UNIVERSITY OF SÃO PAULO

School of Pharmaceutical Sciences

Graduate Program in Pharmacy (Biochemical and Pharmaceutical Technology)

Area of Fermentation Technology

Isolamento e identificação de bactérias ácido-lácticas probióticas produtoras de bacteriocina da truta arco-íris (*Oncorhynchus mykiss*): atividade contra *Flavobacterium psychrophilum*

Wellison Amorim Pereira

Thesis to obtain the title of Doctor

Advisor: Prof. Dr. Ricardo P. Souza Oliveira

São Paulo

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RESUMO

PEREIRA, W.A. **Isolamento e identificação de bactérias ácido-lácticas probióticas produtoras de bacteriocina da truta arco-íris (*Oncorhynchus mykiss*): atividade contra *Flavobacterium psychrophilum*.** 2023. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2022.

O ambiente tem grande influência na determinação da microbiota dos animais. Muitos desses microrganismos apresentam potencial probiótico devido a sua possível aplicação em diversos segmentos da indústria, como aplicações biotecnológicas. Este estudo teve como objetivo a avaliação do potencial biotecnológico de bactérias probióticas após a sua aplicação em diferentes modelos animais. Portanto, os dados apresentados são oriundos de estudo experimental e da construção de revisões de literatura. O primeiro estudo revelou que bactérias isoladas e caracterizadas quanto a sua segurança e atividade antimicrobiana tiveram resultados positivos e podem ser consideradas como candidatas para futuros estudos *in vivo* de suplementação probióticas em modelos animais. Foi observado que as novas cepas isoladas (*Lactococcus lactis* L1 e L2 e *Enterococcus faecium* 135) produziram BLIS (substância antimicrobiana semelhante à bacteriocina) com inibição de diversos patógenos alimentares e aquáticos, principalmente *Listeria monocytogenes*, *Salmonella* Choleraesuis e *Salmonella* Typhimurium. Todos os isolados foram sensíveis a todos os antibióticos testados (ampicilina, clindamicina, estreptomicina, cloranfenicol, rifampicina, gentamicina, vancomicina). Em teste de adesão com Caco-2, as cepas apresentaram percentual de adesão superior a 60%, não sendo observada expressão de fatores de virulência (gelatinase e hemolisina). Nos testes de resistência, EF foi resistente a pH 2,5 e 3,0 e EF/L2 foi resistente a sais biliares. Genes para bacteriocinas como Nisina (L1 e L2) e Enterocina A, B, P e Mundticin KS (EF) foram observados. Assim também, os demais estudos trazem uma análise ampla e detalhadas dos principais resultados publicados nos últimos anos relacionados aos benefícios do uso de probióticos para a aquicultura em geral, para a tilapicultura e para a suinocultura. Foram analisados aspectos relacionados ao efeito no sistema imunológico, crescimento, resistência a patógenos e estresse, dentre outros. Portanto, foi demonstrado que probióticos podem ser considerados para a suplementação alimentar de modelos *in vivo*, sendo possíveis agentes auxiliares no controle de patógenos e na promoção do crescimento, podendo ser utilizados na formulação de alimentos que também beneficiarão a saúde dos consumidores finais.

Palavras-chave: bactérias ácido lácticas, *Lactococcus lactis*, *Enterococcus faecium*, BLIS, potencial probiótico.

ABSTRACT

PEREIRA, W.A. **Isolation and identification of bacteriocin-producing probiotic lactic acid bacteria from the rainbow trout (*Oncorhynchus mykiss*): activity against *Flavobacterium psychrophilum*.** 2023. Thesis (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2022.

The environment has a great influence on the determination of the microbiota of animals. Many of these microorganisms have probiotic potential due to their possible application in various industry segments, such as biotechnological applications. This study aimed to evaluate the biotechnological potential of probiotic bacteria after their application in different animal models. Therefore, the data presented come from an experimental study and the construction of literature reviews. The first study revealed that bacteria isolated and characterized for their safety and antimicrobial activity had positive results and can be considered as candidates for future *in vivo* studies of probiotic supplementation in animal models. It was observed that the newly isolated strains (*Lactococcus lactis* L1 and L2 and *Enterococcus faecium* 135) produced BLIS (antimicrobial substance similar to bacteriocin) with inhibition of several food and aquatic pathogens, mainly *Listeria monocytogenes*, *Salmonella* Choleraesuis and *Salmonella* Typhimurium. All isolates were sensitive to all tested antibiotics (ampicillin, clindamycin, streptomycin, chloramphenicol, rifampicin, gentamicin, vancomycin). In an adhesion test with Caco-2, the strains showed a percentage of adhesion greater than 60%, with no expression of virulence factors (gelatinase and hemolysin) being observed. In resistance tests, EF was resistant to pH 2.5 and 3.0 and EF/L2 was resistant to bile salts. Genes for bacteriocins such as Nisin (L1 and L2) and Enterocin A, B, P and Mundticin KS (EF) were observed. Likewise, the other studies bring a broad and detailed analysis of the main results published in recent years related to the benefits of using probiotics for aquaculture in general, for tilapia farming and for pig farming. Aspects related to the effect on the immune system, growth, resistance to pathogens and stress, among others, were analyzed. Therefore, it was demonstrated that probiotics can be considered for dietary supplementation of *in vivo* models, being possible auxiliary agents in the control of pathogens and in the promotion of growth, and can be used in the formulation of foods that will also benefit the health of final consumers.

Keywords: lactic acid bacteria, *Lactococcus lactis*, *Enterococcus faecium*, BLIS, probiotic potential.

Wellison Amorim Pereira

Isolation and identification of bacteriocin-producing probiotic lactic acid bacteria from the rainbow trout (*Oncorhynchus mykiss*): activity against *Flavobacterium psychrophilum*

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3rd Examiner

São Paulo

2023

DEDICATION

To H.P. Thanks for changing everything.

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My sincere and profound thanks to everyone who has been with me in this cycle that is now ending. Thanks to all of you for participating in this unique moment.

I dedicate this thesis to my Creator, my family, my advisors (Dr. Ricardo Pinheiro, Dr. Elias Figueroa and Dr. Jorge Farias), to the friends that my doctorate brought me (especially Carlos Mendonça, Anna Piazentin, Thamires Simões, Pamela Azevedo... and so many others), the friends/family that welcomed me in the cold days of Chile, the employees and professors at USP, UCT and UFRO.

I would also like to thank FAPESP for funding the project through doctoral and BEPE scholarships (2021/12258-9 and 2021/01570-1).

To all of you my thanks.

I will keep you all forever in my heart.

EPIGRAPH

“Não creio ser um homem que saiba. Tenho sido um homem que busca, mas já agora não busco mais nas estrelas e nos livros: começo a ouvir os ensinamentos que o meu sangue murmura em mim.

Mas na realidade não há nenhum eu, nem mesmo no mais simples, não há uma unidade, mas um mundo plural, um pequeno firmamento, um caos de formas, de matizes, de situações, de heranças e possibilidades”.

Herman Hesse

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ABREVIATIONS

ATP: adenosine triphosphate

AU: arbitrary units

BLIS: bacteriocin like substances

CFU: colony forming unity

CFS: cell-free supernatant

EF: *Enterococcus faecium* 135

GRAS: Generally Recognized As Safe

HPLC: high performance liquid chromatography

LAB: lactic acid bacteria

L1 and L2: *Lactococcus lactis*

MALDI-TOF: Optical Microscopy and Ionization Mass Spectrometry by Laser Desorption Matrix

MRS: Man, Rogosa and Sharpe

O.D.: optical density

RPM: rotations per minute

g: centrifugal force

v/v: volume per volume

w/v: weight per volume

SUMMARY

BACKGROUND

The rainbow trout (*Oncorhynchus mykiss)* is among the fish species with high potential for aquaculture (Valdebenito, 2017). Brazil and Chile are the major producers of fish in Latin American. Chile exports more than 1,000,000 tons of aquaculture products per year; Brazil produced 500,000 tons in 2014 and expects to reach a total production of 2,000,000 tons per yearby 2020 (Valladão *et al.,* 2016). The main bacterial pathogens that cause most of the health problems in fish farming are: *Vibrio anguillarum* (Chai *et al.,* 2018), *Streptococcus faecalis* (Djellouli *et al.,* 2017), *Streptococcus agalactiae* (Zhu *et al.,* 2018), *Enterococcus* spp. (Novais *et al.,* 2018), *Flavobacterium psychrophilum* (Nematollahi *et al.,* 2003), among others. In the lastdecades, the prevention and control of fish diseases have focused on the use of chemical additives and drugs, especially antibiotics. Nevertheless, there has been controversy over the impact of theuse of these substances on the antimicrobial resistance in humans (Jensen *et al.,* 2010).

Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit on the host (FAO / WHO, 2001). The benefits promoted by probiotics as food additive are related to the modulation of the balance and activity of the intestinal microbiota, as well as the strengthening of its resistance to pathogenic bacteria (Choct, 2009). The beneficial effects caused by these microorganisms are lineage-specific (Sanders, 2010), andspecific to each host (Nader-Macias & Tomás, 2015).

In this scope, the present project is innovative and extremely important, since it is pioneer in proposing the study of the efficiency of a probiotic strains mixture, investigating possible interactions between them by compatibility tests. The use of *F. psychrophilum* as bioindicator isconsidered of greater relevance to the present study, once

this bacterium is the main agent of therainbow trout syndrome and diseases in cold waters (Nematollahi et al., 2003). It is worth notingthat the standardization of techniques used to achieve the aims of the present study, could be the basis for producing probiotic supplements for other animal species.

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JUSTIFICATION

The resulting data of this study will contribute to a better understanding of the interaction between the multiple probiotic lineages, to ensure the expected benefits of each of these microorganisms. In addition, the use of compatible probiotic strains in a mixture, as well as their biomolecules (bacteriocins), represents a natural alternative to antibiotics in fish production. In this regard, the present proposal will result in a technological advance, transferring and applying the beneficial effects provided by nature to the industrial purpose. Likewise, the other studies bring a broad and detailed analysis of the main results published in recent years related to the benefits of using probiotics for aquaculture in general, for tilapia farming and for pig farming. Aspects related to the effect on the immune system, growth, resistance to pathogens and stress, among others, were analyzed.

OBJECTIVE

The results of this study are primarily focused on the isolation and identification of new probiotic bacteria strains from rainbow trout excrement samples. The samples were provided by the Aquaculture School of the Catholic University of Temuco, Chile and Fisheries Institute of São Paulo. Likewise, other literature review studies were carried out in order to evaluate the impacts of the use of probiotics on the health of different animal models. To achieve these results, the following specific objectives were established:

- Isolation of bacteriocin-producer probiotic LAB from rainbow trout intestinal microbiota;
- Mixture formulation of probiotic strains;
- Literature review study regarding different animal models;
- Consolidation of an international and interdisciplinary team for the developmentof applied research in high potential aquaculture of Brazil and Chile.

PRESENTATION

This thesis is organized in the form of scientific articles (published and submitted for publication) and is divided into the following chapters:

Main publications

- Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens;
- Use of Probiotic Bacteria and Bacteriocins as an Alternative to Antibiotics in Aquaculture;
- The international tilapiculture market: potential, challenges, and the growing use of probiotic bacteria;
- Beneficial effects of probiotics on the pig production cycle: An overview of clinical impacts and performance;
- Improved productivity: Application of the quality management plan and tools in the field of university research

Annex – Other Publications

- New Insights in to the Antimicrobial Action of Cinnamaldehyde towards *Escherichia coli* and Its Effects on Intestinal Colonization of Mice;
- Long-term survive of *Aliarcobacter butzleri* in two models symbiotic interaction with *Acanthamoeba castellanii*;
- Characterization of levan produced by a *Paenibacillus* sp. isolated from Brazilian crude oil;
- Use of tunable copolymers in aqueous biphasic systems for extractive bioconversion aimed at continuous fructooligosaccharides production;
- Tracking new insights into antifungal and anti-mycotoxigenic properties of a biofilm forming *Pediococcus pentosaceus* strain isolated from grain silage;
- Creatine in sustainable fish aquaculture;

CHAPTER 1

scientific reports

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OPEN

Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens

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The conditions of aquatic environments have a great influence on the microbiota of several animals, many of which are a potential source of microorganisms of biotechnological interest. In this study, bacterial strains isolated from aquatic environments were bioprospected to determine their probiotic profile and antimicrobial effect against fish and food pathogens. Two isolates, identified via 16S rRNA sequencing as *Lactococcus lactis* (L1 and L2) and one as *Enterococcus faecium* 135 (EF), produced a bacteriocin-like antimicrobial substance (BLIS), active against *Listeria monocytogenes*, *Salmonella* Choleraesuis and *Salmonella* Typhimurium. Antimicrobial activity of BLIS was reduced when exposed to high temperatures and proteolytic enzymes (trypsin, pepsin, papain and pancreatin). All strains were sensitive to 7 types of antibiotics (vancomycin, clindamycin, streptomycin, gentamicin, chloramphenicol, rifampicin and ampicillin), exhibited a high rate of adherence to Caco-2 cells and expressed no hemolysin and gelatinase virulence factors. EF showed some resistance at pH 2.5 and 3.0, and L2/EF showed higher resistance to the action of bile salts. Finally, the presence of bacteriocin genes encoding for proteins, including Nisin (L1 and L2), Enterocin A, B, P, and Mundticin KS (EF) was detected. The molecular and physiological evidence suggests that the bacterial isolates in this study could be used as natural antimicrobial agents and may be considered safe for probiotic application.

Probiotics are defined as live microorganisms, which when administered in adequate amounts confer a health benefit on the host[1]. However, to be considered a probiotic, these microorganisms must undergo experiments to attest safety for use in food. Probiotics isolated from aquatic animals are spread through water and via other living organisms, and once they reach the host's intestines, these microorganisms perform vital functions. Several anatomical structures are potential growth sites for microorganisms, such as the skin, gills and gastrointestinal tract[2,3]. Intestinal content is thus an important source of potential probiotic microorganisms that can subsequently be used as food supplements[4].

Proper nutrition is intrinsically associated with correct development and efficient immunological defenses. Thus, studies have shown that both in humans and animals, the microbiota plays an essential role in the proper development and defense against pathogens[2]. Probiotic use in feed improves the health of aquatic animals, without the presence of negative side-effects[5]. Among the studies that have demonstrated the benefits of probiotic

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use, different mechanisms of action have been noted, that differ according to the species and environmental conditions that the microorganism encounters[6,7]. Probiotics used in aquaculture have included specific strains of yeasts and especially bacteria, including representatives of *Lactococcus* sp., *Enterococcus* sp., among others[8]. Some species belonging to the lactic acid bacteria (LAB) are considered safe (GRAS, Generally Reported as Safe)[9] and can be producers of natural antimicrobials, such as bacteriocins[7].

LAB are commonly recommended for aquaculture, and dietary supplementation results in an improved activity of digestive enzymes, immune response, development and even water quality[4,10]. Stimulation of the production of digestive enzymes, such as amylase, protease, lipase and lysozyme, can be an important consequence of probiotic use[11]. In healthy animals, these enzymes are intrinsically associated with improved digestibility, nutritional intake and weight gain[12,13]. Colonization induction and the development of beneficial strains in the intestinal tract also lead to the production of other beneficial substances in addition to enzymes[2].

As previously mentioned, an important characteristic of LAB is the ability to produce bacteriocins that play a key role in controlling pathogens[14]. These are conceptualized as small, cationic, heterogeneous, hydrophobic antimicrobial peptides produced by different microorganisms, with high isoelectric points, an amphipathic character, and a variety of modes of action and biochemical properties[14,15]. Since 1925, with the discovery of colicin, research on bacteriocins has received great attention[16] and by 1995 more than a hundred different types of bacteriocins had been identified[17]. Bacteriocins provide an important competitive advantage for the species that produce them[18]. Probiotics of interest can remain in the intestinal tract while producing bacteriocins, exerting synergistic effects, since they are not toxic to the host and the LAB exert various beneficial functions[19]. Most of the bacteriocins that have been tested to date were isolated from LAB and are generally used in foods for their high antimicrobial potential[18].

The major goal of producing bacteriocins is to increase bacteria's competition for food and ecological niches in the microbiota [18]. The antimicrobial effect of bacteriocins is related to their action on anionic lipids present in the membrane, which results in the formation of pores as well as disrupting ATP synthesis and amino acid transport[20]. For this reason, most studies evaluating bacteriocins are carried out with Gram-positive bacteria, as they have membranes that are richer in anionic lipids. The same effect can be observed in Gram-negatives; however, bacteriocin needs to cross the complex structure of the outer membrane[18]. An example is the bacteriocin microcin C7–C51 which has already been described to be effective against strains of the genus *Escherichia*, *Enterobacteria*, *Klebsiella*, *Salmonella*, *Shigella*, *Proteus*, among others[14]. Studies also point to the possibility of using bacteriocins as an alternative to combat antibiotic-resistant microorganisms, since their mode of action is different[20].

Bacterial diseases can affect various sectors, such as food production and fish farming. In this regard, some pathogens of interest belong to the genus *Streptococcus*, *Staphylococcus*, *Listeria* and *Salmonella*. Streptococcosis is a disease caused by the genus *Streptococcus* and it is triggered by stress and high density in fish culture, which can lead to considerable production losses[21]. Staphylococcal outbreaks are food poisoning caused by *Staphylococcus aureus*, an enterotoxins producing bacteria. Despite not being part of the microbiota of aquatic animals, its presence may be associated with diseases[22]. Thus, bacteria of the *Salmonella* genus are important pathogens known in the literature for their dissemination via water and/or contaminated food and difficult control[23]. Finally, *Listeria monocytogenes* is a pathogen difficult to control with a high incidence in fish processing facilities and has shown some resistance to several antimicrobials[24].

Experiments with aquatic animals have yielded promising results and feed supplementation effectiveness can be optimized if different approaches for the use of probiotics are tested[25]. Recent studies have shown that the future of probiotic research in aquaculture lies in the use of new supplementation techniques, such as the mixing of two or more strains. Indeed, mixing different probiotic microorganisms increases the product efficacy, which opens up the possibility of researching new lines aimed at investigating the interaction of these microorganisms as well as their joint action for the benefit of animal health. But as few examples have been analyzed in detail, specific studies are needed to test each of the strains used and their impact on individual animal models[2].

Therefore, the aim of this study was to evaluate the probiotic and bacteriocinogenic potential of bacteria isolated from an aquatic environment and their antimicrobial potential against important fish and food pathogens.

Materials and methods

Sampling and ethical aspects. Samples were obtained by field collection carried out at the Salmoniculture Experimental Station of the São Paulo Fishing Institute (Campos do Jordão, Brazil). Rainbow trout (*Oncorhynchus mykiss*), approximately 16 weeks old, were selected for the start of bioprospecting. After capture, the animals were sacrificed respecting biosafety and anesthesia rules validated by the institutions themselves, and then, under aseptic conditions, the cecum was removed, stored in a sterile flask in thermal boxes (~ 4 °C), and transported to the laboratory for immediate analysis. This study was analyzed and approved by the Ethics Committee of São Paulo Fishing Institute (registration number 07/2020). For fish anesthesia, an aqueous solution of benzocaine (100 mg/L^{-1}) was used until the loss of balance and reduction of opercular movements. Testing was done following guidelines and regulations.

EF was obtained from the collection belonging to the Laboratory of Bacterial Biotechnology (Universidad Nacional de la Patagonia, Argentina). The strain was isolated from starfish (order *Forcipulatida*) in Playa Unión, Rawson-Chubut (Patagonia, Argentina) and donated by Prof. Marisol Vallejo, National University of Patagonia San Juan Bosco (Argentina).

Bioprospecting and identification by biochemical tests and MALDI-TOF. The protocols described below were used for the isolation and identification of samples present in the cecum content of rainbow trout and starfish. The isolation was carried out according to the methods described by Schirru et al.[26] with minor

modifications. Samples of 25 g of excrement were homogenized in 225 mL of peptone water in a Stomacher. Serial dilutions were performed and cultivated in Man, Rogosa and Sharpe (MRS) and M17 media (BD Difco, New Jersey, USA) with cycloheximide (0.1 g/L). The plates were incubated under different temperatures (15, 25, 32 and 37 °C), for up to 48 h in anaerobic and aerobic conditions. After this period, approximately 300 CFUs were randomly chosen on each plate and replicated in the same culture medium and conditions. Then, biochemical tests were carried out for the classification of isolated microorganisms, such as Gram test (Gram method), production of Catalase (addition of hydrogen peroxide), and analysis by MALDI-TOF (Optical Microscopy and Ionization Mass Spectrometry by Laser Desorption Matrix assisted with flight time analyzer). For MALDI-TOF analysis, isolates defined as Gram-positive, Catalase-negative and with morphology corresponding to cocci and/or bacilli were selected. The protocol described by Alves et al.[27] was used for this test.

Therefore, the isolated strains were grown according to their isolation conditions in plates with 1.5% MRS/M17 medium for 24 h, as previously described. Isolates and 200 µL of sterile distilled water were mixed into a 1.5 mL microtube, being homogenized for 1 min using a vortex device. A volume of 900 µL of ethanol was transferred into the tubes, and centrifugated at 12,000×*g* for 5 min. The supernatant was discarded, and the samples were dried at room temperature for the loss of alcohol residues. 50 µL of formic acid (70%) and 50 µL of acetonitrile were added to the tubes, with a vortex homogenization. Subsequently, a matrix of α-cyano-4-hydroxycinnamic acid was prepared as a solution saturated in 50% acetonitrile and 2.5% trifluoroacetic acid. In a steel target plate, 1µL of treated samples and 1µL of matrix solution was added for drying at room temperature. Finally, the selected strains were cryopreserved in glycerol (20% v/v) at – 80 °C. For identification by mass spectrometry, the ItrafeXtreme MALDI-TOF equipment (Bruker Daltonics, Germany) was used, operating in the positive linear ion mode. The mass spectra were acquired in a mass range of 2 to 20 kDa with ions formed by intelligent beam radiation using a frequency of 2000 Hz, PIE 100 ns, 7 kV lens. The voltages for the first and second ion sources were 25 kV and 3 kV, respectively. The bacteria were identified using the Biotyper 3.1 database. Cut-off values greater than 2 and 1.7 were used to identify species and genera, respectively[27].

16S rRNA sequencing. For the identification of species at the molecular level, isolates L1, L2 and EF were subjected to partial sequencing of the 16S gene (rRNA) using the following primers: (PLB16) AGAGTTTGATCCTGGCTCAG and (MLB16) GGCTGCTGGCACGTAGTTAG. Genomic DNA was extracted using the PrepMan Ultra® kit protocol (Applied Biosystems, Carlsbad, CA, USA), following the manufacturer's instructions. The DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and used for amplification reactions with PCR Master Mix (Promega, San Luis Obispo, CA, USA) under the following thermal cycling conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 55 °C and 72 °C for 1 min, followed by a final extension of 7 min at 72 °C. PCR products were purified with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced in both directions using a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After contig assembly and edition, 16S sequences were used to conduct BLAST search analysis for species identification. All sequences generated in this study were deposited in the GenBank database (Table 1).

Screening for the presence of bacteriocin genes. To assess the presence of bacteriocin-specific genes in L1, L2 and EF, a PCR reaction was performed targeting genes encoding for nisin, lacticin, lactococcin, enterocin, mundticin, and hiracin (Table 1). Amplification reactions were performed with PCR Master Mix (Promega, San Luis Obispo, CA, USA) and the same thermal cycling conditions as described above, modifying the annealing temperature when appropriate. The amplified PCR products were analyzed by 1.2% agarose gel electrophoresis at 100 V for 50 min and bands were visualized with UV light equipment.

Agar diffusion: evaluation of the antimicrobial effect of BLIS. To assess the potential antimicrobial effect of BLIS from probiotic strains and its possible ability to produce antimicrobial peptides, such as bacteriocins, BLIS sensitivity tests against important bioindicator strains were performed using the agar diffusion test. FIOCRUZ (Rio de Janeiro, RJ, Brazil) provided the pathogen *S.* Typhimurium 5551/16, the Fishing Institute of São Paulo (São Paulo, SP, Brazil) provided the pathogen *S. agalactiae*, whilst the strains *L. monocytogenes* CECT 934, *S. aureus* CECT 237 and *S.* Choleraesuis CECT 724 were acquired from the Spanish Type Culture Collection (CECT) (Valencia, Spain). All isolates were reactivated 24 h before the start of the experiments, followed by pre-inoculum preparation. The optical density (OD_{600nm} 0.8) was determined, the inoculum diluted 100 times (~ 10^6 CFU/mL), and then incubated according to the initially described growth conditions. After a period of 24 h, the samples were centrifuged at 4470×*g* at 4 °C for 15 min, with 10 mL of the supernatant being removed for subsequent filtration through a 25 µm hydrophilic PVDF membrane (Filtrilo, Colombo, Brazil). The product resulting from this process was the BLIS.

Before testing for antimicrobial activity, the pH of BLIS was adjusted to ~ 6 using NaOH (1 M) and exposed to high temperatures (80 °C/10 min) to stabilize the substance and inactivate possible acids present in the sample. For the agar diffusion test, 1 mL of the inoculum of the pathogens *S.* Choleraesuis and *S.* Typhimurium was added to Petri dishes (90 × 15 mm) containing 10 mL of TSB (Difco, Michigan, USA) and 1 mL of *L. monocytogenes*, *S. aureus* or *S. agalactiae* on BHI agar (Difco, Michigan, USA) in a semi-solid state (supplemented with 0.75% agar). After solidification, 10 µL of the BLIS were pipetted onto the agar, and the plates were incubated for 18 h at 37 °C. Subsequently, inhibition halos were measured with the aid of digital calipers. Antimicrobial activity was expressed as arbitrary units per milliliter (AU/mL) using the formula described below (1), in which π. R^2 is the area of the inhibition zone (cm^2) and V is the volume (mL) of BLIS used[28,29].

Strains	Molecular identification	Accession number	Bacteriocin genes	Results	Reference
L1	*Lactococcus lactis*	MZ926851	Nisin	+	Alegría et al. [59]
			Lacticin 3147	−	Alegría et al. [59]
			Lacticin 481	−	Alegría et al. [59]
			Lactococcin 972	−	Martínez et al. [60]
			Lactococcin A, B, M	−	Alegría et al. [59]
			Lactococcin G and Q	−	Alegría et al. [59]
L2	*Lactococcus lactis*	MZ926852	Nisin	+	Alegría et al. [59]
			Lacticin 3147	−	Alegría et al. [59]
			Lacticin 481	−	Alegría et al. [59]
			Lactococcin 972	−	Martínez et al. [60]
			Lactococcin A, B, M	−	Alegría et al. [59]
			Lactococcin G and Q	−	Alegría et al. [59]
EF	*Enterococcus faecium*	MZ735396	Enterocin A	+	De Vuyst [61]
			Enterocin B	+	De Vuyst [61]
			Enterocin P	+	De Vuyst [61]
			Enterocin LB50A	−	De Vuyst [61]
			Enterocin LB50B	−	De Vuyst [61]
			Enterocin 96	−	Henning et al. [62]
			Enterocin 31	−	Henning et al. [62]
			Enterocin 1071	−	Martín et al. [63]
			Enterocin Q	−	Belgacem et al. [63]
			Mundticin KS	+	Almeida et al. [64]
			Hiracin JM79	−	Almeida et al. [64]

Table 1. Molecular identification (16S rRNA) and screening for presence of bacteriocin genes in *L. lactis* (L1 and L2) and *E. faecium* strains (EF). + target gene detected, − target gene not detected.

$$AU/mL = \frac{\pi .R2}{V} \quad (1)$$

Absorbance microplate reader. An absorbance microplate reader (BioTech, Vermont, USA) was used to assess the mode of action of BLIS against the pathogens tested at different stages of bacterial growth. For this, the BLIS and pathogens were prepared according to the pre-established conditions and incubated in a Microplate Reader (Bioteck Instruments, Vermont, USA) at 37 °C. The OD_{600nm} was determined automatically every hour for 24 h. From this experiment, it was possible not only to confirm the results obtained in the agar diffusion test, but also to determine the stages of bacterial growth that BLIS interfere with. Subsequently, in a sterile 96-well plate (TPP, Trasadingen, Switzerland) all combinations of variables necessary for this analysis were considered, such as positive (BLIS) and negative controls (saline 0.85%), and associations between the BLIS and different pathogens[28,30].

Tolerance of isolates to bile salts and low pH. The tolerance to acid pH and bile salts was evaluated based on the methodology described by Tan et al.[31]. L1, L2, and EF previously grown in MRS broth (~ 10^8 CFU/mL), were centrifuged (4,470 g), washed and resuspended in MRS with pH adjusted to 2, 2.5, 3 and 6 (negative control) with sterile 1 N HCl (Labsynth, Diadema, Brazil). The samples were then incubated at 37 °C, and 1 mL aliquots were taken after 0, 1, 2 and 3 h for CFU counting on MRS 1.5% (w/v) agar.

To evaluate the effect of bile salts, LAB were grown in MRS broth and incubated with bile salts (Sigma-Aldrich, Missouri, USA) at different concentrations (0.1%, 0.2%, 0.3% and the control, without addition) at 37 °C. Aliquots (1 mL) were taken at 0, 2, 4 and 6 h for CFU counting on MRS 1.5% (w/v) agar plates.

Tolerance of BLIS to low pH, high temperatures and proteolytic enzymes. To verify the stability of BLIS against different temperatures and pH, the method described by Todorov and Dicks[32] was used. To this end, BLIS were subjected to heat treatments (30, 50, 70 or 90 °C for 1 h; 121 °C for 15 min) and pH treatments adjusted to pH 2, 4, 6, 8 or 10 with 1 N NaOH and HCl; Labsynth, Diadema, Brazil) at 30 °C for 1 h. To evaluate the proteinaceous nature of BLIS, samples were subjected to 1% (w/v) trypsin, pepsin, papain or pancreatin (Inlab, Alamar Tecno Científica Ltda, São Paulo, Brazil) and incubated at 30 °C for 2 h. After this period, the stability of BLIS was verified using the diffusion agar technique against *L. monocytogenes*.

Hemolytic activity. The production capacity of the extracellular protein hemolysin was evaluated in Petri dishes containing BHI agar supplemented with 5% sheep's blood. After preparing the inoculum, the isolates were

spread on the surface of the sheep's blood agar and incubated according to the pre-established growth conditions. The activity of hemolytic hemolysin protein was confirmed by the formation of different types of halos, whose interpretation was performed by their coloring: α-hemolysin when there were greenish areas around the colonies, β-hemolysin when the zones were light-colored, and γ-hemolysin in the absence of such zones[33].

Gelatinase production. For the gelatinase production test, the inoculum was cultivated on the surface of Petri dishes containing BHI supplemented with skimmed milk (1.5%) and incubated according to the respective growth conditions described above. According to Tan et al.[31], a clear halo around the colony indicates a positive result for gelatinase production.

Coexistence test. This test investigates the possibility of co-cultivation between the three probiotic bacteria evaluated in this study. The tests were carried out according to the method described by Guo et al.[34]. Specifically, the bacteria were grown in their respective growth conditions for 24 h, and then samples were streaked perpendicularly to each other on the surface of plates containing 1.5% MRS (w/v) agar. After a 24-h incubation period, plates were examined for possible antagonistic effects.

Antibiotic resistance. Antibiotics of clinical importance were used, including vancomycin (30 µg), clindamycin (2 µg), streptomycin (10 µg), gentamicin (30 µg), chloramphenicol (30 µg), rifampicin (5 µg) and ampicillin (10 µg) (all provided by LABORCLIN, São Paulo, Brazil) loaded onto disks. Therefore, isolates were reactivated in the conditions mentioned above and, after 24 h of cultivation, bacterial growth at OD_{600nm} was determined and adjusted to 0.8. Finally, the samples were streaked on the surface of a Petri dish containing Mueller Hinton agar (Difco, Michigan, USA) and, after drying, the antibiotic-containing disks were added to the plates. Following incubation at 37 °C for 24 h, the presence or absence of inhibition halos around the disks was interpreted[35].

Adherence to intestinal epithelial cells. The method described by Jensen et al.[36] was used, with minor changes. For this, DMEM medium (Vitrocell Embriolife, Campinas, Brazil) was added to 24-well culture plates with 2105 human colon adenocarcinoma cells (Caco-2; ATCC HTB-37, Manassas, USA) with low content glucose, 20% (v/v) fetal bovine serum (Vitrocell Embriolife, Campinas, Brazil) and 100 U/mL antibiotics (penicillin/streptomycin) (Sigma-Aldrich, St. Louis, USA). Then, the plates were incubated at 37 °C (humidified atmosphere, 5% CO_2 and 95% air) for three days, until the appropriate growth point was reached. To perform the adhesion test, the isolated bacteria were grown for 24 h in suitable conditions and centrifuged (10,000×*g* for 10 min), and the pellet was resuspended in DMSO medium (without antibiotics). The monolayer formed by the growth of Caco-2 cells was washed twice with PBS before the start of the adhesion test, so that there was complete removal of the antibiotic used in the cell growth medium.

Thus, 1 mL of each bacterial culture (10^7 CFU/mL) was transferred individually to the wells and the plates were incubated at 37 °C for 1, 2 or 4 h, to optimize the assay. Subsequently, the cell monolayers were washed twice (PBS) to remove bacteria that were unable to adhere, and lysis of the monolayer was performed by adding PBS with 0.1% Triton-X100 (Sigma-Aldrich, St. Louis, USA). The resulting suspension (viable adherent bacteria) was diluted in different concentrations and incubated in MRS medium (pouring plate method) for 48 h. At the end of the experiment, the number of CFU/mL was determined and results were expressed as a percentage. Additionally, the ratio between the number of bacterial cells that remained adhered to the monolayer and the total number of bacterial cells added was measured.

Statistical analysis. The mean and standard deviation were used to express the results. The counts of viable bacteria were transformed into log values. The values in the tolerance test were compared using the software Statistica 12.0 (TIBCO, Palo Alto, CA, USA) applying the Tukey test with a level of significance $p < 0.05$.

Results

Isolation and identification by MALDI-TOF and 16S rRNA sequencing. A substantial number of CFU isolated from the cecum content of rainbow trout (*Oncorhynchus mykiss*) and starfish (order *Forcipulatida*) were observed. Subsequently, the isolated bacteria were collected and used in biochemical and morphological identification tests. All isolates belonging to the LAB group were selected for the next stages of this study and the bacteriocin-like inhibitory substances (BLIS) of each one were evaluated for their antimicrobial effect against important pathogens of fish and food. From rainbow trout samples, two isolates identified via MALDI-TOF as *Lactococcus lactis* (L1) and another as *Lactococcus garvieae* (L2), and one isolate from starfish identified as *Enterococcus faecium* 135 (EF) were selected for further molecular identification. The results obtained using the 16S rRNA method confirmed the previous data obtained by MALDI-TOF for isolates L1 and EF; however, the molecular analysis indicated that isolate L2 is *L. lactis*. Sequences generated in this study were deposited at GenBank (NCBI) under accession numbers MZ926851, MZ735396 and MZ926852, respectively.

Tolerance of the isolates to low pH and bile salts. To assess the resistance of the isolates to environments that reflect the adverse conditions of the gastrointestinal tract, they were exposed to different pH and concentrations of bile salts (Fig. 1). In the test of tolerance to different pH (Table S1), L1 was able to grow only in control conditions (pH 6). The same behavior was observed in the test with bile salts, where after 1 h of incubation there was no growth of L1 in any of the concentrations tested. Therefore, L1 was sensitive to low pH and high concentrations of bile salts, indicating that it must be protected by, for example, microencapsulation, if it is

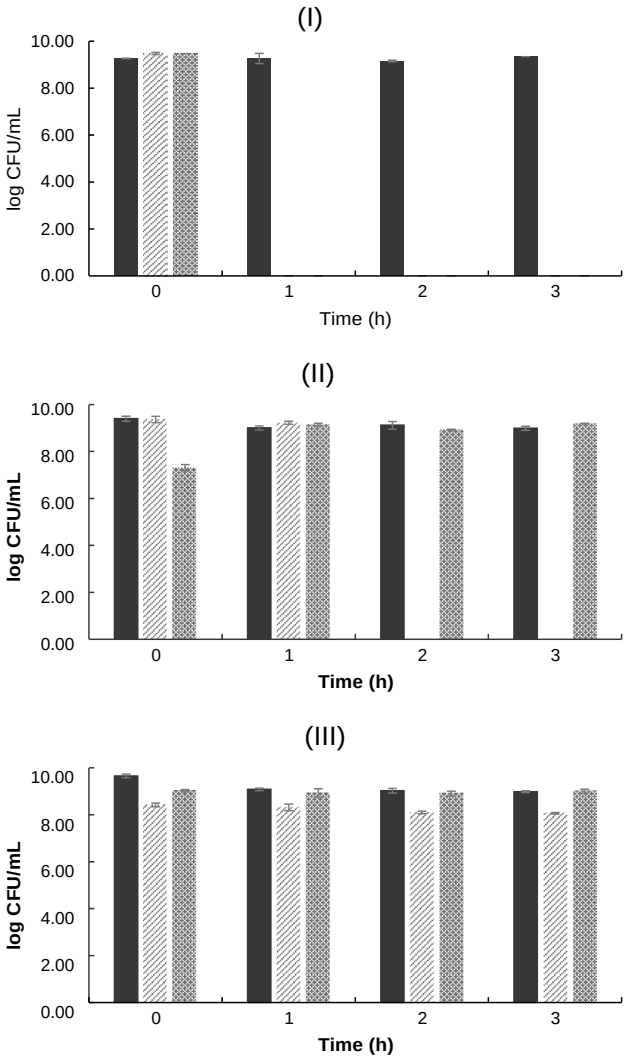

Figure 1. Tolerance of L1 (**I**), L2 (**II**) and EF (**III**), to pH 3 (▨) and 0.3% bile salts (▩). Strains without treatment of acid and bile salts were used as controls (filled black square). Bars represent means ± standard deviation, n = 3.

to be used as a probiotic. In contrast, the L2 isolate grew until 1 h of incubation at pH 3, and no negative effect was observed in the test with bile salts, with good growth observed in all concentrations tested. Finally, EF was resistant to low pH and bile salts during all evaluated periods (Table S2), with the test data with 0.3% bile similar to the results in control conditions.

Hemolysin and gelatinase virulence factors. The capacity of the isolates to produce the extracellular proteins gelatinase and hemolysin was evaluated. None showed α or β-hemolytic profiles and there was also no gelatinolytic activity, since the physical properties of the agar remained unchanged.

Antibiotic susceptibility testing. The susceptibility of isolates to the main antimicrobials of clinical interest was evaluated. In this sense, the three isolates possessed different sensitivity profiles, as observed from the measurement of inhibition halos when cultivated with the different antibiotics tested. Of note is that L1 was especially sensitive to ampicillin and clindamycin, and L2 and EF to clindamycin and rifampicin. When gentamicin was tested against EF, it was observed that the isolate is not very sensitive; however, its degree of resistance was considered low[37], so it could not be defined as resistant (Table 2).

Adhesion test to intestinal cells. All three isolates adhered to Caco-2 cells (Fig. 2). After the first hour of the experiment, L2 presented an adhesion of 94.2%, L1 77.1% and EF 65.6%. In the second hour, the adhesion percentages of L2 and EF were statistically similar (83%, $P > 0.005$), whilst L1 adhesion fell only marginally (76.6%, $P > 0.005$) compared to the first hour. It was observed that after the fourth hour of the experiment, all

Isolated probiotic strains	Antibiotic			
	Name	Disc concentration (µg)	Inhibition zone (mm)	Results *
L1	Ampicillin	10	64.30	S
	Vancomycin	30	39.92	S
	Streptomycin	10	32.88	S
	Gentamicin	10	45.35	S
	Rifampicin	5	18.21	S
	Chloramphenicol	30	52.75	S
	Clindamycin	2	60.03	S
L2	Ampicillin	10	37.08	S
	Vancomycin	30	35.42	S
	Streptomycin	10	15.54	S
	Gentamicin	10	24.60	S
	Rifampicin	5	46.48	S
	Chloramphenicol	30	44.46	S
	Clindamycin	2	48.84	S
EF	Ampicillin	10	25.50	S
	Vancomycin	30	23.00	S
	Streptomycin	10	8.50	R
	Gentamicin	10	12.50	MS
	Rifampicin	5	30.50	S
	Chloramphenicol	30	29.50	S
	Clindamycin	2	31.50	S

Table 2. Sensitivity of isolates to antibiotics by diffusion in agar. *S* susceptible, *R* resistant, *MS* mostly resistant. *Charteris et al.[37].

