**Maria Thereza Carlos Fernandes**

# CARACTERIZAÇÃO BIOLÓGICA E QUÍMICA DE COMPOSTOS BIOATIVOS DA JUÇARA (*Euterpe edulis*) ANTES E APÓS A FERMENTAÇÃO POR *Limosilactobacillus reuteri* E *Bifidobacterium animalis* subsp lactis BB-12.

Londrina
2022

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Tese apresentada ao Programa de Pós-Graduação em Ciência de Alimentos, nível Doutorado, da Universidade Estadual de Londrina, como requisito parcial à obtenção do Título de Doutor em Ciência de Alimentos

Orientadora: Profa. Dra. Sandra Garcia
Co-orientadora: Profa. Dra. Karla Bigetti Guergoletto

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Londrina, 31 de março de 2022

Aos meus pais Luiz e Lucia, por sempre me motivarem e proporcionarem todo o amor, apoio e confiança. Ao meu irmão Guilherme, pelo amor, amizade e carinho. Ao Milton meu companheiro de todas as jornadas da vida. Amo todos vocês.

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*“A verdadeira viagem de descobrimento não consiste em procurar novas paisagens, mas em ter novos olhos”.*

Marcel Proust

Fernandes, Maria Thereza Carlos. Caracterização biológica e química de compostos bioativos da juçara (*Euterpe edulis*) antes e após a fermentação por *Limosilactobacillus reuteri* e *Bifidobacterium animalis* subsp lactis bb-12. 2022. 128 f. tese (doutorado em ciência de alimentos), Universidade Estadual de Londrina, Londrina, 2022.

## RESUMO

A Juçara (*Euterpe edulis*), é uma palmeira pertencente à família *Arecaceae* e ao gênero *Euterpe*, no Brasil está distribuída em áreas da Mata Atlântica. Seu palmito é amplamente consumido no país, porém a extração clandestina deste palmito expõe esta palmeira ao risco de extinção. Desta forma, seu manejo sustentável, através da utilização de seu fruto, torna-se necessário para a conservação da espécie, além de ser considerada uma estratégia rentável economicamente. O fruto da palmeira juçara é rico em compostos fenólicos principalmente antocianinas e sua polpa apresenta-se como um adequado meio fermentativo para bactérias probióticas. Dentre essas bactérias destacam-se *Limosilactobacillus reuteri* e *Bifidobacterium animalis subsp* lactis BB-12 capazes de trazer benefícios ao hospedeiro através da colonização intestinal. Considerando que a fermentação ocasiona mudanças no meio fermentativo, com a utilização de compostos presentes para crescimento bacteriano, e a necessidade de maiores informações sobre a biodisponibilidade de compostos bioativos presentes no fruto juçara, este trabalho teve como objetivo elucidar quais desses compostos são utilizados pelos microorganismos em seus crescimentos, além de quantificar os compostos bioativos antes e depois da fermentação durante a simulação gastrointestinal *in vitro*. Para a biodisponibilidade dos compostos bioativos a polpa de juçara de juçara foi fermentada por 24 horas a 37 °C por *L. reuteri* e BB-12. separadamente e, submetida a digestão *in vitro* pelo método BARGE. A quantificação dos compostos fenólicos foi realizada por UPLC-MS/MS e os compostos que apresentaram maiores concentrações em ambas as amostras foram os ácidos protocatecuico e 4-hidroxibenzóico, demostrando que durante a fermentação novos compostos fenolicos são produzidos. Na quantificação de minerais o

método utilizado foi espectrometria de absorção atômica com chama, onde foi possível observar maior biodisponibilidade de cobre e zinco nas amostras fermentadas. A técnica de RMN foi aplicada nas frações de juçara fermentada em meio basal para identificar o perfil de fermentação delas, sendo possível observar um melhor aproveitamento dos açúcares presentes na polpa por BB-12 quando comparado com *L. reuteri*. Como resultado da fermentação singleto em 1.92 ppm indica a produção de acetato. Desta forma este trabalho demonstra que a polpa de juçara é um excelente meio fermentativo, e que a fermentação por bactérias de interesse proporciona transformações nos compostos bioativos.

Palavras-chave: polifenóis, polissacarídeos, BARGE, bioacessibilidade.

Fernandes, Maria Thereza Carlos. Biological and chemical characterization of bioactive compounds from juçara (*Euterpe edulis*) before and after fermentation by *Limosilactobacillus reuteri* and *Bifidobacterium animalis* subsp lactis BB-12. 2022. 128 p. thesis (PhD in food science), State University of Londrina, Londrina, 2022

## ABSTRACT

Juçara (*Euterpe edulis*), is a palm belonging to the Arecaceae family and the Euterpe genus, in Brazil it is distributed in areas of the Atlantic Forest. Its palm heart is widely consumed in the country, but the clandestine extraction of this palm exposes it to the risk of extinction. In this way, its sustainable management, using its fruit, becomes necessary for the conservation of the species, in addition to being considered an economically profitable strategy. The fruit of the juçara palm is rich in phenolic compounds, mainly anthocyanins, and its pulp presents itself as a suitable fermentation medium for probiotic bacteria. Among these bacteria, *Limosilactobacillus reuteri* LR 92 and *Bifidobacterium animalis* subsp lactis BB-12. capable of bringing benefits to the host through intestinal colonization. Considering that fermentation causes changes in the fermentation medium, with the use of compounds present for bacterial growth, and the need for more information about the bioavailability of bioactive compounds present in the juçara fruit, this work aimed to elucidate which of these compounds are used by microorganisms in their growth, in addition to quantifying the bioactive compounds before and after fermentation during *in vitro* gastrointestinal simulation. For the bioavailability of the bioactive compounds, the juçara pulp was fermented for 24 hours at 37 °C by *L. reuteri* and BB-12. separately and subjected to in vitro digestion by the BARGE method. The quantification of phenolic compounds was performed by UPLC-MS/MS and the compounds that presented the highest concentrations in both samples were protocatechuic and 4-hydroxybenzoic acids, demonstrating that during fermentation new phenolic compounds are produced. In the quantification of minerals, the method used was flame atomic absorption spectrometry, where it was possible to observe greater bioavailability of copper and zinc in the fermented samples. The NMR technique was applied to the fermentation fractions of medium basal with to identify their fermentation profile, observing a better use of the sugars present in the pulp by

BB-12 when compared to *L. reuteri*. As a result of fermentation, a singlet in the region of 1.92 ppm indicates acetate production. Thus, this work demonstrates that juçara pulp is an excellent fermentation medium, and that fermentation by bacteria of interest provides transformations in bioactive compounds.

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# 1-INTRODUÇÃO

A palmeira *Euterpe edulis*, denominada como juçara pertence à família *Arecaceae* e ao gênero *Euterpe*. Nativas da Floresta Atlântica do Brasil, conhecida popularmente como juçara, jiçara ou palmiteiro, contém aproximadamente 7 espécies distribuídas nos trópicos ocorrendo desde o Rio Grande do Sul até a Bahia. Possui importante função ecológica especialmente em interação com a fauna, na composição das matas ciliares e na conservação de mata secundária (MEIRA NETO et *al.*, 2003; RIBEIRA et *al.,* 2020).

Conhecida principalmente pela extração predatória de seu palmito, que apresenta qualidade e sabor superiores em comparação com outras espécies do gênero *Euterpe* (Borges et al., 2013), a palmeira juçara produz frutos roxos e esféricos semelhantes aos das palmeiras *Euterpe oleracea* e *Euterpe precatoria*, conhecidos por produzir o açaí (CARDOSO et *al*., 2018). Desta forma objetivando diminuir o risco predatório, órgãos governamentais e organizações sem fins lucrativos procuram disseminar opções de exploração sustentável de espécies vegetais, sendo o uso de fruta para consumo humano e produção de novos produtos, uma das principais alternativas. (WENJUN, 2007; SILVA, et *al*., 2014; Schulz et *al*., 2016).

O fruto da juçara vem se destacando no meio científico por sua interessante composição nutricional, rica em antioxidantes. Atividades biológicas importantes têm sido associadas ao fruto da juçara, e seu uso na alimentação tornou-se uma alternativa nutricional, ambiental e econômica. Semelhante nutricionalmente ao fruto do açaizeiro (*E. oleracea*), a juçara apresenta quantidades superiores de antocianinas quando comparada ao açaí e outros frutos tropicais como a acerola (*Malphigia emarginata*), jambolão (*Syzygium cumini*) e guajiru (*Chrysobalanus icaco*) (IADEROZA et al., 1992; DE BRITO et al., 2007). Entre as antocianinas identificadas no fruto juçara destaca-se cianidina-3-glicosídeo e cianidina-3-rutinosídeo. Estudos apontam que os frutos de juçara (*E. edulis*) são excelentes fontes de polifenóis, fibras, proteínas, lipídeos e vitamina C (HUI, 2006; RUFINO et *al*., 2010; BORGES et *al.*, 2013; INADA et al., 2015).

Estudos demonstraram que a suplementação de juçara na dieta pode ter efeitos positivos na peroxidação lipídica, modulação do estado inflamatório, melhora dos níveis de glicose no sangue e um possível efeito benéfico sobre os microrganismos

probióticos (CARDOZO et *al.*, 2018). O efeito da fruta nos parâmetros do perfil lipídico e enzimas antioxidantes ainda parece ser um pouco controverso, pois estudos como os de Morais e colaboradores (2014) e Cardoso et *al.*, 2015 mostram efeitos positivos, porém os de De Castro *et al.*, 2014 e Oyama *et al.*, 2016 não apresentaram resultados positivos relacionados ao consumo do fruto juçara para melhoras no perfil lipídico, assim como na atividade de enzimas antioxidantes.

Embora os trabalhos apresentem resultados promissores, ainda é necessário padronizar estratégias importantes: níveis de concentração das frutas preparadas, a utilização de suco, polpa ou pó liofilizado e principalmente o tempo de consumo. Os resultados positivos encontrados após a administração da juçara são atribuídos à sua interessante composição nutricional. São apontados como responsáveis por esses efeitos os componentes do fruto da juçara, como compostos fenólicos, especialmente antocianinas e ácidos graxos insaturados. Ressaltando que os compostos fenólicos são metabolizados pela microbiota intestinal, e seus metabólitos podem exercer efeitos sobre a permeabilidade intestinal e contribuir para os efeitos biológicos dos compostos originais. A biodisponibilidade e o metabolismo dos polifenóis são importantes parâmetros analisados para melhor esclarecer os efeitos da polpa de juçara, para a prevenção ou tratamento de doenças crônicas (CARDOZO et *al.*, 2018).

A fermentação de produtos de origem vegetal tem sido amplamente considerada para a incorporação de probióticos em matrizes não lácteas (SOCCOL et al., 2010) visto que é crescente em todo o mundo a quantidade de consumidores que não utilizam produtos lácteos devido à intolerância à lactose, alergias à proteína do leite e dietas à base de produtos de origem vegetal (SOCCOL et al., 2010; SILVA et *al.*, 2017). Porém as matrizes de vegetais podem constituir meios desafiadores para a viabilidade probiótica e merecem investigação. Àcidos orgânicos e compostos fenólicos comumente presentes em frutas podem apresentar propriedades antimicrobianas (ESPÍRITO SANTO et *al.*, 2011). Entretanto o teor de compostos fenólicos pode contribuir para a sobrevivência de bactérias probióticas em alimentos e até mesmo exercer um efeito prebiótico no intestino humano (SUCCI et al., 2017; SOUZA et al., 2018).

Deste modo, visto que estudos posteriores demonstraram que a polpa de juçara é um bom meio fermentativo e visando alternativas que possam contribuir com a maior utilização do fruto da palmeira juçara de maneira sustentável, contribuindo com a

preservação desta espécie, o objetivo deste trabalho foi isolar frações da polpa dos frutos da juçara, caracterizar compostos presentes na polpa *in natura* e derivados da fermentação transformados (metabolitos) pelos probióticos *L. reuteri* e *Bifidobacterium spp*. Adicionalmente foi estudada a bioacessibilidade dos compostos bioativos deste fruto antes e após a fermentação da polpa de juçara.

## 2-OBJETIVO

### 2.1 Objetivo Geral

Avaliar os compostos bioativos da polpa de juçara *in natura* e após a fermentação por *Limosilactobacillus reuteri* e *Bifidobacterium animalis* subsp lactis BB-12. Avaliar as transformações e a bioacessibilidade causadas pelo processo fermentativo, a ação destes compostos no crescimento bacteriano e atividade antioxidante.

### 2.2 Objetivos Específicos

- Extrair a polpa do fruto da palmeira juçara e determinar a composição centesimal;
- Separar e identificar os compostos fenólicos da polpa da juçara *in natura;*
- Cultivar *L. reuteri* e *Bifidobacterium animalis* subsp. lactis BB-12 em meio basal, na presença de compostos isolados da juçara para elucidar o mecanismo do processo fermentativo;
- Acompanhar o crescimento bacteriano por contagem em placas e decréscimo do pH durante a fermentação;
- Caracterizar os metabólitos produzidos pelas bactérias através de ressonância magnética nuclear (RMN) e análise de componente principal;
- Determinar a atividade antioxidante dos compostos isolados antes e após o processo de fermentação;
- Avaliar o perfil de minerais e sua bioacessibilidade antes e após a fermentação da polpa de juçara;
- Determinar a bioacessibilidade de compostos fenólicos fermentados por HPLC-MS/MS

# CAPÍTULO I

# 3- REFERENCIAL TEÓRICO

## 3.1 Palmeira Juçara (*Euterpe edulis* Martius*)*

A família das palmeiras (*Arecaceae*), constitui-se numa das maiores famílias vegetais do mundo. São divididas em 6 subfamílias, que apresentam 200 gêneros dentre os quais o gênero *Euterpe*. Este gênero congrega cerca de 28 espécies, com destaques para: *oleracea, edulis* e *precatoria* que ocorrem em maior frequência (GALOTTA; BOAVENTURA, 2005). A principal diferença entre as três espécies é a localização de crescimento e as características das palmeiras (DE LIMA YAMAGUCHI et *al*., 2015).

*Euterpe edulis* Martius é uma palmeira nativa da Floresta Atlântica do Brasil, conhecida popularmente como juçara, jiçara ou palmiteiro. Contém aproximadamente 7 espécies distribuídas nos trópicos ocorrendo desde o Rio Grande do Sul até a Bahia. Possui elevada produção de flores, frutos e plântulas, tendo importante função ecológica especialmente em interação com a fauna, na composição das matas ciliares e na conservação de áreas perturbadas ou de mata secundária (LORENZI et *al*., 2010).

O palmito extraído da juçara é comestível, e muito apreciado por consumidores. A extração ilegal ocorre desde meados de 1940, tendo seu ápice em 1970, motivada pelo valor econômico do produto. Devido à intensa exploração predatória e o corte indiscriminado, a regeneração natural e conservação da palmeira juçara foi gravemente prejudicada (BOURSCHEID et *al.*, 2011).

O emprego de plantas nativas, tais como a palmeira juçara, como parte da produção local de alimentos pode ajudar no desenvolvimento de uma agricultura mais sustentável, que ocorre em paralelo com a preservação da biodiversidade (SHELEF, WEISBERG, & PROVENZA, 2017). Desta forma, o uso do fruto desta palmeira para o consumo humano torna-se uma forma de exploração sustentável e economicamente viável (TREVISAN, et *al*., 2015; SCHULZ et *al.*, 2016).

## 3.2 Fruto Juçara

O fruto da palmeira juçara, também denominado de juçara, é constituído de drupa esférica com pericarpo pouco espesso e liso, possui coloração que passa do

verde ao roxo negro durante a maturação e são caracterizados por uma única semente, que constitui cerca de 80% do volume total. São revestidos por uma camada fibrosa, uma fina cobertura oleosa e um mesocarpo comestível. A frutificação dessa palmeira ocorre de maneira abundante entre os meses de março e junho (CERISOLA et *al*., 2007).

Sensorialmente muito semelhante ao fruto do açaí, porém com um consumo muito menor, o fruto juçara pode ser utilizado como matéria-prima para produção de polpa ou suco com excelentes valores nutricionais, rico em ácidos graxos insaturados, lipídeos e elevados teores de compostos fenólicos e antocianinas (De BRITO et *al.*, 2007, BORGES et *al.*, 2011). Diversos estudos apontam que os frutos de juçara (*E. edulis*) são excelentes fontes de polifenóis, uma vez que o conteúdo fenólico total representa de 5672 a 7500 mg equivalente de ácido gálico (GAE) 100 $g^{-1}$ com base no peso seco (BORGES et *al*., 2013; INADA et *al*., 2015; RUFINO et *al*., 2010). Esses valores são maiores do que os encontrados no açaí das espécies *E. oleracea* e *E. precatoria* e de algumas frutas tais como mirtilo, cereja e framboesa, reconhecidas pelo seu poder antioxidante (DE BRITO et al., 2007).

Dentre os fenóis já identificados no fruto da palmeira juçara estão: ácidos fenólicos, benzóico, cafeico, clorogênico, siríngico e vanilico e os flavonoides: quercetina e rutina. Quanto a presença de antocianinas no fruto da palmeira juçara destacam: cianidina 3-O-glicosídeo [C3G] como a principal antocianina, e cianidina-3-O-rutinosideo, cianidina-3,5-diglucosídeo, pelargonidin-3-O-rutinosídeo, peonidina-3-O-glucosídeo, peonidina-3-O-rutinosídeo, cianidina-3-sambubiosídeo e cianidina-3 ramnosídeo em menores quantidades (BICUDO et *al,* 2014; BORGES et *al.*, 2011; GUERGOLETTO et *al*., 2016).

Vários estudos *in vivo* com indivíduos saudáveis, modelo animal e cultura de células Vero apontaram que a fruta juçara possui um efeito positivo no estado antioxidante. Devido à sua capacidade antioxidante excepcionalmente alta, a fruta juçara foi ultimamente mencionada como um “superalimento” (OYAMA et *al.*, 2016; CUNHA JÚNIOR et *al.*, 2015; BORGES et *al.*, 2013)

Em relação aos lipídeos os frutos da palmeira juçara apresentam alto teor. O fruto possui cerca de 20 vezes mais lipídios, comparando-se com outras frutas tropicais brasileiras, tais como araça, grumixama, uvaia e jabuticaba (DA SILVA et *al.*, 2014, INADA et *al.*, 2015). O fruto também se destaca como importante fonte vegetal

de proteína dietética, pois pode fornecer em peso fresco, até dez vezes mais proteína do que frutas como maçãs, manga, pera e melão, (HUI, 2006).

O consumo do fruto da palmeira *Euterpe edulis Martius* pode fornecer boa quantidade de vitaminas e minerais. Com alto teor de vitamina C, o fruto juçara contribui com a absorção de ferro pelo organismo, sendo que o consumo de 100 g da fruta apresenta cerca de 147 mg de ácido ascórbico e garante um valor acima da ingestão diária recomendada para todas as idades e sexos (entre 15 e 120 mg / dia) (RUFINO et *al.*, 2010). O potássio é o principal mineral da fruta e polpa de juçara, porém estão também presentes: sódio, cálcio, magnésio, ferro, zinco, manganês, fósforo, enxofre, cobre, níquel, cobalto, selênio, cádmio, boro, alumínio e molibdênio (SCHULZ et *al.*, 2016). No Quadro 1, estão apresentadas as informações nutricionais do fruto juçara em relação ao açaí:

Quadro 1: Informação nutricional dos frutos juçara e açai

| **Para cada 100 ml** | **Juçara** | | **Açaí** | |
|---|---|---|---|---|
| | | VD* | | VD* |
| Valores energéticos | 63,8 Kcal | 3,44 | 51,4 Kcal | 2,55 |
| Carboidratos totais | 5,7 g | 1,9 | 4,3 g | 1,4 |
| Proteínas | 0,67 g | 0,9 | 0,77 g | 1,03 |
| Lipídeos (Gorduras totais) | 3,5 g | 6,4 | 1,3 g | 0,24 |
| Gorduras saturadas | 0 g | | 0 g | |
| Gorduras trans | 0 g | | 0 g | |
| Fibra alimentar | 3,23 g | 12,9 | 2,2 g | 8,8 |
| Antocianinas | 61,85 mg | | 17,50 mg | |
| Fósforo | 12,85 mg | | 42,82 mg | |
| Potássio | 101,07 mg | | 77,08 mg | |
| Cálcio | 33,96 mg | | 28,26 mg | |
| Magnésio | 9,42 mg | | 10,27 mg | |
| Enxofre | 11,14 mg | | 11,14 mg | |
| Ferro | 0,59 mg | | 0,39 mg | |
| Manganês | 0,31 mg | | 0,92 mg | |
| Cobre | 0,12 mg | | 0,25 mg | |
| Zinco | 0,23 mg | | 0,21 mg | |
| Sódio | 3,51 mg | | 2,44 mg | |
| Boro | 0,08 mg | | 0,02 mg | |
| Cobalto | 1,525 mg | | 0,007 mg | |
| *valores diários de Referência com base em uma dieta de 2000 Kcal ou 8400 Kcal. Seus valores diários podem ser maiores ou menores dependendo de suas necessidades | | | | |

Fonte: Cartilha da juçara 2015.

### 3.3 Polissacarídeos

Os polissacarídeos são polímeros naturais, formados por um ou vários tipos de monossacarídeos. Podem ser classificados como homo-, co- e hetero-polissacarídeos tais como celulose, alginato e goma arábica respectivamente. Esses polímeros são constituídos de monossacarídeos, sendo possível diferenciá-los por meio da quantidade de átomos de carbono presente nessas unidades, pelo grau de ramificação, posição das ligações glicosídicas entre as unidades e comprimento de suas cadeias (LEHNINGER; NELSON, 1995).

Essas macromoléculas são encontradas em todos os organismos vivos, podendo ser obtidas a partir de bactérias ou de seres mais complexos. Os polissacarídeos possuem aplicações nas mais diversas áreas, e desta forma são materiais de grande interesse comercial desde os mais simples, tais como amido e celulose, até os mais complexos como xantanas, quitosanas e quitina. Para aplicações industriais os polissacarídeos normalmente são extraídos de plantas (frutos, sementes, exsudatos e tubérculos), fungos, animais e fermentação microbiana.

Quanto às atividades biológicas atribuídas aos polissacarídeos destacam-se seus efeitos na atividade imunológica modulando a resposta de células do sistema imune, sendo classificados como modificadores exógenos de respostas biológicas (ABBAS; LICHTMAN, 2005). Estudos apontam que os polissacarídeos podem também promover proteção gástrica e intestinal, visto que apresentam potencial cicatrizante de feridas e atividade antiulcerogênica e antidiarreica (SRIKANTA et *al.*, 2007; SCHIRATO et *al.*, 2006; DAMASCENO et *al.*, 2013).

