UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia de Alimentos

FÁBIO JÚNIOR RODRIGUES

Limosilactobacillus reuteri: The *in situ* reuterin production in film-forming solutions, immobilization in natural polymer blends and inhibitory effect against pathogenic and spoilage microorganisms

Limosilactobacillus reuteri: Produção *in situ* de reuterina em soluções formadoras de filme, imobilização em blends de polímeros naturais e efeito inibitório contra micro-organismos patogênicos e deteriorantes

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RESUMO

Limosilactobacillus reuteri é uma bactéria heterofermentativa, não patogênica que apresenta capacidade de produzir reuterina via fermentação anaeróbica do glicerol. Esse composto apresenta características antimicrobianas sendo relacionado à atividade probiótica da bactéria. Assim, e em particular considerando a crescente preocupação com a saúde humana, tanto a suplementação nutricional utilizando probióticos quanto novas estratégias de biopreservação de alimentos vêm sendo estudadas. Neste sentido, são inúmeros os estudos envolvendo soluções filmogênicas à base de polissacarídeos como suporte para entregar e proteger células microbianas, ou ainda para produção de filmes e revestimentos comestíveis para alimentos, visando prevenir ou reduzir a contaminação por micro-organismos. Neste contexto, essa tese possui vasta literatura sobre *L. reuteri*, reuterina, probióticos, encapsulamento de micro-organismos, aprisionamento em revestimentos comestíveis, além de técnicas e materiais utilizados para essas finalidades. Ademais, utilizando cepas de *L. reuteri* (DSM 20016 e 17938) a produção de reuterina *in situ* em solução filmogênica à base de alginato e glicerol foi otimizada, atingindo concentrações de até 68,39 mmol/L. Em seguida, partindo das condições ótimas pré-estabelecidas, foram produzidas novas soluções filmogênicas contendo em sua composição materiais mucilaginosos pouco ou ainda não explorados para essa finalidade (psyllium, konjac, mutamba, cassia tora ou tamarindo). Essas soluções foram utilizadas para encapsular por extrusão células de *L. reuteri* e a reuterina produzida *in situ*. O uso dos materiais mucilaginosos associados ao alginato produziu partículas que melhoraram a eficiência de encapsulamento tanto para células de *L. reuteri* quanto para reuterina, atingindo taxas de 97,4 e 45 %, respectivamente. Além disso, foi aprimorada a sobrevivência de células de *L. reuteri* durante armazenamento refrigerado (4 ± 2 °C), especialmente em armazenamento prolongado de 20, 30 e 60 dias, e em condições gástrica e entérica simuladas (76.6 %). Devido seus possíveis efeitos antimicrobianos e utilização em tecnologias de conservação de alimentos, foi investigado o efeito inibitório de *L. reuteri* e da reuterina contra micro-organismos patogênicos e deteriorantes de origem alimentar. Desta forma, além dos ensaios antimicrobianos utilizando sobrenadantes de *L. reuteri*, foram produzidos e utilizados para mesma finalidade filmes comestíveis a partir de solução mucilaginosa à base de alginato e goma de konjac, contendo células de *L. reuteri* e nos quais a produção de reuterina foi induzida. Tanto os sobrenadantes como os filmes comestíveis demonstraram efeitos antimicrobianos contra *Bacillus cereus*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, além dos fungos *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Alternaria alternata* e *Penicillium digitatum*. Diante ao exposto, essa tese traz informações sobre novas soluções mucilaginosas como material de suporte para compostos biológicos e/ou químicos, possibilitando suas aplicações na entrega de micro-organismos ou no desenvolvimento de revestimentos comestíveis. Além disso, os efeitos antimicrobianos do *L. reuteri* e da reuterina podem ampliar o potencial de aplicação em alimentos.

Palavras-chave: *Lactobacillus reuteri*; 3-hidroxipropionaldeído; microencapsulação; mucilagem; goma; atividade antimicrobiana; filme comestível.

ABSTRACT

Limosilactobacillus reuteri is a heterofermentative, non-pathogenic bacterium able to produce reuterin via glycerol anaerobic fermentation. This compound has antimicrobial characteristics and is related to the probiotic activity of the bacterium. Thus, and in particular considering the growing concern with human health, both nutritional supplementation using probiotics and biopreservation strategies for foods have been studied. Currently, there are numerous studies involving polysaccharides-based film solutions as support to deliver and protect microbial cells or even to produce edible films and coatings for foods, aiming to prevent or reduce the contamination by microorganisms. In this context, this thesis has a wide literature on *L. reuteri*, reuterin, probiotics, encapsulation of microorganisms, entrapment in edible films, besides techniques and materials used for these purposes. In addition, using *L. reuteri* strains (DSM 20016 and 17938), the *in situ* reuterin production in alginate- and glycerol based-film-forming solution was optimized, reaching 68.39 mmol/L. Then, starting from the pre-established optimal conditions, new film-forming solutions containing in their composition mucilaginous materials few or not yet explored (psyllium, konjac, mutamba, cassia tora, or tamarind) were produced. These solutions were used to encapsulate *L. reuteri* cells and the *in situ*-produced reuterin by extrusion. The use of mucilaginous materials with alginate produced particles that improved the encapsulation efficiency for both *L. reuteri* and reuterin, reaching 97.4 and 45 %, respectively. In addition, the survival of *L. reuteri* cells during refrigerated storage (4± 2 °C), especially in prolonged storage of 20, 30 and 60 days, and under simulated gastric and enteric environments (76.6 %) were enhanced. Due to their possible antimicrobial effects and use in food preservation technologies, the inhibitory effect of *L. reuteri* and reuterin against foodborne pathogenic and spoilage microorganisms was investigated. Thus, in addition to antimicrobial assays using *L. reuteri* supernatants, for the same purpose, were produced alginate-konjac gum edible films containing *L. reuteri* cells and in which the reuterin production was induced. Both supernatants and edible films showed antimicrobial effects against *Bacillus cereus*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, besides the fungi *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, and *Penicillium digitatum*. In view of our findings, this thesis brings insights on new mucilaginous solutions as support material for biological and/or chemical compounds, enabling their applications in the delivery of microorganisms or in the development of edible films. In addition, the antimicrobial effects of *L. reuteri* and reuterin may expand the potential for food application.

Keywords: *Lactobacillus reuteri*; 3-hydroxypropionaldehyde; microencapsulation; mucilage; gum; antimicrobial activity; edible film.

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GENERAL INTRODUCTION

Lactobacillus reuteri (Kandler et al., 1980) is a recognized bacterium that has gained prominence in the scientific community. It has been acknowledged by European Food Safety Authority as a dietary supplement for enhancing gastrointestinal well-being (EFSA, 2008). This microorganism promotes beneficial effects on the health of the host and, as a result, is classified as a probiotic bacterium (Mu, Tavella, & Luo, 2018). The Food and Agriculture Organization of the United Nations (FAO), in collaboration with the World Health Organization (WHO), defines the concept of probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). In October 2013, an expert panel convened by the International Scientific Association for Probiotics and Prebiotics (ISAPP) reinforced the significance of this concept for current applications (Hill et al., 2014).

Simultaneously, it is worth noting that recent advancements in research have brought about significant changes in the classification of the *Lactobacillus* genus. Through a thorough analysis of phylogenetic, phenotypical, and habitat specificities, the genus has been divided into 25 genera. As a result, *Lactobacillus reuteri* has been reclassified and is now known as *Limosilactobacillus reuteri* (Zheng et al., 2020) The relevant characteristic of this non-pathogenic bacterium lies in its remarkable ability to produce and accumulate substantial quantities of 3-hydroxypropionaldehyde (3-HPA) when subjected to anaerobic conditions and a medium containing glycerol. This reaction is accomplished via an enzymatic reaction driven by the glycerol dehydratase enzyme (Vollenweider & Lacroix, 2004; Sardari et al., 2013).

The 3-HPA belongs to HPA system, a dynamic system comprising 3-HPA, its hydrated form, and its dimer (Vollenweider et al., 2003). However, Engels et al. (2016) have proposed a recent study that suggests the inclusion of acrolein in the HPA system. It has been observed that in aqueous solution, 3-HPA can undergo dehydration, leading to the formation of acrolein. Produced by *L. reuteri* microbial cultures, 3-HPA has been patented under the name reuterin (Dobrogosz & Lindgren, 1995) and has attracted significant scientific interest due to its antimicrobial properties,

including broad-spectrum activity against both Gram-positive and Gram-negative bacteria, as well as antifungal activity (Talarico & Dobrogosz, 1989; Vollenweider & Lacroix, 2004). These properties remain effective even under challenging conditions, such as acid pH, elevated saline concentrations, and refrigeration temperatures (Rasch et al., 2007; Arqués et al., 2008). However, the precise mechanism by which reuterin inhibits microbial cells is not yet fully understood. Talarico & Dobrogosz (1989) proposed that reuterin may inhibit the ribonucleotide reductase enzyme, thereby interfering with DNA synthesis. Subsequently, Schaefer et al. (2010) emphasized that reuterin can deplete thiol groups in important cellular components like glutathione, proteins, and enzymes. This depletion leads to oxidative stress within the microorganisms, resulting in cell death.

From another perspective, there is a growing interest within the scientific community in developing strategies for food bioconservation (Barcenilla et al., 2022) and encapsulation techniques capable of promoting the effective protection and delivery of probiotic bacteria aimed at enhancing human health and well-being (Rodrigues et al., 2020). Consequently, several studies have been conducted on the production of film-forming solutions used as a support for microorganisms, which act in the production of particles responsible for preserving the viability of encapsulated cells under adverse conditions, such as prolonged storage, acidic environments, high osmotic pressure, etc. (Cedran, Rodrigues & Bicas, 2021). Furthermore, these film-forming solutions are also used in the production of edible films and coatings for food, serving as an alternative in preventing or reducing microbial contamination, factors directly associated with the increased shelf life of coated foods (Díaz-Montes & Castro-Muñoz, 2021). It is common that glycerol to be employed in these solutions to enhance gel properties. However, although glycerol can be utilized as a substrate by *L. reuteri* for the production of reuterin, the *in situ* synthesis of this relevant antimicrobial compound in film-forming solutions has not been reported so far.

In general, film-forming solutions can be produced using various hydrocolloids. Alginates were widely used; however, their utilization often leads to the production of gels with higher porosity and susceptibility to disintegration, factors that can affect the efficacy of encapsulated agents' protection. Consequently, there is a growing interest in combining alginate with other hydrocolloid materials, such as gums

and mucilages, to improve the properties of the formed gels (Rodrigues et al., 2020). Derived from natural sources, mucilaginous materials obtained from konjac tuber (*Amorphophallus konjac*) and seeds of tamarind (*Tamarindus indic*a), mutamba (*Guazuma ulmifolia*), psyllium (*Plantago ovata*) and cassia tora (*Cassia tora*) are hydrocolloids that have been underexplored or not yet explored in the composition of film-forming solutions (Pawar et al., 2014; Fernandes, Acharya, & Bhatt, 2018; Mu et al., 2018; Pereira et al., 2019; Crispín-Isidro et al., 2019).

Based on the stated, this thesis investigated the utilization of *Limosilactobacillus reuteri* to optimize the *in situ* production of reuterin in alginate-based film-forming solutions through the bioconversion of glycerol. Furthermore, the impact of mucilaginous solutions derived from mutamba, tamarind, konjac, cassia tora, and psyllium on alginate particles carriers of *L. reuteri* cells and reuterin, encapsulated via the extrusion technique was examined. From these hydrocolloid blends, we also prepare an edible film with reuterin content *in situ*-produced and comprehensively investigated the antagonistic activity of *L. reuteri* and the edible film against foodborne pathogenic and spoilage microbial cells. In general, these investigations have not only demonstrated the successful incorporation of novel hydrocolloids derived from natural sources in microorganism encapsulation methods but also highlighted their potential for producing valuable edible films and coatings for the food and pharmaceutical industries.

OBJECTIVES

General objective

This thesis aimed to produce film-forming solutions based on hydrocolloid blends containing reuterin-producing *Limosilactobacillus reuteri* and glycerol, aiming to understand and optimize the *in situ* reuterin production. These solutions were extensively investigated in studies involving the encapsulation of *L. reuteri* and the production of edible film, both through crosslinking with calcium ions. Additionally, the antimicrobial efficacy of *L. reuteri* strains and the edible film against foodborne pathogenic and spoilage microorganisms was evaluated.

Specific objectives

- To present current knowledge and perspectives on the encapsulation of probiotics and hydrocolloid materials available for this purpose;
- To fill a knowledge gap in the scientific community, confirming the suitable potential of new hydrocolloids as carrier materials for the delivery of biological or chemical agents;
- To optimize the *in situ* reuterin production in film-forming solutions based on alginate, varying the concentration of glycerol and inoculum of *L. reuteri* DSM 20016 and DSM 17938;
- From film-forming solutions optimized for reuterin production, immobilize *L. reuteri* strains in beads produced with mucilaginous solutions extracted from mutamba (*Guazuma ulmifolia*), psyllium (*Plantago ovata*), cassia tora (*Cassia tora*), tamarind (*Tamarindus indica*) and konjac (*Amorphophallus konjac*), assessing the effectiveness of polymeric matrices into parameters, which include measurements of encapsulation efficiency, monitoring the viability of cells over time, and subjecting them to simulated digestive environments;
- To assess the supernatants of *L. reuteri* strains for their inhibitory effects against foodborne pathogenic and spoilage microorganisms;

- To assess the inhibitory effects of edible films containing reuterin *in situ*-produced against foodborne pathogenic and spoilage microorganisms.

CHAPTER I

REVIEW ARTICLE

Encapsulated probiotic cells: relevant techniques, natural sources as encapsulating materials and food applications – a narrative review

Fabio Júnior Rodrigues, Marina Felix Cedran, Juliano Lemos Bicas, and Hélia Harumi Sato

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Encapsulated probiotic cells: relevant techniques, natural sources as encapsulating materials and food applications – a narrative review

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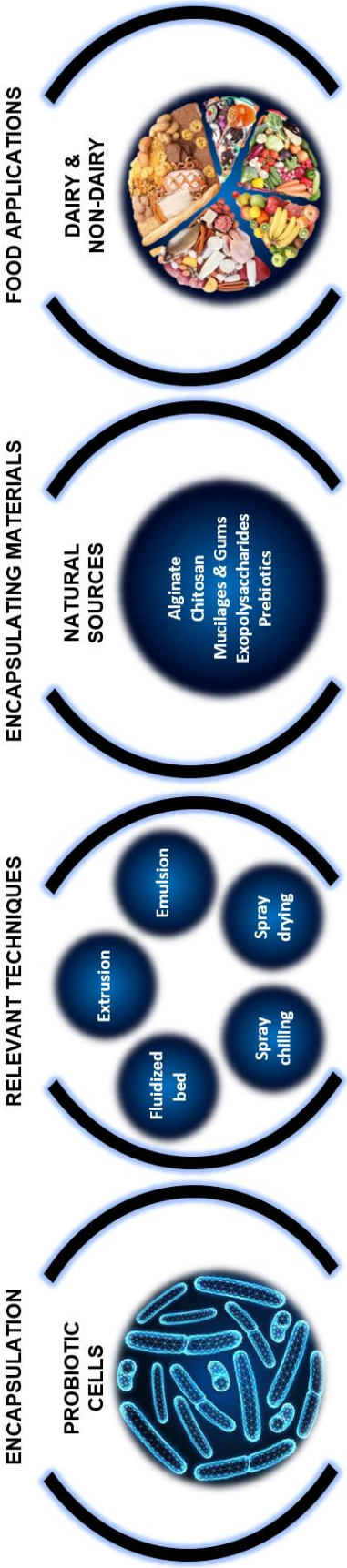
Highlights

- Alginate and extrusion technique are still widely used to encapsulate probiotics.
- Gums/mucilages as encapsulating material improves the survival of encapsulated cells.
- Cell encapsulation improves the survival of probiotics in food matrices.
- Encapsulation turns non-dairy food products into alternative to deliver probiotics.

Graphical abstract



Abstract

The administration of probiotic microorganisms in adequate amounts is constantly related to health benefits. To promote beneficial effects, these microorganisms must not be affected by exposure to environmental factors and must be able to adhere and colonize the human gastrointestinal tract. Several encapsulation techniques and encapsulating materials are available to produce probiotic particles, however, it is essential that the process must not be aggressive, reducing or preventing injuries and cell losses, besides, the particle properties obtained must be adequate for the proposed purpose. At the same time, the global market for supplements and probiotic foods has been growing significantly, and cell encapsulation appears as an alternative to incorporate probiotics into different food matrices. This review discusses and updates the main techniques, and the traditional and emerging polysaccharides for encapsulation of probiotic cells, as well as the advantages and possibilities of incorporating produced particles into food matrices. Currently, various scientific studies report the use of different encapsulation techniques, such as extrusion, emulsion, spray drying, spray chilling and fluidized bed to encapsulate probiotics properly. The alginate is still widely used to produce probiotic particles, however, there has been a growing interest in its total or partial substitution with others polysaccharides, such as gums, mucilages, prebiotic compounds and microbial exopolysaccharides, which improve the protection and survival of encapsulated cells and allow their incorporation into dairy and non-dairy food products.

Keywords: microencapsulation; immobilization; wall material; gastrointestinal survival; functional food; non-dairy food; mucilage; gum

1 Introduction

Recognized for promoting health and wellness, probiotics have been related to beneficial effects, including the modulation of the intestinal microbiota, through the inhibition of pathogenic microorganisms; the production of anti-carcinogenic compounds; the modulation of immune responses, etc (Prakash et al., 2011; Reis et al., 2017; Markowiak & Śliżewska, 2017). However, to promote beneficial effects, microorganisms with probiotic claims must survive exposure to environmental factors, being able to colonize and maintain the metabolic activity in the human intestinal tract (Collins, Thornton, & Sullivan, 1998; Saarela et al., 2000).

In this sense, the cell encapsulation may improve the resistance of probiotic microorganisms to adverse conditions (Kim et al., 2017; Rodrigues et al., 2017), reducing cell losses of encapsulated microorganisms in hydrocolloid matrices. Currently, different probiotic encapsulation techniques are used, in which particles of different properties are obtained (Cavalheiro et al., 2015). Among the main techniques used to encapsulate probiotic cells are extrusion (Krasaekoopt & Watcharapoka, 2014; Rodrigues et al., 2017; Kim et al., 2017; Silva et al., 2018a; Dimitrellou et al., 2019), emulsion (Zhang, Lin, & Zhong, 2016; van der Ark et al.,2017; Raddatz et al., 2020), spray drying (Rajam & Anandharamakrishnan, 2015; Bustamante et al., 2017; Santos et al., 2019; Aragón-Rojas et al., 2020), spray chilling (Pedroso et al., 2012; Bampi et al., 2016; Arslan-Tontul & Erbas, 2017; Silva et al., 2018b; Arslan-Tontul, Erbas & Gorgulu, 2019) and fluidized bed (Silva et al., 2018a; Horison & Surono, 2020).

The selection of suitable encapsulating materials is also essential for the stability of the particles produced. These materials must not present toxicity and protect the encapsulated microbial cells in environments that favor cell injury. In addition, they must control the release during passage through the human gastric and intestinal tract (Rathore et al., 2013; Chen et al., 2017a). Although polysaccharides, proteins and lipids have been used to immobilize probiotics (Rajam & Anandharamakrishnan, 2015; Rodrigues et al., 2017; Arslan-Tontul & Erbas, 2017), natural water-soluble polysaccharides extracted from mucilages and gums have been increasingly exploited (Nami, Haghshenas, & Yari Khosroushahi 2017; Bustamante et al., 2017; Rodrigues et al., 2017; Mu et al., 2018; Rodrigues, Cedran, & Garcia, 2018).

At the same time, due to its potential health benefits, the global market for supplements and probiotic foods has increased significantly each year (Grand View Research, 2019). However, the production of foods with probiotic claims is a challenge, especially due to difficulties of survival and maintenance of the probiotic cells added to the foods under processing, storage, distribution and consumption conditions (Min et al., 2019). In parallel, the cell encapsulation consolidates as an alternative to improve the survival of probiotics added to different food matrices (Angiolillo, Conte, & Del Nobile, 2017; Rodrigues, Cedran, & Garcia, 2018; Zanjani et al., 2017; Dimitrellou et al., 2019; Cavalheiro et al., 2019; Bambace, Alvarez, & Moreira, 2019).

Thus, this review has important concepts concerning probiotics and probiotic encapsulation. In addition, some field trends were discussed, such as the use of alginate as an encapsulating material and the interest in its total or partial replacement by other polysaccharides, especially gums, mucilages, microbial exopolysaccharides and prebiotics, which can modify the particle properties, improving the protection and survival of encapsulated cells. In parallel, non-dairy food matrices are highlighted as alternative vehicles for the probiotic cell delivery.

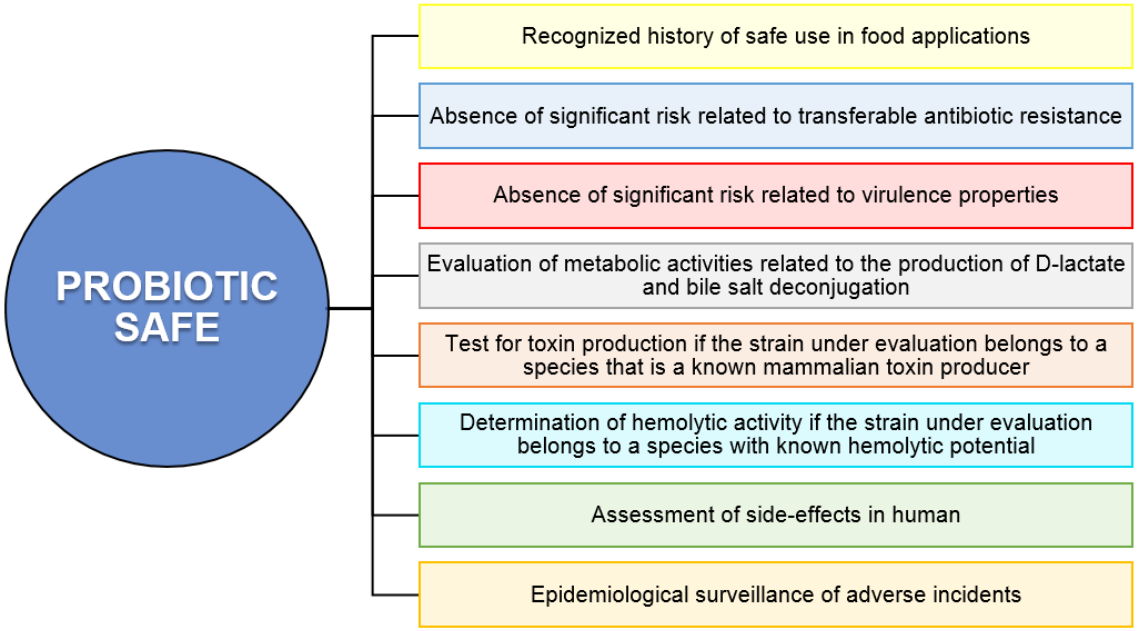
2 Probiotics: History, definition and characteristics

In 1907, Elie Metchnikoff, a Russian Nobel Prize-winning scientist and professor at the Pasteur Institute in Paris, assumed that the lactic acid bacteria could promote health benefits and lead to longevity. He defended that both "intestinal self-poisoning" and aging derived from it could be suppressed by modifying the intestinal microbiota, using useful microorganisms to replace proteolytic microorganisms, such as *Clostridium* spp., which are producers of toxic substances (Anukam & Reid, 2007).

The concept of probiotic, initially proposed by Fuller (1989), suggested a food supplement, consisted of living microorganisms which was capable to benefit the host through intestinal microbiota balance. Currently, the Food and Agriculture Organization of the United Nations (FAO), supported by the World Health Organization (WHO), defines probiotics as bacteria and yeasts that, when administered in adequate amounts, confer health benefits to the host (FAO/WHO, 2002). Regarding the biosafety

of probiotic microorganisms, most lactic acid bacteria are recognized as food-grade, rarely related to infections in humans. In addition, many of these bacteria are not only naturally present in commonly consumed animal and vegetable foods but also found in the gastrointestinal tracts of healthy humans. However, considering possible vulnerabilities in human health, the safety assessment of probiotics (Fig. 1) is essential for a microorganism to achieve the probiotic status (Pradhan, Mallappa, & Grover, 2020).

Figure 1. FAO/WHO (2002) guidelines for establishing safety assessment of probiotics for human use.



The beneficial effects of probiotics include the production of metabolites that inhibit the adhesion and prevalence of pathogenic microorganisms, directly modifying the intestinal microbiota composition; the production of compounds with anticarcinogenic activity, such as short chain fatty acids and conjugated linoleic acid; the degradation of toxins and their respective receptors; and the modulation of immune responses, improving the absorption of micronutrients and stimulating the generation of organic acids and amino acids (Prakash et al., 2011; Reis et al., 2017; Markowiak & Śliżewska, 2017).

Due to these characteristics, probiotics have been investigated in the prevention of colorectal cancer (McIntosh, Royle, & Playne, 1999; Jacouton et al., 2017; Chang et al., 2018; Heydari et al., 2019), inflammatory bowel disease (Zaylaa et al., 2018) and irritable bowel syndrome (Cremon et al., 2018), the reduction of the risk associated with cardiovascular disease (Liu et al., 2017), the improvement of the anti-hypercholesterolemia and anti-hyperlipidemic effect (Bharti et al., 2017; Park et al., 2018), as well as in the non-alcoholic fatty liver disease (Liang et al., 2018), and other conditions. It is important to emphasize that the beneficial health effects to the host can vary according to the probiotic strain administered. In human nutrition, the main types of probiotic bacteria used are *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*, *Enterococcus* and *Bacillus*. However, some species of yeast of genus *Saccharomyces* are also studied (Markowiak & Śliżewska, 2017).

To promote beneficial effects to the host, these microorganisms must present stability against stomach acid and bile salts, ability to adhere to the intestinal mucosa, colonization capacity of the human gastrointestinal tract; production of antimicrobial compounds and maintenance of metabolic activity in the intestine (Collins, Thornton, & Sullivan, 1998; Saarela et al., 2000). Although widely used, probiotic microorganisms presented limitations, since many bacteria with this claim can be affected by several factors such as oxygen concentration, presence of hydrogen peroxide, pH and temperature variations, among others. Therefore, the encapsulation has become a strategy to protect probiotic cells and to support their beneficial effect.

3 Encapsulated probiotics

The encapsulation process involves cells with suitable wall materials to protect and control the release of encapsulated cells (Mortazavian et al., 2008). Thus, the bacterial cell encapsulation is an approach that may improve the resistance of encapsulated microorganisms to adverse conditions (Kim et al., 2017; Rodrigues et al., 2017). In general, it is either a physicochemical or a mechanical process in which bacterial cells are entrapped in encapsulating materials with different properties that can reduce or inhibit the injury or cellular losses of the encapsulated microorganisms,

especially, whereas for the expected beneficial effect on the host be exerted is essential that the probiotics are viable in the colon (Wu & Zhang, 2018).

Nowadays, various encapsulation techniques are used to produce probiotic particles. However, it is extremely necessary that when choose a technique, it should be taken into account that the process should not be aggressive, ensuring sufficient viability of the encapsulated cells and have mechanical stability compatible with the application purpose (Rathore et al., 2013). Several techniques are currently available to encapsulate probiotic cells, such as extrusion, emulsion, spray drying, spray chilling, fluidized bed, freeze drying, spray-freeze drying, coacervation, electrospraying, etc (Frakolaki et al., 2020). Some of the main ones (Table 1) are discussed in the next sections.

Table 1. Main techniques used to encapsulate probiotic bacteria.

TECHNIQUE	ENCAPSULATED MICROORGANISM	REFERENCE
Extrusion	*Lactobacillus casei* 01 *Lactobacillus casei* BGP93	Rodrigues et al. (2017)
	Lactobacillus acidophilus La3	Silva et al. (2018a)
	Lactococcus lactis spp. *cremoris*	Ramos et al. (2018)
	Lactobacillus acidophilus KBL409	Lee et al. (2019)
	Lactobacillus casei ATCC 393	Dimitrellou et al. (2019)
	Lactobacillus rhamnosus GG	Hugues-Ayala et al. (2020)
	Faecalibacterium prausnitzii	Raise et al. (2020)
Emulsion	*Akkermansia muciniphila*	van der Ark et al. (2017)
	Lactobacillus rhamnosus GG	Singh et al. (2018)
	Saccharomyces boulardii *Enterococcus faecium*	Qi et al. (2019)
	Lactobacillus paracasei spp. *paracasei*	Shafizadeh et al. (2020)
	Lactobacillus acidophilus LA-5	Raddatz et al. (2020)
	Lactobacillus acidophilus PTCC 1643	Dehkordi et al. (2020)
Spray drying	*Lactobacillus rhamnosus* GG	Guerin et al. (2017)
	Lactobacillus plantarum ATCC 8014 *Bifidobacterium infantis* ATCC 15679	Bustamante et al. (2017)
	Lactobacillus acidophilus NCDC 016	Arepally & Goswami (2019)
	Lactobacillus plantarum NCIM 2083	Yoha, Moses & Anandharamakrishnan (2020)
	Kluyveromyces marxianus VM004	Vanden Braber et al. (2020)
	Lactobacillus fermentum K73	Aragón-Rojas et al. (2020)

TECHNIQUE	ENCAPSULATED MICROORGANISM	REFERENCE
Spray chilling	*Bifidobacterium animalis* spp. *lactis* BI-01 *Lactobacillus acidophilus* LAC-04	Pedroso et al. (2012)
	Lactobacillus acidophilus LAC-04	Okuro et al. (2013)
	Lactobacillus acidophilus *Bifidobacterium animalis* spp. *lactis*	Bampi et al. (2016)
	Saccharomyces boulardii *Lactobacillus acidophilus* LA-5 *Bifidobacterium bifidum* BB-12	Arslan-Tontul & Erbas (2017)
	Lactobacillus acidophilus La3 *Bifidobacterium animalis* spp. *lactis* BLC1	Silva et al. (2018b)
	Saccharomyces boulardii *Lactobacillus acidophilus* LA-5 *Bifidobacterium bifidum* BB-12	Arslan-Tontul, Erbas & Gorgulu (2019)
Fluidized bed	*Saccharomyces boulardii*	Joshi & Thorat (2011)
	Lactobacillus casei subsp. paracasei LMG P-21380	Semyonov, Ramon & Shimoni (2011)
	Lactobacillus reuteri C 10	Azim et al. (2012)
	Lactobacillus acidophilus La3	Silva et al. (2018a)
	Enterococcus faecium IS-27526	Horison & Surono (2020)

3.1 Extrusion

Widely used to encapsulate bacterial cells, the extrusion technique is simple, easy to use and presents a low cost, besides being a relatively mild process, which ensures high viability of the encapsulated cells (Krasaekoopt, Bhandari, & Deeth, 2003, Rodrigues et al., 2017). Basically, this technique involves the use of hydrocolloid solutions containing microbial cultures, which are extruded through a nozzle in crosslinking solution, providing the instantaneous transition of the hydrocolloid solution to gel, culminating in the formation of beads. The resulting gel is commonly stable in acidic mediums, yet disintegrates under alkaline environment (Favaro-Trindade, Heinemann, & Pedroso, 2011; Etchepare et al., 2016; Rodrigues et al., 2017).

Despite the favorable conditions mentioned, the disadvantages of this technique are that it is slow, which hampers its application on a large scale; is inefficient to produce microspheres smaller than 500 μm; and requires the use of low to moderate

viscosity hydrocolloid solutions (Reis et al., 2006). Among the factors that can influence the size of the produced beads are the diameter of the nozzle, the distance between the outlet of the hydrocolloid solution and the cationic cross-linker solution, and the viscosity/flow rate of the hydrocolloid–microbial cell mixture (Dong et al., 2013).

Various polysaccharides can be used to encapsulate bacterial cells by extrusion, including alginate, chitosan, and those extracted from gums and mucilages. It is necessary to emphasize that the polysaccharide concentration can influence both shape and size of the produced beads, and also in the protection of the encapsulated cells. Haghshenas et al. (2015) encapsulated *Lactobacillus plantarum* 15HN using alginate, alginate-psyllium blend and alginate-feno-greek blend. To produce the beads, the solutions were extruded through a 21-gauge nozzle in a sterile calcium chloride solution. The use of different polysaccharide sources and their concentrations directly influenced the diameter of the beads obtained, varying between 80 to 1000 μm. In general, lower concentrations of alginate produced smaller diameter particles.

To minimize the disadvantages of the extrusion technique, methods have been developed and adapted. Etchepare et al. (2016) encapsulated *Lactobacillus acidophilus* La-14 in resistant starch, chitosan and alginate by extrusion in a calcium chloride solution using an aerograph coupled to an air compressor. The size of the beads varied from 55 to 70 μm, and the addition of chitosan increased the particle size, yet provided better protection for probiotic cells under gastrointestinal conditions.

In addition, the extrusion technique is widely used to produce particles, based on blends containing alginates and other natural polysaccharides, which can extend the viability of encapsulated cells under storage and simulated gastric and enteric conditions. Rodrigues et al. (2017) encapsulated strains of *Lactobacillus casei* 01 and BGP 93 by extrusion using alginate combined with natural polysaccharides present in linseed and okra mucilages, botryosphaeran (exopolysaccharide produced by the endophytic fungus, *Botryosphaeria rhodina* MAMB-05) and fructooligosaccharides. The produced beads had a protective effect on the cell viability of the encapsulated probiotic during 15 days of refrigerated storage. In addition, the probiotic particles extruded with prebiotic compounds increased the resistance of microorganisms both at acid pH and in the presence of bile salts. Although promising,

to date, the extrusion technique and its adaptations still present difficulties for large-scale applications.

