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AUGUSTO TASCH HOLKEM

**Study of encapsulation and anticancer properties of plant extracts with
probiotics**

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*I dedicate this work to my family, mainly to my father José Iloirton Machado Holkem
(In memoriam). Without his help from sunrise to sunset none of this would be possible.*

BIOGRAPHY

Augusto Tasch Holkem, son of José Iloirton Machado Holkem and Gislaine Tasch Holkem, was born in the city of Santa Maria, Rio Grande do Sul, on August 18, 1993. He graduated in Food Technology in 2014 and graduated in Professional Education in 2016 in the Universidade Federal de Santa Maria. During his undergraduate studies, he received a REUNI scientific initiation scholarship for three years, gaining experience in the area of food microbiology.

He obtained a master's degree in Food Science and Technology in the Universidade Federal de Santa Maria. He received a scholarship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and acquired experience in the area of microencapsulation, freeze drying, probiotics and prebiotics.

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“No one walks without learning to walk – without learning to walk by walking, without learning to remake, to retouch, the dream for whose cause the walks have set off down the road.”

(Paulo Freire – Pedagogy of Hope: Reliving Pedagogy of the Oppressed).

RESUMO

HOLKEM, A. T. **Estudo da encapsulação e das propriedades anticâncer de extratos vegetais com probióticos**. 2020. 185 f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2020.

Os probióticos e o extrato de canela rico em proantocianidinas (ECRP) são sensíveis as condições de processamento e armazenamento nos alimentos em que são adicionados e para promoverem efeitos benéficos nos indivíduos, principalmente na prevenção do câncer colorretal (CCR), devem resistir à passagem pelo trato gastrointestinal. As tecnologias de microencapsulação têm sido utilizadas há décadas para proteger materiais bioativos. Primeiramente, o objetivo deste trabalho foi produzir e avaliar as micropartículas lipídicas sólidas recobertas por interações eletrostáticas de polímeros em que *Lactobacillus paracasei* (BGP1) (*Lacticaseibacillus paracasei*) e *Bifidobacterium animalis* subsp. *lactis* (BLC1) foram encapsulados sozinhos ou co-encapsulados com ECRP. Nesse caso, micropartículas com BLC1 e 5 g/100 g de ECRP apresentaram maior eficiência de encapsulação e maior retenção dos materiais bioativos armazenados a 7 °C por 120 dias. Em seguida, o segundo objetivo deste trabalho foi avaliar as propriedades anticâncer do ECRP livre e em combinação com BLC1 e sua resistência em condições gastrointestinais simuladas *in vitro*. A co-encapsulação foi vantajosa, pois protegeu esses materiais bioativos em condições gastrointestinais, permitindo a liberação deles no intestino, onde poderiam atuar nas fases iniciais do CCR. Assim, o terceiro objetivo deste trabalho foi produzir e avaliar um caldo de cana-de-açúcar funcional enriquecido com BLC1 e PRCE, na forma livre e encapsulada. Foi possível produzir o caldo com BLC1 não encapsulado, mas não com a adição de PRCE livre que reduziu a viabilidade desse microrganismo para <1 log UFC / mL após 7 dias. As micropartículas foram eficazes na proteção do BLC1 durante o armazenamento do caldo e na manutenção de altos teores de compostos fenólicos e de proantocianidinas, embora os produtos contendo micropartículas tiveram sua viscosidade alterada e foram menos aceitos sensorialmente do que os materiais bioativos livres. O último objetivo deste trabalho foi estudar a atividade antiproliferativa, antirradical e antioxidante, ensaio de quinona redutase e ensaio de apoptose dos extratos de mirtilo e jabuticaba (sozinhos ou em combinação com probióticos). O extrato que apresentou maior efeito quimiopreventivo em relação aos demais foi o extrato rico em compostos fenólicos solúveis em

água da jabuticaba com BGP1 (JL), mas deve haver mais estudos para entender a interação entre eles. Conclui-se que a co-encapsulação de probióticos e PRCE foi bem-sucedida, proporcionando a esses materiais bioativos maior retenção durante o armazenamento, proteção em condições gastrointestinais simuladas e incorporação no caldo de cana-de-açúcar. A combinação de PRCE e BLC1 exibiu uma propriedade anticâncer nos estágios iniciais do CRC, enquanto JL demonstrou uma ação em diferentes estágios.

Palavras-chave: *Lactobacillus*. *Bifidobacterium*. Fenólicos. Coacervação complexa. Câncer colorretal.

ABSTRACT

HOLKEM, A. T. **Study of encapsulation and anticancer properties of plant extracts with probiotics**. 2020. 185 f. PhD. Thesis – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2020.

Probiotics and proanthocyanidin-rich cinnamon extract (PRCE) are sensitive to the processing and storage conditions in the foods in which they are added and to promote beneficial effects in individuals, especially in the prevention of colorectal cancer (CRC), they must resist passage through the gastrointestinal tract. Microencapsulation technologies have been used for decades to protecting bioactives materials. First, the objective of this work was to produce and evaluate the solid lipid microparticles covered by electrostatic interactions of polymers in which *Lactobacillus paracasei* (BGP1) (*Lacticaseibacillus paracasei*) and *Bifidobacterium animalis* subsp. *lactis* (BLC1) were either encapsulated alone or co-encapsulated with PRCE. In this case, microparticles with BLC1 and 5 g/100 g PRCE showed greater encapsulation efficiencies and greater retention of the bioactive materials stored at 7 °C for 120 days. Then, the second objective of this work was to evaluate the anticancer properties of free PRCE and in combination with BLC1 and their resistance under *in vitro* simulated gastrointestinal conditions. The co-encapsulation was advantageous because it protected these bioactives under gastrointestinal conditions, allowing their release into the intestine, where they could act in the early stages of CRC. So, the third aim of this work was to produce and evaluate a functional sugarcane juice enriched with BLC1 and PRCE, both in free and encapsulated forms. It was possible to produce sugarcane juice with non-encapsulated BLC1, but not with the addition of free PRCE, which in its free form reduced the viability of this microorganism to <1 log CFU/mL after 7 days. The microparticles were effective to protect BLC1 during juice storage and to maintain high contents of phenolic and proanthocyanidin compounds, although the products containing these had their viscosity altered and were less sensorially accepted than with free bioactives. The last aim of this work was to study the antiproliferative, antiradical and antioxidant activity, quinone reductase assay and apoptosis assay of extracts from blueberry and jabuticaba (free or in combination with probiotics). The extract that presented a greater chemopreventive effect compared to the others was the extract rich in water-soluble phenolic compounds from jabuticaba with BGP1 (JL), but there should be more studies to understand the

interaction between them. It is concluded that the co-encapsulation of probiotics and PRCE was successful, providing these bioactives greater retention during storage, protection in simulated gastrointestinal conditions and incorporation in sugarcane juice. The combination of PRCE and BLC1 exhibited an anticancer property in the early stages of CRC, while JL demonstrated an action at different stages.

Keywords: *Lacticaseibacillus*. *Bifidobacterium*. Phenolics. Complex coacervation. Colorectal cancer.

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ACRONYMS AND ABBREVIATIONS

a^* – Redness

b^* – Yellowness

B2 – Procyanidin B2

°C – Degree Celsius

CFU – Colony-forming unit

DMEM – Dulbecco's Modified Eagle
Medium

DPPH – 2,2-diphenyl-1-picrylhydrazyl

FRAP – Ferric reducing ability of plasma

FTIR – Fourier transform infrared
spectroscopy

g – Gram

g – Gravitational force equivalent

GAE – Gallic acid equivalent

h – Hour

HPLC – High performance liquid
chromatography

L – Liter

L^* – Lightness

log – Logarithm

mg – Milligram

min – Minute

mL – Milliliter

MPa – Megapascal

MRS – Man, Rogosa and Sharpe

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-
Diphenyltetrazolium Bromide

rpm – Revolutions per minute

s – Second

SCS – Sodium citrate solution

μg – Microgram

μm – Micrometer

μmol – Micromole

v/v – Volume/volume

w/v – Weight/volume

w/w – Weight/weight

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1 INTRODUCTION

The development of new food products is increasingly becoming a major challenge as consumers are looking for more attractive and healthier foods. In this context, functional foods play a specific role. These foods are not only intended to satisfy hunger and provide the necessary nutrients, but beneficially affect one or more functions of the human body by improving the general state of health and well-being or a reduction in the disease risk (DIPLOCK et al., 1999). They can be classified according to the food itself or according to the bioactive components present in it, such as: vitamins, minerals, herbs, fatty acids, with emphasis on probiotics and phenolic compounds (SILVA; BARREIRA; OLIVEIRA, 2016).

Probiotics are live microorganisms that when administered in adequate amounts confer benefits to the host (HILL et al., 2014). However, there is an ongoing controversy over whether cultures should be viable for effectiveness in all cases referring to paraprobiotics, postbiotics and psychobiotics (inactivated probiotics, metabolites or cell-wall components) (BARROS et al., 2020). The most widely studied bacteria in the field of probiotics, because they are present in the intestinal microbiota and have a long tradition of safe application, are lactic acid bacteria (LAB) and bifidobacteria (XIAO et al., 2019), examples such as: *Lactobacillus paracasei* (BGP1) (*Lacticaseibacillus paracasei*) and *Bifidobacterium animalis* ssp. *lactis* (BLC1). The health claims attributed to these genres are diverse and mainly linked to conditions involving an alteration of the normal microbiota, but it can also be highlighted an improvement in the immune response, reduction of anxiety symptoms, depression and cancer prevention (NG et al., 2018; RAGUL et al., 2020).

Another class of bioactives studied is phenolic compounds. The aromatic ring with hydroxyl groups is a characteristic of the family of these compounds, and these structures include high molecular weight polymers or small phenolic molecules (BALASUNDRAM; SUNDRAM; SAMMAN; 2006). They are natural antioxidants that can react with reactive oxygen species (ROS) reducing the effects of oxidative stress, which gives them cardioprotective, neuroprotective, anti-inflammatory, anti-aging, anti-cancer properties, among others (PAVLIDOU et al., 2018; DAS; MANDAL, 2018). They are substances distributed in nature and part of the constituents of a variety of vegetables, fruits and spices, examples such as: cinnamon, jabuticaba and blueberry, that was

selected for being evaluated in this work. These three materials are notorious sources of phenolic compounds.

Cinnamon, from the genus *Cinnamomum* and Lauraceae family, is a spice derived from the inner bark of the tree. There are a large number of species, such as Ceylon cinnamon (*Cinnamomum zeylanicum*) and China cinnamon (*Cinnamomum cassia*), which are the most well-known and commercialized, mainly in the form of bark, powder, leaves or essential oil extracted from the bark (RAVINDRAN; BABU, 2004). Cinnamon consumption is related to health benefits, such as: anti-obesity, type-2 anti-diabetes, antimicrobial, anticancer, etc (VALLIANOU et al., 2019). Among the compounds related to these effects are polyphenols, mainly proanthocyanidins (JIAO et al., 2013). The previous work of our research group studied the production, evaluation and encapsulation of proanthocyanidin-rich cinnamon extract (PRCE) (SOUZA et al., 2020; SOUZA et al., 2018; TULINI et al., 2017).

Studies have shown that consuming berries exhibit a wide range of positive biological health effects (TIAN et al., 2018). Blueberries (*Vaccinium myrtillus* L.) are berries that grow in shrubs and come from North America. While, jabuticaba (*Myrciaria cauliflora* Berg.) is a native plant from Brazil, known for producing globular fruits with a purple peel and a sweet pulp (WU; LONG; KENNELLY, 2013). In both fruits, there is a high antioxidant capacity due to the large amount of anthocyanins, phenolic acids, flavonoids and tannins (ABE; LAJOLO; GENOVESE, 2012; WANG et al., 2017). Some studies have highlighted the anti-cancer properties present in the extracts of these fruits (CORREA-BETANZO et al., 2014; REYNERTSON et al., 2006).

However, to exert beneficial effects, phenolic compounds must be absorbed in the intestinal tract and to be bioavailable. When they are not absorbed in the small intestine, phenolic compounds reach the colon, where they can undergo extensive biotransformation by the microbiota (MORAIS et al., 2019). The probiotics that have been shown to be able to modify these compounds through the action of different glycosyl hydrolases and release of conjugated glycol aglycones from phenolics improving their absorption and bioavailability (PEREIRA-CARO et al., 2015). Therefore, it can be suggested that there is a synergistic effect between them, since phenolics can exert a prebiotic function by increasing the population of bacteria, while probiotics make them more bioavailable (SOUZA et al., 2019).

Colorectal cancer (CRC) is among the most frequent types in the world population (ARNOLD et al., 2017). Evidences show that the bad eating habits may be associated with the

development of CRC and other dysbiosis (RAMAN et al., 2013). Modulation of the gut by probiotics and phenolic compounds alone or in combination could positively influence the immune system and is beneficial in the preserving of CRC. In this study, different extracts (blueberry, jabuticaba and Ceylon cinnamon) were evaluated and it was investigated what would be the action of chemopreventive alone or with probiotics in CRC cells. The research internship abroad provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (BEPE / FAPESP) allowed these analyzes to be carried out and with the immense contribution of Professor Monique Lacroix and her team at the INRS Armand-Frappier Health and Biotechnology Center (Laval / Canada). So, the scholarship and the collaboration allowed to achieve this aim of the work.

PRCE and probiotics are sensitive to changes in pH, high temperatures, the presence of oxygen, processing, storage and passage through the gastrointestinal tract. The sensation of astringency when PRCE is ingested is also one of the drawbacks, due to the property of these molecules to bind with salivary proteins, precipitating them (SOUZA et al., 2020; HOFMANN et al., 2006). In this sense, the immobilization or encapsulation of these bioactives prevent their interaction with salivary proteins, promote their protection against adverse environmental conditions, in addition to their release in their site of action and under specific conditions.

Microencapsulation is an efficient technology, which has been developed for use in the food industry and allows the protection of bioactive substances until releasing in the intestine. (HOLKEM et al., 2017; SILVA et al., 2019). Some techniques have been used such as: spray drying, spray chilling, extrusion, internal ionic gelation and complex coacervation. The complex coacervation method consists of spontaneous phase separation due to the formation of a complex that can be insoluble, between two or more polymers resulting from electrostatic interactions. This technique does not use organic solvent or drastic temperature conditions, besides having a low cost and, therefore, it is specifically advantageous in relation to other encapsulation processes (MATOS-JR et al., 2019; HOLKEM et al., 2019).

Considering that the first stage of complex coacervation is the preparation of an oil-in-water (O/W) emulsion, this technique is suitable for encapsulating nonpolar materials, such as oils (COMUNIAN et al., 2018) and carotenoids (GOMEZ-ESTACA et al., 2016). Aiming at the encapsulation of microorganisms, whose membrane hydrophobicity is not always high, and plant extracts, which are composed of compounds of different polarities, this research proposed an innovation in the classic coacervation technique, with the use of fat with a high melting point as a

nucleus, to immobilize the two bioactives by the formation of solid lipid particles at room temperature, which in turn would be covered by the two polymers, first by whey protein concentrated (amphiphilic and amphoteric molecule) and then by gum arabic (anionic molecule).

Traditionally, probiotics are added to yogurts and other dairy products. However, there is a growing demand for non-dairy products with probiotic properties (DIAS et al., 2018). Fruit juices are a suitable carrier for these bacteria, as they have beneficial nutrients, a pleasant taste and are appreciated by people of all age groups (COLÍN-CRUZ et al., 2019). An example of this is the sugarcane juice that is extracted from the stem of the sugar cane using mechanical compression. Sugarcane juice is a good source of energy, antioxidants and minerals and has a high therapeutic value (KARTHIKEYAN; SAMIPILLAI, 2010).

In this context, the aims of this work were to co-encapsulate probiotics and PRCE, to evaluate the production of sugarcane juice with them, and to study the anticancer properties of plant extracts alone or in combination with these bacteria.

This thesis was drafted in chapters, in order to facilitate its reading and comprehension. The first step of this research (Chapter 2) consisted of studying the encapsulation of probiotics with PRCE to determine the best treatment; In this case, BLC1 with 5% (w/w) PRCE was chosen. Therefore, the second step (Chapter 3) aimed the study of anticancer properties PRCE in combination with BLC1 and resistance of these free and co-encapsulated bioactives under *in vitro* simulated gastrointestinal conditions. The third step (Chapter 4) was the application of these microparticles in sugarcane juice. Meanwhile, the fourth step (Chapter 5) was the study of chemopreventive properties of extracts obtained from blueberry (*Vaccinium myrtillus* L.) and jabuticaba (*Myrciaria cauliflora* Berg.) in combination with probiotics developed in the research internship abroad provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (BEPE / FAPESP). A schematic representation showing all the steps of this work (Figure 1).

Figure 1 – Schematic representation of each step of this research

Reference: Own source.

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2 CHAPTER 1 – PROBIOTICS AND PHENOLICS: A PROMISING SYNERGY AND MICROENCAPSULATION¹

2.1 PROBIOTICS

Probiotics are defined as live microorganisms, which confer benefits to host (human) health when administered in adequate quantities (FAO/WHO, 2002; HILL et al., 2014). For a microorganism to be considered probiotic, it must have the following characteristics: 1) The culture must be produced on an industrial scale; 2) The culture must tolerate the intestinal environment of the host; 3) The culture must survive during production, storage, and have effects on consumer health (FULLER, 1989; SINGH et al., 2011). These microorganisms should be characterized of human origin and of safe use for the host. Proof of its beneficial physiological effects is required. In addition, it should be effectively able to adhere to the target tissue (OYETAYO; OYETAYO, 2005; PICARD et al., 2005).

The main genera of probiotics are *Lactobacillus* and *Bifidobacterium*. The genus *Lactobacillus* had more than 250 species, but many of them were distinct in terms of genotypic, phenotypic and ecological factors (SALVETTI et al., 2018). As a result, there was a need for taxonomic readjustment, allowing for more detailed communication and a stable nomenclature. The genus *Lactobacillus*, defined in 1901 around the species of *Lactobacillus delbrueckii*, is restricted to 35 species. The remaining species were removed from this genus and allocated to 23 new genera and the genus *Paralactobacillus* (ZHENG et al., 2020). These microorganisms play a multifunctional role for food production, such as probiotics and early dairy cultures (GIRAFFA; CHANISHVILI; WIDYASTUTI, 2010). There are several specificities of action, such as *Lactobacillus reuteri*, which is identified as a *Helicobacter pylori* antagonist that is associated with stomach and ulcer cancer (TYAGI; PRASAD, 2015). *Lactobacillus plantarum* PH04 can reduce cholesterol and triglyceride levels (NGUYEN; KANG; LEE, 2007), just as *Lactobacillus acidophilus* ATCC 4356 is considered anti-atherosclerotic (CHEN et al., 2013). Kailasapathy and Chin (2000) presented the beneficial effects of *L. acidophilus* on human health, such as improving immunity against intestinal infections and preventing diarrhea and colon cancer.

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Bacteria of the genus *Bifidobacterium* are anaerobic rods, Gram-positive. In 1900, Tissier isolated a bacterium from infantile feces and named it as *Bacillus bifidus*. He reported that the presence of this bacterium contributed to a lower incidence of diarrhea in breastfed children. This bacterium was considered an acid-lactic, however, it was reclassified as an independent *Bifidobacterium* genus, due to its distinct characteristics (KWAK et al., 2016; LEE; O'SULLIVAN, 2010). These microorganisms are considered as one of the most important groups of probiotic bacteria and mainly have been incorporated in fermented foods and dairy products.

Some microorganisms of the genus *Bacillus*, characterized as Gram-positive have shown a probiotic effect. Some advantages of the application of probiotic species of *Bacillus* (spore formers) in foods in relation to probiotics of other genera (non-spore forming) are: being thermal process resistant (JAFARI et al., 2017); having extended viability in ambient temperatures (CUTTING, 2011); and survival in high concentrations of bile salts and low pH (NITHYA; HALAMI, 2013). More information on *Bacillus* probiotics and their applications in food can be found in the review elaborated by Cutting (2011). Dudonné et al. (2015) found that supplementation of *Bacillus subtilis* CU1 spores with cranberry phenolic compounds in mice increased the bioavailability of these compounds, due to the microbial metabolism of the phenolics in the intestine. In addition, these metabolites and the probiotic studied may have influenced the modulation of the intestinal microbiota, although their mechanisms of action are not elucidated.

Yeasts are used mainly for fermentation processes and also have some strains characterized as probiotic (MCFARLAND, 2010). The most common yeast with proposed probiotic effects is *Saccharomyces boulardii* and also known as *Saccharomyces cerevisiae* var. *boulardii* or *Saccharomyces cerevisiae* Hansen CBS 5926. The optimal temperature for multiplication is 37 °C, and the pH range is between 4.5-6.5 (CZERUCKA; PICHE; RAMPA, 2007). Studies on the beneficial effects of using these microorganisms are mainly limited to improving disorders of the gastrointestinal tract. *S. boulardii* and *Kluyveromyces fragilis marxianus* may be recommended for adults use as it can prevent diarrhea associated with antibiotic use and traveler's diarrhea, in addition to preventing recurrent disease by *Clostridium difficile* (MACCAFERRI et al., 2012; MCFARLAND, 2010). However, some studies have explored the impact of yeasts on infections that cause liver disorders (DUMAN et al., 2013; EVERARD et al., 2014).

The health effects of probiotics are evaluated using *in vivo* models, as shown in Table 1. Several studies have demonstrated the beneficial effects of the administration of probiotics on

gastrointestinal and immune systems, such as the maintenance of the normal intestinal microbiota and protection against gastrointestinal pathogens (STAVROU; GIAMARELLOS-BOURBOULIS; KOTZAMPASSI, 2015), prevention of diarrhea in adults and children (MEGO et al., 2015), improvement of the bowel transit time and stool consistency (JAYASIMHAN et al., 2013), relieving discomfort related to lactose intolerance (PAKDAMAN et al., 2016) and multiple beneficial effects are exerted on the metabolic syndrome (FESTI et al., 2014). Moreover, recent studies have been investigating that probiotics may influence the mental health, acting on brain regions related with the processing of emotion and sensation (TILLISCH et al., 2013), affecting the behavior and physiological responses and reducing the stress-related disorders (BRAVO et al., 2011), as well as the improvement of the cognitive health (KIM et al., 2020).

In addition, there are significant differences between the administration of probiotic by foods and supplements in relation to the health benefits, indicating that the intervention using only probiotic would not be totally efficient (DONG et al., 2019). Thus, the composition of food matrix may enhance the survival of probiotics during digestion, besides contributing with other nutritional benefits. In order to exert their health benefits, and taking into account the fact that the minimum effective dose is suggested to be 10^8 – 10^9 CFU/ g or mL of cells/ day (CHAMPAGNE; GARDNER; ROY, 2005; KAILASAPATHY; CHIN, 2000), the minimum concentration of live probiotic should be at least 10^6 – 10^7 CFU/g of food at the time of consumption.

Table 1 – Health benefits related to the administration of probiotics using different model approach.

Probiotic strain	Model approach	Health benefits	Reference
®Lacto Levure® - <i>S. boulardii</i> , <i>B. lactis</i> , <i>L. acidophilus</i> and <i>L. plantarum</i>	Mice	Avoided the development of infection by pathogens and improved the immune response	Stavrou, Giamarellos-Bourboulis and Kotzampassi (2015)
Colon Dophilus® - <i>B. breve</i> HA-129, <i>B. bifidum</i> HA-132 HA, <i>B. longum</i> HA-135, <i>L. rhamnosus</i> HA-111, <i>L. acidophilus</i> HA-122, <i>L. casei</i> HA-108, <i>L. plantarum</i> HA-119, <i>Streptococcus thermophilus</i> HA-110, <i>L. brevis</i> HA-112, <i>B. infantis</i> HA-116 and inulin.	Patients with colorectal cancer	Reduction of the incidence of diarrhea	Mego et al. (2015)
Hexbio® - <i>L. acidophilus</i> , <i>L. casei</i> , <i>L. lactis</i> , <i>B. bifidum</i> , <i>B. longum</i> and <i>B. infantis</i> and FOS	Constipated adults	Improved the consistency and frequency of stool.	Jayasimhan et al. (2013)
<i>Clostridium butyricum</i> MIYAIRI, <i>B. longum</i> BB536 and digestive enzymes	Patients subjected to gastric bypass	Improvement of symptomatic GI episodes after surgeries	Chen et al. (2016)
Fermented milk with <i>B. animalis</i> subsp <i>lactis</i> , <i>Streptococcus thermophilus</i> , <i>L. bulgaricus</i> , and <i>Lactococcus lactis</i> subsp <i>lactis</i>	Healthy women	Influenced the brain activity in regions related with the processing of emotion and sensation	Tillisch et al. (2013)
<i>L. rhamnosus</i> JB-1	Mice	Affected the behavior and physiological responses and reduced the stress-related disorders	Bravo et al. (2011)
<i>B. bifidum</i> BGN4 and <i>B. longum</i> BORI	Healthy elders	Improved the cognitive and mental health	Kim et al. (2020)
<i>L. acidophilus</i> DDS-1	Healthy volunteers with complaining about the lactose intolerance	Improved the abdominal symptoms in relation to the diarrhea, vomit and cramp	Pakdaman et al. (2016)

Reference: Own source.

In the last decades, large quantities of dairy products containing probiotics, such as fermented milks, cheeses, cream, whey drinks, and frozen dairy desserts, among others, have been developed, since they are usually consumed within days of manufacturing. However, other food matrices are currently being developed to serve all consumers, especially those with lactose intolerance. Amongst these are drinks (PAIM et al., 2016), meat products (RUBIO et al., 2014), cereal-based products (BAMPI et al., 2016; KEDIA et al., 2007), soy-based products (MATIAS et al., 2014), confectionery products (SOUKOULIS et al., 2014), vegetable-based products (ANEKELLA; ORSAT, 2013; CHEN; MUSTAPHA, 2012), and chocolate (SILVA et al., 2017). The expected annual growth rate of the global probiotics market is approximately 7.4% per year in the period between 2019 and 2024. In addition, food and beverage segment will grow from \$32.9 billion in 2019 to \$46.8 billion in 2024 (KUMAR, 2020). These market forecasts may change in the face of Covid-19 pandemic, that is causing economic crises in many countries, but the claim that these products can strengthen the immune system can attract new consumers.

2.2 PLANT EXTRACTS

Since antiquity, people have used plant and spice extracts for nutritional purposes, but since 1900 that there was an increase in the attempt to characterize them. With the discovery of medicinal properties, plants have become a great source of compounds important in preventing or treating disease and improving health. In the early 1990s, the World Health Organization (WHO) reported that 65-80% of the population in developing countries depended on medicinal plants and their bioactive compounds as the only form of access to basic health care (FARNSWORTH et al., 1985).

The whole living being, from the bacterial cell to the different species of plants, processes several chemical substances for their survival and subsistence in the environment. The metabolites produced can be divided into two major groups. Primary metabolites are chemicals intended for development and growth, such as carbohydrates, proteins and lipids. Meanwhile, secondary metabolites are substances with low molecular weight capable of increasing survival and protection, such as phenolic compounds, alkaloids or terpenes (HARBORNE, 1993).

Phenols are a class of chemical compounds characterized by the presence of at least one aromatic ring attached to a hydroxyl. They are classified according to their chemical structures: 1) Simple phenols - only one aromatic ring as examples are all phenolic acids; 2) Polyphenols -

phenolic compounds that have several phenolic rings in their structure, such as flavonoids, which have thousands of compounds, including flavonols, flavones, flavan-3ol (catechins), flavanones, anthocyanidins and isoflavones (BRAVO, 1998).

Phenolic compounds are called bioactive compounds because they have pharmacological or toxicological effects in humans and animals. The health effects are numerous, as shown in Table 2. The significant functional property of phenolic compounds is their ability to eliminate free radicals. The structure of phenolics is a determining factor for their functional activity, followed by their bioavailability, stability, molecular size, and capacity to form complexes with other compounds, such as phytochemicals and other phenolics. The number and position of the hydroxyl groups relative to the carboxyl groups determine their functional activity. As an example, hydroxyninamic acids have higher activity than hydroxybenzoic acids (with -COOH group), due to their $\text{CH} = \text{CH-COOH}$ group, which guarantees greater capacity for hydrogen donation and radical stabilization (BALASUNDRAM; SUNDRAM; SAMMAN, 2006; KUPPUSAMY et al., 2015).

Currently, there is great interest in methods for obtaining these bioactive properties or functional components of plants from extracts of roots, stem, bark, leaves, flowers, fruits and seeds. However, for economic and sustainable reasons, residues from fruit and vegetable processing are also being used, because they are cheap and often highly concentrated in some bioactive (ALBUQUERQUE et al., 2019). These extracts are used mainly as ingredients in foods, food supplements or as cosmetics (LEAL et al., 2020). Studies show that a good extract qualitatively and quantitatively depends mainly on the selection of suitable extraction methods (AZMIR et al., 2013). These methods are affected by factors such as the botanical source, solvents, temperature, time and pressure (HERNÁNDEZ; LOBO; GONZÁLEZ, 2009).

Table 2 – Health benefits related to the administration of phenolic compounds using different model approach.

Polyphenols source	Model approach	Health benefits	Reference
Green tea (<i>Camellia sinensis</i> L.)	Mice	Alleviated <i>Salmonella</i> Typhimurium-induced ileal injury; Regulated intestinal flora disorder; Decreased inflammation and oxidative stress and preserved tight junction	Zhang et al. (2020)
Cocoa (<i>Theobroma cacao</i> L.)	90 elderly individuals	Regular consumption can reduce some measures of age-related cognitive dysfunction, possibly through an improvement in insulin sensitivity.	Mastroiacovo et al. (2015)
Chokeberry juice (<i>Aronia melanocarpa</i> L.)	Twenty-three subjects (12 men and 11 women) aged 33-67.	Awake systolic and diastolic blood pressure were significantly decreased; Significant reduction in triglyceride level and a reducing effect on total and low-density lipoprotein cholesterol were also found.	Kardum et al. (2015)
Spearmint (<i>Mentha spicata</i> L.)	Subjects with age-associated memory impairment	Spearmint extract may be a beneficial nutritional intervention for cognitive health in older subjects with age-associated memory impairment.	Herrlinger et al. (2018)
Pomegranate Juice (<i>Punica granatum</i> L.)	Healthy male volunteers and in atherosclerotic apolipoprotein E-deficient (E(0)) mice	Antiatherogenic effects in healthy humans and in atherosclerotic mice that may be attributable to its antioxidative properties.	Aviram et al. (2000)
Blackberry juice (<i>Rubus fruticosus</i> L.)	Rats	Protective effect against behavioural changes that are induced by acute stress	Fernández-Demeneghi et al. (2019)
Apple pomace (<i>Malus domestica</i> 'Shampion')	Rats	Apple pomace had beneficial effects on intestinal health and the cholesterol-lowering effect was related to increased production of short-chain fatty acids and excretion of bile acids.	Ravn-Haren et al. (2018)

Reference: Own source.

There are many techniques for recovering bioactive compounds from plants. The existing classical techniques are soxhlet extraction, maceration and hydrodistillation. Their efficiency depends on the choice of suitable solvents, according to polarity of the target compounds. Despite the wide variety, organic solvents are very effective for extraction, but can affect human health if residues are left in the final product. Therefore, care is needed to remove all traces of solvents. Due to these obstacles, unconventional extraction techniques are being widely used as ultrasound, microwave, pulsed electric field, supercritical fluid and pressurized liquid extraction (ARCEUSZ; WESOŁOWSKI; KONIECZYNSKI, 2013).