Alguns tipos de polissacarídeos naturais não são digeríveis pelo trato gastrointestinal, e desta forma, são possíveis substratos para o crescimento de microrganismos probióticos tais como *Bifidobacterium* e *Lactobacillus,* beneficiando o hospedeiro. Wang e colaboradores (2015) encontraram evidências de que os polissacarídeos extraídos de plantas possuíam atividades prebióticas, sendo que polissacarídeos de diferentes espécies podem ter composições variadas, o que resulta em diferentes características físico-químicas e funcionais.

## 3.4 Compostos Fenólicos

Os compostos fenólicos são metabólitos reativos que podem ser divididos em quatro grupos: ácidos fenólicos, flavonóides, estilbenos e taninos. De origem vegetal, funcionam como receptores de radicais livres e quelantes de íons metálicos com capacidade de catalisar a oxidação lipídica, e desta forma, considerados antioxidantes naturais (OZCAN et *al.*, 2014).

Formados por um anel aromático (Figura 1), que comporta um ou mais grupos hidroxila, os fenólicos podem ter estruturas que variam de uma simples molécula fenólica a um polímero complexo de alto peso molecular. Frequentemente chamados de polifenóis (Figura 2), são metabólitos secundários derivados das vias das pentoses fosfato, chiquimato e fenilpropanóide dos vegetais (ESCARPA; GONZALEZ, 2001).

São inúmeros os benefícios dos fenólicos para a saúde humana. Estudos apontam os efeitos anti-carcinogênicos e anti-mutagênicos dos ácidos fenólicos, taninos hidrolisáveis e flavonoides. Esses compostos podem atuar como agentes protetores do DNA contra os radicais livres carcinogênicos, além de inibir as enzimas envolvidas na ativação pró-carcinogênica. A ingestão de flavonoides favorece a diminuição do LDL, e estão relacionados, em conjunto com as catequinas e seus derivados, como agentes terapêuticos para prevenir o processo de envelhecimento do cérebro e doenças neurodegenerativas, servindo como possíveis agentes neuroprotetores em doenças neurodegenerativas como Parkinson e Alzheimer (OZCAN et *al.*, 2014; DEL RIO et *al.*, 2013).

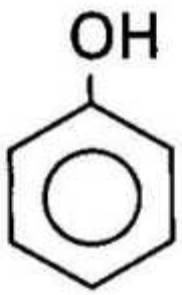



Figura 1: Fenol (estrutura básica)

Fonte: OZCAN et al., 2014.

Os benefícios para a saúde proporcionados pelos compostos fenólicos dependem da sua absorção e metabolismo, o que por sua vez são determinados por sua estrutura, conjugação com outros compostos fenólicos, grau de glicosilação/ acilação, tamanho da molécula e solubilidade (CROZIER; JAGANATH & CLIFFORD, 2009). São diversas as vias de absorção existentes para compostos fenólicos presentes no trato gastrointestinal com o envolvimento de microrganismos, enzimas e em alguns casos transportadores de glicose (ACOSTA-ESTRADA; GUTIÉRREZ-URIBE & SERNA-SALDÍVAR, 2014). Guergoletto et *al.*, (2016) monitorou o conteúdo de fenólicos presentes no fruto juçara durante a simulação gastrointestinal (GI). O estudo apontou que as antocianinas, a quercetina e a rutina sofreram degradação durante a passagem ao trato gastrointestinal. Entretanto, 46% dos teores de fenólicos totais resistiram à digestão e, desta forma, poderiam ser utilizados pelas bactérias benéficas do cólon. Adicionalmente, estudos demonstram que compostos fenólicos promovem o crescimento de bactérias benéficas existentes no colón tais como *Bifidobacterium e Lactobacillus* spp, (CARDONA et *al.,* 2013 HIDALGO et *al.*, 2012; GUERGOLETTO et *al.*, 2016, ZHOU et *al.*, 2016).

Os mecanismos da ação dos polifenóis como substâncias prebióticas ainda não são totalmente elucidados, alguns estudos apontam que diferentes tipos de bactérias possuem enzimas que são capazes de metabolizar esses compostos e utilizá-los como fonte de energia (GARCÍA-RUIZ et *al.*, 2008). Pereira-Caro e colaboradores (2015) apontaram em seu trabalho que ao atingirem o colón, os polifenóis sofrem biotransformações extensas pela microbiota intrínseca, melhorando a absorção e consequentemente sua biodisponibilidade (PLANADA & VODNAR, 2022). Culturas probióticas foram capazes de promover esta biotransformação de compostos fenólicos através da ação de enzimas glicosil hidrolases. As enzimas promovem a liberação de agliconas em substâncias fenólicas que estavam conjugadas com compostos glicóis (GOMES et *al.*, 2018).

Outros estudos demonstram atividades antimicrobianas ou bacteriostáticas dos compostos fenólicos. Ao serem ingeridos na forma de alimento, quando não absorvidos, inibem de forma seletiva o crescimento de bactérias patogênicas e consequentemente permitem uma maior colonização de bactérias benéficas,

influenciando na composição da microbiota intestinal (LEE et al., 2006; SELMA; ESPÍN; TOMÁS-BARBERÁN, 2009; LAPARRA; SANZ, 2010).

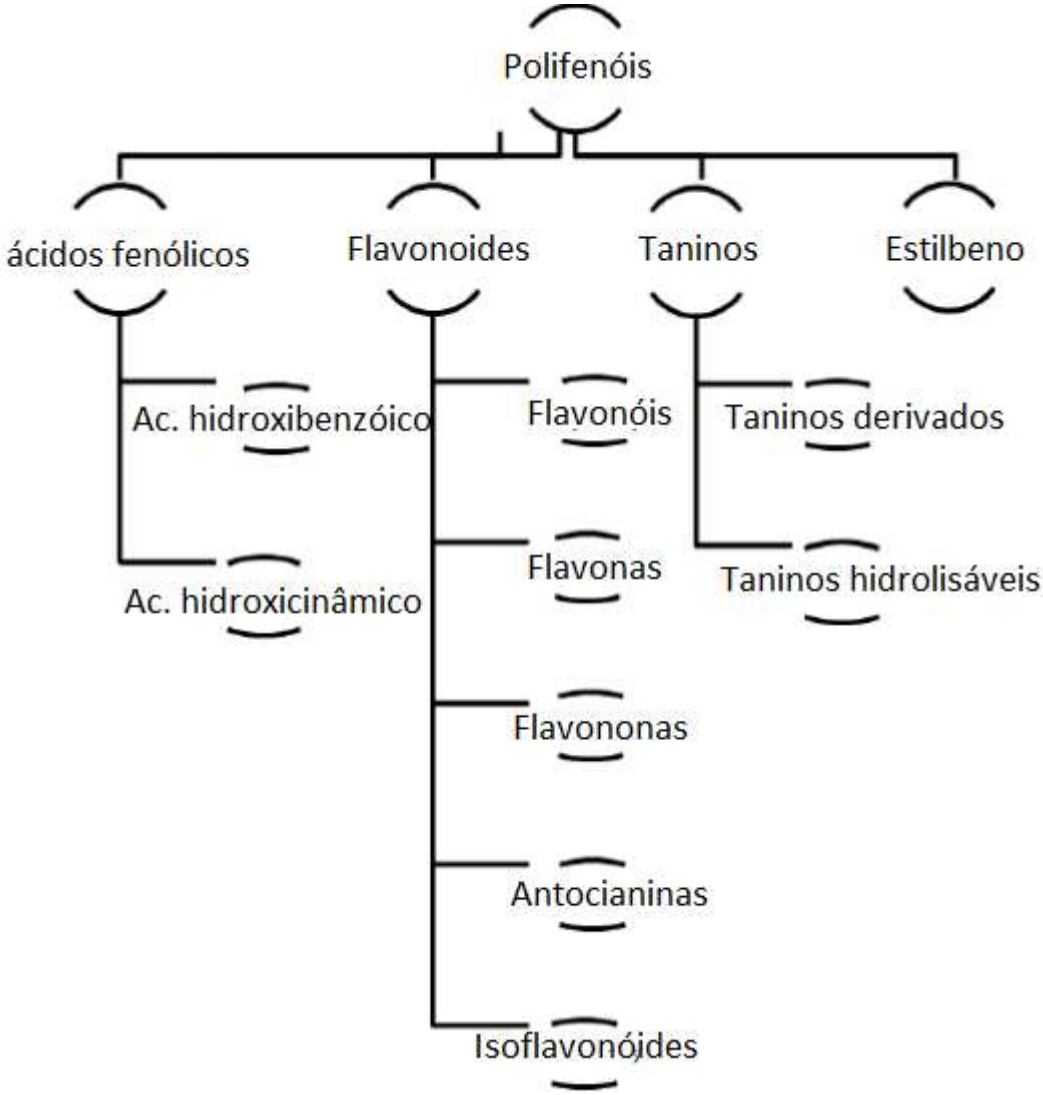



Figura 2: Principais classes de compostos polifenólicos (adaptado de OZCAN et *al.*, 2014).

### 3.4.1 Antocianinas

As antocianinas são pigmentos amplamente distribuídos na natureza e responsáveis por grande parte das cores de flores e vegetais, que vão do vermelho ao azul. Pertencente a subclasse dos flavonoides, constituem um grupo de pigmentos solúveis em água e sensíveis ao calor. (VILLIERS et *al.*, 2009; AZEVEDO et *al.*, 2010, FERREIRA, 2013). As diferenças estruturais das antocianinas são atribuídas ao

número de hidroxilas, a natureza e o número de açúcares ligados à estrutura flavílio, ligantes alifáticos ou carboxilatos aromáticos, e a posição dessas ligações. A estabilidade das moléculas é favorecida pela forma glicosilada, forma na qual é encontrada na natureza (VILLIERS et *al.*, 2009; OREN-SHAMIR, 2009).

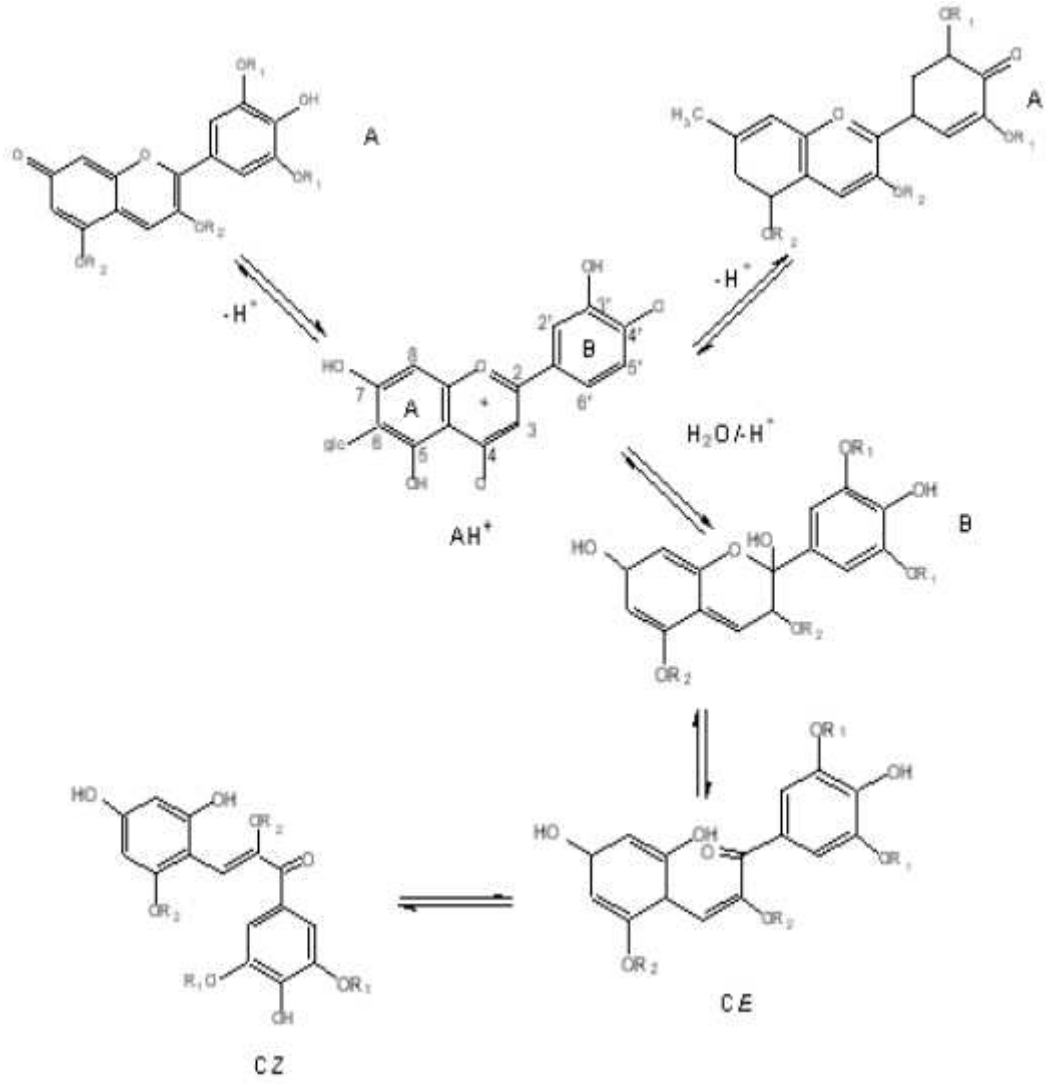
As antocianinas possuem atividade anti-inflamatória e antioxidante e são encontradas em alimentos, como uvas, amoras e mirtilos. Exemplos de antocianinas glicosiladas detectadas, nesses alimentos são: malvidina, delfinidina, pelargonidina, cianidina, peonidina e petunidina (AQIL et *al.*, 2006; FERNANDES et *al.*, 2014). Reconhecidos por suas propriedades antioxidantes, esses flavonóides possuem importante papel na prevenção ou no retardamento do aparecimento de várias doenças (KUSKOSKI et *al.*, 2004; MARTÍNEZ-FLÓREZ et *al.*, 2002). Estudos relatam que as antocianinas podem proteger contra o risco de doença cardiovascular, além de proporcionar melhoras na visão, cognição e pressão arterial (KALT et *al.,* 2014; CASSIDY; FRANZ & RIMM, 2016).

Quando metabolizadas, as antocianinas formam glucurono, sulfo ou metil-derivados no trato gastrointestinal (KAMILOGLU et *al.*, 2015). Pesquisas demonstraram que as antocianinas são pouco absorvidas pelo organismo. Após seu consumo, quando estão ligadas a matrizes alimentares, pequenas frações intactas são capazes de atravessarem a parede gastrointestinal, por meio de transportadores ativos tais como os transportadores de glicose. Ao atingirem o intestino grosso e o cólon, são absorvidas e metabolizadas. Os metabolitos gerados são biologicamente ativos, podendo ser utilizados pela microbiota intestinal (FANG, 2014).

O consumo de 100 g de frutos como mirtilo, framboesa e amora podem prover quantidades maiores que 500 mg de polifenóis e 200 mg de antocianinas (WANG et *al.*, 2013). Frutos típicos do Brasil como juçara, amora silvestre, amora preta e grumixama fornecem de 32 a 260 mg de antocianinas em 100g (HASSIMOTTO et *al.*, 2008; TEIXEIRA et *al.*, 2015).

A utilização desse pigmento em alimentos é desejável, pois não apresenta efeitos adversos à saúde, possuem propriedades químicas e sensoriais desejáveis, além de sua funcionalidade que agrega valor ao produto (FALCÃO et al., 2007). Porém suas características como a baixa estabilidade ao pH e a luz, a presença de enzimas e a temperatura em que o pigmento é exposto no processamento dos alimentos, são fatores que comprometem a sua aplicação. Como exemplo: o cátion flavilium

apresenta-se predominantemente em tom vermelho somente em condições ácidas (Figura 3). Desta forma, são necessários mais estudos para garantir a estabilidade desses compostos, possibilitando sua ampla utilização (CONSTANT, 2003; MALACRIDA & MOTTA, 2006).




A: bases quinoidais.

B: hemiacetal ou pseudobase carbi-nol. AH +: cátion flavilium. CE e CZ: Chalconas.

R1: H, CH3

R2: H, CH3, açúcar, açúcar acilado.

Figura 3: Estruturas de antocianinas em equilíbrio em meio aquoso ácido (FIGUEIREDO et al., 1996)

### 3.5 Minerais

Para uma alimentação balanceada, uma dieta deve fornecer além de quantidades adequadas de nutrientes essenciais, como carboidratos, gorduras e proteínas, outros nutrientes como vitaminas e minerais. Os minerais encontrados nos alimentos possuem inúmeros benefícios para a saúde. Participam da manutenção dos tecidos, formação e saúde dos ossos e dentes, são cofatores e coenzimas para vários sistemas enzimáticos, participando da regulação e coordenação da maioria das funções do corpo e outras funções bioquímicas e fisiológicas (GENAND et *al.*, 2016; TUCKER et *al.*, 2016).

São responsáveis por grande parte da estrutura de ossos (Ca, Mg, Mn, P, B e F), e dentes (Ca, P e F). A transmissão de sinais pelas células nervosas também é influenciada pelos minerais (Ca, Mg, P, Na, K), ainda exercem funções importantes na formação das células sanguíneas (Co, Fe) bem como na regulação dos níveis de glícose do organismo (Cr). Ressaltando o papel do cálcio (Ca) e do potássio (K) no controle da pressão arterial (GHARIBZAHEDI & JAFARI 2017).

São vinte os minerais considerados nutricionalmente essenciais Estes podem ser divididos em macrominerais: sódio, potássio, cloreto, cálcio, magnésio e fósforo; e como microminerais: ferro, cobre, zinco, manganês, selênio, iodo, cromo, cobalto, molibdênio, fluoreto, arsênio, níquel, silício e boro.

O conteúdo mineral em alimentos de origem vegetal depende de fatores como o conteúdo natural de oligoelementos no ambiente, seu nível de fertilizantes minerais e as doses de fertilizantes utilizadas no plantio. No solo, uma fonte natural desses metais é a rocha-mãe, já solos utilizados para fins agrícolas, algumas quantidades de metais são introduzidas juntamente com fertilizantes, tanto orgânicos como minerais (principalmente cálcio e fosfatos) (Zwolak, et *al.*, 2019).

Apesar de muitos produtos vegetais serem ricos em minerais, estudos demonstram que fatores como a forma química, ligantes e atividade redox em componentes do alimento, interações entre minerais, interferem na biodisponibilidade dos nutrientes (DAMODARAN; PARKIN; FENNEMA, 2010). Os antinutrientes são

componentes naturalmente presentes em frutas, grãos e vegetais interferindo na digestibilidade, absorção ou uso de nutrientes no trato gastrointestinal (ETSUYANKPA et *al.*, 2015). O ácido fítico (PA) é um destes antinutrientes e tem potencial para reduzir a absorção de minerais, proteínas e vitaminas ($Zn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Mg^{2+}$, $Ca^{2+}$) devido ao seu efeito quelante, formando complexos insolúveis principalmente com minerais, reduzindo o aproveitamento pelo organismo (UDOMKUN et *al.*, 2019).

### 3.6 Microbiota intestinal

A microbiota é uma população de microrganismos que habitam vários locais do corpo humano, como nariz, boca, pele e intestino (THOMAS & GREER, 2016). Os seres humanos são colonizados por trilhões de microrganismos que possuem um metagenoma estimado de pelo menos 100 vezes o das células humanas. As capacidades metabólicas e enzimáticas desses microrganismos são muito maiores do que as de seus hospedeiros (FLINT et *al.*, 2012; ASSELIN & GENDRON, 2014).

O trato gastrointestinal é densamente povoado por microrganismos, destacando-se o intestino. O ambiente intestinal possui diferentes regiões anatômicas em termos de fisiologia, tempo de digesta, disponibilidade de substrato, tipos de secreções, pH e tensão de oxigênio. O intestino grosso devido a suas características (fluxo lento e pH neutro a levemente ácido) possui a maior comunidade microbiana, sendo esta formada predominantemente por microrganismos anaeróbios obrigatórios. Já o intestino delgado possui uma microbiota que consiste principalmente de anaeróbios facultativos, incluindo Gram-positivos (Estreptococos, Lactobacilos e Enterococos) e Gram-negativos (Proteobactérias e Bacteróides) (BOOIJINK et *al.,* 2010; ZOETENDAL et *al.,* 2012).

A microbiota intestinal (MI) contribui significativamente para a saúde humana e pode desempenhar importante papel no desenvolvimento e / ou na prevenção de doenças. Primeiramente os microrganismos presentes formam uma barreira natural de defesa, evitando a colonização por bactérias potencialmente patogênicas, leveduras (RAMIREZ, 2017).

Importante para a integridade da mucosa, a microbiota presente contribui também para o desenvolvimento e regulação das respostas imunes intestinais, incluindo a tolerância oral às estruturas de antígeno de origem alimentar (DELZENNE

& WILLIAMS, 2002; MAZMANIAN et *al.,* 2005). O impacto da microbiota na inflamação crônica da mucosa ainda não foi totalmente elucidado, mas efeitos protetores anticâncer, por probióticos e prebióticos, via modulação da expressão da ciclooxigenase (COX) presente na mucosa *in vitro* foram apontados em estudos de Maikivuokko et *al.,* (2005) e Nurmi et *al.*, (2010).

Em relação a atividade metabólica muitos carboidratos que não são digeridos pelo homem, quando chegam ao intestino são fermentados pelas bactérias presentes. Essa fermentação é responsável pela formação de ácidos graxos de cadeia curta (AGCC) (short-chain fatty acids – SCFAs), possibilitando obtenção de energia a partir de substratos que seriam eliminados com as fezes. A produção de AGCCs no cólon corresponde a 10% da energia diária necessária ao organismo humano (CONTERNO et *al.*, 2011). Outras funções metabólicas da MI é a absorção de cálcio, magnésio e ferro e a síntese de vitaminas (RAMIREZ, 2017).

A obesidade e a síndrome metabólica também estão relacionadas a microbiota intestinal. A associação de uma dieta irregular com as bactérias que residem no trato gastrointestinal humano pode ser responsável pelo ganho excessivo de peso. A abundância e a proporção relativa de duas linhagens de bactérias, *Bacteroidetes* e *Firmicutes*, podem afetar o potencial metabólico da microbiota gastrointestinal, aumentando a capacidade de extrair energia da dieta (SARTOR, 2008).

A microbiota humana é formada desde seu nascimento, sofrendo diversas alterações ao longo da vida. Esta é influenciada por fatores como idade, alimentação e localização geográfica. Por meio da alimentação é possível aumentar o número de microrganismos promotores da saúde, a ingesta de microrganismos probióticos e de suplemento alimentar prebiótico, modificam seletivamente a composição da microbiota. Desta forma há um crescente interesse nas interações funcionais entre MI, substratos alimentares ingeridos e o metabolismo do hospedeiro (ZEYREK et al., 2006; SEISKAR et al., 2007; RAMIREZ, 2017).

