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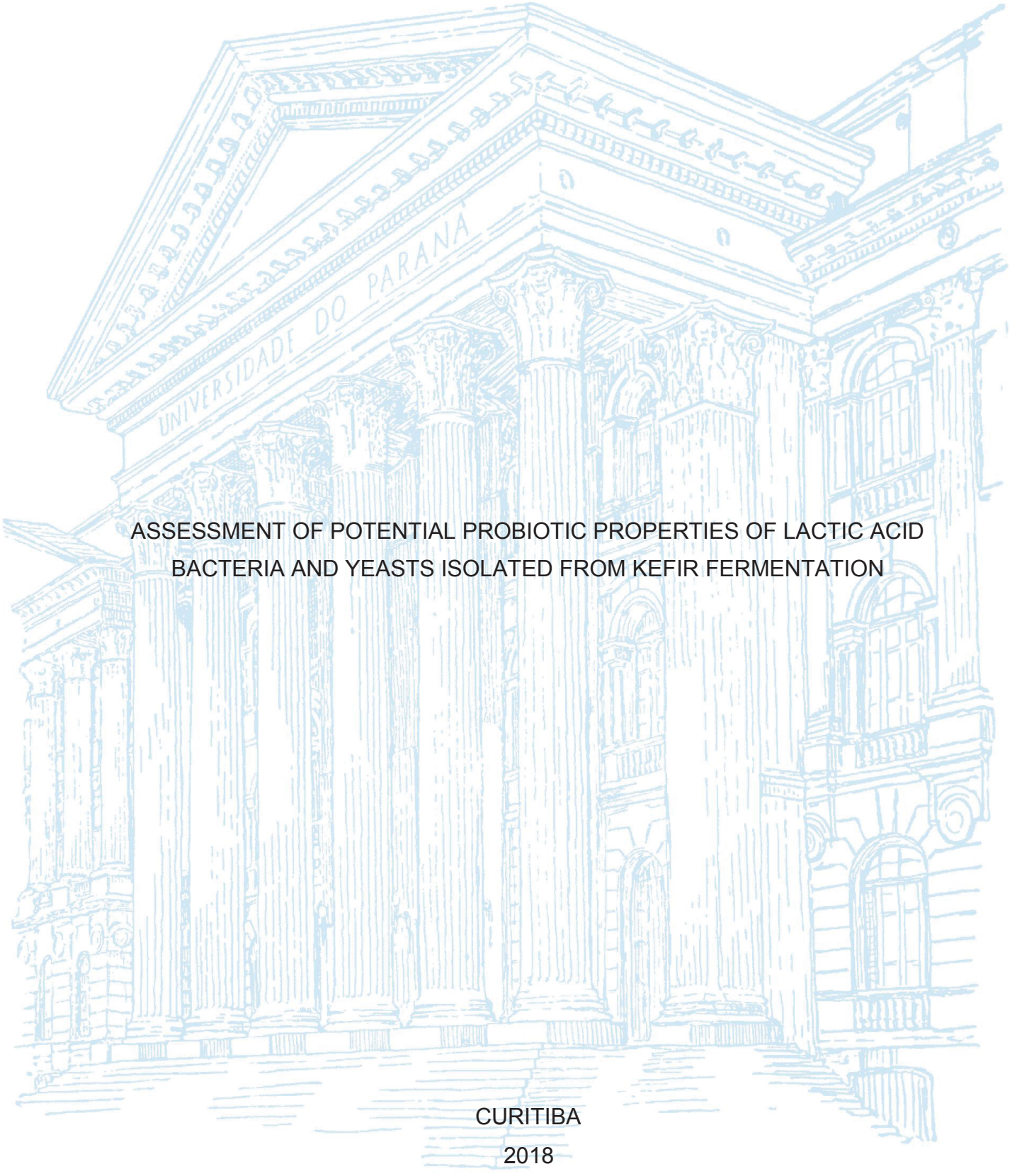
BRUNA DE OLIVEIRA COELHO



ASSESSMENT OF POTENTIAL PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA AND YEASTS ISOLATED FROM KEFIR FERMENTATION

CURITIBA

2018

BRUNA DE OLIVEIRA COELHO

ASSESSMENT OF POTENTIAL PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA AND YEASTS ISOLATED FROM KEFIR FERMENTATION

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientadora: Profa. Dra. Vanete Thomaz Soccol
Coorientador: Gilberto Vinícius de Melo Pereira

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **BRUNA DE OLIVEIRA COELHO** intitulada: **ASSESSMENT OF POTENTIAL PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA AND YEASTS ISOLATED FROM KEFIR FERMENTATION**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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Dedico essa dissertação aos meus
pais, Lindinéia e Fernando.

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"Saber muito não lhe torna inteligente.
A inteligência se traduz na forma que você
recolhe, julga, maneja e, sobretudo,
onde e como aplica esta
informação.” (Carl Sagan, 1995)

RESUMO

A seleção de microrganismos probióticos segue o modelo estabelecido pela Organização Mundial da Saúde (OMS) desde 2002. Esse guia inclui testes básicos, como agregação, co-agregação, hidrofobicidade, resistência as condições do trato gastrointestinal e resistência a antibióticos. Todo microrganismo isolado para fins probióticos requer essas validações. Porém, desde 2002 novas tecnologias e metodologias vem sendo utilizadas e desenvolvidas para avaliação de outras características pertinentes, como produção de antioxidantes, produção de enzimas digestivas e capacidade de proteção ao DNA. Apesar de se tratar de técnicas com alto valor tecnológico e industrial, ainda são negligenciadas em muitos trabalhos, e espécies com características únicas são desprezadas. Esse trabalho teve como objetivo propor um novo modelo de seleção, incluindo técnicas de biologia molecular para identificação de novas espécies probióticas e validar esse método com cepas derivadas do kefir. O trabalho foi dividido em dois capítulos, sendo que o primeiro contém a revisão bibliográfica de técnicas utilizadas para seleção e proposta do novo modelo, e a validação do método de isolamento e seleção no segundo capítulo. De acordo com o levantamento de novas técnicas, é possível observar que bactérias láticas e leveduras possuem capacidade de proteção ao DNA, produção de antioxidantes, e produção de diversas enzimas que podem ser utilizadas de diversas maneiras na indústria. Sendo assim, um novo modelo de seleção foi proposto, incluindo novas técnicas e aplicações. Em seguida, o modelo foi utilizado para isolar e caracterizar cepas isoladas da fermentação de mel por grãos de kefir. Três cepas foram capazes de sobreviver através do trato gastrointestinal, sendo elas *Lactobacillus satsumensis* (LPBF1), *Leuconostoc mesenteroides* (LPBF2) e *Sacharomyces cerevisiae* (LPBF3). Através da técnica molecular Cometa foi possível verificar que as cepas foram capazes de proteger o DNA contra o estresse oxidativo, além de produzirem antioxidantes e possuirem atividade antimicrobiana. Com isso é possível afirmar que o modelo proposto é capaz de selecionar microrganismos probióticos com características específicas.

Palavras-chave: Seleção de probióticos. Bactérias acido láticas. Kefir.

ABSTRACT

The probiotic microorganisms selection follows the model stablished by the World Health Organization (WHO) since 2002. This guide includes basic methods, such as aggregation, co-aggregation, hydrophobicity, survival in the gastrointestinal tract, and antibiotic resistance. Every microorganism isolated for probiotic proposes requires this validation. However, since 2002 new technologies and methodologies have been used and developed to evaluate other relevant characteristics, like the production of antioxidants, digestive and sensorial enzymes, and DNA protective capacity. Despite the fact these techniques possess high technologial and industrial values, they are still negligenciated in some studies, and species with unique characteristics are despised. This work's objective was to propose a new selection model, including molecular biology techniques for identification of new probiotic species, and to validate this method through kefir strains. This work was divided in two chapters; the first has the bibliographic review of techniques used for selection and the new method propose. The isolation and selection validation are included in the second chapter. According to the new techniques review, it is possible to observe that lactic acid bacteria and yeasts have the capacity to protect the DNA against damages, antioxidant and enzymes production that can be used in several industrial applications. Therefore, a new selection model was suggested including novel techniques and applications. Followed by that, the model was used to isolate and characterize strains from the fermentation of honey by kefir grains. Three strains were able to survive through the gastrointestinal tract; *Lactobacillus satsumensis* (LPBF1), *Leuconostoc mesenteroides* (LPBF2) and *Sacharomyces cerevisiae* (LPBF3). By the molecular biology technique, the comet assay, it was possible to evaluate the DNA protection against oxidative stress, besides the antioxidant production, and antimicrobial activity. With this it can be affirmed that the proposed method can select probiotic microbes with specific characteristics.

Key-words: Probiotic selection. Acid lactic bacteria. Kefir.

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LISTA DE ABREVIATURAS

BAI – Beck Anxiety Inventory

BDI - Beck Depression Inventory

DNA – Ácido Desoxirribonucleico

ELISA – Enzyme Linked Immunosorbent Assay

FAO – Food and Agriculture Organization

GALT – Gut Associated Lymphoid Tissue

GAP – Global Action Plan

GIT – Gastrointestinal Tract

HAMA – Hamilton Anxiety Scale

LAB – Lactic Acid Bacteria

MATH – Microbial Adhesion to Hydrocarbons

MRS – de Man, Rogosa and Sharpe **OMS –** Organização Mundial da Saùde

PCR – Polimerase Chain Reaction

ROS – Reactive Oxygen Species

SCGE – Single Cell Gel Eletrophoresis

WHO – World Health Organization

SUMÁRIO

1 INTRODUÇÃO

Microrganismos probióticos são considerados benéficos por produzirem efeitos positivos no hospedeiro em determinadas concentrações. Esses organismos incluem bactérias do ácido lático, bifidobactérias e algumas leveduras, como *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Leuconostoc, Bifidobacterium animalis, Saccharomyces cerevisiae* e *Kluyveromyces marxianus.* Estes microrganismos possuem a capacidade de sobreviver às condições adversas do trato gastrointestinal de humanos e outros animais, colonizando o intestino e auxiliado na saúde do organismo (Liong et al., 2015; Liu, 2016).

A influência dos probióticos na saúde foi primeiramente associada exclusivamente ao sistema digestivo, atuando na prevenção e diminuição de sintomas de doenças como diarréia, intolerância à lactose e doenças autoimunes. Porém, recentemente esses microrganismos também estão associados a prevenção de doenças cardiovasculares, ansiedade, depressão e câncer (Zoumpopoulou et al., 2017).

Apesar de serem amplamente utilizados na indústria e estudados há muito tempo, somente em 2002 que a Organização Mundial da Saúde (OMS) publicou um guia com os requisitos necessários para um microrganismo ser considerado probiótico. Esses requisitos incluem capacidade de sobreviver ao trato gastrointestinal, colonização do intestino, hidrofobicidade, atividade antimicrobiana e sensibilidade a antibióticos (FAO, 2002).

Esse modelo tem sido a base para a seleção de probióticos desde então, porém novas técnicas e características foram desenvolvidas e descobertas depois desse guia ser publicado. Estudos revelam que bactérias e leveduras probióticas produzem diversas enzimas digestórias e sensoriais com alto valor industrial, além da produção de antioxidantes e serem capazes de proteger o DNA contra radiação ultra-violeta (UV) e estresse oxidativo. Sendo assim, o método proposto pela OMS encontra-se desatualizado, por não possibilitar a identificação de microrganismos com características específicas (Fiorda et al., 2016; Chang et al., 2010).

1.2 OBJETIVOS

1.1.1 Objetivo Geral

O estudo teve como proposta buscar novas metodologias para seleção de microrganismos probióticos e propor um novo modelo capaz de selecionar características específicas, validando o método com cepas isoladas da fermentação de mel com grãos de kefir.

1.1.2 Objetivos Específicos

a) Pesquisar as metodologias de seleção de probióticos mais recentes;
b) Propor um modelo atualizado de seleção de microrganismos probióticos;
c) Validar o método com cepas isoladas da fermentação de mel com grãos de kefir.

2 ARTIGO 1

How to select a probiotic? A review update of methods and criteria

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ABSTRACT

International competition within the dairy market and increasing public awareness about the importance of functional foods consumption are providing new challenges for innovation in the probiotic sector. In this context, countless references are currently dedicated to the selection and characterization of new species and more specific strains of probiotic bacteria. In general, basic selection criteria include host-associated stress resistance, epithelium adhesion ability and antimicrobial activity. These aspects are adopted to progressively reduce the number of candidate probiotic strains. However, it cannot be assumed that these novel microbial strains are apt to fulfill several functional benefits claimed to probiotics, including anticarcinogenic, antidepression, antioxidant and cholesterol-lowering activities. In addition, safety-associated selection criteria, such as plasmid-associated antibiotic resistance spreading and enterotoxin production, are often neglected. The purpose of this update was to review strategies for selecting improved probiotic microbes and to assist researchers in choosing methods and criteria for selection.

Keywords: Probiotic selection, lactic acid bacteria, dairy market, functional foods

Introduction

Probiotics are defined as viable microorganisms (bacteria or yeasts) that, when ingested in an appropriate concentration, exert various beneficial effects on the host. Among the known probiotic microorganisms, species of lactic acid bacteria (e.g., *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Enterococcus*) and *Bifidobacterium* have a long history of safe use (Doron and Snydman, 2015). These microbial groups possess the ability to withstand extreme conditions of the human body (e.g., salivary enzymes, low pHs and pancreatic juice), colonizing gut epithelial cells and exercising biological activities, such as prevention of chronical diseases (e.g., Crohn's disease, ulcerative colitis, and pouchitis), increasing the bioavailability of nutrients to the host and antimicrobial properties. In addition, currently, new biological proprieties have been claimed to probiotics, including anticarcinogenic, antidepression, antioxidant and cholesterol-lowering activities (Marchesi et al., 2015; Zoumpopoulou et al., 2017; Liong et al., 2015).

Although diverse functional lactic acid bacteria are already known and applied in commercial probiotic fermented foods worldwide, the market for biofunctional products is continuously in need of implementation and diversification of the available products. For this purpose, there is a growing of scientific works selecting new strains with different and specific functional properties. New microbial groups (e.g., yeast, and *Bacillus*) and more specific LAB strains are constantly identified. These new microbes are usually isolated from humans due to being consider a safe isolation source of microorganisms for product development. However, novel isolation sources are being currently used, such as dairy products, fruits, grains and waste (Plessas et al., 2017; García-Hernández et al., 2016; El-Mabrok et al., 2012; Zendo, 2013; Siddiqee et al., 2013; Sornplang and Piyadeatsoontorn, 2016).