3.2 Emulsion

Emulsions have been used in pharmaceutical and food industries to improve the solubility, the physiological activity and stability of interest compounds. An emulsion is the dispersion of two immiscible liquids with a stabilizing agent, which usually presents a greater affinity to the continuous phase than to the dispersed phase (Alemzadeh et al., 2020). In addition, a solidifying agent can be used to separate the dispersed phase droplets from the continuous phase (Zhang, Lin, & Zhong, 2016). If the dispersed phase is aqueous, the emulsion is named a water-in-oil (W/O) emulsion, whereas the opposite is known as oil-in-water (O/W) emulsion or reverse phase. Both cases, formed by two phases only, are called simple emulsions. By adding of one more phase, double emulsions are obtained, such as water-in-oil-in-water (W/O/W) or oil-in-water-in-oil (O/W/O) emulsions (Goibier et al., 2020).

These emulsified systems can be used to encapsulate probiotics, improving the protection of encapsulated cells. Therefore, the dispersed aqueous phase (W/O or W/O/W emulsion) is preferably used, due to the hydrophilic character of bacterial cells (Wang et al., 2020). Pandey et al. (2016) reported the use of simple emulsion to encapsulate *Lactobacillus plantarum* 299v and metronidazole using xanthan and guar gum in the aqueous phase, and sunflower oil in the lipidic phase. This encapsulation strategy improved the cell viability during storage and was considered suitable for a controlled release of the encapsulated agents.

The encapsulation of probiotics in double emulsion has been reported for *Lactobacillus rhamnosus* LC705 using de Man, Rogosa and Sharpe broth, grape oil and whey protein concentrate ($W_1/O/W_2$, respectively). The protective effect of the emulsifying system under the encapsulated probiotic was evidenced under osmotic stress, by the satisfactory cell survival (>8 log CFU / mL) of the probiotic encapsulated in a sucrose hypertonic solution (Huerta-Vera et al., 2017). In another study, Wang et al. (2020) emphasized that emulsions allow the use of encapsulated probiotic

microorganisms in liquid form. The authors reported the encapsulation of *Lactobacillus acidophilus* AS 1.2686 through a double emulsion formed by alginate (W_1), soybean oil (O) and cellulose solution (W_2). The viability of the probiotic bacteria was improved during 14 days of storage as compared to free cells. Under simulated gastrointestinal conditions approximately 84 % of the encapsulated cells remained viable after digestion, while free cells showed a rapid decline and total loss of viability, especially due to poor tolerance to acid pH.

The use of hydrocolloids in emulsifying systems to encapsulate probiotics is known as internal ionic gelation. In this technique, the hydrocolloid material is used as an encapsulator in the presence of a solidifying agent. Thus, the hydrocolloid solution containing the microbial cells and a non-ionized solidifying agent is added to the oil continuous phase of a water-in-oil emulsion (W/O). The change in the medium pH causes the ionization of the solidifying agent and the consequent formation of particles that can be separated from the continuous phase of the emulsion (Zhang et al., 2020). The internal ionic gelation using alginate and calcium carbonate as encapsulating and solidifying agents, respectively, was employed to encapsulate *Bifidobacterium* BB-12. The particles obtained were effective to protect the probiotic exposed to simulated gastric and enteric juices. In addition, the encapsulation improved the stability and survival of the bacteria during storage of 60 days at 25 ºC (Holkem et al., 2016).

The internal ionic gelation technique may also be performed with two or more materials, which act in synergy as an encapsulating material, improving the resulting particle properties. This approach was carried out to encapsulate *Lactobacillus acidophilus* LA-5 in blends based on pectin, inulin and rice bran. The particles obtained 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 ºC. However, it was observed that the use of combined materials could increase the particle size (Raddatz et al., 2020).

The particle size of the encapsulated probiotic is extremely important for the sensory characteristic of food products. Therefore, smaller particle sizes were achieved by internal ionic gelation using blends of alginate and whey protein isolate to

encapsulate *Lactobacillus acidophilus* PTCC 1643. The particle size ranged from 33 to 180 μm, and was suitable for application in food products. In addition, this encapsulation approach also improved the survival of encapsulated bacteria under simulated gastric and enteric conditions (Dehkordi et al., 2020).

3.3 Spray drying

Commonly used in the food industry, the spray drying technique presents a low cost, fast processing and high productivity. In general, this technique consists of the atomization of a solution containing the encapsulated agent in a high-temperature gas, instantly forming a powder (Ray, Raychaudhuri, & Chakraborty, 2016). To encapsulate bacterial cells by the spray drying technique, various natural polymers can be used, especially arabic gum and starches, due to their recognized ability to form spherical particles after the drying process. However, other materials, such as inulin, fructooligosaccharides, alginates, gums and mucilages have also been used (Avila-Reyes et al., 2014; Arslan et al., 2015; Kingwatee et al., 2015; Rajam & Anandharamakrishnan, 2015; Sarao & Arora, 2017; Hadzieva et al., 2017; Bustamante et al., 2017). Polysaccharide encapsulating materials exhibit good solubility, low viscosity at high concentrations, high glass transition temperature and rapid drying, which are desirable features for the spray drying process. In addition, the combined use of different polymers can improve the survival of the encapsulated cells (Liu et al., 2019).

Key process conditions, such as air flow, feed temperature, inlet/outlet air temperature, are important to optimize processes or the obtained particles. The temperatures used are extremely important, since low temperatures reduce the rate of water evaporation, which forms aggregates of microspheres, while high temperatures can damage bacteria membrane substances, which drastically reduce the cellular viability of the encapsulated microorganisms (Rathore et al., 2013). However, the survival of bacterial cells encapsulated by the spray drying technique is more dependent on the outlet temperature than the inlet temperature (Martín et al., 2015). To evidence this, Gardiner et al. (2000) encapsulated *Lactobacillus paracasei* NFBC 338 using skimmed milk as an encapsulating material, and obtained survival rates after

spray drying ranged from 97 % at an outlet temperature of 70 to 75 °C to 0 % at 120 °C.

Lactobacillus acidophilus La-5 has been recently encapsulated in inulin by spray drying using inlet and outlet temperatures of 120 and 55 °C, respectively. The survival rate of the microorganism after the encapsulation process reached 86.5 % (Santos et al., 2019). Similarly, the encapsulation of *Bifidobacterium* BB-12 in a mixture of skimmed milk powder and prebiotics at inlet and outlet temperatures of 150 and 55 ºC resulted in an encapsulation efficiency higher than 70 % (Fritzen-Freire et al., 2013).

To overcome the negative effects of high temperatures in the spray drying process and improve the resistance and stability of the encapsulated cells, the addition of prebiotics, soluble fibers, gums and mucilages to the encapsulating material as thermal protectors was proposed by research groups (Ross et al., 2005; Rajam & Anandharamakrishnan, 2015; Santos et al., 2019). Rodríguez-Huezo et al. (2007) used the spray drying technique to encapsulate *Bifidobacterium bifidum* BB-02 in whey protein concentrate, mosquita gum, maltodextrin, and aguamiel, which according to the authors can induce a symbiotic effect, improving the resistance of encapsulated bacteria against environmental factors and increasing viability.

3.4 Spray chilling

Also known as spray cooling and spray congealing, this process is analogous to spray drying, especially due to the production of small droplets. However, in this technique the encapsulated agent is dispersed in a molten matrix, formed by lipids, which is atomized in a chamber where cold air is injected, enabling the solidification of the particles. Although this method is not new, spray chilling is less exploited than spray drying and ionic gelling, for instance. However, its process conditions represent an excellent alternative to encapsulate microbial cells, especially due to the low cost and industrial-scale application (Okuro, Matos Junior, & Favaro-Trindade, 2013).

In this process, triglycerides, fatty acids, oils, waxes and other lipid matrices could be used as encapsulating materials (Queirós et al., 2020). However, hydrophilic

compounds such as polysaccharides can be employed by using emulsions with a suitable melting point (Zuidam & Shimoni, 2010). To obtain uniform particles, some process parameters should be considered, such as the temperature of the molten matrix during processing, chamber and atomizing air temperatures, atomizing air pressure, and feeding flow (Okuro, Matos Junior, & Favaro-Trindade, 2013). Moreover, the process does not require the use of organic solvents and high temperatures, which favors the use of the technique to encapsulate thermosensitive agents. The use of lipid matrices as a wall material can prolong the cell viability during storage and allow the controlled release of the encapsulated cells in the gastrointestinal tract after ingestion due to intestinal lipases action (Đorđević et al., 2015).

On the other hand, this technique is also associated with low encapsulation capacity and possible expulsion of the encapsulated cells from the matrix during storage due to the lipid solidification and crystallization processes. Moreover, spray chilling also requires specific carrier materials, which must have a hydrophobic character and melting temperatures lower than those harmful to the encapsulated cells. In combination, these are challenging features for wide applications of this technique, especially in food systems, where the effects on texture and the possibility of particles floating in liquid systems are commonly not negligible (Pedroso et al., 2013; Okuro, Matos Junior, & Favaro-Trindade, 2013).

Despite the disadvantages, *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* were effectively encapsulated by spray chilling using vegetable fat as an encapsulating material. The obtained particles were added to savory cereal bars and did not interfere in the sensorial attributes. In addition, the encapsulated microorganisms presented high viability during refrigerated storage at least of 90 days (Bampi et al., 2016). Similarly, single- and double-layered particles containing *Saccharomyces boulardii*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were produced by spray chilling for the production of cakes. The single-layered particles produced by using hydrogenated palm oil as a wall material presented the best protective effect on the encapsulated probiotics during storage time (Arslan-Tontul, Erbas, & Gorgulu, 2019).

The spray chilling technique has also been studied to preserve the viability of encapsulated cells under gastric and enteric conditions and in different thermal

situations. Arslan-Tontul & Erbas (2017) reported a high-efficiency encapsulation of *Saccharomyces boulardii*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* by spray chilling. The particles produced improved the survival of the encapsulated cells in a simulated gastric system and at temperatures up to 80 ºC, while free cells were detected only at 50 ºC. Silva et al. (2018b) encapsulated strains of *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* in vegetable fat matrices. The authors emphasized that the lipid particles can be effective to protect encapsulated cells under environmental stress and simulated gastrointestinal conditions.

3.5 Fluidized bed

The fluidized bed technique is used for coating, granulation or drying, in which a coating is atomized over solid particles in suspension. This technique is a fast process, which presents a low cost and high production. Moreover, several encapsulating materials, such as lipids, proteins and polysaccharides can be used (Zuidam & Shimoni, 2010). The process consists of a heated chamber, where the particles that will be covered are kept in constant motion due to the air flow. The coating material is pulverized and wets the particle surface, coalescing and solidifying successively, and then forming a solid and homogeneous layer. The coating properties are influenced by the injection angle (top, bottom or tangential spray types), evaporation rate, solidification and viscosity of the encapsulating material, as well as the particle wettability, velocity and temperature of the fluidization air, and the number of coating cycles, i.e. particle passages in the coating zone, which is the collision area (Manojlović et al., 2010; Avilés-Avilés, Dumoulin & Turchiuli, 2015).

In this way, for the probiotic encapsulation, the cells require a previous treatment that promote a solid particle which can be suspended and covered, characterizing the fluidized bed as a co-encapsulation technique (Manojlović et al., 2010; Chávarri, Marañón & Villarán, 2012; Ozdal, Yolci-Omeroglu & Tamer, 2020). Using the fluidized bed technique and cellulose as an encapsulating material to encapsulate *Enterococcus faecium* IFANo.045 and *Lactobacillus plantarum* IFANo.278, Strasser et al. (2009) emphasized that adding non-reducing disaccharides

(sucrose and trehalose) to wall material increased cell protection rates during the production and storage of particles.

The fluidized bed process was also employed to encapsulate *Enterococcus faecium* M74 using microcrystalline cellulose as an encapsulating material. The obtained particles presented excellent physical properties, such as fluidity and good handling, contrasting with the particle properties obtained by other drying encapsulation methods (Stummer et al., 2012). At the same time, the technique was effective to produce alginate-chitosan microcapsules containing *L. plantarum* NCIMB 8826, improving the storage cell survival of encapsulated probiotic as compared to free cells (Albadran et al., 2015).

In parallel, the fluidized bed technique was employed to encapsulate *Lactobacillus acidophilus* TISTR 1338 using encapsulating materials from different sources. The microcapsules, consisted of stearic acid, albumin, alginate and cassava starch, presented an encapsulation efficiency above 90 %. Moreover, it attributed moist-heat-resistance (70 ºC with 100 % relative humidity) to probiotic cells, indicating that the use of the fluidized bed technique to encapsulate thermosensitive compounds favors its incorporation in heat-treated foods (Pitigraisorn et al., 2017).

4 Encapsulating materials for probiotic cells

The selection of the appropriate material for the encapsulation of microbial cells is essential for the stability and properties of the produced particles (Table 2). The encapsulating agent must not present toxicity, as it can directly influence the morphology, diameter and permeability of the particles. In addition, it should protect microbial cells against environmental factors and be sufficiently satisfactory in the controlled release (Rathore et al., 2013; Chen et al., 2017a). During probiotic particle storage, both temperature and moisture content can affect the cell viability, mainly due to the cell membrane lipid oxidation. Therefore, the use of materials with moisture retention capacity improves the survival of encapsulated cells. In addition, materials that completely release encapsulated cells when suspended in gastric juices may not

be suitable for protecting cells during passage through the gastrointestinal tract (Rajam & Anandharamakrishnan, 2015).

Table 2. Recent reports about effects of encapsulating materials used to entrap probiotic cells.

ENCAPSULATING MATERIAL	PROBIOTIC	TECHNIQUE	EFFECT	REFERENCE
microcrystalline cellulose–trehalose–maltodextrin–vegetable wax	*Lactobacillus casei* subsp. *paracasei* LMG P-21380	fluidized bed	high rates of encapsulation efficiency protection enhanced during the encapsulation process and greater stability during storage	Semyonov et al. (2012)
chitosan–alginate–inulin	*Lactobacillus rhamnosus* GG	extrusion	no adverse organoleptic effect on apple juice improvement in the cell survival in apple juice in 90 days of storage	Gandomi et al. (2016)
alginate–shellac	*Lactobacillus paracasei* BGP-1	fluidized bed	improved the microcapsule structure by reducing the porosity provided protection during storage at room temperature and in *in vitro* gastrointestinal simulation	Silva et al. (2016)
alginate–flaxseed or okra mucilage alginate–botryosphaeran	*Lactobacillus casei* LC 01 *Lactobacillus casei* BGP93	extrusion	high encapsulation efficiency improvement in the stability of encapsulated cells in refrigerated storage	Rodrigues et al. (2017)
celulose–alginate	*Lactobacillus plantarum* IS-10506	fluidized bed	increased the probiotic survival rate during gastric simulation	Surono et al. (2018)
alginate–arabinoxylan	*Lactobacillus plantarum*	extrusion	high encapsulation efficiency and resistance to gastrointestinal condition that than alginate beads	Wu & Zhang (2018)
alginate–goats' milk–inulin	*Bifidobacterium animalis* spp. *lactis* BB12	extrusion	inulin addition resulted in compact structure capsules protection of the probiotic in simulated gastrointestinal condition	Prasanna & Charalampopoulos (2019)
Alginate	*Lactobacillus casei* ATCC 393	extrusion	protection of the probiotic in simulated gastrointestinal condition	Dimitrellou et al. (2019)

ENCAPSULATING MATERIAL	PROBIOTIC	TECHNIQUE	EFFECT	REFERENCE
alginate–chitosan	*Bifidobacterium longum* DD98	emulsification; internal gelation	improvement of heat tolerance of the encapsulated cells significantly protection under gastric acid and bile salt	Ji et al. (2019)
maltodextrin–sucrose maltodextrin–sorbitol	*Saccharomyces cerevisiae* KTP *Issatchenkia occidentalis* ApC *Saccharomyces cerevisiae* var. *boulardii*	spray drying	components did not alter the characteristics of maltodextrin encapsulation sucrose and sorbitol enhanced the yeast survival in simulated gastric and bile juices	Suryabhan, Lohith & Anu-Appaiah (2019)
alginate–calcium carbonate	*Bifidobacterium pseudocatenulatum* G7	extrusion	probiotic survived throughout gastrointestinal tract when co-encapsulated with calcium carbonate	Gu et al. (2019)
amidated low–methoxyl pectin	*Faecalibacterium prausnitzii*	extrusion; freeze-drying	stabilization of encapsulated bacteria for 14 days cell protection to stomach and distal jejunum simulated conditions	Raise et al. (2020)
alginate–persian gum–prebiotics	*Lactococcus lactis* ABRIINW-N19	extrusion	high encapsulation efficiency probiotic cell stability during refrigerated storage in orange juice	Nami et al. (2020)
alginate–flaxseed mucilage	*Lactobacillus paracasei* spp. *Paracasei*	emulsion	high encapsulation efficiency resistance against the harmful effects of the simulated digestive system	Shafizadeh et al. (2020)
whey protein–chitosan	*Kluyveromyces marxianus* VM004	spray-drying	increase the viability during storage for 90 days at room temperature improvement of the tolerance to simulated conditions of gastrointestinal digestion	Vanden Braber et al. (2020)

Different polysaccharides, proteins and lipids have been used to encapsulate probiotic cells (Rajam & Anandharamakrishnan, 2015; Rodrigues, et al., 2017; Arslan-Tontul & Erbas, 2017), particularly, water-soluble natural polymers and their combinations have also been widely adopted for this purpose. Their applications allow the use of milder techniques, such as the extrusion technique, and consequently improves the maintenance of cellular integrity of encapsulated microorganisms (Rodrigues, et al., 2017). Examples and characteristics associated with materials are described in the next sections.

4.1 Alginate

Extracted from brown algae, the alginate is a linear heteropolysaccharide consisted of D-manuronic acid residues (M) united by β-(1→4) bonds and L-guluronic acid residues (G) united by α-(1→4) bonds. Therefore, the basic structure of the alginate is composed of unbranched polymer units consisted of monomers arranged in M and G residue blocks interspersed with regions containing alternate M-G sequences (Donati & Paoletti, 2009; Ching, Bansal, & Bhandari, 2017).

The ratio between M and G units and the amount of the three blocks present in the polymer may vary according to the source and influence on the properties of gels formed from this polymer. Alginates with a higher percentage of G blocks tend to give rise to more rigid and brittle gels, while higher amounts of M blocks form less rigid and more flexible gels. The oscillation of alginate gel properties also depends on the polymer concentration, pH and presence of divalent cations that promote intermolecular ionic bonds with the anionic blocks present in the alginate (Gacesa, 1988).

The affinity of the anionic blocks present in the alginate with divalent cations may vary according to the cation used in the crosslinking process, which decreases in order of Pb > Cu > Cd > Ba > Sr > Ca > Fe > Co, Ni, Zn > Mn. However, the use of cations that exhibit toxicity is limited and must not be employed in pharmaceutical or food purposes. Thus, the non-toxic Ca^{2+} ion is widely used in the formation of calcium alginate gels (Haug & Smidsrød, 1970; Mørch, Donati, & Strand, 2006).

Among the reasons for the widespread use of alginate in probiotic encapsulation studies, and in the food and pharmaceutical industries, is due to the polymer be classified as safe for use in the European Union (E 401 and E 404) and in the United States (GRAS, 21 CFR 184.1187 and GRAS, 21 CFR 184.1724), and therefore, it can be used as a functional ingredient (Qin et al., 2018).

Alginates are commonly used as encapsulating materials for microbial cell encapsulation. However, the use of this polymer results in the formation of porous matrices, which are susceptible to disintegration in the presence of excess monovalent ions and Ca^{2+} chelating agents. Thus, many researchers have blended alginate with different polymers, such as natural polysaccharides extracted from plants and their seeds, to improve particle properties (Rodrigues et al., 2017; Chen et al., 2017a; Dokoohaki, Sekhavatizadeh, & Hosseinzadeh, 2019).

4.2 Chitosan

Commercially obtained from the partial deacetylation of chitin extracted from crustaceans, chitosan is a cationic polysaccharide composed of residues of D-glucosamine and N-acetyl-glucosamine linked by β-(1→4) bonds (Silva, Souza, & Lacerda, 2019). It has a Generally Recognized As Safe (GRAS) status by the Food and Drug Administration (FDA) and is biocompatible and soluble at acid pH. In addition, under acidic conditions, the positively charged amine groups (pKa ~6.5) present in chitosan allow electrostatic interaction with anionic polymers (Qi et al., 2020).

The use of chitosan as an encapsulating material for probiotic bacteria can present disadvantages, since this polysaccharide has an inhibitory effect against microorganisms (Goy, Britto, & Assis, 2009), including lactic acid bacteria (Groboillot et al., 1993). However, the effect of this material on encapsulated probiotics is not fully elucidated.

Chitosan was used as a coating material in alginate beads containing *Bifidobacterium longum* DD98 by the internal gelation technique. The chitosan coating improved the survival of encapsulated *B. longum* under gastrointestinal fluid and high-temperature conditions (Ji et al., 2019). In parallel, the encapsulation of *Lactobacillus*

rhamnosus ASCC 290 and *Lactobacillus casei* ATCC 334 in alginate-chitosan by the extrusion technique provided an encapsulation efficiency of >76 %, and both bacteria were protected under simulated gastrointestinal conditions, however, different encapsulated strains can present different behaviors (Farias et al., 2019).

4.3 Mucilages and gums

In recent decades, water-soluble natural polysaccharides extracted from plants and seeds have been studied and widely used in the food and drug industries. Due to their numerous technological and dietary properties, these polymers have been used as food additive, food packaging and edible coating material, gelling agents, thickeners, emulsifiers and stabilizers, as well as encapsulating material to microbial cells and excipients in drug administration (Kumar & Gupta, 2012; Soukoulis, Gaiani, & Hoffmann, 2018; Rodrigues, Cedran, & Garcia, 2018; Salehi, 2019; Hamdani, Wani, & Bhat, 2019; Pereira et al., 2019a).

Also known as plant gums, these polymers are obtained from plants through natural exudation produced in response to either a mechanical injury or a microbial attack (exudates) or extracted from different tissues by appropriate extraction process (non-exudate) (Rana et al., 2011; Hamdani, Wani, & Bhat, 2019).

Gums or mucilages extracted from plant or its seeds are classified in three groups: I) non-starch endosperm fractions, such as galactomannans; II) mucilaginous components of the seed coat; and III) endosperm cell wall material, such as hemicelluloses, mannans, galactomannans, glucomannans, and xyloglucans (Otegui, 2007; Soukoulis, Gaiani, & Hoffmann, 2018).

Various plants and seeds are capable of producing exudates containing high hydrocolloid concentrations. Due to their highly hydrophilic nature, gums and mucilages can be easily extracted by soaking the seeds or their peels in water for predetermined periods (Soukoulis, Gaiani, & Hoffmann, 2018). However, in some cases, only water immersion is not sufficient for complete mucilage dissociation, requiring the use of agitation, temperature control, or ultrasonic extraction methods (Pereira et al., 2019b).

The encapsulation of microbial cells using components extracted from plants as encapsulating materials is of interest for various research groups around the world. These compounds are related to an improvement of the viability of encapsulated cells during storage and passage through the gastric and intestinal tract (Rodrigues et al., 2017; Bustamante et al., 2017). Besides, these are abundantly available materials in nature, and can be sustainably obtained.

4.3.1 Psyllium mucilage (*Plantago ovata* Forssk)

From the *Plantago ovata* Forssk seed husks, it is possible to extract a mucilaginous material known for its gelling capacity and water-absorbing property (Fernandes, Acharya, & Bhatt, 2018). The polymers present in this material are natural antioxidants and anticarcinogenic agents (Patel et al., 2019), and their consumption is associated with beneficial health effects, such as the effective improvement of conventional and alternative lipid markers, which delays the risk of atherosclerosis associated with cardiovascular disease (Jovanovski et al., 2018).

Psyllium mucilage is composed of arabinoxylans that consist of xylan structures with multiple side chains consisting of xylose and arabinose residues (Yu et al., 2017). Arabinoxylan is the main non-starch polysaccharide in plants and in human nutrition has role of fermentable substrate for intestinal microbiota (Broekaert et al., 2011). The psyllium mucilage has been recently reported to consist of three fractions containing multiple arabinoxylans of similar composition and molecular weight. The external fraction is rich in galacturonic acid residues, which favors its solubility in water (Yu et al., 2017).

Psyllium mucilage is used as a polymeric additive, especially in filmogenic solutions for microbial cell encapsulation. Psyllium mucilage–alginate blends were used to encapsulate *Lactobacillus plantarum* 15HN by extrusion technique, reaching high encapsulation efficiency (>98 %), cell viability maintenance during storage for 28 days, cell survival under low pH and in the presence of bile salts (Haghshenas et al., 2015). The compatibility of probiotic particles based on psyllium mucilage and alginate added to food systems was reported by Nami, Haghshenas & Yari Khosroushahi

(2017). The particles produced by the extrusion technique improved the stability and survival of encapsulated *Enterococcus durans* IW3 incorporated in traditional yogurt and under simulated gastric and enteric conditions. Esmaeilzadeh et al. (2016) postulated that one of the possible reasons for the effective improvement of the particle properties produced with psyllium mucilage and alginate was in the surface topography of the obtained spheres, whose integrity was improved.

4.3.2 Konjac Gum (*Amorphophallus konjac*)

Native to subtropical lands in Southeast Asia, konjac is a plant of the Araceae family and genus *Amorphophallus*. A low-cost hydrocolloid gum composed mainly of glucomannan, which is a soluble dietary fiber, can be extracted from its tubers (Devaraj, Reddy, & Xu, 2019). Konjac glucomannan consists of a main chain composed of β-(1→4) bonds linking D-mannose and D-glucose (1.6:1), and side chains connected via β-(1→6) glycosyl units, with approximately one acetyl group in C6 every 17~19 sugar units (Du et al., 2019).

Recognized by the European Union as food additive (E 425), konjac glucomannan is used as a thickener in foods and has its consumption related to the prevention of heart disease and diabetes. In addition, this hydrocolloid is also known by prebiotic properties, since it can be metabolized by groups of probiotic microorganisms promoting its multiplication, being that the depolymerized glucomannans are more effective as compared to the native ones (Al-Ghazzewi et al., 2007; Al-Ghazzewi & Tester, 2012).

Hydrophilic and biodegradable, the konjac gum produces viscous and stable gels with film-forming capacities that can be used in the pharmaceutical and food industries (Mu et al., 2018; Du et al., 2019). Moreover, the high viscosity solutions produced from the konjac gum can be used in synergism with other hydrocolloids for food application, enabling changes in the physicochemical characteristics of the obtained solutions according to the process needs (Liang et al., 2011).

Konjac gum-based hydrogels can be used as drug carriers and as encapsulating materials to immobilize microbial cells by improving the stabilization of

encapsulated agents and ensuring that they effectively achieve their objectives (Devaraj, Reddy, & Xu, 2019). Konjac glucomannan hydrogels were employed to encapsulate *Lactobacillus acidophilus* by the emulsion technique, resulting in an encapsulation efficiency of 62.5 %, improvement of the cell viability during storage at 4 and 25 ºC and under exposure to simulated gastric fluids. In addition, konjac oligosaccharides were used as prebiotic and antifreeze agents (Mu et al., 2018). In parallel, hydrolyzed konjac glucomannans were used to immobilize *Lactobacillus casei* 01 by the spray drying technique. The glucomannan concentrations studied promoted encapsulation efficiency in a range of 78 to 95 %. The obtained particles were added in ice cream and the survival rate of the encapsulated cells was >90 % during storage of 28 days at −18 ºC (Yanprapasiri et al., 2018). Besides these traditional gums and mucilages employed for probiotic encapsulation, novel materials extracted from plants have emerged as alternatives for this procedure. Examples will be discussed in the following section.

4.3.3 Novel mucilages and gums for cell encapsulation

Mutamba, a popular name for *Guazuma ulmifolia* Lam., belongs to the Sterculiaceae family and is found mainly in Latin America, especially in Brazil, Mexico, Venezuela and Peru. The fruit has a black color, dry skin and seeds inserted in a mucilaginous pulp. In the ripe stage, the fruit represents a source of fibers, proteins, vitamins, minerals and phenolic compounds (Morais et al., 2017; Pereira et al., 2019b; Assis et al., 2019). The seeds present in the fruit, if soaked in water, release abundant amounts of mucilage forming “gelatinous capsules”. This mucilage is not easily extracted with conventional stirring procedures, requiring the application of ultrasonic methods for its effective recovery (Pereira et al., 2019b). In a brief characterization, the mutamba mucilage extracted with ultrasonic technology presented a profile of monosaccharides containing galactose, galacturonic and glucuronic acids, rhamnose, glucose and smaller amounts of arabinose/mannose. According to the surface charge density (ζ potential), which ranged from −30.4 to −39.5 mV, the hydrocolloid mucilage system is stable and presents an anionic character (Pereira et al., 2019b).

Cassia tora mucilage, also known as panwar gum, is a low-cost gum widely available in India. It is extracted from dark-colored seeds of *Cassia tora* Linn. and *Cassia obtusifolia* shrub pods, which belong to the Leguminosae family and are commonly found in Asian countries with tropical weather (Meena et al., 2010; Pawar & D'mello, 2011). Chemically, this hydrocolloid is a neutral heteropolysaccharide basically consisted of mannose and galactose in a ratio of approximately 5:1, a desirable ratio for galactomannans used as additives in foods. These galactomannans have a linear main chain consisted of β-(1→4)-D-manopyranosyl bonds with various random side chains of α-(1→6)-D-galactopyranosyl randomly branching from C6 to mannopyranose units (Mathur, 2016). The polymer present in the cassia tora mucilage is soluble in warm water and practically insoluble in organic solvents, has a satisfactory water absorption capacity and the colloidal solution has a pH close to neutrality (Pawar et al., 2014). Approved as food additive in Japan, the purified cassia tora mucilage has received the European Union number E 499 and listed as a stabilizing, thickening and gelling agent (Mathur, 2016).

Tamarind gum is extracted from the powder of tamarind seeds, which is extensively cultivated in Asia, Oceania, Africa and Latin America. It has a high concentration of polysaccharides, mainly galactoxyloglucans containing glucose, galactose and xylose in the proportion of 3:1:2 (Alpizar-Reyes et al., 2018; Crispín-Isidro et al., 2019). Non-toxic, biodegradable and stable in acidic pH environments, tamarind gum can be obtained by chemical method, homogenizing the seed powder in water to extract the mucilage, which is subsequently filtered and precipitated with acetone, or extracted through the enzymatic method by mixing the seed powder with ethanol, followed by treatment using proteases (Dey et al., 2019). The hydrocolloids obtained from the tamarind seed are related to its ability to form high viscosity solutions with thermal and chemical stability. These solutions contain concentrations of essential amino acids, making their use in food applications relevant (Alpizar-Reyes et al., 2018).

Although its structural characteristics indicate great potential as emerging biopolymer in food and pharmaceutical applications (Nayak & Pal, 2017; Mathur, 2016), until now there have been no literature records regarding the use of mutamba, cassia tora and tamarind mucilages to encapsulate probiotic microorganisms.

4.4 Microbial exopolysaccharides

Xanthan gum, discovered in the 1950s, is industrially produced by *Xanthomonas campestris*. Non-toxic and biodegradable was approved by FDA in 1969. It is a heteropolysaccharide constituted by repeated pentasaccharide units containing glucose, mannose and glucuronic acid, in the molar ratio of 2:2:1. It also presents water-solubility in cold and hot water, and has been used in the food industry as a thickening and viscosity agent (Kumar, Rao, & Han, 2018; Kavitake et al., 2018). The use of xanthan gum in synergy with other polysaccharides can improve the particle properties obtained. Complexes based on chitosan–xanthan gum improved the survival of *Bifidobacterium* BB01 in storage and under simulated gastric fluids (Chen et al., 2017b). Similarly, the addition of xanthan gum to alginate systems to encapsulate *Lactococcus lactis* subsp. *lactis*, changed the structure of the beads obtained due to bonds between hydroxyl groups of xanthan gum and alginate carboxylate groups, improving the protection of encapsulated bacteria (Bekhit et al., 2016).

Gellan gum is a water-soluble linear polysaccharide, negatively charged, which is industrially produced by *Sphingomonas elodea* and *Pseudomonas elodea*. It is a heteropolysaccharide constituted by repeated tetrasaccharide units containing two residues of D-glucose, one of L-rhamnose and one of D-glucuronic acid. This gum is heat-resistant and stable at acidic pH, besides being non-toxic and biocompatible with high capacity to establish ionic bonds with positively charged polymers. Due to its gelling property, it is possible to produce hard and translucent gels, whose properties vary according to extrinsic parameters, such as temperature and pH of the medium, presence of ions and the concentration of the polymer (Zia et al., 2018). Gellan gum was employed with alginate, gelatin, fructooligosaccharides and peptides to encapsulate *Lactobacillus plantarum* and *Lactobacillus casei* Shirota by extrusion technique, promoting the protection of probiotic cultures under environmental stress, such as simulated gastric and intestinal juices (Unal Turhan, 2019). Li et al. (2019) encapsulated *Lactobacillus casei* BNCC 134415 using gellan gum, whey protein isolate and cellulose acetate phthalate as encapsulating materials under lyophilization conditions. The tested conditions improved the cell viability of the immobilized probiotic during cold storage, pasteurization and under simulated gastrointestinal fluid.

