Production, characterization and food application of lactic acid bacteria-derived exopolysaccharides with health-promoting benefits

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Production, characterization and food application of lactic acid bacteria-derived exopolysaccharides with health-promoting benefits

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LIST OF PUBLICATIONS

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IV. Jurášková, D., Ribeiro, S. C., & Silva, C.C.G. (2025). Prebiotic and Health-Promoting Benefits of Dextran-Type Exopolysaccharide Produced by Leuconostoc mesenteroides SJC113. *Foods*, *14*(15), 2635.

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"Never give up on a dream just because of the time it will take to accomplish it. The time will pass anyway." **Earl Nightingale**

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ABSTRACT

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) are multifunctional carbohydrate polymers that have attracted increasing attention due to their dual role in food systems and human health. Despite the wide diversity of LAB-derived EPS, systematic research connecting their structural features to both techno-functional and health-promoting properties remains scarce.

This PhD thesis aimed to advance the understanding of LAB-derived EPS by combining a broad review of the state of the art with original experimental studies on selected EPS-producing strains. The work was structured around four main objectives: (i) to review the structural diversity, biosynthetic pathways, and functional roles of LAB-produced EPS with emphasis on their application in food and health; (ii) to characterize the EPS produced by *Leuconostoc mesenteroides* SJC113 (isolated from São Jorge cheese) and evaluate its technological and functional properties, including the beneficial effects (as a prebiotic) on human health; and (iv) to screen goat milk-derived *Streptococcus thermophilus* strains for EPS production and safety, leading to the selection and characterization of the EPS from strain GM4.

An extensive literature review was conducted to summarize the latest advances in LAB-derived EPS, including: (i) the structure, biosynthesis, and properties of EPS; (ii) modern methods for their characterization; (iii) their application in improving the quality of dairy and low-fat food products; and (iv) their documented health-promoting effects, including prebiotic, antioxidant, and cholesterol-lowering activities.

The first experimental work focused on EPS produced by *Ln. mesenteroides* SJC113. Structural characterization confirmed a dextran-type homopolysaccharide resistant to dextranase hydrolysis and of high molecular weight. Functional assays revealed antioxidant and cholesterol-binding properties, while the strain itself exhibited β-galactosidase activity and tolerance to gastrointestinal conditions. Safety assessment demonstrated the absence of virulence genes and appropriate antibiotic susceptibility. When applied in fat-free fresh cheese, the EPS improved water-holding capacity, rheology, and sensory perception, validating its role as a natural fat replacer.

The second study investigated the potential health-promoting effects of the dextran-type EPS. *In vitro* experiments demonstrated its ability to support the growth of probiotic LAB, enhance mucin adhesion, and positively modulate fecal microbiota composition. Antioxidant capacity and cholesterol-lowering properties further supported its functionality as a bioactive dietary

component. These results underline the prebiotic nature of dextran-type EPS and their potential role in gut microbiota modulation and cardiovascular health.

The third study characterized four exopolysaccharide (EPS)-producing *S. thermophilus* strains (GM1-GM4), isolated from goat milk, to evaluate their potential for use in functional dairy products. Safety of GM4 was confirmed by testing for hemolytic, DNase, and gelatinase activities, and by screening for virulence and antibiotic resistance genes. Strain GM4 exhibited a high β-galactosidase activity and its probiotic potential was emphasized by a high auto-aggregation capacity and co-aggregation with pathogens like *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The purified EPS from GM4 was identified as a heteropolysaccharide composed primarily of glucose and mannose with high dextranase resistance and antioxidant activity. GM4 emerged as a safe, technologically versatile, and functional strain, with its EPS possessing bioactive properties, making it a strong candidate for incorporation into functional foods.

Altogether, this thesis demonstrates that LAB-derived EPS are multifunctional molecules bridging technological innovation and health promotion. By combining structural, functional, and application-oriented studies, it provides new insights into the dual role of EPS as natural food structuring agents and bioactive ingredients. These findings contribute to the development of clean-label, functional foods and highlight the potential of LAB strains as a source of health-supporting biomolecules.

Keywords: Lactic acid bacteria, Exopolysaccharides, Food application, Health-promoting benefits

RESUMO

Os exopolissacáridos (EPS) produzidos por bactérias do ácido láctico (BAL) são polímeros multifuncionais que têm atraído uma atenção crescente devido à sua dupla função nos sistemas alimentares e na saúde humana. Apesar da grande diversidade de EPS derivados das BAL, a investigação sistemática que relaciona as suas características estruturais com as propriedades tecno-funcionais e promotoras de saúde, permanece escassa.

Esta tese de doutoramento teve como objetivo estudar os EPS produzidos por estirpes de BAL selecionadas, combinando uma revisão do estado da arte com estudos experimentais. O trabalho foi estruturado em torno de três objetivos principais: (i) fazer uma revisão bibliográfica sobre a diversidade estrutural, biossíntese e propriedades funcionais dos EPS produzidos por BAL, com ênfase na sua aplicação nos alimentos e na saúde; (ii) caracterizar o EPS produzido pela estirpe *Leuconostoc mesenteroides* SJC113 (isolada do queijo de São Jorge) e avaliar suas propriedades tecnológicas e funcionais, bem como investigar os efeitos benéficos (como prebiótico) para a saúde humana (iii) caracterizar as estirpes produtoras de EPS de *Streptococcus thermophilus* isoladas do leite de cabra, ao nível tecnológico e segurança, com especial relevo à caracterização do EPS da estirpe GM4.

Uma extensa revisão de literatura foi conduzida apresentando os últimos avanços dos EPS produzidos pelas LAB, incluindo: (i) a estrutura, biossíntese e propriedades dos EPS; (ii) os métodos mais recentes utilizados na sua caracterização; (iii) a aplicação do EPS nos laticínios e em alimentos com baixo teor de gordura; e (iv) os efeitos do EPS na promoção da saúde, incluindo atividades prebióticas, antioxidantes e redutoras de colesterol.

O primeiro estudo experimental abordou o EPS produzido pela estirpe *Ln. mesenteroides* SJC113. A caracterização estrutural confirmou ser um homopolissacárido do tipo dextrano, resistente à hidrólise pela dextranase e de elevado peso molecular. Este EPS apresentou propriedades antioxidantes e de ligação ao colesterol, enquanto a estirpe exibiu atividade de β-galactosidase e resistência às condições gastrointestinais. A avaliação de segurança demonstrou a ausência de genes de virulência e suscetibilidade a antibióticos. Quando aplicados no queijo fresco sem gordura, o EPS melhorou a capacidade de retenção de água e as propriedades reológicas e sensoriais, confirmando o seu papel como substituto natural de gordura.

O segundo estudo investigou os potenciais efeitos promotores de saúde do EPS produzido por esta estirpe. Estudos *in vitro* demonstraram a sua capacidade em promover o crescimento de BAL probióticas, aumentar a adesão à mucina e modular positivamente a composição da microbiota intestinal. A capacidade antioxidante e as propriedades de redução do colesterol

reforçaram a sua funcionalidade como um componente bioativo. Estes resultados apontam para a natureza prebiótica deste EPS e a sua potencial função na modulação da microbiota intestinal e na saúde cardiovascular.

O terceiro estudo caracterizou quatro estirpes de *S. thermophilus* produtoras de EPS (GM1-GM4), isoladas de leite de cabra, para avaliar seu potencial para uso em produtos lácteos funcionais. A segurança de GM4 foi confirmada por testes de atividade hemolítica, DNase e gelatinase, e pela presença de genes de virulência e resistência a antibióticos. A estirpe GM4 exibiu alta atividade da β-galactosidase e seu potencial probiótico foi confirmado pela elevada capacidade de autoagregação e coagregação com patógenos como *Escherichia coli, Listeria monocytogenes e Staphylococcus aureus*. Os EPS purificados foram identificados como heteropolissacáridos compostos essencialmente por glucose e manose, com alta resistência à dextranase e atividade antioxidante. A estirpe GM4 destacou-se pela sua segurança, sendo tecnologicamente versátil e funcional, produzindo EPS com propriedades bioativas, sendo por isso uma forte candidata para incorporação em alimentos funcionais.

No conjunto, esta tese demonstrou que os EPS derivados de BAL são moléculas multifuncionais que associam a inovação tecnológica e promoção da saúde. Ao combinar estudos estruturais, funcionais e orientados para a aplicação, ela fornece novas perceções sobre o papel duplo dos EPS como agentes estruturais e ingredientes bioativos nos alimentos. Essas descobertas contribuem para o desenvolvimento de alimentos funcionais "clean label" e destacam o potencial das estirpes de BAL como uma fonte de biomoléculas com benefícios para a saúde.

Palavras-chave: Bactérias do ácido láctico, Exopolissacáridos, Aplicação alimentar, Benefícios para a saúde.

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Chapter 1: General Introduction

The growing demand for "clean label" products and functional foods has accelerated the search for natural ingredients that can improve food quality while providing health benefits. In this context, exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) have emerged as a promising class of biopolymers. These high molecular weight carbohydrates not only improve the texture and stability of various food matrices, but they can also provide health-promoting effects. The dual functionality of LAB-derived EPS makes them an ideal candidate for the development of a new generation of functional foods that meet modern consumer preferences.

This work provides a comprehensive exploration of these versatile compounds. The research focuses on the characterisation of EPS and EPS-producers to their final application in food systems, with particular attention to their beneficial effects on human health.

The production of EPS by LAB is a complex process influenced by genetic, physiological, and environmental factors. EPS biosynthesis is primarily regulated by a cluster of genes known as the *eps* gene cluster [1]. The diversity of EPS structures is immense, ranging from simple chains of glucose (glucans) or fructose (fructans) to highly complex, branched polymers with repeating units of various monosaccharides [2–5].

Homopolysaccharides (HoPS) are typically linear or branched polymers of a single type of monosaccharide, such as glucose or fructose. Examples include dextrans (α-glucans) and levans (β-fructans), which are often produced by strains of *Lactobacillus* and *Leuconostoc*. These simple EPS are known for their gelling and thickening properties.

Heteropolysaccharides (HePS) are composed of repeating units of two or more different monosaccharides such as glucose, galactose, mannose, rhamnose, and uronic acids. Their complex structure can lead to unique functional properties. The structure and composition of HePS can be highly variable, even among closely related bacterial strains, which highlights the need for a thorough characterization of each new EPS-producing strain [2–5].

The technological functionality of LAB-derived EPS is a primary reason for their application in the food industry. They can significantly impact the rheological properties of food systems, acting as thickening, gelling, and emulsifying agents. For instance, in fermented dairy products, EPS can reduce syneresis (the expulsion of whey), improve viscosity, and create a smoother texture, which is particularly beneficial for low-fat or fat-free products. This helps in enhancing the mouthfeel and overall consumer acceptance [6–8].

Beyond their technological roles, LAB-derived EPS have been extensively studied for their health-promoting benefits, which are often linked to their prebiotic nature and potential to modulate the gut microbiota. Firstly, the EPS matrix provides a protective encapsulation for producer bacteria, significantly increasing their resilience to gastrointestinal stresses and facilitating their delivery to the colon in a viable state [9,10]. Secondly, these EPS polymers function as selective prebiotics, preferentially stimulating the growth and metabolic activity of beneficial gut genera like *Bifidobacterium* and lactobacilli. The resulting microbial modulation promotes the synthesis of short-chain fatty acids (SCFAs), including butyrate, which are crucial for maintaining gut health [7].

The chemical structure of EPS, particularly the presence of hydroxyl and carboxyl groups, gives them the ability to scavenge free radicals and reduce oxidative stress in the body. Oxidative stress is implicated in various diseases, including cardiovascular and neurodegenerative disorders, making this a highly valuable property of EPS [9,10]. In addition, some studies suggest that specific EPS can bind to bile acids and cholesterol in the small intestine. This binding prevents the reabsorption of bile acids and cholesterol, thus leading to a reduction in serum cholesterol levels [11].

The main objectives of this thesis are to: (i) isolate and identify novel LAB with EPS-producing capability; (ii) elucidate the structure-function relationships of the synthesized polymers; (iii) screen selected isolates for safety (e.g., absence of virulence determinants, antibiotic susceptibility) and key technological properties (e.g., milk acidification, carbohydrate fermentation and enzymatic activity); (iv) evaluate the efficacy of purified EPS as natural bio-texturizers to improve the rheological properties of low-fat dairy matrices; and (v) assess the biofunctional potential of EPS, including their prebiotic activity through the selective stimulation of beneficial gut bacteria, and their direct health-relevant properties such as cholesterol-binding capacity, antioxidant activity, and ability to inhibit pathogen adhesion in vitro.

The work presented in this thesis is divided into six chapters that explore the production, characterization, and food application of LAB-derived EPS.

- **Chapter 1:** Introduces the topic of the thesis.
- **Chapter 2:** A literature review that provides an overview of EPS produced by LAB, detailing their biosynthesis and diverse health-promoting properties.

- **Chapter 3:** This chapter characterizes the functional properties of EPS produced by *Leuconostoc mesenteroides* SJC113 and evaluates its application in fat-free cheese.
- **Chapter 4:** This chapter investigates the health benefits and prebiotic activity of EPS produced by *Ln. mesenteroides* SJC113.
- **Chapter 5:** This chapter investigates and characterizes four *Streptococcus thermophilus* strains isolated from goat milk, their EPS production with different sugars, their safety and their probiotic potential and health benefits.
- **Chapter 6:** This chapter synthesizes the key findings, discusses their implications for the food industry, and provides direction for future research.

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Chapter 2: Exopolysaccharides Produced by Lactic Acid Bacteria: From Biosynthesis to Health-Promoting Properties

1. Introduction

It is well-known that lactic acid bacteria (LAB) can synthesize a variety of polysaccharides. These comprise a large group of high-molecular-weight molecules consisting of monosaccharide units linked by a glycosidic bond, which exhibit a variety of structures, functional properties, and biological activities [1,2]. Polysaccharides are one of the main components involved in the formation of the extracellular biofilm matrix. They play an important role not only in protecting bacteria from adverse environmental factors, but also in the attachment of microbial cells to solid surfaces [3-6]. The polysaccharides produced by bacteria can be found in structures on the cell surface classified as capsular polysaccharides or lipopolysaccharides [7]. They form the outermost layer of the bacterial cell and provide a mechanism to protect the cell, mediating direct interactions with the environment. Due to their attachment to the cell surface, they are unlikely to be isolated or separated from the cell biomass, although some capsular polysaccharides can be released from the cell. In contrast, exopolysaccharides are polysaccharides that are loosely associated with the cell surface or released into the extracellular medium [8,9]. They can either be produced extracellularly by enzymes secreted by the bacterium, or synthesized intracellularly and secreted outside the cells [10]. Considering the protective mechanisms for the microbial cell, such as protection against abiotic or biotic stress, competition, pH, and temperature, exopolysaccharides (EPS) have particular physicochemical properties that most likely have potential for the food and pharmaceutical industries. Nevertheless, most of the research is focused on the application of EPS in the food industry due to their structural properties, such as emulsification, texturization, sweetening, gelling, water-binding capacity, or bioactive properties. This is one of the reasons why EPS has attracted the attention of researchers. Recent studies have also demonstrated the health-promoting potential of EPS, including immunomodulatory, prebiotic, anti-inflammatory, anti-biofilm, and antioxidant activities [11-15]. In this review, the structure of EPS produced by LAB, their biosynthesis, and the methods currently used to isolate and characterize EPS are described. In addition, some food and pharmaceutical applications of EPS are discussed, as well as the recent evidence of health-promoting effects of EPS.

2. Structure of Exopolysaccharides (EPS)

Bacterial EPSs have diverse and complex chemical structures that strongly influence their functional properties and biological functions. They can be divided into homopolysaccharides (HoPS), which contain a single type of monosaccharide, and heteropolysaccharides (HePS),

which consist of two or more types of monosaccharides [16-18]. Most of the EPS produced by LAB are HePS and are synthesized intracellularly, while some LAB species/strains produce HoPS by extracellular enzymes [10,19]. The HoPS produced by LAB (Table 1) can be classified as glucans, fructans, or galactans, which consist of D-glucose, D-fructose, or D-galactose, respectively [10,20]. Glucans consist of glucose residues as the main backbone structure with different degrees of branching and binding sites that vary from bacterial strain to bacterial strain. Glucans can be classified as either α-glucans or β-glucans, and are produced by a variety of LAB species in the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus,* and *Weissella* [21]. Alpha-glucans can be divided into four groups: (i) dextran is water-soluble and mostly has α-(l→6) bonds, although some branching may occur at α-(l→2), α-(l→3) or α-(l→4) [22,23]; (ii) mutan is generally water-insoluble and contains mainly α-(l→3) bonds with branching at α-(l→6) [21]; (iii) reuteran is a water-soluble branched α-glucan consisting of α-(l→4)-linear fragments linked by α-(l→6) bonds [24]; and (iv) alternan exhibits alternating α-(l→3) and α-(l→6) bonds and shows lower viscosity and higher solubility in water [25]. Beta-glucans are also HoPS, produced by *Pediococcus* and *Streptococcus* spp., and consist of D-glucose linked by β-(l→3) bonds together with β-(l→2) branches [19,26]. Fructans are water-soluble fructose polymers produced by strains of *Streptococcus salivarius*, *Leuconostoc mesenteroides*, *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), *Lactobacillus johnsonii* and *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*). They can be divided into (i) levan with β-2,6 link(ages and (ii) inulin with β-2,1 linkages [27]. The water-soluble galactans are less abundant, have α-(1→6)-linked galactose units, and are produced by a few LAB strains belonging to *Weissella confusa*, *Lactococcus lactis* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [19,28].

Table 1. Homopolysaccharides produced by lactic acid bacteria.