Due to the range of target functions and technological applications, selection and evaluation of new probiotic candidates require a comprehensive approach with multiple steps.

Prior to 2002, there is no international regulation to affirm the efficiency and safety of probiotic microorganisms. Because of this, FAO/WHO (FAO, 2002) published the "Guidelines for Evaluation of Probiotics in Food", which establishes safety and effectiveness standards for probiotics. In this guideline are suggested several probiotic criteria including resistance to body conditions, epithelium adhesion ability and antimicrobial activity.

Recently, several methods have been created to evaluate the efficiency of new probiotic microorganisms. These include molecular methods for detecting DNA protection activity, enzymes production, hydrophobicity, antimicrobial activity, and antibiotic resistance. In this update review, we reported strategies and methods for probiotic strains selection with the objective of support next probiotic microbes' evaluation.

Sources

The vast majority of probiotics available on the market today were isolated from healthy humans since it is considered a safe environment, in addition to increase the compatibility and survival in the gastrointestinal tract (GIT) (Rivera-Espinoza and Gallardo-Navarro, 2010). However, functional food market development is confronted by challenges. It is necessary to search new probiotic strains with better industrial performance or to attend the demand of vegans, vegetarians and lactose intolerant consumers. Thus, the search of unconventional sources for isolation of probiotic microorganisms is increasing significantly. Probiotic strains isolated from freshwater fish, and kefir, respectively (Table 2) show more adaptation for production of new non-dairy based products, such as honey, soy, and corn meal (Prado et al., 2008). In general, probiotic strains that are isolated from non-conventional sources don't produce bacteriocins; instead, they can produce hydrogen peroxide and propionic acids against other pathogens (Sornplang and Piyadeatsoontorn, 2016).

Table 1. Examples of conventional sources for isolation of probiotic strains.

Source	Isolated strains	Reference
Camel milk	*L. plantarum, L. pentosus*, and *Lactococcus lactis*	Yateem et al., 2008
Human milk	*L. fermentum, Leuconostoc mesenteroides and L. delbrueckii*	Serrano-Niño et al., 2016
Sheep milk	*Enterococcus faecium, E. durans* and *E. casseliflavus*	Acurcio et al., 2014
Feta-type cheese	*L. paracasei*	Plessas et al., 2017
Feces of infants	*L. rhamnosus, L. paracasei,* and *Bifidobacterium breve*	Munoz-Quezada et al., 2013
Human stomach	*L. gasseri, L. fermentum, L. vaginalis, L. reuteri,* and *L. salivarius*	Ryan et al., 2008
Italian and Argentinean cheeses	*L. plantarum*	Zago et al., 2011

Table 2. Examples of unconventional sources for isolation of probiotic strains.

Source	Isolated strains	Reference
Fermented Koumiss	*L.casei, L. helveticus, L. plantarum, L. coryniformis, L. paracasei, L. kefiranofaciens, L. curvatus, L. fermentum,* and *L. kandleri*	Wu et al., 2009
Oreochromis mossambicus digestive tract	*Bacillus* sp.	Vijayabaskar and Somasundaram, 2008
Sow milk	*L. reuteri, L. salivarius, L. plantarum, L. paraplantarum, L. brevis,* and *Weissella paramesenteroides*	Martín et al., 2009
C. auratus gibelio intestine gut	*Bacillus* spp.	Chu et al., 2011
Broiler chickens GIT	*Enterococcus faecium*, and *Pediococcus pentosaceus*	Shin et al., 2008

Cocoa	*L. plantarum*	Ramos et al., 2013
Kefir grains	*Saccharomyces cerevisiae, Saccharomyces unisporus, Issatchenkia occidentalis*, and *Kluyveromyces marxianus*	Diosma et al., 2014
Kuruma shrimp	*L. plantarum, Lactococcus lactis, Vagococcus fluvialis,* and *Lactococcus garvieae*	Maeda et al., 2014
Tarkhineh	*L. plantarum, L. fermentum, L. pentosus, L. brevis,* and *L. diolivorans*	Vasiee et al., 2014
Wistar rats feces	*L. intestinalis, L. sakei, L. helveticus* and *L. plantarum*	Jena et al., 2013
Opuntia ficus-indica fruits	*L. plantarum and Fructobacillus fructosus*	Verón et al., 2017
Quinoa and amaranth seeds	*L. reuteri, L. casei, L. sakei, L. plantarum, L. brevis, Leuconostoc lactis*, and *Pediococcus pentosaceus*	Vera-Pingitore et al., 2016
Soy sauce	*Bacillus amyloliquefaciens*	Lee et al., 2017

Probiotic microorganisms

Probiotic agents are defined as microorganisms which exhibit a beneficial effect on host health after ingestion, including Lactic acid bacteria (LAB), *Bifidobacterium*, *Bacillus* and yeast. Among these, *Lactobacillus*, under LAB group, was the earliest discovered probiotic. This genus of rod-shaped or rod-like-shaped Gram-positive bacteria comprises 183 recognized species, applied to various industrial processes as preservatives, acidulants and flavorings in foods, as intermediates in drug and cosmetic manufactures and in the manufacture of biodegradable polylactic acid polymers (König and Fröhlich, 2017). *Lactobacillus*, including *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. casei*, *L. paracasei*, *L.reuteri*, *L. rhamnosus, L. satsumensis,* and *L. johnsonii*, is the dominant LAB group in the animal and human

gastrointestinal and urinary systems, possessing proven action in the maintenance and recovery of health. Others LAB genus with proven probiotic action includes *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and *Leuconostoc* (Holzapfel and Wood, 2012). Metabolically, LAB are known to produce high amounts of lactic acid and other lower metabolites from a diverse source of carbon, including glucose, fructose, lactose and galactose. From glucose metabolism, LAB are classified as homofermentative, which produce high concentrations of lactic acid and carbon dioxide by the Embden-Meyerhof-Parnas pathway, or heterofermentative, which, in addition to lactic acid, produces several other metabolites including ethanol, acetic acid, and carbon dioxide by the pentose monophosphate pathway (Carr et al., 2002). All LAB also produces secondary metabolites including bacteriocins, exopolysaccharides and enzymes, used to increase quality and microbial shelf life of fermented foods (Leroy and Vuyst, 2004).

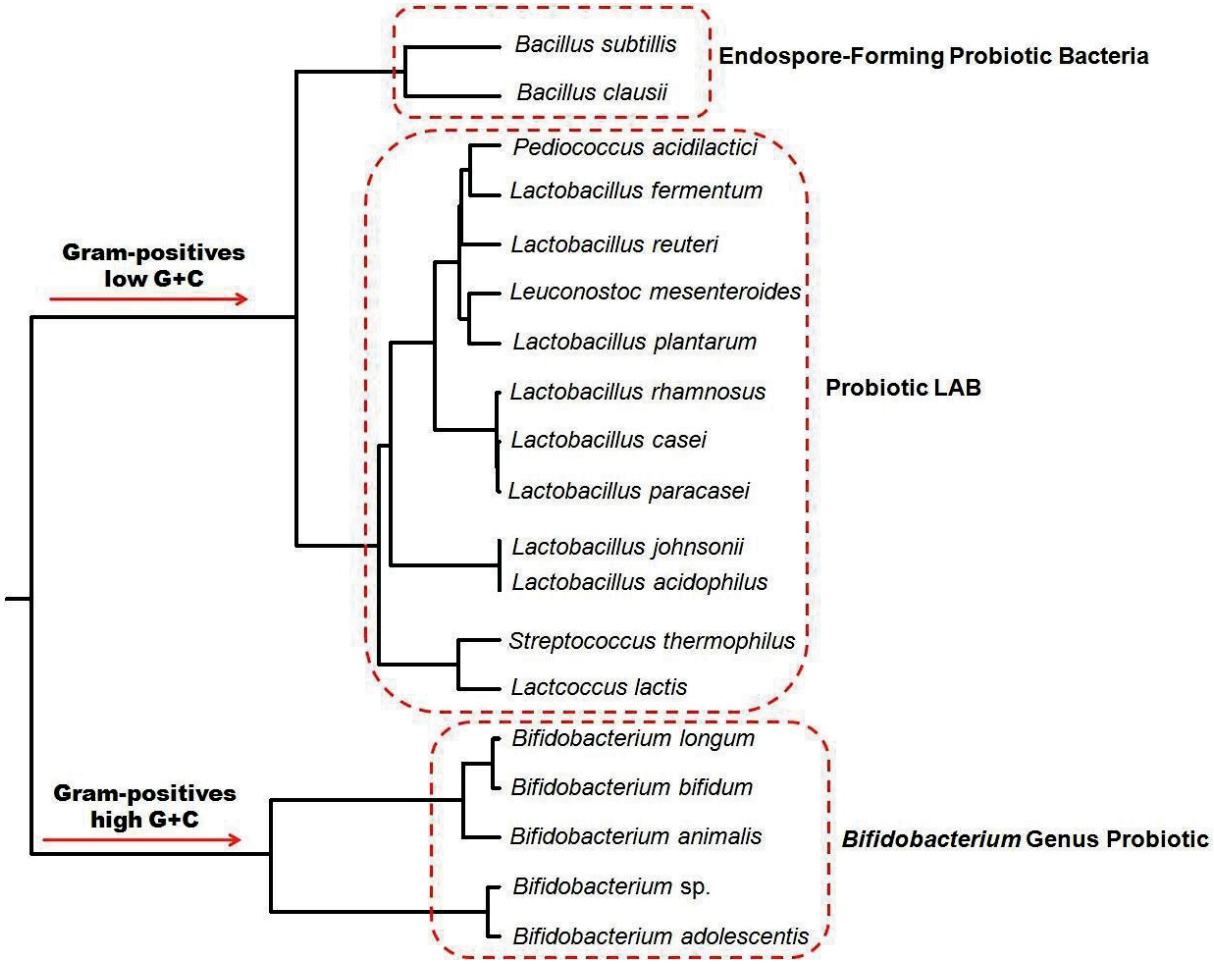
Bifidobacterium was first isolated in the late 19th century by Frenchman Henry Tissier and were inserted in the actinomycetes order mainly due to the high DNA content of guanine and cytosine, which ranges from 42% to 67%. These microbes are heterofermentatives, no spore forming, non-motiles, catalase-negative and anaerobes, with the ability to metabolize glicose, galactose, lactose and frutose (Russell et al., 2011). Nowadays, the genus *Bifidobacterium* includes 30 species, where 10 are from human origin (tooth decay, stool and vagina), 17 from animals, 2 from residual waters and 1 from fermented milk (Russell et al., 2011; Picard et al., 2005). The species *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* are reported for diverse probiotic effects and widely used in yogurts, milk, cheese, and other dairy products (Russell et al., 2011; Picard et al., 2005).

The *Bacillus* genus is widely used as probiotic in food and pharmaceutical industry. The main feature of this Gram-positive, aerobic bacteria group is the formation of endospores and many enzymes, being some toxic (Cutting, 2011). Certain *Bacillus* species (e.g., *B. clausii*, and

B. subtilis) were recently applied as probiotics through studies conducted by Ripert et al (2016) and Liu et al (2018), respectively. The applications include protection of cytotoxic and toxins effects, and disease resistance. The spores formation permits the viability maintance for long shelf periods. However, some species (e.g., *Bacillus anthracis*, *B. cereus*, *B. thuringiensis*, *B. pseudomycoides*, and *B. weihenstephanesis*) are known to produce enterotoxins, proteins that target the intestines causing food poisoning and emetic toxins (Hong et al., 2008; Sorokulova et al., 2008).

The yeasts constitute a large and heterogeneous group of eukaryotic microorganisms widespread in natural environments, including GIT of humans, plants, airborne particles and food products (Foligné et al., 2010). However, this microbial group represents less than 0.1% of the normal microbial flora of humans due to their low resistance through the gastrointestinal tract. Thus, currently, only the species *Saccharomyces cerevisiae* var. *boulardii* fulfill the major criteria for probiotic definition and are commercially exploited mainly in animal nutrition (Czerucka et al., 2007). However, interest in probiotic yeasts has increased due to the various biological activities attributed to this microbial group. In addition, yeasts have the advantages of non-susceptibility to antibiotics, tolerate diverse conditions of industrial processing (i.e., lyophilization and high temperatures) and with important biochemical properties such as fermentation or assimilation of lactose, production of extracellular proteolytic and lipolytic enzymes, and assimilation of lactic and citric acid (Abdel-Rahman et al., 2013; Joshi and Thorat, 2011; Morgunov et al., 2013; Fleet, 2011). *Kluyveromyces marxianus,* for example, is known for the production of *β-galactosidase* and its assimilation of lactose in the milk, and *Debaryomyces hansenii* has a good tolerance to salt, an important component of cheese production. Both strains have the capacity to produce proteolytic and lipolityc enzymes to metabolize the fat and protein from the milk (Tokuhiro et al., 2008; Lane and Morrissey, 2010; Banjara et al., 2015).

Figure 1. Neighbor-joining tree showing the phylogenetic relationship of the different probiotic bacteria groups through16S rRNA gene sequences retrieved from GenBank database. Sequences were aligned with ClustalW and the phylogenetic tree was constructed using MEGA 4 program.