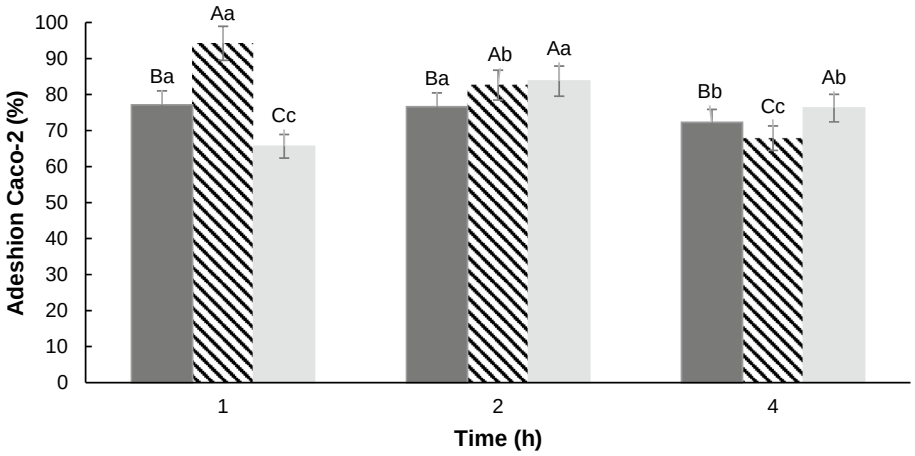

Figure 2. Adhesion (%) of L1 (filled black square), L2 (▨) and EF (filled gray square) to Caco-2 cells, after 1, 2 and 4 h of incubation. Different uppercase letters indicate statistically significant differences for all cultures taken at the same time ($P < 0.005$). Different lowercase letters indicate statistically significant differences for the same strain at different timepoints ($P < 0.005$). Bars represent means ± standard deviation, n = 3.

isolates tested suffered a reduction in adherence, ranging from 67.9% (L2) to 76.2% (EF). L1 possessed the most stable adherence over the time course of the assay. For L1 and L2, only one hour was necessary for the cells to adhere to the Caco-2 cells, whilst the best adherence of EF was obtained after 2 h. With the high percentages of adherent cells, we conclude that if these isolates were administered to a host, they would probably adhere to intestinal cells, and exert a probiotic effect.

Coexistence test. After plating L1, L2 and EF in crossed lines, plates were incubated for 48 h at 37 °C. At the end of the experiment, it was observed that there was a substantial growth of all isolates tested and no antagonistic effects were evident (Fig. 3).

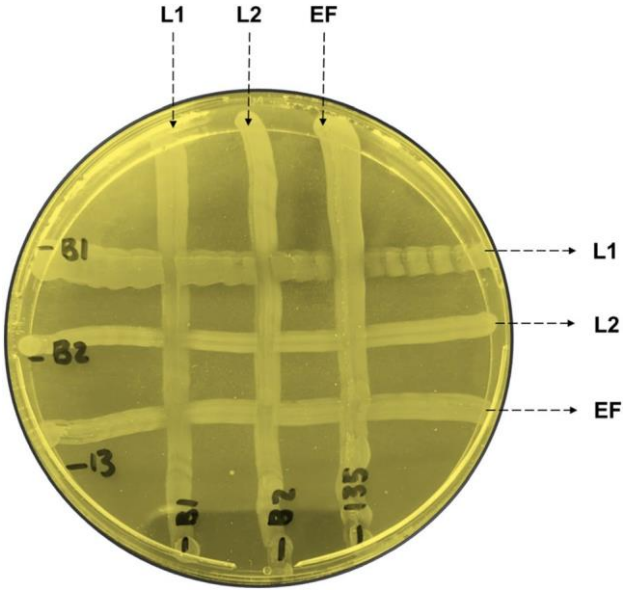

Figure 3. Coexistence test between isolates *L. lactis* (L1 and L2) and *E. faecium* (EF). No antagonist effects were observed.

Bioindicator strains	BLIS of L1		BLIS of L2		BLIS of EF	
	Inhibition zone (cm)	Quant. (AU/mL)	Inhibition zone (cm)	Quant. (AU/mL)	Inhibition zone (cm)	Quant. (AU/mL)
S. agalactiae	1.300	132.660	1.460	167.420	–	–
L. monocytogenes	1.035	162.338	1.629	255.596	2.282	408.790
S. aureus	1.025	160.768	1.014	159.198	–	–
S. Choleraesuis	–	–	0.898	140.986	1.263	125.220
S. Typhimurium	–	–	–	–	–	–

Table 3. Average diameter (cm) and quantification (AU/mL) of the BLIS inhibition halos against pathogens. "–" no inhibition.

Bacteriostatic effect of BLIS and interference with different growth stages. To assess the antimicrobial potential of BLIS produced by isolates and their possible capacity to produce bacteriocins, BLIS sensitivity tests against important pathogens were performed. After the incubation period, the formation of inhibition halos was observed. These were measured, and the antimicrobial effect of BLIS was determined by quantifying the area of the halo, considering the amount of BLIS used (Table 3). The BLIS of L1 had a good inhibitory effect against *Listeria monocytogenes* and *Staphylococcus aureus*, L2 against *L. monocytogenes*, *S. agalactiae*, *S. aureus* and *Salmonella* Choleraesuis and EF against *L. monocytogenes and S.* Choleraesuis. Furthermore, the quantification of BLIS produced by the isolates revealed that L2 was the largest producer, particularly inhibiting the pathogens *L. monocytogenes* and *S. aureus*. None of the three isolates was able to inhibit the growth of *Salmonella* Typhimurium in this agar diffusion test.

These preliminary findings were corroborated by using a microplate reader, as a means of assessing BLIS mode of action against the tested pathogens. From this experiment, it was possible not only to confirm the positive results obtained in the agar diffusion test, but also to pinpoint the specific bacterial growth stage that was affected by BLIS. In general, it was observed that there was interference by the BLIS of all isolates in all growth phases of the pathogens, especially in the delay of the LAG phase and the early stages of the LOG phase, which is equivalent to the full exponential multiplication phase of microorganisms. In this experiment, *L. monocytogenes* was the most sensitive pathogen and the BLIS produced by L2 was the most potent (Fig. 4).

In the test with *L. monocytogenes* (Fig. 4a), BLIS of all isolates delayed the initial growth phases. Notably, the BLIS of EF and L2 delayed the end of the LAG phase of *L. monocytogenes* for up to 13 h/OD_{600nm} 0.07 and 12 h/OD_{600nm} 0.06, respectively, longer than the control (2h50/OD_{600nm} 0.08). In the group treated with the BLIS of EF, *L. monocytogenes* reached the beginning of the stationary phase at 21h50/OD_{600nm} 0.45 compared to 5h50/OD_{600nm} 0.70 in the control group. When general pathogen growth data were compared with those of the control, it was noted that the BLIS of the isolates effectively slowed pathogen growth, an important indication of their potential use and of the possible presence of molecules with antimicrobial effects similar to bacteriocins.

Challenges with *S.* Choleraesuis and *S.* Typhimurium had similar results in this test. When exposed to BLIS of L1 and L2, the time needed by *S.* Choleraesuis to reach the end of the LAG and LOG phase increased (Fig. 4b).

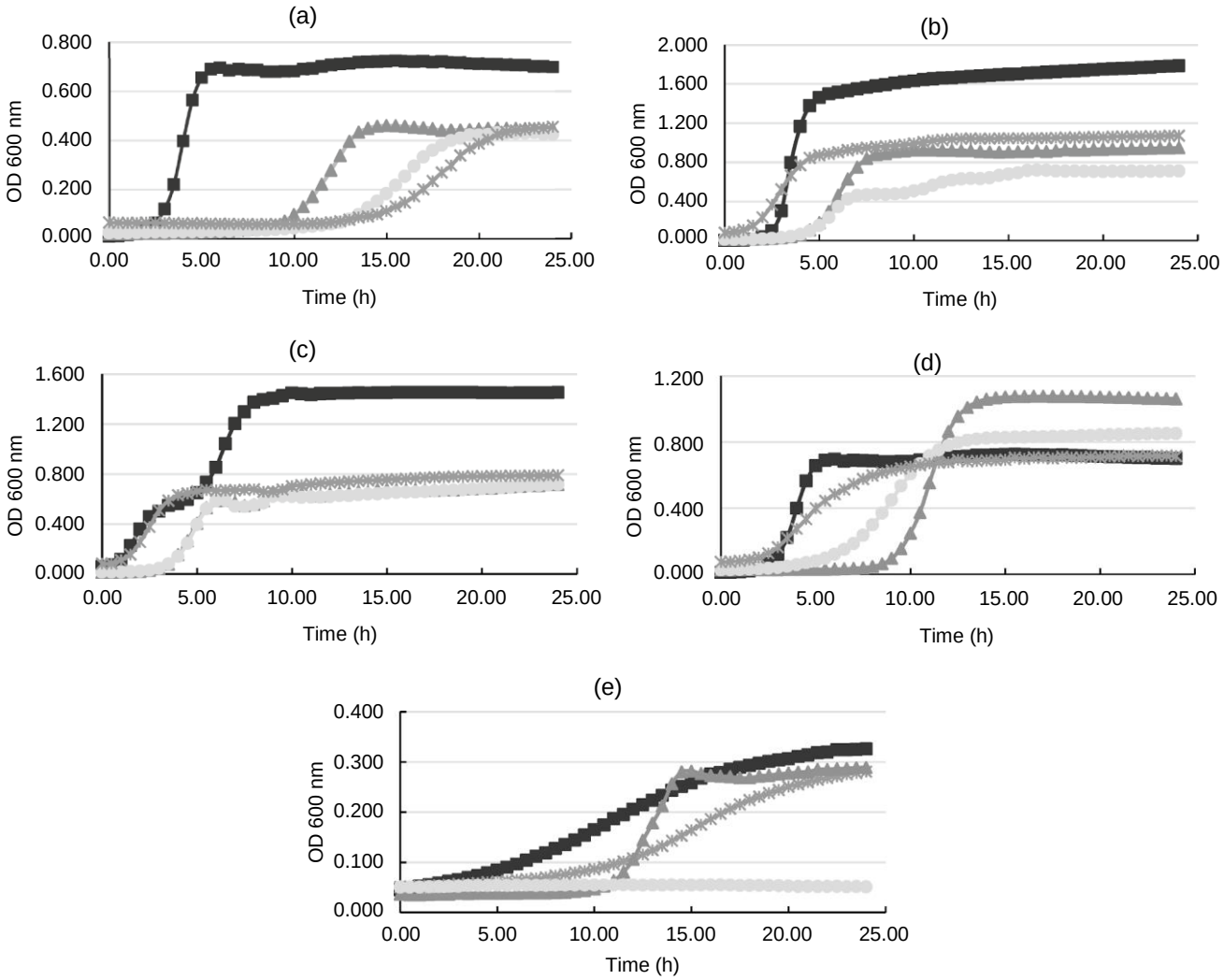

Figure 4. Antimicrobial activity of BLIS produced by L1 (filled black triangle), L2 (filled gray circle), and EF (cross symbol) against the pathogens *L. monocytogenes* (**a**), *S. Choleraesnius* (**b**), *S. Typhimurium* (**c**), S. *aureus* (**d**), and S. *agalactae* (**e**). Assays performed with positive controls (filled black square). The results are represented as an average of three readings.

The BLIS of EF influenced the growth of both pathogens similar to the control, but it was able to maintain an OD_{600nm} of 1.0. However, the BLIS of L2 had the most potent antimicrobial effect, maintaining not only the microbial population at levels below the control amounts, but also delaying the end of the LOG phase from 5 h/OD_{600nm} 1.4 to 16 h/OD_{600nm} 0.71 in the treated group.

Unlike the agar diffusion test results, BLIS derived from the three isolates inhibited *S.* Typhimurium growth (Fig. 4c). EF was able to reduce the OD_{600nm} by half when compared to the control, and L1 and L2 had similar effects of prolonging the LAG phase. Once again, a significant reduction in absorbance and an increase in the time of the LAG and LOG phase of bacterial growth, compared to the control, was observed. L2 proved to be the most potent; in the treated group, the stationary phase was reached at 6 h/OD_{600nm} 0.58 versus 9 h/OD_{600nm} 1.4 in the control group. Despite this, BLIS of L2 exerted its bacteriostatic effect throughout the period, limiting growth to just half of the OD_{600nm} seen in the control group.

The interference in the microbial growth phases occurred differently in tests with *S. aureus* (Fig. 4d). When exposed to BLIS, especially from L1, more time was required for *S. aureus* to reach the end of the LAG phase (9 h/OD_{600nm} 0.09, against 3 h/OD_{600nm} 0.12 in the control). However, growth superior to that of the control was observed, a finding repeated in multiple independent assays. This may be because after the delay in the start of the exponential phase, there may have been an increase in the consumption of substrates present in the medium; alternatively, BLIS might boost growth when these biomolecules lose their inhibitory effect. Further investigations are thus needed to clarify the causes.

Regarding the test with *S. agalactiae* (Fig. 4e), all the different BLIS used were able to prolong the LAG phase. Of special note is that the bactericidal effect of the BLIS of L2 prevented pathogen growth, as observed by the maintenance of OD_{600nm} below 0.1 throughout the experiment.

Treatment	Inhibition zone *		
	L1	L2	EF
Control	+++	+++	+++
Enzymatic treatment			
Trypsin	++	++	–
Pepsin	+	+	–
Papain	+	+	–
Pancreatin	+	+	–
pH resistance			
2, 4, 6, 8 and 10 for 1 h	+++	+++	+++
Heat treatment			
30, 50, 70 or 90 °C for 1 h	+++	+++	+++
120 °C for 15 min	–	–	–

Table 4. Effect of enzymatic treatment, pH and temperature on the stability of the BLIS produced by *L. lactis* (L1 and L2), and *E. faecium* 135 (EF). *(+++) > 12 mm, (++) 10–11.99 mm, (+) 8–9.99 mm, and (–) did not show inhibition zone. The bioindicator strain used to evaluate antimicrobial activity was *Listeria monocytogenes* CECT 934. Control: BLIS without any treatment. The concentration of the enzymes used in the experiments was 1% (w/v).

Tolerance of BLIS to low pH and high temperatures. The tolerance of BLIS to low pH and high temperatures was also investigated. In this sense, the cell-free supernatant of the isolates was recovered and subjected to different pH (2, 4, 6, 8 and 10) and temperature (30, 50, 70, 90 and 120 °C) treatments and then tested against *L. monocytogenes* (Figs. S1 and S2). It was observed that the BLIS of L1 and L2 maintained their activity against the pathogen up to 70 °C, while EF maintained its activity up to 90º C. In the exposure test to different pH, none of the BLIS lost activity at any of the different pH values tested.

Assessment of the protein nature of BLIS. An important step in the characterization of BLIS is the use of proteolytic enzymes to assess their possible protein nature. As already described, since bacteriocins are characterized as antimicrobial peptides, it is expected that there is a loss of antimicrobial activity after treatment with enzymes such as trypsin, pancreatin, papain and pepsin (Fig. S3). In such assays, when compared to the control group, EF BLIS had a total loss of inhibitory activity after incubation with all enzymes tested (Table 4). In turn, L1 and L2 BLIS had a considerable loss of inhibitory activity after treatment with all 4 enzymes. These data strongly suggest the presence of protein molecules with antimicrobial activity in BLIS from all three aquatic isolates.

Presence of genes for different bacteriocins. As a preliminary approach, a study was carried out to detect the main bacteriocins that have been described in the literature in recent years for bacteria of the genus *Lactococcus* and *Enterococcus*. Primers were designed and synthesized to amplify the most well-studied bacteriocins of these genera, which were subsequently used for amplification in PCR reactions. The PCR amplicons were analyzed, revealing the presence of promising amplicons (Fig. S4) for Nisin in L1 and L2 and for different Enterocins A, B and P, and for Mundticin KS in EF (Table 1).

Discussion

In this study, we demonstrated that the intestinal tracts of two aquatic animals are an important source of probiotic bacteria with bacteriocinogenic potential[38]. These results corroborate the data of Sarika et al.[39], where the authors report that a strain of *L. lactis* PSY2 isolated from marine perch (*Perca flavescens*) had a bacteriocinogenic profile and a significant antimicrobial effect against several Gram-negative and Gram-positive bacteria, such as *L. monocytogenes* and *S. aureus*. The authors also emphasize that such a bacteriocinogenic profile can assist in food preservation; in tests carried out with the strain there was an increase of more than 21 days of shelf-life, useful for the preservation of high-value seafoods. Thus, concerning our study, it is important to emphasize that once the antimicrobial potential of the BLIS identified in the three isolates has been demonstrated, specific studies will be carried out to evaluate their possible use in seafood preservation.

After confirming the presence of genes for bacteriocins in all isolates (such as Nisin and Enterocin) and the loss of BLIS activity after enzymatic treatment, their bacteriocinogenic potential should be evaluated further. Indeed, the preliminary tests demonstrated that the L2 and EF isolates from rainbow trout and starfish, respectively, are not only bacteriocin producers, but also have substantial probiotic potential, as they can resist pH 3 and various concentrations of bile salts. In this study, among the pathogens analyzed, *L. monocytogenes*, *S.* Choleraesuis and *S.* Typhimurium were the most sensitive to the bacteriostatic effect of the isolates. The BLIS of L2 had the best results in the inhibition tests, including a bacteriostatic effect against *S. agalactiae*.

The *Salmonella* pathogen is a major concern for the food industry, as it is transmitted through contaminated food and water. In recent years, probiotic bacteria have been studied for the control of the pathogen with promising results[40]. The preliminary inhibition results observed in our study need to be further evaluated. Nevertheless,

they are promising, as they indicate that bacteriocins could be used as a possible non-chemical containment strategy for these pathogens. In a similar survey, Sahnouni et al.[41] investigated the antagonistic effect of 38 LAB isolates against several pathogens, including *Salmonella* sp. The BLIS tested were found to be ineffective against Gram-negative bacteria such as *Salmonella* sp. and *Escherichia coli*, compared to the others. However, in an in vivo study, Mulaw et al.[42] observed a different result. These authors tested a mix of probiotic bacteria (*Lactobacillus plantarum* K132, *Lactobacillus paracasei* K114 and *L. lactis* E124) against infection by *S.* Typhimurium DT104 in mice. They observed that, compared to the control group, treatment with a mix of probiotics led to a reduction in *S.* Typhimurium DT104 counts in feces and the survival rate was significantly higher.

In the test of tolerance to low pH and different concentrations of bile salts, isolates EF and L2 had the best results, with EF resisting all ranges of pH and bile salts tested. As in our study, Yerlikaya[43] evaluated isolated probiotic bacteria in order to select strains for the production of functional foods. During the characterization phase of isolated *L. lactis* strains, the researchers evaluated their ability to resist bile salts and found that none of the tested strains managed to grow in their presence, an important indicator of the high sensitivity of the genus *Lactococcus* to such substances. In turn, Jawan et al.[43] also evaluated the susceptibility of *L. lactis* Gh1 to these factors and found that the strain was tolerant to pH 3 and bile salts at a concentration of 0.3%, indicating that resistance against these factors is strain-specific. Moreover, Dowdell et al.[44] demonstrated the ability of *E. faecium* and *L. lactis* to survive a simulation of adverse conditions in the gastrointestinal tract. The results were similar to those present in our study, and the authors also demonstrated the superior ability of EF to survive acidic environments when compared to *L. lactis*.

In this study, we have emulated the gastrointestinal tract conditions similar to the ones observed in the host, such as high acidity and the presence of bile salts, where probiotic strains can grow and survive. By demonstrating resistance in these tests, the probiotic strain becomes an important candidate to demonstrate its potential in in vivo studies[45]. Thus, Fahim et al.[46] state that a viable alternative would be the use of microencapsulation to increase cell viability. According to the authors, the use of microencapsulation with alginate in association with chitosan offers protection to both the probiotic and biomolecules in the passage through the gastrointestinal tract. Other studies, such as those of Rodklongtan et al.[47], Song et al.[48] and Zohri et al.[49], also report increased cell viability after using different microencapsulation techniques.

Considering that one of the longer term objectives of the present work is the biotechnological application of isolated bacteria and their biomolecules in the formulation of, for example, functional foods, the expression of hemolysin and gelatinase virulence factors in the isolates needed to be investigated. This is because the presence of microorganisms with such characteristics in food matrices is a problem, as these virulence factors may be associated with the development of serious diseases and death[31,33]. Therefore, the absence of expression of such virulence factors in this study is encouraging, although the presence of other virulence genes also needs to be evaluated before performing experiments in vivo.

In the same sense, one of the most undesirable characteristics of a probiotic microorganism is the ability to withstand exposure to antibiotics. In our study, none of the isolates showed resistance to the antibiotics tested, all of which are of clinical importance. Therefore, our results are of great importance and reflect what has also been previously described by other studies with LAB[33,50,51].

After evaluating the expression of these important virulence factors, future work should focus on the possibility of using the isolates in a probiotic mixture. Indeed, Mariam et al.[52] isolated probiotic strains belonging to the LAB group and, after several tests, raised an important issue. Specifically, according to the authors, co-culture in mixtures was not only possible but also increased BLIS antimicrobial action, which started to inhibit pathogenic bacteria such as *L. monocytogenes* (a microorganism that can resist common food preservation methods) more effectively, thus reducing cell count to much lower levels than the control group. For this reason, the authors encourage studies with new probiotic strains to assess their interaction in mixed cultures.

In the experiment with Caco-2 cells, the percentage of adherence was high for all isolates tested (> 70%), a finding which is encouraging for future in vivo studies. Although promising, the high adhesion potential of *L. lactis* and *E. faecium* is well described in the literature. Nascimento et al.[53] and Downdell et al.[44] carried out similar studies and obtained good adherence percentages, but lower than those observed in our study. Vasiee[54], in turn, evaluated the adherence potential of the recombinant strain *L. lactis* NZ1330 to Caco-2 cells and its antagonistic effect on *E. coli*. In the end, a good adhesion potential and ability to compete and prevent the adhesion of *E. coli* to Caco-2 cells were observed. Furthermore, He et al.[55] demonstrated the ability of *E. faecium* WEFA23 to compete and inhibit (> 50%) the adherence of *L. monocytogenes* and *S.* Typhimurium to Caco-2 cells.

The results observed in this study suggest that using bacteriocinogenic probiotic strains as a food supplement might be a feasible strategy for the control of infectious diseases. The increase in recently published studies in the area demonstrating the beneficial effects on health and disease resistance after supplementation with probiotics reveals the great scientific potential of this segment[56,57].

In summary, based on the promising results obtained in this study, a bacteriocin purification study, as well as an evaluation of the protective potential of microencapsulation on the isolates and the individual and concomitant (mix) probiotic effect in an in vivo test, will be performed.

Conclusions

The aquatic environment proved to be an important source of bacteriocinogenic probiotic bacteria. All isolates evaluated in this study harbor genes for bacteriocins, showed antimicrobial activity against important fish and food pathogens, were sensitive to all antibiotics tested, had a high rate of adherence to Caco-2 cells and did not express hemolysin and gelatinase virulence factors. It was shown that isolates L1 and L2 from rainbow trout were not able to resist low pH. However, isolates L2 and EF (from starfish) demonstrated good resistance to the action of bile salts, and EF was also resistant to pH 2.5 and 3. For this reason, future tests to evaluate the protective

effect of microencapsulation on the viability of the isolates and their effect on an animal model will be carried out. There is no doubt that the new discoveries in the field of probiotics will bring countless changes in this area of study, which will result in ever higher quality foods and consumer health, whilst lowering impacts on nature. One of the main advances brought about by research with individual and mixtures of probiotics, is the gradual replacement of antibiotics, a decreased in new episodes of microbial resistance and better responses to production diseases, commonly treated with chemicals or antibiotics.

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Author contributions

Conceptualization: R.P.S.O.; Methodology, investigation, and data curation: W.A.P., A.C.M.P., C.M.N.M., Y.A.T., M.A.M., R.A.F., E.N.M., M.V.; Writing original draft preparation: W.A.P., A.C.M.P.; Writing review and editing: R.P.S.O., B.C., E.F.V., R.C.O.; Supervision, project administration, and funding acquisition: R.P.S.O. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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CHAPTER 2

microorganisms

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Review

Use of Probiotic Bacteria and Bacteriocins as an Alternative to Antibiotics in Aquaculture

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Abstract: In addition to their use in human medicine, antimicrobials are also used in food animals and aquaculture, and their use can be categorized as therapeutic against bacterial infections. The use of antimicrobials in aquaculture may involve a broad environmental application that affects a wide variety of bacteria, promoting the spread of bacterial resistance genes. Probiotics and bacteriocins, antimicrobial peptides produced by some types of lactic acid bacteria (LAB), have been successfully tested in aquatic animals as alternatives to control bacterial infections. Supplementation might have beneficial impacts on the intestinal microbiota, immune response, development, and/or weight gain, without the issues associated with antibiotic use. Thus, probiotics and bacteriocins represent feasible alternatives to antibiotics. Here, we provide an update with respect to the relevance of aquaculture in the animal protein production sector, as well as the present and future challenges generated by outbreaks and antimicrobial resistance, while highlighting the potential role of probiotics and bacteriocins to address these challenges. In addition, we conducted data analysis using a simple linear regression model to determine whether a linear relationship exists between probiotic dose added to feed and three variables of interest selected, including specific growth rate, feed conversion ratio, and lysozyme activity.

Keywords: probiotic; bacteriocin; antibiotic; aquaculture; biotechnology

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1. Introduction

There has been a growing global demand for animal protein, with fish representing a particularly important source. However, systematic and unbalanced human exploitation has led to an 80% reduction of the wild fish populations in the oceans. In parallel, the strong expansion of fish farming and aquaculture production has created a set of new challenges far beyond those involving the growth of the sector and its food supply chains [1]. To continue to grow, the aquaculture sector must focus on resolving difficulties through the demarcation of new breeding areas, accessing highly nutritious feed, developing new technologies and technical support, addressing logistic management limitations, and, very importantly, optimizing the ability to predict, avoid, and contain infections and diseases [2].

Fish consumption has grown in recent decades. It is estimated that a 3.2% increase occurred between 1961 and 2016, a figure that surpassed the corresponding rises in terrestrial animal protein production (2.8%). The estimated annual consumption per person

has also increased significantly; for example, in 1961, average global consumption was less than 9 kilograms (kg), but by 2015, it had increased to 20.2 kg, with an additional growth from 20.3–20.5 kg estimated from 2016–2017 [3]. Most of production derived from aquaculture is intended for human consumption. By 2030, aquaculture is expected to be responsible for producing about 109 million tonnes for human consumption, compared with a predicted 74 million tonnes from exploratory fishing [3], a level of growth that is supported by low taxation levels [4,5]. However, many obstacles may hamper the predicted growth of aquaculture. Of these, the failure to predict and contain infections, diseases, and antibiotic resistance is the most perturbing [6].

As a strategy to minimize production losses due to infectious bacterial outbreaks, the use of antibiotics has been widely employed in recent decades [7]. However, their use is not sustainable and other options must be examined.

The objective of this review is to provide recent information relating to the importance of aquaculture in the animal protein production sector and its global economic impacts and growth prospects, as well as its present and future challenges generated by outbreaks and antimicrobial resistance, while highlighting the potential merits of employing probiotics and bacteriocins within this industry. Beneficial microorganisms (probiotics) and bacteriocins are novel solutions that could help reduce the use of antibiotics in aquaculture.

2. Antibiotics and Fish Infection Control

Along with their therapeutic applications to treat and control the spread of bacterial disease in juvenile and adult fish, antibiotics could be used as tools to avoid and prevent future infections beginning from the first days of fish development, when used as growth factors in feeding formulations [7]. This is sustained by farmers' perception that the continuous presence of small doses of antibiotics in the fish growth environment helps to significantly reduce production costs. Due to the perception established between the decreased proliferation of pathogenic microorganisms with lower production losses and decreased time required to attain market weights, the abusive and unregulated use of these important therapeutic agents has expanded worldwide [7,8]

This is particularly worrying since, according to data reported by the World Health Organization (WHO) [9], a significant proportion of these antibiotics are also used as essential therapeutic agents for the treatment of bacterial diseases in humans. Therefore, the uncontrolled application of these antibiotics in animal protein production presents an enormous risk to human health [10]. Antibiotics can kill beneficial microorganisms, cause disturbances in the microbiota [11], affect nutrition and immunity [12], and their use can lead to the selection of resistant bacteria and the zoonotic transmission of resistance genes to the human microbiota [13]. Due to concerns relating to the global emergence of antibiotic resistance, global authorities and several developed countries, such as Canada, Japan, the United States, and members of the European Union, have implemented strict rules on the use of antibiotics in fish breeding [14]. Restrictions were officially approved, selecting a limited and smaller group of antibiotics that can be used in fish breeding, such as erythromycin, amoxicillin, florfenicol, oxytetracycline, oxolinic acid, flumequine, and combinations of sulphonamides [15]. Notably, a number of these antibiotics are considered essential for disease control in humans [9]. Even more importantly, these restrictions may have little impact globally as the majority of fish production is located in countries that have not adopted similar laws to regulate the use of antibiotics in animals. Thus, one can have extremes whereby, for example, Chile uses approximately 900 g of antibiotics for each tonne of fish while Norway uses only 0.17 g [14,16]. Furthermore, in Brazil, one of the top 25 aquaculture producers, many producers have increased the size of their production areas without following international standards of good environmental management practices. As a result, negative environmental effects and antibiotic-contaminated fish are common [17].

Ultimately, the continued extensive use of antibiotics by some countries is not sustainable, and as the number of bacterial disease outbreaks associated with the artificial

environmental conditions of aquaculture increases and restrictive antibiotic use policies are implemented at an international level, new infectious control and prevention protocols are needed [7]. These new protocols are required to control the most common cause of fish diseases, i.e., bacterial infections. These include infections caused by *Aeromonas salmonicida* [15], *Vibrio anguillarum* [18], *Streptococcus agalactiae* [19], *Flexibacter columnaris* [20], *Aeromonas hydrophila* [21], *Aeromonas caviae* [22], *Pseudomonas aeruginosa* [23], *Enterococcus* spp. [24], *Francisella noatunensis* [25], and *Flavobacterium psychrophilum* [26].

Naturally, producers of non-antibiotic antimicrobials have received great attention as an alternative to the use of antibiotics [27]. In particular, probiotic microorganisms have been increasingly investigated as a means of improving fish defenses, especially as they are considered safe and are also frequently producers of antimicrobial peptides, such as bacteriocins [7].

3. Probiotic Use in Aquaculture

Probiotics are defined as live microorganisms that, when administered in adequate amounts, have the ability to confer health benefits on their host [28]. However, there is no consensus as to the value of applying probiotics to aquaculture. According to Wang et al. (2019), the way these animals relate to and are influenced by the environment is different from other animals, and so strains specifically tailored for aquaculture use need to be evaluated. Verschuere et al. (2000) proposed a new concept when defining probiotics for aquacultural use. Their concept differs from the standard definition of probiotics in that it suggests that probiotics for aquaculture use must have a beneficial action on both the host microbiota and the environment where the fish is located, optimizing the effect of food, animal health, and weight gain [29]. It is also important to note that chemical and physical factors, such as water quality (level of oxygen and carbon dioxide, temperature, pH, and presence of organic matter), fish density, or physical injury during handling, can lead to physiological reactions that culminate in the development of disease [30]. Furthermore, environmental changes or stress exposure can negatively affect fish development via immunosuppression. Thus, probiotic administration may also be targeted towards providing a protective response against these external stimuli [1].

Water and other living organisms might spread microorganisms from the gut microbiota of fish and probiotics. After reaching the host's intestinal mucosa, these microorganisms perform vital functions. Several anatomical structures of aquatic animals are sites for the growth of microorganisms, such as the skin, gills, and especially the gastrointestinal tract [1,31]. Feces and intestinal mucus of fish are the main sources of microorganisms with probiotic potential. After isolation, these microorganisms are tested and can be used as a supplement in the feeding of aquatic animals [32]. The larval stage of growth is optimal with respect to probiotic use in aquaculture, and the consequences of early colonization of these microorganisms can be amplified throughout a fish's life stages [33,34].

The probiotic microorganisms used in aquaculture have included specific strains of yeasts, algae, and especially bacteria, including representatives of *Bacillus* sp., *Lactococcus* sp., *Micrococcus* sp., *Carnobacterium* sp., *Enterococcus* sp., *Lactobacillus* sp., *Streptococcus*, and *Weissella* sp. [35]. Bacteria belonging to the group of LAB are considered GRAS, i.e., generally reported as safe [36] and can produce natural compounds with antimicrobial potential and also stimulate the immune system; thus, most probiotic studies are conducted with strains of LAB [37].

The use of probiotic microorganisms in experiments with aquatic animals has achieved promising results (Table 1), and feed supplementation effectiveness can be optimized if different approaches for the use of probiotics are tested (Figure 1) [38], including the use of mixtures of probiotics where complementary effects can be obtained. Supplementation with prebiotics, which are nondigestible food components that benefit colonization by providing nutrients and protection to probiotic and other desirable strains, or synbiotics, which are combinations of probiotics and prebiotics in the same product, can also have

value [38–40]. Finally, postbiotics, which are the products of probiotic growth, including bacteriocins, can also have a key role [41].

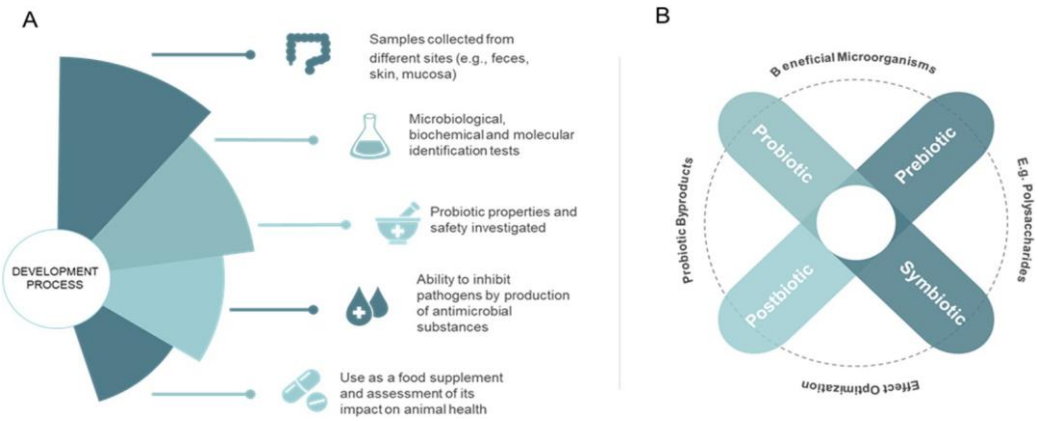

Figure 1. Probiotics development processes for feed and techniques to improve probiotic supplementation effects. (**A**) The different stages before probiotic bacteria use in aquaculture. From a sample, tests to identify genus and species are performed. Then, tests with and without the use of living organisms evaluate its properties and use as a food additive in animal feed. (**B**) In order to optimize aquaculture production processes, different techniques have been used. Probiotic microorganisms are those that confer benefits to the host; prebiotics are nondigestible food components that benefit the colonization of certain bacteria, such as probiotics; synbiotics are the combination of probiotics and prebiotics in the same product; mixtures of probiotics are prepared from the combination of more than one probiotic microorganism to potentiate their action; and postbiotics, dead probiotics or byproducts, are commonly associated with safety [38–40,42].

Table 1. Overview of probiotic effects on fish health or against aquaculture pathogenic bacteria.