### 3.7 Probióticos

A palavra probióticos tem origem grega e significa “para a vida”. Segundo a organização mundial da saúde, os probióticos, são microrganismos vivos que quando administrados em quantidades adequadas conferem benefícios a saúde do

hospedeiro (FAO/WHO, 2001). A definição deste termo foi atualmente reafirmada pela Associação Científica Internacional de Probióticos e Prebióticos (ISAPP), uma vez que o conceito é suficientemente amplo para permitir que uma grande gama de produtos seja desenvolvida e adequadamente restrita para impor alguns requisitos fundamentais (HILL et *al.,* 2014).

No Brasil, a ANVISA (Agência Nacional de Vigilância Sanitária) segue a designação da FAO/WHO. A ANVISA também ressalta que os efeitos benéficos atribuídos pelos probióticos são linhagem dependente, ou seja, nos produtos adicionados de probióticos, o benefício deve ser comunicado por meio da alegação de propriedade funcional ou de saúde aprovada para a linhagem específica (BRASIL, 2018).

Desta forma tanto a legislação brasileira quanto os órgãos reguladores internacionais, não definem a quantidade mínima de células probióticas adequadas que proporcionam efeitos benéficos a saúde, porém, a ISAPP e diversos estudos preconizam a ingestão diária de pelo menos $10^9$ UFC/porção do produto (HILL et *al.*, 2014; MENG et *al.*, 2016).

As bactérias mais conhecidas e comumente usadas como probióticos são principalmente membros dos gêneros *Lactobacillus*, *Streptococcus* e *Bifidobacterium* sendo que cada vez mais linhagens desses gêneros estão sendo reconhecidas como microrganismos probióticos (GRANATO *et al.*, 2010; KECHAGIA *et al.*, 2013). Leveduras *Saccharomyces boulardii* e *Saccharomyces cerevisiae* também são utilizadas em preparações farmacêuticas como probiótico.

Os benefícios gerais dos probióticos são devido ao desenvolvimento de um ambiente intestinal mais favorável, através de mecanismos tais como: colonização por bactérias benéficas e mais resistentes, regulação do trânsito intestinal, produção de ácidos graxos de cadeia curta, exclusão competitiva de patógenos, uma maior renovação de enterócitos, entre outros mecanismos, ainda em estudo. Esses microrganismos de forma geral contribuem para um sistema digestivo mais saudável e melhores respostas do sistema imune (HILL *et al.,* 2014).

Os alimentos que contém probióticos são chamados de alimentos funcionais, como, por exemplo, os leites fermentados. O consumo desse tipo de alimento é recomendado em pacientes com alguns tipos de patologia tais como: doença de Cronh, diarreias causadas por vírus e bactérias, e diarreia do viajante. Porém, a

eficácia dessas bactérias probióticas depende que sejam resistentes ao suco gástrico e à bile, alcançando o intestino (KECHAGIA *et al.,* 2013).

### *3.7.1 Bifidobacterium spp.*

As bifidobactérias foram isoladas pela primeira vez por Tissier (1900), e classificadas inicialmente como *Bacillus bifidus communis*. Somente em 1967, De Vries e Stouthamer sugeriram a reclassificação como gênero distinto (*Bifidobacterium*) devido à presença de frutose-6-fosfato fosfoquinase (F6 PPK) e a ausência das enzimas presentes em lactobacilos: glicose-6-fosfatase desidrogenase e aldolase (BALLONGUE, 2004; CHEIKHYOUSSEF *et al.,* 2008). Pertencentes ao filo de actinobactérias, as bifidobactérias são Gram-positivas, não formadoras de esporos, não móveis, catalase-negativas e são capazes de fermentar glicose produzindo os ácidos láctico e acético. São descritas como microrganismos anaeróbios obrigatórios, embora algumas linhagens possam tolerar pequenas concentrações de oxigênio (MARTINEZ et al., 2013).

Com linhagens classificadas como probióticas, o gênero *Bifidobacterium,* é um dos mais estudados e aplicados em produtos. São responsáveis por 25,6% da microbiota do leite materno, possuindo um papel importante na colonização primária humana afetando a saúde e a homeostase do hospedeiro durante o desenvolvimento do sistema digestivo e imunológico infantil (SOTO *et al.,* 2014). Em adultos, a população intestinal de bifidobactérias tende a diminuir gradativamente representando cerca de 3-7%, enquanto em recém-nascidos, pode representar até 91% da população bacteriana (BALLONGUE, 2004; CHEIKHYOUSSEF *et al.,* 2009).

Entre as várias *Bifidobacterium spp* as *B. infantis* e *B. longum, B. bifidum, B. breve*, são bactérias comumente encontradas no trato gastrointestinal de bebês amamentados (O'SULLIVAN *et al.*, 2015) e durante o crescimento, o perfil das espécies constituintes sofrem alterações, sendo que as *B. infantis* e *B. breve*, tipicamente infantis, são substituídas por *B. adolescentis* em adultos, enquanto *B. longum* persiste ao longo da vida (VASILJEVIC & SHAH, 2008).

Dentre os efeitos benéficos à saúde proporcionados pelas bifidobactérias estão: a estimulação do sistema imunológico, produção de vitamina B, inibição da multiplicação de patógenos, a redução da concentração de colesterol no sangue. Seu

consumo é indicado após a utilização de antibióticos por ajudarem a restabelecer a microbiota, em tratamentos ou prevenção de infecções do trato respiratório e urogenital, além da prevenção de alergias e doenças atópicas na infância. Desta forma esses microrganismos são comumente utilizados em intervenções dietéticas que visam à melhoria da saúde dos indivíduos (LEAHY *et al.,* 2005, SAXELIN *et al.,* 2005).

Algumas linhagens de *Bifidobacterium spp.* também são capazes de produzir bacteriocinas, porém poucas foram purificadas e caracterizadas (bifidocina B, bifidina I, termofilicina B67 e bisina), sendo que algumas podem afetar culturas iniciadoras e probióticas, sendo necessário cuidado ao selecionar as linhagens produtoras de bacteriocinas na utilização em alimentos fermentados para garantir que bactérias importantes não sejam inibidas (SOTO *et al.,* 2014).

*B. animalis* subsp. *lactis* Bb12 (BB-12®) é a bifidobactéria mais documentada dentre as linhagens dessa espécie. Ela tem sido amplamente utilizada em fórmulas infantis, suplementos dietéticos e produtos lácteos fermentados, inclusive por sua alta tolerância em condições ácidas. Seus efeitos benéficos à saúde têm sido demonstrados por meio de ensaios clínicos há mais de 30 anos (JUNGERSEN *et al.,* 2014; RAEISI *et al.,* 2013).

### *3.5.2 Lactobacillus reuteri (Limosilactobacillus reuteri*)

Classificadas até 1980 como *L. fermentum* Biotype II, *Lactobacillus reuteri* recebeu posteriormente este nome em homenagem a G. Reuter, um bacteriologista alemão que primeiro isolou este microrganismo. Recentemente Zheng et al., 2020 propuseram uma nova classificação para os gêneros *Lactobacillus*, incluindo as cepas de *L. reuteri,* que por razões taxonômicas foram denominados de *Limosilactobacillus reuteri*.

*L. reuteri* são bactérias Gram-positivas, ácido-láticas, heterofermentativas (produzem além de lactato, etanol e $CO_2$), anaeróbias facultativas ou aerotolerantes, capazes de usar diferentes fontes de carbono e energia para a fermentação. Este microrganismo possui uma ampla gama de hospedeiros, residindo no trato

gastrointestinal (GI), vaginal e oral do homem e de outros animais de sangue quente (HAMMES & HELTER, 2006).

*L. reuteri* possui o certificado de “presunção qualificada de segurança” (QPS) pela Autoridade Europeia para a Segurança dos Alimentos (EFSA) e é amplamente utilizado na indústria farmacêutica e alimentícia. Segundo um dos seus fabricantes, laboratório Aché, a linhagem *L. reuteri* DSM 17938 possui ação no trato gastrointestinal que define sua ação sobre determinadas patologias, sendo elas: inibição de microrganismos patogênicos, efeito modulador sobre a resposta imune, auxílio na maturação e na motilidade gastrointestinal e equilíbrio da microbiota intestinal, prevenindo e tratando diarreias e cólicas infantis (PROVANCE, 2014). Gutiérrez-Castrellón e colaboradores (2017) através de uma análise sistemática de evidências e levantamento de dados sobre o uso de *L reuteri* DSM 17938 concluíram que este possui uma intervenção significativa para reduzir a duração do tempo de choro em cólica infantil.

Ainda destacando sua importância para a saúde humana, estudos apontaram que *L. reuteri* contribui para a digestão da lactose em indivíduos intolerantes, previne diarreias, câncer de cólon e hipercolesterolemia, além de contribuir com a síntese de proteínas e absorção de cálcio devido ao desenvolvimento de vilosidades mais longas e criptas significativamente mais profundas no íleo. Sendo desta forma compreensível o interesse crescente na incorporação dessa espécie em alimentos e medicamentos (GUTIÉRREZ-CASTRELLÓN *et al.,* 2017).

A ação probiótica de *L. reuteri* é atribuída à combinação de diversos mecanismos, incluindo-se a produção de ácido lático, peróxido de hidrogênio e à capacidade de algumas linhagens em produzir reuterina (β-hidroxipropionaldeído; β-HPA) durante o metabolismo anaeróbico de glicerol (LANGA et al., 2013). A produção de reuterina por algumas cepas de *L. reuteri* é uma vantagem competitiva em nichos ecológicos, tais como o trato gastrointestinal (MORITA et al., 2008). Desta forma supõe-se que cepas de *L. reuteri* produtoras de reuterina são candidatos potenciais para aplicações adicionais na medicina ou saúde animal e humana (SOLTANI *et al.* 2021).

Em seus primeiros estudos, estas cepas foram utilizadas como espécie modelo para determinar a adaptação de lactobacilos intestinais ao hospedeiro e a co-adaptação de espécies de *Limosilactobacillus* e *Lactobacillus* que coexistem em

biofilmes intestinais e demais substratos (DUAR *et al.,* 2017). *L. reuteri* é dividido em linhagens adaptadas ao hospedeiro que são equivalentes à classificação taxonômica de subespécies e diferem em sua capacidade de formar biofilmes no estômago de camundongo (FRESE *et al.,* 2013). Poucas cepas de *L. reuteri* produzem o composto antimicrobiano reutericiclina, um ácido tetrâmico, primeiro antimicrobiano de baixo peso molecular de bactérias lácticas, uma habilidade única entre os lactobacilos (LIN *et al.,* 2015).

### 3.6 Bioacessibilidade

Bioacessibilidade é definida como a fração de um composto ingerido que se torna acessível para absorção através da camada epitelial do trato gastrointestinal (TGI). Para ocorrer a absorção esses compostos devem primeiro ser liberados da matriz alimentar e/ou nanotransportados e então solubilizados em micelas de associação para assim tornarem-se bioativos, ou seja, disponíveis para a utilização do organismo (DIMA et al., 2020).

Bioatividade é a capacidade de um composto de exibir um efeito biológico. Para serem bioativos, os compostos absorvidos pela camada epitelial intestinal devem ser distribuídos para órgãos e tecidos onde estão envolvidos em diversos processos bioquímicos que geram uma resposta fisiológica (benefício para a saúde). A validação da resposta fisiológica de um composto bioativo é feita por meio da avaliação de parâmetros farmacocinéticos como: liberação, absorção, distribuição, metabolismo e eliminação (LADME). Desta forma a bioacessibilidade têm uma influência importante na bioatividade. Por exemplo, embora frutas com alta concentração de biocomponentes sejam consumidas, alguns dos biocomponentes ainda apresentam baixa bioatividade devido às pequenas quantidades que chegam aos órgãos e tecidos. Isso acontece com polifenóis, fitoesteróis ou algumas vitaminas (CHEN, CAO & XIA, 2018; GYLLING, & SIMONEN, 2015; SAID, & NEXO, 2018).

Assim, muitos biocomponentes tem uma baixa biodisponibilidade devido à sua baixa estabilidade no fluido gastrointestinal (GI) e sua má absorção através da camada epitelial intestinal (ROSTAMABADI, FALSAFI, & JAFARI, 2019). Desta forma alguns pesquisadores estudaram a possibilidade de melhorar a biodisponibilidade, incorporando agentes bioativos em vários sistemas de entrega coloidal, como:

emulsões, nanoemulsões, microemulsões, nanopartículas de lipídios sólidos, nanopartículas de biopolímero, microgéis, entre outras formulações (JAIN et al., 2018; GUERGOLETTO et al., 2016; LUO et al., 2014; SILVA et al., 2019; SOUZA-SIMÕES et al., 2017; YANG et al., 2015).

Nos últimos anos uma variedade de simuladores do trato gastrointestinal tem sido empregada como ferramenta para prever a bioacessibilidade de vários compostos bioativos ou nutrientes durante a digestão no trato gastrointestinal. São diversos os tipos de sistemas empregados, desde os estáticos aos dinâmicos multicompartimentais (WU & CHEN, 2021). Dentre as vantagens dessas abordagens destacam-se a reprodutibilidade, facilidade de amostragem em qualquer local de interesse, economia de tempo, menor custo e, mais importante, sem restrições éticas que os experimentos *in vivo* apresentam (MACKIE, CABERO & TORCELLO-GOMEZ, 2020).

A chave para essas determinações é a simulação mais realista das condições químicas, bioquímicas e mecânicas dos quatro setores do GIT: boca, estômago, intestino delgado e cólon (ALMINGER *et al.,* 2014). A bioacessibilidade é expressa como uma porcentagem e é calculada como a razão entre a quantidade de um biocomponente solubilizado na fase micelar e a quantidade de biocomponente no digerido (DIMA et al., 2020).

Dentre as metodologias de bioacessibilidade *in vitro* utilizadas, a Unified BARGE Method (UBM) foi desenvolvido pelo BioAccessibility Research Group of Europe (BARGE), para padronizar o uso da bioacessibilidade em avaliações de risco para a saúde humana para solos contaminados na Europa. Esta metodologia foi considerada a mais representativa das condições físico-químicas do trato gastrointestinal humano para garantir um teste *in vitro* robusto e aplicável (DENYS et al., 2012).

### 3.7 Ressonância Magnética Nuclear

Baseada na interação entre a matéria e a radiação eletromagnética, a Ressonância Magnética Nuclear (RMN) é uma técnica espectroscópica. Esta apresenta-se como uma poderosa ferramenta na determinação de estrutura de substâncias químicas orgânicas, seguida da elucidação estrutural e dinâmica de macromoléculas e, mais recentemente, determinação e quantificação de metabólitos

no campo da metabonômica (impressão digital de uma perturbação e a partir dela identificar quais metabólitos endógenos está relacionada) e metabolômica (a análise qualitativa e quantitativa de todos os metabólitos de um sistema) (FAN & LANE, 2016).

Como toda técnica de análises, RMN apresenta vantagens e desvantagens em sua aplicação. Entre as vantagens temos: alta reprodutibilidade; uma única referência interna é suficiente para a quantificação absoluta de metabólitos; é possível a identificação inequívoca de estruturas de metabólitos desconhecidos utilizando uma combinação de técnicas de RMN (visto que a maioria dos metabólitos detectados em misturas biológicas complexas é desconhecida); a análise de biofluidos e tecidos intactos, sem necessidade de separação ou preparação de amostras, diminuindo o "gargalo" da variação analítica causada pelo preparo de amostras; é uma técnica não destrutiva, permitindo posteriormente sua utilização (GOWDA & RAFTERY, 2015). Alé disso, a RMN consegue rastrear vias metabólicas e medir fluxos metabólicos utilizando substratos marcados com isótopos; a RMN oferece vantagens para compostos que são difíceis de ionizar ou requerem derivatização para demais técnicas, além de permitir a identificação de compostos com massas idênticas; os perfis de metabólitos obtidos por RMN são virtualmente independentes do operador e do instrumento utilizado, o que proporciona um alto grau de confiabilidade aos resultados derivados (MARKLEY et al., 2017).

Como desvantagem podemos apontar a resolução limitada e a baixa sensibilidade que associadas com as dificuldades de identificação de metabólitos desconhecidos (principalmente os de baixa concentração), são grandes desafios para desvendar a complexidade das misturas biológicas. Por exemplo, em uma amostra de plasma sanguíneo o número de metabólitos detectáveis por RMN é restrito a 30, ou menos, o que é muito menor do que o número real de metabólitos no sangue (GOWDA & RAFTERY, 2015).

Dentre as técnicas desenvolvidas, a RMN de hidrogênio (RMN de $^{1}H$) é umas das mais estudadas devido ao seu alto desempenho, uma vez que muitos metabólitos contêm átomos de hidrogênio em sua composição. RMN de $^{1}H$, baseia-se na capacidade de detectar simultaneamente, até em estruturas biológicas mais complexas, todas as moléculas que contêm átomos de hidrogênio e associar

diretamente as intensidades de cada pico ao número de hidrogênios implícitos àquele sinal (SCANO *et al.,* 2019).

Devido a sua robustez e a capacidade de analisar rapidamente misturas em nível molecular sem a necessidade de etapas de separação e / ou purificação, RMN mostra-se ideal para aplicações em ciência de alimentos. Porém apesar de sua crescente popularidade entre os cientistas de alimentos, RMN ainda é uma metodologia subutilizada nesta área, principalmente devido ao seu alto custo, sensibilidade relativamente baixa e à falta de especialização em RMN por muitos cientistas de alimentos (HATZAKIS, 2018).

Por serem matrizes muito complexas, os alimentos consistem em centenas e até mesmo milhares de compostos. Estes compostos são produzidos pelo metabolismo de plantas, bactérias e animais, além da composição do próprio alimento que sofre o impacto de fatores externos, como armazenamento, transporte e processamento. Da mesma forma, a utilização de alimentos por humanos por meio do metabolismo pode criar milhares de novas moléculas adicionais (LAGHI *et al.,* 2014). Todas essas características são refletidas em seus espectros de RMN e nas informações que estes transmitem. Por conterem uma grande quantidade de informações, estas são difíceis de serem extraídas e interpretadas por métodos estatísticos univariados convencionais, tais como testes t de Student e análise de variância (ANOVA). Essas análises se concentram em apenas uma variável de cada vez, sendo inadequadas para descrever a situação global do sistema alimentar, aumentando o risco de falsa tomada de decisão, bem como a perda de informações significativas. Ademais, a sobreposição de sinal que frequentemente ocorre neste tipo de espectro de RMN pode impedir a identificação e / ou quantificação do composto de interesse aumentando a incerteza na seleção de moléculas que estão ligadas a certas propriedades alimentares (HATZAKIS, 2018).

A metabolômica baseada em alimentos é uma alternativa, que leva a uma interpretação mais eficiente da informação espectral. Tal abordagem é combinada com análise estatística multivariada (MVSA) para caracterizar completamente um produto alimentar como um todo e coletar informações sobre sua origem, qualidade, processamento, histórico de armazenamento e percepção sensorial. É possível aplicar a metabolômica na investigação do impacto de uma abordagem nutricional no metabolismo e na saúde. As duas abordagens principais usadas em estudos de

metabolômica baseados em RMN são análises direcionadas e não direcionadas (ROBERTS et al., 2012). Na análise direcionada, os biomarcadores são frequentemente predefinidos. O objetivo é quantificar o maior número possível de metabólitos e a análise estatística é realizada usando concentrações de compostos. Na análise não direcionada, os metabólitos individuais não são quantificados, mas a comparação do padrão espectral é usada para diferenciação entre amostras e grupos (CLOAREC *et al.,* 2005).

A quimiometria é a aplicação de MVSA nos dados espectrais. Utilizando o maior número de variáveis possível simultaneamente, ajuda a extrair o máximo de informações significativas de um grande conjunto de dados que consiste em muitas variáveis, reduzindo sua dimensionalidade (MONAKHOVA, KUBALLA, & LACHENMEIER, 2013).

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# CAPÍTULO 2[1]

[1]-Artigo a ser submetido na Revista ***Food Technology and Biotechnology (FTB)***

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**CAPÍTULO 2-** EFFECT OF FERMENTATION BY PROBIOTIC BACTERIA ON THE BIOACCESSIBILITY OF BIOACTIVE COMPOUNDS FROM THE FRUIT OF THE JUÇARA PALM (*EUTERPE EDULIS* MARTIUS).


## Abstract

The little explored fruit from the juçara palm tree **(***Euterpe edulis* Martius) has bioactive compounds with antioxidant activities such as phenolic acids and anthocyanins. This fruit´s pulp presents itself as an appropriate fermentation medium for probiotic bacteria growth**.** Therefore, this study was conducted to evaluate the effects of fermentation by *Limosilactobacillus reuteri* LR92 (JLR) and *Bifidobacterium animalis* ssp. *lactis* BB-12 (JBB) on the bioactive compounds contents of the juçara pulp, before and after a gastrointestinal simulation. The pulp of the juçara fruit showed probiotic counts of 8.70 ± 0.07 log UFC/mL for JLR and 8.44 ± 0.09 log UFC/mL for JBB, after 24 hours of fermentation. Fermentation with the strains used modified the proportions of fatty acids (fatty acids esters were quantified using a gas chromatography equipment) and fibers when compared to the non-fermented pulp. Antioxidant capacity determined by DPPH, FRAP and ABTS showed significant reduction after the gastrointestinal simulation for samples. Phenolic compound analysis by UPLC-MS/MS showed after fermentation, greater amount of ferulic, protocatechuic and catechin acids in the samples. These results show changes in the bioactive compounds due to the fermentation of the juçara pulp by probiotics. However, these compounds showed bioactive potential and were bioaccessible after the gastrointestinal simulation, with the pulp being a potential mean for bacteria growth, which may bring health benefits.


**Keywords:** protocatechuic acid, 4-hydroxybenzoic acid, gastrointestinal simulation, Unified BARGE Method *Limosilactobacillus reuteri* E *Bifidobacterium animalis* subsp lactis BB-12.

## *Introduction*

The Juçara palm tree (*Euterpe edulis* Martius) is a native species from the Brazilian Atlantic Forest and belongs to the Arecaceae family (Bicudo et al., 2015). This palm tree is an endangered species due to the exploitation in the production of hearts of palm, a highly appreciated delicacy. A sustainable form of use would be the consumption of its fruits, which have high nutritional value, are rich in sugars, proteins and lipids with high proportions of polyunsaturated fatty acids. In addition, they present bioactive molecules such as vitamins, minerals, fibers and antioxidants (Cardoso et al., 2018).