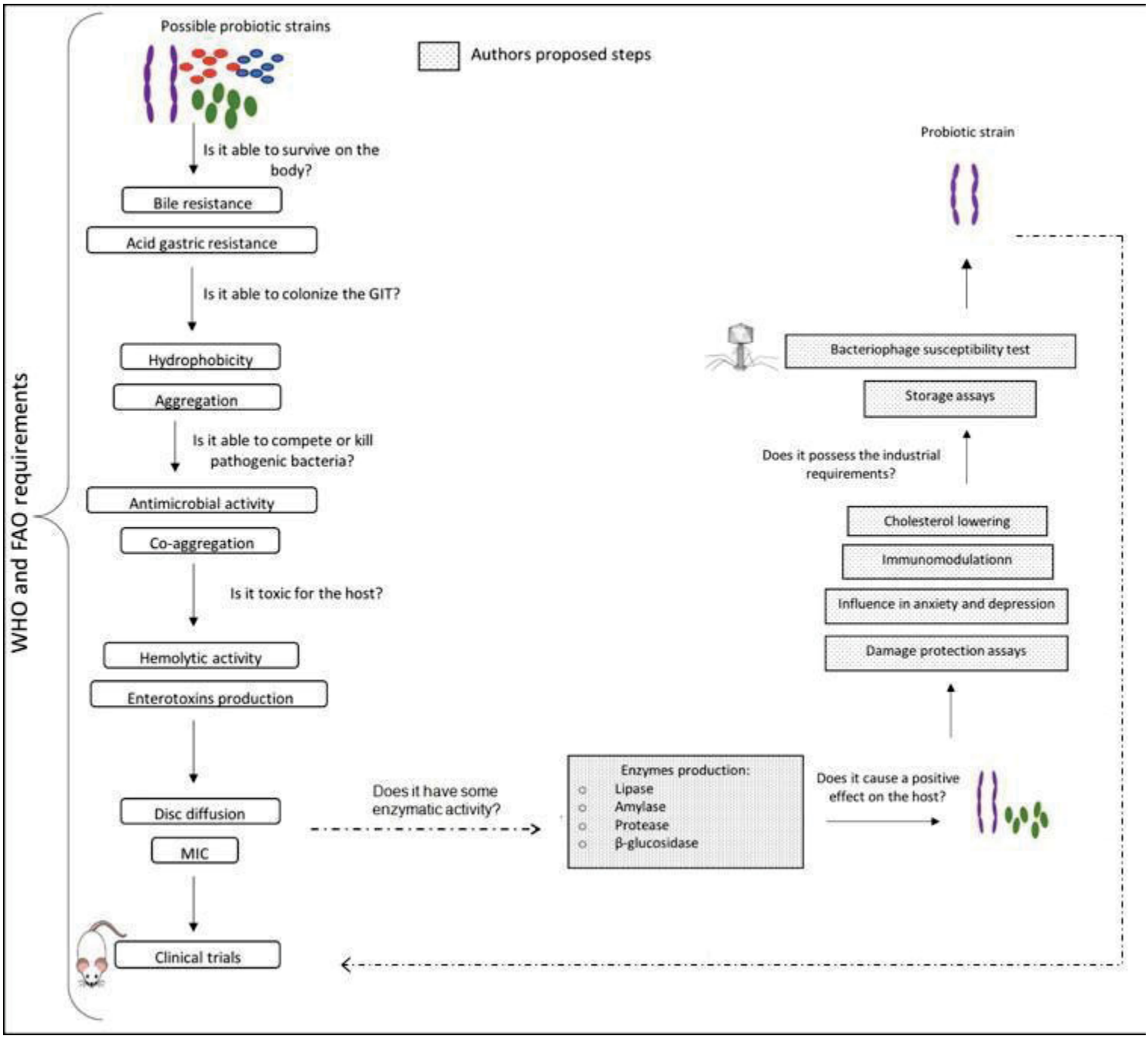
Evaluation of probiotic candidates

Due to the range of target functions and technological applications, selection and evaluation of new probiotic candidates require a comprehensive approach with multiple steps (Figure 2). According to FAO/WHO guide (FAO, 2002), the first step is a taxonomic identification of the candidate to ensure the safety consumption. There are various molecular biology techniques used to identify probiotic microorganisms, such as Polymerase Chain Reaction (PCR), DNA-sequencing, Random Amplified Polymorphic DNA (RAPD), and SDS-

PAGE (McCartney, 2002). For more methods and identification criteria details, the readers are directed to review works carried out by Temmerman et al (2004), Amor et al (2007) and Bagheripoor-Fallah et al (2015).

After the identification, the functional properties must be evaluated by means of *in vitro* and in *vivo* assays, including resistance to oral cavity enzymes, survival along the GIT, and antimicrobial activities (Giraffa, 2012). After these evidences, additional tests include enterotoxins production, hemolytic activity, anticarcinogenic effect and DNA stress protection (Venugopalan et al., 2010; Pieniz et al., 2014; Abushelaibi et al., 2017; Ji et al., 2015) may be performed. Ultimately, it is needed to perform an animal/human trial, to delimitate any side effects and lack of infectivity.

Figure 2. Polyphasic screening approach for characterization of probiotic strains



Polyphasic screening approach

In most cases, the large number of potential probiotic candidates leads to a necessity in the use of a "polyphasic approach", which consist of a sequence of tests (e.g. tolerance to bile

and acids) for progressively reduction the number of probiotic strain candidates At the end of this procedure, the strains that present the highest number of functional properties, and, concomitantly, without any negative traits, are selected.

Firstly, the candidate strains must be able to resist to the stress conditions imposed by the human gastrointestinal tract. It includes the ability to resist oral cavity enzymes, pancreatic juice and bile and to support low stomach pH (Divya et al., 2012). This characteristic can be tested by cultivating the strain of interest in different pH, with the presence of enzymes like pepsin, lysozyme and amylase, phenol, NaCl, Oxgall, porcine gastric juice, pancreatic *USP,* and taurodeoxycholic acid. The resistance to these compounds is measured by the colony counts or by absorbance in different time intervals (Maragkoudakis et al., 2006; Divya et al., 2012; Lin et al., 2007; Martín et al., 2005). The gastrointestinal resistance varies according to species. *Lactobaccillus* are broadly resistant, while *Bifidobacteria* are extremely sensitive to low pH, exhibiting low or no survival rates at pH 2 and pH 3 (Fontana et al., 2013; Sanz, 2007; Takahashi et al., 2004).

The tolerance to inhibitory conditions generally excludes a considerate number of isolated microorganisms. From 29 *Lactobacillus* strains evaluated by Maragkoudakis et al (2006), only six were able to survive 1 hour at pH 1, and eight strains could not survive with pepsin solution at pH 2. A similar result was also reported by Lim et al (2004), where from 100 bacterial strains, including *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*, only 51 were able to survive at pH 2,5 and pH. Yu et al (2013) demonstrated that between seven isolated strains of *L. plantarum*, only S2-5 and S4-1 could survive at pH 2.

The next step is to guarantee that the resistant strains are able to colonize the epithelium walls from GIT. This is necessary to ensure the probiotic strain permanence, so it can act with its functional properties. The cell membrane adhesion to epithelial cells is a complex contact process involving the two membranes that depends on the chemical and physiochemical

composition of the probiotic strain cell surface. This behavior depends on the balance of electrostatic and Van der Waals interactions on the target surface, but studies suggest that bacterial extracellular components and the surrounding composition can influence in adhesion (Boonaert and Rouxhet, 2000; Duary et al., 2011).

The microbial affinity to hydrocarbons has been a useful method to measure the hydrophobicity of cells surfaces for probiotic strains, also known as MATH (microbial adhesion to hydrocarbons) (Chelliah et al., 2016; Del Re et al., 2000; Duary et al., 2011; Collado et al., 2008; Wang and Han, 2007). The method consists in mixing water, a hydrocarbon, and the strain suspension. The two phases solutions are mixed and the hydrophobicity of the strain is measured by absorbance at 600 nm. It is a simple test that requires just a simple spectrophotometer, and the cells can be readily observed in a microscope at 100X (Rosenberg, 2006).

A direct method to analyze if the probiotic strain is able to adhere to epithelial intestinal cells is the evaluation of its adhesion to cells cultures. Mammalian epithelial cells like Caco-2, HT-29, fetal I-407, and IPEC-J2 are used as an *in vitro* evaluation of adhesion ability (Fontana et al., 2013; Dicks and Botes, 2009). Ramos et al. (2013) evaluated the adhesion ability of *Lactobacillus* strains isolated from cocoa fermentation to Caco-2 cells, and only two of six isolated strains showed high percentage of adhesion, and three strains showed moderate adhesion. Leite et al. (2015) isolated 34 acid lactic bacteria from Brazilian kefir and tested its adhesion to Caco-2 cells, and selected a *Lb. paracasei* with significant adhesion ability as a probiotic candidate.

To produce its beneficial effects on the host, the microbial strain need to achieve a certain mass by aggregation. It can be achieved through a simple method where the absorbance of a strain suspension with phosphate buffer solution (PBS) is measured in different time interval (Ogunremi et al., 2015; Kos et al., 2003). The aggregation ability may vary even in the

same genus, as shown on the study of Tuo et al. (2013), where the aggregation of 22 different *Lactobacillus* varied from 24.16% to 41.39%.

Next, antimicrobial capacity against pathogenic bacteria should be evaluated for probiotic candidate. Probiotic microorganisms have this characteristic through different systems, like competition for binding sites and nutrients with other microorganisms or by the production of antimicrobial metabolites. The extracellular antimicrobial components are produced by probiotic strains through the conversion of carbohydrates, proteins, and non-nutritive compounds, forming important substances capable of killing other pathogenic bacteria, such as organic acids, hydrogen peroxide, bacteriocins, and low-molecular-mass peptides or proteins. The strains are tested in agar plates, and the inhibition zones are evaluated (Cueva et al., 2010; Divya et al., 2012). Another method to measure the antimicrorial activity is by the co-aggregation assay. It evaluates the strains capacity to compete or inhibit pathogenic bacteria growth by direct space competition. The method is similar to the aggregation assay, with the suspension being a combination of the two strains. The co-aggregation studies can be performed in combination to *E. coli*, *S. aureus*, *Candida* spp., *Listeria monocytogenes, Salmonella choleraesuis,* and other pathogenic bacteria (Ocaña and Nader-Macías, 2009; Ekmekci et al., 2009; Soleimanil et al., 2010; Vidhyasagar and Jeevaratnam, 2013; Olivares et al., 2006).

Probiotic strains produce volatile substances, such as aromatic hydrocarbons, peroxides, ketones, amides, and alcohols. These compounds change the aroma and flavor profile of products, but can also act like antimicrobial substances. The detection of these substances is by gas chromatography–mass spectrometry (GC–MS) (Sreekumar et al., 2009; Salmeron et al., 2009).

Songisepp et al. (2004) developed a probiotic cheese with *Lactobacillus fermentum* that presented high antimicrobial activity against *E. coli*, *Shigella sonnei*, *Staphylococcus aureus*,

Salmonella enteritidis and *Salmonella typhimurium*. In the study developed by Urdaci et al. (2004), the objective was to evaluate the antimicrobial and immunomodulation of *B. clausii*, and found out that the antimicrobial substance produced by this strain was thermostable and resisted to subtilisin, proteinase K, chymotrypsin, lipase, and α-amylase, demonstrating how these substances can be explored and have several applications.

Host-associated functional criteria selection

The methods previously described in this review are generally performed in all scientific studies for selection of probiotic microbes. However, several health benefits are associated with consumption of probiotics which can be included to select improved strains, such as anticarcinogenic effects, attenuation of immunoinflammatory disorders and lactose intolerance symptoms, immune stimulation, lowering of cholesterol levels and anti-diarrhoeal properties. Some of these effects are due to metabolites excreted in the GIT, such as folic acid, riboflavin, cobalamin, propionic acid, and peptides (Stanton et al., 2005; Kumar et al., 2013).

Stimulation of the immune system by probiotic microorganisms occurs in the gut associated lymphoid tissue (GALT), regulating the local and systemic immune response. These organisms lead to the production of IgA and IgM-secreting cells, IFN-γ, IL-1, TNF-α, IL-10, IL-12, IL-18, TGF-β, and leads to the activation on innate response. Several studies reported the production of these components and its benefits in combating allergic diseases, Crohn's disease and ulcerative colitis. The production of some of these components can be measured by the Enzyme-Linked Immunosorbent Assay (ELISA), a method that combines antibodies with simple enzyme assays, to detect and quantify the presence of peptides, proteins, antibodies, and hormones. Depending on the objective and target of the product, strains able to induce certain immune response can be selected (Gill and Prasad, 2008; Shah, 2007; Dicks and Botes, 2009;

Erickson and Hubbard, 2000; Delcenserie et al., 2008; Prescott and Björkstén, 2007; MacFarlane and Cummings, 2002; Borruel et al., 2003).

Probiotics are able to produce antioxidants that can protect DNA from damage and stress. The reactive oxygen species (ROS), released through cellular metabolism, can interact and damage lipids, proteins, and chromosomes if not inactivated. Several studies have reported the probiotics capacity to produce antioxidants (e.g. superoxide dismutase, catalase, glutathione dismutase, ascorbic acid, melatonin, and glutathione) that can decrease the oxidative stress. The production of antioxidants can be identified by DPPH, ABTS, and Orac assays able to detect and measure the antioxidants production and activity (Amaretti et al., 2012; Sah et al., 2014; Persichetti et al., 2014; Nyanzi et al., 2015).

The direct protection of DNA can also be detected by molecular biology techniques. Fiorda et al (2016) tested DNA protection utilizing a plasmid in contact with probiotic agents against H_2O_2. The protection was visualized in agarose gel, where it could be observed the plasmid DNA. The plasmid has three forms on agarose gel, the supercoiled circular DNA form, open circular, and linear form. The developed probiotic bevarage was able to protect the DNA against hydroxyl radical compared to the negative control. Another technique was performed by Chang et al (2010) that tested the kimchi protection study through the comet assay, also known as Single Cell Gel Eletrophoresis (SCGE), to analyze and quantify DNA damage in individual cells. The authors observed that the selected strain was able to protect the DNA against tumor initiation and DNA damage with immunostimulation characteristic.

Recent discoveries link probiotics with the prevention of heart diseases by lowering the cholesterol serum levels (Ooi and Liong, 2010). This ability can be measured by *in vitro* tests, using cholesterol-phosphatidylcholine micelles, MRS broth supplied with cholesterol, or by water-soluble cholesterol (polyoxyethylene cholesteryl sebacate); all in contact with the probiotic suspension and measuring the residual amount of cholesterol by the o-phthalaldehyde

method. *In vivo* studies to select lowering-cholesterol strains are performed by detecting it in samples like blood, urine, and stool after the ingestion of the probiotic. In addition, molecular biology techniques can detect the expression of cholesterol metabolism-related genes in mice liver of rats with hypercholesteromia that ingested probiotics (Damodharan et al., 2016; Liong and Shah, 2006; Ouwehand et al., 2002; Ooi and Liong, 2010; Kumar et al., 2012; Ding et al., 2017; Wang et al., 2014; Costabile et al., 2017;).

Probiotics influence in anxiety and depression can be detected by the lowering symptoms according to the scales of Anxiety Inventory (BAI), Hamilton Anxiety Rating Scale (HAMA), and Beck Depression Inventory (BDI) in patients supplemented with probiotics. Stress hormones (e.g. adrenocorticotropic, and cortisol) are also dosed from serum, urine, and saliva. Lower levels of these hormones compared to placebos are indicators of probiotics influence in anxiety and depression (Foster and Neufeld, 2013; Desbonnet et al., 2008; Luna and Foster, 2015; Dinan and Cryan, 2013; Collins et al., 2012; Tillisch et al., 2013; Pirbaglou et al., 2016).

