be found in water, sediment, and organisms. The success of dominance in the system will depend on its concentration and whether the physical-chemical-biological factors are favorable for its development. Potential routes of entry of probiotics to fish may be more than one among which stand out: culture water; skin; food through microalgae, rotifers and/or *Artemia*.

4. Considerations for the selection of probiotics in aquaculture

According to Dawood et al. [22], a probiotic microorganism can meet the needs to develop successful aquaculture because it increases the key factors of yield in growth and disease resistance. Microorganisms intended to be used as probiotics in aquaculture should perform functions that should be considered safe not only for aquatic hosts but also for their environments and humans [23]. According to FAO [24], the probiotic effect on food can have the desired impact only if it contains at least 106–107 live probiotic bacteria per gram or milliliter.

Marine microorganisms have been recognized as potential sources of relatively more stable enzymes than homologous enzymes in terrestrial microorganisms; among them, the salinity, pressure, temperature, and lighting conditions differ. Marine microbial enzymes may enhance host digestion or molecular signals involved in the quorum perception in pathogens for aquaculture disease control [25].

It is essential that the strain selected as probiotic does not pose a risk to the host because of the secretion of antibacterial toxins. The preselection measures are very important and should be taken to evaluate their safety before being categorized as probiotic. In this regard, there are countries that have developed standards for the application of food additives with microorganisms [26]. Some of these norms are related to favoring potential probiotic bacteria isolated from the organism of interest to treat, mainly of the digestive system, since they have a greater capacity of adhesion to gastrointestinal mucus and tissues, compared to the foreign bacteria that are usually transient [27] as well as resistant to low pH.

The mechanism of action of each probiotic in specific is difficult to elucidate, because there are a variety of factors that interact between the probiotic and the surrounding environment. However, **Table 1** highlights essential properties to qualify as a probiotic candidate.

	Properties to consider to qualify as a probiotic candidate
+ Degree of importance −	– **Absence of hemolysins**, safety for the host [28]. With in vitro techniques such as hemolytic activity and mannitol's ability to use, the biosafety of selected bacterial strains can be checked, as well as in vivo tests (fish supplemented with probiotics) to confirm the non-pathogenic activity of the selected candidates [19] – **Absence of antibiotic resistant genes** [28] – **Pathogen antagonist:** **Competitive exclusion:** can bind to colon and mucosal cell lines, helping to colonize the intestinal system [28–30] **Ability to produce inhibitory metabolites:** such as protease, amylase, cellulose, phytase, chitinase, and lipase [19]. As well as small (peptide)/major (protein) bacteriocins; lysozyme; proteases and hydrogen peroxide) [9, 28, 31]. Or secretion of antimicrobial proteolytic enzymes (aminopeptidase Bs, trypsin-like serine protease, and enzymes reactive against substrates for cathepsin G- and caspase 1-like proteases) [32] – **Resistant to bile salts and low pH**: one of the routes of introduction of the probiotic is through food [28] – **Rapid growth and adequate to host/crop temperature** [28] – **Capacity of adhesion and compete for adhesion sites**: modulates the host's microbiota [9, 31, 33] – **Improve host immune response** [9, 31, 34–36]. When a pathogen enters the body, the adaptive immune system (B cell and T cell responses) and the complement system are activated [37–39]. Upon attachment to the surface of the mucosa, the probiotic modulates immunity of the mucosal of the fish [34]. The exact mechanism/working path of probiotics in the fish immune system is unclear to date [5] – **Supplementing essential nutrients,** such as vitamins and enzymes [9, 31]. – **Competing for essential** [9, 31] – **Regulating neuropeptides involved in signaling pathways to improve reproductive performance and fecundity** [40] – **Good interaction to apply mix of probiotics:** Variety of probiotic species may exert greater benefit than individual) [15, 41] – **Viability to storage conditions** [28]

Table 1. Degree of importance of the properties that a probiotic candidate must have.

5. Application of probiotics in aquaculture of fish

There is currently a variety of research focused on the probiotic search for fish culture. **Table 2** below provides information based on a review of the last 5 years of research on the use of probiotics in fish aquaculture.

Probiotic	Fish tested	Activity	Reference
Bacillus licheniformis (TSB27) *Lactobacillus thuringiensis* *Bacillus Plantarum* *Bacillus subtilis* (B46).	*Sparus aurata* L.	Enhances the immune	[46]
Shewanella putrefaciens Pdp11	*Solea senegalensis*	Modulates the digestive microbiota, an increase in growth	[51]
Bacillus pumilus H2	Fish	Anti-*Vibrio* activity	[47]
Bacillus subtilis WB60	Anguilla japonica	Increased in weight, efficiency in food and protein	[52]
Lac. pentosus BD6, *Lac. fermentum* LW2, *Bacillus subtilis* E20, *Saccharomyces cerevisiae* P13	Asian seabass	Improved either the growth performance or disease resistance of Asian seabass against *A. hydrophila*	[53]
Pseudoalteromonas sp.	*Seriola lalandi*	Increased larval survival	[54]
Pseudoalteromonas sp.	*Seriola lalandi*	Increased larval survival	[55]
Lactobacillus plantarum	*Oreochromis niloticus*	Decreases mortality and improves growth	[56]
Bacillus subtilis *Lactobacillus rhamnosus*	*Labeo rohita*	Increased the value of biochemical components	[57]
Lactobacillus casei	*Keureling* fish (Tor tambra)	Growth performance and feed efficiency increased	[58]
Bacillus sp. MVF1	*Labeo rohita*	Decreased susceptibility to disease	[59]
Bacillus subtilis *Bacillus licheniformis*	Juvenile rainbow trout	Resistance against *A. salmonicida*	[60]
Kocuria SM1 *Rhodococcus* SM2	*Oncorhynchus mykiss*	Produces extracellular enzymes that may have a role in the host digestive processes	[50]
Vibrio lentus	*Dicentrarchus labrax*	Protective effect against Vibriosis caused by *V. harveyi* in sea bass larvae	[61]
Lactobacillus plantarum	Tilapia	Enhanced the growth performance and modulated some hematological parameters.	[45]
Bacillus megaterium PTB 1.4	Catfish	Increased the activity of digestive enzymes and the growth of catfish	[44]
Lactobacillus rhamnosus	*Pagrus major*	Growth-promoting agent and Increases growth	[22]

Probiotic	Fish tested	Activity	Reference
Lactobacillus acidophilus *Bacillus subtilis* *Lactobacillus bulgaricus* *Saccharomyces cerevisiae*	*C. gariepinus*	Increases larval survival	[43]
Bacillus megaterium, *Bacillus polymyxa* *Lactobacillus delbrueckii*	*Oreochromis* sp.	Increased the performance of zootechnical parameters	[42]
Enterococcus casseliflavus	*Oncorhynchus mykiss*.	Capability of improving growth performance and enhancing disease resistance by inmunomodulation	[62]
Pseudoalteromonas sp.	Fish	Inhibitory activity against fish pathogens	[63]
Pseudoalteromonas sp. Cepa MLms gA3	Fish	Inhibitory activity against the pathogen *V. anguillarum*	[48]
Bacillus sp. *Pediococcus* sp.	*Solea senegalensis*	Improvement protection against pathogen outbreaks and	[64]
Lactobacillus plantarum (LP20)	*Seriola dumerili*	Improves immune response and stress	[65]
Lactobacillus mesenteroides SMM69 *Weissella cibaria* P71	*Scophthalmus maximus* L.	Antimicrobial activity against the turbot pathogens *T. maritimum* and *V. splendidus*	[66]
Bacillus subtilis *Bacillus licheniformis* *Bacillus* sp. *Pediococcus* sp.	*Oreochromis* sp.	Resistance to *S. agalactiae*	[67]
Enterococcus faecalis	*Oncorhynchus mykiss*	Favoring growth, stimulation of the immune system and protection of diseases	[68]

Table 2. Bibliographic review of research published in the last 5 years (2013–2017) on the use of probiotics in aquaculture of marine fish.