Juçara is considered an important source of bioactive compounds, especially phenolic compounds and anthocyanins, which are responsible for the fruit coloration (Fang, 2014; Bicudo et al., 2015). Studies have shown that the anthocyanins cyanidin-3-glucoside and cyanidin-3-rutinoside had a cytoprotective effect against oxidative damage *in vitro* culture of Vero cells (Borges et al., 2013; Cardoso et al., 2015). Other types of phenolic compounds such as rutin, quercetin and caffeic, chlorogenic and ferulic acids are associated with reductions in non-communicable chronic diseases and delay in premature aging (Paredes-López, Cervantes-Ceja, Vigna-Pérez & Hernández-Pérez, 2010).

In recent years, studies of fruit-based probiotic fermented products have been increasing. These microorganisms used in fermentation can produce changes in the profile and types of bioactive compounds present in plant matrices, such as in the production of short-chain fatty acids, consumption of polysaccharides, bioaccessibility and bioavailability of phenolic compounds. Bacteria of the genera *Bifidobacterium* and *Lactobacillus* (*Limosilactobacillus*) are members of the intestinal microbiota, but they can also be used in the elaboration of products, with some strains characterized as probiotics. *Bifidobacterium* and *Lactobacillus* (*Limosilactobacillus*) species have been shown to have β-glucosidase activity that participates in the hydrolysis of plant β-glycosides (Ávila et al., 2009). Thus, in recent decades, there has been a growing interest in the bioconversion of phenolics by lactic acid bacteria (Li et al. 2018).

In addition to being able to modify bioactive compounds, fermentation processes can be influenced by the food matrix and the digestion process, differing quantitatively and qualitatively from the values obtained with solvent extraction used in most studies (Mosele et al., 2015; Seraglio et al., 2015; Seraglio et al., 2018). The

stability of these compounds during gastrointestinal digestion and their release from food matrices are associated with their bioaccessibility after the digestive process and can be observed and quantified by *in vitro* methods (Guergoletto, Costabile, Flores, Garcia & Gibson, 2016; Bouayed, Hoffmann & Bohn, 2011). Thus, studies that evaluate the changes that bioactive compounds can undergo during the fermentation process and their bioaccessibility in fruits, such as juçara, are relevant.

For *in vitro* results to be similar to those obtained *in vivo* digestion, conditions such as pH, the chemical composition of the digestive fluids and the time the food is kept at each stage need to be similar to those of the digestive process (Hornero-Méndez & Mínguez-Mosquera, 2007). Biological fluids frequently used are composed of digestive enzymes (pancreatin, pepsin, trypsin, chymotrypsin, peptidase, α-amylase and lipase) and bile salts (Lee, Lee, Chung & Hur, 2016; Schulz et al., 2017). *In vitro* simulated gastrointestinal digestion is a widely used method, presenting important advantages (reproducibility, speed, low cost and no ethical restrictions) compared to *in vivo* methods (Kamiloglu, Pasli, Ozcelik and Capanoglu, 2014; Minekus et al., 2014).

Thus, the objective of this work was to verify the influence of the fermentation process by *Limosilactobacillus reuteri* LR92 and *Bifidobacterium animalis* ssp. lactis BB-12, both potentially probiotic bacteria, on the bioactive compounds profile, besides to determine if the fermentative process affects bioaccessibility of phenolic and antioxidant compounds after *in vitro* gastrointestinal simulation of the juçara pulp.

## Material and methods

### *Microorganisms*

Strains lyophilized used are *L. reuteri* LR92 (Sacco DSM 26866-Cadorago, Italy) and *Bifidobacterium animalis* ssp. lactis BB-12 (Christian Hansen- Denmark). The pre-inoculum consisted of the addition of 0.1% (w/v) of the lyophilized culture in juçara pulp containing 20% (v/v) glycerol, both previously pasteurized. Before use, 1% of the pre-inoculum was activated in two consecutive stages in juçara pulp at 37 °C for 24 hours in anaerobiosis (Probac, Brazil).

### *Juçara Pulp Preparation*

Juçara fruits (SisGen A880DD5) were harvested in Rolândia-PR, (Latitude: 23 ° 18 '38' 'South, Longitude: 51 ° 22' 10 '' West), Parana, Brazil, in April of 2018. The

pulp (2.5 °Brix) was obtained and pasteurized according to Guergoletto *et al.* (2016). After this process, 1% (v / v) of pre-inoculum was added, with *L. reuteri* or *Bifidobacterium* BB-12. Fermentation was carried out in sterilized glass containers, incubated at 37 °C in anaerobiosis (Probac, Brazil), for 24 hours. The analysis were performed on samples of juçara pulp without fermentation (JP), juçara pulp fermented by *L. reuteri* (JLR) and juçara pulp fermented by *Bifidobacterium* BB-12 (JBB). All samples were freeze-dried (Freeze dryer L101 - Liobras, Brazil).

*Characterization of samples before and after fermentation*

*Counts of probiotics.*

*L. reuteri* and BB-12 counts were determined in fermented juçara pulps, on MRS agar and plates incubated under anaerobiosis (Anaerobac) at 37 °C for 72 h. For the BB-12 count, 2 g $L^{-1}$ of lithium chloride and 3 g $L^{-1}$ of sodium propionate were added to the MRS agar (Lapierre et al., 1992).

*pH determination*

The pH measurements were made with a digital pH meter (Kasvi K39-2014, Brazil).

*Fibers*

The analysis of dietary fibers, soluble and insoluble, was carried out using a combination of enzymatic and gravimetric methods, according to method 991.43 (AOAC, 2005).

*Fatty acids*

For the analysis, JP, JBB and JLR samples were prepared according to Bligh & Dyer, (1959), and the hydrolysis and transesterification of fatty acids according to ISO Method 5509, (1978). Fatty acid esters were quantified using a gas chromatography equipment (Shimadzu 14 A®, Japan), with a flame ionization detector and fused silica capillary column with 100m in length, 0.25 mm in internal diameter and 0.20 µm. Carbowax 20M; flow of 1.2 mL / min of $H_2$ (carrier gas), 30 mL $min^{-1}$ of $N_2$ (auxiliary gas), 30 and 300 mL $min^{-1}$, for $H_2$ and synthetic air for the detector flame. The initial temperature of the column was set at 150 ºC for 3 min, and progressively increased to

240 ºC, with an elevation rate of 10 ºC /min. Fatty acid profile was expressed as a percentage of total fatty acid, with certified values for 11 fatty acid standards.

*Total phenolic compounds, antioxidant activity and anthocyanins*

The JP, JBB and JLR samples were extracted according to Singh *et al.* (2009), with adaptations. The lyophilized pulp was weighed (500 mg) and placed in 15 mL falcon tubes plus 10 mL 80% methanol. The mixture was shaken vigorously using a vortex at 50 Hz (Labnet´s Vortex Mixer VX-200) for 2 min and then taken to a rotary shaker (Tecnal, Brazil) for 30 minutes. Samples were then centrifuged at 5000 rpm for 15 min and the supernatant reserved. The residues were extracted again twice with an additional 10 mL of 80% methanol. All three extracts were combined and subsequently filtered using Whatman filter paper (42). Extracts obtained were used for phenolic determination by UPLC-MS / MS, antioxidant activity by ABTS, DPPH and FRAP and determination of anthocyanins by the pH difference method (Giusti & Wrolstad, 2001).

*In vitro* gastrointestinal simulation (GIS)

The GIS test was performed according to the unified bioaccessibility method (UBM; Wragg et al., 2011). This test was carried out in three stages, simulation the digestive processes in the mouth, stomach and small intestine. The preparation of digestive fluids was according to Broadway *et al.* (2010). For the analysis, 0.3 g of JP, JBB and JLR samples were placed separately in vials and mixed with 4.5 mL of simulated saliva and 6.75 mL of simulated gastric solution (adjusted to a pH of 1.2 ± 0.05). The flasks were shaken at 37 °C for 1 h corresponding to the gastric phase. After this first stage, for the intestinal phase, 13.5 mL of simulated duodenal fluid and 4.5 mL of simulated bile solution were added to the gastric solution, and the pH adjusted to 6.30 ± 0.05. Samples were then placed under stirring at 37 °C for 4 h. After centrifugation, the resulting JP, JBB and JLR supernatants were removed and filtered for phenolic determination by UPLC-MS / MS, antioxidant activity by ABTS, DPPH and FRAP and determination of anthocyanins by the pH difference method. For the control, analytical blanks (only the solvents) were performed in parallel.

*Analysis of phenolic compounds by ultra-efficient liquid chromatography coupled with mass spectrometry (UPLC-MS / MS)*

For the analysis of phenolic compounds, the Acquity UPLC System coupled to the Triple Quadrupole mass spectrometer (TQD) - Waters was used. The Acquity UPLC HSS C18 Column (2.1 mm x 100 mm, 1.8 µm, Waters) with oven temperature of 40 °C and injection volume: 10 µL with a flow rate of 0.4 m. $min^{-1}$. The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B).

The detection by mass spectrometry (MS / MS) was through electrospray ionization (ESI) in a positive mode. Capillary voltage of 1 kV with 400 °C desolvation temperature and 150 °C source temperature. The desolvation gas used was nitrogen (flow rate 700 L $min^{-1}$) and argon was used for collision. Phenolic compounds were detected using multiple reaction monitoring (MRM).

*Analysis of antioxidant activity*

The antioxidant activity was determined by the ABTS, DPPH and FRAP methods. The ABTS •+ (2,2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) cation assay was prepared and conducted according to Sanchez-Gonzales *et al.* (2005). Quantification was by the standard Trolox curve (2.5 to 20 mM) and results expressed in µM Trolox / g of sample.

For the DPPH • radical method (2,2′-diphenyl-β-picrylhydrazyl), an aliquot (100 µL) of the samples was added to the DPPH • 0.1 mM ethanolic solution (1.0 mL). The mixture was stirred in the dark for 60 min and then the absorbance determined at 517 nm. Results were expressed in µM Trolox / g of sample (Brand-Williams *et al.*1995).

The total antioxidant activity of the samples was also determined using the plasma iron-reducing capacity (FRAP) according to Rufino et al. (2006). A 90 µL aliquot of each sample was mixed with 270 µL of distilled water and 2.7 µL of FRAP reagent (in the dark). The tubes were vortexed and incubated at 37 °C for 30 min. Absorbance was determined at 595 nm, using the FRAP reagent as blank and 500–2000 mM ferrous sulfate solutions. Results were expressed in mM of ferrous sulphate / g of sample.

*Determination of anthocyanins*

The pH difference method (Giusti & Wrolstad, 2001) was used to quantify total and monomeric anthocyanins. Samples (extracts and after SGD) were diluted in two

buffer systems: potassium chloride - hydrochloric acid pH 1.0 (0.025 M $L^{-1}$) and sodium acetate pH 4.5 (0.4 M). Samples were diluted in buffer solutions to the required dilution factor to obtain optical density in the range of 0.1-1.2, at 514 nm, and measurements at absorption maximums in the visible region and at 700 nm were carried out.

Absorbance was calculated according to equation 1:

$$A = (A_{vismax} - A_{vis}700nm)_{pH1} - (A_{vismax} - A_{vis}700nm)_{pH4,5} \quad (1)$$

The concentration of monomeric pigments in the extract was calculated and represented in cyanidin-3-glycoside. Then, the concentration of monomeric anthocyanins was calculated using equation 2:

$$MA = \frac{A \times MW \times DF \times 10^2}{E \times L} \quad (2)$$

where "*MA*" are monomeric anthocyanins (mg.100 $g^{-1}$); "*A*" the absorbance difference calculated by eq. (1); "*MW*" is the molecular weight of cyanidin-3-glycoside = 449.2; "*DF"* is the dilution factor; "*E*" molar absorptivity = 26900; and "*L*" the optical path = 1.0 cm.

***Bioaccessibility***

The percentage (%) of bioaccessibility (BF) was calculated according to Equation 3 (Leufroy *et al.* 2012),

$$BF\ (\%) = (BE/TE) \times 100 \quad (3)$$

where *BE* is the fraction of bioaccessible element (mg 100 $g^{-1}$), given by the concentration of an element found in the gastrointestinal extracts, and *TE* is the total element concentration (mg 100 $g^{-1}$).

*Statistical analysis*

The data was expressed and analysed by the analysis of variance (ANOVA), and the differences between the means were detected by the Tukey test with a significance level of 5%. Correlations between the data obtained for antioxidant capacity and phenolic compound were established using the Pearson's correlation coefficient. All data are presented as mean ± standard deviation (S.D.) for samples

that were analysed in genuine triplicate. The data were analysed using the Statistica 10.0 software (Statsoft Inc., Tulsa, OK, USA).

## Results and Discussion

*Probiotic counts and pH values*

After 24 h of juçara pulp fermentation, the probiotic count was 8.70 ± 0.07 log CFU/mL for *L. reuteri* and 8.44 ± 0.09 log CFU/mL for JBB. The pH, which initially was 5.50 for both, showed values of 3.80 for JLR and 3.78 for JBB, respectively, after fermentation, indicating cellular metabolism of bacteria. This result agrees with Guergoletto, Mauro and Garcia (2018) who reported that juçara pulp can be used as a growth medium for probiotic bacteria, resulting in a satisfactory number of viable bacteria to provide benefits to consumers.

*Fatty Acids*

The juçara pulp has a lipid fraction with a high content of polyunsaturated fatty acids such as oleic acid and lower levels of saturated lipids (Borges et al., 2011). The highest percentages of fatty acids found in juçara pulp before the fermentation process (Table 1) were palmitic acid, oleic acid and linoleic acid, similar to those described by Oyama et al. 44% to 55% and 18% to 25%, respectively. Schulz et al. (2015) also reported oleic acid as the main fatty acid in dried samples of juçara, representing about 35% to 42%.

After the fermentation process, it was possible to observe that the linoleic acid reduced from 24.31% to 20.73% and 20.38% for the JLR and JBB samples, respectively. Alpha-linolenic acid was also reduced in percentage from 3.62% (JP) to 1.20% (JLR) and 1.31% (JBB). This reduction may be related to the fact that probiotic microorganisms can express hydratase, the enzyme responsible for catalyzing hydration reactions, using oleic, linoleic and linolenic acids as substrates.

This reaction can generate derived fatty acids such as 9-hydroxy-fatty and 10-hydroxy-fatty acids, which are desired in food, cosmetic and pharmaceutical industries due to their economic interest (Serra, Simeis, Castagna & Valentino, 2020).

Table 1: Juçara pulp: Characterization of samples before and after fermentation

| Analysis | | JP | JLR | JBB |
|---|---|---|---|---|
| **Probiotics log CFU/mL** | - | - | 8.70 ± 0.07 | 8.44 ± 0.09 |
| **pH** | - | $5.50^{a}$ ± 0.01 | $3.80^{b}$± $0.05^{b}$ | $3.78^{b}$± $0.02^{b}$ |
| **Fatty Acids (% m/m)** | Palmitic, C16:0 | $24.74^{a}$ ± 0.02 | $23.72^{b}$ ± 0.10 | $23.49^{b}$± 0.01 |
| | Palmitoleic, C16:1n7 | $1.80^{b}$± 0.45 | $2.22^{a,b}$± 0.15 | $2.53^{a}$ ± 0.22 |
| | Stearic C18:0 | $3.09^{a}$ ± 0.12 | $2.56^{b}$ ± 0.03 | $2.03^{b}$ ± 0.44 |
| | Oleic, C18:1n9 | $42.39^{b}$ ±0.00 | 49.42 ± 0.50 | $50.18^{a}$ ± 0.30 |
| | Linoleic, C18:2n6 | $24.31^{a}$ ± 0.11 | $20.73^{b}$ ± $0.12^{b}$ | $20,38^{b}$± 0.00 |
| | Alpha-linolenic C18:3n3 | $3.62^{a}$ ± 0.56 | $1.20^{b}$ ± 0.13 | $1.31^{b}$ ± 0.17 |
| **Fibers g $100g^{-1}$** | soluble | $7.21^{a}$± 0.20 | $2.91^{c}$± 0.12 | $3.73^{b}$± $0.11^{b}$ |
| | insoluble | $21.24^{c}$± 0.03 | $27.81^{a}$± 0.30 | $25.73^{b}$± 0.07 |
| | Total | $28.45^{a}$C 0.11 | $30.72^{A}$± 0.16 | $29.27^{B}$± 0.13 |

JP: juçara pulp; JLR: juçara pulp with *Lactobacillus reuteri* LR92;

JBB: juçara pulp with *Bifidobacterium animalis* ssp. lactis

[a]Values on the same line followed by different letters indicate significant differences ($p < 0.05$)

*Fibers*

The total fiber content per gram in freeze-dried juçara pulp was 34.45 g $100g^{-1}$ (JP), similar to the 34.20 g $100g^{-1}$ reported by Guergoletto et al. (2016). Values of 30.72 g $100g^{-1}$ and 29.27 g $100g^{-1}$ were found for JLR and JBB respectively. This drop in the number of total fibers can be attributed to the reduction of soluble fibers in the fermented pulp. JBB presented 2.91 g $100g^{-1}$ of soluble fiber and JLR 3.73 g $100g^{-1}$ and the pulp without fermenting 7.21 g $100g^{-1}$.

This result is justified due to the composition of soluble fibers, which contain glucose and galactose as main sugars, which are potentially fermentable by *Bifidobacterium* and *Lactobacillus* (Rufino et al., 2011). Fermentation of soluble fibers leads to the production of short-chain fatty acids such as acetate and propionate (Rufino et al., 2011; Guergoletto et al., 2016).

*Bioaccessibility*

*Phenolic compounds*

The content of each phenolic compound from the JP, JLR and JBB samples, before (extracts) and after the *in vitro* simulated gastrointestinal digestion (GIS), is presented in Table 2. Out of the 12 phenolic compounds detected in the samples, 7

are phenolic acids. For the JP extract, the compounds with the highest concentrations were: rutin (60.24 mg 100 $g^{-1}$), ferulic acid (37.26 mg 100 $g^{-1}$), 4-hydroxybenzoic acid (34.94 mg 100 $g^{-1}$) and protocatechuic acid (24.75 mg 100 $g^{-1}$). The extracts from the fermented samples showed higher amounts of protocatechuic acid, catechin and ferulic acid. Schulz et al. (2016) also reported the presence of protocatechuic acid and ferulic acid among the main phenolics present in unfermented juçara pulps, in concentrations from 8.57 to 22.94 mg 100 $g^{-1}$ and 2.59 to 18.12 mg 100 $g^{-1}$ of dry matter, respectively. This same study reported significant amounts of aromadendrin and vanillic acid, which were not detected in our study. These differences can be explained due to the influence that the maturation and soil and climatic conditions of the fruit exert on the content of bioactive compounds, and the antioxidant activity (Borges et al., 2011).

Ascorbic acid was identified only in the JP extract, not being identified in any of the extracts of the fermented samples (Table 2). The fermentation process can compromise the stability of ascorbic acid. This compound assist in the viability of bifidobacteria and lactic acid bacteria due to its potential to reduce both the dissolved oxygen content in the medium and its redox potential, providing a favorable environment for anaerobic and microaerophilic bacteria (Ku et al., 2015).

Another factor capable of influencing the phenolic content before and after fermentation is the presence of enzymes produced by microorganisms. Microbial enzymes can hydrolyze glycosides, glucuronides, sulfates, amides, esters, lactones and act in ring cleavage, reduction, decarboxylation, demethylation and dehydroxylation of phenolic compounds (Dall'asta et al., 2012). α-L-rhamnosidase is an enzyme found in several types of potentially probiotic bacteria, such as *L. reuteri* and *Bifidobacterium animalis* (Mueller et al., 2017).

In a previous study, the appearance of rhamnose was reported after 16 hours of fermentation of juçara pulp by *L. reuteri*, evidencing the presence of this enzyme (Guergoletto et al., 2017). Glycosyl hydrolase cleaves terminal α -L-rhamnose from many natural glycosides, present for example in rutin (Zhang et al., 2015). In this study, there was a 75% and 80% reduction in rutin for the JBB and JLR extracts, respectively, when compared to the phenolic content in the pulp without fermenting and before the gastrointestinal simulation (Table 2). The hydrolysis of rutin allows the production of flavonoid glycosides, such as isoquercitrin, desirable because it has an important role

in anti-aging and anti-allergic effects in humans (Paulke, Eckert, Schubert-Zsilavecz, & Wurglics, 2012, Valentova, Vrba, Bancířová, Ulrichová & Křen 2014).

Despite causing the reduction of some phenolic compounds, as demonstrated, fermentation by both bacteria favored the increase of protocatechuic acid in the juçara pulp extracts (Table 2). Anthocyanins and flavonols can be precursors of this acid and the transformation can be caused by bacteria. Anthocyanins are hydrolyzed to form the corresponding anthocyanidins, which are transformed into protocatechuic acid and acetic acid (Stevens & Maier 2016). Flavonols are extensively hydrolyzed to their products derived from metabolites in the A and B rings because of cleavage of the C ring. This cleavage causes the formation of intermediates. These metabolites enter the catabolic route of phenyl and benzoic acids to generate protocatechuic acid and 2-(3,4-dihydroxy) -phenylacetic acid as the main metabolites (Ozdal et al., 2016; Stevens & Maier 2016).

The ferulic acid was also quantified in extracts from samples JP, JLR and JBB (Table 2). Aerobic or anaerobic biotransformations of this acid have already been reported, such as the oxidative degradation of its aromatic ring, which results in the formation of protocatechuic acid (Rosazza, Huang, Dostal, & Rousseau, 1995). It was also possible to observe that ferulic acid had a higher content in the JP extract (37.26 mg/100 $g^{-1}$) when compared to JBB extracts (19.46 mg/100 $g^{-1}$) and JLR (16.39 mg/100 $g^{-1}$). This degradation may also have contributed to the increase in protocatechuic acid content in the fermented samples present in the extract even before GIS (Table 2).