HoPS		LAB	Mw/Structure	Reference
α-D-glucans	Dextran	*Leuconostoc mesenteroides* *Leuconostoc citreum* *Leuconostoc pseudomesenteroides* *Lentilactobacillus parabuchneri* (formerly *Lactobacillus parabuchneri*) *Limosilactobacillus fermentum* (formerly *Lactobacillus fermentum*) *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) *Latilactobacillus sakei* (formerly *Lactobacillus sakei*) *Latilactobacillus curvatus* (formerly *Lactobacillus curvatus*) *Lactobacillus hordei* *Lactobacillus nagelli* *Lactobacillus mali* *Lactobacillus satsumensis* *Weissella confusa* *Weissella cibaria* *Streptococcus mutans* *Streptococcus salivarius*	Mw: 10^3–10^7 Da α-D-Glc(1,6)	[29-47]
	Mutan	*Limosilactobacillus reuteri* *Streptococcus downei* *Streptococcus mutans* *Streptococcus salivarius*	Mw: >10^6 Da α-D-Glc(1,3)	[35,36,40,48-50]
	Alternan	*Leuconostoc mesenteroides* *Streptococcus salivarius* *Leuconostoc citreum*	Mw: >10^6 Da (α-D-Glc(1,6)/α-DGlc(1,3)	[51-53]

	Reuteran	*Limosilactobacillus reuteri*	Mw: 10^7 Da α-D-Glc(1,4)/α-DGlc(1,6)	[40,54]
β-Glucans		*Lactobacillus suebicus* *Pediococcus parvulus*	Mw: 10^5–10^6 Da [β-D-Glc(1,3) with side chain linked (1,2)]	[55,56]
Fructans	Levans	*Leuconostoc mesenteroides* *Limosilactobacillus reuteri* *Streptococcus mutans* *Bacillus subtilis*	Mw: 10^4–10^8 Da β-D-Fru(2,6)	[47,57]
	Inulin-type	*Streptococcus mutans* *Limosilactobacillus reuteri* *Leuconostoc citreum* *Lactobacillus johnsonii*	Mw: 10^3–10^7 Da β-D-Fru(2,1)	[20,55,58,59]
Polygalactan		*Lactococcus lactis* *Lactobacillus delbruecki*	pentameric repeating unit of galactose	[47]

HePS (Table 2), unlike HoPS, have a more complex structure as they are composed of several repeating units of sugars, such as pentose (D-ribose, D-arabinose, D-xylose), hexose (D-glucose, D-galactose, D-mannose), N-acetylated monosaccharides (N-acetylglucosamine and N-acetyl-galactosamine), or uronic acids (D-glucuronic acid, D-galacturonic acid), and may be branched or unbranched [8,60,61]. They are produced by members of the genera *Lactobacillus*, *Lactococcus*, and *Streptococcus* [10]. HePS are produced in relatively small amounts by LAB but exhibit high thickening power at low concentrations [20,62,63]. Kefiran is an example of HePS produced by several *Lactobacillus* species in kefir grains, including *L. kefiranofaciens*, *L. kefirgranum*, *L. parakefir*, *L. kefir*, and *L. delbrueckii* subsp. *bulgaricus* [64]. Kefiran is a water-soluble branched glucogalactan with a complex structure consisting of D-glucose (Glc) and D-galactose (Gal) in approximately equal amounts, with (1→6)-linked Glc, (1→3)-linked Gal, (1→4)-linked Gal, (1→4)-linked Glc, and (1→2, 6)linked Gal [65]. Because of these types of linkages, kefiran cannot be hydrolyzed by the digestive enzymes of the human gastrointestinal tract, but it can be fermented by colon bacteria [64]. Other water-soluble HePS include gellan and xanthan, but they are produced by non-LAB, such as *Sphingomonas paucimobilis* and *Xanthomonas campestris* [66].

Table 2. Heteropolysaccharides produced by lactic acid bacteria.

LAB	HePS Composition	Molecular Weight	Reference
Streptococcus thermophilus CC30	Glucose, galactose	58 to 180 kDa	[67]
Streptococcus thermophilus CH101	Glucose, galactose	8.5×10^5	[68]
Streptococcus thermophilus LY03	Glucose, galactose, N-acetylgalactosamine	1.8×10^6	[68]
Streptococcus thermophilus S-3	N-acetylgalactosamine, galactose, glucose	5.7×10^5 Da	[69]
Streptococcus thermophilus NIZO2104	Galactose, ribose, N-acetylgalactosamine, glucose	0.9×10^6 Da	[70,71]
Streptococcus thermophilus AR333	Galactose, glucose, galactosamine	3.1×10^5 Da	[72]
Lactobacillus delbruecki subsp. *bulgaricus* CNRZ 1187	Rhamnose, arabinose, mannose, galactose, glucose	10^4–10^6 Da	[73,74]
Lactobacillus delbruecki subsp. *bulgaricus* DGCC291	Glucose, galactose	1.4×10^6 Da	[70,71]
Lactobacillus delbruecki subsp. *bulgaricus* NCIMB702074	Glucose, galactose	1.8×10^6 Da	[70,71]
Lacticaseibacillus casei (formerly *Lactobacillus casei*) WXD030	Glucose, glucosamine, mannose	37.37 kDa	[75]
Lactobacillus gasseri FR4	Glucose, mannose, galactose, rhamnose, fucose	1.9×10^5 Da	[76]
Lactobacillus helveticus MB2-1	Glucose, mannose, galactose, rhamnose, arabinose	1.83×10^5 Da	[77]
Lactobacillus kefiranofaciens WT-2B	Kefiran: glucose, galactose	7.6×10^5 Da	[78]
Lactobacillus johnsonii 142	D-glucose and D-dalactose	1.0×10^5 Da	[79]
Latilactobacillus sakei O-1 (formerly *Lactobacillus sakei*)	Glucose, rhamnose	6×10^6 Da	[80]
Lactiplantibacillus plantarum (formerly *Lactobacillus plantarum)* JLK0142	Glucose, galactose	1.34×10^5 Da	[81]

Lactiplantibacillus plantarum WLPL04	Xylose, glucose, galactose	6.61×10^4 Da	[82]
Lactiplantibacillus plantarum YW11	Glucose, galactose	1.1×10^5 Da	[83]
Lactiplantibacillus plantarum JLAU103	Arabinose, rhamnose, fucose, xylose, mannose, fructose, galactose, glucose	12.4 kDa	[84]
Lactiplantibacillus plantarum EP56	Glucose, galactose, rhamnose	8.5×10^5 Da	[85]
Lactiplantibacillus plantarum C88	Glucose, galactose	1.2×10^6 Da	[86]
Lactiplantibacillus plantarum C70	Arabinose, mannose, glucose, galactose	3.8×10^5 Da	[87]

3. EPS Biosynthesis

Regardless of their different structures, the biosynthetic pathways of EPS are remarkably similar between different species of microorganisms [88-90]. It is known that HoPS are synthesized by only one enzyme encoded by one gene. In contrast, the genetic sequence of HePS encodes multiple glycosyltransferases, polymerization proteins, and regulatory proteins [90,91]. From a biochemical point of view, there are two major groups of enzymes involved in the production of EPS. The first group consists of enzymes necessary for the synthesis of basic sugar nucleotides used by other cellular metabolic pathways [92,93]. The second group consists of EPS-specific enzymes, such as glycosyl- and acetyltransferases or enzymes responsible for polymerization and export, that regulate the whole process. However, there are also some EPS-specific enzymes whose function is not yet known [92,94]. EPS-specific enzymes are regulated by genes that are usually arranged in clusters on chromosomes or plasmids [55,95,96]. The biosynthesis of HoPS is a relatively simple process because there are no active transport steps and no unnecessary energy is consumed except for the biosynthesis of the extracellular enzymes. Therefore, HoPS is usually synthesized extracellularly mainly from sucrose, although recent reports describe the use of maltodextrins and starch substrates as donors of glucosyl units by some LAB species/strains [8,21]. The enzyme that catalyzes the polymerization of HoPS is a glycosylhydrolase that cleaves the glycosidic bond of its substrate (sucrose) and couples the glucosyl or fructosyl units to synthesize either α-glucans or β-fructans. The energy released by cleavage of the energetic glycosidic bond is used to transfer the monosaccharide units to the reducing end of the polymer [8,97-100]. However, β-glucans are synthesized intracellularly by a membrane-associated glucosyltransferase [19]. The process of biosynthesis of HePS is more extensive and energy-consuming. The reaction steps for the synthesis of HePS are as follows: (I) internalization of the sugar, (II) synthesis of sugar nucleotide precursors from glucose-1-phosphate and fructose-6-phosphate, which provide the energy for the polymerization reaction, (III) transport across the membrane via a flippase, and (IV) polymerization by various types of glycosyltransferases located at the cytoplasmic membrane adds the monosaccharide moiety to the reducing end of the chain [90,92,101,102]. Due to variability in composition and molecular weight, HePS exhibits large differences in their stiffness, charges, spatial arrangement, and ability to interact with proteins. The variability in composition and molecular weight also affects the physicochemical properties, such as viscosity and solubility [8,36,103,104]. As for the LAB production of EPS, the yield is generally higher for HoPS. Several factors can affect

the yield of exopolysaccharides, such as the initial pH, temperature, incubation time, composition of the medium, and so forth [105]. Numerous studies indicate that optimization of various conditions during the production of exopolysaccharides can lead to an increase in exopolysaccharide yield [106,107]. The production of exopolysaccharides by lactic acid bacterial strains varies from 10 mg/L to 400 mg/L if the production process is not optimized. In the case of optimization, this amount can multiply [60,108].

4. Methods of EPS Screening, Isolation and Characterization

Despite the large number of researchers working on EPS-producing LAB, there is still insufficient information on the kinetics of EPS synthesis, where variations in production yield and EPS composition can lead to complete loss of production, as numerous variables can affect production and structure [95,109]. These variables include cultivation time, environmental factors, and carbon source, as they can affect the chemical composition, structure, and even biological properties of EPS [110]. In addition, each microorganism has its own requirements for its ability to produce EPS [2,5,55,111]. Applying the correct methodology for EPS screening, isolation, and purification is critical because, as mentioned previously, EPS production is strain-specific and often depends on the composition of the surrounding media and incubation conditions (e.g., temperature, humidity). A variety of methods for studying EPS produced by LAB, including screening, extraction, and assessment of composition and structure, have been developed and optimized in recent years. The screening methods to evaluate the microbial ability to produce EPS are based on the cultivation of the LAB in a medium enriched with different sugars (glucose, fructose, sucrose, galactose, or lactose). The easiest way to assess EPS production is to visually observe the phenotypic characteristics of the colonies: slimy or ropy phenotypes. The slimy phenotype is characterized by mucilaginous colonies, while the ropy phenotype is characterized by the formation of long filaments when an inoculation loop is lifted from the colony surface or cell pellet (Figure 1). For a more objective test, ruthenium red staining is used in milk agar plates and produces red colonies of non-ropy EPS producers, while ropy colonies remain white [112].

(a)  (b) 

Figure 1. Characteristics of EPS produced by LAB: (**a**) Mucoid colonies—slimy phenotype; (**b**) Ropy phenotype.

Qualitative methods, such as confocal laser scanning microscopy (CLSM) and electron microscopy (EM), are usually used for the detection of EPS in food. CLSM is used to study the presence and distribution of fluorescently labeled EPS in food matrices and has the advantage of visualizing changes in food microstructures without the need for sample preparation [74]. In contrast, EM requires high vacuum conditions and dehydration of the sample. However, EM is useful to determine the structural features at nanometer scale due to the high resolution of the images produced [74].

Quantitative evaluation of EPS produced by LAB requires extraction from the culture media or food matrices. Extraction of EPS involves several steps, including centrifugation to recover the EPS-containing supernatant, addition of acid (e.g., TCA) to remove the high protein content, and precipitation with cold ethanol (Figure 2). The precipitated EPS is then collected and dissolved in water before membrane filtration (usually dialysis or ultrafiltration) to remove the small neutral sugars, salts, and small proteins [19,113-116]. Finally, the dialyzed EPS solution is freeze-dried to obtain a pure EPS solid, which has a white color and a soft, spongy texture (Figure 2). In this step, the weight of the obtained EPS gives an approximate indication of the EPS yield [117]. Colorimetric methods such as the phenol-sulfuric acid method [118] or the sulfuric acid-UV method, which is more accurate and faster, are usually used to quantify the EPS [119]. Sometimes a second purification step is required to ensure the high purity of EPS [110]. This may involve treatment with proteases and nucleases to remove contaminating proteins and DNA. In addition, anion exchange chromatography and/or size exclusion chromatography are recommended as they contribute to high-purity samples [120]. The number of steps used for EPS isolation depends on the complexity of the media used for production and the type of EPS (HoPS or HePS). Therefore, a prior study is essential to optimize and improve the fermentation conditions.

A complete description of the structural features of EPS includes the identification of monosaccharide composition, ring conformation, linkage, degree of branching, and molecular weight. Several methods have been used to identify and analyze EPS molecular weight, composition, and structure. No single method is capable of assigning all of these parameters, so a combination of several techniques is usually required. Among the most commonly used methods for the determination of EPS molecular mass are size exclusion chromatography (SEC) and ion exclusion chromatography (IEC) [74]. To evaluate the monomer composition, the glycosidic bonds must be previously hydrolyzed by acids or enzymes, and the released monomers are then subjected to high-performance liquid chromatography (HPLC) with refractive index detection (HPLC-RI), high-performance ion exchange chromatography (HPAEC) with pulse amperometric detection (HPAEC-PAD), or gas chromatography [1] coupled with mass spectrometry (GC-MS) [121]. The properties and functionality of EPS also depend on the functional groups and the nature of the bond, which can be detected by Fourier transform infrared spectroscopy (FTIR). Nuclear magnetic resonance (NMR) spectroscopy is also used to analyze the glycosidic bonds, ring configuration, and anomeric configuration of the monomeric units [74,92,122].

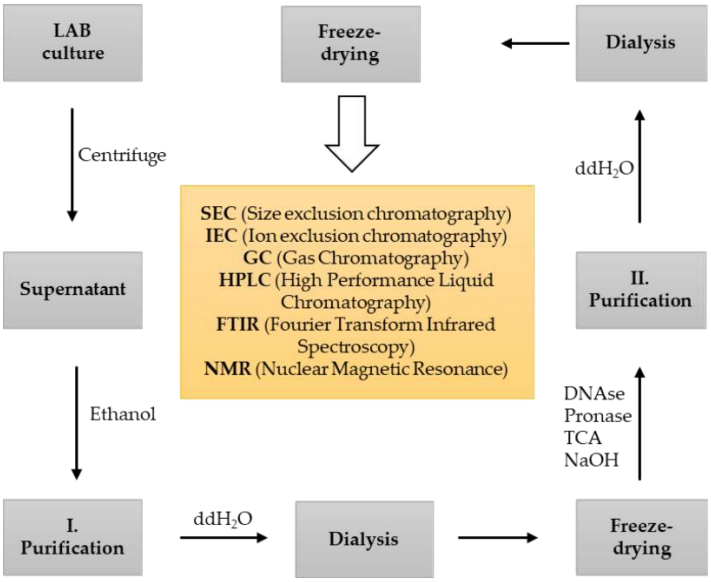

Figure 2. Flow chart for isolation, purification steps (I—first purification, II—second purification), and characterization of EPS.

5. Application of EPS-Producing LAB in Food Products

5.1. Yoghurt

EPS produced by LAB can be used to thicken and stabilize fermented dairy products. Lowering the pH during the fermentation process of yoghourt can lead to syneresis resulting from destabilisation of casein micelles. In situ production of EPS has been used to overcome this problem, as it leads to better rheological properties than when EPS is added as one of the components [92,123]. The starter cultures used for yoghourt production, *Lactobacillus delbruecki* subsp. *bulgaricus* and *Streptococcus thermophilus*, were selected to produce exopolysaccharides, generally 60–150 mg/L and 30–890 mg/L, respectively [8,124]. In recent years, studies have been conducted on new EPS-producing starter or adjunct cultures to be used in yoghourt production [8,125]. For example, Han et al. [126] evaluated 19 high EPS-producing *Str. thermophilus* strains isolated from traditional Chinese fermented milk products and used in yoghourt production. They selected a starter with high EPS production (SH-1), which exhibited lower syneresis, better texture, and better sensory evaluation than the samples fermented with a commercial yoghourt starter culture [126]. Fermented dairy products with low fat content can also be improved by using starters with high EPS production, as milk fat plays an important role on the taste, texture, and overall rheological properties of these products [127]. For example, the performance of EPS-producing *Limosilactobacillus mucosae* (formerly *Lactobacillus mucosae*) DPC 6426 as an adjunct culture in the production of low-fat yoghourt has been evaluated and shown to reduce syneresis and improve the functional properties of yoghourt [128].

5.2. Cheese

A variety of LAB cultures are used in cheese-making as starters and/or adjunct cultures [129]. Nowadays, customers are looking for healthier, low-fat cheeses, and this is where bacterial strains that can produce EPS come into play. As with any other dairy product, the amount of fat is critical to the texture and flavor of the cheese. Therefore, EPS produced by starter/adjuvant cultures can be used as a fat substitute and texturizer in the production of low-fat cheese [8]. Several studies have shown that EPS improves the texture and quality of low-fat

cheese, resulting in a product that has similar properties to its full-fat counterpart. For example, EPS-producing strains of the genus *Lactobacillus* have been shown to increase moisture content and improve the melting of low-fat mozzarella cheese [8,130]. In a study by Costa et al. [131], an EPS-producing strain of *Lactococcus lactis* was used on semi-fat cheddar cheese. Several positive effects of the exopolysaccharide were observed, such as an increase in cheese yield. There was no negative interaction between the starter strain or the ripening strain and the exopolysaccharide-producing strain. After 3 months of ripening, some tests showed that the addition of the EPS-producing strain resulted in a semi-fat cheddar cheese with similar characteristics to a full-fat cheddar cheese without any change in taste [131]. For low-fat cheddar cheese, the addition of the EPS-producing culture of *Lb. plantarum* resulted in cheeses with higher moisture content, higher proteolysis and better sensory values, as well as lower hardness and cohesion compared to the control cheese [132]. In addition, the use of a mixed starter culture containing EPS-producing strains in the production of low-fat Kasar cheese improved the textural properties by producing a less compact protein matrix and a spongy structure [133]. The type of EPS was also shown to affect the rheological properties of the cheese. It was shown that the lower branching ropy EPS reduced protein particle size and decreased creaminess in a model of a low-fat fresh cheese, while the capsular EPS mainly contributed to the reduction of syneresis [134]. The use of EPS-producing LAB in full-fat cheeses has also been studied by several authors. In the production of Prato cheese, the use of exopolysaccharide-producing cultures was shown to improve yield and increase moisture content without affecting proteolysis, pH, melting ability, and sensory acceptability [135]. Rehman et al. [136] used a high EPS-producing strain of *Lactobacillus kefiranofaciens* isolated from Tibetan kefir grain to improve the chewiness and hardness of mozzarella cheese. In a recent study, in situ production of EPS was used to improve the production yield and rheological properties (hardness, elasticity and adhesiveness) of sour whey cheese-requesón [137].