Usually, bioactive compound is extracted from the source in order to be concentrated, fractionated and purified to investigate the therapeutic effect (SEKHON-LOODU et al., 2013). However, this effect can be the result of the combination of several bioactive compounds and the isolation process can lead to loss or reduction. Thus, one of the advantages of plant extracts is their complex composition. Their components have several biological activities that result in a greater total activity and this can occur due to synergy, cumulative effects, addictive properties of the constituents or bioavailability (CARMONA; PEREIRA, 2013; KAHARUDIN et al., 2020).

Regarding to extract compounds solubility, it depends on their chemical structure, and some are not very soluble in water, which may hinder the application of extracts rich in phenolics in food, for example. Another drawback is the complexes formed by these compounds with salivary proteins, which results in the formation of precipitates, and this consequently reduces the lubricating properties of saliva, resulting in an often unpleasant sensation. This unpleasant sensation is called astringency and is defined as roughness, wrinkling, and dryness in the oral cavity (BACON; RHODES, 2000). However, for astringency to occur, it will depend on the phenolic type and structure, the pH value, and the family of salivary proteins (OBERQUE-SLIER; PENA-NEIRA; LÓPEZ-SOLÍS, 2012; PEREZ-GREGORIO; MATEUS; DE FREITAS, 2014). These factors hinder the development and production of foods that contain or are enriched with extracts containing these compounds, so encapsulation can be an alternative.

The main contributions of plant extracts to foods include that they can serve as natural additives (colorant or flavor enhancer) and / or functional ingredient (antioxidant or antimicrobial agents). In the packaging area, extracts can be inserted with biopolymers to be used as antioxidant (or antimicrobial) coatings to prevent prepackaged foods from oxidation. Among many plant extracts that have well-known properties, the ones prepared with cinnamon, blueberry and

jabuticaba will be highlighted due to they were chosen, because they present a profile of phenolic compounds of interest and that can be further explored.

2.2.1 Cinnamon

Cinnamon is among the best known spices by different peoples around the world, having also been one of the most sought after during the great sailings in the Middle Ages. This plant belongs to the genus *Cinnamomum*, whose origin of the word comes from the Greek kinnamon or kinnamomon, which means “sweet wood” (RAVINDRAN; BABU, 2004).

The genus *Cinnamomum* comprises about 250 species, which are distributed in Australia and Asia. The forms of commercialization of cinnamon are mainly in bark and powder. But other products such as leaves, some isolated compounds and essential oil are also important. According to statistics from the Food and Agriculture Organization (FAO), the world's largest producers of cinnamon in 2013 were Indonesia, China, Vietnam and Sri Lanka (FAO, 2016). The best known varieties are China cinnamon (*Cinnamomum cassia*), Java cinnamon (*Cinnamomum burmannii*) and Ceylon cinnamon (*Cinnamomum zeylanicum*), other species originating in India are also used (DAYANANDA; SENANAYAKE; WIJESEKERA, 2004).

Ceylon cinnamon (*C. zeylanicum* Blume synonym *C. verum* J. Presl) is also known as real cinnamon. It is native to Sri Lanka where it is grown on a large scale and exported. In Brazil and in several other countries, cinnamon from China (also called false cinnamon) is sold, which is cheaper than that of Ceylon. However, there are great differences between these two varieties, both in chemical composition and sensory aspects. Visually, Ceylon cinnamon has a light brown color, less thick and a mild aroma when compared to China cinnamon (*C. cassia*). Another major difference between *C. zeylanicum* and *C. cassia* is the content of coumarins (1,2-benzopyrones) which is higher in China's cinnamon, offering health risks if consumed regularly and in large quantities, due to their toxic effects (ABRAHAM et al., 2010; RANASINGHE et al., 2013).

In addition to the use of cinnamon as a spice it is also used for therapeutic purposes by traditional medicine. Some effects are described as anti-spasmodic, anti-diabetes, carminative, stimulant, antioxidant and antimicrobial, among others (JAYAPRAKASHA et al. 2007; SHARAFELDIN; RIZVI, 2015; UNLU et al., 2010). These beneficial effects are usually attributed

to its essential oil or aqueous extracts, obtained with or without the addition of organic solvent (CHENG et al., 2012; JIAO et al., 2013).

Cinnamon extracts obtained with ethanol, water and acetone are rich in phenolic compounds, mainly by proanthocyanidins. In Ceylon cinnamon (*Cinnamomum zeylanicum*) type-A and type-B protoanthocyanidins have been identified with different degrees of polymerization, from dimer to undecamer (2-11 subunits). Other molecules have also been detected, such as prodelfinidines and propelargonidins, due to the presence of different subunits of flavan-3-ols (HO; RAFI; GHAI, 2010).

In short, Ceylon cinnamon extract has many therapeutic properties since it is a good source of bioactive compounds, so, an excellent choice for preparing functional foods.

2.2.2 Blueberry

The blueberry is known as the “longevity fruit”. It is a small globular berry of a dark blue color, inside it houses small seeds and grows in a 1 to 2 meter tall shrub of the *Ericaceae* family within the genus *Vaccinium* (LUBY et al., 1999). Originating in the forests of North America and Northern Europe, however with the abundance of species and its wide distribution, it has made this fruit very popular being produced in almost all countries. Depending on the growing and harvesting season, several types of blueberries are commonly more accessible, for example: highbush blueberry (*Vaccinium corymbosum* L.), rabbiteye blueberry (*Vaccinium ashei* Reade), lowbush blueberry or wild blueberry (*Vaccinium angustifolium* Aiton), and bilberry (*Vaccinium myrtillus* L.) (MÄÄTTÄ-RIIHINEN et al., 2004).

Blueberries have a high content of several phytochemicals, including ascorbic acid and polyphenols. They differ from other fruits, such as strawberries, raspberries and jaboticaba, because they contain different types of polyphenols. For example, blueberries do not contain ellagitannins unlike raspberries and strawberries but they are particularly high in anthocyanins (SEERAM, 2008). At least, 25 structurally different anthocyanins have been identified in blueberries, which included five of the most common aglycones: cyanidin, delphinidine, peonidine, petunidine and malvidin (SILVA et al., 2020). These compounds have a high antioxidant value, as they prevent free radicals from taking electrons and causing damage, triggering a series of diseases, including cardiovascular diseases, type 2 diabetes mellitus and neurological decline (KALT et al., 2020). In

the study by Boespflug et al. (2018) in a randomized, double-blind, placebo-controlled trial evaluated the effect of blueberry supplementation on regional brain activation in older adults at risk for dementia. It was observed that an improvement in the neural response during work tasks for adults treated with blueberry.

In addition to anthocyanins, blueberries are also a source of phenolic acids and their bioactivities have been studied in extracts or fractions of both fruit and leaves. Chlorogenic acid is the main compound in all cultivated *Vaccinium* species and, in fact, is one of the most abundant phytochemicals in blueberries (RODRIGUEZ-MATEOS et al., 2012). This high concentration can contribute to the anti-inflammatory effects of blueberries, due to its ability to inhibit Cox2 and NF- κ B which can suppress the release of inflammatory cytokines. This was confirmed in the study Eladwy et al. (2018) indicated that the blueberry leaf extract had a protective effect against cardiac hypertrophy via suppressing oxidative stress, inflammation and fibrosis.

A stilbene compound originally found in grapes is resveratrol or 3,5,4-trihydroxystilbene, which has also been detected in some blueberry varieties (RIMANDO et al., 2004). Some studies have reported that resveratrol sensitizes some types of cancer to radiation (FANG; DEMARCO; NICHOLL, 2012). In the study by Davidson et al. (2019) demonstrated that blueberry extract sensitized cervical cancer cells to radiation therapy by inhibition of proliferation and promotion of apoptosis.

The food industry has shown interest in blueberry extracts not only for its health-promoting properties, but also due to the unusual pigments of anthocyanins in acidic conditions that present bluish and orange hues. Another fruit that has similar properties is the jabuticaba that will be discussed in the next section.

2.2.3 Jabuticaba

Jabuticaba is a plant native to Brazil, known as the “Brazilian grape tree”. Although, there are several species of jabuticaba with the genus *Myrciaria*, there are two that are more distributed in Brazil that are *Myrciaria cauliflora* (Mart.) O. Berg or jabuticaba assú and *Myrciaria jaboticaba* (Vell.) O. Berg or jabuticaba sabará. Both species produce globular edible fruits on tree trunks with a purple peel and sweet white pulp, maturing in around 40-60 days (LORENZI et al., 2000).

The consumption of this fruit has been associated with a variety of health benefits that are linked to its chemical composition with a large abundance of polyphenols (WU et al., 2012). However, these nutrients are in greater concentration in their peel and seeds, parts that are generally discarded both by consumption in natura and industry through the manufacture of jellies, liquors, vinegar and wine (MORALES et al., 2016). Therefore, studies on extracts from peels and seeds have attracted the attention of academia and the food industry to use them as additives or functional ingredients.

In particular, jabuticaba peels are a source of anthocyanins, which are flavonoids with powerful antioxidant properties responsible for their dark color (ALBUQUERQUE et al., 2020; LEITE-LEGATTI et al., 2012). These molecules are made up of an aglycone (anthocyanidin), a group of sugars and often a group of organic acids (TREVISAN; BOBBIO; BOBBIO, 1972). To date, there are five anthocyanins that have been isolated and identified from jabuticaba which are peonidine, peonidin-3-O-glucoside, cyanidin-3-O-glucoside, delphinidin-3-O-glucoside and pyranocyanin B (WU; LONG; KENNELLY, 2013). In a study by Plaza et al. (2016), jabuticaba peel was used as a source of phenolics in an investigation of the effect on parameters of antioxidant activity and glucose in a double-blind, placebo-controlled cross-over study. The antioxidant capacity of the serum was significantly higher when the subjects had consumed the meal containing jabuticaba. Serum insulin decreased after the second meal in 4 hours after consumption of the jabuticaba peel.

The seeds have shown different phenolic compounds such as castalagin, vescalagin, procyanidin A2 and ellagic acid (FIDELIS et al., 2021). The high content of ellagic acid is even greater than other fruits such as cranberry, blueberry and strawberry, however these high levels are derived from ellagitanins (ABE; LAJOLO; GENOVESE, 2012). This group of compounds has been widely studied for its antimicrobial, anti-proliferative and anti-inflammatory activity (LIPÍŃSKA; KLEWICKA; SÓJKA, 2014). However, in the study by Inada et al. (2019) had an unexpected laxative effect on normoweight, overweight and obese Brazilians after consumption of jabuticaba skin and seed extract, possibly due to the composition of the intestinal microbiota and metabolism of ellagitanins.

As well as Ceylon cinnamon and blueberry, extracts produced using jabuticaba are good sources of bioactive compounds and could be used to produce a natural pigment (SILVA, M. et al.,

2014). This product satisfies the huge industrial and consumers demands for new, low-cost sources of natural additives.

2.3 THE INTERACTION BETWEEN PHENOLICS AND PROBIOTICS

The normal intestinal microbiota represents a dynamic, complex, and diverse set of microorganisms that normally inhabit the human gastrointestinal tract (SERBAN, 2011). Usually, this microbiota, working together with the host, has a beneficial symbiotic relationship in the long term, where the human being contributes nutrients essential for the microbiota survival, while the latter fulfills multiple functions in the intestine.

The quantitative and qualitative variations of the microorganisms along the gastrointestinal tract are due to nutrients and drugs, environmental factors, and the factors intrinsic to the host, such as pH of the intestine, bowel transit time, presence of bile acids, digestive enzymes, and mucus in the intestine, in addition to the factors intrinsic to the microorganisms, such as adhesion capacity, enzymes, and metabolic activity (SERBAN, 2014).

During the life of the human host, a bacterial imbalance called dysbiosis may occur and can cause various diseases. However, it is not always easy to determine whether microbiota modifications are a cause or a consequence of the disease, however, this relationship between microbiota and disease has been suggested by several authors. This microbial-health relationship has raised interest in positive modulation through the administration of live bacteria (probiotics) or substrates (phenolic compounds) for the prevention or even cure of some diseases (BUTEL, 2014).

The mode of action of probiotics may be related to the modulation of the host microbiota. One of the first suggested modes of action is the "barrier" effect, also called resistance to colonization, exerted against pathogenic bacteria, which prevents or limits its colonization. Bacterial inhibition may be due to the production of bacteriocins or metabolites, such as short chain fatty acids, inducing a decrease in pH and, thus, rendering the medium unfavorable for bacterial growth or production of biosurfactants with antimicrobial activity. This barrier effect can also act through various mechanisms, such as competition for binding sites and inhibition of adhesion.

Phenolic compounds also contribute readily to the modulation of the composition of the intestinal microbiota reaching the colon. In addition, phenolic compounds are emerging prebiotics, that can be converted by the gut microbiota into metabolites, which affect the bacterial ecology

functioning in the gastrointestinal system as prebiotics (GIBSON et al., 2017). They are promising supplements for the control of inflammatory and atherogenic disorders associated with diet-induced obesity. There is evidence from both animal and human studies that certain doses of phenolic compounds inhibit certain groups of pathogenic bacteria, while probiotic microorganisms may thrive in the available niche of the intestinal ecosystem (LEE et al., 2006; VAN DORSTEN et al., 2010). The mechanisms responsible for this bacteriostatic or bactericidal effect are complex, as there are different mechanisms of inhibition for the pathogenic bacteria that are involved, such as a decrease in pH values, depletion of metal ions, and inhibition of enzymatic activity (NEYRINCK et al., 2013).

Recent research indicates that rich sources of flavan-3-ols, such as green tea, coconuts, and grape seed extracts, can modulate the intestinal microbiota, both *in vitro* and *in vivo* studies (KANTACHOTE et al., 2017; SAVARD et al., 2011). In research conducted by Yamakoshi et al. (2001) demonstrated that a proanthocyanidin-rich extract obtained from grape seeds and offered to healthy adults for 2 weeks was able to significantly increase the number of *bifidobacteria*. The same result was observed in the study by Vendrame et al. (2011), where a blueberry diet drink was administered in humans to evaluate the consumption and the microbiota modulation. Bacteria of the genus *Bifidobacterium* spp. significantly increased, in addition to *Lactobacillus acidophilus*, however, microorganisms of the genus *Enterococcus* spp. and *Clostridium* spp. did not present significant differences. This study suggested that regular consumption of a wild blueberry drink may positively modulate the composition of the intestinal microbiota.

The administration of antibiotics often causes disturbances in the balance between host and microorganisms of the intestinal microbiota (SULLIVAN; EDLUND; NORD, 2001). Such disorders depend on the dose of the drug, the route of administration, and the properties of these compounds. The incomplete absorption of orally administered drugs can influence the microbiota at different stages of the gastrointestinal system, reducing the viability of these microorganisms (RASHID; WEINTRAUB; NORD, 2012). Studies have shown that the treatment of natural phenolic compounds is an alternative, since it has no adverse influence on the count of probiotic microorganisms in the intestine and still has a stimulatory effect on them (CHINA et al., 2012; ZHOU et al., 2016).

Moreover, human intestinal microorganisms are able to convert phenolics into more bioavailable compounds (LEE et al., 2006). The currently statement about the capacity of

probiotics to increase the bioavailability of phenolic compounds is widely discussed, whereas colon microbiota may act on these bioactive compounds by degradation or biosynthesis of bioavailable derivatives. In this way, metabolic pathway of phenolic compounds is quite challenged to evaluate, considering a wide diversity of each gut microbiota, as well as this analyzes should be supported using sophisticated devices, like HPLC-MS and GC-MS.

Several bioactive compounds are not bioavailable due to the glucoconjugations. Thus, phenols are biotransformed by the intestinal microbiota, initially by deglycosylation. This is followed by disintegration of the flavonoids into relatively simple aromatic carboxylic acids, generally referred to as phenolic acids. These metabolites are known to mediate many physiological effects; for example, 3,4-dihydroxyphenylacetic acid generated from flavonoid partitioning can inhibit platelet aggregation and also provide antioxidant protection near the intestinal wall (PARKAR; TROWER; STEVENSON, 2013).

Pereira-Caro et al. (2015) applied *in vitro* fermentation system aiming to evaluate the catabolism pathway of hesperetin, naringenin and ferulic acid, which are polyphenols found in orange juice. Colonic microbiota increased the bioavailability of these compounds by the activity of O-demethylase and hydrolase, producing phenylpropionic acids. Despite this deepening about the metabolic pathways, it is not possible to correlate these transformations with the performance of probiotics.

Some probiotic strains present glycosyl-hydrolases, such as the β -glucosidase, that breaking down the aglycones (ROSSI et al., 2013). However, authors suggested that enzyme location, mainly cytosolic, may be one of the reasons that hinder the biotransformation of polyphenols by probiotic strains. Other study reported the ability of specific probiotics to degrade phenolic compounds, such as *Lactobacillus plantarum* and *Lactobacillus casei*, known as commercial starters for fermentation of vegetables and fruits (RODRÍGUEZ et al., 2009). *L. plantarum* produces β -glucosidase and esterase, which are involved in the metabolic pathway of oleuropein from olives. First, the β -glucosidase hydrolyzes oleuropein, hence forming an aglycone, which is transformed in hydroxytyrosol and elenolic acid by esterase (MARSILIO; LANZA; POZZI, 1996). In addition, Landete et al. (2008) verified that *L. plantarum* metabolized only oleuropein and protocatechuic acid out nine phenolic compounds degraded.

Tannase is another enzyme produced by *L. plantarum*, which presents technological importance for food industry (RODRÍGUEZ et al., 2009). In this sense, Rodríguez et al. (2008)

verified the capacity of *L. plantarum* tannase degrade the tannic acid by HPLC analysis, indicating the metabolic pathway by the hydrolysis of tannic acid to gallic acid and glucose. Similarly, Llano et al. (2017) reported the catabolism of wine polyphenols by probiotics, increasing the concentration of benzoic and cinnamic acids. In contrast, probiotic strains did not produce derivative of flavan-3-ol monomers, demonstrating that not all phenolic compounds are biotransformed by probiotics. Several questions still need to be answered, such as which phenolic compounds are preferable for bioconversion by probiotic strains, and whether they could act similarly in the colon.

In this sense, Barroso et al. (2014) evaluated the influence on gut microbiota by the supplementation of *L. plantarum*, a specific strain that can metabolize flavan-3-ol, during the long-term feeding with red wine extract using a dynamic Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The initial feeding of red wine extract caused an antimicrobial effect on the gut microbiota, which was reverse during the establishment of continuous feeding-flow. Besides that, the addition of *L. plantarum* increased the production of lactic and butyric acids, as well as decreased the concentration of flavan-3-ol, producing their metabolites.

Other studies correlated the production of specific compounds with the bacterial growth in fermentation processes. Mustafa et al. (2020) evaluated the increment of quercetin-3-glucoside during fermentation of pomegranate juice by *L. casei*. Authors suggested the occurrence of glycosylation, which is triggered when a specific flavonoid may be toxic to the micro-organism (SLANA et al., 2011). The fermentation of red pitaya pulp by *L. acidophilus* LA-05 and *B. lactis* BB-12 decreased the phenolic content, correlating that these compounds were metabolized by probiotics (MORAIS et al., 2019). Rupasinghe, Parmar and Neir (2019) reported the catabolism of proanthocyanidin molecules from cranberry pomace by *L. rhamnosus*, producing catechin, epicatechin, benzoic acid, 4-hydroxyphenylacetic acid and 3-(4-hydroxyphenyl) propionic acid after 48 h of incubation. In addition, they confirmed that biotransformation of phenolic compounds increased the biological activity of derivatives. This approach is a good strategy to delivery more bioavailable phenolic compounds, extending the health benefits.

Taken together all the information discussed in this section, Figure 2 shows the schematic representation of the interaction between probiotics and phenolic compounds. As known, phenolic compounds prevent the colonization of pathogenic, thus the simultaneous consumption of probiotics increases the potential of their adhesion in the gut. Furthermore, a healthy gut microbiota

containing probiotics may biotransform the phenolic compounds, enhancing their bioavailability and benefits. However, additional research on integrative approaches (different polyphenol structures, sensitivity of bacteria to phenolic compounds, intestinal microbial diversity and individual differences in metabolism) and metabolomics are needed to fully understand the interactions between phenolic compounds and intestinal bacteria, as well as the benefits the health of the host.

Figure 2 – Schematic of the interactions of phenolic compounds with probiotics.

Reference: Own source.

In this context, with the evidence emerging from the mutualistic association between probiotics and phenolics and their modulatory effects on the intestinal microbiome, it will attract consumer interest in foods that contain them, it will boost research regarding their symbiotic effect, as well as a potential market. Despite the indisputable functional value of probiotics and rich-phenolic materials, the development of products with them is not an easy task because they are very sensitive and also can alter negatively the sensory characteristics of foods in which they are

added. So, the co-encapsulation of probiotics and rich-phenolic extracts seems to be a good proposal and it'll be explored in this study.

2.4 MICROENCAPSULATION

Probiotics may have reduced viability in food matrices, as phenolic compounds may lose their bioactivity, due to factors inherent to the food as intrinsic factors (pH, water activity (a_w) and chemical composition of food) and those inherent to the environment like extrinsic factors (light, temperature, humidity, presence of gases). These factors can be limiting, interfering in the multiplication of the microorganisms and in the structure of the phenolics.

Microencapsulation is a promising alternative, as it protects a bioactive material or a mixture (solid, liquid, gaseous) inside a microparticle, which can release its content at the ideal time at controlled rates under specific conditions, avoiding inappropriate exposure. The material to be coated is usually called a core or internal phase, while the material that forms the particle is called a shell, carrier or wall material (FÁVARO-TRINDADE; PINHO; ROCHA, 2008).

Microparticles can be classified into: microcapsules (reservoir system) - bioactive material surrounded by a shell; microspheres (matrix system) - bioactive material is dispersed in the shell. In addition, microparticles may have other morphologies, such as mononuclear, multinuclear or agglomerates and this depends on the core and wall materials, as well as the technique involved (SILVA, P. et al., 2014). Usually, microparticles have a diameter of 1 to 1000 μm . The particle size has an influence on sensory properties when it is intended to apply them to food. The presence of particles in foods can induce a feeling of sand in the mouth that is undesirable for the consumer (MERKUS; MEESTERS, 2014). Therefore, a first characterization regarding morphology is essential through the analysis of particle size, optical, confocal and scanning electron microscopy.

The selection of the wall materials is important to achieve a microparticle with adequate physical and chemical properties. The selection depends on a number of factors, among them: do not be reactive with the material to be encapsulated during the process and storage; the mechanism for releasing the encapsulated material; its rheological properties; ability to disperse or emulsify; ability to promote maximum protection against adverse conditions and be economically viable. Some tests such as Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis

(TG) and differential scanning calorimetry (DSC) are useful for evaluating wall materials and their interactions.

However, one of the factors that must be taken into account for the use of microparticles in food, the materials used must be certified as "generally recognized as safe" (GRAS). The most widely used wall materials include carbohydrates (starch, modified starch, maltodextrins, chitosan, dextran and cyclodextrins), proteins (gluten, casein, gelatin, albumin, peptides, whey protein and soy protein), lipids (wax, paraffin, beeswax, oils and fats), gums (gum arabic, agar, sodium alginate and carrageenan) and cellulose (carboxymethylcellulose, methylcellulose and ethylcellulose).

There are numerous microencapsulation methods in existence and increasingly on the rise through the discovery of new wall materials and bioactive materials. However, in order to choose which technique is most appropriate, tests should be carried out with these materials, such as water, humidity, hygroscopicity, solubility as well as encapsulation efficiency and stability of the bioactive material. Above all, an important factor is knowing which food will be added and whether the microparticles will interfere with the characteristics inherent in this product. For this, sensory tests through descriptive methods are important tools.

The main existing methods are divided into three categories. The physical methods are the most used by the industry, due to the simplicity of the process, relatively low cost, continuous operation and high stability due to the drying of the product (MAZZOCATO; THOMAZINI; FAVARO-TRINDADE, 2019). Examples include spray drying and spray chilling, but currently new techniques are being developed as microfluidic devices and supercritical fluids giving good results (FENG; LEE, 2019). The chemical methods, despite being simple, have some limitations such as the toxicity of some monomers and an example is polymerization (SILVA et al., 2002). The physical-chemical methods present high efficiency of encapsulation and control of particle size. Examples include coacervation, micelles, liposomes and emulsions (COMUNIAN et al., 2016).

Complex coacervation is one of the oldest techniques used. Some studies are showing modifications to improve the process and encapsulate different bioactive materials. This can be seen in the next section.

2.4.1 Microencapsulation by complex coacervation

The term coacervation is derived from the Latin '*co*' and '*acervus*' indicating an associative phenomenon between two reagents. The coacervation process was first described in 1911 by F.W.Z. Tiebackx. However, it was studied more thoroughly by Bungenberg de Jong and Kruyt during the 1920s and 1940s using gelatin and gum arabic as model reagents (BUNGENBERG DE JONG; KRUYT, 1949).

Complex coacervation is a three-phase system involving the solvent, bioactive material and coating material. The process basically involves four main steps: (a) preparation of the polymer solutions; (b) The bioactive material is emulsified in the polymer solutions; (c) With the pH changes, the polymers charges are modified becoming opposite, enabling electrostatic interaction between them. At this moment, the bioactive material is encapsulated by the deposition of the complex formed. (d) The complex cover is solidified by cooling the system or using a cross-linker and, consequently, phase separation and particle collection occur. Depending on the application of the microparticles, drying methods such as spray-drying or freeze-drying are employed (COMUNIAN; FAVARO-TRINDADE, 2016).

The interactions between polymers play a role in the structure, texture and stability of coacervates and also of the encapsulated material (EGHBAL; CHOUDHARY, 2018). Among the parameters that influence can be mentioned the temperature, ionic strength, pH of the reaction medium, polymer mixing ratio, their molecular weights, total concentration and charge densities (TIMILSENA et al., 2019). These parameters will vary depending on the type of polymeric pair chosen as wall materials. Turbidimetric titration is a widely used analysis to determine which pH and protein: polysaccharide ratio is ideal for the complex coacervation.

The first and most used polymers were gelatin and gum arabic, but several proteins and polysaccharides have been used as new pairs for the production of microparticles by complex coacervation (MOSCHAKIS; BILIADERIS, 2017). Animal proteins can be cited as whey proteins, egg albumin and silk fibroin, but vegetable proteins have been used widely as wheat protein, soy protein, pea protein, lentil protein and chia protein. Regarding polysaccharides, alginate, chitosan, chia seed gum, cashew gum, pectin, agar, carrageenans, carboxymethylcellulose (CMC) can be mentioned. However, biopolymers must be evaluated first, because not all combinations always result in an opposition of charges. It is necessary to observe the gelation temperature, isoelectric

point of the protein, zeta potential so that there is an adequate combination and later interaction between them.

The complex coacervation method is easily used for the encapsulation of hydrophobic compounds, since one of the first steps is the formation of an oil-in-water (O / W) emulsion. This has already been demonstrated in several studies in the encapsulation of omega 3 fatty acids, phytosterols, carotenoids, tocopherols and vitamin D (COMUNIAN et al., 2018; GOMEZ-ESTACA et al., 2016).

However, some studies have shown success in protecting compounds whose hydrophobicity is not as high as probiotics and plant extracts, through a modification in the classic coacervation technique. The use of a high-melting vegetable fat as a core, for immobilizing the bioactive material by the formation of solid lipid particles at room temperature and later covered by the electrostatic interaction of the polymers (Figure 3). In the study by Holkem and Favaro-Trindade (2020) encapsulated probiotics and cinnamon extract in solid lipid particles covered by the electrostatic interaction of whey protein and gum arabic, achieving an encapsulation efficiency close to 100% of bioactive materials.

The use of a high melting vegetable fat as nucleus for complex coacervation allow the encapsulation of: I) particles - probiotics can be considered particles since they have properties of functional biocolloids ranging from 1 μm (lactic acid bacteria) to 10 μm (yeasts) of average particle size; II) two or more materials and/or compounds in one microparticle; III) materials and/or compounds with different polarities. In addition, this minor adjustment in the traditional method of complex coacervation improved the protection of bioactive materials. This is because there will be two layers present in the microparticles, preventing possible inappropriate exposure through gastrointestinal passage and in the food or nutraceuticals (during their process and storage).

Figure 3 – Production of solid lipid microparticles covered by electrostatic interaction of polymers.

(1) Bioactive material is dispersed in the vegetable fat melted at 50 °C. (2) Addition of the protein solution to obtain an emulsion (O / W). (3) Addition of an oppositely charged polysaccharide solution. (4) Formation of microparticles after pH adjustment and cooling. (5) Precipitation of the microparticles. Reference: Own source.

2.4.2 Microencapsulation of probiotics

There are several problems with the low viability of probiotics in food. Some factors interfere in their viability, such as pH, titratable acidity, storage temperature, dissolved oxygen concentration, lactic and acetic acid concentrations. Survival is essential for these organisms to populate the human intestine and provide benefits for human health. Currently, the protection of probiotics inside a microparticle is being studied a lot (DE PRISCO; MAURIELLO, 2016; FÁVARO-TRINDADE; HEINEMANN; PEDROSO, 2011).

The most used technologies for microencapsulation of probiotics are emulsion, spray drying and extrusion because they can be applied on both laboratory and industrial scale. However, some factors, such as heating, can injure the microorganism during the encapsulation process, which can occur with spray drying (ALVES et al., 2016). As a result, many studies have used thermoprotectants for the medium with the bacteria before drying. Examples of this are trehalose, non-fat milk solids and adnitol. In the study by Etchepare et al. (2020) produced calcium alginate particles by ionic gelation and to coat them with different multilayers of whey proteins and sodium alginate to protect *L. acidophilus* La-14. In the thermal resistance tests, the multilayer particles promoted greater protection for probiotic cultures in relation to the free cells that did not resist the tested conditions.

Other techniques that are gaining attention are complex coacervation, spray chilling and vibrational extrusion technology. It has shown good results on encapsulation efficiency, morphology and functional properties (FÁVARO-TRINDADE, 2011). As demonstrated in the

study by Silva et al. (2019) encapsulated *Lactobacillus acidophilus* by complex coacervation followed by transglutaminase crosslinking. The treatments offered high encapsulation efficiency (68.20–97.72%) and the probiotic maintained its viability at 9.59 log CFU / g during storage for up to 60 days at -18 °C.

After the microparticles are produced and dried, a powder is obtained. So, it is necessary to store it for later analysis. Often, depending on the wall material and encapsulation method used, the powder can have high hygroscopicity, high water activity (a_w) or high moisture. These characteristics of powder are harmful to the bioactive material that is a probiotic, since the microorganisms can remain metabolically active, consequently decreasing their survival in the microparticle. In the study by Silva et al. (2018) encapsulated *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 by spray chilling. All samples absorbed moisture from the surrounding atmosphere and presented a w in the range of 0.911–0.984. Thus, the microorganisms did not survive with a count above 6 log / CFU until the end of storage, which was 90 days in both temperatures of -18 °C and 25 °C.