Enzymes production

Probiotic microorganisms are characterized by the release of various enzymes. These enzymes induce synergistic effects on digestion, alleviating deficiency symptoms in nutrient absorption. Bacterial enzymatic hydrolysis can increase the bioavailability of proteins and fat and increase the release of free amino acids (Parvez et al., 2006). Probiotic strains can be selected by the production of specific enzymes for different proposes. Examples of strains and its enzymes are shown in Table 3.

Table 3. Examples of digestive enzymes production/activity of probiotic strains.

Microorganism (s)	Enzyme (s)	Reference
P. manshurica, S. cerevisiae, C. boidinii, G. reessii, R. glutinis, and *R. graminis*	Lipase, Catalase, Amylase and β-glucosidase	Oliveira et al., 2017
Lactobacillus spp.	Trypsin, Amylase, and lipase	Suzer et al., 2008
Lactobacillus spp.	Amylase	Jin et al., 2000
Debaryomyces hansenii	Amylase	Tovar et al., 2002
Bacillus sp.	Protease, amylase, lipase an cellulase	Wang, 2007

Lipases contribute to the improvement of digestion of lipids to short chain fatty acids. The higher concentration of short chain fatty acids assists in maintaining an appropriate pH in the lumen of the colon, crucial for the expression of many bacterial enzymes on foreign compounds and on the metabolism of carcinogens in the intestine. Amylase promotes the hydrolysis of polysaccharides facilitating the breakdown of starch and glycogen, while proteases catalyze the breakdown of proteins (Bairagi et al., 2002).

Enzymes can be detected by qualitative assays, supplementing the agar media with carboxymethlycellulose, starch, peptone-gelatin, and tributyrin for activity of cellulases, amylase, protease, and lipase respectively, and the supplementation can vary according to the study objective. The halos around the colonies indicate the enzymes activity. Quantitative assays utilize different substrates to react with the cultures that were grow in enriched media, and the activity specific enzyme activity is measured by spectrophotometry (Suzer et al., 2008; Bairagi et al., 2002; Dutta and Ghosh, 2015).

Antibiotic Resistance

The constant use of antibiotics for treatment of microbial diseases increased its resistance in bacteria, and became a current public health problem. This issue has become a globalized problem, and in 2017 the World Health Organization and partners started a Global Action Plan (GAP) on antimicrobial resistance, to raise awareness of the need of taking actions and what society can do to oppose antibiotic resistance. The concern increased with the possibility of horizontal transference of resistance genes to other bacteria (Sharma et al., 2014).

Probiotic bacteria may have several antibiotic resistance genes that can be transfer to other bacteria due to its broad use. This aspect has been negligenciated by some selection studies (Ornellas et al., 2017; Sánchez et al., 2010; Verso et al., 2017). Nawaz et al (2011) isolated LAB from fermented foods, and analyzed its resistance to antibiotics, concluding that antibiotic resistance is well dispersed in Chinese food products. Toomey et al. (2010) isolated 37 LAB from Irish pork and beef abattoirs, and found 33 resistant strains to one or more antibiotics. Several other studies evaluate LAB resistance and gene transference, highlighting the importance of checking their resistance before food development (Sharma et al., 2014; Schjørring and Krogfelt, 2011; Klein, 2011; Wang et al., 2012).

The susceptibility to antibiotics can be measured by the minimum inhibitory concentration (MIC) assay, which determines the minimum necessary concentration of an antimicrobial to inhibit the microorganism growth, and by disc-diffusion, that utilizes antibiotic discs with inhibitory concentrations in agar plates (Mathur and Singh, 2005; Gullberg et a.l, 2011; Ashraf and Shah, 2011).

Molecular techniques such as PCR can be used to locate these resistance genes. The location is crucial to know if the horizontal transference is possible, since it occurs when the gene is located on the plasmid. The sequence of antimicrobial resistance genes and its primers

are extensively reported at literature, making this technique a simple, fast and very specific method for detection of antibiotic resistance genes (Fiórez et al., 2016; Fiórez and Mayo, 2017; Klein et al., 2000; Shevtsov et al., 2011; Garofalo et al., 2007; Giovanetti et al., 2003; Pillai et al., 2012; Whiley et al., 2007; Rojo-Bezares et al., 2006; Ouoba et al., 2008). Hummel et al (2007) investigated resistance genes of 45 lactic acid bacteria, including *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, and *Leuconostoc*. There was low resistance to erythromycin, tetracycline, and chloramphenicol, but for gentamicin, ciprofloxacin and streptomycin the rate of resistance in the strains was 70%, what could indicate intrinsic resistance. The study also indicated problems with conventional resistance tests, due to wrong breakpoint values.

Clinical trials

Clinical trials are required to validate in vivo the actual functionality of probiotics before its use. It is necessary to evaluate the selected strain presence on stool after the patient received the probiotic supplementation, to prove that the strain was able to resist the GIT and effectively colonized the intestines. These trials treatments are administrated in rats or humans and their effects are compared with placebo treatments (Hedin et al., 2007; Fox et al., 2015; Miller at al., 2017). Studies with animal models treated with *Lactobacilli* strains had immunomodulating activity and promising effects in the chronic inflammatory bowel disease, pouchitis, and ulcerative colitis (Schultz and Sartor, 2000). Studies involving children showed that compared to placebo treatments, probiotics reduced significantly the risk and duration of diarrhea (Szajewska and Mrukowicz, 2001). The probiotic treatment can be the combination strains and its effect is dose-dependent. Different doses should be performed in different time intervals, ranging from 7 to 28 days (Gou et al., 2014).

Industrial requirements and technological properties

In the case of probiotics that are added to industrialized foods, candidate strains must survive food processing and biological stresses, which include tolerance to temperature, pH, as well as oxidative and osmotic stress. In addition, genetic stability is essential for safety proposes and production in order to avoid developing pathogenicity or loss of productivity. The probiotic cultures should also not have adverse effects on the taste or aroma of the product and should not increase the acidity over the shelf life of the production (Champagne et al., 2005). Ranadheera et al (2012) evaluated probiotic products stability and its sensory properties, and observed that the addition of some substrates can control non-desired flavors and aroma, like juice fruits, that enhanced sensory aspects and decreased viscosity of the product. Goodarzi (2016) studied the maintain of texture, flavor and acidity of cold-sensitive *L. delbrueckii* products and observed that during a month the sensory properties did not change, therefore this strain could be used as an alternative for shelf life of probiotic products.

Probiotic strains with good industrial properties need to have a high rate of growth in milk. This growth rate is often affected by bacteriophages infections. Bacteriophages are obligate parasites and generally its infection results in cell lysis and the release of new virions that will infect nearly cells. Bacteriophages are a strong concern for acid lactic bacteria in food industry. Besides LAB be susceptible to the attack of these viruses, there is the sanitary conditions concern, due to the contamination by bacteriophages, that decrease or inhibit completely the probiotic production. The industry strategy is to select bacteriophage resistant strains, and through air filtration, direct vat inoculation, and the use of closed vats (Leroy and Vuyst, 2004; Lucchini et al., 2000; Garneau and Moineau; 2011; Konings et al., 2000).

Bacteriophages can be detected by classic methods, like plaque assays or acidification monitoring, or by more sophisticated methods, like qPCR, biosensors, and flow cytometry

(Garneau and Moineau; 2011). Suárez et al (2002) isolated 61 *Streptococcus thermophilus* and *Lactobacillus delbrueckii* phages from yogurt and cheese samples. The study demonstrated the high phage virulence, but also discovered resistant strains as an option to new industrial production.

Safety aspect

Some microbial species are known to produce enterotoxins, proteins that target the intestines causing food poisoning and emetic toxins. For probiotic strains, enterotoxins production is generally reported by the *Bacillus* species, while no production by *Lactobacillus* e *Bifidobacteirum* have been reported. Species as *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. pseudomycoides*, *B. weihenstephanesis,* and *B. cereus* are known to be pathogenic and enterotoxins producers, which drew the concern from WHO and the European Commission (Hong et al., 2008; Sorokulova et al., 2008). This genus is different from *Lactobacillus* spp. because contrary to *Lactobacillus,* the *Bacillus* belongs to the transitory bacteria of the GIT (Sorokulova et al., 2008). Several studies reported toxigenic potential from *Bacillus* genus and special assays like enterotoxin genes detection, enterotoxins detection by kit, cytotoxicity assays, and in vivo studies, should be performed for this genus (Phelps and McKillip, 2002; Sorokulova et al., 2008; Rowan et al., 2003; Guinebretière and Broussolle, 2002).

The hemolytic activity is considered a safety aspect for the selection of probiotic strains (FAO/WHO, 2002). It measures the breakdown of red blood cells, responsible for the transport of oxygen from the lungs to the cells. The evaluation of hemolytic activity is a technique which uses agar plates containing a percentage of blood, and inhibition zones indicate hemolytic activity (Sánchez-Ortiz et al., 2015; Santini et al., 2010).

Storage

Storage stability is considered a quality control measure (Forssten et al, 2011). For probiotic effectiveness, it is required populations of 10^6 to 10^8 CFU/g by the time of consumption. Some products can show modifications during shelf life, such as postacidification, and the strains can lose the viability. The presence of oxygen during some process and storage can also affect the cell's viability (Antunes et al., 2005; Pereira et al., 2016). Viable cells control for production and validation of a new probiotic product requires specific methods to determine which strain can be used for the respective production. To verify cell viability by classic methods such as incubation in plates, the choice of the medium strongly depends on the strain taxonomy and desired product. Agar MRS is widely used, because it contains all vitamins and proteins necessary for the growth of lactic acid bacteria. Plates for *Biffdobacterium* sp. incubation must be done by anaerobic conditions, and the incubation temperature can also change according to the strain. Mesophilic strains must not be incubated in temperatures above 30º C, but for thermophilus organisms temperatures above 37º C are recommended (Davis, 2014).

Real time PCR (qPCR) with propidium monoazide for quantification of probiotics has been reported as an efficient alternative for probiotic cells quantification. Propidium monoazide (PMA) has the capacity to penetrate the cell membrane of dead cells and bind to DNA after photo induction of azido group, inhibiting its amplification through the PCR. Futhermore, the viable cells DNA does not suffer the intercalating agent action. These two intercalants are useful to the differentiation of viable and dead cells of gram-positive and gram-negative bacteria. For this, specific primers are developed with a cellular concentration curve versus melting temperature. Another vantage of this method is that it detects viable cells, but not in the cultivate state. However, the use of this intercalant agent can show limitations. PMA cannot completely inhibit the DNA amplification by PCR of dead cells when the target sequences are short, but it can be overcome by using nested-PCR. Besides that, several variables must be

considered in this technique standardization, like: determination of PMA concentration, dead cells obtainment method, time of incubation on the dark, photo activation, and light potency (Davis, 2014; Radulović et al., 2012).

Conclusion

Different strains can present different probiotic properties, and studies involving isolated strains from non-common sources are crucial for innovation in new products, leading to a whole new range of probiotics application. An important factor that limits the use of new microorganisms is related to their cost and investments with detection and characterization of probiotic candidates, creating the need for development of different test to their selection. Several studies perform probiotic strains selection, but there is not a standardization of methods that detect advanced properties of these microorganisms. Conventional tests and properties just ensure if the microorganism can be considering a probiotic, but they don't select strains with technological potential.

The assays and steps reported on this review are extremely useful for isolation and selection of non-usual strains. Besides these microorganisms present different characteristics it is still crucial to evaluate their safety and antibiotic resistance, as well as they growth rate and storage stability.

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3 ARTIGO 2

IN VITRO PROBIOTIC PROPERTIES AND DNA PROTECTION ACTIVITY OF YEAST AND LACTIC ACID BACTERIA STRAINS ISOLATED FROM KEFIR FERMENTATION

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Abstract

Recent studies have demonstrated the potential use of honey for the production of kefir-like beverages with functional properties (e.g., high antioxidant capacity, exopolysaccharides content and DNA protection effect) and higher sensory quality. In this study, microorganisms isolated from this beverage were evaluated for their probiotic characteristics, such as survive passage through the gastrointestinal (acidic conditions, bile salts concentrations and survive in the presence of simulated gastric juice), pathogen inhibition, hemolytic activity, hydrophobicity, aggregation, co-aggregation with pathogens, antibiotic resistance, antioxidant production, and DNA protection. The results demonstrated the ability of three microbial strains, namely *Lactobacillus satsumensis* (LPBF1), *Leuconostoc mesenteroides* (LPBF2), and *Sacharomyces cerevisiae* (LPBF3) to resist acidic conditions (pH 2.0, 3.0, 4.0 and 7.0), bile salts concentrations (0.3% and 0.6%) and survive in the presence of simulated gastric juice with no hemolytic activity. In the same way, the inhibitory effect on pathogen growth (*E. coli* and *S. aureus*) was observed for all strains, but with LPBF1 being the most effective. High aggregation was observed in the three strains (LPBF1 72%, *Leuconostoc mesenteroides* 93% and *Sacharomyces cerevisiae* 94%). LPBF1 did not aggregate with *E. coli*, but presented co-aggregattion with *S. aureus* (22%). *S. cerevisiae* and *L. mesenteroides* presented 51 and 52% of co-aggregation with *E.coli* respectively. Antioxidant effect was observed on the three strains, but *Sacharomyces cerevisiae* demonstrated the highest result, inhibiting 28% of DPPH. *Leuconostoc mesenteroides* did not present hydrophobic affinity, but it can still cause positive effects on host. The comet assay results indicate that LPBF1, *Leuconostoc mesenteroides* and *Sacharomyces cerevisiae* have DNA protection abilities against H_2O_2 compared to the positive control. LPBF1 was susceptible to almost all antibiotic tested, but *Leuconostoc mesenteroides* was not tested due to its low hydrophobicity. The observed characteristics confer potential probiotic properties of these isolates and should be further evaluated in *in vivo* assays.