From this literature review, we can highlight the novel investigations carried out in *Oncorhynchus mykiss, Seriola dumerili, and Sparus aurata* in which it is shown that the probiotics of the genera *Bacillus, Lactobacillus,* and *Enterococcus* have the capacity to influence the immune system. In this regard, the most widely used probiotics in aquaculture are *Bacillus* and *Lactobacillus* because they have better yield in feed conversion, growth rate, weight gain [22, 42, 43], increase in digestive activity [44], increase in the growth performance of the fish [45], immunostimulant [46] and antagonistic activity against *Vibrios* [47]. In addition, according to the literature, it is common to find probiotics of the genus *Pseudoalteromonas* sp. [48, 49]. However, this genus has not yet been explored at the biotechnological level. On the other hand, there are probiotic strains of the genus *Kocuria* and *Rhodococcus*, which have shown a great resistance to the antibiotics and are able to produce extracellular enzymes [50]. This bibliographic review allows us to verify that the study of probiotics for use in fish aquaculture

is an issue of current interest. The use of specific probiotics will allow controlling organism diseases, water quality of culture, improve survival, and in this way develop a sustainable aquaculture production avoiding the use of antibiotics.

6. Preliminary results of the probiotic search and application in *Seriola lalandi*

In this section, we will introduce the results of research carried out in our laboratory regarding the use of probiotics in *S. lalandi* larvae. This study emerged with the interest of promoting the cultivation of this species in northern Chile, an area not yet developed on an industrial scale.

6.1. Isolation of the probiotic *Pseudoalteromonas* sp. (SLP1-MESO)

The yellowtail *S. lalandi* is a marine species of high commercial demand. However, this species have persistent difficulties with respect to larval survival. Based on the bibliographic background of the benefit of probiotic bacteria in larval fish culture, we isolated and identified bacteria from the gonads microbiota of *S. lalandi* juvenile. The results showed that 42% belong to the genus *Pseudoalteromonas* of the total isolated bacteria (46 strains), nine of which had inhibitory activity against pathogenic bacteria. Of these, *Pseudoalteromonas* sp. (SLP1-MESO) presented inhibitory activity against *Yersinia ruckeri* (35 mm inhibition halo by Dopazo technique) (**Figure 4**) and was the only one that was negative for hemolysis, proteolysis, and lipolysis. These properties make it a good candidate to use as a probiotic in the larval phase of fish culture, which can be incorporated into the fish through the food [63].

6.2. Increased survival of *S. lalandi* using *Pseudoalteromonas* sp. (SLP1-MESO) as probiotic

In order to evaluate the effect of the probiotic potential of *Pseudoalteromonas* sp., isolated from Seriola specimens, this bacterium was added as a probiotic supplement in the culture of *S. lalandi* larvae. For this, larvae of *S. lalandi* cultivated in ponds of 450 lt were fed with rotifers (*B. rotundiformis* and *B. plicatilis*) and *Artemia* sp., which were previously fed with microalgae

Figure 4. From left to right, juvenile *S. lalandi* used for isolation of *Pseudoalteromonas* sp. (SLP1-MESO) image of inhibitory activity by the Dopazo technique observed from the probiotic and the pathogen interaction.

Nannochloropsis gaditana supplemented with the probiotic *Pseudoalteromonas* sp. (SLP1-MESO). The results showed that rotifers and *Artemia* were good vectors of probiotics because *S. lalandi* larvae fed probiotic supplement that had higher survival (**Figure 5**) and length than control at the end of the experiment. These findings show that the probiotic *Pseudoalteromonas* sp. is a good candidate for use in larval cultures of *S. lalandi* [54].

6.3. Cultivation of *S. lalandi* larvae supplemented with probiotics in a mesocosmos system

In order to verify the probiotic effectiveness of *Pseudoalteromonas* sp., on a larger scale, it was evaluated that the survival of *S. lalandi* larvae cultured in a mesocosmos system (50 m^3 Pool) in submerged cages whose cubic structure support (800 lt volume) was composed of PVC pipes and the walls and bottom by mesh (450 μm of Swiss nylon) inoculated *S. lalandi* larvae and fed with *B. rotundiformis* and *B. plicatilis* and *Artemia* sp., supplemented with the probiotic bacterium *Pseudoalteromonas* sp. (SLP1-MESO) and the microalga *N. gaditana*. The survival of the larvae was evaluated until before the change of diet from live food to pellet. The results showed that the addition of the *N. gaditana* microalgae rich in fatty acids and the probiotic bacterium *Pseudoalteromonas* sp. (SLP1-MESO) inoculated in live food of rotifers and *Artemia* improved the survival of *S. lalandi* larvae (**Figure 6**), making it a good dietary alternative to optimize larval survival of this species, being able to be applied to other crops of interest commercial [69].

6.4. Use of biofilm as transfer vector of the probiotic *Pseudoalteromonas* sp. (SLP1-MESO)

The use of fixed biofilms meshes (Nylon Sefar Switzerland, 450 μm) was evaluated as a vector to incorporate specific microalga-probiotic food and as a biological control for the benefit of *S. lalandi* larvae. Biofilms were composed of a mixture of diatoms dominated by *Navicula phyllepta* and bacteria of the family Rhodobacteraceae that were previously isolated from biofilms formed in culture cages of *S. lalandi* larvae. In addition, these specific biofilms were tested with the addition of the probiotic *Pseudoalteromonas* sp. (SLP1-MESO). The meshes with biofilms

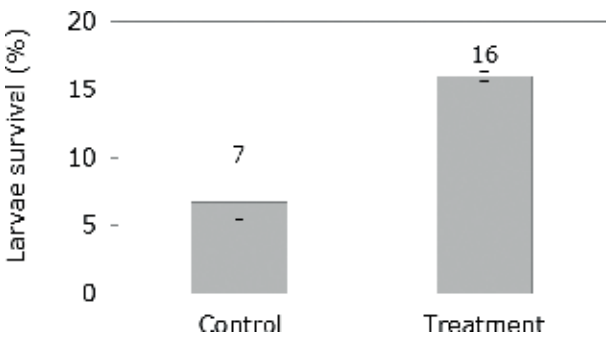

Figure 5. Evaluation of the survival at day of *S. lalandi* larvae fed with probiotics. Supplemented with probiotic bacteria (treatment) and without probiotic bacteria (control). Bars represent ± standard error of the mean. (Figure obtained from Leyton et al. [54]).