An alternative that has been proposed by several researchers to improve the viability of encapsulated probiotics during the process of encapsulation or/and storage is the simultaneous encapsulation with prebiotics (OKURO; MATOS JR; FÁVARO-TRINDADE, 2013). Prebiotics are compounds that induce the growth or activity of probiotics, examples include inulin, polydextrose, wheat dextrin, fructooligosaccharides-FOS and galactooligosaccharides-GOS. Okuro et al. (2013), immobilized *Lactobacillus acidophilus* and prebiotics (inulin or polydextrose) in solid lipid microparticles using spray chilling technology. This study proved that the combination of spray chilling process and the presence of polydextrose in the microparticles is an interesting approach for the protection, controlled delivery and for improving the viability of *Lactobacillus acidophilus*.

In recent years, the main criterion used to determine the efficiency of microencapsulation technique was the ability to keep probiotic viability. Resistance to simulated gastrointestinal conditions and viability during the storage period are evaluated. Thus, greater the protection, better the efficiency. However, the importance of evaluating the functionality of these bacteria after the microencapsulation process and the interactions after the addition in food have been questioned. In this context, Matos-Jr et al. (2019) immobilized *Lactobacillus paracasei* BGP1 (*Lacticaseibacillus paracasei*) and *Lactobacillus rhamnosus* 64 in solid lipid microparticles and covered them by a

complex of gelatin and gum arabic. These authors reported that the technique utilized for encapsulation improved the stability of these microorganisms in the presence of salt, on gastrointestinal conditions *in vitro* and their viability during storage of the microparticles. The dosage of S-IgA and cytokines in mice indicated that encapsulated microorganisms maintained their functionality.

2.4.3 Microencapsulation of probiotics and phenolics

The survival of probiotics is influenced for many factors, including the physical-chemical characteristics and the ingredients used in the formulations of the food in which they are inserted. Foods rich in phenolic compounds or supplemented by them are being considered good carriers for incorporating probiotics by the action of synergisms between them and growth or maintenance of probiotic viability. Table 3 presents some studies that investigated the application of these bacteria in food matrices.

It can be observed that some studies show an increase in the amount of phenolics in the presence of probiotics (DEMIRCI et al., 2020; XIA et al., 2019; ÖZTÜRK; DEMIRCI; AKIN, 2018; VIVEK et al., 2019), while others there was a decrease (CAMPOS et al., 2019; WU et al., 2020). This is due to many factors such as, the composition of the food matrix, pH, storage time/temperature of the product, type of probiotic strain and the effect of depolymerization of macromolecular phenolics or the conversion of individual phenolic compounds conducted by probiotics. To quantify the total phenolics in foods, the colorimetric assay with the Folin-Ciocalteu reagent is widely used. However, together with this technique, the study of the profile of phenolic compounds in extracts or foods before and after the addition of the bacteria using high performance liquid chromatography (HPLC) is necessary. The quantification of total phenolic compounds can suffer interference from other reducing substances present in the sample, such as ascorbic acid, proteins and reducing sugars (IKAWA et al., 2003), that are all also capable of reducing Folin-Ciocalteu reagent.

Regarding antioxidant activity, there was a variation observed between the studies showed (Table 3). One reason for this is that different extracts or food matrices have specific phenolic compounds that are responsible for the increase or decrease in antioxidant properties. In the study by Vivek et al. (2019) was demonstrated an increase in the antioxidant properties in sohiong juice

during fermentation with probiotic *L. plantarum* and this may be due to acylation of anthocyanin with phenolic acid or diacylation of anthocyanins. Another reason for this variation in antioxidant properties may be related to the ability of probiotics chelating metal ions and the elimination of reactive oxygen species (LIN; YEN, 1999). However, this characteristic is dependent on the type of strain (BUJNA et al., 2018; SENGUN et al., 2019).

For probiotics to have a beneficial effect on health, they must reach populations around 10^6 - 10^7 CFU / g or mL in the product (KNEIFEL; SALMINEN, 2011). We observed, in the studies mentioned (Table 3), that most of them presented the quantities recommended, above 6 log CFU / g. This may be due to the addition of phenolics or the insertion of the bacteria in some foods rich in these compounds, which had a positive effect possibly due to the synergistic effect between them. However, it was observed in the study by Duru et al. (2019) when adding *Lactobacillus acidophilus* and *Streptococcus thermophilus* in oat flour, a decrease in viability during storage probably due to acid production and a decrease in pH that affected probiotics. Large amounts of extract added to the product can be harmful due to the pro-oxidant effect that causes oxidative stress and death of the bacteria. This occurred in the study by Holkem et al. (2019) evaluated the addition of proanthocyanidin-rich cinnamon extract with *Bifidobacterium animalis* subsp. *lactis* (BLC1) in sugarcane juice and observed a decrease in viability during storage due to the high concentration of phenolic compounds.

Table 3 – Probiotic foods rich in phenolics or supplemented with phenolics, and the main effects of combining both bioactive materials. Reference: Own source

Food	Probiotics	Polyphenols source	Main effects, including viability during storage	References
Yogurt	<i>Lactobacillus paracasei</i> F19	Hot and cold break tomato powders	↑ Total phenolic; ↑ Antioxidant activity; ↑ Titratable acidity; ↓ firmness; ↓ viscosity; ↓ pH; Probiotic counts were greater than 6 log CFU/g at the end of storage.	Demirci et al. (2020)
Soymilk	Kombucha culture: <i>Pichia</i> and <i>Dekkera</i> are the most dominant yeast species, and <i>Acetobacter</i> and <i>Lactobacillus</i> are the key bacteria.	Soybean	↑ Total phenolic; ↑ Antioxidant activities; ↑ Inhibition activities to α -glucosidase and α -amylase; ↑ score for sensory acceptability test; ↑ Growth of acetic acid bacteria, lactic acid bacteria and yeast; Viability studies during storage not done and/or shown.	Xia et al. (2019)
Goat milk ice cream	<i>Lactobacillus casei</i> 431	White and dark blue myrtle pulps (<i>M. communis</i>)	↑ Total phenolic; ↑ Antioxidant activities; ↑ score for sensory acceptability test; ↑ viability during storage at -20°C for 8 weeks by sustaining 7 - 8 log CFU/g.	Öztürk, Demirci and Akın (2018)
Sohiong juice	<i>Lactobacillus plantarum</i> MCC 2974	Sohiong (<i>Prunus nepalensis</i>)	↑ Total phenolic; ↑ Antioxidant activities; ↑ anthocyanin; ↓ fructose; ↓ glucose; Counts greater than 6 log CFU/ml even after four weeks of storage.	Vivek et al. (2019)
Apple juice	<i>Lactobacillus plantarum</i> 90 (Lp90), <i>Lactobacillus helveticus</i> 76 (Lh76), <i>Lactobacillus casei</i> 37 (Lc37), <i>Lactobacillus paracasei</i> 01 (Lpc01), <i>Lactobacillus acidophilus</i> 85 (La85) and <i>Bifidobacterium lactis</i> 80 (Bla80).	Fresh Fuji (<i>Malus pumila</i> Mill.) apples	↑ Antioxidant activities; ↑ Malolactic conversion capacity of Lp90, La85 and Bla80; ↓ Total phenols; ↓ Flavonoids; Counts greater than 8 log CFU/mL.	Wu et al. (2020)
Orange juice	<i>Lactobacillus rhamnosus</i> ATCC 53103	Nettle (<i>Urtica dioica</i> L.)	↑ Total phenolic; ↓ Antioxidant activity; No change in pH during storage; ↓ Total acidity; Counts greater than 6 log CFU/mL during the storage.	Sengun et al. (2019)
Pineapple and jussara juice	<i>Lactobacillus rhamnosus</i> GG	Pineapple and jussara pulps	↓ Total phenols; ↓ pH; ↑ Acidity; No change in Total anthocyanins and Antioxidant capacity; Counts greater than 7 log CFU/mL during the storage.	Campos et al. (2019).
Oat flour	<i>Lactobacillus acidophilus</i> (LA-5®, strain number: DSM13241) and <i>Streptococcus thermophilus</i> (TH-4®, strain number DSM15957)	Isoflavones rich extract	↑ Antioxidant activity; ↓ pH; ↑ Titratable acidity; ↓ viability of the probiotic bacteria during storage; ↓ Value for the aroma attribute; Counts 10^6 CFU/mL during 21 days.	Duru et al. (2019)
Jelly candies	<i>Bacillus coagulans</i> GBI-30 6086	Pulp of Juçara and Passion fruit	Counts greater than 6.4 log CFU/mL over 90 days; No change in total anthocyanins; No change in antioxidant capacity; No change in physicochemical characteristics; Excellent acceptability; Counts greater than 6 log CFU/g over 90 days.	Miranda et al. (2020)

The encapsulation of probiotic microorganisms and phenolic compounds is usually performed separately and has been shown to be efficient in the protection and release at the desired site (HALWANI et al., 2008; HOLKEM et al. 2017; SHOJI et al. 2013). In fact, there is justification for avoiding the co-encapsulation of probiotics and high concentrations of phenolics. Indeed, it is controversial, since some phenolics have antimicrobial activity, and then, they could reduce the viability of probiotics. On the other hand, and, on the contrary, phenolics could protect and/or promote multiplication of probiotic microorganisms because of their prebiotic and antioxidant actions. Thus, if the presence of high concentrations of phenolics does not impair the viability of probiotics, something to be evaluated is the particles or microparticles produced by co-encapsulation. These are promising systems, where the health benefits of the two materials are combined with their possible synergistic effects as shown in Table 4.

Thus, there are several possibilities for combining phenolic and probiotic compounds for the benefit of the host microbiota and improving the viability of these bacteria. However, further studies on the co-encapsulation of these bioactive compounds and their interactions, mainly within the microparticles, are still needed.

Likewise, there is also a lack of studies detailing this probiotic-phenolic interaction in the microparticles. This synergy could be evaluated in the following stages, as shown in Figure 4: I) during the storage period of the microparticles, the phenolics could act to avoid oxidative stress, thus prolonging the probiotics viability; II) after application of the microparticles in food matrices, which, depending on their physicochemical characteristics (high moisture content, high A_w , and temperature suitable for multiplication), will allow for the interaction between probiotics and phenolics, increasing the bioavailability of phenolics by metabolism of the probiotics and, at the same time, improve the state of the cells by the antioxidant effect of phenolics to prebiotics; III) in the stomach and small intestine the structure of the microparticle would provide protection from factors harmful to probiotics, such as low pH and presence of bile salts; and IV) in the large intestine, where near neutral pH conditions and the presence of enzymes, such as proteases and lipases, can liberate the bioactive compounds (depending on the characteristics of the capsules) to promote its interaction with increased bioavailability of the phenolics, in addition to the prebiotic action that could favor the multiplication and colonization of probiotics.

For the initial investigation of possible probiotic-phenolic interaction, *in vitro* methods are recommended, and afterwards, *in vivo* studies are needed for further clarification.

Table 4 – Some studies about the co-encapsulation of probiotics and phenolics.

Encapsulation Technique	Probiotics	Polyphenols source	Main effects	References
Co-extrusion	<i>Lactobacillus acidophilus</i>	Apple skin polyphenol extract	Microencapsulation efficiency >96 %; Total phenolics > 2.61 ± 0.12 mg Cat eq./g; Total antioxidant activity > 3.05 ± 0.05 mg Trolox eq./g; Counts greater than 6 log CFU/mL	Shinde, Sun-Waterhouse & Brooks (2014)
Extrusion	<i>Lactobacillus casei</i> 01, <i>Lactobacillus acidophilus</i> LA5, <i>Bifidobacterium lactis</i> Bb-12	Cashew flower, pennywort, yanang and green tea extracts	Cashew flower and green tea extracts improved the stability of probiotic beads in all the products; Encapsulated <i>L. casei</i> 01 and <i>B. lactis</i> Bb-12 showed better survival than encapsulated <i>L. acidophilus</i> LA5.	Chaikham (2015)
Emulsification/internal gelation	<i>Lactobacillus helveticus</i> R0052	Green tea extracts	Bacteria encapsulation yield > 71.5 %; Polyphenol encapsulation yield > 36.1 %; DPPH radical scavenging activity > 10%; The extract in pectin-based particles does not alter survival of a probiotic culture in simulated gastric conditions.	Gaudreau et al. (2016)
Emulsification internal ionotropic gelation	<i>Lactobacillus plantarum</i> UAM17, <i>Enterococcus faecium</i> UAM18, <i>Aerococcus viridans</i> UAM21b, <i>Pediococcus pentosaceus</i> UAM22a	Apple marc, cactus pear peel flour and inulin	A stronger prebiotic effect of inulin and apple marc flour enhanced bacteria viability.	Serrano-Casas et al. (2017)
Spray drying	<i>Lactobacillus acidophilus</i>	Blackberry juice	Encapsulation efficiency of total phenolic compounds > 31.1 %, anthocyanins > 35.8 % and probiotic bacteria > 30.2 % after 10 weeks storage of microcapsules.	Colín-Cruz et al. (2019)
Spray drying	<i>Bifidobacterium animalis</i> spp. <i>lactis</i>	Jussara juice	Viable cell counts in all powders were above 6.0 log CFU/g; Highest anthocyanin and phenolic retention after spray drying.	Paim et al. (2016)
Complex coacervation	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BLC1	Proanthocyanidin-rich cinnamon extract	↑ Quinone reductase of combination of probiotic and extract compared to free extract; Greater resistance of encapsulated materials than free ones.	Holkem, Favaro-Trindade, Lacroix (2020)

Reference: Own source.

Figure 4 – Proposed steps for the evaluation of synergism between phenolics and probiotics.

Reference: Own source.

2.5 CONCLUSION AND OUTLOOKS

Although there are still few, the results of some research indicate that the association of probiotics and phenolics is promising because it combines the benefits of both in health. There is a synergy between them because phenolics have a prebiotic effect, while probiotics increase the bioaccessibility of phenolics. However, further research is needed to clarify the magnitude of the health benefits of this association, as well as establish the safety range of its consumption in association to maximize the consumer benefits from both.

It will still be necessary to elucidate the possible mechanisms of action and interaction between them. Further progress in assessing effects on humans depends on the development of new experimental systems. Exploration at the cellular level allows a better understanding of the underlying mechanisms that regulate functions in normal and pathological states. The development of molecular biology techniques and easier access to tools such as mass spectrometry with more representative models, along with the development of good predictive biomarkers, will allow for a better understanding of how phenolic compounds and probiotics interact with endogenous systems and exogenous factors.

Another challenge is the delivery of phenolic compounds and probiotics, due to their instability under adverse conditions. In this sense, microencapsulation stands out as an alternative to protect them during processing, storage, shelf-life, and passage through the gastrointestinal tract, in addition to minimizing the astringency sensation promoted by some phenolics, and enabling controlled release at the action site. Thus, future work should evaluate the release kinetics of these compounds from the encapsulating matrices.

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3 CHAPTER 2 – POTENTIAL OF SOLID LIPID MICROPARTICLES COVERED BY THE PROTEIN-POLYSACCHARIDE COMPLEX FOR PROTECTION OF PROBIOTICS AND PROANTHOCYANIDIN-RICH CINNAMON EXTRACT²

ABSTRACT

Probiotics and proanthocyanidin-rich cinnamon extract (PRCE) have numerous potential health benefits, but they are very sensitive to degradation in various environmental conditions. Additionally, the combination of these two materials into a single structure could possibly enhance their therapeutic properties. Thus, the aim of this study was to produce and evaluate the solid lipid microparticles covered by electrostatic interactions of polymers in which *Lactobacillus paracasei* (BGP1) and *Bifidobacterium animalis* subsp. *lactis* (BLC1) were either encapsulated alone or co-encapsulated with PRCE. Through turbidimetric titration and zeta potential measurement, the optimum coacervates were obtained at a pH of 4.2 with the protein:polysaccharide mixing ratio of 6:1. Along with quantification of the probiotics, total phenolic compounds, and proanthocyanidins, morphological and physicochemical characterizations were performed during storage for 120 days at both 7 and 25°C. All the produced powders had similar morphological and physicochemical properties. The treatments with BLC1 and 5% PRCE presented greater encapsulation efficiencies for probiotic, phenolics, and proanthocyanids with $98.59\% \pm 0.45$, $119.49\% \pm 4.21$, and $81.25\% \pm 1.9$, respectively. Additionally, there was greater viability for BLC1 (9.30 ± 0.16 log CFU / g) after 120 days of storage at 7°C. In conclusion, solid lipid particles with BLC1 and 5% PRCE are a promising solution for the preservation and consumption of both materials.

Keywords: *Lactobacillus*; *Bifidobacterium*; Ceylon cinnamon; Complex coacervation; Viability; Storage.

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3.1 INTRODUCTION

Functional foods containing probiotics and bioactive compounds are being produced in great demand due to increasing consumer awareness to maintain a healthy diet (HOLKEM et al., 2019; RIBEIRO et al., 2020). Probiotics can be defined as living microorganisms that confer a health benefit to the host when administered in adequate amounts (HILL et al., 2014). Cinnamon, a plant of the genus *Cinnamomum*, has been used across the world since ancient times either as a spice or in medicine. Cinnamon consumption has numerous recognized health benefits, such as being an agent in anti-obesity and type-2 anti-diabetes, and it also presents antimicrobial and antioxidant properties. Among the compounds conferring these effects are polyphenols, which are mainly proanthocyanidins (JIAO et al., 2013).

In order for phenolics to exercise such benefits, these compounds need to be released from the food matrix and become absorbed during gastrointestinal digestion. Since probiotics act by enzymatically converting the glycosides present in these compounds that release the aglycones that are biologically more active and can be easily absorbed, they can contribute to this action to occur more quickly (LÓPEZ DE LACEY et al., 2014). Therefore, the combination of these bioactive materials would be interesting because of the possibility to act together.

However, degradation effects such as wide variations in pH and high temperatures can affect the beneficial properties of these bioactive materials. Another drawback is the astringent sensation of proanthocyanidins when we ingest them due to the property of these molecules, which bind with salivary proteins that cause precipitation (HOFMANN et al., 2006).

Microencapsulation comes as an efficient technology, which has been developed for use in the food industry and allows for the protection of bacteria and polyphenols up to their place of action (HOLKEM et al., 2019; TULINI et al., 2016). The complex coacervation method consists of spontaneous phase separation because of the formation of an insoluble between two or more polymers, which results from electrostatic interactions (EGHBAL; CHOUDHARY, 2018). This research proposed encapsulation of microorganisms, whose membrane hydrophobicity is not always high, and plant extracts, which are composed of compounds of different polarities. This is an innovation to the classic coacervation technique by using vegetable fat as a nucleus to immobilize the two bioactive materials in the formation of solid lipid particles at room temperature

in turn, these substances would be covered by the two polymers, primarily by a protein (amphiphilic and amphoteric molecule) and an anionic polysaccharide.

In this context, microparticles that contain a proanthocyanidin-rich cinnamon extract (PRCE) with probiotics have the potential to be used as additives in food products and new functional foods since it is possible to obtain a synergistic action of different phenolics compounds with probiotics.

Previously, researchers of our group study the encapsulation of PRCE by complex coacervation (SOUZA et al. 2018) and probiotics by using solid lipid microparticles covered by the protein-polysaccharide complex (MATOS-JR et al. 2019). However, to the best of our knowledge, this is the first article that aimed to develop, characterize, and evaluate the potential of solid lipid microparticles covered by the protein-polysaccharide complex, which contain probiotic bacteria alone or together with extract obtained from cinnamon (*Cinnamomum zeylanicum* Blume).

3.2 MATERIAL AND METHODS

3.2.1 Materials

Ceylon cinnamon (*C. zeylanicum* Blume) in bark was obtained from WG Products (Waskaduwa, Sri Lanka). Lyophilized *Lactobacillus paracasei* BGP1 and *Bifidobacterium animalis* subsp. *lactis* (BLC1) were kindly donated by Sacco do Brasil (Campinas/ SP, Brazil). Vegetable fat (AL CONF P45X LT) presenting a melting point of 46 °C was donated by Cargill Agrícola S/A (Itumbiara/Goiás, Brazil). Lacprodan 80 whey protein concentrate (WPC) was provided by Arla Foods Ingredients (São Paulo/SP, Brazil). Gum Arabic (GA) was purchased from Nexira (São Paulo/SP, Brazil).

3.2.2 Preparation of probiotics and production of PRCE

The *L. paracasei* BGP1 and *B. animalis* subsp. *lactis* BLC1 were replicated three times in MRS broth for 18 h at 37 ± 1 °C. Then, the cells were centrifuged at $5752 \times g$ for 10 min at 10 °C and washed in 2% sodium citrate solution twice. The cells were suspended in this solution to obtain approximately 10 log CFU/mL.

The procedure for the production of PRCE was based on the methodology of Souza et al. (2018). The cinnamon was triturated and added in 50% (w / w) aqueous ethanol at a solid:solvent ratio of 1: 7.5 (w / v) for 30 min at 60 °C. The extract was centrifuged for 5 min /25 °C / 7500 rpm in a Model 5430 centrifuge (Eppendorf, Hamburg, Germany). The spray dryer model MSD 1.0 (Labmaq of Brasil Ltda, Ribeirão Preto, Brazil) dried the ethanolic extract by using an inlet air temperature of 150 °C with a drying air velocity of 2.5 m/s and feed flow rate of 16 mL/min; the model used a 1.2 mm double spray atomizer nozzle and compressed air flow of 40 L/min. The obtained powder was stored in a polyethylene bottle, covered with aluminum foil, and stored in a freezer at -20 °C until the moment of use.

3.2.3 Hydrophobicity of probiotic cell wall

To evaluate the hydrophobic profile of the cell wall of *Lactobacillus paracasei* BGP1 and *Bifidobacterium animalis* ssp. *lactis* BLC1, bacterial growth was promoted in 10 mL of MRS broth for 16 h at 37°C. At the end of the growth period, centrifugation was performed at 6000 rpm for 6 minutes and double washed with phosphate buffer (0.1 M, pH 7.0). The cultures were suspended in the same buffer and the optical density (D0) was adjusted to approximately 1.0 at 560 nm. Then, 0.6 ml of η -hexadecane (Sigma-Aldrich, St. Louis, USA) was added to 3.0 ml of the bacterial suspension and, after vortex homogenization for 2 minutes, incubated at 37°C for 1 hour for phase separation. The bottom aqueous phase was carefully removed with a sterile Pasteur pipette and absorbance (D1) was recorded at 560 nm following the method described by Geertsema-Doornbusch; Van der Mei; Busscher (1993). To determine the results and express them as a percentage of bacterial adhesion to the solvent, the following equation was used eq. (1):

$$H \% = \left(\frac{1-D1}{D0} \right) \times 100 \quad (1)$$

3.2.4 Turbidimetric titration and zeta potential measurement

Turbidimetric titration and zeta potential measurement were performed to determine the pH and the proportion of the ideal polymeric pairs that should be used in the coacervation. A mixture of WPC with a concentration of 0.1% (w/v) and GA with a concentration of 0.1% (w/v) was

prepared. According to the methodology of Lv et al. (2012), the turbidimetric titration of the protein and polysaccharide dispersions (1:2, 1:1, 2:1, 4:1, 6:1, 8:1) were measured at different pH values (ranging from 2.5 to 5.5) by using a Genesys 10S UV-vis spectrophotometer (Thermo Scientific, São Paulo / SP, Brazil) at 600 nm at 25°C.

The zeta potentials of the encapsulating materials and the polymeric pair that obtained the highest turbidimetric titration were measured in a pH range from 3 to 6.5 in zeta potential analyzer (BTC - Brookhaven instrument corporation, New York, United States).

3.2.5 Production of microparticles and determination of encapsulation efficiency

According to the methodology proposed by Holkem et al. (2019), microparticles were produced by complex coacervation. 1.5 g of either BGP1 or BLC1 (1×10^{10} CFU/mL) alone or with the PRCE mass were mixed with the molten vegetable fat at 50 °C and homogenized by using an ultra-turrax at 3500 rpm for 60s. Based on total solids of the microparticle composition, the preliminary tests defined the values as: 0% - 0 g; 5% - 1.12 g; 10% - 2.25 g; or 15% - 3.37 g. This mixture was homogenized with a WPC (6% w/v) solution at 7000 rpm in ultra-turrax for 90s to obtain an emulsion (O/W). Subsequently, a GA (1% w/v) aqueous solution was added to the emulsion under constant magnetic stirring (500 rpm) and heating (50 °C) to prevent the solidification of the vegetable fat. The pH of the mixture was adjusted to 4.2 to promote the interaction between polymers. Afterwards, the temperature of the mixture was reduced to 10°C by using an ice bath, and the coacervated material was maintained at 8°C for 12 h to decant. The microparticles were frozen and dehydrated by sublimation in the freeze dryer model L 202 (Liobras, São Carlos, São Paulo, Brazil) for 72 h. The powders were stored in high density polyethylene bags and stored at -20°C.

Eight treatments were differentiated in relation to the concentration of PRCE. These were as follows: **BGP1-0** – encapsulated BGP1; **BGP1-5** - encapsulated BGP1 with 5% PRCE; **BGP1-10** - encapsulated BGP1 with 10% PRCE; **BGP1-15** - encapsulated BGP1 with 15% PRCE; **BLC1-0** - encapsulated BLC1; **BLC1-5** - encapsulated BLC1 with 5% PRCE; **BLC1-10** - encapsulated BLC1 with 10% PRCE; **BLC1-15** - encapsulated BLC1 with 15% PRCE.

Encapsulation efficiencies (EE) were calculated as suggested by Souza et al. (2018), considering eq. (2):

$$EE (\%) = \left(\frac{\text{Bioactive materials content in microparticles} - \text{Bioactive materials content on the surface}}{\text{Theoretical bioactive materials content in microparticles}} \right) \times 100 \quad (2)$$

Where:

The concentration of bioactive materials in microparticles was determined experimentally according to section 3.2.6.

The concentration of bioactive materials on the microparticles surface was determined after washing (1 g of microparticles with 9 ml of ethanol for proanthocyanidins and phenolics, while 9 ml of distilled water was used for probiotics. The tubes were vortexed and an aliquot was collected and analyzed according to section 3.2.6).

Theoretical bioactive materials content in microparticles was the theoretical amount of phenol added at the beginning.

3.2.6 Quantification of probiotics, total phenolic compounds, and proanthocyanidins

The quantification of probiotics was determined by the drop plate method, using De Man medium, Rogosa and Sharpe (MRS). One gram of microparticles was dispersed in 9 mL of 2% sodium citrate solution at 48 °C to completely dissolve the lipid matrix in microparticles. The plates were incubated under anaerobiosis (Anaerobac, Probac, Brazil) at 37 °C for 72 h.

For quantification of total phenolic compounds and proanthocyanidins, one gram of microparticles was dispersed in 9 mL of a 0.5 M NaOH solution at 48 °C for 15 min and centrifuged at 7500 rpm/4 °C for 5 min. The aqueous phase was collected, and the precipitate was washed with 0.5 M HCl to extract any remaining compounds.

At an absorbance of 750 nm, the Genesys 10S UV-vis spectrophotometer (Thermo Scientific, São Paulo / SP, Brazil) was used to determine the total phenolic compounds (SINGLETON; ORTHOFER; LAMUELA-RAVENTOS, 1999). The phenolic content of the samples was expressed in milligrams of gallic acid equivalent (GAE) per mL or g of dry matter.

The proanthocyanidins content were determined by the Genesys 10S UV-vis spectrophotometer (Thermo Scientific, São Paulo / SP, Brazil) at an absorbance of 640 nm for 5 min at 10s intervals in order to obtain the maximum absorbance value (PAYNE et al., 2010). Proanthocyanidins were expressed in milligrams of Procyanidin B2 equivalent per ml or g of dry matter.

3.2.7 Characterization of the powders and microparticles

3.2.7.1 Morphology

The wet microparticles were deposited on a glass slide, thoroughly air dried, and evaluated by using an optical microscope (Bel Photonics, Monza, Italy), a digital camera, and an imaging program (Bel MicroImage Analyzer, Monza, Italy).

For the visualization of probiotics after their release from particles, one gram of microparticles was dispersed in 9 mL of 2% sodium citrate solution at 48 °C to completely dissolve the lipid matrix in microparticles. A smear of bacteria was deposited on a glass slide and thoroughly air dried. It was stained for 1 min in Crystal Violet solution, 1 min in iodine solution, washed for 10 s in ethanol, and lastly, counterstained with safranin for 1 min, according to the gram staining methodology. The glass slide was examined under oil immersion at 100× magnification by using an optical microscope (Bel Photonics, Monza, Italy), a digital camera, and an imaging program (Bel MicroImage Analyzer, Monza, Italy).

The dehydrated microparticles were fixed on metallic supports with conventional conductive double-phase adhesive tape and this system analyzed with scanning electron microscopy (SEM) using Tabletop Microscope TM3000 (Hitachi, Japan).

3.2.7.2 Average particle size and powder density

The average size of the microparticles was analyzed by dispersing the samples in distilled water and inserting it into the Shimadzu SALD-201 V laser diffraction particle analyzer (Kyoto, Japan). The density was measured by inserting the powders into the multi-pycnometer (Quantachrome Instruments, Boynton Beach, Florida, USA).

3.2.7.3 Water activity, moisture, hygroscopicity and solubility

Water activity and moisture content of the powders were determined by using AQUALAB equipment (Decagon Devices, Pullman, WA) and a moisture analyzer (Ohaus, model MB 35, Ohio, USA). The hygroscopicity was measured according to the methodology used by Cai and Corke

(2000). Samples of 0.2 g were stored for one week in a desiccator that contained a saturated NaCl solution with a relative humidity of 75%. The hygroscopicity was determined by the mass of water absorbed per 100 g of sample after 7 days of storage.

Solubility was determined according to the method proposed by Eastman and Moore (1984). Samples of 0.5 g were homogenized with 50 mL of distilled water using an orbital shaker (Tecnal, Piracicaba, Brazil) at 100 rpm for 30 min at room temperature. The solution was then centrifuged at 3500 rpm for 5 min in a Centrifuge 5430 R (Eppendorf, Germany); 25 mL aliquot of the supernatant was transferred to a previously weighed porcelain dish and maintained in an oven at 105 °C until complete evaporation of the water.

The solubility was calculated according to eq. (3).

$$S = \left(\frac{\text{Final weight} \times 2}{\text{Initial weight}} \right) \times 100 \quad (3)$$

3.2.7.4 X-ray diffraction

The structure of these ingredients and powders was analyzed by x-ray diffraction (diffractometer Siemens 5100). The measurements were obtained by 2 θ scanning between 5 and 55° at a scanning rate of 6 °C/min, tension of 40 kV, and current of 30 mA.

3.2.7.5 Fourier transform infrared spectroscopy

The spectra of the ingredients and the powders were obtained in the region of 4000 to 600 cm⁻¹ by using a Perkin Elmer FTIR spectrometer (Massachusetts, USA) with Spectrum One software version 5.3.1.

3.2.8 Evaluation of probiotics, total phenolic compounds, and proanthocyanidins in storage for 120 days

The samples were stored in glass jars with relative humidity controlled at 33% and kept at 7 °C and 25 °C for 120 days. The quantification of bioactive materials was performed on days 0 and 120 in accordance with section 3.2.6.

3.2.9 Data statistical analysis

The data were statistically analyzed for analysis of variance (ANOVA) and by Tukey's test at the significance level of 5% using the statistical program Statistica version 7.0 software (Statsoft Inc., Tulsa, OK, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Hydrophobicity of probiotic cell wall

It is advisable to study the hydrophobicity of probiotic cell walls because the greater capacity allowed for greater possibility to successfully encapsulate and keep them in the lipid particles. Figure 5 illustrates the hydrophobicity profile of the cell walls of the studied microorganisms.