Produced by the endophytic fungus, *Botryosphaeria rhodina* MAMB- 05, botryosphaeran is a (1→3)(1→6)-β-D-glucan, with a high molecular weight and water-soluble (Barbosa et al., 2003). It is not genotoxic and the rheological properties describe this β-glucan as presenting non-Newtonian behavior with pseudoplastic characteristics (Bongiovani et al., 2009). Due to its ability to form gels, it was used by Rodrigues et al. (2017) to encapsulate *Lactobacillus casei* 01 and *Lactobacillus casei* BGP 93 by the extrusion technique. Its use blended with alginate improved the encapsulation efficiency, which varied from 87 to 98.5 % and was effective to protect encapsulated probiotics.

4.5 Recognized prebiotics

First described by Gibson & Roberfroid (1995), prebiotics were defined as non-digestible food compounds that promote specific changes in the intestinal colon. These compounds are used as substrates for groups of endogenous bacteria present in the colon, which are associated with health promotion. However, a new definition and scope of prebiotics have been recently proposed by a panel of experts in microbiology, nutrition and clinical research convened by the International Scientific Association for Probiotics and Prebiotics. Aware of recent scientific and clinical developments, the consensus statement defined prebiotics as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Therefore, the current concept of prebiotic is not limited to carbohydrates and their role in the gastrointestinal tract, but also expands to other compounds and body site applications (Neri-Numa & Pastore, 2020). Among the recognized carbohydrates with prebiotic effects are the fructans such as inulin and fructo-oligosaccharides (FOS), and also xylo-oligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), etc (Vazquez-Olivo, Gutiérrez-Grijalva, & Heredia, 2019).

Inulin is a linear polysaccharide that can be extracted from plant sources, and is constituted of fructose monomers linked by β-glycosidic bonds. Due to the β-configuration, inulin is resistant to hydrolysis by the digestive system. From its partial hydrolysis using endo-glycosidase enzymes can be obtained FOS, which have 2~9 fructose units in their structure (Mitmesser & Combs, 2017). The use of these fructans

in the encapsulation of probiotic microorganisms has been studied. The use of inulin-alginate-chitosan to encapsulate *Lactobacillus acidophilus* 5 and *Lactobacillus casei* 01 provided protection for bacteria under simulated gastric and enteric juices (Krasaekoopt & Watcharapoka, 2014). In parallel, the stability of *Lactobacillus plantarum* MTCC 5422 in storage and tolerance at stress environment were improved by spray drying encapsulation using FOS and whey protein isolate (Rajam & Anandharamakrishnan, 2015).

XOS is another class of oligosaccharides, which are obtained by enzymatic hydrolysis of xylan. They are constituted by 2~10 units of xylose residues linked by β-(1→4) bonds (Aachary & Prapulla, 2011). Oligosaccharides, such as XOS, FOS and inulin were used as cryoprotectants of *Bifidobacterium bifidum* BB01 during the freeze-drying process (Shu et al., 2012). In addition, Liao et al. (2019) reported that the use of XOS as a co-encapsulating agent for the immobilization of *Lactobacillus fermentum* L7 by the extrusion technique promoted the survival of encapsulated cells under stress conditions and did not negatively influence the size of the particles.

Similarly, AXOS is also obtained from natural sources, specifically from the enzyme treatment of arabinoxylans. It is mostly constituted of xylose and arabinose, and may have lower fractions of mannose, galactose, glucose, and ferulic, glucuronic and phytic acids (Swennen et al., 2006). AXOS is more resistant to acid hydrolysis as compared to fructans, such as inulin and FOS. In addition, fractions of xylobiose linked by β-(1→4) bonds are not hydrolyzed by digestive enzymes present in the saliva and gastric human tract (Broekaert et al., 2011). *Lactobacillus plantarum* was encapsulated in the alginate-AXOS blend by the extrusion technique. The obtained particles provided a high encapsulation efficiency and improved both gastric stability and bile salt resistance (Wu & Zhang, 2018).

5 Probiotics in food matrices

Especially due to its potential health benefits, the global market for supplements and probiotic foods has been growing rapidly in the past years. In 2019 the global probiotic market was estimated at US$ 48.4 billion and is expected to reach

US$ 77.09 billion by 2025 (Grand View Research, 2019). The most common probiotic strains used in food matrices belong to the *Lactobacillus* and *Bifidobacterium* genera, but other lactic acid-producing bacteria, such as streptococci, enterococci and lactococci can also be used. In addition, some *Bacillus* spp. and yeasts of the genus *Saccharomyces* have also been related to probiotic properties (Sarao & Arora, 2017; Min et al., 2019). To promote health benefits to the host, it is widely accepted that the number of viable probiotic cells present in the food matrix must reach the minimum concentration of 10^6 – 10^7 CFU per gram or mL (FAO/WHO, 2002). Thus, maintaining the cell viability of the microorganism added to the food under processing, storage, distribution and consumption conditions, is a technological challenge for the production of foods with probiotic claims.

Several food matrices have been used to deliver probiotic cells. The NextFoods, through its GoodBelly Probiotics brand, produces juices, supplements, yogurts and cereals containing strains of *Lactobacillus plantarum* 299v or *Bifidobacterium animalis* subsp. *lactis*. According to the company, the concentration of bacteria is tested in all products and regular consumption can help promote digestion (GoodBelly Probiotics, n.d.). Similarly, KeVita produces fermented kefir beverages containing *Bacillus coagulans* GBI-30, 6086, a natural probiotic ingredient (KeVita, n.d.). The Korean company, Binggrae produces fermented milk, known as Dr.Capsule 1000, which according to the company has strains of *Lactobacillus* that present a survival rate of 1000 folds higher than strains of powdered *Bifidobacterium* (Binggrae, n.d.). However, these companies do not inform whether the probiotics are encapsulated and which process is used.

Despite the difficulty in obtaining information regarding processes in the production of commercial food products containing encapsulated probiotics, the Micropharma Inc. (Canada) and Danone Research have developed a fermented milk containing Cardioviva™, which is a culture of encapsulated *Lactobacillus reuteri*. In parallel, the Mexican Yoplait Inc. produced a yogurt containing bididobacteria capsules. The particles were visible and contrasted with other probiotic products, which aims to not modify sensory aspects (Champagne & Kailasapathy, 2011). The Institut Rosell & Lal'food produced chocolate containing encapsulated probiotic cells by the Probiocap® technology. Balchem Encapsulates and the Institut Rosell have produced

nutrients and chocolate bars, which reach microorganism delivery rates close to 100 %. The Kerry group in Ireland, in partnership with Chr Hansen developed an orange juice containing encapsulated probiotic strains (Probio-Tec®) and the probiotic remained viable during the shelf life of the product (Burgain et al. 2011). Thus, the cell encapsulation is suggested as an alternative to improve the survival of probiotics added in food matrices (Table 3), especially under adverse conditions (Angiolillo, Conte, & Del Nobile, 2017; Rodrigues, Cedran, & Garcia, 2018; Zanjani et al., 2017).

Table 3. Recent reports about encapsulated probiotics carried in food matrices.

PROBIOTIC	TECHNIQUE	ENCAPSULATING MATERIAL	FOOD MATRIX	REFERENCE
Lactobacillus acidophilus LA-5 *Bifidobacterium* BB-12	fluidized bed	whey protein hydrolysate–xantham gum	ready-to-reconstitute functional beverage	Kumar et al. (2010)
Lactobacillus curvatus MBSa2	extrusion	Alginate	salami	Barbosa et al. (2015)
Lactobacillus rhamnosus GG	extrusion	chitosan–alginate–inulin	apple juice	Gandomi et al. (2016)
Lactobacillus casei ATCC 39392 *Bifidobacterium adolescentis* ATCC 15703	emulsion	alginate, wheat, rice, and high amylose corn starches with chitosan and poly L-lysine coatings	ice-cream	Zanjani et al. (2017)
Lactobacillus plantarum ATCC 2331	emulsion	alginate–starch	dry fermented sausage	Bilenler, Karabulut & Candogan (2017)
Lactobacillus reuteri	spray drying	Alginate	tuna burger	Angiolillo, Conte & Del Nobile (2017)
Lactobacillus plantarum HM47	spray drying	maltodextrin, *moringa oleifera* gum, tender coconut water	milk chocolate	Nambiar, Sellamuthu & Perumal (2018)
Bifidobacterium animalis ssp. *lactis* BB12	spray drying	maltodextrin and/or inulin	powdered passion fruit juice	Dias et al. (2018)
Saccharomyces boulardii *Lactobacillus acidophilus* LA-5 *Bifidobacterium bifidum* BB-12	spray drying/ spray chilling	arabic gum–β-cyclodextrin / hydrogenated palm oil–Tween 80	Cake	Arslan-Tontul, Erbas & Gorgulu (2019)
Lactobacillus acidophilus LA-5	spray drying	Inulin	Mousse	Santos et al. (2019)
Lactobacillus rhamnosus ATCC 53103	extrusion	alginate–quince seed mucilage	dairy dessert	Dokoohaki, Sekhavatizadeh & Hosseinzadeh (2019)
Lactobacillus reuteri strains	extrusion	alginate–inulin-lecithin	chewing gum	Qaziyani et al. (2019)
Lactobacillus casei ATCC 393	extrusion	Alginate	fermented milk	Dimitrellou et al. (2019)
Lactobacillus rhamnosus	extrusion	Alginate	reduced-fat cream cheese	Ningtyas et al. (2019)
Lactobacillus rhamnosus GG	spray drying	whey protein isolate and modified huauzontle's starch	ready-to-drink green tea beverage	Hernández-Barrueta et al. (2020)

5.1 Encapsulated probiotic in dairy products

Historically, probiotic microorganisms have been carried in fermented or unfermented dairy foods. These foods, especially fermented milks, are considered ideal to deliver bacteria with probiotic claims, since the amounts of carbohydrates, proteins and lipids available in this food matrix can improve the survival of the bacteria added to milk. Thus, the food industry has an interest in developing dairy foods containing probiotic cells (Kumar, Vijayendra, & Reddy, 2015). In this sense, Alvarado-Reveles et al. (2019) emphasized that to overcome consumption limitations by consumers due to possible intolerances or allergies to compounds present in cow's milk, raw materials with high digestibility, such as goat's milk, can be an alternative to develop dairy foods containing probiotics.

In general, dairy foods are acidic and, according to the pH of the medium, they may not be the ideal environment for the stability of probiotic microorganisms. Thus, encapsulation techniques can be used to improve the survival of encapsulated bacteria exposed to stress conditions, ensuring that they fulfill their purpose. Dimitrellou et al. (2019) investigated the production and refrigerated storage of fermented milks containing *Lactobacillus casei* ATCC 393 encapsulated in alginate by the extrusion technique. The encapsulated cells showed greater cell viability than the free cells after 28 days of refrigerated storage. In addition, fermented milks showed improvements in their sensory characteristics due to the production of aromatic compounds produced by *L. casei*. Afzaal et al. (2019b) reported the production of ice cream with free and microencapsulated *Lactobacillus acidophilus* ATCC 4356 using alginate and carrageenan as wall materials. The encapsulation improved the survival of the encapsulated probiotic in the ice cream as compared to non-encapsulated cells under cold storage and passage through the simulated gastrointestinal tract. The authors considered both wall materials effective, however, alginate particles showed a better release profile as compared to carrageenan-based particles. Similar behavior was reported by the same research group in samples of traditional fermented yogurt containing immobilized *L. casei (*Afzaal et al., 2019a).

Dimitrellou et al. (2019) reported the production of fermented milk containing probiotics by spray drying. The cell viability of the probiotic was improved during the production, and the addition of encapsulated cells to the product did not influence its

sensory aspects. In parallel, Nale et al. (2018) reported satisfactory sensory results for ready-to-eat powdered dairy foods produced through the encapsulation of commercial kefir culture in maltodextrin-arabic gum blends by the spray drying technique. However, the use of particles containing probiotic bacteria produced by spray drying in foods with high moisture and water activity is complex, since the number of materials available to be used as an encapsulating agent is limited and present solubility in water, allowing that entrapped cells present in the particles easily migrate to the product under hydration conditions (Ray, Raychaudhuri, & Chakraborty, 2016).

5.2 Encapsulated probiotic in meat products

Meat and meat products are important foods in human nutrition and are part of the diet of consumers worldwide. They are sources of proteins with high biological value and essential micronutrients, such as iron, zinc and vitamin B_{12}, besides having considerable amounts of monounsaturated and polyunsaturated fatty acids (Ekmekcioglu et al., 2018).

However, the current excessive consumption of meat and meat products has been leading to health disorders, such as cardiovascular diseases, type 2 diabetes and cancer, especially colorectal (Ekmekcioglu et al., 2018). Thus, several studies have been conducted to develop meat foods enriched with beneficial compounds in order to minimize the negative effects that such products offer to the health (Cavalheiro et al., 2015).

The use of probiotic microorganisms is among the available approaches used to add value and increase healthy claims in meat products, in this sense, the encapsulated probiotics can be used. In general, fermented meat foods that are consumed without cooking are considered efficient vehicles for probiotic bacteria, since non-heating favors the maintenance of the cellular viability of microorganisms (Cavalheiro et al., 2015). However, Pérez-Chabela et al. (2013) reported the effect of the spray drying encapsulation of thermotolerant lactic acid bacteria on cooked meat batters. Spray-dried lactic acid bacteria increased its initial count and reduced the number of Enterobacteria in the samples, suggesting that the spray drying

encapsulation was effective to protect the lactic acid bacteria. Thus, the particles obtained can be used in other emulsified cooked meat products, as well as in bioprotective cultures to improve microbial quality.

The application of encapsulated probiotic in salami was studied by Barbosa et al. (2015). The report revealed that the use of alginate to encapsulate bacteriocin-producing *Lactobacillus curvatus* by the extrusion technique did not affect the production of bacteriocin and the viability of the bacteria during product storage was maintained. Cavalheiro et al. (2019) have recently reported the incorporation of *Lactobacillus plantarum* encapsulated by the extrusion and emulsion techniques in chorizo, which is a typical Spanish dry-fermented sausage. Alginate particles produced by the extrusion exhibited higher counts of the encapsulated probiotic during the ripening and storage of the product. In addition, the chorizo containing these particles presented a higher score in the overall acceptability than the ones produced using particles obtained by emulsion.

Therefore, the incorporation of encapsulated probiotic cells in meat products is an alternative to conventional products. However, it is essential to consider the intrinsic characteristics of meat foods and probiotic strains, as well as food processing conditions and appropriate encapsulation approaches.

5.3 Encapsulated/ entrapped probiotic in edible films and coatings

While edible films are defined as thin layers used as a cover or wrap, edible coatings are formed directly on the surface of products. Both are prepared with polysaccharides, proteins and/or lipids, and can be classified as any packaging or coating material applied to food, in order to prolong its shelf-life and safety, maintaining and/or improving their nutritional and sensory qualities. Thus, the use of edible films and coatings makes it possible to aggregate different functionalities to food products (Guimarães et al., 2018).

The literature does not have a clear definition of the concept of "encapsulated or entrapped" probiotic cells in edible films and coatings. Guimarães et al. (2018) treated the use of microorganisms in edible films and coatings as a method

of entrapping cells that is easier and less expensive than encapsulation techniques, which are easy to handle and allow easy quantification of the number of encapsulated microorganisms. On the other hand, Pop et al. (2020) considered in their critical analysis that in one of the pioneering studies, conducted by Tapia et al. (2007) involving the dispersion of probiotic bacteria in a polysaccharide-based solution to coat fresh-cut fruits, the bacteria entrapped in edible packaging were, in fact, encapsulated.

Research groups have been studying the use of edible films and coatings carriers of microorganisms with probiotic claims in different food matrices. Shahrampour et al. (2020) reported the production of alginate-pectin-based edible film containing *Lactobacillus plantarum* KMC 45. The addition of the bacteria to the films had no significant impact on parameters, such as thickness, water activity and solubility. Moreover, the viable cell count showed that the *L. plantarum* strain survived during film storage, suggesting that they could be used as active packagings for foods. In parallel, alginate coatings enriched with inulin and oligofructose containing *Lactobacillus rhamnosus* CECT 8361 were used to coat fresh blueberries. The use of prebiotic compounds in the edible coating improved the survival of *L. rhamnosus*, maintaining its viability above 6.2 log CFU/g during coated product storage. The addition of bioactive coating to blueberries did not affect sensory characteristics and conferred antimicrobial activity to the final product, reducing the *Listeria innocua* counts (Bambace, Alvarez, & Moreira, 2019).

Edible coatings were also applied to conserve and increase the shelf-life of fresh-cut vegetables. An alginate edible coating containing *Lactobacillus acidophilus* La-14 was applied in fresh-cut carrots, conserving the moisture content and decreasing color changes during the refrigerated product storage, which are determining factors for the commercialization of this product type. In addition, the coating was efficient support for the probiotic, showing viable cell count >7 log CFU/g at the end of the storage period (Shigematsu et al., 2018).

The use of edible films and coatings containing probiotic bacteria have also been reported in meat and fish products. The application of these active coatings is related to the extension of the shelf-life of processed meat and fish, mainly due to the maintenance of physical-chemical and sensory parameters, such as water activity, moisture and color, or through antagonistic activity exerted by probiotic

microorganisms entrapped into the film/coating against food spoilage bacteria (Pereira et al., 2018; Lacey, López-Caballero, & Montero, 2014). Pereira et al. (2018) reported that the edible coating based on whey protein isolate, containing *Bifidobacterium animalis* Bb-12 and *Lactobacillus casei* 01 in sliced ham, presented high and constant probiotic bacteria counts during its shelf-life, suggesting that the consumption could promote beneficial effects to consumers.

6 Conclusions

The encapsulation of microorganisms with probiotic claims is an efficient alternative to maintain both viability and stability of encapsulated cells. Various reports describe positive results when different encapsulation techniques and encapsulating materials are used. However, both technique and encapsulating material must be carefully chosen, in order to reduce losses during the particle production and their application.

The alginate is still the most used wall material to entrap probiotics, especially due to its properties and application conditions, which are relatively mild and favor the encapsulation of thermosensitive agents, such as microbial cells. However, a growing interest has been observed regarding the total or partial substitution of this anionic polysaccharide by other polysaccharides obtained from natural sources, such as plants and microorganisms, which can change particle properties and improve the protection and survival of encapsulated cells during storage, under food processing conditions and passage through simulated gastric and intestinal tracts.

At the same time, the growth of the global market for supplements and probiotic foods demands new products, in order to meet consumers' aspirations. Therefore, besides the traditional dairy products, meat- and vegetable-based foods have also been studied as carriers of encapsulated probiotics. Despite technological challenges, several studies have reported that a suitable encapsulation approach turns non-dairy food products into alternative matrices to deliver probiotic cells.

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CHAPTER II

RESEARCH ARTICLE

Reuterin-producing *Limosilactobacillus reuteri*: Optimization of *in situ* reuterin production in alginate-based filmogenic solutions

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Reuterin-producing *Limosilactobacillus reuteri*: Optimization of *in situ* reuterin production in alginate-based filmogenic solutions

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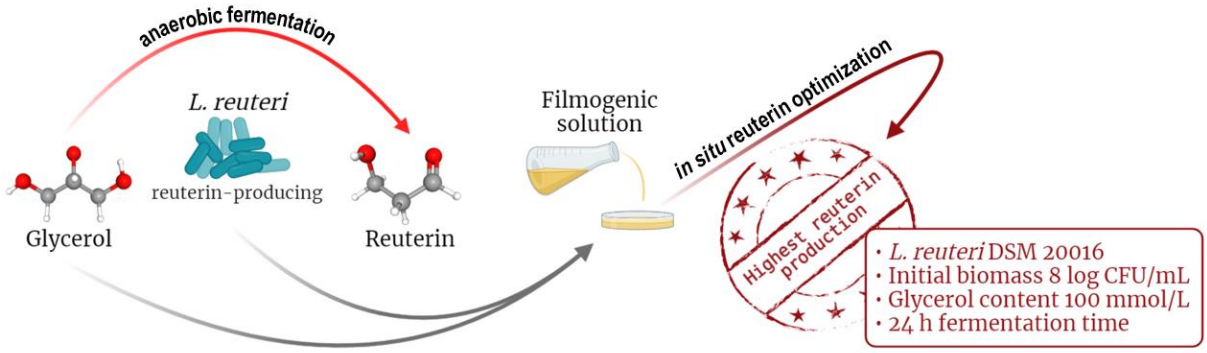
Highlights

- *L. reuteri* strains DSM 20016 and DSM 17938 produce reuterin.
- *In situ* reuterin production was detected in filmogenic solution.
- Reuterin production varied with initial microbial biomass and glycerol concentration.

Graphical abstract



Abstract

Limosilactobacillus reuteri produces reuterin via glycerol anaerobic fermentation. This compound has antimicrobial properties and is used for food preservation purposes. Filmogenic solutions constituted of polysaccharides and glycerol are also employed, however, reuterin synthesis in filmogenic solutions has not yet been reported. Thus, the aim of this study was to optimize the *in situ* reuterin production by *L. reuteri* in alginate- and glycerol based-filmogenic solution, evaluating the survival of reuterin-producing bacteria during fermentation. The study consisted of a completely randomized design employing two *L. reuteri* strains (DSM 20016 and DSM 17938). The filmogenic solutions were obtained using sodium alginate (20 g/L) and two independent variables were studied: glycerol (0–300 mmol/L) and initial biomass of *L. reuteri* (≅6, 7, and 8 log CFU/mL). The samples were analyzed every 24 h for 72 h of anaerobic fermentation (37 ºC). Both *L. reuteri* strains confirmed the potential for reuterin production and were susceptible to the metabolite produced. The highest reuterin production was achieved using *L. reuteri* DSM 20016. The initial microbial biomass of 8 log CFU/mL and 100 mmol/L of glycerol increased the reuterin production. However, higher conversion yields from glycerol to reuterin were obtained using 50 mmol/L of substrate.

Keywords: 3-hydroxypropionaldehyde; *Lactobacillus reuteri*; film-forming solution; *in situ* reuterin production.

1 Introduction

First described by Kandler et al. (1980), *Lactobacillus reuteri* is a heterofermentative bacterium commonly found in humans, mainly in the gastrointestinal tract, vagina, and oral cavity (Hou et al., 2015). Recently, the genus *Lactobacillus* has been divided into 25 new genera according to its phylogenetic, phenotypical, and habitat specificities, and *Lactobacillus reuteri* has been reclassified as *Limosilactobacillus reuteri* (Zheng et al., 2020). This non-pathogenic bacterium is accepted by the European Food Safety Authority (EFSA, 2008) as a food supplement to improve gastrointestinal health. In addition, under anaerobic conditions and in glycerol- containing medium, *L. reuteri* strains can produce and accumulate high contents of 3-hydroxypropionaldehyde (3-HPA) through an enzymatic reaction catalyzed by glycerol dehydratase (Vollenweider & Lacroix, 2004). In 1988, the 3-HPA produced by *L. reuteri* was patented as reuterin (Dobrogosz & Lindgren, 1995) and is constantly related to the probiotic activity of this microorganism (Mu et al., 2018).

In fact, reuterin is a dynamic system, also named as HPA system, which contains 3-HPA, its hydrated form 1,1,3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (Vollenweider et al., 2003). However, it has recently been suggested to include acrolein in the reuterin system since the 3-HPA in an aqueous solution can suffer spontaneous dehydration to acrolein (Engels et al., 2016). The reuterin system has several antimicrobial characteristics, such as antifungal activity (Schmidt et al., 2018; Vimont et al., 2019), broad spectrum of activity against Gram-positive and negative bacteria (Ávila et al., 2014; Montiel et al., 2014; Langa et al., 2018; Asare et al., 2020), and antagonistic effect against some protozoa (Vollenweider & Lacroix, 2004). The exact mechanism of reuterin inhibition against microorganisms is not fully understood. However, Talarico and Dobrogosz (1989) postulated that reuterin can compromise the DNA synthesis by inhibiting ribonucleotide reductase. Schaefer et al. (2010) then suggested that reuterin may cause depletion of thiol groups in glutathione, proteins, and enzymes, inducing cells to oxidative stress that can result in cell death.

Both reuterin and filmogenic solutions have also been associated with prolonged shelf life of foods. Reuterin is used to preserve foods by the inhibition of pathogenic and spoilage microorganisms (Angiolillo et al., 2017; Langa et al., 2018;

Ortiz-Rivera et al., 2017), while an increasing number of studies have described the production of polysaccharide-based filmogenic solutions with or without microbial cells as edible films and coatings for foodstuff (Salinas-Roca et al., 2016; Guerreiro et al., 2017; Rodrigues et al., 2018). Filmogenic solutions are also used as a vehicle to encapsulate microbial cells and/or drugs, improving the stability of encapsulated agents under adverse and storage conditions (Rodrigues et al., 2017; Rodrigues et al., 2020; Uyen et al., 2020).

Sodium alginate is widely used in the production of filmogenic solutions especially due to its colloidal properties. It is a linear heteropolysaccharide constituted of D-mannuronic acid residues united by β-(1→4) bonds and L-guluronic acid residues united by α-(1→4) bonds (Ching et al., 2017). This polysaccharide is considered as safe for consumption in the European Union (E 401 and E 404) and the United States (GRAS, 21 CFR 184.1187 and GRAS, 21 CFR 184.1724), and is used as a functional ingredient in foods (Qin et al., 2018).

Glycerol is also present in the composition of alginate-based filmogenic solutions (Shigematsu et al., 2018; Rodrigues et al., 2018). It can modify mechanical and permeability properties in the obtained solutions (Paixão et al., 2019). Although glycerol can be metabolized by *L. reuteri* for reuterin production under suitable conditions (Vollenweider & Lacroix, 2004), in filmogenic solutions this approach was not very studied. Malmo et al. (2013) reported the reuterin production by *L. reuteri* DSM 17938 coated in alginate-chitosan beads obtained by spray-drying. However, the *in situ* synthesis of this antimicrobial compound in filmogenic solutions has not yet been reported.

Thus, the aim of this study was to optimize the *in situ* reuterin production by *Limosilactobacillus reuteri* strains DSM 20016 and DSM 17938 in a filmogenic solution using alginate and glycerol, evaluating the cell viability of the reuterin-producing bacteria during fermentation.

2 Material and Methods

2.1 Material

L. reuteri strains DSM 20016 and DSM 17938 were obtained from Coleção de Cultura Tropical (Fundação André Tosello) under the number CCT 3433 and isolated from PROVANCE® (Aché Laboratórios Farmacêuticos S.A.), respectively. To produce the filmogenic solutions, high viscosity sodium alginate (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil) and glycerol ≥ 99.5 % (Anidrol Produtos para Laboratórios Ltda., Diadema, SP, Brazil) were used. Bacteriological peptone and yeast extract were purchased from HiMedia (Mumbai India). Both de Man, Rogosa and Sharpe broth and agar were obtained from Merck (Darmstadt, Germany). The sodium chloride, tryptophan, sodium citrate and ethanol were acquired from Dinâmica Química Contemporânea Ltda (Diadema, SP, Brazil). Acrolein standard was obtained from Riedel-de Haën (Seelze, Hannover, Germany).

2.2 Microbial growth conditions

The stock cultures of *L. reuteri* were kept frozen at −18 ºC in a medium of glycerol (130 g/L), bacteriological peptone (4.3 g/L), yeast extract (2.6 g/L), and NaCl (4.3 g/L), and then were activated in de Man, Rogosa and Sharpe sterile broth. The cultures were incubated at 37 ºC for 24 h under aerobic static conditions. Subsequently, *L. reuteri* biomass was obtained by centrifugation at 9800 x *g* for 10 min (Sorvall Legend XTR, Thermo Scientific™, Germany) and used in the production of filmogenic solutions.

2.3 Optimization and determination of *in situ* reuterin production by *L. reuteri* in filmogenic solutions

The filmogenic solutions were prepared using sodium alginate (20 g/L) and different glycerol concentrations. The contents of sodium alginate and glycerol were homogenized in distilled water using a digital disperser (Ultra-turrax, IKA T25, Brazil)

at 710 x *g* for complete dissolution. The solutions were subjected to the vertical autoclave sterilization process (AV-SD 137, Phoenix, Brazil) at 121 ºC for 15 min. After cooling to 42 ºC, *L. reuteri* was inoculated to reach the desired proportions.

The study to optimize the reuterin production was conducted by a completely randomized design and both *L. reuteri* strains (DSM 20016 or DSM 17938) were tested separately. Two independent variables were tested: the glycerol concentration (0, 25, 50, 100, 200, and 300 mmol/L) and the initial biomass of *L. reuteri* (approximately 6, 7, and 8 log CFU/mL). The samples were analyzed at 0 h and after 24, 48, and 72 h of anaerobic fermentation at 37 ºC. The anaerobic conditions were achieved by insufflating nitrogen inside in the sealed flasks of 100 mL containing the reactional medium, whose were purged with nitrogen gas for 7 minutes to ensure anaerobic conditions. The encoding of the treatments is in Table 1.

Table 1. Distribution and encoding samples of optimization study of *in situ* reuterin production in filmogenic solutions.

glycerol (mmol/L)	*L. reuteri* DSM 20016 (≅ log CFU/mL)			*L. reuteri* DSM 17938 (≅ log CFU/mL)		
	6	7	8	6	7	8
0	A60	A70	A80	D60	D70	D80
25	A61	A71	A81	D61	D71	D81
50	A62	A72	A82	D62	D72	D82
100	A63	A73	A83	D63	D73	D83
200	A64	A74	A84	D64	D74	D84
300	A65	A75	A85	D65	D75	D85

The reuterin quantification in filmogenic solutions was carried out indirectly according to the colorimetric method proposed by Circle et al. (1945), with adaptations. Initially, 1320 µL of the samples was homogenized with 300 µL of a 0.1 M tryptophan solution (dissolved in 0.05 M HCl) and 600 µL of ethanol (95 %). The samples were diluted in sodium citrate (20 g/L) and incubated at 40 ºC for 50 min. Then, the absorbances were measured by spectrophotometry (DU 640, Beckman Coulter, CA, USA) at 560 nm. The reuterin content was determined by comparing the absorbance of the samples with an acrolein standard curve previously constructed in the range

from 2 to 100 mmol/L, assuming that 1 M of dehydrated reuterin corresponded to 1 M of acrolein.

2.4 *L. reuteri* cell viability

In order to relate the *L. reuteri* viability with the reuterin production, the number of *L. reuteri* viable cells present in the filmogenic solutions was determined before (0 h) and after fermentation at 37 ºC (24, 48, and 72 h). Thus, 1 mL of each assay solution was diluted in a sterile peptone solution (1 g/L) followed by pour plating in de Man, Rogosa, and Sharpe agar. The samples were incubated at 37 ºC for up to 72 h under aerobic conditions. Posteriorly, the *L. reuteri* viability was estimated by counting of the number of colony-forming units (CFU/mL).

2.5 Statistical analysis

All samplings were made in genuine triplicate. The data were analyzed with an assumption of a normal distribution and subjected to ANOVA and the means were compared by the Tukey test using the Statistica 10 software (Tulsa, OK, USA). The t-test was applied when the comparison of means between two sample groups was required. The results were considered significant at $p<0.05$.

3 Results and Discussion

3.1 Optimization of *in situ* reuterin production in filmogenic solutions

Fig. 1 and 2 present the results obtained from the optimization study of the *in situ* reuterin production by *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938, respectively. The highest reuterin production in the filmogenic solutions was 68.39 mmol/L using *L. reuteri* DSM 20016 in the fermentation process, while the *L. reuteri* DSM 17938 strain produced a maximum of 30.00 mmol/L. Thus, it indicates that different *L. reuteri* strains have different reuterin production capacities through the

bioconversion of glycerol. This difference can occur due to particularities in the gene expression, cell age, and the presence of heterologous cells (Spinler et al., 2008; Ortiz-Rivera et al., 2017). Thus, besides the choice of *L. reuteri* strain, both the preservation and maintenance of microbial cultures require careful attention to ensure that recovered cultures works as the originals.

The effects of the glycerol concentration on the reuterin production were tested by varying the substrate concentration from 0 to 300 mmol/L in the filmogenic solutions. The production of the antimicrobial compound was not detected during the periods analyzed in the controls (samples without glycerol) and in D61, D62, and D71. In general, higher bioconversion rates from glycerol to reuterin were obtained using 50 and 100 mmol/L of glycerol. The highest reuterin production was obtained using 100 mmol/L of glycerol in A83 ($p<0.05$), in which the conversion yields from glycerol to reuterin were directly calculated and estimated at 68.39, 68.02, and 66.17 % after 24, 48, and 72 h of anaerobic fermentation, respectively. Even higher yields were obtained in A82 (83.7 and 92.32 % after 48 and 72 h of fermentation, respectively), the reuterin content produced under these conditions was about 1.5 folds lower than A83. Glycerol concentrations below 50 mmol/L (25 mmol/L) or above 100 mmol/L (200 and 300 mmol/L) were not considered effective in the process. Although the reuterin production was detected in fimogenic solutions prepared with 200 mmol/L of glycerol in A74 and A84, which are close to the highest amounts obtained using 100 mmol/L of glycerol, the bioconversion rates were lower than A83 (32.28 and 30.49 %, respectively). In addition, they were only achieved after 48 and 72 h. Similar results were reported by Doleyres et al. (2005) using *L. reuteri* ATCC 53608. The authors observed a decrease in the conversion rate from glycerol to reuterin at increased glycerol concentrations (84, 82, 77, and 62 % for 200, 250, 300, and 400 mmol/L of glycerol, respectively). The use of high glycerol concentrations during catalysis can inactivate the glycerol dehydratase, which is responsible for the production of electron acceptors in the fermentation of glycerol and the consequent reuterin production (Liu & Yu, 2015).