Keywords: functional beverage, antagonistic activity, lactic acid bacteria, probiotic properties

INTRODUCTION

Probiotics are defined as living microorganisms, which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition (Guarner, & Schaafsma, 1998). Promising probiotic strains include members of the genera *Lactobacillus, Bifidobacterium, Leuconostoc* and *Sacharomyces* (Shori, 2015; Liu, 2016; Castro-Rodríguez et al., 2015; Buntin et al., 2008). Kefir is used as an excellent source of probiotics and beneficial health effects. Kefir is a beverage commonly manufactured by fermenting milk with kefir grains, which supports a complex microbial symbiotic mixture of bacteria and yeasts (Magalhàes, de Melo Pereira, Campos, Dragone, & Schwan, 2011). The result is a naturally carbonated beverage (associated with yeast metabolism) with acid taste and creamy consistency due to lactic acid bacteria (LAB) metabolism. The consumption of kefir beverage has been associated with beneficial effects on human health, and several bacteria and yeasts found in kefir are recognized as probiotics (Diosma et al., 2014; Puerari et al., 2012; Zanirati et al., 2015).

Probiotic microorganisms are subject to stresses before they reach the target site (Ramos et al., 2014). The acid and bile tolerance and resistant to degradation of hydrolytic enzymes are fundamental properties that indicates the ability of a probiotic microorganism to survive through the upper gastrointestinal tract (GIT) (Erkkila & Petaja, 2000; Hyronimus et al., 2000). The ability of probiotic bacteria to survive the harsh environments encountered during processing and gastrointestinal transit has been a major factor in their selection criteria (Ramos et al., 2014). In addition, antagonism against different pathogenic bacteria is a crucial property for probiotic action, which occurs either by production of antimicrobial substances or by competitive exclusion during its growth (Lee & Salminen, 1995).

After reaching the GIT, probiotic strains should be able to colonize, to remain in the intestine and to co-aggregate with other bacteria. These characteristics are evaluated by hydrophobicity and aggregation tests, where the affinity for organic solvents determines the adhesion percentage to tissues. Co-aggregation studies demonstrated that probiotic strains compete for adhesion sites with pathogenic bacteria and therefore interfere on their growth (Kos et al., 2003; Ramos et al., 2013). Lactic acid bacteria are usually associated with DNA protective competence against several range of events, such as UV radiation, H_2O_2, and faecal water, and with antioxidant production (Burns and Rowland., 2004; Chang et al., 2009; Jagtap et al., 2011). As probiotics bacteria have been widely used for its applications and benefits, the concern about its antibiotic resistance and the possibility to pass them to pathogenic bacteria increased. These possible resistances became an important quality control requirement for its application in food industry (Toprak et al., 2012; Sundh et al., 2012).

Since different microorganisms can have different probiotic properties, the prospective study of strains isolated from different products/processes becomes essential. The possibility of including strains isolated from non-dairy sources of probiotic preparations can extend the range of available strains to be candidates for use as probiotics. Recently, we have evaluated the use of honey as an alternative substrate to design a novel probiotic beverage using kefir grains as starter culture (Soccol et al., 2014; Fiorda et al., 2016 a,b). These studies provided evidence indicating that honey can serve as a raw substrate for the production of kefir-like beverages with functional properties (high antioxidant capacity, exopolysaccharides content and DNA protection effect) and with a high sensory quality compared to traditional kefir beverage. Additionally, some known probiotic species, e.g., *Lactobacillus statsumensis, Leuconostoc mesenteroides, Bacillus megaterium* and *Saccharomyces cerevisiae*, were identified in this beverage (Fiorda et al., 2016a). In this way, the aim of this study was to validate the selection method proposed by our previous work, and study the probiotic potential of microbial strains

(yeasts and LAB) isolated from honey kefir beverage, through acid and bile salts resistance, hemolytic activity, aggregation, co-aggregation, hydrophobicity and also to evaluate its *in vitro* antimicrobial properties against growth of two strains of pathogenic microorganisms conveyed by foods and DNA protection.

MATERIALS AND METHODS

MICRORGANISM AND GROWTH CONDITIONS

A total of seventy-five strains (39 bacteria and 36 yeasts), isolated from honey kefir beverage, were used in this study (Fiorda et al., 2016). Among these, LPBF1, LPBF2 and LPBF3 strains were pre-selected, based on their ability to tolerate the effects of low pH, for the tests described below. The identification of these three potential probiotic strains was confirmed by 16S rRNA gene and ITS region sequencing, for bacteria and yeast, respectively (Lott et al. 1993; Barszczewski and Robak 2004; Wang et al. 2006). The nucleotide sequences of microorganism strains were deposited in the GenBank database under access numbers KF747750, KF747751, KF747752, KF747753, KF747754, KF747755, KF747756 and KF747757. The strains were maintained as frozen (-80 ^{o}C) stock cultures in MRS broth (for bacteria) and YM broth (for yeast) containing 20% (v/v) glycerol.

ACID TOLERANCE

The resistance under acid conditions was carried out according to Pieniz et al. (2014) with some modifications. Cells were grown in MRS broth at 37 ^{o}C (for bacteria) and YM broth at 30 ^{o}C (for yeast) without shaking for 24 h. Then, the cultures were standardized at an optical density (OD600) = 1.0 ± 0.05. One milliliter of standardized culture was added into tubes containing 9 mL of respective sterile broth with the following pH values: 2.0, 3.0, 4.0 and 7.0 (adjusted with HCl), in which pH 7.0 was used as a control. Viable cell counts were determined

after exposure to acidic condition for 0, 1, 2, 3 and 4 h. The experiment was performed in triplicate. Survival cell counts were expressed as log values of colony-forming units per mL (CFU/mL) by pour plate method after serial dilutions. The survival percentage was calculated as follows: % survival = final (CFU/mL)/intial (CFU/mL) x 100.

RESISTANCE TO BILE SALTS

After strains were grown in MRS broth (for bacteria) and YM broth (for yeast), cells were harvested by centrifugation (10,000 x g for 10 min at 4 ^{o}C) washed three times with 0.1 M phosphate buffered saline (PBS) (pH 7.2) and suspended in 0.5% NaCl solution. The cultures were standardized at an optical density (OD600) = 1.0 ± 0.05. Then, a 0.2 mL aliquot of suspensions were inoculated into 1.0 mL of YM broth (yeast) and MRS broth (LAB) with 0% (control - pH 7.0), 0.3 and 0.6% (w/v) of bile salts (Sigma-Aldrich®), at pH 7.4. Total viable counts were determined after exposure to bile salts solution at 0, 1, 2, 3 and 4 h of incubation, by pour plate method after serial dilutions and incubated at 37 ^{o}C (for bacteria) or 30 ^{o}C (for yeast) for 24 h. Values were expressed as log CFU/mL and the experiment was performed in triplicate (Perelmuter et al., 2008).

HEMOLYTIC ACTIVITY

The strains were tested for hemolytic activity using blood agar (7% v/v sheep blood) for 48 h incubation at 37 ^{o}C (Foulquié Moreno et al., 2003). Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) were considered non hemolytic. Strains displaying blood lyses zones around the colonies were classified as hemolytic. The experiment was performed in triplicate (β-hemolysis).

SURVIVAL IN SIMULATED GASTROINTESTINAL TRACT

Survival in simulated gastrointestinal tract was performed according to Pieniz et al. (2014). After 24 h of incubation in MRS broth at 37 ^{o}C (for bacteria) or YM broth at 30 ^{o}C (for yeast), cells were harvested by centrifugation (10,000 x g for 10 min at 4 ^{o}C), washed three times with 0.1 M phosphate buffered saline (PBS) (pH 7.2) and suspended in 0.5% NaCl solution. The cultures were standardized at an optical density (OD600) = 1.0 ± 0.05. Then, a 0.2 mL aliquot of suspensions were inoculated into 1.0 mL of simulated gastric or intestinal juices and incubated at 37 ^{o}C for 4 h. Survival cell counts were determined at initial time (0 h) and 1, 2, 3 and 4 h for the gastric tolerance and intestinal tolerance. Values were expressed as log CFU/mL.

Simulated gastric juice was prepared fresh daily containing 3 mg of pepsin (Sigma), 1 mL of NaCl solution (0.5%) and acidified with HCl to pH 3.0. Simulated intestinal juice was consisted of 1 mg of pancreatin (Merck), 1 mL of NaCl solution (0.5%) and adjusted to pH 8.0. Both solutions were sterilized by filtration through 0.22 mm membranes (Millipore, Bedford, USA).

ANTIMICROBIAL ACTIVITY

Antimicrobial capacity of selected strains and of honey kefir beverage were evaluated. *Escherichia coli* JM109 and *Staphylococcus aureus* ATCC® 6538 belonging to the collection of Biorefining Research Institute (Lakehead University, Thunder Bay, Canada), were used as pathogenic microorganisms. They were grown in nutrient broth at 37 ^{o}C for 24 h and suspended in 0.85% NaCl solution standardized to OD600 of 0.150 in spectrophotometer, which corresponded to a 0.5 McFarland turbidity standard solution. One aliquot of 50 µL of culture containing grown LPBF1, LPBF2 and LPBF3 was applied onto Mueller Hinton plates previously inoculated with a swab soaked in a culture of each indicator bacteria. A 50-µL of honey kefir beverage was also evaluated in this step to analyze if antimicrobial activity would increase or decrease when the strains are in symbiosis. The plates were incubated at 37 ^{o}C and

inhibition zones were measured after 24 h. Ampicillin (50 mg mL^{-1}) was used as standard. The diameter of inhibition zones was measured using a caliper rule and halos ≥ 7 mm were considered inhibitory (Bromberg et al., 2006). The experiment was performed in triplicate.

HYDROPHOBICITY

The hydrophobicity of strains is directly related to its ability to attach to cells membranes and human gut. The test was conducted according to Chelliah et at. (2016) in triplicate with some modifications. A culture of 48 h of each strain was harvested by centrifugation (4,000 g for 10 minutes at 4 ºC). The pellets were washed twice with PBS and resuspended in the same buffer. The OD600 was adjusted to 0.6-0.8, and 5 mL of each suspension transferred to two tubes, containing 1 mL of xylene and 1 mL of toluene each. The tubes were agitated in a vortex (Biomixer ql-901) and incubated at 37 ºC. The absorbance of the solutions' superior and inferior phase was measured with 30 and 60 minutes in a spectrophotometer (HINOTEK SP-1105) at 600 nm. The hydrophobicity was determinate by Equation 1:

$$\text{(Eq. 1) Hydrophobicity (\%)} = \frac{\text{Solvent layer absorbance} - \text{Aqueous layer absorbance}}{\text{Solvent layer absorbance}}$$

AGGREGATION

The aggregation capacity is an important characteristic to a probiotic strain, meaning that this microorganism is able to colonize the intestine. Aggregation was ascertain as described by Ogunremi et al. (2015) with few modifications. LPBF1 and LPBF2 were growth in MRS broth medium and LPBF3 in YPD broth medium for 48 h at 37 ºC. The cultures were centrifuged at 3500 g for 5 min and ressuspended with PBS 1x. The OD600 was adjusted to 1, and 4 mL of each suspension was transferred to round bottom tubes and agitated in a vortex. The absorbance was measured immediately, at 5 and 24 h. Aggregation was determined according Equation 2:

(Eq. 2) $(1-At / A0) \times 100$

Where At corresponds to the absorbance values obtained on different times points (t= 5 h, 24 h); and A0 corresponds to the initial time absorbance (0 h).

The suspensions triplicate were stained with metilene blue at 24 h, and monitored by contrast microscopy at 100 X magnification.

CO-AGGREGATION

Probiotic and pathogenic cultures were prepared in triplicate at the same conditions described in the aggregation assay and according to Ogunremi et al. (2015). A volume of 2 mL from *E. coli* and *S. aureus* suspensions were transferred to 2 mL of each probiotic strain tubes. The mixtures were agitated at a vortex and the absorbance was measured immediately, after 5 and 24 h. Tubes containing only probiotic strains were used as negative controls. Samples were stained with metilene blue as described below. Coaggregation was calculated according to Equation 3:

$$\text{(Eq. 3)}\quad \text{Co-aggregation (\%)} = \frac{[(Ax + Ay)/2] - A(x+y)}{Ax + (Ay/2)} \times 100$$

Where A, corresponds to absorbance; X and Y to each strain at negative control tubes; X + Y to the mixture of probiotic and pathogenic strains.

DPPH

The production of antioxidants by the strains and intracellular contents were measured according to Li et al. (2012), with some modifications. For extraction of intracellular antioxidants, 1 mL of each strain suspension were adjusted to Macfarland's 0.5 scale and the intracellular content was obtained by ultrasonic homogenizer for five 1 min intervals (1 min on/1 min off, 35% amplitude) with constant cooling. Cell debris were removed by

centrifugation at 5000 g for 10 min, and the supernatant was used for the antioxidant assay. First 1 mL of the supernatant was added to 1 mL of DPPH solution (0.15 mM in methanol). The mixture was incubated for 30 min in the dark and the absorbance was measured at 517 nm. The same procedure was performed to evaluate antioxidant production of each strain suspension, adjusted to Macfarland's 0.5 scale. The control was methanol and DPPH solution and the blank contained the suspension and methanol. The antioxidant production by the strains in triplicate was estimated according to Equation 4 and intracellular antioxidant production is measured by Equation 5:

$$\text{(Eq. 4) Scavenging activity (\%)} = [1 - (A_{sample} - A_{blank})/A_{control}] \times 100$$

$$\text{(Eq. 5) Scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Where Asample corresponds to the absorbance of the sample; Ablank to the absorbance of the strain suspension and methanol; and Acontrol to the absorbance of methanol and DPPH.

ANTIBIOTIC RESISTANCE

The disk diffusion test was performed to evaluate the strains susceptibility to gram positive antibiotics according to ANVISA (2003) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) protocols. The cellular suspension of LPBF1 and a commercial strain (*Lactobacillus casei*) were adjusted to Macfarland's 0.5 scale, and inoculated on petri plates containing MRS agar. The industrial gram positives antibiotics tested were: Cefepime (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Clindamycin (2 µg), Erythromycin (15 µg), Gentamicin (10 µg), Oxacillin (1 µg), Penicillin G (10 µg), Rifampicin (5 µg), Sulfatrim (25 µg), Tetracycline (30 µg), and Vancomycin (30 µg) (Laborclin, Brazil). The plates were incubated for 48 h at 37 °C, and the zone of inhibition was measured in millimeters and in triplicate. The halo was interpreted as sensitive, S (≥ 21 mm); intermediate, I (16-20 mm) or resistant, R (≤ 15 mm).