5.3. Kefir

Kefir is a self-carbonated, slightly alcoholic beverage made from fermented milk, traditionally from Eastern Europe. Kefir preparation requires kefir grains, which are composed of proteins and polysaccharides and contain a symbiotic association of homofermentative and heterofermentative lactic acid bacteria, acetic acid bacteria, and yeasts [127]. During the fermentation process, exopolysaccharides known as kefiran are produced and act as viscosity

regulators. Kefiran is a branched water-soluble glucogalactan composed of equal parts glucose and galactose, and its production is mainly due *to Lactobacillus kefiranofaciens* [8,127]. Within the complex community of kefir grains, other species of LAB have been isolated from kefir and found to produce EPS, such as *Lactiplantibacillus plantarum*,

Lacticaseibacillus paracasei, *Lactobacillus helveticus*, *Lactiplantibacillus pentosus*, *Lactococcus lactis* subsp. *Lactis*, and *Leuconostoc mesenteroides* [138-141]. In the last decade, selected strains of mesophilic LAB have been investigated for their ability to ferment milk and produce EPS to improve the rheological properties of fermented milk-based foods [83,138,139,142]. In addition, much attention has also been paid to non-dairy products fermented with kefir grains. Due to the wide variety of sugars and subtracts used in the fermentation process, the microbial diversity of non-dairy kefir grains may be greater than that of traditional kefir grains. Several authors reported 45 different bacterial species and 23 yeasts [143]. However, due to its great complexity, the relative composition of bacteria and yeasts may vary during the kefir fermentation process, making it difficult to control and obtain uniform products for industrial use [143].

5.4. Application of EPS-Producing LAB in Plant-Based Beverages

In recent years, interest in vegetarian and vegan diets has increased for many reasons (e.g., health concerns, ethical concerns, sustainability). The challenge for manufacturers of plant-based milk alternatives is to produce products with acceptable taste and texture for customers [92,144]. The application of EPS-producing LAB has emerged to improve the sensory and organoleptic analysis of such products, as EPS can positively influence texture, mouthfeel, and syneresis [92,145]. Several studies have focused on the use of in situ production of EPS in fermented plant-based beverages, as it is possible to develop a large number of products with improved sensory properties. Li et al. [14] studied the fermentation process of soy milk with an EPS-producing *L. plantarum* strain over a period of 21 days. The fermented soy milk maintained the apparent viscosity and EPS content, exhibited satisfactory technological properties, and improved the taste of soy milk [14]. In another study, Hickisch et al. [144] attempted to produce a vegetable yoghourt alternative from lupins, which proved to be a good plant choice. A different strain of EPSproducing *Lactiplantibacillus plantarum* (TMW 1.460) was used for the fermentation process, which achieved better sensory values,

such as appearance, texture, aroma, and taste [144]. Beverages mimicking the characteristics of cow's milk yoghourt were also developed using an aqueous extract of quinoa flour and *Weissella* spp. as EPS producers and showed high acceptability in terms of acidity, sweetness, texture, and overall appearance [146,147].

5.5. Application of EPS in Bakery

EPS produced by LAB has been proposed as a promising alternative to replace the use of polysaccharides in bakeries as an additive for sourdough because they have thickening properties. EPS-producing LAB can be incorporated into fermented sourdough to have a positive effect on the techno-functional properties of baked goods [148-150]. The in situ production of EPS has been shown to improve the handling and stability of the dough, but also to increase the technological properties of the final product, such as the texture and volume of the bread [150,151]. Moreover, the use of selected EPS-producing LAB strains in sourdoughs has been investigated as a novel technological approach to compensate for quality losses in the functional properties of reduced-sugar products or to replace the added fat in bakery products [149,152]. In addition, some studies have shown that the use of EPS-producing LAB has a cold-protective effect and overcomes the quality loss of frozen dough products [153]. The use of polysaccharides has gained interest in recent years with the increasing production of gluten-free products. Not only are customers looking for healthier products, but people with a chronic autoimmune disease—celiac disease—are also looking to bakeries for better-quality gluten-free products [8,92,154]. The beneficial effect of EPS produced by LAB is to improve the structure and volume of gluten-free or glutencontaining bread, as they are able to bind water and form a high-quality network with other dough components. This later leads to softer bread crumb and longer shelf life [10,155]. The in situ production of EPS was demonstrated in a recent study by Zheng et al. [45], in which the EPS-producing *Limosilactobacillus reuteri* was used during the fermentation process. Its ability to produce fructans and glucans resulted in better-quality gluten-free bread.

5.6. Application of EPS in Meat Industry

While EPS-producing LAB are widely used in the dairy industry, their use in the meat industry is still a relatively new area of research [74]. Meat production dates back many millennia, and the use of lactic acid bacteria has given us an enormous variety of traditional foods around the world. By producing lactic acid or acetic acid in raw meat, we can control food safety, texture, color of meat, and much more [92,94]. Meat products contain many important nutrients (protein, iron, zinc, vitamins, etc.), but consumption of high-fat meat products may correlate with diseases such as diabetes or cardiovascular disease. Customer demand for low-fat meat products challenges the meat industry to develop new products to serve this market. This poses many problems for the meat industry because low-fat processed meat products may be deficient in terms of technological and sensory properties [74,92,94,156]. Hydrocolloids are widely used as food additives in the food industry. In the meat industry, they are known to improve the water-holding capacity and gelling properties of meat proteins and can improve the texture of low-fat meat products, but nowadays customers reject food additives on the ingredient list [157-160]. Therefore, in situ production of EPS by LAB during meat processing could be an interesting alternative. Few studies have been conducted on the in situ production of EPS by LAB in meat products. Meat products evaluated for the use of LAB for in situ production of EPS included cooked ham, raw fermented sausages (sucuk and salami), and spreadable raw fermented sausages [74]. Although the processing conditions for ham production showed a negative effect on EPS production, the use of in situ EPS-forming LAB was more promising for the production of fermented sausages [74,161]. In the study by Hilbig et al. [46], selected LAB strains (*L. plantarum* and *L. curvatus* strains) were able to produce sufficient amounts of EPS during sausage fermentation to allow reduction of the high fat content of fermented raw sausages [46]. Moreover, Dertli et al. [162] used EPS-forming LAB cultures in a Turkish fermented sausage to improve its textural properties, which became harder, less sticky, and tougher.

6. Health-Promoting Effects

Several pieces of evidence have shown that EPS produced by LAB are associated with numerous functional roles and health benefits, such as immunomodulatory, antioxidant,

anticancer, antiulcer, anti-biofilm, and cholesterol-binding effects [5,20,163-169]. However, it has been found that the biological activity of different EPS is influenced by their chemical structure (main chain, branching and molecular weight) [170,171]. Fermented foods can provide LAB-derived EPSs with prebiotic properties that promote colonization of the gut with beneficial bacteria [90,172,173]. One of the beneficial capabilities of probiotic bacteria is the formation of biofilms that support their colonization and maintenance of their population in the harsh conditions of the human gastrointestinal tract (GIT) [174]. Thus, attachment of probiotic LAB to epithelial cells in the GIT has been shown to prevent colonization by pathogenic organisms, stimulate the host immune system, and protect epithelial cells from toxic compounds (e.g., carcinogens and toxic metal ions) [102,175]. In addition, LAB-derived EPS can reduce or inhibit biofilm formation by pathogenic bacteria, thereby preventing infectious diseases [175,176]. Other studies have shown that these EPS have the potential to affect the gastrointestinal tract by protecting intestinal cells from toxins and lowering cholesterol levels through increased secretion of bile acids [165,168,177].

The multiple functional activities of LAB-produced EPSs are also very useful and find application in therapeutic medicine as drug carriers and in adjuvant therapy for the treatment of inflammation and cancer due to their high ability to form hydrogels [92,178-183]. For example, Moscovici [184] attempted to incorporate biomolecules and therapeutics into the internal structure of EPS for use as an intelligent drug delivery system for the treatment of brain tumors or other neurological diseases due to their biocompatibility and apparent lack of toxicity. Most studies on the health benefits of EPS have been conducted in vitro, and there is limited information on in vivo experiments with oral administration [167]. However, as new EPS and EPS-producing LAB have been identified, there are emerging studies that provide positive evidence for the use of EPS in medicine and functional foods (Figure 3).

6.1. Prebiotic Activity

Although a growing number of studies confirm the health-promoting effects of EPS, no mechanism of action has yet been proposed that clearly explains how they can exert such activities on biological tissues [168,185]. However, some authors propose that EPSs remain longer in the gastrointestinal tract to exert their effects as prebiotics. Since EPSs are not degraded by human digestive enzymes, they can exert their prebiotic effect and promote colonization by probiotic bacteria, such as lactobacilli and bifidobacteria [20,169,186]. For

example, Abedfar et al. [187] found that symptoms of lactose intolerance decreased with the presence of EPS in the gastrointestinal tract, which was attributed to the proliferation of lactase-producing lactobacilli. Hamdy et al. [188] evaluated the prebiotic activity of EPS in a rat model over 60 days and found an increase in *Lactobacillus* counts while *Escherichia coli* counts decreased, confirming that the increase in beneficial bacteria was related to the presence of EPS. Recently, Pan et al. [189] reported the modulation of the gut microbiota of mice by feeding a linear dextran produced by a strain of *Leuconostoc pseudomesenteroides*. EPS altered the structure of the gut microbiota and decreased the ratio of *Bacteroidetes* to *Firmicutes* in treated mice.

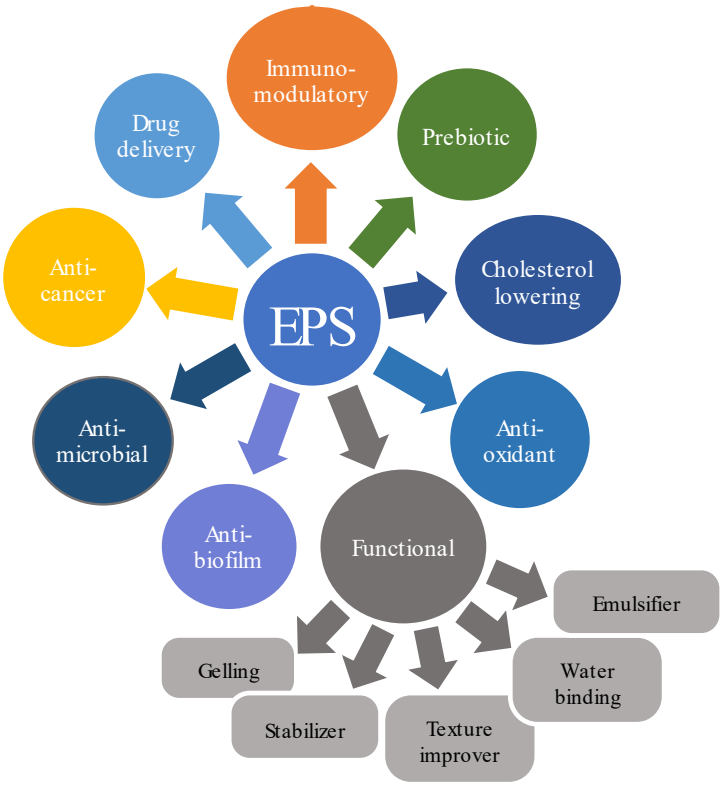

Figure 3. Biological and functional activities exerted by EPS produced by LAB.

6.2. Immunomodulatory Activity

Many food components (called immunomodulators) are known to modulate the innate and adaptive immune system. EPSs, proteins, peptides, glycoproteins, lipopolysaccharides and others are among the many molecules that have immunomodulatory properties. These immunomodulators alter the activity of immune function by regulating cytokines and are able to suppress infections and prevent immunodeficiency-related diseases (e.g., inflammatory bowel disease) of the gastrointestinal tract [168,171,190-192]. Acidic HePS, characterized by

having phosphate (i.e., a negative charge) in their position, seem to be well-able to trigger the immune response [193]. This has been confirmed by studies showing that phosphate is the molecule that triggers the immune response, as chemical dephosphorylation of these HePS leads to a decrease in the stimulatory effect [168,171,190,194,195]. However, immunomodulatory activity has also been associated with high molecular weight HePS, which appears to suppress the immune response [168,171,185,190,196-198]. In addition, the monosaccharide composition of EPS has been shown to influence its anti-inflammatory properties, possibly by association with the recognition of receptors on the surface of immune cells [199,200]. Several studies have shown that EPS produced by LAB stimulates the production of cytokines and antigen-presenting cells [201,202]. Dilna et al. [203] reported the promoting effect of EPS produced by *L. plantarum* on the production of cytokines by macrophages, especially tumor necrosis factor α (TNF-α), interleukin 6 (IL -6), IL -1b and IL -12. In a particular study, (1→6)-α-D-glucan with low Mw from *Lactobacillus confusus* was shown to modulate the immune system to produce the pro-inflammatory mediator nitric oxide (NO) and cytokines [195]. In a rat-induced allergic asthma model using ovalbumin (OVA), Domingos-Lopes et al. [204] demonstrated that the orally administered EPS producer *Leuconostoc citreum* induced immune tolerance to the allergen OVA, specifically by downregulating the overproduction of IgE and reducing the Th2-mediated allergic response. Moreover, in a study using a similar mouse model of respiratory allergy, intranasal administration of EPS from *Bifidobacterium longum* was shown to reduce the recruitment of eosinophils in the lungs [205]. In a similar study, EPS from *L. rhamnosus* was also shown to effectively control T-cell-dependent immune responses in various inflammatory diseases [206].

Other studies also demonstrated the important role of EPS in maintaining the balance of the immune system during infection or inflammation [185]. In the study by Matsuzaki et al. [207], it was shown that the EPS-producing *Leuconostoc mesenteroides* strain could induce the secretion of immunoglobulin A (IgA), indicating a potential use against mucosal pathogens.

6.3. Antioxidant Activity

The accumulation of reactive oxygen species (ROS) leads to oxidative stress in living organisms, which damages biological macromolecules such as DNA, RNA, proteins, and lipids, and can result in tissue damage that can later lead to the development or progression of various diseases (obesity, cancer, neurodegenerative disorders, etc.) [168,171,196,203,208,209].

Exogenous regulation with natural antioxidants (ascorbic acid, α-tocopherol and carotenoids) has been proposed as a way to reduce oxidative stress and delay oxidative damage. Therefore, EPS derived from LAB can be considered as effective natural antioxidants for preventing oxidative stress caused by free radicals. There are numerous studies reporting the antioxidant activity of EPSs produced by LAB, which were evaluated based on their ability to scavenge superoxide anions and hydroxyl radicals [210]. A recent study by Liu et al. [211] reported that sulfonation of EPS produced by *Lactiplantibacillus plantarum* increased antioxidant activity, thereby protecting epithelial cells.

6.4. Cholesterol Lowering Abilities

Major risk factors for cardiovascular disease include high blood pressure and elevated blood cholesterol. There are a growing number of studies showing the ability of EPS from LAB to regulate serum cholesterol levels through intestinal adsorption of this molecule [185]. In this sense, Soh et al. [212] showed the adsorption of cholesterol through an in vitro enzymatic reaction and a polysaccharide precipitation process. Studies in animals and humans have also shown the effect of LAB-derived EPS in lowering blood cholesterol levels. For example, Dilna et al. [203] showed that EPS isolated from *L. plantarum* RJF4 had a cholesterol-lowering effect. In another study, mucilage EPS produced by *L. lactis* subsp. *cremoris* was shown to have a beneficial effect on cholesterol metabolism in rats by lowering serum cholesterol levels [213]. In a similar study, Ai et al. [214] showed that EPS has an antihypertensive effect in an in vivo rat model.

6.5. Anti-Biofilm Formation

Many bacterial species, including pathogenic bacteria, become more resistant to extracellular stress conditions by building biofilms [174]. Biofilms are arrays of bacterial cells bound to a surface in an extracellular polymer matrix composed of EPS, proteins, and extracellular DNA [176]. Biofilms formed by pathogenic bacteria can cause many problems, such as food safety issues or ineffective treatment of infectious diseases [168,215,216]. Several authors reported the potential of LAB -derived EPS to reduce or prevent the formation of biofilms by pathogenic bacteria and thereby control infections caused by the pathogens [176]. For example, an HePS composed of mannose, glucose and galactose was shown to have

considerable anti-biofilm activity against six bacterial pathogens [217]. Several authors have proposed different mechanisms by which EPS can inhibit biofilm formation. Lynch et al. [10] hypothesized that EPS may mask adhesion molecules in the intestinal mucus, thus preventing adhesion of other bacteria. On the other hand, Abdalla et al. [176] proposed that biofilms produced by LAB promote their own colonization in the host mucosa, thus inhibiting the formation of biofilms by bacterial pathogens.