Aquatic Specie	Probiotic	Pathogen or Challenge	Clinical Impact	Reference
Oreochromis niloticus	Mixture of LAB	*Trichodina* sp.	Improved growth rate and antiparasitic activity	[43]
Cyprinus carpio	*Pediococcus pentosaceus*	*Aeromonas hydrophila*	Probiotic increases digestive enzyme activity; enhancement of growth rate and immune response; resistance against bacterial infection	[44]
Litopenaeus vannamei	Mix of commercial probiotics (e.g., *Bacillus* spp., *Lactobacillus* spp., *Saccharomyces* spp.)	Not evaluated	The probiotics did not change water quality or growth parameters when compared with control group	[45]
Salmonids	*Vibrio alginolyticus*	*A. salmonicida*, *V. anguillariim*, *V. ordalii*	Pathogen inhibition	[46]
Salmo salar	*Tetraselmis suecica*	*A. salmonicida*, *S. liquefaciens*, *V. anguillariim*, *V. salmonicida*, *Y. ruckeri*	Suppress pathogen growth	[47]
Salmo tutta	*Lactococcus lactis*, *Leuconostoc mesenteroides*	*Aeromonas salmonicida*	Higher survival rate	[48]

Table 1. *Cont.*

Aquatic Specie	Probiotic	Pathogen or Challenge	Clinical Impact	Reference
Mystus cavasius	*Saccharomyces cerevisiae*	*Pseudomonas fluorescens*	Better weight gain, low mortality; resistance against tested pathogen	[49,50]
Labeo rohita	Probiotic mixture (*Bacillus subtilis, Pediococcus acidilactici,* yeast *Saccharomyces cerevisiae*) and symbiotics (*Bifidobacterium, Lactobacilli, Saccharomyces cerevisiae,* microalgae *Spirulina* sp., phytase)	Not evaluated	Better survival and growth rate; probiotic action is best if administered to developing fish in their first days	[50]
Litopenaeus vannamei	*Bacillus subtilis*	Not evaluated	Significant secretion of hepatopancreatic metabolites; expression of genes linked to antioxidant enzymes	[51]
Oreochromis niloticus	*Aspergillus oryzae*	*Aeromonas hydrophila*	Improvement of immune response and growth rate	[52]
Oreochromis niloticus	*Lactobacillus plantarum* L-137	Exposition to deltamethrin toxicity	Reduction of the toxicity	[52]
Pagrus major	*Pediococcus pentosaceus*	Not evaluated	Increased weight gain, mucus secretion, growth rate, bacterial resistance, and blood parameters	[53]
Pagrus major	*Lactobacillus plantarum*	Not evaluated	Immunostimulant property (innate defenses)	[54]
Pagrus major	*Lactobacillus rhamnosus* and *Lactococcus lactis*	Not evaluated	Better growth, feed utilization, serum lysozyme activity, bactericidal property, and lower triglycerides and cholesterol	[55]
Oreochromis niloticus	*Bacillus subtilis* and *Bacillus licheniformis*	Not evaluated	Enhanced immunological parameters (hematocrit, total leukocytes count, monocytes, and globulin), improved growth and feed utilization	[56]
Oreochromis niloticus	*Lactobacillus* sp., *Bacillus* sp., *Bifidobacterium* sp. (probiotic mixture)	Not evaluated	Antimicrobial activity, better growth rate	[57]
Oreochromis niloticus	*Lactobacillus plantarum*	*Enterococcus faecalis*	Modulation of gut microbiota, immune response, and resistance against pathogenic bacteria	[58]
Atlantic salmon	*Candida utilis*	*Chlorella vulgaris*	Counteracts intestinal inflammation	[59]
Salmon salar	Lactic acid bacteria	*Aeromonas salmonicida*	Higher mortality	[60]
Gadus morhua (Atlantic cod),	*Carnobacterium divergens*	*V. anguillarum*	Disease resistance	[61]

Table 1. *Cont.*

Aquatic Specie	Probiotic	Pathogen or Challenge	Clinical Impact	Reference
Cyprinus carpio	*Pseudomonas aeruginosa*	*Aeromonas hydrophila*	Antioxidant and immune action; better infection control with probiotic treatment	[62]
Oreochromis mossambicus	*Bacillus licheniformis* Dahb1 (105 and 107)	*Aeromonas hydrophilain*	Weight and specific growth rate improvement; high mucosal activity of enzymes; resistance to the infection	[63]
Pangasius hypophthalmus	*Bacillus licheniformis*	*Vibrio parahaemolyticus*	Increased immune, antioxidant and growth parameters; protected against infection	[64]
Ctenopharynodon idellus	*Bacillus subtilis*	*Aeromonas hydrophila, Aeromonas punctata, Edwardsiella ictaluri, Aeromonas punctate, Vibrio flurialis* and *Streptococcus agalactiae*	Inhibitory activity against all pathogenic bacteria tested	[65]
Cyprinus carpio	*Paenibacillus polymyxa*	*Aeromonas hydrophila*	Improved survival rate and immune response; disease resistance against pathogenic bacteria tested	[66]
Litopenaeus vannamei	*Bacillus subtilis, Bacillus pumilus, Bacillus tequilensis, Enterococcus faecalis*	Not evaluated	Significant difference in growth rate, weight gain, and survival	[67]
Acipenser baerii	*Lactobacillus* spp. *Bacillus subtilis, Bifidobacterium bifidum* (probiotics mixture)	Not evaluated	Immunity and growth improvement	[68]
Oreochromis niloticus	*Bacillus licheniformis*	*Streptococcus iniae*	Better survival rate	[69]
Heteropnuestes fossilis	*Bacillus subtilis*	*Aeromonas hydrophila* and *Aphanomyces invadans*	Bacterial treatment leads to a health improvement; fungi treatment does not	[70]
Oncorhynchus mykiss	*Lactobacillus rhamnosus*	*Yersinia ruckeri*	Improved growth rate, immune response, and antioxidant activity; pathogen inhibition	[71]
Litopenaeus vannamei	*Lactobacillus plantarum* and galactooligosaccharide (symbiotic)	*Vibrio harveyi* and *Photobacterium damselae*	Improvement in growth and health parameters; infection control; significant changes in intestinal microbiota of shrimp	[72]
Salmonids	*Carnobacterium Inhibens* K1	*Vibrio anguillarum, Aeromonas salmonicida*	Suppress pathogen growth	[73]
Oreochromis niloticus and *Cyprinus carpio*	*Lactococcus lactis* subsp. *lactis, Lactobacillus plantarum, Lactobacillus brevi*	*Vibrio* sp., *Staphylococcus* sp., *Pseudomonas aeruginosa, Salmonella enterica, Listeria monocytogenes*	Antimicrobial action	[74]

Table 1. *Cont.*

Aquatic Specie	Probiotic	Pathogen or Challenge	Clinical Impact	Reference
Cyclopterus lumpus	*Aliivibrio* sp.	*Moritella viscosa* (contamination)	Resistance against infection caused by *M. viscosa*; low incidence of mortality and ulcers	[75]
Oreochromis niloticus	*Bacillus velezensis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*	*Aeromonas hydrophila*	Improvement of immune response; antimicrobial activity	[76]
Paralichthys olivaceus	*Bacillus* sp. and β-glucan (symbiotic)	*Edwardsiella tarda*	Strain has significant antimicrobial activity; symbiotic effect improved growth performance; resistance against tested pathogen (antibiotic replacement)	[77]
Apostichopus japonicus	*Metschnikowia* sp.	Not evaluated	High activity of lysozyme, total nitric oxide synthase, trypsin, and phenoloxidase	[78]
Lates calcarifer	*Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus fermentum*, *Enterococcus faecium*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*	*Aeromonas hydrophila*	The probiotic mixture improved growth and health status of Asian Seabass	[79]
Oplegnathus fasciatus	*Bacillus subtilis* E20	*Vibrio alginolyticus*	Better growth rate and immune response; pathogen resistance	[80]
Salmon salar	*Pediococcus acidilactici*	IPN virus	Antiviral response	[81]
Pangasius bocourti	*Bacillus aerius* B81	*Aeromonas hydrophila*, *Streptococcus agalactiae*	Antimicrobial effect against tested pathogens, high immune response	[82]
Oreochromis niloticus	*Lactobacillus plantarum*	Environmental challenges	High mucosal immune response	[83]
Oncorhynchus mykiss	*Lactobacillus acidophilus*	*Lactococcus garvieae*	Better growth rate, digestive enzyme production, resistance against tested pathogen	[84]
Cyprinus carpio	*Lactobacillus casei*, β-glucan and mannan oligosaccharide (symbiotic)	*Aeromonas hydrophila*	Symbiotic improves the digestibility; elevation in important enzymes (lipase, amylase, trypsin, and protease); low mortality	[85]
Haliotis midae	*Vibrio midae*	Not evaluated	Increase in growth performance and survival rate	[86]
Labeo rohita	*Bacillus* sp.	*Aeromonas hydrophila*	Improved hematological serum an immunological parameter	[87]
Oncorhynchus mykiss	*Gordonia bronchialis*	Not evaluated	Enhanced growth performance	[88]

Table 1. *Cont.*

Aquatic Specie	Probiotic	Pathogen or Challenge	Clinical Impact	Reference
Penaeus indicus	*Bacillus subtilis*	*Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp., *Micrococcus* sp.	High bacteriocin production; diet with bacteriocin enhances shrimp growth; antibiotic potentials (well diffusion method)	[89]
Salmon salar	*Carnobacterium divergens*	*Aeromonas salmonicida, Vibrio anguillarum*	Prevent pathogen-induced damage	[90]
Salmon salar	*Methylococcus capsulatus*	Not evaluated	No inflammation with soybean meal	[91]
Oncorhynchus mykiss	*Enterococcus casseliflavus*	*Streptococcus iniae*	Elevated digestive enzyme activity, humoral immunity (IgM), total serum protein, and albumin production	[92]
Salmon salar	*Lactobacillus delbruckii*	*Aeromonas salmonicida*	Prevent pathogen damage	[93]
Oreochromis niloticus	*Bacillus* sp.	*Aeromonas hydrophila, Micrococcus luteus, Pseudomonas fuorescence, Enterococcus faecalis*, and *Streptococcus agalactiae*	Probiotic potential (resistance to adverse stomach condition, production of important enzymes)	[94]
Etroplus suratensis and *Oreochromis Mossambicus*	*Bacillus* sp., *Micrococcus* sp.	Not evaluated	Better growth performance and nutritional efficiency	[95]
Danio rerio	*Bacillus subtilis* (transgenic probiotic)	Not evaluated	The transgenic probiotic (phytase) can improve fish nutrition	[96]
Dicentrarchus labrax	*Vibrio lentus*	Not evaluated	Immunomodulation and activation of genes associated to cell proliferation	[97]
Oreochromis niloticus	*Bacillus amyloliquefaciens*	*Yersinia ruckeri, Clostridium perfringens*	Improved immune status (IL-1 and TNF-α mRNA) and disease resistance	[98]
Litopenaeus vannamei	*Enterococcus faecium* and *Lactobacillus pentosus*	*Vibrio harveyi, Vibrio parahaemolyticus*	High antibacterial activity and survival rate; improved humoral immune response	[99]
Oncorhynchus mykiss	*Lactobacillus plantarum*	*Yersinia ruckeri*	High activity of lysozyme and alkaline phosphatase; no interference in the production of immunological proteins	[100]
Oreochromis niloticus	*Enterococcus faecium*	*Aeromonas hydrophila*	Better growth rate and immune defenses	[101]
Oreochromis niloticus	*Bacillus* sp.	Streptococcosis (*Streptococcus agalactiae*)	Controlled the Streptococcosis caused by pathogenic bacteria tested	[102]
Rutilus caspicus	*Enterococcus faecium*	*Aeromonas hydrophila, Yersinia ruckeri*	Better growth rate, immune response, and pathogen resistance	[103]

Table 1. *Cont.*

Aquatic Specie	Probiotic	Pathogen or Challenge	Clinical Impact	Reference
Ictalurus punctatus	*Bacillus velezensis*	Not evaluated	Induction of growth in fingerling and water quality improvement	[104]
Litopenaeus vannamei	*Bacillus subtilis*	Not evaluated	Better growth performance and feed utilization	[105]
Carassius auratus	*Enterococcus faecium*	*Aeromonas hydrophila*	High survival rate as a result of *E. faecium* probiotic proprieties; quorum sense potential	[106]
Atlantic salmon	*Pediococcus acidilactici*		Improvements in the gut health	[107]
Oncorhynchus mykiss	*Lactobacillus fermentum, Lactobacillus buchneri, Saccharomyces cerevisiae* (probiotics mixture)	Not evaluated	Immunity improvement	[108]
Danio rerio	*Pseudomonas aeruginosa*	*Vibrio parahaemolyticus*	Reduced mortality, inhibited biofilm, high level of phagocytic cells, superoxide dismutase activity, and lysozyme	[109]
Oreochromis niloticus	*Bacillus cereus, Alcaligenes faecalis*	Environmental challenges	High production of immune proteins and decrease of phosphorus water concentration	[110]
Ctenopharyngodon idellus	*Shewanella xiamenensis* and *Aeromonas veronii*	*Aeromonas hydrophila*	Enhancement of phagocytic, lysozyme activity, and expression of immune genes	[111]
Rhamdia quelen	*Lactococcus lactis*	*Aeromonas hydrophila, Streptococcus agalactiae*	Antimicrobial activity against tested pathogens	[112]
Carassius auratus	*Bacillus velezensis*	*Aeromonas hydrophila*	Improved survival rate and immune response	[113]
Nile tilapia	Probiotic mixture	Aluminum exposition	Probiotics regulated gut microbiota structure and function	[114]
Oreochromis niloticus	*Lactobacillus plantarum*	Aluminum intoxication	Enhanced feed utilization and growth; decreased deaths caused by aluminum and its accumulation	[115]
Ctenopharyngodon idellus	*Bacillus paralicheniformis*	Not evaluated	High adhesion and colonization capacity	[116]

4. Mode of Action and Benefits of Probiotic

Among the studies that have demonstrated the benefits of probiotic use, different mechanisms of action have been noted, differing by species specificities and environmental conditions that the microorganism encounters [37,117]. Probiotics have been shown to be able to decrease lactose intolerance and infant diarrhea in humans, and many promising studies have shown that they can stimulate the immune system and prevent numerous diseases, including mucosal inflammation, obesity, diabetes, heart and neurological diseases, and certain types of cancer. In this current review, the focus will be placed on the prevention of pathogenic microorganisms in aquacultural settings. Beneficial strains can

function by blocking pathogenic microorganisms due to competition for space on host cell surfaces (Figure 2) [118]. Probiotic use in feed improves the health of aquatic animals and no negative effects have been observed after consumption [14]. Strains of *Lactobacillus* are commonly recommended for aquaculture, and dietary supplementation results in better enzyme activity, immune response, development, weight gain, and even water quality improvement [32,119]. The stimulation of digestive enzyme production, such as amylase, protease, lipase, and lysozyme, can be an important consequence of probiotic use [118]. In healthy animals, these enzymes are intrinsically associated with improved digestibility, nutritional intake, and weight gain [120]. Improving the digestibility of certain compounds may reduce blood lipid rates and even address problems arising from the intolerance to certain compounds [32].

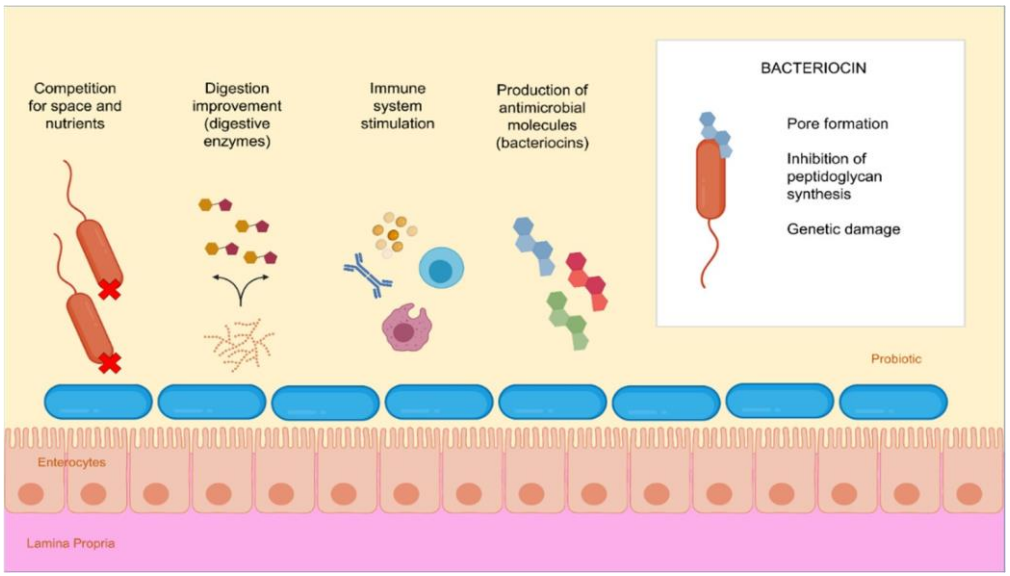

Figure 2. Probiotics and bacteriocins mode of action. Probiotics beneficial effects come from several mechanisms. They secrete digestive enzymes that contribute to macronutrients breakdown, increasing absorption by the host. They can act by blocking pathogens due to competition for space and nutrients, by stimulating the immune system (without the presence of disease) and via the production of antimicrobial substances (such as lactic acid and bacteriocins). Bacteriocins mode of action may vary according to their characteristics. They can lead to death via pore formation, preventing the action of peptidoglycan transporters and, consequently, cell wall synthesis, and via damage to genetic material and protein synthesis. Probiotics, bacteriocins, and the host nutritional improvement contribute to pathogens elimination and diseases control [121,122].

The benefits of probiotics in aquaculture extend beyond animal health and can also be used to improve water quality. The accelerated fish production process creates a stressful environment favorable to pathogenic microorganisms and diseases. However, probiotic use in fish farm systems can modify the aquatic environment and, by reducing the populations of undesirable microorganisms, reduce the chances of disease development [123].

In this review, we conducted data analysis using a simple linear regression model (GraphPad Prism version 9.0, GraphPad Software, San Diego, CA, USA) to determine whether a linear relationship between probiotic dose added to feed and three variables of interest selected, including specific growth rate (SGR; 38 studies), feed conversion ratio (FCR; 32 studies), and lysozyme activity (8 studies), exists. For analysis purposes, we have only taken into account the presence or absence of probiotics without considering the type of probiotic as well as whether they were used as single or multiprobiotic treatment.

Probiotic dose added to feed was transformed to log10 for graphic representation purposes. Data analysis revealed no significant correlation (p = 0.085) between probiotic dose in feed and SGR in fish (R^2 = 0.0182; Figure 3). However, we detected a significant

correlation (p = 0.014; p = 0.017) between probiotic dose in feed and FCR as well as lysozyme activity (R^2 = 0.048; R^2 = 0.163, respectively; Figures 4 and 5) in fish. These results suggest adding probiotics to the diet improves the utilization efficiency of feed in fish and thus contributes to improving the economy and well-being of fish farming. This is especially true since feed is considered to be the highest cost in aquaculture facilities, particularly in intensive culture systems where feed costs represent close to 50% of the variable production cost [124].

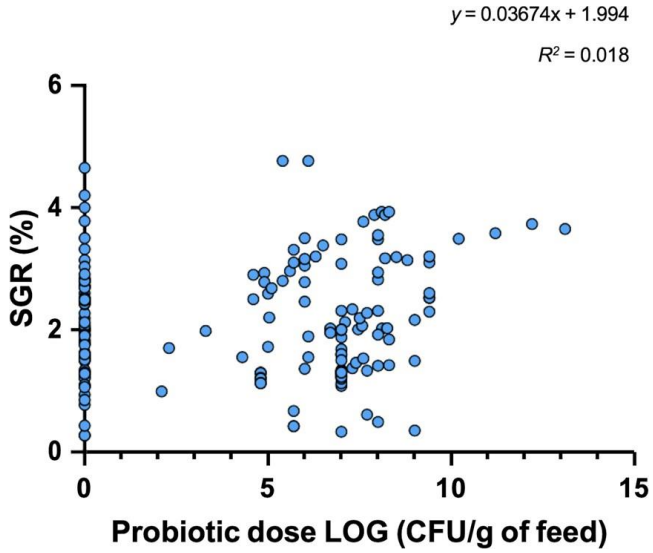

Figure 3. Data analysis revealed no significant correlation between probiotic dose in feed and SGR in fish. The circles represent the mean of experimental groups (n = 3; either control group or probiotics treatment group) tested in the studies considered for the regression analysis.

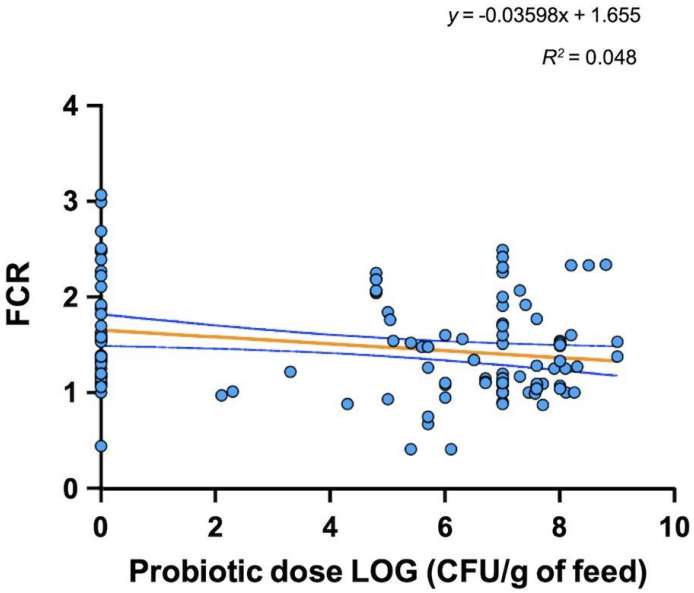

Figure 4. Data analysis revealed significant correlation between probiotic dose in feed and FCR. The circles represent the mean of experimental groups (n = 3; either control group or probiotics treatment group) tested in the studies considered for the regression analysis.

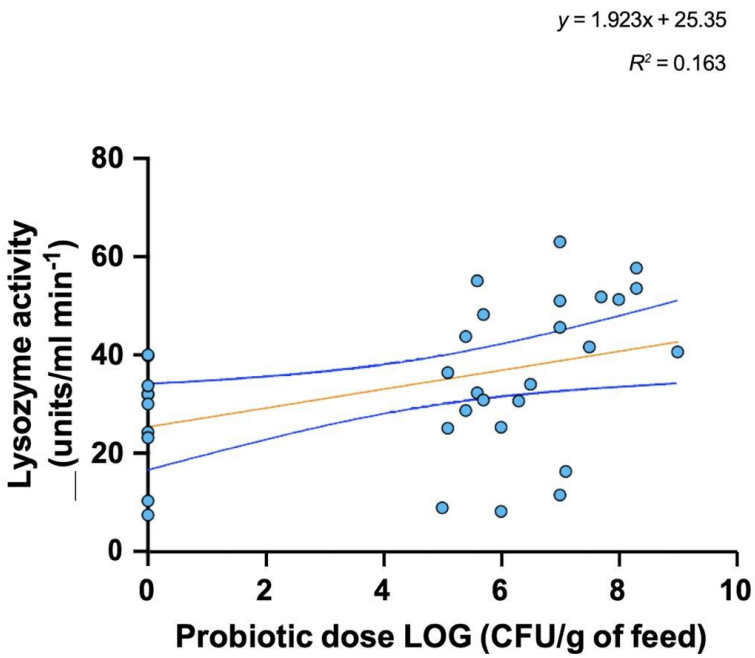

Figure 5. Data analysis revealed significant correlation between probiotic dose in feed and Lysozyme activity. The circles represent the mean of experimental groups (n = 3; either control group or probiotics treatment group) tested in the studies considered for the regression analysis.

The improvement in fish feed utilization could be a consequence of probiotic microbes contributing directly or indirectly, via induced changes in gut microbiota composition, to metabolize undigested nutrients via microbial enzyme activity. However, an enhancement of nutrient absorption surface/capacity due to a stimulatory effect of probiotic microbes on gut epithelium development and gut health might contribute to this outcome as well. For example, short chain fatty acids (SCF) derived from probiotic metabolism influence epithelial cell metabolism, helping with busting diverse energy-demanding cellular processes in enterocytes, such as producing mucin and tight junction enterocyte proteins, which contribute to the integrity of the intestinal barrier [125].

For its part, our analysis revealed that SGR was not affected by adding probiotics to the diet of fish. A possible explanation of this lack of significance is due to the exponential function of SGR, showing some imprecision when determining fish growth efficiency using either long-term data or data over different life stages. Thus, SGR should be used when fish are exactly of the same age, since the growth performance of fish during different life stages introduces a bias into the calculation. Because the studies included in our analysis covered different life stages and trial periods, SGR may have been an unsuitable mathematical model for comparing growth performed in these heterogenous data analysis environments [126].

Finally, the significant positive correlation between lysozyme activity and probiotic dose added to feed found across the studies included in the analysis supports the idea that probiotics provide health benefits to fish (Figure 5). Lysozyme is a hydrolytic glycosidase [(β-) glycoside hydrolase that exerts several important functions related to innate immunity, including the lyse of Gram-positive and Gram-negative bacterial cell membranes (acting as an antimicrobial agent) and activation of the complement system and phagocytes. It is ubiquitously distributed in several tissues, mucus, lymphoid tissue, plasma, and other body fluids [127]. Hence, increasing lysozyme activity by adding probiotics to feed might play an important role in enhancing fish disease resistance in intensive culture systems.

5. Bacteriocin Use in Aquaculture

In recent years, bacteriocins have received substantial attention as antimicrobial compounds. Although bacteriocins have been predominantly used as food preservatives, they are now receiving better attention as potential clinical antimicrobials and as possible immune-modulating agents. Hence, bacteriocin use is another important strategy to control antibiotic-resistant bacteria and improve health [121]. Bacteriocins are a heterogeneous group of small, ribosomally-synthesized antimicrobial peptides. They can have a wide variety of producers, spectrums of action (Figure 2), and biochemical properties [121,128].

Since 1925, with the discovery of colicin, research on bacteriocins has received considerable attention [129], and by 1995, more than a hundred different types of bacteriocins had been identified [130]. Bacteriocins can provide an important competitive advantage for the species that produce them [131]. Probiotics of interest can produce bacteriocins at their site of action [132].

Several classes of bacteriocins have been evaluated [133]. Many of the bacteriocins tested for food-related applications are isolated from LAB [131]. These include nisin, which is produced by *L. lactis* and has been widely used as a food preservative for more than fifty years [134,135]. Others, such as pediocin PA-1, produced by *Pediococcus acidilactici* have been extensively studied due to their activity against Listeria monocytogenes in meat and dairy products [131]. Bacteriocins have also been investigated for their pharmaceutical application [129] because they could serve as a possible alternative to antibiotics to combat pathogenic microorganisms in live organisms [121]. As production losses in aquaculture due to bacterial diseases and bacterial resistance to antibiotics have increased [7,121], bacteriocins have been applied in aquaculture production systems due to their antimicrobial proprieties (including Gram-positive/Gram-negative inhibition) (Table 2). However, the application of probiotics and bacteriocins in fish feed supplementation requires rigorous testing to avoid any unexpected effects. Safety is essential to current research progress [136].

Table 2. Overview of bacteriocin effects in fish health or against aquaculture pathogenic bacteria.

Aquatic Specie	Bacteriocin	Pathogen or Challenge	Clinical Impact	Reference
Epinephelus areolatus	CAMT2	*Listeria monocytogenes, Staphylococcus aureus*	Antimicrobial activity against tested pathogens	[137]
Labeo rohita	Bacteriocin produced by *Bacillus subtilis LR1*	*Aeromonas hydrophila, Aeromonas salmonicida, Bacillus mycoides, Pseudomonas fluorescens*	In vitro antimicrobial activity against tested pathogens	[138]
Oncorhynchus tshawytscha	Enterocina AS-48	*Lactococcus garvieae*	Antimicrobial activity against tested pathogen (in vitro and in vivo)	[139]
Penaeus monodon	Bacteriocin 99% homologous to that produced by *Bacillus* sp.	*Vibrio alginolyticus, Aeromonas hydrophila, Pseudomonas stutzeri*	In vitro inhibitory activity against tested pathogens	[140]
Pseudosciaena croce	Coagulina L1208	*Escherichia coli, Shewanella putrefaciens, Staphylococcus aureus*	Bacteriostatic antimicrobial activity against tested pathogens	[141]
Litopenaeus vannamei	Bacteriocin produced by *Lactobacillus plantarum* FGC-12	*Vibrio parahaemolyticus*	Pathogen inhibition	[142]
Perca sp., *Tuna* sp., *Platax* sp.	PSY2	*Listeria monocytogenes*	In vitro pathogen inhibition; possible biopreservative against degradation	[143]

Table 2. *Cont.*

Aquatic Specie	Bacteriocin	Pathogen or Challenge	Clinical Impact	Reference
Odontesthes platensis	Mundticin KS	*Pseudomonas aeruginosa*, *S. putrefaciens*	In vitro antimicrobial activity against tested pathogen and Gram-positive bacteria	[144]
Odontesthes platensis	Nisin Z	*Lactococcus garvieae*	Pathogen growth inhibition	[145]
Fermented fish roe	Bacteriocin produced by *Enterococcus faecium* CN-25	*Listeria monocytogenes*	In vitro pathogen inhibition	[146]
Tilapia sp., *Catla catla*, *Cyprinus carpio*	Bacteriocin isolated from *Pediococcus acidilactici*	*Listeria monocytogenes*	In vitro antimicrobial activity against tested pathogen	[147]
Acipenseridae, *Oncorhynchus clarkii*	Plantaricin LPL-1	*Listeria monocytogenes*	In vitro antimicrobial activity against tested pathogen and Gram-positive bacteria	[148]
Pangasius bocourti	7293	*Listeria monocytogenes*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium	Gram-positive and Gram-negative growth inhibition	[149]
Oxyeleotris lineolata	L49	*Streptococcus iniae*	In vitro antimicrobial activity against tested pathogen	[150]
Mimachlamys nobilis	PE-ZYB1	*Listeria monocytogenes*	In vitro antimicrobial activity against Gram-positive and Gram-negative bacteria; pathogen inhibition	[151]
Litopenaeus vannamei	Nisin	*Listeria monocytogenes*	Antimicrobial activity against tested pathogen (in vitro and in vivo)	[135]

6. Safety

It is important that probiotics be properly developed and that new products be verified using validated scientific research. In some countries, probiotics have been approved for use based only on initial tests that generally attest to their antimicrobial and immunostimulatory activity. Furthermore, in 2017, during inspections by the US FDA (Food and Drug Administration, Silver Spring, MD, USA), more than 50% of the establishments visited in the probiotic industry had serious violations, all related to failures during the development process, including misidentification and even contamination of supplements, which compromises product efficacy and safety [136].

The transfer of resistance genes to the host microbiota is another growing concern that could result in a loss of commercial interest. In an in vitro experiment, it was observed that Lactobacillus plantarum M345 was able to transfer a resistance gene to Listeria monocytogenes [152]. In 2005, it was reported that a probiotic product that was approved by the FDA contained a strain with resistance to an important clinical antibiotic (tetracycline) and that the gene could be transmitted [136]. The presence of resistance genes in probiotics has already been described in the literature and has been studied. As one of the main advantages of using probiotics is their safety, it is necessary to pay more attention to this problem. If not controlled, it can represent a loss of consumer interest and economic losses to the sector [120].

However, it is important to emphasize that health problems resulting from the use of probiotics are very rare, both for animals and for humans. These microorganisms are already part of the host's microbiota and any problems related to the use of probiotics

are generally related to host immunity and other pre-existing diseases [153]. In addition, many countries already have very strict laws that ensure that the development and sale of probiotic products takes place safely [4,154].

7. Conclusions and Future Perspectives

Bacterial disease outbreaks in aquaculture systems have increased in the last few decades, and policies that restrict antibiotic use have been implemented. To avoid production losses, new therapeutic fish farming technologies and new infectious control and prevention protocols are required. The benefits of specific probiotics and bacteriocins which trigger directly or enhance the immune structure of aquatic species with respect to fish health and controlling pathogenic bacteria in aquaculture are clear. Further advancements in this area have the potential to cause a paradigm shift in aquaculture, resulting in higher quality foods, improved consumer health, increased sustainability (including environmental sustainability), and increased economic value.

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CHAPTER 3

The international tilapiculture market: potential, challenges, and the growing use of probiotic bacteria

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Abstract

Tilapia culture is the second largest production segment of aquaculture and has great growth potential. However, high mortality rates have been reported in several countries, with bacterial infections being the main cause. If the scenario does not change, large tilapia producers such as China may have stagnant production in the future. With the constant use of antibiotics to treat diseases, bacterial resistance has become a major problem for this industry, which lacks effective alternatives. Research on probiotics has advanced and has shown its potential for use in tilapiculture, as they are non-pathogenic microorganisms that have beneficial effects on host health. Probiotics are known to act by promoting the growth of tilapia, stimulating its appetite and optimizing the nutritional efficiency of the rations. In addition, they act as immunostimulants, generating a pro- and anti-inflammatory response, and produce antimicrobial peptides such as bacteriocins, factors that help fight pathogenic microorganisms. In this work, we have updated the data on the international tilapiculture market, its main potential and challenges, and discussed the possible use of probiotics and their benefits to the health and development of tilapia.

Keywords: *tilapia, antibiotics, probiotics, bacteriocins.*

1. Introduction

Aquaculture consists of the rearing of various aquatic animals in a controlled environment (Lucas et al., 2019), generally intended for food (FAO, 2018). It is a prominent activity in the current food production scenario, being the sector whose productivity has shown the greatest growth in the field of animal protein production (FAO, 2018; FAO 2020a). The development of this activity can be associated with the growth of human population, which in recent decades has increased the demand for protein sources alternative to conventional ones (Woods, 2019). Aquaculture contributes significantly to global food security and poverty reduction (Kassam and Dorward, 2017), since it is developed on a global scale and has low aggregate cost, wide distribution, and commercialization, without causing major environmental impacts. Therefore, it is an important tool for ecosystem preservation efforts (Siqueira, 2018).

The Asian continent is the greatest exponent in this activity, accounting for 89.4% of the world production of aquaculture products in 2016 (FAO, 2018). China, the world's largest producer, passed the 47 million tons mark of aquaculture products in 2018 (SOFIA, 2020).This activity receives priority investments compared to other economic sectors in the country (FAO, 2020b) and, if growth rates are maintained, China is expected to be responsible for 62% of world's aquaculture production by 2025 (SOFIA, 2016). The great importance of Asia in aquaculture is due to the fact that this activity was initially developed on the continent. The origin of aquaculture dates to the ancient Chinese civilization, around 2000 B.C. (Lucas et al., 2019), a period in which carp (*Cyprinus carpio*) began to be domesticated and used as food and ornament (Calado et al., 2017).

World trade in fish farmed by aquaculture started in the 1950s, due to farming improvements and reduction of marine population of fish available for capture provoked by intensive fishing (Siqueira, 2018). Despite the promising market and favorable prospects for the intensification of tilapia farming (PEIXE BR, 2020), there are factors that hinder productivity and constitute challenges for the development of this activity (Schulter and Vieira Filho, 2018). Among the obstacles faced by the sector, the great variety of diseases stands out, especially those of bacterial origin, which are often widespread among tilapias and cause costly losses for producers (Hassan et al., 2020). A further problem is associated with the use of antibiotics to treat these diseases, the indiscriminate use of which can constitute a danger to the environment and human health. Therefore, it is essential that alternative methods are validated

so that diseases caused by resistant microorganisms are controlled in a safe and effective manner (Foysal et al., 2020).

This review article updates the most relevant information on the prospects of tilapia aquaculture for the global animal protein market, the challenges generated by losses due to infections, the development of antibiotic resistance, and the positive impact of using probiotics and bacteriocins on tilapia health.

2. Largest global producers, their potential and challenges

Present in more than 125 countries, tilapia culture is an activity with wide global distribution (El-Sayed, 2019). Its high diffusion is the result of tilapia adaptability to various production systems (FAO, 2020c), especially Nile tilapia (*Oreochromis niloticus*), a species that accounts for more than 70% of tilapiculture (El-Sayed, 2019). Tilapia culture is the second most productive segment of fish culture in the world, with a production of 6.4 million tons in 2019, second only to the Chinese Carp (*Cyprinus carpio*) market (FAO, 2018; Milanez et al., 2019).

Among the characteristics that facilitate the appreciation of tilapia by the consumer is the absence of "Y" thorns that are difficult to remove, which makes the product suitable for the industrial filleting (de Andrade and de Azevedo, 2018).

Tilapia culture is an economic activity present especially in countries of Asia, Africa, and America (El-Sayed, 2019), with China, Indonesia, Egypt, and Brazil being the leading world producers in this order (PEIXE BR, 2019).

2.1. China: a process of refinement of production techniques

China became the world leader in this business in the 1990s, having held this place ever since (Gu et al., 2019). Tilapia culture is present in more than 30 Chinese provinces (Yuan et al., 2017), among which Guangdong stands out with 40% of national production, because it has the ideal conditions for the development of this activity, such as strategic geographical position and adequate climate (Gu et al., 2019). The country has a large coastal area and a wide water potential due to the presence of lagoons, lakes, streams and about one hundred rivers (Yuan et al., 2020). Yuan et al. (2017), who reported farming systems in China of varying sizes, from more rudimentary to more sophisticated systems, concluded that, despite the differences in

expected profit for each system and regardless of the adopted model, tilapia cultivation in the southern region is more profitable, offsetting the investments made.

The Southern region concentrates more than 90% of Chinese production, due to the abundance of water resources and favorable climate for the activity development. Southern aquaculture farmers frequently cultivate tilapia in polyculture with other aquatic species, mainly with Chinese carp followed by shrimp species, which makes this economic activity even more profitable and attractive (Yuan et al., 2020).

Recent growth in activity in northern provinces such as Shandong and Beijing has also been reported; however, due to the need to provide electricity to keep breeding tanks warm, production costs are higher (Xu and Ming, 2018). A study by Phiri and Yuan (2018) revealed that most of the country's facilities operate with high technological efficiency; however, average yield is estimated to increase by up to 9% through improvements in fish feeding and training to instruct workers to adopt more effective resource management and strategies (Yuan et al., 2020). To maintain a balance between the domestic and international markets, the Chinese government encourages storage and trade of part of the production from the southern to the north provinces, avoiding product shortages (Xu and Ming, 2018).

The main breeding system adopted in China is intensive, and the most used facilities are closed tanks and cages, demonstrating a process of refinement of production techniques (Xu, 2004; Xu and Ming, 2018). Starting from 2009, a tilapia culture industrialization project came into force, supported by funding from central and provincial governments, which resulted in a first growth phase, followed by a period of stagnation attributed to increased incidence of streptococcosis due to high stocking density and stressful breeding conditions (Xu and Ming, 2018; El-Sayed, 2019).

In addition to the importance of these products for domestic supply, China also stands out as a supplier of tilapia to foreign market (FAO, 2020a). Chinese exports are mostly addressed to the United States, which have a strong demand for the product (FAO, 2020a). Until 2014, the export rate increased year over year; however, in the following three years it decreased from 69.00 to 63.23% (Dai et al., 2020). Competition from Indonesian products is believed to be one of the most important causes of the drop in exports. There are significant differences in price and quality, since Chinese products are classified as unsatisfactory by the international market (Dai et al., 2020) due to the presence of drug residues in tilapia (Yuan et al., 2017).

Between 2005 and 2016, tilapia production in tons per year increased by 45.03% at an average growth rate of 7.8% per year (Yuan et al., 2020). According to the survey of FAO (2019), tilapia production between 2010 and 2016 went from 1.28 to 1.56 million tons, and in 2019 reached 1.93 million tons (PEIXE BR, 2019). However, there are factors that may contribute to Chinese tilapia farming stagnation in the future. The instability of climatic conditions in the country is a major limiting factor. Moreover, another challenge is the fluctuation in product prices, which affects the demand in international trade and reduces the competitiveness of Chinese products (Yuan et al.,2017). Another worrying factor is diseases spread in the breeding environment (Yuan et al., 2017) such as bacteriosis caused by *Salmonella* spp. (Li et al., 2017).

2.2. Indonesia: the association between tilapiculture and rice farming

Indonesia is the third largest supplier of tilapia to the United States, behind China and Colombia. The prospects for tilapia culture in the country are promising, since exports have been showing increasing rates in the last twenty years (Dai et al., 2020). The activity started in the country in 1930, with the introduction of Mozambique tilapia (*Oreochromis mossambicus*) without great economic significance. In the 1960s, Nilotic tilapia was introduced after the adoption of the GIFT genetic improvement program, which led Indonesia to stand out in this sector (Fathi et al., 2017), and from 2004 annual growth reached 20% (Wati et al., 2020). Between 2010 and 2017, tilapia production grew from 458 thousand tons to 1.10 million tons (FAO, 2019; El-Sayed, 2019). The country continued to grow sharply between 2017 and 2019, reaching a total of 1.35 million tons (PEIXE BR, 2019).

Among the most cultivated species, the Nile tilapia, including GIFT, and Red tilapia (hybrid of *Oreochromis niloticus* and *Oreochromis mossambicus*) stand out (El-Sayed, 2019). Red tilapia culture can be carried out both in net tanks (Wati et al., 2020) and in cages installed in brackish waters or lagoons (Wijayanto et al., 2018). According to FAO (2020c), the possibility of breeding in cages is a good alternative for the needs of farmers living in rural areas, such as in Indonesia. In addition to the importance of the international market, tilapia culture also benefits the Indonesian domestic market, especially in small communities away from large metropolises, such as villages on Lake Sentani in eastern Papua. Other important producing regions are found in Sumatra, Java, Bali, and Borneo (Anshary et al., 2014).

The main breeding systems adopted in the country are the polyculture of tilapia and other aquatic species as well as tilapia culture in rice fields (Fathi et al., 2017). The association between tilapiculture and rice farming, also widely applied in Egypt, is considered a promising option, as it allows optimizing the use of water resources and assisting in the control of insects and pests ingested by fish (Shaalan et al., 2018). Goada et al. (2015) also reported that plants can increase water quality by absorbing phosphorus and nitrogenous substances, increasing the yield of fish production and harvest. The production modalities are variable, from intensive to extensive systems; however, it is estimated that semi-intensive practices are more recurrent. In this system, strategies characteristic of the intensive model are adopted, such as the use of feed additives and fertilizers, maintaining facilities like those of extensive practice (Setiadi et al., 2018).

Despite the high potential for expansion, there are still limitations that mainly relate to the high cost of feed, in addition to high waste production among small farms (Parata et al., 2020; Mo et al., 2018), which highlights the need to apply investments and government support to encourage small producers, especially those in remote regions (Wati et al., 2020). Another challenge for production in the country is the problem of the spread of bacterial diseases, mainly caused by *Aeromonas hydrophila* (Fadjar et al., 2020).

2.3. Egypt: good results from production industrialization

The tilapia production in the country showed an accelerated growth between 1995 and 2000, when it went from 21 thousand tons to 157 thousand tons, and then jumped to 557 thousand tons in 2010 (FAO, 2019; El-Sayed, 2019) and to 967 thousand tons in 2017, accounting for 79% of African production (El-Sayed, 2019). The rapid productivity growth was due to the replacement of extensive practices with the intensive system, accompanied by the prioritization of the aquaculture sector by the government that invested in the introduction of new techniques (El-Sayed, 2013; Shaalan et al., 2018). Since 2015, regional governments have provided workshops and training aimed at instructing tilapia producers on best management practices, an intervention that led to an increase in tilapia production, a greater profitability, and a reduction in the environmental impact in the regions surrounding the facilities (Dickson et al., 2016).

The main producing region is located along the Nile river, in reservoirs present in the lakes of the north coast (FAO, 2020c), but there are also tank cultivation systems installed

mainly in desert areas (FAO, 2018). It is worth mentioning that some tributaries of the Nile river, such as Lake Manzala located in the river delta, are affected by severe pollution from nearby industrial plants. A histological study on Nilotic tilapia reared in the region demonstrated degenerative changes in the seminiferous tubules, in addition to deformations in the ovaries (Mansour et al., 2018). High levels of heavy metals such as aluminum, iron, nickel, and chromium have also been found in gills and muscles of tilapias reared in the Salam canal (Donia et al., 2017), whose waters are drained from the Nile's Damietta distributary (Badawy et al., 2018).