Fig. 1 Reuterin production and *L. reuteri* DSM 20016 cell viability according to initial biomass concentration at 6 **(a)**, 7 **(b)** and 8 log CFU/mL **(c)** at 0 and after 24, 48 and 72 hours of anaerobic fermentation (37 ºC), without (● – A60, A70, A80) and with glycerol at 25 (● – A61, A71, A81), 50 (■ – A62, A72, A82), 100 (▲ – A63, A73, A83), 200 (■ – A64, A74, A84) and 300 mmol/L (▲ – A65, A75, A85). *Each data point represents the mean of assays performed in triplicate.

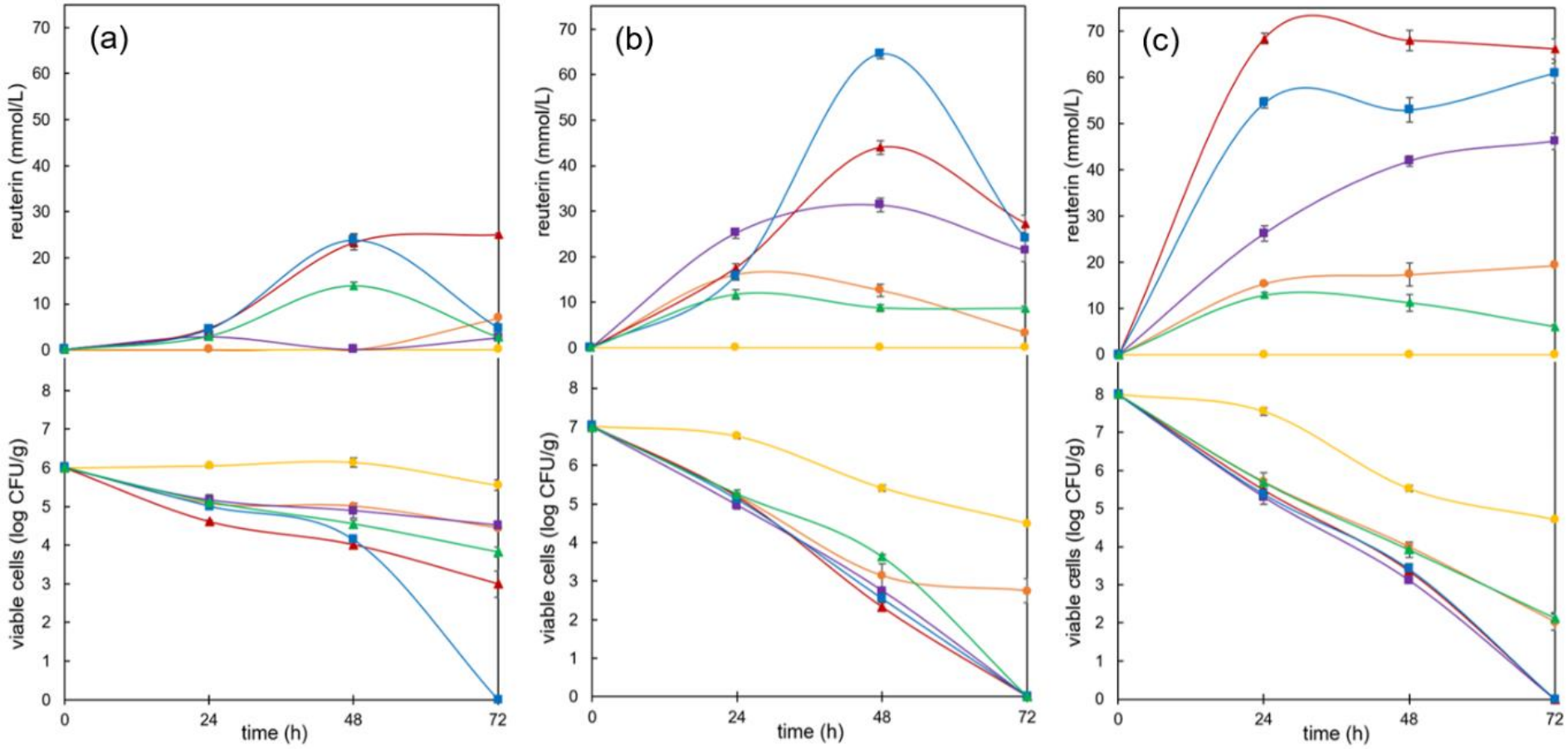
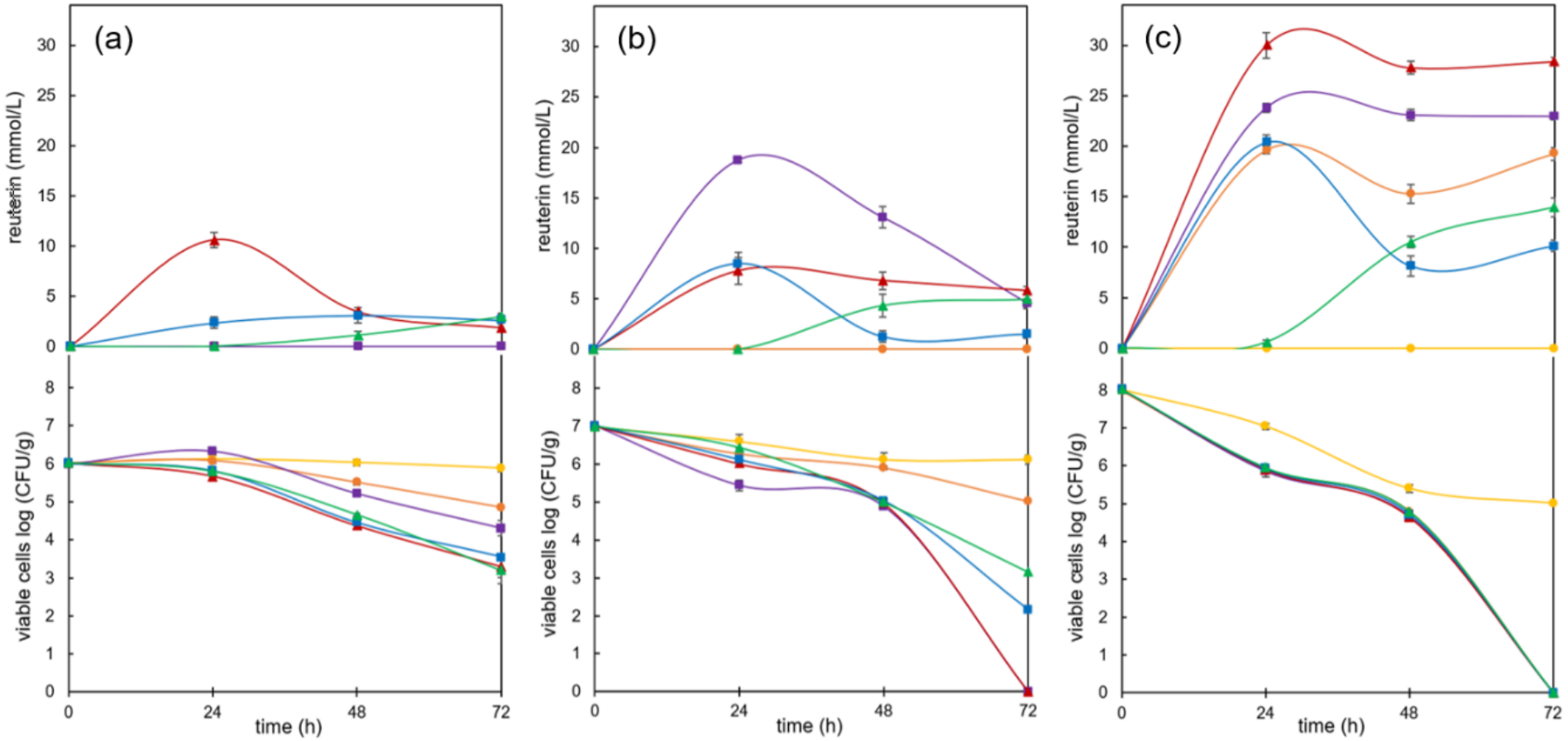


Fig. 2 Reuterin production and *L. reuteri* DSM 17938 cell viability according to initial biomass concentration at 6 **(a)**, 7 **(b)** and 8 log CFU/mL **(c)** at 0 and after 24, 48 and 72 hours of anaerobic fermentation (37 ºC), without (● – D60, D70, D80) and with glycerol at 25 (● – D61, D71, D81), 50 (■ – D62, D72, D82), 100 (▲ – D63, D73, D83), 200 (■ – D64, D74, D84) and 300 mmol/L (▲ – D65, D75, D85). *Each data point represents the mean of assays performed in triplicate.



The fermentation time (24, 48, and 72 h) did not have a significant effect ($p>0.05$) considering the highest reuterin rates (A83), showing a total reduction of 2.22 mmol/L from 24 to 72 h of fermentation. Martín-Cabrejas et al. (2017) reported the *in situ* reuterin production in cheeses supplemented with glycerol (100 up to 500 mmol/L) and *Lactobacillus reuteri* INIA P572 between 4.42 and 4.71 μmol/g on the first day of storage. However, the authors emphasized that the increase in the storage time resulted in a decrease in the reuterin concentration, reaching less than 0.6 μmol/g after 30 days. The decrease in reuterin content may be due to its interaction with the cellular material and/or free amino groups in the medium. Thus, prolonged incubation or storage can result in a decrease in the free metabolite concentration (Doleyres et al., 2005). In addition, the reuterin production can be interrupted due to its accumulation in the medium, resulting in cell death in *L. reuteri* caused by either the inhibition of ribonucleotide reductase, which compromises the DNA synthesis (Talarico & Dobrogosz, 1989), or the interaction and modification of thiol groups in active peptide structures, such as glutathione, thioredoxin, and glutaredoxin, which can induce cells to oxidative stress (Doleyres et al., 2005; Schaefer et al., 2010).

The effect of the initial biomass of *L. reuteri* on the bioconversion from glycerol to reuterin was tested at approximately 6, 7, and 8 log CFU/mL. The increase in the *L. reuteri* biomass from 7 to 8 log CFU/mL increased the reuterin production from 17.52 (A73) to 68.38 mmol/L (A83) and from 7.77 (D73) to 30.00 (D83) mmol/L after 24 h of anaerobic fermentation using *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938, respectively. Besides being associated with an increase in the reuterin production, it was also observed that the increase in the initial biomass of *L. reuteri* may be related to the decrease in the fermentation time to obtain the higher productions of reuterin. In general, the use of the initial biomass concentration of *L. reuteri* at approximately 8 log CFU/mL coincided with the highest production of the reuterin content (A83) immediately after 24 h of fermentation (68.30 mmol/L), with no significant difference ($p> 0.05$) regarding the increase in the fermentation time to 48 and 72 h. In contrast, the initial biomass of *L. reuteri* at 7 log CFU/mL led to a slower biosynthesis from glycerol to reuterin, mainly in A73 and A74, in which the increase in the fermentation time to 48 h resulted in an increase in the reuterin production from 17.52 to 43.94 mmol/L (A73) and from 15.76 to 64.56 mmol/L (A74), representing 2.5- and 4-fold higher metabolite content, respectively. A similar behavior was reported by Doleyres

et al. (2005) in a study to optimize the production of 3-HPA using a two-step fermentation process with *L. reuteri* ATCC 53608. In this study, the increase from 7 × 10^8 to 1.6 × 10^{10} CFU/mL in the initial inoculum led to an increase from 26 mmol/L to 160 mmol/L in the production of 3- HPA, besides decreasing the incubation time from 2 h to 45 min. Ortiz-Rivera et al. (2017) have recently reported the *in situ* reuterin production in fermented milk using the initial inoculum of *L. reuteri* ATCC 52608 at approximately 5 × 10^9 CFU/mL and 200 mmol/L of glycerol. After fermentation, the highest reuterin content was 33.97 mmol/L. However, the authors emphasized that the production was 107.5 mmol/L in aqueous solution, suggesting that the physical and chemical changes of the reaction medium possibly influences the metabolite production, either by the presence of cofactors that favored the conversion from glycerol to reuterin or interferences in the glycerol diffusion reducing its availability to *L. reuteri* cells.

3.2 Survival of *Limosilactobacillus reuteri* in filmogenic solutions

The susceptibility of *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938 to the *in situ*-produced reuterin in filmogenic solutions after anaerobic fermentation at 24, 48, and 72 h was tested. In general, the increase in the fermentation time and the consequent increase in the time of exposure to reuterin significantly decreased ($p<0.05$) the number of viable *L. reuteri* cells present in the filmogenic solutions as compared to the controls (samples without glycerol) (Fig. 1 and 2). Martín-Cabrejas et al. (2017) optimized the reuterin production in cheese by *L. reuteri* INIA P572 initially inoculated at more than 6.3 log CFU/g. The authors reported a gradual decrease in the cell viability during 30 days of cheese ripening, reaching cell counts lower than 4.5 log CFU/g in samples with 100–500 mmol/L of glycerol, while the control (without glycerol) kept a viable cell count of 6.34 log CFU/g after the same storage time. However, the highest reuterin concentrations present in these cheeses were between 4.42 and 4.71 μmol/g, contrasting with the highest results obtained in the present study.

The use of the initial biomass of *L. reuteri* at approximately 7 and 8 log CFU/mL, usually associated with a high production of reuterin content, showed a sharp decrease in the bacterial cell viability during the bioconversion of glycerol. After 72 h

of anaerobic fermentation, no viable *L. reuteri* cells were detected by the standard pour plate method employed in A72, A73, A74, A75, A82, A83, A84, D72, D73, D81, D82, D83, D84, and D85, while a low number of viable cells was detected in A71, A81, A85, D74, and D75 (2.75, 2.02, 2.13, 2.17, and 3.15 log CFU/mL, respectively). Although the cellular survival of *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938 during fermentation showed a similar behavior, *L. reuteri* DSM 20016 produced higher reuterin contents (Fig. 1), suggesting that this specific strain may have greater resistance to the *in situ* reuterin produced.

In previous studies, *L. reuteri* DSM 20016 and SD 2112 showed resistance to reuterin, reaching ranges between 30-50 mmol/L and 60-120 mmol/L for the minimum inhibitory concentration and minimum bactericidal concentration, respectively (Cleusix et al., 2007). On the other hand, *L. reuteri* ATCC 53608 viable cells were not detected in the presence of 8.5 mmol/L of reuterin (Ortiz-Rivera et al., 2017). In the present study, it was observed that in filmogenic solutions with a reuterin content ranging from 7.77 to 68.39 mmol/L, the *L. reuteri* viability cell decreased rapidly after 48 h and no viable cells were detected after 72 h at 37 ºC (Fig. 1 and 2).

Langa et al. (2013) reported the *in situ* reuterin production by *L. reuteri* INIA P579 in dairy products supplemented with 50 mmol/L of glycerol. In cheeses initially inoculated at 5.85 log CFU/mL, *L. reuteri* presented a decrease in the number of viable cells (>3 log CFU/g) during 30 days of storage at 12 ºC, in which the highest reuterin content estimated was 5.52 mmol/L. In parallel, the same authors reported the *in situ* reuterin production in yogurt, which did not show a significant decrease in the *L. reuteri* viability during 28 days of storage, however, in this case the storage temperature was 6 ºC.

The temperature of the medium during the conversion from glycerol to reuterin may be directly related to the decrease in the *L. reuteri* cell viability. Doleyres et al. (2005) reported that reuterin can be less reactive at low temperatures, maintaining the metabolite yield or even the bacterial cell viability under these conditions. The authors reported that the use of temperatures at 5 and 15 ºC during the conversion from glycerol to reuterin did not cause loss in the cell viability of *L. reuteri* ATCC 53608 and no viable cells were detected at 30 and 37 ºC after 1.5 and 2 h of fermentation. However, high reuterin productions were obtained from 5 to 37 °C,

demonstrating the wide activity range of the glycerol dehydratase. du Toit et al. (2010) emphasized that high temperatures increase the spontaneous non-enzymatic dehydration from 3-HPA to acrolein. This dehydration process may be related to the lower reuterin reactivity suggested by Doleyres et al. (2005). Thus, different temperatures can be adopted in the fermentation process according to the application purpose. Therefore, the fermentation temperature (37 ºC) used in the present study may be associated with the increase in the reuterin reactivity or even with greater cellular sensitivity of *L. reuteri* strains as compared to the metabolite produced in filmogenic solutions, especially at temperatures close to the ideal growth temperature of the microorganisms used (Doleyres et al., 2005).

As far as we know, this is the first report on reuterin production in filmogenic solution. Furthermore, the findings of present study establish conditions for *in situ* reuterin production in filmogenic solutions constituted of alginate or another similar natural hydrocolloid. Enabling its application for different purposes, such as, edible coatings for extend the shelf life of foods and/or support material for delivery of chemical or biological compounds.

4 Conclusion

This study confirmed the potential of *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938 for the *in situ* reuterin production in alginate-based filmogenic solutions using glycerol as substrate. Both *L. reuteri* strains studied were susceptible to reuterin produced, being that in the presence of higher reuterin concentrations an evident decrease in the cell viability of *L. reuteri* was detected. The highest reuterin production was obtained using *L. reuteri* DSM 20016. Furthermore, the use of the initial microbial biomass at 8 log CFU/mL was related to an increase in the reuterin production and a decrease in the fermentation time to obtain a higher metabolite concentration. In parallel, the use of 100 mmol/L of glycerol also improved the reuterin production, but even though approximately 1.5 folds lower, higher conversion yields from glycerol to reuterin were obtained using 50 mmol/L of the substrate.

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CHAPTER III

RESEARCH ARTICLE

Effective encapsulation of reuterin-producing *Limosilactobacillus reuteri* in alginate beads prepared with different mucilages/gums

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Effective encapsulation of reuterin-producing *Limosilactobacillus reuteri* in alginate beads prepared with different mucilages/gums

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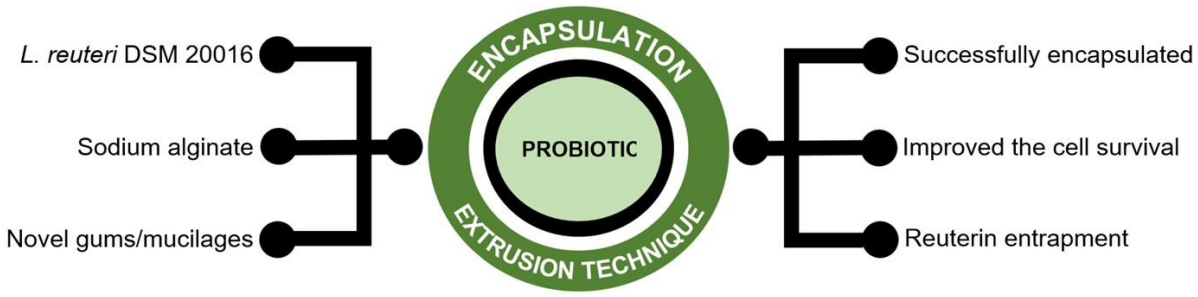
Highlights

- Novel and promising gums/mucilages for microbial encapsulation was firstly reported.
- *L. reuteri* was successfully encapsulated in alginate-mucilaginous solutions blends.
- Alginte-gums/mucilages blends improved the survival of encapsulated *L. reuteri*.
- *L. reuteri* produced reuterin from glycerol in all in film-forming solutions.
- Reuterin entrapment was improved using mucilaginous material in bead's composition.

Graphical abstract



Abstract

The mainly aim of this study was to use mucilaginous solutions obtained from tamarind, mutamba, cassia tora, psyllium and konjac powdered to encapsulate reuterin-producing *Limosilactobacillus reuteri* in alginate beads by extrusion technique. In the particles were determined the bacterial encapsulation efficiency, cell viability during storage and survival under simulated gastric and intestinal conditions. Moreover, the reuterin production, its entrapment into the beads and the influence on viability of encapsulated microorganism were evaluated. Scanning electron microscopy and Fourier Transform Infrared spectroscopy were employed to characterize the produced particles. The beads showed a relatively spherical shape with homogenous distribution of *L. reuteri*. The use of gums and mucilages combined with alginate improved the encapsulation efficiency (from 93.2 to 97.4 %), the viability of encapsulated bacteria during refrigerated storage (especially in prolonged storage of 20, 30 and 60 days) and the survival after exposure to gastric and enteric environments (from 67.7 to 76.6 %). The *L. reuteri* was able to produce reuterin via bioconversion of glycerol in the film-forming solutions, and the entrapment of the metabolite was improved using konjac, mutamba and tamarind mucilaginous solutions in the encapsulation process (45, 44.57 and 41.25 %, respectively). Thus, our findings confirm the great potential of these hydrocolloids to different further purposes, enabling its application as support material for delivery of chemical or biological compounds.

Keywords: probiotic; 3-hydroxypropionaldehyde; *Lactobacillus reuteri*; immobilization; gastrointestinal environment.

1 Introduction

Accepted by the European Food Safety Authority (EFSA, 2008) as a food supplement to improve gastrointestinal health, *Limosilactobacillus reuteri* is recognized for its ability to produce and accumulate, under anaerobic conditions, high contents of 3-hydroxypropionaldehyde (3-HPA) through bioconversion from glycerol (Vollenweider & Lacroix, 2004; Sardari et al., 2013). The 3-HPA belongs to a dynamic system, named as HPA system, which contains 3-HPA, its hydrated form 1,1,3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (Vollenweider et al., 2003). This system was patented with the name reuterin (Dobrogosz & Lindgren, 1995). Its scientific interest is notable, since reuterin has antimicrobial properties, such as antifungal activity and broad spectrum of activity against Gram-positive and negative bacteria (Talarico & Dobrogosz, 1989; Vollenweider & Lacroix, 2004) being related to the probiotic activity of *L. reuteri* (Mu et al., 2018).

Currently, the concept of probiotic is defined by Food and Agriculture Organization of the United Nations (FAO), supported by the World Health Organization (WHO) as bacteria and yeasts that, when administered in adequate amounts, confer health benefits to the host (FAO/WHO, 2002). Due to the benefits conferred to human health, probiotics have been investigated in the prevention and treatment of various conditions (Cremon et al., 2018; Park et al., 2018; Zaylaa et al., 2018; Heydari et al., 2019). However, to promote beneficial effects probiotics must be stable to environmental conditions, enabling its adherence to the intestinal mucosa, colonization of the human gastrointestinal tract, production of antimicrobial compounds and maintenance of metabolic activity in the intestine (Collins et al., 1998; Saarela et al., 2000). Even though the evident health-promoting properties related to the administration of probiotics, the use of these microorganisms is limited by extrinsic factors, such as oxygen concentration, presence of hydrogen peroxide, pH and temperature variations, etc. In this sense, bacterial encapsulation is a useful approach to protect encapsulated cells under adverse conditions (Raddatz et al., 2020; Rodrigues et al., 2020).

Several techniques are currently available to encapsulate probiotic cells, but these procedures should not be aggressive, ensuring sufficient viability of the encapsulated cells (Rathore et al., 2013; Rodrigues et al., 2020;). Thus, due to low

cost, easy application and possibility of use of several hydrocolloid materials, the extrusion technique, which is a relatively mild process is widely used (Krasaekoopt & Watcharapoka, 2014; Etchepare et al., 2016; Rodrigues et al., 2017; Cedran et al., 2021). A diversity of hydrocolloid materials can be used to encapsulate bacterial cells by extrusion, for instance alginate, gums and mucilages. Alginates are commonly used in the probiotic encapsulation. However, its use results in the formation of porous particles, which are susceptible to disintegration (Rodrigues et al., 2020). In contrast, the combination of alginate with others hydrocolloid compounds, such as gums and mucilages is related with the improvement of the viability of encapsulated microorganisms (Rodrigues et al., 2017; Dokoohaki, et al., 2019; Cedran et al., 2021).

Abundantly available in nature, gums and mucilages have been used in partial substitution of alginates to change the particle properties obtained, improving the protection and survival of encapsulated cells during storage, under food processing conditions and passage through simulated gastric and intestinal tracts (Rodrigues et al., 2017; Nami et al., 2020; Shafizadeh et al., 2020). Recently, Rodrigues et al. (2020) reported traditional and novel materials extracted from plants for probiotic encapsulation, such as psyllium (*Plantago ovata* Forssk) mucilage, konjac (*Amorphophallus konjac*) gum, mutamba (*Guazuma ulmifolia*) mucilage, cassia tora (*Cassia tora* L. and *Cassia obtusifolia*) gum and tamarind (*Tamarindus indica* L.) gum. Despite its structural characteristics indicate great potential as emerging biopolymer for probiotic encapsulation (Mathur, 2012; Esmaeilzadeh et al., 2016; Nayak & Pal, 2017; Mu et al., 2018; Pereira et al., 2019), until now the available literature related is scarce. Besides, there have been no literature records regarding the use of mutamba, cassia tora and tamarind mucilages to encapsulate probiotic microorganisms.

This study evaluated the influence of mucilaginous solutions obtained from tamarind, mutamba, cassia tora, psyllium and konjac in alginate beads carriers of reuterin-producing *Limosilactobacillus reuteri* DSM 20016 obtained by extrusion technique. The encapsulation efficiency, cell viability during storage and survival under simulated gastrointestinal conditions of encapsulated bacteria was evaluated and compared among the samples. Moreover, the reuterin production, its entrapment into the beads and influence on viability of encapsulated *L. reuteri* was also determined.

The particles were characterized by scanning electron microscopy and Fourier Transform Infrared spectroscopy.

2. Materials & Methods

2.1 Microorganism and chemicals

Limosilactobacillus reuteri DSM 20016 was obtained from Tropical Culture Collection (André Tosello Foundation), classified in this collection by the number CCT 3433. The following substances were employed for preparing the beads: High viscosity sodium alginate (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil); mutamba mucilage, extracted from the seed of mutamba (*Guazuma ulmifolia* Lam.) fruits collected between August and September 2018 in natural areas of the Cerrado Biome located in the municipality of Uberlândia (18°55′07″ South latitude, 48°16′38″ West longitude and 863 m altitude), Minas Gerais, Brazil; psyllium (*Plantago ovata* Forssk) mucilage (SARGOL 99/100), cassia tora (*Cassia tora* L.) gum (LB COL-200) and tamarind (*Tamarindus indica* L.) gum (TEMCOL 30), kindly provided from Sarda Biopolymers Pvt. Ltd. (Maharashtra, India); and konjac (*Amorphophallus konjac*) gum, supplied by Fooding Group Limited (Shanghai, China). Other chemicals and solvents used were: tryptophan, hydrochloric acid, calcium chloride, sodium citrate (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil); ethanol 95 % (Vetec Química Fina Ltda., RJ, Brazil), glycerol ≥99.5 % (Anidrol Produtos para Laboratórios Ltda., SP, Brazil), and acrolein standard obtained from Riedel-de Haën (Seelze, Hannover, Germany). The pepsin (swine gastric mucosa), lipase and pancreatin (swine pancreas), besides bovine bile, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Microbial growth conditions

L. reuteri DSM 20016 were kept frozen at −18 °C in a medium composed of glycerol (130 g/L), bacteriological peptone (4.3 g/L), yeast extract (2.6 g/L), and NaCl (4.3 g/L), and then was activated in de Man, Rogosa and Sharpe sterile broth (Merck,

Germany). The culture was incubated at 37 °C for 24 h under aerobic static conditions. Subsequently, *L. reuteri* biomass was obtained by centrifugation at 9800 ×*g* for 10 min (Sorvall Legend XTR, Thermo Scientific™, Germany) followed by its resuspension in sterile buffered peptone water (1 g/L at pH 6.5). Cell count was adjusted at 10^9 CFU/mL before the encapsulation.

2.3 Extraction of mutamba mucilage

The method used to extract the mucilage from mutamba seeds was adapted from Pereira et al. (2019). Precisely 10 g of seeds were weighed into centrifuge tubes containing 180 mL of ultrapure water (OS50LXE, Gehaka®, SP, Brazil). The mixture was kept at room temperature for 20 hours to ensure complete seed hydration. The mucilaginous layers were extracted using a 13 mm diameter, 19 kHz ultrasonic probe (Unique, Disruptor, 800 W, Indaiatuba, SP, Brazil) at a power of 475 W for 13 min. The mucilage was obtained by separating the mucilaginous liquid from the seeds with stainless steel sieves followed by centrifugation at 5000 ×*g* for 10 min (Sorvall Legend XTR, Thermo Scientific™, Germany). Subsequently, the mucilage obtained was lyophilized and kept frozen (−18 °C) until use.

2.4 Encapsulation of reuterin-producing *L. reuteri*

Using a digital disperser (Ultra-turrax, IKA T25, Campinas, SP, Brazil) the sodium alginate was homogenized (6000 rpm) with or without the addition of 100 mmol/L of glycerol in 50 mL of distilled water or mucilaginous solution from mutamba, psyllium, cassia tora, tamarind and konjac, which were previously hydrated overnight at 5 °C. The composition and nomenclature of alginate-mucilaginous solutions are presented in Table 1. Due to the high apparent viscosity, the concentration of psyllium and konjac mucilaginous solutions were adjusted to 5 g/L, which allowed the use of the proposed extrusion technique. The film-forming solutions were sterilized in an autoclave at 121 °C for 15 minutes and after cooling (42 °C) the biomass suspension of *L. reuteri* DSM 20016 was aseptically added to reach an initial cell count between 8.3–8.7 log CFU/mL. Then, *L. reuteri* was encapsulated by extrusion technique. The

film-forming solutions was dripped using sterile needles (ø 0.7 mm) into a sterile $CaCl_2$ solution (20 g/L). The beads obtained was kept for 30 minutes for complete crosslinking and gelling, being later removed by aseptic filtration, washed with sterile distilled water and stored at 4 °C ± 2 °C.

Table 1 – Composition and nomenclature of alginate-mucilaginous solutions blends used for encapsulate *L. reuteri*.

Composition			**Nomenclature**
alginate (20 g/L)	–	–	Control
		glycerol (100 mmol/L)	Control-G
	tamarind gum (10 g/L)	–	TG
		glycerol (100 mmol/L)	TG-G
	mutamba mucilage (10 g/L)	–	MM
		glycerol (100 mmol/L)	MM-G
	cassia tora gum (10 g/L)	–	CG
		glycerol (100 mmol/L)	CG-G
	psyllium mucilage (5 g/L)	–	PM
		glycerol (100 mmol/L)	PM-G
	konjac gum (5 g/L)	–	KG
		glycerol (100 mmol/L)	KG-G

2.4.1 Scanning electron microscopy (SEM)

The wet particles were dried in an oven with air circulation (Tecnal, TE-394/1, Piracicaba, SP, Brazil) at 45 °C for 36 hours. Then, the surface and cross-sectional morphology of the *L. reuteri*-loaded particles were observed using a scanning electron microscope (SEM; Leo 440i, LEO Electron Microscopy, Cambridge, UK) with X-ray dispersive energy detector (EDX; 6070, LEO Electron Microscopy, Cambridge, UK). Prior to observation, the samples were coated with 200 Å-thick layer of gold using a sputter coater (K450, Kent, UK).

2.4.2 Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectra of the particles and the powdered hydrocolloid materials were recorded with a IRPrestige-21 Fourier transform infrared spectrometer (Shimadzu, Kyoto, Japan) in the wavelength range of 4000–400 cm^{-1}. The KBr pellets were prepared with a sample to KBr ratio of 1:100 (w/w) (except for powdered konjac gum and KG, whose were prepared in 1.5:100 ratio) in stainless steel molds (13 mm of diameter) compressed into transparent discs using an SSP-10A hydraulic press at 80 kN for 10 min under vacuum (Shimadzu, Kyoto, Japan).

2.4.3 *L. reuteri* encapsulation efficiency (EE)

The EE was determined by counting of the *L. reuteri* viable cells before and after extrusion in calcium chloride. The number of *L. reuteri* viable cells present in the film-forming solutions or in the beads were determined by direct counting of the number of colony-forming units (log CFU/g) employing the pour plating method. Thus, 1 g of each sample was homogenized and diluted using a vortex mixer (Model K45-2810, Kasvi, Brazil) with periodic agitations (10 seconds every minute) in 9 mL of sterile sodium citrate solution (30 g/L) with pH adjusted to 6.0 and temperature of 45 °C for 15 minutes, followed by serial dilution and plating in de Man, Rogosa and Sharpe agar (Merck, Germany). Then, the samples were incubated at 37 °C for up to 72 h under aerobic conditions. The data obtained were applied in Equation (1), where X_t referred to the total number of viable cells encapsulated; X_i referred to the total number of live cells inoculated into film-forming solution.

$$EE(\%) = \frac{Xt}{Xi}.100 \qquad \textbf{(1)}$$

2.4.4 *L. reuteri* survival in refrigerated storage

The produced beads were analyzed to determine the number of *L. reuteri* viable cells during storage (0, 3, 6, 9, 12, 15, 20, 30 e 60 days) at 4 °C ± 2 °C, according

to direct counting of the number of colony-forming units (log CFU/g) proposed in section 2.4.3.

