UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(ÁREA: BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

AVALIAÇÃO DE DIFERENTES PREBIÓTICOS, PROBIÓTICOS E SEUS METABÓLITOS NA INIBIÇÃO DE BACTÉRIAS PATOGÊNICAS E POTENCIAIS APLICAÇÕES NA PRESERVAÇÃO DE ALIMENTOS E BENEFÍCIOS À SAÚDE.

FRANCIANE CRISTINA DE FIGUEIREDO

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Prof. Dr. PEDRO DE OLIVA NETO (Participaçao Virtual)
Departamento de Biotecnologia / Faculdade de Ciencias e Letras UNESP Assis

Prof. Dr. MICHEL BRIENZO (Participaçao Virtual)
Laboratório de Caracterização de Biomassa / Instituto de Pesquisa em Bioenergia de Rio Claro - UNESP

Profa. Dra. PATRICIA BLUMER ZACARCHENCO RODRIGUES DE SÁ (Participaçao Virtual)
Centro de Tecnologia de Laticínios / Instituto de Tecnologia de Alimentos

Prof. Dr. CRISTIANO RAGAGNIN DE MENEZES (Participaçao Virtual)
Departamento de Ciência e Tecnologia de Alimentos / Universidade Federal de Santa Maria

Prof. Dr. JOÃO CARLOS MONTEIRO DE CARVALHO (Participaçao Virtual)
Departamento de Tecnologia Bioquímico-Farmacêutica / Universidade de São Paulo

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Dedico este trabalho aos

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ABSTRACT

Intestinal microbiota has a crucial role in health, with food supplements and other products that provide balance to the intestinal microbiota being thoroughly studied in the present day. Among such products, probiotics, prebiotics and postbiotics stand out. Probiotics are defined as living microorganisms that, when ingested in adequate quantities, positively affect the health of the host. Prebiotics are substrates that reach the intestinal microbiota intact and are capable of selectively stimulating the growth of probiotics. Postbiotics, on the other hand, is a new concept, defining extracts or supernatants of probiotic origin, cell-free and beneficial to health. These ingredients are presented as possible alternatives to the usage of antibiotics or may act as food preservatives, preventing the growth of pathogenic bacteria such as *Salmonella enterica* serovar *Typhimurium*, *Escherichia coli* and *Staphylococcus aureus*, responsible for severe infections. The present thesis aimed to assess the inhibition of these pathogenic bacteria by these food ingredientes, applied either singly or in mixtures. In addition, the stability of antimicrobial activity was assessed in different conditions, along with the characterization of organic acids and scale-up tests. Probiotic strains were *Bifidobacterium animalis, B. breve, B. lactis, B. longum*, *Lactobacillus acidophilus* and *Levilactobacillus brevis*. *E. coli* was inhibited by all postbiotics, both singly and in mixtures. No postbiotic at dosages of 6% (v/v) was capable of inhibiting *S. aureus*. For *Salmonella typhimurium*, the postbiotics of *B. animalis*, *B. breve* and *B. lactis* successfully inhibited the pathogen's growth. Tests with the mixture of the prebiotics fructooligosacharides (FOS) and xylooligosacharides (XOS) showed that, with 10h of culture, only *L. br*evis and *B. longum* were stimulated, exceeding the growth of control (glucose). The postbiotic mixture of all *Bifidobacterium* strains successfully inhibited *Salmonella*'s growth as well; however, adding postbiotics from *Lactobacillus* and *Levilactobacillus* strains to the mixture lessened its inhibitory activity, showing a possible antagonism between genera. No postbiotics inhibited the growth of any probiotic bacteria, showing the potential for combined use of postbiotics and prebiotics. The stability tests revealed inhibitory activity of postbiotics after treatments with high temperature, except for the postbiotics of *B. longum*, probably due to its non-thermo-resistant bacteriocin. Four organic acids (acetic, lactic, succinic and formic) were present in all postbiotics, with acetic and lactic acid present in higher concentrations. The tests in different pH ranges revealed that organic acids show inhibitory activity when in acid pH, due to their presence in their undissociated form, capable of entering the pathogen's cell-wall and dissociating inside the cell, killing the pathogen. Scale-up tests to 2-liter fermenters showed that postbiotics from the *B. breve* strain with satisfactory inhibitory activity can be produced in 24 hours. The concentration of 6% (v/v) of this postbiotic in BG medium containing commercial sugars successfully inhibited the growth of *Salmonella typhimurium*, even in the presence of easily fermented sugars. Oven-dried (105º C) *B. breve* postbiotics showed similar inhibitory activity with concentrations of 0.9% (m/v). Although further studies are necessary to clarify the complex interaction of metabolites present in postbiotics, the stability of the inhibitory activity of these substances after treatments with high temperatures, low pH and after drying is an attribute that highlights their potential usage in the food industry.

Keywords: Probiotics, Postbiotics, Prebiotics, Organic Acid, Inhibition.

RESUMO

A microbiota intestinal tem papel fundamental na saúde, sendo os suplementos alimentares e outros produtos que proporcionam equilíbrio à microbiota intestinal amplamente estudados nos dias atuais. Dentre esses produtos, destacam-se os probióticos, prebióticos e pós-bióticos. Probióticos são definidos como microrganismos vivos que, quando ingeridos em quantidades adequadas, afetam positivamente a saúde do hospedeiro. Os prebióticos são substratos que alcançam a microbiota intestinal intacta e são capazes de estimular seletivamente o crescimento de probióticos. Postbiotics, contudo, é um novo conceito que define extratos ou sobrenadantes de origem probiótica, livres de células e benéficos à saúde. Esses ingredientes são apresentados como possíveis alternativas ao uso de antibióticos ou podem atuar como conservantes de alimentos, evitando o crescimento de bactérias patogênicas como *Salmonella enterica* serovar *Typhimurium*, *Escherichia coli* e *Staphylococcus aureus*, responsáveis por infecções graves. A presente tese teve como objetivo avaliar a inibição dessas bactérias patogênicas por esses ingredientes alimentícios, aplicados isoladamente ou em mistura. Além disso, a estabilidade da atividade antimicrobiana foi avaliada em diferentes condições, juntamente com a caracterização de ácidos orgânicos e testes de aumento de escala. As cepas probióticas foram *Bifidobacterium animalis*, *B. breve*, *B. lactis*, *B. longum*, *Lactobacillus acidophilus* e *Levilactobacillus. brevis*. *E. coli* foi inibida por todos os postbiotics, tanto isoladamente quanto em misturas. Nenhum postbiotic em dosagens de 6% (v / v) foi capaz de inibir *S. aureus*. Para *Salmonella typhimurium*, os postbiotics de *B. animalis*, *B. breve* e *B. lactis* inibiram com sucesso o crescimento do patógeno. Testes com a mistura dos prebióticos frutooligossacarídeos (FOS) e xilooligossacarídeos (XOS) mostraram que, com 10h de cultivo, apenas *L. brevis* e *B. longum* foram estimulados, superando o crescimento do controle (glicose). A mistura postbiotic de todas as linhagens de *Bifidobacterium* inibiu com sucesso o crescimento de *Salmonella*, entretanto, a adição de postbiotic de *Lactobacillus* e *Levilactobacillus* à mistura diminuiu sua atividade inibitória, mostrando um possível antagonismo entre os gêneros. Nenhum postbiotic inibiu o crescimento de qualquer bactéria probiótica, mostrando o potencial para o uso combinado de postbiotic e prebióticos. Os testes de estabilidade revelaram atividade inibitória dos postbiotics após tratamentos com alta temperatura, exceto para os postbiotics de *B. longum*, provavelmente devido à sua bacteriocina não termo-resistente. Quatro ácidos orgânicos (acético, lático, succínico e fórmico) estiveram presentes em todos os postbiotics, com os ácidos acético e lático presentes em maiores concentrações. Os testes em diferentes faixas de pH revelaram que os ácidos orgânicos apresentam atividade inibitória quando em pH ácido, devido à sua presença na forma indissociada, capaz de entrar na parede celular da bactéria e se dissociar no interior da célula, matando o patógeno. Testes de aumento de escala para fermentadores de 2 litros mostraram que postbiotics da linhagem de *B. breve* com atividade inibitória satisfatória podem ser produzidos em 24 horas. A concentração de 6% (v / v) deste postbiotic em meio BG contendo açúcares comerciais inibiu com sucesso o crescimento de *Salmonella typhimurium*, mesmo na presença de açúcares de fácil fermentação. Postibiotic secos de *B. breve* em estufa (105º C) apresentaram atividade inibitória semelhante com concentrações de 0,9% (m / v). Embora mais estudos sejam necessários para esclarecer a complexa interação dos metabólitos presentes nos postbiotics, a estabilidade da atividade inibitória dessas substâncias após tratamentos com altas temperaturas, baixo pH e após secagem é um atributo que destaca seu potencial de utilização na indústria alimentícia.

Palavras-chaves: Probiótico, Postbiotic, Prebiótico, Ácido Orgânico, Inibição.

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INTRODUCTION

In recent years there has been an increasing interest in ingredients that promote health benefits and, at the same time, prevent the appearance of diseases. These ingredients can be naturally present in foods or added to industrialized products. Currently, researchers seek to discover and develop new products that help or modulate the proper functioning of the organism.

Among these ingredients are probiotics, prebiotics and symbiotics. Probiotics are microorganisms, usually *Bifidobacterium* and *Lactobacillus*, which, when ingested in adequate amounts, positively affect the health of the host (Wasilewski et al., 2015). These bacteria are directly related to the prevention of inflammatory bowel diseases, the formation of neoplastic cells and allergic reactions (Quigley, 2010). Recent researches show that health benefits are not exclusively related to the bacterial viability of probiotics, thus, two new terms have emerged: Postbiotics and paraprobiotics. These terms refer to extracts, cell-free supernatants or even non-viable cells of probiotics that have the ability to promote several positive effects such as immunomodulation, anti-inflammatory and antimicrobial effects (Aguilar-Toalá et al., 2018; Cuevas-González et al., 2020).

Prebiotics, on the other hand, are oligosaccharides not digestible by the consumer, these ingredients therefore reach the intestine of human and other animals intact and selectively stimulate the growth of probiotic bacteria in the intestine (Rastall and Gibson, 2015; Hutkins et al., 2016). The main oligosaccharides marketed as prebiotics are fructo-oligosaccharides, xylo-oligosaccharides and galacto-oligosaccharides (Wasilewski et al., 2015). Studies point to many benefits related to the consumption of prebiotics, such as improvements in intestinal balance, increased absorption of mineral salts, inhibition of pathogens and stimulation of the immune system (Wasilewski et al., 2015; Hutkins et al., 2016; Sanders et al. , 2019).

Finally, symbiotics are formulated products that combine prebiotic and probiotic ingredients (Markowiak and Śliżewska, 2018). This formulation ensures that the microorganisms ingested survive and adapt easily to the host's intestine due to the presence of prebiotics (Quigley, 2010).

Probiotics, prebiotics and symbiotics are considered alternatives to the use of antibiotics in the treatment of some diseases caused by pathogenic bacteria such as *Salmonella enterica* serovar *Typhimurium* (*Salmonella typhimurium*), *Escherichia coli*

and *Staphylococcus aureus*, responsible for many infection cases in the world (Dayan et al., 2016; Madigan et al., 2016; Mirsepasi-Lauridsen et al., 2019).

In this thesis, a bibliographical survey was carried out, covering the main functional ingredients and the most recent studies on the importance of the intestinal microbiota on the health of the host. Laboratory tests were also developed to evaluate the effect of single or mixed postbiotics from *Lactobacillus, Levilactobacillus* and *Bifidobacterium* in the inhibition of important pathogenic bacteria. Furthermore, an assessment of the stability of postbiotics and scale-up tests were performed, studying the potential of these substances as ingredients for food preservatives in the food industry.

AIM AND OBJECTIVES

Aim

To assess the effect of different prebiotics and of probiotics of the *Lactobacillus, Levilactobacillus* and *Bifidobacterium* genera, as well as their metabolites (postbiotics), on the inhibition of the pathogens *Salmonella typhimurium*, *Escherichia coli* and *Staphylococcus aureus*.

Objectives

- To test the inhibition of *Salmonella typhimurium, E. coli e S. aureus* by postbiotics from the following probiotic strains: *Bifidobacterium breve, B. lactis, B. longum, B. animalis, Levilactobacillus brevis* and *Lactobacillus acidophilus*;
- To assess a possible synergism or antagonism between the postbiotics on the inhibition of pathogens;
- To determine the minimum inhibitory concentration of postbiotics on the inhibition of pathogens;
- To verify the inhibition of *Salmonella typhimurium* in mixed culture with probiotic strains.
- To assess the stability of postbiotics in different pH, temperature and drying conditions.

- To identify and quantify the organic acids in the postbiotics, verifying their effects on the growth of pathogenic bacteria.
- To assess a possible synergism or antagonism between postbiotics and prebiotics on the inhibition of *Salmonella typhimurium*.
- To estimate the potential use of postbiotics in larger scales performing tests with 2 L fermenters.

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CHAPTER 1

Advances and new perspectives in prebiotic, probiotic and symbiotic products for food nutrition and feed

Franciane Cristina de Figueiredo[a,b*], Pedro de Oliva-Neto[b]

Chapter 10 “Hemicellulose Biorefinery: A Sustainable Solution for Value Addition to Bio-Based Products and Bioenergy”. Michel Brienzo (Eds.).

ABSTRACT

The gastrointestinal tract is colonized by several bacteria that are directly associated with the well-being of the host. Bacteria related to the balance of intestinal flora are often used as dietary supplements, known as probiotics. Other products marketed as a functional ingredients are the prebiotics, non-digestible oligosaccharides known to selectively stimulate the growth of beneficial bacteria. Prebiotics such as xylooligosaccharides (XOs), fructooligosaccharides (FOs) and galactooligosaccharides (GOs) can be found naturally in fruits, vegetables, honey and milk or industrially produced, such as XOs, obtained by the extraction and hydrolysis of hemicellulose derived from lignocellulosic materials. There are also symbiotic products, which consist of added probiotic strains in prebiotic foods or ingredients. These types of ingredients can maintain homeostasis in the intestinal microbiota preventing the onset of some diseases, in addition to being considered an alternative to the use of antibiotics to treat some infections. Comprehending the action mechanisms of these ingredients is important for understanding various diseases and for formulating products that are effective in maintaining equilibrium of microbiota. The present review seeks to bring the most up-to-date research and advances in food nutrition involving intestinal microbiota as well as prebiotics, probiotics and symbiotics.

Keywords: Intestinal microbiota, *Lactobacillus*, *Bifidobacterium*, Xylooligosaccharides, Fructooligosaccharide, Galactooligosaccharides.

1. INTRODUCTION

The intestinal microbiota is largely related to health and the manifestation of diseases in animals. Important advances in the health field have shown that the microbiota is linked to important metabolic activities, and changes in its composition can lead to serious diseases (Wang et al. 2017, Quigley 2019). Currently, researchers seek to discover and develop new products that help or modulate the proper functions of the organism. Therefore, the target of many researches is to try to maintain the homeostasis of the intestinal microbiota. Among the ingredients that develop such functions are prebiotics, probiotics and symbiotics (Sanders et al. 2019a).

These types of ingredients are considered an alternative to the use of antibiotics in the treatment of some diseases, for example, salmonellosis, which are infections caused mainly by *Salmonella enterica* serovar *Typhimurium* (*Salmonella typhimurium*) and *Clostridium difficile*, with more than one million cases reported annually (Sanders et al. 2019a, Sanders et al. 2019b).

Recent discoveries show that maintaining the homeostasis of the microbiota is fundamental to the host's well-being, but little is known about how these microorganisms act to generate such benefits, nor how the composition of the intestinal flora influences certain diseases. Furthermore, many of the modulators of intestinal flora (prebiotics, probiotics and symbiotics) do not have all their mechanisms of action well understood. These types of ingredients still need to be deeply studied to elucidate relevant issues such as the specificities of action in different genera of microorganisms and the doses stipulated for their proper functioning.

The purpose of this review is to present the most up-to-date concepts of prebiotics, probiotics and symbiotics, as well as updating the understanding of the mechanisms of action related to them and situating the latest advances in research that assess the microbiota-health-disease interaction.

2. PREBIOTICS

2.1. Definition and classification

Gibson and Roberfroid (1995) used the term "prebiotic" to define non-digestible oligosaccharides capable of stimulating the beneficial bacteria growth (probiotics). Although this concept has been revised a few times, the main idea has been maintained over the years (Rastall and Gibson 2015, Hutkins et al. 2016). Recently in 2016, the term was updated in a consensus of experts gathered by the International Scientific Association for Probiotics and Prebiotics (ISAPP). The new definition presents prebiotics as "substrate that is selectively used by host microorganisms conferring health benefit" (Gibson et al. 2017). Generally these oligosaccharides have short chains of 2 to 12 simple linked sugars, are sweet and soluble in water depending on the degree of polymerization and molecular mass, however substances such as polyphenols and polyunsaturated fatty acids can also have a prebiotic effect (Manning and Gibson 2004, Markowiak and Śliżewska 2018, Sanders et al. 2019b).

The presence of β-type bonds is what makes these oligosaccharides non-digestible, since most living beings do not have enzymes capable of degrading this type of bond (Carvalho et al. 2013). Because prebiotics reach the intestine intact, they serve as a substrate for non-pathogenic bacteria, usually of the genus *Bifidobacterium* and

Lactobacillus, promoting a change in the composition of the intestinal flora (Markowiak and Śliżewska 2018).

These oligosaccharides can become an alternative to the use of probiotics, because their handling is less laborious, besides being more economically accessible, such as fructooligosaccharides and galactooligosaccharides (Macfarlane et al. 2006).

Interest in the commercialization of prebiotics is growing and an estimation for 2024 the global market will reach $7.2 billion for these produtcs (Global Market Insights 2019). However, to be considered as a prebiotic, the oligosaccharide must exhibit some criteria such as: a) not being susceptible to the action of enzymes of the gastrointestinal tract presenting resistance to the acidity of gastric juice, b) being selectively fermented by the intestinal microflora and c) stimulating the growth or activity of bacteria linked to health. These substances must also exercise beneficial effects on the host health and present favorable characteristics to the manufacturing process (Wang 2009, Markowiak and Śliżewska 2018).

2.2. Mechanisms of action

In general, the beneficial effects provided by ingesting prebiotics described in the literature (Gibson and Roberfroid 1995, Manning and Gibson 2004, Macfarlane et al. 2006, Wang et al. 2014, Sanders et al. 2019a, Sanders et al. 2019b) are as follows:

a) Maintenance of the intestinal flora and increase of the fecal bolus that stimulates the intestinal transit, by the presence of fibers.

b) Aid in the absorption and production of vitamins, such as B complex, vitamin K and folic acid.

c) Increased absorption of minerals, such as iron, calcium and magnesium, decreasing the risk of osteoporosis.

d) Decrease in cases of diarrhea, gastrointestinal, respiratory and urogenital tract infections by stimulating probiotic bacteria and, consequently, the production of SCFAs that lead to a decrease in pH, in addition to competition for nutrients and adherence to the intestinal epithelium by pathogenic bacteria.

e) Modulation of the immune system by the growth of probiotic bacteria responsible for the stimulation of anti-inflammatory cytokines.

f) Decreased risk of cancer, especially of the intestinal colon.

g) Control of glucose and cholesterol levels, which contributes to reduce cases of obesity. Prebiotics can act to decrease the pH in the cecum, which contributes to increase the excretion of bile acid, this causes the lipids stored in the liver to be required for new synthesis of bile acids, decreasing the levels of free cholesterol (Vanhoof and De Schrijver 1995, Al Sheraji et al. 2013).

Prebiotics can also act by increasing the viscosity of the intestine, which creates a barrier that prevents the absorption of fats (Al Sheraji et al. 2013). The control of glycemic indexes occurs through the modulation of insulin levels by a mechanism still unknown (Sousa et al. 2011).

2.3. Main action molecules

Prebiotics can be found naturally in milk, honey and some vegetables such as beans, soy beans, chicory, garlic, tomatoes, bananas, leeks, onions and asparagus (Mussatto and Mancilha 2007, Al Sheraji et al. 2013). In the industry, prebiotics are being used as ingredients for dairy beverages, desserts and low-calorie, low-glycemic index foods for the consumption of people with diabetes (Gibson and Roberfroid 1995, Mussatto and Mancilha 2007).

Industrially produced prebiotics need to present the appropriate daily dosage, because if ingested in excess it can cause diarrhea (Markowiak and Śliżewska 2018). Studies show that there is no prebiotic capable of stimulating a wide range of probiotics, that is, different strains of beneficial bacteria are capable of fermenting specific prebiotics, being necessary a mix of prebiotics to increase its effect on the intestinal microbiota (Moura et al. 2007, Mäkeläinen et al. 2010, Figueiredo et al. 2020).

The prebiotics that stand out in the commercialization as food supplements are: lactulose, fructooligosaccharides (FOs), inulin, raffinose, mannanooligosaccharides (MOs), galactooligosaccharides (GOs), xylooligosaccharides (XOs), isomaltooligosaccharides (IMOs), Human Milk Oligosaccharides (HMOs) (Manning and Gibson 2004, Markowiak and Śliżewska 2018, Sanders et al. 2019a). Their chemical composition, natural sources and main beneficial effects are summarized in Table 1.

Table 1- Chemical compositions, natural sources and health benefits of the main prebiotics studied.