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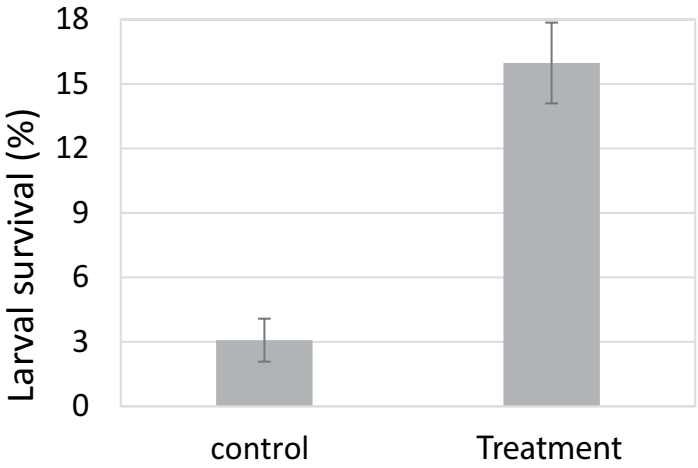

Figure 6. Survival (%) of *S. lalandi* larvae grown in cages in mesocosmos systems. Significant differences were observed between bacteria supplemented with probiotic (treatment) and bacteria without probiotic (control) (t-test = 4.896, $p < 0.05$). Control: *N. gaditana* + *B. rotundiformis* + *B. plicatilis* + *Artemia* sp. + larvae. Treatment: *N. gaditana* + *B. rotundiformis* + *B. plicatilis* + *Artemia* sp. + *Pseudoalteromonas* sp. SLP1 + larvae. Bars represent ± standard error of the mean. (Figure obtained from Plaza et al. [69]).

were immersed in ponds of 200 lt; during 10 days, the consumption and larval survival were evaluated. The results showed that the larvae consumed 70% of the biomass at 72 h in treatment and control without any negative effects on larvae or significant differences. However, a positive survival effect was observed in the biofilms treatments with probiotics obtaining 31% of survival compared to 13% of the control (**Figure 7**). These results demonstrated that this pathway of probiotic entry could be a good alternative for improving the survival of *S. lalandi* larvae [56].

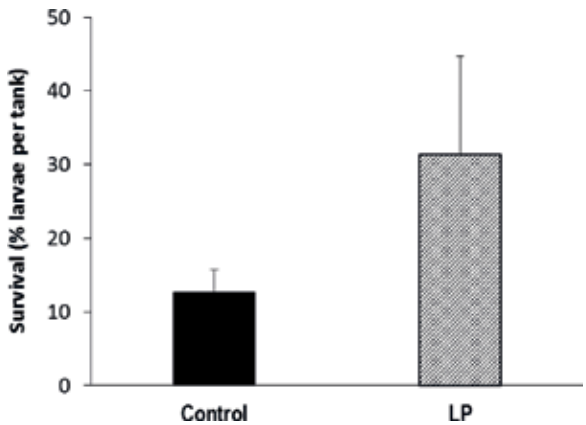

Figure 7. Survival of larvae at the end of the experiment in 200 lt tank. LP: larvae tank treated with probiotics and biofilm. Control: larvae tank with probiotics without biofilm. Data represent the mean and standard deviation of larvae for two replicate tanks for each condition and a triplicate mesh per tank in 400 lt tank and three replicate tanks for each condition and a triplicate mesh per tank in 200 lt tank. (Figure obtained from Mata et al. [55]).

Finally, the analysis of the results obtained in these four research works would indicate that the bacterium *Pseudoalteromonas* sp. (SLP1-MESO) isolated from gonads of healthy juveniles of *S. lalandi*, is a good candidate to be used as a probiotic in the initial larval stages of this species, that is, before the transition from live food to pellet. Our results supported the background of this chapter on the benefits of probiotic bacteria to improve the survival of fish larvae. The different investigations on probiotics in aquaculture have been validating their use to improve the survival of organisms in culture. Probiotic production will be necesary por the future of aquaculture industry.

7. Conclusion and future perspectives

The marine microbial world does not stop surprising us, for its varied potential beneficial to animal health. Based on the literature cited in this chapter, it is evident that the probiotic search for fish application is wide. However, research must be strengthening with new biotechnological processes that allow the mass production and application of probiotics on an industrial scale at an attractive cost. In order to advance in this area and transfer the results of the research from laboratory to the industry, we must overcome some non-minor gaps, such as the legal permit that involves working with living organisms for human consumption. Despite this, it is comforting the increase in worldwide support of respect to the use of probiotics, is becoming a trend in the search for natural solutions to care the environment and to take advantage of what nature offers us.

The authors of this chapter continue to concentrate their research on the application of probiotics in the larval phase of organisms of commercial importance such as fish, molluscs, and currently echinoderms. We have the complete conviction that our specific marine wealth, located in front of the most arid desert in the world, will provide us with the solution to optimize aquaculture in phase larval stages, which will allow to increase the sustainability of aquaculture activity in Chile and South America.

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Section 5

Antimicrobal Resistance

Chapter 8

Heavy Metal Pollutome and Microbial Resistome Reciprocal Interaction and Its Impact on Human and Animal Matrices

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Additional information is available at the end of the chapter

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Abstract

The chapter aims to reveal the complex relationships between antibiotic resistance in bacteria and heavy metal pollution at the human/animal interface. The antibiotic resistance is a continuously growing threat for both people and animals. Animals could represent a source for zoonotic microbial contamination of humans as subject for consumption and also as contacts (companion, sports, zoo animals, etc.). Antimicrobial treatments in animals, if uncontrolled or injudicious, could raise antibiotic-resistant strains to be transferred to humans where they can cause even more severe diseases. Moreover, the environment has its own microbiome, including some nonpathogenic but antibiotic-resistant species. Human industrial activities are carried out in certain environments, with particular microbiomes and also where animals bearing antibiotic-resistant bacteria are present. Thus, the degree of pollution with heavy metals, as part of the global pollutants to the environment, could impact on the bacteria and their resistome with severe consequences for inhabitants of the area.

Keywords: antibiotic resistance, heavy metals, zoonotic bacteria, pollution

1. Introduction

Heavy metal pollution represents a significant part of general pollution subsequent to human activities. Lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) are some of the most widespread pollutants, which pose serious threats to human, farmed animal, and wildlife

health [1, 2, 4–8]. These pollutants interact with the genome of the exposed individuals and interfere with the development/survival of species. Humans are positioned at the crossroad of several exposure routes, such as (a) habitat exposure [10]; (b) consumer exposure; and (c) work place exposure [9]. Heavy metals, as pollutants in various microbial habitats, could change the plasmids, inducing antibiotic resistance. In spite of numerous researches dealing with heavy metal pollution and its influence on humans, less of the studies concern the impact of heavy metal pollution on farmed and especially wild animals and their bacteriome, beyond the effects on their productions: meat, milk, or eggs.

Similar to heavy metal pollution, termed "pollutome" in this chapter as in Refs. [11, 12], resistance to antibiotics stands lately for an issue of broad community concern [13–15]. Biased and exaggerated antibiotic treatments, applied with no clinical reasoning, in both humans and animals, have subjected the microbial community to a strong selective pressure that led to adaptation and appearance of resistance plasmids looked at as "resistome." Recent investigations suggested the intervention of various factors changing the bacterial metabolism and subsequently, the ultrastructure and, eventually, the resistance to antibiotics. The "resistome" is continuously increasing, due to the further replacement of older generation antibiotics with newer ones and concurrent influence of other environmental factors. Due to their continuously increasing numbers, exploration was dedicated to the transfer of multidrug-resistant (MDR) bacteria from farmed animals/food products to humans [16, 17].

The spread of pathogens in the environment subsequent to human activities may cause diseases in humans, livestock, and wildlife [18–20]. The intensive technologies for food animal raising still use the antimicrobial agents at a large scale, in order to treat the infectious diseases in animals and to reduce the mortality and economic impact of diseases. Abusive use as well as misuse of antibiotics induced increased percentages of resistant bacteria. Infections by resistant and MDR bacteria endanger the human population and animals exposing those to sometimes uncontrollable threats [20, 21, 22].