COMET ASSAY

The Comet Assay has the capacity to evaluate and measure the damage a substance can cause to DNA. The ability of LPBF1, LPBF2, and LPBF3 strains to protect DNA against damages caused by hydrogen peroxide was investigated according to Sigh et al. (1988) with modifications. The slides were covered with agarose one day before for overnight dry, and the strains were tested separately. A suspension of 10^8 of each strain was prepared and combined with lymphocytes separated from whole blood (donated by the same lab volunteer). The suspensions were exposed to hydrogen peroxide (30%) for 1 and 24 h. As negative control a suspension containing only lymphocytes and without hydrogen peroxide were added, and for positive control it was tested only the lymphocytes and hydrogen peroxide. After exposure, agarose low melting point was added, and the mixture suspension plus agarose was placed in slides. The slides were treated with a lyse solution (1 mL Triton-X + 10 mL DMSO + 89 mL stock solution: 2,5 M NaCl; 100 mM EDTA; 10 mM Tris; 8 g NaOH; 1% Na lauroyl sarcosinate; pH 10) for 1 h in the fridge. The slides were washed with PBS 1x and placed in an electrophoresis cube. The running conditions were 22V and 300 mA for 20 minutes. Slides were stained with silver nitrate and dried at room temperature. For damage classification, it was considered cells with circular shape as no damaged and cells with “comet” shape with DNA damage. The cells were classified in five categories corresponding to the quantity of damages: 0, no damages (<5%); 1, low level of damages (5-20%); 2, medium level of damage (20-40%); 3, high level of damage (40-95%); and total damage (>95%). The damage index (DI) is calculated by Equation 6:

$$\text{(Eq.6)} \qquad DI = \frac{SCORE}{TOTAL\ OF\ CELLS}$$

SCORE was calculated with the following formula:

SCORE = DAMAGE 0 + DAMAGE 1 + DAMAGE 2 + DAMAGE 3 + DAMAGE 4

Where: Damage 0 = 0 x nº of cells; Damage 1= 1 x nº of cells; Damage 2 = 2 x nº of cells; Damage 3 = 3 x nº of cells and Damage 4 = 4 x nº of cells.

STATISTIC ANALYSES

The results obtained in the study were expressed as mean ± standard deviation from 3 replicate determinations. Differences were analyzed with the software Statistica using one-way analysis of variance (ANOVA) followed by Tukey´s post-hoc test. P-values < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

ACID TOLERANCE AND RESISTANCE TO BILE SALTS

In the first step of this study, a total of 39 LAB (including strains of *Leuconostoc mesentereoides, Lactobacillus satsumensis* and *Lysinibacillus sphaericus*) and 36 yeast (including strains of *Hanseniaspora uvarum, Issatchenkia orientalis, Lachancea fermentati, Pichia membranifaciens, P. kudriavzevii, Saccharomyces cerevisiae* and *Zygosaccharomyces fermentati)*, isolated from honey kefir beverage (Fiorda et al., 2016), were prescreened based on their ability to tolerate the effects of low pH (data not show). In this assay, LPBF1, LPBF2 and LPBF3 strains were pre-selected, for further evaluation. Firstly, these three potential probiotic strains were further analyzed *in vitro* for their ability to survive in a particular period of time under acidic conditions and the results are shown in Figure 1.

The tested isolates survived in all times tested (1, 2, 3 and 4h) at pH 2, pH 3, pH 4 and pH 7, maintaining high counts at pH 3 for 2 h, which are considered to be the standard values of acid tolerance of probiotic cultures (Usman et al., 1999). The viability of isolates was satisfactory when exposed to pH 3 and 4, although it was observed a decrease in viable cell counts in pH 2 in the first hour (until 4 log CFU mL^{-1}). However, the viable count of all isolates

remained up to the limit of 10^3 CFU mL^{-1} (dotted line) after 4 h even at pH 2, and acording to Likotrafiti et al. (2013), this is the limit of detection for acid-tolerance of probiotic strains.

The pH of the stomach is between 2.5 and 3.5, although it may be lower during prolonged fasting (pH 1.5), or higher after a meal (pH 4.5) (Huang & Adams, 2004). Thus, the fact that the strains survived for a short time at pH 2 should not interfere with the probiotic ability, because it is intended to apply the strain concomitantly with the beverage, and thus the pH of the stomach is likely to be greater than 2. Hence, the ability to survive at pH 3.0 over approximately 3 h is an essential criterion for micro-organism has probiotic action (Usman et al., 1999). The highest percentage of survival was observed for LPBF2 (10^5 CFU mL^{-1} at pH 2 after 4 h). The survival residual cells were between 50 and 90% of the initial cells even after 2 h of incubation at the pH 3.

In order to survive in the digestive system, probiotic microorganism should resist and grow in the presence of bile salts, which are present in the gastrointestinal tract. In humans, taurocholic acid and glycocholic acid (derivatives of cholic acid) and taurochenodeoxycholic acid and glycochenodeoxycholic acid (derivatives of chenodeoxycholic acid) are the major bile salts in bile and are roughly equal in concentration (Hoffman, 1999). Thus, the three pre-selected strains were evaluated for their ability to grow in the presence of 0.3 and 0.6% bile salts. The results are presented in Figure 1 showed that all tested strains were able to survive at all bile salt concentrations tested (0.3 and 0.6%) to give an exponential growth from the inoculation (0 h) until 4 h of incubation. The survival at 0.3% bile concentration is essential for probiotic microrganims withstand the conditions of the gastrointestinal tract (Sahadeva et al., 2011). In addition, the viable count of all isolates remained up to the limit of 10^3 CFU mL^{-1} (dotted line) after 2 h, and acording to Likotrafiti et al (2013), this is the limit of detection for bile salts resistence of probiotic strains.

Bile tolerance by probiotics has been revealed to be dependent on bile type and the strain, with resistance levels ranging from bile concentrations of 0.125 - 2.0 % (Lian et al., 2003). It has been hypothesized that deconjugation of bile salts is a detoxification mechanism and bile salt hydrolases enzymes play a role in bile tolerance of probiotic organisms in the GIT. Hence, the resistance of probiotics to bile salts is due to the ability of certain species of microorganisms have to reduce the effect of the detergent for producing enzymes capable of hydrolyzing bile salts. However, the LPBF3 strain tested in the present study was more sensitive to bile salts than bacteria. Probably owing to the capsule present in prokaryotic cells that causes protection effect in probiotic bacteria and not in probiotic yeasts. Nevertheless, LPBF3 reached up to 10^4 CFU mL^{-1} after 4 h of incubation even at 0.6% of bile salts.

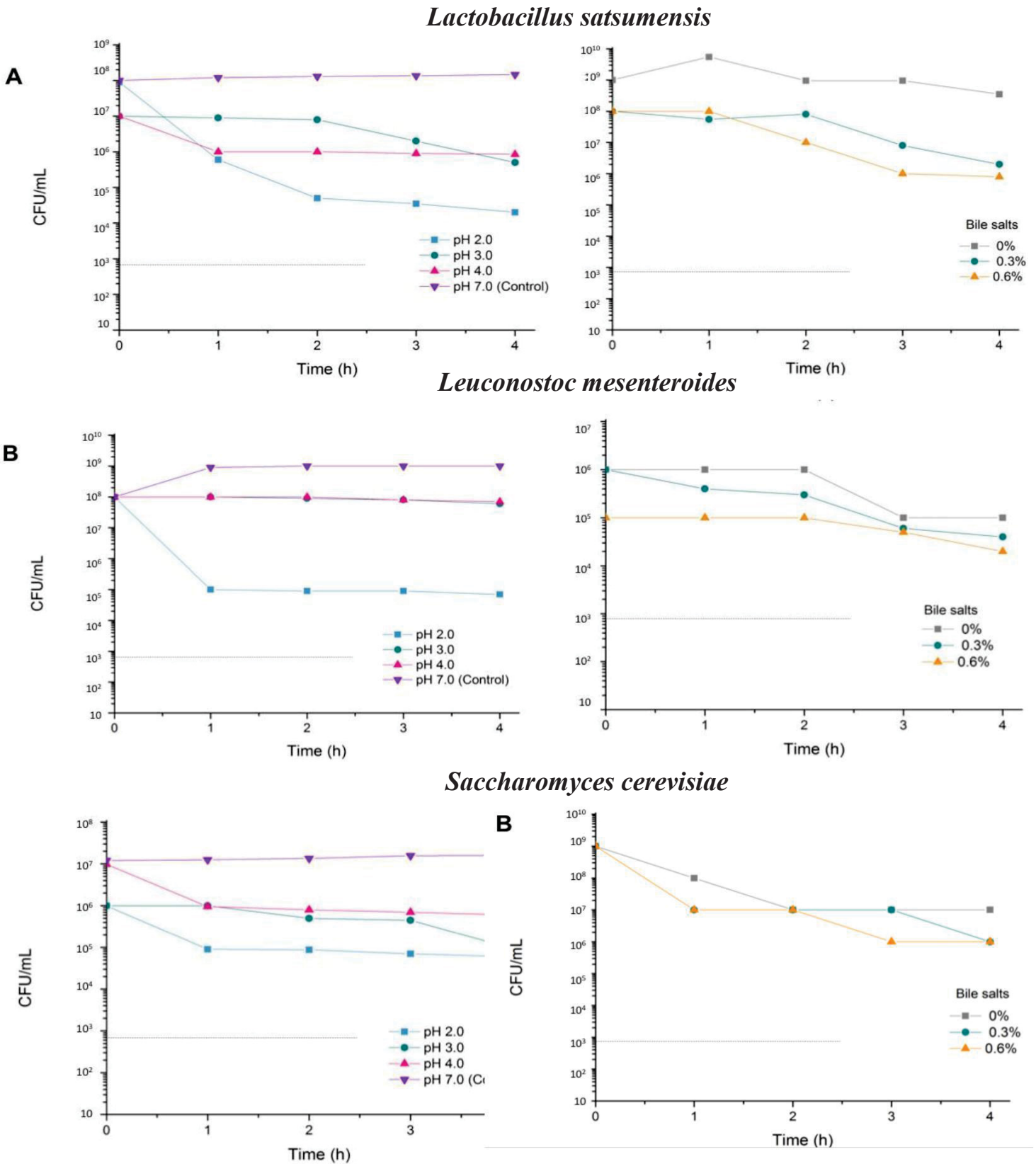


Figure 1. Acid tolerance and resistance to bile salts of LPBF1, LPBF2 and LPBF1. Dotted line is detection limit. Error bars not shown due to low standard deviation.

HEMOLYTIC ACTIVITY

The determination of hemolytic activity is considered a safety aspect for the selection of probiotic strains (FAO/WHO, 2002), and this activity was also investigated in this study. The isolates did not exhibit any effect (γ-hemolysis); green area (α-hemolysis), and/or inhibition zone (β-hemolysis) after 48 h incubation in blood agar plates. Thus, our results showed that none of the isolates exhibited hemolytic activity and this is a good result as the hemolytic activity is the nonspecific killing of blood cells by metabolic by-products of bacteria and yeasts (Ryan et al. 2014).

TOLERANCE TO GASTROINTESTINAL JUICES

Exposure to gastric and intestinal fluids along the digestive tract is the main stress that could decrease the viability of ingested probiotics (Liong & Shah, 2005). Hence survival to pass through the gastrointestinal tract is a desirable characteristic in the choice of probiotic microorganisms since viability plays a significant role in certain of their beneficial properties (Romanin et al., 2010; Saad et al., 2013). The potential ability of the identified isolates to survive under the conditions of transit through the gastrointestinal tract as assayed indirectly in vitro is demonstrated by the results presented in Figure 2.

When exposed to both simulated gastric and intestinal conditions for 4 h, the strains analyzed exhibited cell count nearby 10^7 CFU.mL^{-1}, that would allow it to pass through the stomach. LPBF3 was the most sensitive - but not low resistance - among the strains, while the two others had better resistance properties in both gastric and intestinal conditions.

This indicate that LPBF1, LPBF2 and LPBF3 demonstrated high ability to survive in the presence of simulated gastric juice containing pepsin or pancreatin. Therefore, they can be classified as tolerant to the gastrointestinal secretions and can be used as potentially probiotic microorganisms.

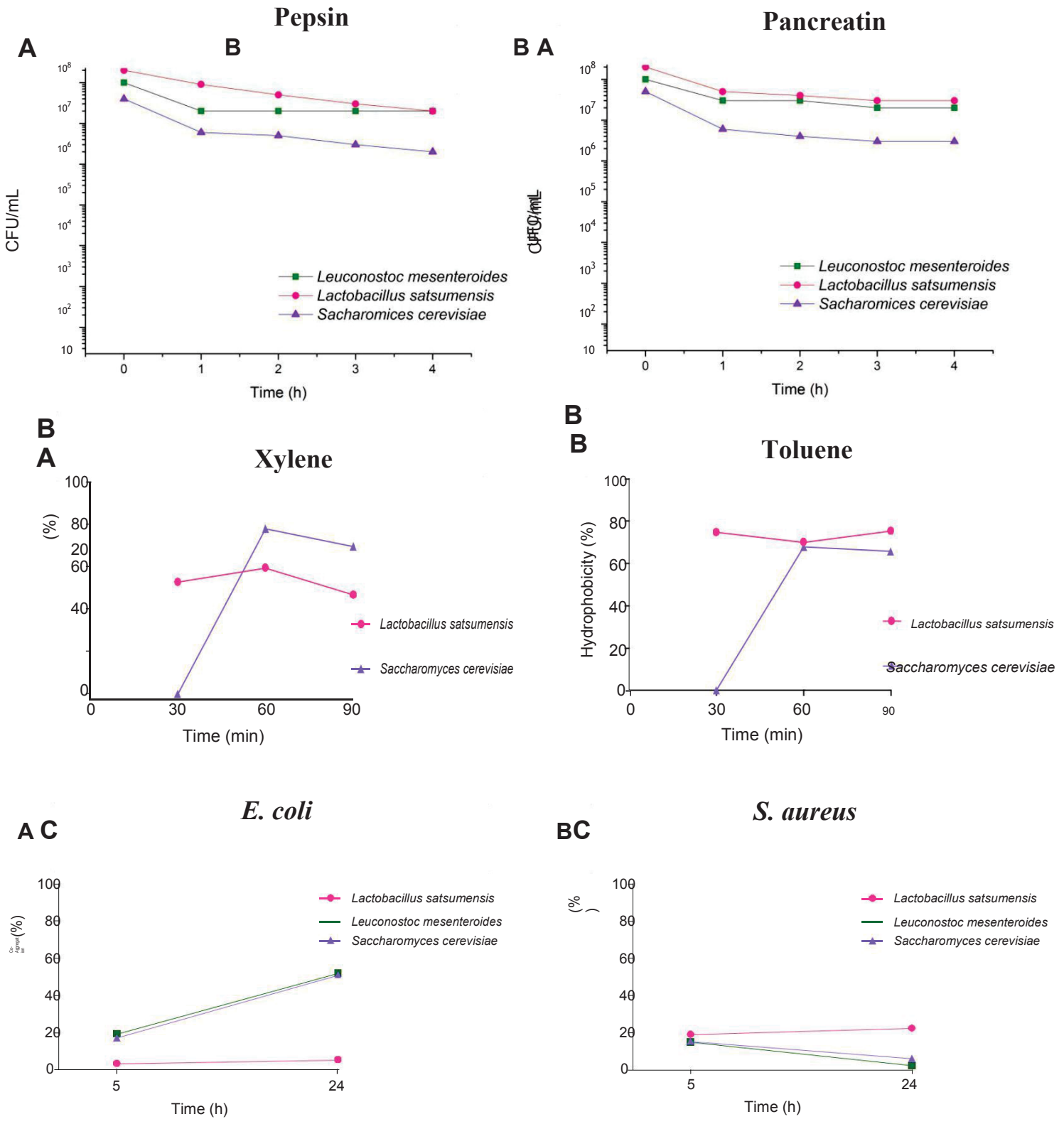


Figure 2. Performed assays of the selected yeast and bacteria. Resistance to simulated Gastric Juice containing pepsin and Intestinal Juice containing pancreatin (A), Hydrophobicity with

different solvents (B), and co-aggregation with pathogenic bacteria (C). Error bars not shown due to low standard deviation.