Figure 5 – Percentage of hydrophobicity of BGP1 and BLC1 cell walls.

The bars identified with different lowercase letters differ statistically ($p \leq 0.05$). Reference: Own source.

BLC1 showed a hydrophobicity value of $37\% \pm 2.0$, which was significantly higher than BGP1 at $29\% \pm 2.0$. The results are very varied because it depends on several intrinsic factors (e.g. cell wall composition - amino acids and sugars) and extrinsic factors (e.g. variation of pH and

temperature); therefore, it can be said that this characteristic of probiotics is strain-dependent. The results found for BLC1 are lower when compared to those obtained by Zuo et al. (2016), who isolated and analyzed bacteria of the genus *Bifidobacterium* from feces and reported values of 98.84%. However, the results obtained for BGP1 in the present work are superior to those obtained by Kotzamanidis et al. (2010), who evaluated the hydrophobicity and self-aggregation of twelve species of *Lactobacillus* isolated from the gastrointestinal tract and found values of 1.4%.

Although it is desirable that a probiotic has a hydrophobic cell wall, this characteristic should not limit the selection of these microorganisms and their encapsulation. Each probiotic strain has a number of other beneficial effects on intestinal morphology, microbiota population, absorption of nutrients, water and electrolytes, body antioxidative capacity, and immune response.

3.3.2 Turbidimetric titration and zeta potential measurement

The complex coacervation between protein and polysaccharide is mainly due to the electrostatic interaction of these biopolymers. Additionally, some secondary interactions can occur, such as hydrophobic interactions, hydrogen bonding, and Van der Waals forces (SINGH et al., 2007). One of the most important parameters for coacervation to occur is pH because the formation of complexes is mainly attributed to electrostatic interactions that depend on the degree of ionization of the polymers (SCHMITT et al., 1998). Turbidimetric titration was used to determine which pH and protein:polysaccharide ratio was ideal for the complex coacervation encapsulation process.

Figure 6A shows the tendency for turbidity to change in six different WPC:GA ratios. The initial pH evaluated was around 5.5, and at this time, both WPC and GA probably carried similar negative charges, which could be represented by the zeta potential in Figure 6B.

Figure 6 – Effect of biopolymers mixing ratio and pH on the turbidity (600 nm) for the system of WPC:GA (A) and zeta potential measurements as a function of pH (B).

Reference: Own source.

By gradually reducing the pH of the medium, the amphoteric protein presented a reduction in negative charges while its positive charges increased, due to the protonation of amino groups. When the pH value gradually decreased, the turbidity in the 6:1 ratio increased from 0.1 to 0.9 (the highest value found in the tested ratios), which was the result of the greater electrostatic attraction between WPC and GA with a phase separation in the solution. This can also be seen in the zeta potential (Figure 6B), where the charges were zero close to the pH of greater turbidity. This is because the polymers in this medium interact by attraction and the insoluble complexes formed between the polymers are concentrate in the liquid droplets, settle, and combine to form a coacervated phase at the bottom (WEINBRECK; TROMP; DE KRUIF, 2004). It can be observed that after reaching the optimum pH value of 4.2, there was a sharp drop in the turbidity values, due to the addition of the acid. This phenomenon can be considered as the transition from insoluble complexes to soluble complexes.

Consequently, the basic theory of microencapsulation by complex coacervation depends only on the formation of insoluble complexes that involves the core material and the sedimentation of the microparticles (LIU; LOW; NICKERSON, 2009). Therefore, the 6:1 mixture ratio was determined to be the most suitable ratio for the microencapsulation process by complex coacervation. In addition, a pH of 4.2 was considered the best for the formation of microparticles.

3.3.3 Characterization of obtained powders and their microcapsules

3.3.3.1 Morphology

Figure 7 shows the powder samples obtained after the production of the microparticles covered by electrostatic interaction and dehydrated by lyophilization. The fine powders presented a whitish or brown color for both treatments with and without PRCE, respectively. Furthermore, the presence of some typical clusters in the freeze-dried samples was observed. It should be emphasized that the appearance and color of all formulations were similar to that described above, regardless of the probiotic used.

Figure 7 – Image of the powders obtained covered by electrostatic interaction followed by lyophilization.

(1) Treatment with 0% PRCE; (2) Treatment with 5% PRCE; (3) Treatment with 10% PRCE; (4) Treatment with 15% PRCE. Reference: Own source.

Figure 8 shows the morphology of the microparticles before the drying process. It is possible to observe the spherical shape with well-defined contours. An inner layer was formed by vegetable fat and another outer layer by WPC/GA complex, which demonstrates that the encapsulation process was effective. When both the probiotic and PRCE are immobilized in vegetable fat and has a second layer for the electrostatic interaction of WPC and GA, this type of microparticle is called a reservoir.

Figure 8 (Q) and Figure 8 (R) show BGP1 and BLC1, respectively, being released from the particles after they were dispersed in 2% sodium citrate solution at 48 °C and gram staining. It can be seen that the constituted wall material and the bacteria stained purple because both are gram-positive. In Figure 8, microparticle structures can be observed through micrographs obtained by scanning electron microscopy (SEM). The lyophilized powders showed solid bridges forming a network between the microcapsules, losing the spherical shape (before lyophilization), and consequently resulting in agglomeration. This previously described structure is very common in microparticles that have gone through the freeze-drying process as shown by other studies (COMUNIAN et al., 2013; SANTOS et al., 2015). In this microscopy, no morphological differences were observed due to the encapsulated microorganism or the concentration of PRCE inside the particle. Despite the characteristics of agglomeration and loss of structure, they return to a spherical shape after the rehydration of the microparticles produced by electrostatic interaction; the outlined contours and the evident nucleus are observed again by optical microscopy.

Figure 8 – Optical and scanning electron microscopy of the treatments.

Optical microscopy of the treatments (A) BGP1-0, (C) BGP1-5, (E) BGP1-10, (G) BGP1-15, (I) BLC1-0, (K) BLC1-5, (M) BLC1-10, (O) BLC1-15, (Q) Free BGP1, (R) Free BLC1. Scanning electron microscopy of the treatments (B) BGP1-0, (D) BGP1-5, (F) BGP1-10, (H) BGP1-15, (J) BLC1-0, (L) BLC1-5, (N) BLC1-10, (P) BLC1-15. Reference: Own source.

3.3.3.2 Encapsulation efficiencies

Table 5 shows that the encapsulation efficiencies for probiotics were between $81.74\% \pm 0.54$ and $98.59\% \pm 0.45$, depending on the formulation. Formulations with BLC1 had a significantly higher efficiency than formulations with BGP1. This can be explained by the difference in hydrophobicity of the cell wall between the probiotics, since BLC1 showed greater retention inside the particle due to its higher hydrophobicity; however, both the temperature of the melted fat where the probiotic was inserted and the homogenization process that decrease the viability of bacteria may also have influenced this process (OKURO et al., 2013).

Table 5 – Percentage of encapsulation efficiency for probiotics, phenolic compounds and proanthocyanids.

Treatments	Encapsulation efficiency % - probiotics	Encapsulation efficiency % - phenolic compounds	Encapsulation efficiency % - proanthocyanids
BGP1-0	81.74 ± 0.54^c	-	-
BGP1-5	87.11 ± 0.96^b	114.97 ± 2.90^{ab}	78.08 ± 2.90^{ab}
BGP1-10	83.11 ± 2.70^c	99.89 ± 1.48^d	72.49 ± 3.41^b
BGP1-15	85.14 ± 0.77^{bc}	107.93 ± 0.63^c	74.21 ± 2.45^{ab}
BLC1-0	95.66 ± 1.72^a	-	-
BLC1-5	98.59 ± 0.45^a	119.49 ± 4.21^a	81.25 ± 1.91^a
BLC1-10	96.11 ± 1.81^a	103.33 ± 1.56^{cd}	72.49 ± 3.41^b
BLC1-15	96.07 ± 0.54^a	108.66 ± 1.21^{bc}	71.59 ± 1.17^b

Means followed by the same lower-case exponent in the same column are not different ($p > 0.05$) by the Tukey test.

Reference: Own source.

Encapsulation efficiencies for total phenolic compounds were $99.89\% \pm 1.48$ and $119.49\% \pm 4.21$, while the encapsulation efficiency for proanthocyanidins did not exceed $81.25\% \pm 1.91$. This increase in phenolic compounds may be related to the depolarization of proanthocyanidins in monomers that may have occurred during the encapsulation process, which increase the amount of total phenolics. Souza et al. (2020) also observed this behavior when studying encapsulation of proanthocyanidins and the polyphenolic compounds from cinnamon extract by complex coacervation. Although complex coacervation is a milder method, there are some steps that can cause degradation, such as homogenization, pH, and temperature variation in the same process.

3.3.3.3 Average particle size and powder density

The values for average particle size were in a range of $40.84 \mu\text{m} \pm 5.52$ to $51.75 \mu\text{m} \pm 4.14$ (Table 6). There was no significant difference between treatments, which suggest that the amount of probiotics and PRCE had no influence on the average particle size. Usually, particles obtained by complex coacervation can have sizes that vary between 1 to $500 \mu\text{m}$ (FÁVARO-TRINDADE; PINHO; ROCHA, 2008). The particles in this study presented sizes smaller than $100 \mu\text{m}$, which makes them interesting for application in food products. For instance, these sizes make mixing within the product easier and decrease the possibility of consumers noticing these particles during ingestion.

Regarding powder density, the values were similar between treatments and ranged from 1.41 ± 0.008 to $1.55 \pm 0.01 \text{ g/cm}^3$ (Table 6); this demonstrates that the variation in the composition of the microparticles did not affect their density.

3.3.3.4 Water activity, moisture, hygroscopicity and solubility

The values of water activity in the different treatments were less than 0.290 ± 0.004 , which is considered satisfactory (Table 6). The materials with values less than 0.6 are considered microbiologically stable (FENNEMA; DAMODARAN; PARKIN, 2010). The moisture values were less than $4.83\% \pm 0.1$, and with respect to hygroscopicity, values showed variation from 4.22 ± 0.25 to $6.68 \pm 0.22 \text{ g water/100 g of sample}$. These values facilitate the storage and application of the powders (COMUNIAN et al., 2016).

Table 6 shows that the solubility varied from $8.05\% \pm 0.44$ to $19.70\% \pm 0.48$. The complexes formed by coacervation are usually insoluble. The low solubility is a good indicator, as it shows that the complex remained stable in water; therefore, it offers greater protection of the bioactive materials and, thus, can be released under specific conditions (e.g. when there is a pH change).

Table 6 – The average particle size, water activity (Aw), moisture, hygroscopicity, density and solubility for each treatment.

Treatments	Average particle size (µm)	Aw	Moisture (%)	Hygroscopicity (g of water/ 100 g of sample)	Density (g/cm ³)	Solubility (%)
BGP1-0	45.61 ± 2.32 ^a	0.282 ± 0.003 ^{ab}	4.37 ± 0.10 ^c	4.22 ± 0.25 ^d	1.43 ± 0.01 ^{de}	8.05 ± 0.44 ^d
BGP1-5	47.79 ± 0.69 ^a	0.270 ± 0.002 ^{bc}	4.58 ± 0.04 ^b	5.28 ± 0.28 ^c	1.50 ± 0.01 ^b	17.90 ± 0.50 ^{ab}
BGP1-10	43.82 ± 0.65 ^a	0.254 ± 0.006 ^d	4.50 ± 0.03 ^{bc}	5.51 ± 0.07 ^{bc}	1.45 ± 0.003 ^{cd}	17.40 ± 0.85 ^b
BGP1-15	46.03 ± 2.50 ^a	0.280 ± 0.007 ^{ab}	4.64 ± 0.08 ^{ab}	6.29 ± 0.37 ^{ab}	1.47 ± 0.008 ^{bc}	15.01 ± 0.98 ^c
BLC1-0	47.62 ± 3.61 ^a	0.290 ± 0.004 ^a	4.49 ± 0.08 ^{bc}	5.54 ± 0.64 ^{bc}	1.41 ± 0.008 ^e	8.41 ± 0.54 ^d
BLC1-5	41.16 ± 0.41 ^a	0.289 ± 0.006 ^a	4.03 ± 0.07 ^d	5.59 ± 0.12 ^{bc}	1.55 ± 0.01 ^a	16.39 ± 1.01 ^{bc}
BLC1-10	40.84 ± 5.52 ^a	0.256 ± 0.003 ^d	4.83 ± 0.1 ^a	5.77 ± 0.22 ^{bc}	1.49 ± 0.01 ^b	19.70 ± 0.48 ^a
BLC1-15	51.75 ± 4.14 ^a	0.273 ± 0.004 ^c	4.6 ± 0.03 ^b	6.68 ± 0.22 ^a	1.41 ± 0.03 ^e	17.93 ± 0.68 ^{ab}

Means followed by the same lower-case exponent in the same column are not different ($p > 0.05$) by the Tukey test. Reference: Own source.

3.3.3.5 X-ray diffraction and Fourier transform infrared spectroscopy

X-ray diffraction analyses are used in studies of the parameters for encapsulation and structure of materials (crystalline or amorphous).

Figure 9 presents the obtained diffractograms. It is observed that fat and all powders samples have similar diffractograms with three peaks, which are characteristics of β' type crystal structure of fat. β' type fat crystals may undergo to phase transition to β type that is more stable and has greater crystals. This rearrangement can expel the probiotics; however, as these lipid particles have a second cover that are constituted for the polymer complexes, the probiotics will be ideally protected under the microcapsules.

The formation of β' crystals during production is what confers the interesterified fat qualities, such as plasticity and different qualities of melting and cooking (ROUSSEAU; MARANGONI, 2002).

Figure 9 – X-ray diffraction (A) of the treatments with BGP1, (B) of the treatments with BLC1 and (C) of the core and shell materials.

Reference: Own source.

The Fourier Transform Infrared Spectroscopy (FTIR) can be used to determine compounds or possible radicals formed by interactions during the encapsulation process. The spectra were obtained for wall materials, bioactive materials, and powders (Figure 10).

For vegetable fat, an elongation of saturated compounds C-H is observed at absorptions of 2916 cm^{-1} and 2849 cm^{-1} . There was an elongation of ester, aldehyde, and ketones C=O at the absorption of 1727 cm^{-1} , an elongation of the groups C-O , and flexion of the groups $\text{-CH}_2\text{-}$ in the absorption of 1173 cm^{-1} . The intensities and groups found corroborate with studies that have already evaluated the main bands of the spectrum for vegetable oils and fats (GUILLÉN; CABO, 1997; ZHANG et al., 2012).

Figure 10 – Spectra obtained by FTIR for (A) the treatments with BGP1, (B) the treatments with BLC1 and (C) the core and shell materials.

Reference: Own source.

The spectrum of BGP1 and BLC1 showed an absorption of 1098 cm^{-1} , and according to Feng et al. (2018), these correspond to bacterial proteins and nucleic acids. The PRCE showed an absorption at 3299 cm^{-1} that possibly corresponds to phenolic compounds and carbohydrates. The absorptions at 1606 and 1282 cm^{-1} correspond to great aromatic compounds and proanthocyanidins. These results are similar to the studies by Souza et al. (2018) and Ping et al. (2012).

Four main peaks were observed in the WPC spectrum, and according to Andrade et al. (2019), these are classified as the following: Amide A, representative of -OH and NH stretching in the band 3277 cm^{-1} ; Amide B, representative of =C-H and -NH_3^+ stretching in the 2923 cm^{-1} band; Amide I, representative of C=O stretching and NH bending in the 1633 cm^{-1} band; and Amide II,

representative of N-H bending and C-N stretching in the 1520 cm^{-1} band. Turgeon and Laneuville (2009) explained that the structure of the random coil (Amide B) of a protein has a more favorable conformation, and it can facilitate the interaction of biopolymers during the complexation process.

GA presented three typical bands at 3310 cm^{-1} , which is representative of -OH stretching, and both 1598 and 1411 cm^{-1} , which are representative of asymmetric and symmetric stretching of carboxyl acid salt -COO^- . Anvari and Chung (2016) and Souza et al. (2018) also observed this behavior.

In the powders, a spectrum common to all treatments was observed. There was a disappearance of the WPC band 2923 cm^{-1} (Amide B) and the GA bands 1598 and 1411 cm^{-1} (asymmetric and symmetric -COO^- stretching) that, according to Anvari and Chung (2016), indicate electrostatic interaction between amino groups of the protein and carboxylic groups of the polysaccharide. It is possible to observe the presence of bands at 2916 , 2849 , 1727 , and 1173 cm^{-1} , corresponding to the vegetable fat compounds, and the band at 1096 cm^{-1} represents the stretching of the C-O bond present in the proanthocyanidins and the presence of the probiotic in this region. There was a change in the wavelength in the region of amide A, I, and II in the coacervates with respect to the WPC; this may indicate the presence of bonds of other species between the phenolic compounds, probiotics, and wall materials of the particle.

3.3.4 Evaluation of probiotics, total phenolic compounds, and proanthocyanidins over 120 days of storage

Table 7 shows the quantification of probiotics, total phenolic compounds, and proanthocyanidins during powder storage for 120 days at 7 and $25\text{ }^{\circ}\text{C}$.

In the present study, the viability of probiotics stored at $7\text{ }^{\circ}\text{C}$ ranged from 6.03 ± 0.05 to $9.30 \pm 0.16\text{ log CFU / g}$ on day 120, which shows statistically higher values ($p \leq 0.05$) than those stored at 25°C , and these ranged from 4.31 ± 0.19 to $6.38 \pm 0.26\text{ log CFU / g}$ on day 120. The good results obtained from storage at 7°C can be explained by the fact that at this temperature cell viability rates improve because lower temperatures reduce the rates of harmful chemical reactions to microorganisms, such as fatty acid oxidation.

Table 7 – Quantification of encapsulated probiotics, total phenolic compounds and proanthocyanidins on days 0 and 120 at 7 °C and 25 °C.

Temperature		7 °C				
Treatments	Probiotics (log CFU/g)		Total phenolics (mg GAE/g)		Proanthocyanidins (mg B2/g)	
	Day 0	Day 120	Day 0	Day 120	Day 0	Day 120
BGP1-0	7.35 ± 0.08 ^{cA}	6.03 ± 0.05 ^{fB}	-	-	-	-
BGP1-5	7.35 ± 0.08 ^{cA}	6.27 ± 0.05 ^{efB}	32.66 ± 0.49 ^{cA}	29.3 ± 0.42 ^{efB}	10.21 ± 0.19 ^{cA}	7.83 ± 0.27 ^{fgB}
BGP1-10	7.35 ± 0.08 ^c	NS	52.10 ± 1.34 ^{bA}	45.94 ± 0.65 ^{cB}	17.26 ± 0.10 ^{bA}	17.56 ± 0.89 ^{dA}
BGP1-15	7.35 ± 0.08 ^{cA}	6.64 ± 0.10 ^{cdB}	82.98 ± 0.93 ^{aA}	76.97 ± 1.32 ^{aB}	26.46 ± 0.40 ^{aA}	21.82 ± 0.78 ^{bB}
BLC1-0	8.61 ± 0.19 ^{aA}	8.20 ± 0.10 ^{bB}	-	-	-	-
BLC1-5	8.61 ± 0.19 ^{aA}	9.30 ± 0.16 ^{aB}	32.94 ± 1.38 ^{cB}	37.25 ± 0.33 ^{dA}	10.16 ± 0.23 ^{cA}	10.44 ± 0.27 ^{eA}
BLC1-10	8.61 ± 0.19 ^{aA}	8.09 ± 0.04 ^{bB}	56.14 ± 3.00 ^{bA}	47.47 ± 0.90 ^{cB}	17.37 ± 0.81 ^{bA}	17.35 ± 0.99 ^{dA}
BGP1-15	8.61 ± 0.19 ^{aA}	8.21 ± 0.09 ^{bB}	83.77 ± 2.04 ^{aA}	64.88 ± 2.59 ^{bB}	26.16 ± 2.22 ^{aA}	26.19 ± 0.45 ^{aA}
Temperature		25 °C				
Treatments	Probiotics (log CFU/g)		Total phenolics (mg GAE/g)		Proanthocyanidins (mg B2/g)	
	Day 0	Day 120	Day 0	Day 120	Day 0	Day 120
BGP1-0	7.92 ± 0.08 ^b	NS	-	-	-	-
BGP1-5	7.92 ± 0.08 ^b	NS	32.66 ± 0.49 ^{cA}	25.66 ± 0.39 ^{fB}	10.21 ± 0.19 ^{cA}	9.46 ± 0.25 ^{efB}
BGP1-10	7.92 ± 0.08 ^{bA}	4.66 ± 0.11 ^{gB}	52.10 ± 1.34 ^{bA}	33.21 ± 4.14 ^{deB}	17.26 ± 0.10 ^{bA}	16.32 ± 0.20 ^{dB}
BGP1-15	7.92 ± 0.08 ^b	NS	82.98 ± 0.93 ^{aA}	62.21 ± 0.58 ^{bB}	26.46 ± 0.40 ^{aA}	21.82 ± 0.78 ^{bB}
BLC1-0	8.69 ± 0.15 ^{aA}	6.38 ± 0.26 ^{cdeB}	-	-	-	-
BLC1-5	8.69 ± 0.15 ^a	NS	32.94 ± 1.38 ^{cA}	34.63 ± 4.32 ^{dA}	10.16 ± 0.23 ^{cA}	7.44 ± 0.20 ^{gB}
BLC1-10	8.69 ± 0.15 ^a	NS	56.14 ± 3.00 ^{bA}	37.21 ± 0.95 ^{dB}	17.37 ± 0.81 ^{bB}	19.28 ± 0.25 ^{cA}
BLC1-15	8.69 ± 0.15 ^{aA}	4.31 ± 0.19 ^{hB}	83.77 ± 2.04 ^{aA}	61.17 ± 0.45 ^{bB}	26.16 ± 2.22 ^{aA}	27.58 ± 0.73 ^{aA}

Means with the same lower case letter in a column and means with the same upper case letter in a row are not statistically different ($p > 0.05$). *NS: not significant (microbial population below the detection limit of the method that is 500 CFU/g). Reference: Own source.

It was observed that, at a temperature of 7 °C (day 120), treatments with BLC1 showed a significantly higher viability ($p \leq 0.05$), ranging from 8.09 ± 0.04 to 9.30 ± 0.16 log CFU / g, than treatments with BGP1, which ranged from 6.03 ± 0.05 to 6.64 ± 0.10 log CFU / g. Depending on the conditions of the medium and water activity of the microcapsules, the viability of the probiotic may change during storage. The results of the present study were superior to those found by Silva et al. (2018), which encapsulated *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 by complex coacervation between gelatin and gum arabic and showed a count of less than 6 log CFU/g for both probiotics after 90 days of storage.

The addition of PRCE in the microparticles increased the viability of probiotics, especially when 5% of PRCE was added and stored at 7°C. BGP1-0 and BLC1-0 stored at 7°C (day 120) showed a count of 6.03 ± 0.05 log CFU / g and 8.20 ± 0.10 log CFU / g, respectively, but it was a value significantly lower than BGP1-5 and BLC1-5, which had a count of 6.27 ± 0.05 log CFU/g and 9.30 ± 0.16 log CFU / g, respectively. This demonstrates that the addition of PRCE can have a protective effect on the probiotic during storage, as it contains large amounts of phenolic compounds that can stimulate the growth of these bacteria due to the elimination of free radicals and oxygen.

The phenolic compounds present in the particles stored at 7°C (day 120) had a higher value than those stored at 25°C and varied between 29.3 ± 0.42 to 76.97 ± 1.32 mg GAE / g with BGP1, while phenolics with BLC1 obtained a variation from 37.25 ± 0.33 to 64.88 ± 2.59 mg GAE / g. It can be observed that the lower temperature favored the conservation of these compounds; another possible cause is that the greatest values occurred in treatments that had a greater viability of probiotics, which demonstrates that the insertion of bacteria allowed for an increase in the amount total phenolic compounds by the depolymerization of proanthocyanidins possibly carried out by these microorganisms. Thus, the present results suggest that small phenolic compounds can be more effective in protecting probiotics from oxidative stress than polymers, such as proanthocyanidins.

These results corroborate the study by Holkem et al. (2019) that investigated microencapsulated PRCE and BLC1 and applied it to sugarcane juice, observing an increase in the amount of phenolic compounds over 21 days of storage.

The proanthocyanidins present in the microparticles ranged from 7.44 ± 0.20 to 27.58 ± 0.73 mg B2/g (day 120). It was observed that treatments with BLC1 stored at 7 °C did not show

any significant difference ($p \leq 0.05$) at the end of storage compared to day 0. The degradation or depolymerization possibly caused by probiotics during storage did not decrease the levels of proanthocyanidins in some treatments. Prior et al. (2010) indicated that the degree of polymerization of these compounds can undergo great variations, which decrease and increase throughout the analysis period; furthermore, oligomers and monomers can be generated and quantified by the colorimetric method, thus increasing these levels.

3.4 CONCLUSION

With these results, it can be concluded that the solid lipid microparticles with probiotics and PRCE coated by electrostatic interactions that use the combination of WPC-GA were successfully formed, and they presented similar morphological and physicochemical characterizations for all treatments. Through turbidimetric titration and zeta potential measurement, the optimum coacervates were obtained at pH 4.2 with the WPC:GA mixing ratio of 6:1. Compared to the other treatments, the BLC1-5 treatment showed greater encapsulation efficiencies and large amounts of bioactive materials when stored at 7 °C after 120 days, which is explained by the high hydrophobicity of the BLC1 cell wall.

This study proves that co-encapsulation is promising because of the trapping two bioactive materials, which can be released together and present possibilities for synergies. These microparticles have potential applications in foods that need to be stored under refrigeration.

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4 CHAPTER 3 – STUDY OF ANTICANCER PROPERTIES OF PROANTHOCYANIDIN-RICH CINNAMON EXTRACT IN COMBINATION WITH *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BLC1 AND RESISTANCE OF THESE FREE AND CO-ENCAPSULATED MATERIALS UNDER *IN VITRO* SIMULATED GASTROINTESTINAL CONDITIONS³

ABSTRACT

Bifidobacterium animalis subsp. *lactis* (BLC1) and proanthocyanidin-rich cinnamon extract (PRCE) have many beneficial health properties. However, they are very sensitive materials; co-encapsulation is one alternative to protect them. Therefore, the objective of this work was to evaluate the anticancer properties of free PRCE and in combination with BLC1 and the resistance of these free and co-encapsulated materials under *in vitro* simulated gastrointestinal conditions. In terms of anticancer proprieties, PRCE had an IC₅₀ value close to 30 mg extract/mL for Hepa 1c1c7 and HT-29 cells and resulted in a significantly higher percentage ($p \leq 0.05$) of total apoptotic and necrotic cells compared to treatment in combination with BLC1 (PRCE + BLC1), with values above 31.66% in both cells. For the quinone reductase (QR) assay, there was a significant increase only for PRCE + BLC1 treatment, with a fold induction of 5.11 ± 0.56 for HT-29. The resistance of the encapsulated materials was greater than for the free form after 240 min of simulated gastrointestinal conditions. The combination of these materials in a microcapsule is advantageous because it protects them under gastrointestinal conditions, allowing them to be released into the intestine and act in the early stages of colon cancer.

Keywords: Probiotic; Cinnamon; Encapsulation; Gastrointestinal; Cancer.

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4.1 INTRODUCTION

Bifidobacterium is one of the genera of probiotics used in the production of fermented dairy products that may contribute to the maintenance or improvement of health and to disease prevention in humans. Their benefits are numerous, especially with regard to the gastrointestinal microbiota (KONG et al., 2019). Another important group of materials is proanthocyanidins, which are polyphenolic compounds (oligomers and polymers of flavan-3-ols) present in various plant extracts such as grape seeds. However, proanthocyanidins from cinnamon are less common because they have more type A double bonds than other sources (ANDERSON et al., 2004). To these molecules have been attributed several capacities such as: anti-obesity, antioxidant and anti-diabetes type 2, among others (ZARE et al., 2019).

For proanthocyanidins to exert their benefits, they must be released from their matrix and become absorbable in the intestine. Probiotics may contribute to this action occurring more rapidly, as they act by enzymatically converting the glycosides present in these compounds by releasing the more biologically active aglycones, that can be easily absorbed (MUELLER et al., 2016). Thus, the combination of these materials would be interesting because of the possibility of their acting in synergy.

Nonetheless, probiotics and proanthocyanidins are susceptible to degradation by pH changes, high temperatures, humidity, gastrointestinal environment, etc. Therefore, they require protection from rapid degradation and inactivation until they are delivered to the desired location; microencapsulation is one alternative for this protection. One of the widely used techniques is complex coacervation that consists of the interaction of polymers of opposite charges to form a coating. One of the advantages is that this technique does not require the use of high temperatures or organic solvents and so it is interesting for the encapsulation of bacteria and proanthocyanidins (COMUNIAN et al., 2016).

In a previous work (HOLKEM et al., 2019), we have studied the coencapsulation of *Bifidobacterium animalis* subsp. *lactis* BLC1 and proanthocyanidin-rich cinnamon extract added in sugarcane juice. The results were very promising, since it was possible to encapsulate these materials and incorporate them in a beverage. As far as we know, this was the first study to co-encapsulate probiotics and cinnamon proanthocyanidins.

In addition, to date, no study has evaluated the anticancer properties of proanthocyanidin-rich cinnamon extract (PRCE) alone and in combination with *Bifidobacterium animalis* subsp. *lactis* BLC1, as well as the resistance of these microencapsulated materials under *in vitro* simulated gastrointestinal conditions, which was the aim of this study.

4.2 MATERIAL AND METHODS

4.2.1 Materials

Ceylon cinnamon (*Cinnamomum zeylanicum* Blume), from the inner bark of trees, was obtained from WG Products (Waskaduwa, Sri Lanka). Lyophilized *Bifidobacterium animalis* subsp. *lactis* BLC1 was kindly donated by Sacco do Brasil (Campinas, SP, Brazil). AL CONF P45X LT vegetable fat with a melting point of 46 °C was donated by Cargill Agrícola S/A (Itumbiara, Goiás, Brazil). Lacprodan 80 whey protein concentrate (WPC) containing 80% protein was provided by Arla Foods Ingredients (São Paulo, SP, Brazil). Gum Arabic (GA) was purchased from Nexira (São Paulo/SP, Brazil).

4.2.2 Preparation of BLC1 inoculum

The inoculum was prepared according to Silva et al. (2017). The BLC1 was cultured in 10 mL in De Man, Rogosa and Sharpe (MRS) broth for 18 h at 37 ± 1 °C. The cells were transferred to 90 mL of MRS broth and cultured for 18 h at 37 ± 1 °C. Then, cells were centrifuged at $5752 \times g$ for 10 min at 10 °C and washed twice in 2% sodium citrate solution (SCS) being resuspended in 10 mL of 2% SCS and added to 500 mL of MRS broth being incubated under the same conditions. In this last stage, the culture was centrifuged under the same conditions and resuspended in 2% SCS to obtain approximately 10 log CFU / mL.