Prebiotic	Composition	Natural sources	Health benefits	References
Fructooligosaccharides (FOs)	Fructose (dp 2-10)	Garlic, onions, bananas, chicory and tomatoes	Satiety. Increasing the abundance of bifidobacteria. Prevention of allergies and infections. Related to metabolic health and minerals absorption in the bowel.	Gibson et al 2017, Sanders et al. 2019a.
Xylooligosaccharides (XOs)	Xylose	Vegetables, fruits, milk and honey	Stimulation of beneficial bacteria, reduction of blood glucose, cholesterol and pro-carcinogenic enzymes. Enhanced minerals absorption and immune-stimulation.	Samanta et al. 2015, Figueredo et al. 2020.
Galactooligosaccharides (GOs)	Galactose	Human's milk and cow's milk	Improved minerals absorption, antipathogenic activities and immunomodulatory effects. Prevention of IBS and infections. Related to urogenital health.	Gibson et al. 2017, Young et al. 2019.
Mannanooligosaccharides (MOS)	Mannan	Yeast cell wall	Improved insulin response and nutrients absorption. Prevention of infections. Increasing of SCFAs.	Al Sheraji et al. 2013, Gibson et al. 2017, Markowiak and Śliżewska 2018.
Isomaltooligosaccharides (IMOs)	Glucose	Starch	Prevention of infections. Increasing the abundance of *Bifidobacterium* species.	Al Sheraji et al. 2013, Markowiak and Śliżewska 2018.
Raffinose	Glucose, fructose and galactose	Vegetable seeds, lentils, peas, beans, chickpeas, mallow composite, and mustard	Improving the growth of *Bifidocbacterium* and *Lactobacillus.*	Martinez-villaluenga et al. 2004, Al Sheraji et al. 2013.
Human Milk Oligosaccharides (HMOs)	Glucose, galactose, N-acetylglucosamine (GlcNAc), l-fucose, and sialic acid (N-acetylneuraminic acid	Human Milk	Stimulation of bifidobacteria growth in breast-fed infants. Preventing the adhesion of pathogens.	Gibson et al. 2017, Sanders et al. 2019a.
Inulin	Fructose (dp 10-60).	Garlic, onions, bananas, chicory and tomatoes	Related to metabolic health. Improvement in minerals absorption. Prevention of constipation and inflammatory bowel diseases.	Gibson et al. 2017.
Lactulose	Galactose and Fructose	Lactose (Milk)	Stimulation of beneficial bacteria. Prevention of inflammatory bowel diseases.	Al Sheraji et al. 2013, Gibson et al. 2017.

2.3.1. Fructooligosaccharides

FOs are found naturally in plants, such as garlic, onions, bananas, chicory and tomatoes, and consist of fructose units joined by β 2-1 bonds (Figure 1) (Sangeetha et al. 2005, Bali et al. 2015, Sanders et al. 2019a). FOs, oligofructose and inulin are differentiated by the degree of polymerization (dp) (Gibson and Roberfroid 1995). FOs are called oligofructose when they have dp 2 to 9, on the other hand, they are called inulin when they have a dp between 10 and 60 (Gibson and Roberfroid 1995, Cummings et al. 2001).

Inulin can be obtained directly from vegetables by hydrothermal treatment, while oligofructose can be obtained through inulin or through sucrose by the action of the enzyme β-D-fructofuranosidase (Bali et al. 2015, Sanders et al. 2019a, De la Rosa et al. 2019).

The caloric value of oligofructose and inulin is estimated between 1.5 to 2 Kcal / g, and because it provides low caloric value, since they are rarely hydrolyzed by digestive enzymes, it can be included in food used by diabetics (Roberfroid 1999, De La Rosa et al. 2019). In vivo studies have shown that ingestion above 20 g / day can promote abdominal discomfort and diarrhea and consumption of only 2 to 10 g / day is sufficient to stimulate the growth of probiotic bacteria and maintain intestinal balance (Bouhnik et al. 1999, Rivero-Urgell and Santamaria-Orleans 2001, Al Sheraji et al. 2013).

Among the functional properties of FOs, the potential to control glucose and cholesterol levels stands out (Sousa et al. 2011). These oligosaccharides have a sweet taste about 50% lower than sucrose, are considered low-calorie products, with anticariogenic properties and do not have genotoxicity and mutagenicity. For these reasons, they are marketed as dietary and low-calorie sweeteners (Biedrzycka and Bielecka 2004).

FOs reach the intestine and selectively stimulate the growth of bacteria of the genera *Bifidobacterium*, in addition to *Lactobacillus* and some *Streptococcus*, which, by competition, inhibit the growth of putrefactive bacteria, such as those of the genera *Clostridium*, *Salmonella*, *Shigella*, *Listeria*, *Campilobacter* (Crittenden and Playne 1996). Mäkeläinen et al. (2010) and Figueiredo et al. (2020) tested the inhibitory effect of FOs (Beneo - Belgium) on *Salmonella* growth, where practically no growth in the

medium containing FOs was possible to observe, indicating a possible inhibition of *Salmonella* growth by these oligosaccharides.

Studies indicate that FOS also contribute to the absorption of minerals (calcium and magnesium) in the intestine (Roberfroid 2002, Bornet et al. 2002). Supplementing the diet with FOs stimulated the decrease in blood lipid rates, probably due to the production of fatty acids by the fermentation of FOs by probiotic bacteria (Roberfroid 2002, De La Rosa et al. 2019).

2.3.2. Xylooligosaccharides

XOs are nondigestible oligosaccharides found in vegetables, fruits, milk and honey, and are composed of xylose units joined by β 1-4 bonds (Figure 1), which can have different branches, such as α-D-glucopyranosyl uronic acid, 4-O-methyl derivative, acetyl groups, or arabinofuranosyl residues (Amorim et al. 2019). XOs are marketed as a white powder and their composition and stability basically depends on the extraction process used, in addition to the types of bonds and residual sugars (Carvalho et al. 2013).

The industrial production of xylooligosaccharides is carried out using lignocellulosic materials (LCMs), such as corn cob, rice husks, barley straw, tobacco, cotton stalks, sunflower stems, wheat straw and sugar cane bagasse (Carvalho et al. 2013). XOs can be obtained directly by acid hydrolysis and subsequent purification (Otieno and Ahring 2012), or by using acid, alkaline pre-treatments or auto-hydrolysis to extract hemicellulose (Carvalho et al. 2013, Figueiredo et al. 2017). The hydrolysis of hemicellulose for the production of XOs is usually performed by acid hydrolysis or enzymatic treatment using xylanases (Carvalho et al. 2013).

In comparison with FOs, XOs have some advantages over commercial production, as they demonstrate greater resistance to wide pH ranges (2.5 to 8) and stability at high temperatures (above 100ºC) (Amorim et al. 2019).

The recommended daily consumption for healthy adults is on average 8 to 12 g / day (Samanta et al. 2015). However, Finegold et al. (2014) evaluated that the intake of only 1.4 g / day of XOs is sufficient to increase the number of bifidobacteria present in the intestine without increasing the levels of constipation and diarrhea when compared to the group that did not ingest XOs. The maximum tolerated dose for XOs consumption by

humans is 12 g / day, which can cause side effects such as nausea, flatulence, diarrhea and distension of the abdominal region (Xiao et al. 2012, Samanta et al. 2015).

XOs also showed a possible inhibitory effect on *Salmonella typhimurium* proliferation, as low growth was observed mainly in the first hours in media containing XOs with dp 2 (xylose) (Trademark Longlive - China) (Mäkeläinen et al. 2010, Figueiredo et al. 2020).

Another trend towards the use of XOs is linked to animal feed in substitution to antibiotics, since the excessive use of these drugs can induce the resistance of bacteria, including pathogenic ones (Carvalho et al. 2013).

Many studies indicate the ability of XOs to stimulate the growth of bacteria of the genus *Bifidobacterium* and *Lactobacillus*, inhibiting and / or decreasing the proliferation of pathogenic bacteria in the intestine (Rycroft et al. 2001, Crittenden et al. 2002, Gullón et al. 2008, Mäkeläinen et al. 2010, Chen et al. 2016).

Ingestion of XOs is linked to the prevention of diabetes, cholesterol and colon inflammation (Amorin et al. 2019). Because it has a sweet taste and having low calories, XOs can be consumed by patients with diabetes or on diets with restricted calories (Samanta et al. 2015).

Studies indicate that supplementing foods with XOs improves intestinal functions, such as calcium absorption, providing positive effects on the immune and cardiovascular system and triggering anti-allergic and anti-inflammatory activities (Grootaert et al. 2007, Chung et al. 2007, Aachary and Prapulla 2009).

2.3.3. Galactooligosaccharides

GOs are oligosaccharides found naturally in human milk, and also in bovine milk, they are composed of galactose units joined to lactose by β bonds (Figure 1) (Al Sheraji et al. 2013, Ferreira-Lazarte et al. 2019, Young et al . 2019). It has a sweetening power of 0.3 to 0.6 times in relation to sucrose (Torres et al. 2010).

GOs are obtained from the action of β-galactosidases using lactose as a substrate, depending on the origin of the enzyme different mixtures of GOs can be formed (Botvynko et al. 2019). The consumption of 10 g / day is adequate to balance the intestinal flora, although some authors claim that the consumption of 2 to 3 g / day is already sufficient to increase the levels of *Bifidobacterium* in the intestine (Torres et al. 2010,

Sousa et al. 2011, Al Sheraji et al. 2013). The maximum tolerated dose is around 0.3 to 0.4 g / kg of the consuming individual (Sako et al. 1999).

Studies indicate that diets enriched with GOs positively affected the composition of the intestinal flora, such as the increase in the number of *Bifidobacterium* and *Lactobacillus* in the intestine (Searle et al. 2009, Torres et al. 2010).

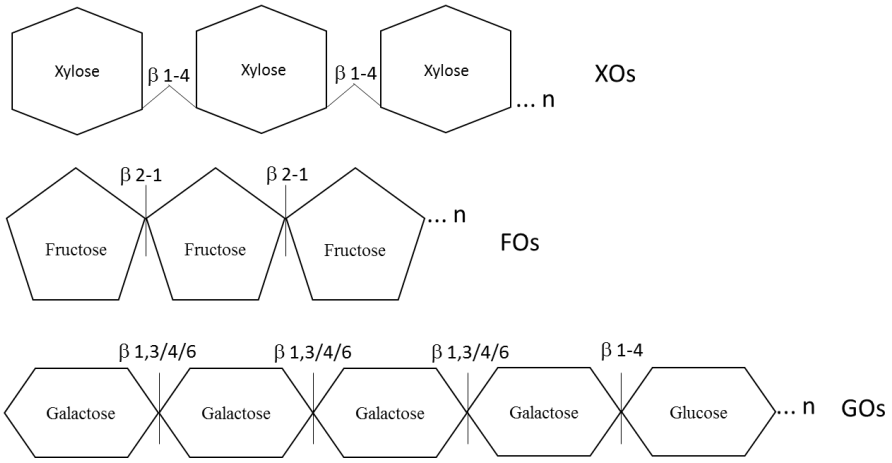
"In vitro" tests with tissue cultures simulating the intestinal environment showed that the presence of GOs avoided adhesion and invasion by *Salmonella typhimurium*, preventing colonization by this type of pathogen (Searle et al. 2009). The inhibitory effect of GOs (Danisco) on the growth of *Salmonella typhimurium* was evaluated by an "in vitro" test, where Mäkeläinen et al. (2010) observed an inhibition of *Salmonella typhimurium* growth for this prebiotic.

GOs also acts as a prebiotic in patients with IBS, stimulating the activity of *Bifidobacterium* in the intestine and, consequently, relieving the symptoms of the disease (Silk et al., 2008). The intake of GOs also helps in the absorption of mineral salts, mainly calcium, and is related to the reduction of toxic metabolites produced by pathogens, and an increase in lactose tolerance (Sousa et al. 2011, Young et al. 2019).

Because the beneficial effects, GOs has been a frequent ingredient added to foods, even used as a possible complement of human milk for infants (Botvynko et al. 2019).

In general, the study of different prebiotics and their methods of action are important factors in understanding their influence on the composition of the intestinal microbiota.

Figure 1- Schematic structures of xylooligosaccharides (XOs), fructooligosaccharides (FOs) and galactooligosaccharides (GOs).



Source: Own work.

3. INTESTINAL MICROBIOTA

The gastrointestinal tract is intensely colonized by several microorganisms, presenting a complex and dynamic interaction between different populations of bacteria, fungi, archaea and viruses (Wang 2017, Tang et al. 2019). This community of microorganisms that inhabits the gut is often called a "microbiota". The microbiota, also called microbiome, is constituted of approximately 10 trillion microorganisms, presenting from 300 to 500 species covering around two million genes (Quigley 2010, Tang et al. 2019).

3.1. Composition and colonization of the microbiota

Colonization of the intestine occurs during the birth process, as part of the microbiota is derived from the mother to the newborn (Maslowski and Mackay 2011). Some factors are essential to determine the composition of the intestinal microbiota such as the type of delivery, breastfeeding, environmental factors and the use of antibiotics during the first days of life (Derrien et al. 2019).

In general, the gastrointestinal tract is inhabited by microorganisms along its entire length, however, the colon has a greater colonization of bacteria. The phyla of microorganisms most frequently found in the intestines of adult individuals are: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia (Guarner and Malagelada 2003, Tang et al. 2019).

In-depth analysis of the microbiota composition requires detailed techniques for the cultivation of bacteria in different growth media and methods for taxonomic identification of the isolates (Guarner and Malagelada 2003). In humans, there are some prominent genera such as *Bacteroids, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus, Prevotella* and *Ruminococcus* (Guarner and Malagelada 2003, Derrien et al. 2019). In addition, other genera can be frequently found, such as *Escherichia*, *Enterobacter*, *Enterococci*, *Klebsiella*, *Lactobacillus* and *Proteus* (Guarner and Malagelada 2003).

The type of diet, lifestyle, geography, use of antibiotics and infections, can determine the variation in genera found in different individuals. For example, a diet rich in fiber may be associated with a greater diversity of the microbiota, as well as a diet rich in fats, very common in developed countries, is correlated with an increase in *Bacteroides* and a decrease in *Prevotella* and *Succinivibrio* abundance (Derrien et al. 2019). Age is also an important factor. In healthy children, the intestinal microbiota has a different composition when compared to adults because it tends to develop slowly, in the earlier stages of life *Bifidobacterium* spp. are very abundant and show a reduction in their number in the adult phase (Derrien et al. 2019).

3.2. Importance of intestinal microbiota

Colonization of the intestinal tract by specific types of bacteria has some benefits for the host health. The microbiota plays an important role in nutrition and nutrient absorption, in the stability of the intestinal epithelial protection barrier against pathogens and in the development of the immune system (Russel et al. 2011, Guarner and Malagelada 2003, Tang et al. 2019). The metabolic function of the intestinal flora is related to the fermentation of carbohydrates that escape the action of enzymes and gastric juice present in the digestive system. Such function results in the main source of energy in the form of fatty acids available in the colon, and also in improving the absorption of ions by the host's intestinal epithelium (Gibson and Roberfroid 1995, Wang et al. 2017). The microbiota is also involved in the production of vitamins, mainly vitamin K, responsible for blood clotting (Guarner and Malagelada 2003, Quigley 2010).

The formation of short-chain fatty acids (SCFAs) (acetate, propionate and butyrate), during the fermentation of the microbiota, provides the host with benefits

linked to the control of the differentiation and proliferation of intestinal epithelial cells, preventing the formation of neoplastic cells, being key in preventing colon cancer (Sanders et al. 2018, Tilg et al. 2018). The balanced intestinal flora helps keeping the intestinal epithelium intact, which may represent one of the barriers against the invasion of pathogens. In addition, the roles of the microbiota against microorganisms related to infections may also occur through competition for nutrients and adhesion sites present in the intestine, through the decrease in pH as a result of the production of acids by the fermentation of carbohydrates, and finally through the release of bacteriocins (Guarner and Malagelada 2003, Leahy et al. 2005).

3.3. Microbiota and diseases

Dysbiosis in the intestinal microbiota, a condition of microbial community imbalance, is strongly associated with the appearance of diseases, mainly infections (Tang et al. 2019, Wang et al. 2017). Some situations directly affect intestinal balance: changes in eating habits, stressful situations, usage of antibiotics, environmental changes; all may lead to an increase in the number of pathogenic microorganisms. Colonization by pathogens generates important anti-inflammatory responses, and treatments for these types of infections can also lead to a very large change in the composition of the microbiota. The excessive proliferation of the group of pathogenic bacteria may cause the host disturbances like diarrhea, infections, release of toxic or carcinogenic substances and even pathologies related to the immune system, as, for example, autoimmune diseases and allergies (Gibson and Roberfroid 1995, Johannsen and Prescott 2009).

Disturbances in the intestinal microbiota are the main cause of antibiotic-associated diarrhea (AAD), a common complication in hospitalized patients undergoing antimicrobial therapy (Velasco et al. 2018). Infections with *Clostridium difficile* are often associated with AAD, where dysbiosis caused by antibiotics leads to a sharp drop in resistance to toxins produced by *C. difficile*, enabling its rapid infection (Wang et al. 2017).

Many species of *Salmonella enterica* can induce gastrointestinal diseases known as salmonellosis, caused by the ingestion of contaminated water or food. After intestine colonization by *Salmonella*, the cells of the intestinal epithelium are attacked causing changes in the microvilli, which provokes a reduction in the enzymatic activities of the

brush border (Symonds et al. 2012). In this way, some intestinal functions are affected, for example, the absorption of ions, which results in diarrhea (Symonds et al. 2012).

Colon cancer is also involved in the imbalance of intestinal flora. A diet rich in fats and red meats, mainly processed food and high body weight significantly affects the microbiota metabolic activity and composition (Tilg et al. 2018). The anaerobic metabolism of peptides and proteins by some proteolytic bacteria can generate toxic substances such as ammonia, amines and phenols. These compounds are known as colon cancer initiators or promoters (Gibson and Roberfroid 1995, Guarner and Malagelada 2003). Bacteria of the genus *Clostridium* have been associated with an increased incidence of colon cancer and tumors (Tilg et al. 2018).

Metabolic disorders are also related to intestinal microbiota dysbiosis, such as type 2 diabetes and obesity. Research has intensified in order to assess how food factors directly influence the population of microorganisms that inhabit the intestine, and concludes that diet modulates the composition and function of the microbiota (Wang et al. 2017). A microbiota with altered diversity and resilience was found as a common characteristic among patients with type 2 diabetes, for the reason it can alter the metabolism and induce insulin resistance, for example (Wang et al. 2017).

The manipulation of intestinal microbiota for the treatment of ulcerative colitis and other gastrointestinal diseases has been shown to be very advantageous, however, regarding Crohn's disease, there is little evidence that this approach can act to induce remission of the disease and studies in this area are still inconclusive (Limketkai et al. 2020).

Cardiovascular diseases, anxiety symptoms, allergic sensitivities, asthma and eczema may be more effective in treatments when used together with ingredients that regulate the intestinal microbiota (Tang et al. 2019, Yang et al. 2019, Zimmermann et al. 2019). These studies have contributed to the knowledge that a good balance of the intestinal flora can contribute to prevention and even a possible treatment associated with the existing treatments for certain diseases.

The use of antibiotics, a common practice in the treatment of these types of diseases, has aroused the concern of researchers and the medical community due to the risk of inducing resistant strains and AAD. For this reason, research has been carried out to seek the replacement of antibiotics with treatments with beneficial bacteria (probiotics) (Guarner and Malagelada 2003, Velasco et al. 2018, Tilg et al. 2018).

The evident importance of the intestinal microbiota corroborates the great interest in the development of foods that preserve or stimulate the growth of probiotic microorganisms.

4. PROBIOTICS

4.1. Definition and classification

In 1965, Lilly and Stillwell presented the first definition of probiotics referring to substances produced by microorganisms related to promoting the growth of other microorganisms. The widely quoted Food and Agricultural Organization, of the World Health Organization, defined and recognized the benefits of probiotics in 2001. Currently, this definition has been revised and probiotics are described as living microorganisms that, when ingested in adequate amounts, are related to the balance of intestinal flora and the well-being of the host (Hill et al. 2014). The growing interest in the use of probiotics, mainly in the food industry for food supplementation, makes this ingredient of high added value, with an estimated value in the probiotics market of above $69.3 billion in 2023 (Markets and Markets 2019).

In order to be classified as suitable and safe for consumption, it is extremely important that the microorganism: is taxonomic identified; preferably of human origin; is able to survive in the intestinal colon; resists acid pH, enzymes and bile salts; colonizes the intestine; is not pathogenic and does not release toxins. Showing antagonism to pathogens, bringing benefits to the host and remaining viable throughout industrial processes, as well as during the period of use and storage are also important characteristics (Plaza-Diaz et al. 2019, Sanders et al. 2019a).

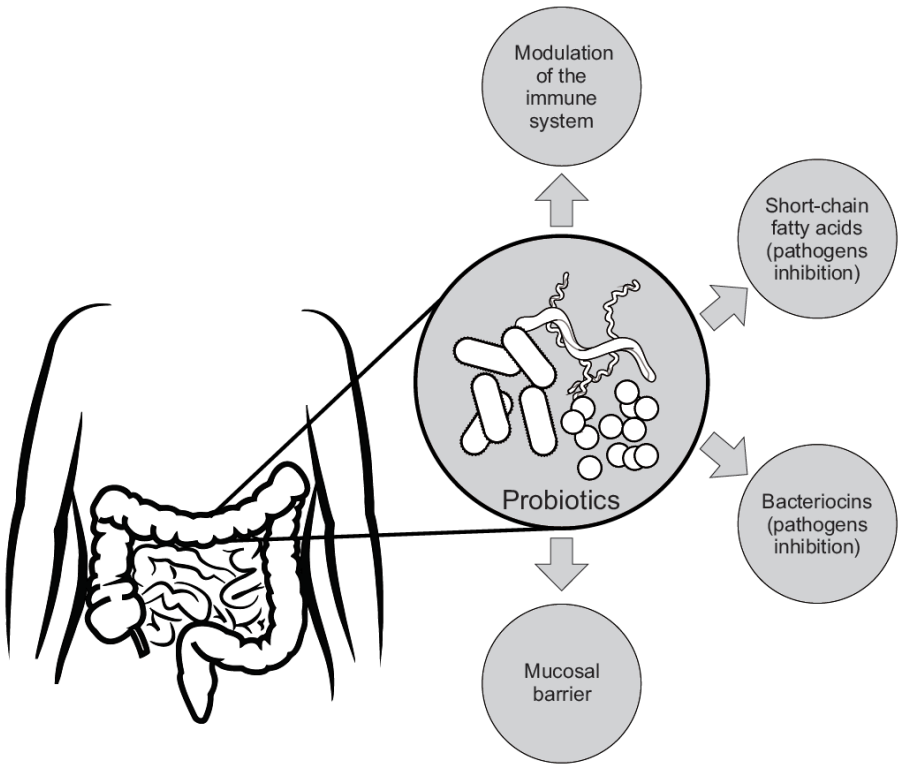
It is very important to ensure safety in the use of probiotics, since their mechanisms of action are not yet fully understood.

4.2. Mechanisms of action

The mechanisms of action by which probiotics play their beneficial role are known through "in vitro" tests, cell cultures, humans and other animal models. However, in clinical circumstances these benefits are not yet fully understood (Sanders et al. 2019a).

Current knowledge shows that probiotics can beneficially influence beyond the colonizing microbiota and their mechanisms of action depend on several factors, such as the types of microorganisms present in the intestine and the ability of the probiotic to act according to the host's needs (Sanders et al. 2019b). Probiotics can have several mechanisms of action in a single strain and the mode and spectrum of action of these mechanisms can also vary between different strains (Sanders et al. 2019b). The probiotics mechanisms of action are summarized in figure 2.