Bacteria associated with human bacteriosis have also been found including *Escherichia coli*, *Streptococcus agalactiae, Streptococcus iniae* and *Aeromonas hydrophila* (Nicholson et al., 2017), demonstrating the urgency of implementing measures to treat the canal and monitor water quality (Donia et al., 2017). The high incidence of infections is a major problem for tilapia farming in Egypt, with viral diseases being the main culprits in tilapia mortality, especially during the summer (Ali et al., 2020).

Another challenge faced by small Egyptian producers is the difficult access to quality feed with an adequate formulation to promote growth and strengthen tilapia immunity (El-Sayed, 2013). For this reason, small producers often associate the farming of tilapia with that of other fish such as carp or mullet (El-Sayed, 2013; FAO, 2018). However, industrialization of farming systems has given good results in the country, which already has a global prominence (El-Sayed, 2013).

2.4. Brazil: favorable natural characteristics

Intensive livestock and poultry farming are the main sources of animal protein in Brazil (Milanez, 2019), which has made large investments in recent years (Schulter and Vieira Filho, 2018). Among the potentialities in the country, tilapia culture emerges as one of the fastest growing activities (Castilho-Barros et al., 2020). The first tilapia species introduced in Brazil in 1952 was Congo tilapia (*Tilapia rendali*); however, its low growth rate resulted in low productivity, which made the first tilapiculture insertion initiative in the country ineffective (Raghiante et al., 2017). Tilapia culture introduction began to take hold in the 1970s, when the *Oreochromis niloticus* and *Oreochromis hornorum* species were simultaneously introduced in the country by the National Department of Works Against Drought (DNOCS) (Schulter and Vieira Filho, 2018).

Among the more than 70 known and cataloged tilapia species, only four species, all belonging to the genus *Oreochromis*, are expressive in the world aquaculture market (Mello et al., 2017). In Brazil, the *Oreochromis niloticus* species is predominant (Raghiante et al., 2017), corresponding to 45% of the fish produced on the continent (de Andrade and de Azevedo, 2018). The country showed rapid growth between 2014 and 2019; at the beginning the production was 200 thousand tons, and by the end of this period the total production achieved 450 thousand tons (FAO, 2019; PEIXE BR, 2019).

Studies have shown that Nile tilapia is the species with the greatest productivity potential in Brazil, due to its a) adaptation to the tropical climate, b) tolerance to intermediate salinity environments (Barroso et al., 2018) as well as low levels of oxygen dissolved in water (de Andrade & de Azevedo, 2018), and c) adaptation to different cultivation systems (Mello et al., 2017). Tilapiculture feeds a national and international market with high demand (Milanez et al., 2019). In Brazil, the export of tilapia alone accounted for 81.35% of all fish from aquaculture, with Mato Grosso do Sul being the main exporting state (PEIXE BR, 2020). According to data from the annual survey of the Brazilian Association of Fish Culture, the top destinations for domestic tilapia production are Japan, China, and the United States (PEIXE BR, 2019).

The domestic consumption of fish in Brazil in 2018 reached an average of 11 kg per capita, indicating that this is a promising market; however, this value is lower than that recommended by the World Health Organization (12 kg) (CNA, 2018).

The favorable characteristics of tilapia culture in Brazil are many water resources, especially river channels (Kubitza, 2015), an adequate climate to support tilapia's metabolic activities (Raghiante et al., 2017) and growing trend of private investments in management technology (Pedroza Filho et al., 2015). The exploitation of this activity also generates socioeconomic benefits in Brazil, since it contributes to reduce the food deficit (Igarashi, 2018), providing cheap and nutritious food to local communities (Nowland et al., 2020), and has a rather low cost of the production stages (Asche et al., 2018).

Tilapia culture allows the implementation of production models based on associativism and cooperativism, another factor closely associated with socioeconomic gains (Schulter and Vieira Filho, 2018). These production models have already been successfully implemented in the country, especially in Santa Catarina state, the fourth largest tilapia producer in Brazil

(PEIXE BR, 2020). This type of production has had a positive impact on producing cities, seen in the improvement of the human development index (Barroso et al., 2018).

Although the growth of Brazilian tilapia culture has been notable in the last decades, much remains to be explored (Schulter and Vieira Filho, 2018). In this sense, it is necessary to face some obstacles, among which the lack of governmental policies to encourage production centers, low producer qualification on proper management, and natural resources preservation are the most urgent (Kubitza et al., 2010). In addition, there is a great deal of bureaucracy for granting water use permits and environmental permits for the construction of fish farms, which makes it difficult to expand facilities (Milanez et al., 2019) and hampers new investments in the sector (Schulter and Vieira Filho, 2018). An important source of expenses associated with the production process is related to the feed purchase, which accounts for 70-80% of production cost, depending on the region and production system employed (Milanez et al., 2019). Another problem is the occurrence of diseases (Wamala et al., 2018), whose incidence increases in proportion to the fish stocking density and depends on the culture system (Raghiante et al., 2017).

3. Main pathogens for tilapia

Bacterial infections affect several sectors of aquaculture (Hamom et al., 2020) and are a major problem for tilapiculture companies, since some bacteria are the pathogens responsible for great production losses around the world (Hassan et al., 2020). The distribution of pathogenic species has a different profile depending on the region (Guerrero-Carbrera, 2020) and the tilapia species (Wanja et al., 2020), and the main bacterial diseases affecting tilapiculture are streptococcosis, francisellosis, aeromonosis, vibriosis and columnariosis (Soto et al., 2016).

3.1. Streptococcosis

Streptococcosis is the main infection responsible for the mortality among tilapias globally, affecting several species including Nile tilapia (Liu et al., 2016) and Red tilapia (Ismail et al., 2016). Outbreaks of this disease are estimated to result in a loss of up to 40 million dollars to the tilapia industry each year (Sun et al., 2016). Between 2009 and 2019, recurrent outbreaks of streptococcosis spread across China, compromising the profitability of the

business, as fish mortality rate ranged between 30 and 90%, mainly affecting farms in the southern region (Ye et al., 2011; Li et al., 2019).

Streptococcus spp. are Gram-positive bacteria (Bueno and Neto, 2019), with coccus shape, that preferentially grow under aerobic conditions, but can tolerate anaerobiosis (Veselá et al., 2019). Under experimental conditions, the optimum temperature for the incubation of *Streptococcus* spp. is between 26 °C (Palang et al., 2020) and 37 °C (Bal et al., 2019). For this reason, outbreaks of contamination occur mainly in the summer, and higher temperatures tend to result in higher mortality rate (Palang et al., 2020). Hu et al. (2017), who compared cultures of *Streptococcus agalactiae* incubated at temperatures of 25 °C and 35 °C, observed that strains grown at higher temperatures exhibited greater pathogenicity, evidenced by an increase in secretion of virulence factors, nucleotides and compounds associated with stress regulation such as oxidized glutathione and glyceraldehyde-3-phosphate. In particular, virulence factors induced an accelerated bacterial growth and increased the adhesion capacity of pathogens, in addition to producing pores in the host cell membrane and damaging the tissues (Palang et al., 2020).

The main etiological agent is the species *Streptococcus iniae*, which is the major cause of the increase in tilapia mortality worldwide (Laith et al., 2019; Saleh et al., 2019; Suhermanto et al., 2019). In addition to damaging tilapia culture, this pathogen also affects other fish species such as Crucian carp (*Carassius auratus*) (Geng et al., 2012) and mammals, including humans (Palang et al., 2020). Several studies (Iregui et al., 2016; Soto et al., 2016; Vásquez-Machado et al., 2019) have shown that infection occurs mainly through the gastrointestinal tract: the pathogen, after passing the stomach and intestinal mucosa, adheres to the gastrointestinal epithelium and, after this stage of infection, its cells can spread to other organs, causing systemic septicemia (Iregui et al., 2016).

Soto et al. (2016), who tested different ways to infect Nilotic tilapia to mimic the natural conditions of disease development, found that the infection proceeded through intramuscular injection, which suggests that lesions in the epidermis may represent an entry route for the pathogen. In culture facilities, the high stocking density, in addition to the aggressive behavior of the fish, can increase the likelihood of skin ulcers development, favoring this type of infection. The most common symptoms of streptococcosis include erratic swimming, exophthalmos, corneal opacity, and skin lesions (Ye et al., 2011). Septicemia and

meningoencephalitis (Soto et al., 2016) are frequently observed, as well as complications that compromise the functioning of liver, kidneys, and spleen (Nicholson et al., 2020).

3.2. Francisellosis

Another disease that stands out in the tilapia culture is francisellosis (Raghiante et al., 2017). Although its epidemiological distribution is more restricted (Bueno and Neto, 2019), the high mortality rates and its ability to persist in the environment (Soto et al., 2015) severely affect the productivity in endemic regions (Bueno and Neto, 2019). Francisellosis is frequently found in the United States, Indonesia (Raghiante et al., 2017), United Kingdom (Assis et al., 2017) and Latin America, with southern Brazil being one of the regions most affected by the outbreaks of this disease (Leal et al., 2014; Facimoto et al., 2019); the first confirmed cases of francisellosis in Brazil date back to 2014, and the mortality rate is around 60% in the country (Facimoto et al., 2019).

The main causative agent belongs to the species *Francisella noatunensis* subsp. *orientalis*, which shows coccus-rod morphology, is Gram-negative, is strictly aerobic and may have facultative intracellular growth (Soto et al.,. 2011, Raghiante et al., 2017). Although its pathogenic mechanisms have not been completely clarified (Assis et al., 2017), it is known that temperatures below 25 °C favor disease outbreaks (Sebastião et al., 2017). For this reason, in Brazil, winter is the main period in which there is an increase in the incidence of disease and mortality, especially among fry and young tilapias (Assis et al., 2017).

Intensive farming conditions, such as high stocking density and poor quality of culture water, can increase susceptibility to the development of francisellosis (Amal et al., 2015; Assis et al., 2017). Transmission can be horizontal, through direct contact of the pathogen present in the water with the animal's skin and through direct contact between infected animals (Bueno and Neto, 2019). Another possibility is vertical transmission between contaminated breeding tilapia and fry or eggs. The entry routes for *F. noatunensis* subsp. *orientalis* are the skin, peritoneum (Fernandez-Alarcon et al., 2019) and gastrointestinal tract, through the consumption of contaminated food (Iregui et al., 2016; Bueno and Neto, 2019).

The disease can manifest itself acutely, presenting symptoms that include anorexia, erratic swimming, anemia, and exophthalmos (Raghiante et al.,2017). The chronic or sub-acute condition is also possible, which involves a milder manifestation of the disease symptoms and results in lower mortality rates (Raghiante et al., 2017; Bueno and Neto, 2019). The

differentiation among clinical conditions depends on the amount of intracellular infiltrates in the central nervous system (Bueno and Neto, 2019). The most frequent complications are related to the appearance of multifocal granulomas containing the pathogen (Fernandez-Alarcon et al., 2019). The main regions affected by granulomas are the spleen, kidneys, liver, and skeletal muscle tissue (Raghiante et al., 2017).

3.3. Aeromonosis

The most common bacterioses in freshwater aquaculture are related to the genus *Aeromonas* spp. (Dong et al., 2017). These pathogens primarily affect freshwater fish (Bueno and Neto, 2019), and a wide variety of bacterial species behave as opportunistic parasites in tilapia, with *Aeromonas hydrophila*, a rod-shaped, Gram-negative (Fernandes et al., 2019), anaerobic facultative and motile bacterium (Rai et al., 2020), being the main agent responsible for the primary transmission of aeromonosis (Dong et al., 2017) and the development of co-infection with Tilapia lake virus (Amal et al., 2018; Salem et al., 2020).

The predisposition to initiate the infection depends on certain environmental stimuli that activate the secretion of virulence factors (Abdel-Tawwab et al., 2018; Farias et al., 2020). Pathogenicity is stimulated by the presence of certain pollutants in the culture water, hypoxia, high stocking density of fish, pre-existing infections in the host and high temperatures (Abdel-Tawwab et al., 2018). It has been reported that the optimum temperature for bacterial growth and disease development is 28 °C (Nicholson et al., 2020). The disease has a high incidence in Asian countries such as Indonesia and Malaysia (Basri et al., 2020), but is also distributed in other tropical regions such as Latin America (Grajales-Hahn, 2018; Espinosa-Chaurand et al., 2019) and African countries, including Egypt (Elsheshtawy et al., 2019).

The mucosal surface is one of the main entry routes for *A. hydrophila* (Farias et al., 2020). Addo et al. (2017a) reported that tilapia infected through intraperitoneal injection showed erratic swimming, multiple foci of hemorrhage and depigmentation as well as skin erosions on the fins. Aeromonosis is known to develop into motile *Aeromonas* septicemia when associated with other secondary infections (Addo et al., 2017a), with potentially fatal consequences due to deep ulcerations in internal organs, necrosis of the cells in liver, brain, kidneys, and blood flow congestion (Pauzi et al., 2020; Hal and Manal, 2020). Faced with highly virulent strains, contaminated fish can die before showing clinical signs, making diagnosis and taking measures to contain the bacterium spread difficult (Pauzi et al., 2020).

3.4. Vibriosis

The term vibriosis refers to a series of diseases that affect a wide variety of aquatic species (Ceballos-Francisco et al., 2020). Pathogens include several species of the genus *Vibrio* spp., including *Vibrio harveyi*, *Vibrio anginolyticus*, *Vibrio cholerae* (Aboyadak et al., 2017) and *Vibrio vulnificus* (Sumithra et al., 2019). Members of this genus are rod-shaped, Gram-negative bacteria (Ceballos-Francisco et al., 2020) provided with flagella that aid in locomotion (Zhu et al., 2013). Although these bacteria are more abundant in saline environments (Novriadi, 2016), the growth of some species is also possible in freshwater (Ceballos-Francisco et al., 2020).

The geographical distribution of vibriosis extends mainly from coastal regions of European countries (Baker-Austin et al., 2018) to Asian countries (Sumithra et al., 2019). Outbreaks are seasonal in nature and occur mainly in summer (Baker-Austin et al., 2018); however, the expression of virulence can even be detected in cold waters at temperatures above 15 °C (Mabrok and Wahdan 2018; Sumithra et al., 2019). Although the mode of transmission and evasion from the host's immune system has not yet been fully clarified, it is known that the virulent bacterium can adhere to the host's skin and penetrate the tissues (Novriadi, 2016). Clinical manifestations include lethargy, damage to fish development, tissue necrosis, malformation, discoloration of scales, and erythema near the oral cavity (Novriadi, 2016; Eissa et al., 2017).

3.5. Columnariosis

Columnariosis is caused by the bacterium *Flavobacterium columnare*, a Gram-negative species, with shape of long, non-flagellated bacilli (Sebastião et al., 2011). The disease has a worldwide distribution and is highly infectious (Bueno and Neto, 2019) with higher incidence in tropical countries, being a recurring problem for Brazilian tilapia farming (Sebastião et al., 2017). Resistance to infection depends on the stage of tilapia development, with fry and young fish being more susceptible to the most severe symptoms of the disease (Wonmongkol et al., 2018; Bueno and Neto, 2019). The occurrence of columnariosis is more frequent in summer, as temperatures above 20 °C favor the growth of the pathogen (Sebastião et al., 2011), however the optimum temperature for the development of virulence is between 28 and 30 °C (Bueno and Neto, 2019).

Other conditions, such as low dissolved oxygen concentration, high stocking density and high ammonia concentration in the aquatic environment, stimulate the secretion of virulence factors, facilitating the infection (Sebastião et al., 2011). The disease can be transmitted orally through the gastrointestinal tract and the contact of pathogenic bacteria with pre-existing lesions (Leal et al., 2010; Bueno and Neto, 2019). Initial symptoms, including lethargy, erratic swimming, and accelerated opercular movements, are nonspecific and can be confused with clinical manifestations also shown by other bacterioses (Bueno and Neto, 2019). As the infection progresses, more features related to columnariosis are observed, including corrosion of the dorsal and caudal fins, presence of yellowish or gray skin erosions close to a reddish hyperemic zone, and tissue necrosis in the cranial and branchial region (Sebastião et al., 2011).

4. Antibiotics use in disease control

Measures to control bacterial diseases are crucial for maintaining tilapia culture productivity, and antibiotics are the most common tools to treat these diseases. However, antibiotics are not always used with technical monitoring, and large doses are often used without even identifying the pathogen responsible for the infection (Khoi et al., 2008). The absence of accurate diagnoses for bacteriosis and a surveillance system to ascertain the need for antibiotics application is a reality in many countries (FAO, 2016; Brunton et al., 2019). Even in regions where there is regulation and inspection, these measures are mainly applied to systems whose production is destined for export (Khoi et al., 2008; Brunton et al., 2019).

The indiscriminate use of antibiotics can cause serious problems for tilapia culture and for human health. The development of resistance to antimicrobials, for example, leads to ineffectiveness in disease control by favoring the selection of resistant strains (Mannan et al., 2020). The consequences of this selection can be compounded by the fact that bacteria have mechanisms that allow for the exchange of genetic material (Singh et al., 2017), which can result in the possible transfer of resistance genes to antimicrobial sensitive strains and then in the inefficiency of currently used treatments (Gastalho et al., 2014; Islam and Yuan, 2019). In this context, several studies have evaluated the incidence of antibiotic resistant microorganisms in tilapia culture. The harms resulting from indiscriminate use of these drugs can also be associated with ecological losses (Limbu et al., 2018).

It is estimated that more than 80% of antibiotics used in aquaculture remain in the aquatic environment for decades after their use (Makled et al., 2019); during the exposure period, they can cause a reduction in the population of phytoplankton and green algae, organisms responsible for the primary production of organic matter that are crucial for the biochemical cycle maintenance in aquatic ecosystems (Song et al., 2016). To minimize the impact of the use of these compounds, the World Health Organization (WHO), World Organization for Animal Health (OIE) and Food and Agriculture Organization (FAO) established global guidelines for the use of antibiotics in animals and the monitoring of resistant strains. This information has helped governments and their regulatory agencies make decisions (FAO/ WHO, 2008).

Table 1 provides an overview of some tilapia pathogens that show resistance to antibiotics used in farming systems. From the analysis of the data gathered in the table, it is possible to notice that some of the main bacteria that cause diseases in tilapia have developed resistance to most of the drugs currently used to treat bacteriosis. This is the case of *S. agalactiae*, as most of the antibiotics to which its resistance has been detected are the most used for treating infections (Lulijwa et al., 2020). In addition, most studies indicate that persistence in the use of prohibited drugs or use without a technician's prescription is a problem of great relevance in the breeding systems, especially in China.

In addition to therapeutic use, many aquaculture farmers use these drugs as a preventive measure, as they understand that administration of prophylactically medications reduces mortality and accelerates animal development (Gaunt et al., 2011). The growth improvement may be an indirect result of pathogens control; however, the indiscriminate use of antimicrobials has risks. Therefore, new promising alternatives to antibiotics such as probiotics must be investigated, as they demonstrated ability to combat pathogenic microorganisms and have great metabolic efficiency (Gaskins et al., 2002).

5. Benefits of probiotics, prebiotics and bacteriocins in tilapiculture

5.1. Probiotics in tilapia culture

Due to the risks that excessive exposure to antibiotics can produce, both on the environment and on human health (Foysal et al., 2020), the use of probiotics as growth promoters has been evaluated to replace antibiotics (Kuebutornye et al., 2020). Probiotics are

living microorganisms capable of producing beneficial effects for the host if administered in adequate amounts (FAO / WHO, 2008; Hasslöf and Stecksén-Blics, 2020). They can regulate the intestinal microbiota by competing with enteropathogens for nutrients and space (Umu et al., 2017), but not with the bacteria that constitute the normal host microbiota (Musa et al., 2009).

Probiotics can be used in tilapia culture, as they act as growth promoters and potentially produce antimicrobial peptides (Kuebutornye et al., 2020). In addition, they stimulate the immune response and resistance of tilapia to pathogens; these two functions are very important since the confinement conditions present in breeding systems can act as stressors and contribute to immunosuppression (El-Sayed, 2019).

Improvement in animal growth can occur through several different mechanisms (Begum et al., 2017). These microorganisms can stimulate the appetite and optimize the host nutrition by competing with bacteria responsible for amino acid deamination, which reduce nitrogen uptake (McDonald et al., 2011). In addition, probiotics secrete fatty acids, essential amino acids, biotin and enzymes capable of cleaving carbohydrates, lipids and proteins into smaller fragments in the animal digestive tract, thus facilitating their absorption (Wiëers et al., 2020). Endogenous enzymes secreted by tilapia are in fact considered insufficient to guarantee a satisfactory use of feed, therefore the enzymes of probiotics improve nutrients absorption (Banerjee et al., 2017).

Table 2 provides a list of recent studies that reported enzymes secretion by probiotics used in tilapia. Although few, the most recent studies that aimed to assess the impact of these enzymes on tilapia health were selected, which highlighted that probiotics are important sources of exogenous enzymes, mainly digestive ones. In addition, it was observed that such enzymes played a beneficial role in promoting fish health, having as main clinical impacts the improvement in the immune response, increased resistance to diseases and faster weight gain. It was noted, in particular, that after the administration of probiotics in the tilapia diet there was an increase in the nutritional efficiency of the feed, since most of the main secreted enzymes are related to the digestion of complex nutrients. This suggests an improvement in development and response to diseases, as well-fed animals are known to have less chance of disease and a higher growth rate.

Most of the selected studies used concentrations in the range between 10^7 and 10^8 CFU/g with an administration period of 7 to 60 days. Growth promotion was the main effect observed,

especially in studies with shorter administration periods, indicating that the benefits can be seen in the first few days. Dawood et al. (2020a), using concentrations of the probiotic *Aspergillus oryzae* close to the range normally reported in studies on tilapia (~ 10^8 CFU), detected at the end of a long dosing period (60 days) important enzymes, including superoxide dismutase and catalase, possibly responsible for improvements in the immune response and protection against the tested pathogen (*Aeromonas hydrophila*). The same pattern was observed by Gobi et al. (2018), who also found similar benefits when increasing the administration period. On the other hand, Selim and Reda (2015) observed only lysozyme production when administering the probiotic *Bacillus amyloliquefaciens* for 8 weeks and did not report any growth promotion, because, despite the prolonged use, the concentration of the probiotic was significantly lower than the standard dosage.

Some strains of *Saccharomyces cerevisiae* are added to the tilapia diet (Abdel-Aziz et al., 2020) for their ability to bring improvements in microbiota regulation (Navarrete and Tóvar-Ramirez, 2014) through the production of polyamines involved in metabolite biosynthesis (Zorriehzahra et al., 2016; Madibana and Mlambo, 2019). Results of previous research have revealed that the intestinal tract morphology may be affected by the composition of the host's microbiota (Welker and Lim, 2011). The greater distribution of commensal bacteria increases the nutrient absorption capacity and leads to the development and maturation of mucins and epithelial cells (Hamdan et al., 2016).

To assess the safety of a probiotic candidate before using it in the food industry, FAO and WHO recommend evaluating criteria such as antibiotics sensitivity, absence of toxin production and hemolytic activity (Byakika et al., 2019). Another aspect that must be considered when selecting a probiotic is its ability to colonize the host's intestinal tract, resisting stomach acids, bile salts and enzymes (Liao and Nyachoti, 2017). The efficiency of a probiotic also depends on its ability to attach to the gastrointestinal tract and its antagonism against pathogenic species (Cho et al., 2011). Since many pathogenic bacteria need to adhere to the epithelium to cause harmful effects to the host, the insertion of probiotic species into the microbiota implies their exclusion through competition for receptor sites (Chauhan and Singh, 2019), thereby reducing susceptibility to infections (Yirga, 2015).

Some probiotic bacteria can increase their ability to adhere by synthesizing glycol conjugates on the gastrointestinal tract wall that serve as receptors for bacteria fixation (Wegner et al., 2018; Liao and Nyachoti, 2017). The adhesion of probiotics is favored over that of

pathogens because the host's immune system recognizes probiotic antigens as harmless and does not develop an inflammation mechanism (Oriá and Brito, 2016). Furthermore, probiotics can induce the regulation of mucins and proteins belonging to tight junctions, such as claudins, occludines and ZO-1, preventing the fixation of harmful bacteria (Yirga, 2015). Nwanna (2015) reported that probiotics belonging to the genus *Lactobacillus* are able to prevent the adhesion of *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas aeruginosa* strains in host intestinal cells (Chauhan and Singh, 2019).

The competition promoted by probiotics also extends to nutrients necessary for pathogens metabolism (Zorriehzahra et al., 2016). Some probiotics can produce siderophores, iron chelating agents that can capture medium metal ions, reducing their availability to pathogenic bacteria (Chauhan and Singh, 2019). Probiotics can even increase tilapia resistance to disease development by providing improvement in water quality (Kuebutornye et al., 2019).

For instance, Gram-positive bacteria, mainly belonging to the genus *Bacillus* spp., are recognized for their ability to supply nutrients to the aquatic ecosystem by degrading organic matter with high efficiency (Farizky et al., 2020). Other parameters such as salinity, pH and ammonia concentration in water can also be modulated using probiotics (Elsabagh et al., 2018).

In addition to reducing the chances of contracting diseases, probiotics induce an improvement in the tilapia immune response, resulting in increased survival from infection (Zorriehzahra et al., 2016; Chauhan and Singh, 2019). Some studies on the effect of probiotics on the expression of genes related to inflammatory pathway activity and the regulation of the levels of immunological markers (Thomas and Versalovic, 2010; Suez et al., 2019) revealed that probiotic bacteria significantly influence gene expression; even when the probiotic is dead, its secreted metabolites can produce immunomodulatory effects (Oelschlaeger, 2010).

Gram-positive probiotics mainly stimulate the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, tumor necrosis factor α (TNF-α), gamma interferon (IFN-γ) and anti-inflammatory cytokines such as IL-10, hence increasing the phagocytic activity of leukocytes, the levels of antibodies and the activity of enzymes associated with the innate immune system, while Gram-negative probiotics mainly stimulate cell immunity to the detriment of humoral response, associated with serum immunity and mucus production (Zorriehzahra et al., 2016).

Table 3 lists the main information about recent investigation on the effects of probiotics on tilapia. The probiotics most frequently used in tilapiculture belong to the genus *Bacillus*

(Opiyo et al., 2019), followed, to a lesser extent, by LABs (Hoseinifar et al., 2018; Dias et al., 2020). The recurrent use of *Bacillus* spp. can be associated with the sporulating capacity of these bacteria, which facilitates the handling and application of probiotics, as the spores tend to pass easily through the stomach. Furthermore, sporulation provides greater resistance to harmful storage conditions, such as drying, exposure to heat and UV radiation, thus increasing their viability (Liao and Nyachoti, 2017). On the other hand, LABs are Gram-positive, catalase and oxidase negative cocci or bacilli (Ismail et al., 2018) that do not form spores (da Silva et al., 2020). Due to the synthesis of lactic acid, these bacteria secrete metabolites such as acidoline, acidophylline, lactocidine and lactonin that contribute to the reduction of medium pH, hence affecting hydrogen peroxide metabolism in enteropathogens (Ewing, 2008), in addition to producing molecules with antagonistic effects on other bacteria such as bacteriocins (see section 5.3) (Pacheco et al., 2018). The concentration of probiotics applied was quite variable, being the focus of some studies. In particular, Xia et al. (2019) concluded that the concentration of 10^8 CFU/mL brought more benefits to the host compared to the other diets with lower probiotic levels.

Other important results refer to the promotion of growth, weight gain, improvement of feed conversion ratio, and immune response. Something in common among these studies, which could explain such improvements in the health tilapia parameters, was the increase in the production of digestive enzymes and metabolites involved in the regulation of inflammatory responses. Most studies maintained a dosage of 10^8 CFU/mg and an application period of two months; however, Chen et al. (2019), Abarike et al. (2018) and Gobi et al. (2018) managed to obtain a series of benefits (growth promotion and improvement in immunological parameters) by administering lower concentrations (10^5 and 10^7 CFU/mg).

Chu et al. (2020) found that one week of *Enterococcus avium* administration was sufficient to increase the survival rate of tilapia due to the production of exogenous enzymes; however, results on growth promotion were not reported in that study. Xia et al. (2019) also obtained positive results of resistance to the development of diseases after 15 days of *Lactococcus lactis* administration. However, only the standard concentration of 10^8 CFU/mg resulted in these benefits, while the diet with 10^4 CFU/mg only impacted the regulation of the tilapia microbiota.

The use of *Bacillus subtilis* alone was found not to be effective in promoting the growth of Nilotic tilapia in any of the selected studies. Adeoye et al. (2016) found growth promotion

and improvement in feed conversion when administering *B. subtilis* combined with other *Bacillus* species; this finding corroborates with evidence reported in the literature that multiple probiotics may be more efficient than a single strain, but the mechanisms promoting these synergistic effects have not yet been fully elucidated (McFarland, 2020). Xia et al. (2020) reported that the combined application of *B. subtilis* and *Bacillus cereus* brought benefits in feed conversion, while not having a considerable impact on growth rate.

According to Han et al. (2015) and Gobi et al. (2018), the use of *Bacillus licheniformis* improves the absorption of nutrients and production of exogenous enzymes, thereby increasing both growth rate and feed conversion. While the former authors used a concentration of 4.4×10^6 CFU/g for 70 days, the latter used a concentration range of 10^5 to 10^7 CFU/g over a period of only 28 days, obtaining similar results. Reda and Selim (2015) reported that *Bacillus amyloliquefaciens* did not impact the growth of tilapia after 30 days of administration, but growth performance and weight gain increased even at sub-standard concentrations after 2 months.

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Foysal et al. (2020) and Hamdan et al. (2016) conducted experiments with *Lactobacillus plantarum* strains administered in concentrations (10^9 to 10^{10} CFU/g) higher than the standard for more than one month. Both research-groups reported that the immune response to the infection was amplified through the stimulus in cytokines production and intestinal flora regulation, expanding commensal microorganisms' diversity and reducing potentially pathogenic bacterial populations such as *Vibrio* spp. (Lauzon et al., 2010). Guimarães et al. (2019) found no improvement in the immune response or expression of genes responsible for the production of tumor necrosis factors when using a combination of *Lb. plantarum* and *B. subtilis*; however, it can be inferred that this discrepant result was due to lower concentration of *Lb. plantarum* (1.51×10^6 CFU/g) and shorter research time compared to other studies.

Several authors have reported increased rate of tilapia survival to different pathogens. Chu et al. (2020) found that administration of 10^7 CFU/g of *E. avium* for 7 days was sufficient to increase the secretion of protease, amylase and lipase and the survival rate. Samson et al. (2020) also observed higher survival of tilapia and digestive enzymes production, using a combination of different species of *Bacillus* at concentrations of 10^7 CFU/g and 10^8 CFU/g for a period of two weeks. Dawood et al. (2020a), Tan et al. (2019) and Addo et al. (2017a) also

reported similar results administering probiotics at concentration in the range 10^6-10^8 CFU/g, even though the treatment was prolonged for about 2 months in these studies.

Guimarães et al. (2019), Van Doan et al. (2018) and Ayyat et al. (2014) included in their assays *Lb. plantarum* alone or in association with other probiotics at concentrations equal to or below the standard dosage and concluded that there was no variation in the survival rate compared to the control groups. In disagreement with these results, Foysal et al. (2020) proved that the use of *Lb. plantarum* increased the survival rate; however, these authors employed 1.02×10^9 CFU/mL/kg, which suggests that *Lb. plantarum* must be used at high concentration to produce relevant effects on the survival of infected fish. To confirm this trend, further studies should be carried out following these application conditions.

Abarike et al. (2018) observed that administration of 10^7 CFU/g *Bacillus* spp. for one month reduced the incidence of mortality among fish affected by *S. agalactiae*. This result is consistent with those of Addo et al. (2017a,b), who observed a reduction in mortality in a group of tilapia treated with *B. subtilis*. Gobi et al. (2018) and Han et al. (2015) found that the administration of *B. licheniformis* at concentrations below the standard dosage culminated in resistance to disease development, due to the increase in the lysozyme level. These results are in line with previous evidence that strains of *Bacillus* spp. can stimulate humoral parameters associated with the response of innate immune system, the main defense mechanism of fish against infections (Han et al. 2015). Xia et al. (2020), Abarike et al. (2018), Addo et al. (2017b) and Selim and Reda (2015) reported production of lysozyme following the use of probiotics belonging to the genus *Bacillus*. These studies shared concentrations between 10^6 and 10^8 CFU/g and administration periods of no more than two months.

The study by Guimarães et al. (2019) revealed that the inclusion of *B. subtilis* and *Lb. plantarum* mixture in Nile tilapia diet during the sexual reversal phase did not result in significant differences in growth, survival rate and expression of the TNF-α and HSP-70 genes, while it caused changes in fish gut microbiota. Tachibana et al. (2020) reported the beneficial effect of *Enterococcus faecium* as a promoter of tilapia growth and its potential to boost the immune system if continuously administered over a period of 7 days.

Recent studies have evaluated the effectiveness of the probiotic *Lb. plantarum* as growth promoter of fish species, including Nile tilapia (Aboul-El-Atta et al., 2019; Hoseinifar et al., 2018; Van Doan et al., 2018). Its use is also especially recommended for prevention and control of bacterial diseases, as tilapia treated with this probiotic has shown a better immune response

to infections (Hoseinifar et al., 2018). Other strains with probiotic characteristics that have been considered promising candidates in this respect belong to the species *Lactococcus lactis* (Zhou et al., 2010; Kaktcham et al., 2018) and *Pediococcus acidilactici* (Standen et al., 2013), which, in addition to offering benefits to tilapia immune system, are bacteriocinogenic (Kaktcham et al., 2018; Sudarsanan and Thangappan, 2017).

To optimize the effects of probiotics, there are other parameters that must be considered when assessing their applicability, such as introduction method, animal age, time of use, dosage, and application frequency (Wang et al., 2019; Van Hai, 2015; Welker and Lim, 2011). For instance, the dosage of probiotics used in tilapia culture is not the same when compared to other aquatic organisms (Welker and Lim, 2011). Studies claim that the ideal dosage for tilapia, between 10^5 and 10^9 CFU/mL, is higher than the value normally used for other fish species (10^5 CFU/mL) (Van Hai, 2015).

Probiotics are often used to feed tilapia in the form of pellets, flours, granules, or flakes that are easily incorporated into the animal feed (Van Hai, 2015). Another possibility is the administration made directly in the culture water; however, recent studies have shown that this method has limited action when compared to the feeding application method, presenting less effectiveness in stimulating growth (; Padmavathi et al., 2012; Sutthi et al., 2018; Wang et al., 2019).

To increase the viability of probiotics included in diet, encapsulation methods in matrices that do not have nutritional value for fish, such as calcium alginate, can be used (Welker and Lim, 2011; Pinpimai et al., 2015). Bioencapsulation is an alternative that allows probiotic survival and adhesion optimization (Van Hai, 2015).

As previously seen, another way to increase probiotic efficiency is the use of a mixture of probiotics. Some studies have shown that the use of this technique tends to produce better effects than single strains (Welker and Lim, 2011; Standen et al., 2016). General information about time and frequency of probiotic use is still scarce (Welker and Lim, 2011; Dias et al., 2020), so it is recommended to investigate these variables in future studies, as probiotics are believed to have an ideal period of use that can magnify the effects on the host (Van Hai, 2015).

5.2. Probiotics in combination with probiotics (synbiotics)

It was also found that the association of probiotics with prebiotics in the diet can also optimize growth promotion and immunological parameters (Van Hai, 2015). Prebiotics are

substrates that confer benefits to the health of the host, as they favor the selective growth of microorganisms, aiding in the modulation of the intestinal microbiota. In addition, they can secret substances that can reduce the intestinal pH stimulating the absorption of minerals (Tachibana et al., 2020) and the release of microbial metabolites, such as short chain fatty acids including butyrate (Ballan et al., 2020). When there is evidence that the combination of probiotics and prebiotics produces more efficient effects on the health of the host, these supplements are called synbiotics (Swanson et al., 2020).

Cavalcante et al. (2020) found that the application of the synbiotic consisting of DBA® (*Bifidobacterium* spp., *Lactobacillus acidophilus* and *Enterococcus faecium*) and MOS (mannan oligosaccharides) promoted a relative protection index against infection of Nile tilapia by *A. hydrophila* of 40%, while Dawood et al. (2020) reported that the *Aspergillus oryzae* plus β-glycan synbiotic improved growth, production of antioxidants and immunomodulation in the same fish. Addo et al. (2017a,b) opted for a probiotic application strategy associated with the prebiotic Previda®. Whereas the performance in fish growth was not significantly altered by *Bacillus subtilis* administration compared to the control group, the association of the prebiotic Previda® with this probiotic drastically reduced fish mortality (Incorporar referencia¡¡¡¡).

5.3. Bacteriocins in tilapiculture

Bacteriocins are bacterial ribosomally-synthesized peptides (Yang et al., 2014) that have bactericidal or bacteriostatic activity against strains phylogenetically close or distant from the bacteriocinogenic strain (Yang et al., 2014), which instead possesses an immune mechanism (Cotter et al., 2013). The mode of action of most bacteriocins is based on membrane permeabilization (Ogaki et al., 2015), induced by the formation of pores deriving from the interaction of bacteriocins with anionic lipids (Yang et al., 2014), which affects the transport of amino acids as well as the dissipation of the proton motive force necessary for ATP synthesis (Ogaki et al., 2015). Therefore, bacteriocins are mainly active against Gram-positive bacteria that have a higher proportion of anionic lipids in the composition of their membrane structures, whereas to inhibit Gram-negative species bacteriocins must be able to cross the wall outer membrane (Yang et al., 2014).

LAB bacteriocins are often used in the food industry as food additives, as they exhibit potential against pathogenic bacteria (Ogaki et al., 2015). Since bacteriocins are present in foods that contain bacteriocinogenic probiotics and there is no evidence of adverse effects on humans

(Liong, 2008), they are excellent candidates for use in different segments of tilapia culture. Moreover, since the mechanisms associated with the acquisition of resistance to these compounds are different from those of antibiotics (Cunha et al., 2006), they may be used to fight infections caused by bacteria resistant to antibiotic action that may be sensitive to bacteriocins. It is noteworthy that the use of a single bacteriocin is not as efficient as the combined use of a variety of bacteriocins; therefore, the use of bacteriocinogenic probiotics may allow better prevention and containment of diseases than the treatment based on simple bacteriocins (Yang et al., 2014).

Considering these perspectives, it is believed that, although LABs are not the predominant probiotics used in tilapia culture, there is great interest in strains that can be incorporated into this activity (Standen et al., 2013), because the antimicrobial potential of these microorganisms against pathogens and their safety are reported in the literature (Kuebutornye et al., 2020).

Table 4 summarizes the results of *in vitro* and *in vivo* studies on the production of bacteriocins or not yet fully characterized bacteriocin-like inhibitory substances (BLIS) from probiotics. In general, all the bacteriocins/BLIS considered in the selected studies contributed to the inhibition of pathogenic strains, however the details about the characterization and properties of these substances were variable, depending on the focus of each study.

Assessing the antibacterial effect of bacteriocins/BLIS in *in vivo* studies tends to be more difficult. It is known that the many benefits provided by supplementation of probiotics lead to improvement in several health parameters, including the host's resistance to disease. Therefore, it is difficult to determine which of these effects come exclusively from bacteriocins. Abdelfatah and Mahboub (2018) reported that the protection against *Staphylococcus aureus* is due not only to the action of BLIS, but also to other effects exerted by the probiotic *Lactococcus garvieae*. In *in vitro* studies, however, it is necessary to distinguish whether the bactericidal and bacteriostatic effects are due to protein compounds such as bacteriocins or to organic acids, hydrogen peroxide or other metabolites secreted by bacteria. Rahman et al. (2018) and Etyemez and Balcazar (2016) stated that the inhibitory action against pathogens is probably due to the action of bacteriocins, because treatments with proteinases resulted in the loss of the antibacterial effect.

Abdelfatah & Mahaboub et al. (2018) and Loh et al. (2017) found that the concentrations of BLISs were low, producing moderate antibacterial effects. These results are consistent with

the fact that bacteriocins are released at low levels depending on environmental stimuli. Among the studies taken into account, the only bacteriocin detected was nisin, which reveals that studies associated with the identification of bacteriocins used in tilapiculture are scarce.

It is important to note that these microorganisms must have safety and efficiency evaluated for each animal model, since it cannot be assumed that an effective probiotic for other aquatic animals is also effective for tilapia. This is due to the metabolic diversity of these organisms and the fact that bacteria considered pathogenic for some fish species may not be for others (Van Hai, 2015).