A recent study has shown that in soil samples, in particular in those where the phenomenon of crossing species-specific barriers is present, the occurrence of heavy metals is associated with elevated antibiotic resistance. It was also indicated that the geo-chemical metal conditions innately influence the potential for antibiotic resistance in the soil [23]. The importance of understanding the soil resistome in the preservation of antibiotics for the treatment of infections was highlighted [24]. The elevated frequency of both metal and antibiotic tolerances in bacterioplankton from metal contaminated sites has been identified by [25]. The associations between the types and levels of metal contamination and specific patterns of antibiotic resistance indicated several mechanisms that underlie this coselection process, including coresistance and cross-resistance [2, 26, 27]. Therefore, it was suggested that metal contamination represents a selective pressure with both environmental and clinical importance that potentially contributes in maintaining and spreading antibiotic resistance factors [2] and also increasing the risk for both humans and animals in specific areas (**Figure 1**).

Due to peculiarities of multiplication and physiological traits of bacteria such as rapid growth, this antimicrobial resistance could spread to naive microbial populations. Identification of the relationship that exists between heavy metal pollutants, which by themselves negatively affect

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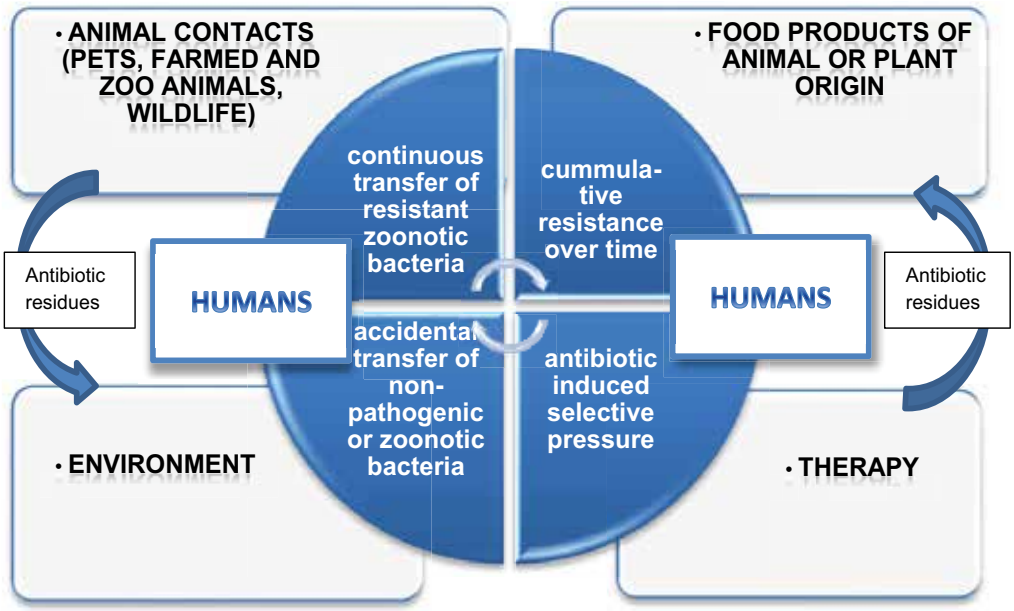

Figure 1. Main sources for human exposure to antibiotic-resistant bacteria.

human and animal health, and pathogenic or potentially pathogenic bacteria showing various degrees (MDR or HARS) of antimicrobial resistance could substantially improve the health care programs and allow the identification of crucial points where implementing a better control strategy is of utmost importance. Thus, not only the human and animal welfare will be improved but a substantial progress toward environment and ecosystem protection could be accomplished.

2. Heavy metal pollutome and its impact on health

The most common heavy metal pollutants are arsenic, cadmium, chromium, copper, nickel, lead, and mercury; because of the environmental and health effects in human and animal ontogeny, lead, mercury, and cadmium are of the greatest concern.

Present naturally or secondary from anthropogenic sources in the environment, the heavy metals are mostly nonessential to humans and other organisms and only some of them (e.g. copper, selenium, zinc, etc.) are essential in small quantities for the metabolism of the living organisms [1]. The main characteristic of heavy metals consists of their toxicity at low concentrations. Heavy metals enter the organism by ingestion via food, drinking water and, in some particular cases, soil, and by inhalation via air.

The lifetime of heavy metals in the environment can vary, but some of them can persist in soils for tens or even hundreds of years. There are no doubts that the soil and water ecosystems are the most impacted by heavy metal contamination.

Even though the most important disasters caused by heavy metals have been due to the massive contamination of water and soil and secondary contamination of food (Minamata, Japan, 1932; Sandoz, Germany, 1986; Coto de Donana, Spain, 1998), the historical (persistent) pollution of the soil with heavy metals still remains a problem of global concern.

In this framework, Romania represents an important example. Here, hot spots for heavy metal contamination of the environment are operational and former mining sites (extractive industry), particularly smelters, contribute to air, soil, and water pollution. In these areas, a number of studies have shown that the soils are polluted by heavy metals, especially in the proximity of metallurgical smelters and tailing dams [4], also representing a significant contamination source for water and vegetation [4, 8, 28]. Frequently, heavy metals are dispersed to long distances affecting broad land surfaces, which include agricultural land and forest funds. The increase of the natural soil acidity and soil contamination with heavy metals contributed to soil base depletion, microbiological disturbance, organic matter degradation, soil structure deterioration, and others [7, 29, 30].

Generally speaking, soil contamination is an actual universal problem. In this frame, pollution of soils by heavy metals in urban areas is not an exception and is mostly due to former industrial activities and traffic. The redistribution of heavy metals in urban areas is known to be strongly correlated with historical pollution, the best example in this regard being the lead contamination. Recent evidence [31, 32] indicates that urban soils have been moderately to highly polluted by Cd, Zn, Cu, and Pb originating in current industrial activities (e.g. steel industry). Also, topsoils near the smelting plants of molybdenum concentrate have had moderately to extremely high contamination levels for Mo and Pb. When it comes to urban public places, several studies [31–33] have showed that a site polluted by metals (e.g. Pb, Zn, Ni, As, and Mo) could pose a high noncarcinogenic health risk, or, on the contrary, the noncarcinogenic and carcinogenic risks could be insignificant or within acceptable limits, depending on the contamination degree and land use. On the other hand, in children, the most sensitive population, the risk assessment showed that they are at high multielemental noncarcinogenic risk [32].

Another source of soil pollution and transfer to different environmental components (water, plants, and animals) from urban areas is represented by waste materials. All types of municipal solid waste and sewage sludge contain many heavy metals with a significant impact on the environment, primarily increasing their levels in soil. Such wastes and sewage sludge added to agricultural and other soils lead to higher heavy metal content and the availability of heavy metals for transfer into crop plants with implicit risk to human health. It has to be mentioned that the composting process reduces the metal availability and the impact on the ecosystems [34, 35].

As it has been indicated, heavy metal pollution had a significant impact on the environment and ecosystems, causing the emergence of negative effects in all forms of life. Lead, cadmium, mercury, and arsenic are toxic for plants, wildlife, experimental animals, and humans. Bioavailability and bioaccumulation are key factors in their toxicity in live organisms and that is why most diseases associated with heavy metal pollution are the chronic ones, sometimes systemic, and generally are results of long-term/low level of exposure [1, 10, 35]. Due to their transfer chain between different matrices, bioavailability, and accumulation in live organisms, heavy metals are potentially harmful at some level of exposure and absorption, this property conducting to severe disorders [1, 5, 34–36].