Figure 2- Probiotics mechanisms of action.



Source: Own work.

4.2.1. Modulation of the immune system

Probiotics are related to the modulation of the immune system by stimulating the production of anti-inflammatory molecules. The probiotic interacts via receptors in the intestinal mucosa regulating the production of antibodies, phagocytes and natural

killer cells (Sanders et al. 2019b). These microorganisms can increase the levels of anti-inflammatory cytokines and reduce production of pro-inflammatory cytokines in the intestine, this regulatory system decreases the incidence of colon cancer and colitis (Sanders et al. 2019b, Plaza-Diaz et al. 2019). Some studies show that the regulation of the system can suppress chronic inflammation, presenting beneficial effects for the treatment of irritable bowel syndrome (IBS) (Quigley and Flourie 2007, Wasilewski et al. 2015, Quigley 2019).

4.2.2. Mucosal barrier

The intestinal mucosa barrier is very important for the individual's health, as it presents selective permeability allowing the entry of some nutrients (Sanders et al. 2019a). The intestinal mucosa is also responsible for secreting mucins, proteins that prevent the adhesion of pathogens on epithelial cells (Plaza- Diaz et al. 2019). The adhesion of probiotics provides an improvement in the mucosal barrier, as they stimulate a greater production of intestinal epithelial cells and protective substances, such as mucin (Sanders et al. 2019a).

4.2.3. Inhibition of pathogens

Probiotics act in inhibiting pathogens due to their important role in the mucosal barrier of the intestine as discussed in the previous item. Furthermore, these microorganisms can compete for nutrients and adhesion sites present in the intestine, preventing the proliferation of pathogens (Sanders et al. 2019b). The production of short-chain fatty acids (SCFAs) and bacteriostatic or bactericidal substances, such as bacteriocins, is being widely studied as the main competitive exclusion mechanism for invading microorganisms from the natural microbiota (Nagpal et al. 2018, Sanders et al. 2018).

Short-chain fatty acids (SCFAs)

Organic acids are the main end products from the carbohydrate metabolism of some probiotic bacteria, mainly *Bifidobacterium* and *Lactobacillus* (Sanders et al.

2019b). SCFAs including lactic, butyrate, acetic and formic acids, have a great inhibitory potential of microorganisms that cause important infections, mainly Gram-Negative bacteria (Sanders et al. 2018).

The undissociated form of these organic acids passively pass through the bacterial cell wall of the pathogen and dissociate (Vieco-Saiz et al. 2019). The decrease in pH in the cytoplasm, or the accumulation of the ionized form of the acid inside the bacteria, promotes its death, since it modifies the proton gradient and the electric charge in relation to the extracellular medium, inhibiting its metabolic functions (Carpenter and Broadbent 2009).

This hypothesis implies that there would be a change in the transport of amino acids and phosphates, in addition to inactivating endogenous enzymes in the cell (Carpenter and Broadbent 2009). In addition, it causes an increase in the osmotic pressure of the cell due to the electrical charge, since there would be an increase in the concentrations of sodium and potassium (Carpenter and Broadbent 2009). In this way, an intracellular ionic force is created that promotes the disruption of the microorganism's cell wall.

Among the classes of acids, weak ones are more efficient at penetrating and acidifying the interior of the cell than strong acids, due to their dissociation capacity (Wang et al. 2014).

Bacteriocins

Bacteriocins are peptide compounds derived from the ribosomal synthesis of some bacteria and secreted in the extracellular environment, which have antimicrobial effects (Vieco-Saiz et al. 2019, Gao et al. 2019).

Due to its bactericidal or bacteriostatic effect, it has often been used in the food industry as a natural preservative to replace chemical preservatives (Salman et al. 2020). Bacteriocins have been studied as a possible complement for treatments of infections or even as an alternative to the use of antibiotics, since in contrast to the latter, bacteriocins act in certain species without affecting the composition of the microbiota (Vieco-Saiz et al. 2019).

The classification of bacteriocins takes into account characteristics such as, structure, mode of action, size, antimicrobial potential, immunity mechanism and target cell receivers (Salman et al. 2020).

Cotter et al. (2005) presented a classification that divides these molecules into classes, as follows:

Class I: Lantibiotics, that is, bacteriocins that have the amino acid lanthionine that can be further subdivided into two types A and B. They are small and thermostable peptides whose molecular weight is less than 5 kDa. The classic example of this Class is Nisin, produced by strains of *Lactococcus lactis*, usually isolated from dairy products. It is the most studied and accepted bacteriocin in the food industry.

Class II: These are bacteriocins that do not have lanthionine, thermostable, small (<10kDa). They have a large concentration of small amino acids such as glycine, are cationic and amphiphilic, culminating in its great ability to permeabilize membranes. They are produced by a wide variety of species, including *Enterococcus* and *Lactobacillus.* They can be subdivided into four subgroups: IIa (Pediocin-like bacteriocins), IIb (Bacteriocins from two peptides), IIc (Cyclic bacteriocins) and IId (Linear bacteriocins that are not pediocin).

Class III: They are larger (> 30 kDa) and thermolabile compounds. They were identified predominantly among the genus *Lactobacillus*. Unlike the other groups, these bacteriocins are sensitive to high temperatures, being inactivated in the range of 60-100 ºC in heat treatment that lasts from 10 to 15 minutes.

The bacteriocins of the first two groups are those of greatest technological interest due to their greater applicability in industrial processes and their greater abundance

4.3. *Probiotic properties of Bifidobacterium, Levilactobacillus and Lactobacillus*

Bifidobacterium, *Levilactobacillus* and *Lactobacillus* are genera of facultative anaerobic Gram-positive bacteria, or microaerophiles, often found in the colon and commonly associated with health of the host (Leahy et al. 2005, Makras and De Vuyst 2006). Both genera are commonly considered as probiotics.

These bacteria are saccharolytic, they ferment carbohydrates from the host's food, in addition to having no pathogenic representatives (Macfarlane et al. 2006, Quigley 2010).

The population number of probiotic bacteria in the intestine is relatively high, around 10^7-10^{10} CFU x g $^{-1}$, this number can vary according to the individual's age, use of antibiotics and diet (Makras and De Vuyst 2006, Markowiak and Śliżewska 2018, Nagpal et al. 2018).

A study using meta-analysis of probiotic strains such as *Saccharomyces boulardii* and *Lacticaseibacillus rhamnosus* GG, showed efficiency of these strains in preventing AAD (McFarland 2006). So far, these two probiotic strains are the only ones that have been shown to be efficient in the treatment of AAD (Velasco et al. 2018).

Some studies indicate that *L. rhamnosus* GG, *L. plantarum, L. acidophilus, L. paracasei, B. animalis, B. lactis, B. breve* and *B. longum* are able to alleviate the symptoms of inflammatory bowel disease, such as bloating, diarrhea and nutritional deficiencies (Coqueiro et al. 2018).

"In vivo" studies show that *Bifidobacterium infantis* 35624 attenuated the levels of infection in the brush-border enzymatic activities caused by *Salmonella*, probably due to the modulation of the host's immune system (Symonds et al. 2012; Scully et al. 2013). Ren et al. (2013) demonstrated that strains such as *L. salivarius* and *L. plantarum*, had the potential to prevent inflammatory diseases, including those caused by *Salmonella typhimurium*.

Related to colorectal cancer, *L. rhamnosus, B. lactis, B. longum, L. helveticus,* and *L. acidophilus,* can act to reduce the formation of tumors and inflammatory responses, in addition to reducing the effects of cytotoxic and genotoxic, ammonia concentration, β - glucosidase and oxidative stress (Eslami et al. 2019).

Many products have been commercialized for many years containing probiotics as food supplements, mainly in fermented drinks, such as *L. acidophilus, L. casei, L. delbruekii, L. plantarum, L. rhamnosus, B. adolescentis, B. bifidum, B. longum, B. infantis, B breve, B. lactis* and *B. animalis* (Gibson and Roberfroid 1995, Holzapfel et al. 1998).

5. SYMBIOTICS

Symbiotics consist of a combination of probiotic microorganisms and prebiotic ingredients. Gibson and Roberfroid (1995) defined symbiotics as "mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and / or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare ".

The mechanisms of action and criteria for the selection of symbiotics are the same studied in probiotics and prebiotics (Markowiak and Śliżewska 2018). Symbiotics production can take two different approaches according to the prebiotic chosen to compose the product (Kolida and Gibson 2011, Sanders et al. 2019a). It can be considered "complementary", where the prebiotic choice has no relation with the probiotic of the elaborated product, or it can be considered "synergistic", when the prebiotic is chosen specifically to stimulate and guarantee the survival of the selected probiotic (Kolida and Gibson 2011, Sanders et al. 2019a).

Because they present different approaches, each "complementary" and "synergistic" symbiotic shows different formulations according to their purpose. For example, in the first approach, the target of the prebiotic is to cause health benefits to the host by maintaining the balance of the intestinal flora, regardless of the probiotic selected (Kolida and Gibson 2011). Therefore, this symbiotic composition generally presents high concentrations of prebiotics to obtain the desired purpose (Kolida and Gibson 2011). In the "synergistic" approach, the prebiotic's target is to guarantee the survival and implantation of the probiotic that makes up the product, in this case, the prebiotic concentration may be lower, as long as it fulfills the proposed effect (Kolida and Gibson 2011).

Some studies show the effect of symbiotic ingestion on the intestinal microbiota. Morelli et al. (2003) analyzed the feces of 12 healthy adults and demonstrated that the combination of inulin, *Lacticaseibacillus paracasei* and *Lactobacillus gasseri* ($0.5x10^9$ CFU - colony-forming units) ingested for 15 days increased the populations of probiotics in the intestine.

A study of 51 healthy elderly people ingesting a symbiotic containing *L. acidophilus* ($2x10^9$ CFU) with 5g of lactitol, demonstrated by real-time PCR tests an increase in the populations of *Lactobacillus* and *Bifidobacterium* (Ouwehand et al. 2009). The combination of XOs and *Bifidobacterium lactis* can confer additional benefits to the

intestinal microbiota and the immune system, due to the increased survival of live bacteria present in the food supplement (Childs et al. 2014).

Studies with commercial symbiotics, Flortec (Bracco Co), which contains *L. paracasei*, XOs, glutamine and arabinogalactan, and Zir Fos® which contains *B. longum* and FOs, have shown efficiency in reducing symptoms, such as pain and diarrhea, of patients with irritable bowel syndrome. (Dughera et al. 2007, Adriulli et al. 2008).

Symbiotic products are also related to decrease risk of colon cancer, as the daily consumption of fibers associated with the maintenance of intestinal flora plays a fundamental role in the proper functioning of cell division, preventing the formation of tumor cells (Quigley 2019). Le Leu et al. (2005) evaluated the combination of resistant starch with strains of *B. lactis* and *L. acidophilus*, alone or in mixtures, and observed an increase in *Lactobacillus* and *Bifidobacterium*, in addition to the combination of starch and *B. lactis* increasing the acute apoptotic response of tumor cells.

The interest in the development and use of new symbiotics has risen due to studies that prove the benefits related to alternative treatments for intestinal diseases, in addition to its use as a preventive for such diseases, although determining the minimum concentrations of the components is still a challenge for the researchers.

6. EVALUATION MODELS OF PREBIOTICS, PROBIOTICS AND SYMBIOTICS.

6.1. Prebiotics

6.1.1. "In vitro" tests

Some "in vitro" methodologies are proposed to evaluate characteristics such as resistance to the action of digestive enzymes, fermentability by the intestinal flora and, finally, the selectivity of the prebiotic by beneficial bacteria. It is very common that in these types of tests the chemical simulation of environments such as the small intestine is performed, where salivary, pancreatic and intestinal enzymes are added and after a few hours the degradation of the oligosaccharide is evaluated (Figueiredo et al. 2020).

Often pure cultures or fecal samples are used to determine the fermentation and selectivity of prebiotics by intestinal bacteria (Kolida and Gibson 2011). Both tests have

advantages and disadvantages, since in pure cultures it is not possible to identify the inhibitory effects of pathogens by the growth of probiotics, however, tests with fecal samples are not always completely conclusive in relation to the identification of the species present (Roberfroid 2007).

"In vitro" tests are very important tools to determine the efficiency and safety of prebiotics, with lesser costs, ease of handling and without involving many ethical aspects, while "in vivo" tests are being questioned progressively. The "in vitro" tests generally provide greater predictability for the next stage that generally involves the "in vivo" tests, which are mainly aimed at observing the action of prebiotics in relation to intestinal diseases.

6.1.2. "In vivo" tests

Candidates for prebiotics must pass "in vivo" tests in rats and later in humans with experiments using double-blind, control and placebo (Kolida and Gibson 2011). A very common "in vivo" method for assessing digestibility is through oral administration of the prebiotic to rats free of infection or treated with antibiotics to suppress the intestinal flora, after this process the feces are collected and the presence of the administered product is analyzed (Nilsson and Bjorck 1988). Some methodologies make it possible to determine digestibility in humans, after ingestion of the prebiotic. In such cases, blood tests are done to observe the increase or not of glycemic rates in the circulatory system (Molis et al. 1996, Gibson et al. 2004). The same tests are performed to assess fermentability and selectivity, however the flora is kept intact and the evaluation consists of the administration of prebiotics and subsequent measurement of the concentration of gases (mainly oxygen) produced, or performed by collecting fecal samples and analyzing the amount of prebiotics recovered in feces after oral administration (Gibson et al. 2004, Roberfroid 2007). Molecular biology tools can also be used to study the growth stimulation of beneficial bacteria in the intestine by ingesting prebiotics (Kolida and Gibson 2011).

The low number of individuals in "in vivo" studies with patients with gastrointestinal diseases impairs the reliability and interpretation of results (Kolida and Gibson 2011).

6.2. Probiotics

6.2.1. "In vitro" tests

For the evaluation of probiotics by "in vitro" tests it is important that they are characterized as safe for human consumption. Thus, many commercialized probiotics are isolated from the gastrointestinal tract of humans, as they do not present risks to human health, in addition to being more effective in adapting to the intestinal environment (Kolida and Gibson 2011). Molecular techniques should make it possible to assess the probability of probiotic strains to acquire pathogenicity and resistance to antibiotics (Quigley 2010).

Some tests are performed to determine the resistance of probiotics to technological processes, such as durability and viability of the microorganism (Quigley 2010, Kolida and Gibson 2011). Another important step in this type of test is to determine the probiotic's ability to resist the acidity of gastric juice and simulate the intestinal environment, allowing the assessment of the ability of the microorganism to adhere to the intestinal epithelium (Kolida and Gibson 2011).

6.2.2. "In vivo" tests

The "in vivo" tests of probiotics are more focused on analyzing their effects in individuals diagnosed with gastrointestinal diseases. In rats and humans, these tests ensure the efficiency of probiotics in fighting or preventing certain diseases, especially in the gastrointestinal tract (Kolida and Gibson 2011, Tong et al. 2013).

Few studies including "in vitro" or "in vivo" tests demonstrate the efficacy of mixtures of probiotic strains, moreover, the mechanism that results in synergism in terms of increased bioactivity is poorly understood (Rosenfeldt et al. 2003, Kim et al. 2003, Viljanen et al. 2005, Chapman et al. 2011, Chapman et al. 2012). In many studies, the comparison between pure and mixed cultures is not always performed by standardizing the inoculum concentration, which can generate unreliable results (Chapman et al. 2012). Further studies are needed to define the ideal combination of probiotics, without an antagonistic effect between species, in order to expand the benefits to the host and enhance the inhibition of a greater variety of pathogens.

6.3.Symbiotics

6.3.1. "In vitro" and "In vivo" tests

The establishment of a new symbiotic formulation must pass all the "in vitro" and "in vivo" evaluation criteria for prebiotics and probiotics described above (Kolida and Gibson 2011). The components of the symbiotic formulation need to be evaluated separately to determine whether the positive effects of the symbiotic were superior to the separate ingredients (Kolida and Gibson 2011). Determining the minimum dose of each component and the storage conditions are fundamental studies for creating a new product (Kolida and Gibson 2011). Currently, few "in vivo" studies are being carried out to demonstrate the effects of symbiotic ingestion in patients with diseases that affect the intestine, although the trend is that these studies will increase over the years due to the potential that these products have in preventing certain diseases.

7. CONCLUSION AND FUTURE PERSPECTIVES

Many researches seek to understand the mechanisms that lead to an important relationship between the intestinal microbiota and the health of the host. The development and consumption of probiotic, prebiotic and symbiotic ingredients has increased both in human food and in other animals. Consequently, the market for these products has grown over the years all over the world.

The next steps in researches of this field aim to fully clarify the mechanisms of action in the microbiota, as well as the effect of the synergism between several prebiotics mixed with several probiotics, combined or not, in the control of specific diseases and to characterize the benefits for the health and to avoid futures diseases. As consequence, to develop new products that can reach a wide range of disease control and/or avoiding futures diseases, replacing the classic medicines. In addition, to stimulate the formulation of new foods with some special ingredients (prebiotics and probiotic) which can increase the healthy benefits.

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CHAPTER 2

Understanding the antimicrobial properties of metabolites from probiotic bacteria

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ABSTRACT

Postbiotics is a new concept, defining cell-free extracts of microbial origin that confer health benefits. The positive benefits of postbiotics are related to immunomodulatory, anti-inflammatory and antimicrobial effects. *Salmonella enterica* serovar *Typhimurium*, *Escherichia coli* and *Staphylococcus aureus* are pathogenic bacteria that can cause diverse infections. Probiotic bacteria control or inhibit such pathogens by competition mechanisms or release of antimicrobial compounds. The goals of this work were to verify and understand the antimicrobial activity of metabolites from different culture extracts of *Bifidobacterium*, *Levilactobacillus* and *Lactobacillus* strains against these pathogens. All postbiotics inhibited *E. coli*, while none inhibited *S. aureus*. *Bifidobacterium* species seem to have a stronger inhibitory activity against *Salmonella typhimurium* than *Lactobacillus* or *Levilactobacillus.* Temperature tests showed that all postbiotics are stable at high temperatures, except those of *B. longum*, with its bacteriocin acting as its major antimicrobial metabolite. Neutralization of pH canceled the inhibitory activity, while acidification improved the inhibition by postbiotics, highlighting the role of organic acids and their synergism with low pH. Although further studies are necessary to explain the complex synergism of the metabolites involved in the antimicrobial activity, the stability at high temperatures and low pH shows great potential for postbiotics as natural food preservatives in the food industry.

Keywords: *Salmonella typhimurium*, *E. coli*, *S. aureus*, Bacteriocin, Organic Acids.

1. INTRODUCTION

Numerous studies highlight the role played by intestinal microbiota on health. Such interest is justified mainly by the increase in health problems related to the quality of food, also known as "21st century diseases", such as obesity, food poisoning and allergies. Researchers seek to discover and develop new products that help or modulate the proper functioning of the organism, such as probiotics, prebiotics and symbiotics (Markowiak and Śliżewska, 2018; Figueiredo, Ranke, Oliva-Neto, 2020). In general, these ingredients can be naturally present in food or added to industrialized products.

Probiotics can be defined as microorganisms, usually *Bifidobacterium* and *Lactobacillus*, which, when ingested in adequate quantities, positively affect the health of the host (Wasilewski et al., 2015). These bacteria are directly related to the prevention of inflammatory bowel diseases, the formation of neoplastic cells and allergic reactions (Vieco-Saiz et al., 2019).

Bifidobacterium and *Lactobacillus* are genera of Gram-positive and facultative anaerobic bacteria, or microaerophiles, with the human colon as their natural habitat (Makras and De Vuyst, 2006). The population of probiotic bacteria in the intestine is

relatively high, around 10^7–10^{10} CFU x g^{-1}, however, this number may vary according to the individual´s age and diet (Makras and De Vuyst 2006; Markowiak and Śliżewska, 2018; Nagpal et al., 2018).

The use of antibiotics can frequently cause diarrhea in patients, due to the reduction in the population of intestinal flora and its fermentation capacity. In this context, many probiotics stand out as an additional treatment for infections (Velasco et al., 2018; Mekonnen et al., 2020), with reports of effective prevention of antibiotic-associated diarrhea in hospitalized patients by a combination of *Lactobacillus acidophilus*, *Bifidobacterium animalis* and *Lacticaseibacillus casei* (Velasco et al., 2019).

In addition, the consumption of probiotics is a possible replacement to the use of antibiotics in the treatment of infections caused mainly by *Salmonella enterica* serovar *Typhimurium* and *Escherichia coli*, bacteria present in contaminated water and foods and responsible for more than one million intestinal infection cases annually (Madigan et al., 2016; Mirsepasi-Lauridsen et al., 2019). *Staphylococcus aureus* is another important pathogenic bacterium, responsible for many cases of hospital-associated infections and capable of causing food poisoning with its toxins (Dayan et al., 2016).

According to the definition of probiotics, these ingredients must contain live microorganisms. However, the new concept of postbiotics has emerged, defined as extracts/supernatants free from viable cells that confer health benefits like immunomodulatory, anti-inflammatory, antioxidant and antimicrobial effects (Cuevas-González, Liceaga and Toalá, 2020).

Probiotics produce antimicrobial substances such as bacteriocins, hydrogen peroxide, amines and short-chain fatty acids (SCFAs) that can be associated with inhibition of pathogens that often cause infections (Nagpal et al., 2018; Sanders et al., 2018). Even though their mechanisms of action are not yet fully understood, there is a consensus among researchers that these mechanisms vary between different species (Sanders et al., 2018).

Bacteriocins, one of the metabolites produced by lactic acid bacteria, are polypeptides produced during ribosomal synthesis, mainly in the exponential growth phase or in the beginning of the microorganism's stationary phase (Vieco-Saiz et al., 2019; Gao et al., 2019). These compounds have bactericidal and / or bacteriostatic activities and have been frequently used as a natural food preservative, in addition to being studied as an alternative to traditional antibiotics (Salman et al., 2020).

Other biomolecules produced by lactic acid bacteria, including probiotic bacteria, are organic acids, the main product of their metabolism (Sanders et al., 2018). Organic acids act in the reduction of pH, affecting metabolic functions and leading to the death of the pathogen (Vieco-Saiz et al., 2019).

In view of the growing demand of probiotic ingredients and postbiotics as possible replacements of antibiotics and as natural food preservatives, understanding the role of the metabolism of probiotics in inhibiting the growth of pathogenic bacteria is extremely important for the future of prevention and cure of enteric diseases and for the protection of foods against pathogenic microorganisms. The goals of this work were to identify which cell-free probiotic extract of recognized probiotic strains has the strongest inhibitory action against *Salmonella typhimurium, E. coli* and *S. aureus* and to understand their mechanisms of action.