2.4.5 *L. reuteri* survival under simulated gastrointestinal conditions

Prior to assay, a *L. reuteri* free cell culture (FC) was previously obtained according to the method proposed in section 2.2. Then, the survival of FC and encapsulated *L. reuteri* was evaluated in different buffer solutions and at predetermined times. Thus, 3 g of each sample were placed in falcon tubes containing 30 mL of simulated gastric juice (0.85 % saline solution with pH adjusted to 2.3–2.6 with 1 M HCl) containing pepsin and lipase, at a final concentration of 3 g/L and 0.9 mg/L, respectively. The tubes were incubated at 37 °C for 2 hours, simulating the gastric phase. In the next step – enteric phase I, the pH of the reaction medium was adjusted to 5.4–5.7 using an alkaline solution (150 mL of 1 M NaOH and 14 g of $NaH_2PO_4.2H_2O$ for 1 L of distilled water). Bile and porcine pancreatin were added to the reaction medium to reach a concentration of 10 g/L and 1 g/L, respectively. After a period of 2 hours at 37 °C, the pH of the samples was adjusted to 6.8–7.2 using the same alkaline solution used in the enteric phase I, maintaining the concentrations of bile and pancreatin. Then the samples were incubated at 37 °C for another 2 hours, simulating the enteric phase II (Bedani et al., 2014). The *L. reuteri* survival was performed with aliquots of sample dilutions (from 1 g of sample) after 0, 2, 4 and 6 hours of *in vitro* digestion, which were plated in de Man, Rogosa and Sharpe agar (Merck, Germany) by the pour plate technique. The samples were incubated at 37 °C for up to 72 hours under aerobic conditions.

2.5 Reuterin production

The ability of *L. reuteri* DSM 20016 for the *in situ* reuterin production in alginate-mucilage/gum-based film-forming solutions using glycerol as substrate was tested (samples Control-G; PM-G; KG-G; MM-G; CG-G and TG-G described in Table 1). These film-forming solutions produced according to method described in section 2.4 were subjected to anaerobic fermentation at 37 °C for 24 hours, according to

Rodrigues et al. (2021). At the end of fermentation *L. reuteri* and reuterin-content produced present in the film-forming solutions were encapsulated by extrusion technique in calcium chloride cross-linking solution (20 g/L) according to section 2.4.

2.5.1 Determination of *in situ* reuterin production

The reuterin quantification in the film-forming solutions and in the produced beads were carried out indirectly according to the colorimetric method proposed by Circle et al. (1945), with adaptations. Initially, 1 g of each sample analyzed was diluted in 9 mL of sodium citrate (20 g/L). Posteriorly, 320 µL of the samples was homogenized with 300 µL of a 0.1 M tryptophan solution (dissolved in 0.05 M HCl) and 600 µL of ethanol (95 %). The samples were incubated at 40 °C for 50 min. Then, the absorbances were measured by spectrophotometry (DU 640, Beckman Coulter, CA, USA) at 560 nm. The reuterin content was determined by comparing the absorbance of the samples with an acrolein standard curve previously constructed in the range from 2 to 100 mmol/L, assuming that 1 M of dehydrated reuterin corresponded to 1 M of acrolein.

2.5.2 Reuterin entrapment

The reuterin entrapment was estimated via the comparison of reuterin-content present in the film-forming solutions to the reuterin detected after extrusion in calcium chloride and consequent formation of hydrocolloid particles. The data obtained were applied in Equation (2), where Rt referred to the reuterin detected in the beads; Ri referred to the reuterin-content present in the film-forming solutions.

$$Reuterin\ Entrapment\ (\%) = \frac{Rt}{Ri}.100 \qquad \textbf{(2)}$$

2.6 Statistical analysis

All samplings were made in triplicate, except the *L. reuteri* survival under simulated gastrointestinal conditions, which was performed in duplicate. The data were subjected to ANOVA and the means were compared by the Tukey test using the Statistica 10 software. The t-test was applied when the comparison of means between two sample groups was required. The results were considered significant at $p<0.05$.

3 Results & Discussion

3.1 Scanning electron microscopy (SEM)

Details obtained by SEM to evaluate the structure, surface morphology and the microstructure of the beads are presented in Figs. 1 and 2. The particles produced by the extrusion technique showed a spherical or relatively spherical shape independently of the addition of gums or mucilages (Fig. 1 A, C, E and G). Homogenous distribution of *L. reuteri* was observed in the surface and cross-sectional morphology of the particles (Fig. 1 E and H). Similar results were reported by Karimi et al. (2021) that produced *L. reuteri* particles based on alginate. However, the addition of asafoetida and zedo gum make the beads surface smoother and improved the physical barrier to the encapsulated cells.

The particle surface of the Control sample was smoother then observed into the beads containing gum or mucilage, suggesting that fractions of these hydrocolloids gelled on the surface of the particles, slightly increasing the roughness (Fig. 1 A, C, E and G). This result is in accordance with data reported by Nasiri et al. (2021), which encapsulated *Lactobacillus casei* in alginate microcapsules containing wild sage seed mucilage by emulsion technique. Their results demonstrated spherical beads with roughness surface due to wild sage seed mucilage incorporation. In this sense, the use of glycerol in the particle's composition changed the structure of the produced beads making them smoother (Fig. 2). In this sense, Jouki et al. (2021) related the softer structure of quince seed gum-alginate beads to plasticizer capacity of glycerol used in the production of microcapsules. In addition, to characterize the structure of

the bead's polymeric network, FTIR assays were conducted and discussed in the next section.

Fig. 1 – Scanning electron microscopy (SEM) of the *L. reuteri*-loaded particles. Surfaces of the particles and cross-sectional morphology of Control (A & B), TG (C & D), CG (E & F), and KG (G & H) at 150× magnification. The images inserted in B, D, E and H were taken at 5000× magnification. *Refer to Table 1 for details of each of the samples.

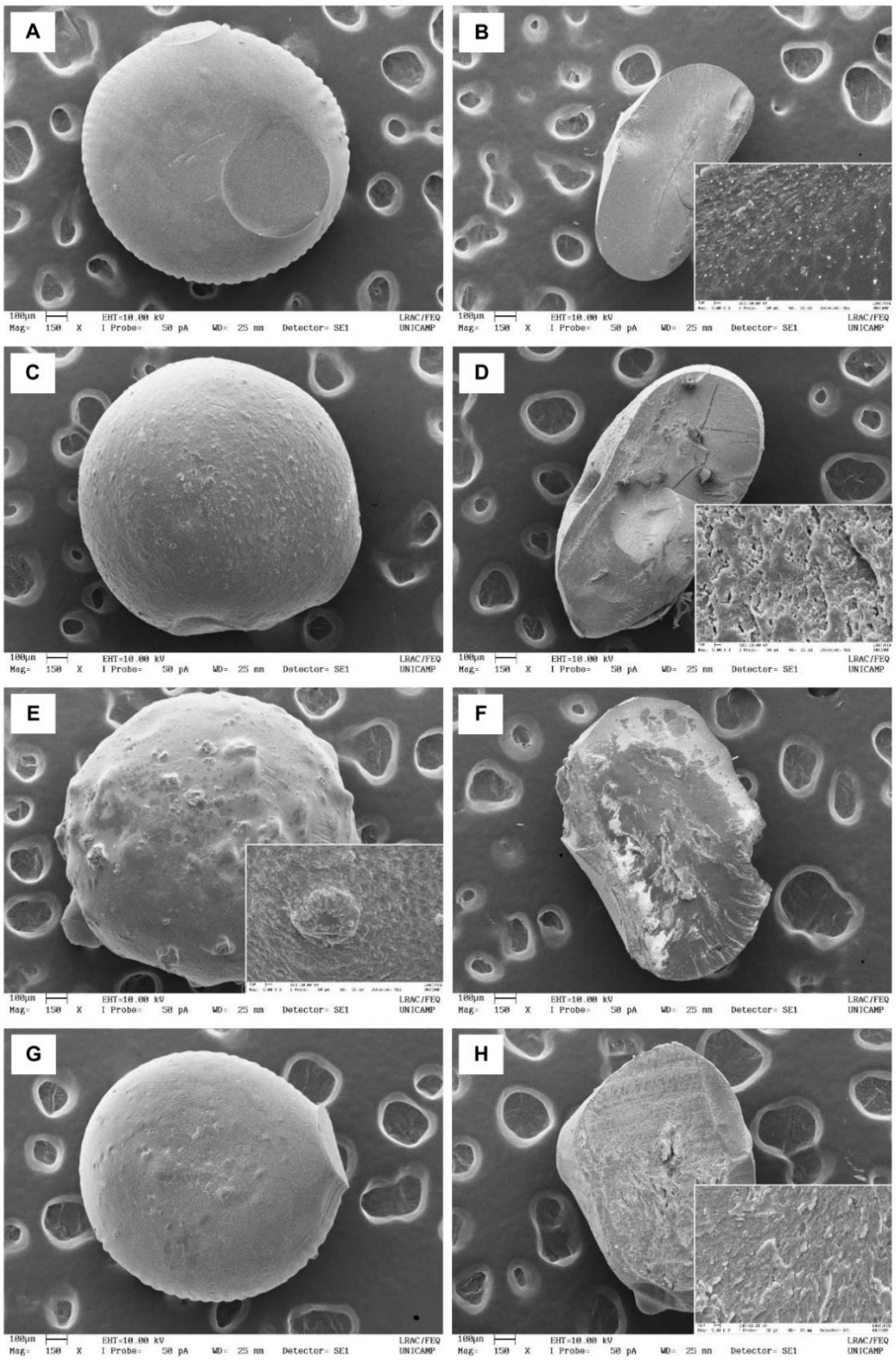
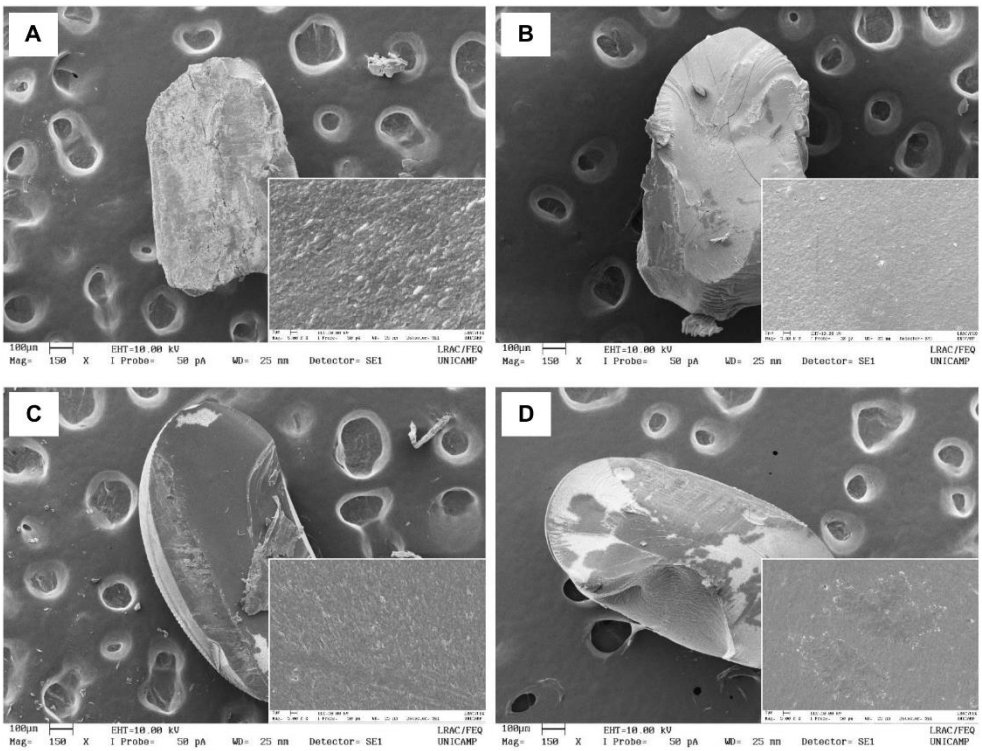


Fig. 2 – Scanning electron microscopy (SEM) of the *L. reuteri*-loaded particles. Cross-sectional morphology of PM (A), PM-G (B), MM (C) and MM-G (D) at 150× magnification. The inserted images were taken at 5000× magnification. *Refer to Table 1 for details of each of the samples.



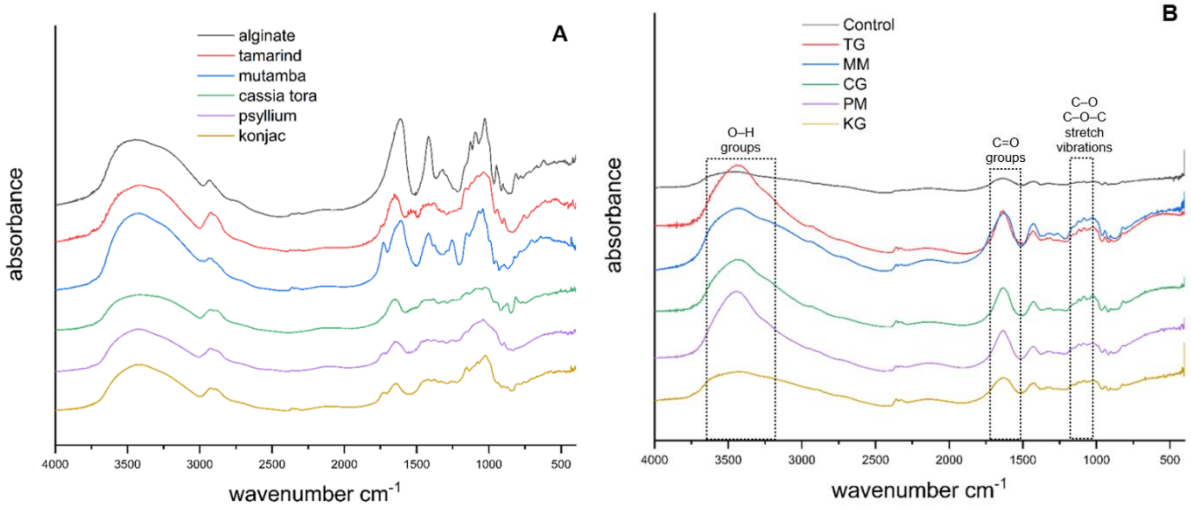
3.2 Fourier-transform infrared spectroscopy (FT-IR)

In general, the FT-IR spectra of the powdered hydrocolloid materials (Fig. 3A) showed a characteristic band of O–H stretching and intra- and intermolecular hydrogen bonds between 3650 cm^{-1} and 3000 cm^{-1}, being a broad band centered at 3430 cm^{-1}. Another weaker peak was detected in the same samples in the region between 2950 cm^{-1} and 2800 cm^{-1}, centered at 2910 cm^{-1} due to C–H stretching vibrations. These two bands are characteristics of all polysaccharides (Pereira et al., 2019).

The powdered alginate FT-IR spectra showed two characteristic bands at 1600 and 1400 cm^{-1} corresponding to a carbonyl group of carboxylic acid (Belščak-Cvitanović et al., 2015) and asymmetric and symmetric stretching vibrations of C–O bond of carboxylate salt ion (Rather et al., 2017), characteristic of an alginate structure. In addition, the spectral bands between 1320 and 1025 cm^{-1} suggest the presence of

guluronic acid (Ramos et al., 2018), with peaks between 1150 and 1045 cm^{-1} being previously related to C–O stretching vibrations of the pyranosyl ring (Rather et al., 2017). At the same time, were detected asymmetric stretching vibrations between 900 and 800, whose usually correspond to mannuronic acid units (Ramos et al., 2018).

Fig. 3 – FT-IR spectra of the powdered hydrocolloid materials (A) and of the alginate-mucilage/gum beads (B). *Refer to Table 1 for details of each of the samples.



The FT-IR spectra of powdered tamarind gum presented three bands at 1730, 1640 and 1540 cm^{-1}, which are regions with stretching vibrations related to proteins and carbohydrates. Being the 1730 and 1640 cm^{-1} wavenumbers related to C=O stretching characteristic of amide I ($–CONH_2$ groups), while 1540 cm^{-1} was associated to amide III ($–NH_3^+$ groups) (Alpizar-Reyes et al., 2017). The peak at 1440 cm^{-1} is related to C–C stretching vibrations, and the bands between 1400 and 1050 cm^{-1} are characteristic of xyloglucan backbone, being related to CH_2 bending (1370 cm^{-1}) and C–O bond stretching of the xyloglucan ring (1161 and 1050 cm^{-1}) (Crispín-Isidro et al., 2019).

Although until now there have been few records available, the FT-IR patterns of the lyophilized mutamba mucilage detected bands at 1732 and 1143 cm^{-1}, which are related with stretching vibrations of C=O and C–O–C present in the pyranose ring. In addition, the bands at 1600 and 1417 cm^{-1} its due to presence of the

carboxylate ion from the uronic acids, as well as the peak observed at 1253 cm^{-1} may represent uronic acid contents. Asymmetrical vibrations were also detected between 1075–1045 cm^{-1}, whose are assigned to C–O–C stretching of glycosidic bonds and C–O–H bending (Pereira et al., 2019). In parallel, the FT-IR spectra of cassia tora gum showed an absorption peak at 1654 cm^{-1} indicating the presence of carbonyl groups (Wu et al., 2017), while stretching vibrations at 1022 cm^{-1} are attributed to C–H bending, indicating carbohydrate fractions in the mucilage (Deore et al., 2020).

The FT-IR spectrum of psyllium mucilage showed bands at 1726 cm^{-1} and 1649 cm^{-1}, whose are characteristics vibration of ester carbonyl group and carboxylic group (Belščak-Cvitanović et al., 2015). The stretching vibration of C–O–C group was detected at 1049 cm^{-1}, suggesting the presence of arabinoxylans (Belščak-Cvitanović et al., 2015; Monge Neto et al., 2017). In parallel, the konjac gum FT-IR spectra detected bands at 1730 and 1651 cm^{-1} with stretching vibrations that are characteristics assigned to acetyl groups (C=O) and the presence of amide groups (–CONH–), respectively (Silva et al., 2020). Bending vibration correspondent to symmetric carboxylate group and C–O at 1390 and 1174 cm^{-1}, respectively were detected, while the peak at 1014 cm^{-1} is related to C–O–C stretching bond (Özbaş et al., 2021).

The FT-IR spectra of alginate-mucilage/gum interactions were studied (Fig. 3B). In the presence of Ca^{2+} ions, the beads presented a similar FT-IR spectra with the powder results. Besides, the presence of lower intensity peaks in Control indicates the interaction of alginate with calcium ions. In the samples, the O–H groups had been detected in the region between 3600 cm^{-1} and 3200 cm^{-1}, however the peak was narrower, and the intensity was reduced. According to Daemi & Barikani (2012) the hydroxyl and carboxylate groups present in the alginate participate to form chelating structure to the calcium ion, which decrease in hydrogen bonding between hydroxyl functional groups in calcium alginate. The peaks at 1627 and 1417 cm^{-1}, also narrower and lesser intensity, were assigned to the stretching vibrations of carbonyl group. In contrast, Ramos et al. (2018) reported peaks of greater intensity in the same region in calcium alginate particles. The authors attributed this change to the substitution of sodium ions by calcium ions, which can result in a change in the charge density of carboxyl groups. In addition, the shifting of COO^- peaks to higher wavenumber are

related to the satisfactory interaction between calcium and anionic hydrocolloid fractions (Rather et al., 2017). The asymmetric bands in the region between 1150 and 1020 cm^{-1} were assigned to the stretching vibrations C–O and C–O–C, which are the characteristic of the natural hydrocolloids (Nayak et al., 2013). Overall, the FT-IR spectra of alginate-mucilage/gum beads showed characteristic peaks without any significant shift. Thus, no interaction between the hydrocolloids was confirmed.

3.3 *L. reuteri* encapsulation efficiency

In present study, the evaluation of EE according to the hydrocolloid materials used to produce the *L. reuteri* particles ranged from 85 to 97.4 % (Table 2). In general, the encapsulation technique was sufficiently satisfactory to entrap the *L. reuteri* cells in the hydrocolloid matrices. There was no critical difference in EE when mucilages and gums were used with alginate.

Table 2 – *L. reuteri* encapsulation efficiency (EE) in alginate-mucilage/gum beads.

Samples	Log CFU.g^{-1} during encapsulation		EE (%)
	before[I]	after[II]	
Control	8.54 ± 0.080	7.80 ± 0.020	91.44^{bc}
Control-G	5.50 ± 0.142	4.68 ± 0.036	85.09 ^{c}
TG	8.61 ± 0.066	8.25 ± 0.058	95.93^{ab}
TG-G	5.76 ± 0.020	5.37 ± 0.108	93.23^{ab}
MM	8.51 ± 0.046	8.12 ± 0.034	95.42^{ab}
MM-G	6.31 ± 0.270	5.90 ± 0.020	93.50^{ab}
CG	8.45 ± 0.075	8.23 ± 0.191	97.40 ^{a}
CG-G	5.94 ± 0.025	5.58 ± 0.095	93.94^{ab}
PM	8.47 ± 0.026	8.15 ± 0.136	96.10 ^{a}
PM-G	6.47 ± 0.198	6.18 ± 0.113	95.36 ^{a}
KG	8.34 ± 0.068	8.04 ± 0.050	96.28 ^{a}
KG-G	6.73 ± 0.115	6.46 ± 0.030	95.99 ^{a}

[I]*L. reuteri* cells detected in the film-forming solution. [II]*L. reuteri* cells detected in the beads. *Refer to Table 1 for details of each of the samples. **Means followed by the same lowercase letter in the column do not differ by the Tukey test (p>0.05). ***Each data point represents the mean of assays performed in triplicate.

The highest EE was obtained in CG, PM, PM-G, KG and KG-G (p<0.05). In contrast, beads produced only alginate-based showed lower EE (Control and Control-

G). Despite widely employed for microbial encapsulation, the use of alginate can result in the formation of porous particles. Therefore, the use of hydrocolloids extracted from plants in combination with alginate can improve particle properties and enhance the entrapment of the cells (Rodrigues et al., 2020). Besides exhibiting high colloidal stability, several mucilaginous solutions present anionic characteristics, which can increase the electrostatic interactions in the formation of the beads (Waghmare et al., 2021), improving the EE.

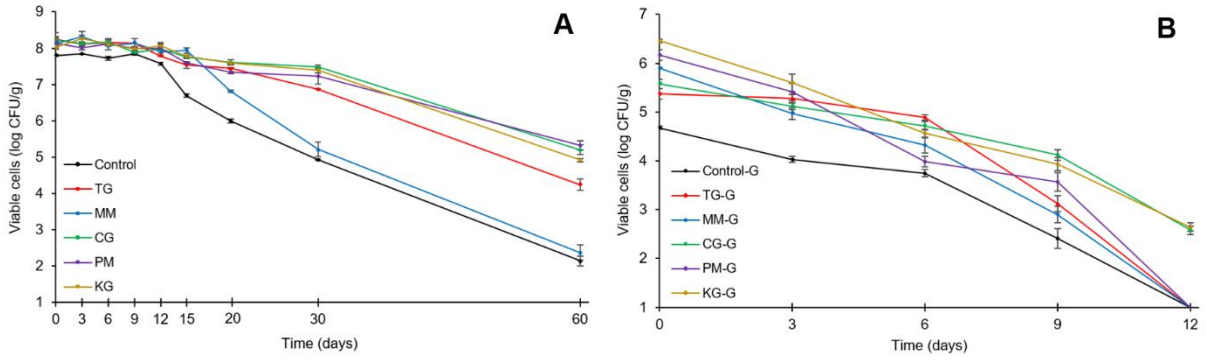
Similar to our results, Kahieshesfandiari et al. (2021) and Nami et al. (2020) reported the encapsulation by extrusion of *Enterococcus faecium* ABRIINW.N7 and *Lactococcus lactis* ABRIINW-N19 in alginate-basil-fenugreek and alginate-persian gum, respectively. The produced particles showed an EE >98 %. In contrast, Shah et al. (2016) reported EE between 71–79 % using β-glucan to encapsulate *Lactobacillus plantarum* NCDC 012, *Lactobacillus casei* NCDC 297, and *Lactobacillus brevis* NCDC 021 by emulsion technique. In parallel, Rajam & Anandharamakrishnan (2015) reached an EE between 70 and 73 % when encapsulating *Lactobacillus plantarum* MTCC 5422 using fructooligosaccharides by spray drying technique. As consequence, it is evident that both material and technique applied affect the entrapment of bacterial cells in the obtained particles.

3.4 *L. reuteri* survival in refrigerated storage

The *L. reuteri* survival in refrigerated storage was investigated. In fact, the most of probiotic content-products must be kept at refrigeration temperatures to maintain the microorganism survival, avoiding sharp decreases in its microbial population (Dianawati et al., 2016). Fig. 4 shows that the viable cell count of encapsulated *L. reuteri* decreased during storage ($p<0.05$). However, the decrease was smaller in samples without glycerol and no subjected to anaerobic fermentation process before encapsulation. The glycerol bioconversion can result in reuterin production and its accumulation in the medium can cause *L. reuteri* cell death by either the inhibition of ribonucleotide reductase or the interaction and modification of thiol groups in active peptide structures, which can compromise the DNA synthesis and induce cells to oxidative stress, respectively (Talarico & Dobrogosz, 1989; Doleyres et

al., 2005; Schaefer et al., 2010). In this sense, the *L. reuteri* beads, in which film-forming solutions were subjected to previous anaerobic fermentation, showed a strong decrease in the encapsulated bacterium viable cell count (Fig. 4B). In 12 days of refrigerated storage no viable *L. reuteri* cells were detected in Control-G, TG-G, MM-G and PM-G, while a low number of viable cells ($p>0.05$) was detected in CG-G and KG-G (2.65 and 2.58 log CFU/g, respectively).

Fig. 4 – *L. reuteri* survival in alginate-mucilage/gum beads during refrigerated storage. Samples with (B) or without (A) the induction of reuterin production. *Refer to Table 1 for details of each of the samples. **Each data point represents the mean of assays performed in triplicate.



In contrast, the samples in which the *in situ* reuterin production was not induced showed viable cell counts after 60 days of refrigerated storage (Fig. 4A), although a significant reduction, compared to the initial count ($p<0.05$), was noticed. The total reduction in viable cell count after 60 days of refrigerated storage was averaged 2.99 log CFU/g in KG, PM, and CG, while the Control showed a sharp decrease of 5.66 log CFU/g ($p<0.05$). This indicates that the use of hydrocolloids, such as konjac gum, cassia tora gum or psyllium mucilage combined with alginate can improve the survival of encapsulated *L. reuteri* cells over time. It is important to emphasize that cassia tora mucilage have not been reported to encapsulate probiotics. This hydrocolloid extracted from dark-colored seeds of *Cassia tora* L. and *Cassia obtusifolia*, basically constituted of mannose and galactose (Rodrigues et al., 2020), demonstrated to be an efficient support for encapsulating *L. reuteri*. In addition, no

decrease in the number of *L. reuteri* viable cells in KG, PM and CG was detected for 12 days of refrigerated storage ($p>0.05$).

Mu et al. (2018) employed konjac glucomannan hydrogels to encapsulate *Lactobacillus acidophilus* by the emulsion technique. The cell viability of the encapsulated bacteria was evaluated for 4 weeks at 4 and 25 °C. The hydro microcapsules produced presented stability during storage, with decreases of 1.7 and 2.5 log CFU/g at 4 and 25 °C, respectively. The authors emphasized the improvement of *L. acidophilus* viability when konjac oligosaccharides were applied as prebiotic, besides the influence of high temperatures in the particle protective effect, which can be weakens due to decomposed of polymer material inducing changes in the permeability of the beads. In parallel, Peredo et al. (2016) reported high survival in storage conditions (4 °C for 30 days) of *Lactobacillus plantarum* (Lp33 and Lp17) encapsulated in alginate-psyllium mucilage blend by extrusion. The total reduction in the number of viable cells of the encapsulated probiotics at the end of storage ranged from 0.75 to 0.77 log CFU/g. A similar trend was evidenced in the present study, the *L. reuteri* beads constituted of alginate-psyllium mucilage showed a total reduction in the number of viable cells of 0.91 and 2.82 CFU/g in 30 and 60 days of refrigerated storage, respectively. The psyllium mucilage is composed of arabinoxylans that consist of xylan structures with multiple side chains consisting of xylose and arabinose residues. Although it was not fully explored, the improvement of the alginate-psyllium mucilage particle properties and consequent bacteria protection can be related with the improvement of integrity of surface topography of the beads (Rodrigues et al., 2020), or due to herbal-based polymers, such as psyllium present prebiotic characteristic which can favor encapsulated probiotic cells (Haghshenas et al., 2015).

On the other hand, the survival of *L. reuteri* in beads consisting of alginate-mutamba mucilage (MM) showed a maintenance behavior of probiotic cell viability for 15 days of refrigerated storage ($p>0.05$), showing total reduction on 0.18 log CFU/g. However, after 20 days a sharp decrease was detected, reaching at the end of storage (60 days) on 5.74 log CFU/g; not differing from the Control ($p>0.05$). According to Pereira et al. (2019), the mutamba mucilage present a profile of monosaccharides containing galactose, galacturonic and glucuronic acids, rhamnose, glucose and smaller amounts of arabinose/mannose. The surface charge density (ζ potential)

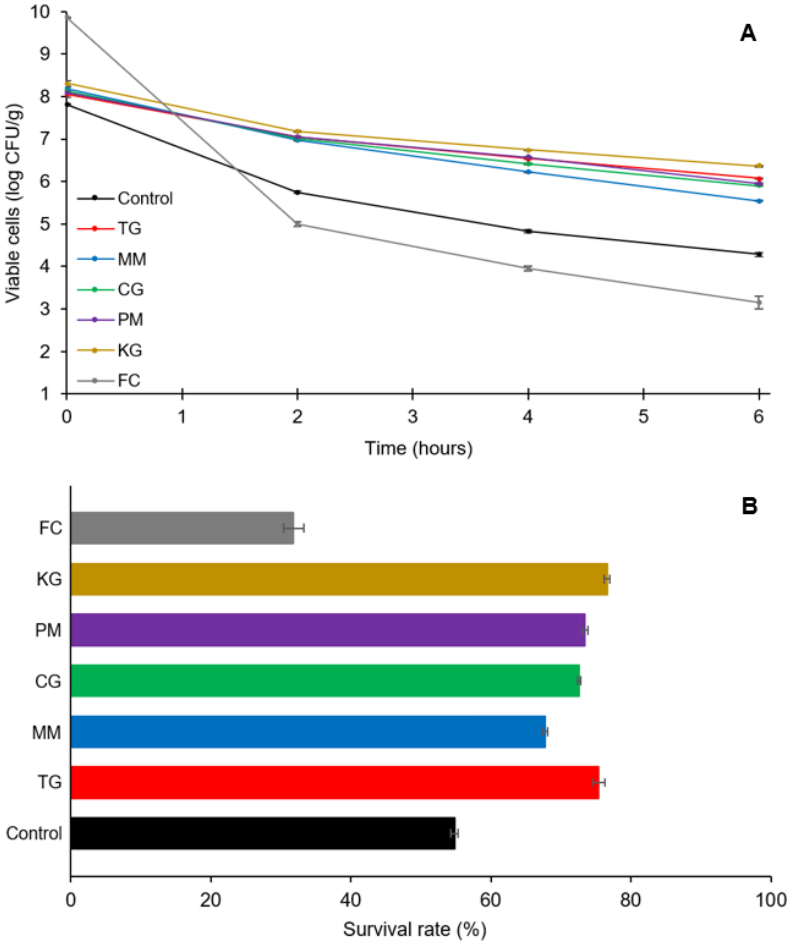
confers a stable hydrocolloid mucilage system with an anionic character, which according to Manzoor et al. (2020) may favor the ionic crosslinking of the polymer chains by divalent cations, like the calcium cations used in extrusion technique. Despite producing stable gels to temperature changes, they can present dryness. Until now, the mutamba mucilage has not been explored to encapsulate microorganisms, but Pereira et al. (2019) reported the encapsulation of the volatile compounds from orange peel oil in emulsion systems. The mutamba mucilage could efficiently retain volatile compounds after 72 hours, especially if compared with other biopolymers widely used by food industry (gum acacia, Hi-cap 100 and Snow-Flake E6131).

Tamarind gum, another hydrocolloid not yet explored to encapsulate bacteria, was tested. Constituted mainly by galactoxyloglucans containing glucose, galactose and xylose, the hydrocolloid from tamarind seed is related to its ability to form high viscosity solutions with thermal and chemical stability (Rodrigues et al., 2020). Moreover, due to its valuable potential functional and a low-cost, the tamarind gum should be applied in encapsulation procedures (Alpizar-Reyes et al., 2020). The same authors reported that the tamarind gum was successfully employed as a novel wall material for sesame oil encapsulation, protecting it against oxidation. In contrast, the tamarind gum present low solubility in cold water and biodegradability, which can limit its efficiency in forming microparticles, mainly caused by the droplet aggregation (Guo et al., 2020). To overcome these disadvantages, Khounvilay et al. (2019) suggested a carboxymethylation process for crude tamarind gum, which can confer it an anionic character. In our study, the results obtained during refrigerated storage showed that the beads constituted of alginate-tamarind gum (TG) were capable to maintain the number of viable cells of encapsulated *L. reuteri* for 9 days of storage ($p>0.05$); result also observed in Control. Even though the total decrease observed in the *L. reuteri* cell viability in TG was greater than KG, PM and CG ($p<0.05$), reaching 4.01 log CFU/g after 60 days of refrigerated storage, this decrease was smaller ($p<0.05$) when compared to MM and Control samples, especially in prolonged storage conditions (20, 30 and 60 days). In addition to maintain the cell viability during storage, microorganisms with probiotic claims must be stable under gastrointestinal conditions (Rodrigues et al., 2020). In this way, the *L. reuteri* survival in acid and enteric simulated environments are discussed in the next section.