6.6. Antimicrobial Activity

In recent years, antimicrobial resistance has become a very serious health problem, increasing the demand for new antimicrobial drugs. Several authors have shown that LAB - derived EPSs can exhibit antagonistic effects in vitro against Gramme-positive and Gramme-negative pathogens or against pathogenic bacteria in the gastrointestinal tract [47,176,218]. For example, HePS produced by *Lactobacillus gasseri* inhibited in vitro antibacterial activity against several foodborne pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* [76]. The EPS produced by a *Lactobacillus* strain also showed considerable antibacterial activity against the pathogenic bacteria *Salmonella enterica* and *Micrococcus luteus* [219]. In a similar study, HePS isolated from *L. kefiranofaciens* was shown to exert bactericidal activity against *Listeria monocytogenes* and *Salmonella enteritidis* [220]. In another study, silver nanoparticles with EPS produced by LAB were used against pathogenic bacteria. These were found to have antimicrobial activity against Gramme-positive and Gramme-negative bacteria, with Gramme-positive bacteria being more sensitive [221]. The antimicrobial activity of EPSs in vivo can also be explained by the prebiotic effect that helps probiotics to colonise the surface of the gastrointestinal tract, thus enhancing their competitive inhibition of pathogenic bacteria in the host [90]. Recent studies showed remarkable antifungal activity of some EPS produced by *Lactobacillus* strains [176]. Moreover, Álvarez et al. [222] used the strain *L. plantarum* for the production of EPSs included in an edible coating as an antifungal agent. EPSs have also been reported to exert antiviral activities, especially the sulfated EPSs [90]. It is believed that the immunomodulatory effect of EPSs produced by LAB is responsible for the antiviral activity [176,182]. In a study by Nagai et al. [223], EPS produced by *Lactobacillus bulgaricus* in fermented yoghourt was fed to mice infected with influenza virus. They found an increase in natural killer cell activity and an increase in immunoglobulins IgG1 and IgA, which led to a reduction in viral infection.

6.7. Anti-Cancer Activity

Cancer is an abnormal form of tissue growth due to irreversible damage to DNA caused by mutations in certain proto-oncogenes and tumor suppressor genes, resulting in a progressive increase in the number of dividing cells. Highly effective chemotherapies are cytotoxic/immunotoxic, which affects tumor development and impairs patient recovery [168,224]. The discovery and identification of new antitumor drugs with low side effects on the immune system has become an important goal in many immunopharmacology studies. EPS from safe natural sources such as LAB usually have low cytotoxicity and side effects and can serve as good substitutes for synthetic antitumor agents [196,216,224]. Liu et al. [202] demonstrated the antiproliferative effect of *L. casei* EPS on HT -29 cell line.

Al-so, the EPS produced by *L. plantarum* strain showed remarkable antitumor activity on HepG-2, BGC-823 and especially HT -29 cancer cells [216]. EPS produced by *L. casei*, *L. plantarum* and *L. acidophilus* showed antitumor properties against different cell lines depending on the dose [225]. Self-assembled nanoparticles from EPS loaded with anticancer drugs, such as epirubicin, increase the uptake of the drug in mice bearing tumors while decreasing the uptake in heart and kidney. They show a sustained release pattern and a 70% reduction in tumor volume [226]. Another study by Liu et al. [208] showed the antioxidant and antiproliferative effects of EPS produced by LAB on hepatoma cells, HepG2.

6.8. Drug Delivery Systems

EPSs are promising candidates for drug delivery systems due to their bioactive function and ability to transport drugs. Bacterial EPSs not only serve as bioactive agents but can also be potential carriers for valuable drugs, including growth factors and antitumor agents. Although their function as carriers is similar, the production of EPSs as drug carriers is simpler than the production of biological scaffolds loaded with viable cells. As drug carriers, EPS can be modified to facilitate the controlled release of drugs, increase the shelf life of drugs in the body, and improve drug efficacy. Antibiotics are often used as a model for drug release by bacterial EPS [168,227,228]. In a study by Wang et al. [229], it was reported that encapsulation with kefiran was able to protect ciprofloxacin from gastric conditions. In another study, sulfated EPS of bacterial origin were used as vaccine adjuvants for the treatment and prevention of hepatitis B virus in animal models [230].

7. Conclusions

The diversity of EPS produced by LAB has increased and become the focus of many researchers in recent years. The non-toxic behavior of EPSs offers tremendous opportunities for applications in food, medicine, and even pharmaceuticals. LAB -derived EPSs hold remarkable properties, improving stability, flavor, texture, shelf life, and rheological properties of several food products. In addition to technological benefits, recent research shows the health benefits of EPSs, such as immunomodulatory, antioxidant, prebiotic, antibiofilm, cholesterol-lowering, antimicrobial, and anticancer activities. Therefore, the use of LAB cultures for in situ production of EPS meets the consumer demand for health-promoting functional foods. The major limitation for the industrial use of EPS is the low yield and wide variation in the production capacity of EPS-producing strains. Therefore, it is crucial to explore new ideas to optimize the production of EPS for its future industrial use. The type of EPS (HoEPS or HeEPS), molecular weight, monosaccharide composition, and functional groups are also crucial factors affecting technological and biological activities. Most of the research conducted in recent years has focused on the evaluation of EPS structures as well as novel functionalities. However, the mechanisms by which EPSs exert their effects in biofilm formation, immune system modulation, and pathogen inhibition are very complex and still not fully understood. A better understanding of the underlying mechanisms could lead to the development of tailored EPSs with desired/specific properties for use in functional foods or as therapeutic agents.

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Chapter 3: Exopolysaccharide (EPS) Produced by *Leuconostoc mesenteroides* SJC113: Characterization of Functional and Technological Properties and Application in Fat-Free Cheese

1. Introduction

Lactic acid bacteria (LAB) are an extensive group of food-grade bacteria that comprise many genera and are widely distributed in dairy products during the fermentation process [1]. Due to their probiotic and technological properties, they have attracted widespread interest in research [2]. One of these properties is the ability to produce exopolysaccharides (EPS) with different structures and properties. Microbial EPS generally exist in two forms, depending on their location. EPS produced by LAB are often distinguished as ropy or slime EPS [3,4].

EPS can be synthesized extracellularly or excreted into the extracellular environment by the producing microorganisms [5]. They can appear as capsular exopolysaccharides associated with the cell surface or as exopolysaccharides secreted into the environment as free polymers, with a filamentous appearance or in viscous secretions. Although both homopolymeric and heteropolymeric EPS can be produced by LAB, the interesting technological properties belong to homopolymeric EPS [6]. They can be divided into four groups—α-glucans, β-glucans, fructans and polygalactan. The α-glucan group contains dextrans, which consist of linear (1→6)-linked α-D-glucose units that can be branched with (1→2), (1→3) or (1→4)-linked α-D-glucose units. These dextrans have been used in various industrial and commercial applications, for example, to thicken and stabilize fermented dairy products and to improve the production yield and rheological properties of low-fat cheeses [6-9].

According to Park et al., the predominant LAB group of EPS producers are *Leuconostoc* species. In the early 1950s, it was discovered that *Leuconostoc mesenteroides* strains produce high molecular weight dextran when sucrose is added to the culture medium [10]. Indeed, *Leuconostoc* spp. have been shown to produce EPS with different structures and properties that influence the sensory and rheological properties of food [11,12].

In the food industry, the in-situ production of EPS has attracted particular interest due to their functional properties as thickening, stabilizing, emulsifying, texturizing and gelling agents [6]. These EPS improve the texture, stability and sensory properties, such as creaminess, of fermented dairy products, which include yogurt, fermented beverages, cheese and desserts [13]. In addition to the technological benefits, certain EPS exhibit physiological effects that are beneficial to the consumer. These health benefits include the stable growth of bacteria during gastrointestinal stress, microbial immunomodulation and antimicrobial properties against pathogens in the digestive tract [5,14-19].

In recent years, consumers have shown an increasing interest in reducing the fat content of dairy products [20,21]. One of the main characteristics of reduced-fat cheese is a lower moisture content, resulting in a more compact and rubbery texture [22,23]. It is necessary to compensate for the deficiencies caused by fat removal and to prepare low-fat cheese that has characteristics comparable to full-fat cheese to improve consumer acceptance. To compensate for the reduced fat content in the matrix, the moisture content of the cheese can be increased using various techniques, e.g., by adding ingredients that increase the water-holding capacity [24,25]. Several studies have shown that the water-holding capacity of low-fat cheese can be increased by polysaccharide hydrocolloids, whether they are produced in situ by LAB or used as additives from microbial, algal or plant sources [24]. Polysaccharide-based fat replacers have been shown to alter the microstructural properties of low-fat cheese and mimic some of the textural and sensory properties of fat molecules [26]. For example, the addition of beta-glucan from the mushroom *Pleurotus ostreatus* has been shown to improve the texture and organoleptic properties of low-fat white-brined cheese [27]. However, some studies suggest that EPS produced in situ have advantages in increasing moisture retention and thus improving the textural properties of fat-reduced cheese [28-30]. In this context, the addition of an EPS-producing culture to lowfat Kasar cheese resulted in a less compact protein matrix and a sponge-like structure [31]. Similarly, the addition of a ropy EPS producer in the manufacture of a low-fat cheddar cheese resulted in a cheese with the same texture and melting properties as the full-fat type [26]. Moreover, the use of LAB cultures producing EPS in situ during fermentation could be a suitable alternative for products whose addition of polysaccharides requires labeling as food additives, which is not appreciated by consumers [14].

Recently, a strain of *Leuconostoc mesenteroides* (SJC113) was isolated from an artisanal cheese with a protected designation of origin (São Jorge cheese from the Azores, Portugal) and was found to produce large amounts of EPS from sucrose. In the present study, the EPS produced by this strain was characterized, and the functional and technological properties of this strain were evaluated. In addition, the application of in-situ-produced EPS as a fat substitute in fresh cheese was investigated.

2. Materials and Methods

2.1. Materials

The following materials were used: MRS broth and agar (Biokar Diagnostics, Allonne, France), skim milk (VWR Chemicals, Leuven, Belgium), PCA (Biokar Diagnostics, France), sucrose (Sigma, Steinheim, Germany), glucose (Sigma, Saint-Quentin-Fallavier, France), lactose (Scharlau, Barcelona, Spain), fructose (Sigma, Burlington, MA, USA), TCA (Pan-Reac AppliChem ITW reagents, Darmstadt, Germany), DNAse type I (Amresco, Solon, OH, USA), pronase E (Amresco, USA), *Chaetomium erraticum* (Sigma, Søborg, Denmark), pancreatin (Sigma), DPPH (Sigma, Germany), brilliant green (Sigma, India), ascorbic acid (Riedel-de Haën, Seelze, Germany), BHT (Sigma, Spain), cholesterol (Sigma-Aldrich, USA), NaCl (Merck, Darmstadt, Germany), methanol (Fisher Chemical, Loughborough, UK), PBS (Sigma, USA), Fe_2SO_4 (Riedel-de Haën, Berlin, Germany), H_2O_2 (Panreac Química SAU, Barcelona, Spain), dialysis membrane (10 kDa, Spectrum Laboratories Inc., Piscataway, NJ, USA), $CaCl_2$ (Merck, Germany, Darmstadt) and rennet (Lusocoalho, Portugal).

2.2. Bacteria

The strain *Ln. mesenteroides* SJC113 was isolated from the curd during cheese-making of São Jorge cheese (The Azores, Portugal). The 16S rDNA sequences of the strain were deposited in the GenBank under accession number MT742947. Stock cultures were kept at −80 °C in 30% (*v/v*) glycerol and propagated twice in MRS broth with 1% (*v/v*) of inoculum at 30 °C for 24 h.

2.3. Production and Purification of EPS

The bacterial culture (1%) was inoculated into 50 mL MRS broth and incubated overnight at 30 °C. The culture was then transferred to 1 L of modified MRS broth, in which the glucose was replaced with 10% (*w/v*) sucrose and incubated at 30 °C for 48 h. After fermentation, the EPS was extracted according to Domingos-Lopes et al. [32] with some modifications. Briefly, the medium was centrifuged at 9000× *g* for 5 min at 4 C to remove the

bacterial cells. The EPS was precipitated by adding 2 volumes of chilled ethanol at 4 ˚C for 48 h. After centrifugation at 10,000× *g* for 20 min at 4 ˚C, the precipitate was dissolved in Milli-Q water and dialyzed against Milli-Q water at 4 ˚C for 3 days. The water was changed twice daily, the crude EPS was obtained by freeze-drying (Scanvac Coolsafe 55-4 Pro, Labogene, Allerød, Denmark) and the dialysate sample was stored at −20 ˚C for further purification. The extraction procedure was repeated four times to determine the EPS yield, which was quantified using the phenol-sulfuric acid method [33]. In the second purification (to analyze EPS composition and linkage), the crude EPS (5 mg/mL) was dissolved in 50 mM Tris–HCl, 10 mM $MgSO_4.7H_2O$ (pH 7.5) and was incubated with DNAse type-I (2.5 µg/mL) for 6 h at 37 ˚C and Pronase E (50 µg/mL in 50 mM Tris–HCl, 2% EDTA, pH 7.5) for 18h at 37 ˚C, as described by Domingos-Lopes et al. [32].

2.4. Characterization of EPS

2.4.1. Monosaccharide Composition and Linkage Analysis

The neutral sugar composition and linkage analysis were determined by gas chromatography (GC) and alditol acetates, as described by Nunes et al. [34]. Briefly, EPS hydrolysis was performed with 2M trifluoroacetic acid (TFA) for 1 h at 120 ˚C. The monosaccharides were reduced by $NaBH_4$ and were acetylated with acetic anhydride and 1-methylimidazole. To separate the alditol acetate derivatives, dichloromethane was used, and after the separation, they were analyzed by GC with an FID detector. Determination of uronic acids was performed using the colorimetric method described by Blumenkrantz and Asboe-Hansen [35] with pre-hydrolysis of samples, as described by Coimbra et al. [36]. The linkage analysis of the monosaccharides was performed by methylation, as reported by Ciucanu and Kerek [37] with modifications [36]. The partially methylated alditol acetates were separated and analyzed by gas chromatography quadrupole mass spectrometry (GCqMS) (GC-2010 Plus, Shimadzu, Kyoto, Japan) using a non-polar column HT5 (30 m length, 0.25 mm internal diameter and 0.10 µm stationary phase, Trajan, Melbourne, Australia), as described by Concórdio-Reis et al. [38].

2.4.2. Dextranase Resistance

The susceptibility of the EPS to dextranase was performed using 1,6-α-D-glucan 6-glucanohydrolase from *Chaetomium erraticum* (D0443, Sigma), as described by DomingosLopes et al. [32].

2.4.3. Protein Content

Protein content was determined using an elemental analyzer Truspec (630–200-200) with a thermal conductivity detector, as described by Bastos et al. [39]. The conversion from % N to protein content was performed considering the Kjeldahl factor of 6.25.

2.4.4. EPS Production in Milk Whey and Skim Milk

The production of EPS was evaluated in milk whey and skim milk with different concentrations of sucrose (5%, 10% and 20%). The bacterial culture (1%) was inoculated into 50 mL of MRS broth and incubated overnight at 30 °C. The medium was centrifuged at 3500× *g* for 30 min at 4 °C, and the pellet was dissolved in either milk whey or skim milk containing 5%, 10% or 20% sucrose and incubated at 30 °C for 48 h. After fermentation, the EPS was extracted as follows: 25 mL of the fermented sample was diluted with MiliQ water (1:1). The casein fraction was precipitated by adding 4 mL of 0.2 M TCA and centrifuging at 3500× *g* for 30 min at 4 °C. The supernatant was collected, and the pH value was adjusted to 6.8 with 4 M NaOH. Then, the solution was boiled for 30 min and centrifuged at 3500× *g* for 30 min at 4 °C to remove the whey proteins. The volume of the supernatant was measured, and an equal volume of cold ethanol was added to further precipitate the carbohydrates overnight at 4 °C. The sample was centrifuged at 3500× *g* for 30 min at 4 °C. The pellet was resuspended in 10 mL MiliQ water and sonicated for 1 h at room temperature. The sample was vortexed to a uniform solution and dialyzed against MiliQ water for 7 days at 4 °C, changing the water twice daily. The dialysate sample was then stored at −20 °C. The EPS concentration in the suspension was quantified using the phenol-sulfuric acid method [33].

