PROGRAMA DE PÓS-GRADUAÇÃO EM ALIMENTOS E NUTRIÇÃO
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
UNIVERSIDADE FEDERAL DO ESTADO DO RIO DE JANEIRO

DESENVOLVIMENTO DE BEBIDA NÃO LÁCTEA COM PROBIÓTICO

DEVELOPMENT OF NON-DAIRY BEVERAGE WITH PROBIOTICS

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Rio de Janeiro
2022

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Tese de Doutorado apresentada ao
Programa de Pós-Graduação em Alimentos e Nutrição,
da Universidade Federal do Estado do Rio de Janeiro
como requisito parcial para obtenção do título de
Doutora em Alimentos e Nutrição.

Orientador: Profº Dr. Otniel Freitas Silva

Co-orientadora: Dra. Janine Passos Lima

Rio de Janeiro

2022

A633 Alves Gomes, Izabela

DESENVOLVIMENTO DE BEBIDA NÃO LÁCTEA COM PROBIÓTICO / Izabela Alves Gomes. -- Rio de Janeiro, 2022.

154

Orientador: Otniel Freitas Silva.
Coorientadora: Janine Passos Lima.

Tese (Doutorado) - Universidade Federal doEstado do Rio de Janeiro, Programa de Pós-Graduação em Alimentos e Nutrição, 2022.

1. Suco de maçã. 2. Probióticos. 3. Lactobacillus acidophilus. 4. Bebida não láctea. 5. Sobrevivência gastrointestinal. I. Freitas Silva, Otniel, orient.

II. Passos Lima, Janine, coorient. III. Título.

Izabela Alves Gomes

DESENVOLVIMENTO DE BEBIDA NÃO LÁCTEA COM PROBIÓTICO

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Aprovada em: 24/02/2022

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30/03/2022 12:56:55 - Assinatura realizada por simone.ribas@unirio.br, IP 177.25.177.145
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Aos meus pais Iêda e Evandro, e à minha irmã
Isadora por me darem o suporte necessário,
caminhando sempre ao meu lado.

AGRADECIMENTOS

A Deus, por ter plantado este sonho em meu coração e por ter me dado condições para realizá-lo.

Aos meus pais, Evandro e Iêda, por terem me ensinado o caminho por onde eu deveria andar, por tantos sacrifícios feitos em prol da minha formação, por todas as orações que me sustentaram e me fizeram chegar até aqui.

A minha irmã Isadora por ser a minha maior fã, melhor amiga e maior incentivadora. Obrigada por todas as vezes em que você me disse que eu era capaz, mesmo quando eu nem acreditava que conseguiria, você estava lá, mesmo longe, você sempre se fez perto!

Aos meus orientadores, mais do que incríveis, Janine e Otniel, por terem acreditado em meu potencial. Obrigada por terem investido o tempo de vocês, me ensinando, me guiando, chamando a minha atenção sempre que necessário e me acompanhando de perto. Quando eu cheguei na Embrapa em 2013, eu sabia que seria um lugar que marcaria a minha vida, só não imaginava que seria tanto. Eu não teria conseguido metade disso se não fosse a dedicação de vocês. Vocês são mais do que professores e orientadores, são influenciadores, que mudaram a minha vida! Serei eternamente grata!

Ao professor Armando Venâncio e toda a equipe do LAMG que me recebeu tão bem na Universidade do Minho, em Braga, que me permitiu viver esse projeto com uma outra dimensão e um outro olhar, obrigada professor, por ter transmitido todo o seu conhecimento de forma tão humilde e acolhedora. Obrigada Andreia, Enrique, Eva, Ana Carolina, Jéssica, Fábio, Carol e Hugo, pelas risadas no laboratório, as dicas de passeios e o auxílio em vários momentos durante a execução deste projeto! Vocês são maravilhosos e espero ter a oportunidade de recebê-los no Rio, em breve!

A toda a Equipe da Embrapa Agroindústria de Alimentos, em especial, Flávia Gomes, Renata Tonon, Erika Fraga, Ana Paula Ribeiro, Lêda Gottschalk, Daniela de Grandi, Edna Oliveira, Simone Duarte, Chorão, Filé e Adriano que contribuíram e muito, para a execução deste projeto! Obrigada pela disponibilidade de tempo em me atender e tirar as minhas dúvidas, sempre que eu precisei! Obrigada, por serem exemplos de profissionais!

Aos alunos da graduação e pós-graduação da Embrapa, com quem dividi grandes momentos, risadas, momentos de tensão e que muito me ajudaram tanto na execução do trabalho de mestrado, como agora. Obrigada, Carol, Leílson, Aline, André, Nátali, Mariana Ribeiro, Priscila, Felipe, Cínthia, Gabi, Maraísa e tantos outros, nós nos encontraremos por aí.

Aos amigos que sempre entenderam a minha ausência e compartilharam risos, lágrimas, vitórias, medos e angústias da pós-graduação e da docência, em especial, Lana Rosa e Victor Hugo Cordeiro Rosa. Nossa viagem para Acapulco, agora precisa acontecer!

A família que fiz em Portugal, durante o período sanduíche, que hoje não são mais simples amigos, são irmãos de alma. Vocês tornaram essa jornada mais leve! Eu não poderia ter tido pessoas melhores ao meu lado para viver essa etapa da minha vida! Obrigada Mika, Débora, Fander, Jéssica, Luara e Fran!

As professoras que prontamente aceitaram o convite para participar da banca examinadora, tenho certeza que as contribuições servirão para enriquecer ainda mais este trabalho.

Aos professores e coordenadores do PPGAN, por sempre batalharem pelo sucesso do programa! Vocês são uma inspiração.

Aos meus alunos da graduação do curso de Nutrição. Lecionar é uma “via de mão dupla”, obrigada por terem me ensinado tanto. Contribuir para a formação de vocês é uma honra!

“Consagre ao Senhor tudo o que você faz, e
os seus planos serão bem-sucedidos.”
Provérbios 16:13

RESUMO

A maçã (*Malus domestica*) é o fruto da macieira, uma planta perene, planta decídua e dicotiledônea da família *Rosaceae Rose*; isso é uma das frutas mais populares e abundantes em todo o mundo. O suco de maçã tem recebido atenção, por ser uma matriz alimentícia promissora para carrear probióticos, devido ao seu conteúdo de nutrientes essenciais, juntamente com seu apelo a um nicho de consumidores que se preocupam com hábitos mais saudáveis. Sendo assim, este trabalho teve como objetivo principal, desenvolver uma bebida com a adição de probióticos, a partir de suco de maçã integral. A bebida de maçã foi desenvolvida a partir de suco de maçã integral e adição de *Lactobacillus acidophilus* encapsulado. Foram avaliados a sobrevivência da cepa durante armazenamento em temperaturas de refrigeração e temperatura ambiente, assim como a sobrevivência da passagem da cepa pelo trato gastrointestinal e aceitabilidade sensorial da bebida. Com base nos resultados encontrados em nosso estudo, o suco de maçã com adição de probióticos apresentou níveis de *L. acidophilus*, com células viáveis, acima do valor estabelecido pela legislação, mesmo após 180 dias de armazenamento e, que a microencapsulação foi capaz de proteger as cepas durante o armazenamento da bebida e na passagem pelo trato gastrointestinal, com liberação do probiótico somente no intestino. Em relação à aceitabilidade quanto à aparência, fragrância, sabor, textura e impressão geral dos sucos, as notas mais frequentes recebidas pelo suco de maçã com adição de probióticos ficaram entre 9 e 8 em uma escala hedônica de 9 pontos, indicando que os consumidores gostaram dos produtos de " moderadamente" a "muito". Os sucos de frutas, como a maçã, são considerados matrizes candidatas à incorporação de probióticos, pois são ideais para consumidores que se interessam por produtos não lácteos, no entanto, mais estudos são necessários para garantir a manutenção da viabilidade probiótica em matrizes alimentícias não lácteas, assim como para que seja avaliado o potencial das bactérias em adsorver a patulina presente em suco de maçã

Palavras-chave: Suco de maçã; bebida não fermentada; probiótico encapsulado

ABSTRACT

Apple (*Malus domestica*) is the fruit of the apple tree, a perennial plant, deciduous and dicotyledonous plant of the *Rosaceae Rose* family; this is one of the most popular and plentiful fruits all over the world. Apple juice has received attention as a promising food matrix to carry probiotics, due to its content of essential nutrients, together with its appeal to a niche of consumers who are concerned with healthier habits. Therefore, this work had as main objective, to develop a drink with the addition of probiotics, from whole apple juice. The apple drink was developed from whole apple juice and the addition of encapsulated *Lactobacillus acidophilus*. The survival of the strain during storage at refrigeration and room temperature, as well as the survival of the strain passing through the gastrointestinal tract and sensory acceptability of the beverage were evaluated. Based on the results found in our study, apple juice with the addition of probiotics showed levels of *L. acidophilus*, with viable cells, above the value established by legislation, even after 180 days of storage, and that microencapsulation was able to protect strains during beverage storage and passage through the gastrointestinal tract, with probiotic release only in the intestine. Regarding the acceptability regarding the appearance, fragrance, flavor, texture and general impression of the juices, the most frequent grades received by apple juice with the addition of probiotics were between 9 and 8 on a 9-point hedonic scale, indicating that consumers liked it. of products from "moderately" to "very". Fruit juices, such as apple, are considered candidate matrices for the incorporation of probiotics, as they are ideal for consumers who are interested in non-dairy products, however, more studies are needed to ensure the maintenance of probiotic viability in non-dairy food matrices, as well as to evaluate the potential of bacteria to adsorb patulin present in apple juice.

Keywords: Apple juice; non-fermented beverage; encapsulated probiotic

SUMARY

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INTRODUÇÃO

Frutas e vegetais são parte essencial de uma dieta diversificada e nutritiva. Uma dieta composta por mais de 400 g de frutas e vegetais por dia tem efeitos preventivos contra doenças crônicas, como doenças cardíacas, câncer, diabetes e obesidade. Dentre os vários tipos de frutas que são consumidas, a maçã é rica em fitoquímicos e consumida mundialmente (BABOLI, WILLIAMS, CHEN, 2020).

A maçã é o pseudofruto pomáceo da macieira (*Malus domestica Bork*.) pertencente à família *Rosaceae* e subfamília das *Pomoidae*. É uma planta que tem seu centro de origem proveniente do Cazaquistão e da Ásia Central e possui grande abundância de espécies selvagens com distintas formas, cores e sabores (SILVA, 2021). Tanto a fruta, quanto os seus subprodutos, apresentam uma grande fonte de nutrientes devido aos altos níveis de substâncias bioativas em sua composição. Nos últimos anos o consumo de maçãs e suco de maçã aumentou, pois esta fruta e seus componentes possuem diversos benefícios a saúde em humanos (DIAS *et al*., 2019).

A maçã é a segunda fruta mais consumida no mundo e seu suco é considerado um dos mais populares em países da Europa e nos Estados Unidos. Seu consumo tem sido associado a efeitos benéficos à saúde, principalmente pela disseminação do dito popular "*An apple a day keeps the doctor away*" (GARCIA, 2019). É classificada como a terceira fruta mais produzida em todo o mundo, depois da banana e da melancia, com uma produção que atingiu 75 milhões de toneladas em 2018–2019 (DACCACHE *et al*., 2020). Em 2016, a China liderava a produção mundial de maçãs, sendo responsável por aproximadamente 56% do total produzido. Os Estados Unidos apareciam em segundo lugar com 6%, seguidos pela Polônia com 5% e o Peru com 4%. O Brasil ocupava a décima terceira posição com 1,3%, equivalente a uma produção de 1,04 milhões de toneladas no ano (ABPM, 2019).

O cultivo da maçã no Brasil é uma atividade relativamente recente. Com incentivos fiscais e apoio à pesquisa, sul do Brasil aumentou a produção de maçã em quantidade e qualidade, tornando o país autossuficiente e potencial exportador (DIAS *et al*., 2019; SILVA, 2021). O cultivo comercial da maçã no Sul do Brasil começou na década de 1960, no estado de Santa Catarina, com a implantação de pomares por imigrantes europeus e seus descendentes. O cultivo é favorecido nestas regiões devido as melhores condições climáticas, em virtude da sua exigência de temperaturas abaixo de 7,2°C e 9,7°C, favorecendo assim o seu desenvolvimento vegetativo e reprodutivo (ZANDONÁ, 2017).

Atualmente, o estado de Santa Catarina é o maior produtor de maçã, com cerca de 54% da produção total do Brasil, durante a safra 2014/2015, representando quase 612.000 toneladas (DIAS *et al*., 2019). Em 2020/2021, a safra de maçã no Brasil alcançou a marca de 1,276 milhão de toneladas, de acordo com Associação Brasileira que reúne os produtores da fruta (ABPM).

No Brasil, a safra de maçã tem início no final de dezembro, em regiões com temperaturas mais altas, com a colheita dos cultivares de baixo requerimento de frio; e, se mantém, até o início de maio, em regiões de baixas temperaturas, com a colheita dos cultivares de requerimento de frio, sendo as frutas armazenadas em temperaturas baixas para manter a conservação por mais tempo (SILVA, 2021).

As cultivares que predominam são Gala e Fuji, as quais produzem frutos com algumas características apreciadas pelos consumidores do mercado interno para o consumo *in natura*, como por exemplo, a epiderme avermelhada e a baixa acidez de polpa. Além disso, as tecnologias atualmente disponíveis permitem que as maçãs Gala sejam colhidas em fevereiro e estocadas em câmara fria até dezembro, e as variedades Fuji sejam colhidas em abril e estocadas até o mês de fevereiro do ano seguinte, tendo o abastecimento garantido ao longo de todo ano (GARCIA, 2019).

Por esses fatores, as cultivares Gala e Fuji junto com suas diversas mutações concentram quase toda a produção brasileira. Juntas representam aproximadamente 90% da produção, enquanto os 10% restantes são distribuídos entre as cultivares *Golden Delicious, Brasil, Anna, Condessa, Catarina e Granny Smith* (GARCIA, 2019).

Cerca de 20 a 25% da safra brasileira de maçã é destinada à produção de sucos e, do volume total produzido, a participação do mercado interno vem aumentando, em paralelo à tendência de maior consumo de alimentos saudáveis. Esta foi uma das maiores razões de interesse pelo suco da fruta, uma vez que com a mudança na legislação, que exigiu maior presença de produtos naturais do que artificiais nas bebidas, o líquido extraído da maçã ganhou espaço por ser mais neutro e acessível. Este aumento interno no consumo de suco representa um crescimento de 5% para 15% do produto disponível para população (BASSO, 2019).

O suco de maçã é o produto extraído da fruta por moagem ou prensagem (pressão), passando por um processamento de clarificação, adição de antioxidante, desaeração, pasteurização e envase, sem adição de açúcar, adoçante ou conservantes. O produto final se apresenta como um líquido límpido, claro e brilhante (SILVA, 2021).

O suco de maçã tem recebido atenção, por ser uma matriz alimentícia promissora para carrear os probióticos, devido ao seu conteúdo de nutrientes essenciais, juntamente com seu apelo a um nicho de consumidores que já se preocupam com hábitos mais saudáveis. O suco de maçã é pouco consumido no Brasil em comparação à Europa e aos Estados Unidos, onde é considerado um dos sucos mais populares (SILVA, 2021). Recentemente, bebidas feitas de frutas, vegetais e cereais estão sendo pesquisadas, por pertencerem ao grupo das bebidas que mais crescem no mercado (HAFFNER, PASC, 2018).

A maçã pode ser uma via importante de exposição a contaminantes e devido a isso hoje em dia a segurança dos alimentos é uma grande preocupação, pois causa um significativo impacto econômico. A contaminação de alimentos por micotoxinas também pode comprometer a segurança dos alimentos e suprimentos de ração e afetam adversamente a saúde humana e animal. As micotoxinas são metabólitos secundários produzidos sob condições ambientais por certos fungos e podem ser compostos altamente tóxicos (DIAS *et al*., 2019). Uma ampla gama de espécies de fungos do gênero *Penicillium*, *Aspergillus* e *Byssochlamys* podem produzir a micotoxina patulina, uma das micotoxinas mais importante devido à sua alta toxicidade combinada às grandes dificuldades durante sua determinação (DIAS *et al*., 2019).

A fonte mais comum de patulina é o *Penicillium* expansum e este fungo pode ser encontrado em diferentes tipos de frutas, mas é especialmente encontrado em maçãs, peras, pêssegos e seus produtos processados, como sucos e purê, devido ao ácido ambiental necessário para a estabilidade da patulina (DIAS *et al*., 2019). A contaminação pode acontecer por motivos como diferenças geográficas, condições climáticas, práticas agrícolas, cultivares e ambiente pós-colheita, os quais podem afetar a composição da maçã (SILVA, 2021).

Sendo assim, é preciso tomar cuidado com a presença de agentes contaminantes nos frutos e produtos de maçã, visto que a presença da micotoxina patulina tem sido frequentemente relatada (GARCIA, 2019).

Devido à sua toxicidade e alta frequência de contaminação, a Organização Mundial da Saúde OMS, alguns países europeus e a China estabeleceram uma concentração máxima recomendada de 50µg/kg de patulina no suco de maçã. A Comissão Europeia também estabeleceu níveis máximos permitidos, incluindo 50 µg/kg para sucos de maçã, 25 µg/kg para produtos sólidos de maçã e 10 µg/kg para alimentos frutados para bebês (LAI *et al*., 2022). No Brasil, os limites máximos constituídos pela legislação visam proteger os consumidores das prováveis decorrências tóxicas que agentes contaminantes como as micotoxinas e resíduos de fungicidas podem gerar à saúde. A Agência Nacional de Vigilância Sanitária (ANVISA) determina o LMT para micotoxinas em alimentos através da Resolução da Diretoria Colegiada (RDC) Nº 7 de 18 de fevereiro de 2011; sendo o nível máximo de contaminação estabelecido para PAT em suco e polpa de maçã é 50 µg/L (SILVA, 2021).

Recentemente, tem ocorrido um crescente interesse pela produção de alimentos probióticos não-lácteos, o que tem impulsionado a realização de pesquisas sobre a incorporação de probióticos em matrizes alimentares de origem vegetal, visto serem produtos com boa aceitação no mercado. Como os fatores que fizeram com que houvesse este aumento de interesse, podemos citar a crescente quantidade de pessoas com intolerância à lactose, ou alergia à proteína do leite de vaca, ou até por aversão a quantidade de colesterol que os produtos lácteos apresentam. Outros fatores como culturais e/ou econômicas também podem influenciar negativamente o consumo de produtos lácteos fermentados (RODRIGUES, 2021).

Bebidas feitas de frutas, vegetais e cereais têm sido investigadas como transportadores de probióticos promissores devido ao seu conteúdo de nutrientes essenciais, juntamente com o seu apelo a um nicho de consumidores que já se preocupam com hábitos mais saudáveis (HAFFNER, PASC, 2018).

Os probióticos são microrganismos que, quando ingeridos em quantidades adequadas, conferem benefícios ao hospedeiro. Esses microrganismos têm sido associados a vários efeitos benéficos, principalmente relacionados a problemas gastrointestinais e o sistema imunológico, bem como diabetes, obesidade, hipercolesterolemia, câncer, entre outros (PIMENTEL *et al*., 2021).

O mercado global de probióticos atraiu a atenção da indústria de alimentos para produzir novos produtos contendo probióticos, além de pesquisadores para estudar características dos probióticos e seus efeitos na saúde humana (PIMENTEL *et al*., 2021).

Durante décadas, o mercado de probióticos se concentrou em laticínios (iogurte e outros produtos fermentados), porém o aumento do veganismo e intolerância à lactose e / ou indivíduos hipercolesterolêmicos exigiram mudanças neste cenário (PIMENTEL *et al*., 2021). Assim, matrizes alimentares contendo como probióticos estão inseridas no mercado de alimentos funcionais. Além disso, a relação de extratos de frutas com benefícios para certas condições de saúde reforça seu interesse positivo nestes produtos (HAFFNER, PASC, 2018).

Alguns pesquisadores, já vem estudando a sobrevivência da bactéria *L. acidophilus* na produção de suco de maçã com probióticos, e, como a adição dessa cepa afeta as características do suco (ALALEH *et al*., 2019)

Assim esta tese foi dividida em 4 capítulos, conforme Figura 1.

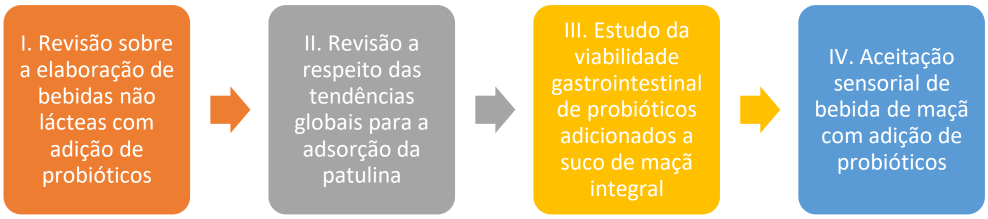

Figura 1. Divisão da tese

Assim, os objetivos deste trabalho foram desenvolver uma bebida com a adição de probióticos a partir de suco de maçã integral; avaliar a viabilidade destes probióticos durante o armazenamento e passagem pelo trato gastrointestinal e, avaliar a aceitabilidade sensorial da bebida produzida.

INTRODUCTION

Fruits and vegetables are an essential part of a diverse and nutritious diet. A diet of more than 400g of fruits and vegetables a day has preventive effects against chronic diseases such as heart disease, cancer, diabetes and obesity. Among the various types of fruits that are consumed, apple is rich in phytochemicals and consumed worldwide (BABOLI, WILLIAMS, CHEN, 2020).

The apple is the pomaceous pseudofruit from the apple tree (*Malus domestica Bork*.) and it belongs to the *Rosacea* family and the *Pomoid* subfamily. It comes from Kazakhstan and Central Asia, and has a great abundance of wild species with all kinds of different shapes, colors, and flavors (SILVA, 2021). Both the fruit and its by-products are great sources of nutrients due to the high levels of bioactive substances in its composition. In the recent years, the consumption of apples and apple juice has increased, as this fruit and its components have health benefits in humans (DIAS *et al*., 2019).

Apple is the second most consumed fruit in the world and its juice is considered to be one of the most popular in European countries and the United States. Its consumption has been associated with beneficial effects on health, mainly due to the popular saying “An apple a day keeps the doctor away” (GARCIA, 2019). It is ranked as the third most produced fruit in the whole world, after bananas and watermelon, with a production that has reached 75 million tons in 2018–2019 (DACCACHE et al., 2020). In 2016, China led the world production of apples, accounting for approximately 56% of the total produced. The United States appeared in second place with 6%, followed by Poland with 5% and Peru with 4%. Brazil occupied the thirteenth position with 1.3%, equivalent to a production of 1.04 million tons in the year (ABPM, 2019).