2. MATERIAL AND METHOD

2.1. Microorganisms

Some recognized probiotic strains were obtained via culture collections. *Levilactobacillus brevis* ATCC 367 and *Lactobacillus acidophilus* ATCC 4356 strains were obtained from the Collection of Reference Microorganisms in Sanitary Surveillance - CMRVS (Oswaldo Cruz Foundation - FIOCRUZ, RJ, Brazil). These microorganisms were maintained as a stock culture at 7 °C on MRS medium. Other probiotics were obtained through the donation of companies: *Bifidobacterium animalis* BB-12 Christian Hansen (Hoersholm, Denmark), *Bifidobacterium lactis* BI-07 Danisco (Madison, USA), *Bifidobacterium longum* BL-05 Danisco (Madison, USA) and *Bifidobacterium breve* BB-03 Danisco (Madison, USA). These strains were maintained as a stock culture at 7 °C on MRS medium with cysteine.

Salmonella enterica subsp. *enterica* serovar *Typhimurium* ATCC 14028 (hereafter called *Salmonella typhimurium*), *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* ATCC 25923 were used as models of pathogenic strains. *Salmonella* typhimurium ATCC 14028 was obtained from the Tropical Culture Collection (CCT), André Tosello Foundation, Campinas, SP, Brazil, registered with the code CCT 1478. The cultures were maintained in the Laboratory of Industrial Biotechnology (UNESP-Assis / SP) at 7 ºC in Nutrient Agar.

2.2. Growth medium for bacteria of the genera *Levilactobacillus, Lactobacillus* and *Bifidobacterium*

Static liquid cultures with formulated MRS medium (De Man, Rogosa, & Sharp, 1960) were used, with the medium prepared at pH 6 and autoclaved for 20 minutes at 121 °C. *L. acidophilus* was grown at 37 °C for 48 hours and *L. brevis* was grown at 30°C for 48 hours. *Bifidobacterium* cultures were grown in modified MRS medium with the addition of L-cysteine at 37 °C for 48 hours.

2.3. Growth medium for *Salmonella typhimurium, Escherichia coli* and *Staphylococcus aureus*

The culture medium for *Salmonella typhimurium* was Brilliant Green medium (BG) (Kauffmann, 1935) prepared with slight modifications. The composition was (g / L): 3 g of yeast extract, 10 g of proteose-peptone, 5 g of sodium chloride, 10 g of lactose and 10 g of sucrose. *E. coli* was cultured in pH 7 Nutrient Broth (NB) and *S. aureus* was grown on Brain Heart Infusion (BHI) medium. The culture media were prepared and autoclaved for 20 minutes at 121 °C. All strains were incubated at 37ºC for 48 hours.

2.4. Tests of antimicrobial activity against pathogenic bacteria by postbiotics

The "in vitro" tests were carried out according to Chapman and Gibson (2013). Cultures incubated in liquid MRS (item 2.2) were adjusted to values between 0.8 and 0.9 of optical density in a spectrophotometer at 650 nm in order to represent approximately equal biomass. After growth, they were centrifuged at 2000 x g for 10 minutes at 25 °C and the supernatants were microfiltered through a 0.22 μm membrane filter (Advantec) in order to obtain cell-free probiotic extracts. Cell-free probiotic extracts were called in this paper as postbiotics.

The pathogenic strains, *Salmonella typhimurium*, *E. coli and S. aureus,* were grown in their corresponding liquid medium (item 2.3) at 37 ° C for 48 hours. A 50 μl aliquot of pathogenic cultures was added in 90 ml of their respective medium. Thereafter, for each medium, 10 ml of postbiotics were added. Tests were performed in triplicate (n = 3). Three controls were developed. In the first one, pathogenic cultures were incubated

in 100 mL of their respective medium. In the second one, 10 mL of water, instead of postbiotics, were added to 90 mL of medium. Finally, in the third control, in order to evaluate the influence of pH on the pathogenic strains growth, 10 ml of HCl solution with similar pH to those observed in the postbiotics were added to 90 ml medium (Chapman and Gibson, 2013). Growth curves of pathogenic strains were carried out from the turbidity analysis in a spectrophotometer using aliquots obtained at 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours.

2.5. Influence of pH, heating and freezing on the antimicrobial activity of postbiotics against pathogenic bacteria.

Additional tests were carried out similarly to the previous item. In order to assess whether the inhibition was due to the production of acids and / or bacteriocins or other components by the probiotic strains and to test the influence of pH, prior to the addition to the medium, each of the postbiotics had their pH adjusted. Two treatments were performed, one where all postbiotics were neutralized with sodium hydroxide (NaOH) until pH 7 and a second treatment where the postbiotics were acidified to pH 2 using HCl.

To verify whether temperature could change the pattern of inhibitory action observed with postbiotics, tests were made where, prior to the addition to the medium as described in item 2.4, the postbiotics were subjected to different temperature treatments. For testing the inhibitory action after freezing, each of the postbiotics were frozen for seven days after microfiltration, and for testing their heat stability, postbiotics were sterilized in autoclave at 121º C for 15 minutes instead of filtering.

2.6. Analytical methods

2.6.1 Determination of the microorganisms growth curve

Optical density (O.D.) was used as a rapid measurement of bacterial biomass (Shuler & Kargi, 2005). Growth curves of probiotic and pathogenic bacteria were determined by optical density analysis (O.D.). Sterile growth medium (MRS medium for probiotic and BG, BHI and NB medium for pathogenic bacteria) were used as standard for the calibration curve. The absorbance was measured at 650 nm in a spectrophotometer

(Fento 700 Plus) (Moura et al., 2007; Chapman and Gibson, 2013). Aliquots of pathogenic bacteria were collected after 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours.

2.6.1. Calculation of the growth inhibition of the cultures by the postbiotics

The calculation of the percentage of inhibition growth of the cultures was performed by the following equation (1):

$$\text{Inhibition \%: } \frac{OD\ control - OD\ treatment}{OD\ control} * 100 \quad (1)$$

OD control: Optical density of control after 48h of incubation.
OD treatment: Optical density of the experiments after 48h of incubation.

2.6.2. Determination of organic acids

Organic acids from postbiotics were analyzed through liquid chromatography (WATERS – HPLC) after being filtered through a 0.22 μm membrane filter (Advantec). An HPX-87H column was used for the chromatographic ion exchange with 5 mM eluted H_2SO_4. The flow rate was maintained at 0.6 mL / min for 18 minutes (De Keersmaecker et al., 2006).

2.6.3. Statistical analyses

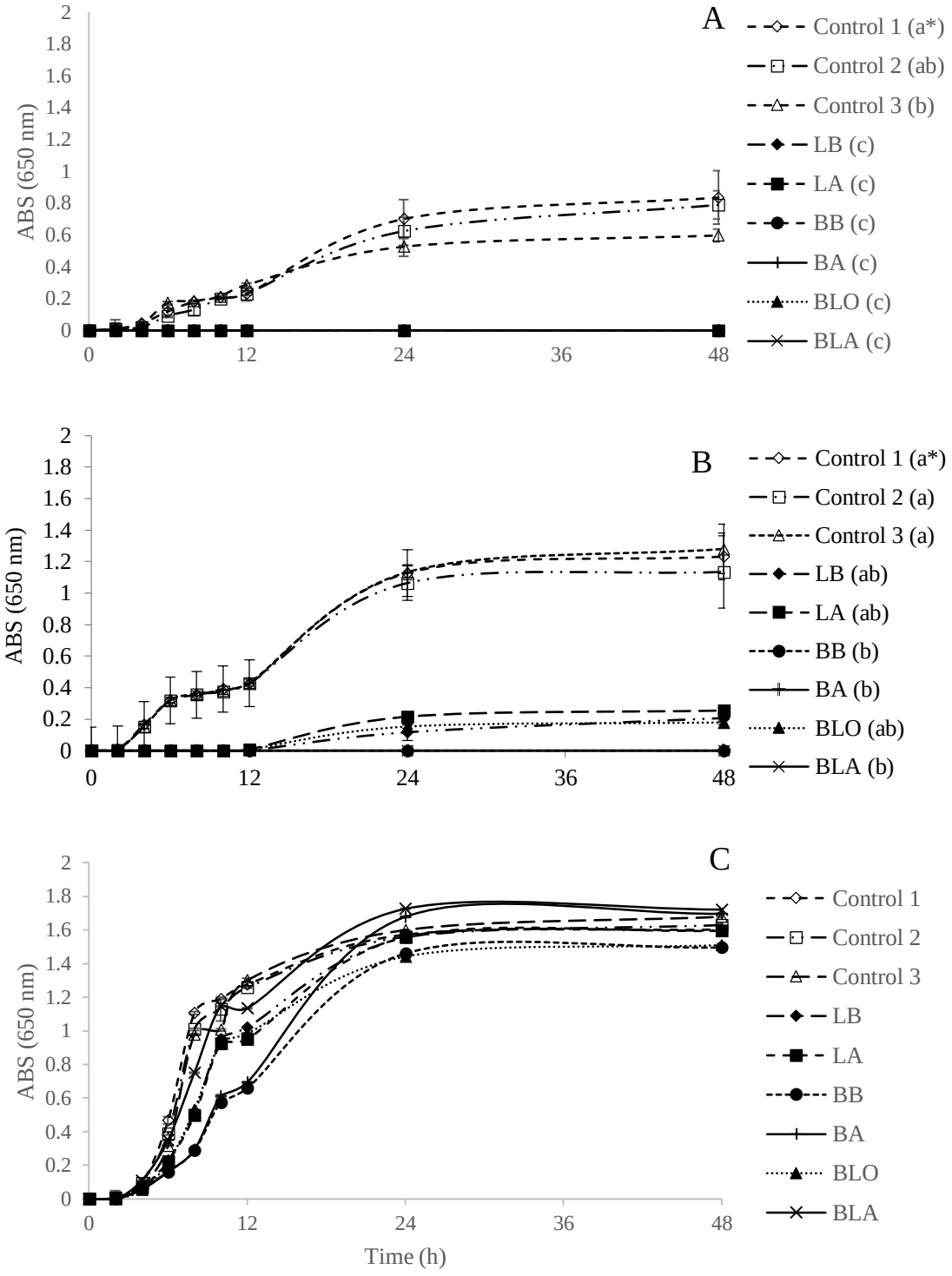
Statistical analyses were performed to compare the inhibition of the three pathogenic bacteria by different postbiotcs using Kruskal-Wallis ANOVA through the BioEstat 5.3 software.

3. RESULTS AND DISCUSSION

3.1. Inhibition of pathogenic bacteria by postbiotics and evaluation of antimicrobial activity after pH and temperature treatments.

Tests with postbiotics were performed to understand the inhibition mechanisms of pathogenic microorganisms by the metabolites of probiotic strains. The tested pathogenic bacteria grew in all controls (Fig 1), with the growth in control 3 (pH 4.0) revealing that only an acidic pH value is not sufficient to inhibit the growth of these pathogens. *E. coli* did not grow in the presence of any of the postbiotics (Fig 1A). In addition, the tests showed that the postbiotics of *B. animalis*, *B. breve* and *B. lactis* fully inhibited *Salmonella typhimurium* growth, with statistically significant difference ($p < 0.05$) from controls (Fig 1B). *B. longum*, *L. acidophilus* and *L. brevis* postbiotics inhibited the growth of *Salmonella typhimurium* in the first 12 h, and significantly ($p < 0.05$) lowered the pathogen's growth after 12h in comparison with controls (Fig 1B). None of the postbiotics inhibited the growth of *S. aureus* (Fig 1C).

Figure 1- Growth profile of *Escherichia coli* (A), *Salmonella typhimurium* (B) and *Staphylococcus aureus* (C) in NB, BG and BHI medium respectively, containing micro filtered cell-free probiotic supernatants from strains at 37º C for 48h. Control 1 = 100 ml of medium. Control 2 = 90 ml of medium + 10 ml of H2O. Control 3 = 90 mL of medium + 10 mL of H2O at pH 4. LB = *L. brevis*, LA = *L. acidophilus*, BB = *B. breve*, BA = *B. animalis*, BLO = *B. longum* and BLA = *B. lactis.* *Groups followed by the same letter in brackets were not significantly different in Kruskal-Wallis ANOVA followed by Student-Newman-Keuls test.



Source: Own work.

Genus *Lactobacillus* presents a strong inhibition against *E. coli*, as observed by Chapman et al. 2013. *Bifidobacterium* strains are also capable of inhibiting the growth of *E. coli*, especially through the release of organic acids in the medium (Makras and De Vuyst, 2006). In contrast to our findings, *B. longum* has been described as a strong inhibitor of *Salmonella typhimurium* (Makras and De Vuyst, 2006), however, the postbiotics of this strain showed only a partial inhibition of *Salmonella typhimurium* in our results. Another study observed a sharp drop in *Salmonella* cell viability, mainly in its exponential phase, in the presence of *Lactobacillus* strains (Fayol-Messaoudi et al., 2005), an inhibition pattern confirmed in the present work (Fig 1B). *S. aureus*, on the other hand, is a Gram positive pathogenic strain, well known for its antibiotic resistance and virulence, with previous reports of its growth being inhibited by pure probiotics (Dayan et al., 2016), but weak growth inhibition by the supernatants of probiotic bacteria (Agostini et al., 2018).

The antimicrobial activity of lactic acid bacteria is strongly related to the production of organic acids, however, it is believed that part of their inhibitory action is associated with other antimicrobial compounds, such as bacteriocins (Vieco-Saiz et al., 2019). Most bacteriocins are proteinaceous in nature, and may denature under certain temperature or pH conditions (Sidooski et al., 2019). Considering this information, our tests aimed to verify whether different pH and temperatures would degrade any bacteriocins and change the inhibition pattern observed with postbiotics.

Regarding the temperature treatments, the inhibitory activity of the postbiotics did not suffer any interference after being frozen (-10 ºC) for seven days, since the inhibition pattern of the three pathogens tested remained the same after freezing and thawing the postbiotics. For the treatments with high temperature using autoclaved postbiotics, the growth inhibition were similar to those of the postbiotics that were not treated by heating at 121ºC (Fig 2), with the exception of the postbiotics of *B. longum*, which presented a significantly lower inhibition of *Salmonella typhimurium* after autoclaving (Fig 2B). These results suggest that either the organic acids or other thermostable biomolecules from the metabolites of probiotic are enough to maintain the inhibitory action and/or the bacteriocins produced by these specific probiotic strains (except *B. longum*) are stable at high temperatures (121ºC).

Table 1- *Lactobacillus, Levilactobacillus* and *Bifidobacterium* bacteriocins and their main characteristics.

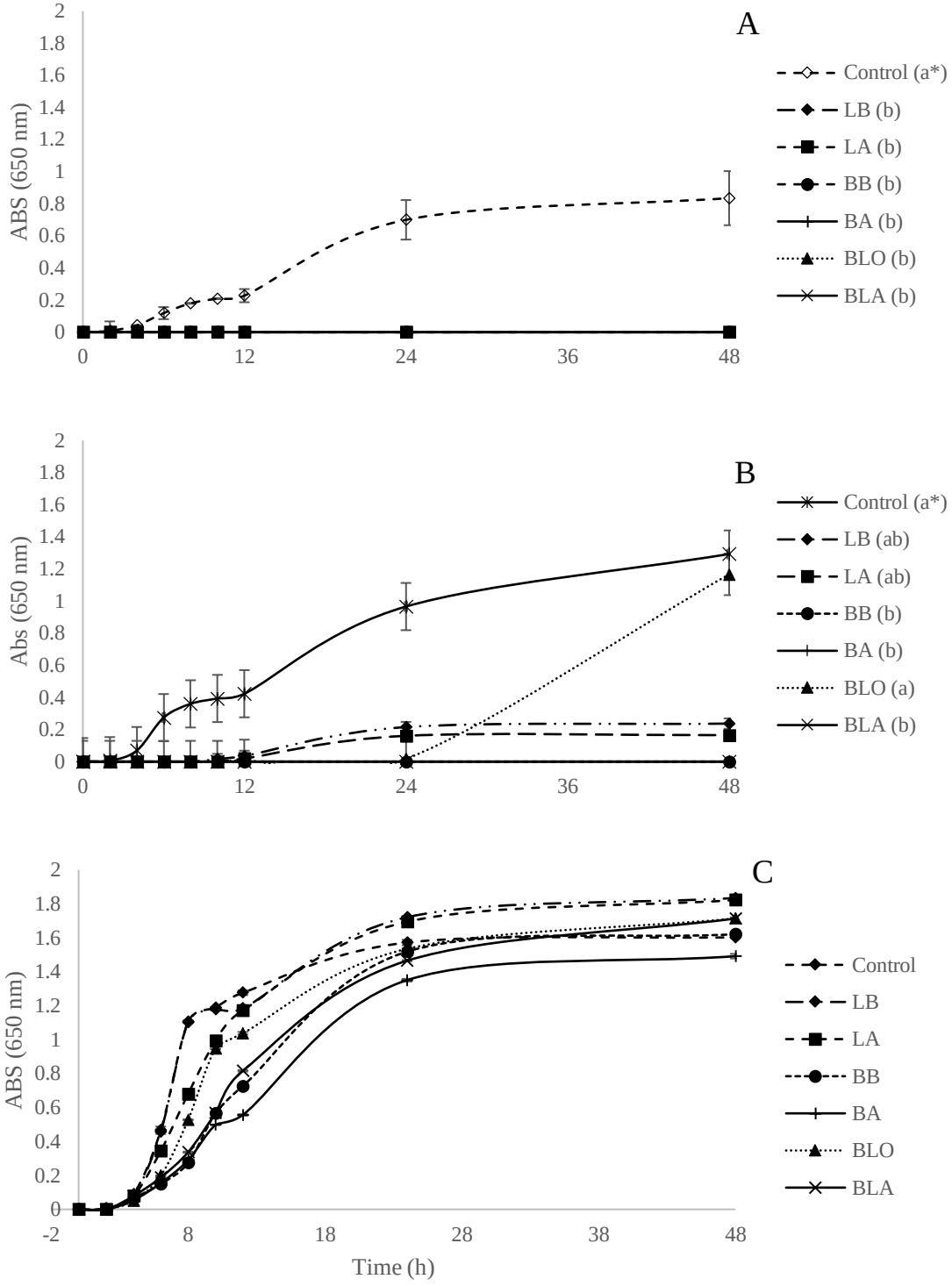
Bacteriocin	Species	Heat range stability	pH range stability	Inhibitory spectrum	Reference
Acidocin d20079	*L. acidophilus*	121°C – 30 min	2 – 7	*Bacillus cereus*	Deraz et al. (2005); Salman et al. (2020)
Brevicin ns01	*L. brevis*	20 - 100°C	3 – 8	*E. coli*, *S. aureus*, lactic acid-producing bacteria, *B. cereus*, *Listeria*, *Salmonella*, and the fungi *Candida albicans* and *Penicillium citrinum*	Duraisamy et al. (2015)
Bifidocin a	*B. animalis*	121°C – 15 min	2 – 10	Gram-positive and Gram-negative bacteria	Liu et al. (2015)
Bifidobrevicin LHM	*B. breve*	100 ºC – 30min	3 – 8	*Streptococcus agalactiae*	Mahdi (2017)
Bifilact bb-12	*B. lactis*	Unstable at high temperatures	4 – 7	*Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus*, *E. coli*	Saleh and El-Sayed (2004)
Bifilong	*B. longum*	100°C – 30 min	2 – 5	Gram-positive and Gram-negative bacteria	Kang et al. (1989)

Table 1 summarizes the temperature stability, pH ranges and inhibitory spectra of some bacteriocins produced by the probiotic strains used in this study. The bacteriocins produced by *L. acidophilus* and *B. animalis*, acidocin and bifidocin A, respectively, show stability in higher temperatures, demonstrating that in this study they were probably not degraded by heat and thus, the inhibitory action of these two strains on *Salmonella typhimurium* and *E. coli* growth may have occurred due to the action of their bacteriocins with organic acids.

Bacteriocins produced by *B. lactis* and *L. brevis* (Bifilact Bb-12 and Brevicin NS01, respectively), on the other hand, do not show stability above 100º C. Therefore, the results with autoclaved postbiotics (121 ºC) show that the inhibitory effect of these strains has their main action mechanism probably associated with the production of organic acids. *B. longum*'s bacteriocin (Bifilong) presents similar stability to those of *B. lactis* and *L. brevis*, since, in the present study, the percentage of inhibition of *Salmonella typhimurium* dropped significantly from 85.49 to 9.81% (Table 2) with *B. longum*'s autoclaved postbiotics. This result shows that its bacteriocin was probably the primarily responsible for the antimicrobial effect against *Salmonella typhimurium*.

Organic acids have a fundamental role in the antimicrobial potential of probiotic bacteria, therefore, the pH study was necessary to understand if this performance was due to the organic acids specifically or due to pH.

Figure 2- Growth profile of *Escherichia coli* (A), *Salmonella typhimurium* (B) and *Staphylococcus aureus* (C) in NB, BG and BHI medium respectively, containing autoclaved (121º C) cell-free probiotic supernatants from strains at 37º C for 48h. Control 1 = 100 ml of medium. LB = *L. brevis*, LA = *L. acidophilus*, BB = *B. breve*, BA = *B. animalis*, BLO = *B. longum* and BLA = *B. lactis.* *Groups followed by the same letter in brackets were not significantly different in Kruskal-Wallis ANOVA followed by Student-Newman-Keuls test.



Source: Own work.

Table 2- Percentage inhibition of pathogenic bacteria by micro filtered and autoclaved (121ºC / 15min) cell-free probiotics supernatants. *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* grew in NB, BG, BHI and media respectively, at 37º C for 48 h.