2.5. Functional and Technological Properties of Ln. mesenteroides SJC113

2.5.1. β-Galactosidase Activity

The enzymatic activity of the strain SJC113 was performed according to Miller [40] with some modifications [41]. The strain was grown in MRS broth overnight at 30 °C, and the OD was measured at 600 nm. Freshly prepared (80 µL) permeabilization solution (100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/mL hexadecyltrimethylammonium bromide—CTAB, 0.4 mg/mL sodium deoxycholate and 5.4 µL/mL β-mercaptoethanol added immediately before use) was added to 20 µL of bacterial culture and 600 µL of substrate solution (1 mg/mL *o*-nitrophenyl-β-D-galactoside—ONPG in phosphate buffer pH 7.0, and 2.7 µL/mL β-mercaptoethanol added just before use). The microtubes were incubated at 30 °C for approximately 30 min or until sufficient color developed. The reaction was stopped by adding 700 µL of stop solution (1 M Na_2CO_3) and centrifuged at 16,100× *g* (model 5415D; Eppendorf AG, Eppendorf, Hamburg, Germany) for 5 min. The tubes were carefully removed from the centrifuge, and the absorbance of the top layer was measured at 420 nm. β-galactosidase activity was calculated as follows:

$$\beta - \text{galactosidase activity (Miller units)} = 1000 \times \frac{\text{Abs420}}{(\text{Abs600} \times 0.2 \times \text{reaction time})}$$

2.5.2. Cholesterol-Reducing Ability

The in vitro cholesterol-lowering ability of the strain SJC113 was evaluated, as described by Domingos-Lopes [42]. MRS broth was supplemented with 0.5 mg/mL (*w*/*v*) of cholesterol, inoculated with log phase grown bacterial culture and incubated at 30 °C for 24 h. The percentage of cholesterol reduction was calculated by measuring the cholesterol at the end of the incubation (cholesterol final) using the enzymatic colorimetric method (CHOD-PAP cholesterol kit; NS Biotec, Cairo, Egypt) and calculated as follows:

$$\text{Cholesterol removal (\%)} = \frac{\text{Absorbance control} - \text{Absorbance spent broth}}{\text{Absorbance control}} x100$$

2.5.3. DPPH Scavenging Activity

The free radical scavenging activity or 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was performed according to Aarti and Khusro [2], with some modifications. Overnight grown cells were freshly centrifuged and resuspended in dipotassium hydrogen phosphate buffer. The cells were added to a 1 mL DPPH solution (0.2 mM), and the mixture was incubated for 30 min at room temperature in the dark. During the assay, cells at the same concentration with methanol were used as blank, and the DPPH solution was used as control. Ascorbic acid was used as reference. All mixtures were read at 517 nm, and DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging capacity (\%)} = \left[(A_{sample} - A_{blank})/A_{control}\right] \text{x } 100$$

2.5.4. Hydroxyl Radical Scavenging Assay

The methodology previously described by He et al. [43] was used, with some modifications to estimate the hydroxyl scavenging activity of the strain. The reaction mixture consisted of brilliant green (0.5 mM; 1 mL), $FeSO_4$ (0.5 mM; 2 mL) and H_2O_2 (1.5 mL). The bacterial cells were previously centrifuged and inoculated into the reaction mixture. This was incubated for 15 min at room temperature, and the absorbance was read at 624 nm. Butylated hydroxytoluene was used as a standard.

$$\text{Hydroxyl radical scavenging activity (\%)} = \left[(A_1 - A_0)/(A - A_0)\right] \text{x } 100$$

A_1 is the absorbance of a solution with the sample present, A_0 is the absorbance of a solution where the sample was not present and A is the absorbance without the sample and the Fenton reaction system.

2.5.5. Tolerance to Sodium Chloride (NaCl)

Overnight culture of LAB was inoculated into an MRS broth supplemented with either 2%, 5% or 10% (*w*/*v*) NaCl, as described by Aarti and Khusro [2]. Incubation was done at 30 °C for 48 h. After incubation, the survival potential was estimated by a plate counting method and expressed in log CFU/mL.

2.6. In Vitro Safety Evaluation of Ln. mesenteroides SJC113

2.6.1. Antibiotic Susceptibility

Antibiotic susceptibility was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute [44]. Antibiotic discs (Oxoid, Basingstoke, UK) were used to determine the susceptibility of the strains to 9 antibiotics: penicillins—ampicillin (2 µg per disc) and oxacillin (1 µg per disc); aminoglycosides—kanamycin (30 µg per disc), gentamycin (30 µg per disc) and streptomycin (300 µg per disc); glycopeptidesvancomycin (30 µg per disc); tetracyclines—tetracycline (30 µg per disc); macrolides—erythromycin (15 µg per disc) and amphenicols—chloramphenicol (30 µg per disc). The analyses were performed in duplicate. The discs were placed on the surface of inoculated Mueller-Hinton (AES, Liévin, France) agar plates colonized with LAB strains previously grown in MRS broth for 24–48 h at 30 °C. After the 24 h incubation at 30 °C, the diameter of the inhibition zones around the discs was measured using a digital caliper (Absolute Digimatic Caliper; Mitutoyo, Neuss, Germany). The strain was categorized as sensitive (S) or resistant (R) based on the diameter of the inhibition zones. Antibiotic susceptibility and resistance were interpreted as no growth and growth, respectively, as there are no CLSI guidelines for interpretation criteria for this genus.

2.6.2. Virulence Genes

The strain was tested for the presence of *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (endocarditis antigen), *ace* (adhesion of collagen), *vanA* and *vanB* (vancomycin resistance), *hdc1* and *hdc2* (histidine decarboxylase), *tdc* (tyrosine decarboxylase) and *odc* (ornithine decarboxylase) according to Ribeiro et al. [45]. The amplified fragments were separated by electrophoresis with 0.8–2% (*w*/*v*) agarose gel in 0.5 TAE buffer, and the gels were stained with SybrGreen (15 µg/mL).

2.7. Application of Ln. mesenteroides SJC113 in Fat-Free Fresh Cheese

2.7.1. Effect of Temperature on EPS Production in Skim Milk

The LAB were previously cultured in MRS broth for 48 h. They were then added to skim milk (10% *w*/*v*) and fermented overnight (approx. 16 h). Subsequently, this mixture was

added to freshly prepared skim milk containing 0% sucrose, 5% sucrose or 10% sucrose to study the acidification and EPS production of *Ln. mesenteroides* at refrigeration temperatures (4 ˚C and 8 ˚C). The total number of colony-forming units (CFU/mL) was evaluated by plating appropriate dilutions on De Man–Rogosa–Sharpe agar (MRS), as described in Section 2.7.8. Viscosity (V-72 measuring system, speed: 12 rpm, time: 90 s) was determined using the RM100 Plus viscometer (Lamy Rheology Instruments, Lyon, France). These measurements were carried out during fermentation (0–120 h) in skim milk at 4 ˚C and 8 ˚C.

2.7.2. Cheese Making

Before starting cheese production, 1% of an overnight culture of *Ln. mesenteroides* was inoculated into skim milk media and left at 30 ˚C for 16 h. This inoculum was then used to ferment skim milk with 0% or 5% sucrose at 8 ˚C for 5 days (to promote EPS production). This fermented milk was later incorporated into skim milk (1:4) to produce fresh cheese. Fresh cow's milk from the Azores University farm (Chegalvorada, Angra do Heroísmo, Portugal) was used for the production of fresh cheese according to Coelho et al. [46]. Whole milk (4.64% fat, 3.71% protein) was used for the production of full-fat cheese. For the production of fat-free cheese (no-fat cheese), the milk was skimmed. Four different types of fresh cheese were produced: full-fat cheese (FF), no-fat cheese (NF) made with skim milk, no-fat cheese with *Ln. mesenteroides* fermented with skim milk (NFLn0) and no-fat cheese with *Ln. mesenteroides* fermented in skim milk with 5% sucrose (NFLn5). The cheeses were stored at 4 ˚C until they were analyzed on day 1 and day 7.

2.7.3. Cheese Composition

The moisture content was determined using the oven-drying method [47]. The ash content was determined according to the AOAC method 945.46 [48]. Fat content was analyzed using the Van Gulik method [49], and total protein was quantified using the Kjeldahl method [50]. The pH values were measured directly with a potentiometer (HannaFoodcare HI99161, Hanna Instruments, Amorim, Portugal) on the 1st and 7th days of storage by inserting the electrode into the cheese.

2.7.4. Weight Loss

To determine the weight loss, the weight of each cheese was measured during the storage period using a precision scale (AE200, Metler Toledo, Columbus, OH, USA) according to the following calculation:

$$\text{Weight loss (\%)} = \frac{\text{Wi} - \text{Wf}}{\text{Wi}} \text{x} 100$$

where W_i is the initial weight (day 0) and W_f is the weight measured at different times during storage (day 1 and day 7).

2.7.5. Water-Holding Capacity

The water retention capacity of fresh cheese was measured according to Linares et al. [51] using the following calculation:

$$\text{WHC (\%)} = 100 \text{ x } (\text{CW} - \text{DW weight})/\text{CW}$$

where C_W is the weight of the cheese and D_W is the weight of the decanted whey.

2.7.6. Rheological Parameters

The viscosity was measured with a Viscometer RM100 Plus (LAMY Rheology Instruments). The device was equipped with an RV-7 spindle (dimensions—Ø3.20; reference—111007). The viscosity was measured for 60 s at a speed of 30 rpm. Texture properties of fresh cheese samples were measured in a texture analyzer TMS-PRO (Food Technology Corporation, Sterling, VA, USA) equipped with a cylindrical probe (35 mm diameter) with a flat surface, a penetration depth of 15 mm and a probe speed of 1 mm/s. All analyses were performed in four independent experiments.

2.7.7. Determination of EPS Content in Fresh Cheese

The EPS of fresh cheese (25 g cheese sample) were extracted according to the method described by Kearney et al. [52]. The EPS content of the extracted suspension was estimated

using the phenol-sulfuric acid method and expressed as glucose equivalent. All analyses were performed in triplicate.

2.7.8. Microbial Analysis

Microbial growth was assessed on the 1st and 7th day of storage at 4 °C. Microbial enumeration was performed by plating appropriate dilutions on De Man–Rogosa–Sharpe agar (MRS), a selective medium for enumeration of LAB (including *Leuconostoc* sp.), and plate count agar (PCA) a non-selective medium for enumeration of total aerobic microorganisms. Plates were incubated for 48–72 h at 30 and 37 °C for MRS and PCA, respectively. Results were expressed as log CFU/g. All analyses were performed in triplicate.

2.8. Statistical Analysis

The effect of sucrose on EPS production in whey and skim milk was tested using a factorial analysis of variance (ANOVA) with the treatments (milk whey and skim milk) and sucrose concentration (5%, 10% and 20%) as factors. The factorial ANOVA design was also used to investigate the effects of fermentation of skim milk with 0%, 5% and 10% sucrose at different temperatures (4 °C and 8 °C) by *Ln. mesenteroides* SJC113. Post-hoc multiple comparisons were performed using the Tukey test. An analysis of variance (ANOVA) was performed to evaluate the differences between the cheese formulations for the different parameters evaluated. For each value, the means ± standard error of the means (SEM) were reported. In cases where ANOVA revealed statistically significant differences ($p < 0.05$), Tukey's test for multiple comparisons was applied. All statistical analyses were performed using the SPSS program (version 28; IBM SPSS Statistics, Armonk, NY, USA).

3. Results and Discussion

3.1. EPS Yield and Characterization

The estimated EPS production of *Ln. mesenteroides* SJC113 was 7.4 ± 0.9 g/L when cultured (1%) in MRS broth containing 10% (*w*/*v*) sucrose for 48 h at 30 °C. Several studies reported similar yields when using different *Leuconostoc* strains [32,53,54], although the yield

of EPS may vary with the fermentation conditions, purification and quantification methods [55,56]. The carbohydrate composition and glycosidic linkages of EPS are presented in Table 1. The *Ln. mesenteroides* SJC113 produced a mucoid EPS that can be classified as glucan since glucose was the main monosaccharide detected (92 mol%). Moreover, taking into account the high percentage (84.5%) of (1→6) α-glycosidic linkages, the EPS is mainly a dextran with a low percentage of α-3,6 branched glycosidic linkages (Table 1). This result was further confirmed by the dextranase resistance since glucans presenting 85% of α-1,6 glycosidic linkages were shown to produce approx. 50% of resistance to dextranase [57].

Table 1. Total carbohydrate and protein concentration, dextranase resistance (%), monosaccharide composition (mole percentage) and relative abundances (mole percentage) of glycosidic linkages of purified EPS produced by *Ln. mesenteroides* SJC113.

Carbohydrate (mg/g)	352.2 ± 47.9
Protein (mg/g)	415.2 ± 10.4
Dextranase resistance (%)	52.1 ± 2.3
Monosaccharide	Mol%
Mannose (Man)	2
Glucose (Glc)	92
Uronic acids (UA)	5
Glycosidic linkage	Mol%
t-Man*p*	1.3
2-Man*p*	0.3
2,6-Man*p*	0.7
3,6-Man*p*	0.7
Total Man	3
t-Glc*p*	6.3
4-Glc*p*	0.7
6-Glc*p*	84.5
3,6-Glc*p*	5.6
Total Glc	97

EPS may be characterized by its susceptibility to dextranase. Dextranase resistance was shown to range from 4.3 to 37.4% for classical dextrans (>92% of α-1,6 glycosidic linkages), from 37.9 to 82% for dextrans containing α-1,2 linkages and from 64.0 to 97.8% for glucans with high percentage of α-1.3 linkages [57]. A novel water-soluble polysaccharide extracted from *Leuconostoc citreum* strains showed dextranase resistance ranging from 9.32 ± 0.23 to 88.96 ± 1.06 [58]. Also, higher dextranase resistance (82–95% resistance) expressed by *Ln. mesenteroides* strains were also reported [59]. Several *Leuconostoc* spp. were described to

produce dextran from sucrose with high levels of (1→6)-linked α-D-glucopyranosyl units and low levels of (1→3)-linked α-Dglucopyranosyl units [9,57]. According to the study of London et al. [56], the percentages of α-1.6 linkages in EPS produced by *Leuconostoc* vary from 50% to 97.9%.

3.2. EPS Production in Milk Whey and Skim Milk

Ln. mesenteroides was cultured in two different types of media—skim milk and sweet whey (derived from the manufacture of fresh cheese), as shown in Figure 1, with three different concentrations of sucrose (5, 10 and 20%). The production of EPS in sweet whey with 5% sucrose was twice as high as the production of EPS in skim milk with 5% sucrose, namely 0.96 ± 0.11 g/L and 0.44 ± 0.14 g/L, respectively. However, this trend changed when 10% and 20% sucrose were added to skim milk and sweet whey, respectively. EPS production was 1.34 ± 0.19 g/L in sweet whey supplemented with 10% sucrose and 3.36 ± 0.15 g/L in sweet whey supplemented with 20% sucrose. In contrast, EPS production in skim milk supplemented with 10% sucrose doubled to 3.13 ± 0.04 g/L, and in skim milk supplemented with 20% sucrose, the yield was 6.81 ± 0.88 g/L. According to other authors, the highest EPS yields were produced by *L. rhamnosus* RW-9595 M, which was able to produce 2.7 g/L EPS in whey medium. However, the authors assume that due to the high variability in the chemical composition of whey, EPS could be produced with inconsistent biological and technological properties [60,61].

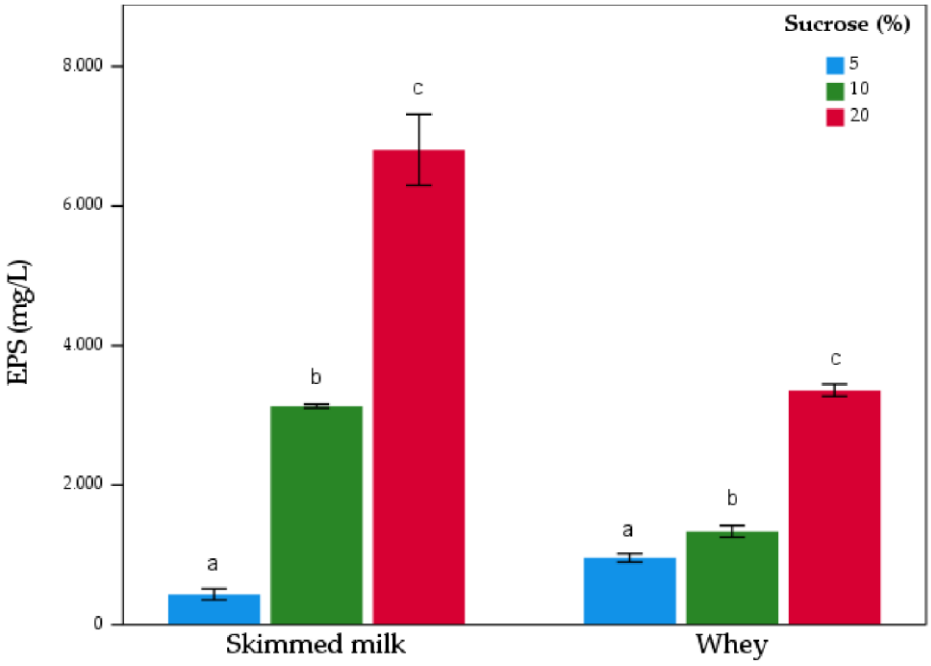

Figure 1. EPS production (mg/L) of *Ln. mesenteroides* SJC113 in skim milk and sweet whey with 5%, 10% and 20% sucrose. Different lowercase letters indicate a significant difference ($p < 0.05$) between sucrose concentrations.

3.3. Functional and Technological Properties of Ln. mesenteroides SJC113

Strain SJC113 showed a high tolerance to NaCl, as it was able to grow in the range of 2% to 10% NaCl. The maximum growth (7.35 ± 0.08 log CFU/mL) was attained at 2% NaCl was), but the strain also showed high growth (6.33 ± 0.04 log CFU/mL) at 10% NaCl. Similar high tolerances to NaCl of LAB were reported by several studies [62-64]. Tolerance to high osmotic concentrations of NaCl could be an important requirement of bacteria to be used in food as commercial strains [62,64]. *Ln. mesenteroides* SJC113 presented high β-galactosidase activity (2368.4 ± 24.4 Miller units, Table 2). The β-galactosidases belong to a group of enzymes involved in the breakdown of lactose into glucose and galactose, and their activity in dairy products has beneficial effects in lactose-intolerant individuals [65,66]. In addition, the β-galactosidase activity expressed by LAB presents technological applications such as improving the technological and sensorial characteristics of dairy foods by overcoming the low solubility of lactose and providing greater sweetening power [67]. In recent years, several studies have been conducted to determine the production of β-galactosidase by LAB, reporting a wide range of activities. While some authors reported similar high values of β-galactosidase activity [68], others reported much lower β-galactosidase activities [69,70]. For example, Shukla et al. [70] reported reduced β-galactosidase activity (94.24 Miller units) for a *Ln. mesenteroides* strain. *Ln. mesenteroides* SJC113 showed an ability to reduce cholesterol in vitro by approx. 15% (Table 2). Although there is not a high reduction in cholesterol, as different LAB strains were shown in other studies [42,71], this is a useful trait that can be used in the reduction of cholesterol in foods [72]. The antioxidant activity of LAB can be an important characteristic for the preservation of food. Low antioxidant activity was shown by the *Ln. mesenteroides* SJC113 using both a radical scavenging activity and hydroxyl scavenging activity (Table 2). The strain inhibited 11.7 ± 0.7% of DPPH radical and presented 15.7 ± 0.4% of hydroxyl scavenging activity, corresponding to 1.08 ± 0.04 mg/m BHT equivalents. These results are consistent with other authors that reported antioxidant activities of intact bacterial cells ranging from 4.5% to 19.7% and 8.8% to 30.6% of DPPH and hydroxyl radical scavenging activity, respectively [73]. In similar studies, LAB strains isolated from traditional artisanal milk cheese showed DPPH scavenging activity ranging from 2.79% to 39.3% [74]. However, higher DPPH and hydroxyl scavenging activities were reported by other authors (32.9–63.8%

and 46–51.5% for DPPH and hydroxyl scavenging activities, respectively), presumably due to differences in protocol assays [75].