Apple cultivation in Brazil is a relatively recent activity. With tax incentives and support for research, southern Brazil have increased the production of apples in quantity and quality, making the country self-sufficient and a potential exporter (DIAS *et al*., 2019; SILVA, 2021).

The state of Santa Catarina is the largest apple producer, with about 54% of the total production in Brazil, during the 2014/2015 harvest, representing almost 612,000 tons (DIAS *et al*., 2019). In 2020/2021, the apple harvest in Brazil reached the mark of 1.276 million tons, according to the Brazilian Association that brings together fruit producers (ABPM).

In Brazil, the apple harvest starts in the end of December, in regions with higher temperatures, with the harvest of cultivars with low cold requirements. The harvest is maintained until the beginning of May, in regions of low temperatures, with the harvest of cultivars that require cold weather, with the fruits being stored at low temperatures to maintain conservation for a longer period of time (SILVA, 2021).

The Gala and Fuji Cultivars produce fruits with some characteristics appreciated by consumers in the domestic market for fresh consumption, such as reddish epidermis and low pulp acidity. In addition, currently available technologies allow Gala apples to be harvested in February and stored in a cold chamber until December. For the Fuji varieties, they are harvested in April and stored until February of the following year, with guaranteed supply throughout every year (GARCIA, 2019).

Due to these factors, the Gala and Fuji cultivars, together with their various mutations, concentrate almost the entire Brazilian production. Together they represent approximately 90% of production, while the remaining 10% are distributed among the cultivars *Golden Delicious, Brazil, Anna, Condessa, Catarina* and *Granny Smith* (GARCIA, 2019).

Around 20 to 25% of the Brazilian apple crop is destined for the production of juices and, of the total produced volume, the participation of the domestic market has been increasing in parallel with the trend towards greater consumption of healthier foods. This was one of the main reasons for interest in the fruit juice, since with the change in legislation, which required a greater presence of natural products than artificial ones in beverages, the liquid extracted from the apple has gained space for being more neutral and accessible. This internal increase in juice consumption represents an increase from 5% to 15% of the product available to the population (BASSO, 2019).

Apple juice is the product extracted from the fruit by grinding or pressing (pressure), undergoing clarification processing, addition of antioxidant, deaeration, pasteurization and packaging, without any addition of sugar, sweetener or preservatives. The final product is a clear, bright and shiny liquid (SILVA, 2021).

Apple juice has received attention as a promising food matrix for carrying probiotics due to its essential nutrient content, along with its appeal to a niche of consumers who are already concerned about healthier habits. Apple juice is little consumed in Brazil compared to Europe and the United States, where it is considered one of the most popular juices (SILVA, 2021). Recently, beverages made from fruits, vegetables and cereals are being researched, as they belong to the group of the fastest growing beverages in the market (HAFFNER, PASC, 2018).

The apple can be an important route of exposure to contaminants and because of this, food safety is a major concern nowadays, as it causes a significant economic impact. Mycotoxins, which are secondary metabolites produced under environmental conditions by certain fungi and can be highly toxic compounds (DIAS *et al*., 2019), can also compromise the safety of food and feed supplies and adversely affect human and animal health. A wide range of fungal species of the genus *Penicillium, Aspergillus* and *Byssochlamys* can produce the mycotoxin patulin, one of the most important mycotoxins due to its high toxicity combined with great difficulties during its determination (DIAS *et al*., 2019).

The most common source of patulin is *Penicillium expansum* and this fungus can be found in different types of fruits, but it is especially found in apples, pears, peaches and their processed products, such as juices and purees, due to the patulin environmental acid needs for stability (DIAS *et al*., 2019). Contamination can happen for some reasons, such as geographic differences, climatic conditions, agricultural practices, cultivars and post-harvest environment, which can affect the composition of the apple (SILVA, 2021).

Therefore, it is necessary to be careful with the presence of contaminants in apple fruits and products, as the presence of the mycotoxin patuline has been frequently reported (GARCIA, 2019).

Due to its toxicity and high frequency of contamination, the World Health Organization WHO, some European countries and China have established a maximum recommended concentration of 50μg/kg of patulin in apple juice. The European Commission has also set maximum allowable levels, including 50 μg/kg for apple juice, 25 μg/kg for solid apple products and 10 μg/kg for fruity baby foods (LAI *et al*., 2022). In Brazil, the maximum limits established by the legislation aim to protect consumers from the probable toxic consequences that contaminating agents such as mycotoxins and fungicide residues can generate to health. The Agência Nacional de Vigilância Sanitária (ANVISA) determines the LMT for mycotoxins in food through Resolution of the Collegiate Board of Directors (RDC) no. 7 of February 18, 2011; the maximum level of contamination established for PAT in apple juice and pulp is 50 μg/L (SILVA, 2021).

Recently, there has been a growing interest in the production of non-dairy probiotic foods, which has led to research on the incorporation of probiotics in food matrices of plant origin, as they are products with good market acceptance. As the factors that caused this increase in interest, we can mention the growing number of people with lactose intolerance, or allergy to cow's milk protein, or even an aversion to the amount of cholesterol that dairy products present. Other factors such as cultural and/or economic can also negatively influence the consumption of fermented dairy products (RODRIGUES, 2021).

Beverages made from fruits, vegetables and cereals have been investigated as promising probiotic carriers due to their content of essential nutrients, along with their appeal to a niche of consumers who already care about healthier habits (HAFFNER, PASC, 2018).

Probiotics are microorganisms that, when ingested in adequate amounts, provide benefits to the host. These microorganisms have been associated with several beneficial effects, mainly related to gastrointestinal problems and the immune system, as well as diabetes, obesity, hypercholesterolemia, cancer, among others (PIMENTEL *et al*., 2021).

The global market for probiotics has attracted the food industry attention to produce new products containing probiotics, in addition to researchers to study characteristics of probiotics and their effects on human health (PIMENTEL *et al*., 2021).

For decades, the probiotics market has focused on dairy products (yoghurt and other fermented products), but the increase in veganism and lactose intolerance and/or hypercholesterolemic individuals have required changes in this scenario (PIMENTEL *et al*., 2021). Thus, food matrices containing probiotics are inserted in the functional food market. Furthermore, the relationship of fruit extracts with benefits for certain health conditions reinforces their positive interest in these products (HAFFNER, PASC, 2018).

Some researchers have already been studying the survival of the bacterium *L. acidophilus* in the production of apple juice with probiotics, and how the addition of this strain affects the characteristics of the juice (ALALEH et al., 2019)

Thus, this thesis was divided into 4 chapters, as shown in the following figure:

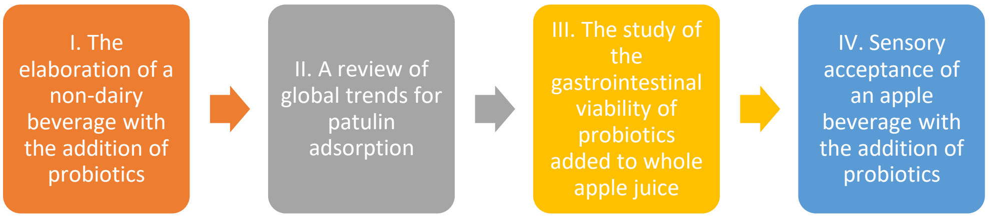

Figure 1. Division of the thesis

Thus, the objectives of this work were to develop a beverage with the addition of probiotics from whole apple juice; to evaluate the viability of these probiotics during storage and passage through the gastrointestinal tract, and to evaluate the sensory acceptability of the beverage produced.

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CHAPTER 1

The use of non-dairy fruit beverages to improve gut microbiota: a new approach for probiotics

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This chapter was published on Advances on Biological Chemistry
(DOI: http://dx.doi.org/10.4236/abc.2021.116021)

Abstract

The growing interest of consumers in using foods that improve health has motivated researchers and the food industry to develop new functional products such as foods with probiotics. Probiotic cultures, for example, from lactic acid bacteria and bifidobacteria have been highlighted for their ability to promote balance in the intestinal microbiota as well as other benefits such as anticarcinogenic and antimutagenic effects, reduced plasma cholesterol levels, decreased symptoms of lactose intolerance, and stimulation of the immune response. Traditionally, probiotics are incorporated into dairy products. However, because of the growing number of individuals affected by lactose into-lerance and/or vegans, other food matrices have been studied as potential carriers for these microorganisms. Considering all the facts mentioned above, cereals, legumes, fruits, and vegetables could be potential substrates, where probiotic bacteria can be used for the development of non-dairy beverages. This review aimed to highlight the research carried out on 1) probiotic microorganisms, including the more recent reclassification according to their phylogenetic position, 2) probiotic beverages from non-dairy sources which emerged as an alternative for lactose-intolerant consumers and, 3) the aspects of improving the gut microbiota.

Keywords

Functional Foods, Fruit Juice, *Lactobacillus*, Non-Dairy Beverages, Gut Microbiota

1. Introduction

The term "functional food" seems generic, but the history of the term can be highlighted in the late 1960s, for example, with researches that showed that polyunsaturated fatty acids could control the level of cholesterol in the blood [1].

Initially, the concept of functional foods was to seek food with the ability to treat diseases [2]. The concept of functional food emerged in Japan in 1984, with the disclosure of information from the beneficial effects of foods enriched with special components, such as probiotics by Japanese scientists [3]. From 1984 until now, functional food has changed its meaning due to different cultural origins [2]. In 1991, the Japanese Ministry of Health, Welfare, and Labor established “Food for Specified Health Uses” (FOSHU) as a regulatory system for functional foods. FOSHU was the result of a program financed by the Japanese authorities which aim at the reduction of the financial resources spent on public health, containing the progress of chronic diseases. After the introduction of the FOSHU regulation, the number of functional food products increased, especially between 1997 and 2007 [4].

In 2014, at the 17th International Conference on Functional Foods in Health and Diseases, functional foods were granted a new definition as natural or processed foods, containing known or unknown bioactive components in the non-toxic efficacy of performing clinically proven or documented health benefits [3].

In 2015 a new regulatory system for functional foods was established based on the system of the Food Supplement Health and Education Act, already established in the USA. With the introduction of this system, many new functional foods were developed due to the more flexible health claims when compared to FOSHU. This fact provided a growing increase in functional foods, which in 2018 reached an appreciation of around 1.8 billion dollars and this market is still undergoing an exponential and impactful expansion [4].

The European Commission has adopted a definition stating that “a food can be considered “functional” if it is satisfactorily determined to benefit one or more target functions in the body, in addition to having adequate nutritional effects to improve health, health, and well-being and/or reduced risk of disease” [5].

In Brazil, the current legislation on functional foods, approved by the National Health Surveillance Agency (ANVISA) in 1999, does not define the term “functional foods”, but rather a functional property claim that is “related to the metabolic or physiological role that the nutrient or non-nutrient has in the growth, development, maintenance and other normal functions of the human organism”. As long as ANVISA’s General Management of Food and its safety of use evaluate it and its efficacy is proven, the food that bears the claim may be made available on the consumer market [6]. Brazilian legislation prevents the attribution of medicinal and therapeutic effects to foods; therefore, claims cannot be associated with prevention, treatment, or cure of diseases. Specific claims are desirable as they communicate the claimed benefit more clearly to the consumer. This type of allegation should also not be too general, at the risk of not being able to obtain evidence capable of proving the effect and properly communicating about the claimed benefit [6].

The fastest-growing sector of functional food worldwide contains probiotics. Probiotic foods consist in represent 60% to 70% of the functional food market [7]. In 2015, it was predicted that the food market containing probiotics would increase from $35 billion up to $48 billion in 2020 [8].

Currently, the demand for functional foods containing bacteria with probiotic properties is growing rapidly due to increased public awareness of the benefits of probiotics for health, maintaining the balance of the intestinal microbiota, and improving mucosal defenses against pathogens [9].

This study aimed to summarize the current state of non-dairy beverages with the addition of probiotics, as well as to demonstrate the potential of the application of probiotics in juices from fruits.

2. Probiotics

The intestinal microbiota is a complex ecosystem, which is composed of microorganisms associated with various nutritional, metabolic, endocrine processes, immunological and psychological mechanisms. This complex of microorganisms maintains and regulates some endogenous functions such as nutrient metabolism, immunomodulation, synthesis of bioactive compounds and vitamins, and the fermentation of non-digestible carbohydrates, in short, they serve as a more efficient intestinal selective barrier. Thus, due to the importance of the intestinal microbiome in maintaining health, the search for new probiotics products with single, multi-strain or multi-species strains, associated or not with prebiotics, are sure bets on the market [10].

Romans and Greeks in the past used fermented dairy foods to ensure and maintain health [11]. The definition of probiotics (Greek; Pro: promotion, biotic: life) as living microorganisms, which when administered in adequate quantities, can offer a benefit to the health of the host, was established by the United Nations Food and Agriculture Organization (FAO) and by World Health Organization (WHO) in 2001 [12].

In Brazil, the National Health Surveillance Agency (ANVISA) defines probiotics as live microorganisms which are capable of improving the intestinal microbial balance, producing beneficial effects on the health of the individual when administered in adequate doses [13] [14].

Probiotics are defined by The World Gastroenterology Organization as live microorganisms that, when administered in quantity, confer health benefits on the host. The species of *Lactobacillus* and Bifidobacterium are the most used as probiotics, but the yeast *Saccharomyces boulardii* and some species of *E*. *coli* and *Bacillus* are also used. The new agents also include *Clostridium butyricum*, recently approved as a novel food in the European Union. Government regulations differ between countries, however, the status of probiotics as a component in foods is not currently established on an international basis. For most countries, probiotics come in dietary and dietary supplements because most come in the form of foods [15].

Probiotics also called living biotherapeutic products (LBP), are products that contain living organisms, such as bacteria, found naturally in humans. Government regulation of probiotics in the United States is complex. Depending on the intended use of a probiotic product, the Food and Drug Administration (FDA) may regulate it as a dietary supplement, food ingredient, or drug [16].

The mechanisms of action of probiotics are not always well understood, which are one of the problems considered by the European Food Safety Authority (EFSA), which in 2014 rejected the health claims of marketed probiotics due to lack of sufficient evidence [17].

Historically, the concept of probiotics was developed around 1908 by the Nobel Prize winner Elie Metchnikoff, who discovered that the consumption of live bacteria (*Lactobacillus bulgaricus*) in yogurt or fermented milk could improve some biological characteristics of the gastrointestinal tract [7] [12]. In his study, Dr. Metchnikoff concluded that a bacterium helps control the effects caused by enteric pathogens and toxemia, which play an important role in aging and mortality. This research resulted in an increase in the production and consumption of yogurt all over the world [11].

In 1965, Lilly and Stillwell used the term probiotic for the first time. Over the next decade, the term was used by Fujii and Cook in 1973 and denoted chemicals in mice that protected against *Staphylococcus aureus* infection. In 1974, the term was used by Parker in a broader sense to refer to interactions of microorganisms with the animal or human host, that is, “organisms and substances which contribute to balance microbial proliferation”. Finally, in 2013, the consultation of experts from international scientists at the meeting of the International Scientific Association of Probiotics and Prebiotics provided minor grammatical corrections and reformulated the previous definition as “living microorganisms that, when administered in adequate quantities, confer a benefit to the health of the host”, which is now widely accepted and used. Several studies on probiotics have been published since this discovery [11].

The daily recommendation for ready-to-eat probiotics must contain at least a minimum amount of viable cells in the range of 10^8 to 10^9 colony forming units (CFU). Smaller values are acceptable, as long as its effectiveness is proven [18]. The viability of probiotics should be ensured during processing and storage, aiming to keep their counts at high levels (10^6 - 10^7 CFU/mL or g of food) until consumption [19].

The World Gastroenterology Organization considers that a required dose of probiotics varies greatly depending on the strain and the product. Although many products provide between 1 - 10 billion CFU/dose, some products are effective at lower levels, while others are at higher amounts. It is not possible to establish a general dose for probiotics; since the dosage has to be based on human studies that show a health benefit [15].

In Brazil, the ANVISA legislation on foods with alleged functional and/or health properties, new foods/ingredients, bioactive substances, and probiotics has such probiotic microorganisms approved for use in food: *Lactobacillus acidophilus*; *Lacticaseibacillus casei shirota*; *Lacticaseibacillus casei* variety rhamnosus; *Lacticaseibacillus casei* variety defensis; *Lacticaseibacillus paracasei*; *Lactococcus lactis*; *Bifidobacterium bifidum*; *Bifidobacterium animallis* (including the subspecies *B. lactis*); *Bifidobacterium longum*; *Enterococcus faecium* [20].

All probiotic species are considered safe for the general population by the Eu-ropean Food Safety Authority (EFSA). The U.S. Food and Drug Administration (FDA) classifies probiotics individually but also classifies them as safe for food use [10]. For a single strain to reach probiotic status, it is necessary to assess its resistance to the digestion process and its ability to promote health benefits [10]. Probiotics have been used in food with the main objective of strengthening the natural intestinal microbiota. Its effectiveness in improving health status depends mainly on its ability to provide viable functional bacteria, overcoming the harsh effects of the intestinal tract [6]. Raising their health benefits, probiotic bacteria with activity have been increasingly added to a range of products, including yogurts, cheeses, ice cream, powdered milk, and frozen desserts [6].

Viable microorganisms such as *lactobacilli* and *bifidobacteria* that benefit the host by improving intestinal bacterial balance are the most used by the industry, as they have several typical characteristics such as metabolic stability, adherence to intestinal cell walls, without promoting antibiotic resistance and not pathogenic, safe for the consumption and effective. Besides that, these bacteria must be active in the product, survive throughout the upper digestive tract, resist gastric juice, oxygen, and enzymes, and can co-aggregate as a part of the natural intestinal microbiota and have beneficial effects after adhering to the host's intestine [21] [22].

The world market for probiotics including their use as an ingredient, supplements, and their incorporation in food products is increasingly expanding [11] and according to Transparency Market Research was estimated to reach U$12,753 million by 2026.

Preclinical and clinical studies on the benefits of probiotics to gastrointestinal health has shown effects on 1) prevention of acute diarrhea associated or not with antibiotics, 2) symptomatic relief in irritable bowel syndrome, 3) treatment of hepatic encephalopathy, and 4) prevention of necrotizing enterocolitis in premature babies [23]. Probiotics act as antagonists to pathogens such as *Enterococcus faecalis*, *Salmonella enterica* subsp. enteric serotype *Enteritidis*, *Listeria monocytogenes*, *E. coli* and *Staphylococcus aureus* by immunological, hormonal and neuronal manipulation [24]. Specific probiotics also have gained a place in the treatment of ulcerative colitis and are useful to combat overweight, obesity and are recommended as options in the main clinical guidelines [25].

To avoid miscommunication about all living species, biological knowledge on taxonomy and nomenclature should be considered when choosing probiotics. [26]. In the strictest sense, taxonomy deals with the theory and classification practice, including principles, rules, and methods. When new species or higher taxa are discovered or in case of revised or reclassified taxon, taxonomists publish a formal description of each change to establish names and circumscribe the corresponding taxonomic concepts or to amend existing ones to reflect their discoveries [27].

When DNA-DNA hybridization method, used since 1960 for bacterial taxonomy, suffers from reproducibility problems and cannot provide an accurate measurement of the actual sequence identity between genomes [28], new strategies need to be found. For exemple the genus *Lactobacillus* was proposed by Beijerinck in 1901 and includes Gram-positive microorganisms, fermentative, optionally anaerobic, and non-spore-forming, which can also be mobile, catalase-negative when cultivated without heme on the medium, usually oxygen tolerant, aciduric or acidophilic, mandatorily saccharolytic with at least 50% of the final carbohydrate product being lactate and other fermentation products consisting of acetate, ethanol, CO_2 and succinate. Several types of fermentation can be recognized, such as metabolisms that are mandatory homofermentative, optionally heterofermentative, and mandatorily heterofermentative, based on the types of fermented sugars (hexoses and pentoses) and fermentation products [29]. The genus is classified in the phylum *Firmicutes*, class *Bacilli*, order *Lactobacilalles*, family *Lactobacillaceae*, which contains the genera *Lactobacillus*, *Paralactobacillus*, and *Pediococcus* [30]. The *Lactobacillaceae* family contains the genera *Lactobacillus* and *Pediococcus*, which are phylogenetically mixed. The ancient genus *Paralactobacillus* was recently included in the genus *Lactobacillus*, although this inclusion is questionable. More than 150 species are recognized in the genus *Lactobacillus* and are heterogeneous in several properties [29].

In the past two decades, sequencing of entire bacterial genomes has become widely available and the mean values of the nucleotide identity (INA) of the genes shared between different bacterial genomes has been introduced as the “gold standard” for the design of new bacterial species [30]. Due to the relatively small size of the bacterial genome and the greater availability of high-throughput sequencing DNA technology, phenotypic testing has now been replaced by genome sequencing as the main source of taxonomic information [26].

Since 1983, the similarity between the 16S rRNA genes has been used in bacterial taxonomy to provide phylogenetic schemes as a backbone for classification and nomenclature. Limitations to the 16S rRNA gene approach; for example, many recently divergent species that have undergone intense evolutionary pressures may have highly similar 16S rRNA gene sequences that can, however, ignore a wide phylogenetic gap between such taxa [28]. Based on parameters as the Average Aminoacid Identity (AAI) and the Percentage of Conserved Proteins (PCPO), this genus has a wide range that far exceeds the normal spread of a genus [26]. From the available genome sequences, 16 groups were discriminated within the *Lactobacillus* genus. Forteen (14) stable phylogenetic groups were described within the genus, based only on 16S rRNA sequence similarity. According to the taxonomic subcommittee on *Bifidobacterium*, *Lactobacillus* and related organisms this taxonomic aspect was discussed and they decided for a formal division of the genus, creating a working group to collect all available genotypic and phenotypic information that would allow defining a new, reliable and stable structure for the genus *Lactobacillus* [26].

In the First International Taxonomy Congress, held in 1930 in Paris, France, the first "Bacterial Code" was developed. This code updates qualitatively the bacterial nomenclature, reducing the duplicate names and including a better description of the different species and corresponding Type strains [26]. The Procaryotes International Nomenclature Code defines rules for the nomination of bacterial taxa based on their taxonomic classification. Although it covers many exceptions and particular cases, its principles are relatively simple; but, as the Code stands, it is not easy to find reasons for name changes of the genus *Lactobacillus* [26]. In the *Lactobacillus* genus the species were included based on several common phenotypic characteristics. For a reclassification of the genus, the demonstrated genetic distinctions within the genus should be supported by a discriminative set of parameters which, in addition to genome sequence, will allow a reliable and consistent description of a new genus. So, it is necessary to collect available information on all known *Lactobacillus* species, exploring different bioinformatic tools, and eventually arrive to a possible reclassification for the genus [26].