6. Conclusion

Tilapia breeding has grown steadily in recent years, as has its global economic impact. Losses caused by bacterial infections have been observed in several countries, as well as cases of inefficiency of some antibiotics due to resistance development in bacteria responsible for the infection. Biotechnological methods, such as the use of probiotics and bacteriocins, have been used successfully, although they cannot fully replace the use of antibiotics. To expand probiotic use in tilapiculture, it is necessary to further investigate current microorganisms used and candidates as probiotics as well as to evaluate their modes of action and proper conditions of application to offer better results. Further research aimed at identifying and attesting safety of bacteriocins and bacteriocin-like inhibitory substances can represent a significant advance in the productivity and disease control of tilapia breeding in the coming years.

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Caption of Figures and Tables

Figure 1. Effect on the intestinal microbiota and immunological parameters of fish after probiotic bacteria use. (1) When probiotics reach the intestine, they start competing for space and nutrients. (2) They produce vitamins and bacteriocins, which inhibit the growth of pathogens and produce (3) digestive enzymes, which improve the host nutrition. (4, 5) Due to their antagonistic effect, they may be associated with Microbe-associated Molecular Pattern (MAMPs) by Pattern Recognition Receptors (PRRs) and Toll-like Receptors (TLRs), which lead to the activation of immune system cells . T cells produce cytokines, B cells produce antibodies and they active phagocytes responsible for neutralizing and destroying pathogens**.**

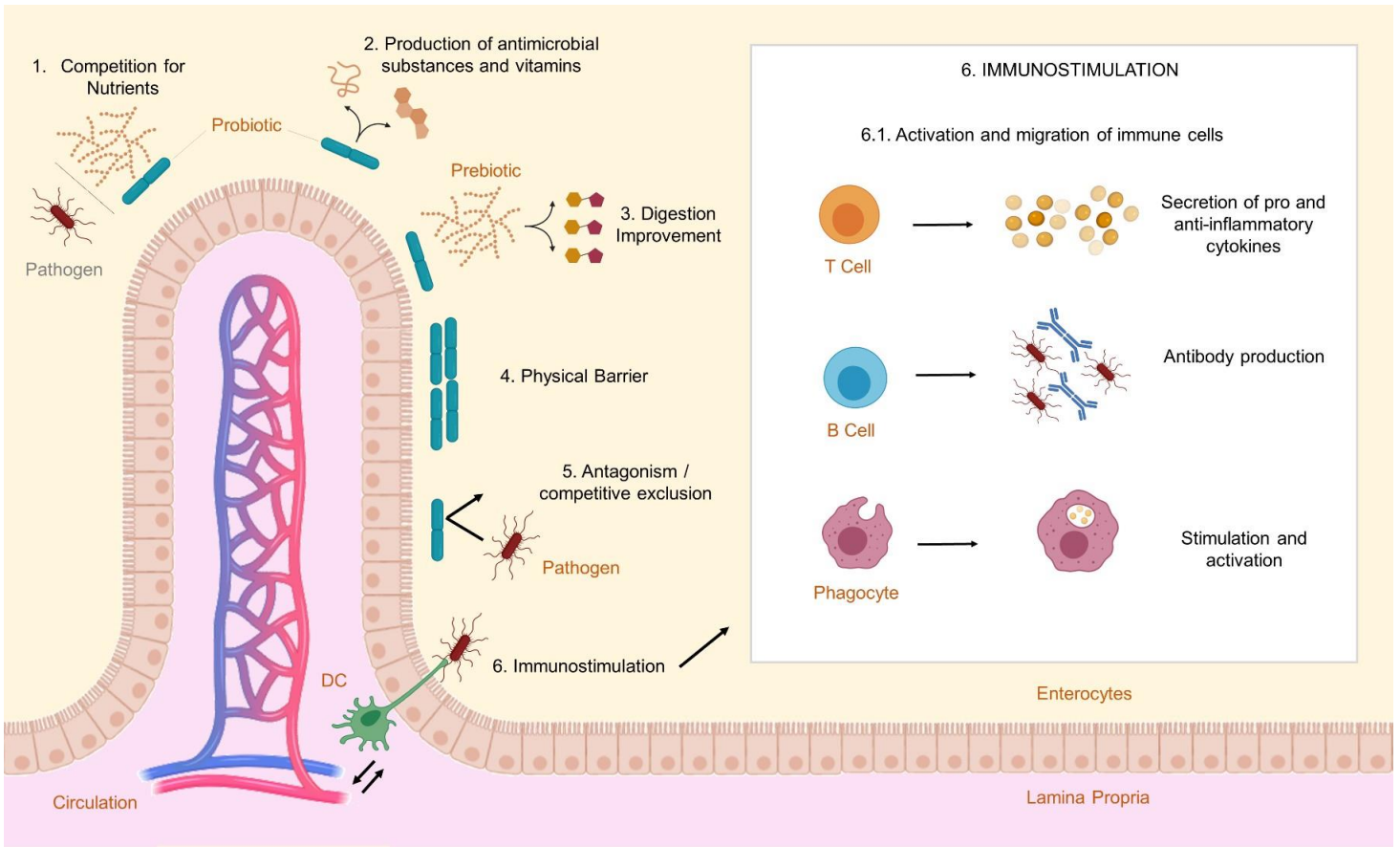

Table 1. Tilapia pathogens that showed resistance to antibiotics and other antimicrobials used in farming systems.

Resistant Pathogen	**Antibiotic**	**Tilapia origin**	**Reference**	**Most common antibiotics detected in the country**	**Reference**
Pseudomonas aeruginosa	Ampicillin, sulfamethoxazole /trimethoprim, tetracycline and nalidixic acid	Giza (Egypt)	Osman et al., 2019	Ciprofloxacin and florfenicol	Lulijwa et al., 2020
Klebsiella pneumoniae, *Escherichia coli*, *Proteus mirabilis*	Penicillin and ampicillin	Bangkok (Thailand)	Thongkao and Sudjaroen, 2019	Enrofloxacin, norfloxacin, amoxicillin, oxolinic acid, penicillin, florfenicol, tetracycline, oxytetracycline, sulphadiazine, trimethoprim, ormetoprim, sulfadiazine + trimethoprim, sulfadimethoxine + trimethoprim, sulfaguanidine	FAO, 2017a
Vibrio spp.	Erythromycin and chloramphenicol	Sri Tujuh (Malaysia)	Hamdan et al., 2018	Oxolinic acid, virginiamycin, chloramphenicol and sulphonamides, tetracyclines, nitrofurans*	FAO, 2017a; Lulijwa et al., 2020
Enterococcus spp.	Tetracycline	Chennai (India)	Arumugam et al., 2017	Erythromycin, chloramphenicol, sulphadiazine, sulfadimethoxine, sulfamethazine, sulphapyridine, sulphamethoxypyridazine, sulphadoxine,	Lulijwa et al., 2020

				sulfamethoxazole, sulphanilamide, sulphathiazole	
Aeromonas hydrophila	Tetracycline, sulfathiazole	Solteira Island (Brazil)	Monteiro et al., 2016	Florfenicol, tetracycline, oxytetracycline and enrofloxacin**	Lulijwa et al., 2020
Streptococcus agalactiae	Oxytetracyclines, trimethoprim, oxolinic acid, gentamicin, and sulfamethoxazole	Nakhon Si Thammarat (Thailand)	Dangwethnangam et al., 2016	Enrofloxacin, norfloxacin, amoxicillin, oxolinic acid, penicillin, florfenicol, tetracycline, oxytetracycline, sulphadiazine, trimethoprim, ormetoprim, sulfadiazine + trimethoprim, sulfadimethoxine + trimethoprim, sulfaguanidine	Lulijwa et al., 2020
Acinetobacter spp.	Sulfamethoxazol, tetracycline	Tianjin (China)	Gao et al., 2012	Neomycin sulphate, doxycycline hydrochloride, thiamphenicol, florfenicol, sulfadiazine, sulfamethoxazole + trimethoprim, sodium sulfamonomethoxine, enrofloxacin, flumequine, oxolinic acid, oxytetracycline, ciprofloxacin, norfloxacin, ofloxacin, amoxicillin, cephalexin, cefradine, cefotaxime, erythromycin, gentamicin S, neomycin,	Lulijwa et al., 2020; FAO, 2017b

				tetracycline, lycomicin, sulfamethoxazole***		

*According to Lulijwa et al. (2020) report, there were no updated data showing specifically which drugs are used in farming systems in Malaysia. The FAO survey (2017a) maily registered most used classes of antibiotics.

** Although enrofloxacin is not allowed in aquaculture in Brazil (Guidi et al., 2018), some studies (Lulijwa et al., 2020) have reported this antibiotic in fish samples.

*** According to FAO (2017b), only 13 antibiotics are allowed in Chinese aquaculture; however, Lulijwa et al. (2020) reported that 33 different drugs were detected in farming systems, some of the most recurrent of which are listed in this table.

Table 2. Summary of enzymes produced by probiotics and health impacts in studies with tilapia.

Tilapia specie	**Probiotic**	**Concentration**	**Duration**	**Secreted enzyme**	**Results**	**Reference**
Oreochromis niloticus	*Aspergillus oryzae*	10^6 and 10^8 CFU/g	60 days	PR, AP, LY, SD and CA (Results obtained under hypoxia)	IR, AA, PP, FCR, GR ↑; BC, SR, AN←; CH, PC, ROS↓	Dawood et al., 2020a
Oreochromis niloticus	*Rummeliibacillus stabekisii*	10^7 CFU/g	7 days	PR, CE, AM and XY	GR, FE, WG ↑	Tan et al., 2019
Oreochromis spp.	*Lactobacillus plantarum, Bacillus velezensis*	10^8 CFU/g- *Lb. plantarum* 10^7 CFU/g- *B. velezensis*	15 and 30 days	PE and LY	IR, GR, DR ↑	Van Doan et al., 2018

Oreochromis niloticus	*Bacillus* spp.	10^7 CFU/g	14 days	LY, SD, CA, MPO and AP	WG, GR, IR, DR, FCR, PP↑	Abarike et al., 2018
Oreochromis niloticus	*Lactobacillus plantarum* (KC426951)	10^5 and 10^7 CFU/g	14 and 28 days	ALP, MPO and LY	WG, GR, IR, DR, FCR, PP, ROS↑	Gobi et al., 2018
Oreochromis niloticus	*Bacillus amyloliquefaciens*	10^4 and 10^6 CFU/g	56 days	LY	IR, DR, PP ↑	Selim and Reda, 2015
Oreochromis niloticus	*Bacillus subtilis* V1TNJ1	Not specified	Not specified	PR	PI ↑	Efendi, 2014

Results: Significant change ←; No significant change →; Increase/Growth ↑; Decrease/Reduction ↓.

Parameters evaluated: Antioxidants activity (AA), Anti-protease activity (AP), Bacterial community (BC), Cholesterol (CH), Disease resistance (DR), Feed conversion ratio (FCR), Feed efficiency (FE), Growth rate (GR), Immune response (IR), Plasma cortisol (PC), Plasma glucose (PG), Protection against pathogen (PP), Proteolysis index (PI), Reactive oxygen species (ROS), Survival rate (SR), Weight gain (WG).

Enzymes: Amylase (AM), Catalase (CA), Cellulase (CE), Lysozyme (LY), Myeloperoxidase (MPO), Peroxidase (PE), Protease (PR), Superoxide dismutase (SD), Xylanase (XY).

Table 3. Summary of probiotic effects against some pathogens in tests with tilapia.

Tilapia species	Probiotic	Concentration	Pathogen	Duration	Results	Reference
Oreochromis niloticus	*Enterococcus faecium*	10^{10} CFU/g	*Aeromonas hydrophila*	84 days (CON); 7 days (P7); 14 days (P14)	SR, HE, HM, PG, PC, MO (regardless the period) →; PP (CON)↑; GR, WG (P7) ↑; RB (P14) ←	Tachibana et al., 2020
Oreochromis niloticus	*Enterococcus avium*	10^7, 10^8, 10^9 and 10^{10} CFU/g	*Streptococcus agalactiae*	7, 14 and 21 days	SR, AM, PR, LA ↑ (10^7 CFU/g during 7 days)	Chu et al., 2020
Oreochromis niloticus	*Lactobacillus plantarum*	1.02×10^9 CFU/mL/kg	*Enterococcus faecalis*	56 days	GR →; IR (innate), DR, IG (cytokines), SR↑; MO↓; BC←	Foysal et al., 2020
Oreochromis niloticus	*Bacillus spp.*	10^7 and 10^8 CFU/g	*Aeromonas hydrophila*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *Enterococcus faecalis and Streptococcus agalactiae*	14 days	SR↑ DE←	Samson et al., 2020

Oreochromis niloticus	*Aspergillus oryzae*	10^6 and 10^8 CFU/g	*Aeromonas hydrophila*	60 days	FCR, GR, GLx, IR (immunoglobulin M), SR, LY, PP, TP, PA, CA↑; AN←; PG, CH↓	Dawood et al., 2020a
Hybrid tilapia *(Oreochromis niloticus x Oreochromis aureus)*	*Clostridium butyricum*	1.50×10^8 CFU/g	*Aeromonas hydrophila*	56 days	PRE, LR, ADC, VH, GR, FCR↑; BC←; MO↓	Poolsawat et al., 2020
Oreochromis niloticus	*Bacillus subtilis* and *Lactobacillus plantarum*	1.51x 10^6 CFU/g for *Lb. plantarum* 1.34x10^7 CFU/g for *B. subtilis*	*Aeromonas hydrophila*	28 days	GR, SR, IG →; BC←	Guimarães et al., 2019
Oreochromis niloticus	*Bacillus cereus* NY5 and *Bacillus subtilis*	10^8 CFU/g	*Streptococcus agalactiae*	42 days	WG, FCR, SR→ (only *B.subtilis*); FCR↑ (*B.cereus* alone, and *B.cereus* + *B. subtilis*); DR, LY, ML, MD↑; BC←	Xia et al., 2020

Oreochromis niloticus	*Rummeliibacillus stabekisii*	10^7 CFU/g	*Streptococcus iniae* and *Aeromonas hydrophila*	56 days	WG, FCR, GR, FE, DE, SR, DR, IR, IG (cytokines)↑; PA, RB, LY←	Tan et al., 2019
Oreochromis niloticus	*Lactococcus lactis* subsp. *lactis* JCM5805	10^4 CFU/mL (T1) and 10^8 CFU/mL (T2)	*Streptococcus agalactiae*	15 days	DR, GR, IG, SR↑ (only in T2); BC ←	Xia et al., 2019
Oreochromis niloticus	*Lactobacillus plantarum CR1T5*	10^8 CFU/g	*Streptococcus agalactiae*	84 days	WG, GR, FCR, PA, PE, RB, LY, IR↑; SM←; SR→	Van Doan et al., 2019
Oreochromis niloticus	*Paenibacillus ehimensis* NPUST1	10^6 and 10^7 CFU/g	*Streptococcus iniae* and *Aeromonas hydrophila*	70 days	WG, FCR, FE, PA, RB, SD, LY, IG (TNF-α and IL-1β), PY, LA, AM, PR↑	Chen et al., 2019
Oreochromis spp.	*Lactobacillus rhamnosus*	10^8 CFU/g	*Aeromonas veronii*	30 days	GR, FCR, LY, WG, CH, PG, VH, VW, GC, AB, MP↑; AL, TR, AST→; ALT, BUN, MO↓	Sewaka et al., 2019
Oreochromis niloticus	*Lactococcus lactis*	Not specified	*Staphylococcus* spp., *Vibrio* spp., *Pseudomonas aeruginosa*	Not specified	AN ←	Kaktcham et al., 2018

Oreochromis niloticus	*Lactococcus coryniformis* subsp. *torquens* MTi1 and MTi2	1.50×10^8 CFU/mL	*Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus*	Not specified	AN←	Rahman et al., 2018
Oreochromis niloticus	*Bacillus* spp.	10^7 CFU/g	*Streptococcus agalactiae*	30 days	GR, WG, LY, PR, CA, SD, ALP, MPO, ROS, GC, PP↑; IR, IG ← MO↓	Abarike et al., 2018
Oreochromis niloticus	*Bacillus licheniformis*	10^5 and 10^7 CFU/g	*Aeromonas hydrophila*	14 or 28 days	GR, WG, FCR, IR, DR, ROS↑; ALP, LY←	Gobi et al., 2018
Oreochromis niloticus	*Bacillus subtilis*	3.9×10^7 CFU per fish	*Aeromonas hydrophila*	56 days	WG, GR, FCR, LY, RB→; SR, PP↑; MO↓ (even lower when probiotic was combined with Previda® prebiotic)	Addo et al., 2017a
Oreochromis niloticus	*Bacillus subtilis*	4 x 10^7 CFU/g	*Streptococcus iniae*	21 days	GR→; AN, LY↑; MO↓	Addo et al., 2017b

Oreochromis niloticus	*Lactobacillus plantarum* AH78	10^{10} CFU/ml	*Aeromonas hydrophila*	40 days	GR, IR, FCR, AL, GLx↑; IG (cytokines), VH, BC, ABA, ←	Hamdan et al., 2016
Oreochromis niloticus	*Bacillus subtilis, Bacillus licheniformis* and *Bacillus pumilus*	Not specified	*Cetobacterium spp.* and *Plesiomonas*	49 days	GR, FCR, LY, GC, WG, ABA, VH↑	Adeoye et al., 2016
Oreochromis niloticus	*Bacillus amyloliquefaciens*	10^4 and 10^6 CFU/g	*Yersinia ruckeri* and *Clostridium perfringens*	30 days	LY, NO, PA, IR, DR, IG, PP (at higher concentration) ↑	Selim and Reda 2015
Oreochromis niloticus	*Bacillus amyloliquefaciens*	10^4 and 10^6 CFU/g	Not evaluated	30 and 60 days	WG, GR (after 60 days), SR, GC; GB, AL, TP (at higher concentration) ↑VH, BC←	Reda and Selim 2015
Oreochromis niloticus	*Bacillus licheniformis*	4.4×10^6 CFU/g	*Streptococcus iniae*	70 days	WG, GR, DR↑; LY, ML←; FCR, SD, SR→	Han et al., 2015
Oreochromis niloticus	*Lactobacillus acidophilus, Bifidobacterium*	Not specified	*Aeromonas hydrophila*	98 days	GR, DR, AL, GC, FCR↑; MO↓; ALT,	Ayyat et al., 2014

	bifidum and *Streptococcus thermophilus*				AL, GB, SR→; AST←	
Oreochromis niloticus	*Bacillus subtilis*	5×10^6 CFU/g	Not evaluated	84 days	GR, PC, PG→; LY, PA, HE↑; HM↓; IR←	Telli et al., 2014
Oreochromis niloticus	*Bacillus subtilis*	0.1 g/mL (in water), 0.2 g/mL (in diet)	*Flavobacterium columnare*	60 days	MO↓; WQ→; DR↑	Mohamed and Refat, 2011

Results: Significant change ←; No significant change →; Increase/Growth ↑; Decrease/Reduction ↓;

Parameters evaluated: Absorptive area (ABA), Apparent digestibility coefficient (ADC), Albumin (AL), Alanine aminotransferase (ALT), Amylase (AM), Antibacterial activity (AN), Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Bacterial community (BC), Blood urea nitrogen (BUN), Catalase (CA), Cholesterol (CH), Digestive enzyme activities (DE), Disease resistance (DR), Feed conversion ratio (FCR), Feed efficiency (FE), Globulin (GB), Goblet cells (GC), Glutathione (GLx), Growth rate (GR), Hematocrit (HM), Hemoglobin (HE), Immune-related genes expression (IG), Immune response (IR), Lipase activity (LA), Lipid retention (LR), Lysozyme (LY), Microvilli density (MD), Microvilli length (ML), Mucin production (MP), Myeloperoxidase (MPO), Mortality (MO), Nitric oxide (NO), Phagocytic activity (PA), Plasma cortisol (PC), Peroxidase (PE), Plasma glucose (PG), Protection against pathogen (PP), Protease (PR), Protein retention (PRE), Phytase activity (PY), Respiratory burst (RB), Reactive oxygen species (ROS), Superoxide dismutase (SD), Skin mucous (SM), Survival rate (SR), Total blood protein (TP), Triglyceride (TR),Villus height (VH), Villus width (VW), Water quality (WQ), Weight gain (WG).

Table 4. Bacteriocins or BLIS with antimicrobial effects against some pathogens in tests with tilapia.

Tilapia Species	Bacteriocin or BLIS	Pathogen	Research mode	Results	Reference
Oreochromis niloticus	Nisin	Enterobacteriaceae	*In vitro* study, the capacity of preserving tilapia meat was evaluated	Biopreservation effect, the bacteriocin did not affect sensory properties of the product, there was no biogenic amine production.	Mohammed and Ammar, 2020
Oreochromis niloticus	BLIS produced by *Paenibacillus ehimensis* NPUST1	*Streptococcus iniae* and *Aeromonas hydrophila*	*In vivo* study, administration of probiotics with BLIS production	Low pH tolerance, high thermal tolerance, broad spectrum, BLIS had antibacterial activity and improved fish immunity	Chen et al., 2019
Oreochromis niloticus	BLIS produced by *Lactococcus lactis* subsp. *lactis* 3MT	*Vibrio* spp.	*In vitro* study, evaluation of the biopreservation capacity of bacteriocin isolated from tilapia in fish pâté	Stable to heat and pH, antibacterial properties, free of virulence, no production of biogenic amines	Kaktcham et al., 2019a
Oreochromis niloticus	Nisin Z	*Staphylococcus aureus* ATCC 25923	*In vitro* study, screening for bacteriocin production in LAB isolates and identification	High stability to heat, resistance to pH variations, detergents and NaCl, wide range of antibacterial activity	Kaktcham et al., 2019b

Oreochromis niloticus	BLIS produced by *Bacillus* spp.	*Aeromonas hydrophila, Salmonella typhi*	*In vitro* study, purification and evaluation of antibacterial capacity of BLIS extracted from tilapia gut	BLIS was not resistant to acid treatment and denatured in ammonium sulfate (20% of saturation), antibacterial activity against both tested pathogens.	Pacheco et al., 2018
Oreochromis niloticus	BLIS produced by *Lactococcus garvieae*	*Staphylococcus aureus*	*In vivo* study, administration of probiotics with BLIS production	BLIS showed moderate zones of inhibition against closed related species, fish that received bacteriocinogenic probiotics were protected against pathogens and had improved immune response.	Abdelfatah and Mahboub, 2018
Oreochromis niloticus	BLIS produced by *Lactococcus coryniformis* subsp. *torquens* MTi1 and MTi2	*Escherichia coli*	*In vitro* study, purification and evaluation of antibacterial capacity of BLIS extracted from tilapia gut	BLIS exhibited antibacterial activity, but when submitted to enzymatic treatment, the inhibitory properties were inactivated, implying proteic nature of these compounds	Rahman et al., 2018
Oreochromis niloticus	BLIS produced by *Lactococcus lactis* subsp. *lactis* CF4MRS	*Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli,*	*In vitro* study, purification and evaluation of antibacterial activity of BLIS extracted from tilapia intestine	BLIS concentration was too low to significantly inhibit the pathogens.	Loh et al., 2017

		Aeromonas hydrophila, *Edwardsiella tarda* and *Serratia marcescens*			
Oreochromis niloticus	Supernatant produced by *Pediococcus pentosaceus* NP6	*Salmonella enterica* serovar *typhimurium*	*In vitro* study, the capacity of preserving tilapia by-products	Supernatant exhibited antibacterial activity; partially purification indicates that it may be a bacteriocin	Tasaku et al., 2017
Oreochromis niloticus	BLIS produced by *Bacillus endophyticus*, *Bacillus flexus*, *Bacillus mojavensis*, *Bacillus sonorensis* and *Bacillus subtilis*	*Streptococcus iniae*	*In vivo* study, administration of probiotics with BLIS production	BLIS exhibited antibacterial activity; the enzyme treatment suggests that the inhibitory substance may be a bacteriocin	Etyemez and Balcazar, 2016
Oreochromis niloticus	BLIS produced by *Lactococcus lactis* RQ516	*Aeromonas hydrophila*	*In vivo* study, administration of probiotics with BLIS production	Immunostimulant effect and antibacterial activity against a wide spectrum of bacteria, including *A. hydrophila*.	Zhou et al., 2010

Table 5. Production systems and technological resources employed by the 12 largest tilapia producers.

Country	Production in 2018 (in millions/ton)	Reference	Installations	Integration with other economic activities	Other technologies	Reference
China	1.86	Peixe BR (2019)	Floating cage (high-density), net, ponds (in hydroelectric reservoirs)	Polyculture with carp, mullet or shrimp; rice culture	Hydroponics, GIFTs and ProGIFT*, RAS (Recirculating systems), hatcheries	El-Sayed (2019); Xu & Ming (2018); Gui *et al.* (2018)
Indonesia	1.25	Peixe BR (2019)	Floating net cage, two-net cage	Polyculture with carp; rice culture	Biofloc technology, RAS, GIFT and other genetic improved tilapias, nanobubble technology, dual-cage	El-Sayed (2019); Nugroho *et al.* (2020); Mahasri *et al.* (2018)
Egypt	0.86	Peixe BR (2019)	Pond-farm, tank, earthen-ponds	Polyculture with mullet; rice culture	RAS, In-pond raceway system (IPRS), dual-cage, aquaponics, hatcheries, improved-feeds, GIFT and GIANT*, seed production, Automated Monitoring and Control System (AMCS)	El-Sayed (2019); Helal *et al.* (2020)
Brazil	0.40	Peixe BR (2019)	Earthen-ponds, tank-net, cages (in hydroelectric reservoirs, high-density),	Polyculture with pirapitinga or shrimp	Aerator, automatic feeding, Biofloc technology, GIFTs and GST	El-Sayed (2019); Milanez *et al.* (2019)

			periphyton pond			
Philippines	0.33	Peixe BR (2019)	Earthen-ponds, floating cages and fixed cages, tank	Most farmers adopt monoculture system, however, there are integration with swine, rabbit and poultry cultures in lesser extent	GIFT and other genetic improved tilapias, monosex tilapia, supermale technology, Biofloc technology	El-Sayed (2019); Caipang & Avillanosa (2019); Prabu *et al*. (2019)
Thailand	0.32	Peixe BR (2019)	Floating-cages (high-density), Bamboo cages	Integration with poultry culture; rice culture	GIFT and other genetic improved tilapias, supermale technology, improved seaweed	El-Sayed (2019); Romana-Eguia *et al.* (2020); Trono & Largo (2020)
Bangladesh	0.22	Peixe BR (2019)	Pond-dike, cages	Polyculture with carp, rice culture (rotational)	GIFT, feed supplements, improved seeds, water-saving technologies	El-Sayed (2019); Majumder *et al.* (2017); Uddin *et al.* (2019)
Vietnam	0.20	Peixe BR (2019)	Cages, net	Polyculture with silver barb and carp or shrimp, rice culture	RAS, GIFT	El-Sayed (2019); Tran *et al.* (2020)

Colombia	0.077	FAO (2018)	Cages (in hydroelectric reservoirs, high-density), pond, tanks, raceways	Polyculture with carp or bocachico	Improved seeds, Biofloc technology	El-Sayed (2019); Camero-Escobar & Calderón-Calderón (2018); Jimenéz-Ojeda *et al.* (2018); Reyes-Serna (2018); García *et al.* (2011)
Uganda	0.070	FAO (2018)	Earthen-ponds, Cages (low-density), cage/pens, tank/raceways	Most farmers adopt monoculture system, however there is integration between farming/aquaculture activities	Hatcheries, improved seeds	El-Sayed (2019); Safina *et al.* (2018); Clough *et al.* (2020); Oyebola *et al*. (2021); Hyuha *et al.* (2017)
Taiwan	0.062	FAO (2018)	Cages, octogonal tanks/raceway, ponds	Polyculture with shrimp	Aerator, automatic feeders, RAS	El-Sayed (2019); Prabu *et al.* (2019); Hoang *et al.* (2020)
Mexico	0.052	FAO (2018)	Net-pens, cages	Polyculture with Mayan cichlids shrimp or prawn	RAS, Biofloc technology	El-Sayed (2019); Asiain *et al.* (2020); Suárez-Puerto *et al.* (2021)

*GIANT and ProGIFTs are types of genetic improved tilapia.

Table 6. Tilapia pathogens that showed resistance to antibiotics and other antimicrobials used in farming systems in some countries.

Resistant Pathogen	Antibiotic	Tilapia origin	Reference	Most common antibiotics detected in the country	Reference	Antibiotic allowed in the country (active principle)	Reference
P. aeruginosa	Ampicillin, sulfamethoxazole /trimethoprim, tetracycline and nalidixic acid	Giza (Egypt)	Osman et al. (2019)	Ciprofloxacin and florfenicol	Lulijwa *et al.* (2020)	Florfenicol, ciprofloxacin	Rezk *et al*., 2015
Klebsiella pneumoniae, *E. coli*, *Proteus mirabilis*, and *S. agalactiae*	Penicillin, ampicillin, oxytetracyclines, trimethoprim, oxolinic acid, gentamicin, and sulfamethoxazole	Bangkok and Nakhon Si Thammarat (Thailand)	Thongkao & Sudjaroen (2019), Dangwethn angam et al. (2016)	Enrofloxacin, norfloxacin, amoxicillin, oxolinic acid, penicillin, florfenicol, tetracycline, oxytetracycline, sulphadiazine, trimethoprim, ormetoprim, sulfadiazine + trimethoprim, sulfadimethoxine + trimethoprim, sulfaguanidine	FAO (2017a), Lulijwa *et al.* (2020)	Oxytetracycline, tetracycline, sulphadimethozine, trimethoprim, sulphadimethoxine-ormethoprim and amoxicillin	Lulijwa *et al.*, 2020
Vibrio spp.	Erythromycin and chloramphenicol	Sri Tujuh (Malaysia)	Hamdan et al. (2018)	Oxolinic acid, virginiamycin, chloramphenicol and sulphonamides, tetracyclines, nitrofurans	FAO (2017a); Lulijwa *et al.* (2020)	Amoxicillin, oxytetracycline, flumequine and florfenicol, oxolinic acid, virginiamycin and tetracyclines	NPRA, 2017
Enterococcus spp.	Tetracycline	Chennai (India)	Arumugam et al. (2017)	Erythromycin, chloramphenicol, sulphadiazine, sulfadimethoxine, sulfamethazine, sulphapyridine, sulphamethoxypyridazine, sulphadoxine, sulfamethoxazole, sulphanilamide, sulphathiazole	Lulijwa *et al.* (2020)	Sulfadimethoxine, sulfabromomethazin, erythromycin, oxytetracycline, althrocin, ampicillin, sparfloxacin, and	CDDEP, 2016

						enrofloxacin and sulfaethoxypyidazine	
A. hydrophila	Tetracycline, sulfathiazole	Solteira Island (Brazil)	Monteiro et al. (2016)	Florfenicol, tetracycline, oxytetracycline and enrofloxacin	Lulijwa *et al.* (2020)	Florfenicol, oxytetracycline and neomycin (ornamental fish)	MAPA, 2020
Acinetobacter spp.	Sulfamethoxazole, tetracycline	Tianjin (China)	Gao et al. (2012)	Neomycin sulphate, doxycycline hydrochloride, thiamphenicol, florfenicol, sulfadiazine, sulfamethoxazole + trimethoprim, sodium sulfamonomethoxine, enrofloxacin, flumequine, oxolinic acid, oxytetracycline, ciprofloxacin, norfloxacin, ofloxacin, amoxicillin, cephalexin, cefradine, cefotaxime, erythromycin, gentamicin S, neomycin, tetracycline, lycomicin, sulfamethoxazole	Lulijwa *et al.* (2020), FAO (2017b)	Neomycin sulphate, doxycycline hydrochloride, thiamphenicol, florfenicol, sulfadiazine, sulfamethoxazole + trimethropim, sodium sulfamonomethoxin, enrofloxacin, flumequine, oxolinic acid, oxytetracycline	FAO, 2017

CHAPTER 4

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Beneficial effects of probiotics on the pig production cycle: An overview of clinical impacts and performance

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ABSTRACT

In pig nutrition, antibiotics are used to promote growth and/or to treat diseases in order to improve animal performance. However, due to the potential risk of cross selective pressure for antibiotic resistance among bacterial pathogens, the development of new nutritional additives is needed. Among them, probiotics are of great interest since they could improve the immune response, maintain animal intestinal health, and improve nutritional efficiency. Studies with probiotics have also demonstrated their antimicrobial effects on several pathogenic strains, emphasizing that the form of administration can enhance the beneficial effects. In view of the promising advances in probiotic research, it is opportune to highlight their capacity to modulate health and improve performance at all stages of pig production. Therefore, in this review, we will discuss the benefits of probiotics on physiological, immunological, and clinical aspects during different stages of the pig's life cycle. Specifically, probiotics improve performance during pregnancy, parturition and lactation in sows, they can improve immunohematological parameters and defenses in the growing phase, they can influence the quality of meat in the finishing phase and can also help in the reduction of environmental pollutants.

1. Introduction

In recent years, increased demand for improvements in productivity, quality, cost reduction and environmental impacts has led to greater pressure on the pig production chain. Moreover, it is essential to have in place effective measures to guarantee the control of human and animal pathogens. Antibiotics have been intensively used in pig production because they help to fight diseases and can play a role as growth promoters, resulting in higher productive efficiency and animal growth (Carlson and Fangman, 2018).

However, the use of antibiotics has been questioned due to the loss of effectiveness and selection of resistant bacterial strains (Yirga, 2015) as evidence suggests that resistance genes can be transmitted from animal to human microbiota. This is of great concern because it may narrow the therapeutic options available to treat human bacterial infections (Ma et al., 2018; McEwen and Collignon, 2018).

Therefore, it is necessary to search for other strategies to combat diseases inherent to swine production. Unlike antibiotics, probiotic supplementation promotes the general health of pigs and increases the number of desirable microorganisms in the intestine (Liao and Nyachoti,

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2017). Thus, the use of probiotics and/or antimicrobial metabolites from beneficial microbes, offers an alternative to improve animal health, to modulate the intestinal flora and to limit the spread of multi-drug resistant genes, in addition to their potential to be used in food bio-preservation (O'Connor et al., 2020).

In swine production, the administration of probiotics can be carried out during different stages of growth (Liu et al., 2020), but in the literature there is no consensus about the selection of microbial strains, doses and duration recommended for treatment. However, it has been demonstrated that the administration of probiotics impacts the intestinal microbiota, restores and improves pig resistance to diseases and results in better performance (Liao and Nyachoti, 2017).

2. Probiotics can be an alternative to the use of antibiotics

The use of probiotics in animal feed for safe production has been taken into consideration by several researchers in recent years. Nowadays, significant efforts are being made by researchers in order to clarify the benefits of probiotics in the intensive production of pigs. From a microbiological point of view, the use of probiotics has been targeted at substantial improvements in animal health and welfare parameters, with benefits in both, the decrease of specific microorganims during breeding and the presence of some foodborne pathogens known to proliferate along the processing chain. Resistance to antibiotics and the presence of antibiotic residues in foods of animal origin is still a major concern in the field of animal nutrition. In this way, the use of probiotics in animal feed for safe production and as a potential alternative to the use of antibiotics has grown and gained prominence in current research (Liao and Nyachoti, 2017; Carlson and Fangman, 2018; Li et al., 2019a).

Probiotics are described as microorganisms, such as bacteria or yeasts, which when ingested in sufficient quantities exert positive effects on host health for its ability to reduce the harmful effect of pathogenic microorganisms (Dubreuil, 2017). Unlike antibiotics, which do not distinguish between harmful and beneficial bacteria, probiotics are designed to encourage benign strains over unwanted ones (Liao and Nyachoti, 2017). For a probiotic to be considered effective, it must have characteristics such as resisting gastric acid, bile salts and pancreatic enzymes, in addition to the ability to adhere to the intestinal mucosa in order to colonize it (Dubreuil, 2017). According to the International Scientific Association for Probiotics and Prebiotics, metabolicby-products, dead microorganisms or microbe-based nonviable productsdo not fall under the probiotics' characteristics, but these should be considered since several studies have demonstrated that dead bacteria and bacteria molecular components display probiotics properties (Plaza-Díaz et al., 2017a, 2017b, 2018, 2019). Nowadays, the term "post-biotics" is the option to be used to soluble components with biological activity instead of the use of whole bacteria (Tsilingiri et al., 2012; Plaza-Díaz et al., 2019).

To be considered a probiotic, the microorganism must have the ability to colonize the gastrointestinal tract (GIT), have a fast growth rate, especially on a large scale under commercial conditions, a low requirement for nutrients, suppress enteric pathogens and also their metabolites, and be able to survive in-feed and after the manufacturing process, maintaining its viability and activity stable. In order to exert a

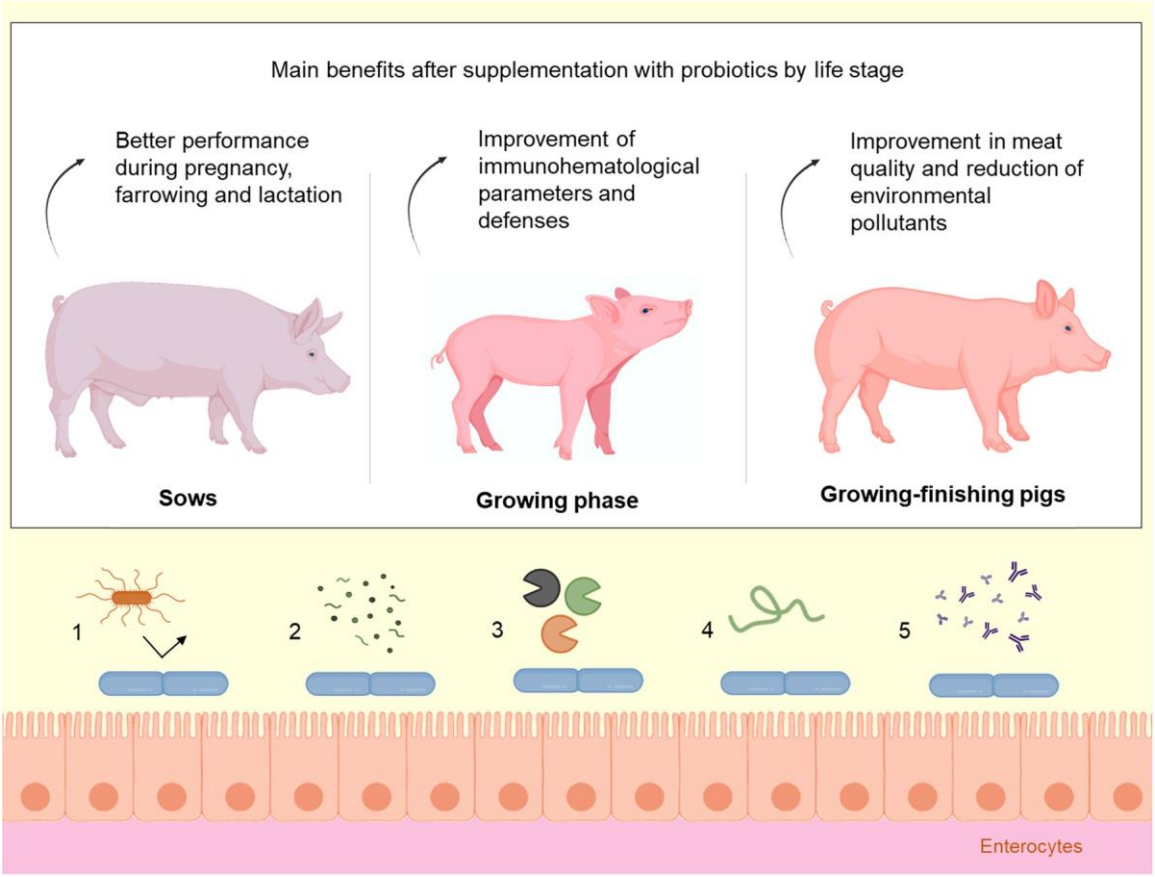

Fig. 1. Overview of the main beneficial effects of the use of probiotics in pig production at different stages of life cycle. In general, (1) after the supplementation of probiotics there is a constant growth in the number of probiotic strains present in the intestine, which leads to an increase in competition for space and nutrients with other microorganisms, which can reduce the presence of pathogens in the intestine. (2) This reduction may also be associated with the ability of probiotics to produce molecules with antimicrobial potential, such as bacteriocins and organic acids. (3) The use of probiotics can also improve the nutritional status of pigs, (4) as they have the ability to synthesize vitamins and digestive enzymes. (5) In addition, probiotics have immunomodulatory effects, as they stimulate the immune system without causing disease.

positive effect on pig performance, a probiotic in the GIT must stimulate the development of healthy microbiota, especially beneficial bacteria, prevent the colonization of enteric pathogens, increasing digestive capacity, decreasing pH, improving mucosal immunity or enhancing gut tissue maturation and integrity (de Lange et al., 2010).