Table 2: Concentration (mg 100 $g^{-1}$ of dry matter) and bioaccessible fractions (%) of phenolic compounds in juçara pulp

| Phenolics compounds (mg 100 $g^{-1}$) | | JP | JBB | JLR |
|---|---|---|---|---|
| Trigonelina | Extract | 0.15 $^{c}$ ± 0.00 | 0.24 $^{b}$ ± 0.01 | 0.30 $^{a}$ ±0.01 |
| | GIS | 0.35 $^{a}$ ±0.02 | 0.25 $^{b}$ ±0,00 | 0.24 $^{c}$ ±0,00 |
| | BF (%) | 228 | 104 | 77,41 |
| Ascorbic acid | Extract | 12.39 ± 0.10 | ND | ND |
| | GIS | ND | ND | ND |
| | BF (%) | ND | ND | ND |
| Nicotinic acid | Extract | 7.36$^{a}$ ± 0.08 | 6,09$^{b}$ ± 0.05 | 7,01$^{c}$ ± 0.02 |
| | GIS | ND | ND | 8,01 |
| | BF (%) | ND | ND | 114 |
| Protocatechuic acid | Extract | 24.75$^{c}$ ± 0.02 | 37.60$^{a}$ ± 0.33 | 34,52$^{b}$ ± 0.22 |
| | GIS | 95.18$^{c}$ ± 1.01 | 159.57$^{a}$ ± 3,32 | 134.57 $^{b}$ ± 7.83 |
| | BF (%) | 384 | 424 | 390 |

| | | | | |
|---|---|---|---|---|
| 4-hydroxybenzoic acid | Extract | 32.7 [a] ± 2.10 | 14.44[b] ± 0.53 | 13.1[c] ± 0,44 |
| | GIS | 34.94 ±3,35[c] | 83.01[a] ± 7.61 | 68.29 [b] ± 5.11 |
| | BF % | 106 | 574 | 521 |
| Catechin | Extract | 22.80 [b] ± 0.80 | 23.70 [a] ± 0,43 | 17.24[c] ±1.09 |
| | GIS | ND | ND | ND |
| | BF % | ND | ND | ND |
| Chlorogenic acid | Extract | 2.32 ± 1,13[c] | 7.02 [a] ± 0.65 | 5.67[b] ± 0.43 |
| | GIS | < LQD | < LQD | < LQD |
| | BF % | < LQD | < LQD | < LQD |
| Synaptic acid | Extract | 6.78 ± 0.05[a,b] | 6.64 [b] ± 0.11 | 7.88[a] ± 0.10 |
| | GIS | 2.16[a] ± 0.31 | 2.79[a] ± 0.23 | 2.87[a] ± 0,12 |
| | BF % | 31.96 | 42.14 | 36.67 |
| Quercetin | Extract | 1.08 ± 0.02[c] | 2,30[b] ± 0,17 | 5,03[a] ± 0.36 |
| | GIS | ND | ND | ND |
| | BF % | ND | ND | ND |
| Rutin | Extract | 60.24 [a] ± 1.87 | 14.9[b] ± 0.98 | 11.63 ± 1.45[c] |
| | GIS | 9.10 [a] ±0,49 | 9.83 [a,b] ± 0.72 | 8.73[b] ± 0,91 |
| | BF % | 15.10 | 65.97 | 75.07 |
| Ferulic Acid | Extract | 37.26 [a] ± 4,16 | 19.46 [b] ± 3.65 | 16.39 [b] ± 4.54 |
| | GIS | 19.24 [a] ± 0.43 | 17.57 [b] ± 0.67 | 14.70 [c] ± 0,76 |
| | BF % | 51.65 | 90.30 | 89.72 |
| Naringenin | Extract | 0.47 [a] ± 0,27 | 0.47 [a] ± 0,21 | 0.65 [a] ± 0.23 |
| | GIS | < LQD | < LQD | < LQD |
| | BF % | < LQD | < LQD | < LQD |

JP: juçara pulp; JP: juçara pulp; JLR: juçara pulp with Lactobacillus reuteri LR92; JBB: juçara pulp with *Bifidobacterium animalis* ssp. lactis; LQD: detection limit; ND: Not detected; GIS: After the gastrointestinal simulation; BF: Bioaccessible fraction. Values on the same line followed by different letters indicate significant differences ($p < 0.05$).

Decarboxylation or reduction metabolism towards phenolic acids is probably the main mechanism for detoxification of compounds harmful to lactic acid bacteria (LAB) in plant substrates. Among these compounds are 4-hydroxybenzoic acid, which in the extracts of the fermented samples in our work showed a significant reduction when compared to the extract without fermenting before going through the gastrointestinal simulation.

After the gastrointestinal simulation, eight phenolic compounds were detected (Table 2). However, quercetin, catechin, nicotinic acid and ascorbic acid were not found after this step. The highest bioaccessible fractions were found for protocatechuic acid, 4-hydroxybenzoic acid and trigonelline. The bioaccessibility value for protocatechuic acid was on average 4 times higher in the three samples, JP, JLR and JBB after GIS, when compared to the extracts before the simulation. Highlighting that for the fermented samples the concentration of this compound was 159.57 mg 100 $g^{-1}$ for JBB and 134.57 mg 100 $g^{-1}$ for JLR against 95.18 mg 100 $g^{-1}$ of the sample without

fermenting after GIS. The 4-hydroxybenzoic acid showed the highest bioaccessible fractions in the fermented samples. In this way, the fermented samples presented themselves as a greater source of these compounds when compared to JP at the end of the gastrointestinal simulation.

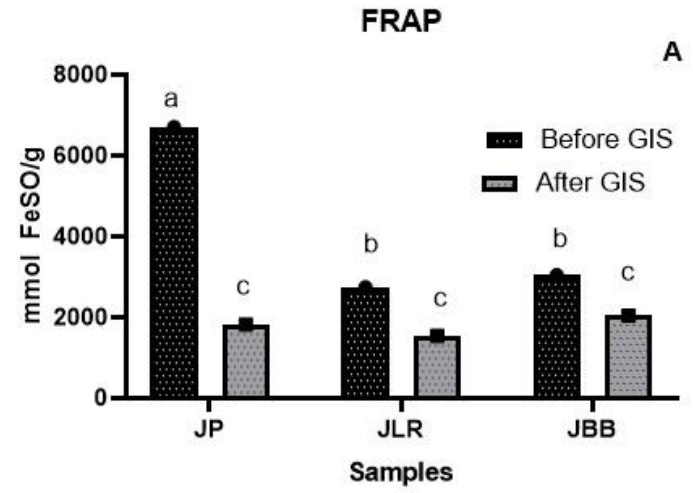
Rutin, synaptic acid and ferulic acid showed bioaccessibility below 100%, and the compounds naringenin, chlorogenic acid were detected after analysis, but below the limit of quantification in samples after GIS (Table 2). These different values of bioaccessibility are due to the chemodiversity of phenolic compounds, which range from simple molecules to highly polymerized molecules. Most phenolic compounds are found in glycosylated forms or as esters or polymers, which are hydrolyzed during gastrointestinal digestion, due to the acidic environment of the stomach and the alkaline environment of the intestine, and also by the action of digestive enzymes (Alminger et al., 2014; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Conditions that cause several structural changes in the compounds can be: hydroxylation, methylation, isoprenylation, dimerization and glycosylation, as well as the formation of phenolic derivatives by partial degradation of the combined forms or by the loss of portions between phenols and sugars (Schulz et al., 2017; Chen et al., 2016).

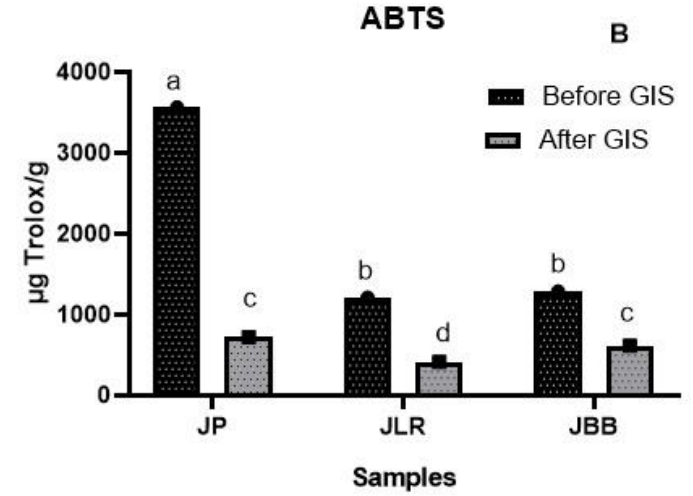
Phenolic structures are also susceptible to interaction with other dietary constituents and released during this process, such as iron, fiber or dietary proteins (Bouayed, Hoffmann, Deuber, & Bohn, 2012; Chen et al., 2016). Thus, changes in the structure of phenolics is a hypothesis that may justify the higher content of some compounds in the bioaccessible fraction compared to the gross extract (Schulz et al., 2017; Seraglio, 2018).

*Antioxidant Capacity*

The antioxidant capacity of JP, JBB and JLR samples from extracts and after GIS were evaluated using ABTS, DPPH and FRAP assays (Figure 1). The JP extract showed the highest antioxidant activities, 6711.47 mM of FeSO4 /g, 2479.66 µg Trolox/g and 3564.39g µg Trolox/g for FRAP, DPPH and ABTS respectively. For JBB 3049.1 mM of FeSO4 /g (FRAP), 815.99 µg Trolox/g (DPPH) and 1287.01 µg Trolox/g (ABTS) was found. For the JLR sample, 2747.07 mM of FeSO4/g (FRAP), 753.74 µg Trolox/g (DPPH) and 1211.8 µg Trolox/g (ABTS) were presented.

We can observe (Figure 1) that GIS also influenced the bioaccessibility of the antioxidant capacity. The decrease in the values obtained is justified by the decrease in the content of phenolic compounds or their transformation into different structural forms with other chemical properties. Changes in pH and the interference of other matrix constituents on phenolic compounds with antioxidant capacity also exert influences (Celep et al., 2015; Chen et al., 2016; Schulz et al. 2017).


FRAP
A
8000
6000
4000
2000
0
mmol FeSO/g
a
c
b
c
b
c
Before GIS
After GIS
JP
JLR
JBB
Samples


ABTS
B
4000
3000
2000
1000
0
µg Trolox/g
a
c
b
d
b
c
Before GIS
After GIS
JP
JLR
JBB
Samples

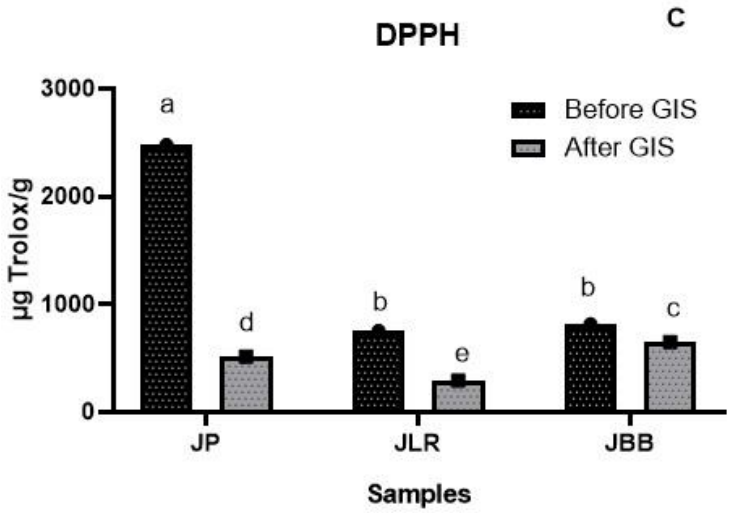

DPPH
C
3000
2000
1000
0
µg Trolox/g
a
d
b
e
b
c
Before GIS
After GIS
JP
JLR
JBB
Samples

Figure 1: Antioxidant capacity of JP, JLR and JBB; (A): FRAP mmol of FeSO4 / g; (B) ABTS µg Trolox / g; (C) DPPH µg Trolox / g; before and after gastrointestinal simulation (GIS).*Different letters indicate significant differences ($p < 0.05$).

The correlation is indicative of which quantified phenolic compounds contributed to the antioxidant capacities of juçara pulp (Schulz, et al. 2017). Correlations between the antioxidant capacity and the contents of bioaccessible phenolic compounds in the samples were calculated. The correlation was positive and significant for the compounds rutin and ferulic acid ($r > 0.95$; $p < 0.01$), for the three methods tested, DPPH, ABTS and FRAP, indicating that the concentration of these phenolics is directly proportional to the activity antioxidant measured by the methods. On the other hand, protocatechuic acids and trigonelline showed a negative correlation ($r > - 0.85$; $p < 0.01$) in the antioxidant activity assays, that is, the increase in the concentration of these acids did not present an increase in the antioxidant activity in this study.

*Anthocyanins*

Anthocyanins are found in large amounts in juçara fruits. Levels of 93.60 mg.100 $g^{-1}$ for JP, 54.40 mg.100 $g^{-1}$ for JLR and 50.4 for JBB were found in the fruits before GIS. The results obtained are within the range that Borges et al. reported in their study (409.85± 2.33 to 14.84± 2.1 mg 100 $g^{-1}$). The results of anthocyanins also decreased after fermentation, results similar to those found in the analysis of antioxidant activities in this study. These results are justifiable, as according to Borges et al., (2013) and Schulz et al., (2015) there is a high correlation between anthocyanin content and *in vitro* antioxidant activity of the juçara fruit.

There is a bioaccessibility reduction during passage through the simulated gastrointestinal tract (Figure 2). The change from the gastric acid-containing medium to the alkaline intestinal environment results in a decrease in the amount of bioaccessible anthocyanins, because these pigments are highly unstable at intestinal pH, where the oxidation of anthocyanins or the transformation of their secondary structures occurs (Tagliazucchi et al., 2010).

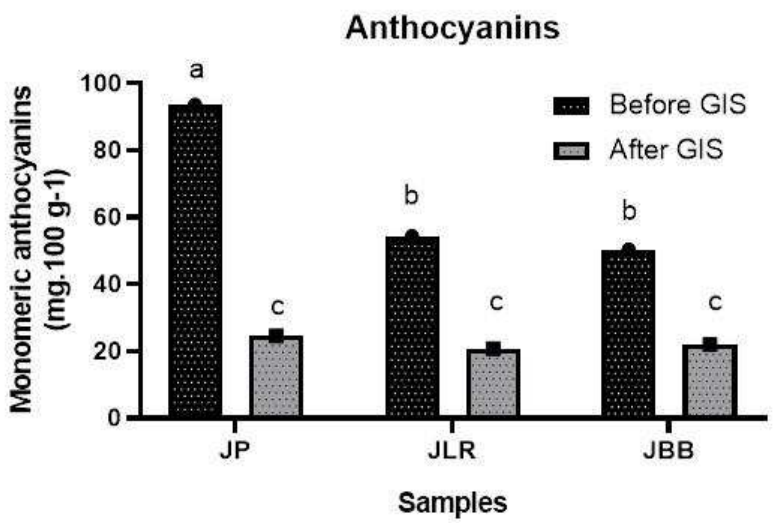



Figure 2: Monomeric anthocyanins (mg.100g-1) before (extracts) and after gastrointestinal simulation. * Different letters indicate significant differences ($p < 0.05$)

Correlations between antioxidant capacity and anthocyanins were also calculated. The correlation was positive and significant for the three tested methods, DPPH ($r > 0,96$; $p < 0,01$) ABTS ($r > 0,93$; $p < 0,01$) and FRAP ($r > 0,96$; $p < 0,01$), indicating that the concentration of anthocyanins is directly proportional to the antioxidant activity measured by the methods and responsible for part of the antioxidant activity found in this fruit.

Table 3: Bioaccessible fraction of antioxidant capacity and anthocyanins

| Analysis | Bioaccessible fraction % | | |
|---|---|---|---|
| | JP | JLR | JBB |
| ABTS | 20,22 | 34,20 | 47,68 |
| DPPH | 20,84 | 38,97 | 79,86 |
| FRAP | 27,21 | 56,15 | 67,03 |
| Anthocyanins | 26,28 | 38,23 | 43,65 |

*Conclusion*:

Juçara pulp presented as a favorable medium for the growth of *L. reuteri* LR92 and *Bifidobacterium animalis* subsp. lactis BB-12. Fermentation caused alterations in phenolic compounds, as well as in antioxidant activities and anthocyanins concentration, highlighting the increase in the bioaccessibility of protocatechuic and 4-hydroxybenzoic acids when compared to unfermented juçara pulp. In this way, even with the reduction of the antioxidant activity of the pulps after the fermentation process,

it is still presented as a source of antioxidant compounds, and that associated with probiotic bacteria, can confer health benefits.

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# CAPÍTULO 3*

[2]Artigo a ser submetido na Revista ***LWT Food Science and Technology***

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**CAPÍTULO 3 –** Evaluation of *in vitro* bioaccessibility of minerals and phenolic compounds in juçara pulp (*Euterpe edulis* Martius) fermented by probiotic bacteria

Abstract

Juçara (*Euterpe edulis* Martius) is a palm tree distributed in the Atlantic Forest and its fruit stands out for their high nutritional value, which contains different kinds of nutrients, including minerals and bioactive compounds such phenolic acids, which promote health benefits. Its pulp proved to be a suitable medium for probiotic bacteria growth. Thus, the objective of this work was to present the changes in bioaccessibility of minerals and phenolic compounds in juçara pulp fermented by *Lactobacillus reuteri* (JLR) and *Bifidobacterium* BB-12 (JBB). In general, the highest concentrations (mg $100g^{-1}$) obtained for mineral content were: Ca (403.03 - 213.02) > Mg (308.64 - 279.40) > Fe (9.37 - 8.65). The highest bioaccessibility percentage were obtained for Mg (116.80 - 110.26), followed by Mn (95.11 - 62.06), Cu (93.80 - 79.37) and Zn (77.28 - 72.57). The fermentation favored the bioaccessibility of Mn, Zn and Cu, whereas Ca was not detected. The total phenolics suffered reductions after fermentation but presented high bioaccessible fraction 63.10 % for JBB and 64.35% for JLR against 60.35 % in the unfermented sample. Thus, the results obtained showed that JLR and JBL are good sources of minerals and phenolic compounds.

Keywords: bioactives, *Lactobacillus reuteri*, *Bifidobacterium,* UBM methodology

## 1.Introduction

Juçara palm (*Euterpe edulis* Mart.) is native to the Brazilian Atlantic Forest, occurring from the south of Bahia to the north of Rio Grande do Sul. This palm tree has great economic and ecologic importance in Brazil (Garcia et al, 2019). However, the large and continuous palm heart production resulted in near extinction of the species, since palm heart extraction involves the death of the plant Thus, the consumption of juçara fruits can present a sustainable and economically viable alternative for the exploitation and preservation of this palm tree (Schulz, Borges, Gonzaga, Costa, & Fett, 2016).

Juçara fruits are globose purple berries and are not consumed *in natura*, as they present high perishability and a very thin epicarp/mesocarp that is not juicy (Garcia et al., 2019). Therefore, juçara fruit pulp is extracted with water and used for the development of products such as juice, ice cream and fermented milk (Machado et al 2020; Fernandes et al, 2020). The juçara pulp has an important

nutritional energy value, high in caloric content, proteins, sugars, fibers, and lipids. In its composition there are bioactive compounds, such as phenolic compounds that include anthocyanins (Guergoletto et al., 2016). The phenolic compounds help protect against degeneration diseases, and their collaboration in health has been mainly attributed to its antioxidant properties (Bicudo, Ribani, & Beta 2014).

The juçara pulp also stands out for its mineral content, which is around 8.8% (w/w) on a dry weight basis (Da Silva et al., 2014; Inada et al., 2015). Potassium, calcium, magnesium, phosphorus and sodium are the major macro-minerals in juçara fruit pulp, while iron, manganese and zinc are the predominant microminerals (Inada et al., 2015). Juçara fruit iron content is similar to that found in açai, which is known as a natural good source of iron (Oliveira. 2019).

Although these compounds are present in the juçara fruit, after ingestion they may quantitatively and qualitatively differ from the values obtained with the chemical extraction used in most studies. *In vitro* and *in vivo* digestion tests assist to a better understanding of the release of nutrients in the digestive tract (bioaccessibility) (Cardoso et al., 2015; Pereira et al., 2018). The bioaccessible fraction is the maximum amount of a nutrient that can be absorbed through the intestinal epithelium, reaching the bloodstream (Souza et al., 2018). The Unified Bioaccessibility Method (UBM) was developed by the BARGE group to study *in vitro* bioaccessibility of toxic elements in contaminated soils (Broadway et al., 2010; Denys et al., 2012). This unified method has been widely used for different kind of samples, including fruit pulp, vegetables and flours (Ferreira e Tarley, 2020).

Some strains of *Lactobacillus* spp and *Bifidobacterium ssp* are described as probiotics. It has been demonstrated that the species of *Bifidobacterium ssp* and *Lactobacillus spp* have enzymes that participate in biotransformations in the composition of fruits and vegetables, but little is known about their potential for biotransformation of bioactive compounds (BRAGA et al. 2017) In this context, the objective of this study was to evaluate the *in vitro* bioaccessibility of total minerals and phenolics of juçara pulp before and after fermentation by probiotic bacteria.

## 2. Material and methods

### *2.1 Microorganisms*

*Lactobacillus reuteri* LR92 (Sacco DSM 26866-Cadorago, Italy) and *Bifidobacterium animalis* ssp. lactis BB-12 (Christian Hansen-Dinamarca) in lyophilized form were stored at a concentration of 0.1% (w / v) in pasteurized juçara pulp added 20% (v / v) sterile glycerol (Synth, Brazil). Before inoculation, the pre-inoculum was activated in juçara pulp, twice, for 24 h at 37 °C, in anaerobiosis (Probac, Brazil).

### *2.2 Pulp Preparation*

Juçara fruits (SisGen A880DD5) were harvested in Rolândia-PR, (Latitude: 23 ° 18 '38' 'S, Longitude: 51 ° 22' 10 '' W), Parana, Brazil, in April of 2018. The pulp was obtained and pasteurized according to Guergoletto et al. (2016). After this process, 1% (v / v) of pre-inoculum active cells containing *L. reuteri* or *Bifidobacterium* BB-12 were added. Fermentation was carried out in sterilized glass containers, incubated at 37 °C in anaerobiosis (Probac, Brazil), for 24 h. The analyzes were performed on samples of juçara pulp without fermentation (JP), juçara pulp fermented by *L. reuteri* (JLR) and juçara pulp fermented by *Bifidobacterium* BB-12 (JBB). All samples were freeze-dried up to constant weight (Freeze dryer L101 - Liobras, Brazil).

### *2.3 Physicochemical analysis*

The analysis for determining moisture content, ash, proteins, and lipids were performed according to AOAC standard method (2012), and the carbohydrate content was calculated by difference. The results expressed as a percentage.

The tannins concentration was determined according to Folin-Danis and this procedure consisted on the principle of oxireduction, being extensively used in the quantitative determination of tannins (Santos, Varejão, Nascimento, 2012).

### *2.4 Determination of the total concentration of Mg, Ca, Zn, Mn, Cu and Fe*

Microwave-assisted mineralization of samples was carried out using the heating program described by Brito et al. (2017), with minor modifications. Initially, 400 mg of dry sample was weighed on an analytical balance and transferred to Teflon® vials from the microwave oven, followed by the addition of 12.0 mL of concentrated $HNO_3$. The following heating ramp was used: heating to 120 °C in 13 min, heating to 170 °C in 16 min, heating to 190 °C in 10 min and maintained at that temperature for 10 min. During all heating program the power was set as 700W. After mineralization, the samples were heated to near dryness in a heating plate, followed by addition of 2.0 mL of $H_2O_2$ (29%). Then, the samples were transferred to 10.0 mL volumetric flasks and the volume was made up with ultrapure water. The same procedure was performed for the certified material (SRM) of Brown Bread (BCR - 191). The procedure was performed in triplicate (n = 3) for both samples and analytical blanks. The characteristic parameters of the analytical curve, including the regression coefficient (R2), limit of detection (LOD), and limit of quantification (LOQ), calculated according to IUPAC recommendation (Iupac, 1997) and described by Ferreira and Tarley (2020).