All metal pollutants can reach the aquatic environments and the concentrations of heavy metals in trophic chain (e.g. fish) can be much higher than those found in aquatic environment or in sediments [37]. Even without major contamination sources, heavy metals are found in sediments (deposits) due to contamination and transportation from the river basins, higher concentrations being related to the lower velocity of the water flow. Sediment contamination affects primarily benthonic organisms but, indirectly, aquatic beings as well, due to their higher trophic level or by heavy metal remobilization to the overlying water [38]. The bioaccumulation of some metals (Hg, Cd, Cr, Cu, and Pb) in two species of fresh water fish from Yonki Reservoir has been reported, even though the concentration has been safe for human health [39]. As for the marine environment contaminated by several heavy metals (Cu, Zn, and Cr), some concerns were noticed, particularly in terms of safety for human consumption of aquatic animals captured from different areas in North-Eastern Mediterranean Sea [37].

Heavy metal pollution is noteworthy because of the risks it poses to human health. Lead, cadmium, mercury, and arsenic affect in different proportions, and mostly in an irreversible way, the central and peripheral nervous systems and cardiovascular, renal, reproductive, hematopoietic, and immune systems. These effects have been extensively studied. The concern on the frequency and magnitude of health effects is very high, as long as the body burden of cadmium, mercury, and lead depends mostly on the dietary intake of these elements.

Recent data indicate that adverse health effects of lead, cadmium, and mercury may occur at lower exposure levels than previously anticipated, especially for lead and methylmercury, which generate neurotoxic effects such as developmental delays, neurobehavioral dysfunction, attention deficit, hyperactivity disorder in susceptible population, and a decrease of intelligence quotient (IQ) in children. One still incompletely answered question is "what are the interactions when an individual is exposed to the combination of lead and methylmercury?" [40]. Moreover, a very recent study emphasizes the economic benefits of methylmercury exposure control in Europe [3]. The renal tubular dysfunction, a critical effect of long-term exposure to cadmium, is irreversible. Long-term drinking-water arsenic exposure is mainly related to increased risks of skin and other cancers, as well as skin lesions [40]. Thus, growing environmental pollution by heavy metals probably contributes to the enhanced incidence of allergic diseases and cancers in urban populations.

In the last years, several studies have been focusing on the reproductive and immunotoxic effects of heavy metals, looking for the mechanisms of these effects with severe consequences on human and animal health. In contrast with the numerous studies that have observed reproductive effects in occupationally exposed humans and experimental animals with high exposure, the studies concerning the effects of low levels of these metals on male reproductive outcomes are limited. Even so, the evidence for the effects of low exposure was the strongest for cadmium, lead, and mercury and less certain for arsenic [41].

There is proof that heavy metals influence the immune response of the body and most of the studies were focused on lead and cadmium because of the number of exposed individuals worldwide. For example, [42] was among the first authors to publish on lead, cadmium, and methylmercury immune toxicity in experimental animals, demonstrating the dose-response relationship of lymphocytes' memory to antigen. A very important observation refers to subclinical

amounts of the administrated metal, which could affect the T lymphocyte when the secondary immune response is altered. In copper smelters, a lower production of IgA and IgG can be detected in targeted subjects, predisposing them to infections and cancers [43].

The higher intensity of exposure in recent studies on the work-related environment [9] suggests that occupational exposure to lead may disrupt the immune response and diminish immune prevention in exposed individuals.

Related to mercury, there is evidence that both inorganic and organic forms of it cause immune suppression and induction of autoimmunity. A study was conducted [44] to monitor the effect of inorganic mercury in an autoimmune heart disease model induced by infection with Coxsackievirus B3 in mice. The exposure to inorganic mercury before Coxsackievirus B3 infection functioned as an aggravating factor for the severity of the cardiac pathology induced by a macrophage infiltrate and mixed cytokine response in the heart.

More recently [45], it has been shown that lead up to 5.0 μg/dL affected the immune competence against pathogens, depending on bacterial species (*Escherichia coli* or *Salmonella typhimurium*), suggesting that Toll-like receptors, TLR4, were targets for the lead effect.

3. Antimicrobial resistance: patterns of human-animal-environment connections

At broad public and scientific community level, nationally and internationally, the expanding problem of antimicrobial resistance has been identified and tackled for several years, since antimicrobial resistance transpasses frontiers. The factors that favor bacterial resistance were partly investigated. Nevertheless, the therapy of bacterial infections and transmissible diseases still relies on antibiotic use and it is mostly being done without a previous testing of the resistance of the agent to various antimicrobials, regardless of environmental variables. Similarly, heavy metal pollution and preservation of ecosystems represented a central point of numerous national and international organisms' interest in developing strategies to ensure health preservation and drug production, marketing and use worldwide.

The appearance and expansion of antimicrobial resistance [19, 46] have become lately one of the main public health worldwide concerns. The detection, improvement, and placement on market of compounds with killing or growth-inhibiting effects on bacteria and other microorganisms, modernized the treatment of infectious diseases, helping with a dramatic diminishment of morbidity and mortality rates in humans and animals [14] and have substantially contributed to population health improvements. Antibiotics belong to the group of these compounds.

Nonetheless, disease-causing organisms have an outstanding ability to adapt, markedly to acquire and transmit antimicrobial resistance [47, 48]. Natural ecosystems, also including human gut, contain a large number of elements that can confer resistance to antimicrobials [46].

The antibiotic resistome concept indicates a dynamic process as a cause of resistance. This process involves microbial interactions in multiple environments, taking place ahead of the so-called antibiotic-era [26, 27].

The fact that resistant microorganisms can explore a wide range of potential niches and acquire optimal adaptations for life in alternative hosts is threatening human or animal health, by amplifying the capacity of bacteria to acquire new virulence and resistance determinants, meanwhile adapting to the habitat. Since the variability of these resistance determinants and their expression in different hosts are broader in nature than in human and animal pathogens, bottlenecks conditioning the transfer, spread, and stability of antibiotic-resistant genes must exist [49]. Moreover, the abusive and uncontrolled use of antimicrobial agents in both human and veterinary medicine facilitates the continuously increasing numbers of resistant organisms. These organisms have the capability to "commute" from animals to humans and back [16, 17] (**Figure 2**).

Considering that the origin of antibiotic resistance is the environmental microbiota [46], recent studies indicated that urban wastewater treatment plants are among the main sources of antibiotics' release into the environment and treated wastewater contains antibiotic-resistant bacteria or genes encoding virulence or antimicrobial resistance leading to the emergence of new pathogens [20, 50, 51]. Even though antimicrobial resistance from natural sources existed before antimicrobial agents were introduced into medical treatment [15], a strong connection can be established between the amounts of antimicrobials used and the increase of numbers of species resistant to them. Despite ongoing research to find new groups of drugs to combat