By March 2020, 261 species of *Lactobacillus* had already been described. The genus *Lactobacillus* is very heterogeneous and in the last decades, more than 250 species have been attributed to this genus [30].

The previous taxonomy of lactobacilli was based on phenotypic factors and characteristics such as the ideal temperature of growth, use of sugar, and range of metabolites produced [30]. Later in the 20th century, genotypic and chemo-taxonomic criteria (e.g., chemical structure of peptidoglycans) were used to design new bacterial species [30].

A new perspective for taxonomy, the Total Nucleotide Identity (TNI) was found between the expected values between order and family and the INA values between Order and Class for the 237 species of *Lactobacillus* (208, excluding synonyms and subspecies). In the same study, Pot *et al.* [26] showed that representatives of the *Pediococcus* genus, and members of *Leuconostocaceae* family are mixed with species within the *Lactobacillus* genus. But the genus continues to grow, and new isolates are continuously being added, which the latter authors find scientifically unacceptable. rRNA gene sequence analysis introduced a tool for a more exhaustive and robust taxonomy for the genus with the introduction of 16S, but ended by revealing fewer correlations between traditional classification based on phenotype and the new phylogenetic one. The continuous description of new species of *Lactobacillus*, led to the recognition of an increasing number of variable phylogenetic subgroups [26].

Zheng *et al.* [30] states that it is recognized according to the level of genetic diversity found for the *Lactobacillus* genus exceeds what is commonly found for other bacterial genera and even for bacterial families. The availability of complete genomes of *Lactobacillus* strains, representing the main families of the *Lactobacillales* order, has allowed a more definitive analysis of their evolutionary relationships [29].

Recommendation 30b of the Bacteriological Code of Nomenclature (1990 Review), as modified at the 1999 meeting of the International Committee for Systematic Bacteriology (ICSB) and its Judicial Commission, calls for the definition of minimum standards to describe new bacterial taxa [29].

With the description of the new genus, the researchers suggested keeping the initial “L” for the new genus names to minimize confusion. This way, considered commercially important species such as *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, and *Limosilactobacillus reuteri*, which will no longer be lactobacilli and will be included in a new genus, will be abbreviated as *L. casei*, *L. plantarum*, and *L. reuteri*. Other commercially important species of *Lactobacillus*, including species and strains with fermentation capacity and others with proven probiotic activity, will be mainly found in the newly defined genera. In this way, the number of species of the genus *Bifidobacterium*, *Lactobacillus*, and related genera has increased considerably over the past 10 - 15 years [29].

Thus, some guidelines have been defined to recommend the labeling of probiotics using a current nomenclature for genera and species, although there is a greater concern from a practical and financial standpoint, there is a clear demand for stability in the new classification system [26].

2.1 Probiotic Products Availability on the Market

After *in vitro* and pre-clinical research, or after large-scale clinical trials, a substantial number of microbial species have been revealed to exhibit potential probiotic properties, however, only the most documented and robust strains can reach the market [31]. Most of the current probiotics are lactic acid bacteria (LAB), which belong to the genus *Lactobacillus* and *Bifidobacterium*, with a smaller number of *leuconostocs*, *pediococci*, *lactococci*, *enterococci*, and *streptococci*. A variety of species of LAB are listed as generally recognized as safe (GRAS) and comprise the probiotic species that are the most used in supplements or food matrices. The most commonly used probiotics are species of *Bifidobacterium* (*B. animalis*, *B. bifidum*, *B. breve*, *B. infantil*, *B. longum*, *B. lactis*), and species of *Lactobacillus* (*L. acidophillus*), *Lacticaseibacillus* (*L. casei*, *L. rhamnosus*), *Lactiplantibacillus* (*L. plantarum*), *Ligilactobacillus* (*L. salivarius*) and *Li-mosilactobacillus* (*L. fermentum*, *L. reuteri*). Other species as *Streptococcus* and *Bacillus* or the yeast *Saccharomyces cerevisiae* are also used as probiotics, and incorporated in non-dairy foods. There has recently been a reclassification of *Lacticaseibacillus*, *Lactiplantibacillus*, *Ligilactobacillus*, and the genus *Limosi- lactobacillus*, which previously comprised the genus *Lactobacillus*. Although the use of some *Bacillus* or *Clostridium spp*. may seem to be controversial from a security perspective, the technological advantage for using spores compared to the most vulnerable plant cells explains the increased interest in research and commercial development for these species [31] [32].

A beneficial effect on the host is obtained if a minimal amount of viable probiotic cells reaches the intestine. The suggested minimum amount in food at the time of consumption is around 10^8 viable cells per mL or g of food, so that the observed survival during exposure in the gastrointestinal tract (GIT) cannot compromise the functionality of probiotics. Despite the differences between the daily amounts recommended by American or European agencies for making health claims, it was proposed that the daily intake should be from 6 to 9 log CFU probiotic g^{-1} or mL^{-1} for its effectiveness. However, to claim specific health effects, the required dose may be lower and it is specific to each strain [32].

Dairy industries and LAB preparation companies in Europe, Japan, and the United States have developed their own internationally renowned strain brands, as well as product brands. And, to assess the probiotic functions, these strains have been submitted to many clinical trials. *Bifidobacterium lactis* BB-12 strain, developed by Chr. Hansen (Denmark), is reported as the most studied *Bifidobacterium* strain in the world. Over the past 80 years, the strain *Lacticaseibacillus casei* Shirota from Yakult Company has undergone a large number of scientific studies and clinical trials. In late May 2015, its survival, efficacy, and safety in the gut were scientifically verified in China, Japan, Thailand, UK, and else- where [33].

Large differences are observed between strains from the same species, as they may have different phenotypes and properties that can lead to different clinical effects [31].

Many studies have found out that the addition of probiotic cultures in foods has not influenced the sensory acceptance of the products [32].

2.2 Dysbiosis

Dysbiosis, an imbalance in the intestinal microbiota composition after the use of antibiotics, has largely boosted the therapeutic application of probiotics [24]. A consideration of the intestinal microbiota is necessary to understand its relevance to human health and the concept of probiotic food. Each individual has in its gastrointestinal tract a unique signature with more than 1000 microbial species. The bacterial cells comprehend half the wet weight of the colon material and its number reaches 10 times over the number of cells in the tissue that forms the human body.

Normally, the stomach contains 10^3 different bacterial species, while the total microbial population of colon bacteria is around 10^{11} and 10^{12} CFU/g. The bacterial colonization of the intestine begins at birth when newborns are exposed for the first time to a non-sterile environment. Thereafter, it evolves and transforms throughout life, depending on a complex and dynamic interaction between the host diet, genome, and lifestyle, as well as the use of antibiotics. The composition of the intestinal microbiota is generally considered to be essentially stable throughout adulthood [25].

The vast majority of clinical trials of probiotics reported in the literature have not given rise to major concerns. However, some examples of serious adverse effects of probiotics have been documented regardless of formulation, dosage, and daily consumption [12].

Lactobacillus and *Bifidobacterium* are common genera of the endogenous mammalian gastrointestinal tract. They induce host immunomodulation and reduce symptoms of a wide range of gastrointestinal disorders, and therefore have been widely used as probiotics. The cell membrane integrity, the intracellular pH, and the functional enzymes of probiotic cells can be constantly attacked by stress factors such as bile acids, digestive enzymes and gastric acidity during their passage through the gastrointestinal tract. As a consequence, probiotic cells can be viable (active and cultivable), inactive (inactive, but cultivable), active (but not cultivable), or dead (inactive and non-cultivable). Cells that experience a high level of stress may still have some metabolic activity, but they may no longer be cultivable [10].

Even inactive probiotic cultures or their fragments are also being investigated by researchers and have been shown to have effects such as an improvement in adverse behaviors (mental health) related to sleep disorders, stress, regulation of intestinal function, and positive effects on the administration of immunity and allergy. These nonviable cells also present advantages for the food industry, making products safer and more stable. These types of applications deserve special attention for patients in critical states for whom the risks of consuming active cultures are quite high [10].

2.3 Mechanism of Action and Probiotic Activity

Probiotics create a favorable environment for the intestine, through their mechanisms of action, which will depend on a variety of factors, such as the type of strain used, the host, and the food. The probiotics can improve the epithelial barrier promoting mucus secretion. Furthermore, some probiotic strains release active peptides, also known as bacteriocins, against bacteria, fungi, and viruses, which may stabilize the intestinal barrier. Other antimicrobial substances are also produced by probiotics, such as lactic and acetic acid, that present an inhibitory effect on Gram-negative bacteria [6].

In this way, the probiotics act in several ways, interfering with the adhesion of pathogens to the intestinal mucosa [6]. As there are different strains and product formulations, there is not a single answer. An intriguing aspect of probiotic strains is the ability of some to confer distant effects at the site of administration. This can occur through the transfer of organisms, for example, from the intestine to the mammary glands of breastfeeding women [34].

The mechanisms by which probiotics exert their effects, in general, are largely unknown, but they may involve changing the intestinal pH, antagonizing pathogens through the production of antimicrobial compounds, competition for binding sites and pathogen receptors, as well as nutrients and factors of available growth, stimulation of immunomodulatory cells and lactase production [23]. As described in Figure 1.1, there may be four different mechanisms in which probiotics can defend the body against pathogens.

Bacteria with probiotic properties are now widely available in the form of foods, such as dairy products and juices, as well as capsules, drops, and powders [12] [25].

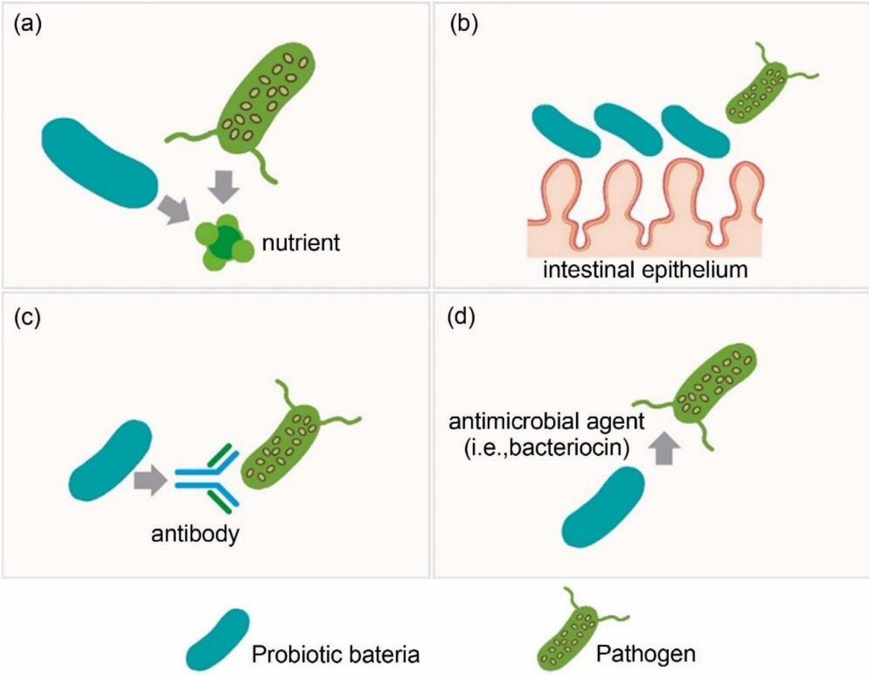

Figure 1.1 Probiotics functions against the pathogen in the intestine by: (a) competing against pathogens for the same essential nutrients, thus making it less available for the pathogen to use, competition for nutrients; (b) attachment to the adhesion sites and, therefore, preventing attachment of the pathogen by reducing the surface area available for colonization of the pathogen, Blocking of adhesion sites; (c) sends a signal to the immune cells that result in the secretion of cytokines, which target the pathogen for destruction, Immune stimulation; (d) attacking pathogenic organisms by releasing antimicrobial agents, such as bacteriocins, that kill them directly (adapted from Fazilah *et al.*, 2018), direct antagonism.

Traditionally, a wide range of fermented foods such as yogurt, kefir, kimchi, sauer-kraut, tempeh, miso, and kombucha are part of the regular diet in different cultures and ethnicities, ranging from eastern to western, serving as a conventional source of probiotic strains [24].

Studies have shown that the specific food matrix can affect probiotics, by allowing their multiplication and protecting the product's shelf life, as well as pro-viding protection in the passage through the gastrointestinal tract. Foods with high pH or high buffer capacity can reduce stomach acidity in humans, promoting the survival of probiotic microorganisms. In addition, liquid foods are digested more quickly than solid foods, consequently reducing the exposure of probiotics to agents of stress, such as stomach acid [35]. Probiotics demonstrate the beneficial effects as shown in Table 1.1.

Table 1.1 Probiotic functions

Strain	Action	Reference
Lactobacillus	Significant decrease in gastrointestinal symptoms, including nausea, postprandial fullness, and gastrointestinal pain	36
Lactobacillus	Decreased inflammation related to *H. Pylori*	36
Lactobacillus	Relief of symptoms of lactose intolerance, cholesterol reduction	37
Sacharomyces boulardii	Anti-inflammatory activity	38
Saccharomyces boulardii	Recommended for the treatment of acute gastrointestinal diseases, such as rotaviral and bacterial diarrhea and chronic conditions, such as inflammatory bowel disease	39

Lactobacillus acidophilus	Changes in the profile of inflammatory cytokine production and regulation of proinflammatory pathways, inhibition of the adhesion of pathogenic bacteria, modification of the microbiota by acidification of the colon due to fermenting agents, improvement in the function of the epithelial barrier, and protection against physiological stress	40
Bifidobacterium longum	Associated with carbohydrate metabolism	41
Bifidobacterium longum	Protection against autoimmune diseases such as inflammatory bowel disease, metabolic syndromes, irritable bowel syndrome colitis and brain disorders	41
Lacticaseibacillus rhamnosus	Beneficial for vaginal and postpartum female health	42
Lactobacillus acidophilus	Decrease in toxins produced by the kidneys	43

The higher saturated fat and cholesterol content in dairy based products compared to plant-based ones is also an inhibiting factor among some health-conscious consumers. The aforementioned concerns have opened a path for probiotic non-dairy products. The flavor and refreshing nature are the main advantages of non-dairy probiotics, as they include fermented products from cereals, soy, meat, fruits, and vegetables, enriched with various nutrients, vitamins, and antioxidants [44].

Dairy beverages have been the most popular source of probiotics. However, due to the market demand for plant-based beverages (e.g., vegetarianism and veganism) and to consumer awareness of adverse reactions to dairy products (intolerance and malabsorption) functional non-dairy beverages became an alternative carrier for probiotics [43]. It is weell knowm the association for the intake of dairy products with lactose and milk protein intolerance. Besides that, the higher saturated fat and cholesterol content in dairy based products compared to plant-based ones is also an inhibiting factor among some health-consciousconsumers. The aforementioned concerns have opened a path for probiotic non-dairy products. The flavor and refreshing nature are the main advantages ofnon-dairy probiotics, as they include fermented products from cereals, soy, meat, fruits, and vegetables, enriched with various nutrients, vitamins, and anti-oxidants [44].

2.3 Clinical Effect of the Consumption of Probiotics

The World Health Organization stated that up to 38 million people died from chronic diseases worldwide in 2012, of which more than 40% died of prematuredeath, and this value was much higher than in 2000 (14.6 million). Inadequate dietary fiber intake is another major cause of chronic diseases, which can lead tothe loss of some intestinal microorganisms, which can later result in various chronic diseases [33]. The growing number of studies shows the association between intestinal microbiota and chronic diseases that helped to develop the hypothesis that modulation of the intestinal microbiota may be a factor that links the environment with the genetics and diseases of the host [45] [46].

The intestine is one of the organs most important organs in the human body. It contains more than 70% of the body’s mucosal immunity. In addition, the intestine is closely related to various parts of the body through complex immune mechanisms. Therefore, considerable attention must be paid to intestinal health to achieve a healthy lifestyle [33].

More than 300 scientific publications describe the benefits of using probiotics, of which more than 130 are related to clinical studies in humans [33]. One study demonstrated that tomato and bean juices fermented with *Lactiplantibacillus plan tarum* LP DSM20205 (formerly known as *Lactobacillus plantarum* LP DSM20205) could have an important effect on the integrity and adherence of the barrier, being the most pronounced effect for fermented tomato juice. Probiotic cultures isolated from plant products could also have *in vitro* effects. For instance, lacticacid bacteria (LAB) isolated from fermented cocoa juice and their metabolites have demonstrated antagonistic activity against *Helicobacter pylori*, which is associated with gastric ulcers. Also, *Pediococcus pentosaceus* SC28 and *Levilactobacillus brevis* KU15151 (formerly known as *Lactobacillus brevis* KU15151) from traditional Korean food (jeotgal octopus and kimchi radish) showed adherence rates of 4.45% and 6.30%, respectively, to HT-29 cells, which is a humancolon adenocarcinoma cell [32].

Vegetable products with the addition of vegan probiotics could have hypocholesterolemia *in vitro* and anticarcinogenic effects. The addition of *Lactiplantibacillus plantarum*-1 (formerly known as *Lactobacillus plantarum*-1) and *Lacticaseibacillus rhamnosus* GG (formerly known as *Lactobacillus rhamnosus* GG) on the blueberry bagasse presented potential benefits on the cholesterol reduction this fact is to the hydrophobic bonding, increasing the excretion of cholesterol [32] [47].

Immunomodulatory and control of diabetes properties have also been associated with plant products added with probiotics. Lychee juice fermented with *Lacticaseibacillus* casei FL (formerly *Lactobacillus casei* (FL) was used to investigate the effects on immunity and intestinal microbiota in mice. Also, consumption of soy milk containing *Lactiplantibacillus plantarum* A7 (formerly *Lactobacillus plantarum* A7) resulted in antioxidant properties and decreased the risk of incompatible base pairs in DNA among patients with type IIdiabetes [32].

3. Non-Dairy Products with the Addition of Probiotics

Probiotic cultures are usually added to dairy products; besides that, consumers are used to the presence of microorganisms in this type of product [15]. However, lactose intolerance, veganism, high cholesterol content, and allergy to milk proteins are limiting factors in the growth of dairy products with probiotics. In total, 75% of the world's population suffers from lactose intolerance [48]. According to previous studies, higher milk fat content has shown inhibitory effects for probiotic cultures, particularly *B. bifidum* in yogurt [49].

The use of probiotics in non-dairy products has increased, probably, due to the growing number of adherents to veganism, thus opening room for demand for products free of ingredients from animals [50]. Therefore, other food matrices are being evaluated as carriers of bioculture, aiming to provide other options to the market, especially to consumers who do not appreciate or cannot consume milk products, including people who are lactose intolerant, allergic to proteins milk or strict vegetarian (vegan) [13].

Probiotic beverages can be made from various raw materials, such as vegetables, corn, legumes, and fruits [51] [52].

Juices (from fruits) can represent an alternative means for adding probiotic cultures because they are considered healthy products and are regularly consumed. In addition, fruit juices are rich in sugars, minerals, and vitamins, which are used as a substrate by probiotics and in combination with a rapid passage through acidic stomach conditions result in the high viability of probiotic cells [13, 37, 52].

Unlike dairy products, fruits and vegetables do not have allergens, lactose, and cholesterol, which adversely affect certain population groups [37]. They are healthy, refreshing, have a good taste, and may be suitable for probiotics. Because they are considered perishable products, fruits require immediate processing to reduce post-harvest losses and the development of probiotic products can be an approach to increase the product's availability and market value. Fruit-based probiotic products are made from pineapple, blackberry, apple, strawberry, lemon, mango, grape, cashew, oranges, carrot, beet, etc., as shown in Table 1.2 [48].

Table 1.2. Probiotic fruit beverages

Strain	Fruit	Shelf life	Reference
Saccharomyces cerevisiae	Cherry	21 days	43
Bifidobacterium animalis subsp. lactis and Lactobacillus acidophilus	The mix of banana, strawberry, and juçara beverage	90 days	52
Lactiplantibacillus plantarum	Apple	3 days	53
Lactiplantibacillus plantarum *Bifidobacterium breve and Streptococcus thermophilus*	The mix of orange, carrot, apple	21 days	54
Lactiplantibacillus plantarum, Lactobacillus delbrueckii	Cabbage juice	-	55
Lactobacillus spp., *Leuconostoc mesenteroides, Bifidobacterium longum*	Carrot and orange juice	-	56
Lactiplantibacillus plantarum, Lacticaseibacillus casei, Lacticaseibacillus paracasei, Lacticaseibacillus rhamnosus	Cherry	12 days	57
Lactiplantibacillus plantarum	Cornelian cherry	28 days	58
Lacticaseibacillus casei	Pineapple	42 days	59

Lacticaseibacillus casei	Apple	42 days	60
Lacticaseibacillus casei	Lychee	28 days	30
Lactiplantibacillus plantarume Lactobacillus. delbrueckii	Pomegranate	28 days	61
Lactiplantibacillus plantarum e Lactobacillus acidophilus	Orange	3 days	62
Lactobacillus e Bifidobacterium	Orange, pineapple, and cranberry	84 days	63

A variety of types of probiotic fruits and vegetables have been developed and marketed including fruit and vegetable juices, dried fruits, fermented vegetables, and desserts for vegetarians. However, studies that show the feasibility of incorporating probiotic bacteria in fruits and vegetables, and found that their feasibility and stability in these foods are highly dependent on several factors [37]. Fruits, such as apples, guava, bananas, and melons, are potential carriers of probiotic bacteria and strong adhesion of these bacteria to fruit tissue [64].

Considering that fruit and vegetable beverages are an excellent source of vitamins, antioxidants, minerals, and bioactive compounds and represent a good alternative to dairy matrices and a good choice. Different fruits and vegetable juices in the fermentation process can increase the nutritional and functional properties, with beneficial effects on health, in addition to increasing the shelf life of beverages [19].

Researches with non-dairy symbiotic beverages fermented beverages, including different types or mixed vegetables or fruits, with different concentrations of inulin, pomegranate juices, and cherry beverages using wheat bran, apple juice with oligofructose, orange juice with oligofructose, orange juice and hibiscus tea mixed with oligofructose and berry (strawberry, blackberry, and papaya) supplemented with three separate prebiotics: FOS, inulin, and galactooligossaccharides has been a constant search [13] [19]. In some cases, LABs can biotransform polyphenols into phenolic compounds with better bioavailability and bioactivity during the fermentation time [65].

However, adding probiotics to fruit juices is more complex when compared to adding to the dairy matrix. The main challenges faced this, are due to some intrinsic properties of these products, such as low pH and high concentration of organic acids, associated with other important factors like storage time and conditions for maintaining the viability of probiotics. In addition, fruit juices are considered highly perishable products and contain a large amount of water, which leads to a higher cost of transportation and production [53]. The disadvantage of non-dairy beverages is some unpleasant flavors caused by probiotics are almost perceived by the consumer [49].