3.5 *L. reuteri* survival under simulated gastrointestinal conditions

The encapsulation technique and the encapsulating materials used should be suitable for that the entrapped bacteria remain viable during passage through gastric and enteric juices (Rodrigues et al., 2020). The survival of FC and encapsulated *L. reuteri* in different hydrocolloid blends before (0 h) and during exposure to gastric (2 h) and enteric (4 and 6 h) conditions is showed in Fig. 5A. It was observed that the total reduction in the number of *L. reuteri* viable cells after exposure to simulated gastric and enteric juices was higher for FC, which showed an expressive decrease by 6.72 log CFU/g on viable cell count ($p<0.05$). This demonstrates that the encapsulation process protected the encapsulated cells under gastric and enteric environments.

Fig. 5 – Viable cell counts (A) and survival rate (B) of free (FC) and encapsulated *L. reuteri* in alginate-mucilage/gum beads before (0 h) and after exposure to simulated gastric (2 h) and enteric (4 and 6 h) conditions. *Refer to Table 1 for details of each of the samples. **Each data point represents the mean of assays performed in duplicate.



After 2 hours of exposure to simulated gastric juice, the viability of FC showed sharped decrease ($p<0.05$) by 4.87 log CFU/g, representing a reduction of 49.39 %. In contrast, the survival of encapsulated *L. reuteri* under the same conditions was improved. The use of gums and mucilage in the composition of the beads conferred even greater protection to *L. reuteri* cells compared to Control ($p<0.05$), which showed reduction of 26.38 %, while TG, MM, CG, PM and KG showed an average reduction of 12.55, 14.67, 13.67, 12.96 and 13.6 %, respectively. The sharped decline in the viability of bacterial cells subjected to gastric juices were generally attributed to the acidic environment and presence of digestive enzymes (Chean et al., 2021; Lai et al., 2021). Similar to our results, Nami et al. (2020) reported the survival of *Lactococcus lactis* ABRIINW-N19 encapsulated in alginate-persian gum-inulin blend under simulated gastric juices. According to the authors, un-encapsulated cells presented a reduction rate (log CFU/g) of 6.52, while cells encapsulated cells only 1.46. In parallel, Lai et al. (2021) reported the encapsulation of *Lactobacillus rhamnosus* GG in blend based in alginate-pectin-flaxseed mucilage by co-extrusion technique. After simulated gastric digestion, free cells showed greater reduction in the *L. rhamnosus* GG cell viability by 31.8 %, while encapsulated cells presented reduction between 5.9 and 10.7 %. Therefore, the use of gums and mucilages as wall material, besides improving the properties of the particles (Waghmare et al., 2021), it can increase the resistance of the encapsulated microorganisms due to possible synergistic effects between the entrapped bacteria and the encapsulating material (Haghshenas, et al., 2015; Rodrigues et al., 2017; Lai et al., 2021).

After exposure to simulated gastric phase, free and encapsulated *L. reuteri* were subject to simulated enteric stages (I and II) to complement the 6-hour *in vitro* assay. Although milder than the behavior observed in gastric phase, all samples also showed reduction in *L. reuteri* viable cell counts after both enteric stages, however the decrease was sharper ($p<0.05$) in FC (1.85 log CFU/g), representing decrease of 37.07 %, evidencing the harmful effects of bile salts on *L. reuteri* cells. In contrast, under the same conditions, higher survival cells rates were obtained in KG, TG, PM and CG, in which the decrease after exposure to simulated enteric juices were of 11.42, 13.78, 15.6 and 15.83 %, respectively. Besides, the protection conferred to the encapsulated microorganisms in these hydrocolloid blends was higher ($p<0.05$) than obtained in the Control, which presented a reduction of 25.57 % in *L. reuteri* viable cell count after the

enteric phases. Although alginate gels are widely used to encapsulate and protect microorganisms against intestinal fluids (Dimitrellou et al., 2019; Prasanna & Charalampopoulos, 2019), the protective effects of the beads depend on size, porosity, and surface properties (Rodrigues et al., 2020). The addition of gums and mucilages to alginate beads can improve the effectiveness of encapsulation process for protecting probiotics in intestinal conditions, mainly because the particles remain in contact with intestinal juices for a relatively long time (Shafizadeh et al., 2020).

Based on *L. reuteri* survival rate after simulated gastrointestinal conditions (Fig. 5B), KG, PM, CG, and TG provided higher protection, which ranged from 72.6 - 76.6 % ($p<0.05$), while the Control and FC showed only 54.8 and 31.84 %, respectively. Konjac glucomannan hydrogel combined with alginate were used to encapsulate *Lactobacillus acidophilus*. Its use improved the stability of the particles and the bacterium survival, mainly by increasing the tightness of the wall material and performing prebiotic activity, which can improve the cell viability by ensuring protection under gastric phase and progressively release it under enteric juices (Mu et al., 2018). In parallel, psyllium mucilage, was related with effective improvement of the alginate particle properties, in which integrity was improved (Esmaeilzadeh et al., 2016), besides it was associated with prebiotic characteristics, which can benefit probiotic bacteria, conditions that can enhances the cell survival under low pH and in the presence of bile salts (Haghshenas et al., 2015), Furthermore, the tamarind and cassia tora gums have been used in pharmaceutical applications, such as drug delivery and sustained release agent (Dey et al., 2019; Verma & Ahuja, 2020) and until now there were no literature records regarding their use to encapsulate microorganisms. In this sense, our study confirmed great potential of these gums to microorganism encapsulate purposes. Moreover, these hydrocolloid blends were also tested as support for the *in situ*-produced reuterin by *L. reuteri* cells. The main findings are discussed in the next section.

3.6 The reuterin production and entrapment

The Table 3 showed the reuterin-content present in the film-forming solutions and the reuterin detected after the encapsulation and consequent formation

of the beads. In all tested conditions, the *L. reuteri* cells were able to produce reuterin. However, the highest reuterin production in film-forming solutions were obtained in MM-G and Control-G reaching 60.12 and 58.52 mmol/L, respectively ($p>0.05$). In parallel, Angiolillo et al. (2017) reported the reuterin quantification in alginate solutions containing *L. reuteri* and glycerol. The reuterin content ranged from 1.92 to 11.42 mmol/L according to alginate concentration. The authors suggested that the presence of vitamin B_{12} in the alginate composition, which is cofactor of the glycerol dehydratase favored the glycerol-reuterin conversion. Therefore, the increase of alginate concentration in the film-forming solution improved the reuterin production.

Table 3 – Reuterin production (mmol/L) in the film-forming solutions and its detection after the entrapment into the beads.

Samples	Reuterin-content		Reuterin entrapment (%)
	solution	bead	
Control-G	58.52 ± 3.533	20.24 ± 0.855	34.63 ^{c}
TG-G	50.00 ± 1.959	20.61 ± 0.565	41.25^{ab}
MM-G	60.12 ± 1.864	26.79 ± 1.190	44.57 ^{a}
CG-G	51.23 ± 0.932	19.75 ± 0.770	38.56^{bc}
PM-G	50.98 ± 1.826	20.24 ± 0.213	39.73 ^{b}
KG-G	50.25 ± 1.496	22.59 ± 0.641	45.00 ^{a}

*Refer to Table 1 for details of each of the samples. **Means followed by the same lowercase letter in the column do not differ by the Tukey test ($p>0.05$). ***Each data point represents the mean of assays performed in triplicate.

Compared to samples MM-G and Control-G, the reuterin production in TG-G, CG-G, PM-G and KG-G was slightly smaller, ranging between 50 and 50.25 mmol/L ($p<0.05$). Chen et al. (2012) reported the reuterin production by *L. reuteri* DPC16 in alginate-chitosan microcapsules. The authors emphasized a markedly lower reuterin content when the particles contained chitosan in their composition. Thus, the addition of other hydrocolloids to the alginate matrix may change the physical and chemical properties of the beads, which may interfere in the glycerol diffusion into the particles, reducing its availability to immobilized *L. reuteri*, decreasing the bioconversion of glycerol into reuterin.

The encapsulation process can result in decrease of reuterin content (Angiolillo et al., 2017). In this study, the evaluation of reuterin entrapment into the beads ranged between 34.63 and 45 % (Table 3), being that the entrapment was

higher in KG-G, MM-G and TG-G ($p<0.05$). Although the high *in situ* reuterin production, the reuterin entrapment in Control-G was the lowest (34.63 %) reported in this study, however this result did not differ from CG-G ($p>0.05$), which present reuterin entrapment of 38.56 %. In parallel, Mishra et al. (2018) encapsulated the reuterin produced by *L. reuteri* BPL-36 using alginate and guar gum by extrusion technique. The reuterin encapsulation efficiency varied between 36.35 and 37.97 %. Similarly, Narsaiah et al. (2014) optimized the encapsulation of nisin in alginate-guar gum particles, reaching nisin entrapment of 36.65 %. The authors suggested that the addition of another hydrocolloid to structure of alginate beads may increase the density of the gel and favor the metabolite entrapment, which can be related with the results obtained in this study.

Currently, the use of reuterin in food preservation technologies gaining ground (Pilote-Fortin et al., 2021). Recently, it was reported the first development of a pectin coating with reuterin as a food preserver (Hernández-Carrillo et al., 2021). In parallel, it is common the use of hydrocolloid-based edible films carried of lactic acid bacteria with the same objective (Rodrigues et al., 2018; Shigematsu et al., 2018). Therefore, the combination of alginate and others hydrocolloids reported in this study establish conditions that enable its application for different purposes, such as edible film/coatings for extend the shelf life of foods and/or support material for delivery of chemical or biological compounds.

4 Conclusion

L. reuteri DSM 20016 was successfully encapsulated in different alginate beads added of mucilaginous solutions obtained from tamarind, mutamba, cassia tora, psyllium, and konjac by extrusion technique. The beads showed a relatively spherical shape independently of the addition of gums or mucilages, besides homogenous distribution of *L. reuteri*. The use of gums and mucilages with alginate improved the EE and the survival of encapsulated bacteria during refrigerated storage, being that konjac gum, cassia tora gum and psyllium mucilage improved the *L. reuteri* survival especially after prolonged storage (20, 30 and 60 days). In contrast, the samples in

which reuterin production was induced showed a strong decrease in the encapsulated cells count and after 12 days of storage no viable cells were detected.

The encapsulation process protected the encapsulated cells under gastric and enteric environments. However, the effects were dependent on the presence of gums or mucilages in the composition of the beads, being that the highest survival rates were obtained in KG, TG, PM, and CG. *L. reuteri* was capable to produce reuterin from glycerol in all in film-forming solutions. The highest production was obtained in Control-G and MM-G. In contrast, the metabolite entrapment was improved when mucilaginous materials from konjac, mutamba or tamarind were used. Thus, our findings confirm the great potential of the hydrocolloids tested as support material for biological or/and chemicals compounds. Enabling its applications for different further purposes, such as for delivery of probiotic microorganisms or for use of these reuterin content-mucilaginous solutions as edible film/coating for foods, a valuable approach to food safety.

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CHAPTER IV

RESEARCH ARTICLE

Inhibitory effect of reuterin-producing *Limosilactobacillus reuteri* and edible alginate-konjac gum film against foodborne pathogens and spoilage microorganisms

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Inhibitory effect of reuterin-producing *Limosilactobacillus reuteri* and edible alginate-konjac gum film against foodborne pathogens and spoilage microorganisms

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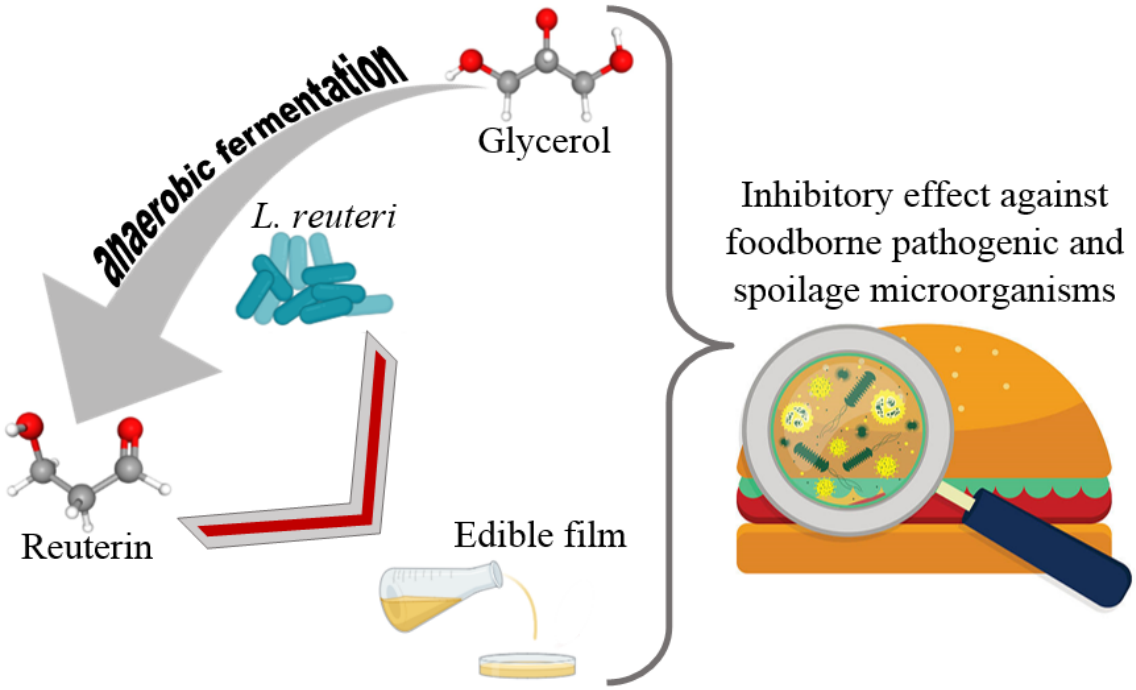
Highlights

- *L. reuteri* was screened for inhibitory effects against foodborne microorganisms.
- *In situ* reuterin production in alginate-konjac gum film-forming solution.
- *L. reuteri* and the reuterin *in situ*-produced were entrapped in the edible film.
- Reuterin production in edible films is a valuable approach to food safety.

Graphical abstract



Abstract

The possible inhibitory effects against foodborne pathogens and spoilage microorganisms of two strains (DSM 20016 and DSM 17938) of reuterin-producing *Limosilactobacillus reuteri*, and an edible alginate-konjac gum film containing *L. reuteri* cells and *in situ* produced reuterin was evaluated. An *in vitro* antimicrobial screening study indicated that both *L. reuteri* strains and the edible film showed inhibitory effects against *B. cereus*, *C. perfringens* and *P. aeruginosa*, and towards the fungi *F. oxysporum*, *C. gloeosporioides*, *A. alternata*, and *P. digitatum*. In addition, *L. reuteri* DSM 20016 was able to produce reuterin *in situ* (46.67 mmol/L) in alginate-konjac gum film-forming solution through anaerobic glycerol bioconversion. Furthermore, after the edible film production by extrusion technique, both *L. reuteri* and reuterin were successfully trapped (98 and 67.6 %, respectively). Further studies must be conducted to detect the exact metabolites related to the inhibition effects against foodborne pathogens and spoilage microorganisms.

Keywords: 3-hydroxypropionaldehyde; *Lactobacillus reuteri*; antimicrobial activity; edible film, konjac gum.

1 Introduction

Latic acid bacteria are widespread in nature, and several species are important representatives of human microbiota. This bacterial group includes several species that can be used in industrial food applications. Moreover, some of these species have demonstrated the capacity to produce organic acids, peroxides, bacteriocins and other molecules which can inhibit foodborne pathogens and spoilage microorganisms (Özogul & Hamed, 2018). Members of the *Lactobacillus* genus are the main representatives of latic acid bacteria. However, this genus has recently been divided into 25 new genera, based on phylogeny, phenotype, and habitat specificities (Zheng et al., 2020).

The *Limosilactobacillus reuteri* (formerly known as *Lactobacillus reuteri*) is a heterofermentative bacterium found in the human microbiome; and is accepted by the European Food Safety Authority (EFSA, 2008) as a food supplement to improve gastrointestinal health. In general, *L. reuteri* has the ability to produce and accumulate high reuterin content through an enzymatic reaction catalyzed under anaerobic conditions by glycerol dehydratase (F.J. Rodrigues et al., 2021). The reuterin produced by *L. reuteri* is a dynamic system, called the HPA system, which contains 3-hydroxypropionaldehyde (3-HPA), its hydrated form 1,1,3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (Vollenweider et al., 2003). The 3-HPA in an aqueous solution can suffer spontaneous dehydration to acrolein. Thus, it was recently suggested to include acrolein into the HPA system (Engels et al., 2016).

Reuterin has antimicrobial characteristics, such as antifungal activity (Vimont et al., 2019) and a broad spectrum of activity against Gram-positive and negative bacteria (Ávila et al., 2014; Langa et al., 2018). In addition, it is resistant to lipolytic and proteolytic enzymes, and maintains its antimicrobial activity at low pH, high saline concentrations (Rasch et al., 2007) and at refrigeration temperatures (Arqués et al., 2008). This dynamic compound has been related to the probiotic capacity of *L. reuteri*, however, its mechanism of inhibition against microorganisms is not fully understood. It was suggested that reuterin can compromise the DNA synthesis, by inhibiting ribonucleotide reductase (Talarico & Dobrogosz, 1989) and/or by induction of oxidative stress in the microbial cells, leading to cell death; due mainly to the depletion of thiol groups in glutathione, proteins, and enzymes (Schaefer et al., 2010).

The potential use of reuterin-producing *L. reuteri* as a biopreservative against food spoilage and pathogens microorganisms has been reported in various food matrices, such as dairy (Langa et al., 2018), meat (Montiel et al., 2016) and vegetable (Asare et al., 2018) products. Recently, F.J. Rodrigues et al. (2021) reported the *in situ* reuterin production in alginate-based filmogenic solutions using *L. reuteri* strains. These solutions can be employed to produce edible films that can be used against pathogens and spoilage microorganism inhibition purposes (Hernández-Carrillo et al., 2021; Mozaffarzogh et al., 2019). Meanwhile, natural polysaccharides extracted from plants and seeds in synergism with alginate have been commonly used to produce edible films (Rodrigues et al., 2018). Konjac (*Amorphophallus konjac*) gum, for instance, is a low-cost hydrocolloid, composed mainly of glucomannan, extracted from konjac tubers (Devaraj et al., 2019). This gum produces viscous and stable gels with film-forming capacities and has been related with food microbial preservation (Hashemi & Jafarpour, 2020, 2021; Li et al., 2021).

Thus, the aim of this study was to evaluate the possible inhibitory effects of two strains (DSM 20016 and DSM 17938) of reuterin-producing *Limosilactobacillus reuteri* and an edible alginate-konjac gum film containing *L. reuteri* cells and reuterin produced in situ against foodborne pathogens and spoilage microorganisms. In addition, the produced film was also evaluated through scanning electron microscopy (SEM) and to determine reuterin content, *L. reuteri* survival, and both entrapment efficiency.

2. Materials and Methods

2.1 Microorganisms and chemicals

The DSM 20016 and DSM 17938 strains of *Limosilactobacillus reuteri* were obtained from the Tropical Culture Collection (André Tosello Foundation), classified in this collection as CCT 3433, and isolated from PROVANCE (Aché Laboratórios Farmacêuticos S.A.), respectively. The microbial cultures used as indicators in assays of antagonistic activity included *Salmonella enterica* subsp. *enterica* serovar Typhimurium IAL 2431, *Staphylococcus aureus* subsp. *aureus* ATCC 13565,

Escherichia coli IAL 2064 (kindly provided from Laboratory of Food Microbiology, Department of Food Science, University of Campinas), *Bacillus cereus* ATCC 11778, *Clostridium perfringens* ATCC 13124, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enterica* subsp. *enterica* serotype Typhimurium ATCC 14028, *Staphylococcus aureus* subsp. *aureus* ATCC 8095, and *Escherichia coli* ATCC 11229. Filamentous fungi cultures included, *Aspergillus flavus* ATCC 16883, *Aspergillus niger* ATCC 1004, *Aspergillus niger* ATCC 22342, *Fusarium oxysporum* ATCC 48112, *Colletotrichum gloeosporioides* ISO024, *Alternaria alternata* ISO34, and *Penicillium digitatum* ISO003 (culture collection of Laboratory of Food Biochemistry, Department of Food Science, University of Campinas).

The film-forming solutions were prepared using high viscosity sodium alginate (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil), konjac (*Amorphophallus konjac*) gum (Fooding Group Limited, Shanghai, China) and glycerol ≥99.5 % (Anidrol Produtos para Laboratórios Ltda., SP, Brazil). Other chemicals and solvents used were: tryptophan, hydrochloric acid, calcium chloride, sodium chloride, sodium citrate, monobasic sodium phosphate (Dinâmica Química Contemporânea Ltda., Diadema, SP, Brazil); ammonium sulfate, Tween 80 (Êxodo Cientifica, Sumaré, SP, Brazil); ethanol 95 % (Vetec Química Fina Ltda., Duque de Caxias, RJ, Brazil), and acrolein standard obtained from Riedel-de Haën (Seelze, Hannover, NI, Germany). The culture media used included: plate count agar (PCA), malt extract and brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hants, England); wheat bran (Natural Life, São José do Rio Preto, SP, Brazil), agar powder/bacteriological grade, bacteriological peptone, yeast extract and *Bacillus cereus* (BC) agar base (HiMedia Laboratories Pvt. Ltd., Mumbai, MH, India); potato dextrose agar (PDA) and SFP agar base (Difco, Sparks, MD, USA); eosin methylene blue (EMB) agar and xylose lysine deoxycholate (XLD) agar (Fluka Analytical, Buchs, SG, Switzerland); both de Man, Rogosa and Sharpe (MRS) broth and agar (Merck KGaA, Darmstadt, HE, Germany); and Mueller-Hinton (MH) agar (Neogen, Lansing, MI, USA).

2.2 Microbial growth and standardization conditions

DSM 20016 and DSM 17938 *L. reuteri* was kept frozen at −18 °C in a medium composed of glycerol (130 g/L), bacteriological peptone (4.3 g/L), yeast extract (2.6 g/L), and NaCl (4.3 g/L) and were activated in de sterile MRS broth. The cultures were incubated at 37 °C for 24 h under static aerobic conditions. Subsequently, *L. reuteri* biomass was obtained by centrifugation at 9800 ×*g* for 10 min (Sorvall Legend XTR, Thermo Scientific, Germany) followed by its resuspension in sterile buffered peptone water (1 g/L of peptone at pH 6.5) (Rodrigues et al., 2022). The cell count of *L. reuteri* cultures were adjusted (according to the need of the assays) and used in antagonistic effect assays, and for the production of edible films.

Bacterial cultures of *S. enterica* subsp. *enterica* serovar Typhimurium (IAL 2431 and ATCC 14028), *S. aureus* subsp. *aureus* (ATCC 13565 and ATCC 8095), *E. coli* (IAL 2064 and ATCC 11229), *B. cereus* ATCC 11778, *C. perfringens* ATCC 13124, and *P. aeruginosa* ATCC 15442 were inoculated in sterile BHI broth and incubated at 37 °C for 24 h. After, bacterial cultures were diluted in sterile buffered peptone water (1 g peptone /L at pH 6.5), and were then standardized for antibacterial activity assays. Simultaneously, *A. flavus* ATCC 16883, *A. niger* ATCC 1004, *A. niger* ATCC 22342, *F. oxysporum* ATCC 48112, *C. gloeosporioides* ISO024, *A. alternata* ISO34, and *P. digitatum* ISO003 were cultured on PDA at 25 °C for 120 h. Then, a 1 mL spore suspension from each was aseptically prepared and sterile erlenmeyer flasks (125 mL) containing 10 g of wheat bran with 4 mL of ammonium sulfate (2 g/L) and monobasic sodium phosphate (1.7 g/L) were inoculated; each flask was incubated at 25 °C for 72 h. Then, the fungi spores ($\cong 1 \times 10^7$ spores mL^{-1}) were extracted using sterile buffered peptone water containing Tween 80 (3 mL/L), and stored at 4 ± 2 °C until use (Cunha et al., 2018).

2.3 *L. reuteri* antibacterial activity in brain heart infusion broth

Bacterial cultures of *S.* Typhimurium (IAL 2431 and ATCC 14028), *S. aureus* (ATCC 13565 and ATCC 8095), *E. coli* (IAL 2064 and ATCC 11229), *B. cereus* ATCC 11778, *C. perfringens* ATCC 13124, and *P. aeruginosa* ATCC 15442, including

the probiotic cultures of *L. reuteri* (DSM 20016 or DSM 17938) were properly diluted in sterile buffered peptone water. Subsequently, the probiotic (6 log CFU/mL) and the test bacterial cultures (4 log CFU/mL) were inoculated in 3 mL of BHI broth and incubated at 37 °C for 24 h under aerobic conditions. Viable cells of *S.* Typhimurium, *S. aureus*, *E. coli*, *B. cereus*, *C. perfringens*, and *P. aeruginosa* were estimated by growth on XLD, BP, EMB, BC, SFP and PCA agar, respectively. The inhibitory effect of *L. reuteri* strains against the studied microorganisms were calculated from the log response ratio (LogRR), which is calculated according to Equation (1); where LR is the mean slope of bacterial growth with the presence of lactic acid bacteria, and C is the mean slope of bacterial growth without lactic acid bacteria (Haraguchi et al., 2019). A LogRR positive value indicates stimulation, and a negative value indicates inhibition of bacterial growth (Schwartz et al., 2017).

$$LogRR = Log\ (\frac{LR}{C}) \qquad (1)$$

2.4 *L. reuteri* antimicrobial activity

Aliquots of 100 µL of each target bacterial culture suspensions (≅ 1 ×10^6 CFU/mL) obtained according to section 2.2 were spread onto MH agar plates. Then, in sterile paper discs (ø 6 mm) were inoculated with 10 µL of *L. reuteri* (DSM 20016 or DSM 17938) were placed on the MH agar plates containing the bacterial cultures. The samples were then incubated at 37 °C for 24 h (adapted from P.R Rodrigues et al., 2021).

The overlayed method (Russo et al., 2017) with adaptations was used to evaluate the antifungal activity of the *L. reuteri* strains. Briefly, 5 µL of the probiotic cultures at the middle exponential phase in sterile paper discs (ø 6 mm) were spotted onto MRS agar Petri plates and incubated at 37 °C for 24 h. Then, the petri plates were overlaid with 10 mL of soft malt extract agar (0.75 g/L of agar) supplemented with 100 µL of malt extract broth containing ≅ 1 × 10^7 spores mL^{-1} of each fungi (obtained according to section 2.2) investigated. The samples were incubated at 25 °C for 120 h. Both antibacterial and antifungal effects were determined by measuring of the halo formed surrounding the discs using a digital caliper (Jomarca, Guarulhos, SP, Brazil);

and were classified as no (–), mild (+), or strong (++) inhibition, in regards to halo zones being lower than 1 mm, ranging from 1 to 3 mm, or more than 3 mm, respectively (Russo et al., 2017).

2.5 Production of film-forming solutions/edible films and reuterin biosynthesis

Two edible films were produced from film-forming solutions based on sodium alginate (20 g/L) and konjac gum (5 g/L, previously hydrated overnight at 5 °C) with (FKGG) or without (FKG) glycerol (100 mmol/L). The mixtures were homogenized (6000 rpm) using a digital disperser (Ultra-turrax, IKA T25, Campinas, SP, Brazil) for complete dissolution. Then, the film-forming solutions were subjected to the vertical autoclave sterilization process (AV-SD 137, Phoenix, Brazil) at 121 °C for 15 min and after cooling (42 °C), the biomass suspension of *L. reuteri* DSM 20016 was aseptically inoculated between 8.48–8.54 log CFU/mL. In order to induce reuterin production, FKGG was subjected to anaerobic fermentation at 37 °C for 24 h (F.J. Rodrigues et al., 2021). A film-forming solution was prepared without the addition of *L. reuteri*, and was used as a control sample.

Subsequently, to produce the films, 20 mL aliquots of the film-forming solutions were distributed in sterile Petri plates (ø 90 mm) and 30 mL of sterile calcium chloride solution (20 g/L) was added; to promote the crosslinking and consequently form the films, and were kept submerged for 30 min. The films were then removed from the $CaCl_2$ solutions, washed with sterile distilled water, and dried at 5 °C ± 2 °C for 12 h.

2.5.1 Reuterin determination and entrapment in edible films

Due to its high reuterin production (F.J. Rodrigues et al., 2021), the *L. reuteri* DSM 20016 strain was used for *in situ* reuterin production in the alginate-konjac gum edible film. Reuterin production was determined before (in the film-forming solution) and after film production (in the film). Quantification was carried out indirectly, according to the colorimetric method proposed by Circle et al. (1945), with adaptations

proposed by F.J. Rodrigues et al. (2021). Briefly, 1 g of each sample was diluted in 9 mL of sodium citrate (20 g/L). Then, 320 µL of the sample was homogenized with 300 µL of 0.1 M tryptophan solution (dissolved in 0.05 M HCl) and 600 µL of ethanol (95 %). The samples were incubated at 40 °C for 50 min. Then, the absorbance was measured by spectrophotometry (DU 640, Beckman Coulter, Fullerton, CA, USA) at 560 nm. The reuterin content was determined by comparing the absorbance of the sample with an acrolein standard curve, previously constructed in the range from 2 to 100 mmol/L, assuming that 1 M of dehydrated reuterin corresponded to 1 M of acrolein.

In addition, reuterin entrapment was determined by comparing the reuterin-content present in the film-forming solution with the reuterin detected in the edible film. The data obtained were applied to Equation (2), where Rt referred to the reuterin detected in the edible films; and Ri referred to the reuterin-content present in the film-forming solutions.

$$Reuterin\ Entrapment\ (\%) = \frac{Rt}{Ri}.100 \qquad (2)$$

2.5.2 *L. reuteri* survival and entrapment in edible films

L. reuteri survival was estimated to relate the probiotic viability with the reuterin production. The number of viable *L. reuteri* cells present in the film-forming solutions was determined before (0 h) and after fermentation (37 °C for 24 h). Furthermore, the entrapment of *L. reuteri* cells in edible alginate-konjac gum films was determined by counting the viable *L. reuteri* cells after crosslinking and consequent formation of edible films. The *L. reuteri* cell viability assays were conducted by direct counting of the number of colony-forming units (log CFU/g), using the pour plating method; with adaptations proposed by Rodrigues et al. (2022). Briefly, 1 g of each sample was diluted in a sterile sodium citrate-peptone solution (20 g/L of sodium citrate and 1 g/L of peptone) followed by plating on MRS agar. The samples were then incubated at 37 °C for up to 72 h under aerobic conditions. The data obtained were applied to Equation (3), where Lt referred to the total number of viable cells entrapped in the edible film; and Li referred to the total number of live cells inoculated in the film-forming solution.

$$L.reuteri\ entrapment\ (\%) = \frac{Lt}{Li}.100 \qquad (3)$$

2.5.3 Scanning electron microscopy (SEM)

The edible alginate-konjac gum film containing *L. reuteri* cells was coated with 200 Å-thick layer of gold using a sputter coater (K450, Kent, UK) and the surface and cross-sectional morphology were observed using a scanning electron microscope (SEM; Leo 440i, LEO Electron Microscopy, Cambridge, UK) with an X-ray dispersive energy detector (EDX; 6070, LEO Electron Microscopy, Cambridge, UK) (Rodrigues et al., 2022).

2.5.4 Inhibitory effect of *L. reuteri* edible films against foodborne pathogens and spoilage microorganisms

The antimicrobial activities of edible alginate-konjac gum films with or without *L. reuteri* cells were evaluated using an adapted method of P.R. Rodrigues et al. (2021); in MH or PDA agar for bacteria and fungi, respectively. The films were aseptically cut to obtain discs (ø 8 mm) that were applied onto agar plates containing 100 µL aliquots of each bacterial culture suspension (≅ 1 × 10^6 CFU/mL) or fungal spores (≅ 1 × 10^7 spores mL^{-1}) obtained according to item 2.2. The samples were then incubated at 37 °C for 24 h and at 25 °C for 120 h for bacteria and fungi, respectively. Both antibacterial and antifungal effects were determined by measuring halo formation around the discs using a digital caliper (Jomarca, Guarulhos, SP, Brazil); and were classified as no (–), mild (+), or strong (++) inhibition, in regards to halo zones lower than 1 mm, ranging from 1 to 3 mm, or more than 3 mm, respectively (Russo et al., 2017).

2.6 Statistical analysis

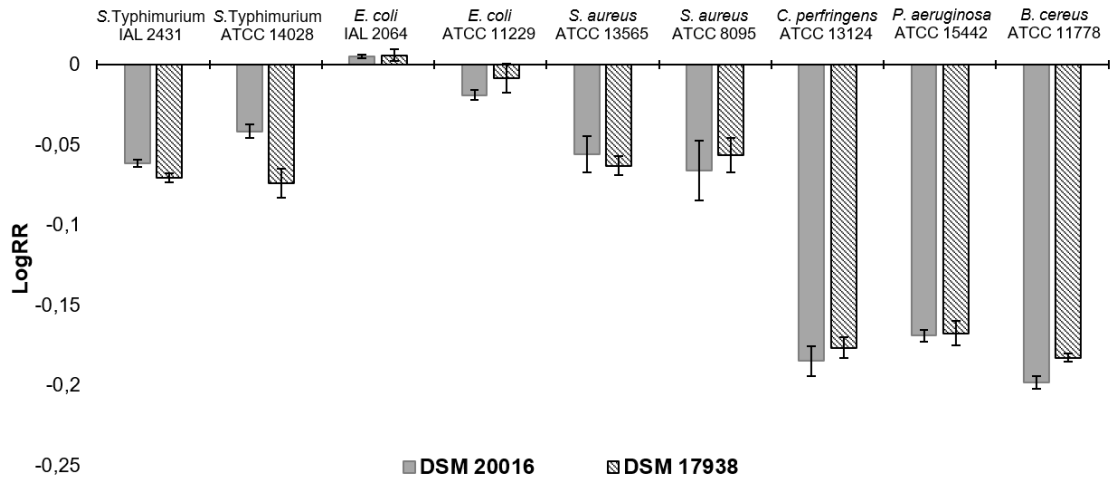
All evaluations were made in triplicate. The data were analyzed with the assumption of a normal distribution and subjected to ANOVA and the means were compared using the Tukey test with the Statistica 10 software (Tulsa, OK, USA). The Student's *t* test was applied when the comparison of means between two sample groups was required. The results were considered significant at $p<0.05$.