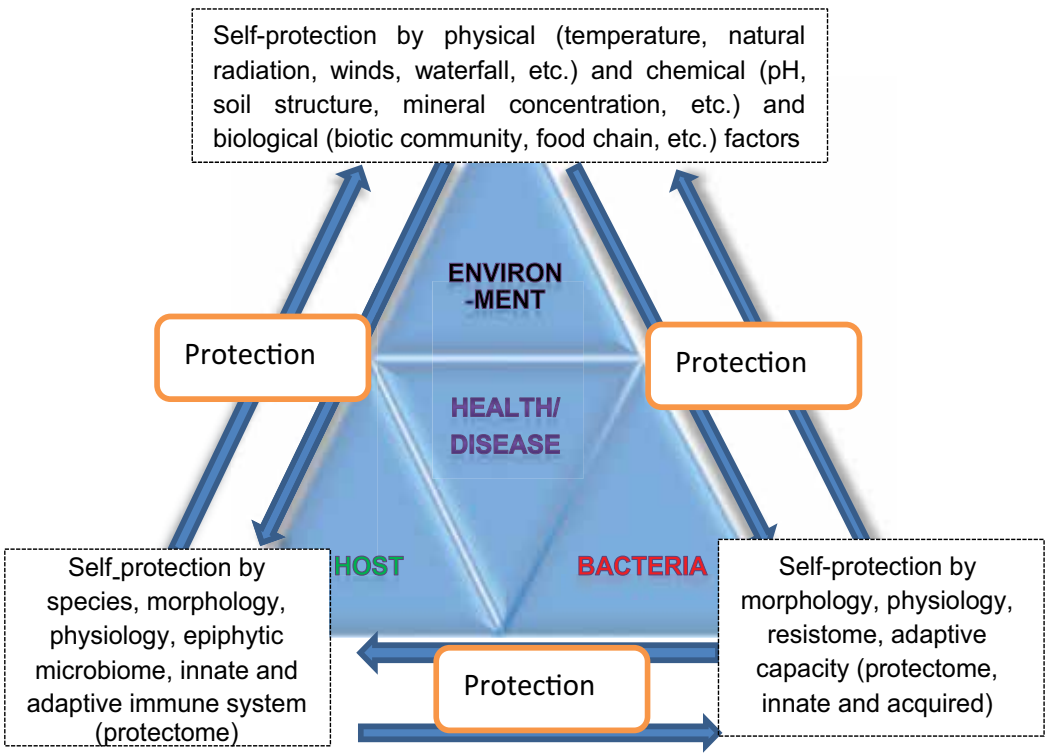

Figure 2. Multilevel regulating interactions within the epidemiological triangle that conditions health/disease outcome for the host.

resistant organisms, it is uncertain if and when such drugs will be available. Therefore, antimicrobial agents must be used prudently in order to limit the further emergence and spread of resistant germs [15].

Along with the manure used as fertilizer and spread onto agricultural fields, both residues of antibiotics and antibiotic-resistant bacteria may spread and pollute the environment [52]. Within this framework, the assessment of bacterial resistance in wild animals and game may be a valuable tool to monitor the environmental health and to manage emerging infectious diseases.

Several studies pointed out that fecal bacteria such as *E. coli* and enterococci of wild animals (wild boars, rabbits, and bison) [53] could be a reservoir of antimicrobial-resistant genes and frequently multiple resistant bacteria that could be transmitted to other animals or even to humans [54–56]. The authors suggest that wild animals can serve as sentinel populations for studying the origin and spreading of antibiotic resistance. Up to now, there are no studies to report on the antibiotic resistance in wildlife or humans from heavy metal–polluted areas.

In clinical practice, bacteria are being monitored for their resistance to antimicrobial drugs, which is an important predictor of treatment outcome. However, it is recognized that there is considerable heterogeneity in the metabolism of bacterial species and, in most of the cases, in their behavior toward the antibiotics. Methods for revealing the support of antimicrobial resistance, sometimes to multiple drugs, have developed from simple diffusion tests to gene detection, plasmid monitoring by molecular methods, and changes in their spectra following treatment by FTIR.

In considering the pollutome/resistome interaction, we identified the following bottlenecks: (a) heavy metal pollutants change the bacterial metabolism, inducing an increased resistance to antibiotics in the absence of antibiotic treatment; therefore, (b) in wild animals inhabiting polluted areas, antibiotic-resistant bacteria could be present without any previous contact with humans or domestic animals, but (c) the simultaneous activity of antibiotic treatment and pollution could induce the presence of an extremely resistant bacterial flora in humans, domestic animals, and wildlife contacts and pose a serious threat to their health/survival.

According to this, it is presumable that the exposure to heavy metals can emerge in severe infections in humans and animals, very difficult to treat because of the depletion of the immune system function and antibiotic resistance of the involved pathogen.

The identification of associations between genotype, resistance, and clinical outcome is obviously important under these circumstances. It could allow the prediction of likely outcomes in terms of aggravation of resistant bacteria–induced diseases and overall pollution, which is of critical importance to preventive strategies.

The identification of significant interactions between genetic loci and lifestyle risk factors could strengthen the evidence that heavy metal pollution factors are causally related to the emergence of MDR and could improve the understanding of the mechanisms through which these risk factors influence microbial diseases.

An effective preventive strategy requires close cooperation and consultation between all involved decision-making parties, especially at international level [13].

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4. Influence of heavy metal pollution on antibiotic resistance

Since 1999, when the complex socioeconomic and behavioral backgrounds were first mentioned in developing countries as a cause of the escalating problem of antibiotic resistance, some studies went further investigating the environmental factors' contribution.

Secondary to pollution by heavy metals, the biology and chemistry of the soil can be influenced regarding the tolerance of bacteria to these toxic elements.

Some researches [57] have shown that plants can harbor different metal-resistant bacterial communities in their rhizosphere soils, *Pseudomonas* and *Arthrobacter* dominating the isolates tolerant to lead. The evaluation of the aerobic soil microbial population demonstrated the presence of considerable numbers of viable bacteria in soil samples from two long-term heavy metal highly contaminated sites [58].

A very recent study [59] on the relationship between the antibiotic and heavy metal tolerance of cultivable bacteria isolated from soils containing different levels of heavy metals showed a high rate of coresistance toward Hg and antibiotics among the Gram-negative isolates and toward Zn, Ni, Hg, and the beta-lactam antibiotics among the Gram-positive bacteria. Along with other factors influencing gene transfer between bacteria, the authors possibly relate higher percentage of isolates with multiple antibiotic resistance to the level of soil heavy metals and the population of soil bacteria. Also, the distribution of antibiotic-resistant genes in surface water (lakes) was mainly attributed to antibiotic and heavy metal coselection as a result of anthropic impact [60]. Other researchers [61] confirmed the links between cadmium accumulation and antibiotic resistance in *Salmonella enterica* serovar Typhi Ty2, because of the influx of heavy metal ions in the environment from where the infection is transmitted (e.g. sludge).

The information on the pollutome/resistome interaction that could intermediate the appearance of antibiotic-resistant strains in nonantibiotic "user" environments, such as wildlife microflora or nonpatogenic microbiota, in case of heavy metal pollution presence is scarce or lacking.

By taking into account various types of changes that could induce antimicrobial resistance, when relating the risk to genetic factors and gene-environment pollution interactions, specific pathways may be picked up, in which the effects of environmental risk factors may prove to be clearer, and therefore this approach may substantially increase the chance of finding relevant gene-environment interactions. In a broader perspective, detailed knowledge of the pollutome-resistome interaction is needed to prepare control plans for avoiding further spreading of such bacteria and eliminating the risk for humans and animals. In addition, genetic factors definitely influence the interaction between such bacteria and the susceptible hosts.

5. Interactions of heavy metal pollutome and bacteria in the Danube Delta Biosphere Natural Reserve: a case study

As a representative case study on the simultaneous presence of heavy metals (Pb, Cd, Hg, and As) and antibiotic-resistant bacteria in water system, toxicological and microbiological

pollution within the Danube Delta (**Figure 3**), a UNESCO Biosphere Reservation from Romania subjected to the broader European contamination transported by the Danube River, is presented. The research was carried out within the frame of a research project supported by the National Research Council of the Ministry of Education (PNII 61/2012).

5.1. Methods

Samples were collected from representative areas of the Danube Delta, but for a comprehensive case study, the area was assigned around the Sfântu Gheorghe settlement toward the mouth of the homonymous river branch. This area was certified and statistically supported by the similarities noticed with the level of the entire Danube Delta, and the same microbial species were isolated in both the research area and the Sfântu Gheorghe river branch.