Patents involve an admirable transfer of knowledge, both in terms of dispersing information about the deposits, and through the diversified use of scientific and technological knowledge necessary to produce the patented technology [66]. Patent-based statistics assume the innovative performance of a country, company, or institution, as well as other aspects that involve the innovation process. There was an effective start of patent filings from the year 2000, this fact may be related to the growth due to the increasing demand for functional foods since they are important for nutrition that helps in improving health and quality of life. Dairy beverages grew by 2.5% between 2008 and 2011 [66].

Currently in Brazil, the panorama regarding the protection of new products characterized as probiotics, focusing on non-dairy food matrices indicates that the products already patented on this topic fall on the production of non-alcoholic beverages fermented with probiotics, mainly of the genus *Lactobacillus* and/or *Bifidobacterium*, with fruits as a food matrix, and a patent on a smoothie, using the same genera of microorganisms and food matrix as the others [67]. It is necessary to encourage mainly in Brazil, the development of technological innovations aimed at patenting of the methods, the preparation of probiotic products, given that these are influencing the quality of life and preventing diseases that affect the world population [68].

Spray drying is the most used technique in the production of juice powders. In addition, it is widely used for microencapsulation of bioactive components, including probiotics, providing protection against adverse environmental conditions and improving processing and stability during storage [52]. In addition to being a quick-drying process, this technique has other advantages, such as relatively low cost, simplicity of use, and continuous operation capability. Although in some cases high temperatures used on processing and the low moisture content can lead to the decreased survival of the probiotic cells, to overcome these limitations polysaccharides especially those with prebiotic properties, such as inulin and oligofructose, can be used for the microencapsulation of probiotics increasing the viability of probiotic cultures [52] [66].

During the processing and storage of the products, oligofructose is the available substrate for the metabolism of these microorganisms and, thus, could increase the stability of probiotics in fruit juices during storage. In addition, oligofructose can be used as a sugar substitute, as it has a sweet taste similar to sucrose [13].

3.1 The Market of Vegetable Beverages with Probiotics

Combining probiotics with fruits and vegetables can be interesting as it provides the probiotics and dietary fiber the body needs, indicating an important direction of development for the probiotic industry in the future [48]. The combination of “probiotics + fruits and vegetables” takes many forms; of these, the direct addition of probiotics to existing traditional fruits and vegetables is the simplest way and the best approach is to ferment raw fruits and vegetables using probiotics strains [33].

The probiotic industry is expanding rapidly and new probiotic products are constantly been developed. The global probiotics market is estimated to be worth $15 billion a year and is growing at an estimated 7% annual rate [45].

This increase has led to a large number of new products including probiotics on supermarket shelves as well as in drugstores [31].

In recent years, there was an increase in vegetarianism and veganism and with that, the consumer demand for products with high nutritional value has increased too. People are increasingly avoiding products derived from animals (*i.e.*, vegetarians and/or vegans) and this has become a growing trend in modern lifestyles. In addition to that, many consumers started to demand plant-based milk alternatives for sustainability, health, dietary, and lifestyle issues or broader social or political reasons, resulting in an abundance of fruits, seeds, nut products or beans. Also, the global market for alternatives to non-dairy beverages has become a multi-billion-dollar business and will account for approximately U$26 billion in 2023 [32, 34].

In this scenario, the development of new products that are nutritionally balanced and/or add value stands out for their practical use as probiotics should be emphasized because of its proven effectiveness and wholesomeness, and the adaptability of probiotic cultures in different food matrices. Teas, fruit juices, or fermented beverages are matrices composed of bioactive compounds, such as vitamins, minerals, and polyphenols, representing interesting matrices for the addition of probiotics. However, there is always a need to assess the survival of the probiotic culture and its impact on the quality characteristics of the product [32].

It is necessary to exhaustively select excellent strains for the fermentation of different fruits and vegetables, as LAB derived from plants such as *L. plantarum* and *L. acidophilus* have gradually come to be used for the fermentation of fruit and vegetable juices. There is also a lack of high-density cultivation technology for fruit and vegetable fermentation varieties suitable for industrial production that needs to be incremented [34]. The industry still faces several important scientific and technological issues. More strains with excellent fermentation performance are needed to develop and the effects of prebiotics, probiotics, and fermented fruit and vegetables on human health, in addition to their mechanisms of action, should be better understood. Research and industries in the field of fruits and vegetables fermented with probiotics will have greater development opportunities if these problems can be effectively addressed [34].

3.2 Study for the Viability of Probiotics

In the development of functional foods with probiotic microorganisms, the formulation, processing, and storage should favor the survival of the microorganisms. Both technologies and the food matrix must aim to protect the microorganism's cells against external stress factors. In addition, once the food is consumed, the effect of digestion through the gastrointestinal system must be taken into account [69]. Foods that contain probiotic microorganisms with beneficial properties represent the largest segment of functional food on the market. For benefits to be obtained, foods containing probiotics must be consumed regularly and the food matrix must contain a minimum amount of viable probiotic microorganisms. The use of fruits and vegetables as vehicles for probiotic microorganisms represents a challenge. However, several factors can influence the viability of the probiotic microorganism. These factors can be inherent to the food matrix, such as fat and protein content, sugar composition, pH, and presence of antimicrobial substances, in addition to those linked to the process (oxygen level, presence of preservatives, storage time, and temperature) [70]. In this regard, probiotic microorganisms must survive not only the shelf life of the food product but also the passage through the gastrointestinal tract (GIT) [35]. The low pH of the stomach combined with the presence of bile in the intestine can affect survival, which could directly influence the proliferation and colonization of probiotics in the intestinal tract [71].

Although the *in vitro* test has limitations to assess the viability of a probiotic strain in humans, it is very useful for selecting the strains that, when introduced into a food matrix, behave more satisfactorily [35] [71]. Table 1.3 shows some studies focused on the gastrointestinal viability of some strains with probiotic activity. The main concern of the industry is ensuring the viability of probiotics is essential, due to the adverse conditions of food matrices, which can affect the viability and gastrointestinal resistance of these microorganisms. Therefore, the results found in several studies strengthen the processing and marketing of these products, ensuring the transmission of probiotics to consumers [71].

Table 1.3. Gastrointestinal viability tests of probiotic strains.

Strain	***Food Matrix***	***Study***	***Reference***
Bifidobacterium animalis	Juçara juice	*In vitro*	53
Ligilactobacillus salivarius	Apple juice	*In vitro*	70
Lacticaseibacillus rhamnosus	Pineapple juice with juçara	*In vitro*	71
Lacticaseibacillus rhamnosus	Guava juice	*In vitro*	72
Lacticaseibacillus casei Lactobacillus acidophilus	Mao luang Juice	*In vitro*	73
Lacticaseibacillus rhamnosus	Apple juice	*In vitro*	74
Lactiplantibacillus plantarum	Apple juice	*In vitro*	75

4. Effects of the Food Matrix on Cell Viability

In dairy-based probiotic foods, the physical-chemical composition of the milk, which is rich in proteins and lipids (fats), acts as a protective matrix for probiotics and these factors help the survival of probiotics from adverse conditions of the stomach and small intestine. However, matrices of non-dairy foods are very different from those based on dairy products; they are more versatile and less understood [65].

The big challenge is the application of probiotic cultures in different beverages based on food matrices. Different probiotic species show different sensitivities concerning substrate acidity, dissolved oxygen, post-acidification in fermented beverages, metabolism products, temperatures, and conditions of the gastrointestinal tract [67].

To exercise their probiotic activity, the live microorganisms must be in an adequate quantity, resisting the adverse conditions of digestion and reaching the intestine in a sufficient dose to effectively develop and promote the benefits to the host. The viability and metabolic activity of the bacteria are important characteristics of the inclusion of probiotics in beverages. This occurs because the bacteria need to survive in the beverage during the expiration date and on the gastrointestinal digestion [67]. Therefore, the choice of the food matrix is an essential part to maintain the probiotic viability in the final product. Fruit juices and smoothies can be challenging matrices and their effects on probiotic viability are worth investigating since organic acids and phenolic compounds commonly present in fruits can exhibit microbiological properties. On the other hand, the content of the phenolic compounds can contribute to the survival of probiotic bacteria in food and even exert an effect similar to a prebiotic in the human intestine [53].

The formulation of beverages can favor their stability. Storage temperature is also a relevant factor in maintaining probiotic activity. There is a general recommendation that probiotic foods should preferably be stored between 4˚C and 5˚C [53].

Sugar supports the multiplication of probiotics, so, in theory, the use of sugar by the probiotic in juices will decrease the sugar content and increase the acidity of the juice [76]. In general, according to previous studies, the growth and viability of probiotic bacteria in fruit and vegetable beverages depends on the species and variety of bacteria used, the pH, and the concentration of lactic and acetic acid in the final production [77]. The applicability of probiotics in food products generally depends on factors such as water activity, processing, and storage temperature, expiration date, oxygen content, pH, mechanical stress, salt content, and content of other harmful or essential ingredients [78].

The non-dairy sources are fortified with acidulants that can increase the shelf life by creating an anaerobic environment that is ideal for probiotic cultures, which is achieved by eliminating the available oxygen. One more advantage is that these juices stay much less time in the stomach and, therefore, probiotic species spend much less time in the acidic environment of the stomach [65].

Strategies can be used to improve the viability of probiotic microorganisms, such as proper selection of acid and bile resistant strains, use of oxygen-impermeable containers, two-stage fermentation, microencapsulation, and incorporation of micronutrients such as peptides and amino acids [37].

Technological advances have made it possible to alter some structural characteristics of the matrices of fruits and vegetables, modifying components of these foods in a controlled manner, which can make them ideal substrates for probiotic strains [78].

The survival of the bacteria within the host and the preservation of dedicated properties remains a problem, even with the use of encapsulation. The optimization of the process and product design, cell viability and probiotic functionality, and strict fermentation quality control (culture medium or food matrix, pH, temperature, carbon source composition, and fermentation time) and post-fermentation processing (spray drying, lyophilization, homogenization, mixing and high-pressure tablets, packaging, etc.). Sublethal stress during production can be useful to improve resistance to probiotics in foods and food additives can be avoided by encapsulation process [31].

5. Consumer Study and Potential Market

Sensory evaluation is a very important issue and has a direct association with product quality, processing characteristics, and consumer acceptability. Therefore, an appropriate selection of substrate composition and formations is necessary [77]. In addition to the above challenges, the sensory characteristics and general acceptance of non-dairy probiotic products also have some limitations. Thus, sensory evaluation of probiotic microorganisms in non-dairy products and consumer acceptance testing are of vital commercial importance [37].

It is important to consider the sensory acceptance by consumers during the development of non-dairy probiotic products, concerning appearance, aroma, texture, or flavor, to convey the direction for the production and ideal formulation of these products, always observing which are the expectations of consumers about these products. The attractive taste and the refreshing profile offered by fruit juices have stimulated a genuine interest in the industry for the development of fruit juices with the addition of probiotics [65]. Interactions between different probiotic strains and food substrates, where textures, flavors, aromas, and colors can be improved or aggravated by the production of different metabolic compounds, such as lactic acid and other metabolites during processing and storage, can influence the sensory properties of probiotic foods non-dairy. When preparing food with the addition of probiotics, the probiotic bacteria ferment the carbohydrates present in fruits, vegetables, cereals, and vegetables, releasing gases and alcohol. Some individuals report that the addition of probiotics to fruit juices can result in flavors described as “milky”, “medicinal”, “acidic”, “salty”, “bitter”, “astringent”, “artificial”, or “earthy”.

Some studies show that probiotics do not affect the general acceptance of fruit juices, this can happen depending on the type of fruit, the probiotic organism, the temperature at which they are stored, and the supplementation of prebiotics [37].

However, inadequate content of aromas (perfumery, dairy products) and flavors (sour, salty) have been reported when *Lactobacillus* plantarum was added to juices. A sensory impact study showed that consumers prefer the sensory characteristics of the conventional orange juice to their functional equivalent (juice containing probiotics), but if their information on health benefits is provided, preference increases over conventional orange juice [78].

The perception of unpleasant flavors in juices, resulting from the addition of probiotics that contribute to consumer dissatisfaction, can be overcome by adding 10% (v/v) of tropical fruit juices [65].

The value of the global market for probiotics is around $15 billion per year and is increasing by 7%. Today, these probiotic products represent between 60% and 70% of the total functional food market, demonstrating their importance. The global probiotic food and beverage market was worth around 24.8 billion euros in 2011 and more than 31.1 billion euros in 2015 [37]. This value is expected to reach up to $69.3 billion by 2023, which also represents the driving force behind the functional beverages market. The estimate in 2019 was that the market for supplements containing probiotics would increase from $48 billion to $62 billion in 2022.

Non-dairy food products have gained popularity in the past decade. It is expected that the food products business reach approximately $26 billion over the next five years. However, the manufacture of fermented probiotic foods at the commercial level faces many challenges, including the selection and identification of economical and abundant substrates, reducing operating expenses, and improving probiotic viability [44]. The willingness of the consumers to buy products incorporated with probiotics explains why they use functional beverages to improve their health [43].

The first non-dairy probiotic was produced by a Swedish company called Skane Dairy in 1994, since then many non-dairy probiotic beverages are already on the market. The basis of this product was oat flour fermented by *Lactiplantibacillus plantarum*. A similar product Good Belly (another company), prepared from oats and *Lactiplantibacillus plantarum*, was the first non-dairy probiotic launched on the US market in 2006 [37].

6. Technological Challenges in the Food Industry with the Addition of Probiotics

The selection of the appropriate probiotic strains in an appropriate dose and food matrix is the first requirement for the development of a food product with the addition of probiotics. Fruits, vegetables, and cereals represent a good matrix of probiotic bacteria with good nutraceutical components. However, some limitations can prevent the production of non-dairy probiotics at an industrial level, such as sensory characteristics, general acceptance, and, most importantly, the survival of probiotics over storage [79, 80]. To achieve these health benefits, the viability of probiotics through different conditions after consumption is crucial. The selected microorganisms must be able to tolerate bile and acid, colonization in the human intestine, good adhesion to people's epithelial cells; good growth characteristics, not being pathogenic and good impact on people's health [48].

The probiotic strains selected for use in the food industry must be stable during storage as chilled, frozen, or dried crops, and suitable for large-scale in industrial production with the ability to survive and maintain their functionality [10].

The greatest difficulty in the production of dairy beverages with probiotic properties is the preservation of the product's physical stability. The optimization of the process of these beverages needs more care, including a selection of concentration and type of stabilizer and optimization of pretreatment conditions, such as homogenization regimes. Storage at room temperature, which is common in many types of non-dairy products, can create a major challenge for probiotic viability [37].

The proper scientific validation of claims for functional food products re- mains a critical issue for food science. Especially issues of safety, biocompatibility, and health claim of functional milk-based and/or non-dairy beverages are often recorded or evaluated with inadequate/insufficient methodologies. An inter-disciplinary approach must be followed to create a solid scientific base for the functionality of the beverages in question [78].

New products need to take advantage of emerging technologies based on nanotechnology, high-pressure homogenization during processing, and methods that better preserve viability during storage, to increase the manufacture and consumption of products with the addition of probiotics [49].

Non-dairy foods must be developed with the addition of probiotics, allowing the consumption of these beneficial microorganisms by people who do not like dairy products or who are intolerant or allergic to milk components. There are two main challenges with a probiotic product: the maintenance of the physic-chemical and sensory characteristics equivalent to conventional products without probiotics and the maintenance of the viability of the microorganism during the lifespan of these products with the guarantee of the passage of the probiotic through the gastrointestinal tract, reaching in a sufficient quantity in the intestine to exert its probiotic activity [81]. Despite the challenges, the future of non-dairy products with probiotics is promising [37].

7. Conclusions

Since fruits and vegetables can be used as raw materials for a probiotic fermentation, this takes into account the biochemical and physicochemical composition of these raw materials. In addition to the content of health-promoting phytochemicals in fruits and vegetables, they also offer several advantages such as adding value to products and extending the shelf life of processed foods.

The development of new technologies that are more economically appropriate, and of matrices with technological potential for the industry is extremely important for the supply of non-dairy probiotic foods according to the demand they have. Although there is great potential for the use of fruit juices as probiotic products, there are few reports on their preparation and production and this needs to be intensified.

Innovative technologies for the preparation of probiotic food products to improve their nutritional value need to be an urgent priority area.

Acknowledgements

This work was carried out with the support of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brazil (CAPES)—Financing Code 001, University of Minho, Rio de Janeiro State Research Foundation (FAPERJ, E-26.202.749/2018) and National Council for Scientific and Technological De- velopment (CNPq, 311936/2018-0).

Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 2

Global Trends for Patulin Adsorption: a review

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This chapter was published on Research, Society and Development
(DOI: http://dx.doi.org/10.33448/rsd-v10i6.16162)

Abstract

Patulin is a toxic metabolite produced by several species of fungi. The species that are responsible for the production of patulin enter fruits through bruised and broken skin, causing contamination. Apple-derived products are considered to be by far the most significant dietary sources of patulin. According to the literature, three strategies have been used to break down or remove patulin in food, such as physical, chemical, and biological methods. Degradation of patulin by microorganisms or biodegradation enzymes is an efficient and promising method for the removal of patulin from food. The incidence of patulin contamination continues to be high, despite global efforts to reduce the levels of this mycotoxin at each stage of the fruit production process. Its transformation into other compounds has been reported. However, the toxicities of its byproducts as deoxypatulinic acid, ascladiol, and hydroascladiol should be subjected to an intensive study.

Keywords: Detoxification; Biological methods; Mycotoxins; Bacteria

Resumo

A patulina é um metabólito tóxico produzido por várias espécies de fungos. As espécies fúngicas responsáveis pela produção de patulina contaminam os frutos por meio de ferimentos naturais e rachaduras, causando a infecção. A maçã e os produtos derivados da maçã são considerados, de longe, as fontes dietéticas mais significativas de patulina. De acordo com a literatura, três estratégias têm sido utilizadas para decompor ou remover a patulina de alimentos, como métodos físicos, químicos e biológicos. A degradação da patulina por microrganismos ou enzimas de biodegradação é um método eficiente e promissor para a remoção da patulina dos alimentos. A incidência de contaminação por paulina continua alta, apesar dos esforços globais para reduzir os níveis dessa micotoxina em cada etapa do processo de produção da fruta. Sua transformação em outros compostos foi relatada. No entanto, as toxicidades destes subprodutos como o ácido desoxipatulínico, ascladiol e hidroascladiol devem ser submetidas a um estudo intensivo.

Palavras-chaves: Descontaminação; Métodos biológicos; Micotoxinas; Bactérias

Resumen

La patulina es un metabolito tóxico producido por algunas especies de hongos. Los hongos responsables de la producción de patulina contaminan los frutos a través de heridas y grietas naturales, provocando infecciones. Las manzanas y los productos derivados de las manzanas constituyen las fuentes dietéticas más importantes de patulina. Según la literatura, se han utilizado estrategias para descomponer o eliminar patulina en los alimentos, como los métodos físicos, químicos y biológicos. La degradación de la patulina por microorganismos y/o enzimas de biodegradación es un método eficaz y prometedor para eliminar la patulina de los alimentos. La incidencia de la contaminación por patulina sigue siendo alta, a pesar de los esfuerzos mundiales para reducir los niveles de esta micotoxina en cada etapa del proceso de producción de la fruta. Se ha informado de su transformación en otros compuestos. Sin embargo, las toxicidades de estos compuestos como el ácido desoxipatulínico, el ascladiol y el hidroascladiol deben someterse a un estudio intensivo.

Palabras clave: Descontaminación; Métodos biológicos; Micotoxinas; Bacterias.

1. Introduction

Patulin (PAT) is a toxic metabolite produced by several species of fungi, and it represents a significant hazard to the food or the food chain. It can be found in a large number of fruits, but it is more commonly found in apples. Generally, the amount of PAT in apple-derived products is seen as a measure of the quality of apples used in the food industries (Zhang et al., 2019).

Among mycotoxins, patulin (4-hydroxy-4H-furo [3,2-c] pyran-2 (6H) -one) is a polyketide lactone, with a molar mass of 154.12 g/mol and a melting point of 110℃. It is soluble in water and stable under acidic conditions and heat, which cannot be thermally denatured (Saleh & Goktepe, 2019a; Zheng et al., 2020). PAT is produced by at least 60 different species of fungi, such as *Penicillium expansum* (*P. leucopus*), *P. crustosum*, *P. patulum* (*P. urticae* and *P. griseofulvum*) and *A. clavatus* (Vidal, et al., 2019; Diao et al., 2018). The contamination of apple juice by PAT is one of the most important food safety issues worldwide being the species of fungi that are responsible for the production of PAT enter the fruits through bruised or broken skin areas, causing contamination (Sajid et al., 2018).

Recently, fifteen genes involved in the patulin biosynthesis have been identified. Among those, the patE and patH genes have shown to be needed for the production of patulin (Saleh & Goktepe, 2019a).

Patulin was first isolated from the *Penicillium griseofulvum* in 1943 by Harold Raistrick (Saleh & Goktepe, 2019a). Shortly after its identification, patulin was studied at the British Medical Research Center under the name "tercinin" as an antimicrobial agent against some gram-positive and gram-negative bacteria. However, it did not take long before researchers at the center had identified its toxic effects in 1944 (Saleh & Goktepe, 2019b).

Its activity as an antimicrobial was seen to be very promising since it proved to be up to ten times more effective in the treatment of infections by *Bacillus* sp. than *Penicillin* G. At the time, the substance was named from *Penicillium patulum* (later named *Penicillium urticae* and today known as *Penicillium griseofulvum*). However, between the years of 1950 and 1960, its toxic aspects became evident, both in plants and in animals, which prevented its clinical use as an antibiotic. Since the 1960s, patulin was reclassified, now being known as a toxic secondary metabolite, of fungal origin, that is, a mycotoxin (Basso, 2019).

PAT is a type of enteropathogenic mycotoxin, is rapidly absorbed and causes ulceration and inflammation of the intestinal mucosa. It causes different health problems for humans and animals, including edema, ulceration, inflammation, vomiting, bleeding, and even death (Bayraç & Camizci, 2019; Xiao et al., 2019).

The analytical determination of PAT represents common challenges observed in the analysis of mycotoxins. One of the biggest issues is sampling, as mycotoxins have a heterogeneous distribution in each sample batch (Vidal et al., 2019).

This work is a review of the literature on the mechanisms of adsorption of patulin where the characteristics of patulin are discussed along with the possible routes of human exposure and the mechanisms of adsorption of this mycotoxin. The review aims to provide food science researchers with information about the levels of patulin in apple-based foods and the possible ways of absorption of this compound. The google scholar, Scopus, and web of science databases between the years 2000-2021 were considered for writing the present review article. This article deals with researches on PAT decontamination, in the sense of recommending ways for PAT mitigation until the finding of food safety for this mycotoxin in food matrices and products.