4.2.3 Production of PRCE

The procedure for the production of PRCE was based on the methodology of Souza et al. (2020). The cinnamon was triturated and added to 50% (w/ w) aqueous ethanol at a solid:solvent

ratio of 1:7.5 (w/v) for 30 min at 60 °C. The extract was centrifuged for 5 min at 25 °C and 7500 rpm in an Eppendorf 5430 centrifuge (Hamburg, Germany). The ethanolic extract was dried in an MSD 1.0 spray dryer (Labmaq of Brasil Ltda, Ribeirão Preto, Brazil). The obtained powder was stored in a polyethylene bottle covered with aluminum foil and stored in a freezer at –20 °C until used. The extract produced had a total phenolic content of 585.38 ± 13.73 mg GAE / g of powder and a proanthocyanidin content of 263.62 ± 12.98 mg procyanidin B2 / g of powder, according to the methodology of the section 4.2.7.

4.2.4 Anticancer properties

4.2.4.1 Colon cancer cell lines and cell maintenance

Hepa 1c1c7 (ATCC number: CRL-2026) and HT-29 (ATCC number: HTB-38) colon cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cell lines were cultivated as described by Fortin et al. (2017) in 75 cm² cellular flasks (Corning, NY, USA) at 37 °C in a humidified incubator under an atmosphere of 5% CO₂ and 95% air. Hepa 1c1c7 and HT-29 cells were grown in complete Dulbecco's Low Glucose Modified Eagle's Medium – DMEM (0.1% essential amino acids, 0.1% sodium pyruvate, 10% fetal bovine serum).

4.2.4.2 Antiproliferative assay for free PRCE

The free PRCE was serially diluted using phosphate-buffered saline (PBS) to give serial concentrations of 50.0, 25.0, 12.5, 6.25, 3.1, 1.55, 0.78 and 0.39 mg/mL in order to select the best concentration through an antiproliferative assay and then combine it with BLC1 for another assay. The antiproliferative effect was based on method of Vistica et al. (1991). The cells were seeded at a concentration of 2×10^4 cells/well of medium in a 96-well plate and incubated for 24 h at 37 °C in 5% CO₂. Then, the medium was removed and 100 µL of fresh medium containing 10 µL of serial concentrations of extracts was incubated for 48 h as mentioned above. The medium was then replaced with 225 µL of fresh medium containing 25 µL of a 0.5% (w/v) solution of thiazolyl blue tetrazolium bromide (MTT) followed by incubation for 4 h at 37 °C in 5% CO₂ in the dark. Finally,

the medium was carefully removed and replaced with 225 μL of dimethyl sulfoxide (DMSO) containing 25 μL of Sorensen 2504 buffer (0.1 mol/L glycine and 0.1 mol/L NaCl at pH 10.5). The microplate was read at 562 nm. PBS and medium were used as negative control and blank, respectively. Cellular growth inhibition was calculated as expressed below eq. (4):

$$\text{Cellular growth inhibition (\%)} = 100 - \left(\frac{\text{optical density of sample}}{\text{optical density of negative control}} \right) \times 100 \quad (4)$$

The IC_{50} value was calculated based on the dose–response curve between the sample concentrations and the percentage inhibition of cell growth. The IC_{50} value was defined as the PRCE concentration (mg/mL) required to inhibit 50% of cellular growth (VISTICA et al., 1991).

4.2.4.3 Quinone reductase (QR) assay for free PRCE and in combination with BLC1

QR assay was based on study of Prochaska and Santamaria (1988). Hepa 1c1c7 and HT-29 cells were seeded at a density of 1×10^4 cells/ mL in 96-well microplates in DMEM medium and were incubated 24 h at 37 °C in 5% CO_2 . Thereafter, the medium was removed and 20 μL of free PRCE (30.88 mg extract/mL for Hepa 1c1c7 and 36.65 mg extract/ mL for HT-29) or 20 μL of PRCE with 20 μL of BLC1 (1×10^9 CFU/mL) were added and completed each well up to a final volume of 200 μL of medium supplemented with fetal bovine serum and incubated for 48 h. Cells were washed three times with 200 μL of PBS solution and 50 μL of 1.6% (w/v) digitonin was added to each well and incubated for 20 min. Then, 20 μL of the sample was removed and used for total protein quantification while 200 μL of complete reaction mixture (0.25 mol/L Tris-HCl pH 7, 4.6% (w/v) bovine serum albumin, 0.01% Tween 20 mmol/L FAD, 1 mmol/L glucose-6-phosphate, 30 mol/L NADP, 34.8 $\mu\text{mol/L}$ MTT, 50 $\mu\text{mol/L}$ menadione and 2 mU/ μL glucose-6- phosphate dehydrogenase) was added to each well. The microplate was read at 595 nm after 5 min at room temperature. The protein test was performed using Pierce® Thermo Scientific reagents using the manufacturer's instructions. Controls consisted of dilution solution whereas medium was used as a blank. Specific activity was calculated and expressed as nanomoles of MTT formed per minute per milligram of protein. Fold induction was calculated as expressed below eq. (5):

$$\text{Fold induction} = \left(\frac{\text{specific activity of treated group}}{\text{Specific activity of negative control group}} \right) \quad (5)$$

A fold induction > 1 indicates that the compound stimulates QR activity whereas a result below 1 indicates that the compound does not stimulate QR.

4.2.4.4 Apoptosis assay for free PRCE and in combination with BLC1

Apoptosis was quantified by using the Annexin V-FITC Apoptosis Detection Kit, according to the manufacturer's instructions and Zhang et al. (1997). Initially, Hepa 1c1c7 and HT-29 were seeded in six-well plates at 1×10^5 cells/well (3 mL of medium/well) incubated as previously described for 24 h. Then, cells were incubated with 300 μ L of free PRCE (30.88 mg extract/mL for Hepa 1c1c7 and 36.65 mg extract/mL for HT-29) or 300 μ L of PRCE with 300 μ L of BLC1 (1×10^9 CFU/mL) completing each well up to a final volume of 3 mL using medium supplemented with fetal bovine serum for 48 h at 37 °C in 5% CO₂. Cells present in the supernatant were centrifuged for $500 \times g$ for 10 min at 4 °C. The adhered cells were treated with $1 \times$ trypsin-EDTA for 8 min at 37 °C. Then, 2 mL of DMEM medium was added and cells were centrifuged under the same conditions. Pellets (from the supernatant and the adhered cells) were washed twice with PBS. The cells were diluted in $1 \times$ binding buffer at 10^6 cells/mL and the evaluation of apoptosis was performed by flow cytometry (a total of 10,000 events; Epics XL-MCL, Beckman Coulter Canada, Inc., Mississauga, ON, Canada) using annexin V-FITC and propidium iodide (PI) for staining. Camptothecin (CPT) and PBS were used as positive and negative controls, respectively.

4.2.5 Production of microcapsules loaded with PRCE and BLC1

Microcapsules were produced by complex coacervation, according to the methodology proposed by Holkem et al. (2019). 1.5 g of BLC1 (1×10^{10} CFU/mL) with 1.12 g of PRCE were mixed with molten vegetable fat at 50 °C and homogenized using an Ultra-Turrax at 3500 rpm for 60 s. This mixture was homogenized with a solution of WPC (6% w/v) at 7000 rpm in an ultra-turrax for 90 s to obtain an emulsion (O/W). A solution of gum arabic (1% w/v) was added to the emulsion under magnetic stirring (500 rpm) and heating (50 °C). The pH of the mixture was

adjusted to 4.2 and the temperature reduced to 10 °C, in an ice bath. The microparticles were dehydrated by lyophilization using a Liobras L 202 freeze dryer (São Carlos, São Paulo, Brazil) for 72 h.

4.2.6 Enumeration of BLC1

Enumeration of BLC1 was determined by the drop plate method as described by Silva et al. (2017), using MRS medium. One milliliter of sample was dispersed in 9 mL of 2% SCS at 48 °C to completely dissolve the lipid matrix in microcapsules. The plates were incubated under anaerobiosis (Anaerobac, Probac, Brazil) at 37 °C for 72 h.

4.2.7 Total phenolic and proanthocyanidin content of PRCE

One milliliter of microcapsules was dispersed in 9 mL of a 0.5 M NaOH solution at 48 °C for 15 min, and centrifuged at 7500 rpm at 4 °C for 5 min. The aqueous phase was collected, and the precipitate was washed with 0.5 M HCl to extract some remaining compound. The total phenolic (SINGLETON; ORTHOFER; LAMUELA-RAVENTOS, 1999) and proanthocyanidin (PAYNE et al., 2010) content was determined using a Genesys 10S UV–Vis spectrophotometer (Thermo Scientific, São Paulo, SP, Brazil) at 750 and 640 nm absorbance, respectively, for 5 min at 10 s intervals in order to obtain the maximum absorbance value. The phenolic content of the samples was expressed in milligrams of gallic acid equivalent (GAE) per milliliter or gram of dry matter. Proanthocyanidins were expressed in milligrams of procyanidin B2 equivalent per milliliter or gram of dry matter

4.2.8 Resistance of free and encapsulated PRCE and BLC1 under simulated gastrointestinal conditions

In vitro digestion experiments were based on the harmonized COST INFOGEST protocol, in which the composition of simulated saliva fluid – SSF (15.1 mmol/L KCl; 3.7 mmol/L KH₂PO₄; 13.6 mmol/L NaHCO₃; 0.15 mmol/L MgCl₂(H₂O)₆; 0.06 mmol/L (NH₄)₂CO₃; 1.1 mmol/L HCl; 1.5 mmol/L CaCl₂(H₂O)₂), simulated gastric fluid – SGF (6.9 mmol/L KCl; 0.9 mmol/L KH₂PO₄;

25 mmol/L NaHCO₃; 47.2 mmol/L NaCl; 0.1 mmol/L MgCl₂(H₂O)₆; 0.5 mmol/L (NH₄)₂CO₃; 15.6 mmol/L HCl; 0.15 mmol/L CaCl₂(H₂O)₂) and simulated intestinal fluid – SIF (6.8 mmol/L KCl; 0.8 mmol/L KH₂PO₄; 85 mmol/L NaHCO₃; 38.4 mmol/L NaCl; 0.33 mmol/L MgCl₂(H₂O)₆; 8.4 mmol/L HCl; 0.6 mmol/L CaCl₂(H₂O)₂) was determined as described by Minekus et al. (2014). In the first step, microcapsules (1 g) or free BLC1 and PRCE (1 g) were added to 3 mL of SSF for 2 min and kept in a shaker at 37 °C and 200 rpm. In this step, salivary α -amylase was not added to the SSF, since the microcapsules did not contain starch. Next, 4 mL of SGF with porcine pepsin stock solution (2000 U/mL in the final mixture) was added to the system for 2 h, followed by the addition of 6.5 mL of SIF with pancreatin solution (100 U/mL of trypsin activity), totalizing 4 h of analysis. The 0.5 mL aliquots were collected every 30 min and the quantity of BLC1, phenolic compounds and proanthocyanidins was enumerated according to sections 4.2.6 and 4.2.7, respectively.

4.2.9 Statistical analysis

The data were submitted to analysis of variance – ANOVA (The data was normally distributed, the variance among the groups was approximately equal and the observations were independent of each other) using Statistica version 7.0 software (2004) (StatSoft Inc., Tulsa, OK, USA), followed by Tukey's means comparison test at a level of 5% significance. All data were expressed as the mean $\pm$ standard deviation.

4.3 RESULTS AND DISCUSSION

4.3.1 Anticancer properties

The antiproliferative assay results for free PRCE against cancer cells are shown in Figure 11. The IC₅₀ values for PRCE against Hepa 1c1c7 and HT-29 cells were 30.88 ± 6.98 and 36.65 ± 8.11 mg/mL, respectively. No significant difference ($p > 0.05$) was observed between cell type. Probably, the PRCE acted by blocking the genes involved in the cell cycle and in DNA replication, reducing cellular growth. This was expected, since numerous researches have indicated the antiproliferative effects of cinnamon against various cancer cells (KOPPIKAR et al., 2010; KWON

et al., 2010; SINGH et al., 2009). The extract produced in this work, rich in proanthocyanidins, showed strong antiproliferative effect against both cancer cells studied

Figure 11 – IC₅₀ (mg extract/mL) values of the antiproliferative assay for free PRCE against the cancer cells (A) Hepa 1c1c7 and (B) HT-29.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). Reference: Own source.

The QR assay results for free PRCE and in combination with BLC1 are shown in Figure 12. The results for free PRCE showed no significant increase ($p > 0.05$) in QR activity compared to the negative control for either cell type, with a fold induction of 0.76 ± 0.06 in Hepa 1c1c7 cells (Figure 12A), while in HT-29 cells (Figure 12B) the value was 1.34 ± 0.04 . Among the various constituents of cinnamon extracts, *trans*-cinnamaldehyde in particular contributes to most cytotoxic functions, especially increased QR activity (HUANG et al., 2011). However, in the present study, treatment with free PRCE did not result in a significant increase in QR activity although this extract contains *trans*-cinnamaldehyde (results for chromatographic profile unpublished). This may be due to correlations between different PRCE constituents, such as cinnamaldehyde, the mixture of oligomeric proanthocyanidins and cinnamic acid, and the different regulatory pathways of QR.

Figure 12 – Induction of quinone reductase (QR) by free PRCE and in combination of BLC1 against (A) Hepa 1c1c7 and (B) HT-29 cells.

Negative control: phosphate-buffered saline (PBS). Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). Reference: Own source.

Treatment with PRCE in combination with BLC1 resulted in a significant increase ($p \leq 0.05$) in QR activity compared to free PRCE, with a fold induction of 1.00 ± 0.08 in Hepa 1c1c7 and 5.11 ± 0.56 in HT29. Although free PRCE did not have a significant influence, the addition of BLC1 contributed to an increase in QR activity. This may have been due to the possible ability of these microorganisms to depolymerize proanthocyanidins into flavan-3-ol units, such as (+)-catechin and (–)-epicatechin, increasing phase II (QR) enzyme generation. In their study evaluating the inhibitory effect of oxidative stress of compounds isolated from ethanol (95% ethanol) extract of cinnamon, Li et al. (2019) confirmed that these flavan-3-ols units exhibit a more potent QR-inducing activity than cinnamaldehyde and its analogs.

The apoptosis assay of free PRCE and in combination with BLC1 in Hepa 1c1c7 and HT-29 cells is shown in Figure 13 and Figure 14, respectively. It was observed that the apoptosis percentage showed a significant difference ($p \leq 0.05$) only for free PRCE compared with the other cell treatments, with a value of $39.36 \pm 1.33\%$ for Hepa 1c1c7 and $31.66 \pm 0.64\%$ for HT-29. Regarding necrotic cells, there was a significant increase ($p \leq 0.05$) also observed for cells treated with free PRCE, with a percentage of $41.53 \pm 0.74\%$ for Hepa 1c1c7 and $51.83 \pm 0.80\%$ for HT-29.

Figure 13 – Representative flow cytometry analysis of apoptosis in Hepa 1c1c7 cells using annexin V / propidium iodide staining.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). (A) Positive control: camptothecin, (B) negative control: phosphate-buffered saline, (C) PRCE, (D) PRCE + BLC1, (E) percentage of total apoptotic cells (early and late apoptotic cells), (F) percentage of necrotic cells. Reference: Own source.

Figure 14 – Representative flow cytometry analysis of apoptosis in HT-29 cells using annexin V / propidium iodide staining.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). (A) Positive control: camptothecin, (B) negative control: phosphate-buffered saline, (C) PRCE, (D) PRCE + BLC1, (E) percentage of total apoptotic cells (early and late apoptotic cells), (F) percentage of necrotic cells. Reference: Own source.

In colon cancer cells, apoptosis is induced by ERF-dependent inactivation of extracellular signal-regulated kinase (NF- κ B), suppression of AP-1 DNA binding activity and down-regulation of c-Fos and c-Jun expression, or inhibition of 26S proteasome activity (SADEGHI et al., 2019). Possibly, free PRCE used one of these pathways to present higher percentages of apoptosis and necrosis; some studies have confirmed the increase of apoptosis by proanthocyanidins in several cancer cells, as demonstrated in the study by Gopalakrishnan et al. (2018) in which the procyanidin B2-enriched fraction of aqueous cinnamon extract had proapoptotic effects in prostate cancer cells. However, in this study, the addition of BLC1 to PRCE decreased apoptosis and necrosis rates; this may have been due to changes made by the microorganism to the proanthocyanidin aromatic ring, preventing one of the possible apoptosis induction pathways.

4.3.2 Resistance of free and encapsulated BLC1 and PRCE under simulated gastrointestinal conditions

Encapsulation provides a protective barrier for materials against the harsh conditions of the gastrointestinal tract, such as low pH and the presence of enzymes and bile salts. Figure 15 shows the resistance of free and encapsulated BLC1 and PRCE under simulated gastrointestinal conditions.

It can be observed that free BLC1 in the SGF phase (Figure 15A) showed a significant ($p \leq 0.05$) reduction of 4.32 log CFU/g up to 240 min under simulated gastrointestinal conditions. This result shows that the bacterial resistance decreased in an acid medium and in the presence of enzymes. In addition, PRCE was unable to act by stimulating the growth of these bacteria and may have acted as a pro-oxidant.

However, under the same conditions and after the same digestion period, a small increase (0.39 log CFU/g) in the population of encapsulated BLC1 was observed. The encapsulated BLC1 was more resistant to gastrointestinal conditions than the free BLC1, as expected, because the particle wall acted as a protective shield, making it difficult for H^+ ions to access cells. In the intestinal phase, due to the pH that disrupts the coacervate, as well as fat-digesting lipases, microparticles may have opened to release the probiotics as shown in the micrographs (Figure 15). These results corroborate those from the study by Silva et al. (2018), in which *B. animalis* subsp. *lactis* BLC1 was encapsulated by the complex coacervation technique; capsules exposed to simulated gastrointestinal conditions had an encapsulated probiotic survival rate above 80%.

Figure 15 – Gastrointestinal release profile of probiotics, phenolic compounds and proanthocyanidines encapsulated and free. Micrographs show the effect on microcapsules.

Means with different letter in a column are significantly different ($p \leq 0.05$). Reference: Own source.

Nonetheless, we observed an increase in probiotic count due to the possible effect of proanthocyanidins in stimulating these bacteria. This result corroborates the one reported by Holkem et al. (2019); they added free BLC1 and PRCE as well as encapsulated forms to sugarcane juice and obtained an increase in probiotic count on the first day of storage for the microcapsules compared to free BLC1.

In respect of the free total phenolic content (Figure 15B), it was observed that there was a significant ($p \leq 0.05$) reduction of 77.18% (8.80 ± 0.51 GAE/mL) of the initial total phenolic content up to 240 min under simulated gastrointestinal conditions. Although most phenolic compounds are stable at low pH, there was a reduction of these compounds, possibly due to SGF composition. Many studies have reported that the phenolic content of various plant extracts tends to decrease during simulated gastrointestinal digestion (JARA-PALACIOS et al., 2018; MARTÍNEZ-LAS HERAS et al., 2017; TAGLIAZUCCHI et al., 2010).

The encapsulated phenolic compounds showed a reduction of 8.87% (35.13 ± 3.04 GAE/mL) after 240 min under simulated gastrointestinal conditions compared to the initial content. It can be observed that the values increased considerably in the intestinal phase, because there was a rupture of the capsule and greater release in this phase resulting in a higher content than in the gastric phase, while the free compounds had already degraded in the gastric phase. This demonstrates that the complex coacervation method was efficient in protecting these compounds and their gradual release profile.

With respect to free proanthocyanidins (Figure 15C), there was a significant ($p \leq 0.05$) reduction of 82.68% (1.20 ± 0.01 mg B2/mL) compared to the initial content up to 240 min under simulated gastrointestinal conditions. As previously reported, these compounds are sensitive to changes in pH, *in vitro* gastrointestinal tract composition, enzymatic activity and structural degradation. For these encapsulated compounds, there was a significant ($p \leq 0.05$) reduction of 34.66% (4.73 ± 0.07 mg B2/mL) compared to the initial content. This can be attributed to the action of probiotics depolarizing these compounds to maintain their viability and this can be supported by the increased amount of BLC1 observed to the end of the simulated digestive process. López de Lacey et al. (2014) determined the survival of different probiotics during incubation in different green tea extracts; they observed that none of the bacteria were able to survive in cinnamon green tea. This corroborates the results of our study, since free PRCE in combination with BLC1 acted as a pro-oxidant, but we observed a different behavior when they were incorporated into microcapsules: the number of bacteria increased, allowing the use of PRCE and BLC1 together for food application, as there may be a synergism between them in the intestine.

4.4 CONCLUSION

This study demonstrated different anticancer effects of free PRCE or in combination with BLC1 against Hepa 1c1c7 and HT-29 cells. The free PRCE had a similar IC₅₀ value for the cell types and significantly ($p \leq 0.05$) increased apoptosis and necrosis but had no significant effect on QR activity in either cell type. The combination with BLC1 resulted in only a significant increase in QR compared to free PRCE.

In simulated gastrointestinal conditions, there was a greater resistance of encapsulated materials than free ones. The co-microencapsulation can deliver BLC1 and PRCE to the human intestinal system with less impact on functional properties and prevent cancer in the early stages.

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5 CHAPTER 4 – SUGARCANE JUICE WITH CO-ENCAPSULATED *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BLC1 AND PROANTHOCYANIDIN- RICH CINNAMON EXTRACT⁴

ABSTRACT

Bioactive compounds are sensitive to many factors, and they can alter the sensory characteristics of foods. Microencapsulation could be a tool to provide protection and allow the addition of bioactives in new matrices, such as sugarcane juice. This study focused on producing and evaluating the potential function of probiotics and proanthocyanidin-rich cinnamon extract (PRCE), both in free and encapsulated forms when added to sugarcane juice. The pure sugarcane juice treatment **T1** was compared to other sugarcane juice to which bioactive compounds had been added: **T2**, a non-encapsulated *Bifidobacterium animalis* subsp. *lactis* (BLC1); **T3**, a non-encapsulated BLC1 and PRCE; **T4**, BLC1 microcapsules, and **T5**, with BLC1 and PRCE microcapsules. The samples were morphologically, physico-chemically, rheologically, and sensorially characterized. Samples were also evaluated regarding the viability of BLC1 during the juice's storage at 4 °C. It was possible to produce probiotic sugarcane juice with non-encapsulated BLC1, but not with the addition of free PRCE, which in its free form reduced the viability of this microorganism to <1 log CFU/mL after 7 days. The microcapsules were effective to protect BLC1 during juice storage and to maintain high contents of phenolic and proanthocyanidin compounds, although the products containing these had their viscosity altered and were less accepted than either the control or those with non-encapsulated BLC1.

Keywords: Microencapsulation; *Bifidobacterium*; Polyphenols; Complex coacervation; Viability; Sensorial acceptance.

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5.1 INTRODUCTION

In addition to providing basic nutrition, functional foods have great beneficial effects on health and the prevention of diseases such as diabetes, cancer, cardiovascular diseases, and obesity (ARVANITOYANNIS; HOUWELINGEN-KOUKALIAROGLOU, 2005). This functionality derives from the bioactive ingredients present, which have an anti-inflammatory, anti-hypertensive action and modulate the intestinal microbiota (QUINTIN et al., 2018). Examples of these are: phenolic compounds, fiber, minerals, vitamins, fatty, carotenoids and probiotics (AYYASH et al., 2019).

Bifidobacteria are an important group of intestinal bacteria, representing about 3-7% of the microbiota in adults and, according to some reports, up to 91% in newborns (CHEIKHYOUSSEF et al., 2009). In particular, *Bifidobacterium animalis* subsp. *lactis* are often applied to probiotic products in combination with other bacteria (MASCO, et al., 2005; AIZAWA et al., 2016). They produce numerous beneficial effects on the host, such as: lowering the pH in the intestine producing a bactericidal effect; participation in the production of B vitamins and influence on the immune response; substrate fermentation in the gut resulting in the production of short chain fatty acids (SCFA); decreased serum ammonia levels by protein fermentation and is highly effective in preventing and treating a wide spectrum of gastrointestinal disorders such as intestinal transit diseases, infections, colon adenomas and cancer (BENGMARK, 1998; BLAUT, 2002). In addition, some studies have demonstrated the beneficial potential of bifidobacteria in reducing obesity, blood lipids and the risk of cardiovascular disease in patients with metabolic syndrome (BERNINI et al., 2016).

It is very common to incorporate probiotic bacteria into dairy products such as yogurts, cheeses and fermented milks. This represents one of the largest food markets (SONG; IBRAHIM; HAYEK, 2012). However, a large part of the population has some lactose intolerance, so having a variety of available probiotic products is of great importance (SILANIKOVE; LEITNER; MERIN, 2015; MORTAZAVIAN et al. 2010). Vegetable and fruit juices are rich in vitamins, carbohydrates and minerals (especially potassium) that contribute to maintaining alkaline reserve, building and repairing the body and are good vehicles for addition of probiotic bacteria (SEPTEMBRE-MALATERRE; REMIZE; POUCHERET, 2018).

Brazil is the world's largest grower of sugarcane (*Saccharum officinarum* L) and this crop is of the utmost importance for Brazilian agribusiness (CONAB, 2017). From cane, it is possible to extract juice that is an energetic, non-alcoholic beverage that has a sweet and pleasant flavor, low acidity and opaqueness and with a coloration ranging from brown to dark

green (BROCHIER; MERCALI; MARCZAK, 2016). Thus, the choice to produce a probiotic sugarcane juice becomes a diversification of the functional products already offered in the international market. As far as we know, this is the first study to co-encapsulate probiotics and proanthocyanidins added in sugarcane juice. Besides, the phenolics present in the sugarcane juice can aid in the preservation of the probiotic.

Some studies have shown that polyphenols (compounds that have the ability to donate protons and chelate metals, which implies high antioxidant capacity) may stimulate the growth of the beneficial microbiota while inhibiting pathogenic strains (DE LACEY et al. 2014). This is because the non-digestible phenolics can act as prebiotics by reducing oxidative stress, as well as the probiotics that degrade these compounds producing simpler phenolics capable of being digested (GONG, L. et al. 2018).

Ceylon cinnamon (*C. zeylanicum* Blume) is a rich source of phenolic compounds, mainly the proanthocyanins. Our research group's previous works studied the production, evaluation and encapsulation of proanthocyanidin-rich cinnamon extract (TULINI et al., 2016; OSTROSCHI et al., 2018; SOUZA, V. et al., 2018). A study by Li et al. (2013) demonstrated that two species of probiotics *Streptococcus thermophilus* and *Lactobacillus casei*-01 were able to metabolize Litchi pericarp type A procyanidins during their growth phase into simpler compounds (isomer, 3,4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, m-coumaric acid and p-coumaric acid). Thus, a vehicle containing these two bioactives may be relevant because of the synergism between them.

Still, both phenolic compounds and probiotics are very sensitive to many factors, so to maintain the stability and viability of these materials in a juice is not an easy task. Microencapsulation therefore becomes an efficient technology to protect these bioactives in microcapsules, microspheres or microparticles and to release them in the desired location (FAVARO-TRINDADE; PINHO; ROCHA, 2008; CHAI et al., 2018).

One of the most common methods of encapsulation is complex coacervation, which has been shown to be very efficient both in the protection of phenolic compounds and also with probiotics (MATOS-JR et al., 2019). It consists of spontaneous phase separation due to the formation of a complex that may be insoluble between two or more polymers resulting from electrostatic interactions (SINGH; BURGESS, 1989). Our group innovated using this method, since we promoted the immobilization of probiotics in a vegetable fat before the electrostatic interaction between the polymers resulting in true microcapsules, where bioactives are in the center of the particles and covered by two protective walls (SILVA et al., 2018; MATOS-JR et al., 2019).

This research work was conducted to develop and evaluate sugarcane juice with *Bifidobacterium animalis* subsp. *lactis* (BLC1) and/or proanthocyanidin-rich extract in both free and microencapsulated forms.

5.2 MATERIAL AND METHODS

5.2.1 Materials

Sugarcane (*Saccharum officinarum*, cultivar SP 3250, 16 months of maturation) was procured in Pirassununga / SP, Brazil. Lyophilized *Bifidobacterium animalis* subsp. *lactis* (BLC1) kindly donated by Sacco do Brasil (Campinas / SP, Brazil). Ceylon cinnamon (*C. zeylanicum* Blume) in bark obtained from WG Products (Waskaduwa, Sri Lanka). Vegetable fat AL CONF P45X LT with melting point of 46 °C was donated by Cargill Agrícola S/A (Itumbiara / Goiás, Brazil). Lacprodan 80 whey protein concentrated (WPC) provided by Arla Foods Ingredients (São Paulo / SP, Brazil). Gum Arabic (GA) by Nexira (São Paulo / SP, Brazil).

5.2.2 Preparation of bacterial suspension

The inoculum was prepared according to Silva et al. (2017). BLC1 was grown three times in MRS broth for 18 hours at 37 ± 1 °C. Then cells were centrifuged at $5752 \times g$ for 10 min at 10 °C and washed in 2% sodium citrate solution twice. The cell pellet (containing 10^{10} CFU/g) was used to prepare microparticles and to add in sugarcane juices in free form.

5.2.3 Production of PRCE

The extract was obtained according to Souza, V. et al. (2018). The bark cinnamon was ground in a blender (model RI 2008, Walita, Varginha, Brazil) obtaining a powder. The ground cinnamon was added in 50% (w/w) aqueous ethanol. The ratio of powdered cinnamon:solvent was 1:7.5 (w/v). The extraction occurred under mechanical agitation (model 713 agitator, Fisaton, São Paulo, Brazil) for 30 minutes, at 60 °C and protected from light. The mixture was filtered through cotton cloth for separation of the coarse particles and centrifuged for 5 minutes at 25 °C and 7500 rpm in a Model 5430 centrifuge (Eppendorf, Hamburg, Germany) for the separation of fine particles.

The ethanol extract was spray dried (spray dryer model MSD 1.0, Labmaq do Brasil Ltda, Ribeirão Preto, Brazil), using the inlet air temperature of 150 °C with a drying air velocity of 2.5 m/s and feed flow rate of 16 mL/min. The double spray atomizer nozzle of 1.2 mm and compressed air flow of 40 L/min was used. The obtained powder was stored in a polyethylene bottle covered with aluminum foil and stored in a freezer at -20 °C until the moment of use.

5.2.4 Microencapsulation

The production of microcapsules was performed using the method described by Matos-Jr et al. (2019). The cell pellet, alone or with 5% (w/w) PRCE relative to the total mass of solids, was mixed with the molten vegetable fat at 50 °C and homogenized using an ultra-turrax at 3500 rpm for 60 s. This mixture was homogenized with a 6% WPC solution at 7000 rpm in ultra-turrax for 90 s to obtain an emulsion. A 1% aqueous solution of GA was added keeping the mixture under magnetic stirring and heating. To promote the coacervation process, the pH of the blend was adjusted to 4.2 to promote interaction between the polymers. After the temperature of the mixture was gradually reduced to 10 °C using an ice bath, the coacervated material was maintained at 8 °C for decantation overnight. The supernatant was removed and the particles were accommodated in Falcon tubes, frozen at -20 °C and dehydrated in freeze dryer model L 202 (Liobras, São Carlos, São Paulo, Brazil) for 72 h. The obtained samples were conditioned in high density polyethylene bags and stored at -20 °C for later addition in the product.