Probiotics are able to provide benefits to the host through several mechanisms, many of which are still unknown (Zimmermann et al., 2016). Taking into account the mechanisms of action of probiotics (Fig. 1), the first step is the colonization of intestinal microbial communities. Probiotics bacteria are able to spread from the digestive tract to extradigestive sites through dendritic cells (DC), penetrating the epithelium and taking the bacteria directly from the intestinal lumen. Once inside DCs or macrophages, the bacteria can be transported to other areas by immune cell circulation through the bloodstream (Martín et al., 2004). The interaction between probiotics and DCs is responsible for immune modulation (D'Amelio & Sassi, 2017). The second step is the adhesion of bacteria to host gut surfaces by transmembrane proteins (integrins and cadherins) and components of the extracellular matrix (collagen, fibronectin, laminin or elastin) (Ribet & Cossart, 2015), enhancing the elimination of pathogens (Plaza-Díaz et al., 2019).

Competitive exclusion of pathogens, bacteriocin production, enzymatic activities and production of volatile fatty acids are also mechanisms of action of probiotics (Fig. 1). Competitive exclusion means that one species of bacteria competes for receptor sites in the intestinal tract more intensely than other species (Bermudez-Brito et al., 2012; Plaza--Díaz et al., 2019). In addition to the ability to reduce or prevent adhesion of pathogens by competitive exclusion, they modulate the host's immune response, contribute to the integrity of the intestinal wall barrier and produce substances that may inhibit Gram-positive and Gram-negative bacteria, such as bacteriocins, organic acids and hydrogen peroxide (Dubreuil, 2017). Bacteriocin and fatty acids (e.g. propionic acid, acetic acid) production by probiotics also contribute as one of the mechanisms of action, since these molecules have antimi- crobial effects and are able to prevent the proliferation of pathogens (Bermudez-Brito et al., 2012). Regarding the enzymatic activity of probiotics, it is known that they can produce important digestive enzymes, which contribute to the development of the animal, especially by improving digestibility, feed efficiency and weight gain (Domingos et al., 2021; Peng et al., 2020; Hao et al., 2020). In humans, probiotics interact with bile acids in the gut lumen through the synthesis of an enzyme called bile salt hydrolase, thus modifying bile acid metabolism and influencing cholesterol absorption (Pavlovic et al., 2012). Probiotic mechanism of action in pigs may be through modulation of the intestinal microbiota, which results in a reduction in diseases and an improvement in growth performance (Yirga, 2015).

Interest in probiotics has increased significantly in recent years due to their possible use as an alternative to low-dose antibiotics, safety, viability in the GIT and because they do not negatively influence the taste of foods (Trukhachev et al., 2021; Suez et al., 2019). In pig farming, the most used probiotic genera are *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Weissella* (Vieco-Saiz et al., 2019). Furthermore, there are other probiotics of interest for pig farming, such as some of the *Bacillus* genus (Yirga, 2015) and the yeast *Saccharomyces* (Domingos et al., 2021; Peng et al., 2020).

Probiotics are used in all stages of swine rearing (from early weaned piglets to growing-finishing pigs) (Barba-Vidal, et al., 2019). In pigs, Huang et al. (2004) showed that dietary lactobacilli supplementation improved performance and changed *Escherichia coli* counts in intestinal microflora after weaning. A healthy GIT and a strategy to fight diseases caused by enteropathogens is necessary for pig raising, which will result in better digestion and absorption of nutrients (Liao and Nyachoti, 2017).

3. Impacts of probiotic administration at different stages of pig production

In recent years, probiotics have received more attention as a nutritional strategy in pig farming. There are important differences in experimental factors for probiotics administration, such as frequency of administration, when it starts to be administered, the animal age, interactions with some medications, accommodation, state of health, nutrition, stress, animal genetics, as well as the use of different doses and different strains of microbial species may influence the clinical benefits. Probiotics can be used in all stages of pig production, such as in sows, neonatal piglets, early weaned piglets and growing-finishing pigs (Yang et al., 2015a). An overview of their application and administration on all those different life development stages can be seen in Table 1.

From the analysis of the results of the published studies, it was observed the lack of a standard in the methodologies adopted for the evaluation of benefits using probiotics in swine. Many authors do not clearly demonstrate the exact concentration of probiotics given to animals daily or their proportion in the ration. Several authors used probiotic mixtures in their studies. In these cases, it was possible to observe that sometimes there is no clarity about the exact amount of each strain that was administered. There is also no clarity in some studies about the time of administration and whether in the different groups analyzed each animal consumed the same amount of feed daily. However, even with the lack of some information, it was possible to identify some patterns. The administration of probiotics provided positive results in several studies, with the standard concentration of 10^8 to 10^9 CFU/g. Mostly, administration occurs twice a day (morning and afternoon), the amount being quite variable (~1–3 kg/animal/day), and a percentage of probiotics per gram of feed is usually calculated.

Probiotic administration in sows is mainly aimed at improving gut health, animal welfare and reproductive performance. Ingestion of probiotics is also associated with a higher rate of feed intake during pregnancy and lactation, which leads to a greater energy supply to the animal during these important phases. In swine production, the nursery phase is the one in which probiotics are most commonly applied (Barba-Vidal et al., 2019). There is evidence that probiotics are more effective in animals during microflora development, which means they are expected to be more beneficial in young animals, as they have not yet developed stable intestinal microflora (Yirga, 2015), especially after weaning (McDonald et al., 2010).

Therefore, based on the analysis in Table 1, studies have shown that for newly weaned piglets there has been an improvement in the intestinal mucosa, such as increased production of digestive enzymes, improvement in performance and digestibility, increased immunity and consequent effects against the main pathogens from swine (*E. coli*, *Salmonella enterica*, *Salmonella* Typhimurium). Finally, in growing-finishing pigs, due to the limited influence of probiotics in the final stage of life (Barba-Vidal et al., 2019), most studies opted for combinations of different strains and the results were mainly related to quality improvement of the final meat, reduction of harmful faecal gases and greater feed conversion efficiency.

In the following sections, we will discuss in more detail the impacts of probiotic supplementation on the main life stages of pigs.

4. Sows: gestation and lactation stages

Probiotic application in sows (gestation and lactation stages) contributes to the improvement of the animal health status and results in improvements both for the reproductive performance and for the developing piglets (Betancur et al., 2021). Table 1 reveals that administration of probiotics in sows provided positive results in several studies, with the majority adopting a standard concentration of 10^8 to 10^9 CFU/g given for ca. four months, starting at late pregnancy up to early lactation. It is not possible to establish a standard dose, since there were variations according to each study. In general, the animals were

Table 1
Overview of probiotic application and administration on swine during different life development stages.

Life development stage	Species	Probiotic	Bacteria concentration in the probiotic preparation *	Dose per day**	Duration	Form of administration	Pathogen or Challenge	Clinical Impacts ***	Reference
Sows	Commercial genetic line (Topigs Norsvin®)	*S. cerevisiae var. boulardii*	2×10^{10} CFU/g	From day 110 until farrowing: 2 kg/day (twice a day); after farrowing: 2 kg on day 1 and reaching 8 kg/day on day 7; after day 7 until weaning: were fed ad libitum; more details not given	From day 90 (gestation) to 24 (lactation)	Feed	Not evaluated	TR, WG, GR, MP, MIQ, PBA, IBW, VFI ↑; HS↓	Domingos et al. (2021)
	Polish Large White × Polish Landrace	*E. faecium, L. rhamnosus, L. fermentum*	10^9 CFU/g	10 g of the probiotic mixture per kg (dose per day and for each strain not given)	147 days	Feed	Not evaluated	WG, WBC, IR ↑	Satora et al. (2021)
	Landrace × Yorkshire	*B. subtilis* PB6	4.0×10^{11} CFU/g	Dose per day to sows not given; piglets: progressively increased (1 kg/day to their maximum feed intake); 0.2% of probiotic	From day 90 (gestation) to 21 (lactation)	Feed	Not evaluated	BC←; GR, GM, PC, PBA, LS↑; IBW↓	Zhang et al. (2020)
	Landrace × Yorkshire	*B. subtilis* and *B. licheniformis*	1.0×10^9 CFU/g / 1.0×10^9 CFU/g	0.1% or 0.2% of probiotics; inclusion rate of probiotic not given	11 days	Feed	*E. coli*	FN, FNG ↓ (not H_2S); RP →; PP ↑	Hu et al. (2020)
	Landrace × Yorkshire	*S. cerevisiae*	1.0×10^{13} CFU/g	Gestation diets: twice daily (3 kg/day); lactation diets: 4 times a day (starting at 2 kg/day and increased by 5 kg/day during the first 5 days, afterwards the sows had free access to diet	108 days	Feed	Not evaluated	PG, TR, PU, MO, IBW→; TB, IG (IgG), MIQ, MP↑; ADFI, PBA, ALT, ALP, AST↓	Peng et al. (2020)
	Polish Large White × Polish Landrace	Bokashi ® (*S. cerevisiae*, *L. casei*, *L. plantarum*, *E. faecium*, *E. faecalis*, *Bifidobacterium bifidum*, *Bifidobacterium pseudolongum*, *B. licheniformis*, *B. cereus var toyoi*, *B. subtilis*, *C. butyricum*)	*S. cerevisiae* (5×10^4 CFU/g), *L. casei* (5×10^8 CFU/g), *L. plantarum* (5×10^8 CFU/g), *E. faecalis* (2.5×10^6 CFU/g), *E. faecium* (5×109 CFU/g), *B. bifidum* (5×108 CFU/g), *B. pseudolongum* (5×10^8 CFU/g), *B. licheniformis* (4×10^9 CFU/g), *B. cereus var. toyoi* (4×10^9 CFU/g), *B. subtilis* (4×10^{11} CFU/g), *C. butyricum* (1×10^8 CFU/g)	Probiotic was added to the feed in the amount of 10 kg/t of feed; inclusion rate of probiotic not given	From day 90 (gestation) to 28 day (lactation)	Feed	Not evaluated	PP, IR, IG (IgA, IgG, TGF-β, IL-10) PBA, IBW↑; MO, ID↓; ADFI→	Laskowska et al. (2019)
	Landrace × Large White	BIO- THREE PZ (*Bacillus mesentericus, C. butyricum, E. faecalis*)	*B. mesentericus* (1×10^6 CFU/g, *C. butyricum* (1×10^6 CFU/	Sows were fed twice a day; 15 g/day of	From day 28 (pre-parturition) to 7	Feed	Not evaluated	DR, RP←; IG (cytokines IgG	Tsukahara et al. (2018)

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Table 1 (*continued*)

Life development stage	Species	Probiotic	Bacteria concentration in the probiotic preparation *	Dose per day**	Duration	Form of administration	Pathogen or Challenge	Clinical Impacts ***	Reference
			g) and *E. faecalis* (1×10^8 CFU/g)	the probiotic was orally administered	(post-parturition)			and IgA), WG, MP↑; ID↓	
	Large White × Yorkshire	*P. acidilactici*	2.40×10^{12} CFU/g	Sows were fed twice a day; from gestation to farrowing: 3 kg/day of diet; lactation: 2 kg/day	From day 90 (gestation) to 28 (lactation)	Feed	Not evaluated	IR, IG (cytokines), TB, WL, PBA, IBW, RP↑; DI, HAP, ALT↓; BC←	Liu et al. (2020)
	Landrace × Yorkshire	*B. subtilis* and *L. acidophilus*	1.2×10^7 CFU/g, 1.15×10^6 CFU/g	Gestation period: 2.5 kg/day; 0.1% or 0.2% of probiotics	4 weeks (day 86–109 of pregnancy and day 110 of pregnancy to weaning)	Feed	*E. coli* and *Salmonella* spp.	WG, GR ↑; FNG↓; MO→	Jeong et al. (2015)
Early weaned piglets	Yorkshire, Landrace and Duroc	*L. planetarium*	1.2×10^{12} CFU/g	Inclusion rate of probiotic not given	6 weeks (42 days)	Feed	*E. coli*	WG, GR↑; PP, ND ←	Yang et al. (2020)
	Duroc × Landrace × Yorkshire	*L. reuteri*	5×10^{13} CFU/g	Inclusion rate of probiotic not given	175 days	Feed	Not evaluated	GR, WG, GU↑; MC, TB →	Tian et al. (2020)
	Landrace × Yorkshire × Duroc	*S. cerevisiae*	Not specified in CFU	0.2% and 0.3% of probiotic; inclusion rate of probiotic not given	96 days	Feed	Not evaluated	WG, GR, FBW, TB, MQ↑; FG, AL, BL, pH→	Dávila-Ramírez et al. (2020)
	Duroc × Landrace × Large White	*L. fermentum* and *P. acidilactici*	9.1×10^8 CFU/g and 5.25×10^8 CFU/g	The pigs were fed 4 times per day (4% of probiotic); more details not given	28 days	Feed	*Treponema* and *Anaerovibrio*	IG (inflammatory cytokines) ↓; WG, FG ↑	Wang et al. (2019)
	Duroc × Landrace × Large Yorkshire	*L. delbrueckii*	5×10^9 CFU/mL	Inclusion rate of probiotic not given	49 days	Feed	Diarrhea	FG, WG, GR, IM, AG, IR↑	Li et al. (2019c)
	Not evaluated	*C. butyricum*	2×10^6 CFU/g and 5×10^5 CFU/g	Inclusion rate of probiotic not given	42 days	Feed	*S.* Typhimurium	PP, SE, IC, FE →	Peeters et al. (2019)
	Landrace × Large White	*L. johnsonii*	10^9 CFU/mL	10 mL/day	7–18 days	Intragastrically solution	*S. enterica*	FG, PP, GR, ↑	He et al. (2019)
	Duroc × Yorkshire × Landrace	Probiotic mix (*B. coagulans, B. licheniformis, B. subtilis,* and *C. butyricum*)	*B. coagulans* (1×10^{15} CFU/g), *B. licheniformis* (5×10^{14} CFU/g), *B. subtilis* (1×10^{15} CFU/g), *C. butyricum* (1×10^{14} CFU/g)	0.1%, 0.2% and 0.3% of probiotic mix; more details not given	42 days	Feed	*E. coli*	GR, PP, DN DM ↑; FN↓	Nguyen et al. (2019)
	Landrace × Desi	*P. acidilactici*	2×10^9 CFU/g	200 g/day/pig	180 days	Feed	Not evaluated	AL, GL, BL ↑; pH, LO ↓	Dowarah et al. (2018)
	Duroc × Landrace × Yorkshir	*S. cerevisiae*	Not specified in CFU	Diet 1: 3 g/kg^{-1} of live yeast; diet 2: 2.66 g/kg^{-1} of heat-killed whole yeast; diet 3: 3 g/kg^{-1} of superfine yeast powders; more details are not available	3 weeks	Feed	*E. coli*	AG, GM, IM, PP ↑; ACP; pH ↓	Cui et al. (2017)
	Polish Landrace × Polish Large White sows mated to Duroc × Pietrain boar	*E. faecium*	3.5×10^{11} CFU/g	Inclusion rate of probiotic not given	day 7–21 and 22–70	Feed	*E. coli* and *C. perfringens*	WG, PP ↑; VFA →	Hanczakowska et al. (2016)
	Landrace × Large White × Pietrain	*B. subtilis* and *B. licheniformis*	4×10^{14} CFU/g	1000 g/tonne; more details not given	28–42 days of age and 42–70	Feed	Not evaluated	GR, FG ↑; BG ←	Jørgensen et al. (2016)

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Table 1 (*continued*)

Life development stage	Species	Probiotic	Bacteria concentration in the probiotic preparation *	Dose per day**	Duration	Form of administration	Pathogen or Challenge	Clinical Impacts ***	Reference
	Large white × Landrace	*L. plantarum*	~1 × 10^{10} CFU/pig/day	Inclusion rate of probiotic not given	10 days	Feed	*S.* Typhimurium	IR, PP ↑	Naqid et al. (2015)
	Not evaluated	*L. reuteri*	10^7 CFU/g	Inclusion rate of probiotic not given	21 days	Feed	*E. coli*	PER ↑; GR, FC, PP←; MO↓	Yang et al. (2015b)
Growing-finishing pigs	Landrace × Yorkshire × Duroc	Probiotic mix (*L. plantarum* CJLP243, *L. fermentum* LF21, *L. salivarius* E4101, *Leuconostoc paramesenteroides* KJP421, *B. subtilis* CJMPB957, *B. licheniformis* CJMPB283)	*L. plantarum* (10^{11} CFU/g), L. fermentum, *L. salivarius*, *L. paramesenteroides, B. subtilis* and *B. licheniformis* (10^9 CFU/g)	2 g/kg of probiotics; more details not given	42 days	Feed	Not evaluated	WG, FC, GM, IG (cytokines) ↑	Kwak et al. (2021)
	Yorkshire × Landrace × Duroc	*B. subtilis*, *B. licheniformis*, and *S. cerevisiae*	*B. subtilis* (1.5 × 10^9 CFU/g), *B. licheniformis* (1.5 × 10^9 CFU/g), *S. cerevisiae* (1.5 × 10^9 CFU/g)	Diet 1: 0.05% probiotics; diet 2: 0.10% probiotics; more details not given	42 days	Feed	Not evaluated	FNG ↓; GR, FG, ACP ↑	Wang et al. (2021)
	Not evaluated	Probiotic mix (*S. cerevisiae, L. casei*, *L. plantarum*)	*S. cerevisiae* (3.3 × 10^5 CFU/mL), *L.casei* and *L. plantarum* (1.95 × 10^7 CFU/mL)	Diet 1: 0.3%; diet 2: 0.5%; more details not given	Data not available	Feed	Not evaluated	WG, pH, GM →;	Rybarczyk et al. (2020)
	Duroc × Landrace × Large White	*B. subtilis* ZJU12 and *P. pentosaceus* ZJUAF-4	3.6 × 10^8 CFU/g, 2.5 × 10^8 CFU/g	5% and 10% probiotics; more details not given	35–39 days	Feed	Not evaluated	FG, WG, GR, TR, CH, MQ ↑; pH, FN ↓	Hao et al. (2020)
	Large White boar × York × Dutch Landrace sow	Probiotic mix *(B. amyloliquefaciens* and *B. subtilis)*	6 × 10^{11} CFU/g	Probiotics diet: 400 mg/kg	102 days	Feed	*Lawsonia intracellularis*	PP, FC↑; DR ↓	Van der Peet-Schwering et al. (2020)
	Hampshire × local	*P. acidilactici* FT28 and *L. acidophilus NCDC 15*	1–2 × 10^9 CFU/g	200 g/day/pig; more details not given	90 days	Feed	Not evaluated	FC, FG, GR, MQ ↑; AL, TB →; TR, CH ↓	Joysowal et al. (2018)
	Landrace × Yorkshire × Talent	Probiotic mix (*S. thermophile, B. animalis, L. acidophilus, L. helveticus, L. paracasei, L. plantarum, L. brevis*)	Not specified in CFU	100 mg/kg; more details not given	12 weeks	Feed	Not evaluated	GY, GR, MQ, IG ↑	Accogli et al. (2018)
	Yorkshire × Landrace × Duroc	*B. coagulans, B. licheniformis B. subtilis* and *C. butyricum*	*B. coagulans* (1 × 10^9 CFU g^{-1}), *B. licheniformis* (5 ×10^8 CFU g^{-1}), *B. subtilis* (1 ×10^9 CFU g^{-1}), *C. butyricum* (1 ×10^8 CFU/g)	0.1 and 0.2 g/kg of probiotic mixture; more details not given	16 weeks	Feed	*E. coli*	FC, FG, GR, PP, DM, FN ↑	Balasubramanian et al. (2018)
	Local × Landrace	*P. acidilactici* or *L. acidophilus*	10^9 CFU/g	200 g/pig/day; more details not given	44 days	Feed	*E. coli*	GR, PP, BC, FN ↑; pH ↓; IM ←	Dowarah et al. (2017)
	Landrace × Large White × Pietrain	*B. subtilis* and *B. licheniformis*	4 × 10^{14} CFU/g	1000 g/tonne; more details not given	From 120–182 days	Feed	Not evaluated	GR ↑ FC; BG ←	Jørgensen et al. (2016)

* Concentrations were standardized to CFU/g.

** Kg/day means kg of supplemented feed/day.

*** Interpretation: Significant Change ←; No significant change →; Increase/Growth ↑; Decrease/Reduction ↓; average daily food intake (ADFI), albumin (AL), alkaline-phosphatase (ALP), alanine aminotransferase (ALT), aspartate - aminotransferase (AST), alternative complement pathway (ACP), antioxidant genes (AG), bacterial community (BC), blood cell (BL), cholesterol (CH), digestibility of nitrogen (DN), disease resistance (DR), dry matter (DM), fecal excretion (FE), fecal NH_3-N (FN), fecal noxious gas emission (FNG), feed conversion ratio (FC), feed/gain (FG), final body weight (FBW), glycoproteins (GY), globulin (GL), glutamine (GU), haptoglobin (HAP), hemoglobin (HE), heat stress (HS), growth rate (GR), gut microbiota (GM), individual born weight (IBW), initial body weight (IBG), intestinal carriage (IC), incident diarrhea (ID), intestinal morphology (IM),

immune response (IR), immune-related genes expression (IG), Immunological parameters (IP), lipid oxidation (LO), litter size (LS), meat color (MC), milk quality (MIQ), meat quality (MQ), milk production (MP), mortality (MO), potential of hydrogen (pH), proportion of born alive piglets (PBA), plasma cortisol (PC), plasma glucose (PG), plasma urea (PU), protein efficiency ratio (PER), protection against pathogen (PP), reproductive performance (RP), serological response (SE), total blood protein / total protein (TB), triglyceride (TR), volatile fatty acid (VFA), voluntary feed intake (VFI), white blood cell (WBC), weight of litter (WL), weight gain (WG).

fed daily with ~2–3 kg of feed and the proportion of probiotic present in the feed was also variable, generally ranging from 0.1% to 0.2%. There is no doubt that these differences observed in the methods of the studies may be an explanation for the differences found in their respective results. However, it is possible to observe some important data, such as the minimum concentration of probiotics required. As mentioned, most studies chose to administer preparations with a concentration of 10^8 to 10^9 CFU/g, and all of them had good results. Even those who opted for higher concentrations obtained positive results, however, in these cases not only the concentration was higher but also the dose. Domingos et al. (2021), used the concentration of 2×10^{10} CFU/g and fed the animals with different doses at each stage, not controlling the amount ingested in the phase close to weaning. Likewise, Li et al. (2019c) used a concentration of 5×10^9 CFU/mL (dose not given) and Zhang et al. (2020) that progressively fed the animals to their maximum feed intake (4×10^8 CFU/kg). Even with good results, the increase in concentration does not seem to be economically viable, especially for small producers, considering that good results can also be achieved at lower concentrations and the increase in the concentration of probiotics may represent a more expensive feed. A good alternative to high probiotic concentration vs high production cost could be the application of low probiotics concentrations in a higher daily dose of feed. Thus, other important findings are shown below.

Domingos et al. (2021) and Peng et al. (2020) evaluated the effects of *Saccharomyces cerevisiae* as probiotic in pigs. The authors found that the use of probiotics during gestation affected positively the production of colostrum and milk, and that there was an increase in the concentration of fatty acids (total, saturated, monounsaturated, polyunsaturated and unsaturated) present in milk, improving its nutritional value. However, as mentioned earlier, the increase in concentration does not seem to be responsible for these improvements, since Peng et al. (2020) used similar dose and lower concentrations, and also obtained positive results.

Jeong et al. (2015) used the probiotics *Lactobacillus acidophilus* and *Bacillus subtilis* from day 86–109 of gestation (i.e. 4 weeks prior to farrowing) until day 21 of lactation, and they reported a decrease in the emission of harmful faecal gases and verified an increase in the average daily feed intake, related to growth promotion and increase in the initial body weight of the piglets. The positive results observed in the study were likely due to the adoption of a combination of probiotics, which is more effective than the application of isolated strains, according to well-established evidence in the literature (e.g. Yirga, 2015). Similarly, Hu et al. (2020) used a probiotic mixture during the lactation phase in sows. It was found that the use of *B. subtilis* and *Bacillus licheniformis* resulted in a decrease in the emission of harmful faecal gas (ammonium hydroxide) and provided protection against *E. coli*. With the exception of the dose/day (which was not specified by Hu et al. (2020)), the methodologies adopted by the authors were quite similar. Although the authors used the standard concentration of 10^9 CFU/g, the duration of the analysis was reduced compared to other studies, lasting only 11 days, and even then, it had significant effects on the sows evaluated. Therefore, the use of a probiotic mixture seems to enhance the effect of supplementation, reduce the time needed to observe good clinical results and also reduce costs. Below we cite other studies with a mixture of probiotics with good results in reducing mortality, weight gain, immunomodulation and defense against pathogens.

Improvement of aspects related to reproductive performance in pigs due to the use of probiotics were reported by Domingos et al. (2021), Laskowska et al. (2019) and Ma et al. (2019), who found reductions in the proportion of stillbirths and demonstrated that the use of probiotics increases the weight of newborn piglets. Zhang et al. (2020) demonstrated that the use of *B. subtilis* resulted in an increased number of low weight piglets that were able to thrive, overcoming possible consequences of poor competition for nutrients and restriction of uterine resources.

In addition to these effects, probiotic strains can stimulate the

production of immunoglobulins, such as IgG (Satora et al., 2021). Laskowska et al. (2019) and Tsukahara et al. (2018) applied probiotic mixtures containing lactic acid bacteria at the end of pregnancy and lactation and reported that there was an increase in the production of IgG in colostrum and milk. Both authors found that there was a decrease in the incidence of diarrhea among the sows and their litters. Furthermore, Laskowska et al. (2019) recorded a reduction in swine mortality and observed an increase in the levels of IgA, IL-10 and IL-4 detected in milk. The same authors also reported an increase in the production of other cytokines such as IL-2, TNF-α and IFN-γ, which indicated the immunomodulatory effect of the probiotic formulation studied. The presence of IgA in the milk can prevent pathogen adhesion to enterocytes and it is an important protective resource for young piglets that do not yet have the fully developed GALT system (Gut-associated lymphoid tissue) (Laskowska et al., 2019; Langel et al., 2020).

Satora et al. (2021) and Tsukahara et al. (2018) found that sows that received combinations of probiotics (*Enterococcus faecium*, *Lactobacillus rhamnosus* and *Lactobacillus fermentum,* and *Bacillus mesentericus*, *Clostridium butyricum* and *E. faecalis*) in concentrations of 10^6 to 10^9 CFU/g produced milk and colostrum with higher concentration of IgG and, as a result, in both studies the litter showed greater weight gain compared to the control group. These results were similar to those of Peng et al. (2020), which used a high concentration of the probiotic *S. cerevisiae* (10^{10} CFU/g).

Liu et al. (2020) used a dietary treatment with *Pediococcus acidilactici* (2.40×10^9 CFU/kg of diet) and found an increase in the total protein concentration in the blood of sows, including the proportion of immunoglobulins. Furthermore, reductions in the concentration of alanine aminotransferase (ALT) and haptoglobin compared to the control group were also described. High levels of ALT in blood plasma can be interpreted as an indication of liver damage/cytolysis in pigs (Hlatini & Chimonyo, 2016; Liu et al., 2020) or presence of viral infections (Xing et al., 2018), while haptoglobin is a hemolysis indicator (Minović et al., 2017), for which a very low concentration may indicate anemia. On the other hand, when associated with a reduction in red blood cell counts, high levels of haptoglobin are associated with inflammatory processes, infections, and injuries (Liu et al., 2020).

Thus, the maintenance of balanced concentrations of haptoglobin in the serum is an important indicator of animal health status. Liu et al., (2020) concluded that the reduction in haptoglobin concentrations is an important parameter in animals supplemented with probiotic, as there is a significant increase in haptoglobin concentration in the serum of pigs that have suffered tissue damage, infections, inflammation or even stress. Similar to this result, Peng et al. (2020) reported a reduction in the levels of transaminases in blood plasma, which indicated that sows treated with the probiotic *S. cerevisiae* apparently had better liver function.

5. Early weaned piglets: separation of pigs from the sow

In pig production, weaning represents a stressful event due to the sudden separation of pigs from the sow, and it may contribute to intestinal, immune dysfunctions (Campbell et al., 2013), digestive disorders and the highest death loss of post-weaned pigs from diarrhea caused by enterotoxigenic *E. coli* (Liao and Nyachoti, 2017). Probiotics can act in this phase by preventing disease, restoring microbiota balance after a transient drop in favorable bacteria and stimulating immunity (Barba--Vidal et al., 2019). Weaned piglets face psychological stress caused by changing their diet and environment (Yang et al., 2015a; Ross et al., 2010), and become more vulnerable to the development of diseases, which can negatively impact the animal's development (Siggers et al., 2008). For this reason, the use of probiotics at this stage can represent an important tool for improving animal health parameters.

As previously described, many differences were found in the methods used in studies with early weaned piglets. The average concentration of microorganisms in the probiotic additives used in this group showed the greatest variation (from 10^9 CFU/mL to 5×10^9 CFU/mL of probiotic product, and 1.2×10^9 CFU/kg to 5×10^{10} CFU/kg of feed), and it was not possible to establish a standard. However, it is possible to determine that the average of microorganisms in probiotic additives is between 10^9 to 10^{10} CFU/kg of product. In addition, most studies did not report the daily dosage administered to animals. In general, studies are limited to just reporting the percentage of probiotic present in the feed (generally 2–4%), without specifying the amount administered. In the studies with sows, most of the work was focused on evaluating health parameters associated with nutrition, milk quality and piglet mortality rate. However, in the group of early weaned piglets, health parameters are also evaluated, but most studies seem to focus on the antimicrobial potential of probiotics against pathogens. Thus, despite the differences found in the methodologies, we describe below the main results found in these studies.

Peeters et al. (2019) administered *C. butyricum* for 42 days and did not significantly reduce fecal excretion of the pathogen, had no serological response, and did not decrease the prevalence of *S.* Typhimurium in ileocecal lymph nodes in pigs challenged experimentally. The authors also did not show the dose/day administered, which makes a deeper discussion about the possible causes of these results difficult. A possible explanation would be the pathogen itself, since it is difficult to control, as the authors themselves claim.

Yang et al. (2020) used *Lactobacillus plantarum* and Tian et al. (2020) *Lactobacillus reuteri* in pigs weaned at 21 days and observed that the supplementation resulted in increased rate of weight gain due to better feed conversion provided by the production of digestive enzymes. However, the study by Yang et al. (2020) used a concentration of 10^7 CFU/g for 42 days, while Tian et al. (2020) used a concentration of 5×10^{10} CFU/kg for 175 days (both authors did not specify the daily dose). However, while the results were promising with different concentrations of probiotic supplementation, the data from both studies do not allow us to conclude that these promising results are due solely to the concentrations of probiotics, because other variables or factors (e.g. study design, pig genetics) were not assessed.

Dávila-Ramírez et al. (2020) and Cui et al. (2017) used *S. cerevisiae* at a dosage of 0.3% yeast culture, for 96 days in 14-days old weaned pigs, respectively. The authors found positive effects on weight gain, concentration of total protein in the blood, meat quality, changes in microbiota and intestinal morphology, decreased pH, increased mucosal immunity, increased IgA activity against pathogens, with a consequent reduction in colonization of pathogenic bacteria (*E. coli*). These results reinforce that *S. cerevisiae* used has several positive effects if added to the swine diet and indicates that it can be an alternative growth promoter (Elghandour et al., 2020).

Jørgensen et al. (2016) carried out a study in different phases of swine production, using the combination of *B. subtilis* and *B. licheniformis* in 28–42-day old and 42–70-day old pigs, and observed that probiotic combination improved the weight gain, digestibility and feed efficiency. The authors also noted that the administration appeared to be more effective in pigs between 42 and 120 days of age.

In another study, administration of 0.3% *B. licheniformis* and *B. subtilis* with *B. coagulans* and *C. butyricum* for 42 days in 28-day old weaned pigs improved weight gain, nutrient digestibility, decreased the emission of harmful gases and reduced the count of *E. coli* (Nguyen et al., 2019). Zhang et al. (2020) highlighted that the use of *Bacillus subtilis* in swine production had positive effects due to its ability to colonize the GIT.

Dowarah et al. (2018) administering *P. acidilactici* at a concentration of 10^9 CFU/g with 28-day-old pigs, observed an increase in globulin and albumin in the blood, as well as a decrease in pH and lipid oxidation. Wang et al. (2019), who also administered *P. acidilactici* in combination with *L. fermentum* at standard concentration for 28 days with piglets weaned at 28 days of age, observed decreased serum levels of IL-6 and IFN-γ, better average daily weight gain, feed gain and inhibition of pathogens growth in the cecal digesta.

Effects against pathogens were observed with probiotics administration in 28-day old nursery pigs. Yang et al. (2020) and Hanczakowska et al. (2016) found that the use of *Lactobacillus plantarum* and *E. faecium*, respectively, had an effect against *E. coli*, increasing the survival rate of pigs. According to He et al. (2019), it is possible to have an effect against *S. enterica* Infantis, reducing mortality, using *Lactobacillus johnsonii* at a concentration of 10^9 CFU/mL (in sterile saline), with pigs in the nursery phase. Li et al. (2019b) using *Lactobacillus delbrueckii,* in pigs weaned at 21 days of age, observed that probiotic administration stimulated the immune response, improved intestinal morphology, promoted growth, and mitigated diarrhea. Furthermore, Naqid et al. (2015) used the probiotic *Lactobacillus plantarum* and obtained increased humoral immune responses against the pathogen *S.* Typhimurium.

Thus, despite the different methodologies used in studies with probiotics, the results show that the administration of probiotics can be considered a strategy to combat gastrointestinal colonization by the main pathogens in swine at this stage of life. Compared to the other stages of pig development, this is one of the ones that presents the best results from supplementation with probiotics. In addition, the clinical impacts resulting from early colonization by beneficial bacteria can positively impact the health of the animal throughout the production cycle, which may represent an advantage of the early use of probiotics (Yang et al., 2020; Peeters et al., 2019; Cui et al., 2017).

6. Growing-finishing pigs: lower impact of probiotic supplementation

Pigs in the rearing and finishing phases have a fully formed GIT, have greater immunological capacity which results in greater resistance to diseases (Yang et al., 2015a). Due to the fact that the adult pig already has its microbiota formed, probiotics impact is lower compared to the use in nursery pigs. So, at this stage of production, probiotics are provided to improve the final quality of the pork meat, such as color and firmness, to improve performance and to decrease environmental pollutants in feces (Barba-Vidal et al., 2019). In this group there are also significant differences between the studies, making it difficult to establish a pattern. The concentrations used are quite varied, as well as the daily dose administered to each animal. At this stage, several aspects are evaluated, especially those related to the development of the animal, such as weight gain.

Combination of probiotics in growing and finishing pigs has been used to enhance their effect (Yirga, 2015). In a study with animals from the 78th day of life, Rybarczyk et al. (2020) administered a combination of three strains (*S. cerevisiae*, *Lactobacillus casei* and *L. plantarum*) and obtained a better weight gain, a significant increase in the LAB count in the microbiota and a decrease in the amount of *Enterobacteriaceae*. Accogli et al. (2018) used a combination of seven strains (*Streptococcus thermophiles*, *Bifidobacterium animalis*, *L. acidophilus*, *Lactobacillus helveticus*, *L. paracasei*, *L. plantarum*, *Lactobacillus brevis*) and obtained better weight gain and improved meat quality. Kwak et al. (2021) using a combination of six strains (*L. plantarum*, *L. fermentum*, *L. salivarius*, *Leuconostoc paramesenteroides*, *B. subtilis*, *B. licheniformis*), observed a reduction in pathogenic bacteria that resulted in better feed conversion, better weight gain and increased expression of genes related to the immune system, especially cytokine concentration, a possible biomarker to examine the host's immune response against bacterial infections. However, despite the promising results, it is important to mention that the studies show some important differences in the physiologies, such as the concentration used, daily dose (not specified in CFU by Accogli et al. (2018)) and supplementation time (not specified by Rybarczyk et al. (2020)).

The authors Van der Peet-Schwering et al. (2020) and Hao et al. (2020) used *B. subtilis* in association with *Bacillus amyloliquefaciens* and *Pediococcus pentosaceus,* with pigs aged 102 days and 39–63 days respectively. The best results from the study of Hao et al. (2020) were obtained by combining *B. subtilis* with *P. pentosaceus* at a concentration of 3.6×10^8 CFU/g and 2.5×10^8 CFU/g, respectively, with increased in concentrations of triglycerides and cholesterol, increased growth rate and weight gain, as well as better final meat quality. Furthermore, that study observed a decrease in pH and ammoniacal nitrogen (NH_3-N) concentration in feces, which indicated a decrease in contamination by this manure pollutant (Barba-Vidal et al., 2019). Van der Peet-Schwering et al. (2020), on the other hand, found results related only to action against the pathogen *Lawsonia intracellularis*, in a longer experiment of 102 days. The differences observed in performance among different probiotic combinations are not completely elucidated, but they may be due to several mechanisms of action possessed by the different strains (McFarland, 2020), and/or their interaction with hosts (Suez et al., 2019).

A study conducted by Dowarah et al. (2017) compared the use of *P. acidilactici* and *L. acidophilus* at a concentration of 10^9 CFU/g for 180 days with finishing pigs. This probiotic concentration was mixed in the basal diet and offered at a dose of 200 g/day/pig. The use of *P. acidilactici* had greater efficacy against *E. coli* and improved swine intestinal health, due to the possible synergistic probiotic effect with the intestinal microbiota. In addition, both strains had positive results in bowel morphology, increased weight gain, decreased NH_3-N concentration in feces and decreased pH, likely due to the production of short-chain fatty acids, which are metabolites of probiotics (Bajagai et al., 2016). The probiotic *P. acidilactici* showed better results compared to *L. acidophilus*, producing more fecal lactic acid and fighting diarrhea, which implies greater host species specificity with this probiotic (Dowarah et al. 2017).

The authors Joysowal et al. (2018) also compared the use of *P. acidilactici* and *L. acidophilus* at a concentration of 2×10^9 CFU/g for 90 days and observed better weight gain with the use of probiotics. The authors also used a dose of 200 g/day/pig of probiotic concentration mixed in the basal diet. *P. acidilactici* provided higher feed conversion efficiency, crude protein digestibility and nitrogen retention, as well as lower serum concentrations of triglycerides and cholesterol, compared to *L. acidophilus* use. It is likely *P. acidilactici* had better effects due to its swine origin, which could favor better interactions with the animal's GIT.

7. Safety

A factor that influences probiotics use is safety. Currently, much of the research related to probiotics addresses the safety of their use (Cohen et al., 2018). For a probiotic to be used in animal nutrition, it must have a good safety record (Yang et al., 2015a). Therefore, probiotics available on the market are considered safe and strictly regulated by organizations such as *Food and Drug Administration* and *The European Food Safety Authority* (Barba-Vidal, et al., 2019). However, the beneficial effects of probiotics are unique under defined experimental conditions, as the effects considered adverse depend on the physiological state of the host and its immunity (Sanders et al., 2010).

For meat consumers, probiotics are not a risk since they are added to the animal feed and their action is restricted to the TGI. In addition, even under prolonged exposure for those who have direct contact with the probiotics, they do not present a risk for human health (Yirga, 2015). There is no evidence that they pose a risk to the environment, as probiotics are partially decomposed and digested like other organic nutrients in the intestine. Only a small proportion is excreted viable in feces and survives in manure to reach fields and pastures (Yirga, 2015; FEFANA, 2005).