	E. coli		*Salmonella typhimurium*		*S. aureus*	
Probiotics	**Micro filtered (%)**	**Autoclaved (%)**	**Micro filtered (%)**	**Autoclaved (%)**	**Micro filtered (%)**	**Autoclaved (%)**
L. acidophilus	100	100	79.35	87.26	0	0
L. brevis	100	100	83.22	81.69	0	0
B. longum	100	100	85.49	9.81	6.3	0
B. animalis	100	100	100	100	0	0
B. lactis	100	100	100	100	0	0
B. breve	100	100	100	100	6.3	0

Table 3 shows the individual pH of each postbiotics from probiotic strains immediately after centrifugation and microfiltration, as well as the pH of the pathogenic culture media (BG, NB and BHI) after the addition of the postbiotics. These data are presented in order to better understand the effects responsible for such inhibitions of *E. coli* and *Salmonella typhimurium*. *Salmonella* species can grow in a pH range of 4.0 to 9.0, with optimal growth in a neutral pH range (6.5 - 7.5) (D'Aoust & Maurer, 2007; Jay, 2005), while *E. coli* can grow in a pH range of 4.02 to 8.28 (Ross et al., 2003) and *S. aureus* in a range of 4 to 7, with an optimum between 6 and 7 (Sutherland, Bayliss & Roberts, 1994). All postbiotics showed an acid pH in the range of 3.92 - 4.73 and after being diluted with culture medium of pathogenic bacteria presented an increase in pH ranging from 4.4 – 5.2. Therefore, these values of pH could contribute to the growth inhibition, especially since this range is not the ideal for the studied pathogenic bacteria.

Table 3- Average pH of cell-free probiotic supernatants after addition of supernatants.

Probiotic	pH (supernatant)	pH (supernatant + medium)
Control 1	-*	6.96±0.01
Control 2	7**	6.91±0.05

Control 3	4.0***	4.54±0.03
L acidophilus	4.31±0.01	4.87±0.06
L. brevis	4.43±0.08	4.88±0.08
B. animalis	4.73±0.02	5.19±0.02
B. lactis	4.51±0.03	4.90±0.03
B. longum	4.40±0.07	4.85±0.04
B. breve	3.92±0.05	4.37±0.09

*no addition of supernatant **water ***HCl solution

In order to test whether the pH variable alone is sufficient to inhibit the growth of pathogenic strains, a test with a pH 4 (acidified by HCl solution) in the control 3 was performed. In this control, with pH close to those of the postbiotics, the pathogen's growth was similar to the other controls, showing that organic acids produced by probiotic bacteria (lactic, acetic and succinic acid) are clearly more effective than HCl in inhibiting the pathogens growth, especially due to their dissociation (Wang et al., 2014). Lactic acid for instance, unlike HCl, is a weak acid (pka 3.85 at 25ºC) and at low pH it appears in its undissociated form and migrates through the cell membrane, where, inside the cytoplasm it dissociates and decreases the cell's internal pH and/or increases the cell's energy demand. High concentrations of dissociated forms of organic acids within the cell cytoplasm generate an energy stress in the attempt to restore homeostasis by pumping out protons. This high energy demand significantly reduces the energy available for important metabolic processes and growth, ultimately leading to the death of the pathogen (Narendranath, Thomas and Ingledew, 2001; Carpenter and Broadbent, 2009; Jacob and Pescatore, 2012). Other possible organic acid effects include membrane disruption, inhibition of the activity of cellular enzymes and accumulation of toxic anions (Narendranath Thomas and Ingledew, 2001).

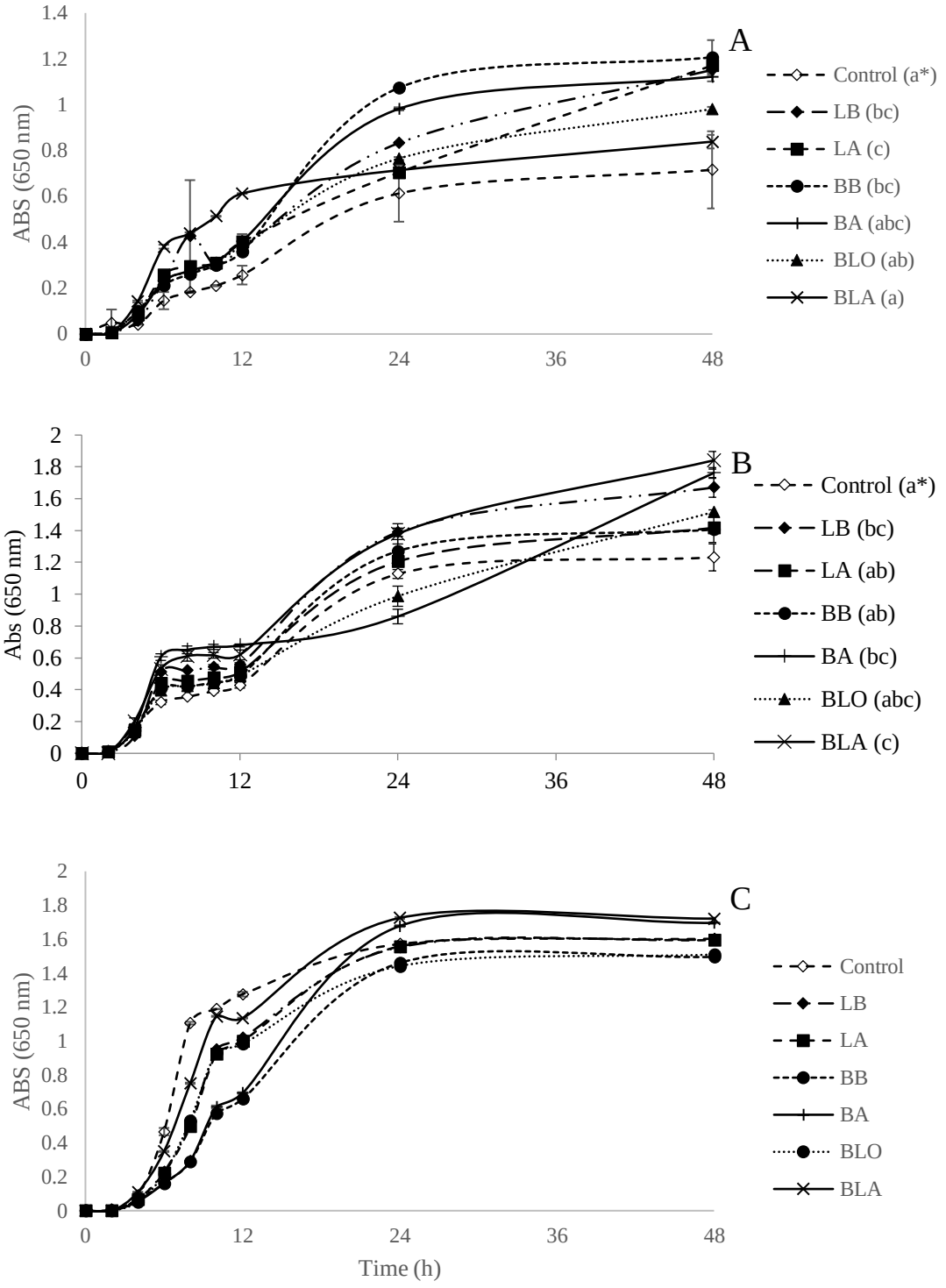
All the cultures of *Salmonella typhimurium*, *E. coli* and *S. aureus* grew in all treatments carried out with neutral pH (Fig 3). In these treatments, there was a growth stimulus, reaching results even above those of controls with statistically significant difference ($p < 0.05$) from controls for *B. animalis*, *B. lactis* and *L. brevis*. In addition to the medium being at optimum pH for the three pathogens growth, the lactic acid is a weak acid that has stronger inhibitory action at lower pHs. On the other hand, in neutral pH, it

is presented in its dissociated form as lactate, an ion form not capable of crossing the cell membrane (Jacob and Pescatore, 2012).

When the postbiotics pH were adjusted to 2, *Salmonella typhimurium* and *E. coli* had their growth completely inhibited, which may have occurred due to the drop in the pH of the medium to 3, which is outside the growth range of both microorganisms (Fig 4A, B) and results in physiological stress. *B. animalis*, *B. breve* and *B. lactis* postbiotics adjusted to pH 2 with HCl were capable of inhibiting the growth of *S. aureus* (Fig 4C), indicating that further acidification of the medium improves the inhibitory action of some organic acids from the strains, probably due to higher concentration of its undissociated form.

Considering this, we can say that the physicochemical conditions of the medium, as well as the buffering effect and dissociation capacity are factors that directly affect the inhibitory efficiency of the organic acid, in other words, a synergism between pH and organic acid is needed for the growth inhibition to occur.

Figure 3- Growth profile of *Escherichia coli* (A), *Salmonella typhimurium* (B) and *Staphylococcus aureus* (C) in NB, BG and BHI medium respectively, with pH adjusted to 7, containing micro filtered cell-free probiotic supernatants from strains at 37º C for 48h. Control 1 = 100 ml of medium. LB = *L. brevis*, LA = *L. acidophilus*, BB = *B. breve*, BA = *B. animalis*, BLO = *B. longum* and BLA = *B. lactis.* *Groups followed by the same letter in brackets were not significantly different in Kruskal-Wallis ANOVA followed by Student-Newman-Keuls test.



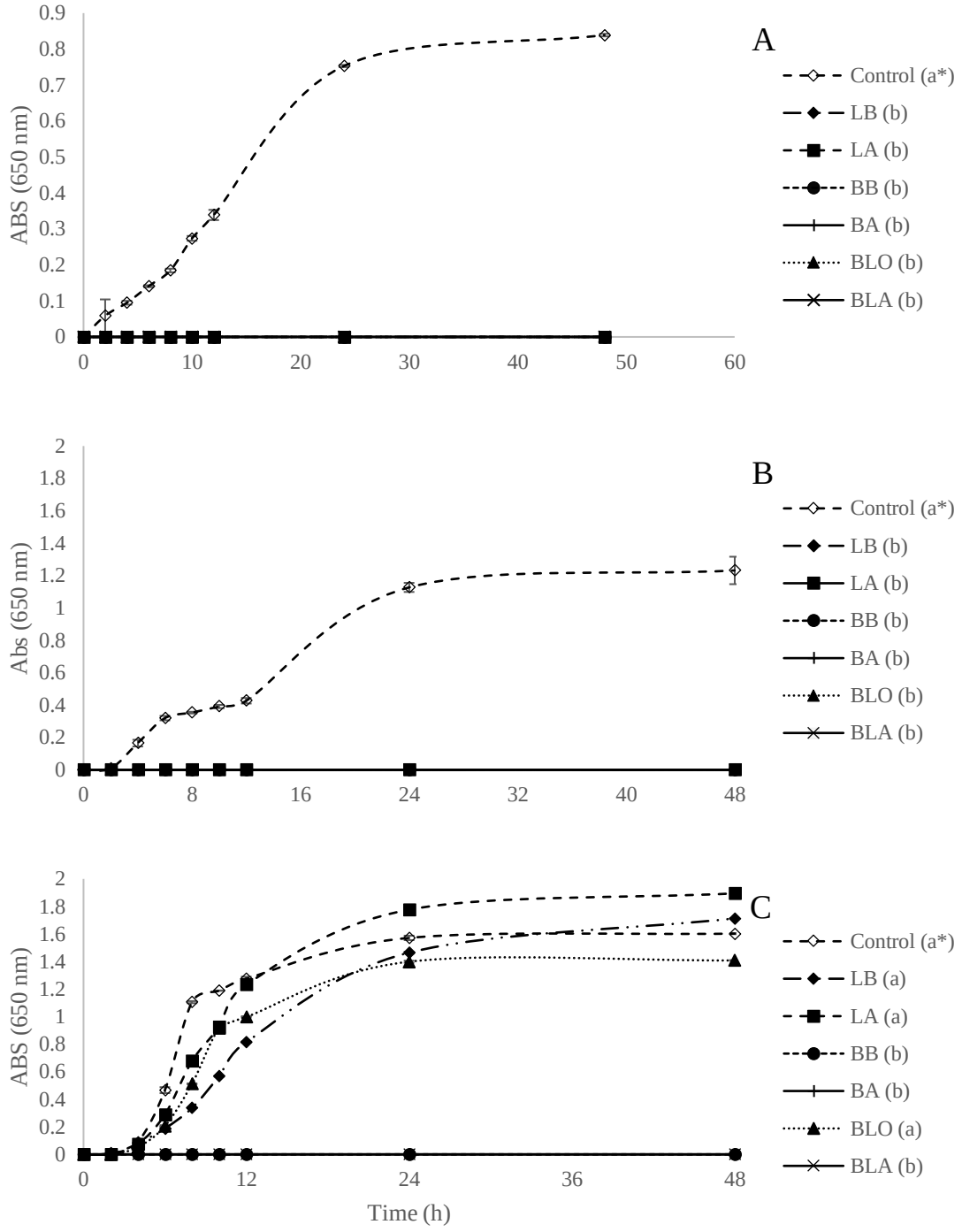
Source: Own work.

The HPLC analysis showed that lactic acid is the most produced acid among the tested probiotic strains, with *B. brevis* producing 27.3 g / L of this organic acid. Acetic acid is the second most produced organic acid, followed by formic acid and small amounts of succinic acid (Table 4). The quantification of organic acids further corroborate the hypothesis that *B. longum*'s inhibitory action is mainly due to its bacteriocins, since this strain was the one that produced the smallest quantity of total organic acids, with only 9.5 g / L, in contrast with other strains, such as *B. breve*, with 32.52 g / L (Table 4).

Of the three strains that successfully inhibited *Salmonella typhimurium* (*B. animalis, B. breve* and *B. lactis*), two of them produced the highest quantity of total organic acids, with concentrations of lactic acid above 26 g / L for *B. animalis* and *B. breve*. While *B. lactis* had a lower concentration of lactic acid (6.1 g / L) in comparison with the aforementioned *Bifidobacterium* strains, among all tested strains, *B. lactis* was the highest producer of acetic acid (7.7 g / L), showing that its inhibitory action is probably due to a synergistic action of lactic and acetic acids. Acetic acid has two to four times more molecules in its undissociated form in pH around 4 when compared to lactic acid, which makes it more lethal to microorganisms (Narendranath, Thomas and Ingledew, 2001). This explains the fact that the minimum inhibitory concentration of acetic acid is twice as low as that of lactic acid (Monte et al, 2014).

Our results show the potential of postbiotics produced by some strains of *Bifidobacterium, Levilactobacillus* and *Lactobacillus* in the inhibition of Gram-negative pathogenic bacteria (*Escherichia coli* and *S. typhimuri*um), even if the postbiotics are submitted to low pH and high temperatures. Further studies elucidating the synergism of organic acids, bacteriocins and other molecules from the metabolism of probiotics can open new perspectives in the development of new ingredients for the preservation of food and new functional ingredients.

Figure 4- Growth profile of *Escherichia coli* (A), *Salmonella typhimurium* (B) and *Staphylococcus aureus* (C) in NB, BG and BHI medium respectively, with pH adjusted to 2, containing micro filtered cell-free probiotic supernatants from strains at 37º C for 48h. Control 1 = 100 ml of medium. LB = *L. brevis*, LA = *L. acidophilus*, BB = *B. breve*, BA = *B. animalis*, BLO = *B. longum* and BLA = *B. lactis.* *Groups followed by the same letter in brackets were not significantly different in Kruskal-Wallis ANOVA followed by Student-Newman-Keuls test.



Source: Own work.

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Table 4- Concentration of organic acids present in the Cell-free probiotic supernatant.

Strain	Succinic acid (g / L)	Lactic acid (g / L)	Formic acid (g / L)	Acetic acid (g / L)	Total (g / L)
B. animalis	0.10	26.69	1.25	3.83	31.87
B. lactis	0.16	6.11	1.65	7.66	15.58
B. longum	0.11	6.93	0.47	1.95	9.46
B. breve	0.00	27.30	1.31	3.91	32.52
L. acidophilus	0.14	13.39	1.06	2.91	17.50
L. brevis	0.11	7.57	0.43	1.75	9.85

4. CONCLUSIONS

Tests with postbiotics showed complete inhibition of *E. coli*. *Salmonella typhimurium* was successfully inhibited by the postbiotics of *B. animalis, B. breve* and *B. lactis.* The postbiotics of *B. longum, L. acidophilus* and *L. brevis* inhibited *Salmonella* mainly in its exponential growth phase (12h), while *S. aureus* grew in all tested postbiotics. The postbiotics showed stability at high and low temperatures with the exception of *B. longum*. Organic acids have proven to be key tools in antimicrobial action and there is a synergism between these molecules and pH, since only in acidic conditions the postbiotics were able to inhibit the Gram-negative pathogenic bacteria. Their main mechanism of inhibition is probably related to their undissociated form. Bacteriocins also act as important molecules in the inhibition of pathogenic bacteria, especially for *B. longum*. Other molecules may participate in the inhibition and more studies are necessary to elucidate these mechanisms. Understanding the synergism of these molecules can open new perspectives for the development of new ingredients for food preservation and new functional ingredients for health.

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CHAPTER 3

Growth inhibition of pathogenic bacteria by single and mixtures of postbiotics from lactic acid bacteria.

ABSTRACT

Probiotic mixtures have gained the interest of researchers for their beneficial effects. There is, however, a need in understanding the interaction between the metabolites of different probiotics strains in such mixtures. The aim of this study was to determine the minimum inhibitory concentration (MIC) of postbiotics from single probiotic strains and of postbiotic mixtures for the inhibition of *Salmonella typhimurium, Escherichia coli* and S*taphylococcus aureus*. The assessment of the co-culture of *Salmonella typhimurium* with probiotics and the inhibition of this pathogen by commercial lactic acid were performed as well. Postbiotics of the following probiotic strains were used: *Bifidobacterium breve*, *B. lactis*, *B. longum*, *B. animalis*, *Lactobacillus acidophilus* and *Levilactobacillus brevis*. Tests were performed with postbiotics (single or mixtures) added to the culture media of the pathogens. *S. aureus* was inhibited only by the postbiotics of *B. breve*, with a MIC of 20% (v/v). Both *E. coli* and *Salmonella typhimurium* were inhibited by all single postbiotics. MICs for these two pathogens ranged from 5% (v/v) to 16% (v/v), with *Bifidobacterium* strains showing lower MICs, in general. The *Bif* mixture (all *Bifidobacterium* strains) completely inhibited both *Salmonella typhimurium* and *E. coli*, with a MIC of 5% (v/v). The addition of postbiotics from the *Levilactobacillus* and *Lactobacillus* strains to the *Bif* mixture removed the inhibitory activity, indicating a possible antagonism between the postbiotics of these genera. This antagonism was confirmed with the loss of inhibitory activity of the *Lac* mixture (*L. acidophilus* + *L. brevis*) when the postbiotics from *B. longum* were added. Although the inhibitory activity dropped with mixtures of postbiotics from different genera, no inhibition of probiotic bacteria by the postbiotics was verified, proving that postbiotics from different strains are safe for probiotic species. In the co-culture tests, *Salmonella typhimurium* was inhibited by *B. animalis*, *B. lactis* and *L. acidophilus*. Concentrations of 0.4 g / L of commercial lactic acid and higher successfully inhibited the growth of this pathogen as well. Determining the minimum concentration of postbiotics necessary to inhibit the growth of pathogens and the composition of the mixture that has greater action against specific or general pathogens, without growth inhibition of probiotics, guarantees the formulation of a mixture of postbiotics with possible application in food preservation or capable of bringing benefits to the host.

Keywords: synergistic performance, metabolites, mixed probiotics, growth inhibition, pathogenic strains.

1. INTRODUCTION

Probiotics are defined as living microorganisms that provide health benefits to the host, usually related to the balance of the intestinal flora when ingested in adequate amounts (Hill et al., 2014). In addition, probiotics are related to the modulation of the immune system and improvement of the intestinal mucosal barrier (Sanders, Goh, & Klaenhammer, 2019).

The ability of these microorganisms to produce bacteriocins and to compete for adhesion sites and nutrients leads to inhibition of other species that try to colonize the intestinal environment (Sanders et al., 2019). Consequently, this information has gained the interest of researchers due to the various pathogens that can occasionally settle and proliferate in the intestine, leading to numerous diseases.

Each probiotic species acts with particular mechanisms, producing metabolites such as bacteriocins, with bactericidal or bacteriostatic action capable of inhibiting some pathogenic species or even other probiotic strains (Sanders et al., 2018; Vieco-Saiz et al., 2019; Gao et al., 2019). One additional inhibitory mechanism may be the production of short-chain fatty acids, including lactic, butyrate, acetate and succinic acids that act decreasing pH and leading to the pathogen's cell death (Sanders et al., 2018).

Researches indicate that some probiotic strains act more efficiently against certain pathogenic species (Chapman, Gibson and Rowland, 2011; Chapman, Gibson and Rowland, 2012; Chapman, et al., 2013, Fredua-Agyeman et al., 2017; Fijan, Sulc, & Steyer, 2018). Considering this, studies have shown that using mixtures of different probiotics is very promising against a wide variety of enteric diseases. However, there is little information regarding a broader study of synergistic performance or even inhibition among the metabolites of probiotic species in a mixture (Chapman, Gibson and Rowland, 2012; (Fredua-Agyeman et al., 2017).

Lately, the metabolites produced by probiotics have gained a new term, postbiotics, which are extracts free from viable cells that when ingested in adequate amounts bring health benefits (Aguilar-Toalá, et al., 2018; Cuevas-González et al., 2020). Among the most known and studied probiotic species are *Lactobacillus* and *Bifidobacterium* (Fredua-Agyeman et al., 2017). These are genera of Gram positive bacteria that have different mechanisms of action within their species. These bacteria are present in several foods and are sold as supplements in single strains or in mixtures (Fredua-Agyeman et al., 2017; Fijan, Sulc & Steyer, 2018).

Salmonella enterica serovar *Typhimurium* is a pathogenic microorganism usually transmitted by contaminated food and capable of causing salmonellosis (Madigan et al., 2016). Salmonellosis is a gastrointestinal disease that can cause severe diarrhea and occasionally lead to death in debilitated patients. Another important pathogen responsible for gastrointestinal infections, mainly in children, is *Escherichia coli*, which can cause mild to severe diarrhea and, in some occasions, lead to death (Mirsepasi-Lauridsen et al., 2019). *Staphylococcus aureus*, on the other hand, is an opportunistic human pathogen

associated with skin infections and possessing a toxin that can cause severe food poisoning (Dayan et al., 2016). Currently, the major concern involving bacterial infections is the resistance developed by several bacteria to various antibiotics, therefore, the use of probiotics has become an important addition to treatments of diseases caused by bacteria (Fijan, Sulc, & Steyer, 2018).

The choice of suitable probiotic species that can act specifically against certain diseases is crucial for product formulations, since adequate concentrations could provide greater efficiency, lower costs and higher safety for the consumer, while also preventing the selected probiotic species of competing against each other (McFarland, 2020). This study aimed to evaluate the synergism between the metabolites of some probiotic species of the genus *Bifidobacterium*, *Levilactobacillus* and *Lactobacillus* in the growth inhibition of *Salmonella typhimurium*, *E. coli* and *S. aureus*.