*2.5 Determination of total phenolic compounds*

The samples were extracted according to Singh *et al.* (2009), with adaptations. The lyophilized pulp was weighed (500 mg) and placed in 15 mL falcon tubes plus 10 ml 80% methanol (Synth, Brazil). The mixture was shaken vigorously using a vortex at 50 Hz (Labnet´s Vortex Mixer VX-200) for 2 min and then taken to a rotary shaker (Tecnal, Brazil) for 30 min. Samples were then centrifuged at 5000 rpm for 15 min and the supernatant reserved. The residues were extracted again twice with an additional 10 mL of 80% methanol. The three extracts were combined and subsequently filtered using Whatman filter paper (0.45 µm). The total content of phenolic compounds was determined by the spectrophotometric method (at 760 nm) described by Swain and Hills (1959). The quantification used the standard analytical curve of gallic acid (Sigma-Aldrich, USA), and the results were expressed in mg of gallic acid equivalent (EAG)/100 g sample.

*2.6 In vitro* bioaccessibility

The *in vitro* bioaccessibility assays was based on three sequential extraction steps: salivary, gastric and intestinal digestion, as described in the UBM methodology (Wragg et al., 2011). The reagents used to prepare fluids: salivary, gastric, duodenal and biliary, are described in the work by Broadway et al. (2010). All determinations were made in triplicate (n = 3), accompanied by analytical blanks. In order to assure an analytical control in the bioaccessibility assays, the mass balance was carried out by comparing the total concentration of the elements with the sum of the bioaccessible and residual fraction. For the simulation of the gastric phase, 300 mg of dry sample was weighed, and 4.5 mL of salivary fluid and 6.75 mL of gastric fluid were added. The pH was adjusted to 1.2 ± 0.05. The mixture was subsequently placed in a thermostatic bath with stirring (168 bpm) at 37 °C for 1 h. For gastrointestinal phase simulation 13.5 mL of intestinal fluid and 4.5 mL of bile fluid were added, the pH was adjusted to 6.3 ± 0.5 and the mixture was placed in a thermostatic bath with stirring (168 bpm) at 37 °C for 4 h. Afterwards, the samples were placed in an ice bath for 10 min and subsequently centrifuged at 2,000 rpm (605 x g) for 30 min. Next, the samples were acidified with 500 µL of concentrated $HNO_3$ and soluble and insoluble fractions were separated. The acidified supernatants, from both the gastrointestinal and gastric phases, were subsequently filtered using qualitative filter paper.

For mineral analysis the residual solids (non bioaccessible fractions) were mineralized using the same procedure described in Section 2.4. After mineralization and heating to near dryness in the heating plate, the volume was made up to 5.0 mL in volumetric flask with ultrapure water. The metallic elements present in bioaccessible (supernatants) and non-bioaccessible (waste) fractions were quantified by flame atomic absorption spectrometry (FAAS). For phenolic compounds methodology described in Section 2.5

The percentage of bioaccessibility (BF) was calculated according to Equation 1:

$$BF\ (\%) = BE/\ TE \times 100 \quad \text{(Equation 1)}$$

Where BE is the fraction of bioaccessible element (mg $100g^{-1}$), given by the concentration of an element found gastrointestinal extracts, and TE is the total element concentration (mg 100g-1).

*2.7 Statistical analysis*

The data were submitted to analysis of variance (ANOVA), and the differences between the means were detected by the Tukey test with a significance level of 5%. Correlations between the data obtained for proximate composition, minerals and and phenolic compound were established using the Pearson's correlation coefficient. The software R was used to obtain the graphs of correlations between the variables. All data are presented as mean ± standard deviation (S.D.) and the experiments were conducted in genuine triplicate. The data were analyzed using the Statistica 10.0 software (Statsoft Inc., Tulsa, OK, USA).

## 3. Results and Discussion

*3.1 Proximate composition and Tannins content*

The proximate composition and tannins content in JP, JBB and JLR are shown in Table 1.

**Table 1.** Values obtained in the analysis of proximate composition and tannins content of juçara pulp (JP), juçara pulp fermented by *L. reuteri* (JLR) and juçara pulp fermented by *Bifidobacterium* BB-12 (JBB).

| Parameters | Samples | | |
|---|---|---|---|
| | JP | JBB | JLR |
| **Moisture (g.100g$^{-1}$)** | $89.76^{a} \pm 0.32$ | $90.42^{a} \pm 0.53$ | $90.62^{a} \pm 0.71$ |
| **Carbohydrate (g.100g$^{-1}$ of dry matter)** | $78.27^{a} \pm 0.31$ | $69.24^{b} \pm 0.11$ | $70.17^{b} \pm 0.13$ |
| **Ashes (g.100g$^{-1}$ of dry matter)** | $2.20^{a} \pm 0.11$ | $2.10^{a} \pm 0.23$ | $2.21^{a} \pm 0.24$ |
| **Lipids (g.100g$^{-1}$ of dry matter)** | $21.63^{b} \pm 0.21$ | $24.74^{a} \pm 1.35$ | $23.52^{a} \pm 0.43$ |
| **Proteins (g.100g$^{-1}$ of dry matter)** | $4.90^{a} \pm 0.08$ | $1.92^{b} \pm 0.02$ | $2.01^{b} \pm 0.13$ |
| **Tannins (mg.100g$^{-1}$ of dry matter)** | $0.19^{c} \pm 0.01$ | $0.42^{b} \pm 0.01$ | $0.55^{a} \pm 0.02$ |

Values followed by different superscripts letters in the same line are significantly different ($p < 0.05$) according to the Tukey HSD test.

The samples showed a mean of 90.26% moisture, with no statistical difference between them. Similar results were obtained by Da Silva et al (2014) and Da Silva et al. (2013) who determined 88.4 and 89.4% (w / w) of moisture in juçara pulp, respectively. The high moisture content of juçara pulp is attributed to the use of water in the pulp extraction process.

The results obtained for lipids varied from 17.6 to 24.7%, where the samples JLR and JBB showed the highest value ($p < 0.05$) compared to the juçara pulp not fermented. Juçara pulp show similar values to the açai for lipids. This fruit stands out for their high fat content, which is higher than other berries from the Brazilian rainforest such as jabuticaba, araça, grumixama and uvaia (Da Silva et al., 2014; Inada et al., 2015).

Juçara fruit may be an important plant source of dietary protein, since it can provide more protein than common fruits such as apples, grapes, pears and melons (Hui, 2006). In our work, we found protein contents (w/w) of 4.90, 1.92 and 2.01 for JP, JBB and JLR, respectively. These results are lower than those described by Da Silva et al (2014) and Inada et al., (2015) who found values between 6–7.5% w / w on a dry basis. The values of proteins and lipids can vary between juçara fruits, and consequently, in the juçara pulp, according to the location where this fruit was grown and the conditions of cultivation (Borges et al., 2013).

Fermentation by both bacteria increased the tannin content in the pulps. The increase in tannin during fermentation was attributed to hydrolysis of condensed tannins such as proanthocyanidin (Emambux & Taylor, 2003). Despite the negative effect of tannin on the nutritional value of certain vegetables, in particular the reduction of digestibility of proteins, the inhibition of the action of digestive enzymes and interference with the absorption of iron, the effects of tannin on human health are considered beneficial. Among the beneficial effects we can mention anti-inflammatory, antioxidant, antidiabetic, anticancer, and cardioprotective effects (Sallan et al., 2021; Vazquez-Fresno, et al., 2017).

Tannins represent a heterogeneous group of high-molecular-weight polyphenols that are widespread among plant families. Hydrolysable and condensed tannins, are the main classes of these

bioactive compounds. Microbiota-mediated hydrolysis of tannins and microbial metabolism, produces highly bioaccessible metabolites, which have been extensively studied and account for most of the health effects attributed to tannins (Sallan et al., 2021).

*4.2 Minerals determination*

4.2.1. Total concentration of Macroelements and microelements

A certified reference material was analyzed to evaluate the accuracy of the microwave-assisted acid digestion method associated with FAAS determination of elements according to Ferreira and Tarley (2020). The results obtained according to Student's t-test (95% confidence level), presented no differences between the certified values and the experimental values.

The mineral contents are summarized in Table 2. There are many minerals present in juçara fruit, considered rich in dietary minerals. At least 17 chemical elements were described in studies (Schulz et al., 2016). The highest concentrations of macro-minerals found in juçara pulp in our study were Calcium (403.03 to 213.02 mg.100g$^{-1}$) and Magnesium (308.64 to 279.40 mg.100g$^{-1}$). This result is similar to that found by Pupin et al. (2018). The fermented samples JLR and JBB showed significant reductions in Ca and Mg concentrations when compared to the sample without fermentation. Pupin et al (2018) described in their work that the different stages of juçara pulp processing could affected the quality parameters, generally promoting a reduction in minerals content.

Table 2: Total concentrations, bioaccessible and residual fractions (mean ± standard deviation, n = 3) of Ca, Mg, Mn, Fe, Zn and Cu in juçara pulp (dry samples)

| | | **Concentration (mg.100 g$^{-1}$)** | | | **%** | |
|---|---|---|---|---|---|---|
| **Elements** | Samples | Total | BE | Residue | BF | MB |
| **Mn** | JP | 7.37$^{b}$ ±0.11 | 7.01$^{a}$ ±0.04 | 1.00$^{b}$ ±0.01 | 95.11 | 109.18 |
| | JBB | 6.22$^{c}$ ±0.20 | 5.55$^{b}$ ±0.11 | 0.98$^{b}$ ±0.01 | 89.22 | 105.05 |
| | JLR | 7.92$^{a}$ ±0.17 | 4.94$^{c}$ ±0.01 | 2.98$^{a}$ ±0.33 | 62.06 | 100.02 |
| **Zn** | JP | 4.23$^{c}$ ±0.01 | 3.07$^{c}$ ±0.05 | 1.75$^{b}$ ±0.08 | 72.57 | 113.94 |
| | JBB | 9.95$^{a}$ ±0.32 | 7.69$^{a}$ ±0.02 | 2.20$^{a}$ ±0.21 | 77.28 | 99.39 |
| | JLR | 4.94$^{b}$ ±0.02 | 3.71$^{b}$ ±0.30 | 0.58$^{c}$ ±0.10 | 75.10 | 86.84 |
| **Cu** | JP | 2.23$^{a}$ ±0.07 | 1.77$^{b}$ ±0.17 | 0.46$^{a}$ ±0.01 | 79.37 | 100.21 |
| | JBB | 2.26$^{a}$ ±0.10 | 2.12$^{a}$ ±0.02 | 0.48$^{a}$ ±0.03 | 93.80 | 115.04 |
| | JLR | 2.31$^{a}$ ±0.01 | 2.02$^{a}$ ±0.08 | 0.42$^{a}$ ±0.05 | 87.44 | 105.62 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Fe** | JP | 9.37$^{a}$ ±0.31 | 3.63$^{a}$ ±0.01 | 4.11$^{c}$ ±0.07 | 38.74 | 85.1 |
| | JBB | 8.65$^{b}$ ±0.23 | 2.49$^{b}$ ±0.11 | 6.63$^{a}$ ±0.11 | 28.78 | 105.47 |
| | JLR | 8.79$^{b}$ ±0.11 | 2.42$^{b}$ ±0.11 | 6.12$^{b}$ ±0.01 | 27.53 | 97.24 |
| **Ca** | JP | 403.03$^{a}$ ±10.0 | 152.20$^{a}$ ±1.21 | 317.70$^{a}$ ±6.60 | 37.76 | 114.99 |
| | JBB | 213.02$^{b}$ ±32.01 | ND | 179.65$^{c}$ ±4.11 | - | 85.33 |
| | JLR | 271.18$^{b}$ ±50.1 | ND | 261.37$^{b}$ ±10.00 | - | 96.38 |
| **Mg** | JP | 308.64$^{a}$ ±8.00 | 340.32$^{a}$ ±5.75 | 13.86$^{b}$ ±0.61 | 110.26 | 114.75 |
| | JBB | 279.40$^{c}$ ±2.0 | 314.12$^{c}$ ±8.01 | 12.23$^{c}$ ±0.98 | 116.80 | 112.42 |
| | JLR | 293.66$^{b}$ ±5.2 | 323.80$^{b}$ ±1.26 | 15.55$^{a}$ ±0.77 | 110.26 | 115.55 |

BE is the fraction of bioaccessible element (mg 100g$^{-1}$), BF: % of Bioaccessible fraction **MB: Mass balance. The values followed by different superscripts in the same column for an element, are significantly different ($p < 0.05$).

The total content of the microelements Mn (7.92 - 6.22 mg.100 g$^{-1}$), Cu (2.31 - 2.23 mg.100 g$^{-1}$), Zn (9.95 - 4.23 mg.100 g$^{-1}$) in our study, were higher to those observed by Silva, Barreto, & Serôdio (2004). The authors reported values of 4.3, 1.4, and 1.2, mg.100 g$^{-1}$ of Mn, Cu, Zn respectively.

The total Fe content found in our samples (9.37 - 8.65 mg.100 g$^{-1}$) were lower than the content found by Pupin et al (2018), which was 254.42 mg.100 g$^{-1}$ in freshly processed pulp. However, these values were higher than those reported by Schulz et al, (2016) who reported a concentration of 4.6 to 7.3 mg.100 g$^{-1}$ of iron in the dry matter of juçara pulp. These differences can be explained by the origin of the fruit and harvest because the mineral composition of the fruit reflects its maturation stage and also concentrations found in the soils (Schulz et al., 2017).

The fat present in the samples showed a negative correlation (Figure 1) in relation to the contents of the minerals Fe, Ca and Mg and a positive correlation for Zn. Thus, the concentration of lipids interfered negatively in the concentrations of Fe, Ca and Mg and contributed to higher concentrations of Zn. The other macronutrients did not show significant correlations with the total minerals.

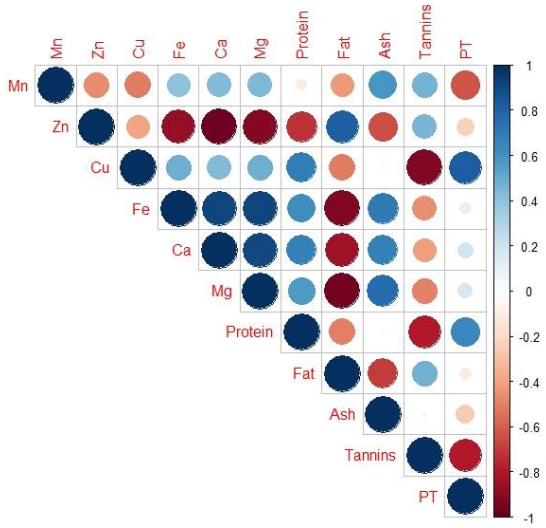


**Figure 1** – Spearman correlation between total mineral concentration with the proximate composition, tannins and total phenolics (PT). Correlation data were obtained by using the average of the values of each parameter for the juçara pulp after and before fermentation

4.2.2 Bioaccessibility

Recovery values were between 85% and 118% (Table 2). Good agreement between determined and certified values of mineral contents indicated adequate accuracy of the method described by Ferreira and Tarley (2020).

Iron presented bioaccessible fractions from 38.74 to 27.53 %, with decreasing rates after fermentation by *L. reuteri* LR92 and *Bifidobacterium* BB-12. The bioaccessibility rate of iron observed in most fruit has correlation with the concentrations of protein, which increased bioaccessibility by reducing and chelating iron (Sandberg, 2002; Sulibursk a & Krejpcio, 2011). In our work, fermentation significantly reduced the concentration of proteins present in the pulp, corroborating with the result of the iron bioaccessibility.

However, an increase in the bioaccessibility of zinc in fermented samples was observed, with emphasis on the JBB sample. Sripriya et al. (1997) also observed increase in zinc content in fermented samples. This could be explained by the process of fermentation, which degrades phytates that complex with minerals. The fermentation process is important for the reduction of phytic acid, which

usually binds to minerals present in food (Gupta, Gangoliya & Singh, 2015). Therefore, after fermentation, minerals become more bioaccessible and available to be absorbed by the human body (Pranoto et al., 2013).

On the other hand, the increase in tannin during fermentation, as observed in our work, can reduce the bioavailability of minerals, as it binds to them (Emambux & Taylor, 2003). As can be observed from Figure 2, Mn and Ca showan accentuated negative correlation with tannin. In fermented samples, calcium did not show bioaccessible concentrations. In addition to the tannins, other components present in foods such as phytates, oxalates, phosphates, carbonates and fibers can act as inhibitors, negatively affecting the bioaccessibility of Ca through the formation of insoluble complexes or by precipitation due to the formation of insoluble salts, such as calcium phosphate (Qu et al., 2018).

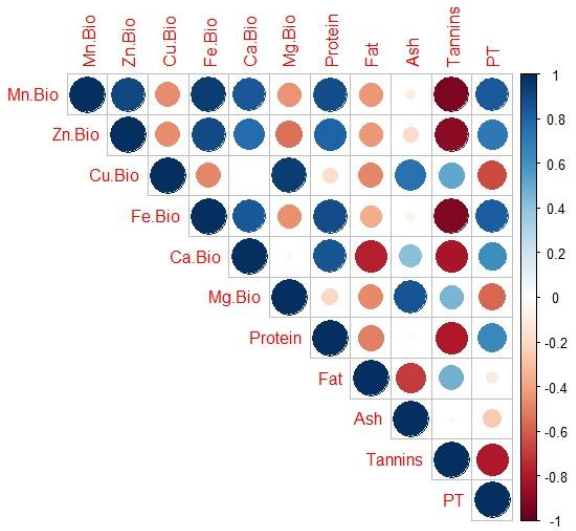


**Figure 2 –** Spearman correlation between minerals bioaccessible fractions concentration with the proximate composition, tannins and total phenolics (PT). Correlation data were obtained by using the average of the values of each parameter for the juçara pulp after and before fermentation.

The bioaccessible levels of copper (2.12 – 1.77 mg. 100 $g^{-1}$ dry matter) in juçara fruit pulp were higher than those found by Schutz et al. (2017) in juçara pulp and reported by Arpadjan, Momchilova, Venelinov, Blagoeva, & Nikolova (2013) for walnuts (0.2 – 0.25 mg.100 $g^{-1}$ dry matter, respectively),

which are considered one of the main contributors in the supply of copper and in the human diet. LAB fermentation improved copper bioaccessibility in juçara pulp compared to unfermented sample.

The juçara pulp showed bioaccessibility above 100% for Mg in the three samples, indicating that during the gastric simulation process these minerals became more available for absorption. The chemical form (Tokalioğlu et al., 2014), the type of food matrix (Menezes et al., 2018), as well as other inhibitors present in foods can influence the bioaccessibility of Mg.

Considering the dietary reference intakes for an adult male (IOM - Institute of Medicine, 2001), the intake of 100 $g^{-1}$ of juçara pulp (dry base) supplies in recommended daily percentage 24–43% of copper, 73- 66 % of magnesium, 40–21% of calcium and 90–38% for zinc. For iron, manganese and copper, the consumption of 100 $g^{-1}$ of juçara pulp provides higher amounts than those recommended (Table 3). In the gastric phase, except for calcium, all minerals had significant daily percentages.

Table 3: Contribution (%) of consumption juçara pulp (JP), pulp after fermentation by *Lactobacillus reuteri* (JLR) and *Bifidobacterium* BB-12 (JBB) to the RDI of the elements for a portion of 100g $day^{-1}$ of flour

| **Elements** | **RDI mg $day^{-1}$** | **Portion (100 g $day^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | Total (%) | | | Gastrointestinal phase (%) | | |
| | | JP | JBB | JLR | JP | JBB | JLR |
| **Ca** | 1000 | 40.30 | 21.30 | 27.11 | 15.22 | - | - |
| **Fe** | 8 | 117.12 | 108.12 | 109.87 | 45.37 | 31.12 | 30.25 |
| **Mg** | 420 | 73.48 | 66.52 | 69.91 | 81.02 | 74.79 | 77.09 |
| **Zn** | 11 | 38.45 | 90.45 | 44.90 | 27.90 | 69.90 | 33.72 |
| **Cu** | 0.9 | 255.55 | 251.11 | 256.66 | 196.66 | 235.55 | 224.44 |
| **Mn** | 2.3 | 320.43 | 270.43 | 344.34 | 304.78 | 241.30 | 214.78 |

*4.3 Total phenolic compounds and bioaccessibility*

In Figure 3 it is possible to observe that the sample without fermenting (JP) presented significantly higher concentrations of total phenolics compared to the fermented samples. In fermentation, the presence of microbial enzymes can hydrolyze glycosides, glucuronides, sulfates, amides, esters, lactones and act in the cleavage of rings, reduction, decarboxylation, demethylation and dehydroxylation of phenolic compounds interfering in their activity (Dall'asta et al., 2012).

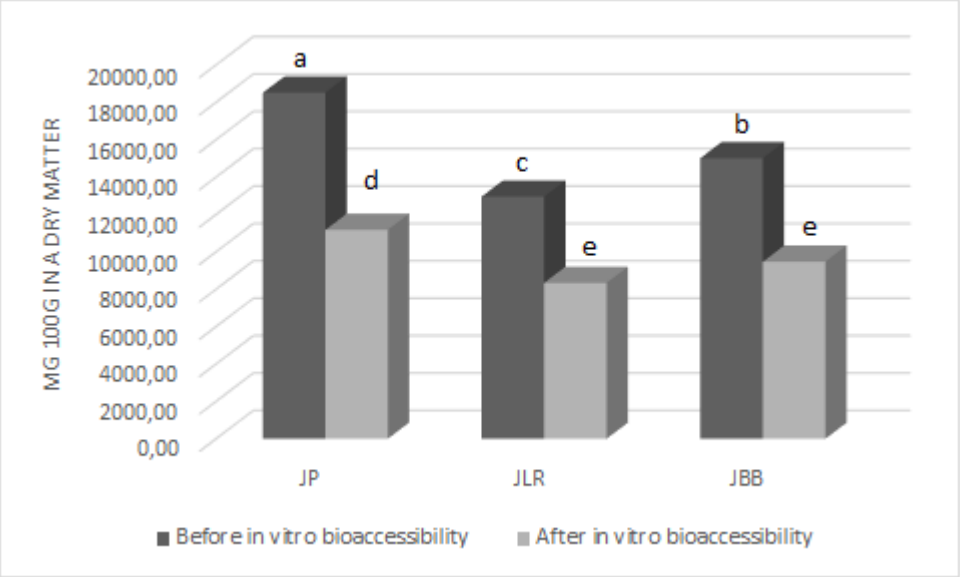


**Figure 3**. Total phenolic content (mg 100 $g^{-1}$ dry matter) before and after *in vitro* gastrointestinal digestion of juçara pulp and fermented juçara pulp.
*Data are presented as mean ± S.D. (n = 3).$^{a-e}$ Different letters in the same series indicate significant differences ($p < 0.05$). JP: Juçara pulp; JLR: Juçara pulp fermented by *L. reuteri;* JBB: Juçara pulp fermented by *Bifidobacterium* BB-12.