2. Tolerable levels of patulin and risks to the population

After England's veterinary crisis in the 1960s, studies on the effects of mycotoxins on plants and animals have expanded (Basso, 2019). Due to the high volatility in water, resistance to heat, and high toxicity, patulin is among a list of mycotoxins in which the levels in food products are regulated (Bayraç & Camizci, 2019). According to the World Health Organization, the maximum acceptable level of patulin in apple juice is set at 50 µg/L (Saleh & Goktepe, 2019a). This value is following the recommendations of the Food and Drug Administration (FDA), the National Health Surveillance Agency (ANVISA), and the European Union (EU), in which the last one also limited the level of patulin in solid apples to 50 µg/kg, and on apple-based foods for children and/or babies at 10 µg/L. In Brazil, only on February 22, 2011, through ANVISA, it was created the first Resolution of the Collegiate Board (RDC) regarding the tolerable limits for patulin in food commercialization through the RDC n. 07/2011, adopting the same values for patulin as the North American and European organizations (Basso, 2019).

According to the recommendation of the European Commission and based on the established level of patulin (43 µg/kg body weight), the provisional maximum permissible daily dose of patulin was fixed at 0.4 µg/kg body weight. This level was adopted by most of the health risk evaluation analyzes carried out on patulin (Saleh & Goktepe, 2019a).

Apple products are considered by far the most significant dietary sources of patulin (Rodríguez-Bencomo, et al., 2020) as shown in Table 2.1. This occurrence is justified due to the physicochemical properties of the fruit, such as the water activity, which are the favoring factors for germination of the fungus spores. Therefore, the concentration of patulin in raw apples, as well as juices and apple-based products is used as a quality indicator (Saleh & Goktepe, 2019a).

Patulin also contaminates several other foods, including apple, dried figs, and corn. Currently, patulin is considered to be a global toxin, and many countries monitor its residues in food (Zhao et al., 2019).

Table 2.1. Food contaminated with patulin

Food	**Amount of patulin**	**Analytical Method**	**LOD* (µg/L or µg/kg)**	**Origin of samples**	**Reference**
Apple	122 µg/L	LC-MS/MS	0.5	Czech Republic	Vidal et al., 2019
Pear	31 µg/L	HPLC-UV	0.01		
Fruit juice mix	56 µg/L	HPLC-UV	3.5		
Apple juice	464 µg/L	HPLC-UV	-	Japan	Li et al., 2019
Apple	270 µg/L	HPLC-UV	0.04	Pakistan	Iqbal et al., 2018
Grape	466 µg/L	HPLC-UV	0.04		
Corn	105 µg/L	HPLC-UV	5.0	Cameroon	Abia et al., 2017
Dried figs	131 µg/L	HPLC-UV	7.5	China	Ji et al., 2017

**LOD: Limit of detection*

The incidence of patulin in recent research has pointed out the importance of strengthening strategies to control patulin during food production. Cleaning, washing, milling, and pressing can indicate a reduction of up to 55 % patulin. Heat treatment showed a limited effect of the reduction of patulin in apple juice, as it was observed a reduction of 26 % of patulin with a treatment of 100 °C for 20 minutes. Based on these results, the reduction of patulin in apple juice under industrial pasteurization conditions (a heat treatment used in fruit and vegetable products) does not allow for greater results, which means that only a low reduction in the patulin content can be expected using pasteurization (Vidal et al., 2019).

Over the past few decades, consumers have become more aware of the health and quality of the food and, as a result, research on food safety has increased. The evaluation of patulin contamination in fruits and vegetables has become an important factor to ensure the quality of these products (Basso, 2019). Therefore, it is of great practical importance to develop a method for the detection of patulin traces in food (Zhao et al., 2019).

Based on the high incidence of patulin, concerns were raised about the toxicological effects of patulin on humans and animals through consumption. Patulin causes several chronic health effects on genetics, immunity, and the central nervous system in animals, even though its effects on humans are not yet clear (Sajid et al., 2018).

Although the information on patulin absorption is rare, the bio-accessibility observed in vitro is large, especially on the oral (87 %) and gastric (82 %) phases (Vidal et al., 2019). Several studies have revealed that patulin accelerates the intracellular production of oxygen reactive species (ORS), leading to the damage of many vital macromolecules, such as proteins, enzymes, and DNA (Ramalingam et al., 2019).

3. Patulin adsorption mechanisms

The patulin content found in the final processed foods is usually lower than the content found in raw materials since processing steps can actively contribute to its reduction. Among these, some procedures stand out, such as the clarification process, the filtration and enzymatic treatment during the juicing process, and the fermentation in the wine production. In general, controls on all the stages of apple processing, such as homogenization, pulping, pasteurization, and aseptic packaging, can contribute to the reduction of the final levels of patulin. The stability of patulin is illustrated by its presence in apple-based products, such as juices, jams, and baby foods, even after all the industrialization process suffered by these foods (Basso, 2019).

Based on the adverse effects of patulin on human health and the quality of agricultural products, researchers have been looking for ideal methods for the degradation or the removal of it from contaminated food (Zheng et al., 2020; Diao et al., 2018). As pre-harvest treatments do not always guarantee sufficiently low levels of patulin in juices, different approaches are being investigated to remove or degrade this toxin during food processing (Rodríguez-Bencomo et al., 2020).

In addition to patulin analysis, detoxification attempts have become an important factor in food safety. There are some methods to reduce the level of patulin during the pre-harvest, harvest, and post-harvest steps of the apple juice manufacturing process to ensure the integrity of the final product. Recently, adsorption methods using different biosorbents have been studied for both aqueous solutions and juices (Bayraç & Camizci, 2019).

Many physical and chemical approaches have been developed for the detoxification of PAT in apple juice and apple-based products. However, some disadvantages, such as safety problems, possible losses in nutritional quality, chemical risks, limited efficacy, and high costs have been registered for these approaches. In addition to the chemical and physical approaches, recent interest has been observed regarding the use of biological methods for the removal of PAT from fruit juices, particularly, the bio-sorption method using inactivated microbial cells (Sajid et al., 2018).

Several factors make the control of patulin and the producing fungi even more complex, such as climatic conditions, geographic location, year of production, pre, and post-harvest treatments, damage to the surface of the fruits, and storage conditions. Sensitive, accurate, and robust analytical procedures are necessary for the qualitative and quantitative analysis of mycotoxins (Vidal et al., 2019).

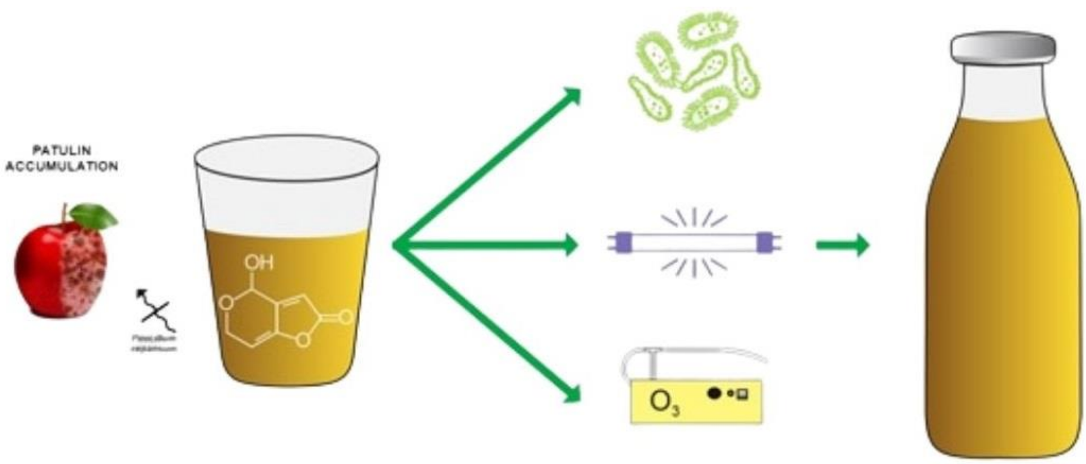
Adsorption is considered to be the most effective way to remove PAT in juices, due to the convenient operation and without introducing any new threats.

With technological advancements, researchers have been developing a series of new adsorbent materials with high adsorption capacity and good stability. Some of them have effectively been used for patulin remotion, such as silica-based materials, carbon nanotubes, and organic metallic structures. Synthetic adsorbents generally have good adsorption effects, but those are limited for practical application due to the high cost, complicated preparation processes, and potential safety risks (Qiu et al., 2018).

Recently, bio-waste from agriculture and industries, including microbial cells, algae, plant biomass, and organic sludge, have been considered as fascinating adsorbents as a result of their cost-benefit, respect for the environment, and some interesting properties (Qiu et al., 2018).

According to the literature, three strategies have been used to break down or remove patulin in food, being those physical, chemical, and biological methods (Figure 2.1). Physical-chemical methods have been extensively studied and some new advanced techniques have been used in practical production. Most of the biological methods were studied only on a laboratory scale even though they have more advantages, such as high safety and efficacy of detoxification (Diao et al., 2018).

Figure 2.1- Currently measures for patulin decontamination



The physical methods involve sorting, washing the fruit with high-pressure water, refrigeration, juice clarification, filtration, adsorption, pasteurization, and radiation to treat raw materials, such as apples, to avoid contamination of patulin. Physical methods work well but require a considerable amount of labor and material resources, which are expensive methods and can even affect product quality (Zheng et al., 2020).

For the chemical methods, it is included ammonia and potassium permanganate, which can reduce the content of patulin in the juice by more than 99 %. This technique is the most effective way of detoxifying patulin, but it leads to serious damage to the quality of the product. In addition, sulfur dioxide, ascorbic acid, N-acetylcysteine and glutathione, and calcium D pantothenate can reduce the amount of patulin in the juice. However, the addition of these chemicals requires a thorough understanding of the reaction mechanism, that is, whether the reaction products are toxic. In addition, some of these chemicals can seriously decrease the quality of the product by destroying its nutritional content and flavor (Zheng et al., 2020).

The biological methods, especially the direct degradation of patulin by an antagonistic microorganism, have shown great prospects for application in the control of patulin contamination. However, several aspects need to be considered before using these microorganisms. Firstly, it is necessary to investigate whether patulin is adsorbed or degraded by the microorganisms. Degradation of patulin by microorganisms could prevent further over-processing if the patulin were transformed into a less toxic compound or a non-toxic compound. For this, the patulin can be reduced through an enzymatic action (Zheng et al., 2020).

The use of yeast and bacteria has also been studied for several years for their ability to degrade or inactivate patulin. These microorganisms are effective in controlling blue mold infection, as biocontrol agents, such as *Rhodosporidium kratochvilovae, Pichia caribbica*, *Metschnikowia* spp., and *Lactobacillus plantarum*. All of these microorganisms demonstrated the ability to protect apples against blue fungi and also to prevent the accumulation of patulin (Basso, 2019).

Microorganisms have been widely used to decrease contamination, for example, heavy metals, dyes, and mycotoxins. Some inactivated microorganisms are effective for the removal of PAT in simulated juice aqueous solutions, while their adsorption capacity decreases noticeably when applied in apple juice. The most probable reason is that some substances in the juice may occupy the PAT binding sites (Qiu et al., 2018).

Although physical-chemical methods may have varying degrees of success, the limited effectiveness and loss of important nutrients still prevent their application in the food industries. Biological methods have been recognized as the promising solution for mycotoxin decontamination, and are being widely studied (Li et al., 2019).

3.1 Physical methods

The physical treatment strategies involve strengthening the selection and the cleaning of raw materials, physical adsorption, and clarification. The selection of high-quality raw materials is the first step to reduce or avoid contamination by PAT or the production in food (Wei et al., 2020).

The processing for the removal of patulin in foods has low-cost and it is easily operable with high detoxification efficiency (Diao et al., 2018).

Currently, the adsorbent is widely used in the physical area for the removal of PAT in fruit juices. Magnetic chitosan, resin, and activated carbon are often chosen for the absorption of PAT in the food industry. The resin adsorption is the most commonly used method to remove PAT during the manufacture of industrial apple juice (Wei et al., 2020).

The application of high-pressure hydrostatic processing (pulsed or continuous) and its effects on the patulin content, leads to degradation of up to 62 %, but only for a low initial patulin content (5 µg/L). The effectiveness of high pressure in the degradation of patulin depends on the composition of the juice, particularly in the presence of compounds containing thiol groups (Rodríguez-Bencomo et al., 2020; Qiu et al., 2020; Gonçalves et al., 2019).

Treatments based on irradiation with light have also been tested, where the researchers have applied ultraviolet (UV) light (20) in contaminated apple juices. It was noticed that the effectiveness of the patulin degradation depended on the wavelength, obtaining 90 % of degradation at 222 nm with minor color changes. In addition, physical methods such as UV light, have the advantage of degrading certain organic contaminants, without the formation of taste or odor during the treatments (Wei et al., 2020; Tang et al., 2019). Meanwhile, UV can lead to the formation of furans, which has been identified as a highly carcinogenic compound for humans. Even though these studies are promising regarding the effectiveness of patulin degradation, especially in the case of light treatments, none of them determined the type of degradation products generated, which is a limiting aspect for its application in food products. The development of physical methods to remove patulin depends on the development of special equipment (Rodríguez-Bencomo et al., 2020).

3.2 Chemical methods

Chemical methods have great potential for detoxifying mycotoxins such as patulin and are probably the most easily suited method for commercial application in the food industry today. In the 1970s, people used effective chemical additives to remove patulin from food. Sulfur dioxide, ascorbic acid or ascorbate, ammonia, acid or alkaline potassium permanganate, sodium benzoate, potassium sorbate, ozone, thiamine hydrochloride, pyridoxine hydrochloride, phenolic compounds, and calcium d-pantothenate have already been used to successfully degrade patulin (Diao et al., 2018; Wei et al., 2020).

Among the chemical additives, ozone has been widely used to detoxify patulin in foods, due to its high safety, low cost, it does not generate toxic waste, is environmentally friendly, and is easy to operate (Diao et al., 2018). The ozone gas can reduce the amount of patulin in food by up to 98 % after 1 minute. On the other hand, it introduced new impurities to it or led to the loss of important nutrients that limit this application in the food industry (Tang et al., 2019).

However, only a few additives have been reported in the literature for removing PAT directly from food. Chemical methods, such as ozone degradation, remained mainly in the experimental stage due to uncertain safety, few results, or other limitations concerning practical applications (Wei et al., 2020).

Patulin can be completely degraded into CO2, oxalic acid, diglycolic acid, and H_2O in high ozone concentration or sufficient ozonation time, attacking two ethylene double bonds conjugated in its chemical structure (Vidal et al., 2019). The presence of oxygen and free radicals is necessary for rapid degradation, which limits its application in juices. Chemical methods are economical and efficient if the presence of unknown reactions or toxic by-products with the use of these chemicals is assured (Rodríguez-Bencomo et al., 2020).

3.3 Biological methods

Biological methods refer to any methods that utilize active or inactive microorganisms or enzymes for the reduction of patulin in food. Based on the patulin reduction method, the biological methods have been classified into two types: adsorption and degradation. There were no significant differences observed in the efficiencies of patulin removal by living or dead bacteria (Vidal et al., 2019; Wei et al., 2020).

The most recent studies have led to the identification of two main products of patulin degradation by microorganisms, which are the Z-/E-ascladiol the deoxypatulin acid, and have revealed the main mechanism of patulin biotransformation/biodegradation (Li et al., 2019; Wei et al., 2020). Recent studies have shown that adsorption using inactivated microbial cells is a promising method for efficient and economical removal of contamination (Qiu et al., 2018).

Inactivated microorganisms with large surface area, adsorptive selectivity, and abundant functional groups are sought after as adsorption agents. Due to the advantages of bio-sorption, numerous microorganisms such as lactic acid bacteria (LAB), *Gluconobacter oxydans*, *Saccharomyces cerevisiae*, and *Alicyclobacillus* spp. have been reported for the adsorption and the removal of PAT. However, filtration or even centrifugation are necessary processes after adsorption for the separation of it from the microbial cells. These operations are inefficient and impractical for large-scale application, which seriously restricts the application of inactivated microorganisms as adsorption agents (Qiu et al., 2018). The adsorption of PAT in apple juice using ten strains (BAL) was influenced by the toxin concentration and temperature. Researchers suggest that heat-inactivated (HI) LAB cells are more likely to have a greater ability to adsorb PAT from an aqueous solution. The magnetic separation technique is considered to be a fascinating method. The modification of microbial cells with magnetic nanoparticles (MNPs) can allow the promptly available bio-absorbents to be easily manipulated under the applied magnetic field (Sajid et al, 2018).

The three possible mechanisms for reducing patulin by microorganisms are absorption in the cell wall, degradation by intracellular or extracellular enzymes (Zheng et al, 2020).

For example, an inactivated *Alicyclobacillus* spp. can efficiently reduce the concentration of patulin in apple juice through biosorption (Yuan et al., 2014). Some yeasts have potential as biocontrol agents since they compete for nutrients and inhibit the growth of opportunistic microorganisms and plant pathogens (Tang et al, 2019). *Saccharomyces cerevisiae*, *Gluconobacter oxydans,* and *Lactobacillus plantarum* are effective in reducing the content of patulin and transforming it into the Z- / E-ascladiol isomer. In addition, *Rhodosporidium kratochvilovae*, strain LS11, and *Rhodosporidium paludigenum* are responsible for the degradation of patulin into deoxypatulin acid, which is a less toxic compound, resulting in significant removal of patulin (Li et al, 2019).

Lactic acid bacteria (LAB), which are widely used as a probiotic for humans, are even more advantageous in the removal of patulin contamination, as they can degrade patulin into non-toxic compounds. The adsorption of patulin across the surface of the LAB is the primary mechanism for removing patulin. Studies have shown that the main functional groups involved in patulin adsorption are polysaccharides and/or proteins. Studies confirm the bacterial surface layer protein as the PAT binding site (Wei et al, 2020). Currently, only a few strains of LAB have been identified as having the potential use to control patulin contamination in food (Zheng et al, 2020). *Lactobacillus brevis* 20023 (LB-20023) removed PAT from a working solution (4.0×10^3 μg/L) at 37 °C for a period of 48 h (Wang et al., 2015). This organism, which has a large surface area and cell wall volume, reduced the concentration of PAT by 65.02 %. In another study, *Rhodotorula mucilaginosa* JM19 significantly degraded PAT (1.0 × 10^5 μg/L) in deoxypatulinic acid (DPA) in a MES buffer at 35 °C for 21 h, with, in some cases, a degradation rate greater than 90 %. Interestingly, another study of *Saccharomyces cerevisiae* reported a 100 % degradation rate for PAT (50 μg/L) after 2 days. In that report, PAT degradation was identified as an enzymatic hydrolysis reaction, even though the enzymes that metabolize PAT were not induced by incubation with PAT (Wei et al, 2020).

It is essential to assess the toxicity of patulin degradation products (Zheng et al, 2018). The biological method not only effectively controls the hazard caused by *Penicillium expansum*, but also removes toxins that already exist in food. Degradation of patulin by microorganisms or biodegradation enzymes is an efficient and promising method for the removal of patulin in food if the microorganisms used and the degradation products are completely non-toxic. However, the toxicities of DPA, ascladiol, and hydroascladiol should be subjected to an intensive study.

4. Conclusions

The incidence of PAT contamination remains high, despite global efforts to reduce the levels of this mycotoxin at each stage of the fruit production process. Several physicals, chemical, and biological technologies have been developed for the adsorption of patulin. However, most of these techniques are less available and due to their high cost, they are not suitable for industrial manufacturing or the introduction of new chemical hazards. Generally, ideal detoxification methods should: destroy or remove the toxin, not produce or leave any new toxic substances, retain nutritional value and product acceptability, not change significantly the product processing technology, if possible, destroy fungal spores, and to be practical to the extent as if it is technologically and economically viable. Nevertheless, all of the aforementioned detoxification techniques have defects that do not completely satisfy the ideal conditions. The development of physical methods to remove patulin depends on the development of new dedicated equipment. The chemical methods are economic and efficient, but only if it is ensured that there are not any unknown reactions or toxic sub-products with the use of those chemicals.

Based on the premises discussed in this article on how patulin can contaminate food, affecting the quality of products as well as the effects on health. Thus, as evidenced in this review, there is no single method that completely satisfies the requirements for the ideal detoxification of patulin in food. Currently, a combination of several methods may be the best choice for removing or degrading patulin from food. Therefore, more attention should be paid to the development of advanced detoxification methods in the future.

Acknowledgements

This work was carried out with the support of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Financing Code 001, University of Minho, Rio de Janeiro State Research Foundation (FAPERJ, E-26.202749/2018) and National Council for Scientific and Technological Development (CNPq, 311936/2018-374 0)

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CHAPTER 3

Viability evaluation of *Lactobacillus acidophilus* applied to non-dairy and non-fermented beverages in simulated gastrointestinal conditions

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This chapter was submitted to Microorganisms

Abstract

Probiotic beverages in Brazilian markets are fermented, of dairy origin, and with an average shelf life of 35 days, as long as they are kept under refrigeration up to 10 °C. In this work, a non-dairy and non-fermented beverage was developed, with a shelf life of 180 days and that can be stored either at room temperature or under refrigeration. The viability of probiotic microorganisms was evaluated for 180 days, as well as its survival to simulated gastrointestinal conditions. In assessing survival, there was no reduction in the viability up to 90 days. After 90 days, the viability reduction was 1 log, both for the beverage under refrigeration and for the beverage at room temperature. A reduction of up to 2 logs would be acceptable, as probiotic microorganisms would still reach the colon in sufficient quantity to exert probiotic activity. The results from this study confirmed the suitability of the ionic gelation technique to encapsulate the probiotic strain evaluated using a prebiotic compound such as oligofructose, especially when the microorganism was incorporated in a symbiotic mixture, leading to an excellent survival rate.

Keywords: gastrointestinal survival; probiotics; apple juice

1 Introduction

Probiotics are defined by the Food and Agriculture Organization of the United Nations (FAO) and by the World Health Organization (WHO) as living microorganisms that have beneficial effects on the host's health when administered in adequate amounts. *Lactobacillus* and *Bifidobacterium* are two common species of probiotics used in food products [1, 2]. These bacteria can be intentionally inserted in industrialized products or be naturally present in foods such as dairy products, meat, or sausages [2].

The preparation of food products has become increasingly challenging due to consumer demand for healthier and more attractive foods. Currently, there is an increase in the consumption of fruit juices and beverages, especially functional beverages with the addition of probiotic bacteria. Functional foods, in addition to contributing to nutritional aspects, also contain biologically active substances that possess health benefits. In recent years, lactic acid bacteria (LAB) have gained ground in many studies, as several of them have distinct probiotic characteristics [2].