2. Material and method

2.1. Microorganisms

The strains *Levilactobacillus brevis* ATCC 367 and *Lactobacillus acidophilus* ATCC 4356 were obtained from Collection of Reference Microorganisms in Sanitary Surveillance - CMRVS (Oswaldo Cruz Foundation - FIOCRUZ, RJ, Brazil) and were maintained as a stock culture at 7 °C on MRS medium.

The following microorganisms of the genus *Bifidobacterium* used in this study were maintained as a stock culture at 7 °C on MRS medium with cysteine: *Bifidobacterium animalis* BB-12 Christian Hansen (Hoersholm, Denmark), *Bifidobacterium lactis* BI-07 Danisco (Madison, USA), *Bifidobacterium longum* BL-05 Danisco (Madison, USA) and *Bifidobacterium breve* BB-03 Danisco (Madison, USA).

Salmonella enterica subsp. *enterica* serovar *typhimurium* ATCC 14028 (hereafter called *Salmonella typhimurium*) was obtained from Tropical Culture Collection (CCT), André Tosello Foundation, Campinas, SP, Brazil, with the code CCT 1478. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were also used as pathogen models. The cultures were maintained in the Laboratory of Industrial Biotechnology (UNESP-Assis / SP) at 7 ºC in Nutrient Agar.

2.2. Growth medium for microorganisms

Formulated MRS medium (De Man, Rogosa, & Sharp, 1960) was used at pH 6 and autoclaved for 20 minutes at 121 °C. *L. acidophilus* strains were grown at 37 °C for 48 hours and *L. brevis* strains were grown at 30 ° C for 48 hours. *Bifidobacterium* cultures were grown at 37 °C for 48 hours in modified MRS medium with the addition of L-cysteine.

Salmonella typhimurium was grown in Brilliant Green medium (BG) (Kauffmann, 1935). The composition had slight modifications (g / L): 3 g of yeast extract, 10 g of peptone protease, 5 g of sodium chloride, 10 g of lactose and 10 g of sucrose. *E. coli* was grown in Nutrient Broth (NB) and *S. aureus* was grown on Brain Heart Infusion (BHI) medium. The culture media were prepared in pH 7 and autoclaved for 20 minutes at 121 °C. Pathogen strains were grown at 37ºC for 48 hours.

2.3. Minimum inhibitory concentration of pathogens using single postbiotics.

The "in vitro" tests were carried out according to Chapman and Gibson (2013). After growth (48h), probiotic cultures incubated in liquid MRS (item 2.2) were adjusted to the same optical density in a spectrophotometer at 650 nm to values between 0.8 and 0.9 in order to represent approximately equal biomass. Probiotics where then centrifuged at 2000 x g for 10 minutes. Then, supernatants were filtered through a 0.22 µm membrane filter (Advantec) to obtain postbiotics. In order to define the minimum inhibitory concentration (MIC) for the growth of *Salmonella typhimurium*, *E. coli* and *S. aureus*, the volume of postbiotics added to each pathogen medium varied until reaching the MIC. A 50 µl aliquot of pathogen culture was added in 100 mL of medium (BG, NB or BHI) + postbiotics. The control was carried out using 100 mL of the respective medium + the 50 µl aliquot of pathogen culture. Growth curves of the pathogenic strains were made from the turbidity analysis in a spectrophotometer using aliquots removed at 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours.

2.4. Inhibition of pathogens using mixtures of postbiotics.

The tests were carried out similarly to the previous item, however, the experiment was developed with mixtures of postbiotics. From the results of the item 2.3, mixtures with a total volume of 10 mL were performed. Mixtures were prepared using

the postbiotics that presented the lowest MIC for each pathogenic bacteria, as well as mixtures using all strains within the same genus and a mixture with postbiotics of all available probiotic strains. All mixtures had equal concentrations of each of the used postbiotics. Mixtures that presented total inhibition of pathogens growth with 10mL were reduced to find the minimum inhibitory concentration of mixtures.

The mixtures presented the following combinations of postbiotics:

- *Lac*: postbiotics from *Lactobacillus* and *Levilactobacillus* (*L. acidophilus* and *L. brevis*)
- *Bif*: postbiotics from all available strains of *Bifidobacterium* (*B. breve, B. animalis, B. lactis* and *B. longum*)
- *Lac+Blo*: strains of *Lactobacillus* and *Levilactobacillus* (*L. acidophilus* and *L. brevis*) with the addition of *B. longum*
- *Bif-Blo*: *B. breve, B. animalis, B. lactis*
- *Bif-Bb+La: B. animalis, B. lactis, B. longum. L. acidophilus*
- *Lac+Bif*: All 6 available probiotic strains (*B. breve, B. animalis, B. lactis, B. longum, L acidophilus* and *L. brevis*).

2.5. Inhibition of probiotic strains using postbiotics.

Postbiotics were obtained similarly to item 2.3. To assess whether the metabolites present in postbiotics were capable of inhibiting the available probiotic strains, postbiotics were added to the MRS medium and probiotic strains were incubated in the same conditions described in item 2.2.

2.6. Determination of total acidity

The postbiotics were analyzed for lactic acid content according to the standard titratable acidity methodology (AOAC, 1990) using NaOH (0.1 N). Equation 1 was used to convert the volume of titrated NaOH into lactic acid (%), in which V represents the volume of NaOH in titrated mL, CF is the NaOH correction factor, and v is the used sample volume (in mL). The calculation was performed using the following equation (1):

% Total acidity: $\frac{V*9.008*CF}{v}$ (1)

The correction factor used was 1.018 for lactic acid.

2.7. Co-culture of Salmonella typhimurium and probiotics.

Salmonella typhimurium and probiotics were grown in BG and MRS medium, respectively, for 48 hours at 37ºC. Cultures were then adjusted to the same optical density in a spectrophotometer at 650 nm to values between 0.8 and 0.9 in order to represent approximately equal biomass. To test the inhibition of the different strains in cocultures, 50 µl of Salmonella and each of the probiotics were inoculated in MRS medium for 48 hours at 37ºC. After this period, Gram staining was performed to differentiate the *Salmonella typhimurium* from probiotic strains. The samples were evaluated through the presence and absence of the tested microorganisms.

2.8. Inhibition of Salmonella typhimurium using commercial lactic acid.

Salmonella typhimurium, used as a model of pathogenic strain, was grown for 48 hours in liquid BG medium at 37 ° C under aerobic conditions. After the optical density was adjusted in spectrophotometer at 650 nm to values between 0.8 and 0.9, aliquots of pathogenic culture (50 µl) were added to 90 ml of Bright Green medium and 10 ml of commercial lactic acid (Synth, Brazil). Different lactic acid concentrations were tested: 1, 2, 3, 4, 5 and 6 g/ L. As a control, *Salmonella typhimurium* cultures were incubated with 100 ml of BG medium, without the addition of lactic acid. A growth curve of the pathogenic strain was made from the turbidity analysis in the spectrophotometer at 650 nm with aliquots taken at 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours. Analyses were performed in triplicate.

2.9. Calculations of percentage of inhibition.

The calculation of percentage of inhibition was performed by the following equation (2):

Inhibition %: $\frac{OD\ control - OD\ mixture}{OD\ control} * 100$ (2)

OD control: Optical density of control at 650nm after 48h of incubation.

OD mixture: Optical density of experiments at 650nm after 48h of incubation.

2.10. Statistical analyses

Statistical analyses were performed to compare the pathogens inhibition with different mixtures of postbiotics using Kruskal-Wallis ANOVA by BioEstat 5.3 software.

3. RESULTS AND DISCUSSION

3.1. Minimum inhibitory concentration of pathogens using postbiotics of single probiotic strains.

Tests with postbiotics show the inhibition by the production of metabolites, such as the organic acids and bacteriocins. Previous results show that 10% (v/v) of postbiotics from single strains fully inhibited the growth of *E. coli*. *Salmonella typhimurium* was fully inhibited by the postbiotics of *B. animalis*, *B. breve* and *B. lactis*, and there was partial inhibition by the postbiotics of other strains (*L. acidophilus*, *L. brevis* and *B. longum* with 79.4%, 83.2% and 85.5%, respectively). *S. aureus* was inhibited only by the postbiotic of *B. breve* at 20% (v/v) or 1.2 % (m/v) in dried base.

Table 1 presents the minimum inhibitory concentration (MIC) of the postbiotics from single strains. Total inhibition of *E. coli* was reached using a concentration of 5% (v/v) of the postbiotics of *B. lactis* and *B. animalis*. For *Salmonella typhimurium*, the MIC by these same strains were observed with concentrations of 6% (v/v). *B. breve* postbiotics showed the lowest MIC for *Salmonella typhimurium*, with 5% (v/v) being able to fully inhibit the growth of this pathogen. For *E. coli*, however, this strain presented the highest MIC (10% v/v). The other postbiotics of *B. longum*, *L. acidophilus* and *L. brevis* inhibited the growth of *E. coli* with concentrations of 6%, 6% and 7% (v/v) respectively; and the growth of *Salmonella typhimurium* with 16%, 15% and 11% (v/v) respectively.

These differences in minimum inhibitory concentrations (MIC) of the postbiotics from the same strains against different pathogens highlight that each pathogenic bacteria reacts differently to the inhibitory mechanisms of different probiotic bacteria, reinforcing the need of studies that evaluate the specificity of anti-microbial

substances against bacteria that cause gastrointestinal infections and the need of an appropriate mixture of postbiotic in order to be effective for a wider spectrum of pathogenic bacteria.

Table 1- Minimum inhibitory concentration of postbiotics against the growth of some important pathogenic bacteria.

	E. coli (ATCC 25922)		*Salmonella typhimurium* (ATCC 14028)		*S. aureus* (ATCC 25923)	
Postbiotics	mL[1]	g[2]	mL	g	mL	G
B. animalis	5.00	0.26	6.00	0.31	> 20.00	> 1.04
B. lactis	5.00	0.26	6.00	0.31	> 20.00	> 1.02
B. breve	10.00	0.60	5.00	0.30	= 20.00	= 1.20
B. longum	6.00	0.30	16.00	0.80	> 20.00	> 1.00
L. acidophilus	6.00	0.32	15.00	0.80	> 20.00	> 1.06
L. brevis	7.00	0.34	11.00	0.54	> 20.00	> 0.98

1 = % (v/v) 2 = % (dried m/v)

Since *S. aureus* was inhibited by 20% (v/v) of *B. breve*'s postbiotics, this result may indicate that this probiotic has specific metabolites for this pathogen or the amount of these metabolites may be higher than the other probiotics. Some studies highlight the possible inhibition of *S. aureus* by probiotic strains (Piewngam & Otto, 2020) by the production of lipopeptides, which further evidences that each probiotic strain has different methods of inhibitory action against different pathogenic strains. While the action of short chain fatty acids (SCFAs) and bacteriocins present in the postbiotics of most probiotic strains proved to be sufficient in inhibiting the growth of *E. coli* and *Salmonella typhimurium*, a more complex set of inhibitory mechanisms is necessary for the inhibition of *S. aureus*. Considering that, in the presence of probiotic cells, the inhibition of *S. aureus* is more accentuated, the competition for adhesion sites and nutrients may represent an important factor in the inhibition of this pathogenic strain.

In 2012, Chapman et al. also demonstrated the difference of inhibitory action mechanisms by postbiotics in some pathogenic bacteria. Their tested *Lactobacillus* species inhibited the growth of *E. coli* and *Enterococcus faecalis*, however, the authors noted that while the total inhibition of the growth of *E. coli* was achieved by the

supernatants of the strains *L. acidophilus, L. fermentum, L. plantarum* and *L. rhamnosus,* only *L. plantarum* was able to fully inhibit *E. faecalis*.

Table 2 shows the total acidity in g / L of the postbiotics of each strain. It is interesting to note that the three strains with the lowest MIC for *Salmonella typhimurium* (*B. breve, B. animalis* and *B. lactis*) had the highest total acidity (3.5%, 3.6% and 2.93%, respectively) compared to the other species tested. This may indicate the strong performance of organic acids that, in their undissociated form, cross the plasma membrane and decrease internal pH, leading to cell death (Carpenter and Broadbent, 2009).

Table 2- Total acidity of the postbiotics from *Levilactobacillus, Lactobacillus* and *Bifidobacterium* after 48 hours of incubation at 37º C and 30 º C.

Probiotic	Total acidity* (%)
L. acidophilus	2.1 ± 0.03
L. brevis	2.0 ± 0.01
B. lactis	2.9 ± 0.05
B. longum	2.0 ± 0.07
B. animalis	3.6 ± 0.04
B. breve	3.5 ± 0.07

* expressed in lactic acid

On the other hand, the strains that presented the lowest MIC for *E. coli* (*B. longum* and *L. acidophilus*) had lower total acidity, corroborating the hypothesis that different methods of action are more effective against different pathogenic microorganisms.

3.2. Inhibition of strains using mixtures of postbiotics.

Figure 1 shows the growth curve of *E. coli, Salmonella typhimurium* and *S. aureus* using mixtures of postbiotics. The *Bif* mixture (*B. breve, B. animalis, B. lactis and B. longum*) fully inhibited *E. coli*'s and *Salmonella typhimurium* with a concentration of 5% (v/v). The *Bif-Blo* (*B. breve, B. animalis, B. lactis*) mixture was developed by adding

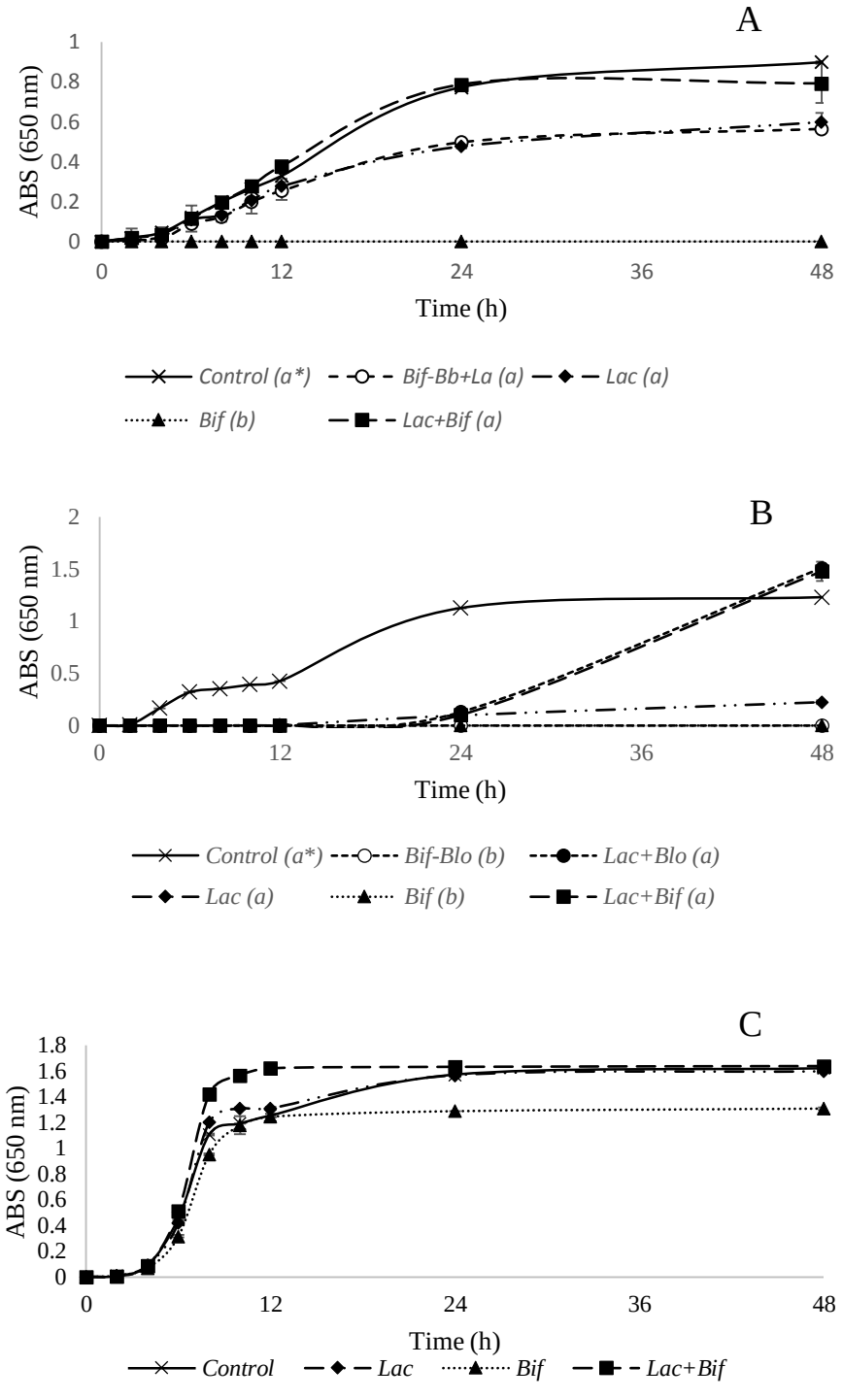
the single strains that presented the lowest MIC for *Salmonella typhimurium*. This mixture, composed of equal parts of postbiotics from different strains, showed total inhibition of the growth of this pathogen with a concentration of 5% (v/v), with statistically significant difference ($p < 0.05$) from controls. The full inhibition of *E. coli* by the *Bif* mixture and the inhibition of *Salmonella typhimurium* by the *Bif* and *Bif-Blo* mixtures shows a synergy among the postbiotics of the *Bifidobacterium* strains, since the mixture contained 1.67% (v/v) of each single postbiotic.

Lac mixture (*L. acidophilus* and *L. brevis*, 15% v/v) showed 83% inhibition of *Salmonella typhimurium* after 48 hours of incubation (figure 1) but no inhibition by this mixture was observed for *E. coli* or *S. aureus*. The total inhibition of *E. coli* and partial inhibition of *E. fecalis* was obtained by Chapman et al. (2013) with single *Lactobacillus* strains and mixture of *Lactobacillus*.

Mixtures *Lac+Blo* (*L. acidophilus, L. brevis, B. longum*), *Bif-Bb+La* (*B. longum, B. animalis, B. lactis, L. acidophilus*) and *Lac+Bif* (*B. breve, B. animalis, B. lactis, B. longum, L acidophilus and L. brevis*) showed no inhibition of the pathogen's growth whatsoever, which may indicate an antagonism between the metabolic products of the probiotic strains from different genera.

The antagonism between the metabolic products is further observed with the removal and addition of the *B. longum* strain to the *Bif* and *Lac* mixtures, respectively. When *B. longum* was present in the mixtures containing only *Bifidobacterium* strains (*Bif*), no antagonistic effect between the metabolites was observed, since the inhibitory activity was similar both in the *Bif* and *Bif-Blo* mixtures. However, while *Lac* was capable of high inhibition of *Salmonella typhimurium*, the addition of *B. longum* reversed this trend, with the *Lac+Blo* mixture showing growth of this pathogen similar to values observed in control.

Figure 1- Growth profile of *Escherichia coli* (A), *Salmonella typhimurium* (B) and *Staphylococcus aureus* (C) in NB, BG and BHI media respectively, containing mixtures of postbiotics at 37º C for 48h. Control = 100 ml medium. Bif-Bb+La: *Bifidobacterium animalis*, *B. longum*, *B. lactis* and *Lactobacillus acidophilus*; Lac: *L. acidophilus* and *Levilactobacillus brevis*; Bif: *B. animalis*, *B. breve*, *B. lactis* and *B. longum*; Lac+Bif: *L. acidophilus*, *L. breve*, *B. animalis*, *B. breve*, *B. lactis* and *B. longum*; Bif-Blo: *B. animalis*, *B. breve* and *B. lactis*; Lac+Blo: *L. acidophilus*, *L. breve* and *B. longum*. *Groups followed by the same letter in brackets were not significantly different in Kruskal-Wallis ANOVA followed by Student-Newman-Keuls test.



Source: Own work.

In general, growth inhibition among probiotic species is often related to the competition for nutrients or release of bacteriocins (Chapman, Gibson, & Rowland, 2012; Fijan, Sulc, & Steyer, 2018). However, since these mixtures were obtained with cell-free supernatants, the antagonistic effect between species cannot be explained by competition for nutrient or adhesion sites. It is possible that the organic acids, bacteriocins, peroxides, enzymes or a combination of factors from one strain could neutralize the action of the other strains' metabolites. In studies analyzing the inhibitory action between probiotic species of the *Lactobacillus* genus, no inhibition was observed between metabolites of different species within the same genus (Chapman et al., 2013). However, studies using probiotic mixtures with viable cells from the genera *Lactobacillus* and *Bifidobacterium* showed inhibition between genera of probiotic species and suggested that strains of *Lactobacillus* have a high ability of inhibiting strains of other genera but are less inhibitory against each other (Chapman et al. 2012). The inhibition of other probiotic species by *Lactobacillus* may be due to their high spectrum of activity. *Bifidobacterium* species, on the other hand, showed greater tolerance between species of other genera, but showed higher degrees of inhibitory action within their same genus (Chapman et al. 2012).

Hydrogen peroxide (H_2O_2) is an important metabolite with antimicrobial activity usually produced by *Lactobacillus* strains. *Bifidobacterium* probiotic species are very sensitive to such metabolites, therefore, in mixtures with cell cultures of these two genera, antagonism may occur due to the action of H_2O_2 on *Bifidobacterium* cells (Oberg et al., 2011). However, in the present work, only metabolites without the presence of cells were mixed. As such, the antagonism observed in the Lac+Bif mixture could be explained by the presence of peptidases often produced by probiotic strains. The action of such enzymes can degrade the bacteriocins, peptides responsible for a large fraction of the antimicrobial activity (Francavilla et al., 2017).

Our results showed no inhibitory activity of postbiotics on probiotic strains, suggesting that the inhibitory activity between probiotic species reported by other works (Chapman et al., 2012) probably happens due to the competition for nutrients.