The results show the bioaccessibility of the phenolic compounds after SGI of 60.35 % (JP), 63.10 % (JBB) and 64.35 % (JLR). The bioaccessibility value is attributed to the chemodiversity of phenolic compounds, which range from simple molecules to highly polymerized molecules. Phenolic compounds have glycosylated forms or as esters or polymers, which are hydrolyzed during gastrointestinal digestion due to the acidic environment of the stomach and the alkaline environment of the intestine, and also by the action of digestive enzymes (Alminger et al., 2014). Conditions that cause several structural changes in compounds can be: hydroxylation, methylation, isoprenylation, dimerization and glycosylation, as well as the formation of phenolic derivatives by partial degradation of the combined forms or by the loss of portions between phenols and sugars (Schulz et al., 2015).

## 5. Conclusion

Fermentation of juçara pulp by bacteria utilized in our study caused changes in the total content of minerals and phenolic compounds, as well as in its bioaccessibility after *in vitro* gastrointestinal digestion. Fermentation provided higher bioaccessibility values for copper and zinc. In

relation to the content of total phenolics, the fermentation caused a reduction, but still showed significant values.

The present study also found that juçara pulp before and after fermentation contributed greatly to the recommended daily intake of minerals, mainly manganese, copper, zinc and iron. To our knowledge, this is the first study to evaluate the effects of *in vitro* gastrointestinal digestion on minerals and phenolic compounds of fermented juçara pulp.

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# CAPÍTULO 4[3]

[3] **Artigo formatado Segundo as regras da ABNT**

**CAPÍTULO 4** –Characterization and fermentation of bioactive compounds isolated from Juçara pulp by probiotic bacteria

Abstract

Juçara (*Euterpe edulis* Martius) is a palm tree widely distributed in the Atlantic Forest, which produces round fruits that stand out for its bioactive compounds and because it is a potential means of growth of beneficial microorganisms. The main objective of this work is to study the isolated bioactive compounds fractions of juçara, to ferment by action of *Limosilactobacillus reuteri* LR92 (LR) and *Bifidobacterium animalis* ssp. lactis BB-12 (BB-12), besides to identify the changes caused by them. For this, separations were performed in fractions of hydrophilic and lipophilic phenolics, soluble and insoluble polysaccharides. Characterization of phenolics, was performed t by UPLC-MS/MS analysis and the compound detected in greater quantity was the protacatechuic acid in the hydrophilic portion, the same fraction that showed better viability of LR and BB-12. This study also showed the fermentation profile of the fractions through the analysis of $^{1}$H NMR, which presented characteristic peaks of the fermentations. In general, this study confirms the juçara pulp as an excellent growth medium for beneficial microorganisms, with production of acetate through fermentation by bacteria of interest, and a good source of bioactive compounds. The most abundant phenolic compounds were protocatechuic acid (14483 µg.L-1), rutin (5143 µg.L -1) and catechin (4295 µg.L -1) for hydrophilic fraction. For lipophilic fraction myricetin, protocatechuic acid, and synaptic acid are those detected with the highest concentrations

Keywords: protocatechuic acid, basal médium, principal component analysis, metabolites.

## 1. Introduction

Studies involving the consumption of certain foods and disease prevention are increasingly recurrent. Fruits, vegetables, cereals and probiotic metabolites have bioactive compounds that effectively play a beneficial role (Cardoso et al. 2015). The consumption of fruits rich in antioxidants, especially berries and dark colored fruits, has been associated with reduced risk of several chronic diseases caused by oxidative

stress (Aravandis et al. 2021). The biological properties of these berries have been largely attributed to their high levels of various phenolic compounds, such as phenolic acids and flavonoids (Garzón, Narváez, Riedl, & Schwartz, 2010; Kaurinovic & Vastag, 2019). The dark-colored fruits are commonly found in North America and Europe, however, international interest in this kind of fruit of the other regions such as South America has increased (Da Silva et al., 2011; Schulz et al., 2020).

Among these juçara dark-colored fruit from Juçara palm (*Euterpe edulis*) stands out. From the Arecaceae family and genus *Euterpe*, popularly known as juçara, içara or palm tree, this palm contains approximately 7 species distributed in the tropics (Barroso et al., 2019). When used, the juçara fruit is often consumed as a pulp and for this, the fruit usually macerated with water and separated from their seeds to obtain a thick, purple drink (Da Silva et al. 2011). Known for being an excellent source of antioxidants, studies have shown that juçara pulp is an excellent medium for propagation and maintenance of probioticbacteria (Guergoletto et al, 2016).

Fermentation is one of the most used methods in food processing (Martí-Quijal et al., 2021), with *Lactobacillus spp* being the best known probiotic microorganismdue to popular fermented dairy products and their consumption is commonly associated with healthy habits (Elmer, Mcfarland, Mcfarland & Russo 2013). For a long time, probiotic foods were found almost exclusively in dairy products. However, fermented products of plant origin have been widely considered to be an excellent vector for incorporation of probiotics (Rasika et al., 2021).

Fermentation with *L. reuteri* and *L. plantarum* to produce a new juçara product, caused a favorable growth and maintenance of these bacteria, with consumption of sugars present in the juçara and production of lactic acid. (Guergoletto et al, 2018). In other studies, it was observed that the fermentation of juçara pulp by intestinal bacteria increases the number of beneficial bacteria in the colon, such as bifidobacteria, with production of short-chain fatty acids and phenolic compounds (Guergoletto et. al., 2016).

However, through these studies it was not possible to conclude whether bacterial growth is promoted by the consumption or use of sugars, fibers or phenolic compounds present in the juçara and which compounds produced by the bacteria exert the observed beneficial effects. Therefore, in-depth studies with isolated and identified compounds are needed to elucidate the metabolic pathway of juçara fermentation and

which compounds are produced. Thus, the purpose of this study was to study the isolated bioactive compounds fractions of juçara, to fermented them by *Limosilactobacillus reuteri* (*L.reuteri* LR92) and *Bifidobacterium animalis* ssp. lactis BB-12, besides to identify the changes caused by these in juçara pulp.

## 2. Materials and methods

### *2.1 Microorganisms*

*Lactobacillus reuteri* LR92 (Sacco DSM 26866-Cadorago, Italy) and *Bifidobacterium animalis* ssp. lactis BB-12 (Christian Hansen-Dinamarca) in lyophilized form were stored at a concentration of 0.1% (w / v) in juçara pulp (2.5 °Brix) pasteurized with 20% (v / v) sterile glycerol (Synth, Brazil). Before inoculation, the pre-inoculum was activated in juçara pulp, twice, for 24 h at 37 °C, in anaerobiosis (Probac, Brazil). Bacterial suspension was centrifuged at 8000× g for 5 min. The supernatant was removed and the cell pellet was washed twice with sterile distilled water and re-suspended in 9 mL the basal nutrient medium.

### *2.2 Pulp Preparation*

Juçara fruits (SisGen A880DD5) were harvested in Rolândia-PR, (Latitude: 23 ° 18 '38' 'S, Longitude: 51 ° 22' 10 '' W), Parana, Brazil, in april of 2018. The pulp was obtained and pasteurized according to Guergoletto et al. (2016).

### 2.3 Separation of phenolic extracts from juçara extract

The separation of the phenolic extracts was carried out in the pulp of juçara according to the methodology of Borges et. al. (2013).

First, 30 g of juçara pulp was submitted to bleaching for 10 min at 85 ºC to inactivate the enzymes. Afterwards, the pulp was dried in an oven at 40 ºC for 12 h and crushed in a food processor. To obtain the lipid fraction, 1 g of ground sample was extracted three times with 50 mL of hexane (Synth, Brazil) by ultrasound in a laboratory water bath at 25 ºC for 15 min and centrifuged at 2000.g for 15 min. The hexane-

soluble fractions were combined and dried under vacuum at a temperature below 35 ºC.

To prepare the lipophilic extract, a part of the lipid fraction was subjected to liquid extraction with methanol using a water bath at 25 ºC for 15 min and then centrifugation at 2000.g for 15 min. This extract was used to determine the total phenolic content and antioxidant activity.

To prepare the hydrophilic extract, the defatted pulp was extracted with 50 mL of methanol (three times) at 25 ºC for 30 min. The extract was then dried in an oven under vacuum at a temperature lower than 35 ºC.

The dry extract was reconstituted in methanol with the aid of a beaker until the volume of 5 mL was used for quantification of total phenolic content and identification of phenolic compounds by HPLC-LC-MS.

To evaluate the effect of fractions with isolated juçara compounds on the fermentation of bacteria these were reconstituted in water with a beaker up to a volume of 5 mL.

### 2.4 Separation of the polysaccharide fraction from juçara pulp

The extraction of the polysaccharide fraction from the juçara was performed according to the methodology described by Holderness et. al., (2011). First 1,500 g of juçara pulp was extracted with 8 L of boiling water for 1 h. The aqueous extract was centrifuged at 2000 x g for 15 min. Ethanol 95% in the amount of 4 times its volume was added to the supernatant for precipitation of polysaccharides, for one night at 4 ºC. The precipitate was pelletized by centrifugation and redissolved in distilled water and centrifuged again at 2000 x g for 15 min. Soluble and insoluble fractions of polysaccharides were used for fermentation by L. reuteri and BB-12.

### 2.5 Fermentation of phenolic and polysaccharide fractions by probiotics

For the fermentation of isolated juçara fractions, the basal nutrient medium (peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), $K_2HPO_4$ (0.04 g/L) ), $NaCO_3$ (2 g/L), $MgSO_4.7H_2O$ (0.01 g/L), $CaCl_2.6H_2O$ (0.01 g/L), Tween 80 (2 mL/L), haemine (50 mg/L) , vitamin K (10 µl/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), and distilled water) were inoculated with 1% (w/v) of the isolated fractions and 1% (w/v) of the active probiotic culture described in item 2.1. After fermentation, the pH and viability

of the microorganisms were measured and the fermented samples were analyzed by NMR to identify fermentation profile of the metabolites produced.

As a control, the addition of 1% glucose in the basal medium (positive), a basal medium without sugar source (negative) was used for fermentation. As a comparison, a medium with the addition of FOS (prebiotic) was also used for the fermentation.

*2.6 Analysis of phenolic compounds by ultra-efficient liquid chromatography coupled with mass spectrometry (UPLC-MS / MS)*

For the analysis of phenolic compounds, the Acquity UPLC System coupled to the Triple Quadrupole mass spectrometer (TQD) - Waters was used. The Acquity UPLC HSS C18 Column (2.1 mm x 100 mm, 1.8 µm, Waters) with oven temperature of 40 °C and injection volume: 10 µL with a flow rate of 0.4 m $min^{-1}$. The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B).

The detection by mass spectrometry (MS / MS) was through electrospray ionization (ESI) in a positive mode. Capillary voltage of 1 kV with 400 °C desolvation temperature and 150 °C source temperature. The desolvation gas used was nitrogen (flow rate 700 L $min^{-1}$) and argon was used for collision. Phenolic compounds were detected using multiple reaction monitoring (MRM).

*2.7 Analysis of antioxidant activity and total content of phenolic compounds*

The antioxidant activity was determined by the method: DPPH. For the DPPH • radical method (2,2′-diphenyl-β-picrylhydrazyl), an aliquot (100 µL) of the samples was added to the DPPH • 0.1 mM ethanolic solution (1.0 mL). The mixture was stirred in the dark for 60 min and then the absorbance determined at 517 nm. The results were expressed in µM Trolox / g of sample (Brand-Williams, Cuvelier & Berset, 1995).

The total content of phenolic compounds was determined by the spectrophotometric method (at 760 nm) described by Swain and Hills (1959) The quantification used the standard analytical curve of gallic acid (Sigma-Aldrich), and the results were expressed in mg of gallic acid equivalent (EAG)/100 g sample.

*2.8 Metabolite analysis by 1H-NMR*

The analysis of metabolites by NMR followed the methodology described by Grimaldi et al 2016. Fermentation supernatants from all time points were defrosted, vortexed and centrifuged at 599.g for 5 min. Supernatants were filtered using 0·22-μm low protein binding durapore polyvinylidene fluoride membrane (Millex; EMD Millipore) and 400 μL was transferred into fresh eppendorf tubes. Filtered samples were then combined with 200 μL of phosphate buffer (0·2 M (pH 7·4) in $D_2O$ plus 0·001% trimethylsilyl propionate (TSP)). The mixture was vortexed and centrifuged at 1136.g for 10 min and then 550 μl was transferred into 5-mm NMR tubes for analysis. All NMR spectra were acquired on Bruker Avance DRX 500MHz NMR Spectrometer (Bruker BioSpin) operating at 500 MHz. They were acquired using a standard 1-dimensional (1D) pulse sequence (recycle delay (RD) − 90° − t1 − 90° − tm − 90° − acquire free induction decay (FID)) with water suppression applied during RD of 2 s, a mixing time tm of 100 ms and a 90 pulse set at 7·70 μs. For each spectrum, a total of 128 scans were accumulated into 64 k data points with a spectral width of 12·001 parts per million. The FID were multiplied by an exponential function corresponding to 0·3Hz line broadening. All spectra were manually phased, baseline corrected and calibrated to the chemical shift of TSP (δ 0·00). Principal component analysis (PCA) was performed with Pareto scaling. The dataset resulting from the experiments was imported into the web-based tool for metabolomic data processing MetaboAnalyst software® (version 5.0) for multivariate statistical analysis.

## 2.9 Statistical analysis

The data was expressed and analyzed by the analysis of variance (ANOVA), and the differences between the means were detected by the Tukey test with a significance level of 5%.

# 3.Results and discussion

### *3.1 Viability of L. reuteri and Bifidobacterium spp of whole juçara pulp and Fractions of Interest in basal medium.*

Cultures of *L. reuteri* and BB-12 were grown in basal medium added with whole juçara pulp and isolated fractions of phenolics and polysaccharides for 72 hours, with maximum viability observed in 24 hours in all samples (Table 1).

Table 1: *Viability of L. reuteri and Bifidobacterium spp in whole juçara pulp and Fractions of Interest in basal medium until 72 hours.*

| hours | pH | Counts (log CFU/mL) | pH | Counts (log CFU/mL) |
|---|---|---|---|---|
| | LR-whole pulp | | BB12- whole Pulp | |
| 0 | 7.53 $^{a}$±0.18 | 6.21 $^{b}$± 0.08 | 8.53 $^{a}$± 0.03 | 6.60$^{d}$ ±0.03 |
| 6 | 7.46 $^{a}$± 0.08 | 6.32 $^{b}$±0.10 | 8.46 $^{a}$± 0.02 | 6.69$^{d}$ ±0.00 |
| 24 | 7.35 $^{a}$±0.34 | 7.47 $^{a}$± 0.02 | 8.50 $^{a}$± 0.21 | 8.12$^{a}$±0.00 |
| 48 | 7.31$^{a}$± 0.07 | 6.07$^{c}$ ± 0.19 | 8.50 $^{a}$± 0.19 | 7.52$^{c}$± 0.01 |
| 72 | 7.30 $^{a}$±0.08 | 6.03$^{c}$ ± 0.04 | 8.50 $^{a}$± 0.11 | 7.97$^{b}$± 0.01 |
| | LR- lipophilic | | BB12- lipophilic | |
| 0 | 8.53$^{c}$± 0.01 | 6.31$^{a}$ ± 0.08 | 8.53 $^{a}$± 0.08 | 6.04$^{a}$ ±0.03 |
| 6 | 8.46$^{c;d}$± 0.13 | 6.22$^{a}$ ±0.10 | 8.46 $^{a}$± 0.00 | 4.3$^{b}$ ±0.00 |
| 24 | 8.35$^{d}$± 0.12 | 6.47$^{a}$± 0.02 | 8.50 $^{a}$± 0.04 | 2.9$^{c}$ ±0.00 |
| 48 | 8.81$^{b}$± 0.08 | 6.47$^{a}$± 0.19 | 8.50 $^{a}$± 0.01 | 2.17$^{d}$ ± 0.01 |
| 72 | 9.00$^{a}$± 0.09 | 6.43$^{a}$ ± 0.04 | 8.50 $^{a}$± 0.01 | - |
| | LR- hydrophilic | | BB12- hydrophilic | |
| 0 | 7.80 $^{a}$±0.08 | 6.12$^{c}$ ±0.06 | 8.53 $^{a}$± 0.08 | 6.04$^{a}$ ±0.02 |
| 6 | 8.00 $^{a}$±0.08 | 6.14$^{c}$ ±0.015 | 8.46 $^{a}$± 0.08 | 5.35$^{b}$ ±0.18 |
| 24 | 7.00 $^{b}$±0.08 | 8.34$^{a}$ ±0.03 | 8.35 $^{a}$± 0.08 | 5.91$^{a}$ ±0.14 |
| 48 | 7.38 $^{b}$±0.08 | 7.05$^{b}$ ±0.63 | 8.51 $^{a}$± 0.08 | 5.01$^{c}$ ±0.00 |
| 72 | 7.39 $^{b}$±0.08 | 5.45$^{d}$ ±0.00 | 8.50 $^{a}$± 0.08 | 4.92$^{c}$ ±0.37 |
| | LR- Soluble Polysaccharides | | BB12- Soluble Polysaccharides | |
| 0 | 7.53 $^{a}$±0.08 | 6.11$^{b}$ ±0.03 | 7.10 $^{a}$ ± 0.08 | 6.2$^{a}$ ±0.01 |
| 6 | 7.35$^{a}$± 0.08 | 6.45$^{ab}$ ±0.08 | 7.00 $^{a}$± 0.08 | 5.03$^{b}$ ±0.22 |
| 24 | 6.90 $^{b}$±0.08 | 6.79$^{a}$±0.07 | 7.10 $^{a}$± 0.08 | 4.99$^{b}$ ±0.31 |
| 48 | 6.45 $^{c}$± 0.08 | 5.29$^{c}$±0.63 | 7.10 $^{a}$± 0.08 | 4.32$^{c}$ ±0.37 |
| 72 | 6.10 $^{c}$± 0.08 | 4.79$^{d}$±0.07 | 7.15 $^{a}$± 0.08 | 4.31$^{c}$ ±0.21 |
| | LR- Insoluble Polysaccharides | | BB12-Insoluble Polysaccharides | |
| 0 | 7.53$^{a}$± 0.08 | 6.12$^{c}$±0.16 | 7.45$^{a}$ ± 0.08 | 6.14$^{b}$ ±0.06 |
| 6 | 7.35$^{a}$± 0.08 | 6.14$^{c}$±0.63 | 7.62$^{a}$ ± 0.08 | 6.31$^{ab}$ ±0.00 |
| 24 | 6.90 $^{b}$±0.08 | 8.34$^{a}$±0.08 | 7.50$^{a}$ ± 0.08 | 6.44$^{a}$ ±0.1 |
| 48 | 6.00 $^{c}$± 0.08 | 7.05$^{b}$±0.63 | 7.55 $^{a}$ ±0.08 | 5.79$^{c}$ ±0.01 |
| 72 | 6.1$^{c}$± 0.08 | 5.45$^{d}$±0.07 | 7.39 $^{a}$ ±0.08 | 4.77$^{d}$ ±0.01 |
| | LR- positive control | | BB12- positive control | |
| 0 | 7.53$^{a}$ ±0.08 | 6.30$^{c}$±0.16 | 7.66 $^{a}$± 0.08 | 6.77$^{a}$ ±0.06 |
| 6 | 7.35 $^{a}$±0.08 | 6.40$^{c}$±0.63 | 7.49 $^{a}$± 0.08 | 6.26$^{b}$ ±0.00 |
| 24 | 6.90 $^{b}$±0.08 | 7.84$^{a}$±0.08 | 6.11 $^{b}$± 0.08 | 6.24$^{b}$ ±0.1 |
| 48 | 6.00 $^{c}$± 0.08 | 7.65$^{ab}$±0.63 | 5.75 $^{c}$± 0.08 | 5.30$^{c}$ ±0.01 |
| 72 | 6.10 $^{c}$± 0.08 | 7.55$^{b}$±0.07 | 5.49 $^{c}$± 0.08 | 5.18$^{c}$ ±0.01 |

| | LR- Negative control | | BB12- Negative Control | |
|---|---|---|---|---|
| 0 | 8.53$^{a}$± 0.01 | 6.10$^{a}$±0.16 | 8.45 $^{a}$± 0.08 | 6.10$^{a}$ ±0.07 |
| 6 | 8.35$^{a}$± 0.11 | 3.60$^{b}$±0.63 | 8.62$^{a}$± 0.08 | - |
| 24 | 8.35$^{a}$± 0.09 | 3.54$^{b}$±0.08 | 8.50$^{a}$± 0.08 | - |
| 48 | 8.35$^{a}$± 0.08 | - | 8.55$^{a}$± 0.08 | - |
| 72 | 8.34$^{a}$± 0.08 | - | 8.39$^{a}$± 0.08 | - |
| | LR-Prebiotic (FOS) | | BB- Prebiotic (FOS) | |
| 0 | 8.43$^{a}$± 0.08 | 6.42$^{b}$±0.16 | 8.45$^{a}$± 0.08 | 6.48$^{c}$ ±0.07 |
| 6 | 8.35$^{a}$± 0.02 | 7.26$^{b}$±0.63 | 8.62$^{a}$± 0.08 | 7.20$^{b}$±0.07 |
| 24 | 7.35$^{b}$± 0.07 | 7.85$^{a}$±0.08 | 7.50$^{b}$± 0.08 | 7.95$^{a}$±0.07 |
| 48 | 7.35$^{b}$± 0.12 | 7.69$^{a}$±0.07 | 7.55$^{b}$± 0.08 | 7.63$^{a}$±0.07 |
| 72 | 7.34$^{b}$± 0.02 | 7.56$^{a}$±0.07 | 7.39$^{b}$± 0.08 | 7.48$^{b}$±0.07 |

*Different letters in the same sample and same column differ significantly ($p < 0,05$).