ANTIMICROBIAL ACTIVITY

The demonstration of antimicrobial activity towards pathogenic species *in vitro* may be considered an imperative attribute of some probiotic bacteria. The pathogens studied in the present work commonly cause different diseases, so they are used as standards in antimicrobial activity tests of potentially probiotic microorganisms (Ramirez-Chavarin et al., 2013; Yamazakia et al., 2012; Ramos et al., 2012; Tsai et al., 2008; Valdéz et al., 2005). In this study, the strains isolated from honey kefir beverage exhibited antimicrobial activity against different indicator microorganisms (Table 1).

Table 1. Antimicrobial activity of strains isolated from honey kefir beverage against indicator microrganisms.

Microrganism	Inhibition zone (mm)*	
	Escherichia coli	*Staphylococcus aureus*
Lactobacillus satstumensis	12.5 ± 0.50^{Ca}	10.5 ± 0.50^{Ba}
Leuconostoc mesenteroides	10.5 ± 0.50^{Ca}	12.0 ± 1.00^{Ba}
Sacharomyces cerevisiae	8.0 ± 0.10^{Ca}	8.5 ± 0.50^{Ba}
Honey kefir beverage	27.5 ± 1.50^{Aa}	19.5 ± 1.50^{Ab}
Control (Ampicilin 50 mg/mL)	42.5 ± 1.50^{Ba}	23.5 ± 0.50^{Aa}

*values represent the mean ± standard deviation of three independent experiments
**Upper-case letters show significant differences between column, and lower-case letters show significant differences between lines, as determined by Tukey´s test ($p < 0.05$).

Highest antimicrobial activities were observed in LPBF1 against *Escherichia coli* and LPBF2 against *Staphylococcus aureus*. At this step, antimicrobial activities of Honey kefir beverage were included against these same pathogens. Interestingly, the results showed high antimicrobial activity against both pathogens This demonstrates that the use of cocultures including LPBF1, LPBF2 and LPBF3 can optimize the antimicrobial activity of the final product.

As *Escherichia coli* and *Staphylococcus aureus* have high pathogenic activity and are of clinical concern globally, these *in vitro* antimicrobial efficacy results from this study highlight the high potential of honey beverage developed with kefir grains containing strains such as LPBF1, LPBF2 and LPBF3.

HYDROPHOBICITY

The ability to attach to human gastrointestinal tract is an important factor for probiotic microorganisms. This characteristic is directly related to the hydrophobicity of strains and its capacity to colonize the intestine (Orlowski & Bielecka., 2006). The colonization of the intestine by probiotic strains is important to maintain the microbiota and avoid the growth of pathogenic microorganisms (Santos et al., 2016). The affinity to hydrocarbons, like xylene and toluene, has been a useful method to measure the hydrophobicity of cells surfaces for probiotic strains (Chelliah et al., 2016). The results from LPBF1 and LPBF3 are shown on figure 2.

The hydrophobicity results from LPBF1 in 30 and 90 min with toluene was the highest among the strains (75%). Cells that have toluene affinity are strong electron donors, with good capacity of intestine colonization (Wodstroum et al., 1987). Lactobacili strains are generally associated with high hydrophobicity, but some studies show different results. Santos et al., (2016) isolated different lactobacillus from cocoa fermentation, and obtained 14, 22 and 16,87% of hydrophobicity for *L. fermentum* and *L. plantarum* respectively. These variations on the same species strains are often related to expression of cell surface proteins and the fermentation substrate. Substrates with high water content tend to influence the expression of surface proteins, changing its solvent solubility (Kaushik et al., 2009). Vinderola and Reinheimer (2003) found values ranged from 38,1 to 67,8% for *L. acidophilus* and 10,9 to 24,1% for *L. casei*, elucidating these variations between same species.

Different solvents can change the results, as it is shown in this study. LPBF1 has more affinity in xylene than in toluene. Martins et al. (2009) evaluated the hydrophobicity of probiotic strains in chloroform and obtained 45,6% for *L. casei* and 81,5% for *S. boulardii.* Yeasts have a high affinity to organic solvents, like toluene and xylene. LPBF3 was 67% hydrophobic in toluene and 78% in xylene with 60 min in this study. Chelliah et al. (2016) obtained 75 and 59% with the same solvents respectively, for *P. kudriavzevii*.

LPBF2 was not included on the graphics because it did not show hydrophobicity. The results were below zero, demonstrating that this strain is hydrophilic. Although some studies describe *Leuconostoc mesenteroides* with hydrophobic profile (Paula et al., 2014) it was not the case in this work. Some strains do not show a good adherence in the intestines but may cause positive effects in hosts (Saarela et al., 2000).

AGGREGATION

The auto-aggregation ability is one of the most important characteristic in probiotic strains. It means that the microorganism is able to colonize the GIT over time and modulate the immune system (Saulnier et al., 2009). The results for LPBF1, LPBF2, and LPBF3 are shown in Figure 3.

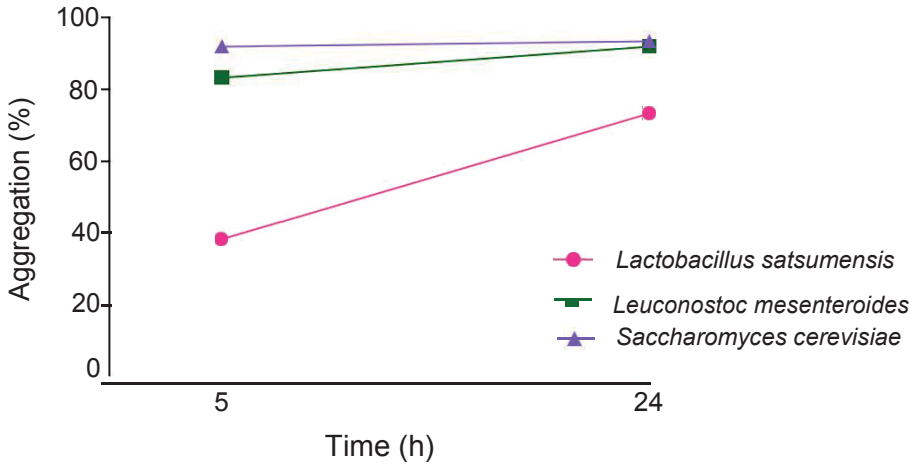


Figure 3. Aggregation results for *L. satsumensis*, *L. mesenteroides*, and *S. cerevisiae* in 5 and 24 hours. Error bars not shown due to low standard deviation.

The values found for LPBF3 were stable for 5 and 24 h (92-94%). Similar results were found by Syal and Vohra (2013) with yeasts isolated from Indian fermented foods. After 20 h of experiment, all the strains showed aggregation percentage ≥ 95%, but not with the same stability. Fakrunddin et al. (2017) isolated a *S. cerevisiae* from fruit and obtained 61,34% of aggregation. *Leuconostoc mesenteroides* was also analyzed for Paula et al. (2014) that obtained 85,64% of aggregation, lower than the 93% found in this study. LPBF1 increased its aggregation over time, passing from 40 to 72% in 24 h. The 5 h result was similar for those exhibited by Tuo et al. (2013) where 20 Lactobacili strains showed results ranging from 24,16 to 41,39%. On the other hand, the 72% aggregation obtained by LPBF1 in 24 h was higher than the 11 strains of *L. fermentum* tested by Bao et al. (2010), where the best aggregation value was 51,5%.

LPBF1, LPBF2, and LPBF3 presented good aggregation parameters even after the wash step by PBS (Figure 4), that removes extracellular components that may be related to aggregation (Kos et al., 2003). The values from LPBF1, LPBF2, and LPBF3 indicate that these strains have a strong aggregation phenotype, related with biofilm production, and with the ability to adhere and persist in the GIT (Vlková et al., 2008).

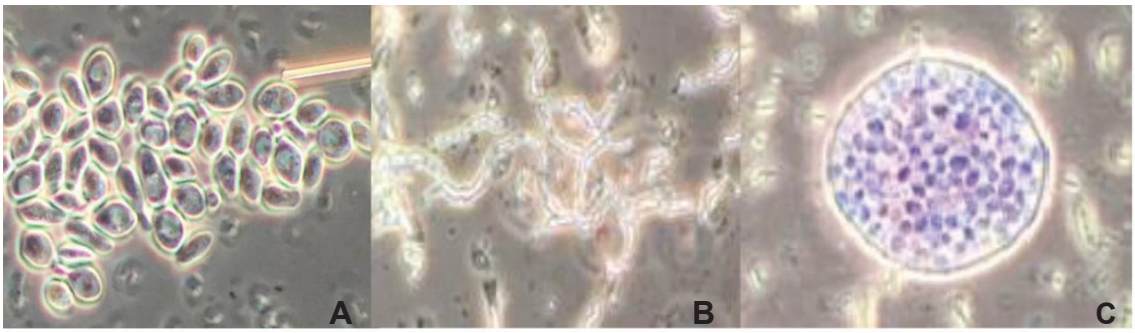


Figure 4. Aggregation after 24 h. (A) *Saccharomyces cerevisiae*, (B) *Lactobacillus satsumensis*, and (C) *Leuconostoc mesenteroides*. Phase contrast microscope at 100 x magnification.

CO-AGGREGATION

The ability of probiotic strains to co-aggregate with pathogenic microorganisms is an important defense to the host, forming a barrier that prevents the colonization by pathogenic microorganisms (Del Re et al, 2000; Rickard et al, 2003). In this work LPBF1, LPBF2, and LPBF3 were tested with *E. coli* and *S. aureus* (Figure 2).

The values for LPBF2 and LPBF3 were similar for both pathogenic strains on the intervals tested, obtaining 52 and 51% with *E. coli* and 2 and 6% with *S. aureus* in 24 h respectively. LPBF1 did not show co-aggregation with *E. coli* even with 24 h. However, with *S. aureus* it obtained 22% of aggregation.

Zhang et al. (2013) isolated a *Leuconostoc lactis* and obtained 24,41% for *S. aureus* and 10,74% for *E. coli* with 20 h. This difference between species was also present in the study by Keller et al. (2011) where eight commercial lactobacilli displayed co-aggregation in a range of 9,3 to 22,7%. LPBF3 presented different co-aggregation values from previous works with yeasts (Chelliah et al., 2016), where it presented higher aggregation with *S. aureus* (31,12%) and lower aggregation with *E. coli* (23,11%).

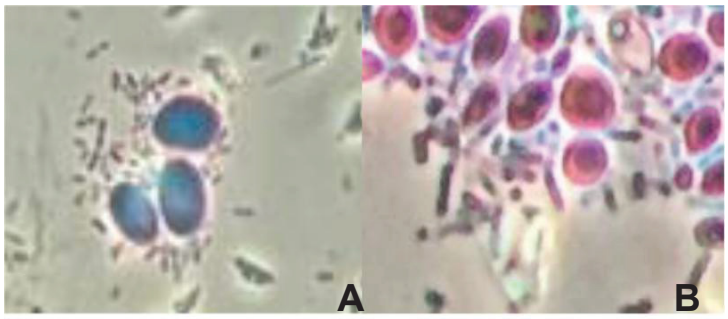


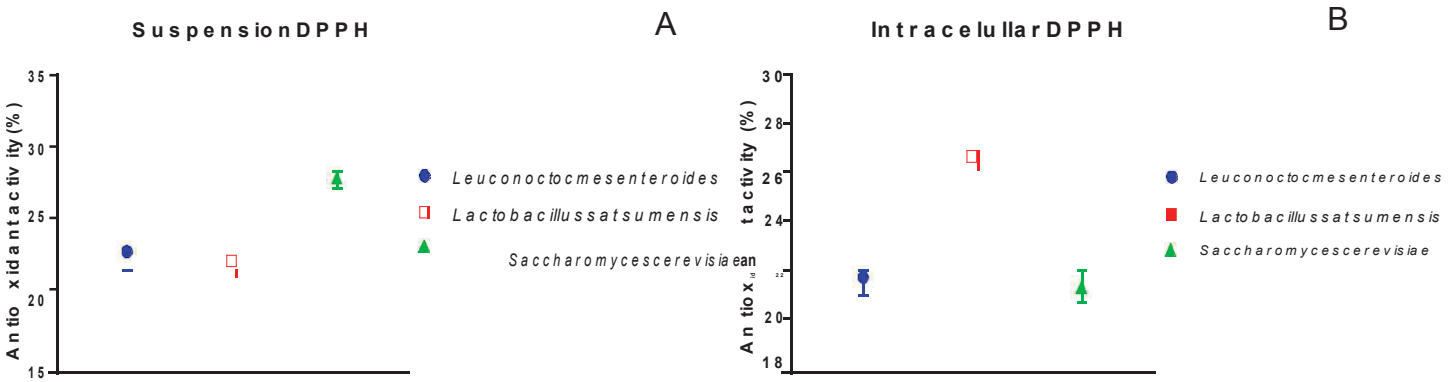
Figure 5. Co-aggregation of *Saccharomyces cerevisiae* (A) and *Leuconostoc mesenteroides* (B) with *E. coli*. Phase contrast microscope at 100 x magnification.