5.2.5 Cane processing

The raw material was processed in a pilot plant at the University of São Paulo (Pirassununga/SP-Brazil). The extraction followed this procedure: The cane was sorted, cut, scraped and decontaminated by immersion in 0.05% (v/v) peracetic acid solution for 25 minutes at approximately 25 °C. The juice was extracted in an electric stainless steel cylinder mill manufactured by Maqtron (Joaçaba/SC, Brazil), pre-filtered in a 28 mesh nylon sieve and double filtered in a 35 mesh stainless steel sieve. The juice was then pasteurized at 90 °C/30 s, cooled to 8 °C and aseptically filled into high density polyethylene (HDPE) bottles. The aseptic filling was carried out in a semi-automatic gravimetric machine made by Polienva Movitron (São Paulo/SP – Brazil), in a unidirectional air-flow cabinet (ISO class 5) manufactured by VECO (Campinas/SP-Brazil). The microcapsules or non-encapsulated BLC1, individually or

together with PRCE, were homogenized in the juice using an ultra-turrax at 3500 rpm for 60s. The end product was stored at 4 °C for 28 days.

Five treatments were differentiated in relation to the presence or absence of microcapsules and free bioactive compounds. These were as follows: **T1** - control sugarcane juice, composed of the basic sugarcane juice formulation; **T2** - juice containing the non-encapsulated BLC1 (concentration of 0.001% (w/v) to result a product with approximately 10^7 CFU/mL); **T3** - juice containing the non-encapsulated 0.001% (w/v) BLC1 and 0.3% (w/v) PRCE in the same ratio as used in the microparticles; **T4** - juice containing 5% (w/v) microcapsules with BLC1; **T5** - juice containing 5% (w/v) microcapsules with BLC1 and PRCE.

5.2.6 Characterization of the sugarcane juice

The analyses to characterize the sugarcane juice were carried out every 7 days during 28 days of storage.

5.2.6.1 Morphological characterization

The evaluation of the morphology was performed only on day 1, when an aliquot of the sugarcane juice was placed on a glass slide that was visualized in an optical microscope (BEL Photonics - Milan, Italy) equipped with camera and BEL View v.62 software.

5.2.6.2 Physical-chemical characterization

5.2.6.2.1 pH and titratable acidity

The pH of juice samples was measured using a pH meter (Marte MB10, São Paulo, Brazil). The total titratable acidity was determined by titrating to pH 8.2 with 0.05 M NaOH and expressed in g citric acid / 100 g sample (AOAC, 2010).

5.2.6.2.2 Total soluble solids (TSS) and density

The total soluble solids content and density were determined with a handheld portable refractometer (Briobrix 103, São Paulo, Brazil) at 20 °C.

5.2.6.2.3 Instrumental color

Samples were analyzed in Hunterlab MiniScan XE Plus colorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA). The CIELab color system was used to measure the parameters L^* (lightness), a^* (green to red), b^* (blue to yellow). The total color difference (ΔE) was obtained by eq. (6) described by Comunian et al. (2017).

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (6)$$

5.2.6.2.4 Quantification of total phenolic compounds and proanthocyanidins

The preparation of samples for total phenolic compounds and proanthocyanidin quantification was based on the methodology reported by Tulini et al. (2016) with modifications in some reagents. One mL of sample was dispersed in 9 mL of a 0.5 M NaOH solution, heated at 48 °C for 15 minutes and centrifuged at 7500 rpm / 4 °C for 5 min. The aqueous phase was collected and the precipitate was washed with 10 mL of 0.5 M HCl to extract some remaining compound. The solutions were pooled and adjusted to 20 mL with distilled water and used for quantification.

The total phenolic content was determined by Genesys 10S UV-vis spectrophotometer (Thermo Scientific, São Paulo / SP, Brazil) at absorbance at 750 nm, according to Singleton, Orthofer and Lamuela-Raventos (1999) and Souza, V. et al. (2018). The content was estimated from the calibration curve ($r^2 = 0.9984$), by plotting known solutions of gallic acid (Acros Organics, New Jersey, USA). The phenolic content of the samples was expressed in milligrams of gallic acid equivalent (GAE) per mL or g of dry matter.

The proanthocyanidins content was determined by Genesys 10S UV-vis spectrophotometer (Thermo Scientific, São Paulo, SP, Brazil) at absorbance at 640 nm for 5 min at 10 s intervals in order to obtain the maximum absorbance value, according to Payne et al. (2010). The content was estimated from the calibration curve ($r^2 = 0.9976$) by plotting known solutions of Procyanidin B2 (EP, Sigma-Aldrich, St. Louis, USA). Proanthocyanidins were expressed in milligrams of Procyanidin B2 equivalent per ml or g of dry matter.

5.2.6.3 Microbiological characterization

The preparation of the samples for microbiological analyses was performed as described by Silva et al. (2017), in which 5 ml of sample were diluted in 45 ml of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$) heated to 48 °C. This solution was then homogenized for 2 minutes in stomacher and 1 ml aliquots were serially diluted.

The psychrotroph counts were performed by the spread plate method, using as culture medium the plate count agar (PCA). The plates were incubated inverted at 7 °C for 10 days before the colony count was enumerated.

The mold and yeast counts were performed by the spread plate method, using Dichloran Rose-Bengal Chloramphenicol (DRBC) as the medium. They were incubated at 25 °C for 5 days, before the colony count.

The probiotic count was performed by the drop plate method, using De Man medium, Rogosa and Sharpe (MRS). The plates were incubated under anaerobiosis (Anaerobac, Probac, Brazil) at 37 °C for 72 h, before the colony count.

5.2.6.4 Rheological characterization

The viscosity of the samples was determined using a rotational rheometer AR 2000, TA Instruments (New Castle, Delaware, USA), with double concentric cylinder geometry (gap 2000 μm) and the software Rheology Advantage Instrument control AR and Rheology Advantage Data Analysis. The rheological measurements were obtained at 4 °C and 37 °C, according to the method of Astolfi-Filho et al. (2011). A 10.77 mL aliquot of sugarcane juice was maintained in equilibrium for 120 s and then analyzed for 6 min (3 min up and 3 min down). Also, the flow curve was analyzed at a shear rate from 4 to 180 s^{-1} and the results modeled, according to the Ostwald-de-Waele or Power-Law equation (eq. (7)):

$$\tau = k(\dot{\gamma})^n \quad (7)$$

Where, k and $\dot{\gamma}$ represent the consistency index (mPa.s) and shear rate (s^{-1}), respectively. Values of n are necessary to define the type of fluid: $n = 1$: Newtonian fluid; $n < 1$: pseudoplastic fluid and $n > 1$: dilating fluid (RAO, 1999).

5.2.6.5 Sensory acceptance and purchase intention

The product's acceptability was evaluated by using the nine-point hedonic scale test, as described by Meilgaard, Civille and Carr (1999). A panel of 120 untrained assessors, of both genders at ages between 17 and 37, was recruited through the availability, frequency and interest in the consumption of sugarcane juice. A sample of approximately 30 mL at a temperature of 10 °C was monadically and randomly served in 3-digit coded plastic cups. The attributes of flavor, texture, color, aroma and overall acceptance were evaluated a structured scale. The purchase intention evaluation was also carried out by using 5-point scale. This study was approved by the Research Ethics Committee of the Faculty of Animal Science and Food Engineering of the University of São Paulo (87062618.4.0000.5422 - Attachment D).

5.2.7 Data statistical analysis

The data were statistically analyzed using the statistical program Statistica version 7.0 software (Statsoft Inc., Tulsa, OK, USA), by analysis of variance (ANOVA) and Tukey's test, at the significance level of 5%.

5.3 RESULTS AND DISCUSSION

5.3.1 Morphological characterization

Figures 16A and 16B evidence the presence of particles originating from sugarcane that was very fibrous in the T1 and T2 treatments. This is due to the fact that, although the juice was filtered, there were still suspended and colloidal particles. The clarification process would be an alternative to remove these impurities; however, we chose to maintain the characteristics of the product frequently found on the market. The Figure 16C also shows higher particles that are non-encapsulated PRCE that contained many hydrophobic compounds, which remained insolubilized.

In Figures 16D and 16E it is possible to visualize the structure of the microcapsules in the sugarcane juice. The microcapsules have spherical shape, different diameters, and they are of the reservoir type, where the core is surrounded by wall material. Similar images have been reported by Silva et al. (2018); Comunian et al. (2013); Souza, C. et al. (2018). This

demonstrated that the homogenization of the microparticles in the juice after the pasteurization did not cause major modifications or ruptures in their structures.

Figure 16 – Micrographs obtained by optical microscopy of sugarcane juice samples.

In this figure: (A) T1: control (whole cane juice); (B) T2: juice containing non-encapsulated BLC1; (C) T3: juice containing non-encapsulated 0.001% (w/v) BLC1 and 0.3% (w/v) PRCE; (D) T4: juice containing 5% (w/v) microcapsules with BLC1; (E) T5: juice containing 5% (w/v) microcapsules with BLC1 and PRCE.
Reference: Own source.

5.3.2 pH and titratable acidity

The Table 8 shows the results of the physicochemical analyzes of each treatment of sugarcane juice stored at 4 ° C for 28 days. Comparison of the values from day 1 to storage on day 28 demonstrated that the pH and titratable acidity of the products did not differ significantly ($p > 0.05$), except for the T3 and T4 formulations for titratable acidity. The stability of pH values and titratable acidity of probiotic sugarcane juice may be related to the buffering capacity of juices as reported by Nualkaekul, Salmeron and Charalampopoulos (2011) and Costa et al. (2017).

T1 treatment had pH 5.05 ± 0.03 on day 1, a significantly higher value ($p \leq 0.05$) in relation to the other treatments, which had values ranging from 3.98 to 4.86. This demonstrated that addition of the non-encapsulated BLC1 and/or PRCE or microparticles reduced the pH values of the juices. The addition of metabolically active probiotic bacteria causes some inherent sugars to metabolize and organic acids to be produced, which decreases the pH of the medium. This also occurred with microparticles due to the presence of some probiotics on the surface.

5.3.3 Total soluble solids (TSS) and density

Regarding the results of TSS and density, there was no significant difference ($p > 0.05$) comparing the values at the beginning of the storage with the final values in all the treatments, so time and temperature variability did not influence these parameters. However, from day 1 to 21, a statistically significant difference ($p \leq 0.05$) was observed in the T4 and T5 treatments in relation to the others for both TSS and density. The addition of microparticles in the sugarcane juice resulted in the increase of soluble solids and density. This was due to the composition of the capsule, which is made up of materials that have an expressive density, such as vegetable fat, protein and polysaccharide.

Table 8 – Values of pH, titratable acidity, TSS (°Brix) and density for each treatment during 28 days at 4 °C.

Treatments / days	1	7	14	21	28
pH					
T1	5.05 ± 0.03 ^{aA}	5.05 ± 0.08 ^{aA}	5.08 ± 0.06 ^{aA}	5.08 ± 0.03 ^{aA}	5.03 ± 0.01 ^{aA}
T2	4.68 ± 0.38 ^{aB}	5.01 ± 0.11 ^{aAB}	4.86 ± 0.23 ^{aB}	4.81 ± 0.29 ^{aB}	4.69 ± 0.37 ^{aB}
T3	4.86 ± 0.04 ^{aAB}	4.86 ± 0.08 ^{aB}	4.89 ± 0.05 ^{aAB}	4.88 ± 0.05 ^{aAB}	4.86 ± 0.02 ^{aAB}
T4	3.98 ± 0.16 ^{cC}	4.22 ± 0.00 ^{aD}	4.16 ± 0.04 ^{abD}	4.08 ± 0.05 ^{abcC}	4.03 ± 0.07 ^{bcC}
T5	4.31 ± 0.10 ^{bcC}	4.50 ± 0.05 ^{aC}	4.43 ± 0.04 ^{abC}	4.32 ± 0.04 ^{bcC}	4.22 ± 0.14 ^{cC}
Titratable acidity (g citric acid / 100 g)					
T1	0.068 ± 0.008 ^{aB}	0.060 ± 0.002 ^{bD}	0.060 ± 0.003 ^{bD}	0.061 ± 0.001 ^{bD}	0.064 ± 0.002 ^{abC}
T2	0.082 ± 0.015 ^{aB}	0.062 ± 0.003 ^{bD}	0.068 ± 0.007 ^{abD}	0.069 ± 0.007 ^{abCD}	0.076 ± 0.010 ^{abC}
T3	0.071 ± 0.001 ^{bB}	0.083 ± 0.005 ^{aC}	0.079 ± 0.001 ^{aC}	0.078 ± 0.001 ^{aC}	0.080 ± 0.005 ^{aC}
T4	0.234 ± 0.019 ^{aA}	0.200 ± 0.004 ^{bA}	0.196 ± 0.001 ^{bA}	0.196 ± 0.009 ^{bA}	0.212 ± 0.007 ^{bA}
T5	0.253 ± 0.060 ^{aA}	0.166 ± 0.003 ^{bB}	0.183 ± 0.004 ^{bB}	0.188 ± 0.004 ^{bB}	0.209 ± 0.013 ^{abB}
TSS (°Brix)					
T1	19.73 ± 0.68 ^{aB}	19.98 ± 1.01 ^{aB}	19.99 ± 0.59 ^{aB}	19.96 ± 0.57 ^{aB}	20.78 ± 1.25 ^{aA}
T2	19.51 ± 0.41 ^{aB}	19.88 ± 0.85 ^{aB}	19.98 ± 0.41 ^{aB}	20.03 ± 0.37 ^{aB}	20.76 ± 1.23 ^{aA}
T3	19.61 ± 0.41 ^{aB}	19.95 ± 0.85 ^{aB}	20.31 ± 0.58 ^{aB}	20.13 ± 0.20 ^{aB}	20.83 ± 1.10 ^{aA}
T4	21.18 ± 0.28 ^{aA}	21.51 ± 0.68 ^{aA}	21.79 ± 0.47 ^{aA}	21.66 ± 0.53 ^{aA}	22.16 ± 1.03 ^{aA}
T5	21.35 ± 0.29 ^{aA}	21.88 ± 0.60 ^{aA}	21.81 ± 0.52 ^{aA}	21.69 ± 0.43 ^{aA}	22.19 ± 1.06 ^{aA}
Density (g/mL)					
T1	1.083 ± 0.003 ^{aB}	1.081 ± 0.003 ^{aB}	1.081 ± 0.002 ^{aB}	1.082 ± 0.002 ^{aB}	1.087 ± 0.006 ^{aA}
T2	1.083 ± 0.003 ^{aB}	1.082 ± 0.002 ^{aB}	1.083 ± 0.001 ^{aB}	1.082 ± 0.002 ^{aB}	1.086 ± 0.005 ^{aA}
T3	1.084 ± 0.002 ^{aB}	1.083 ± 0.002 ^{aB}	1.084 ± 0.002 ^{aB}	1.082 ± 0.001 ^{aB}	1.087 ± 0.004 ^{aA}
T4	1.090 ± 0.003 ^{aA}	1.090 ± 0.002 ^{aA}	1.090 ± 0.002 ^{aA}	1.090 ± 0.003 ^{aA}	1.092 ± 0.005 ^{aA}
T5	1.091 ± 0.002 ^{aA}	1.091 ± 0.001 ^{aA}	1.090 ± 0.003 ^{aA}	1.091 ± 0.001 ^{aA}	1.092 ± 0.005 ^{aA}

Means followed by the same lowercase exponent in the same row. And the same uppercase in the column are not different ($p > 0.05$) by the Tukey test. T1: control (whole cane juice); T2: juice containing non-encapsulated BLC1; T3: juice containing non-encapsulated 0.001 % (w/v) BLC1 and 0.3 % (w/v) PRCE; T4: juice containing 5 % (w/v) microcapsules with BLC1; T5: juice containing 5 % (w/v) microcapsules with BLC1 and PRCE.

Reference: Own source.

5.3.4 Color

The parameters of L^* , a^* , b^* and the total color difference (ΔE) are shown in Table 9 for each treatment during storage. The L^* values ranged from 31.89 to 61.38 and 32.13 to 62.80 for sugarcane juice at day 1 and 28, respectively. There was a significant difference ($p \leq 0.05$) between treatments for the same day, but between the different days there was no significant difference. In T1 and T2 treatments, a small difference was observed throughout the storage, making it possible to state that the addition of the non-encapsulated probiotic bacteria did not result in a large difference. Regarding the T3 treatment, a significant difference ($p \leq 0.05$) was observed compared to the other treatments. This was due to the addition of non-encapsulated PRCE that diminishes luminosity by its own brown coloration. The treatments T4 and T5 had higher values compared to the others due to the presence of the encapsulating materials that have a whitish coloration.

Regarding the parameters, a^* and b^* , all treatments presented positive values for both and indicated a tendency of color for red and yellow, respectively. This was possibly due to the extraction of the juice in the early stages of maturation of sugarcane and the presence of tannins, increasing significantly ($p \leq 0.05$) the values of a^* and b^* , as was also found in the study by Qudsieh et al. (2002). One can highlight the treatment T3, which presented a greater and smaller value significantly in relation to the others for the parameter a^* and b^* , respectively. In this case, the presence of proanthocyanidins contributed to this difference.

Concerning the total color difference (ΔE), it was observed that T1 and T2 treatments presented values of 4.32 ± 1.76 and 4.68 ± 1.66 for day 28, respectively. These values were significantly higher ($p \leq 0.05$) in relation to the other treatments. It was observed that in T1 and T2 there was a higher sedimentation of the compounds and enzymatic browning, which contributed to the higher values. This was also reported by Yusof and Osman (2000). For the treatments T4 and T5, there was a lower value in the difference of total color because the addition of microparticles diluted pigments (probably flavonoids) from sugarcane.

Table 9 – Values of L*, a*, b* for each treatment in the period of 28 days.

Treatments / days	1	7	14	21	28
L*					
T1	38.24 ± 1.17 ^{abC}	39.40 ± 0.58 ^{aC}	36.63 ± 2.01 ^{bC}	39.34 ± 2.54 ^{aD}	37.60 ± 1.68 ^{abD}
T2	39.75 ± 1.69 ^{abC}	38.23 ± 1.45 ^{bD}	37.91 ± 1.56 ^{bC}	41.27 ± 0.52 ^{aC}	41.60 ± 1.23 ^{aC}
T3	31.89 ± 1.37 ^{abD}	33.88 ± 0.17 ^{aE}	30.04 ± 3.93 ^{bD}	31.53 ± 2.51 ^{abE}	32.13 ± 1.48 ^{abE}
T4	61.38 ± 1.31 ^{abcA}	59.44 ± 0.36 ^{cA}	60.94 ± 0.94 ^{bcA}	62.36 ± 0.97 ^{abA}	62.80 ± 2.64 ^{aA}
T5	56.01 ± 0.58 ^{aB}	55.36 ± 0.73 ^{aB}	55.52 ± 0.68 ^{aB}	56.43 ± 1.22 ^{aB}	56.72 ± 2.15 ^{aB}
a*					
T1	5.81 ± 0.19 ^{bcD}	5.95 ± 0.26 ^{cD}	8.23 ± 1.08 ^{aC}	7.62 ± 0.98 ^{abC}	8.22 ± 1.72 ^{aC}
T2	7.02 ± 0.23 ^{abC}	6.63 ± 0.36 ^{bC}	7.18 ± 0.69 ^{aC}	7.11 ± 0.23 ^{abC}	7.18 ± 0.65 ^{abD}
T3	12.45 ± 0.85 ^{abA}	12.24 ± 0.12 ^{bA}	13.88 ± 2.18 ^{aA}	12.06 ± 0.67 ^{bA}	11.63 ± 0.78 ^{bA}
T4	4.56 ± 0.03 ^{abE}	4.55 ± 0.05 ^{bE}	4.98 ± 0.37 ^{abD}	5.10 ± 0.23 ^{abD}	5.34 ± 0.93 ^{aE}
T5	9.95 ± 0.14 ^{abB}	9.77 ± 0.15 ^{bB}	9.86 ± 0.14 ^{bB}	10.01 ± 0.21 ^{abB}	10.16 ± 0.28 ^{aB}
b*					
T1	25.82 ± 0.65 ^{aA}	25.49 ± 0.95 ^{aA}	24.93 ± 2.34 ^{aAB}	26.62 ± 2.10 ^{aAB}	26.22 ± 3.10 ^{aA}
T2	25.46 ± 1.44 ^{aA}	25.49 ± 1.78 ^{aA}	25.00 ± 4.98 ^{aAB}	27.52 ± 0.58 ^{aA}	26.83 ± 2.89 ^{aA}
T3	21.00 ± 1.50 ^{abB}	20.62 ± 0.22 ^{abB}	22.09 ± 2.31 ^{aB}	19.99 ± 2.72 ^{bcD}	18.22 ± 0.61 ^{cB}
T4	24.98 ± 0.35 ^{aA}	24.57 ± 0.08 ^{aA}	25.43 ± 0.31 ^{aA}	24.70 ± 0.79 ^{aC}	25.22 ± 1.37 ^{aA}
T5	25.59 ± 0.03 ^{aA}	25.64 ± 0.40 ^{aA}	25.54 ± 0.45 ^{aA}	25.60 ± 0.51 ^{aBC}	25.59 ± 0.63 ^{aA}
ΔE					
T1	-	1.45 ± 0.80 ^{bBC}	4.28 ± 1.28 ^{aA}	3.98 ± 1.07 ^{aA}	4.32 ± 1.76 ^{aAB}
T2	-	2.28 ± 0.64 ^{bA}	4.90 ± 2.00 ^{aA}	3.87 ± 0.43 ^{aA}	4.68 ± 1.66 ^{aA}
T3	-	2.05 ± 0.19 ^{bAB}	4.86 ± 2.93 ^{aA}	3.80 ± 1.04 ^{abA}	3.39 ± 0.33 ^{abB}
T4	-	1.99 ± 0.34 ^{bAB}	1.29 ± 0.19 ^{bB}	1.65 ± 0.48 ^{bB}	3.32 ± 1.17 ^{aBC}
T5	-	0.98 ± 0.46 ^{bC}	0.84 ± 0.48 ^{bB}	1.35 ± 0.41 ^{bB}	2.15 ± 1.01 ^{aC}

Means followed by the same lowercase exponent in the same row and same uppercase exponent in the same column are not different ($p > 0.05$) by the Tukey test. T1: control (whole cane juice); T2: juice containing non-encapsulated BLC1; T3: juice containing non-encapsulated 0.001% (w/v) BLC1 and 0.3% (w/v) PRCE; T4: juice containing 5% (w/v) microcapsules with BLC1; T5: juice containing 5% (w/v) microcapsules with BLC1 and PRCE.

Reference: Own source.

5.3.5 Quantification of total phenolic compounds and proanthocyanidins

The content of total phenolic compounds and proanthocyanidins are shown in Figures 17A and 17B, respectively. The initial phenolic contents in the T3 and T5 treatments showed mean values of 1.33 ± 0.06 and 1.73 ± 0.11 mg GAE/mL, respectively, and in 21 days the values were 2.05 ± 0.22 and 2.22 ± 0.18 mg GAE/mL, respectively. The results demonstrated that co-encapsulated BCL1 and PRCE as well as for these free bioactives, the phenolic content increased significantly ($p \leq 0.05$) up to 21 days. This effect can be attributed to the probiotics can consume certain polyphenolic compounds, reducing them in derived compounds thus increasing the amount of phenolic compounds in the product. Similar results were reported by Filho et al. (2019), who produced cocoa juice with sucralose and *Lactobacillus casei* and observed an increase in polyphenols from 40.00 ± 0.02 , at the fermentation's beginning to 49.63 ± 0.04 mg / 100mL at the 24 h mark. Regarding the non-encapsulated BLC1 and PRCE, there was a significant increase ($p \leq 0.05$) in the amount of phenolics, but this effect can be attributed to probiotics only from day 1 to day 7, because in the rest of the storage there was no presence of bacteria as shown in Figure 18. The effect of increasing the amount of these compounds can be attributed to degradation of polymeric proanthocyanidins during storage and limitations in the colorimetric detection method used. Corroborating the study by Lee (2010) who observed the degradation kinetics of phenolic compounds such as grape skin and seed proanthocyanidins and noticed that several oxidation reactions and variation in the degree of polymerization of these compounds during storage time decreasing and increasing over the analysis period.

Treatments T1, T2, and T4 presented mean values of 0.32 ± 0.04 , 0.34 ± 0.056 and 0.82 ± 0.04 in 28 days. A certain stability of phenolic compounds was observed during storage for these treatments. This may have happened due to the absence of PRCE, which has proanthocyanidins that can suffer depolymerization during storage. Phenolic compounds are stable at low pH (KWAU et al. 2017) and the pHs of sugarcane juice for T1, T2, T4 presented a low value between 4-5 and did not present significant variation ($p > 0.05$) during storage (Table 8), indicating stability for these compounds.

Figure 17 – Stability of phenolic compounds (A) and proanthocyanidins (B) during storage for 28 days in sugarcane juice.

In this figure: T1: control (whole cane juice); T2: juice containing non-encapsulated BLC1; T3: juice containing non-encapsulated 0.001% (w/v). Reference: Own source.

The content of proanthocyanidins in the T3 treatment did not show significant difference ($p > 0.05$) during the storage. This is due to the fact that probiotics in this treatment did not survive in a considerable amount up to 7 days (Figure 3A) to have any effect on these compounds. Ostroschi et al. (2018) produced particles from PRCE using a spray-dryer and evaluated the content of proanthocyanidins during 90 days of storage for both free and encapsulated extract. These authors observed that in the liquid extract a significant decrease of proanthocyanidins content occurred after 90 days, and they attributed this result, among other reasons, to the presence of high water content. However, Ostroschi et al. (2018) evaluated content of proanthocyanidins every 30 days, but we analyzed them every 7 days during just 28 days, and they were in a completely different medium. Our results demonstrate that proanthocyanidins from cinnamon, even in free form, are stable in sugarcane juice that is stored at refrigeration temperatures for 28 days.

With respect to content of proanthocyanidins in the treatments, T5 presented values on day 1 of 0.19 ± 0.01 mg B2/mL and then on day 28 of 0.12 ± 0.03 mg B2/mL. There was a significant ($p \leq 0.05$) decrease in the amount of these compounds during storage. This can be attributed to the action of the probiotic together with the polyphenols within the particle, which reduces these compounds and maintains the viability of the bacteria, as the counts in Figure 18 prove. Similar results were found by Nematollahi et al. (2016), who investigated the viability of the probiotic in cornelian cherry juice and concluded that there was a decrease in the amount of anthocyanins present in the juice due to the activity of probiotic bacteria.

5.3.6 Microbiological characterization

To be considered probiotic, it is recommended that a product has counts above 10^6 CFU/mL. In this study a population of approximately 10^7 CFU/mL of BLC1 were inoculated in all formulations. The stability of BLC1 during storage is shown in Figure 18.

The treatment T2, containing free BLC1, presented counts of 6.63 ± 0.05 log CFU/mL and 6.56 ± 0.00 log CFU/mL on days 1 and 28, respectively. No significant difference ($p > 0.05$) was observed during storage. It was found that free BLC1 did not lose its viability in sugarcane juice maintained under refrigeration. This result proves that sugarcane juice at mild storage temperature is a favorable environment for maintenance of this strain, probably due to its pH stability during storage and the presence of protective compounds. According to Duarte-Almeida et al. (2006) and Ogando et al. (2019) sugarcane juice has compounds such as polysaccharides, hydroxycinnamic acids (for example caffeic and sinapinic acids), and

flavonoids (eg, vitexin and diosmetine-8-C-glycoside), that can be useful for the maintenance of the viability of the probiotics.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$) for the same time interval and bars with an asterisk differ statistically ($p \leq 0.05$) when compared to the initial population in the same treatment. In this figure: T1: control (whole cane juice); T2: juice containing non-encapsulated BLC1; T3: juice containing non-encapsulated 0.001% (w/v) BLC1 and 0.3% (w/v) PRCE; T4: juice containing 5% (w/v) microcapsules with BLC1; T5: juice containing 5% (w/v) microcapsules with BLC1 and PRCE.

These results corroborated those reported by Costa et al. (2017), which produced a probiotic orange juice supplemented with ascorbic acid and oligofructose. It was noted that the viability of probiotic cultures for the formulation with the bacteria alone was maintained for 28 days, and it was concluded that the compounds contained fibers and acids (malic, citric and ascorbic) that may have aided in stability.

On the other hand, samples with free BLC1 and PRCE (T3) only showed counts at the beginning of the storage (8.25 ± 0.08 log CFU/mL); thereafter no populations were counted until the end of the storage. The addition of PRCE increased the counts at day 1, but afterwards it was detrimental to the viability of the probiotic during storage. These results can be attributed to the high concentration of phenolic compounds in the medium that, in this case, probably acted as a pro-oxidant that promoted oxidative stress and death of the microorganisms. Similar results were found by Nualkaekul et al. (2013) who evaluated the survival of *Bifidobacterium longum* NCIMB 8809 in different fruit juices stored under refrigeration. They observed in the

juices of pomegranate and strawberry that when the concentration of phenols was too high, the cells died within one week of storage.

In the treatments T2, T3, T4 and T5 the mean values of counts after 28 days were 6.56 ± 0.00 log CFU/mL, 0.00 ± 0.00 log CFU/mL, 6.88 ± 0.15 log CFU/mL and 7.26 ± 0.31 log CFU/mL, respectively. The T5 treatment, containing BLC1 and PRCE co-encapsulated, presented a significantly higher count ($p \leq 0.05$) than T2 and T3 treatments at the end of storage. This result demonstrated that the microparticles and the presence of PRCE promoted protection and helped in the maintenance of these cells when compared to the treatments with the free bioactives. A similar effect was also observed in the study by Vodnar and Socaciu (2014) where microencapsulated *Lactobacillus casei* and *Lactobacillus plantarum* with selenium enriched green tea with alginate particles. It was found that the immobilization of *L. casei* and *L. plantarum* with 2 g / 100 mL of green tea improved the viability of the strains during refrigerated storage compared to the free bacteria.

Comparing the T4 and T5 treatments, which contained probiotic microcapsules without and with PRCE, it was found that at the end of the storage there was no significant difference ($p > 0.05$) regarding the viability of the BLC1 for the capsules with or without PRCE. These results allowed the conclusion that PRCE had no effect on the viability of encapsulated BLC1; however, the microencapsulation was effective. The effectiveness of the microencapsulation can be explained by the fact that the microcapsules acted as a barrier, protecting BLC1 against environmental conditions. Another possibility is that BLC1 was capable of using the materials of the microcapsules (polysaccharide, protein, and/or vegetal fat) as nutrients, which helped in its survival. In fact, studies have shown that whey-protein aids in the maintenance or growth of probiotic species, such as *Lactobacillus* and *Bifidobacterium*, which may have occurred in our study (TRIPATHI; GIRI, 2014).

In conclusion, treatments with encapsulated BLC1 and PRCE presented higher mean counts than those being non-encapsulated. This demonstrates that the encapsulation process is essential to maintain the viability of the probiotic in this product. With respect to the addition of PRCE to the particle, no significant difference ($p > 0.05$) was observed in the microparticle without the extract. Given this, we can state that regardless of the function of the extract, the viability will remain the same. However, the production and use of microcapsules containing both BLC1 and PRCE is interesting because it delivers 2 bioactive compounds in the same vehicle.