The research has extended over a period of 3 years (2012–2015) during which samples of water and sediments were collected seasonally from the case study area, along with benthic and pelagic fish organs and microbiological swabs (Deltalab, Eurotubo). The water, sediment, and fish (perch, wels catfish, sander, pike, pontic shad, crucian carp, and common carp) samples were analyzed for heavy metals through atomic absorption spectrometry (AAS) method: Pb and Cd through electrothermal atomic absorption spectrometry (ET-AAS); As and Hg through hydride generation atomic absorption spectrometry (HG-AAS) supplemented with amalgamation and Cold Vapour Atomic Absorption (CVAA) method.

Figure 3. The Danube Delta within Romania and the case study area [62].

Isolation and identification protocols selected for targeted bacteria included initially bacterioscopy directly from the buffers (transport media) by Gram staining and cultivation on Tryptone Soya Agar (TSA, Biolab) medium and afterwards passages to peptone water and Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Oxoid) for isolation of *Vibrio* spp. (*V. cholera* and *V. parahaemolyticus*); CUM (Chromogenic UTI agar, Oxoid) medium for isolation of *E. coli*, enterococci, and coliforms; and BHI and Cetrimide + Nalidixic acid (AES Laboratoire) medium for isolation of *Pseudomonas aeruginosa*. Antibiograms on these isolates were performed by the Kirby-Bauer diffusion method, the disks containing 9 antibiotics (ciprofloxacin, penicillin, streptomycin, erythromycin, oxytetracycline, marbofloxacin, akamycin, enrofloxacin, and ampicillin). Inhibition diameters were read, and the presence of total resistance (R) or resistant colonies (partial resistance, CR) were recorded.

For studying the patterns regarding the spatial distribution of different parameters, we used advanced spatial analysis methods (GIS) that imply geostatistical interpolations (Kriging) with the use of natural barriers (water/land). To reveal seasonal patterns and multiannual tendencies in the measured variables, we used statistical analyses, starting from Pearson's correlation, t-test, or ANOVA, to advanced methods like principal component analysis (PCA) and canonical discriminant analysis (CDA).

5.2. Results and discussion

The environmental assessment held in the case study area showed an inconsistent detection of heavy metals, like Pb, Cd, and Hg, in the water samples, being influenced by the location and the sampling season. However, As was the only metal that was measured constantly, with perceptible, from small to medium, variations. Two metals with high toxicity, Cd and Hg, have exceeded the Romanian limits for surface water quality, according to the EU Directive for water quality, being in some cases even above the maximum class—V.

In sediments, the presence of metals was constant, rendering as follows: Pb > As > Cd > Hg, the last two (Cd and Hg) exceeding the Romanian quality limits for sediments.

By analyzing the seasonal variability of the quality of habitats during this research, the water values for Pb and As on one hand and those for Cd and Hg on the other had similar tendencies, but without a pattern being highlighted within the normal seasonal variation. However, the metal distribution in sediment was normal, with predictable seasonal variations that can be directly compared to the tendency observed in other reference studies. Therefore, with regard to the seasonal variation of heavy metals, it can be stated that the data indicate that sediments act as a reservoir of contaminants due to immobilization and remobilization processes that occur under different conditions depending on seasonal changes, confirming the study of heavy metals in sediments, by occasional, seasonal sampling, as a good method to describe the temporal variations in the status of aquatic systems studied.

Of the studied fish species, five out of six (carp, crucian carp, pike, sander, wels catfish, perch, excluding the pontic shad) showed average values of arsenic and mercury higher or equal in internal compared to external organs. In the case of cadmium, in all investigated species, the concentrations in the internal organs were significantly higher than those in the external

organs. As for the concentration of lead, it was in five out of six cases higher or equal in the external organs than in the internal ones, a situation opposite to the other metals.

The highest bioaccumulations of Pb and Cd were recorded in the nonpredatory species (carp, crucian carp, and carp, respectively). Predatory fish, holding a top position in the food chain, showed higher levels of Hg in both internal and external organs.

The assessment of the microbiome in the water and sediment indicated a high degree of pollution. A total of 197 bacterial strains (85, 43.14% in water and 112, 56.85% in sediment) were isolated. Most strains were isolated in July (a total of 66, 33.50%; 28, 42.42% in water; 38, 57.57% in sediment), followed by November (a total of 50, 25.38%, 19.38%, water; 31.62% in sediment) in March (a total of 44, 22.33%; 23, 52.27% in water; 21, 47.73% of sediment) and May (a total of 37, 18.78%; 15, 40.54% of water and 22, 59.45% of sediment). The highest prevalence of *E. coli* strains/coliforms was identified in July (9, 56.25%), while *Vibrio* spp. (*V. cholerae*/*V. fluvialis*, *V. alginolyticus*/*V. metschnikovii*, *V. mimicus*/*V. vulnificus*, *V. parahaemolyticus*) showed an increased incidence in November (7, 53.84%). The *Pseudomonas* spp. strains were isolated exclusively in November (2, 15.36%) and July (2, 12.5%), while *Proteus* spp. and *Enterococcus faecalis* were also present in large numbers, especially in July. In terms of staphylococcal strains, large seasonal variations were present in July (4, 25.00%).

Summing up the data, mainly coliform bacteria were isolated, more than half of the isolates being confirmed as *E. coli* from water samples. Next were ranked the species belonging to *Pseudomonas* genus, followed by *Vibrio* species, with an average to low level.

In the sediment, the frequency of positive samples with coliform bacteria was much higher, more than half of them being positive for *E. coli* as well. *Pseudomonas* spp. had a frequency close to the one in water, while *Vibrio* spp. had a lower frequency (except V. *cholerae*).

The microbial qualitative assessment of aquatic habitats indicated variability induced by seasonal fluctuations in the percentage of positive samples. Species of the genus *Vibrio* had similar variations in water and sediment, with a high peak in presence and diversity during late-2013 and mid-2014.

The frequency of coliforms was higher in sediment samples than in water samples, the *E. coli* bacteria being present among these in between 20 and 100% of the seasonal cases. The species of genus *Pseudomonas* were detected during all seasons, except the spring of 2013.

The increased frequency of bacteriologically positive samples was influenced by the number of rainy days and the amount of precipitation, statistically supported for all studied bacteria types with correlation coefficients between 0.57 and 0.81.

The microbial population isolated in fish is presented in **Figures 4a** and **4b**. There were no statistically significant differences between the total flora isolated from gills and hepatopancreas or the distinctive bacteria, based on feeding habits and habitat (**Tables 1** and **2**).

Antibiograms performed on these isolates indicated that there were changes in overall antibiotic sensitivity and resistance depending on the season (**Figures 5a** and **5b**). The inhibition diameters were variable, depending on the strain from 11 to 26 mm; nevertheless, the average

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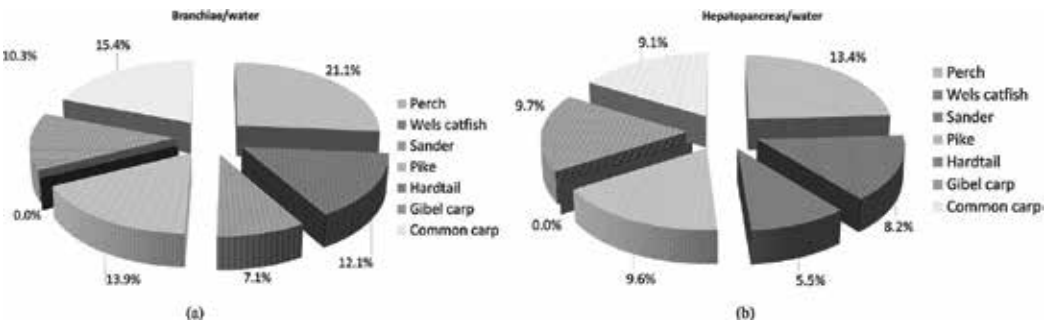

Figure 4. (a) The frequency of bacterial isolates by species, found in gills and also the habitat water. (b) The frequency of bacterial isolates by species, found in hepatopancreas and also the habitat water.