Recently, probiotic beverages have become a crucial growth sector in the exploitation of whey worldwide. A reasonable number of fermented products have been developed by the food industry using probiotic strains. Sufficient amounts of viable probiotic counts in fermented products can bring abundant benefits to human health. The probiotics intake has many health benefits, including improved gut health by resistance to intestinal pathogens, reduced serum cholesterol, alleviated lactose intolerance, reduced risk of cancer, and so on [3].

Several studies demonstrate that dairy products are the main foods that employ probiotic bacteria in their composition. However, with the increase in allergies, lactose intolerance, and other diseases, which do not allow the ingestion of these types of products, the food industry is continually increasing the manufacture of innovative plant-based probiotic beverages. This demand represents a major technological challenge concerning the survival of these probiotics in fruit juices, and their resistance to passage through the gastrointestinal tract is also necessary so that they can be absorbed in the intestine and exert their beneficial effects on health [2, 4].

Recently, beverages prepared from fruits, vegetables, and cereals have been investigated. Fruit and vegetable juices are promising probiotic carriers due to their essential nutrient content, along with their appeal to a niche of consumers who are already concerned with healthier habits. In addition to this, the correlation of fruit extracts with benefits for certain health conditions reinforces their positive interest [4].

Apple consumption has been inversely linked to chronic diseases, including certain types of cancer, cardiovascular disease, asthma, and diabetes. It contains polyphenols, α-amylase, and α-glucosidase inhibitors. Fruit juices also contain oxygen-eliminating ingredients, such as ascorbic acid and high amounts of sugar, which can promote anaerobic conditions and stimulate probiotic growth. Fruit juices like apple juice provide a suitable medium for the growth and survival of probiotics [5]. Apple juice can also promote the survival of probiotics and has a considerable nutritional value as well. After probiotic fermentation, apple juice retains its nutrient content, gains a unique flavor and efficacy, diversifies the market for probiotic products, and expands consumer choices [6].

Lactobacillus acidophilus (La5) is a probiotic microorganism that has been proven to help in the treatment of inflammatory bowel diseases (IBD) such as modulation of the immune response, necrotizing enterocolitis, radiation enteritis and also vaginosis and vaginitis, non-alcoholic fatty liver disease and alcoholic liver disease [6].

Lactobacillus acidophilus (La5), specifically, can suppress free radicals that are involved in the development of diabetes and control blood cholesterol levels. La-5 was tested in patients with Type 2 Diabetes Mellitus and fasting glucose was improved, as was the attenuation of oxidative stress [7].

The consumption of probiotic microorganisms in the treatment of IBD can take the form of dietary supplements or foods. The foods with probiotics most commonly found by the consumer are milk and dairy products, especially yogurts and fermented milk. However, the increase in the incidence of food allergies due to the large number of individuals intolerant to lactose and/or milk protein, causes the demand for new products with probiotics, with emphasis on the need for non-dairy beverages such as fruit juices. which are widely consumed by the Brazilian population, in addition to being nutritious [8].

Apple and its juice can be considered an important food for human health due to its content of bioactive compounds, such as polyphenols with antioxidant function and pectins and acids that promote the balance of gastrointestinal transit [9]. Pectin, when fermented by colonic bacteria such as Lactobacillus acidophilus, which acts in the distal portion of the colon, the region of IBD activity, helps in the formation of short-chain fatty acids, which promote the reduction of inflammation. Like pectin, prebiotics such as oligofructose, when fermented by a probiotic, produce short-chain fatty acids, which are beneficial for reducing inflammation in individuals with IBD ulcerative colitis [10,11]

An apple juice associated with *Lactobacillus acidophilus* (La5) as a probiotic, can generate a functional beverage of great appeal, as it combines the nutritional quality of the fruit with the benefits that the probiotic can bring concerning IBD, diabetes, oxidative stress, and the modulation of blood cholesterol levels [6].

Several technical underpinnings are associated with procedures and the production of functional foods using microorganisms and fruit juice as raw material. Studies involve the composition and preparation, viability, benefits, and efficiency of cultivation, scientific and technological requirements, and storage time after food production to control its safety and durability [2].

To infer health benefits to the host, the probiotic must survive the severe conditions encountered during processing, storage, and in the upper digestive tract to reach the intestines in adequate quantities. To ensure this, different microencapsulation techniques have been used to provide physical barriers against environmental stress [5].

Several approaches have been proposed to improve the viability of some probiotics under adverse conditions, including the selection of acid- and bile-resistant strains, use of appropriate oxygen-impermeable containers, stress adaptation, two-step fermentation, micronutrient incorporation, and microencapsulation. Among them, microencapsulation has attracted significant attention, as it can protect probiotics from environmental stressors [12].

The encapsulation of bacteria with probiotic activity is considered a method to improve these problems. The selection of wall material for encapsulation is always a challenge. Because the viability of encapsulated probiotic cells depends on the physicochemical properties of the capsules. Ionic gelling helps form capsules between polymers and the carrier molecules [13].

In essence, microencapsulation is defined as the process in which small amounts of an active ingredient are packaged within a micrometer-sized capsule to prevent harmful chemicals and physical reactions and to protect the active ingredient from its surrounding environment. To indicate capsules produced independently of their size, the term encapsulation is preferred and will be used hereinafter. This process results in the maintenance of the biological and physicochemical properties of the encapsulated materials. Probiotics can be encapsulated using a variety of methods, the method used in our study was the physicochemical method called ionic gelation [12].

In this method, the polymeric solution (usually hydrocolloid solution) is mixed with microbial cells, and the suspension is projected through a syringe needle into a solution, which results in gelation. Several factors can affect the size of the microspheres produced, such as the viscosity and flow rate of the polymer solution, concentration, and temperature of the polymer solution, orifice diameter, and droplet height or distance from the orifice to the crosslinking solution [14].

In this work, a non-dairy and unfermented apple beverage, which could be stored at room temperature, was developed with apple juice from the cultivar Fuji added of an encapsulated symbiotic [15, 16]. To evaluate the effectiveness of the beverage as a vehicle for a probiotic, the viability of the encapsulated symbiotic during 180 days of storage and on the digestive process for the same period was measured.

2. Materials and Methods

2.1 Source of microorganisms

Lactobacillus acidophilus La-5 were obtained as freeze-dried, concentrated starter cultures from Christian Hansen, A/S (Hrrsholm, Denmark) in the lyophilized form.

Sodium alginate (Sigma-Aldrich, United States of America), gelatin (Royal, Brazil), oligofructose (Orafti®, Beneo, Belgium), and calcium chloride (Sigma-Aldrich, United States of America) were used for the encapsulation process.

2.2 Encapsulation of probiotics

The culture was encapsulated by ionic gelation using a biopolymeric solution containing sodium alginate (1%, m/v), gelatin (1.5%, m/v), and oligofructose (3%, m/v), known for its prebiotic properties. This formulation was based on the work of Silva *et al*. (2017) [15]. The polymers were dispersed in distilled water at 50 °C and kept under magnetic agitation until complete dissolution. The probiotic was previously hydrated in a 0.5% (w/v) sodium chloride solution and then added to the biopolymeric solution, reaching a final concentration of 10% (m/v). The solution was kept under constant agitation, in a magnetic stirrer, for one hour. At the end of this period, the encapsulation solution was ready with a viable cell concentration between 8 and 10 log CFU g^{-1}.

The biopolymeric solution containing the probiotic microorganisms and the prebiotic agent was atomized in a calcium chloride solution (1,6%, m/v), using an atomizing nozzle with 0.5 mm diameter, at a pressure of 450 bar, keeping a distance of 20 cm between the nozzle and the chloride solution calcium.

The particles were kept under magnetic agitation for 30 minutes and then the calcium chloride solution containing the microgels was sieved in a 0.062 mm mesh sieve. The microgels that remained in the sieve were collected in a sterile bottle and stored under refrigeration until use.

2.3 Production of Non-fermented Probiotic Apple Beverage

Apple juices from the Fuji variety, produced at Embrapa Uva e Vinho (Brazil, Rio Grande do Sul) were used to prepare the probiotic apple beverage. The process consists of the addition of 100 g of encapsulated symbiotic [10] to 1 L of whole apple juice. After homogenization, the beverage was pasteurized (FT25D, Armfield, UK) at 90 ° C for 30 seconds, followed by filling in glass bottles at 90 ° C in a storage chamber of aseptic filling. The bottles were cooled in an ice bath for 5 minutes [16]. The resulting product was the apple beverage with probiotic.

2.4 Probiotic viability during the storage period

The beverages were stored at 7 and 30°C and the viability and the resistance to the simulated gastrointestinal conditions of the probiotics were evaluated during 180 days. Probiotic viability was monitored, using the total viable count (TVC) (Log_{10} CFU mL^{-1}) [13], immediately after the preparation and at every 7 days in the first month, and every 15 days to the rest of the experiment (180 days).

2.4.1 Microbiological analysis

Enumeration of viable *L. acidophilus* (LAB) cells was performed as described by Silva et al (2018) [17] first, the dilutions of the culture were made by successively adding 1 mL of the samples to a tube containing 9 mL of peptone water 0.1 g/100 mL, followed by its homogenization and the withdrawal of another 1 mL for the next dilutions. Then, 1mL of each dilution was plated in numbered sectors. After absorbing the drops, the plates were incubated at 37°C for 96 h. Results were expressed as CFU/200 mL. To enumerate viable cells of LAB of gelled systems, a fraction of the microbeads was broken with sodium phosphate in a magnetic stirrer for 45 min and plated as previously described. LAB was enumerated on MRS (Kasvi, Italy) agar after plates were incubated at 37 °C for 96 h under aerobic conditions. The microorganisms count in the probiotic beverage was evaluated at times 0, 7, 14, 21, 28, 45, 60, 75, 90, 105, 120, 135, 155 and 180 days.

2.4.2 Survival of encapsulated *Lactobacillus acidophilus* under simulated gastrointestinal conditions

The survival of probiotics to the in vitro gastrointestinal tract (GIT) conditions was performed according to Madureira *et al*. (2011) [19], with adaptations, following the scheme shown in Figure 3.1. The simulated digestion included a gastric phase and two enteric phases and the survival assay was performed with samples collected at 90, 125, 139, 155, and 183 days of storage.

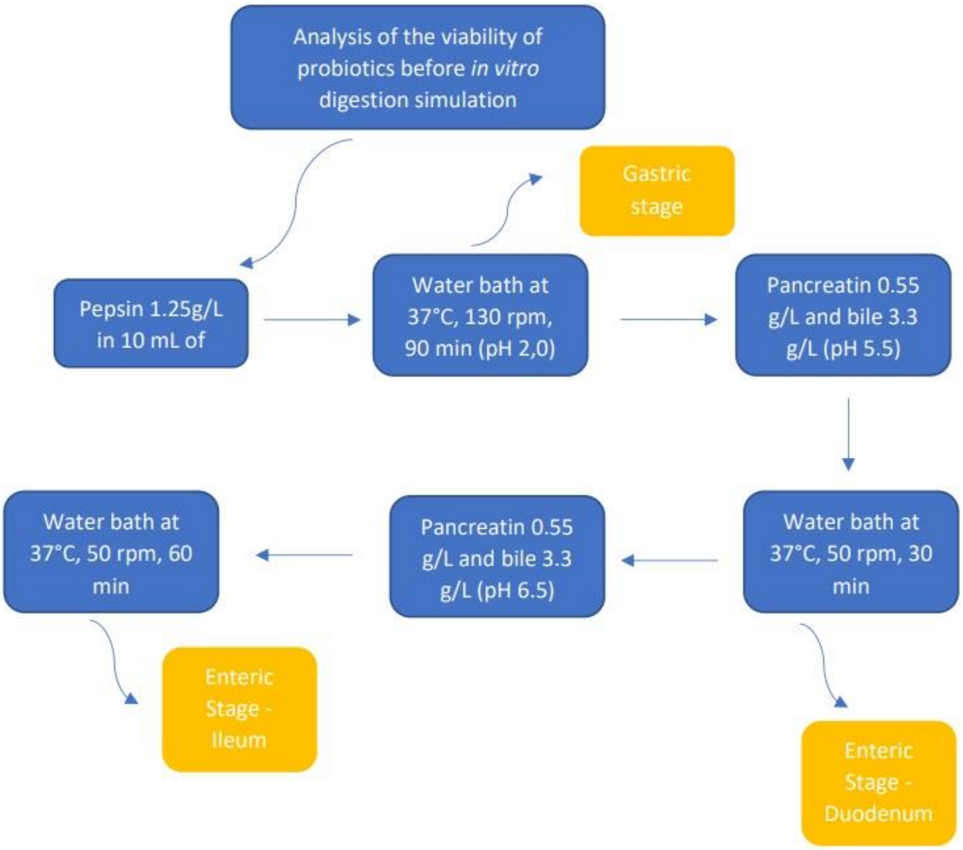

Figure 3.1. Stages of the *in vitro* test, simulating gastrointestinal conditions

To the gastric phase, 10 mL of apple beverage with encapsulated probiotic were distributed in centrifuge tubes, followed by a pH adjustment, using 1 mol L^{-1} hydrochloric acid (Sigma) to pH 2.0. Then, porcine gastric pepsin (Sigma) was added at a concentration of 1.25 g L^{-1} in HCl 0.1 moL L^{-1}. The tubes were incubated in a Dubnoff orbital water bath (Nova Técnica, Brazil) at 37 °C for 90 minutes, under magnetic agitation at 130 RPM.

To simulate the duodenum conditions, the pH adjustment was performed with sodium bicarbonate 1 mol L^{-1} (Sigma) to pH 5.5. Then porcine pancreatin (Sigma) was added at a concentration of 0.55 g L^{-1} and bovine and ovine bile (Sigma) at a concentration of 3.3 g L^{-1}, both in broth in 0.1 M $NaHCO_3$ solution. The tubes were incubated in a water bath at 37 °C for 30 minutes with agitation at 50 rpm, removing aliquots for analysis of the probiotics.

For simulating the conditions of the ileum, the pH adjustment was carried out with sodium bicarbonate 1 mol L^{-1} (Sigma) to pH 6.5. Then, pancreatin and bile were added, so that the concentrations were kept at 0.55 g L^{-1} and 3.3 g L^{-1}, respectively. The tubes were incubated at 37 ° C for another 60 minutes, with agitation at 50 rpm.

The probiotic counts were performed immediately before the *in vitro* assay and after each digestion simulated phase. Probiotic cultures were counted in MRS medium as previously described.

2.5 Physical-chemical analysis

All the probiotic-based beverages were analyzed for soluble solids (°Brix) [20], using a digital bench refractometer, brand Atago model PR 1001 with a scale from 0 to 45°Brix, titrable acidity and pH measured by direct reading in a Methrom brand automatic titrator, model 785 DMP - Titrino [20].

2.6 *Statistical analysis*

The data were analyzed using analysis of variance (ANOVA) and test of averages using the BioStat 5.3 statistical software. The p-value of ≤ 0.05 was considered significant.

3. Results

3.1. Viability of the microencapsulated *Lactobacillus acidophilus* during storage

The viability of microencapsulated *Lactobacillus acidophilus* La-5 in apple juice during storage at 30 °C and 7 °C is presented in Table 3.1.

Table 3.1. The viability of microencapsulated *Lactobacillus acidophilus* La-5 in apple juice

	Samples	
	30°C	**7°C**
T (days)	**Results (log $_{10}$ CFU mL^{-200})**	

0	8.73 ± 0.00^{a,A}	8.73 ± 0.00^{a,A}
7	8.73 ± 0.00^{a,A}	8.73 ± 0.00^{a,A}
14	8.73 ± 0 .00^{a,A}	8.73 ± 0.00^{a,A}
21	7.94 ± 0.01^{a,B}	7.37 ± 0.05^{b,B}
28	6.84 ± 0 .04^{a,B}	6.43 ± 0.01^{b,B}
45	6.52 ± 0.00^{a,B}	6.43 ± 0.00^{b,B}
60	6.11 ± 0.02^{b,B}	6.40 ± 0.03^{b,B}
75	7.56 ± 0.00^{a,B}	6.27 ± 0.03^{b,B}
90	8.00 ± 0.01^{b,A}	8.00 ± 0.02^{b,A}
105	7.37 ± 0.02^{a,B}	6.37 ± 0.01^{b,B}
120	7.07 ± 0 .02^{b,B}	7.07 ± 0.02^{b,B}
135	7.07 ± 0.00^{b,B}	7.07 ± 0.01^{b,B}
155	7.00 ± 0.02^{b,B}	7.00 ± 0.00^{b,B}
180	7.14 ± 0.04^{b,B}	6.89 ± 0.00^{b,B}

$^{A-D}$ Different superscript capital letters in the same column denote significant differences (p<0.05)

$^{a-d}$ Different superscript small letters in the same row denote significant differences (p<0.05)

The above results are expressed in colony-forming units, in 200 mL of the finished product. For juice, nectar, and fruit beverages, the portion to be declared on the nutrition label is 200 mL, following Brazilian legislation [21]. The viable cell count did not change during the first 14 days of storage at both temperatures. After the 28th day of storage, it is possible to observe a drop in the number of viable cells, which lasts until the 60th day, when the counts rise again. However, this increase cannot be attributed to cell multiplication and therefore probably occurred as a consequence of the recovery of sublethally damaged cells after the encapsulation process.

According to the results, it is possible to conclude that the temperature did not influence the number of viable cells in the beverage, with no statistically significant difference between the counts, however, there is a difference between the storage time.

At the end of storage, it is possible to observe that there was a decrease of approximately 1 log in the viable cell count, for the sample stored at 30 °C, and 2 logs for the sample stored at 7°C, this decrease, however, does not affect the quality of the beverage, as at 180 days, the beverages still had a cell count above 6 logs, showing that the microencapsulation of the bacteria exerts a protective effect during storage, as a test performed with the non-encapsulated probiotics, demonstrated a cell count of 9.23 log CFU in 200 mL of apple juice.

3.2 Survival of *Lactobacillus acidophilus* in simulated gastrointestinal conditions

The beverages were submitted to tests to simulate the gastrointestinal conditions so that the survival of the probiotic bacteria was evaluated (Figure 1). The results are shown in Table 3.2.

The initial log indicates the initial content of *Lactobacillus acidophilus* in apple juice. The gastric phase indicates the number of microorganisms in stomach conditions, after a change in pH, and peristaltic movements. And, the enteric phases are the counts of microorganisms after passing through the duodenum.

There appears to be no statistically significant difference in the concentration of viable cells between the temperatures at which the beverages were stored. However, there was a statistically significant difference between the storage times, especially for the sample at 7 °C. It is possible to observe that the resistance of probiotics to the digestion process can be influenced by the storage time.

Table 3.2. Survival of *Lactobacillus acidophilus* in simulating *in vitro* gastrointestinal conditions

Sample	Time (days)	Initial log (CFU/ml $^{-200}$)	Gastric phase (CFU/ml $^{-200}$)	Enteric phase I (CFU/ml $^{-200}$)	Enteric phase II (CFU/ml $^{-200}$)
30°C	90	$8.00 \pm 0.01^{A,a}$	$6.60 \pm 0.01^{B,b}$	$6.40 \pm 0.00^{B,b}$	$6.36 \pm 0.00^{B,b}$
	125	$7.37 \pm 0.02^{A,a}$	$6.84 \pm 0.00^{A,b}$	$6.52 \pm 0.01^{B,c}$	$6.46 \pm 0.01^{B,c}$
	139	$7.07 \pm 0.02^{A,a}$	$6.27 \pm 0.01^{B,b}$	$5.72 \pm 0.02^{C,d}$	$5.74 \pm 0.00^{C,d}$
	155	$7.00 \pm 0.02^{A,a}$	$6.72 \pm 0.01^{A,b}$	$5.62 \pm 0.02^{C,d}$	$5.79 \pm 0.01^{C,d}$
	180	$7.14 \pm 0.00^{A,a}$	$6.72 \pm 0.00^{A,b}$	$5.92 \pm 0.01^{C,d}$	$5.48 \pm 0.02^{C,d}$
7°C	90	$8.00 \pm 0.02^{A},^{a}$	$6.52 \pm 0.01^{B,b}$	$6.27 \pm 0.01^{B,b}$	$6.15 \pm 0.01^{B,b}$
	125	$6.37 \pm 0.01^{B,a}$	$6.51 \pm 0.01^{B,b}$	$6.17 \pm 0.00^{C,b}$	$6.03 \pm 0.01^{B,b}$
	139	$7.07 \pm 0.02^{B,b}$	$6.13 \pm 0.02^{B,b}$	$5.78 \pm 0.01^{C,d}$	$5.16 \pm 0.01^{C,d}$
	155	$7.00 \pm 0.01^{B,b}$	$6.56 \pm 0.00^{B,b}$	$5.86 \pm 0.02^{C,c}$	$5.62 \pm 0.00^{C,c}$
	180	$6.89 \pm 0.00^{B,b}$	$6.88 \pm 0.01^{A,b}$	$6.79 \pm 0.02^{B,b}$	$5.62 \pm 0.01^{C,d}$

[A-C] Different superscript capital letters in the same column denote significant differences for each time ($p<0.05$)

[a] Different superscript small letters in the same row denote significant differences for each phase ($p<0.05$)

Up to the 125th day of storage, both samples had La-5 counts above 6 log in 200 mL of product, after passing through the TGI, showing that microencapsulation can have a protective effect during storage of the beverage and during passage through the TGI. The biggest differences were observed in the passage from the stomach to the intestine. The initial population of *Lactobacillus acidophilus* in the beverages showed a significant reduction of 2 logs, after 139 days of storage, in the enteric phase.

Regarding the final phase of the TGI simulation (enteric phase II), both samples showed a reduction of 2 log in viable cell count within 180 days.