Table 2. β-galactosidase activity, cholesterol-reducing ability (%), radical scavenging activity—DPPH (%) and hydroxyl scavenging activity (% and BHT equivalent) of *Ln. mesenteroides*. Results are presented as mean ± SEM.

β-galactosidase activity (Miller units)	2368.4 ± 24.4
Cholesterol-reducing ability (%)	14.8 ± 4.1
Radical scavenging activity—DPPH (%)	11.7 ± 0.7
Hydroxyl scavenging activity (%)	15.7 ± 0.4
Hydroxyl scavenging activity (mg/mL BHT Eq.)	1.08 ± 0.04

3.4. Antibiotic Susceptibility and Virulence Genes

The safe use of *Ln. mesenteroides* SJC113 was tested by screening for antibiotic resistance to clinically important antibiotics and the presence of virulence genes (Table 3). Remarkably, the strain was sensitive to all antibiotics tested. These results were based on the formation of inhibition zones (>8 mm), as there are no CLSI guidelines for interpretation criteria for *Leuconostoc* sp. However, since genes involved in antibiotic resistance might be present but not expressed, these negative phenotypic results should be confirmed by a molecular biology approach. Regarding the presence of virulence factors, the strain did not harbor any of the tested virulence genes, including the resistance genes for vancomycin *vanA* and *vanB*. The presence of some virulence genes, including the genes for resistance to vancomycin (*vanA*), was observed in several studies using LAB as probiotics [76. In contrast, other authors reported that LAB used as probiotics were negative for virulence genes[77].

Table 3. Resistance/susceptibility to antibiotics and occurrence of virulence genes in *Ln. mesenteroides* SJC113.

Antibiotics [1]		**Virulence Genes** [2]			
Ampicillin	S	*gelE*	-	*vanA*	-
Oxacillin	S	*hyl*	-	*vanB*	-
Kanamycin	S	*asa1*	-	*hdc1*	-
Gentamicin	S	*esp*	-	*hdc2*	-
Streptomycin	S	*cylA*	-	*tdc*	-
Vancomycin	S	*efaA*	-	*odc*	-
Tetracycline	S	*ace*	-		
Erythromycin	S				
Chloramphenicol	S				

[1] S—sensitive (inhibition-zone >8 mm). [2]—absence of virulence gene.

3.5. EPS Production in Skim Milk

To determine the best conditions under which *Ln. mesenteroides* SJC113 can produce a highly viscous EPS, fermentation was tested in skim milk at 4 °C and 8 °C at different concentrations of sucrose (0%, 5% and 10%) for up to 5 days. The results for bacterial viability, pH values and apparent viscosity are shown in Figure 2. Colony counts of *Ln. mesenteroides* SJC113 increased from ~6 log (CFU/mL) to ~10 log (CFU/mL) after 48 h at 8 °C and remained stable for up to 5 days. As expected, growth was lower at 4 °C and reached ~9.5 log (CFU/mL) after 4 days. Bacterial growth was similar for milk with 5% or 10% of sucrose and slight lower for skim milk with no sugar added. As for the change in pH values, a slight decrease was observed during fermentation, with a maximum of 0.28 units for the highest level of sucrose (10%), indicating that this strain is a very low acidifier. This is important for this type of cheese, as it should have a low acidity since no acidification step is performed during cheese production [78].

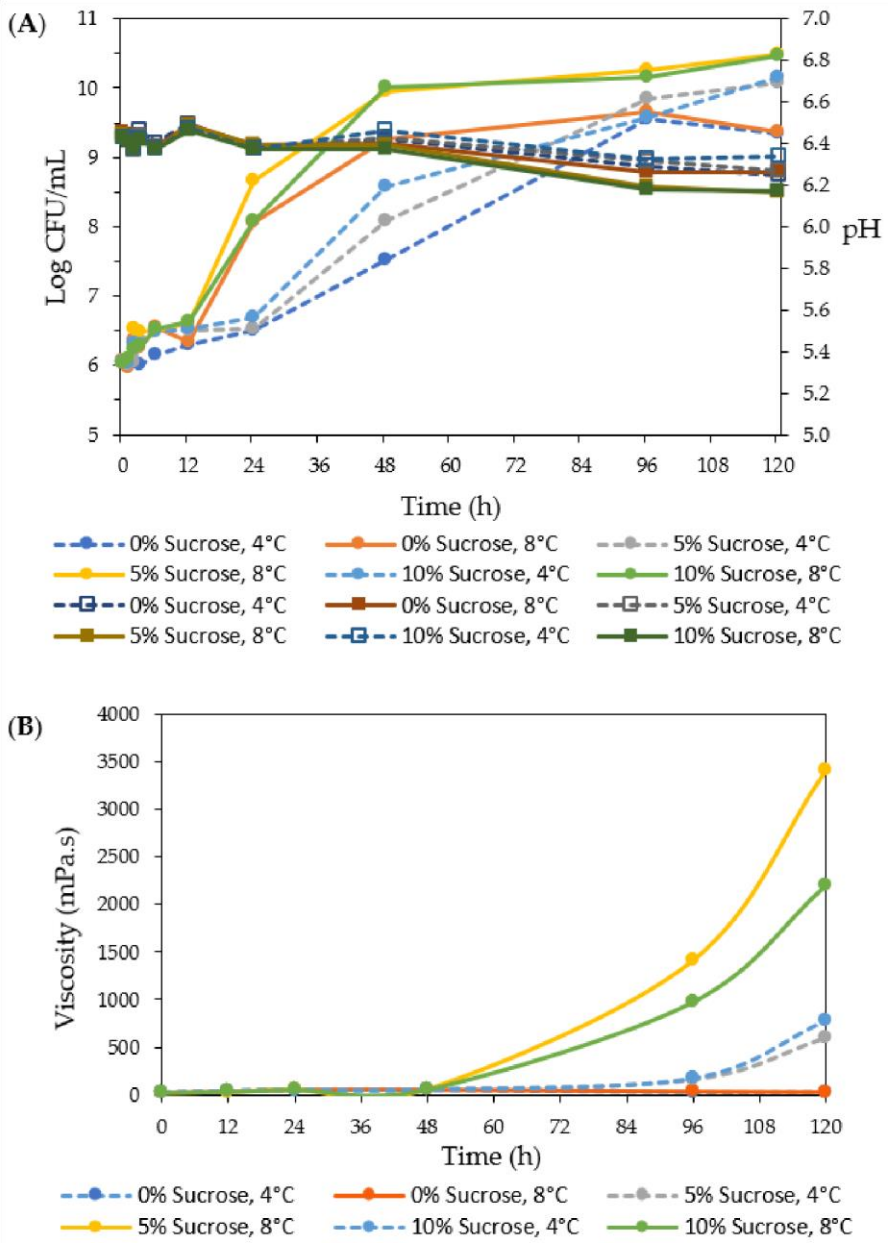

Figure 2. Fermentation of skim milk with 0%, 5% and 10% sucrose by *Ln. mesenteroides* SJC113 at 4 °C and 8 °C. (**A**) Bacterial viability (circles) and pH values (squares) in the skim milk during fermentation time (5 days) at 4 °C (dashed line) and 8 °C (solid line). (**B**) Apparent viscosity (mPa.s) of fermented skim milk at 4 °C (dashed line) and 8 °C (solid line).

The highest viscosity (3405 ± 212 mPa.s) was achieved after a five-day fermentation of skim milk with 5% and 10% sucrose at 8 °C (Figure 2). These results indicate that this strain is capable of producing large amounts of EPS at refrigerated temperatures (especially at 8 °C), as shown by the increase in milk viscosity. The incubation of skim milk at these chilling temperatures is important to limit the growth of undesirable bacteria that survive the

pasteurization process, such as psychrotrophic Gram-positive spore-forming bacteria [79]. Some authors reported that the maximum production of dextran occurs in a logarithmic phase since dextran is a biopolymer associated with bacterial growth [80]. However, in the present study, a delay between bacterial growth and EPS production, which starts after the logarithmic phase, was observed at refrigerated temperatures (Figure 2A,B). It is also noteworthy that EPS production was highest when the strain was fermenting skim milk with 5% sucrose at 8 ◦C. This result could be due to the decreasing pH values (higher at this sucrose concentration), which may influence EPS production. Indeed, several authors reported that pH = 7 is the best pH value for the production of glucan-type EPS by Ln. mesenteroides and other LAB [55,81]. Therefore, the highest pH drop observed during the fermentation of skim milk with 10% sucrose at 8 ◦C could cause slower EPS production under these conditions.

3.6. Application of Ln. mesenteroides SJC113 in Fat-Free Fresh Cheese

Considering the higher EPS production after the fermentation of skim milk with 5% sucrose at 8 ◦C after 5 days, these conditions were used in the next experiments to produce a fat-free fresh cheese. The aim was to replace the fat with the highly viscous EPS produced after fermentation with *Ln. mesenteroides* SJC113 to obtain the same physicochemical properties as a full-fat fresh cheese. The physicochemical analysis of the different types of cheese (full-fat cheese and fat-free cheese) was carried out on day 7 of storage at 4 ◦C and is shown in Table 4. The gross composition of the standard full-fat cheese is similar to the values determined by other authors [78,82]. As expected, the cheeses made with skim milk (NF, NFLn0 and NFLn5) had a higher ($p < 0.05$) moisture content than the full-fat cheeses (FF). The lack of fat in the fat-free cheeses is compensated for by water, protein and carbohydrates (lactose) in the experimental control cheeses (NF and NFLn0). However, the cheese made with the 5% sucrose skim milk fermented by *Ln. mesenteroides* had a lower protein content (6.4%) compared to the fat-free control cheeses ($p < 0.05$). The application of fermented milk with 5% sucrose on cheese-making increased the carbohydrate content as expected, although some of these carbohydrates were presumably EPS. When compared to standard full-fat cheese, this experimental cheese has a protein content that is not significantly different ($p > 0.05$) and a slightly lower ash content ($p < 0.05$), as the fat was replaced by water and carbohydrates (partly as EPS).

Table 4. Composition of fresh cheeses: full-fat cheese (FF), no-fat cheese (NF), no-fat cheese with 0% sucrose skim milk fermented by *Ln. mesenteroides* SJC113 (NFLn0) and no-fat cheese with 5% sucrose skim milk fermented by *Ln. mesenteroides* (NFLn5). Values are means ± SEM of three replicates.

Parameters	FF	NF	NFLn0	NFLn5
Moisture (%)	78.3 ± 1.3 [a]	85.8 ± 0.2 [b]	85.2 ± 0.6 [b]	87.0 ± 0.4 [b]
Crude fat (%)	11.4 ± 0.2	ND [1]	ND [1]	ND [1]
Crude protein (%)	7.5 ± 0.8 [ab]	9.7 ± 0.4 [bc]	10.3 ± 0.6 [c]	6.4 ± 0.1 [a]
Crude ash (%)	1.98 ± 0.06 [b]	2.23 ± 0.03 [b]	2.26 ± 0.10 [b]	1.43 ± 0.08 [a]
Carbohydrates (%)	0.86 ± 0.44 [a]	2.24 ± 0.29 [b]	2.25 ± 0.14 [b]	5.18 ± 0.26 [c]

Different letters as superscripts in the same line indicate a significant difference ($p < 0.05$) between cheese treatments. [1]—Not detected.

The viability of *Ln. mesenteroides* SJC113 in fresh cheese and the in-situ production of EPS were evaluated on day 1 and day 7 of storage at 4 °C and are shown in Table 5. The number of viable cells was similar in MRS and PCA, indicating that only *Ln. mesenteroides* was present in the cheeses until day 7. The initial colony count of *Ln. mesenteroides* was slightly higher in cheese made from fermented skim milk with 5% sucrose and remained stable in both treatments (NFLn0 and NFLn5) until day 7. EPS was not detected in the cheese made from fermented skim milk without sucrose, as the strain requires sucrose for EPS synthesis. However, in fresh cheese made with the addition of fermented skim milk with 5% sucrose, EPS was already detected on day 1, and its concentration increased significantly ($p < 0.05$) to a concentration of 1.76 mg/g during storage at 4 °C (Table 5). These results indicate that at 4 °C, the strain actively converts the remaining sucrose (which was not consumed during the fermentation period) into EPS.

Table 5. Number of viable microorganisms (Log CFU/mL) in MRS and PCA, and EPS content on day 1 and day 7 of storage in no-fat cheese made with *Ln. mesenteroides* SJC113 fermented skim milk without sucrose (NFLn0) and no-fat cheese made with *Ln. mesenteroides* fermented skim milk with 5% sucrose (NFLn5). Values are mean ± SEM of three replicates.

Parameters	Day 1		Day 7	
	NFLn0	NFLn5	NFLn0	NFLn5
MRS Log (CFU/mL)	6.09 ± 0.24 [a]	7.77 ± 0.15 [b]	6.53 ± 0.03 [a]	7.96 ± 0.01 [b]
PCA Log (CFU/mL)	6.06 ± 0.23 [a]	7.74 ± 0.13 [b]	6.50 ± 003 [a]	7.88 ± 0.02 [b]
EPS (mg/g)	ND [1]	0.44 ± 0.02 [A]	ND [1]	1.76 ± 0.28 [B]

Different lower- and upper-case letters in the same line indicate a significant difference ($p < 0.05$) between the cheese treatments and days, respectively. [1]—Not detected.

The presence of the bacterial strain was responsible for the slight decrease in pH values observed in cheeses with fermented milk (NFLn0 and NFLn5), especially in NFLn5 (Table 6). A decrease in pH value was also observed in these cheeses on day 7, possibly caused by the fermentation of residual lactose and the resulting formation of lactic acid [29]. Although the difference in pH compared to the control cheeses (FF) was significant ($p < 0.05$), this represents a decrease of 0.21 units of pH, being not high enough (> 6.3) to affect the organoleptic properties of this cheese, as reported in previous studies [46]

Table 6. Physicochemical characterization of fresh cheeses: full-fat cheese (FF), no-fat cheese (NF), nofat cheese with 0% sucrose skim milk fermented by *Ln. mesenteroides* SJC113 (NFLn0) and no-fat cheese with 5% sucrose skim milk fermented by *Ln. mesenteroides* (NFLn5). Values of pH, water-holding capacity (WHC), weight loss, apparent viscosity and hardness are means ± SEM of four replicates.

	Day 1				**Day 7**			
Cheeses	**FF**	**NF**	**NFLn0**	**NFLn5**	**FF**	**NF**	**NFLn0**	**NFLn5**
pH	6.58 ± 0.03 ^{a}	6.55 ± 0.02 ^{ab}	6.48 ± 0.01 ^{bc}	6.46 ± 0.02 ^{c}	6.57 ± 0.03 ^{a}	6.57 ± 0.03 ^{a}	6.45 ± 0.02 ^{ab}	6.36 ± 0.04 ^{b}
WHC (%)	57.78 ± 5.94 ^{a}	39.59 ± 3.34 ^{b}	41.58 ± 3.89 ^{ab}	57.48 ± 0.76 ^{a}	60.63 ± 1.07 ^{a}	42.35 ± 3.32 ^{bc}	39.95 ± 4.91 ^{c}	55.41 ± 1.13 ^{ab}
Weight loss (%)	2.85 ± 0.33 ^{a}	2.91 ± 0.17 ^{a}	1.75 ± 0.39 ^{ab}	0.71 ± 0.04 ^{b}	10.80 ± 0.62 ^{ab}	12.75 ± 0.48 ^{a}	7.60 ± 1.28 ^{b}	3.75 ± 0.61 ^{c}
Viscosity (Pa.s)	15.06 ± 2.22 ^{ab}	18.14 ± 2.58 ^{b}	14.89 ± 3.05 ^{ab}	5.67 ± 1.60 ^{a}	13.42 ± 1.75 ^{b}	19.64 ± 2.39 ^{b}	12.38 ± 1.39 ^{b}	3.33 ± 1.16 ^{a}
Hardness (N)	0.24 ± 0.03 ^{a}	0.52 ± 0.06 ^{b}	0.26 ± 0.02 ^{a}	0.13 ± 0.04 ^{a}	0.39 ± 0.08 ^{b}	0.62 ± 0.03 ^{c}	0.36 ± 0.03 ^{b}	0.11 ± 0.03 ^{a}

Different letters as superscripts in the same line indicate a significant difference ($p < 0.05$) in each day (day 1 or day 7) between cheese treatments.