Species	1	2	3	4	5	6	7	Total
Perch	0.05	0.21	0.32	0.63	0.74	0.74	0.47	0.45
Wels catfish	0.44	0.41	0.46	0.28	0.47	0.58	0.18	0.40
Sander	0.30	0.20	0.30	0.20	0.70	0.80	0.20	0.39
Pike	0.44	0.50	0.47	0.35	0.50	0.53	0.24	0.43
Pontic shad	0.20	0.40	0.40	0.30	0.60	0.70	0.40	0.43
Crucian carp	0.29	0.24	0.30	0.23	0.37	0.51	0.19	0.30
Common carp	0.24	0.33	0.39	0.31	0.65	0.82	0.20	0.42

1—*V. cholerae, V. fluvialis*; 2—*V. alginolyticus, V. metschnikovii*; 3—*V. mimicus, V. vulnificus*; 4—*V. parahaemolyticus*; 5—*E. coli*; 6—coliform bacteria; 7—*Pseudomonas* spp.

Table 1. The frequency of bacteria from gills of fish that differ by habitat and feeding habits.

Species	1	2	3	4	5	6	7	Total
Perch	0.05	0.11	0.21	0.42	0.68	0.84	0.63	0.42
Wels catfish	0.31	0.38	0.44	0.33	0.54	0.69	0.26	0.42
Sander	0.30	0.30	0.30	0.10	0.40	0.90	0.40	0.39
Pike	0.38	0.41	0.32	0.29	0.50	0.59	0.33	0.40
Pontic shad	0.50	0.40	0.30	0.50	0.70	0.90	0.40	0.53
Crucian carp	0.23	0.26	0.30	0.26	0.39	0.64	0.36	0.35
Common carp	0.16	0.14	0.31	0.29	0.41	0.76	0.39	0.35

1—*V. cholerae, V. fluvialis*; 2—*V. alginolyticus, V. metschnikovii*; 3—*V. mimicus, V. vulnificus*; 4—*V. parahaemolyticus*; 5—*E. coli*; 6—coliform bacteria; 7—*Pseudomonas* spp.

Table 2. The frequency of bacteria from hepatopancreas of fish that differ by habitat and feeding habits.

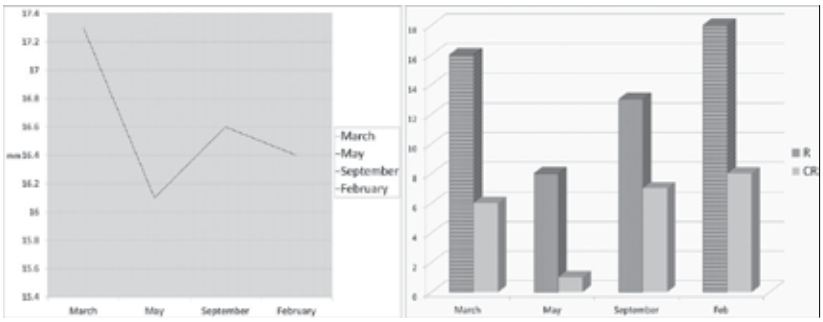

Figure 5. (a) Changes in overall inhibition (average—all species, all antibiotics) diameters (mm) by season. (b) Variation in cumulated total resistance (R) and resistant colony (CR) numbers by season.

diameters calculated by antibiotic type differ significantly for May and February ($p < 0.05$) (14–19 mm in March, 11–21.5 in May, 12–19 in September, and 11.5–20.4 in February).

The highest total resistance was present in March and February, at similar levels, while May, September, and February had the least R and CR. The only antibiotic with no R or CR in any of the seasons was the ciprofloxacin. There were no R and CR to enrofloxacin in February.

The isolated *E. coli*, coliform, and *E. faecalis* strains showed multidrug resistance, to 7 or 8 of the tested antibiotics, at different levels. The presence of resistant colonies stood for the partial inefficacy of the antibiotics used against these strains. This phenomenon is further posing the risk of selective pressure toward MDR in these locations in case of their use and increasing the consumer risk. The results indicated the presence of antibiotic resistance indicator bacteria in the tested samples and also showed that the changes in antibiotic resistance depended on the season, rather than on fish species, their feeding habits, or habitat.

5.3. Conclusions

There were no obvious direct relations observed between the two types of pollution, toxicological and microbial, the only relationship being pointed out after a spatial variation analysis using the geographic information system (GIS) technique, where areas could be ranked by having the lowest or highest pollutant values, and possible input areas.

There were similarities regarding the spatial pattern of the As, Hg, and Pb, together with *Vibrio* species and *E. coli* according to their average water values, having maximum loads in the Sf. Gheorghe branch and its secondary delta. The upstream pollution was the one that characterized the size and intensity of the contaminated areas, with the hydrological conditions influencing the local spatial distribution and the time of retention.

The research regarding the harmful effects of habitat pollution upon the fish population studied in the Danube Delta aimed to examine the bioaccumulation of heavy metals. Studying the most common fish species, it can be stated that arsenic level was predominant, compared to other metals, and by ignoring it for ranking purposes, carp is the first species, due to higher levels of lead and cadmium.

Pontic shad (*Alosa immaculata*) bioaccumulates heavy metals differently, indicating that it had different routes of exposure, being an allochthone species that was not relevant in the ecotoxicological assessment of deltaic habitats. Bioaccumulation of heavy metals was different among species and types of analyzed organs (internal or external), nonpredatory species bioaccumulating mainly Pb and Cd, while predatory species especially accumulating Hg.

In spite of the heavy microbial pollution of the investigated fish, this study could not clearly identify any species to be outstanding in terms of microbial contamination, regardless of samples originating from the gills or hepatopancreas. *E. coli* had the broadest distribution, followed by several species of the *Vibrio* genus.

Several recent studies pointed out the importance of the environmental nonpathogenic microbiome in transferring antibiotic resistance to pathogenic bacteria shed by diseased or convalescent individuals [23]. Since heavy metal and antibiotic resistance are connected [24, 25], tolerance to both of those pollutants is frequent; the higher the level of heavy metal pollution, the more numerous the antibiotic-resistant bacteria [25]. Heavy metal pollution was suggested to exert a selective pressure with both environmental and clinical importance [2]; therefore, in the Danube Delta, the simultaneous presence of zoonotic bacteria in the environment and fish and medium levels of heavy metal pollution in the same areas could contribute to endemic antibiotic resistance and upsurge the risk for both humans and animals.

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The book *Antibiotic Use in Animals* has everything said in the title, but it is not only meant for the veterinarians. It is intended to be used also by the medical doctors, animal owners, consumers of food of animal origin, etc. The book has five sections: "Introduction," "Use of Antibiotics in Animals," "Antibiotics and Nutrition," "Probiotics," and "Antimicrobial Resistance." Each of the sections discusses about one side of the antibiotic usage. Each group of authors has dedicated their work to one of the topics with key roles of antibiotics in the health of animals and public health in general. This book is a work of scientists and researchers in the topic of antibiotic use, and with this book, we hope to open new questions and deepen the research on roles of antibiotics in everyday life.

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