These observations show the importance of studies with mixtures of postbiotics from different probiotic cultures since the antagonistic effect between probiotic species or genera can significantly reduce the efficiency of their metabolic products in the inhibition of pathogens. In addition, it has become clear that the careful choice of strains

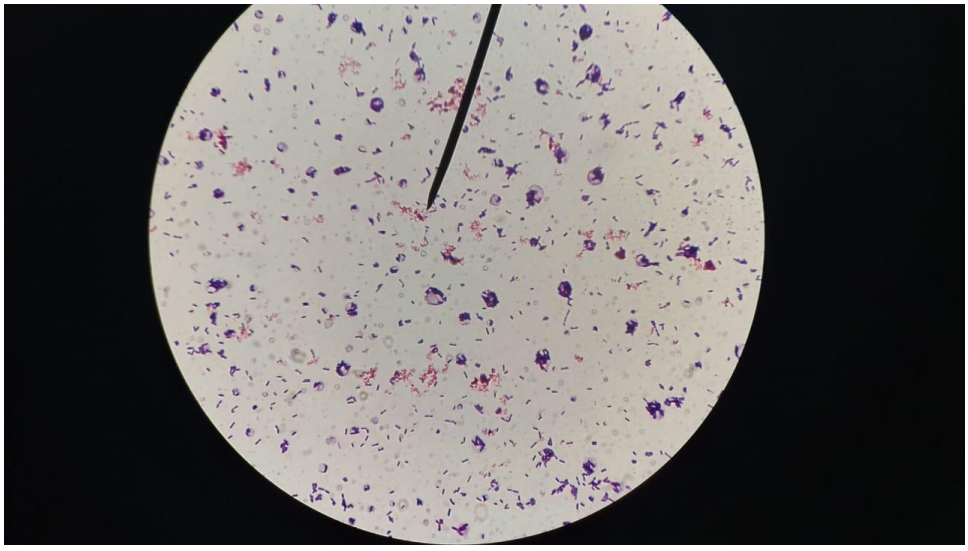
present in the mixture, with careful selection of the action spectrum of each strain, can guarantee specificity in the treatment of certain infections.

3.3. Co-culture of *Salmonella typhimurium* and probiotic strains.

In tests with co-cultures, the *B. animalis*, *B. lactis* and *L. acidophilus* strains inhibited the growth of *Salmonella typhimurium*. In contrast to previous tests with postbiotics, *B. breve* did not inhibit the growth of *Salmonella* in the presence of cells. *L. acidophilus* also presented different results from previous tests, since the metabolites of this strain did not inhibit the pathogen. *B. longum* and *L. breve* did not show any inhibition (Table 3.)

This difference in antimicrobial activity between postbiotics and cultures with living cells highlights the importance of selecting proper strains for each pathogen treatment, or, in a broader point of view, a mixture of postbiotics and probiotics can be more effective in avoiding or controling enteric diseases. In addition, since postbiotics present a high tolerance to industrial conditions like temperature, drying and storage (Aguilar-Toalá, et al., 2018) and still present high inhibitory activity in certain conditions, these ingredients should be considered as food ingredients used to avoid enteric diseases or even in the treatment of bacterial infections, although “in vivo” tests are needed to prove these advantages in further stages.

Figure 2- Gram staining of co-cultures of *Salmonella typhimurium* and *Bifidobacterium breve.*



Source: Own work.

Table 3- Co-cultures of *Salmonella typhimurium* and probiotics in MRS medium for 48h at 37º C. *Bifidobacterium animalis* (BA), *B. breve* (BB), *B. lactis* (BLA), *B. longum* (BLO), *Lactobacillus acidophilus* (LA) and *Levilactobacillus brevis* (LB). (+) means presence of cells and (-) means absence of cells.

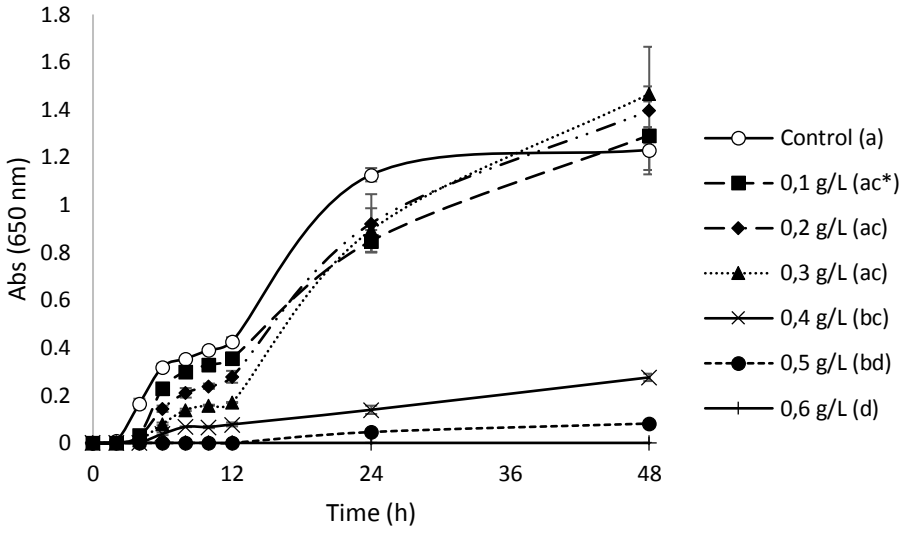
	Salmonella growth	Probiotics growth
Salmonella + BA	-	+
Salmonella + BB	+	+
Salmonella + BLA	-	+
Salmonella + BLO	+	+
Salmonella + LA	-	+
Salmonella + LB	+	+

3.4. Evaluation of commercial lactic acid in the inhibition of *Salmonella typhimurium* growth.

An assay was carried out with different concentrations of commercial lactic acid to verify their effect on the growth of *Salmonella typhimurium* and to possibly compare the results to the effects of organic acids produced by probiotics.

All treatments showed cell growth values lesser than the control at 24 hours of fermentation, measured by optical density (OD) (Fig. 2). However, after such period, two trends were observed: the first, in which treatments with the lowest concentrations of lactic acid (0.1, 0.2 and 0.3 g/L), and consequently with pHs close to neutrality (within the range of 7.5 – 8), presented no statistically significant differences from control and OD values higher than control, indicating non-inhibitory effect on growth (Table 4).

Figure 3- Growth profile of *Salmonella typhimurium* in BG medium containing commercial acid lactic at 37º C for 48h. Control = 100 ml of BG medium. *Groups followed by the same letter in brackets were not significantly different in Kruskal-Wallis ANOVA followed by Student-Newman-Keuls test.



Source: Own work.

On the other hand, in the second trend, treatments with higher concentrations (0.4 and 0.5 g/L) and consequently lower pHs (4 - 4.5) were significantly different from control and showed small growth during the entire time interval. The concentration of 0.6 g / L also fully inhibited the growth during the whole experiment (48h).

Table 4- pH values of the wort with the addition of different concentrations of commercial lactic acid in BG medium after *Salmonella typhimurium* growth at 37ºC for 48h.

Lactic acid (g/L)	% (v/v)	Culture-medium pH*
0.1	0.01	7.5
0.2	0.02	8
0.3	0.03	7
0.4	0.04	4.5
0.5	0.05	4
0.6	0.06	4

*Control pH- 7

Salmonella typhimurium has been shown to have its growth delayed with 2 g/L lactic acid (Ibrahim, Yang and Seo, 2008). In this present study, a smaller concentration

reached similar results, with growth lower than control until 24h and with an increase after such period. Previous studies by De Keersmaecker (2006) have shown that high inhibition of *Salmonella typhimurium* by *L. rhamnosus* GG was related to concentrations of lactic acid at pH 4.5, but the reported concentration for inhibition was much higher than those tested in this study (20 g/L). These differences probably depend on the buffering capacity of the medium and its constituents, since pH 4.5 seems to be an important growth limiting parameter for *Salmonella typhimurium.*

4. CONCLUSIONS

Tests with single postbiotics showed that *Bifidobacterium breve, B. animalis* and *B. lactis* fully inhibited the growth of *Salmonella typhimurium* in 48 hours with the lowest observed MICs. *E. coli* was inhibited, with the lowest MICs, by the postbiotics from *B. animalis, B. lactis, B. longum* and *L. acidophilus.* The acidity analysis of postbiotics reveals the production of large amounts of organic acids as their main inhibition mechanism. On the other hand, the difference in MICs of postbiotics from same strains between pathogens suggests that these mechanisms act in different ways for each pathogenic bacterium.

Mixtures of postbiotics from the *Bifidobacterium* strains proved to be more effective in the growth inhibition of pathogens than the mixture of *Lactobacillus* and *Levilactobacillus*. An antagonistic action between the metabolic products within the postbiotics of different genera (*Bifidobacterium*, *Levilactobacillus* and *Lactobacillus*) was verified, probably due to reactions among metabolites. These results indicate the importance of correct selection of strains in the mixture of postbiotics for the control of *Salmonella typhimurium* and *E. coli* or other enteropathogenic bacteria. In addition, such characteristics highlight the potential use of different postbiotics, either alone or in appropriate mixtures, as food preservatives and/or as ingredients in functional foods, providing benefits to the intestinal microbiota.

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CHAPTER 4

Evaluation of postbiotics and prebiotics in the control of pathogenic bacteria and stimulation of probiotic bacteria.

ABSTRACT

Postbiotics are metabolites, without living cells, from probiotic bacteria that cause health benefits for intestinal microbiota and may be used as food preservatives. The present study evaluated the production of postbiotics from a *Bifidobacterium breve* strain in a 2-liter fermenter and the stability of its antimicrobial activity against *Salmonella typhimurium* after drying. Furthermore, the inhibitory activity of postbiotics in the presence of commercial sugars was analyzed, as well as the synergism between prebiotics and postbiotics. Tests with the mixture of the prebiotics fructooligosacharides (FOS) and xylooligosacharides (XOS) showed that, with 10h of culture, *Levilactobacillus brevis* and *Bifidobacterium longum* were stimulated by the presence of prebiotics, exceeding the growth of control (glucose). The *B. breve* strain showed a small growth reduction (less than 8%), while *L. acidophilus, B. animalis* and *B. lactis*' growth were reduced by 75%, 16% and 39.6%, respectively. *Salmonella typhimurium*'s growth was reduced by 22% when compared with the medium with glucose (control). The scale-up tests showed that postbiotics with antimicrobial action may be produced in 24 hours in non-sterile conditions. The dried product showed inhibitory activity against *Salmonella typhimurium* at a concentration of 0.9% (m/v). Furthermore, the addition of only 6% (v/v) of liquid postbiotics in medium containing commercial sugar significantly prevented the growth of *Salmonella typhimurium*. In conclusion, there is a great potential for the application of postbiotics as functional ingredients in foods, either alone or in combination with prebiotics and/or probiotics, improving the preservation of food and the health of the intestinal microbiota.

Keywords: Postbiotics, scale-up, food preservative, *Salmonella typhimurium*.

1. INTRODUCTION

The composition of the microorganisms in the intestine, the intestinal microbiota, is strongly related to health. A well-balanced microbiota can bring many benefits to the host, since these microorganisms play a key role in absorbing nutrients, developing the immune system and acting as a barrier against pathogens (Russell et al., 2011; Guarner and Malagelada, 2003; Tang, Bäckhed, et al., 2019). The imbalance of the intestinal microbiota, dysbiosis; however, has been related to health problems such as gastrointestinal infections, colon cancer and obesity (Tang, Ding, et al., 2019; Wang et al., 2017). Dysbiosis is caused mainly by the prolonged use of antibiotics, but may originate as a result of stressful situations and changes in eating habits.

In this context, many researches seek to develop food ingredients that act directly on the intestinal microbiota in a positive manner, helping in the maintenance of intestinal balance. One type of such ingredients is prebiotics, substrates used selectively by

microorganisms that stimulate the proliferation of beneficial bacteria and ultimately cause many benefits to the host (Gibson et al., 2017; Markowiak and Śliżewska, 2018; Sanders et al., 2019). Prebiotics are also related to increased absorption of mineral salts, stimulation of the immune system and inhibition of pathogens (Wasilewski et al., 2015; Hutkins et al., 2016; Sanders et al., 2019). Fructooligosaccharides, inulin, galactooligosaccharides and xylooligosaccharides are the most commercialized prebiotics in the world (Wasilewski et al., 2015).

A second type of ingredients is probiotics, live microorganisms that, when ingested in adequate amounts, positively influence the health of the host (Hill et al., 2014). Probiotics have a fundamental role in the mucosal barrier, preventing the proliferation of pathogens (Sanders et al., 2019). Furthermore, the inhibition of harmful bacteria by probiotics may occur due to the production of short-chain fatty acids and other antimicrobial substances (Nagpal et al., 2018; Sanders et al., 2018). After considering the antimicrobial activity of such substances, the new term postbiotics has emerged.

Postbiotics is a new concept and they are metabolites from probiotic bacteria, which can be short-chain organic acids, enzymes, peptides, vitamins, polysaccharides, cell wall proteins and peptidoglycans that can act on the host through immunomodulatory, anti-inflammatory and antimicrobial effects (Cuevas-González, Liceaga, and Aguilar-Toalá, 2020). This kind of ingredient proves that the viability of probiotic bacteria is not the main factor required for obtainment of health benefits (Aguilar-Toalá et al., 2018). In addition, the lack of viable cells in postbiotics is a feature that makes for easier applications of these ingredients on industrial processes, with great potential for use in the food preservative industry.

Scale-up tests in the laboratory constitute an important step in assessing the wide range of difficulties and costs of technological processes in an industrial scale (Crater and Lievense, 2018). An increase in scale from 100 mL flasks to fermenters, although smaller in size in comparison to the equipment of an industry, helps in understanding, predicting and optimizing the yield of the tested product, especially when considering processes with cellular biomass production, where the yield can drop considerably with increase in scale (Xia et al., 2016).

The food industry commonly applies substances that preserve food, since processed products generally contain large amounts of water, sugar and fat, ideal conditions for the contamination by harmful microorganisms (Sharif et al., 2017). However, searching for healthier substances for food preservation is a current concern.

Given the reported inhibition of pathogenic strains by prebiotics and postbiotics, the present work aims to study the production of antimicrobial metabolites (postbiotics) by probiotics in fermenters, in addition to verifying the interaction of postbiotics with prebiotics and other sugars.

2. MATERIAL AND METHOD

2.1. Microorganisms.

The strains *Levilactobacillus brevis* ATCC 367 and *Lactobacillus acidophilus* ATCC 4356 were obtained from Collection of Reference Microorganisms in Sanitary Surveillance - CMRVS (Oswaldo Cruz Foundation - FIOCRUZ, RJ, Brazil) and were maintained as a stock culture at 7 °C on MRS medium.

The following microorganisms of the genus *Bifidobacterium* used in this study were maintained as a stock culture at 7 °C on MRS medium with cysteine: *Bifidobacterium animalis* BB-12 Christian Hansen (Hoersholm, Denmark), *Bifidobacterium lactis* BI-07 Danisco (Madison, USA), *Bifidobacterium longum* BL-05 Danisco (Madison, USA) and *Bifidobacterium breve* BB-03 Danisco (Madison, USA).

Salmonella enterica subsp. *enterica* serovar *typhimurium* ATCC 14028 was obtained from Tropical Culture Collection (CCT), André Tosello Foundation, Campinas, SP, Brazil, with the code CCT 1478. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were also used as pathogen models. The cultures were maintained in the Laboratory of Industrial Biotechnology (UNESP-Assis / SP) at 7 ºC in Nutrient Agar.

2.2. Growth medium for microorganisms.

Formulated MRS medium (De Man, Rogosa, and Sharpe, 1960) was used at pH 6 and autoclaved for 20 minutes at 121 °C. *L. acidophilus* was grown at 37 °C for 48 hours and *L. brevis* was grown at 30 ° C for 48 hours. *Bifidobacterium* cultures were grown at 37 °C for 48 hours in modified MRS medium with the addition of L-cysteine.

Salmonella typhimurium was grown in Brilliant Green medium (BG) (Kauffmann, 1935). The composition had slight modifications (g / L): 3 g of yeast extract, 10 g of peptone protease, 5 g of sodium chloride, 10 g of lactose and 10 g of sucrose. *E.*

coli was cultured in Nutrient Broth (NB) and *S. aureus* was grown on Brain Heart Infusion (BHI) medium. The culture media were prepared in pH 7 and autoclaved for 20 minutes at 121 °C. The pathogen strains were cultured at 37ºC for 48 hours.

2.3. In vitro fermentation of oligosaccharides.

In vitro fermentation of commercial oligosaccharides was carried out using Xylooligosaccharides (XOs) (Shandong Longlive Biotechnology CO., LTD 95P - China) (95% powder of XOs, DP 2–7), Fructooligosaccharides (FOs) (Beneo Orafti 95P - Chile) (95% powder of FOs, DP 2–3), β- Glucan (Yeast cell wall), Mannan oligosaccharides (MOs) (Yeast cell wall).

For the tests with commercial XOs, FOs, MOs and β- Glucan, the carbon source of the medium was replaced by 2% of the respective tested oligosaccharide. In addition, a formulated medium with 2% of carbohydrate and a medium without carbohydrate were used as positive and negative controls, respectively (Figueiredo, Ranke, and Oliva-Neto, 2020). Growth experiments on medium containing different carbon sources were performed in triplicate for *L. acidophilus*, *L. brevis*, *B. animalis*, *B. lactis*, *B. longum*, *B. breve*, *Salmonella typhimurium*, *S. aureus* and *E. coli.*

2.4. Production of postbiotics in a two-liter fermenter.

For the tests aiming to obtain postbiotics on a larger scale, the *B. breve* strain was selected, since good results on the inhibition of *Salmonella typhimurium* were obtained with this strain in previous studies.

The “in vitro” tests were carried out according to Chapman et al. (2013). *B. breve* incubated in liquid MRS containing 200 mL (item 2.2) was adjusted to the same optical density in a spectrophotometer at 650 nm to values between 0.8 and 0.9 in order to represent approximately equal biomass. After 48h of growth, the entire content (10%) was inoculated into a 2 liter fermenter containing MRS medium at 37ºC under agitation at 60 rpm. Simple batch, without nutrient feeding and pH adjustment was performed for 120h and fed-batch, with pH adjustment to 7 was performed for 240h (Bioflo 115, New Brunswick, New Jersey, USA). Aliquots were sampled at 0h, 24h, 48h, 72h, 96h and 120h both for the simple batch and for fed-batch. Further sampling was performed at 144h, 168h, 192h, 216h and 240h for the fed-batch. Then, the aliquots were centrifuged at 2000

x g for 10 minutes and autoclaved for 30 minutes to obtain the postbiotics. The optical density and total acidity of all samples were analyzed as described in Figueiredo, Ranke, and Oliva-Neto (2020) and AOAC (1990), respectively. The inhibitory activity was evaluated by adding 6% (v/v) postbiotics in BG medium and inoculating a 50 µl aliquot of *Salmonella typhimurium*.

2.5. Stability of postbiotics after drying.

The postbiotics, obtained as previously described, were tested for their stability after drying. Samples of 100 mL were oven dried at 80ºC for 24 h and then added to BG medium at concentrations of 0.3, 0.6, 0.9 and 1.2% (m/v). Then, a 50 µl aliquot of *Salmonella typhimurium* was added to assess the inhibitory activity.

2.6. Fractions separation of postbiotics by organic solvents.

In order to better understand the chemical properties of the postbiotics, liquid-liquid extractions were performed with the following organic solvents: butanol, methylene chloride, ethyl acetate, hexane and ethanol. The ratio was 1:2 (v/v - Postbiotic : Solvent). After separation, the liquid fractions were oven dried at 80ºC for 24h, weighed and then added to BG medium with *Salmonella typhimurium* at a concentration of 0.9% (m/v) to evaluate the inhibitory activity.

2.7. Inhibitory activity of postbiotics with commercial sugars

Different commercial sugars (2%) were added to liquid postbiotics (6% v/v). Then, this solution was mixed with BG medium and inoculated with *Salmonella typhimurium* to evaluate the inhibitory activity. Commercial sugars were: fructooligosaccharides (FOS), xylooligosaccharides (XOS), glucose, sucrose, lactose.

2.8. Analytical methods

2.8.1. Percentage of inhibition

The calculation of percentage of inhibition was performed by the following equation (1):

Inhibition %: $\frac{OD\ control - OD\ treatment}{OD\ control} * 100$ (1)

OD control: Optical density of control in 48h of incubation.

OD treatment: Optical density of the experiments in 48h of incubation.

2.8.2. Determination of total acidity

The probiotic supernatant was analyzed for lactic acid content according to the standard titratable acidity method (AOAC, 1990) using NaOH (0.1 N). Equation 2 was used to convert the volume of titrated NaOH into lactic acid (%), in which V represents the volume of NaOH in titrated mL, CF is the NaOH correction factor, and v is the sample volume in mL used. The calculation was performed using the following equation (2):

Total acidity: $\frac{V * 9.008 * CF}{v}$ (2)

The correction factor was 1.018, used for lactic acid.

2.8.3. Statistical analyses

Response means were analyzed using T- student test by BioEstat 5.3 software.

3. RESULTS AND DISCUSSION

3.1. In vitro fermentation of oligosaccharides by probiotics and *Salmonella typhimurium*

All probiotic strains showed significant differences in growth using the prebiotic mixture of FOS and XOS in comparison to control (Fig 1). Despite the statistical differences, the growth reduction in the prebiotic mixture compared with the control, in 48 h culture, was only of 9.9%, 14.7% and 9.7%, for *B. breve*, *B. animalis* and *L. brevis* respectively. *B. lactis* had a reduction of 37.7% in growth, while *L. acidophilus* and *B. longum* had reductions of more than 60%. Interestingly, the probiotic strains that showed

greater reductions in growth with the prebiotic mixture are the strains that, in previous works, showed a preference for FOS as a single carbon source (Figueiredo, Ranke, and Oliva-Neto, 2020). On the other hand, those strains that showed preference for XOS as a single carbon source (*B. breve* and *L. brevis*) continued to show good growth values using the mixture of FOS and XOS.

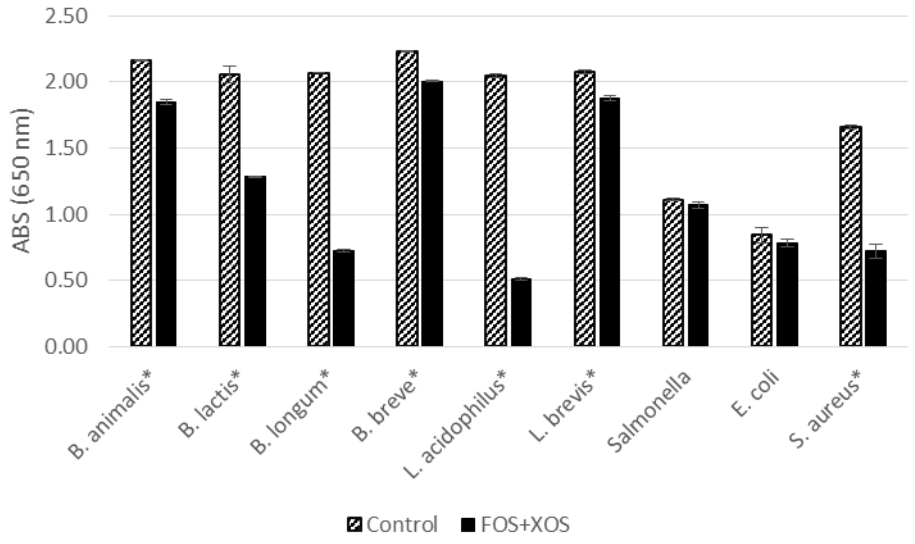
In addition, the evaluation of the effect of prebiotics in the growth of probiotic bacteria also needs to be performed in the log phase, within the first 10hs. In this context, the mixture of FOS and XOS stimulated *L. brevis* and *B. longum*, since their growth values exceeded the growth in control (glucose). *Salmonella typhimurium* had a growth reduction of 22%, while *B. breve* suffered a growth reduction inferior to 8%. However, *L. acidophilus*, *B. animalis* and *B. lactis* had their growth reduced respectively by 75%, 16% and 39.6% when compared to the medium with glucose (control) (Table 1). Therefore, the prebiotics only stimulate some probiotic strains in the first hours of culture, while also showing greater inhibition on the growth of *Salmonella typhimurium* only in the early period of culture.