Table 3.3. Survival of *Lactobacillus acidophilus* in simulating gastrointestinal conditions in vitro (whole capsule)

Sample	Time (days)	Initial log (CFU/ml $^{-200}$)	Gastric phase (CFU/ml $^{-200}$)	Enteric phase I (CFU/ml $^{-200}$)	Enteric phase II (CFU/ml $^{-200}$)
30°C	139	$6.16 \pm 0.04^{a,A}$	$5.22 \pm 0.00^{b,A}$	$5.24 \pm 0.02^{b,A}$	$5.80 \pm 0.04^{c,A}$
	155	$6.47 \pm 0.00^{a,A}$	$5.10 \pm 0.01^{b,A}$	$5.14 \pm 0.02^{b,A}$	$5.72 \pm 0.03^{c,A}$
	180	$5.97 \pm 0.01^{a,A}$	$5.31 \pm 0.00^{b,A}$	$5.51 \pm 0.04^{b,A}$	$5.69 \pm 0.01^{b,A}$
	139	$6.02 \pm 0.02^{a,A}$	$5.72 \pm 0.02^{b,A}$	$5.85 \pm 0.00^{b,A}$	$5.88 \pm 0.01^{b,A}$
	155	$6.01 \pm 0.03^{a,A}$	$5.59 \pm 0.00^{b,A}$	$5.60 \pm 0.01^{b,A}$	$5.62 \pm 0.01^{b,A}$
7°C	180	$6.56 \pm 0.02^{a,A}$	$5.16 \pm 0.05^{b,A}$	$5.60 \pm 0.00^{b,A}$	$5.22 \pm 0.00^{b,A}$

[A-C] Different superscript capital letters in the same column denote significant differences for each time ($p<0.05$)

[a] Different superscript small letters in the same row denote significant differences for each phase ($p<0.05$)

The results in the table above show that the viable cell count remained high even in the ileum, where the opening of the capsule should occur.

These results suggest that, for both temperatures, the resistance to the conditions of the TGI decreases during the storage time of the beverages.

3.3 Physico-chemical characterization

For pH, acidity, and soluble solids, there was a statistically significant difference between beverages stored at different temperatures (p-value = 0.01) at 90 days, as shown in Table 3.3.

There was a reduction in the values of pH and soluble solids, and an increase in the acidity values of the samples. Although statistical differences were observed, this reduction or increase was not substantial in these parameters evaluated, in the time and conditions evaluated.

Table 3.3. Physic-chemical evaluation of the different types of storage of probiotic apple beverages

Parameters	*Initial*	*7 °C*	*30 °C*
		90 days	
pH	4.50[a]	4.38 ± 0.01[a]	4.35 ± 0.00 [b]
Acidity (mg malic acid g^{-1})	4.52[a]	5.20± 0.10[a]	5.55 ± 0.80 [b]
Soluble solids (°Brix)	15.0[a]	13.6 ± 0.05 [a]	11.8 ± 0.00 [a]

Mean ± SD. Data in the same row with different superscript letters are significantly different at p≤0.05.

The highest acidity and pH values were found on the beverages stored at 30°C. The initial value of apple juice acidity, before the addition of the encapsulated probiotics, was 4.52. The results from this work have presented little variation after the addition of the probiotics

4. Discussion

4.1 Viability of the microencapsulated *Lactobacillus acidophilus* during storage

The two probiotic juice formulations have presented high counts after being stored for 180 days at 7 °C and 30 °C. This fact demonstrates the high viability of the probiotic strain under the adopted conditions.

According to Dias *et al*. (2018) [22], encapsulating agents have different physicochemical properties and such differences can influence the degree of protective effect on encapsulated bacteria when they are exposed to thermal stress during pasteurization and, therefore, lead to lactic bacterial survival at different levels.

In the study by Calabuig-Jiménez *et al*. (2019) [23], the content of *L. salivarius spp*. *salivarius* encapsulated and unencapsulated was determined in tangerine juice after 1, 3, 7, and 10 days of storage. The authors reported that after 10 days of storage, the content of *L. salivarius spp*. *salivarius* encapsulated was significantly higher than the unencapsulated one, proving that the microcapsule formed with alginate as a coating seems to be sufficient to guarantee the survival of probiotics in that juice.

Experimental studies have deepened the applicability of probiotics in the production of functional beverages and the highlight has been related to the good results obtained with the multiplication and viability of *Lactobacillus* and *Bifidobacterium* strains in pineapple juice and other fruits, such as apples and oranges [24, 2].

An important criterion in the production of probiotic beverages is the maintenance of the inoculum of at least 10^6 CFU mL^{-1} of the probiotic strain alive at the time of consumption [20].

In the work of Miranda *et al* (2019) [25], the probiotic count decreased on the 14^{th} day and then increased until the end of storage (21 days), which is similar to the results found in the present work. In this study, cells remained viable until the end of the observation period (180 days), with a reduction of approximately 1 log compared to the initial count.

Pimentel *et al* (2015) [27] analyzed the viability of *Lactobacillus paracasei* ssp. *paracasei* not encapsulated in clarified apple juice, and observed the loss of probiotic viability during juice storage, which may be related to the lack of protection of the bacteria. This makes them susceptible to the acidity and the presence of oxygen in the medium with the low level of nitrogenous compounds. When probiotic cells are present in low pH environments (<4.5), increased energy is required to maintain intracellular pH, possibly due to the absence of ATP for other critical functions, causing cell death. Continuous exposure to oxygen under acidic conditions during storage is the main reason for the reduction in probiotic counts.

This finding is in line with previous research papers that reported a decline in total viable cell counts of *Lactobacillus reuteri* and *Biofidobacterium bifidum* in whey-based probiotic beverages during storage at 4 °C after 30 days [28, 3]. However, the beverage proposed in this work presented a viable probiotic count in a sufficiently safe range, and thus be considered as a functional dose for humans, even after 100 days of storage under both conditions.

Haffner and Pasc (2018) [29] encapsulated probiotic bacteria with alginate and silica and added them to apple juice, the researchers highlighted in the study the importance of encapsulation, as the microencapsulated strains with alginate-maintained viability of 4 logs after 6 weeks and non-encapsulated strains did not survive after 5 weeks of storage. The authors concluded that encapsulation protects cells during storage and in a second step during the gastrointestinal passage.

Gandomi *et al* (2016) [30] also evaluated the effect of encapsulation of *Lactobacillus rhamnosus* GG with chitosan and alginate on the bacteria survival in apple juice. They observed that in the control group containing free bacteria, the survival rate decreased over the 90 days of storage; while for encapsulated *L. rhamnosus* was 4.5 times higher than those for free bacteria. The result of this study reinforces that the microencapsulation process exerts a protective effect on bacterial viability and it is in agreement with other studies, indicating the reductions of free probiotic bacteria counts 2-3 times than probiotics encapsulated in fruit juice storage.

Afzaal *et al*., (2019) [31] microencapsulated probiotics with carrageenan and sodium alginate, free and encapsulated cells were added to pasteurized grape juice samples. The initial count of free probiotic cells in grape juice was 9.35 log CFU mL^{-1} and there was a reduction to 6.58 log CFU mL^{-1} after 35 days. Likewise, the count of probiotics, that is, in samples containing encapsulated probiotics, decreased from 8.51 log CFU mL^{-1} to 7.09 log CFU mL^{-1} after the same storage period, demonstrating the protective effect of microencapsulation.

Silva *et al* (2021) [32] attribute that the survival of probiotic strains in fruit juice is related to pH with values close to 4.0 being the most adequate. These authors microencapsulated *Lactobacillus acidophilus* and applied them to different fruit juices, such as orange and apple. Regarding the free and microencapsulated forms, it was observed that *L. acidophilus* showed greater viability when added to fruit juices in the microencapsulated form, the microcapsule was formed by complex coacervation with gelatin-gum arabic as coatings, and was sufficient to protect and prolong their survival. Furthermore, in the case of encapsulated cells, the pH does not appear to affect significantly bacterial cells.

Calabuig-Jiménez *et al*. (2019) [23] observed that microcapsules from alginate were able to protect *L. salivarius*, increasing its survival in tangerine juice.

Mokhatari, Jafari, Khomeiri (2019) [33] encapsulated probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* with sodium alginate, by ionic gelation. The encapsulated cells and free cells were added to pasteurized grape juice and were stored for 60 days. At the end of the storage period, the survival capacity of the bacteria in the samples (P was significant<0.05), encapsulated cells were significantly higher than in the free ones (8.67 ± 0.12 and 7.57 ± 0.08 log CFU mL^{-1} for *L. acidophilus* and 8.27 ± 0.05 and 7.53 ± 0.07 log CFU mL^{-1} for *B. bifidum* for encapsulated and free forms, respectively).

The positive results found in our study for storage at refrigeration temperature can be explained by the maintenance of the bacteria in a latency state at low temperatures, a condition in which substance rates are lower and therefore allows the cells to settle recover from stressful conditions [22].

Impairment of bacterial viability during storage at higher temperatures can be correlated with lipid oxidation of bacterial cell membranes, which leads to changes in their composition and induces DNA damage, resulting in cell death. Furthermore, the use of higher storage temperatures also implies an increase in the metabolic activity of microorganisms and it can result in the accumulation of toxic metabolites, which are harmful to their viability [22].

In this study, the survival rates were observed to be up to 180 days of storage, even when the juice was stored at room temperature (30 °C), which is a good result considering that a non-dairy matrix (apple juice) was employed.

This result may be related to the fact that the cells of the probiotics are encapsulated, which means that the material is protected and isolated from any possible damage [22, 34].

The result found in our study was expected, since microcapsules are used to improve the survival of probiotics in foods, acting as a protective barrier against adverse conditions presented in fruit juices, such as acidity and oxygen [32].

The World Health Organization (WHO) defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host", while in the context of a food product, a minimum of 10^6 colony-forming units per mL (CFU mL^{-1}) must be reached for the food product to be labeled as a probiotic [35,36].

As observed in the current work, our two formulations of apple juice with probiotics, stored at different temperatures, presented counts between 7.14 and 7.12 log CFU in 200 mL of the finished product, after 180 days at 30 °C and 7 °C, respectively.

Thus, the presented results are very promising and can serve as a good basis for the development of an apple juice-based probiotic beverage.

4.2 Survival of *Lactobacillus acidophilus* in simulated gastrointestinal conditions

The resistance to GI stress is considered to be an important requirement for probiotic organisms in food products [34].

It was observed that *Lactobacillus acidophilus* was little affected by the in vitro digestion of beverages during storage up to 125 days. It is evident from Table 2 that the greatest survivability of the probiotic strain occurred in these first 125 days, but the strain remained viable until the end of storage. The efficiency of the encapsulation method and the stability of the protective material could explain the obtained results. However, the solubility of alginate salts at pH above 3.5 can leave the encapsulated microorganisms unprotected in the last stage of gastrointestinal digestion, which explains the results found in our study [23].

The sodium alginate used in the encapsulation makes the bacteria present thermotolerance, based on these properties, the alginate has been widely used in microencapsulation studies of various probiotic cells such as *Lactobacillus casei* Lc01, *Bifidobacterium bifidum* BB-02, *Lactobacillus paracasei* L26, *Lactobacillus acidophilus* KI and *Bifidobacterium animalis* BB-12 [12]. For example, Yeung *et al*. (2016) [37] studied the impact of encapsulating *L. lactis* in calcium alginate microspheres to mitigate sensitivity to environmental factors. The results demonstrated that the microcapsules protected the microorganisms from the stress conditions, resulting in an increase in the viability of the probiotics compared to non-encapsulated cells.

Phuong *et al*., (2021) [38] evaluated the effects of various polysaccharides (alginate, carrageenan, gums, chitosan) and their combination with prebiotic saccharides (resistant starch, lactosucrose, lactulose) on the encapsulation of probiotic bacteria *Lactobacillus casei* 01. To demonstrate the relevance of texture of the gel matrix for the bacterial protection capacity, free and microencapsulated *L. casei* 01 were digested with a sequential model of gastric and intestinal media. The viability of free cells of *L. casei* 01 was reduced by 50% and 80% after incubations for 45 and 135 min, respectively. The microencapsulated cells showed a better degree of gastric tolerance even in the presence of pepsin compared to the free cells. More than 60% of the initial microencapsulated cells were protected after exposure to simulated gastric fluid for 45 minutes.

In our study, where after 90 min of exposure and 90 days of storage, the count for both samples were 6.60 logs CFU/200 mL (for the sample at 30°C) and 6.52 log CFU/200 mL (for the sample at 7°C).

Almeida *et al*., (2021) [39] carried out a study to evaluate the viability of microencapsulated probiotic cells. They compared to non-encapsulated cells in mixed carrot and acerola juice during storage at 5°C for 28 days. After *in vitro* simulation of gastrointestinal conditions, at time 0, the viability of microencapsulated cells was 7.20 log CFU mL^{-1} while that of non-encapsulated cells was 4.07 log CFU mL^{-1}.

In the study by Afzaal *et al* (2019) [31], researchers evaluated the gastrointestinal survival of free and encapsulated probiotic bacteria added to pasteurized grape juice. Free/unencapsulated and encapsulated cells were exposed to simulated intestinal conditions for a specific time interval. A rapid decline in the free cell population was observed compared to cells encapsulated at pH 7.6. A slow logarithmic reduction was observed in encapsulated probiotics, which shows the protective effect of encapsulation.

Tarifa *et al* (2021) [40] investigated the survival of encapsulated *Lactobacillus casei* and *Lactobacillus rhamnosus* and concluded that the encapsulated cells had survival rates after passage through the larger stomach and intestine.

Moreover, Pankasemsuk and co-authors [41], reported that microencapsulated *L. casei* with 2% alginate and 1% hi-maize starch enabled optimal survival in both gastric and bile fluids.

The study by Rodrigues *et al*. (2020) [42], shows that showed that the microencapsulation of *Lactobacillus acidophilus* AS 1.2686 and *Bifidobacterium BB-12*, through a double emulsion formed by alginate and soybean oil, increased the viability of probiotic bacteria during 14 days of storage, compared to free cells, improved the stability and survival of bacteria during storage for 60 days at 25 °C [42]. And, under simulated gastrointestinal conditions, approximately 84% of the encapsulated cells remained viable after digestion, while free cells showed a rapid decline, and complete loss of viability, especially due to a low tolerance to acidic pH [42].

These results show that the particles obtained after microencapsulation are effective to protect the probiotic exposed to a simulation of gastric and enteric juices, as in both samples the bacteria remained viable after passing through the gastrointestinal tract. The particles obtained by microencapsulation protected the microbial cells under simulated gastrointestinal conditions and improved the encapsulation efficiency, reaching rates above 90%. In addition, the bacteria remained viable during storage of 120 days at 25 °C, which was also observed in our study [42].

Betoret *et al*., (2019) [43] observed the survival of *L. salivarius spp. salivarius* encapsulated by high pressure and incorporated into apple and tangerine juice. The samples were evaluated for a period of 30 days, and the sample that contained the encapsulated bacteria had a greater number of viable cells than those that were not encapsulated. The gastrointestinal simulation results showed protection of the microorganism due to the effect of the capsule, showing that encapsulation exerts a significant protective effect on bacteria during passage through the gastrointestinal tract.

Gandomi *et al.* [30] (2016) studied free and encapsulated probiotic *L. rhamnosus* for their stability in the simulated gastrointestinal condition in days 1, 45, and 90 of apple juice storage at 4 and 25°C. Encapsulation of *L. rhamnosus* improved bacterial viability, as 27.7% of the bacterial population survived the gastrointestinal transition model. The researchers found no statistically significant difference in bacterial survival between treatment groups stored at 4°C and those stored at 25°C. Comparing the survival rate of bacteria in apple juice at different storage times indicated that the GI stability of the bacteria decreased both for free bacteria and for encapsulated bacteria. However, the encapsulated groups showed better stability compared to free bacteria.

The ideal situation is that the probiotics were able to survive on low pHs, for example in the presence of bile salts and that they can withstand the conditions of the entire digestive tract [25]. In the present study, it was possible to observe that the *Lactobacillus acidophilus* was able to pass through the complete gastrointestinal tract.

The method used to make the capsule significantly influences the result [43]. In our case, the capsule provided protection. The typical porosity of particles produced with alginate and their degradation during storage may explain the decrease in *L. acidophillus* with storage time and passage through the gastrointestinal tract. The capsules were broken only in the ileum, the ideal place for probiotics to act in the gut.

With an encapsulation material, a controlled release of cells into the intestine takes place, thus ensuring that the polymer matrix structure is maintained until the end of the gastric stage [38].

Some authors [33, 35, 36, 44] reported that encapsulation protected cell viability after passage through the gastrointestinal tract, fermentation, and storage compared to free cells.

4.3 Physico-chemical characterization

The physicochemical properties of the two samples were similar during the period evaluated. The same happened with the study by Betoret *et al*., (2019) [43], where the addition of free and encapsulated probiotics to apple and tangerine juices did not affect the physicochemical properties of the beverages. The pH values of dehydrated apple juice with encapsulated *L. salivarius* were higher, with less variability than those obtained in samples with non-encapsulated microorganisms. Furthermore, the encapsulation process can decrease the activity of *L. salivarius* spp. *salivarius* resulting in a lower fermentation activity of microencapsulated cells that would produce less acidic compounds. At the end of storage (30 days), there were no differences between the two samples.

The content of simple sugars present in fruit juices can be metabolized by probiotics and, consequently, increase the acidity of the product. For example, *Lactobacillus* strains can produce organic acids by converting simple sugars present in juices, in addition, depending on the variations are also related to the metabolic activity of probiotics, which was observed in our study, where the pH of the samples increased from 4.52 to 5.2 and 5.5 (7° C and 30°C) [3, 45].

As the storage period advances, changes in the microbial population can also encourage changes in factors such as pH, reduction in sugar content, and, from there, alter other physicochemical parameters [3].

Gandomi *et al*, (2016) [30] evaluated the survival of *Lactobacillus rhamnosus GG* microencapsulated with inulin during apple juice storage. The pH changes of apple juices containing free and encapsulated bacteria during 90 days of storage at 4 and 25 °C were evaluated. As a result, the initial pH of apple juice significantly decreased after the addition of probiotics, whereas the pH of apple juice without probiotic bacteria remained relatively constant during storage at 4°C. Interestingly, the pH ranges found in juice stored at 25°C were similar to the ranges found in juice stored at 4°C. The same happened in our study, where, despite the juice stored at 30°C having a slightly higher acidity, the value was very close to the acidity found in the juice stored at 4°C.

The study by Silva *et al.,* (2021) [32] compared the effects of the addition of free and encapsulated bacteria on the pH of apple juice and orange juice, stored at 4°C for 63 days, and observed that the greatest pH variations were observed in treatments with the addition of free cells, indicating that microcapsules are efficient in reducing the interactions of probiotics with the environment of fruit juices, highlighting the importance of microencapsulation in probiotic viability.

The reduction in the soluble solids content in our samples was an expected effect, due to the fermentation of sugars present in fruit juices by *L. acidophilus*. The same effect was observed by Hruyia *et al.* (2018) [46] who also observed more marked reductions with the addition of different species of *Lactobacillus* in orange juice and by Calabuig-Jiménez *et al*. (2019), [23] who observed reductions in TSS when more microcapsules were added to the tangerine juice.

Swakhi et al. (2017) [47] evaluated the effects of adding *Lactobacillus acidophilus* and *Bifidobacterium lactis* to the physicochemical characteristics of apple juice, in the period of 0, 10, 20 and 30 days after inoculation, in juices stored at 31°C and 9°C. The authors observed that the soluble solids content decreased and the pH significantly decreased. The acidity of the apple juice also increased, as did the turbidity of the juice.

According to the literature, pH is the most relevant factor for probiotic viability, however, factors such as titratable acidity, presence of oxygen, water activity, presence of salt, sugar, and chemicals such as hydrogen peroxide, bacteriocins, flavorings, and artificial colors can influence the survival of probiotics in fruit juices [45].

Calabuig-Jiménez *et al* (2019) [23] conducted a study to evaluate the physicochemical characteristics of mandarin juice with the addition of microencapsulated *L. salivarius spp*. *salivarius*, and observed that there was a reduction in the soluble solids content after the addition of the encapsulated microorganism. The same happened in our study, where there was a reduction in solids content from 15.0°Brix to 13.6 and 11.8 (7° and 30°C respectively).

Xu *et al* (2019) [48] evaluated the chemical profile and flavor properties of mixed orange, carrot, apple, and Chinese jujube juice fermented with probiotics enriched with selenium, and after fermentation of the beverage, the pH value found was close to 3.29 ± 0.01. After a storage time of 3 weeks at 4 °C, the pH value was 2.80.

Mendes (2020) [49] studied mango juice with the addition of probiotics and concluded that the addition of a microencapsulated probiotic caused an increase in pH and a reduction in acidity, °Brix, and total solids content in the sample. In comparison with this study, our samples had an increase in the initial acidity value (4.52 mg malic acid g^{-1}), and a reduction in the pH value, after the addition of encapsulated bacteria, during storage at temperatures of 7 and 30 ° C, respectively (acidity values after the addition of encapsulated bacteria 5.2 and 5.5).

According to some studies [41, 50, 51, 52], adding probiotics to juices trends to reduce the pH. When the pH is below 4.5, pathogenic and deteriorating microorganisms can be inhibited, prolonging the shelf life of the food. In our study, pH values and soluble solids decreased after the addition of probiotic bacteria in both beverages. However, in the work of Ellendersen *et all.* [53], it was also used a probiotic fermented apple juice, and the cultures *Lactobacillus acidophilus* and *Lactobacillus casei* in apple juices of the Fuji and Gala varieties. After 20 hours of fermentation, the juice of the Fuji variety containing *Lactobacillus casei* showed a significant increase, with an initial pH of 4.01 and a final pH of 4.30.

5. Conclusions

Adding probiotics to fruit juice is an alternative for individuals with lactose intolerance, vegans, and other patients who want to reduce their consumption of dairy products.

Although the use of fruit juices as an alternative non-dairy option has been studied recently, few studies on juices with added probiotics have been published. Based on the results found in our study, apple juice with the addition of probiotics showed levels of *L. acidophilus*, with viable cells, above the value set by regulation, even after 180 days of storage.

Compared to beverages with probiotics available in the Brazilian market, which have a shelf life of 35 days under refrigeration, the encapsulation of the probiotic allowed the microorganism to be protected in the capsule with oligofructose, being able to survive the thermal processing applied to apple juice with probiotic.

This treatment made it possible to increase the shelf life to 180 days. This step also allowed the probiotic to survive to the acidity and pH conditions of apple juice even after passing through to the gastrointestinal tract, and thus reach the intestine at the recommended concentration to exert its probiotic effect.

Author Contributions: I.A.G. drafted and finalized the manuscript. F.S.G; A.P.O.R.; R.V.T.; O.F.S.; J.P.L coordinated the study. All authors have read and agreed to the published version of the manuscript.

Funding: This article was funded by of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Financing Code 001, Embrapa Agroindústria de Alimentos, Rio de Janeiro State Research Foundation (FAPERJ, E-26.202.749/2018) and National Council for Scientific and Technological Development (CNPq, 311936/2018-0).

Acknowledgments: This work was carried out with the support of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brazil (CAPES)—Financing Code 001, Embrapa Agroindústria de Alimentos, Rio de Janeiro State Research Foundation (FAPERJ, E-26.202.749/2018) and National Council for Scientific and Technological Development (CNPq, 311936/2018-0).