3 Results and Discussion

3.1 *L. reuteri* antibacterial activity in brain heart infusion broth

In general, the antagonistic activity of *L. reuteri* DSM 20016 and DSM 17938 against the evaluated indicator microorganisms in BHI broth assay did not vary between the latic acid bacteria strains. In control samples (without the addition of *L. reuteri* strains), the microbial indicator strains with initial concentrations of ≅ 4 log CFU/mL, had increased viable cell counts after incubation in BHI broth; between 8.39–9.49 log CFU/mL. The *L. reuteri* strains' LogRR inhibitory effects against the evaluated microorganisms are shown in Fig. 1.

Fig. 1. Inhibitory effect (LogRR) of *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938 against foodborne pathogenic and spoilage bacteria.



Each data point represents the mean of assays performed in triplicate with standard deviations (error bars).

No bacterial growth stimulation was detected in the indicator microorganisms ($p>0.05$). Except against *E. coli*, both *L. reuteri* strains showed an inhibition of bacterial growth against the foodborne microorganisms tested. In general, *E. coli* has a cysteine-containing tripeptide, named reduced glutathione, an intracellular low-molecular-weight thiol, which protect the cells against oxidative stress from the effects of oxidative damage (Cleusix et al., 2007). This might explain the milder effect observed in this target strain, since the reuterin mechanism of action involves the induction of oxidative stress (Schaefer et al., 2010). In contrast, Ma et al. (2023) reported that a strain of *L. reuteri*, genetically modified through random mutagenesis with atmospheric and room temperature plasma (ARTP), showed 37 % higher antibacterial activity against *E. coli* ATCC 25922 compared to the *L. reuteri* wild strain. The authors emphasized that the antibacterial mechanism of the *L. reuteri* mutant strain on *E. coli* occurs is through membrane disruption, decreased intracellular enzyme activity, and reduced DNA and protein concentrations; therefore, affecting the bacterial growth and metabolism. Among the possible mechanisms for improving of antimicrobial activity are: improvement of reuterin production, ability to secrete novel antibacterial compounds, or increased the levels of other antibacterial compounds. However, these parameters have not yet been determined.

A low inhibitory effect ($p<0.05$) was obtained in assays against *S*. Typhimurium IAL 2431, *S*. Typhimurium ATCC 14028, *S. aureus* ATCC 13565 and *S. aureus* ATCC 8095, where the cell counts were between 9.15 and 15.7 % smaller than their respective control samples. Using the same method, a small antagonistic effect was obtained using strains of *L. plantarum* and *L. lactis* for the inhibition of *S.* Typhimurium NBRC 13245. However, this effect was heightened when *Listeria monocytogenes* Scott A CIP 103575 was used as the target microorganism (Haraguchi et al., 2019). In parallel, Marianelli et al. (2010) reported significant growth inhibition (20% reduction) of *S.* Typhimurium 1344 under microaerobic conditions and at pH 7.5 in BHI medium, using cell-free culture supernatants from *L. reuteri* DSM 17938. However, the antimicrobial effect was even higher (approximately 38 %) under anaerobic conditions. The authors emphasized that in BHI medium *L. reuteri* cells can present longer lag phases and generation times than those of *S.* Typhimurium, favoring *Salmonella* cell growth before the probiotic cells can express its antimicrobial activity.

The highest antagonist effects were achieved with *B. cereus* ATCC 11778, *C. perfringens* ATCC 13124, *P. aeruginosa* ATCC 15442 as indicator strains, achieving bacterial growth inhibitions between 32.25 and 36.67 % ($p<0.05$). Compared to the control sample, the reduction of *Bacillus cereus* was 3.22 and 3.01 log CFU/mL ($p<0.05$) for *L. reuteri* strains DSM 20016 and DSM 17938, respectively. As for, *C. perfringens* the decrease was 2.91 and 2.80 log CFU/mL, while for *P. aeruginosa* the reduction was 2.78 and 2.76 log CFU/mL when using *L. reuteri* DSM 20016 and DSM 17938 as inhibitory agents, respectively. Similar to our results, Greifová et al. (2017) reported that the antibacterial activity of four *L. reuteri* strains inhibited the growth of six target bacteria, with *B. cereus* OPT and *P. aeruginosa* CCM 3955 included and inhibited by 15.1–20.4 and 23.2–33.4 %, respectively.

Using *L. reuteri* as a growth suppressor, the viable cell counts of *C. perfringens* were 5.48 and 5.59 log CFU/mL for the DSM 20016 and DSM 17938 strains, respectively. This is a significant finding, especially with regards to the biocontrol of food systems, as *C. perfringens* can cause food poisoning after the ingestion of 10^8 or more of enterotoxin-producing vegetative cells (Garde et al., 2014). The lack of bacterial growth stimulation can be attributed to the inhibitory activities that arise from *L. reuteri*'s ability to produce various antimicrobial substances besides reuterin; these include lactic acid, acetic acid, and ethanol, which can lead to pH reduction in the environment (Greifová et al., 2017). *L. reuteri* antimicrobial activities through disc diffusion are discussed in the following section.

3.2 *L. reuteri* antimicrobial activity

The antimicrobial activity against foodborne pathogens and spoilage microorganisms are presented in Table 1. The *S.* Typhimurium, *E. coli* and *S. aureus* bacterial strains were not inhibited (–). These results contrast with those obtained from the BHI broth assay, where a low inhibitory effect was observed for *S*. Typhimurium and *S. aureus* (0.04-0.07 and 0.05-0.06 LogRR, respectively). This behavior was also reported by Haraguchi et al. (2019), who evaluated the inhibitory effects of *L. plantarum* and *L. lactis* strains on *S.* Typhimurium and *L. monocytogenes*. Moreover, Moreno et al. (2006) emphasized that the absence of inhibition zones in disk diffusion tests does

not necessarily mean that compounds are inactive, but that the compounds may have difficulty in diffusing into the culture medium, due mainly to its hydrophilic and lipophilic characteristics.

Table 1. Antimicrobial activity of *L. reuteri* strains against foodborne pathogenic and spoilage microorganisms.

Microorganisms	*L. reuteri* DSM 20016		*L. reuteri* DSM 17938	
	mm[1]	act.[2]	mm	act.
Bacterium				
S. Typhimurium IAL 2431	nd[3]	–	nd	–
S. Typhimurium ATCC 14028	nd	–	nd	–
E. coli IAL 2064	nd	–	nd	–
E. coli ATCC 11229	nd	–	nd	–
S. aureus ATCC 13565	nd	–	nd	–
S. aureus ATCC 8095	nd	–	nd	–
C. perfringens ATCC 13124	$1.16^{a} \pm 0.05$	+	$1.00^{b} \pm 0.04$	+
P. aeruginosa ATCC 15442	$1.10^{a} \pm 0.10$	+	$1.11^{a} \pm 0.17$	+
B. cereus ATCC 11778	$1.12^{a} \pm 0.08$	+	$1.14^{a} \pm 0.06$	+
Fungi				
Aspergillus niger ATCC 1004	nd	–	nd	–
Aspergillus niger CCT 3941	nd	–	nd	–
Colletotrichum gloeosporioides ISO 024	$10.00^{a} \pm 0.55$	++	$4.08^{b} \pm 0.57$	++
Aspergillus flavus ATCC 16883	nd	–	nd	–
Penicillium digitatum ISO 003	$2.88^{b} \pm 0.13$	+	$3.26^{a} \pm 0.17$	++
Alternaria alternata ISO 034	$12.29^{a} \pm 1.19$	++	$10.55^{a} \pm 0.57$	++
Fusarium oxysporum ATCC 48112	$6.00^{a} \pm 0.82$	++	$4.34^{b} \pm 0.35$	++

[1]The measured diameter of inhibition zone around the spotted *L. reuteri*. [2]Antimicrobial activity. [3]Not detected. Each data point represents the mean of assays performed in triplicate ± standard deviations. Antimicrobial activity was classified as no (–), mild (+), or strong (++). Means followed by the same lowercase letter in the lines do not differ by Student's *t* test ($p>0.05$).

On the other hand, *B. cereus*, *C. perfringens*, and *P. aeruginosa* were susceptible to the *L. reuteri* DSM 20016 and DSM 17938. The inhibition of these bacteria was classified as mild (+), and ranged from 1.00 to 1.16 mm of inhibition zone diameter. Among the *L. reuteri* strains tested, the antimicrobial activity against *C. perfringens* was higher for DSM 20016 than DSM 17938 strain ($p<0.05$); however, no

difference was detected against *B. cereus* and *P. aeruginosa* ($p>0.05$). Although, Jonkuvienė et al. (2016) reported an antimicrobial effect of *L. reuteri* isolates from spontaneous sourdough against *B. cereus* ATCC 11778 through an agar well diffusion assay, resulting in an inhibition zone of 19.3 mm. However, the same authors also reported effective inhibition of *E. coli* ATCC 25922 (14.3 mm) and *S. aureus* ATCC 25923 (19.7 mm), which were different from our findings. According to the authors, the antimicrobial effects in these cases it was related to substances produced by *L. reuteri* cells of a proteinaceous nature and therefore, sensitive to the action of proteases.

Also, in contrast to our results, El-Hosseiny et al. (2018) reported the inhibitory effect of the culture supernatant *L. reuteri* ATCC 53608 against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 through the disc diffusion test, in which the samples with the pathogens showed an inhibition zone diameter of 11 and 14 mm, respectively. The authors suggested that the antimicrobial substances produced by *L. reuteri* were able to inhibit bacteria when their cell surface structure is injured. Voravuthikunchai et al. (2006) also reported a *L. reuteri* strain with antagonistic effect against *S. aureus* however, but not against *E. coli* and *S.* Typhimurium. In order to relate the antimicrobial interactions of *L. reuteri* strains with target microorganisms, Maccelli et al. (2020) demonstrated that during its growth *L. reuteri* produces membrane vesicles, and in both vesicles and *L. reuteri* supernatants numerous metabolites have been identified. These include organic acids, biogenic amines, neurotransmitters, vitamins and their derivatives, fatty acids and their hydroxyl- or keto-derivatives, antioxidants, antibiotics, and microbial surfactants. Most of these metabolites can participate in antimicrobial activity, moreover these compounds are pH-dependent; being active only at acidic pH values, when the undissociated form is more membrane- or cell wall-permeable.

P. aeruginosa is a common pathogen and source of microbiological food contamination (Liu et al., 2020). Wang et al. (2021) reported the antimicrobial spectrum of lactic acid bacteria strains, including *L. reuteri* P7 and P16 against *P. aeruginosa* ATCC 15692. Under the same conditions, the P7 strain showed an inhibition zone diameter ranging from 10 to 14 mm, while *L. reuteri* P16 had no inhibitory effect against the pathogen. This suggests that the inhibition spectrum and inhibition ability of each *L. reuteri* strain can vary for the same target microorganism. Dec et al. (2016) also

reported *C. perfringens* inhibition through the disc diffusion method. This relevant spore-forming bacterium, which is associated with environments that include soils and foods was effectively inhibited by *L. reuteri* supernatant; however, higher inhibitory activities were obtained using other lactobacilli species, such as *L. salivarius* and *L. ingluviei*.

Regarding of the antimicrobial activity against fungi, *L. reuteri* strains did not display an inhibitory effect against *A. niger* and *A. flavus* (–). However, a strong antagonistic effect (++) was obtained against *C. gloeosporioides*, *P. digitatum* (except for DSM 20016, which as classified as mild), *A. alternata* and *F. oxysporum*. *L reuteri* DSM 20016 showed higher inhibition against *C. gloeosporioides* and *F. oxysporum*, while the DSM 17938 strain presented higher effectiveness against *P. digitatum* ($p<0.05$). *A. alternata* was the mold that displayed the highest sensitivity to the presence of *L. reuteri* cells, with the DSM 20016 and DSM 17938 strains producing inhibition zones of 12.29 and 10.55 mm ($p>0.05$), respectively.

Fungi are capable of growing in several food matrices, causing extensive economic losses, in addition to being health risks to consumers (Cortés-Zavaleta et al., 2014). Fungi, such as *Penicillium digitatum* and *Alternaria alternata* are relevant postharvest pathogens (El-Gazzar & Ismail, 2020; Gerez et al., 2010). Chen et al. (2021) reported strains of *L. plantarum* (32.3 to 48.7 mm), *L. parafarraginis* (41.3 to 43.3), *L. casei* (35 to 43 mm), *L. paracasei* (40.3 mm), *L. buchneri* (34.7 mm), and *W. paramesenteroides* (40.3 mm) inhibited the *P. digitatum* growth under *in vitro* assay. The authors emphasized that the growing number of fungicides or preservatives not authorized, aside from the potential hazards to human health, paves the way for the eco-friendly fungal control, such as biological control using lactic acid bacteria. Fugaban et al. (2022) reported a strain of *E. faecium* (named, ST6019ea) with a strong *in vitro* antifungal activity against the *Alternaria alternata* ATCC MYA-4642, using the overlayed method. According to the authors, identifying the nature of bioactive molecules, which are responsible for the effects against these filamentous fungi, can direct the use of lactic acid bacteria cultures.

In this way, the use of *L. reuteri* cell-free-supernatant for antifungal purposes has been previously reported. Besides latic and acetic acids, *L. reuteri* can produce several other acid-based antifungal compounds, such as benzoic, 4-hydroxybenzoic,

vanillic, caffeic, hydrocaffeic, phenyllactic, OH-phenyllactic, coumaric, ferullic, hydroferullic, and hydrocinnamic acids. The high production and concentration of these acids makes *L. reuteri* R29 an option for the biocontrol of *Fusarium* spores (Oliveira et al., 2015). Furthermore, Jonkuvienė et al. (2016) reported relevant antimicrobial effects of *L. reuteri* isolates and *L. reuteri* cell-free supernatants against the foodborne micromycetes: *C. herbarum* SR-11, *P. chrysogenum* SR-12, *S. brevicaulis* Mi-Gr-5, *A. brasiliensis* Mi-G-21, *A. versicolor* Mi-Pr-4.

In accordance with our results, Greifová et al. (2017) reported a *L. reuteri* strain, named E, with a growth inhibition potential of 52 % against *A. alternata* CCM F-128. Under the same conditions, *A. flavus* CCM F-171, *R. oryzae* CCM F-8284, *M. rouxi* CCM F-220 and *P. chrysogenum* CCM F-432 had their growth potential reduced by 11.6, 19.1, 16 and 17.4 %, respectively. *C. gloeosporioides*, another fungus of relative importance, is related to citrus anthracnose around the world. The use of lactobacilli cell-free-supernatant for *C. gloeosporioides* biological control was reported by Cortés-Zavaleta et al. (2014), where the use of *L. reuteri* NRRL 14171 inhibited growth of the spoilage microorganism by 20.34 %. However, under the same conditions, the inhibition provided by *L. acidophilus* ATCC 4495 cell-free-supernatant it was even higher, reaching 40.57 %. The authors suggested that antifungal activities were related to the ability of lactobacilli to produce heat stable acidic compounds, which can penetrate the plasma membrane; causing cell cytoplasm acidification and inhibiting metabolic activities.

3.3 *L. reuteri* survival and reuterin production in alginate-konjac edible films

Table 2 shows the *L. reuteri* survival (log CFU/g) and the reuterin-content (mmol/L) detected during fermentation and edible film production processes. The *L. reuteri* DSM 20016 was susceptible to the *in situ*-produced reuterin in the alginate-konjac film-forming solution. After 24 h of anaerobic fermentation, the bacterial cell viability decreased by 2.08 log CFU/g, from an initial inoculation concentration of 8.48 log CFU/g in FKGG. Meanwhile, the reuterin produced in FKGG was 46.67 mmol/L. The accumulation of this metabolite in the medium can cause the *L. reuteri* cell death; either by the inhibition of ribonucleotide reductase, which compromises the DNA

synthesis (Talarico & Dobrogosz, 1989), or the interaction and modification of thiol groups in active peptide structures, inducing oxidative stress in cells (Doleyres et al., 2005; Schaefer et al., 2010). F.J. Rodrigues et al. (2021) emphasized that different *L. reuteri* strains may present higher or lower susceptibility to the *in situ* reuterin produced. The authors optimized the *in situ* reuterin production in alginate-based film-forming solutions using the *L. reuteri* DSM 20016. The total reduction in *L. reuteri* viable cell count after fermentation showed a sharp decrease, reaching 2.51 log CFU/mL. However, Cleusix et al. (2007) reported reuterin resistance in *L. reuteri* DSM 20016, which had ranges between 30–50 and 60–120 mmol/L for the minimum inhibitory concentration and minimum bactericidal concentration, respectively.

Table 2. The *L. reuteri* survival (log CFU/g) and reuterin production (mmol/L) in alginate-konjac edible films.

Sample	*L. reuteri*			Reuterin	
	viability during fermentation (log CFU/g)		entrapment (%)	production (mmol/L)	entrapment (%)
	before	after			
FKG	8.54 ± 0.05	NA[1]	97.54	NA	NA
FKGG	8.48 ± 0.04	6.40 ± 0.04	97.97	46.67 ± 2.31	67.61

[1]Not applicable. FKG did not have glycerol (substrate for reuterin production) in its composition and was not subjected to the fermentation process. Edible films were produced from film-forming solutions based on sodium alginate (20 g/L), konjac gum (5 g/L) with (FKGG) or without (FKG) glycerol (100 mmol/L). Each data point represents the mean of assays performed in triplicate ± standard deviations.

After crosslinking and consequent formation of the edible film, reuterin entrapment in FKGG was 67.71 %. Using the extrusion technique, the reuterin produced by *L. reuteri* BPL-36 was trapped in alginate and guar gum particles, which displayed reuterin entrapment between 36.35 and 37.97 % (Mishra et al., 2018). Thus, it was suggested that the use of different hydrocolloids may interfere with the density of the gel; resulting in and edible film that favors the metabolite entrapment. In contrast, the use of other hydrocolloids with alginate can decrease the bioconversion of glycerol into reuterin, due to possible changes in the physical and chemical properties of the film-forming solution interfering with glycerol diffusion into *L. reuteri* cells. In

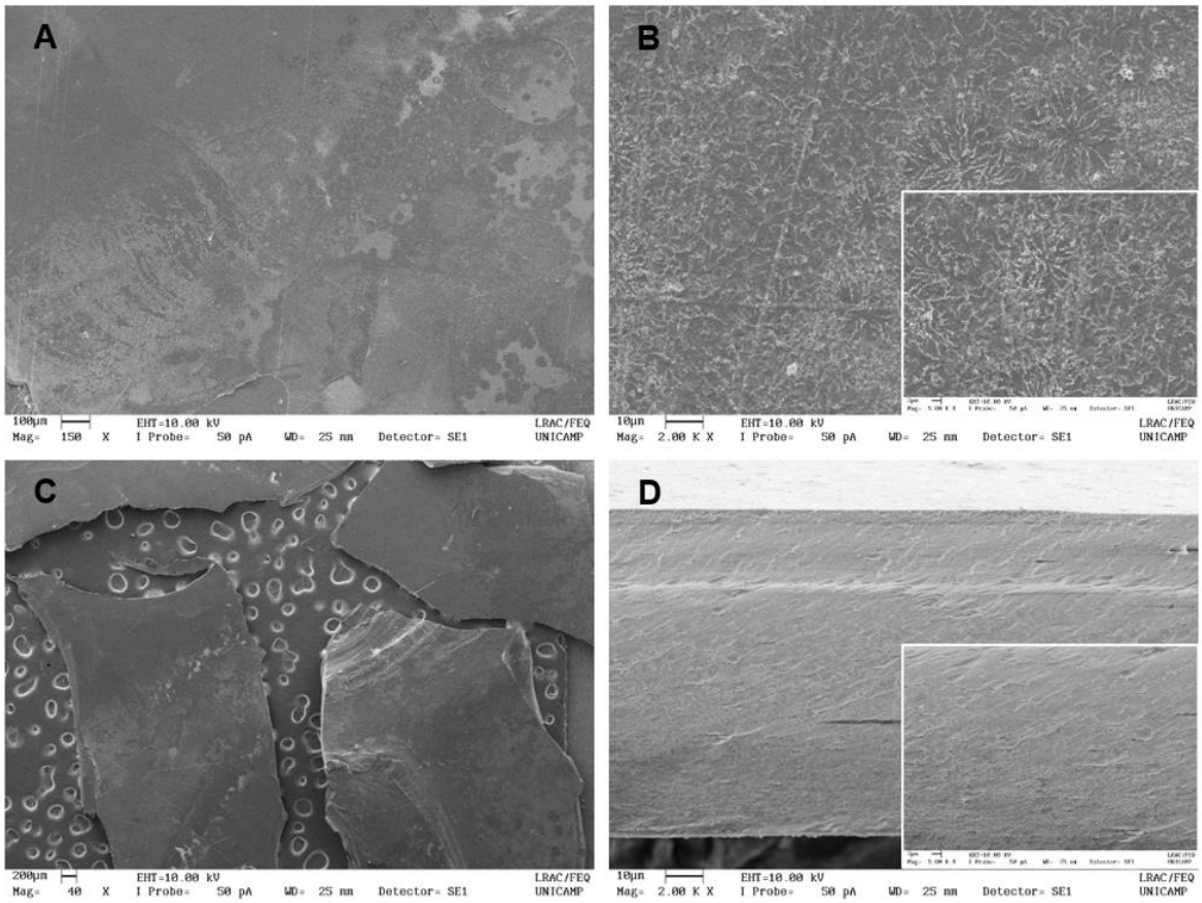
agreement, Chen et al. (2012) reported the reuterin production by *L. reuteri* DPC16 in alginate and alginate-chitosan microcapsules. The authors emphasized that in the film-forming solution, in which chitosan was used for particle production, a markedly lower reuterin content was detected.

In terms of *L. reuteri* cell entrapment in edible films, the percentages determined were higher than 97 % for both FKG and FKGG samples. Similarly, Rodrigues et al. (2022) reported an entrapment of *L. reuteri* cells at ≅ 96 % in alginate-konjac gum beads, also produced through the calcium chloride extrusion technique. At the same time, gel beads produced without konjac gum showed *L. reuteri* entrapment decreased (85 to 91 %). The use of alginates in the production of a film-forming solution can give rise to porous gels, which are susceptible to disintegration. Thus, it has been suggested that the use of other hydrocolloids, such as gums and mucilages, could improve the gel properties and increase the entrapment of the bacterial cells (Rodrigues et al., 2020). Shigematsu et al. (2018) reported the production of an edible alginate coating for fresh-cut carrots, containing *L. acidophilus* strains with an initial inoculum concentration of 7.36 log CFU/g. After film formation, the cell viability of the probiotic bacterium showed a sharp decrease to ≅ 6 log CFU/g. By contrast, in our study, the reduction of viable *L. reuteri* cells after film production was 0.21 and 0.13 log CFU/g for FKG and FKGG, respectively.

3.4 Scanning electron microscopy (SEM)

SEM micrographs were used to evaluate the edible probiotic film (FKGG), through examination its surface morphology and microstructure (Fig. 2). The *L. reuteri* cells were homogeneously distributed throughout the edible film (Fig. 2 B), which explains the high and homogeneous bacterial cell entrapment. In contrast to our results, Ma et al. (2019) reported a poor distribution of *L. lactis* over the edible film which was derived from sodium carboxymethyl cellulose, collagen, and glycerol. According to the authors, the film microstructures are related to the diffusion of compounds inserted in them. Moreover, they emphasized that the use of an alginate-sodium carboxymethyl cellulose blend improved the *L. lactis* viability and, consequently, the antibacterial properties of edible film.

Fig. 2. Scanning electron microscopy (SEM) of the *L. reuteri*-loaded alginate-konjac gum edible film. Film surface (A, B, & C) of and cross-sectional morphology (D) of FKGG. The images inserted in B & D were taken at 5000× magnification. FKGG were produced from film-forming solutions based on sodium alginate (20 g/L), konjac gum (5 g/L) with glycerol (100 mmol/L).



The cross-section (Fig. 2 D) and surface (Fig. 2 A and C) of the film were smooth, relatively homogeneous and compact with no cracks, suggesting that the hydrocolloids used gelled on the surface of edible film satisfactorily. A similar result was reported by Shahrampour et al. (2020), who developed edible alginate/pectin films containing *L. plantarum* KMC 45. The authors highlighted that the addition of the bacteria did not affect the microstructure of films, in addition, the compact structure of the film improved compatibility between hydrocolloid blends. In the same way, the glycerol-content used in the film-forming solution composition is related to much smoother and uniform surface structures, especially due to the plasticizing capacity that the glycerol confers to the produced films (Jouki et al., 2021).

3.5 Inhibitory effect of edible *L. reuteri* films against foodborne pathogens and spoilage microorganisms

According to the results of the antimicrobial activity assays, as well as the success of *L. reuteri* cell immobilization and the *in situ*-produced reuterin, the hypothesis of antimicrobial property maintenance in the films was tested. The antimicrobial activity of edible *L. reuteri* films against foodborne pathogens and spoilage microorganisms is shown in Table 3.

Table 3. Antimicrobial activity of *L. reuteri* edible films against foodborne pathogenic and spoilage microorganisms.

Microorganisms	FKG		FKGG	
	mm[1]	act.[2]	mm	act.
Bacterium				
S. Typhimurium IAL 2431	nd[3]	–	nd	–
S. Typhimurium ATCC 14028	nd	–	nd	–
E. coli IAL 2064	nd	–	nd	–
E. coli ATCC 11229	nd	–	nd	–
S. aureus ATCC 13565	nd	–	nd	–
S. aureus ATCC 8095	nd	–	nd	–
C. perfringens ATCC 13124	$1.93^{a} \pm 0.14$	+	$2.27^{a} \pm 0.26$	+
P. aeruginosa ATCC 15442	$1.35^{b} \pm 0.12$	+	$2.29^{a} \pm 0.22$	+
B. cereus ATCC 11778	$2.07^{b} \pm 0.20$	+	$3.11^{a} \pm 0.11$	++
Fungi				
Aspergillus niger ATCC 1004	nd	–	nd	–
Aspergillus niger CCT 3941	nd	–	nd	–
Colletotrichum gloeosporioides ISO 024	$8.43^{a} \pm 0.38$	++	$8.21^{a} \pm 0.13$	++
Aspergillus flavus ATCC 16883	nd	–	nd	–
Penicillium digitatum ISO 003	$2.24^{b} \pm 0.15$	+	$3.70^{a} \pm 0.10$	++
Alternaria alternata ISO 034	$10.93^{a} \pm 0.25$	++	$11.04^{a} \pm 0.31$	++
Fusarium oxysporum ATCC 48112	$5.92^{b} \pm 0.10$	++	$7.06^{a} \pm 0.14$	++

[1]The measured diameter of inhibition zone around the spotted *L. reuteri* edible films. [2]Antimicrobial activity. [3]Not detected. Each data point represents the mean of assays performed in triplicate ± standard deviations. Edible films were produced from film-forming solutions based on sodium alginate (20 g/L), konjac gum (5 g/L) with (FKGG) or without (FKG) glycerol (100 mmol/L), besides *L. reuteri* DSM 20016 culture. Antimicrobial activity was classified as no (–), mild (+), or strong (++). Means followed by the same lowercase letter in the lines do not differ by Student's *t* test ($p>0.05$).

As in tests using *L. reuteri* supernatants (Table 1), no antagonistic activity (–) against *S.* Typhimurium, *E. coli* and *S. aureus* was detected. However, FKG and FKGG showed a mild inhibition (+) against *C. perfringens*, *P. aeruginosa* and *B. cereus* (except for FKGG, which was classified as strong). Moreover, the antimicrobial activity against *P. aeruginosa* and *B. cereus* using edible FKGG film was higher than FKG, if the inhibition zone diameters are considered ($p<0.05$). In contrast to our results, Mozaffarzogh et al. (2019) reported that the antimicrobial effect of films, based on carboxymethyl cellulose-sodium caseinate containing *L. reuteri* PTCC 1655 against common foodborne pathogenic bacteria; utilizing the disc diffusion test, Petri dishes containing *S. aureus*, *L. monocytogenes*, *S.* Typhimurium and *E. coli* cultures displayed inhibition zone diameters around the *L. reuteri* films ranging 2.87 to 3.71 mm. However, the authors noted that the addition of *L. acidophilus* PTCC 1643 and *B. bifidum* PTCC 1644 to *L. reuteri* films significantly increased the inhibition zones of the target microorganisms. This suggests that the use of lactic acid bacteria co-cultures in edible film production can improve the antimicrobial effects of the films produced.

Furthermore, El-Sayed et al. (2021) reported the development of an eco-friendly edible probiotic film based on alginate for antimicrobial purposes. The edible film contained *B. lactis*, *L. acidophilus*, and *L. casei* and showed high antagonistic effect against *S aureus*, *S.* Typhimurium, *L. monocytogenes*, *E. coli*, *B. cereus*, as well as the fungi *A. niger*, and *A. flavus*, with inhibition zones ranging from 17 to 25 mm. Subsequently, the alginate film was used to coat UF-soft cheese and prevented the growth of fungi and yeasts during storage for 30 days stored at 7 °C. Under the same conditions, the use of films composed of chitosan and carboxymethyl cellulose provided a lower antagonistic effect, due manily to the ability of alginate to allow for the diffusion and release of compounds entrapped in the films.

Regarding the antimicrobial activity of edible *L. reuteri* films (FKG and FKGG) against fungi, no inhibition against *A. niger* and *A. flavus* (–) was detected. In contrast, a strong antagonistic effect (++) was obtained against *C. gloeosporioides*, *P. digitatum* (except for FKG, which was classified as mild), *A. alternata* and *F. oxysporum*. FKGG produced a higher inhibition zone diameter against *P. digitatum* and *F. oxysporum* ($p<0.05$), while no difference was detected against *A. alternata* and *C. gloeosporioides* ($p>0.05$). Despite FKGG having reuterin content produced *in situ* by

L. reuteri, its antimicrobial effect against some of the target microorganisms was not significantly higher than that produced by FKG. In addition to the problems associated with the diffusion of compounds entrapped in the films, it is possible that the high reactivity of reuterin with other matter, or even the high cellular sensitivity of *L. reuteri* to the metabolite produced can lead to no satisfactory antimicrobial *in situ* results (Schmidt et al., 2018; F.J. Rodrigues et al., 2021). In this sense, Hernández-Carrillo et al. (2021) reported the use of *L. reuteri* ATCCC 55730 to produce reuterin from glycerol, the metabolite was recovered by centrifugation and filtration, to transform it into an aqueous extract. Then, the reuterin extract was used to prepare an edible pectin film, that had effective antifungal activity against *Penicillium* spp., and is being used in the preservation of strawberries. Although the use of *L. reuteri* and reuterin have been used as an antimicrobial against pathogens and spoilage microorganisms, to the best of our knowledge, this is the first time that the *in situ* production and evaluation of this metabolite in edible films has been reported. In addition, further studies must be conducted to detect the exact metabolites related to the inhibition effects against foodborne pathogens and spoilage microorganisms.

4 Conclusion

The reuterin-producing *L. reuteri* (strains DSM 20016 and DSM 17938), and edible alginate-konjac gum film containing *L. reuteri* cells and the reuterin produced *in situ* demonstrated inhibitory effects against some foodborne pathogens and spoilage microorganisms; among those evaluated were: the bacterial strains, *B. cereus* ATCC 11778, *C. perfringens* ATCC 13124, and *P. aeruginosa* ATCC 15442, and the fungi *F. oxysporum* ATCC 48112, *C. gloeosporioides* ISO024, *A. alternata* ISO34, and *P. digitatum* ISO003. In addition, we confirmed the potential of *L. reuteri* DSM 20016 for the *in situ* reuterin production in alginate-konjac gum film-forming solution, using glycerol as substrate. Despite being partially inhibited after reuterin production, the *L. reuteri* was effectively entrapped together with the produced metabolite in the edible alginate-konjac gum film. Our findings provide antimicrobial alternatives against specific indicator strains, and indicate that the *in situ* reuterin production in edible alginate-konjac gum film can be a valuable approach to food safety. Further

investigations should be performed to evaluate the effects of edible films on prolonging shelf-life of food matrices.

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GENERAL DISCUSSION

The remarkable scientific interest in microorganisms with probiotic properties for promoting human health, as well as their application in the production of beneficial compounds and biopreservation strategies, has driven us to explore approaches that enhance the survival of these bacteria when exposed to environmental factors. This opens up possibilities for their utilization in various fields of Food Science. Accordingly, we carefully selected strains of *Limosilactobacillus reuteri*, renowned for their probiotic potential and proven ability to produce high levels of reuterin via glycerol bioconversion. Our main objective was to understand and optimize the *in situ* production of reuterin in filmogenic solutions, which could be used in encapsulation processes or the production of edible films.