The WHC evaluates the ability of proteins to bind water molecules when exposed to centrifugal force and is an important parameter for foods with high moisture content such as yogurt and fresh cheese [78]. In the present study, cheeses made with skim milk had lower WHC values, with the exception of NFLn5 cheese (Table 6). For this cheese, the presence of EPS improves the WHC value to similar values as full-fat cheeses. The high WHC of fresh cheeses indicates that the proteins that make up the gel structure of the curd may prevent excessive whey loss. This can be observed in cheeses made from whole milk, as the interaction of the fat with the casein leads to high water binding [24]. In contrast, low-fat cheeses lose an excessive amount of whey over time, resulting in a more compact and drier structure [30]. The extent of syneresis in fresh cheese can also be assessed as the weight loss of the cheese during storage and is an indicator of the quality and shelf life of cheese [78]. The greatest weight loss was observed in fat-free cheese (12.75 ± 0.48%), followed by full-fat cheese (10.80 ± 0.62%), which was considered normal for this type of cheese [78,83]. In contrast, the addition of fermented milk with *Ln. mesenteroides* SJC113 decreased cheese syneresis during the 7-day storage period, resulting in minimal weight loss (3.75 ± 0.61%). With respect to texture, apparent viscosity and hardness were measured on days 1 and 7 and are shown in Table 6. The presence of EPS reduced ($p < 0.05$) the apparent viscosity and hardness of NFLn5 cheese. In contrast, the fat-free (NF) cheese exhibited higher viscosity and hardness. The removal of fat from cheese without the addition of fat replacers usually results in a compact microstructure of the protein matrix, which is drier and harder compared to its high-fat counterpart [24]. Therefore, the use of in-situ-produced EPS could compensate for the changes in texture caused by the reduction in fat content during cheese production. Despite the high moisture content, the texture of the cheese was maintained, and the lack of fat was successfully compensated for by the EPS produced by the strain *Ln. mesenteroides* SJC113. Similar have been reported for low-fat cheddar cheese [26] and Kasar cheese [31]. However, to our knowledge, no studies have been conducted on fresh cheeses in which milk fat was completely replaced by in-situ-produced EPS.

4. Conclusions

Ln. mesenteroides SJC113 was shown to produce a glucan-type EPS from sucrose after fermentation of milk at different temperatures, including refrigeration. This strain showed important technological properties such as high tolerance to NaCl and high activity of β-galactosidase, while its safety was confirmed, as it did not exhibit virulence genes and was sensitive to clinically important antibiotics.

The results also show that EPS produced by fermenting skim milk with 5% sucrose at 8 ˚C for 5 days can improve the rheological properties of fat-free fresh cheese by softening it and reducing syneresis. As far as we know, this is the first report on the use of in-situproduced EPS as a fat substitute in fat-free fresh cheese. The use of fermented skim milk with EPS for the production of fat-free fresh cheese showed great potential and could meet the growing consumer demand for low-fat and healthier dairy products.

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Chapter 4: Prebiotic and health-promoting benefits of dextran-type exopolysaccharide produced by *Leuconostoc mesenteroides* SJC113

1. Introduction

The global increase in lifestyle-related diseases emphasizes the need for innovative approaches to promote gut health and overall well-being. Probiotics and prebiotics have proven to be promising strategies to address these challenges [1,2].

Knowledge of the benefits of the gut microbiota in many physiological processes of the host has opened up new possibilities for the use of certain bacterial strains as probiotics. Current scientific evidence suggests that lactic acid bacteria (LAB), particularly lactobacilli (*Bacillota*) and bifidobacteria (*Actinobacteria*), are beneficial to the host in correcting imbalances in the gut microbiota and consequently in maintaining and restoring health [3,4]. Probiotic bacteria can also produce a variety of bioactive compounds, including exopolysaccharides (EPSs). A growing number of studies indicate that certain EPSs produced by LAB have positive physiological effects and serve as fermentable substrates (prebiotics) for the beneficial commensal microbiota [5]. EPSs produced by LAB may also be important for the survival of probiotic bacteria during gastrointestinal transit. EPSs may act as a protective barrier that increases the bacteria's resistance to harsh conditions such as low pH, bile salts and digestive enzymes, thus increasing their survival rate in the gut [6]. In addition, several authors have investigated the effects of prebiotic compounds on the function of probiotics, as these are not broken down during digestion but have a positive effect on the intestinal tract by being utilized by certain groups of bacteria (usually bifidobacteria and lactobacilli) that colonize the intestine [7].

Due to the great differences in their composition and structure, EPSs can have various health effects, such as the control of blood glucose levels, the absorption of calcium and magnesium as well as antioxidant and anticarcinogenic activities [6,8,9]. Several studies have also shown that EPSs have the potential to influence the gastrointestinal tract, protect intestinal cells from toxins and lower cholesterol levels [10-12].

Leuconostoc mesenteroides is a LAB that has attracted attention due to its probiotic properties, in particular the production of EPSs of the dextran type [13,14]. The structure of these dextrans can vary considerably, affecting their biological activities and functional properties and influencing their solubility and interactions with biological systems [5,6]. Several studies suggest that dextran-type EPSs may play a role in lowering cholesterol levels, potentially

contributing to the treatment of dyslipidemia and cardiovascular health [2,7]. The scientific study of dextran-type EPSs is a dynamic area of research with significant implications for health and well-being [4,8]. In a previous study, a strain of *Ln. mesenteroides* (SJC113) isolated from an artisanal cheese was described, which produced a dextran-type EPS composed of glucose with 85% α-(1→6) glycosidic bonds and a small percentage of α-(3→6) bonds [15]. This strain showed good functional and technological properties, and the EPS produced *in situ* was successfully used as a fat substitute in fresh cheese [15]. The present study addresses the potential of this dextran-like EPS as a novel bioactive compound. Therefore, the specific health-promoting effects of the EPS produced by *Ln. mesenteroides* SJC113 are investigated in this study, focusing on the antioxidant, cholesterol-lowering and prebiotic effects as well as on the modulation of the gut microbiota.

2. Materials and Methods

2.1 Materials

The following materials were used: MRS broth and agar (Biokar Diagnostics, Allonne, France), VRBG agar (Biokar Diagnostics, Allonne, France), PCA agar (Biokar Diagnostics. Allonne, France), Peptone Water (Biokar Diagnostics, Allonne, France), Mucin (Sigma-Aldrich, St. Louis, MO, USA), Trypsin (Sigma, St. Loius, MO, USA), Trichloroacetic acid (PanReac AppliChem ITW reagents, Darmstadt, Germany), DNAse type I (Amresco, USA), Pronase E (Amresco, USA), DPPH (Sigma, Germany), Brilliant green (Sigma, India), L-ascorbic acid(Riedel-de Haën, Seelze, Germany), BHT (Sigma, Spain), Cholesterol (Sigma-Aldrich, Japan), PBS (Sigma, USA), Fe_2SO_4 (Riedel-de Haën, Berlin, Germany), H_2O_2 (Panreac Química SAU,Barcelona, Spain), Dialysis membrane (10 kDa, Spectrum Laboratories Inc., Piscataway, NJ,USA), Sucrose (Sigma, Steinheim, Germany), Peptone (Biokar Diagnostics, Allone, France), Raffinose (Fluka, Switzerland), Lithium Chloride (Panreac, Barcelona, Spain) L-cystein (Sigma, Steinheim, Germany), Pectin (Condi-Alimentar, Malveira, Portugal), Xylose (Difco, Detroit, Michigan, USA), Sodium bicarbonate (Sigma, Steinheim, Germany), Glucose (Sigma, France), Potato starch (Merck, Dermstadt, Germany), Yeast extract (Oxoid, Basingstoke, Hampshire, England), Fresh bile (Himedia, Mumbai, India), Pancreatine (PanReac Applichem, Barcelona, Spain).

2.2 Bacterial strains

Bacterial strains isolated from Azorean cheeses, human feces and milk, and the commercial probiotic strain *Lacticaseibacillus rhamnosus* GG were used in the present study (Table 1). Stock cultures were kept at −80 °C in 30% (v/v) glycerol and propagated twice in MRS broth with 1% (v/v) of inoculum at 37 °C for 24 h.

Table 1. Bacterial strains used in the present study.

Strains	Origin	ATCC/GenBank Accession number	Source
Lacticaseibacillus rhamnosus GG	Human gut	ATCC 53103	Dicofarm
Leuconostoc mesenteroides SJC113	Cheese	MT742947	IITAA Collection
Lactococcus lactis L3A21M1	Cheese	KF193424	IITAA Collection
Lacticaseibacillus rhamnosus FB1	Human feces	OQ713701	IITAA Collection
Lacticaseibacillus paracasei FB2	Human feces	OQ713702	IITAA Collection
Lacticaseibacillus paracasei FB3	Human feces	OQ713703	IITAA Collection
Lactobacillus gasseri BM8	Human milk	OQ713687	IITAA Collection

2.3 Production and purification of EPS

Ln. mesenteroides SJC113 was used to produce EPS by incubation of the bacterial culture (1%) in modified MRS broth (10% sucrose) at 30 °C for 48 h. After fermentation, the EPS was extracted according to Jurášková, *et al.* [15]. Briefly, the medium was centrifuged at 9000 × *g* for 5 min, at 4 °C, EPS was precipitated by adding 2 volumes of chilled ethanol, dissolved in Milli-Q water, dialyzed against Milli-Q water at 4 °C for 3 days, and freeze-dried. The phenol-sulfuric acid method was used to quantify EPS yield. Further purification for composition analysis involved DNAse and Pronase E treatment as described by Domingos-Lopes*, et al.* [16].

2.4 Biological activities of EPS

2.4.1. Cholesterol-binding

Cholesterol-binding ability of EPS was tested by the methodology described by Gawande*, et al.* [17] with some modifications. Purified EPS at 0.01% and 0.1% was resuspended in MRS broth with 3 mg/mL bile salt and 100µg/mL cholesterol. Uninoculated MRS broth was used as control. The samples were incubated for 12h and centrifuged at 10 000 × g for 20 min. The absorbance of the supernatant was measured at 517nm and the cholesterol removing ability was calculated as follows:

$$\text{Cholesterol removal (\%)} = \frac{\text{Absorbance control} - \text{Absorbance spent broth}}{\text{Absorbance control}} x100$$

2.4.2. DPPH scavenging activity

The effect of scavenging DPPH radicals was measured according to the methodology of Hu*, et al.* [18]. EPS was dissolved in 2 mL of distilled water to obtain 1, 2 and 3 mg/mL. Then, 2 mL of 0.1 mM DPPH was mixed with EPS, incubated in the dark for 30 min, and the absorbance was measured at 517 nm (A_1). A_2 was obtained by replacing the DPPH solution with ethanol and A_0 was a mixture of 2 mL distilled water and 2 mL DPPH solution. Ascorbic acid (0.2 – 1 mg/mL) was used as a positive control. DPPH scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100$$

2.4.3. Hydroxyl radical scavenging activity

The scavenging activity of hydroxyl radicals was determined using the Fenton´ reaction according to Zhang*, et al.* [19]. For this proposal, 100 µL EPS solutions (1, 2 and 3 mg/mL) were mixed with 0.435 mM brilliant green (100 µL), 0.5 mM $FeSO_4$ (200 µL) and 3.0% H_2O_2 (150 µL) and incubated for 20 min at room temperature. Before reading the absorbance at 624 nm, the mixture was centrifuged at 5000 × g for 5 min.

The hydroxyl radical scavenging activity was calculated according to the following equation:

$$\text{Hidroxyl radical scavenging activity (\%)}: [(\text{As} - \text{A}_0)/(\text{A} - \text{A}_0)] \times 100$$

As is the absorbance of the mixture with EPS, A_0 is the absorbance of a mixture in which the EPS has been replaced by distilled water and A is the absorbance of the solution obtained by replacing the brilliant green solution with water, labelled A. Ascorbic acid (0.2 – 1 mg/mL) was used as a positive control.

2.5 Prebiotic properties

2.5.1. Mucin adhesion assay

Ln. mesenteroides SJC113 was tested for mucin adhesion as described by Dhanani and Bagchi [20]. The probiotic *L. rhamnosus* GG was also tested for comparison. In brief, 96-well plates were coated overnight at 4 °C with 300 µL porcine mucin (0.5 mg/mL in PBS, pH 7.0). The wells with mucin were washed several times with sterile PBS to remove unbound mucin. The overnight bacterial cultures were harvested and washed with PBS. The cell density was adjusted to 1×10^8 CFU/mL in PBS and 200 µL of the bacterial strain was pipetted into each well and allowed to adhere at 37 °C for 90 min. The wells were then washed at least 5 times with PBS to remove all non-adherent cells. To release the adherent cells, 300µl of Triton X-100 (0.05%) was pipetted into each well and incubated at 37 °C for 20 min. The viable cells were counted after plating on MRS agar and incubation at 37 °C for 48 h.

2.5.2. Effect of EPS on probiotic adhesion to mucin

To evaluate probiotic adhesion in the presence of EPS, the mucin-coated wells were pre-incubated for 15 min at 37 °C with EPS at different concentrations: 0.5, 1 and 2 µg per well. Glucose at a concentration of 1 µg per well was used as a control. After pre-incubation, 200 µL of *L. rhamnosus* GG (1×10^8 CFU/mL) was added to each well and incubated at 37 °C for 90 min. The steps were then repeated as previously described (section 2.5.1).

2.5.3. Stimulation of LAB growth

To investigate the effect of EPS isolated from *Ln. mesenteroides* SJC113 on LAB growth, the bacterial cells were grown in MRS (0.5% glucose) as a control and in a 0.5% glucose with MRS (0.5% glucose) supplemented with 1% EPS, according to Liu*, et al.* [21]. The LAB strains used were *L. lactis* L3A21M1, *L. rhamnosus* FB1, *L. paracasei* FB2, *L. paracasei* FB3 and *L. gasseri* BM8. All LABs were previously cultivated in MRS broth at 37 °C. After 48 h of incubation at 37 °C, growth was quantified by measuring the optical density at 600 nm.

2.6 Effects of EPS on fecal microbiome

2.6.1. Gut nutrient medium

In order to cultivate the diverse microbial communities in the gut, it is necessary to provide them with a nutrient-rich environment, such as the gut nutrient medium (GNM) previously described [22,23]. The composition of the GNM was adapted to contain the EPS: 1 g/L EPS, 2 g/L pectin, 1 g/L xylan, 3 g/L potato starch, 0.4 g/L glucose, 3 g/L yeast extract, 1 g/L peptone, 4/L mucin and 0.5 g/L L-cysteine. This solution was sterilized at 121 °C for 21 min. In addition, a pancreatic juice was prepared with 12 g/L $NaHCO_3$, 6 g/L dehydrated oxgall (fresh bile) and 0.9 g/L porcine pancreatin.

2.6.2. Fecal slurry

In this study, three independent experiments were conducted with stool samples from 1-year-old infants (3 collections of individual fecal samples). The selection of infants was based on a diverse microbiota including the *Bifidobacteriaceae*, *Lactobacillaceae* and *Enterobacteriaceae* families. Fresh stool samples were collected according to a procedure approved by the Ethics Committee of the University of the Azores (reference number CE/ALN/PT/2022/036). This procedure required parents to sign a consent form on behalf of their children to provide their stool sample. The selection criteria for the donor samples used here were as follows: no intake of antibiotics in the last 3 months, no gastrointestinal disorders and no intake of probiotics. At the beginning of the experiment, a 20% (w/v) fecal slurry was

prepared as previously described by Tamargo, *et al*. [23]. Fresh feces were collected under anaerobic conditions. A phosphate saline buffer (0.1M, pH 7) was used to dilute the sample and this mixture was then thoroughly mixed with a stomacher (Stomacher 400 Circulater, Seward, England) at a speed of 230 rpm for 3 min. Each bioreactor was initially filled with 135 mL of the gut nutrient medium (GNM) as previously described by Machado*, et al.* [24]. One reactor, which served as a negative control, contained 135 mL of GNM without EPS. Subsequently, 15 mL of freshly prepared fecal slurry was added to each reactor, resulting in a final volume of 150 mL. The mixture was kept anaerobic and incubated at 37 °C. Samples were taken after 0, 6, 24 and 48 h and analyzed immediately. The pH values of the samples during simulated fermentation were determined by pH meter.

2.6.3. Microbiological analysis

Decimal dilutions were performed and plated on selective media: PCA for total aerobes, MRS agar for LAB, MRS agar supplemented with L-cysteine 0.5g/L, raffinose 1g/L and lithium chloride 0.05g/L for bifidobacteria and VRBG for *Enterobacteriaceae* [23,25,26]. The plates were kept in anaerobiosis at 37 °C for 24 to 48 h, except for the total aerobic count (PCA). Colony counts on all plates were performed in duplicate and results were expressed as colony forming units per milliliter (CFU/mL).

2.7. Statistical analysis

All experiments were performed in triplicate and the results are expressed as mean ± SEM. Analysis of variance (one-way ANOVA) was used to evaluate the effect of the treatments on the different parameters studied. Tukey test was performed to detect significant differences ($p < 0.05$). The statistical analysis was performed using SPSS software, version 27 (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Biological activities of EPS produced by Ln. mesenteroides SJC113

3.1.1. Cholesterol-lowering ability

EPS showed a strong cholesterol-binding effect and effectively reduced cholesterol levels in cholesterol-enriched MRS broth (Table 2). A 0.1% EPS concentration reduced cholesterol levels by over 40%, while a 0.01% EPS concentration achieved a reduction of 33.01 ± 2.78%. These results are in agreement with the findings of several authors who reported similar levels of cholesterol binding of heteropolysaccharides produced by *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* strains [17,27-29]. To our knowledge, there are no previous reports describing the cholesterol-binding ability of α-glucan-type EPS or EPS produced by *Leuconostoc* spp. However, several studies have reported the cholesterol-lowering effect of *Leuconostoc* spp. [30-32]. The cholesterol-binding capacity of EPS may play a key role in reducing intestinal cholesterol absorption, thereby improving cardiovascular health. Recent studies highlight the hypocholesterolemic effect of EPS-producing strains (especially *Lactobacillus* and *Leuconostoc mesenteroides*) *in vivo* by assimilating cholesterol and excreting it via feces [33]. Therefore, the inclusion of these EPS in food formulations could be a useful approach for the production of functional foods with cholesterol-lowering potential.

3.1.2. Antioxidant activity

The EPS produced by *Ln. mesenteroides* SJC113 showed moderate antioxidant activity with scavenging rates of DPPH radicals ranging from 10.94±1.33% to 17.48±1.74% at concentrations of 1-3 mg/mL (Table 2). This activity was lower than the values reported by Amiri*, et al.* [34], where various LAB strains achieved up to 62.33±1.02% radical scavenging at 2 mg/mL EPS, and was significantly lower than the 86.2% activity reported by Hu, *et al*. [18] for EPS-2 at 5 mg/mL. The hydroxyl radical scavenging activity was also modest (6.29±1.59% to 9.34±1.89% at 1-3 mg/mL), in contrast to the activity (59.94%) reported by Amiri, *et al.* [34] for EPS produced by a *Lactobacillus acidophilus* strain. However, other authors also reported reduced antioxidant activity (both DPPH scavenging activity and hydroxyl scavenging activity) for bacterial EPS < 4 mg/mL [35].