The transfer of resistance genes to the host microbiota is a growing concern that could result in loss of commercial interest. In 2005, the FDA allowed the sale of a probiotic product that contained, among others, a strain resistant to tetracycline and could transmit this gene (Cohen, 2018). Resistance gain from probiotic bacteria has already been described and needs to be further studied. If confirmed and not controlled, it may impair the use of probiotics, as their use is mainly

based on their safety and the hope of replacing the use of antibiotics and other chemicals to control infections (Wang et al., 2019). In light of this, many jurisdictions place limits on the levels of antibiotic resistance that can be present in strains under consideration for probiotic use.

Fortunately, complications resulting from probiotics use are extremely rare since most of these microorganisms already belong to human and animal microbiota. In general, such complications result from consumer health problems, such as immunodeficiency (Shanahan, 2012). Health authorities have established strict safety standards that ensure the use of probiotics. In the European Union and several other jurisdictions, sets of standards have been established and are constantly reviewed by experts. From the existing regulations, the approval of new probiotics is a careful process, in which several issues are analyzed, such as identification, specifications, purity criteria, method of production, intention to use, analysis methodology and results of studies that prove its effectiveness and security. In the United States, the FDA's Center for Veterinary Medicine maintains a rigorous notification program for ingredients used in animal feed. To obtain the safe status, the product must have its safety recognized through scientific evidence (FAO, 2016).

8. Conclusion

Probiotic supplementation can be applied in all different phases of swine rearing and has been shown to be efficient in the prevention, control, and treatment of infections, in addition to positively influencing the modulation of the immune response, bowel function and growth rate. However, from the data presented in this review, it was possible to observe that the benefits obtained with the use of probiotics vary at each stage of the animal's life and could be useful for decision-making by producers in rearing systems. Moreover, it is important to emphasize that the administration of probiotics in sows promotes improvement of performance in pregnancy, parturition, and lactation. In the initial phase of growth, there is a beneficial effect on the intestinal mucosa, on immunohematological parameters, as well as effects against pathogens. Finally, in growing-finishing pigs, there is an improvement in pig growth, meat quality and a reduction in environmental pollutants. Thus, the many benefits observed with the administration of probiotics in pigs are satisfactory and there is no doubt that new discoveries in this field will bring numerous changes in pig nutrition that will reflect an increase in productive capacity. Meanwhile, in general, even with all these benefits pointed out, it is still not possible to conclude that these all-beneficial effects come only from probiotic supplementation, since most publications do not adequately describe the methodologies applied, limiting conclusions from the point of view of the concentration of probiotic supplementation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 5

Improved productivity: Application of the quality management plan and tools in the field of university research

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Abstract

The aim of this study was to evaluate the implementation of a quality management (QM) plan based on the PDCA method to increase productivity in a research laboratory. For this, a management plan with the application of Ishikawa Diagram, WBS, PDCA, 5W2H, SWOT and Scrum methods/tools were implemented and monitored by an online work platform. It was observed an increased productivity that could be attributed to changes observed in planning, communication and engagement of the students/researchers. Of the 36 activities proposed 27 were fully completed (75%), 15 papers were published or submitted (65% of the total) and there was an increase in monitoring of all projects and compliance with their respective schedules. With the use of a data management plan, the online project and people management platform, it was possible to develop a new organizational culture and it was feasible to increase project monitoring, schedule compliance, and communication at different hierarchical levels. This is perhaps the first case report of successfully planned, implemented and quantified use of QM tools and techniques in a Brazilian public university laboratory that serve as a baseline model for researchers worldwide.

Keywords: *Organizational culture; monitoring; communication; productivity; quality management; research laboratory.*

1. Introduction

The management science gave rise to what is known today as Project Management, which is project management is defined as the leadership and application of techniques in order to ensure the delivery of the required product within the requirements, deadlines and budget (PMI, 2017). The term "project" is defined by the Project Management Body of Knowledge (PMBOK) as a temporary effort employed in the construction of a unique product, service or result. For a project to run successfully, several factors are necessary, the most important of which is the planning process which is defined by the management. Therefore, a correct and accurate execution of a Project Manager requires a professional with theoretical and practical knowledge that guarantees the integration, good functioning and adaptation of the different lines of work (Gharouni-Jafari & Noorzai, 2021).

For a project to be executed correctly, several work fronts, also called knowledge areas, need to be aligned, namely: scope, schedule, cost, quality, resources, communication, risks, acquisitions, stakeholders and integration (Boydjian, 2019). Thus, Quality Management (QM) is key since it is responsible for adhering to requirements and implementing continuous improvement techniques in accordance with business needs, that are dependent on quality and the demands of the company (Baker, 2018). Therefore, applying methods associated with Quality Management is to improve processes in order to optimize deliveries, both qualitatively and quantitatively, through the application of various tools (Baker, 2018). The Ishikawa Diagram, one of the main methods used, according to Wong et al. (2016), make it possible to unravel, organize and understand the demands of an audience in order to establish the link between an "effect" and its probable "causes".

The Work Breakdown Structure (WBS) plays an important role in the design of the work fronts of a project, it is used to divide a job into deliverables and to subdivide these into smaller parts, making them easier to manage. Its structure highlights the hierarchy and needs of the project and, as it is structured with a focus on deliveries, it is an essential tool for building the scope of projects in the initial phase (Cerezo-Narváez et al., 2020). According to the PMI (2017), another important tool is the definition of the project scope which requires detailed documentation of the project's objectives and stages, as well as its characteristics and stakeholders' requirements.

Once the main problems of an organization and their causes have been identified through the Ishikawa Diagram, and the WBS has been defined, it is necessary to apply

quality management (QA) methods; mainly through the use of the PDCA. The term PDCA derives from the combination of the terms “Plan”, which refers to the action plan to solve a given problem; “Do”, which refers to the implementation and monitoring of the strategy created in the previous step; “Check”, which refers to checking the progress made and possible adjustments necessary after the implementation of the strategy; and “Act”, which refers to the standardization and adoption of lessons learned during the process (Martins & Laugeni, 2005).

The SWOT analysis – that stands for “Strengths”, “Weaknesses”, “Opportunities” and “Threats” – is widely used in projects that focus on improving strategy. This tool allows for a more assertive decision making, reducing risks, optimizing the resources applied and expanding a company's opportunities (Longhurst et al., 2020). In addition, the 5W2H tool is used in conjunction with the PDCA method, especially in the construction of an action plan for a given project. The name “5W2H” represents basic questions that need to be answered before making important decisions about how a problem and/or opportunity will be addressed, with the 5Ws being “What?” (What will be done?), “Who?” (Who will do it?), “When?” (When will it be done?), “Where?” (Where will it be done?) and “Why?” (Why will it be done?); and the 2Hs “How?” (How will it be done?) and “How much?” (How much will it cost?) (do Prado et al., 2021).

Finally, there is the Scrum, which is an agile work methodology that is recognized and widely used by companies around the world (Briscoe, 2006). This methodology's main event is the “Sprint”, which is a cyclical event in which the project and its results must be evaluated and, if necessary, adjusted (Briscoe, 2006). The dynamism of the Scrum methodology allows for a higher success rate in the delivery of project products, especially those related to sectors with constant changes, such as research in the biomedical area. Good project management practices described in the PMBOK and their use in conjunction with the Scrum methodology can represent gains for projects (Briscoe, 2006).

The above-mentioned methods are widely applied in private companies and in industry (Aldenny et al. 2022; Qiu & Du, 2021; Fernandes et al., 2021; Prasetya et al. 2021). However, few reports are observed from experiences in research laboratories at universities. Much of Brazil’s scientific production is carried out in university research laboratories, with science and technology production also associated with academic training. For this reason, these spaces generally use traditional management methods,

with productivity monitoring focused only on the fulfilment of institutional goals, without significant changes in methods (de Almeida and Guimarães, 2017).

Therefore, due to above mentioned circumstances, there are opportunities for improvement in university research laboratories with the adoption of QA techniques through a management plan, using tools/methods such as the Ishikawa Diagram, PDCA, SWOT and Scrum. This implementation would also aid in creating a more management-oriented students which in turn would have a more valued profile. Thus, the aim of this study was to evaluate the implementation of a quality management (QM) plan based on the PDCA method to increase productivity in a research laboratory.

2. Material and methods

This case study was carried out in a research laboratory in the field of pharmaceutical and biotechnology sciences, in the Sao Paulo state. It has a supervisor, 10 graduate students (three post-doctoral students, six doctoral students and one master's student) and 2 undergraduate students.

After all the participants were briefed and introduced to the concepts of project management, its methods and tools, the Agile Scrum technique was used (Hron & Obwegeser, 2022; Schwaber & Sutherland, 2020), with adaptations as follow: The laboratory supervisor became the “Project Owner” (PO), a researcher responsible for implementing the management techniques became the “Scrum Master” (SM) and the other researchers, the “Scrum Team”. Weekly “Sprint” meetings were adopted, in which deliverables had to be presented and analyzed; and fortnightly meetings, in which progress in the projects was monitored and new strategies were adopted. All methods and tools were applied and decisions were taken together, with the “Scrum Master” playing the role of mediator and the “Project Owner” in the decision-making role.

Different approaches were used to diagnose the problems to be solved, construct an action plan focused on quality management (with its respective tools), and create an online platform for monitoring quality based on the following methodologies:

- *Creation of an Ishikawa Diagram:* aimed at identifying the main productivity-related problems in the laboratory and their possible causes. Its construction was a group activity, as described above, where everyone was invited to list the main problems that would prevent productivity

improvement and their causes; models previously described in the literature were used (Wong et al., 2016).

- *Construction of the Work Breakdown Structure of the project:* aimed at identifying the work fronts necessary to achieve the study object. It was built setting using models previously described in the literature (Cerezo-Narváez et al., 2020; PMI, 2017).
- *Application of the PDCA, SWOT and 5W2H methods:* aimed at diagnosing and monitoring the challenges and progress observed, as well as taking better advantage of the opportunities generated (Figure 1). Its construction took place in a group and it was applied through the implementation of the online management platform, as described below. Models previously described in the literature were used (Martins & Laugeni, 2005; Longhurst et al., 2020; do Prado et al., 2021).

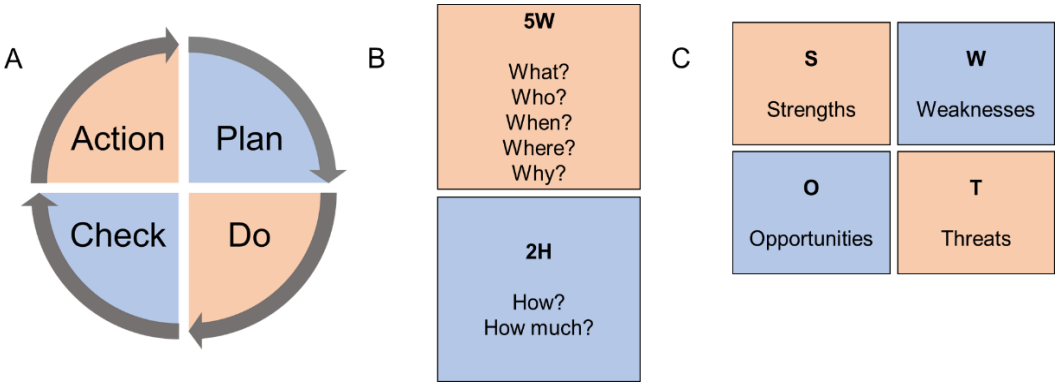

Figure 1. Methods used in this study. (A) PDCA, (B) 5W2H and (C) SWOT.

- *Creation of a data management plan:* aimed at organizing/defining schematically how data is generated, stored, protected and made available. Its creation took place through the work of the Scrum Master and Project Owner, based on the needs observed in the previous stages. This step is especially significant since the governmental entities that fund laboratory research have sought increasingly thorough data management plans.
- *Creation of an online project management platform (Monday, daPulse, TelAviv, Israel):* aimed at monitoring the progress of the improvements implemented in the previous stages.

The combination of the above approaches formed the basis for the construction of the Quality Management action plan. A study was carried out for a period of approximately six months, in which data collection was carried out, the action plan was constructed, the online work platform was created and, finally, talks were addressed to all the employees of the research laboratory in order to make them aware of the guiding concepts of the techniques to be used, their importance, and how to apply them correctly in everyday activities.

2.1. Customer Definition

It is a public institution that aims to finance scientific research, especially in the academic field, based in the State of São Paulo, Brazil. The use of its resources must presuppose the approval of a project in which the expected results of the research are useful in the implementation of socially relevant public policies. The institution chooses its priority areas and, based on that, selects the projects it will finance according to its own criteria. The counterpart for the allocation of resources to a project is, as mentioned, the proposition of alternatives to problems of social relevance, and its materialization takes place through the creation of new forms of diffusion and dissemination of acquired knowledge, that is, the publication of these results. For contractual reasons, the customer will not be identified by name.

2.2. Definition of Productivity

With the definition of the client and the expected objectives with its lines of research funding, it was also possible to specify what would be considered as "productivity", one of the central themes of this project. Thus, it was defined that, in this context, productivity would be the entire result generated from the execution of the research project that could be measured by the client's criteria and the activities necessary for their delivery:

a) *Proposing alternatives to problems of social relevance:* it is understood as the core of the project, that is, it must be built in order to generate a benefit to society. In this sense, only works that strictly follow this rule are approved and, given that the present work has already been approved by the institution, it fits these requirements. For contractual reasons, especially with regard to

intellectual property, the theme of the project will not be described more comprehensively.

b) *Dissemination of acquired knowledge:* it is understood as the publication of articles in international scientific journals, related to the research area, with a high impact factor and classified as Q1, in the largest possible number.

For the project to continue to receive funding, it was also necessary to meet basic requirements with the host educational institution. These were also taken as productivity measures, namely:

c) *Compliance with institutional deadlines:* it is understood as the delivery of internal reports on the results of the projects and their presentations to evaluators.

d) *Delivery of renovations, purchase of inputs and monitoring/repair of equipment: it is understood as the management of basic inputs for the work of researchers:* its monitoring started to be done regularly and, if at each new cycle all the inputs were available, the equipment was working and the renovation deadlines were up to date, the activity was marked as accomplished. At the end, the percentage of activities delivered was measured.

3. Results and discussion

3.1. Diagnosing faults and their causes

Productivity is one of the most important parameters of success in a university research laboratory and is related to the production of knowledge/technologies and their publication through articles and patents. As a starting point for this study, planning meetings were held with the participation of all laboratory members and coordinated by the Scrum Master, in which application of the methods and tools began. The starting point was given using the Ishikawa Diagram. According to Wong et al. (2016), from the Ishikawa Diagram it is possible to unravel, organize and understand the demands of an audience in order to establish the link between an “effect” and its probable “causes”.

At the meeting, the Scrum Master asked the Scrum Team which aspect of the laboratory should be the main target for the application of quality management

techniques. It was unanimously agreed that constant improvement in productivity was considered by all to be the most important aspect for the growth of the laboratory and its members. Next, construction of the Ishikawa Diagram began with everyone's participation. The Diagram was divided into six areas with defined causes (Figure 2), namely:

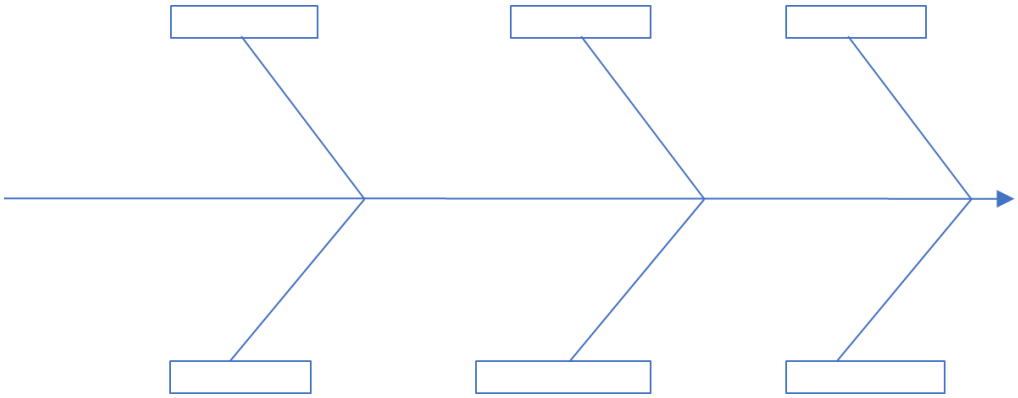

Figure 2. Ishikawa diagram and the identification of problems and their causes.

- *Productivity:* there is a need to define more clearly the collective and individual objectives in the short/medium term and to increase the periodic monitoring of production.
- *Hierarchy:* there is a need for the definition of individual attributions at a general level; for the creation of work centers (with a leader); and for clear, central positioning on certain subjects.
- *Management:* there is a need for greater monitoring of the different aspects related to work, greater rigor in meeting deadlines, and more detailed planning of processes and deliveries.
- *Training:* there is a need to create a training plan and a career plan for all individuals, and implementation/validation of work protocols.
- *Individual Responsibilities:* there is a need to increase personal support and gradually raise individual productivity, eliminating non-compliance with institutional deadlines and increasing proactivity.
- *Organizational Culture:* there is a need for a clear definition of the overall objectives of the laboratory in the medium/long term, and an increase in the participation of agents in solving problems.

Though the analysis of the proposed Ishikawa diagram, it was found that most of the causes of low productivity were related to management and communication failures, especially the lack of a single tool to control the schedule, with goals and short/medium term deliveries, at a general and individual level. Therefore, as observed in the study by Campbell et al. (2020), the lack of communication was pointed out in all the studies analyzed as one of the main causes of the drop-in productivity in work environments. The authors also report that as companies implement measures that make employees engage with other members, productivity tends to increase substantially.

According to Gunasekaran et al. (2019), the application of tools related to quality management has become increasingly indispensable in the contemporary world, given the constant changes in markets, business models, technologies and people. Nasim et al. (2020) state that higher education institutions have a high degree of competitiveness and that they are very different from other sectors of administration, such as industries. The project manager's work in a university environment is challenging, and the application of quality management tools must always consider the specifics of each institution. Therefore, it is of great importance to use tools that allow correct problem diagnosis.

Most scientists who have implemented quality management tools in the laboratories they direct have wondered why structured quality management has not reached the academy (university research laboratories) in a massive way. Unlike clinical practice and R&D in the pharmaceutical industry, structured quality management is virtually unknown in preclinical and basic biomedical research, yet it is fraught with methodological complexities, error proneness, and cumbersome laws and regulations, all added to a highly fluctuating workforce. Scientists, who typically do not have a working knowledge of quality management, find its normative language, nomenclature, and processes aversive. Furthermore, most quality management systems have been developed for companies or service providers and therefore have limited applicability to academic research, making it difficult to motivate scientists to work with quality management systems on a daily basis (Dirnagl et al., 2018). As a result, it is critical that meetings with the entire laboratory team be held as a starting point, where the objective of the quality management system and its operation is explained, and it is emphasized that this new system will help to achieve the goals (individual and team) more expeditiously and within the established times.

After the initial diagnosis, the results were presented and discussed in a meeting, this time with the Project Owner, Scrum Master and Scrum Team, followed by

construction of the WBS (Figure 3). According to Cerezo-Narváez et al. (2020), WBS is a tool used to divide a job into deliverables and subdivide these into smaller parts, making them easier to manage. Its structure highlights the hierarchy and needs of the project; as it is structured with a focus on deliveries, it is an essential tool for building the scope of projects in the initial phase.

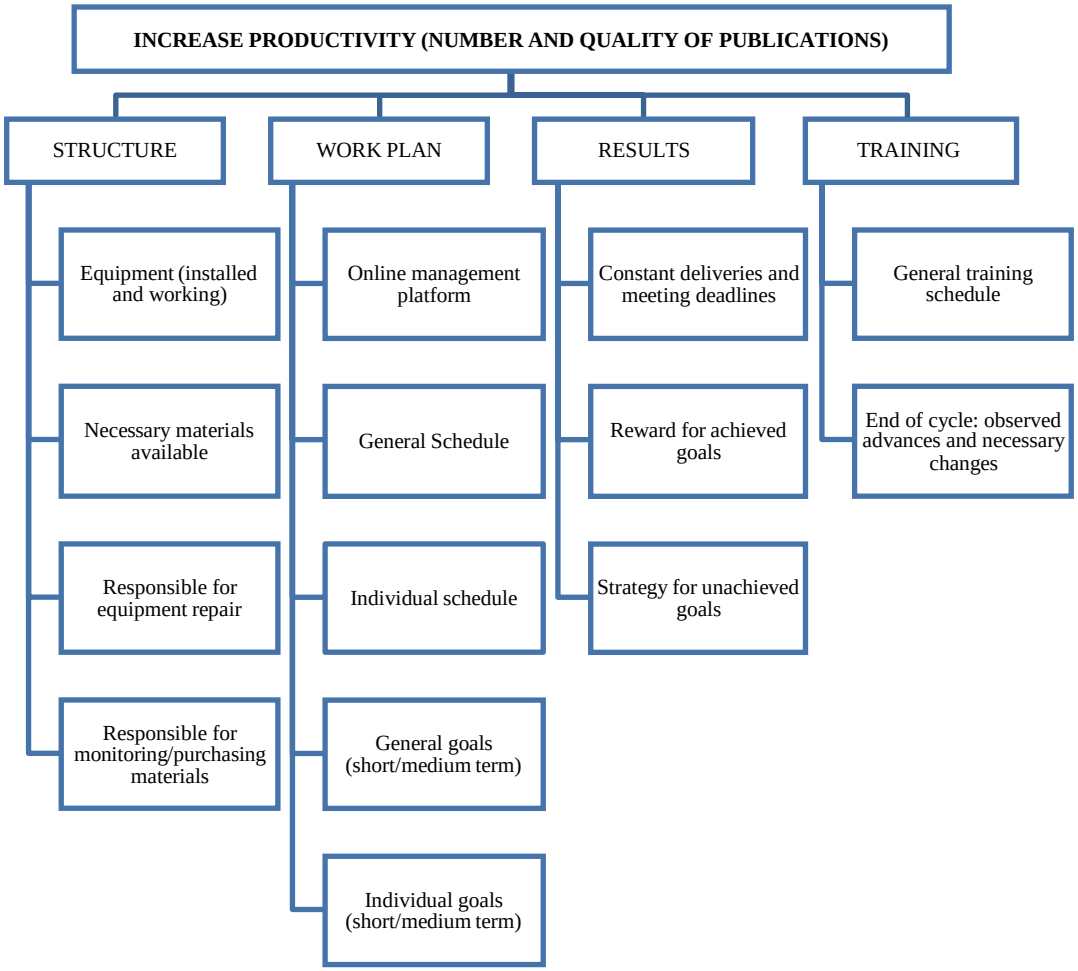

Figure 3. Work Breakdown Structure of the project and division of project work with focus on deliverables.

At this stage, once again all laboratory members were invited to participate. The Scrum Master took the role of moderator and all the participants' doubts about the goals of the WBS were clarified. Members were encouraged to participate in the construction of the WBS, especially in the division into deliverables and their subdivision into work packages. From the construction of the WBS, it was possible to distinguish the priority work fronts within the project and align them with the objectives and deadlines set by the Project Owner, divided into four major areas:

- *Structure:* corresponds to the physical components necessary for the proper functioning of research activities; without these, deliveries (articles, reports and patents) are not feasible.
- *Work plan:* comprises the outlining and monitoring of goals and meeting deadlines in greater detail and with short/medium term goals; it was observed that the existing goals were medium/long term, representing a risk to the fulfillment of delivery deadlines; the construction of an online platform for monitoring the schedule and deliveries was approved.
- *Results / Training:* concerns the main parameters that will be evaluated. Deliveries and training constitute the reason for the existence of the laboratory, since it is expected that the investments applied there will generate results for society and guarantee quality training for students.

The importance of good WBS design has already been mentioned in the literature. Fernandes et al. (2018) carried out a study with the object of identifying and proposing a hybrid management method that would meet the needs of "Stakeholders" in the research and development area on the university-industry axis. Among other methods cited, the authors highlight the importance of WBS as an important tool that can be applied in various contexts at the university-industry interface. They also highlighted that its benefits can be increased when it is used in conjunction with project monitoring software, in this case with a direct impact on meeting project deadlines (schedule).

To finalize the diagnosis stage, the SWOT tool was applied in order to provide important information for improving the strategic planning of the action plan. The method of application of the tool was similar to the previous steps, i.e. including the participation of all individuals, with the Scrum Team having the function of proposing ideas and opinions, the Scrum Master of mediation and the Project Owner of taking the final decision. The result of the meeting held for the application of the SWOT tool is described in detail in Table 1.

Table 1. Application of the SWOT tool.

SWOT	Description
Strengths	Laboratory with funding for activities, good management and participatory agents - and open to changes;

Weaknesses	Constant need to increase productivity, inflexibility of change in aspects related to the university and low knowledge of agents regarding project management techniques;
Opportunities	Increased productivity and management improvements promote increased opportunities for students and funding for the laboratory;
Threats	Short deadlines for delivery of results by the laboratory, need for accurate planning and effective participation of agents for success;

The components related to Strengths and Opportunities were used to build the laboratory development strategy; they were materialized in the quality management tools which were used (described below). The Weaknesses and Threats components received the most attention. The ways found to reduce the Weaknesses were: creation of schedules for delivery of activities/products at a general and individual level; and weekly talks and discussions with the Scrum Team about the importance of quality management tools. The ways found to reduce the Threats were: constant monitoring and maximum attention to delays in schedules; and constant presentation of responses to criticisms and suggestions from specialists.

The next phase of the study took place through the construction of strategies using the PDCA method, which is divided into four stages. The objectives of each stage and the activities carried out to achieve them are described in Table 2.

Table 2. PDCA method according to project specifics.

PDCA	**Objective**	**Strategy Adopted**
Plan	• Definition of the problem to be solved; • Creation of an action plan; • Construction of an online management platform;	• Ishikawa Diagram; • WBS and 5W2H; • Implementation of the Monday platform;
Do	• Execution of the defined plan;	• Use of the Monday platform to centralize project management and improve communication;
Check	• Continuous monitoring of results;	• Adaptation of Scrum; • Definition of the Project Owner, Scrum Master and team; • Deliverables to be made (Product Backlog) based on WBS; • Weekly meetings (evaluation of

		deliveries, Sprint);
Act	• Application of corrective actions	• Weekly meetings (Sprint) and biweekly meetings (readjustment of goals and new plans);

Zhang et al. (2019) state that the traditional teaching methods used in universities no longer meet all the needs of the market, and that universities have had to deal with innumerable new challenges in recent years. The authors also say that one way of meeting these challenges is by implementing new process control methods focusing on QM, for example the PDCA cycle. Gulden et al. (2020) state that universities are under constant pressure from different sectors (political, economic, social), and that the application of practices related to quality management can be useful in improving their internal organization, optimizing processes and results, and attracting investment.

To ensure that the PDCA method was applied successfully, other tools were used to guarantee accurate planning. The 5W2H tool (Table 3) was important for gaining a better understanding of the problems encountered in previous stages, increasing the chances of achieving the expected results (do Prado et al., 2021).

Table 3. Application of the 5W2H tool.

5W2H	Description
What?	Progressive increase in productivity;
Why?	"Productivity" is one of the most important metrics in university research centers;
Who?	PO and Scrum Master (responsible for the action plan);
Where?	Research laboratory at an important public university;
When?	Immediate start after planning approval; duration of 6 months (experimental phase);
How?	Creation of an action plan based on quality management methods and tools;
How long?	There will be no additional costs to the project with the application of the action plan;

3.2. Data management plan and online management platform

In order to guarantee the preservation and integrity of the data collected during the execution of the current project, as well as the correct dissemination of its results, a data management plan was prepared. To this end, some aspects considered vital for correct management were considered, such as the promotion and valuing of the data found, covering all stages of the project from conception to completion of the activities

foreseen in the schedule. The management plan consisted of the following steps: construction, archiving, sharing and security (figure 4).

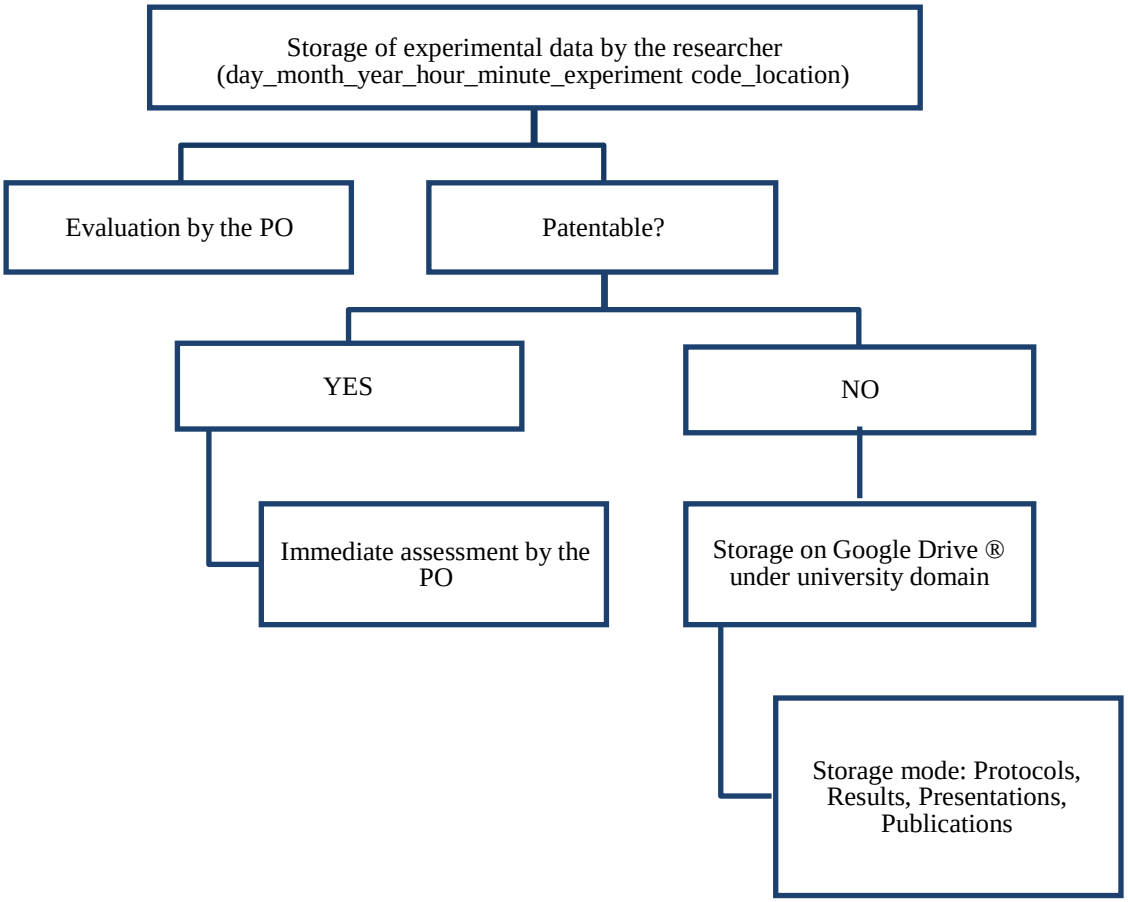

Figure 4. How data will be archived through shared folders.

All laboratory data will/was stored in a google drive, following the below decision algorithm:

It was possible to observe that the implementation of this procedure facilitated access to protocols, results of previous research and other documents of interest to laboratory researchers, and reduced the time that would be lost in the search for the necessary data. The increase in the organization of laboratory data may be one of the factors that had a direct effect on improving laboratory productivity compared to the six months prior to the implementation of this QM plan (discussed below). In addition, it was possible to observe that this organization influenced individual productivity, as none of the researchers/students missed a date set for the delivery of their institutional reports.

It is important to emphasize that a data-management plan explains how researchers will handle their data during and after a project and encompasses creating, sharing, and preserving research data of any type. Many funders are asking grant applicants to provide data plans. Requirements vary from one discipline to another. But in general, scientists will need to explain, before they start any research, what data they will collect, how it will be recorded, described, kept safe, and curated, and who will have access to it after the research is done (Schiermeier, 2018).

3.3. Online management platform

At the end of the planning phase, the present work was presented to the Project Owner and then to the collaborators. At this stage, it was ensured that everyone was aware of the methods and tools that would be used from then on. The online project management platform was also presented and all the doubts raised by employees were answered. The platform was built by the Scrum Master in partnership with the Project Owner, and experimental application was programmed for a period of six months. It was necessary to list the main work fronts and the schedule, both at the general and the individual level. The platform was divided into four work areas, namely:

- *General Activities:* intended for general laboratory activities, such as replacement/repairs, purchase of materials, development/validation of new protocols, installation of equipment, etc. The demands that led to its construction came from specific meetings with all laboratory employees, where everyone was asked about the demands of the workplace.
- *Publications and Reports:* intended for the project's products, that is, publication of articles, reports and patents. It is possible to monitor all products, their respective phases, delivery dates and those responsible. The construction of this work area took place after a meeting between the Scrum Master and the Project Owner, in which the Project Owner defined the publication goals for the semester and the general and individual deadlines of the projects.
- *Individual Projects:* intended to monitor progress/delays at the level of individual projects, that is, academic research projects necessary for employees to obtain an academic title.

- *Protocols and Documents:* intended for the storage and easy access by employees of all the knowledge produced and validated by the laboratory. This area contains validated protocols, reference articles, reagent leaflets, equipment manuals, list of working materials available in the laboratory, etc. To construct this area, the Scrum Master took stock of all the protocols, products, equipment and articles/theses used for reference in the laboratory.

The reliance on the results of laboratory research demands increased traceability and data integrity, ensuring the quality of transferrable results to the clinical setting. In recent years, the scientific community has experienced an awareness regarding a reproducibility crisis related to factors such as the pressure for publication, low statistical power, and insufficient supervision. On the other hand, adequate management, training, and good practices may improve data quality by improving workflow, avoiding errors, and providing traceability (Baker, 2016).

However, academic laboratories experience several critical barriers to developing and implementing a good laboratory practice-compliant infrastructure **(**Adamo et al., 2012). Timóteo et al. (2021) claim that an online management platform at academic centers should explore tools that facilitate supervision and achieve goals. In this context, digital systems are among the most important tools available for efficient management. Laboratory information management systems, specifically online management platforms, offer databases and automation that allow experimental data tracking and storage. These tools offer solutions to laboratory management, coping with other aspects of quality assurance related to communication, staff, multiuser equipment schedule and maintenance, standard procedures, and inventory control, which are fundamental in the full spectrum of a laboratory's workflow (Timóteo et al., 2021).

3.4. Results observed after 6 months of work

After a period of six months in which the quality management tools were applied, especially the use of the online management platform, the following results related to the Ishikawa Diagram were observed:

- *Productivity:* the collective objectives were listed (mainly relating to the publication of articles, approval of reports by government research funding agencies and reforms), with those responsible and the delivery schedule. The

individual objectives (also relating to the publication of scientific articles and delivery of reports to the university), with those responsible and the delivery schedule, were likewise listed (Figure 5, 6). Compliance with the schedule was monitored through weekly and fortnightly meetings.

Figure 5. Area on the online work platform for publications and reports.

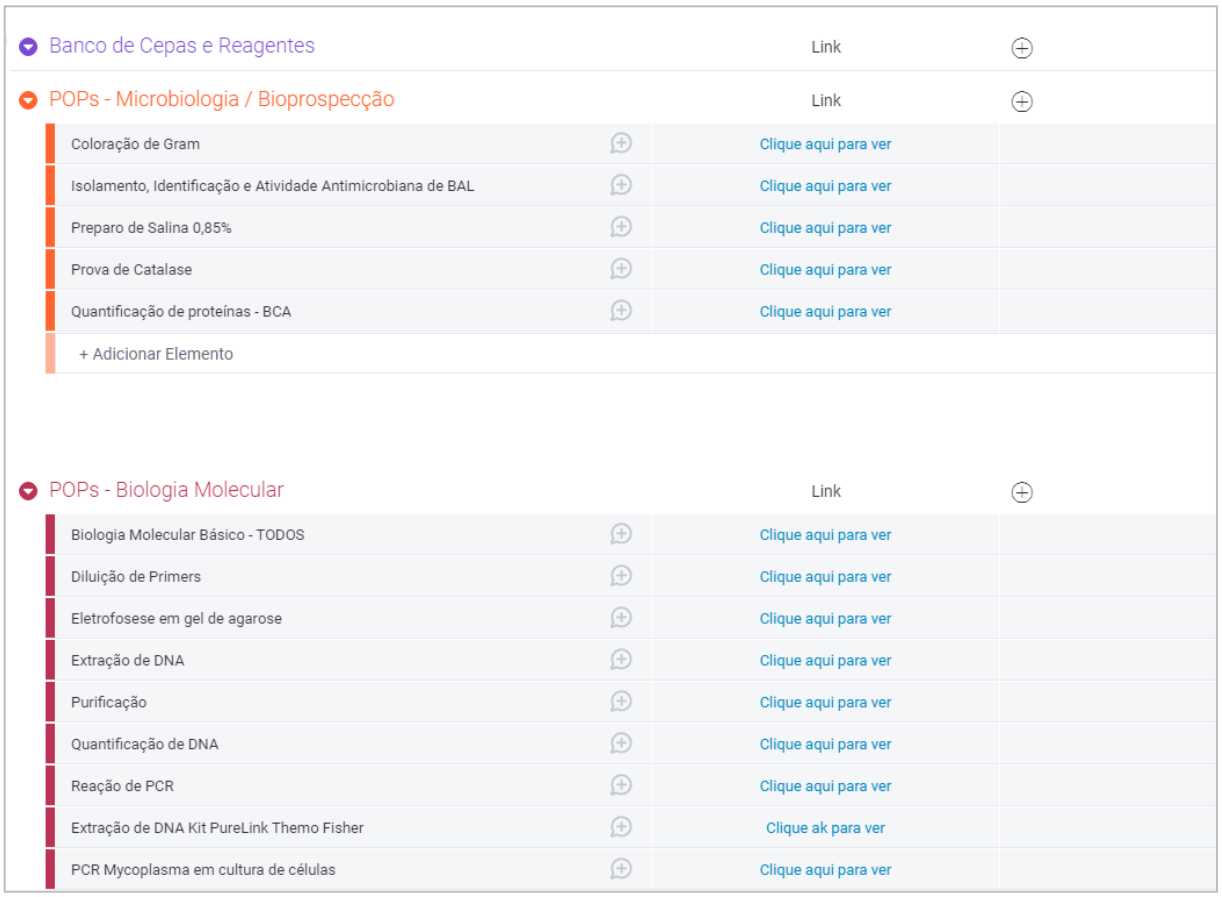

Figure 6. Area of the online work platform for validated experimental protocols and other normative documents.

- *Hierarchy:* hierarchy levels were defined (general coordinator > postdoctoral students > doctoral students > master's students > undergraduate students), and 3 working groups were created led by the 3 postdoctoral students of the laboratory. It was defined that the final decisions on highly important topics would be taken by the general coordinator during the fortnightly meetings.
- *Management*: with the implementation of the online management platform, there was an increase in monitoring of all projects and compliance with their respective schedules.
- *Training:* a monthly schedule of training and presentation of results was created. At these events, students presented their results and were evaluated by external professors. Furthermore, these external teachers were encouraged to share experiences and knowledge. The laboratory protocols

were all validated and made available on the online management platform. The introduction of career planning will be part of a future study.

- *Individual Responsibilities:* with the creation of working groups and the management platform, students now have greater personal support. With the weekly monitoring of compliance with deadlines, there was also an increase in deliveries and, as a consequence, in proactivity. No institutional deadlines were missed during the study period.
- *Organizational Culture:* as "organizational culture" is one of the causes that will require more time to resolve, it was not a focus of this work. It will be discussed in a future study based on the results observed after the implementation of the new methodologies.

Likewise, the following WBS-related results were observed:

- *Structure:* a schedule was created for equipment reforms and repairs; in addition, individuals were designated who were responsible for monitoring equipment in need of repair, and the need to purchase new inputs.
- *Work plan:* schedules were created at a general and individual level, with their respective goals.
- *Results / Training:* constant delivery of results and the achievement of goals were ensured through weekly meetings and the management platform. As a strategy for unachieved goals, it was stipulated that overdue goals should immediately be raised to the status of priority work for the individual concerned (at the individual level) or the group (at the collective level).