Therefore, an extensive literature review was conducted on probiotic cells, relevant encapsulation techniques, and encapsulating materials **(Chapter I)**. We gathered a broad range of literature on the encapsulation of microorganisms, providing a current overview of the most commonly used encapsulation techniques and both traditional and emerging encapsulating materials for this purpose. It is crucial to carefully select both the technique and encapsulating material when considering probiotic cells, as this can help reduce microbial losses during particle production and improve the resistance of microorganisms to adverse conditions. The extrusion technique remains widely used for entrapping probiotics, particularly due to its low cost and relatively gentle process, which is advantageous for encapsulating thermosensitive agents like microbial cells, ensuring their high survival rates. Concurrently, alginate is the most commonly used wall material when employing this technique. This polymer is classified as safe for use by regulatory agencies in the European Union and in the United States, making it widely employed in the food and pharmaceutical industries. Additionally, it exhibits compatibility for use with glycerol to produce film-forming solutions.

With the insights from **Chapter I**, we proceeded to **Chapter II**, where our focus shifted to optimizing the *in situ* reuterin production by *L. reuteri* strains in alginate-based film-forming solutions while evaluating the survival of reuterin-producing

bacteria during fermentation. To the best of our knowledge, this *in situ* reuterin synthesis had not been previously reported. The study design involved the use of two strains of *L. reuteri* (DSM 20016 and DSM 17938) in alginate-based film-forming solutions, with two independent variables (glycerol content and initial biomass of *L. reuteri*) studied over a 72-hour anaerobic fermentation period at 37 °C. Our investigation showcased the ability of *L. reuteri* DSM 20016 and 17938 strains to produce reuterin *in situ* within the developed solutions via glycerol bioconversion. However, strain DSM 20016 exhibited higher reuterin production, suggesting that different *L. reuteri* strains possess varying capacities for reuterin production through glycerol bioconversion. Furthermore, it was observed that an initial inoculum of *L. reuteri* at 8 log CFU/mL resulted in enhanced reuterin production following 24 hours of fermentation. In addition, while higher conversion rates from glycerol to reuterin were achieved at a substrate concentration of 50 mmol/L, reuterin production levels (in mmol/L) 1.5 folds greater were attained when utilizing 100 mmol/L of glycerol.

Nevertheless, despite successful reuterin production, both strains of *L. reuteri* showed susceptibility to the metabolite they produced. Extended fermentation and heightened exposure to reuterin had a notable impact on the viability of *L. reuteri* cells in the film-forming solutions, leading to a significant decrease in their numbers. Considering the results presented in **Chapter II**, which indicated favorable conditions for the production of reuterin in alginate film-forming solutions, our study shifted towards the prospect of encapsulating both *L. reuteri* and the *in situ*-produced reuterin. However, as discussed in **Chapter I**, alginates tend to form porous matrices that are susceptible to disintegration, compromising the efficiency of the encapsulation process. Consequently, there has been growing interest in partially substituting this polysaccharide with polysaccharides derived from natural sources, such as plants and their seeds. Therefore, in combination with alginate, we selected mucilaginous solutions that have been previously studied (psyllium and konjac) or remain unexplored (cassia tora, tamarind, and mutamba) for immobilizing both *L. reuteri* and the *in situ*-produced antimicrobial compound.

Thus, the third phase of this thesis focused on encapsulating *L. reuteri* DSM 20016 in alginate particles prepared using various mucilages/gums, as detailed in **Chapter III**. The extrusion technique yielded moderately spherical particles with

homogeneous dispersion of *L. reuteri* cells. The employ of mucilaginous materials in combination with alginate played an important role in improving the encapsulation efficiency, enhancing bacterium entrapment (93.2 to 97.4 %), as well as the survival of encapsulated cells exposed to simulated gastrointestinal conditions, increasing bacterial survival from 67.7 to 76.6 %. In comparison, control samples and free cells exhibited only 54.8 % and 31.84 % survival rates, respectively.

Regarding the refrigerated storage, beads without glycerol and not subjected to fermentation conditions prior to encapsulation exhibited higher *L. reuteri* survival. Furthermore, the use of mucilages of psyllium, cassia tora, tamarind, and konjac significantly improved *L. reuteri* viability during extended refrigerated storage periods, maintaining viable *L. reuteri* cell counts after 20, 30, up to 60 days. On the other hand, the induction of reuterin production in the samples caused a substantial decrease in viable cell counts of encapsulated *L. reuteri*. Under this condition, after 12 to 15 days (refrigerated storage), the presence of viable *L. reuteri* cells was not detected, confirming that the bioproduction and accumulation of reuterin in the medium can lead to bacterial cell death of this microorganism.

In terms of reuterin production, *L. reuteri* demonstrated its ability to produce the metabolite under all tested conditions, ranging from 50 to 60.12 mmol/L. It is worth noting that the addition of other hydrocolloids in the composition of particles can lead to lower bioconversion rates. These hydrocolloids may alter the film-forming solutions (physical and chemical properties), affecting the diffusion of glycerol into the medium and reducing its availability to *L. reuteri*, consequently impacting the conversion to reuterin. On the other hand, the incorporation of tamarind, konjac, psyllium, and mutamba mucilages in the encapsulation procedure resulted in improved entrapment of reuterin in the beads, with entrapment efficiencies ranging from 39.73 % to 45 %. This indicates that including these wall materials into alginate bead structure may enhance gel density and facilitate the entrapment of the metabolite. It is important to note that the use of tamarind, cassia tora, and mutamba mucilaginous solutions for encapsulating *L. reuteri* or its bioconverted reuterin has not been previously reported. Thus, these hydrocolloids demonstrate their effectiveness as supports for this purpose, confirming the great potential raised in **Chapter I**.

Simultaneously, the use of lactobacilli strains and their metabolites in combating foodborne microorganisms and food preservation technologies is gaining popularity. Therefore, the fourth phase of this thesis focused on studying the antagonistic activity of reuterin-producing *L. reuteri* against foodborne pathogenic and spoilage microorganisms. Additionally, an alginate-konjac gum edible film was developed, incorporating *L. reuteri* cells and *in situ*-produced reuterin for the same purpose **(Chapter IV)**. In the initial stage, an *in vitro* screening was conducted to assess the antagonistic activities of *L. reuteri* (DSM 20016 and 17938) against bacterial strains, such as *P. aeruginosa*, *C. perfringens*, and *B. cereus*. *L. reuteri* produces reuterin and other antibacterial compounds, including lactic/acetic acids and ethanol, which may contribute to the inhibitory effects observed. These substances decrease the environment's pH, thereby hindering bacterial growth. In addition to the bacterial strains, *L. reuteri* supernatants exhibited antifungal effects against molds such as *C. gloeosporioides* ISO024, *P. digitatum* ISO003, *F. oxysporum* ATCC 48112, and *A. alternata* ISO34. It is worth noting that molds not only pose risks to human health but can also cause significant economic losses by growing in various food matrices.

Therefore, and in general, building upon the findings of *L. reuteri* and reuterin immobilization obtained in **Chapter III**, we aimed to investigate the hypothesis of maintaining the antimicrobial properties of an alginate-konjac gum edible film loaded with *L. reuteri* and its *in situ*-produced reuterin. The results revealed that both reuterin-producing *L. reuteri* and its metabolite were successfully encapsulated in the edible film, which exhibited antimicrobial properties comparable to the antimicrobial effect of the supernatants of the microbial culture. However, as compared to films loaded with *L. reuteri* cells only, the *in situ* reuterin production did not significantly enhance the antibacterial action against the target microorganisms. This suggests that aside from potential challenges in reuterin diffusion within the films, the strong reactivity of this compound with other substances, or the heightened vulnerability of *L. reuteri* to the *in situ*-produced metabolite may contribute to suboptimal antimicrobial results. Nevertheless, our findings regarding the edible films offer antimicrobial alternatives against specific pathogenic microorganisms, making reuterin-producing *L. reuteri* edible films a valuable approach to ensuring food safety.

It is important to emphasize that the antimicrobial reuterin (3-HPA) has been recognized for its potential as a biopreservative. This potential is particularly noteworthy in the context of regulatory considerations, as reuterin has been granted Generally Recognized As Safe (GRAS) status in the USA and Qualified Presumption of Safety (QPS) status in Europe. Nevertheless, the inherent issue of the spontaneous conversion of 3-HPA to acrolein, an unsaturated aldehyde known for its reactivity and cytotoxic properties, poses a significant challenge to the practical utilization of reuterin as a viable natural bioprotectant. In this context, particularly when contemplating its application in food products, it is necessary to emphasize the necessity for additional research to estimate the *in vivo* production of acrolein within the human gastrointestinal tract to ensure the safe application (Zhang & Schwab, 2022).

GENERAL CONCLUSIONS AND PERSPECTIVES

The literature review conducted in **Chapter I** revealed several key findings: **1)** Probiotic encapsulation is an effective approach to maintain the viability and stability of the encapsulated cells; **2)** Alginate remains a widely used material for the encapsulation of probiotics, primarily due to its favorable properties and relatively mild application conditions. This makes it suitable for encapsulating thermosensitive agents; **3)** There is a growing interest in substituting or partially substituting alginate with hydrocolloids derived from natural sources, particularly plants. These alternative materials can modify the particle's properties and enhance the protection of the encapsulated cells; **4)** Novel mucilaginous materials extracted from plants (mutamba, cassia tora, and tamarind) have emerged as alternatives for microbial encapsulation. These findings highlight the ongoing research and development in the field of microbial encapsulation, with a focus on improving the effectiveness and applicability of encapsulation techniques for probiotics.

The investigative research executed in **Chapter II** showed: **1)** The study found the ability of two *L. reuteri* strains, DSM 20016 and 17938, for reuterin synthesis via glycerol bioconversion; **2)** The production of reuterin varied depending on the specific strain, initial biomass concentration, and glycerol content used. *L. reuteri* DSM 20016 produced 68.39 mmol/L of reuterin, while strain DSM 17938 produced 30.00 mmol/L. These concentrations were measured immediately after 24 hours of anaerobic fermentation; **3)** Both reuterin-producing *L. reuteri*, exhibited a significant loss in bacterial cell viability throughout the conversion of glycerol, particularly after 48 hours of fermentation. This indicates that the bioconversion process negatively impacts the survival of the bacterial cells, leading to a decline in viability over time. These findings highlight the strain-dependent differences in reuterin production capacity and emphasize the challenge of maintaining bacterial cell viability during the bioconversion process.

In **Chapter III**, several significant findings were observed: **1)** The combination of alginate with gums and mucilages did not have a negative impact on *in situ* reuterin production; **2)** The mucilaginous solutions developed in this thesis demonstrated their

potential for distinct applications, including serving as support materials for the encapsulation of *L. reuteri* cells and reuterin contents. A great potential of hydrocolloids not yet explored in microbial encapsulation purposes by extrusion technique, such as tamarind, mutamba and cassia tora gums/mucilages was revealed; **3)** Scanning electron microscopy analysis revealed that the beads produced through extrusion had a moderate spherical form regardless of the incorporation of mucilaginous material, also indicating a homogeneous distribution of *L. reuteri* within the beads; **4)** The particles' FT-IR spectroscopy revealed typical peaks with no substantial shifts, indicating no major interaction amongst the hydrocolloids; **5)** By incorporating gums and mucilages in conjunction with alginate for the production of *L. reuteri* particles, there was a notable improvement in encapsulation efficiency, with an increase from 93.2 to 97.4 %. Additionally, the bacterial survival during refrigerated storage was enhanced, particularly in particles with konjac, cassia tora, and psyllium mucilaginous wall materials. This improvement was observed even after prolonged storage periods of 20, 30, and 60 days; **6)** Encapsulation procedure effectively preserved the encapsulated *L. reuteri* under simulated gastrointestinal conditions. Beads produced using konjac, psyllium, cassia tora, and tamarind demonstrated *L. reuteri* survival rates ranging from 72.6 % to 76.6 %, while free *L. reuteri* cells exhibited a survival rate of only 31.84 %; **7)** In the film-forming solutions, *L. reuteri* successfully produced reuterin. The entrapment of this antimicrobial compound was enhanced by utilizing tamarind, mutamba, and konjac, with entrapment efficiencies of 41.25 %, 44.57 %, and 45 %, respectively. In general, these findings demonstrate the effectiveness of incorporating gums and mucilages into alginate-based formulations for the encapsulation of *L. reuteri* cells and reuterin.

Following this path, the investigation performed on **Chapter IV** showed: **1)** Capability of *L. reuteri* DSM 20016 for *in situ* reuterin production in alginate-konjac gum film-forming solution was confirmed; **2)** *L. reuteri* (97.97 %) and reuterin (67.61 %) were effectively entrapped in edible film; **3)** SEM micrographs of the edible film revealed that cells of *L. reuteri* were uniformly dispersed within the film matrix. The cross-section and surface displayed a smooth, moderately homogeneous, and compact structure, with no fissures. This observation suggests that the hydrocolloids used in the formulation effectively formed a gel-like network on the surface of the film; **4)** *L. reuteri* (DSM 20016 and 17938) and the edible film produced demonstrated significant inhibitory effects

against various bacterial and fungal strains, including of *P. aeruginosa, B. cereus*, *C. perfringens*, *C. gloeosporioides, F. oxysporum*, *P. digitatum*, and *A. alternata*; **5)** Among the tested molds, *A. alternata* exhibited the highest susceptibility to *L. reuteri*. In contrast, no inhibitory effect was detected against *Aspergillus niger* and *A. flavus*. These findings emphasize the antimicrobial potential of *L. reuteri* and the *in situ*-produced reuterin in edible films.

Overall, this thesis provides valuable insights into the use of mucilaginous solutions as support materials for biological and chemical compounds. It highlights the potential of these solutions in various applications, including the delivery of microorganisms and the development of edible films and coatings. The research conducted in this thesis contributes to the advancement of knowledge in the field and opens up new possibilities for utilizing natural and sustainable materials in food preservation and safety strategies. Furthermore, the antimicrobial effects observed in the context of biological control using lactic acid bacteria and their metabolites offer an eco-friendly alternative for microbial control, especially in an era where the increasing use of preservatives raises potential concerns for human health.

Thus, from the findings, future research objects are following:

- Investigate the chemical composition, bioactive activity, and interaction with probiotic cells of the mucilaginous material extracted from natural sources, such as mutamba, cassia tora, and tamarind seeds;
- Evaluate the use of these mucilaginous materials to encapsulate other microorganisms, enzymes, and bioactive compounds;
- Evaluate the possibility of using these mucilaginous materials to produce particles by other encapsulation techniques, such as emulsification, internal gelation, and spray drying;
- Application of edible film developed to coat food matrices, such as fruits, dairy, and meat products, aiming to prolong the shelf-life of food matrices;
- Study the toxicity of *in situ*-produced reuterin by *L. reuteri in vitro* and *in vivo* models;

- Detect the exact metabolites present in the edible film that contributed to the antagonist activities against the foodborne microorganisms under investigation;
- Investigate the potential of utilizing *L. reuteri* and its metabolites for controlling fungi, including *Alternaria alternata*, *Penicillium digitatum*, and *Colletotrichum gloeosporioides*, in fruits and vegetables.

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ANNEXS

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Review

Encapsulated probiotic cells: Relevant techniques, natural sources as encapsulating materials and food applications – A narrative review

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ABSTRACT

The administration of probiotic microorganisms in adequate amounts is constantly related to health benefits. To promote beneficial effects, these microorganisms must not be affected by exposure to environmental factors and must be able to adhere and colonize the human gastrointestinal tract. Several encapsulation techniques and encapsulating materials are available to produce probiotic particles, however, it is essential that the process must not be aggressive, reducing or preventing injuries and cell losses, besides, the particle properties obtained must be adequate for the proposed purpose. At the same time, the global market for supplements and probiotic foods has been growing significantly, and cell encapsulation appears as an alternative to incorporate probiotics into different food matrices. This review discusses and updates the main techniques, and the traditional and emerging polysaccharides for encapsulation of probiotic cells, as well as the advantages and possibilities of incorporating produced particles into food matrices. Currently, various scientific studies report the use of different encapsulation techniques, such as extrusion, emulsion, spray drying, spray chilling and fluidized bed to encapsulate probiotics properly. The alginate is still widely used to produce probiotic particles, however, there has been a growing interest in its total or partial substitution with others polysaccharides, such as gums, mucilages, prebiotic compounds and microbial exopolysaccharides, which improve the protection and survival of encapsulated cells and allow their incorporation into dairy and non-dairy food products.

1. Introduction

Recognized for promoting health and wellness, probiotics have been related to beneficial effects, including the modulation of the intestinal microbiota, through the inhibition of pathogenic microorganisms; the production of anti-carcinogenic compounds; the modulation of immune responses, etc. (Prakash, Tomaro-Duchesneau, Saha, & Cantor, 2011; Reis et al., 2017; Markowiak & Śliżewska, 2017). However, to promote beneficial effects, micro-organisms with probiotic claims must survive exposure to environmental factors, being able to colonize and maintain the metabolic activity in the human intestinal tract (Collins, Thornton, & Sullivan, 1998; Saarela, Mogensen, Fonden, Mättö, & Mattila-Sandholm, 2000).

In this sense, the cell encapsulation may improve the resistance of probiotic microorganisms to adverse conditions (Kim et al., 2017; Rodrigues et al., 2017), reducing cell losses of encapsulated microorganisms in hydrocolloid matrices. Currently, different probiotic encapsulation techniques are used, in which particles of different properties are obtained (Cavalheiro et al., 2015). Among the main techniques used to encapsulate probiotic cells are extrusion (Krasaekoopt & Watcharapoka, 2014; Rodrigues et al., 2017; Kim et al., 2017; Silva et al., 2018a; Dimitrellou et al., 2019), emulsion (Zhang, Lin, & Zhong, 2016; van der Ark et al., 2017; Raddatz et al., 2020), spray drying (Rajam & Anandharamakrishnan, 2015; Bustamante, Oomah, Rubilar, & Shene, 2017; Santos et al., 2019; Aragón-Rojas, Hernández-Álvarez, Mainville, Arcand, & Quintanilla-Carvajal, 2020), spray chilling (Pedroso, Thomazini, Heinemann, & Favaro-Trindade, 2012; Bampi et al., 2016; Arslan-Tontul & Erbas, 2017; Silva et al., 2018b; Arslan-Tontul, Erbas, & Gorgulu, 2019) and fluidized bed (Silva et al., 2018a; Horison & Surono, 2020).

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Encapsulated probiotic cells: Relevant techniques, natural sources as encapsulating materials and food applications – A narrative review

Author: F.J. Rodrigues,M.F. Cedran,J.L. Bicas,H.H. Sato

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Annex 3: Published manuscript to *Current Research in Food Science*

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Reuterin-producing *Limosilactobacillus reuteri*: Optimization of *in situ* reuterin production in alginate-based filmogenic solutions

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ARTICLE INFO

Keywords:
3-Hydroxypropionaldehyde
Lactobacillus reuteri
Film-forming solution
In situ reuterin production

ABSTRACT

Limosilactobacillus reuteri produces reuterin via glycerol anaerobic fermentation. This compound has antimicrobial properties and is used for food preservation purposes. Filmogenic solutions constituted of polysaccharides and glycerol are also employed, however, reuterin synthesis in filmogenic solutions has not yet been reported. Thus, the aim of this study was to optimize the *in situ* reuterin production by *L. reuteri* in alginate- and glycerol based-filmogenic solution, evaluating the survival of reuterin-producing bacteria during fermentation. The study consisted of a completely randomized design employing two *L. reuteri* strains (DSM 20016 and DSM 17938). The filmogenic solutions were obtained using sodium alginate (20 g/L) and two independent variables were studied: glycerol (0–300 mmol/L) and initial biomass of *L. reuteri* (≅6, 7, and 8 log CFU/mL). The samples were analyzed every 24 h for 72 h of anaerobic fermentation (37 °C). Both *L. reuteri* strains confirmed the potential for reuterin production and were susceptible to the metabolite produced. The highest reuterin production was achieved using *L. reuteri* DSM 20016. The initial microbial biomass of 8 log CFU/mL and 100 mmol/L of glycerol increased the reuterin production. However, higher conversion yields from glycerol to reuterin were obtained using 50 mmol/L of substrate.

1. Introduction

First described by Kandler et al. (1980), *Lactobacillus reuteri* is a heterofermentative bacterium commonly found in humans, mainly in the gastrointestinal tract, vagina, and oral cavity (Hou et al., 2015). Recently, the genus *Lactobacillus* has been divided into 25 new genera according to its phylogenetic, phenotypical, and habitat specificities, and *Lactobacillus reuteri* has been reclassified as *Limosilactobacillus reuteri* (Zheng et al., 2020). This non-pathogenic bacterium is accepted by the European Food Safety Authority (EFSA, 2008) as a food supplement to improve gastrointestinal health. In addition, under anaerobic conditions and in glycerol- containing medium, *L. reuteri* strains can produce and accumulate high contents of 3-hydroxypropionaldehyde (3-HPA) through an enzymatic reaction catalyzed by glycerol dehydratase (Vollenweider and Lacroix, 2004). In 1988, the 3-HPA produced by *L. reuteri* was patented as reuterin (Dobrogosz and Lindgren, 1995) and is constantly related to the probiotic activity of this microorganism (Mu et al., 2018).

In fact, reuterin is a dynamic system, also named as HPA system, which contains 3-HPA, its hydrated form 1,1,3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (Vollenweider et al., 2003). However, it has recently been suggested to include acrolein in the reuterin system since the 3-HPA in an aqueous solution can suffer spontaneous dehydration to acrolein (Engels et al., 2016). The reuterin system has several antimicrobial characteristics, such as antifungal activity (Schmidt et al., 2018; Vimont et al., 2019), broad spectrum of activity against Gram-positive and negative bacteria (Ávila et al., 2014; Montiel et al., 2014; Langa et al., 2018; Asare et al., 2020), and antagonistic effect against some protozoa (Vollenweider and Lacroix, 2004). The exact mechanism of reuterin inhibition against microorganisms is not fully understood. However, Talarico and Dobrogosz (1989) postulated that reuterin can compromise the DNA synthesis by inhibiting ribonucleotide reductase. Schaefer et al. (2010) then suggested that reuterin may cause depletion of thiol groups in glutathione, proteins, and enzymes, inducing cells to oxidative stress that can result in cell death.

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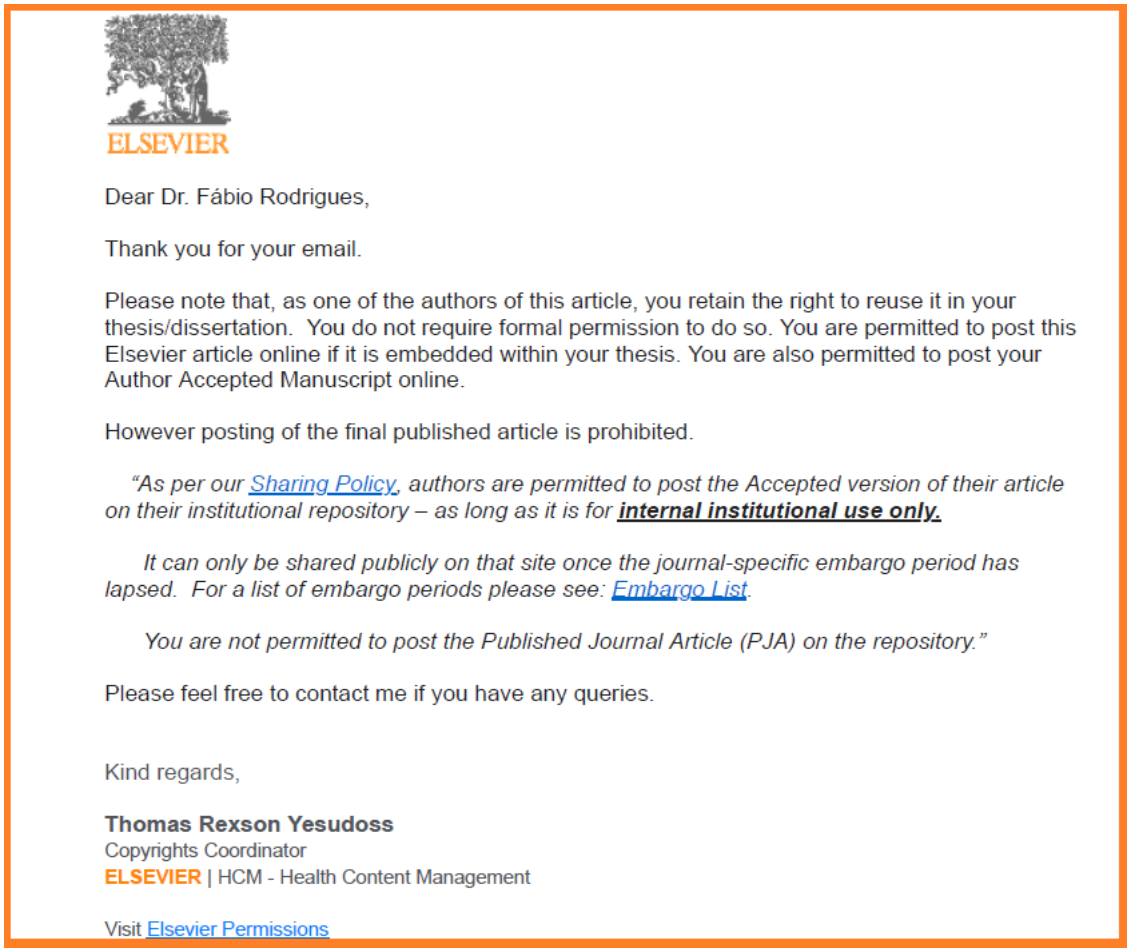

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Annex 5: Published manuscript to *Biotechnology Reports*

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Effective encapsulation of reuterin-producing *Limosilactobacillus reuteri* in alginate beads prepared with different mucilages/gums

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ARTICLE INFO

Keywords:
Probiotic
3-hydroxypropionaldehyde
Lactobacillus reuteri
Immobilization
Gastrointestinal environment

ABSTRACT

The mainly aim of this study was to use mucilaginous solutions obtained from tamarind, mutamba, cassia tora, psyllium and konjac powdered to encapsulate reuterin-producing *Limosilactobacillus reuteri* in alginate beads by extrusion technique. In the particles were determined the bacterial encapsulation efficiency, cell viability during storage and survival under simulated gastric and intestinal conditions. Moreover, the reuterin production, its entrapment into the beads and the influence on viability of encapsulated microorganism were evaluated. Scanning electron microscopy and Fourier Transform Infrared spectroscopy were employed to characterize the produced particles. The beads showed a relatively spherical shape with homogenous distribution of *L. reuteri*. The use of gums and mucilages combined with alginate improved the encapsulation efficiency (from 93.2 to 97.4%), the viability of encapsulated bacteria during refrigerated storage (especially in prolonged storage of 20, 30 and 60 days) and the survival after exposure to gastric and enteric environments (from 67.7 to 76.6%). The *L. reuteri* was able to produce reuterin via bioconversion of glycerol in the film-forming solutions, and the entrapment of the metabolite was improved using konjac, mutamba and tamarind mucilaginous solutions in the encapsulation process (45, 44.57 and 41.25%, respectively). Thus, our findings confirm the great potential of these hydrocolloids to different further purposes, enabling its application as support material for delivery of chemical or biological compounds.

1. Introduction

Accepted by the European Food Safety Authority [1] as a food supplement to improve gastrointestinal health, *Limosilactobacillus reuteri* is recognized for its ability to produce and accumulate, under anaerobic conditions, high contents of 3-hydroxypropionaldehyde (3-HPA) through bioconversion from glycerol [[2],[3]]. The 3-HPA belongs to a dynamic system, named as HPA system, which contains 3-HPA, its hydrated form 1,1,3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane [4]. This system was patented with the name reuterin [5]. Its scientific interest is notable, since reuterin has antimicrobial properties, such as antifungal activity and broad spectrum of activity against Gram-positive and negative bacteria [[6],[3]] being related to the probiotic activity of *L. reuteri* [7].

Currently, the concept of probiotic is defined by Food and Agriculture Organization of the United Nations (FAO), supported by the World Health Organization (WHO) as bacteria and yeasts that, when administered in adequate amounts, confer health benefits to the host [8]. Due to the benefits conferred to human health, probiotics have been investigated in the prevention and treatment of various conditions [[9],[10],[11],[12]]. However, to promote beneficial effects probiotics must be stable to environmental conditions, enabling its adherence to the intestinal mucosa, colonization of the human gastrointestinal tract, production of antimicrobial compounds and maintenance of metabolic activity in the intestine [[13],[14]]. Even though the evident health-promoting properties related to the administration of probiotics, the use of these microorganisms is limited by extrinsic factors, such as oxygen concentration, presence of hydrogen peroxide, pH and temperature variations, etc. In this sense, bacterial encapsulation is a useful approach to protect encapsulated cells under adverse conditions [[15],[16]].

Several techniques are currently available to encapsulate probiotic cells, but these procedures should not be aggressive, ensuring sufficient viability of the encapsulated cells [[17],[16]]. Thus, due to low cost,

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Effective encapsulation of reuterin-producing Limosilactobacillus reuteri in alginate beads prepared with different mucilages/gums

Author: F.J. Rodrigues,M.F. Cedran,G.A. Pereira,J.L. Bicas,H.H. Sato

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Annex 7: Published manuscript to Food Bioscience (Chapter IV)

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Inhibitory effect of reuterin-producing *Limosilactobacillus reuteri* and edible alginate-konjac gum film against foodborne pathogens and spoilage microorganisms

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ARTICLE INFO

Keywords:
3-Hydroxypropionaldehyde
Lactobacillus reuteri
Antimicrobial activity
Edible film
Konjac gum

ABSTRACT

The possible inhibitory effects against foodborne pathogens and spoilage microorganisms of two strains (DSM 20016 and DSM 17938) of reuterin-producing *Limosilactobacillus reuteri*, and an edible alginate-konjac gum film containing *L. reuteri* cells and *in situ* produced reuterin was evaluated. An *in vitro* antimicrobial screening study indicated that both *L. reuteri* strains and the edible film showed inhibitory effects against *B. cereus*, *C. perfringens* and *P. aeruginosa*, and towards the fungi *F. oxysporum*, *G. gloeosporioides*, *A. alternata*, and *P. digitatum*. In addition, *L. reuteri* DSM 20016 was able to produce reuterin *in situ* (46.67 mmol/L) in alginate-konjac gum film-forming solution through anaerobic glycerol bioconversion. Furthermore, after the edible film production by extrusion technique, both *L. reuteri* and reuterin were successfully trapped (98 and 67.6 %, respectively). Further studies must be conducted to detect the exact metabolites related to the inhibition effects against foodborne pathogens and spoilage microorganisms.

1. Introduction

Latic acid bacteria are widespread in nature, and several species are important representatives of human microbiota. This bacterial group includes several species that can be used in industrial food applications. Moreover, some of these species have demonstrated the capacity to produce organic acids, peroxides, bacteriocins and other molecules which can inhibit foodborne pathogens and spoilage microorganisms (Özogul & Hamed, 2018). Members of the *Lactobacillus* genus are the main representatives of latic acid bacteria. However, this genus has recently been divided into 25 new genera, based on phylogeny, phenotype, and habitat specificities (Zheng et al., 2020).

The *Limosilactobacillus reuteri* (formerly known as *Lactobacillus reuteri*) is a heterofermentative bacterium found in the human microbiome; and is accepted by the European Food Safety Authority – EFSA (2008) as a food supplement to improve gastrointestinal health. In general, *L. reuteri* has the ability to produce and accumulate high reuterin content through an enzymatic reaction catalyzed under anaerobic conditions by glycerol dehydratase (Rodrigues, Cedran, et al., 2021). The reuterin produced by *L. reuteri* is a dynamic system, called the HPA system, which contains 3-hydroxypropionaldehyde (3-HPA), its hydrated form 1,1, 3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1, 3-dioxane (Vollenweider et al., 2003). The 3-HPA in an aqueous solution can suffer spontaneous dehydration to acrolein. Thus, it was recently suggested to include acrolein into the HPA system (Engels et al., 2016).

Reuterin has antimicrobial characteristics, such as antifungal activity (Vimont et al., 2019) and a broad spectrum of activity against Gram-positive and negative bacteria (Ávila et al., 2014; Langa et al., 2018). In addition, it is resistant to lipolytic and proteolytic enzymes, and maintains its antimicrobial activity at low pH, high saline concentrations (Rasch et al., 2007) and at refrigeration temperatures (Arqués et al., 2008). This dynamic compound has been related to the probiotic capacity of *L. reuteri*, however, its mechanism of inhibition against microorganisms is not fully understood. It was suggested that reuterin can compromise the DNA synthesis, by inhibiting ribonucleotide reductase (Talarico & Dobrogosz, 1989) and/or by induction of oxidative stress in the microbial cells, leading to cell death; due mainly to the depletion of thiol groups in glutathione, proteins, and enzymes (Schaefer et al., 2010).

The potential use of reuterin-producing *L. reuteri* as a biopreservative

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Inhibitory effect of reuterin-producing Limosilactobacillus reuteri and edible alginate-konjac gum film against foodborne pathogens and spoilage microorganisms

Author: F.J. Rodrigues,M.F. Cedran,J.L. Bicas,H.H. Sato

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Lactobacillus reuteri	
Lactobacillus reuteri	
Título da Atividade:	**Produção de reuterina in situ por Lactobacillus reuteri em soluções filmogênicas**
Equipe	
Hélia Harumi Sato	**UNICAMP**
Fábio Júnior Rodrigues	**UNICAMP**

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