Psychotrophic and mold and yeast counts during storage were also evaluated (data not shown). Mean counts for both psychotrophic and mold and yeast for all treatments were <1.0

CFU/mL during all storage. Currently Brazilian legislation (RDC, 2001) does not establish any standards for the counting of psychotrophic and mold and yeast in sugarcane juice; however, these results indicate that the product exhibited microbiological stability when maintained at 4 °C. Similar results were found by Silva et al. (2016) who produced sugarcane juice for microbiological evaluation of a pilot plant (the same one used in our study). It was observed that both psychotrophic microorganisms such as molds and yeasts presented counts $<1 \log$ CFU/mL after pasteurization.

5.3.7 Rheological characterization

Rheograms of the different treatments are shown in Figure 19. The Ostwald-De Waele model (Power Law) was used, and it was observed that, at the temperatures studied (4 °C and 37 °C), the treatments of sugarcane juice showed a Newtonian behavior, as indicated by the linear dependence of the shear stress on the shear rate and the value of n equal to 1. The Ostwald-De Waele model was suitable in this study for sugarcane juice and is extensively used to describe the rheological behavior of fruit juices (RAO, 1984).

Viscosity values are shown in Table 10. It was observed that, regardless of the treatment at 37 °C, the viscosity was lower than at the temperature of 4 °C. Generally, the viscosity of the liquids decreased with increasing temperature (SARAVACOS, 1970). Corroborating this, the study of Astolfi-Filho et al. (2011) evaluated the rheological characteristics of sugarcane juice in different technological processes and realized that the increase in temperature also influenced the decrease in viscosity. It should be noted that both sugarcane juice with added microparticles and other treatments are products that are easy to swallow at 37 °C (close to body temperature) because they have a lower viscosity that aids in the process of digestion and microparticle disruption in the gut.

Figure 19 – Reograms obtained from different treatments at temperatures of 4 °C and 37 °C on day 1.

In this figure: T1: control (whole cane juice); T2: juice containing non-encapsulated BLC1; T3: juice containing non-encapsulated 0.001% (w/v) BLC1 and 0.3% (w/v) PRCE; T4: juice containing 5% (w/v) microcapsules with BLC1; T5: juice containing 5% (w/v) microcapsules with BLC1 and PRCE.
Reference: Own source.

It was observed that in the T3, T4 and T5 treatments the viscosity increased compared to the T1 treatment for both temperatures. This was because the addition of microparticles or non-encapsulated PRCE increased the viscosity of the sugarcane juice and also increased solids in the medium. Accordingly, many products with similar additive ingredients may increase viscosity, such as the study by Costa et al. (2015), who added cupuaçu to goat milk yogurt and improved viscosity during storage. However, comparing T2 treatment with T1 treatment, there was no significant differences ($p > 0.05$) between them. It was noted that the presence of non-encapsulated probiotic did not modify the rheological characteristics of the original juice, probably because the amount of solids added were not enough for this effect. Similar results were found by Costa et al. (2017), who produced orange juice with *Lactobacillus paracasei* subsp. *paracasei* and prebiotic and no significant differences between the formulations and texture characteristics were observed for 28 days.

Both the T4 and T5 treatment showed a significant decrease ($p \leq 0.05$) in viscosity by comparing day 1 with storage day 28 at 37 °C. At the temperature of 4 °C, similar behavior was observed only in T5. This occurred because the phenolic compounds present in the sugarcane juice may have interacted with the WPC present on the surface of the microparticle, interacting on day 1 by significantly increasing ($p \leq 0.05$) the viscosity, whereas in the proceeding days these interactions were weakened by decreasing the viscosity. Polyphenols have a certain affinity for proteins that lead to the formation of soluble complexes, and these multiple interactions are weak (mainly hydrophobic) between the side chains of the amino acids and the aromatic rings of the polyphenols (CHARLTON et al. 2002). The formation of these complexes is influenced for several reasons: the nature of the polyphenol, the nature of the protein, temperature, and the presence of other compounds (PRIGENT et al., 2003).

Table 10 – Viscosity of treatments during storage at 4 °C and 37 °C.

T / days	Viscosity (η) MPa.s				
	1	7	14	21	28
T1					
4 °C	3.62 ± 0.30 ^{aCD}	4.98 ± 1.76 ^{aB}	4.23 ± 0.32 ^{aCD}	4.26 ± 0.88 ^{aBC}	3.94 ± 0.39 ^{aB}
37 °C	1.97 ± 0.19 ^{aD}	1.87 ± 0.11 ^{aC}	1.85 ± 0.13 ^{aD}	1.73 ± 0.08 ^{aC}	1.85 ± 0.27 ^{aB}
T2					
4 °C	3.86 ± 0.09 ^{aCD}	3.93 ± 0.20 ^{aBC}	3.76 ± 0.40 ^{aC}	3.84 ± 0.51 ^{aBC}	3.98 ± 0.30 ^{aB}
37 °C	1.90 ± 0.15 ^{abD}	2.10 ± 0.42 ^{aBC}	1.86 ± 0.15 ^{abD}	1.68 ± 0.09 ^{bC}	1.75 ± 0.04 ^{abB}
T3					
4 °C	6.94 ± 1.10 ^{bC}	14.10 ± 1.14 ^{aA}	10.89 ± 3.86 ^{abAB}	9.29 ± 4.08 ^{abA}	8.25 ± 3.00 ^{bA}
37 °C	2.11 ± 0.14 ^{bD}	3.64 ± 0.54 ^{aBC}	3.57 ± 0.83 ^{aD}	2.54 ± 0.28 ^{bBC}	3.18 ± 0.77 ^{abB}
T4					
4 °C	20.01 ± 4.02 ^{aB}	12.76 ± 1.10 ^{bA}	8.42 ± 3.45 ^{bBC}	8.92 ± 2.86 ^{bA}	8.69 ± 1.42 ^{bA}
37 °C	2.81 ± 0.43 ^{aCD}	3.45 ± 1.35 ^{aBC}	3.76 ± 0.97 ^{aD}	3.83 ± 1.27 ^{aABC}	3.25 ± 1.93 ^{aB}
T5					
4 °C	27.64 ± 3.83 ^{aA}	11.52 ± 3.21 ^{bA}	12.53 ± 2.84 ^{bA}	7.26 ± 0.44 ^{bAB}	9.05 ± 1.46 ^{bA}
37 °C	3.99 ± 0.67 ^{bCD}	4.00 ± 0.57 ^{abBC}	5.65 ± 0.67 ^{abBCD}	5.02 ± 2.91 ^{abABC}	8.19 ± 0.99 ^{aA}

Means followed by the same lowercase exponent in the same row and same uppercase exponent in the same column are not different ($p > 0.05$) by the Tukey test. T1: control (whole cane juice); T2: juice containing non-encapsulated BLC1; T3: juice containing non-encapsulated 0.001% (w/v) BLC1 and 0.3% (w/v) PRCE; T4: juice containing 5% (w/v) microcapsules with BLC1; T5: juice containing 5% (w/v) microcapsules with BLC1 and PRCE.

Reference: Own source.

5.3.8 Sensory acceptance and purchase intention

The results of the sensorial attributes and purchase intentions are shown in Figures 20A and 20B, respectively. It was observed that the T1 and T2 treatments presented a similar behavior for all the sensorial attributes, with means ranging from 5.88 to 7.21. This result demonstrates that the addition of the non-encapsulated probiotic to the sugarcane juice did not change its sensory acceptance, compared to the control (T1). Further, the free probiotic did not change the sensory characteristics of the product. In T1 treatment, 33% of testers would probably buy this product while T2 30% might buy the product.

It was also observed that the treatments T4 and T5 presented similar behavior for all the sensorial attributes, although the mean the averages had lower values compared to the T1. It can, however, be observed in Fig. 5B that T4 33% of the panelists might buy it, while with the T5 treatment 37% would also perhaps buy the product. Although, the averages of the attributes were lower, the testers had doubts—whether or not they would buy the product with added particles. This happens when a new product reaches the consumer market because it causes many doubts in consumer's considerations.

Regarding T3 treatment, the sensorial attributes presented averages ranging from 4.3 to 5.54. Lower results compared to other treatments. 35% of the testers indicated that they would certainly not buy this product. This indicated demonstrates that the presence of non-encapsulated PRCE had a negative effect due to the change in sensorial characteristics that were manifested mainly by substances that are unpleasant to the taste buds.

Figure 20 – Sensory acceptance test (A) and purchase intention (B) for sugarcane juice treatments.

In this figure: T1: control (whole cane juice); T2: juice containing non-encapsulated BLC1; T3: juice containing non-encapsulated 0.001% (w/v) BLC1 and 0.3% (w/v) PRCE; T4: juice containing 5% (w/v) microcapsules with BLC1; T5: juice containing 5% (w/v) microcapsules with BLC1 and PRCE.
Reference: Own source.

5.4 CONCLUSION

Both co-encapsulation of BLC1 with PRCE and its incorporation into sugarcane juice were successfully performed. Specifically, the microparticles provided oxidative stability for probiotics and high levels of phenolic compounds; however, for proanthocyanidins there was a decrease during the 28 days of storage. In addition, the encapsulation process of lipid particles coated by electrostatic interaction of WPC and GA was effective for the application of these bioactive compounds in sugarcane juice.

It was also possible to produce a sugarcane juice with non-encapsulated BLC1 as the probiotic counts were maintained during the storage time, but without the addition of free PRCE, because this extract affected the viability of the bacteria.

Considering the physicochemical characteristics, the rheological and sensorial properties of sugarcane juice with additive microparticles were not similar to the treatment without added bioactive compounds or those with non-encapsulated BLC1; however, the majority of the tasters were in doubt if it would buy the sugarcane juice with additive microparticles, which is a normal consumer doubt for a functional product that is new on the consumer market. Finally, it is of interest to use a microparticle with BLC1 and PRCE, because in addition to protecting the bioactives, it also allows for their simultaneous delivery.

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6 CHAPTER 5 – CHEMOPREVENTIVE PROPERTIES OF EXTRACTS OBTAINED FROM BLUEBERRY (*VACCINIUM MYRTILLUS* L.) AND JABUTICABA (*MYRCIARIA CAULIFLORA* BERG.) IN COMBINATION WITH PROBIOTICS⁵

ABSTRACT

The association of probiotics and fruit extracts may influence the chemopreventive effect of colorectal cancer. In this context, antiproliferative activity was evaluated to select the best extracts that would be added probiotics, after addition of *Bifidobacterium* or *Lactobacillus* in the extracts the antiradical and antioxidant activity, quinone reductase (QR) assay and apoptosis assay were evaluated. Four extracts were isolated: E1: rich in total phenolic compounds; E2: rich in water-soluble phenolic compounds; E3: rich in most apolar phenolic compounds and E4: rich in anthocyanins. The antiproliferative results showed that the best extracts for blueberry and jabuticaba were respectively the extract E4 and E2. After addition of the probiotic bacteria in these best extracts, it was observed that E2 from jabuticaba presented significantly higher antiradical and antioxidant activity values compared to E4 from blueberry before and after addition of probiotics. There was also a 9-fold increase in activity of QR by the E2 from jabuticaba with *Lactobacillus* (JL). Likewise, this same extract showed a significant increase both in apoptotic and necrotic cells for both cells. In conclusion, extract rich in water-soluble phenolic compounds (E2) from jabuticaba presented a greater chemopreventive effect compared to the others.

Keywords: Fruits; Chemoprevention; Antiproliferative; Antioxidant activity; Quinone reductase; Apoptosis.

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6.1 INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world population with 13,60602 cases in both sexes (GLOBOCAN, 2012). In Canada, this type is the second most diagnosed and in 2017 were estimated 26,800 cases (CANADIAN CANCER SOCIETY, 2017). While in Brazil, this type of cancer is the third most common in the country and in 2018 will estimate 36,360 new cases (INSTITUTO NACIONAL DE CÂNCER, 2018).

The risk factors for the development of this type of cancer are many such as age, race and ethnicity, hereditary factors, inflammatory bowel diseases (ARNOLD et al., 2017; SPÁČILOVÁ et al., 2018). However, a very common factor for increased CRC is the diet, mainly increased consumption of processed and unprocessed red meat (OOSTINDJER et al., 2014; ROSATO et al., 2017). Many studies also have associated alcohol consumption and smoking as major factors for the development of adenomas (FAGUNWA; LOUGHREY; COLEMAN, 2017; MCMENAMIN; MCCAIN; KUNZMANN, 2017; WEI; LIN; CHANG, 2011). Therefore, the interest in the consumption of natural products was increased to prevent the development of this disease (called chemoprevention). Thus, prevention is the best alternative because chemotherapy treatments are very expensive and invasive to patients (CZADEK, 2016; ROLEIRA et al., 2015).

Probiotic bacteria can be defined as live microorganisms which, after ingestion in sufficient quantity, have a beneficial effect on the health of the host by improving and maintaining the balance of the intestinal microbiota. The major genera recognized as probiotics are *Lactobacillus* and *Bifidobacterium* (SOCCOL et al., 2010). Therefore, modifying the composition of the intestinal microbiota by probiotics when ingested in adequate amounts may prevent the development of CRC because these microorganisms reduce intestinal pH, present an immunomodulatory effect and inactivation of carcinogens and mutagenic agents (RAMAN et al., 2013). While phenolic compounds (found in fruits, vegetables, oilseeds, herbs, spices and beverages, among others) have peculiar properties, such as the ability to donate protons and chelate metals, which implies high antioxidant capacity and property of binding to proteins which allows the modulation of the activity of various enzymes, whether digestive or microbial metabolism (ANDERSON et al., 2004). Fruits polyphenols show anticancer effects on colon cancer thanks to their ability to influence carcinogen metabolism, reduce oxidative damage to DNA and scavenge free radicals (AFRIN et al., 2016; THILAKARATHNA; LANGILLE; RUPASINGHE, 2018).

Studies have shown that consuming berries exhibit a wide range of positive biological health effects (TIAN et al., 2018). Blueberries (*Vaccinium myrtillus*) are berries that grow in shrubs and come from North America. They have a high antioxidant capacity, a high concentration of anthocyanins and other phenolic compounds (chlorogenic acid, quercetin, catechin, epicatechin, among others) (WANG et al., 2017). The study of Correa-Betanzo et al. (2014) investigated the effect of blueberry extract on the gastrointestinal system *in vitro* and antiproliferative activity. Crude extracts were observed to inhibit HT-29 and CRL-1790 cells growth by 90% and 60%, respectively. As well as jaboticaba (*Myrciaria jaboticaba* (Vell.). Berg is a native plant to Brazil, known for producing globular fruits with a purple peel and a sweet pulp (WU; LONG; KENNELLY, 2013). In particular, it is the peel that contains a large number of anthocyanins, phenolic acids, flavonoids, and tannins that have a high antioxidant capacity (ABE; LAJOLO; GENOVESE, 2012). Some flavonoid compounds extracted from jaboticaba exhibited antiproliferative effects against HT29 and HCT116 colon cell lines (REYNERTSON et al., 2006).

In fact, probiotics and phenolics had shown some synergistic effect that can be attribute to: I) certain enzymes, such as esterases and glycosities that are synthesized by probiotics, produce activities, including deglicosylation, dehydroxylation, demethylation, or decarboxylation (AURA, 2008), which convert phenolics into more bioavailable or bioactive forms than their original forms (LÓPEZ DE LACEY et al., 2014); II) on the other hand, a notorious function of phenolics is to inhibit pathogenic bacteria, favoring the prevalence and development of the beneficial microbiota in the intestine (BIALONSKA et al., 2009); III) lastly, as one of the main causes of death of probiotics is oxidative stress, it is inferred that phenolic compounds, due to their antioxidant action, could delay this process, increasing the viability of probiotics.

In order to evaluate whether these compounds have the ability to chemoprevention, that is, if they reduce the occurrence of cancer by inhibiting critical pathways important in the process of carcinogenesis, it is necessary to consider all the major stages of the multistage formation of the cancer. The first stage called initiation involves the exposure of electrophilic metabolites or reactive oxygen species (ROS) to normal cells (KLAUNIG; WANG, 2018). It is necessary that the chemopreventive has a high antioxidant capacity, antiradical and quinone reductase (QR), since it is directly related in the protection against ROS (mainly O_2^- , OH^-) (FUCHS-TARLOVSKY, 2013; TALALAY, 2000). The second stage called promotion involves the selective clonal expansion of the modified cell through increased proliferation and cell growth. It is important to evaluate the

antiproliferative properties of the chemopreventive agent, since depending on the dose, the promotion stage can be reverted, since they may act as antipromoters or growth inhibitors of the genetically damaged cells (CARMO et al., 2018). The progression is the third stage presenting a high level of genetic instability leading to chromosomal changes that goes to the neoplastic state, making this stage irreversible. The evaluation of the induction of apoptosis (programmed cell death) is a fundamental analysis to measure the ability of the chemopreventive to prevent progression, as the apoptotic process can remove damaged cells and prevent the development and progression of tumors (TANAKA, 2013; CALL; ECKHARDT; CAMIDGE, 2008).

Therefore, the objective of this work was to evaluate different extracts produced from two berries (blueberry and jabuticaba) from completely different regions, used alone and in combination with *Bifidobacterium* and *Lactobacillus*, comparing by the antiproliferative assays, antiradical and antioxidant activity, quinone reductase and apoptosis.

6.2 MATERIAL AND METHODS

6.2.1 Materials

The raw material for extraction of the phenolic compounds was used Jabuticaba (*Myrciaria cauliflora* B.) that was acquired in the local commerce (Pirassununga/SP, Brazil). The Blueberry (*Vaccinium myrtillus* L.) of the Star variety from MaxFrutas, Ltda (São Paulo, Brazil) imported from Emergosol, Ltda (Alto de Esteveira, Portugal). The probiotics used were *Lactobacillus paracasei* BGP1 and *Bifidobacterium animalis* ssp. *lactis* BLC1, provided by Sacco Brazil (Campinas, Brazil).

6.2.2 Production of extracts

The production of extracts from jabuticaba and blueberry was carried out using four solvent solutions of different polarities for the recovery of four specific classes of phenolic compounds that have different solubilities:

- 1) Extract 1 (E1): extraction of total phenolic compounds with ethanol:water (70:30, v/v) as described by Lazar et al. (2016);

- 2) Extract 2 (E2): extraction of water-soluble phenolic compounds with water:methanol (85:15, v/v) as described by Seeram et al. (2004);
- 3) Extract 3 (E3): extraction of most apolar phenolic compounds with acetone:methanol:water (40:40:20, v/v/v) as described by Neto et al. (2006);
- 4) Extract 4 (E4): extraction of anthocyanins with methanol:water:acetic acid (85:15:0.5, v/v/v) as described by Wu and Prior (2005).

Around 300 g of frozen fruits were crushed in Philips food processor (Walita - Viva RI7630 - Amsterdam, Netherlands) at 6 °C for 1 minute. After, crushed fruits were added to 500 mL of specific solvent solution and three successive extractions were carried out using the same procedure under mechanical agitation (model 713 agitator, Fisaton, São Paulo, Brazil) for 40 minutes at 6 °C and protected from light. The solution was recovered after each extraction and the solvents from the successive extractions were combined and filtered into cotton cloth for the separation of the coarse particles and centrifuged for 5 minutes at 25 °C and 7500 rpm in a model 5430 centrifuge (Eppendorf, Hamburg, Germany) for the separation of fine particles. The filtrate was concentrated on a rotary evaporator (TE-211, Tecnal, Piracicaba, Brazil) at 49 °C until reducing to 1/3 of the initial volume. Finally, the samples were accommodated in Falcon tubes, frozen at -20 °C and dehydrated in freeze dryer model L 202 (Liobras, São Carlos, São Paulo, Brazil) for 72 hours. The samples obtained were packed in high density polyethylene bags and stored at -20 °C.

6.2.3 Cell cultures and antiproliferative assay

6.2.3.1 Colon cancer cell lines and cell culture media

Hepa 1c1c7 (ATCC number: CRL-2026) and HT-29 cell lines (ATCC number: HTB-38) were purchased from the American Type Culture Collection, ATCC, Rockville, MD, USA. All cell lines were cultivated in 75 cm² cellular flasks (Corning, NY, USA) at 37 °C with 5% CO₂ and 95% air in humidified incubator. Hepa 1c1c7 and HT-29 cells were maintained in completed Dulbecco's Low Glucose Modified Eagles Medium – DMEM containing 5% amino acids (Sigma-Aldrich, St. Louis, USA) and 10% fetal bovine serum (Fisher Scientific Lit., Ottawa, Ontario, Canada).

6.2.3.2 Preparation of the samples for antiproliferative assay

Blueberry and jabuticaba extracts were serially diluted using phosphate-buffered saline (PBS) to have serial concentrations of 50.0, 25.0, 12.5, 6.25, 3.1, 1.55, 0.78 and 0.39 mg/ml for each sample.

6.2.3.3 Antiproliferative assay

To select the best extracts to add the probiotics the antiproliferative test was carried out. Hepa 1c1c7 and HT-29 cells were seeded at a concentration of 2×10^4 cells / well of medium in a 96 well plate and incubated for 24 hours at 37 °C in 5% CO₂. Then, the medium was removed and 100 µL of fresh medium containing 10 µL of serial concentrations of extracts were incubated for 48 hours as mentioned above. The medium was then replaced with 225 µL of fresh medium containing 25 µL of a 0.5% (w / v) solution of Thiazolyl blue tetrazolium bromide (MTT) followed by incubation for 4 hours at 37 °C in 5% CO₂. Finally, the medium was carefully removed and replaced with 225 µL of DMSO containing 25 µL of Sorensen buffer (0.1 mol / L glycine and 0.1 mol / L NaCl at pH of 10.5). The microplate was read at 562 nm. Negative control and blank were used PBS and cell-free medium, respectively. Growth inhibition was calculated according to Fortin et al. (2017) eq. (8):

$$\% \text{ Growth inhibition} = 100 - \left(\frac{\text{Optical density sample}}{\text{Optical density negative control}} \right) \times 100 \quad (8)$$

The IC₅₀ value was calculated based on the dose-response curve between the sample concentrations and the percentage of growth inhibition of cells. The IC₅₀ value is defined as the extract concentration (mg / mL) required to inhibit 50% of cancer cell growth to the control.

6.2.4 Treatments

After choosing the best extracts with their respective IC₅₀ values, probiotics were added to the antiradical and antioxidant activity determinations, quinone reductase (QR) assay and apoptosis assay. The formulations of each treatment are shown in Table 11.

Table 11 – Formulation of each treatment for the antiradical and antioxidant activity determinations, quinone reductase (QR) assay and apoptosis assay.

Treatments	Fruit	Extract	Concentration of the extract	Probiotic	Concentration of the probiotic
B	Blueberry	E4: extracted with methanol:water:acetic acid (85:15:0.5, v/v/v).	58.97 mg / mL (against Hepa 1c7c cells) 40.58 mg / mL (against HT-29 cells)	-	-
BB	Blueberry	E4: extracted with methanol:water:acetic acid (85:15:0.5, v/v/v).	58.97 mg / mL (against Hepa 1c7c cells) 40.58 mg / mL (against HT-29 cells)	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BLC1	1×10^9 CFU/mL
BL	Blueberry	E4: extracted with methanol:water:acetic acid (85:15:0.5, v/v/v).	58.97 mg / mL (against Hepa 1c7c cells) 40.58 mg / mL (against HT-29 cells)	<i>Lactobacillus paracasei</i> BGP1	1×10^9 CFU/mL
J	Jaboticaba	E2: extracted with water:methanol (85:15, v/v).	48.63 mg / mL (against the Hepa 1c7c cells) 37.52 mg / mL (against the HT-29 cells)	-	-
JB	Jaboticaba	E2: extracted with water:methanol (85:15, v/v).	48.63 mg / mL (against the Hepa 1c7c cells) 37.52 mg / mL (against the HT-29 cells)	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BLC1	1×10^9 CFU/mL
JL	Jaboticaba	E2: extracted with water:methanol (85:15, v/v).	48.63 mg / mL (against the Hepa 1c7c cells) 37.52 mg / mL (against the HT-29 cells)	<i>Lactobacillus paracasei</i> BGP1	1×10^9 CFU/mL

Reference: Own source.

6.2.5 Measurement of antiradical property

6.2.5.1 Free radical-scavenging activity on DPPH

The DPPH assay was performed according to the method of Brand-Williams, Cuvelier and Berset (1995) with some modifications. Briefly, 1 mL of 80 μ M DPPH previously dissolved in methanol was added in 250 μ L of diluted sample. The solution was stirred and kept at room temperature for 1 hour protected from light. After time the Optical Density (OD) was measured in UV-Vis Spectrophotometer (model S-3100, Scinco, Samseong-dong, Gangnam-gu, Seoul, South Korea) at 517 nm, including methanol as the control and the percentage of inhibition (% I) was determined as eq. (9):

$$\% I = \left(\frac{(\text{OD} - \text{OD}_{\text{Blank}})_{\text{control}} - (\text{OD} - \text{OD}_{\text{Blank}})_{\text{sample}}}{(\text{OD} - \text{OD}_{\text{Blank}})_{\text{control}}} \right) \times 100 \quad (9)$$

With IC_{50} defined as the concentration which results in 50% inhibition.

6.2.6 Measurement of antioxidant property

6.2.6.1 Determination of reducing power (FRAP)

The FRAP assay was performed according to Benzie and Strain (1996) with some modifications. Previously a FRAP solution was made by mixing 10 mL of a solution of 300 mM acetate buffer pH 3.6, 2.4 mL MilliQ water, 2.5 mL of a 20 mM FeCl_3 (Ferric chloride) solution and 1 mL of a 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) solution. In a 96-well microplate, 10 μ L of diluted sample and 200 μ L of the FRAP solution were added. After the plate was incubated for 10 minutes protected from light. The absorbance was measured in Microplate Autoreader (model EL 309, Biotek Instruments, Winooski, VT) at 593 nm. The standard curve was linear between 98 and 1129 μ M ferrous sulfate (FeSO_4). The results are expressed in $\mu\text{mol FeSO}_4 \text{ g}^{-1}$ sample.

6.2.7 Quinone Reductase (QR) assay

Quinone reductase was based on the study of Prochaska and Santamaria (1988) with some modifications. Hepa 1c1c7 and HT-29 cells were seeded at a density of 1×10^4 cells / mL in 96-well microplates in DMEM and incubated 24 hours at 37 °C in 5% CO₂. Thereafter, the medium was removed and 20 µL of extracts were added and each well was filled with 200 µL of medium for 48 hours. PBS (20 µL) was used as negative control. Cells were washed with 200 µL of PBS and 50 µL of 1.6% (w / v) digitonin was added in each well and incubated for 20 minutes. Then, 20 µL of the sample was removed and used for total protein quantification while 200 µL of complete reaction mixture (0.25 mol / L Tris-HCl pH 7, 4.6% (w / v) bovine serum albumin, 0.01% Tween 20 mmol / L FAD, 1 mmol / L glucose-6-phosphate, 30 mol / L NADP, 34.8 µmol / L MTT, 50 µmol / L menadione and 2 mU / µL glucose-6-phosphate dehydrogenase) were added in each well and incubated for 5 minutes at room temperature and the optical density (OD) values were measured on a microplate reader at 595 nm. The protein test was performed using Pierce® Thermo Scientific reagents using the manufacturer's instructions. Specific activity was calculated and expressed as nM MTT formed by per min per mg of protein. Fold induction was calculated as expressed below eq. (10):

$$\text{Fold induction} = \left(\frac{\text{Specific activity of treated group}}{\text{Specific activity of negative control group}} \right) \quad (10)$$

When the fold induction was greater than 1 this indicates that the compound stimulates the quinone reductase activity whereas a result below 1 indicates that the compound does not stimulate quinone reductase.

6.2.8 Apoptosis assay

Hepa 1c1c7 and HT-29 were seeded in 6-well plate at 1×10^5 cells / well (3 ml / well) incubated for 24 hours at 37 °C in 5% CO₂. The cells were then incubated with the extracts in a final volume of 3 ml for 48 hours at 37 °C in 5% CO₂. After, the cells that were in the supernatant were centrifuged for $500 \times g$ for 10 min at 4 °C. The adhered cells were treated with $1 \times$ trypsin-EDTA for 8 minutes at 37 °C, soon after 2 mL of DMEM was added and centrifuged under the

same conditions. Pellets (from the supernatant and the adhered cells) were washed twice with PBS. The cells were diluted in $1 \times$ binding buffer at 10^6 cells / ml and the evaluation of apoptosis was performed using Annexin V-FITC and Propidium Iodide (PI) for staining according to manufacturer's instructions with a total of 10.000 events by flow cytometry Epics XL-MCL, Beckman Coulter Canada, Inc., Mississauga, ON, Canada). Camptothecin (CPT) and PBS were used as positive and negative control, respectively.

6.2.9 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the statistical software Statistica version 7.0 software (Statsoft Inc., Tulsa, OK, USA) and Tukey's test, at the significance level of 5%.

6.3 RESULTS AND DISCUSSION

6.3.1 Antiproliferative assay

In order to select the best extract for the addition of the probiotic bacteria, the IC_{50} (mg / mL) (the concentration of extract that requires 50% inhibition of the growth of the cancerous cells) of the different extracts of blueberry and jabuticaba against the Hepa 1c1c7 and HT-29 cell lines was evaluated and the results are shown in Figure 21. It is noted that a lower IC_{50} value indicates a greater efficiency in inhibiting cells.

The IC_{50} values of the extracts from blueberry for the Hepa 1c1c7 cell (Figure 21A) increased in the order: E4 (58.97 ± 3.07) < E1 (97.75 ± 0.92) < E3 (131.15 ± 12.57) < E2 (141.30 ± 2.18); In the HT-29 cell (Figure 21B), the order of values were: E4 (40.58 ± 0.31) < E2 (52.18 ± 8.93) < E1 (52.77 ± 0.73) < E3 (146.58 ± 8.08). Although our variety is *Mirtillus* the variation of our values corroborates with the study of Wang et al. (2017) who evaluated different varieties of blueberry in relation to different functional properties. It was observed that the highest antiproliferative activity for the HepG2 cell was observed in the Chipava variety (20.55 ± 5.23 mg / mL) whereas the lowest activity was recorded in the Bluecrop (102.3 ± 25.1 mg / mL) variety.

Figure 21 – IC₅₀ (mg extract/mL) values of the antiproliferative assay for blueberry and jabuticaba extracts against the cancer cells (A) Hepa 1c1c7 and (B) HT-29.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). E1: extract rich in total phenolic compounds; E2: extract rich in water-soluble phenolic compounds; E3: extract rich in apolar phenolic compounds; E4: extract rich in anthocyanins. Reference: Own source.

In the present study, it was observed that, for both cell lines, the extract with lower IC₅₀ value was E4. This extract presents as rich in anthocyanins from blueberry, because this fruit has large amounts of pelargonidins, peonidins, malvidins, cyanidines, delphinidins and petunidins (LI et al., 2011). Many studies have confirmed that anthocyanins act to inhibit not only the colon cancer cells but also several others such as lung, liver and skin (BUNEA et al., 2013; HSIEH et al., 2013; ZHU et al., 2016). Corroborating with the study of Correa-Betanzo et al. (2014) who evaluated the stability and biological activity of wild blueberry polyphenols (*Vaccinium angustifolium*) during simulated gastrointestinal digestion *in vitro* and found that crude blueberry extracts as well as isolated anthocyanins (cyanidin 3-glucoside, delphinidin 3-glucoside) inhibited cell growth of normal human colonic epithelial cell line (CRL 1790) and human colorectal cancer cell line (HT 29) by 90% and 60%, respectively.