Table 1- Percentage of inhibition (negative values) or stimulus (positive values) on the growth of bacteria using prebiotic mixture (Fructooligosaccharide + Xylooligosaccharide) in comparison to medium with glucose.

Time (h)	LA* (%)	LB (%)	BB (%)	BLO (%)	BA (%)	BLA (%)	ST (%)
0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
4	-87,5	-95,31	-60,08	-48,32	-100	-100	-51,58
6	65	-12,5	-14,99	-16,95	-100	-66,67	-32,74
8	-3,40	14,47	-7,33	16,86	-44,44	-88	-22,02
10	-48,72	60	-12,10	-58	-27,46	-68,61	-20
12	-62,01	37,36	-23,99	-57,31	-45,56	-27,61	-16,45
24	-74,71	1,86	-11,44	-74,74	-15,93	-39,59	-8,57
48	-74,98	-9,68	-9,93	-64,92	-14,71	-37,95	0

*Symbols: LA = *Lactobacillus acidophilus*, LB = *Levilactobacillus brevis*, BB = *Bifidobacterium breve*, BLO = *B. longum*, BA = *B. animalis*, BLA = *B. lactis*, ST = *Salmonella typhimurium*.

Figure 1- Growth of probiotic strains (*Bifidobacterium*, *Levilactobacillus* and *Lactobacillus*) and pathogenic strains (*Salmonella typhimurium*, *Escherichia coli* and *Staphylococcus aureus*) in medium containing 1% Xylooligosaccharides and 1% Fructooligosaccharides ta 37º C for 48h.



Source: Own work.

Of the pathogenic strains, only *S. aureus* was significantly different from control, with a reduction of 56% in its growth in the prebiotic mixture. *E. coli* and *Salmonella typhimurium* showed no statistical differences between control and prebiotic mixture at least in 48h (Fig 1).

Mäkeläinen et al. (2010) demonstrated a decrease in the growth of *E. coli* and *Salmonella typhimurium* for 24h using FOS and XOS as the single carbon sources, however, no mixtures were tested. Since our tests were performed with in vitro cultures of pure strains in an enriched medium, these bacteria are capable of good growth even in media with the presence of prebiotics, as shown by Figueiredo, Ranke, and Oliva-Neto (2020), where growth of *Salmonella typhimurium* was reported with FOS and XOS and even in medium without any carbon source.

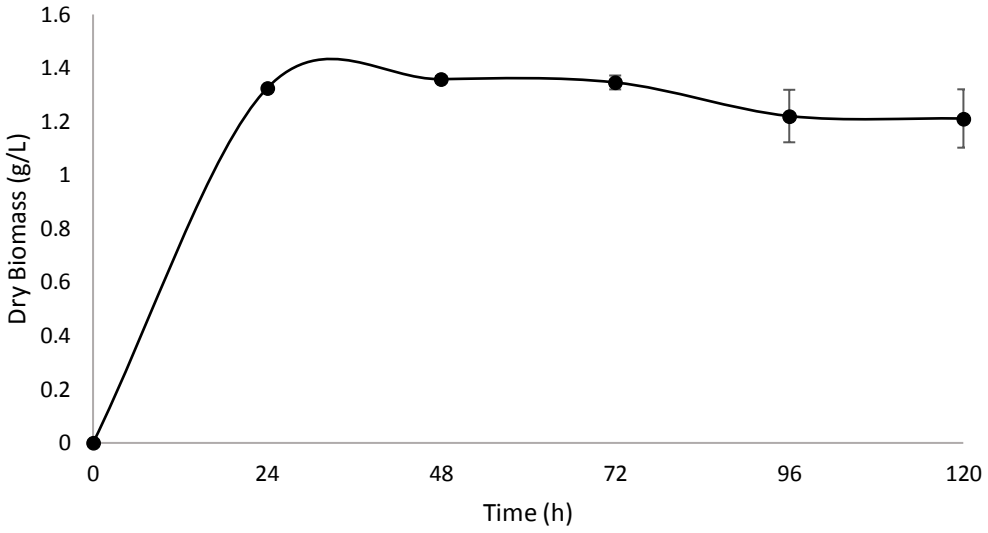
None of the tested microorganisms was able to ferment β glucans and Mannanoligosaccharides (MOS). Both β glucans and MOS are derived from the cell wall of yeasts and present high protein content and low solubility. These two characteristics may have hampered the solubility of these carbon sources and hindered the ability of the tested probiotic microorganisms in using such substances for fermentation processes. The fermentation of MOS by probiotic organisms is also related to the degree of

polymerization (DP), where MOS with low DP is better suitable for fermentation by *Lactobacillus* strains (Suryawanshi and Kango, 2021). Considering that the DP of the tested MOS was not assessed, such parameter may have affected the fermentation of the tested MOS by probiotic microorganisms as well. Regarding the tested pathogenic microorganisms, the lack of growth was expected. MOS and β glucan are reported as not being fermentable by pathogenic bacteria, acting by binding to type1 fimbriae in the pathogens and avoiding their binding to the intestinal epithelium, while, at the same time, agglutinating gram-negative bacteria for later elimination (Borowsky, Corção, and Cardoso, 2009).

3.2. Scaling-up of postbiotics production.

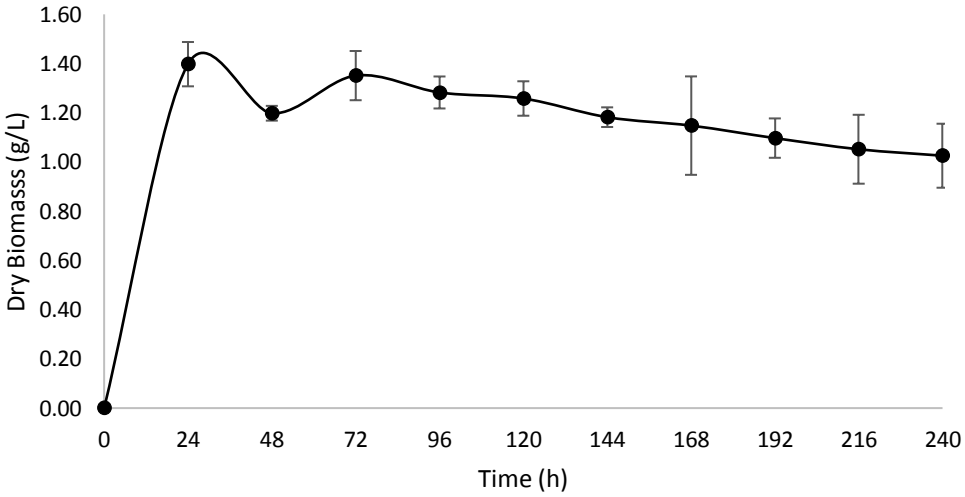
Scale-up of the culture was developed in a two-liter fermenter. This test helps in anticipating some difficulties faced with the increase to the industrial scale. The results of optical density by turbidity and total acidity showed that a period of 24 h is sufficient for the growth and release of essential metabolites for antimicrobial activity (Fig. 2 and Table 2), since there was no significant increase in biomass and total acidity with the increase in fermentation time (48, 72, 96 and 120 h). Tests with fed-batch presented higher maximum biomass values than simple batch in the first few hours; however, no significant increase in biomass or acidity were observed with the increase in fermentation time throughout the experimental period (Fig. 3 and Table 3).

Figure 2- *Bifidobacterium breve* growth after different time periods in a 2 liter fermenter in simple batch under 60 rpm agitation at 37°C for 120h.



Source: Own work.

Figure 3- *Bifidobacterium breve* growth after different time periods in a 2 liter fermenter in fed-batch under 60 rpm agitation at 37°C for 240h.



Source: Own work.

The antimicrobial activity remained the same as those on a laboratory scale obtained from previous works. There was total inhibition of *Salmonella typhimurium* with 6% (v/v) of postbiotics from the simple batch experiment.

Table 2- Total acidity of *Bifidobacterium breve* postbiotics in different fermentation times and percentage of inhibition of *Salmonella typhimurium* with 5% (v/v) and 6% (v/v) of postbiotics at pH 4.5 in simple batch.

		Percentage of inhibition (%)	
Time (h)	**Total Acidity (%)***	**5% of Postbiotics (%)**	**6% of Postbiotics (%)**
0	0 ± 0.00	0	0
24	3.67 ± 0.02	91.7	100
48	3.67 ± 0.01	91.0	100
72	3.63 ± 0.03	91.0	100
96	3.63 ± 0.01	91.3	100
120	3.63 ± 0.06	93.2	100

* expressed in lactic acid

Table 3- Total acidity of *Bifidobacterium breve* postbiotics in different fermentation times and percentage of inhibition of *Salmonella typhimurium* with 6% (v/v) in neutral (6.7) and acidic (4.5) pH in fed-batch.

		Percentage of inhibition (%)	
Time (h)	**Total Acidity (%)***	**Neutral pH (6.4)**	**Acidic pH (4.5)**
0	0 ± 0.00	0	0
24	0 ± 0.00	0	86.49
48	0 ± 0.00	0	89.75
72	0 ± 0.00	0	71.84
96	0 ± 0.00	0	95.02
120	1.8 ± 0.2	0	99.04
144	0 ± 0.00	0	85.56
168	0 ± 0.00	0	83.64
192	0 ± 0.00	0	82.28
216	0 ± 0.00	0	61.97
240	0 ± 0.00	0	55.56

The increase in scale allowed us to observe that with just 24h, obtaining postbiotics with great inhibitory potential is possible, in quantities similar to those obtained using longer fermentation time. This finding is advantageous for industrial production, since faster production leads to savings in energy costs and allows for faster obtainment of the product. In addition, the process showed good results even without previous sterilization of the equipment, possibly due to the low pH caused by the production of organic acids, which blocks the contamination by other microorganisms.

Previous tests showed that treatments with low concentrations of lactic acid (0.1, 0.2 and 0.3 g/L) did not inhibit the growth of *Salmonella typhimurium*. Such treatments

presented pH values close to neutrality (within the range of 7.5 – 8). However, treatments with lower pH values (4 – 4.5) due to higher concentrations of lactic acid (0.4 and 0.5 g/L) decreased the growth of *Salmonella typhimurium*. Lastly, treatments with 0.6 g/L of lactic acid in BG medium fully inhibited the growth of this pathogen during 48h of culture (Table 4).

The inhibition of the growth of *Salmonella typhimurium* can be obtained by the addition of lactic acid and lowering of pH values, as shown by our previous tests and other reported inhibitions in the literature (Ibrahim, Yang, and Seo, 2008). However, previous tests have shown that low pH values, although crucial to the antimicrobial action, are not sufficient to inhibit the growth of pathogen by itself. Lowering the pH to 4.0 using Hydrochloric acid (HCl) did not inhibit the growth of *Salmonella typhimurium*, while low pH values obtained with the addition of postbiotics successfully inhibited this pathogen (Table 4). Such results suggest that the acidic conditions are important for the action of organic acids on the antimicrobial activity.

Table 4- Percentage of inhibition of the growth of *Salmonella typhimurium* (ST) (37ºC, 48h, BG medium) in pH 4 using hydrochloric acid and different pH values using different concentrations of commercial lactic acid.

Lactic acid (g/L)	Lactic acid solution pH*	Inhibition of ST (%) by lactic acid	pH of the solution with hydrochloric acid	Inhibition of ST (%) by hydrochloric acid
0.1	7.5	0	n.a.*	n.a.
0.2	8	0	n.a.	n.a.
0.3	7	0	n.a.	n.a.
0.4	4.5	77.64	n.a.	n.a.
0.5	4	93.40	4.0	0
0.6	4	100	n.a.	n.a.

*n.a. not applicable

Comparing the simple batch with the fed-batch, the fed-batch showed similar biomass values even with the renewal of the culture medium every 24 hours and with the adjustment of pH to neutral values. However, since in neutral pH the activity of organic acids is lost, no inhibitory potential was observed. The tests with simple batch and fed-batch showed that, in an industrial scale, the simple batch process is adequate for a large production of these postbiotics, presenting favorable results.

Dried products constitute another important characteristic for industrial and commercial purposes. Using products with low humidity offers advantages such as increased shelf life, smaller packaging, easier transport and storage (Moses et al., 2014).

Postbiotics produced by simple batch continued to show antimicrobial activity after being oven-dried at 105ºC for 24 hours. The dried product was suspended in the growth medium of *Salmonella* and total inhibition of *Salmonella typhimurium* was observed at a concentration of 0.9% (m/v) of dried postbiotic. Comparing the antimicrobial activity of dried postbiotics and liquid postbiotics, a 2.5-fold drop was observed. The drying process of extracts with antimicrobial activity commonly alters their biochemical properties and may cause changes in their inhibitory potential (Sasidharan and Menon, 2010; Chua et al., 2019). However, this inhibitory concentration of 0.9% (m/v) of postbiotic in the product can still be advantageous for food preservation, although sensorial aspects should be evaluated.

3.3. Separation of postbiotics by organic solvents.

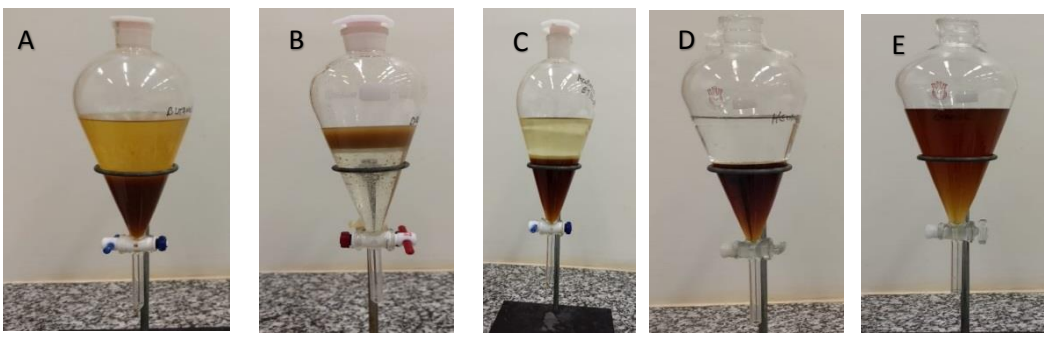
Generally, another important step in the industrial process is the isolation of the active product, through purification and concentration (Zydney, 2016). For a liquid-liquid extraction, the two-phase system with immiscible substances may be used, so that the product will remain in one phase according to its characteristics.

Tests with solvents (butanol, methylene chloride, ethyl acetate, hexane and ethanol) revealed good phase separation, except for ethanol. Both aqueous and organic fractions of all the solvents in the liquid-liquid extraction were oven-dried at 80º C for 24h. Only the aqueous fraction presented mass yields. Dry mass of aqueous fractions are shown in table 5.

The dried aqueous fraction of each of the postbiotics succesfully separated by the solvents were added to BG medium at a concentration of 0.9% (m/v) to test the inhibition of *Salmonella typhimurium*. All dried aqueous fractions showed high values of inhibition, except those separated with butanol. Highest inhibition was obtained with the dried aqueous fraction of hexane (96.92%) while the dried aqueous fraction of the methylene chloride and ethyl acetate solvents showed 92.97% and 85.95% inhibition of *Salmonella typhimurium*, respectively (Table 5).

These results show that postbiotics were mostly in the aqueous fraction of the liquid-liquid separation (Fig. 3), except those separated with butanol or ethanol. Therefore, we can deduce that its composition is mainly of substances that present a polar nature, such as organic acids, peptides and others.

Figure 3- Separation of postbiotic from *Bifidobacterium breve* by organic solvents. A) butanol, B) methylene chloride, C) ethyl acetate, D) hexane and E) ethanol.



Source: Own work.

Table 5- Dry mass of aqueous fractions of postbiotics separated with different solvents and percentage of inhibition of *Salmonella typhimurium* (BG medium, 37º C, 48h) using dried aqueous fraction of the postbiotics (0.9% m/v).

Solvent	Dry mass (g)	Inhibition (%)
Butanol	3.73	0
Methylene chloride	2.36	92.97
Ethyl acetate	4.42	85.95
Hexane	4.67	96.92

3.4. Addition of postbiotics to commercial sugar.

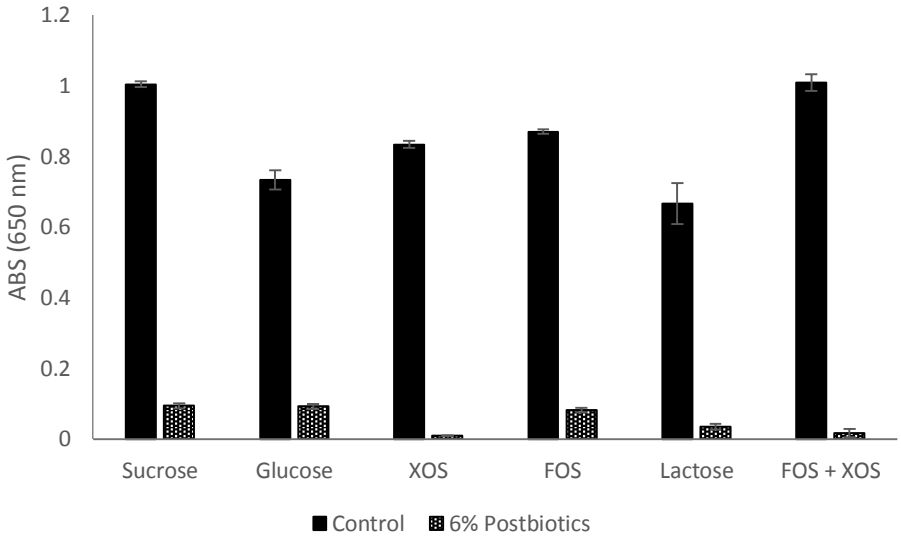
To simulate an addition of postbiotics as a preservative of commercial sugars and to verify a possible synergism of its inhibitory action with prebiotics, solutions of BG medium containing 2% sucrose, lactose, glucose, xylooligosaccharides (XOS) and fructooligosaccharides (FOS) were prepared with the addition of postbiotics.

The postbiotics significantly inhibited the growth of *Salmonella* with a concentration of 0.36% (m/v), regardless of the carbon source used in the BG medium (Figure 4). This value corresponds to 18% (m/m) of the sugar added to the medium, highlighting the inhibitory potential of these ingredients, even in conjunction with commercial sugars easily absorbed by this pathogenic bacterium. Concentrations of postbiotics lower than 0.36% (m/v) did not inhibit the pathogen's growth. No synergism between postbiotics and the prebiotics XOS or FOS in the inhibition of *Salmonella typhimurium* was verified.

Industrial foods such as juices present large quantities of sugar in their composition, offering a suitable environment for the growth of bacteria and other microorganisms, which, in turn, decrease the shelf life of products and may present risks for consumers. Our results showed a potential use of the postbiotics as a food preservative, since, in comparison to the medium containing only commercial sugars, the addition of postbiotics significantly inhibited the growth of *Salmonella typhimurium*. Postbiotics present a well-known benefit to health, these ingredients successfully inhibited pathogenic bacteria and did not inhibit probiotic strains. Further quality assurance tests such as palatability, food safety and recommended dose usage (Fuller,

2004) are needed for a consolidation of the application of *B. breve* postbiotics in well-established food products.

Figure 4- *Salmonella typhimurium* growth in BG medium containing commercial sugars (Sucrose, Glucose, Xylooligosaccharides (XOS), Fructooligosaccharides (FOS), Lactose and FOS + XOS) and in BG medium with commercial sugars with the addition of 6% of *Bifidobacterium breve* postbiotics at 37º C for 48 h.



Source: Own work.

4. CONCLUSION

The scaling up and drying of products are important to assess possible difficulties in industrial processes. The increase in scale to small fermenters has shown that this postbiotic has very good potential for scaling up to industrial processes, since it is a cell-free substance and did not show contamination problems during the non-aseptic process. Furthermore, the inhibitory activity levels were similar to those obtained in scales of 100 mL flasks. The cultures using medium with a mixture of FOS and XOS revealed partial growth inhibition of *Salmonella typhimurium* and stimulation of a few probiotic strains only in the early stages of culture. The drying tests showed a loss in inhibitory activity, but it still shows potential for industrial production, as its activity remained at concentrations of 0.9% (m/v). The addition of postbiotics, combined or not with prebiotics, prevented the growth of *Salmonella* in medium containing commercial

sugars, confirming its potential usage as a food preservative and functional ingredient, without any inhibition on the growth of probiotic bacteria.

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CONCLUSION AND FUTURE PERSPECTIVES

Most tested postbiotics presented inhibitory activity against the pathogenic bacteria *Salmonella typhimurium* and *E. coli* and, when in very acidic conditions, against *S. aureus*. In addition, no inhibitory activity against probiotics was observed. The tests showed that all postbiotics presented antimicrobial activity after treatments with low temperature and in low pH, while most of them remained stable after treatment with high temperature. The inhibitory potential of postbiotics is possibly related to the action of organic acids in acid pH, since the inhibitory activity was lost in neutral pH. Scale-up tests showed a production of *B. breve* postbiotics with satisfactory inhibitory activity in 24 hours. The addition of this postbiotic inhibited the growth of *Salmonella typhimurium* in medium with easily fermented sugars and oven-dried (105º C) postbiotics presented similar levels of inhibition. The stability of postbiotics in high temperatures, low pH and after drying highlights the great potential of postbiotics for the food industry. Further tests should include in-vivo tests in order to verify the health benefits of postbiotics from *B. breve* and the addition of these postbiotics in food, verifying the inhibitory activity against common food contaminants and possible changes in color, flavor or smell.