Regarding the online management platform, the following results were observed:

- *General Activities:* the main gain arising from this area of work was to ensure that all employees had all the necessary working conditions, avoiding waste of time and materials. When a new problem arose, communication with the Project Owner was immediate, facilitating communication in the laboratory and reducing the time needed for repairs and purchase of materials that could

affect laboratory productivity. At the end of the 6 months' application of the tool, of the 36 activities proposed, 27 were fully completed (75%). The other activities were not completed due to factors external to the laboratory – especially replacements, which depend on the release of resources by the university.

- *Publications and Reports:* the main gain resulting from this area of work was to facilitate the monitoring of progress/delays in projects and ensure that the action plan was followed, that deadlines were met, and that the products were delivered; i.e. that the purpose of this study was achieved. At the end of the 6 months' application of the tool, of the 23 articles planned, 15 were published or submitted (65%). In addition, the laboratory production report with a research funding institution was approved (Figure 7).

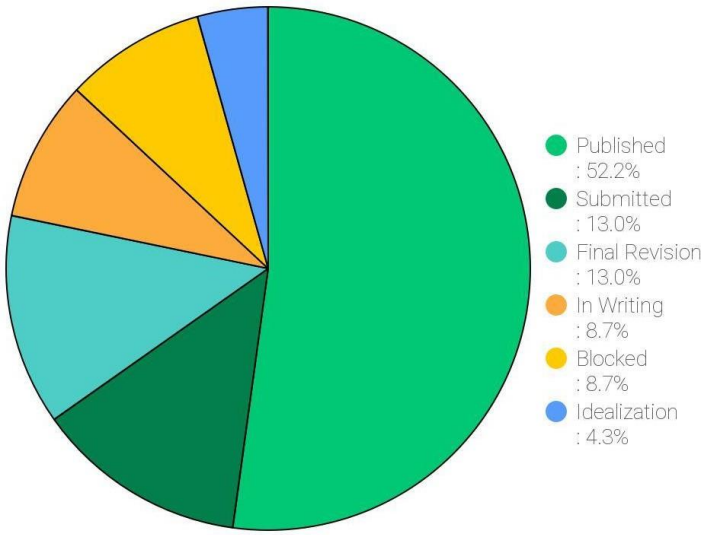

Figure 7. Articles published or submitted after six months of implementing the quality management techniques.

- *Individual Projects:* the main gain resulting from this area of work was to improve the monitoring of progress/delays in individual projects and ensure the success of each employee's projects. In addition, it also ensured that the Project Owner was able to monitor progress in real time and identify employees who needed support.

- *Protocols and Documents*: the main gain from this work area was the standardization of how work was carried out, reducing the possibility of errors and delays.

Our experience after 6 months of work left us convinced that a structured approach to QM has enormous potential to improve the quality of research. We agree with Dirnagl et al. (2018) that QM should have the following desirable features: it should consist of mandatory core elements and optional supplement modules and therefore be scalable and adjustable to research environments; it must be financeable and sustainable; it must support common daily laboratory practices and address prevalent biases and validity threats; it must incorporate various regulations; and it should lead to a more transparent and trustworthy research process.

4. Conclusion

Much of Brazilian scientific production comes from public universities, and productivity is measured, in particular, by the publication of articles and patents. However, there are few reports in the literature of studies on quality management in research laboratories in Brazilian universities. Through the application of Quality Management methods and tools, it was possible to identify problems and build an action plan. It was observed that productivity could be improved by working on aspects related to it, especially, with planning and communication. In addition to the action plan, a data management plan and an online project and people management platform were also built. The platform made it possible to improve the monitoring of work progress, compliance with schedules, and communication at different hierarchical levels of the project, in addition to implementing a new organizational culture. With the full use of the Monday platform, the foundations were laid for substantial advances in the delivery of high-quality, high-impact scientific articles since the research laboratory has begun to work with the same methods used by large companies. Similarly, it will be possible to stagger delivery processes very soon, allowing the research laboratory to transform itself into a small service provider. This change will not only allow the laboratory to expand, but will also provide students with an experience similar to that seen in the corporate world, providing them with better technical training and job opportunities. A study will be conducted in the future to determine how the proposed changes will affect the research laboratory.

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ANNEX 1

biomolecules

MDPI

Article

New Insights into the Antimicrobial Action of Cinnamaldehyde towards *Escherichia coli* and Its Effects on Intestinal Colonization of Mice

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Abstract: *Escherichia coli* is responsible for cases of diarrhea around the world, and some studies have shown the benefits of cinnamaldehyde in the treatment of bacterial disease. Therefore, the objective of this study was to evaluate the effects of cinnamaldehyde in mice colonized by pathogenic *E. coli*, as well as to provide more insights into its antimicrobial action mechanism. After determination of minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations, the interference of cinnamaldehyde in macromolecular pathways (synthesis of DNA, RNA, protein, and cell wall) was measured by incorporation of radioisotopes. The anti-adhesive properties of cinnamaldehyde towards *E. coli* 042 were evaluated using human epithelial type 2 (HEp-2) cells. Intestinal colonization was tested on mice, and the effect of cinnamaldehyde on *Tenebrio molitor* larvae. Cinnamaldehyde showed MIC and MBC values of 780 µg/mL and 1560 µg/mL, respectively; reduced the adhesion of *E. coli* 042 on HEp-2 cells; and affected all the synthetic pathways evaluated, suggesting that compost impairs the membrane/cell wall structure leading bacteria to total collapse. No effect on the expression of genes related to the SOS pathway (*sulA* and *dinB1*) was observed. The compound did not interfere with cell viability and was not toxic against *T. molitor* larvae. In addition, cinnamaldehyde-treated mice exhibited lower levels of colonization by *E. coli* 042 than the untreated group. Therefore, the results show that cinnamaldehyde is effective in treating the pathogenic *E. coli* strain 042 and confirm it as a promising lead molecule for the development of antimicrobial agents.

Keywords: cinnamaldehyde; intestinal colonization; natural products

1. Introduction

Escherichia coli is an important pathogen responsible for numerous cases of diarrhea worldwide, representing a serious problem for immunocompromised individuals, and especially children [1–4]. Several reports have associated diarrhea with significant delays in childhood development [1,3,5].

In a study carried out in South America, Africa and Asia, in children and adults with diarrhea, the predominant pathogen isolated in fecal samples was enteroaggregative *E. coli*

ANNEX 2

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BRIEF REPORT

Long-term survive of *Aliarcobacter butzleri* in two models symbiotic interaction with *Acanthamoeba castellanii*

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Abstract

Aliarcobacter butzleri (formerly known as *Arcobacter* butzleri) is an emerging food-borne zoonotic pathogen that establishes in vitro endosymbiotic relationships with *Acanthamoeba castellanii*, a free-living amoeba. Previously, we described that this bacterium acts as an endocytobiont of *A. castellanii*, surviving for at least 10 days in absence of bacterial replication. Thus, the aim of this study was to evaluate the ability of *A. butzleri* to survive as a long-term endosymbiont of *A. castellanii* for 30 days in two models of symbiotic interaction with *A. castellanii*: (i) endosymbiotic culture followed by gentamicin protection assay and (ii) transwell co-culture assay. The results allow us to conclude that *A. butzleri* is capable of surviving as an endosymbiont of *A. castellanii* for at least 30 days, without multiplying, under controlled laboratory conditions. In addition, in the absence of nutrients and as both microorganisms remain in the same culture, separated by semi-permeable membranes, *A. castellanii* does not promote the survival of *A. butzleri*, nor does it multiply. Our findings suggest that the greater survival capacity of *A. butzleri* is associated with their endosymbiont status inside *A. castellanii*, pointing out the complexity of this type of symbiotic relationship.

Keywords *Acanthamoeba* · *Aliarcobacter* · Endosymbiosis

Acanthamoeba castellanii is a ubiquitous free-living amoeba (FLA) that plays an important role in the ecology of multiple ecosystems due to its participation in nutrient recycling, mainly in aqueous environments (Scheid 2014; Anderson et al. 2005). This protozoan feeds on bacteria, algae and yeasts, controlling the biomass of these organisms in the environment (Yousuf et al. 2013). However, some bacteria are resistant to amoebic phagocytosis and can survive and/or multiply inside FLA, being able to establish endosymbiotic relationships, mainly with *A. castellanii*. Some of these bacteria are considered to be clinically important pathogens for humans and other mammals, being collectively named ARB for amoebae-resistant bacteria (Schuster 2002; Greub and Raoult 2004; Anderson et al. 2005; Garcia-Sanchez et al. 2013; Mella et al. 2016; Balczun and Scheid 2017).

Aliarcobacter butzleri [formerly known as *Arcobacter butzleri* (Oren and Garrity 2014)] is a small, curved, non-spore-forming Gram-negative rod, considered an emerging food-borne zoonotic pathogen worldwide, classified as a serious risk to humans (Vandamme et al. 1992; ICMSF 2002; Ramees et al. 2017). It is the species of the genus most frequently isolated from environmental water, food and human clinical samples, being associated with abortion and enteritis in animals, as well as diarrhea and occasional systemic infections in humans (Collado and Figueras 2011; Ferreira et al. 2015). *A. butzleri* and FLA can be frequently found in environmental water sources, where this bacterium

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ANNEX 3

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Characterization of levan produced by a *Paenibacillus* sp. isolated from Brazilian crude oil

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ARTICLEINFO

Keywords:
Levan
Physicochemical characterization
Thermomechanical stability

ABSTRACT

A levan-type fructooligosaccharide was produced by a *Paenibacillus* strain isolated from Brazilian crude oil, the purity of which was 98.5% after precipitation with ethanol and dialysis. Characterization by FTIR, NMR spectroscopy, GC-FID and ESI-MS revealed that it is a mixture of linear β(2 → 6) fructosyl polymers with average degree of polymerization (DP) of 18 and branching ratio of 20. Morphological structure and physicochemical properties were investigated to assess levan microstructure, degradation temperature and thermomechanical features. Thermal Gravimetric Analysis highlighted degradation temperature of 218 °C, Differential Scanning Calorimetry (DSC) glass transition at 81.47 °C, and Dynamic Mechanical Analysis three frequency-dependent transition peaks. These peaks, corresponding to a first thermomechanical transition event at 86.60 °C related to the DSC endothermic event, a second at 170.9 °C and a third at 185.2 °C, were attributed to different glass transition temperatures of oligo and polyfructans with different DP. Levan showed high morphological versatility and technological potential for the food, nutraceutical, and pharmaceutical industries.

1. Introduction

Fructooligosaccharides (FOS), also known as oligofructans, are a group of oligosaccharides composed of fructosyl oligomers with different chemical structures and degrees of polymerization (DP) [1,2]. Capable of resisting the digestion process in the upper gastrointestinal tract, FOS are known to stimulate the growth of specific endogenous probiotics of gut microbiota (*e.g. Bifidobacterium* spp. and *Lactobacillus* spp.) [3], while suppressing the growth of pathogens [2,4]. Their role in boosting the immune system and reducing the risks of gastrointestinal infection and inflammation, as well as their therapeutic effects against inflammatory bowel disease, obesity-related metabolic disorders, diabetes and diarrhea, has been demonstrated in a significant number of experimental studies [4–7]. Further beneficial effects deriving from the direct interactions of these non-digestible oligosaccharides with host intestinal cells have also been described, in accordance with their recognition as soluble dietary fibers [8]. Based on their natural origin and remarkable health benefits, FOS are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and other regulatory agencies around the word [9,10].

Levan-based FOS gained considerable interest in food and nutraceutical industries due to their biocompatibility, biodegradability, anti-inflammatory and anticarcinogenic effects, bioactivity profiles and organoleptic properties [11,12]. Produced by a small number of plant species as non-structural storage carbohydrates and by a wide range of microorganisms as exopolysaccharides (EPS), these fructose homopolymers exhibit a main glycosidic chain composed of repeating fructofuranosyl units linked mainly or exclusively by β(2 → 6) glycosidic bonds. Although predominantly linear, especially levans with high DP may have some degree of branching through β(2 → 1) fructosyl-fructose bonds [13]. Given the current market demand and growing industrial interest in such biopolymers, large-scale microbial production has been

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ANNEX 4

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Research Article

Use of Tunable Copolymers in Aqueous Biphasic Systems for Extractive Bioconversion Aimed at Continuous Fructooligosaccharide Production

Carlos M. N. Mendonça, Nathalia V. Veríssimo, Wellison A. Pereira, Paula M. Cunha, Michele Vitolo, Attilio Converti, Kiki Adi Kurnia, Fernando Segato, Pamela O. S. de Azevedo, Mara G. Freire, Koen Venema, Joao H. P. M. Santos,[†] and Ricardo P. S. Oliveira*[,†]

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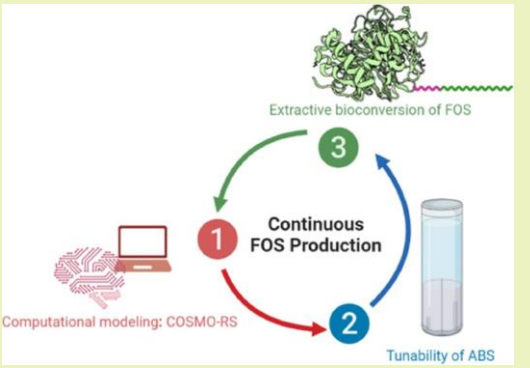
ABSTRACT: Aqueous biphasic systems (ABSs) based on sodium polyacrylate (NaPA), ethylene oxide/propylene oxide (EO/PO) polymers, and $(EO)_x$-$(PO)_y$-$(EO)_x$ triblock copolymers were prepared and applied aiming at continuous fructooligosaccharide (FOS) production and separation. EO/PO hydrophilicity/hydrophobicity balance had a significant effect on ABS formation. To develop an integrated process including the continuous enzymatic (levansucrase) production of FOSs and their purification while improving the production yield by further glucose separation, the potential of these novel polymer-based ABSs as alternative platforms was investigated. They were used to partition different carbohydrates (FOS, sucrose, D-fructose, and D-glucose) and levansucrase. Results revealed a highly polymer-dependent partition of carbohydrates and a poorly dependent one of the enzymes. Changing EO/PO and copolymers, FOS was purified with high yields (72.94−100.0%). Using polypropylene glycol 400 + NaPA 8000-based ABS, the FOS was precipitated in the interphase and separated from the other components. Pluronic PE-6800 + NaPA 8000 was identified as the best ABS for FOS continuous production and in situ purification, while minimizing levansucrase inhibition by D-glucose. This system allowed selective partition of FOSs and D-glucose toward the top phase and that of levansucrase and its substrates toward the bottom one. COnductor-like Screening MOdel for Real Solvent (COSMO-RS) suggested that ABS formation may have been due to NaPA and polymer/copolymer competition to form hydrogen bonds with water molecules. Moreover, the partition of FOSs and sugar may have been the result of a subtle balance between hydrogen bonding of sugar and polymer/copolymer and electrostatic misfit of solute with NaPA. Finally, two integrated processes were proposed to deal with real FOS extracts obtained by chemical or enzymatic hydrolysis of inulin or by transfructosylation of concentrated sucrose solutions using bacterial levansucrases.



KEYWORDS: *fructooligosaccharides, levansucrase, aqueous biphasic systems, polymers, sodium polyacrylate, extractive bioconversion*

1. INTRODUCTION

In recent years, the increased demand of the food and nutraceutical industry for functional fibers has led to renewed interest in diverse types of exopolysaccharides (EPSs) from vegetables, microalgae, and microbial sources.[1,2] From the large plethora of EPSs, fructooligosaccharides (FOSs) have gained special recognition by the scientific community and industry due to their health benefits[2−4] and caloric profiles.[5] Generally regarded as safe for human consumption,[6,7] FOSs have been classified as prebiotics since they (i) are not hydrolyzed/absorbed by the upper part of the gastrointestinal tract, (ii) are a selective substrate for one or a limited number of probiotics, and (iii) are able to alter the colonic microbiota toward a potentially healthier composition and/or activity.[8−10] Since their Food and Drug Administration approval, FOSs entered the food and feed international market as a functional ingredient.[9−11] With daily consumption of 1−4 g in the USA and 3−11 g in Europe,[12] FOS acceptance and application in different food products have extensively increased in the last decades. Based on such consumption trends, the global FOS market was forecast to grow at a rate of 10.4% during the period of 2016−2027 and to reach USD 3.88 billion in 2027.[13] FOSs are industrially produced by either chemical or enzymatic hydrolysis of inulin or by enzymatic trans-

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ANNEX 5

Tracking new insights into antifungal and anti-mycotoxigenic properties of a biofilm forming *pediococcus pentosaceus* strain isolated from grain silage

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ǂ Pamela O. S. Azevedo and Ricardo P. S. Oliveira shared last co-author

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Abstract

The present study offers detailed insights into the antifungal and anti-mycotoxigenic potential of a biofilm forming lactic acid bacteria (LAB) (*Pediococcus pentosaceus*) against one atoxigenic (*Aspergillus flavus*) and two toxigenic (*Aspergillus nomius* and *Fusarium verticillioides*) fungal strains. The antifungal effect of *Pd. pentosaceus* was initially investigated through comparative analysis of fungi physiology by macroscopic visual evaluations and scanning electron microscopy (SEM) examinations. The effects over fungal growth rate and asexual sporulation were additionally accessed. Furthermore, analytical evaluations of mycotoxin production were carry out by HPLC-MS/MS to provide insights on the bacterial anti-mycotoxigenic activity over fungal production of the aflatoxins B1, B2, G1 and G2 as well as fumonisins B1 and B2. Finally, reverse transcription quantitative real-time PCR (RT-qPCR) analysis were employed at the most effective bacterial inoculant concentration to evaluate, at the molecular level, the down-regulation of genes *aflR*, *aflQ* and *aflD*, related to the biosynthesis of aflatoxins by the strain of *Aspergillus nomius*. The effects over mycotoxin contamination were thought to be result of a combination of several biotic and abiotic factors. Several possible mechanisms of action were addressed along with potentially deleterious effects ascribing from *Pediococcus pentosaceus* LBM18 misuse as biopestecide.

Keywords: mycotoxin, aflatoxins, fumonisin, crops contamination, biopestecides, lactic acid bacteria, Pediococcus pentosaceus, biofilm

ANNEX 6

Creatine in sustainable fish aquaculture

Alejandro Villasante [a], Carolina Ramírez [b], Elías Figueroa [b, *], Wellison A. Pereira [c], Madison S. Powell [a], Delbert M. Gatlin III [a], T. Gibson Gaylord [a], Patricio Dantagnan [a], Jaime Romero [a]

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[c] *Nucleus of Research in Food Production, Faculty of Natural Resources, Catholic University of Temuco, Temuco, Chile. 0000-0002-3979-7876.*

Corresponding author: * Elias Figueroa. E-mail: efigueroa@uct.cl

Abstract

Creatine is a non-essential amino acids derivative that is part of the creatine–phosphocreatine–creatine kinase system, which is involved in the high-energy phosphate metabolism, required for buffering, transport and regulation of cellular energy. Hence, it plays a pivotal role in the homeostasis of the energy budget and the complete cellular metabolism in vertebrates, which continuously require a replacement of creatine stores through diet or *de novo* synthesis. The benefits of creatine supplementation are not limited to improve exercise performance and muscle growth. Other beneficial effect, such as antioxidant activity, enhanced flesh quality and improved lipid homeostasis has also been suggested. Natural diets and endogenous creatine synthesis should satisfy total creatine demands in fish. However, differences in the proportions of precursor amino acids consumed in creatine synthesis between fish from different trophic levels are likely to exist; this, since piscivorous species can obtain creatine exogenously from prey in contrast to herbivorous species. Thus, further research to considerate creatine a "conditionally essential nutrient" in carnivorous fish when fed diets formulated with ingredients devoid of creatine, highlighting the need for its dietary supplementation under this nutritional scenario to support efficient growth, optimal health and high-quality fish, is required.

Keywords: creatine; aquaculture; fish development.

OTHER ACTIVITIES

Piracicaba, São Paulo, January, 12, 2023

Escola Superior de Agricultura Luiz de Queiroz – Universidade de São Paulo
DEPARTAMENTO DE ECONOMIA, ADMINISTRAÇÃO E SOCIOLOGIA
Av. Pádua Dias, 11 | Piracicaba, SP | 13418-900
Contato para cursos de especialização e atualização
Tel.: +55 (19) 26603343 | secretariambauspesalq@usp.br | www.mbauspesalq.com

Declaration of Course Completion

We herein confirm that **Wellison Amorim Pereira**, holder of ID card number **0375608720096**, academic record (AR) 267372120340, has completed the **MBA in Project Management - Distance Education - 2 nd half/2020** main knowledge area in Human, held between 21/09/2020 and 30/08/2022, comprising a workload of 400 hours.

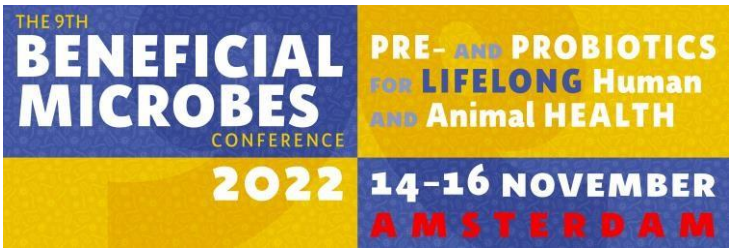
The student has fulfilled all the requirements for the course completion of the course and their Final Paper entitled “Gestão da Qualidade: plano de ação para aumento da produtividade em um laboratório de pesquisa” was approved with a grade 7,75 out of 10.0.

The **MBA in Project Management - Distance Education - 2 nd half/2020** course is in accordance with the Resolution CNE/CES Number 1, issued on April 6, 2018, and it is certified by the University of São Paulo - Luiz de Queiroz College of Agriculture. The mentioned course is also accredited by the Brazilian Ministry of Education according to Ordinance Number 503, issued on July 19, 2022.

The MBA diploma and Academic Student Record will be issued shortly. This document has one year of validity after its issuance.

Digital Validation:

* Use the link to check the validity of this document https://moveurl.me/eYlmSuZpd



Secretariat
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the Netherlands
T +31 30 2294247
BMC@bastiaanse-communication.com
www.BeneficialMicrobes2022.org

CERTIFICATE

Wellison Amorim Pereira

attended the

9th Beneficial Microbes Conference
14-16 November 2022
Amsterdam, the Netherlands

and presented a poster entitled
"*Antimicrobial compounds produced by aquatic lactic acid bacteria inhibit the growth of food and fish pathogens*"

ADVISORY COMMITTEE

Prof. Koen Venema	Maastricht University and Beneficial Microbes Consultancy, the Netherlands (chair)
Dr Frédérique Chaucheyras-Durand	Lallemand, France
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Date: 16 November 2022

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Bilthoven, 21 October 2022

Attn. Mr. Wellison Amorim Pereira
University of São Paulo
Department Biochemical-Pharmaceutical Technology
Av. Prof. Lineu Prestes, 580, B16 – Cidade Universitária
05508-000 São Paulo
Brazil

Subject: 9th Beneficial Microbes Conference

Dear Mr Wellison Amorim Pereira,

Herewith I confirm that the following abstract is accepted for poster presentation at the 9th Beneficial Microbes Conference taking place in Amsterdam, the Netherlands, 14-16 November 2022:

Antimicrobial compounds produced by aquatic lactic acid bacteria inhibit the growth of food and fish pathogens.
Wellison Amorim Pereira, Anna Carolina M. Piazentin, Carlos Miguel N. Mendonça, Marisol Vallejo, Elias Figueroa Villalobos, Ricardo Pinheiro de S. Oliveira

Best regards,
On behalf of the Advisory Board

Secretariat
Helena B. Bastiaanse, M.Sc.

UNIVERSIDAD CATÓLICA DE TEMUCO | DOCTORADO EN CIENCIAS AGROPECUARIAS FACULTAD DE RECURSOS NATURALES

CONSTANCIA

Dr. Claudio Inostroza Blancheteau, Director del Doctorado en Ciencias Agropecuarias de la Universidad Católica de Temuco, deja constancia que el **Sr. Wellison Amorim Pereira**, estudiante del Programa de Doctorado en Tecnología Bioquímica y Farmacéutica de la Universidad de São Paulo, participo como expositor del trabajo titulado "**Evaluating the antimicrobial effect of isolated probiotic strains against *Flavobacterium psychrophilum* and their interference in the health status of rainbow trout (*Oncorhynchus mykiss*)"** en el **I WORKSHOP DEL DOCTORADO EN CIENCIAS AGROPECUARIAS** realizado el 05 de diciembre de 2022 en las dependencias de nuestra universidad.

Se extiende el presente certificado al interesado para los fines que estime conveniente.

UNIVERSIDAD CATOLICA DE TEMUCO
DOCTORADO EN CIENCIAS AGROPECUARIAS
FACULTAD DE RECURSOS NATURALES

Dr. Claudio Inostroza Blancheteau
Director
Programa de Doctorado en Ciencias Agropecuarias

Temuco, 13 diciembre 2022

Processo

Identificação do Processo

Número do Processo	2021/01570-1 - Doutorado
Situação	Interrompido
Grupo de Financiamento	Bolsa no País
Linha de Fomento	Programas Regulares / Bolsas / No País / Doutorado - Fluxo Contínuo
Beneficiário	Wellison Amorim Pereira
Responsável	Ricardo Pinheiro de Souza Oliveira
Data Início	01/10/2021
Duração	6 mês(es)
Período Total Usufruído	6 mês(es)
Período Total Interrompido	9 mês(es) / 8 dia(s)

Instituição de Pesquisa/Empresa	Faculdade de Ciências Farmacêuticas/FCF/USP
Departamento	Departamento de Tecnologia Bioquímico-Farmacêutica
Data de Abertura	23/02/2021

Processo Vinculado

Número	Linha de Fomento	Beneficiário	Responsável	Título
2018/25511-1	Projeto de Pesquisa - Temático	Ricardo Pinheiro de Souza Oliveira	Ricardo Pinheiro de Souza Oliveira	Bioprospecção de bactérias probióticas bacteriocinogênicas: da otimização do cultivo à aplicação em sistemas de produção animal

Resumo

A utilização de micro-organismos probióticos na prevenção e no tratamento de infecções bacterianas em animais destinados ao consumo humano vem sendo considerada uma alternativa eficiente frente ao uso de antibióticos. Adicionalmente, estudos recentes demonstram que determinadas biomoléculas produzidas por estes micro-organismos, tais como bacteriocinas, vitaminas, ácidos graxos, exopolissacarídeos, enzimas, entre outras, podem melhorar a imunidade e o desenvolvimento de seus hospedeiros. Os micro-organismos probióticos mais utilizados atualmente nas indústrias de alimentos e farmacêuticas são os pertencentes ao grupo de bactérias ácido-láticas (BALs), uma vez que são consideradas seguras pelos órgãos reguladores nesta área. No entanto, sabe-se que os efeitos benéficos gerados pelos probióticos são específicos para cada hospedeiro e que, frequentemente, cada biomolécula de interesse é sintetizada, em maior quantidade, por uma determinada linhagem bacteriana. Nesse contexto, o presente projeto tem como objetivo principal o isolamento e a identificação de BALs probióticas presentes na microbiota do intestino de aves, suínos e peixes. Para tanto, serão selecionadas cepas com alta capacidade de produzir bacteriocinas. A partir desta seleção, serão realizados ensaios de compatibilidade entre as cepas e, posteriormente será confeccionado um "mix" de probióticos. As cepas que o compõe serão individualmente micro-encapsuladas e administradas diariamente na dieta dos animais de interesse do setor agropecuário, através de ração e água, a fim de averiguar a eficácia probiótica da mistura. Bacteriocinas sintéticas serão igualmente micro-encapsuladas e administradas na dieta dos animais para compreender seu efeito individual na saúde dos mesmos. Os resultados obtidos com as microcápsulas serão comparados com aqueles obtidos com as mesmas estruturas livres. Ademais, serão realizados estudos imunológicos, análises de microscopia eletrônica e de diversidade da microbiota intestinal desses animais.

Projeto - Identificação

Título em Português

Avaliação do potencial de bactérias láticas bacteriocinogênicas isoladas de truta arco-íris (Oncorhynchus mykiss): efeito antimicrobiano contra Flavobacterium psychrophilum

Título em Inglês

Evaluating the potential of bacteriocinogenic lactic acid bacteria isolated from rainbow trout (Oncorhynchus mykiss): antimicrobial effect against Flavobacterium psychrophilum

Classificação

Grande Área	Ciências Agrárias
Área	Recursos Pesqueiros e Engenharia de Pesca
Sub-área	Aquicultura
Especialidade	Biotecnologia Farmacêutica

Processo

Identificação do Processo

Número do Processo	2021/12258-9 - BEPE - Doutorado
Situação	Em Execução
Grupo de Financiamento	Bolsa no Exterior
Linha de Fomento	Programas Regulares / Bolsas / No Exterior / Bolsa Estágio de Pesquisa no Exterior / BEPE - Doutorado - Fluxo Contínuo
Beneficiário	Wellison Amorim Pereira
Responsável	Ricardo Pinheiro de Souza Oliveira
Data Início	01/04/2022
Duração	12 mês(es)

Instituição de Pesquisa/Empresa	Faculdade de Ciências Farmacêuticas/FCF/USP
Departamento	Departamento de Tecnologia Bioquímico-Farmacêutica
Data de Abertura	21/10/2021

Processo Vinculado

Número	Linha de Fomento	Beneficiário	Responsável	Título
2021/01570-1	Doutorado	Wellison Amorim Pereira	Ricardo Pinheiro de Souza Oliveira	Avaliação do potencial de bactérias láticas bacteriocinogênicas isoladas de truta arco-íris (Oncorhynchus mykiss): efeito antimicrobiano contra Flavobacterium psychrophilum

Resumo

O uso de microrganismos probióticos para a prevenção e tratamento de infecções bacterianas em animais destinados ao consumo humano tem sido considerado uma alternativa eficiente ao uso de antibióticos. Além disso, estudos recentes mostraram que certas biomoléculas produzidas por esses microrganismos, como bacteriocinas, vitaminas, ácidos graxos, exopolissacarídeos e enzimas, melhoram a imunidade e o desenvolvimento de seus hospedeiros. Os microrganismos probióticos mais usados atualmente nas indústrias alimentícia e farmacêutica são o grupo das bactérias ácido lácticas (LAB). Essas bactérias são consideradas seguras pelos reguladores nessa área. No entanto, sabe-se que os seus efeitos benéficos são específicos para cada hospedeiro e que cada biomolécula benéfica é produzida, em grandes quantidades, por uma estirpe específica. Nesse contexto, os principais objetivos do presente estudo são o isolamento e identificação de LABs probióticas da microbiota intestinal da truta arco-íris (Oncorhynchus mykiss). Serão selecionadas linhagens com alta atividade bacteriocina e potencial probiótico e serão preparadas culturas de fermentação, utilizando como substrato resíduos agroindustriais, como o bagaço de cana-de-açúcar. A fermentação será realizada em um biorreator de bancada de 3 L para otimizar o sistema de bioprocessos. Testes de compatibilidade entre as linhagens selecionadas serão realizados e, baseando-se nos resultados, será preparada uma mistura bacteriana probiótica. Para verificar o efeito probiótico da mistura, cada cepa de composição será individualmente microencapsulada e administrada diariamente na dieta de alevinos de truta arco-íris de aproximadamente 5,0 g de peso total, por meio de ração na dosagem de 2% do peso corporal. As bacteriocinas sintéticas também serão microencapsuladas e administradas aos alevinos, a fim de proporcionar uma melhor compreensão dos efeitos individuais dessas biomoléculas na saúde animal. Os resultados obtidos com a utilização das microcápsulas serão comparados com os obtidos sem a administração das microcápsulas. Os alevinos serão mantidos em tanques de fibra de vidro; a concentração e saturação de oxigênio e a temperatura da água serão determinadas diariamente. Uma vez que os animais triplicam seu peso (15g), parâmetros zootécnicos serão avaliados, como aumento de peso, crescimento, fator de conversão e sobrevivência. Além disso, análises histológicas do intestino e do fígado serão realizadas para avaliar alterações morfoestruturais nesses órgãos, bem como a atividade fagocítica in vitro de macrófago

Projeto - Identificação

Título em Português

Avaliação do efeito antimicrobiano de cepas probióticas isoladas contra Flavobacterium psychrophilum e sua interferência no estado de saúde da truta arco-íris (Oncorhynchus mykiss)

Título em Inglês

Evaluating the antimicrobial effect of isolated probiotic strains against Flavobacterium psychrophilum and their interference in the health status of rainbow trout (Oncorhynchus mykiss)

APPROVED SCIENTIFIC INITIATION PROJECTS

Bioprospecção de bactérias ácido láticas probióticas bacteriocinogênicas a partir do trato intestinal de Tilápia (*Oreochromis niloticus*): efeito antimicrobiano contra *Pseudomonas aeruginosas*

Candidato: Iara Santos Reis

Orientador: Prof. Dr. Ricardo Pinheiro de Souza Oliveira (FCF/USP)

Co-orientador: MSc. Wellison Amorim Pereira (Doutorando FCF/USP)

Duração prevista: 01 (um ano) – 2020/21.

Natureza do Projeto: Pesquisa Aplicada.

Financiamento: CNPq (bolsa nº 2020-2577).

RESUMO

A produção de peixe tem desempenhado um papel importante na economia mundial, devido à alta demanda por proteína animal destinada ao consumo humano, sendo a Tilápia (*Oreochromis niloticus*) uma das espécies com alto potencial para a aquicultura. O uso de microrganismos probióticos na prevenção e tratamento de infecções bacterianas em peixes tem sido considerado uma alternativa eficiente ao uso de antibióticos. Além disso, estudos recentes mostraram que certas biomoléculas produzidas por esses microrganismos, como bacteriocinas, melhoram a imunidade e o desenvolvimento. Nesse contexto, o presente projeto tem como objetivo o isolamento e a identificação de bactérias ácido láticas (BAL) probióticas bacteriocinogênicas presentes na microbiota intestinal da Tilápia. Para tanto, será realizada identificação bioquímica e molecular de bactérias probióticas isoladas e o sobrenadante livre de células será utilizado a fim de avaliar a produção, a natureza peptídica e o poder antimicrobiano das bacteriocinas contra Pseudomonas aeruginosas, um importante patógeno na aquicultura. Os resultados obtidos possibilitarão a identificação de novas cepas probióticas seguras para o uso na alimentação animal, assim como de bacteriocinas eficazes no combate a bactérias patogênicas.

Palavras chaves: *probióticos, bacteriocinas, Oreochromis niloticus, Pseudomonas aeruginosas.*

Bioprospecção de bactérias probióticas bacteriocinogênicas a partir do trato intestinal de suínos (*Sus scrofa domesticus*): efeito antimicrobiano contra *Escherichia coli*

Candidato: Sara Mariano Franco

Orientador: Prof. Dr. Ricardo Pinheiro de Souza Oliveira (FCF/USP)

Co-orientador: MSc. Wellison Amorim Pereira (Doutorando FCF/USP)

Duração prevista: 01 (um ano)

Natureza do Projeto: Pesquisa Aplicada

Financiamento: FAPESP (bolsa nº 2020/03071-0)

Resumo

A carne suína é a segunda mais consumida no mundo, sendo importante fonte de recursos e empregos para diversos países. A necessidade de aumento da eficiência alimentar e perda de produtividade por infecções bacterianas, são os maiores entraves ao crescimento da suinocultura. O uso de antibióticos no tratamento de doenças é uma prática recorrente e questionada, por seus impactos na saúde animal e humana. Sabe-se que sua utilização desregulada contribuiu para o aumento do número de micro-organismos resistentes, reduzindo as opções disponíveis para o enfrentamento das enfermidades. Diferentes países adotaram políticas de restrição ao uso de antibióticos em animais saudáveis ou como promotores de crescimento, sendo necessário o desenvolvimento de tecnologias alternativas. Sendo assim, as pesquisas atuais estão voltadas à identificação de novos compostos antimicrobianos para o uso na suinocultura. Estudos com probióticos e bacteriocinas têm demonstrado seu efeito antimicrobiano sobre diversas cepas patogênicas, como melhoria no funcionamento do intestino, aumento da eficiência nutricional e crescimento dos suínos. Neste trabalho, atualizamos as informações acerca do uso de probióticos e bacteriocinas na suinocultura, seus efeitos sobre a saúde animal e perspectivas futuras, assim como os impactos globais gerados nos últimos anos por essa importante atividade econômica.

Palavras chaves: *suínos, probióticos, bacteriocinas, antibióticos, biotecnologia.*

ETHICS COMMITTEE

Documento 09/20

Temuco, 19 de marzo de 2020

Dr.
Iván Valdebenito Isler
Departamento de Ciencias Agropecuarias y Acuícolas
Facultad de Recursos Naturales
Universidad Católica de Temuco
Presente

El Comité de Ética de la Investigación de la Universidad Católica de Temuco ha conocido antecedentes del proyecto denominado **"Isolation and identification of bacteriocin-producing probiotic lactic acid bacteria from the rainbow trout (*Oncorhynchus mykiss*): activity against *Flavobacterium psychrophilum*"**, que usted remitiera a este comité, el que será desarrollado en conjunto con la Universidad de Sao Paulo, Brasil.

El proyecto busca la aislación y sepas de probióticos desde tracto digestivo de juveniles de trucha arcoíris para la elaboración de futuros probióticos, la cuantificación y caracterización parcial de bacteriocinas desde el tracto digestivo de trucha arcoíris y la aplicación de protocolos de genotipificación, identificación, cultivo y administración de probióticos en dietas para juveniles de trucha arcoíris. Cabe hacer presente que sólo el cultivo y administración de probióticos en dietas serán desarrollados en la UCTemuco.

Se trabajará con un total de 14 estanques y un total de 50 peces por estanque utilizando un total de 700 peces para el desarrollo del bioensayo. Se trabajará en la Unidad Experimental de Acuicultura del Depto. de Cs. Agropecuarias y Acuícolas y la Unidad cumple con las normas establecidas por la autoridad Sanitaria (SUBPESCA) y de Fiscalización (SERNAPESCA) para operar según resolución 1594 del año 2019 y está inscrita según consta en Folio RNA#21466. Además, cuenta con agua dulce obtenida de un pozo profundo en circuitos que pueden ser abiertos o cerrados (recirculación). Los mismos están diseñados para garantizar el suministro continuo de aireación para la mantención de concentraciones adecuadas de oxígeno, la eliminación de heces y alimento no consumido. Además, la Unidad Experimental cuenta con un sistema de rejillas que evitan la fuga de los peces mantenidos en el

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sistema experimental y con sistemas de rodiluvios, pediluvios y maniluvios que reducen la probabilidad de ingreso o salida de patógenos desde la unidad experimental.

La Unidad Experimental de Acuicultura posee la infraestructura y ubicación apropiada para el desarrollo de prácticas experimentales que garanticen razonablemente los niveles de bioseguridad y bienestar animal físico y etológico de los peces. La Unidad Experimental está diseñada para animales experimentales BS-1 y según esto, sigue las recomendaciones para un Nivel de Bioseguridad 1, según lo indicado en el punto 9.1 del Manual de Normas de Bioseguridad (CONICYT, 2008). Además, cada estanque tiene su propio sistema de desinfección y limpieza de estanques (escobillas), además, de los utensilios (quechas) utilizados para la captura de peces. Toda persona que ingresa a la UEA debe utilizar maniluvios y pediluvios. La manipulación de los peces se realiza con guantes de latex desechables y delantal para la protección personal.

Cinco peces por estanque (valor mínimo de peces que estadísticamente reflejan la condición de los peces de ese estanque) serán sacrificados para realizar los análisis histológicos y fisiológicos. El sacrificio se realizará mediante sobredosis del anestésico benzocaína (BZ-20 en dosis de 5mL/10L de agua) y la biomasa no utilizada en los análisis será congelada a la espera de su retiro por parte de la Empresa LOGISAMB Soluciones Ambientales, compañía que se encarga de la incineración del material biológico retirado.

La Unidad experimental de Acuicultura de la UCTemuco funciona bajo la resolución 1594 del año 2019 y está inscrita según consta en Folio RNA#21466.

Por las razones expresadas, el Comité de Ética de la Investigación de la Universidad Católica de Temuco avala la ejecución del proyecto mencionado.

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