In the study by Olsson et al. (2004) found that the anthocyanin fraction from blueberry had lower inhibition activity against HT-29 cells than the total ethanol extract, but greater inhibition for other MCF-7 cells. In our study we observed an opposite effect, since the anthocyanins rich E4 extract against the HT-29 cells obtained a lower IC_{50} (40.58 ± 0.31) compared to the same extracts against the Hepa 1c7c cells. This may be related to the anthocyanins composition of the extract or to the susceptibility (absorption) of the cell lines to the extracts. Studies have shown that different sources of anthocyanins and glycosylations present in their chemical structures may affect the growth of several cells (MAZZUCATO et al., 2013; ZHANG; VAREED; NAIR, 2005).

In relation to the extracts from jabuticaba for Hepa 1c1c7 cell (Fig. 1A), the order of the values was: E2 (48.63 ± 4.15) < E1 (48.74 ± 1.22) < E4 (60.42 ± 0.56) < E3 (66.05 ± 0.92). The mean values for HT-29 cell (Fig. 1B) were: E2 (37.52 ± 8.03) < E3 (49.58 ± 0.07) < E4 (52.13 ± 4.75) < E1 (58.59 ± 4.18). No significant difference was observed between the extracts for both cells.

It was observed that the extract from jabuticaba presented a lower IC_{50} value was E2 for both of HT-29 and Hepa 1c1c7 cells. This extract presents as rich in water-soluble phenolic compounds because the jabuticaba has several compounds that can be extracted as: ellagic acid, gallic acid, elagitannins and their derivatives (ABE; LAJOLO; GENOVESE, 2012). However, it is also known that it is possible to extract water-soluble anthocyanins in these solvents (BALDIN et al., 2016). In addition, both phenolic acids and anthocyanins have demonstrated a high antiproliferative and antioxidant effect (LEITE-LEGATTI et al., 2012; REYNERTSON et al., 2006). For example, in the study by Mertens-Talcott, Talcott and Percival (2003) ellagic acid significantly potentiated the effects of quercetin (at 5 and 10 $\mu\text{mol} / \text{L}$ each) on reducing proliferation and viability of MOLT-4 cell lines. Also, in the study by Seeram et al. (2005) demonstrated that both pomegranate juice and extract of the isolated compounds (punicalagin, ellagic acid and total pomegranate tannin) inhibited up to 100% of human oral (KB, CAL27), colon (SW460; SW620; HT-29; HCT116) and prostate (RWPE-1; 22Rv1) tumor cells.

Jabuticaba is a fruit that is also rich in anthocyanins (mainly cyanidin 3-glucoside and delphinidin 3-glucoside) and its derivatives. However, it can be observed that the extract E4 (rich in anthocyanins) did not present a lower value of IC_{50} compared to the other extracts. Some studies have suggested that anticarcinogenic activity of anthocyanins is associated with the number of hydroxyl groups on the B-ring, if a change in this structure occurs, it may influence the effect

against the cells (HOU, 2003). This may have occurred with the extract E4 from jabuticaba. Corroborating with the study by Hou et al. (2004) concluded that anthocyanins (pelargonidin, peonidine and malvidin) lacking the ortho-dihydroxyphenyl structure failed to show significant inhibitory effects on the tumor in mouse JB6 cells.

Thus, the best extracts were chosen because they presented the lowest values of IC₅₀ indicating greater inhibition of the cells. E4 extract (rich in anthocyanins) from blueberry at a concentration of 58.97 ± 3.07 mg / mL against Hepa 1c1c7 cells and a concentration of 40.58 ± 0.31 mg / mL against HT-29 cells was selected. In contrast, the E2 extract (rich in water-soluble phenolic compounds) from jabuticaba, with a concentration of 48.63 ± 4.15 mg / mL against the Hepa 1c1c7 cells and a concentration of 37.52 ± 8.03 mg / mL against the HT-29 cells was chosen.

6.3.2 Antiradical and the antioxidant properties

The antiradical and the antioxidant properties of the extracts from blueberry and jabuticaba were evaluated by the elimination capacity of the DPPH IC₅₀ radical and by the iron ion reduction power FRAP, respectively (Table 12).

Table 12 – Determinations of antiradical DPPH IC₅₀ (μg / mL extract) and antioxidant activity FRAP (μmol FeSO₄ / g) of extracts from blueberry and jabuticaba.

Extracts	DPPH IC ₅₀ (μg/mL)	FRAP (μmol FeSO ₄ /g)
B	641.45 ± 6.24^a	185.90 ± 4.04^c
BB	544.03 ± 3.82^c	212.32 ± 7.41^b
BL	623.18 ± 4.10^b	204.41 ± 3.97^b
J	236.73 ± 0.93^d	263.94 ± 3.78^a
JB	203.20 ± 1.22^e	270.24 ± 1.83^a
JL	211.90 ± 3.33^e	263.57 ± 3.48^a

Means followed by the same lowercase exponent in the same column are not different ($p > 0.05$). B: extract rich in anthocyanins from blueberry; BB: extract rich in anthocyanins from blueberry with *Bifidobacterium*; BL: extract rich in anthocyanins from blueberry with *Lactobacillus*; J: extract rich in water-soluble phenolic compounds from jabuticaba; JB: extract rich in water-soluble phenolic compounds with *Bifidobacterium*; JL: extract rich in water-soluble phenolic compounds with *Lactobacillus*. Reference: Own source.

It can be observed that the extract rich in anthocyanins from blueberry (B) before probiotic addition had values of DPPH IC₅₀ and FRAP of 641.45 ± 6.24 μg / mL and 185.90 ± 4.04 μmol FeSO₄ / g, respectively. Our extract (B) presented values that corroborate with several studies, since the antioxidant activity of blueberry and its extract originate mainly from the anthocyanins (responsible for 84% of the total antioxidant activity) that have a polyphenolic ring structure with the capacity to reduce reactive oxygen species (ROS) (SCALZO et al., 2005). Our DPPH IC₅₀

values corroborated the variation of the study of Saral, Ölmez and Şahin (2015) compared the antioxidant properties of wild blueberries (*Vaccinium arctostaphylos* L. and *Vaccinium myrtillus* L.) with varieties of cultivated blueberries (*Vaccinium corymbosum* L.) and had a DPPH IC₅₀ values between 229-1178 µg / ml. The same occurred for the FRAP values that are in the study variation of Pertuzatti et al. (2014) that analyzed ten cultivars of Highbush and Rabbiteye Brazilian blueberries (*Vaccinium corymbosum* L. and *Vacciniumashei* Reade, respectively) with high concentrations of anthocyanins and obtained a variation of FRAP values between 132 - 312 µmol TE / g DW between the harvests 2010 and 2012.

In the addition of *Bifidobacterium* (1×10^9 CFU / mL) in the extract rich in anthocyanins from blueberry (B) there was a significant reduction compared to the extract before in the DPPH IC₅₀ values that decreased from 641 ± 6.24 µg / mL to 544.03 ± 3.82 µg / mL, relative to FRAP there was a significant increase from 185.90 ± 4.04 µmol FeSO₄ / g to 204.41 ± 3.97 µmol FeSO₄ / g. In the case of the addition of *Lactobacillus* (1×10^9 CFU / mL) in the extract rich in anthocyanins from blueberry (B), there was a significant reduction compared to the extract before with a reduction of DPPH IC₅₀ from 641 ± 6.24 µg / mL to 623.18 ± 4.10 µg / mL, whereas for FRAP there was a significant increase from 185.90 ± 4.04 µmol FeSO₄ / g to 204.41 ± 3.97 µmol FeSO₄ / g.

This shows that for the blueberry anthocyanin-rich extract (B) there was an increase in antioxidant activity when the bacteria were inserted. The presence of probiotics in extract rich in anthocyanins contributes to the simple conversion and depolymerization of these high molecular weight compounds, thus contributing to the increase of the antioxidant activity by the large amount of bioactive compounds present. Our study corroborates with Oh et al. (2017) investigating the fermentation of blueberry fruits with probiotic bacteria (*Bacillus amyloliquefaciens* and *Lactobacillus brevis*) and yeast (*Starmerella bombicola*). They observed that both probiotics and yeast had a reduction of the phenolic compounds, flavonoids and a highlight for a greater reduction in the anthocyanins level (6.11 mg CGE / 100 g dw of the control) with *Lactobacillus brevis*. This may have been due to the bioconversion of anthocyanins and phenolic compounds to more bioactive compounds, thus increasing antioxidant activity. Similar studies also showed that the presence of probiotics in yogurts and juices contributed to the increase in antioxidant activity (FILHO et al., 2019; MCCUE; SHETTY, 2005; TAVAKOLI; NAJAFI; MOHEBBI, 2019).

Regarding the extract rich in water-soluble phenolic compounds from jabuticaba (J) before the addition of the probiotic presented values of DPPH IC₅₀ and FRAP of $236.73 \pm 0.93 \mu\text{g} / \text{mL}$ and $263.94 \pm 3.78 \mu\text{mol FeSO}_4 / \text{g}$, respectively. To date, 51 phenolic compounds have been found in jabuticaba fruits, among which we can mention anthocyanins, but water-soluble compounds (present in our extract) such as valonic acid dilactone, ellagic acid and its pentosid, gallic, gallotannins, ellagitans, glycosides derived from quercetin and, to a lesser extent, from myricetin, even the 3-glycosides of isorannetine and kaempferol contribute to a high antioxidant activity (ALEZANDRO et al., 2013). Our DPPH IC₅₀ values are in the range of the study by Morales et al. (2016) that evaluated the chemical composition of the unfermented and fermented pomace of jabuticaba and resulted in DPPH EC₅₀ of $262 \pm 8 \mu\text{g} / \text{ml}$ extract and $130 \pm 2 \mu\text{g} / \text{ml}$ extract, respectively. The same occurred for the FRAP values that corroborated with the variation of the study by Seraglio et al. (2018) that characterized the compounds present in jabuticaba, guabiju and jambolan during maturation (intermediate and mature stages) presenting FRAP values for the intermediate stage of $266.67 \pm 1.43 \mu\text{M Fe}^{+2} / \text{g DW}$ and in the mature stage of $249.56 \pm 7.02 \mu\text{M Fe}^{+2} / \text{g DW}$.

In the addition of *Bifidobacterium* (1×10^9 CFU / mL) in extract rich in water-soluble phenolic compounds from jabuticaba (J) there was a significant reduction ($p \leq 0.05$) compared to the extract before, in the values of DPPH IC₅₀ that decreased from $236.73 \pm 0.93 \mu\text{g} / \text{mL}$ to $203.20 \pm 1.22 \mu\text{g} / \text{mL}$, with respect to FRAP there was an increase, but not significant, from $263.94 \pm 3.78 \mu\text{mol FeSO}_4 / \text{g}$ to $270.24 \pm 1.83 \mu\text{mol FeSO}_4 / \text{g}$. In the case of the addition of *Lactobacillus* (1×10^9 CFU / mL) in extract rich in water-soluble phenolic compounds from jabuticaba (J) there was a significant reduction ($p \leq 0.05$) in the DPPH IC₅₀ values from $236.73 \pm 0.93 \mu\text{g} / \text{mL}$ to $211.90 \pm 3.33 \mu\text{g} / \text{mL}$ compared to the extract before, whereas for FRAP the value remained from $263.94 \pm 3.78 \mu\text{mol FeSO}_4 / \text{g}$ to $263.57 \pm 3.97 \mu\text{mol FeSO}_4 / \text{g}$. This demonstrated that the insertion of *Bifidobacterium* and *Lactobacillus* significantly increased the values of DPPH IC₅₀, but there was no significant difference for the FRAP values ($p > 0.05$). A similar study demonstrates this increase when using extract from jabuticaba and probiotics. Pereira et al. (2016) evaluated the action of extract from jabuticaba throughout the processing of the probiotic cheese Petit Suisse and it was observed that the increased treatments of cysteine and extract from jabuticaba skin presented higher values of antioxidant activity (DPPH and FRAP), but also provided stability of the probiotic culture over the storage period of 28 days.

It was also observed that both the extract rich in water-soluble phenolic compounds from jabuticaba (J) alone as well as in combination with probiotics showed significantly higher antioxidant activity ($p \leq 0.05$) in relation to extracts rich in anthocyanins from blueberry (B) and combination with probiotics. This may have occurred because of the diversity of phenolic compounds present in jabuticaba as mentioned above and alteration that may have occurred in these compounds with probiotics. Corroborating with the study of Reynertson et al. (2008) showed that the extract of jabuticaba showed a higher antioxidant activity (DPPH IC₅₀ of $19.40 \pm 0.28 \mu\text{g} / \text{mL}$) compared to other species such as *Eugenia* and *Syzygium* and a slightly higher amount than the average of phenolic compounds (31.6 mg GAE / g) and total anthocyanins (2.78 mg / g).

6.3.3 Quinone Reductase (QR) assay

The activity of quinone reductase (QR) for extracts from blueberry and jabuticaba are shown in Figure 22. In the extract rich in anthocyanins from blueberry (B), a significant increase ($p \leq 0.05$) was observed compared to the negative control of the specific QR activity, with a value of 2.64 ± 0.33 in the Hepa 1c7c cells (Figure 22A), whereas in HT-29 cells (Figure 22B) the value was 1.38 ± 0.39 , however there was no significant difference compared to the control. This demonstrates that depending on the cell type the extract will exert different effects. Our study corroborated with Srivastava et al. (2007), who evaluated the effect of anthocyanin fractions on selected cultivars of cultivated blueberries grown in Georgia on phase II enzymes and apoptosis, concluded that QR activity was lower in all HT-29 cells treated with an anthocyanin fraction of the cultivars *Tifblue*, *Powderblue*, *Brightblue* and *Brightwell* than in control cells. However, our results differed from Bomser et al. (1996) evaluated the *in vitro* anticancer activity of Hepa 1c7c cells in different extracts of different *Vaccinium* species and found that extracts of anthocyanins were not highly active in the induction of QR.

In relation to the extract rich in anthocyanins from blueberry with *Bifidobacterium* (BB), there was a significant increase ($p \leq 0.05$) in the activity of QR compared to the control in both cells with a value of 2.52 ± 0.44 for Hepa 1c71 and 4.17 ± 1.55 for the HT-29 cells. In the extract rich in anthocyanins from blueberry with *Lactobacillus* (BL) no significant difference was observed ($p > 0.05$) compared to the control, presenting values of 2.22 ± 0.08 for Hepa 1c7c cells and 0.93 ± 0.29 for HT-29 cells. It is already known that some probiotics have a biotransformation

capacity of some antioxidant compounds when they are inserted in certain media, allowing for an increase in the induction of QR by the presence of a double bond at the binding position of the heterocyclic ring (C) 2 and 3 (SEPTEMBRE-MALATERRE; REMIZE; POUCHERET, 2018; UDA et al., 1997). The study by Ávila et al. (2009) investigated the enzymatic potential of different probiotic strains selected for the bioconversion of glycosides of the anthocyanins delphinidin and malvidin in their metabolites and observed degradation of these compounds mainly by *Bifidobacterium lactis* BB-12 and the production of metabolites more bioavailable or bioactive than the original molecule.

Figure 22 – Induction of quinone reductase (QR) by extracts of blueberry and jabuticaba against (A) Hepa 1c1c7 and (B) HT-29 cells.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). Negative control: phosphate-buffered saline (PBS); B: extract rich in anthocyanins from blueberry; BB: extract rich in anthocyanins from blueberry with *Bifidobacterium*; BL: extract rich in anthocyanins from blueberry with *Lactobacillus*; J: extract rich in water-soluble phenolic compounds from jabuticaba; JB: extract rich in water-soluble phenolic compounds with *Bifidobacterium*; JL: extract rich in water-soluble phenolic compounds with *Lactobacillus*. Reference: Own source.

In the extract rich in water-soluble phenolic compounds of jabuticaba (J) a significant increase in the QR activity ($p \leq 0.05$) was observed compared to the negative control with a value of 4.25 ± 0.06 in Hepa 1c7c cells (Figure 22A), while that in the HT-29 cells (Figure 22B) the value was 1.59 ± 0.16 , however there was no significant difference compared to the control ($p > 0.05$). This difference may be related to the cell type, but also the spatial position of some phenolic compounds resulting in different binding affinities, consequently to different QR inducing activities. As demonstrated in the study by Li et al. (2012), which evaluated fractionation of the black chokeberry extract (*Aronia melanocarpa*) and it was observed that the methyl ester of the

neo-chlorogenic acid was more active in the induction of QR than the methyl ester of chlorogenic acid.

In the case of the extract rich in water-soluble phenolic compounds from jabuticaba with *Bifidobacterium* (JB) occurred a significant increase ($p \leq 0.05$) in the activity of QR compared to the control with a value of 2.60 ± 0.09 for Hepa 1c71, while the HT-29 cells did not present significant difference with a value of 1.23 ± 0.09 ($p > 0.05$). In the extract rich in water-soluble phenolic compounds from jabuticaba with *Lactobacillus* (JL) there was a significant difference ($p \leq 0.05$) in the activity of QR compared to control for both cells, presenting values of 3.93 ± 0.03 for Hepa cells 1c7c and 9.70 ± 0.89 for HT-29 cells. It is observed that the bacteria that had the greatest effect under the activity of QR was *Lactobacillus*, differently from extract rich in anthocyanins from blueberry, it was *Bifidobacterium*. Studies have shown that there are different effects of probiotics on different tumor cells (BALDWIN et al., 2010; DALLAL et al., 2015). This is due to the fact that there are different ways of regulating cell proliferation, especially when dealing with different species and genera of bacteria (LIONG, 2008). However, there is no literature that discusses the relation of probiotics to extracts from jabuticaba under the activity of QR, but our study corroborates with other studies to show a protective potential of *Lactobacillus* against cancerous cells by increasing the activity of QR (DESROUILLÈRES et al., 2016; FOTIADIS et al., 2008).

6.3.4 Apoptosis assay

The percentage of apoptosis and necrosis of the extracts against the Hepa 1c1c7 and HT-29 cells are shown in Figure 23 and Figure 24, respectively. In the extract rich in anthocyanins from blueberry (B), it was observed that the percentage of apoptosis presented significant difference ($p \leq 0.05$) for both cells compared to the negative control of $11.04\% \pm 0.15$ for Hepa 1c1c7 cells (Figure 23A), whereas for the HT-29 cells (Figure 24A) was $17.36\% \pm 1.33$. Regarding the necrotic cells, there was no significant difference in both cells compared to the negative control with a percentage of $4.62\% \pm 0.16$ for Hepa 1c1c7 and $11.14\% \pm 1.57$ for HT-29. Our study corroborates with Zhan et al. (2016) who investigated the effects of blueberry juice consumption on rats under human hepatocellular carcinoma (HCC) cells and it was observed that the percentage of apoptosis in the groups receiving blueberry juice dosages was significantly higher compared to

the control group. *In vitro* studies have shown that the effects of blueberry on the protopic effect of anthocyanins in different cancer cell lines have been reported (VUONG et al., 2016; YI et al., 2006). This is due to the number of OH groups on ring B, and the ortho-dihydroxyphenyl structure on ring B of anthocyanins causing an increase in apoptosis (HOU et al., 2003).

Figure 23 – Effect of extracts of blueberry and jabuticaba on the percentage of (A) apoptotic cells and (B) necrotic cells in Hepa 1c1c7.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). Positive control: Camptothecin (CPT); Negative control: phosphate-buffered saline (PBS); B: extract rich in anthocyanins from blueberry; BB: extract rich in anthocyanins from blueberry with *Bifidobacterium*; BL: extract rich in anthocyanins from blueberry with *Lactobacillus*; J: extract rich in water-soluble phenolic compounds from jabuticaba; JB: extract rich in water-soluble phenolic compounds with *Bifidobacterium*; JL: extract rich in water-soluble phenolic compounds with *Lactobacillus*. Reference: Own source.

In the extract rich in anthocyanins from blueberry with *Bifidobacterium* (BB) did not present significant difference in the percentage of apoptosis with respect to the negative control for Hepa 1c1c7 cells with a value of $7.42\% \pm 0.20$, but a significant increase ($p \leq 0.05$) was observed for HT-29 with a value of $15.31\% \pm 0.07$. There was no significant difference in the percentage of necrosis compared to the negative control, presenting values of $7.97\% \pm 0.92$ for Hepa 1c1c7 cells and $7.98\% \pm 0.40$ for HT-29 cells ($p > 0.05$). In the extract rich in anthocyanins from blueberry with *Lactobacillus* (BL) presented a significant difference ($p \leq 0.05$) in the percentage of apoptosis compared to the control for both cells with values of $17.96\% \pm 0.2$ for Hepa cells 1c1c7 and $29.47\% \pm 0.17$ for HT-29 cells. In the case of the percentage of necrotic cells, there was a significant increase ($p \leq 0.05$) for both cells compared to the control with a value of $59.44\% \pm 0.24$ for Hepa 1c1c7 cells and $56.73\% \pm 0.02$ for HT-29 cells. There are not many studies on the effect of extracts rich in anthocyanins from blueberry in combination with probiotics under increased apoptosis in

cell lines. But our study has shown an increase in apoptosis and necrosis when adding *Lactobacillus* and this may already be an indication of some synergism between them. Zhu et al. (2016) have already shown that the combination of blueberry juice with probiotics has a synergistic effect against the progression of alcoholic fatty liver disease (AFLD) by protecting liver cells.

Figure 24 – Effect of extracts of blueberry and jabuticaba on the percentage of (A) apoptotic cells and (B) necrotic cells in HT-29.

Bars identified with the same lowercase letters do not differ statistically ($p > 0.05$). Positive control: Camptothecin (CPT); Negative control: phosphate-buffered saline (PBS); B: extract rich in anthocyanins from blueberry; BB: extract rich in anthocyanins from blueberry with *Bifidobacterium*; BL: extract rich in anthocyanins from blueberry with *Lactobacillus*; J: extract rich in water-soluble phenolic compounds from jabuticaba; JB: extract rich in water-soluble phenolic compounds with *Bifidobacterium*; JL: extract rich in water-soluble phenolic compounds with *Lactobacillus*. Reference: Own source.

With respect to the extract rich in water-soluble phenolic compounds from jabuticaba (J), it was observed that the percentage of apoptosis and necrosis in Hepa 1c1c7 cells did not have significant difference compared to the control with values of $4.43\% \pm 0.15$ and $5.27\% \pm 0.32$, respectively. Whereas, in the HT-29 cells we observed a significant increase ($p \leq 0.05$) in the percentage of apoptosis and necrosis with values of $20.27\% \pm 1.11$ and $28.10\% \pm 2.94$, respectively. It has already been observed in the study by Wang et al. (2014) that the aqueous extract of the seed of the jabuticaba induced in 57.1% oral cancer cell apoptosis by decreasing the expression of surviving pointing to a potential chemopreventive agent. This is because this fruit presents large amounts of different antioxidant compounds, thus promoting an inhibitory effect on carcinogenesis and tumor growth (COSTA et al., 2013).

In the extracts rich in water-soluble in phenolic compounds from jabuticaba with *Bifidobacterium* (JB) the percentage of apoptosis and necrosis in the Hepa 1c1c7 cells was not significant compared to the negative control with values of $5.49\% \pm 0.59$ and $7.66\% \pm 0.03$, respectively. In HT-29 cells a significant increase ($p \leq 0.05$) in apoptosis was observed with values of $19.83\% \pm 1.48$ and no significant difference was observed in the percentage of necrosis with a value of $7.02\% \pm 0.76$. In the extracts rich in water-soluble in phenolic compounds from jabuticaba with *Lactobacillus* (JL) there was a significant increase ($p \leq 0.05$) in the percentage of apoptosis and necrosis in Hepa 1c1c7 cells with values of $14.25\% \pm 0.06$ and $70.25\% \pm 0.13$, respectively. In the HT-29 cells there was a significant increase ($p \leq 0.05$) in the percentage of apoptosis and necrosis with values of $45.13\% \pm 1.16$ and $40.10\% \pm 0.63$, respectively. It can be stated that the insertion of *Lactobacillus* significantly increased ($p \leq 0.05$) the percentage of apoptosis and necrosis in both cancer cells. No studies were found to show the effect of jabuticaba extracts with probiotics on the percentage of apoptosis. Some studies have demonstrated that specific microbial communities such as probiotics have the ability to produce metabolites with high bioactivity from phenolic compounds (KEPPLER; HUMPF, 2005; SOUZA et al., 2018). Our extract is rich in water-soluble phenolic compounds from jabuticaba (eg anthocyanins, ellagic acid and derivatives) and this combination with probiotics can considerably influence several biochemical and molecular pathways that inhibit mutagenesis, proliferation and induce apoptosis.

6.4 CONCLUSION

This study demonstrated different chemopreventive effects of extracts of blueberry and jabuticaba alone or in combination with probiotics against Hepa 1c1c7 and HT-29 cells. In relation to the antiproliferative activity, it was verified that for the extract from blueberry had the best results with extract E4: extract rich in anthocyanins, as for the jabuticaba extract was extract E2: extract rich in water-soluble phenolic compounds.

The highest FRAP and DPPH IC₅₀ values were found in extract rich in anthocyanins from blueberry with *Bifidobacterium* (BB) and extract rich in water soluble phenolic compounds from jabuticaba with *Lactobacillus* (JL) and *Bifidobacterium* (JB). There was also a significantly higher increase in activity of QR for the extract rich in water-soluble phenolic compounds from jabuticaba with *Lactobacillus* (JL) for both cells, the same occurs with extract rich in anthocyanins from blueberry with *Bifidobacterium* (BB).

With respect to assay apoptosis, both the extract rich in water-soluble phenolic compounds from jabuticaba with *Lactobacillus* (JL) and the extract rich in anthocyanins from blueberry with *Lactobacillus* (BL) presented significantly higher percentages of apoptosis and necroses compared to extracts alone or with *Bifidobacterium*. In conclusion, the extract that presented a greater chemopreventive effect compared to the others was the extract rich in water-soluble phenolic compounds from jabuticaba with *Lactobacillus* (JL), but there should be more studies to understand the interaction between them.

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7 CONCLUSION

This study demonstrates that the anticancer properties of plant extracts alone or in combination with probiotics have different effects. The extract rich in water soluble phenolic compounds from jabuticaba with BGP1 has a superior effect to the other extracts studied in the different cells of the CRC. This combination should be better evaluated in *in vivo* tests and also the other possible health benefits. However, PRCE with BLC1 increases the induction of quinone reductase in HT-29 cells, showing a possible effect in the early stages of carcinogenesis.

Due to this synergism between PRCE and BLC1, it is necessary to protect them to ensure that they can act together in the intestine. So, the solid lipid microparticles covered by electrostatic interactions of polymers demonstrate an effective alternative for the protection of these bioactive materials providing high values of encapsulation efficiency, irregular shapes and stable physico-chemical characteristics. The treatment BLC1 and 5% PRCE presents a good stability of the bioactive materials during storage, as well as during the simulated gastrointestinal conditions, which concludes that these microparticles can be incorporated in several food matrices.

The application of these microparticles in the sugarcane juice is effective in protecting BLC1 during the storage of the juice and in maintaining high levels of phenolic compounds and proanthocyanidins from PRCE. Nevertheless, the presence of this type of microparticle in drinks alters viscosity and sensory characteristics, which is not an advantage. Therefore, application in solid foods can be a proposal to be investigated.

ATTACHMENT A – PAPER PUBLISHED IN FOOD RESEARCH INTERNATIONAL

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Potential of solid lipid microparticles covered by the protein-polysaccharide complex for protection of probiotics and proanthocyanidin-rich cinnamon extract

Author: Augusto Tasch Holkem, Carmen Sílvia Favaro-Trindade

Publication: Food Research International

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Study of anticancer properties of proanthocyanidin-rich cinnamon extract in combination with Bifidobacterium animalis subsp. lactis BLC1 and resistance of these free and co-encapsulated materials under in vitro simulated gastrointestinal conditions

Author: Augusto Tasch Holkem, Carmen Silvia Favaro-Trindade, Monique Lacroix

Publication: Food Research International

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ATTACHMENT C – PAPER PUBLISHED IN PROBIOTICS AND ANTIMICROBIAL PROTEINS

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Institution name	Universidade de São Paulo/Faculdade de Zootecnia e Engenharia de alimentos
Expected presentation date	Dec 2020
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**ATTACHMENT D – ACCEPTANCE OF THE RESEARCH ETHICS COMMITTEE FOR THE
EVALUATION OF SUGARCANE JUICE SAMPLES.**

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação sensorial de caldo de cana enriquecido com probiótico e/ou extrato de canela(C. zeylanicum)

Pesquisador: AUGUSTO TASCH HOLKEM

Área Temática:

Versão: 1

CAAE: 87062618.4.0000.5422

Instituição Proponente: UNIVERSIDADE DE SAO PAULO

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.611.903

Apresentação do Projeto:

O trabalho esta bem apresentado e contentem todos os arquivos necessários para sua avaliação. O trabalho destaca a importância dos alimentos funcionais contendo probióticos e compostos bioativos devido à crescente conscientização dos consumidores em manter uma alimentação saudável.

Objetivo da Pesquisa:

O projeto visa elaborar caldo de cana enriquecido com probióticos e/ou extrato da canela na forma livre e encapsulada, e caracterizar sensorialmente os produtos obtidos.

A técnica utilizada será a de microencapsulação a qual é uma tecnologia eficiente, que tem sido desenvolvida para utilização na indústria alimentar e permite a proteção das bactérias e dos polifenóis até seu local de ação. Os materiais encapsulantes utilizados serão: gordura vegetal com ponto de fusão de 48

°C, proteína do soro do leite e goma arábica.

Avaliação dos Riscos e Benefícios:

A avaliação dos riscos foi muito bem descrita destacando todos os cuidados necessários na elaboração do produto final. O desconforto pode ser pela utilização da canela e sua sensação de adstringência, porem está destacado no TCLE.

Endereço: Avenida Duque de Caxias Norte, 225
Bairro: Campus Universitário da USP **CEP:** 13.635-900
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USP - FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS DA

Continuação do Parecer: 2.611.903

Comentários e Considerações sobre a Pesquisa:

Esta assessoria acredita que este trabalho deva ser desenvolvido, pois apresentará novas possibilidades ao mercado de alimentação saudável.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos apresentados estão corretos.

Recomendações:

Recomendo a aprovação deste projeto

Conclusões ou Pendências e Lista de Inadequações:

Nada a declarar

Considerações Finais a critério do CEP:

Este CEPH FZEA aprova o desenvolvimento do projeto e ressalta a necessidade de se informar, no TCLE, o nome, e-mail e telefone do CEP responsável pela aprovação.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMACOES_BASICAS_DO_PROJETO_1097626.pdf	05/04/2018 16:34:32		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLEnovo.pdf	05/04/2018 16:33:25	AUGUSTO TASCH HOLKEM	Aceito
Projeto Detalhado / Brochura Investigador	Projetonovo.pdf	05/04/2018 16:33:09	AUGUSTO TASCH HOLKEM	Aceito
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ATTACHMENT E – PAPER PUBLISHED IN NUTRITION AND CANCER

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Chemopreventive Properties of Extracts Obtained from Blueberry (*Vaccinium myrtillus* L.) and Jaboticaba (*Myrciaria cauliflora* Berg.) in Combination with Probiotics
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