Fermentation of whole juçara pulp in basal medium by *L. reuteri* presented counts that did not differ significantly from the positive control containing glucose in 24 hours (table 2). For BB-12, the juçara pulp in basal medium provided significantly higher counts when compared to the positive control and similar to the medium supplemented with FOS, which is recognized as a prebiotic. Guergoletto et al. (2016) reported that after fermentation of juçara pulp by bifidobacterium, beneficial modulations in terms of bifidogenic effects were observed, indicating the degradation of juçara phenolic compounds during digestion and simulated fermentation.

Table 2: Comparison between samples with 24 hours of fermentation by *L. reuteri (LR)* and BB-12

| Sample in 24 hours | Counts (log CFU/mL | Sample in 24 hours | Counts (log CFU/mL |
|---|---|---|---|
| LR-Whole Pulp | 7.47 $^{a}$± 0.02 | BB12- Whole Pulp | 8.12$^{a}$±0.00 |
| LR- Positive Control | 7.84$^{a}$±0.08 | BB12 – Positive Control | 6.24$^{c}$ ±0.1 |
| LR- Negative Control | 3.60$^{b}$±0.63 | BB12- Negative Control | ND |
| LR-Prebiotic (FOS) | 7.85$^{a}$±0.08 | BB12- Prebiotic (FOS) | 7.95$^{b}$±0.07 |

* Same letters in the same column no differ significantly ($p < 0.05$). ND: not detected

The culture medium containing the insoluble fraction of polysaccharides had the highest count on average after 24 hours of fermentation, being 8.34 ± 0.18 log CFU/mL for *L. reuteri* and 6.44 ± 0.1 log CFU/ mL forBB-12. The hydrophilic fraction of phenolic compounds also showed good counts after 24 hours of fermentation, being 6.16 log

CFU/mL for *L. reuteri* and 5.91 log CFU/mL for *Bifidobacterium* spp. As the greatest viability of the microorganisms was within 24 hours, we chose this parameter for the other analyzes.

3.2 Profile of phenolic compounds

The analysis results of phenolic compounds fractions by ultra-efficient liquid chromatography coupled with mass spectrometry (UPLC-MS / MS) are shown in Table 3. The total phenolic content was highest in hydrophilic fraction, with 18 compounds compared with 9 compounds detected in lipophilic fraction.

Table 3: Fractions of phenolic compounds by ultra-efficient liquid chromatography coupled with mass spectrometry (UPLC-MS / MS)

| **Phenolic compounds** | **µg.L$^{-1}$** | |
|---|---|---|
| | Hydrophilic | lipophilic |
| **Trigonelline** | 4031 | 30 |
| **Ascorbic Acid** | 2096 | ND |
| **Nicotinic Acid** | 1029 | 13 |
| **Protocathecuic Acid** | 14483 | 205 |
| **Theophyline** | 7 | ND |
| **4-Hidroxybenzoic Acid** | 3748 | ND |
| **Catechin** | 4295 | ND |
| **Chlorogenic Acid** | 751 | ND |
| **Caffeine** | 33 | 41 |
| **Caffeic Acid** | 1 | ND |
| **Epichatechin** | 852 | ND |
| **p-Coumaric** | 57 | 36 |
| **Ferulic Acid** | 1637 | 47 |
| **Sinapic Acid** | 674 | 93 |
| **Rutin** | 5143 | 12 |
| **Myricetin** | 1065 | 284 |
| **Quercetin** | 474 | ND |
| **Kaempferol** | 189 | ND |

ND: not detected

The most abundant phenolic compounds are protocatechuic acid (14483 µg.L$^{-1}$), rutin (5143 µg.L$^{-1}$) and catechin (4295 µg.L$^{-1}$) for hydrophilic fraction. For lipophilic fraction myricetin, protocatechuic acid, and synaptic acid are those detected with the highest concentrations. Da Silva et al 2011, studying juçara fruit from the state of

Santa Catarina, reported higher concentrations of protocatechuic acid and catechin like those found in our study.

Protocatechuic acid, phenolic compound determined in greater quantity in juçara pulp is a strong antioxidant, exhibit hypoglycemic and insulin-like effects, also has been reported for its cardiovascular-protective effects and recent studies have reported its cardioprotective effect (Safaeian et al. 2018; El-Sonbaty et al. 2019). Still highlighting the importance of phenolics found in juçara pulp, flavonoids such as catechin and rutin are studied for their antidiabetic, antioxidant and antiinflammatory activities (Mechchate et al. 2021).

*3.3 Determination of Antioxidant activity and Total Phenolic Content (TPC).*

The results for total phenolics and DPPH are shown in Table 4. All samples showed significantly lower results after 24 hours of fermentation independent of the microorganism. Tang et al (2021) reported the decreased antioxidant activity (DPPH and FRAP) might be partly related to microbial growth. This reduction is related to chemical and enzymatic degradation of phenolic compounds (Hashemi et al, 2017) results also observed in our study.

Table 4: Determination of Antioxidant activity (DPPH) and Total Phenolic Compounds (TPC) initial contents and after fermentation of whole pulp, hydrophilic and lipophilic fractions.

| | LR | | BB | |
|---|---|---|---|---|
| **TPC** | **0** | **24** | **0** | **24** |
| **whole Pulp** | 15,98 $^{a}$±0.01 | 13,02 $^{b}$±0.06 | 14,01 $^{a}$±0.00 | 13,02 $^{b}$±0.07 |
| **Hydrophilic** | 11,26 $^{a}$±0.21 | 9,95 $^{b}$±0.02 | 10,59 $^{a}$±0.03 | 8,60 $^{b}$±0.17 |
| **Lipophilic** | 3,09 $^{a}$±0.04 | 2,78 $^{b}$±0,11 | 4,74 $^{a}$±0.02 | 4,40 $^{b}$±0.11 |
| **DPPH** | **0** | **24** | **0** | **24** |
| **whole pulp** | 47,43 $^{a}$±0.26 | 39,16 $^{b}$±0.18 | 47,43 $^{a}$±0.05 | 34,98 $^{b}$±0.07 |
| **Hydrophilic** | 34,78 $^{a}$±0.05 | 24,71 $^{b}$±0.05 | 34,50 $^{a}$±0.02 | 22,38 $^{b}$±0,05 |
| **lipophilic** | 28,68 $^{a}$±0.04 | 27,52 $^{b}$±0.25 | 32,65 $^{a}$±0.05 | 29,50 $^{b}$±0.11 |

*Different letters in the same sample and same line differ significantly ($p < 0,05$).

The hydrophilic fraction had a higher content of total phenolics and were also proportionally more biotransformed when compared to the lipophilic fraction. This result is consistent with the amount of phenolics detected and quantified by HPLC-MS/MS (Table 3). Regarding the different cultures used for fermentation, BB-12

showed greater reductions in antioxidant activity when compared to samples fermented by *L. reuteri.* These differences probably reflect different microbial enzymatic activities that modulate the composition of fruit juice during fermentation.

Fessard et al (2017) when studying different types of fermented tropical juices also observed a reduction in the content of total phenolics in mango pulp. Like juçara pulp, mango pulp is rich in hydroxybenzoic acids (gallic acid, vanillic acid, protocathecuic acid); in chlorogenic acid and in hydroxybenzoic glycoside derivatives, phenolics that had their concentration reduced during fermentation in the cited work.

### *3.4 $^{1}$H NMR Spectroscopy*

Figure 1 shows the $^{1}$H NMR spectra of juçara fractions fermented in basal medium. The different spectral regions are characterized by the resonance of specific compounds, highlighting the regions of aliphatic compounds (0.00 - 3.00 ppm), carbohydrates (3.00 - 6.00 ppm) and aromatic compounds (6.00 - 10.00 ppm) (Del Campo et al., 2016).

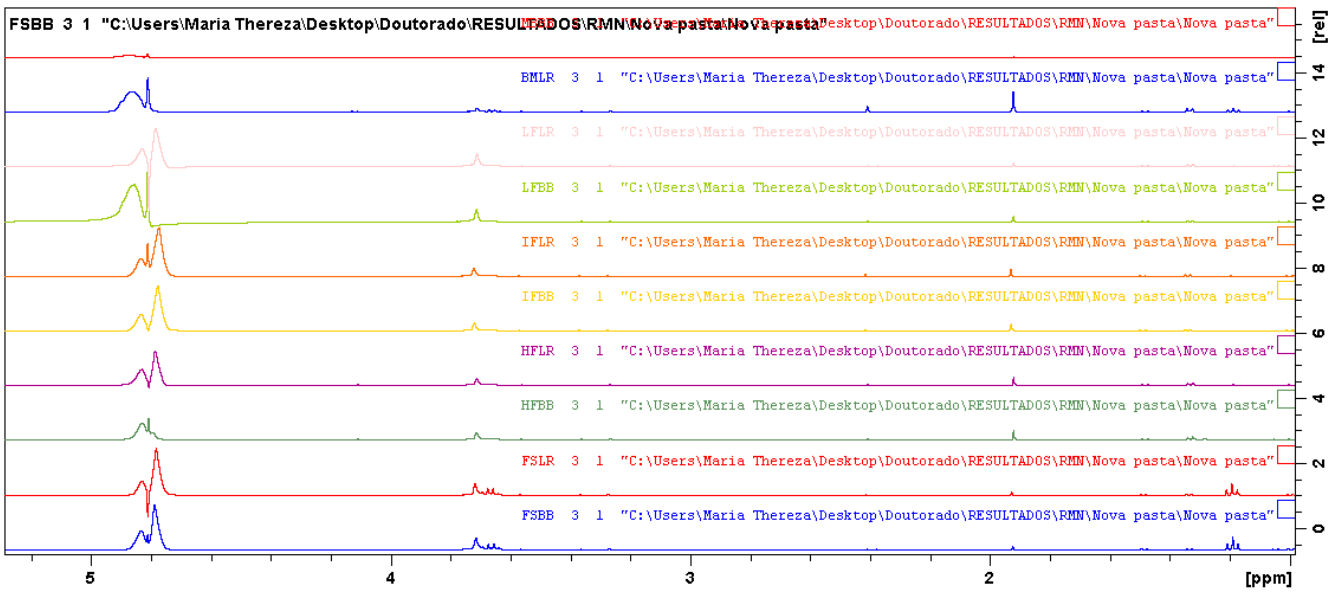


Figure 1: $^{1}$H NMR spectra of juçara fractions fermented in basal médium.

Most of the samples studied showed peaks in the region of 3 to 4.5 ppm. According to Cheng & Neiss (2012) the $^{1}$H NMR spectrum tends to have overlapping lines in the 3.0–4.5 ppm region and may also exhibit line broadening, especially for high-molecular-weight polysaccharides, and the coupling information may not always be available, although multidimensional methods can be very helpful here.

All the samples after fermentation showed a singlet in the region of 1.92 ppm, this peak was not present in controls without fermentation, indicating a product generated by the fermentation of juçara and whole juçara fractions, by *L. reuteri* and BB-12. (Lamichhane et al., 2016) This singlet is characteristic of acetate, providing evidence of ongoing saccharolytic activity by bacteria. The short chain fatty acid (SCFAs), such as acetate, butyrate and propionate are products of fermentation of complex carbohydrates such as dietary fiber, and the production primarily depends on the availability of substrates and of the microorganism (Lamichhane et al., 2016; Besten et al. 2013; Macfarlane and Macfarlane 2003).

Compared to other samples, the basal medium containing whole juçara and fermented by BB-12 (MBBB), it did not show peaks in the region of 3 to 4 ppm, a characteristic region of carbohydrates, mainly cellulose (3.5 to 5.5 ppm), indicating a possible better use by BB-12 of these nutrients present in whole juçara. The same sample stands out for presenting a doublet in 5.22 referring to a-Glucose. The presence of glucose is justified because cellulosic material is first converted into

glucose either by chemical or by enzymatic process during fermentation (Del Campo et al., 2016, Cerqueira et al., 2010).

For a better understanding of samples behavior Metabolomics studies have been presented using non-targeted mode. This way has been widely employed in fingerprinting of many medicinal and food plants. In contrast to targeted analyzes, untargeted metabolomics allows the uncovering of as many assortments of metabolites as possible without necessarily identifying or quantifying a particular compound (Chung et al. 2018). Multivariate statistical analyzes have also been designed to compare the chemical composition of individual samples, of which PCA is the most used tool, as shown in Figure 2

The first two CP explained 59% of the total variance contained in the data, and CP1 explained 32.1 % of this variance against 26.9 % of CP. Figure 3 shows the projection of the metabolites identified in the fractions fermented by *L. reuteri* through the $^{1}$H NMR overlaid on the samples of the fractions fermented by BB-12. With a high reproducibility, minimum of sample preparation, and fast experimental time, this methodology it can be a superior approach for detection of metabolic changes associated with probiotics (Fan and Lane, 2016).

By analyzing Figure 3, referring to the projection of the variables on the factorial plane of the PCA, it is possible to verify that the samples fermented by BB-12 were all in the upper quadrant, whereas the samples fermented by LR, with the exception of HFLR, were projected in the lower quadrant, indicating a difference in metabolites resulting from the fermentation of juçara fractions by different microorganisms. The analysis of the profile changes of the endogenous metabolites allows a “fingerprint" of a particular intervention focusing on qualitative evaluation and not so much in the quantification of a metabolite, important to present a fermentation profile of juçara fractions by the different strains used in our study

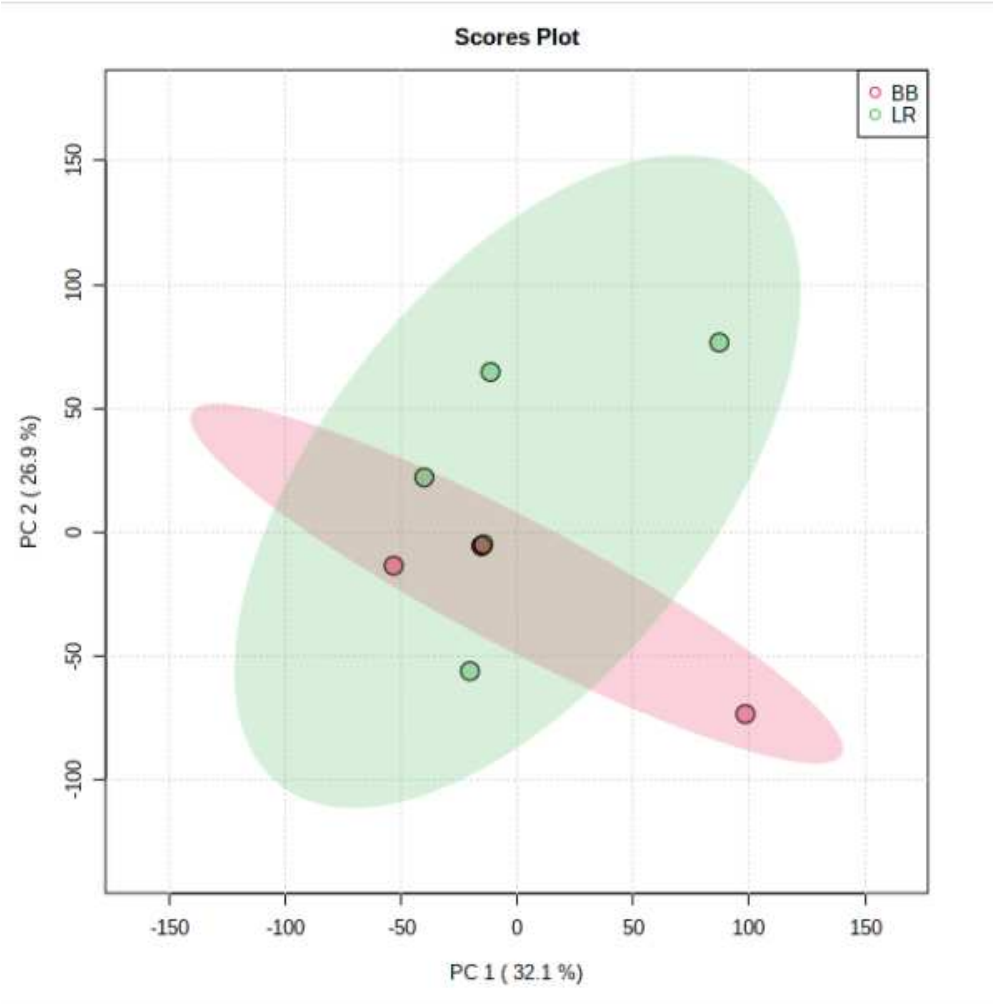


Figure 2: principal component analysis (PCA)

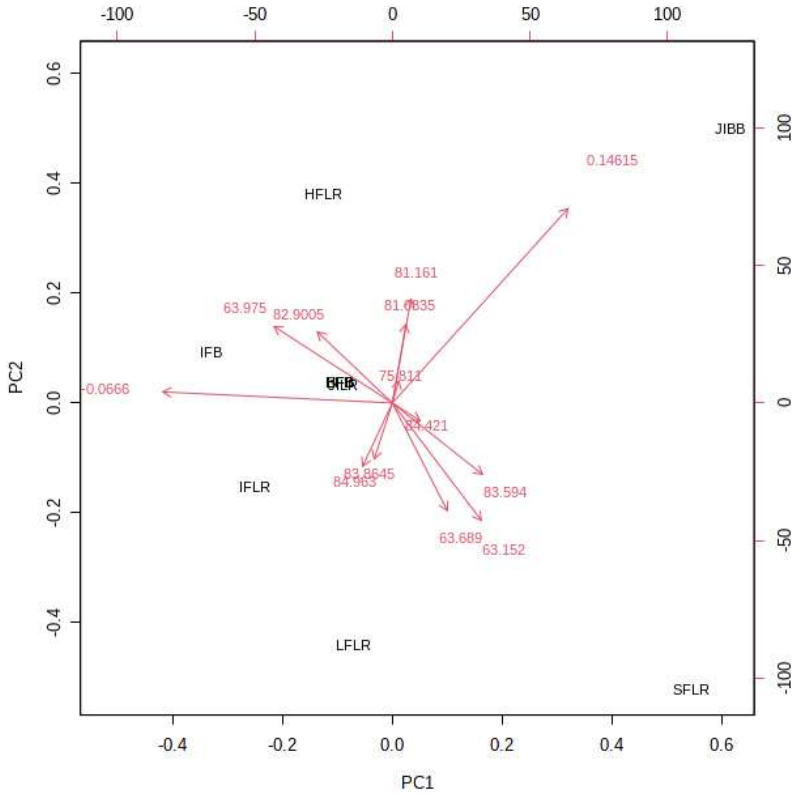


Figure 3: Projection of the metabolites identified in the fractions

FSLR: soluble fraction fermented by *L. reuteri*; FSBB: soluble fraction fermented by BB-12; HFLR: hydrophilic fraction fermented by *L. reuteri*; HFBB: hydrophilic fraction fermented by BB-12; IFLR: insoluble fraction fermented by *L. reuteri;* IFBB: insoluble fraction fermented by BB-12; LFLR lipophilic fraction fermented by *L. reuteri*; LFBB: lipophilic fraction fermented by BB-12; BMLR: Juçara pulp fermented by *L. reuteri*; BMBB: Juçara pulp fermented by BB-12

## Conclusion

It was possible to observe that in the hydrophilic and insoluble fractions *L. reuteri* obtained better growth rate. However, when fermenting whole fruit BB-12 obtained better results, reinforcing a possible and desirable bifidogenic effect in this fruit. The data obtained evidenced the use of carbohydrates, possibly even those present in polyphenol molecules, by microorganisms.

The results obtained in this study reinforced the excellent nutritional and functional value of the juçara fruit, which serves as support for future research in animal and human trials, in addition to presenting alternatives for increasing the production and consumption of juçara, developing of fermented products with high antioxidant activity using this fruit as a base.

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## CONSIDERAÇÕES FINAIS

Nos últimos anos estudos sobre fermentados à base de fruta estão ganhando destaques, porém estudos sobre a fermentação de polpa de juçara e as transformações que as bacterias causam em seus compostos bioativos são escassos. Nosso estudo apontou que a polpa de Juçara é um meio favorável ao crescimento de *L. reuteri* LR92 e *Bifidobacterium animalis* subsp. *lactis* BB-12, possibilita a viabilidade de 8,70 e 8,44 log UFC/mL respectivamente em 24 horas de fermentação.

A fermentação pelas bacterias estudadas provocou alterações nos compostos fenólicos, bem como nas atividades antioxidantes e na concentração de antocianinas, destacando-se o aumento da bioacessibilidade dos ácidos protocatecuico e 4-hidroxibenzóico quando comparado à polpa de juçara não fermentada.

Em relação ao teor de fenólicos totais, assim como nas atividades antioxidantes (ABTS, Frap, DPPH) a fermentação, por ambas as bacterias, provocou redução em seus valores, mas ainda apresentou teores significativos, desmonstrando que a polpa de juçara fermentada é uma excelente fonte desses compostos.

Alterações nos conteúdos de minerais totais também foram observadas após a fermentação da polpa de juçara pelas bacterias de interesse. A bioacessibilidade após digestão gastrointestinal *in vitro* nas amostras fermentadas foi maior para os minerais cobre e zinco. O presente estudo também constatou que a polpa de juçara antes e após a fermentação pode contribuir para a ingestão diária recomendada de minerais, principalmente manganês, cobre, zinco e ferro. Até onde sabemos, este é o primeiro estudo a avaliar os efeitos da digestão gastrointestinal *in vitro* sobre minerais da polpa de juçara fermentada.

Dentre os compostos presentes na polpa de juçara os disponivies nas frações hidrofilicas e insoluveis apresentaram melhores condições para o crescimento de *L. reuteri* No entanto, ao fermentar a polpa de fruta integral, BB-12 obteve melhores resultados, reforçando um possível e desejável efeito bifidogênico nesta fruta. Os dados obtidos evidenciaram o uso de carboidratos, possivelmente até mesmo aqueles presentes em moléculas de polifenóis. Na análise de componentes principais observamos que as amostras fermentadas por BB-12 estavam todas no quadrante superior, enquanto as fermentadas por LR, com exceção de HFLR, foram projetadas no quadrante inferior, indicando uma diferença de metabólitos resultantes da fermentação de frações de juçara por diferentes microrganismos. Entretanto ao

analisar os picos de RMN é possivel detectar a presença de acetato nas amostras fermentadas.

De modo geral os resultados obtidos neste estudo elucidaram as transformações que a fermentação pelas bactérias utilizadas provoca nos compostos bioativos assim como na bioacessibilidade dos mesmos. As análises de RMN possibiliram a visualização do perfil de fermentação e de como os compostos são utilizados pelas bacterias.

Reforça-se ainda o excelente valor nutricional e funcional do fruto da juçara, que serve de subsídio para futuras pesquisas em testes com animais e humanos, além de apresentar alternativas para o aumento da produção e consumo de juçara, desenvolvimento de produtos fermentados, com alta atividade antioxidante e boa fonte de minerais, utilizando esta fruta como base.