DPPH

Antioxidant production is an important characteristic of probiotic yeast and bacteria. Reactive oxygen species (ROS) are related with various diseases, like cancer, cirrhosis, atherosclerosis, and other chronic pathogenesis, causing damage in proteins, DNA mutations,

and oxidation of phospholipids membrane. ROS are produced during the passage of nutrients and its metabolic reactions in the GIT (Ljung and Wadström, 2006). On figure 6 the DPPH results for the isolated stains are shown.

Figure 6. Strains suspension (A) and intracellular (B) and antioxidant activity.



Studies have demonstrated that antioxidant activity is strongly strain related. Even though for the suspension DPPH LPBF2 and LPBF1 showed similar results with no statically difference, and LPBF3 obtained the highest percentage, with 27,96% of inhibition. The opposite happened with the intracellular antioxidant production where LPBF2 and LPBF3 had similar results of 22,51 and 20,73%. LPBF1 obtained the highest significant result of 27% intracellular antioxidant production.

Lactobacillus species are extensively reported to produce antioxidants by the synthesis of extracellular polysaccharides (EPSs), but other species are also able to produce antioxidants substances. Amaretti et al. (2012) tested thirty-four probiotic strains for they antioxidant activities, including *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, and *Streptococcus thermophilus*, and obtained the highest result with lactobacilli (82%) and bifidobacteria (32%) emphasizing the strain related activity. Prabhakar and Sen (2008) isolated studied the

antioxidant activity of an EPS produced by isolated *Bacillus coagulans* and obtained 82.2% of inhibition with this EPS.

Li et al. (2012) tested eleven *Lactobacillus plantarum* strains for their antioxidant activities and observed an inhibition range of 44-53%. Even though these results are higher than the ones obtained in this study, the value is lower than other studies found on literature, which indicates that not all strains have high activities, like the ones isolated in this study.

ANTIBIOTICS RESISTANCE

Different methods can be applied to test strains susceptibility to antibiotics. The disc-diffusion in agar method is usually used to evaluate fast growth bacteria. The results for LPBF1 and a commercial bacteria (*L. casei*) are on table 2. The choose antibiotics were selected for gram positive bacteria.

Table 2. Inhibition zones of *Lactobacillus satsumensis* and a commercial strain.

Mechanism of action	Antibiotics	Inhibition zone (mm)*	
		L. satsumensis	Commercial strain
Cell wall inhibitor	Oxacillin	0 ± 0.00	0 ± 0.00
	Penicilin-G	48 ± 1.00	40 ± 0.50
	Cefepime	17.5 ± 2.5	0 ± 0.00
	Vancomycin	0 ± 0.00	0 ± 0.00
Protein synthesis inhibitors	Chloramphenicol	35 ± 0.50	35 ± 0.10
	Clindamycin	45 ± 1.00	35 ± 0.50
	Erythromycin	40 ± 0.50	40 ± 0.50
	Gentamycin	0 ± 0.00	0 ± 0.00
	Tetracycline	42 ± 0.47	35 ± 0.50
RNA-polymerase inhibitors	Rifampicin	35 ± 0.50	35 ± 0.50
Inhibition of folic acid synthesis	Sulfatrim	0 ± 0.00	0 ± 0.00

*values represent the mean ± standard deviation of three independent experiments

The lactobacilli vancomycin-resistance phenotype is present in almost all species of *Lactobacillus*. This intrinsic resistance replaces the D-alanine residue on the cell wall by D-lactate or D- serine, preventing the antibiotic binding (Delcour et al. 1999). On this work, LPBF1 and the Commercial *Lactobacillus* (*L. casei*), showed resistance to vancomycin, oxaciliin, gentamycin, and sulfatrim. The LPBF1 strain presented more sensitivity against the antibiotics then the commercial one. Antibiotic generally act on the inhibition synthesis of cell wall, proteins, folic acid and action of DNA gyrase. The commercial and LPBF1 strains were susceptible to almost all protein synthesis inhibitors, but gentamycin. Besides the constant

concern for probiotic passing resistance genes to pathogenic bacteria at the GIT, few problems and side effects have been reported (Alvarez-Olmos et al., 2001).

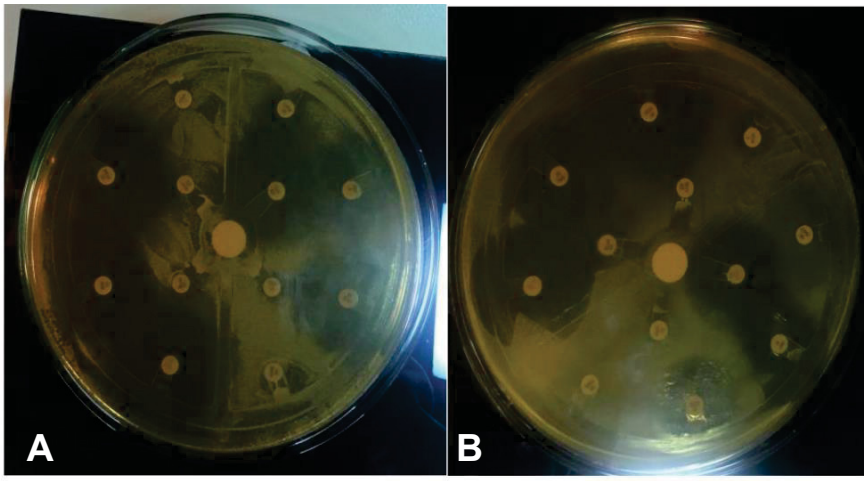


Figure 7. MRS plates showing inhibition zones of *Lactobacillus satsumensis* (A), and *Lactobacillus casei* (B).

Antibiotic treatments often affect the GIT microflora balance, leading to intestinal disorders. Ingestion of antibiotic resistant bacteria could be used as parallel treatment to restore the normal bacterial ratio or its faster restoration (Sabir et al., 2010). Although antibiotic and transferable resistances be one of the main criterion for determination of QPS status (Qualified Presumption of Safety) by EFSA (European Food Safety Authority), approved standards for the genotypic and phenotypic determination of food isolated antibiotic resistances are scarce (Hummer et al., 2007). The guidance report for products and additives used in animal feed by EFSA classified antimicrobial resistance in three distinguish categories: as intrinsic or natural resistance inherent to a bacterial species, as acquired resistance caused by the mutation of indigenous genes, or as acquired resistance due to the acquisition of exogenous resistance genes. Microorganisms carrying an exogenous resistance gene cannot be used as animal feed additive. It also states that for use of resistant strains, the genetic bases for this resistance needs

to be revealed, as well as the transfer to the GIT microbiota (Sundh et al., 2012). *L. mesenteroides* was not evaluated due to its low hydrophobicity.

COMET ASSAY

The strains capacity to protect DNA against harmful agents was tested with comet assay, a fast and sensitive method to evaluate DNA damage before and after cell repair. Its principle is that damages loops containing a break lose the supercoiling and go through agarose gel toward the anode (Collins., 2004).

Lactic acid bacteria have been constantly investigated for its possible role as dietary antimutagens, protection against oxidative damage (ROS), and UV radiation (Guéniche et al., 2006; Renner and Münzner, 1991; Koller et al., 2008). Results of LPBF1, LPBF2, and LPBF3 are presented on table 3.

Table 3. Damage index of LPBF1, LPBF2, and LPBF3 up to 1 and 24 h.

Microorganism	**Damage index***	
	1 h	24 h
Lactobacillus satsumensis	3.05 ± 0.25^{aA}	3.45 ± 0.13^{aA}
Leuconostoc mesenteroides	3.26 ± 0.21^{aA}	3.65 ± 0.24^{aA}
Saccharomyces cerevisiae	2.44 ± 0.06^{bA}	2.39 ± 0.16^{bA}
Positive control	3.79 ± 0.19^{cA}	3.86 ± 0.06^{aA}

*values represent the mean ± standard deviation of three independent experiment.
** Means of triplicate in each column bearing the same lower case letters or the same capital letters in each row are not significantly different (P > 0.05) from one another using Tukey's Test (mean ± standard variation).

The strains LPBF1 and LPBF2 did not show significant difference between their values, but both were lower than the positive control damage, that reached an index of 3.79 in 1 h of

exposure, indicating a protective ability from these bacteria. LPBF3 presented the best protective value, reaching in 1 h a damage index of only 2.44, the lowest result between the strains. Even after 24 h LPBF1, LPBF2, and LPBF3 did not lost their viability, showing stability in protection rate. LPBF3 repeated the low damage level in 24 h with significant difference from positive control, which indicates high permanent protection from this yeast (Figure 8).

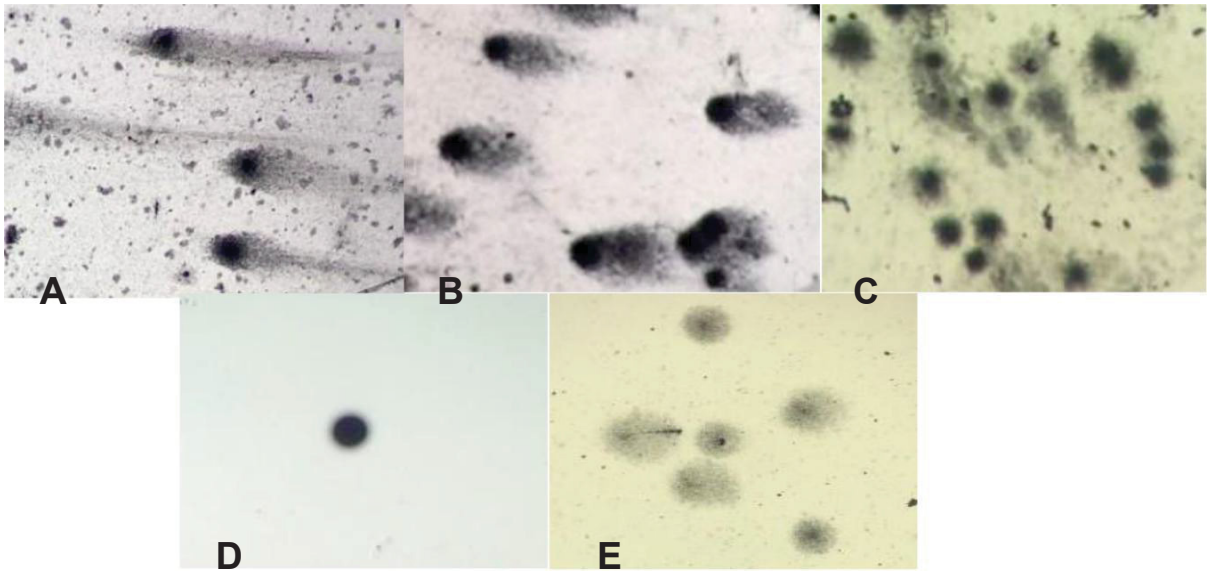


Figure 8. Comet tails of 24 h treatment of lymphocytes with H_2O_2 and isolated strains. (A) *L. mesenteroides,* (B) *L. satsumensis,* (C) *S. cerevisiae,* (D) Negative control, and (E) Positive control.

On figure 8 it can be observed the supercoiled DNA containing no damage on negative control, with round shape. The difference between positive and negative control is on the absence of supercoiled DNA where total damage can be observed. The presence of integrate DNA on the strains treatment indicates that the strains were able to preserve part of the genetic content.

There are several studies describing probiotics capacity to prevent diseases, such as colorectal cancer. Not only because the competition for adhesion site with pathogenic bacteria that cause inflammatory host response and possibly a tumor, but some strains are able to bind and hydrolyze carcinogenic compounds, such as N-nitroso, heterocyclic aromatic amines,

mycotoxins and cyanobacterial toxins (Geier et al., 2006; De Moreno de LeBlanc, A., and G. Perdigón, 2005; Goldin et al., 1980; Oelschlaeger, 2010). Many of these carcinogenic compounds are food bourne, formed during the cooking of meat and fungal contaminants. Zsivkovits et al. (2003) investigated different yogurt *Lactobacillus* strains effects on DNA damaging heterocyclic aromatic amines, and obtained complete dose depended inhibition of DNA breaking, providing a possible explanation to reduced colon cancer rates found in previous studies (Zsivkovits et al., 2003; Burns and Rowland., 2004).

Since the major cause of colorectal cancer are derivate from food and inflammation caused by pathogenic bacteria present in the GIT, the consumption of probiotic strains is a valid prevention and treatment for these disease range.

CONCLUSION

The results obtained in this study suggest that LPBF1, LPBF2 and LPBF3 strains isolated from honey kefir beverage, are resistant strains to pass through the gastrointestinal tract and did not show hemolytic activity. These strains also showed strong antimicrobial activity against important pathogens, produce antioxidants, are able to colonize the intestines, and have DNA protection abilities. These characteristics show that these strains have great potential as new probiotics with potential for producing non-dairy probiotic products, since they were isolated from honey matrix. However, *in vivo* assays must be performed to elucidate the potential of these new isolates, such as immunomodulatory capacities in animal models.

Most commercialized probiotics are bacteria. Only two yeasts are used: *S. boulardii* in human medicine and *S. cerevisiae* in veterinary medicine, in cattle. The advantage of working with yeast is that it can be lyophilized, it is rapidly eliminated after discontinuation of therapy, and is not affected by the use of antibacterial. This study demonstrated the potential of probiotic strain *S. cerevisiae* (LPBF3) through its ability to tolerate bile salts, acidy conditions and be resistant to pass through the gastrointestinal tract and validates the selection method proposed

in the previous article to select strains with specific characteristics, such as DNA stress protection, and antioxidants production.

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4 CONSIDERAÇÕES FINAIS

O isolamento e seleção de microrganismos probióticos derivados do kefir através do modelo proposto, se mostrou efetivo para as cepas isoladas, que além de possuirem todos os requesitos necessários para serem consideradas probióticas, apresentaram produção de antioxidantes e a habilidade de proteger o DNA contra danos oxidativos. Isso indica que o método é válido e pode ser direcionado de acordo com o objetivo de cada estudo.

O modelo também indica que o guia da OMS pode limitar o uso de cepas isoladas, pois este só especifica se o microrganismo é probiótico ou não, e não revela todo o potencial de uma cepa.

4.1 RECOMENDAÇÕES PARA TRABALHOS FUTUROS

As cepas *Lactobacillus satsumensis* e *Saccharomyces cerevisiae* podem ser utilizadas no desenvolvimento de novas bebidas probióticas com diferentes substratos para auxiliar na manutenção da microbiota intestinal e sua reposição em paralelo ao uso de antibióticos.

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