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 4

Evaluation of the acceptability of a probiotic beverage elaborated from whole apple juice

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Abstract

Consumer's demand for safe, fresh, healthy, and natural food has increased over the past two decades. Fruit juices are considered to be excellent sources of energy, fiber, and nutrients and are consumed by every age group. They are also sources of polyphenolic compounds and carotenoids, and many consumers include them as part of their diet. Foods with probiotics are highly sought after by consumers due to the associated health properties that make them the most popular functional foods. Probiotics have been used primarily in dairy products. However, non-dairy foods are increasingly being used as carriers of probiotics since the population has been presenting higher levels of lactose intolerance. Sensory quality is very important for the manufacture and consumption of products with added probiotics. Therefore, the aim of this study was to evaluate the sensory acceptability of an apple beverage with the addition of *Lactobacillus acidophilus*. The test was carried out in a supermarket in the city of Rio de Janeiro, with 114 participants. The beverage was well accepted by the participants, with an average acceptance score of 8.05. The addition of probiotics to apple juice did not change its acceptance. These results have indicated that apple juice can be used as a matrix for the production of a non-dairy probiotic beverage.

Keywords: fruit juice; non-dairy beverage; *Lactobacillus acidophilus*

1. Introduction

Apple (*Malus domestica*) is the fruit from the apple tree, a perennial, deciduous, and dicotyledonous plant in the *Rosaceae Rose* family. Apples are one of the most popular and abundant fruits in the whole world (Park *et al*., 2020).

The production of apples in Brazil has begun in the mid-70s, when it took advantage of the large granting of agricultural credits by the civilian-military regime, which aimed to “modernize” Brazilian agriculture through inputs, machinery, and pesticides (Motta; Motta, 2019).

Apples and its by-products are great sources of nutrients due to the high levels of bioactive substances in the composition. The consumption of apples and apple juice has increased in recent years, as this fruit and its components help improve the health of humans (Dias *et al*., 2019).

Apple cultivation in Brazil is a relatively recent activity. With tax incentives and support for research, southern Brazil has increased apple production in both quantity and quality, making the country self-sufficient and a potential exporter (Rajão *et al*., 2020).

Worldwide, the apple production chain plays an important economic and social role. With the production of close to 77 million tons, the fruit has an average annual per capita consumption of around 10 kilos. In this context, although accounting for only 1.4% of world production, in 2018 Brazil was among the ten largest apple producers, with an exploited area of about 33,500 hectares (Lazzarotto, 2018).

The Brazilian production is heavily concentrated in the states of Rio Grande do Sul (46.1%) and Santa Catarina (50.9%), reaching about 1.25 million tons of product (IBGE, 2018). The state of Santa Catarina is the largest apple producer, with around 54% of Brazil's total production during the 2014/2015 harvest, representing almost 612,000 tons. The state of Rio Grande do Sul appears as the second-largest apple producer, with about 493,000 tons, almost 43% of national production (Dias *et al*., 2019; Motta; Motta, 2019).

In terms of fruit consumption within the country, there is still great potential for expansion, given that, on average, this consumption is only 5.0 kg/person/year (Lazzarotto, 2018).

Despite its relevance, the national apple production chain is faced with a series of factors and risks that can significantly affect the technical performance and economic and financial viability of many production systems. Such factors are productivity level of orchards, quality and prices received for produced fruits, weather conditions, market issues, especially involving aspects of logistics, post-harvest, and imports of apples from competing countries. Thus, to offer apples competitively, it is necessary that producers make important investments in production, post-harvest, and management technologies (Lazzarotto, 2018).

The apple harvest campaign in the 2019/2020 season started, for the Gala, in February 2020 and, for Fuji, in April 2020, with a slight delay compared to previous crops, as the cold hours were lower than expected in the 2019 winter. In November 2019, the rainy weather in the southern orchards, especially in Santa Catarina, resulted in a higher incidence of fungal diseases (Brazil, 2020).

Data from the 2013 harvest has indicated that only 5% of the apples consumed by Brazilians are from other countries and 25% of the total countrywide produced apples go to the foreign market, mainly in the form of apple juice. A small portion (20% of the total exported)is in the unprocessed form, in addition to jelly and purees (Dias *et al*., 2019; Park *et al*., 2020).

In the production chain, apples are destined for fresh consumption and industrialization, the latter one being a way of adding value to the raw material that has been rejected for fresh commercialization. In recent years, around 30% of the Brazilian apple crops have been directed to industrialization and, within that, around 20 to 25% was destined for the production of juice. The Brazilian market for industrialized fruit juice has been growing, given that, in 2018, 15% of juice production was destined for the domestic market, whereas, a few years ago, this number was at 5%. In addition, the responsibility for this expansion is the ready-to-beverages juices (Ortiz, 2020).

Ready-to-beverages juice is characterized by having a number of soluble solids of 11.5 °Brix and can be made from freshly extracted juice or by diluting concentrated juice. The technique for preserving juice is the pasteurization process, which is characterized by the application of heat at a temperature of 90-95°C for 15-30 seconds. However, the use of heat treatment at high temperatures generates sensory and organoleptic losses for the product (Ortiz, 2020).

As consumer preferences usually evolve towards a greener environment and natural products, the consumer appeal for apple juice has increased. Therefore, apple juice has natural antioxidants such as polyphenols, which attractconsumer attention (Park *et al*., 2020).

The consumption of fruits, especially apples, has high acceptability. However, this consumption is also associated with a high degree of perishability. Thus, due to seasonal periods, it is necessary to apply technologies that can provide a better use of these raw materials. The use of new processes that can add value and also increase the shelf life of products generated from fruit, along with the diversity of research in the agro-industrial area, has been awakening a more innovative profile in the food market (Albuquerque *et al*., 2021).

Currently, foods that bring with them biological defense mechanisms, prevention, treatment, or delay of diseases, in addition to nutrition, are said to be functional, which are notable for being similar to conventional food and for being consumed as a normal part of the diet, but with proven capacity to promote physiological benefits with clinical evidence (Banwo *et al*., 2021).

Among the most diverse types of functional foods, probiotics stand out. Normally they are used in dairy products, as the objective of several recent studies relating to their health benefits has shown. For a food to be considered probiotic, it is recommended that it contain at least 10^8 to 10^9 CFU g^{-1} of product, with the bacteria of the genus *Lactobacillus* and *Bifidobacterium* being the most widely used for human consumption (Reque & Brandelli, 2021).

Traditionally, dairy products are used in the preparation of probiotic foods. However, there has been an increase in the demand for products that do not have an animal origin, due to the growth in the number of vegan consumers, as well as those with lactose intolerance and milk proteins allergies. The inclusion of probiotics in non-dairy foods is becoming an increasingly attractive option for the food industry. Therefore, these microorganisms have already been incorporated into several products, whether in the form of beverages or even as supplements in capsules (Cosme, Inês & Vilela, 2022).

In this context, it has been observed that traditionally probiotics are incorporated into dairy products, such as yogurt, fermented milk, among others. As these are products are directly linked to the proper functioning of the microbiota present in the intestinal microbiota, which is a complex communication system in the body, there is a clear need for the development of fruit juices with added probiotics, representing an option for diversification of products for industry, using a new plant matrix (Pimentel *et al*., 2020).

Due to the importance of this subject, the objective of this work was to elaborate an apple beverage with the addition of probiotics and to evaluate its sensory acceptability.

2. Materials and methods

2.1 Apple juice

Apple juices of the Fuji variety, produced at Embrapa Uva e Vinho (Brazil, Rio Grande do Sul) were used to prepare the apple beverage with probiotic.

2.2 Microorganisms

The probiotic culture *Lactobacillus acidophilus* La-5 LA-5 (ATCC 4356) was obtained from Chr. Hansen A/S (Hrrsholm, Denmark) in the lyophilized form, and was encapsulated by the ionic gelation technique using biopolymeric solution containing probiotic microorganisms and prebiotic agent Orafti®Oligofructose

(Beneo, Belgium). The solution was atomized directly in a gelling solution (calcium chloride) to produce the symbiotic microparticles (SILVA *et al*., 2017b).

2.3 Preparation of the beverage with probiotics

The process consists of adding 100 g of symbiotic encapsulated (Silva et al., 2017a) for each 1 L of whole apple juice from the cultivar Fuji. After homogenization, the beverage was pasteurized at 90 ° C for 30 seconds, followed by filling in glass bottles at 90 °C. The bottles were cooled in an ice bath for 5 minutes (Silva et al., 2017b). The resulting product was the apple beverage with probiotics. The beverages were stored at temperatures of 7°C.

2.4 Sensory Analysis

The sensory test was carried out in a supermarket located in the Barra da Tijuca neighborhood, in the city of Rio de Janeiro. A point with a table and banner was set up as a point of attraction for participants for the sensory analysis. The previously prepared beverage was stored in glass bottles and placed in thermos boxes with artificial ice sheets.

Participants were approached by the team, informed about the probiotic apple beverage and invited to participate in the research.

The beverage was stirred and served in individual portions of up to 30 mL, at ± 10°C 10 mL after the individuals informed that they would like to participate in the analysis (Guo *et al*., 2020). Consumers evaluated the beverage and rated it, answering a beverage acceptance questionnaire.

The questionnaire also contained questions to assess consumption habits and basic knowledge about probiotic microorganisms. These participants also answered a socioeconomic questionnaire assessing sex, age, education level, family income, and the frequency of probiotic food consumption.

2.5 Statistical analysis

Analysis of variance (ANOVA) was performed using the BioStat software. Differences at $p < 0.05$ (Tukey test, to perform a test of the difference between means) were considered statistically significant (Barbosa et al., 2020).

3 Results and Discussion

A study on the consumer perception of foods with probiotics was carried out with 114 consumers in a supermarket branch in the city of Rio de Janeiro.

Consumers evaluated the beverage developed and answered a questionnaire. The participants were mostly women (77%), with completed high school (25.4%), or university students (24.56%) or with a graduate degree (23.68%). Of these, 33.3% did not or rarely consumed foods with probiotics, and 53.5% consumed it from time to time, always or every day. Tables 4.1- 4.5 show the socioeconomic data of the test participants.

Table 4.1. Gender of participants

Male	***Female***
26	88

Table 4.2 Degree of education

Degree of education	**Number of participants**
Complete Elementary	3
Incomplete Elementary	3
Incomplete High School	3
Complete High School	29
Incomplete College	4
Complete College	28
Complete Post Graduation	27

Incomplete Post Graduation	1

Table 4.3 Age of participants

Age Group	***Number of participants***
<18	4
19-25	5
26-35	12
36-45	31
46-55	17
56-65	13
>65	18

Table 4.4 Family income

Family Income	***Number of participants***
1 to 5 minimum wages	7
> 5 to 10 minimum wages	16
> 10 to 20 minimum wages	11
>20 to 30 minimum wages	7
> 30 minimum wages	4

Table 4.5 Consumption of probiotics

Consumption frequency	***Number of participants***
Never	22
Rarely	15
Sometimes	24
Always	20

Every day	16

The beverage was well accepted by the participants, with an average acceptance score of 8.05, which is equivalent to the term "very good" in the evaluation scale used, as shown in table 4.6. Regarding the scores, 96% of consumers have given grades between 7, 8, and 9 (referring to the terms “good”, “very good” and “super good”, respectively).

Table 4.6. Consumers' assessment of acceptance

Grade awarded	Consumers (%)	Acceptance
9	35.09	Pretty good
8	41.23	Very good
7	20.18	Good
6	1.75	Just a little good
5	0.8	Maybe good or maybe bad
4	0.8	Just a little bad

p-value = <0.01

Pimentel, Madrona, and Prudencio (2015) have conducted a study aiming the evaluation of the sensory profile and acceptability of clarified apple juice, with the addition of *Lactobacillus paracasei* ssp. *Paracasei*, oligofructose and sucralose. The authors have concluded that the sensory profiles of the clarified apple juice formulations indicated that the addition of probiotic cultures, oligofructose, and sucralose do not substantially change the intensity of the intrinsic attributes of apple juices (flavor of apple, apple flavor, sour taste). This fact is of vital importance in the reformulation of juices, as consumers want functional products with reduced sugar content that have similar characteristics to conventional products on the market.

Regarding acceptability in terms of appearance, fragrance, flavor, texture, and overall impression of the juices, the most frequent scores received by apple juice were between 7 and 8 on a 9-point hedonic scale, indicating that consumers liked the products from "moderately" to "a lot". As for purchase intent, the results were found close to 4 on a 5-point scale for all formulations tested, indicating that consumers would likely buy the juices (Pimentel, Madrona, and Prudencio, 2015). The high acceptability of the products by consumers was an interesting result, which was also found in our study, considering that Brazilians are not used to consuming apple juice.

Studies evaluating the incorporation of probiotic cultures in fruit juices and/or nectars were contradictory, with some indicating no change in product acceptance (Bevilacqua *et al*., 2013) and others reporting a loss of acceptance due to the presence of an unpleasant taste (Luckow & Delahunty, 2004a, 2004b; Saeed *et al*., 2013). This fact, which was observed in the study conducted by Fonseca et al (2021), which aimed to investigate the effect of *Lactiplantibacillus plantarum* CCMA 0743 in monoculture and co-culture on volatile compounds and sensory profiles of fermented passion fruit juice. However, the sensory profile of passion fruit juice was modified by simple fermentation and in co-culture. The fermented samples were mainly correlated with the terminology “salty, acidic and bitter flavors” and “sweetening aftertaste”. In this study, despite the passion fruit juice has shown to be an adequate food matrix to deliver the evaluated strains, these strains affected the fermented product. These differences may be related to the type of fruit juice and the probiotic culture used.

Chavan, Gat, Harmalkar and Waghmare (2018) have compared three non-dairy probiotic beverages and have observed that the overall acceptability score was around 7.1 and 8.9, and that the acceptability of the probiotic beverage based on coconut extract was higher when compared to distilled water sample, soy vegetable beverage, almond vegetable beverage.

Yuasa *et al*., (2021) have compared in their study, the sensory acceptance of two fermented beverages, both made from citrus juices (hyuganatsu juice, tangerine juice, and orange juice) and *L. plantarum* SI-1 and *L. pentosus* MU-1. The obtained results suggested that some citrus juice preferences were not altered by the lactic acid fermentation. The researchers concluded that fermented beverages made from citrus juices and lactic acid bacteria can be suitable probiotic food for people who do not like the taste of fermented milk. However, the chemical composition of long-term fermented citrus juice may be different. Therefore, the effects of long-term fermentation on citrus juices should be investigated in future studies.

In the study by Islam *et al*. (2021), the aim was to develop a probiotic beverage from whey and pineapple juice, 15 people have evaluated the sensory parameters such as color and appearance, flavor, tastiness, sweetness, and general acceptability. The sample that received the highest score for color and appearance, and flavor and tastiness among the prepared beverages was the sample with 75% pineapple juice and 25% whey in its composition. This shows that the use of fruit juice in the production of probiotic beverages has good acceptability. In addition, this can be important knowledge for the probiotic beverage industries.

In our study, consumers were given the opportunity to express their perceptions about the sensory characteristics of products. The format of the CATA questionnaire was used, as it allowed consumers to choose all possible attributes to describe the product, from a presented list. The main attributes responsible for this acceptance were "apple flavor" and "ideal sweetness", as shown in table 4.7.

Table 4.7. Attributes that consumers liked the most in the beverage

Attributes	***Number of participants***
Apple Flavor	48
Swetness	21
Nothing	39
No sugar	4
Lightness	4
Everything	1
Natural Flavor	3
Addition of probiotics	1
No bitter	1

Freshness	1
Healthy	1
Not cloying	1

There was no significant difference ($p > 0.05$) for the attributes evaluated, as shown in Table 4.7.

In this study, the evaluators were not asked to indicate the intensity of the selected terms. It was chosen a shortlist of selected terms, as a long list could cause a “dilution” effect, harming the results. Therefore, different terms were chosen that referred to the relevant sensory characteristics for the evaluated product, with the aim of recognizing the consumer's heterogeneity (Alcântara & Freitas-Sá, 2017).

Xu *et al* (2019) in their study, evaluated the flavor properties of the mixture of orange, carrot, and apple juice, fermented by probiotics and enriched with selenium. As a result, the researchers concluded that the process of probiotic bacteria fermentation affects the composition flavor, as there is an increase in the number of alcohols and esters during fermentation, and high levels of alcohols can potentially further increase the fragrance characteristic of fermented juices. Fermentation time is a significant factor that can modify metabolic activity and control flavor attributes.

During the test, only 8.8% of the interviewees sought information about the beverage they were tasting, the information presented on the banner available for viewing, or with the team of analysts present. This fact is in agreement with data already described in the literature that when choosing products in the supermarket, the consumer bases his choices on the product's ease of "use", its sensory properties, palatability, and, ultimately, advertisements, with nutritional and health claims. In most cases, those claims have not been proven by scientific evidence, but they make the consumer feel good and consider their diet to be very healthy (Muñoz, 2018).

However, 94.5% of them said they considered the probiotic beverage to be healthy, and 93.3% said they considered the beverage to be able to prevent diseases, as it contains live microorganisms that reduce the multiplication of pathogenic bacteria, preventing changes in the intestinal microbiota. The socioeconomic level changes the perception of whether one food is healthier than another is. Therefore, ultra-processed foods are perceived as healthier by consumers with a lower socioeconomic level, compared to less processed foods, which obtain this health attribute by individuals belonging to the classes with higher purchasing power (Muñoz, 2018). In this study, 48.1% of respondents had completed or incomplete undergraduate and/or graduate degrees, which indicates that the health benefits arising from the “probiotic” claim, are already established among consumers (mainly among those with more access to information).

It is important to emphasize that, according to the Guide for the assessment of functional and health property claims for bioactive substances, present in foods and food supplements, published by the Agência Nacional de Vigilância Sanitária (ANVISA), both the health property claims, which is one that "affirms, suggests or implies the existence of a relationship between the consumption of a food or ingredient and a disease or health-related condition, aiming at reducing the risk of the disease", as for the claim of functional property, must be proven and supported by consistent and reliable scientific evidence (Brazil, 2021).

When asked if they would regularly consume the beverage they tasted, 7% answered yes, just because they liked it; 18% answered yes, considering only its beneficial properties to health; while 73% answered yes, considering both their sensory characteristics and their health-beneficial properties. This shows that the sensory factor (palatability) is still relevant in foods with functional health claims.

Probiotic beverages can be made from a variety of raw materials such as cereals, corn, legumes, fruits, and vegetables (Chavan, Gat, Harmalkar, Waghmare, 2018). Recent technological advances have made it possible to change and improve the characteristics of fruits and vegetables, modifying the internal and external components of these foods in a controlled manner. They are a good substrate for probiotics due to the presence of antioxidants, dietary fiber, minerals, and vitamins (Ilango & Antony, 2021).

4. Conclusions

With the trend of increasing consumer preference for foods with a fresher taste and higher nutritional value, the production of ready-to-beverages that promote health and with satisfactory organoleptic properties has been increasing.

Fruit juices have been found suitable for addition to probiotic cultures since they contain beneficial nutrients, have flavor profiles considered pleasant by people of all ages, are considered healthy and refreshing beverages, and are consumed regularly, a quality that is essential for obtaining the benefits attributed to probiotics.

The development of non-dairy beverages with the addition of probiotics has been considered a focus of study, aiming at the improvement of the nutritional value and the variety of choices for vegetarian and lactose-intolerantconsumers.

In the development of new functional products, it is necessary to understand the sensory impacts of these components and determine how their addition to products can influence the acceptability and consumer preference.

This study has provided important contributions for the apple juice industries, and for researchers in the field of functional foods. Apple juice was considered a suitable medium for the incorporation of *Lactobacillus acidophilus*, as a probiotic culture, providing products with satisfactory sensory characteristics.

This study has also provided important knowledge for the continuity of the work of developing probiotic beverages based on non-dairy food matrices, especially using fruits, in relation to the nutritional and functional properties, which are important for consumers and for the food industry.

Thus, it was concluded that it is possible to prepare a non-dairy beverage with the addition of probiotics, having whole apple juice as a base, which can please consumers.

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CONCLUSÃO E PERSPECTIVAS FUTURAS

Os sucos de frutas, como a maçã, são considerados matrizes candidatas à incorporação de probióticos, pois são ideais para consumidores que se interessam por alimentos com baixo teor de colesterol, sofrem de intolerância à lactose e/ou são vegetarianos. Um desafio que a indústria tem para o desenvolvimento de sucos probióticos é a sobrevivência das cepas probióticas durante o armazenamento do produto. O baixo pH e a presença de fenólicos têm sido citados como os principais fatores associados à baixa sobrevivência de probióticos em sucos de frutas.

No entanto, estudos anteriores relataram que a sobrevivência de probióticos cepas em sucos de maçã, podem ser aumentadas por constituintes naturais do suco bem como por técnicas de microencapsulação que protegem os probióticos de condições adversas. A manutenção da viabilidade probiótica em matrizes alimentares antes do consumo é fundamental e continua sendo um desafio para a entrega bem-sucedida das células nos intestinos.

Os resultados encontrados em nosso estudo, indicam que o suco de maçã é um veículo adequado para fornecer as cepas de *Lactobacillus acidophilus*, pois as bactérias apresentaram a capacidade de sobreviver nos sucos armazenados sob refrigeração e em temperatura ambiente ao longo do tempo.

O suco de maçã foi considerado um meio adequado para a incorporação de *Lactobacillus acidophilus*, como cultura probiótica, proporcionando produtos com características sensoriais satisfatórias, além de ter um tempo de prateleira maior do que os produtos encontrados no mercado atualmente.

Mais estudos devem ser realizados com as cepas de *Lactobacillus acidophilus* para que seja avaliado o potencial dessas bactérias em adsorver a patulina presente em suco de maçã.

CONCLUSION AND FUTURE PERSPECTIVES

Fruit juices, such as apples, are considered candidate matrices for the incorporation of probiotics, as they are ideal for consumers who are interested in low-cholesterol foods, suffer from lactose intolerance, and/or vegetarians. One challenge that the industry has for the development of probiotic juices is the survival of probiotic strains during product storage. Low pH and the presence of phenolics have been cited as the main factors associated with the low survival of probiotics in fruit juices.

However, previous studies have reported that the survival of probiotic strains in apple juice can be increased by natural constituents of the juice as well as by microencapsulation techniques that protect the probiotics from adverse conditions. Maintaining probiotic viability in food matrices prior to consumption is critical and remains a challenge for the successful delivery of cells to the intestines.

The results found in our study indicate that apple juice is a suitable vehicle to deliver Lactobacillus acidophilus strains, as the bacteria showed the ability to survive in juices stored under refrigeration and at room temperature over time.

Apple juice was considered an adequate medium for the incorporation of Lactobacillus acidophilus, as a probiotic culture, providing products with satisfactory sensory characteristics, in addition to having a longer shelf life than products found on the market today.

More studies should be carried out with strains of Lactobacillus acidophilus to assess the potential of these bacteria to adsorb patulin present in apple juice.