Table 2. Cholesterol-binding (%), radical scavenging activity—DPPH (% and equivalent concentration of ascorbic acid (mg/mL)), and hydroxyl scavenging activity (% and equivalent concentration of ascorbic acid (mg/mL)) of EPS produced by *Ln. mesenteroides* SJC113. Ascorbic acid (1 mg/mL) is presented as positive control. Results are presented as mean ± SEM (n=3).

EPS*	0.1 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL
Cholesterol-binding (%)	33.01±2.86[a]	40.18±2.42[b]	-	-
Radical scavenging activity—DPPH (%)	-	10.94±1.03[a]	13.15±0.81[ab]	17.48±1.74[b]
Ascorbic acid (%)	-	89.3±4.8	-	-
Eq. ascorbic acid (mg/mL)	-	0.092±0.017[a]	0.119±0.011[ab]	0.164±0.015[b]
Hydroxyl scavenging activity (%)	-	6.29±1.46[a]	8.57±2.29[a]	9.34±2.06[a]
Ascorbic acid (%)	-	65.0±1.2	-	-
Eq. ascorbic acid (mg/mL)	-	0.065±0.024[a]	0.111±0.039[a]	0.128±0.033[a]

* A hyphen (-) indicates that testing for that EPS concentration was not performed. Different letters as superscripts in the same line indicate a significant difference ($p < 0.05$).

Studies on the antioxidant activity of EPS show that it does not correlate directly with the amount of EPS, but depends on both the bacterial strain and the culture conditions [36,37]. These results emphasize that the antioxidant potential of EPSs varies greatly depending on the LAB strains and is strongly influenced by its composition. Huang*, et al.* [38] demonstrated that the antioxidant effect of purified EPS is primarily due to the protein components rather than the polysaccharide components, emphasizing the importance of protein-associated mechanisms. In addition, the molecular weight (MW) of EPS has a significant influence on its bioactivity [39]. Hydroxyl groups, which serve as important free radical scavengers, are less accessible in polysaccharides with high MW, which impairs their antioxidant efficacy. The high viscosity and steric hindrance in larger EPS molecules further limit mobility and interactions with radicals in solution. The lower antioxidant performance of EPS produced by *Ln. mesenteroides* SJC113 may therefore be due to a combination of high molecular weight, limited exposure of hydroxyl groups and low protein content of the purified sample.

3.2 Prebiotic properties

3.2.1. Adhesion to immobilized mucin

Table 3 presents the mucin-binding capacity of *Ln. mesenteroides* SJC113, evaluated alongside the probiotic reference strain *L. rhamnosus* GG. Mucin adhesion is a critical determinant of probiotic functionality, as it enhances gut colonization potential and host-microbe interactions. *Ln. mesenteroides* SJC113 demonstrated high adhesion levels (74.1%) comparable to *L. rhamnosus* GG (75.5%), suggesting strong probiotic potential for this strain.

Table 3. LAB adhesion to immobilized mucin expressed as viable counts (Log CFU/mL) after 90 min of incubation. Values are the average of three independent experiments ± SEM.

Strain	**Log CFU/mL**	
	0 min*	**90 min**
Ln. mesenteroides SJC113	11.64±0.07	8.62±0.08
L. rhamnosus GG	10.63±0.06	8.03±0.25

* Initial counts.

3.2.2. Adhesion to mucin in the presence of EPS

The role of the EPS in modulating probiotic adhesion to human intestinal mucus was also investigated in this study. Using *L. rhamnosus* GG as a probiotic model strain, we assessed the mucin binding capacity in the presence of different EPS concentrations (0.5 – 2 mg/mL). As shown in Figure 1, EPS significantly ($p < 0.05$) enhanced the adhesion of *L. rhamnosus* GG to mucin in a dose-dependent manner. The maximal adhesion of 5.6×10^6 and 1.1×10^7 CFU/mL, occurred at 1 and 2 mg/mL EPS, respectively, and was significantly higher ($p < 0.05$) than the positive control (1 mg/mL of glucose) and negative control (PBS). The 7.04 Log CFU/mL adhesion at 2 mg/mL exceeds values reported for *L. rhamnosus* GG in EPS-free system (PBS) in the present study and by other authors (typically 5.8–6.2 Log CFU/mL) [40,41], indicating synergistic effects of the EPS in adhesion to mucin. These results suggest that EPS may serve as an important mediator of probiotic-mucin interactions, possibly through surface modification or direct binding facilitation.

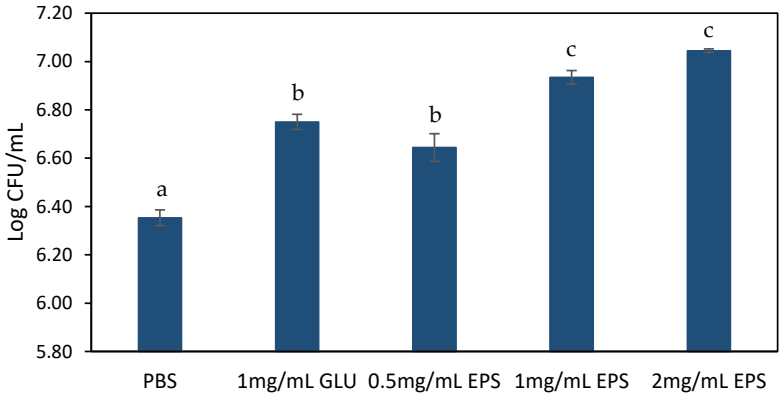

Figure 1. Mucin adhesion of *L. rhamnosus* GG in the presence of EPS or glucose. The values are the average of three experiments ± SEM. Different letters (a-c) indicate significant differences ($p < 0.05$) between treatments.

3.2.3. Stimulation of LAB growth

The changes in LAB growth in the presence of EPS are shown in Figure 2. Overall, the exponential growth phase — characterized by a rapid increase in optical density (OD) — was significantly more pronounced in the EPS-supplied cultures than in the untreated controls. The results also indicate a strain-dependent enhancement of LAB growth after EPS treatment, with the effect being more pronounced in lactobacilli (Fig. 2B-E) than in *Lactococcus* (Fig. 2A). The sustained increase in OD values in the EPS-treated groups indicates a direct stimulatory effect of EPS on LAB growth and emphasizes its potential as a microbial growth promoter. The observations in the present study are consistent with previous reports by Zhao*, et al.* [42], which showed that LAB cultured in MRS medium supplemented with both glucose and dextran or commercial prebiotics exhibited significantly better growth kinetics than LAB in glucose-only medium. In addition, these authors reported that the effect of prebiotics on different probiotic bacteria was not only strain-dependent but also depended on the type of polysaccharides. These results suggest that EPS-mediated growth promotion may be a generalizable phenomenon that depends on the EPS type and the LAB strain. Although reference prebiotics such as inulin, FOS, or GOS, were not tested in the present study, several studies reported that carbohydrates with longer chain lengths are fermented more slowly by LAB strains [43]. For example, inulin showed limited growth stimulation of *L. casei* strains compared to FOS [44]. Further investigation of the strain-specific mechanisms underlying EPS utilization could provide valuable insights into the optimization of probiotic production and functional food applications.

The measurement of OD (at 600 nm) was employed as the primary growth assessment method, providing real-time, non-destructive monitoring of bacterial strain dynamics under EPS exposure. While this method captures total biomass accumulation, its correlation with cellular growth remains robust in controlled monocultures, making it ideal for comparative analysis of EPS-mediated growth patterns across LAB strains [43,45,46]. However, for specific investigations of viability cell quantification under EPS treatment, complementary plating assays (CFU) should be included.

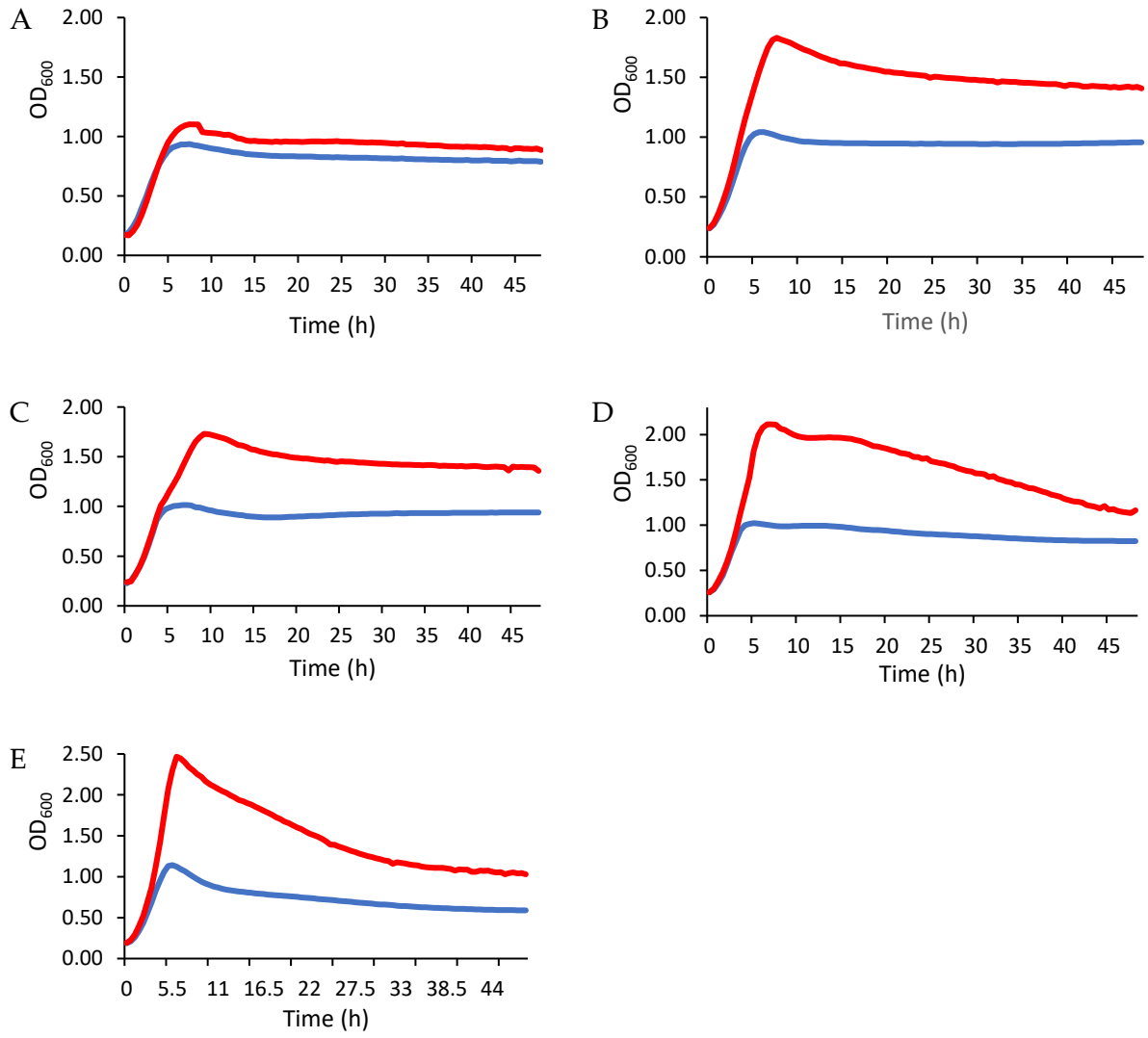

Figure 2. Growth of LAB evaluated by OD at 600nm in MRS medium supplemented with glucose only (blue line) or with glucose and EPS (red line). LAB strains: A) *L. lactis L3A21M1*, B) *L. paracasei* FB2, C) *L. paracasei* FB3, D) *L. rhamnosus* FB1, and E) *L. gasseri* BM8.

3.2.4. Effects of EPS on selected bacterial groups in fecal fermentation

Adhesion to intestinal mucus is the first step of microbial colonization of the gut and plays a crucial role in modulating immune function and improving the integrity of the intestinal barrier. Despite their importance, the influence of dietary polysaccharides on the gut microbiota of infants is still poorly understood. Dextrans that resist digestion can promote beneficial bacteria such as *Bifidobacterium*— a dominant microbial genus in infants that plays a key role in establishing a healthy infant gut microbiota health [47]. In this study, the prebiotic potential of a dextran-like EPS using infant stool samples were investigated, focusing on *Bifidobacterium* spp. and LAB. The number of bacteria over time (Fig. 3) showed that EPS significantly increased *Bifidobacterium* spp. after 24h and 48h ($p < 0.05$; Fig. 3A) and LAB after 6h ($p < 0.05$; Fig. 3B). In contrast, EPS reduced *Enterobacteriaceae* after 24 h and 48 h ($p < 0.05$; Fig. 3C), with no significant effect on the total number of aerobes ($p > 0.05$; Fig. 3D). These results are consistent with previous reports showing the stimulatory effect of dextran on the growth of bifidobacteria and lactobacilli [48]. In the present study, selective plate cultures were used to evaluate the effects of EPS on the fecal microbiota *in vitro*. While the use of selective media is an established approach to evaluate specific bacterial groups, this method is not without limitations. Selective media can lead to false-positive results and a lack of resolution at the species level, which may not fully capture the actual growth dynamics of the target bacterial species. Therefore, the accuracy of the results should be interpreted with caution considering this methodological limitation. For accurate profiling, molecular methods (16S rRNA sequencing, qPCR) or colony validation (MALDI-TOF MS) should complement traditional cultivation to account for media-related biases. A notable observation was the significant pH reduction during 48-h fermentation ($p < 0.05$; Fig. 4), likely attributable to microbial production of acidic metabolites such as short-chain fatty acids (SCFAs). Although SCFA quantification was not performed in this study, the pH trend is consistent with previous reports in which EPS fermentation by the gut microbiota resulted in increased acetate and propionate levels [49,50]. However, the lack of direct SCFA measurements limits the ability to conclusively link the pH drop to specific metabolic pathways, highlighting an important area for further investigation.

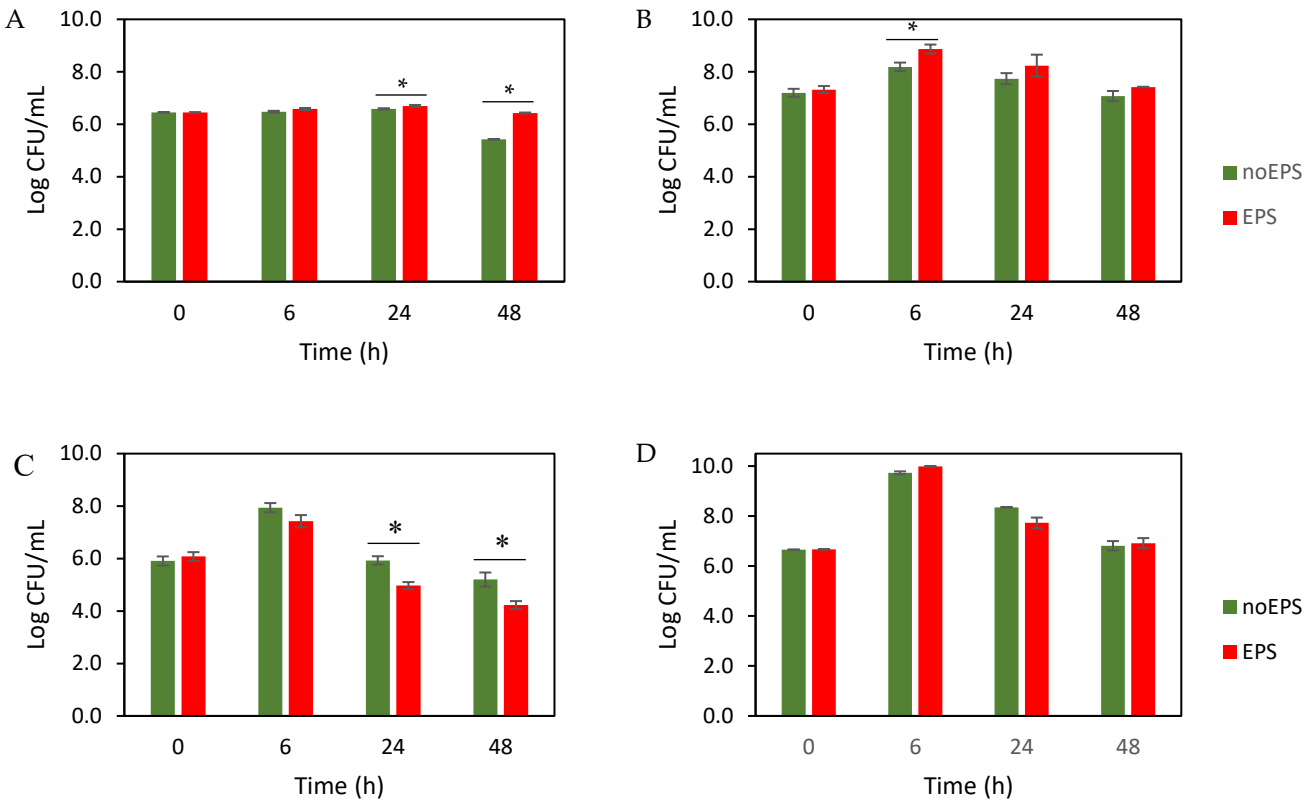

Figure 3. Effect of EPS on microbial growth (Log CFU/mL) of (A) *Bifidobacterium,* (B) LAB, (C) *Enterobacteriaceae*, and (D) Total aerobes. The values are the average of three experiments ± SEM. * indicates significant difference at $p < 0.05$.

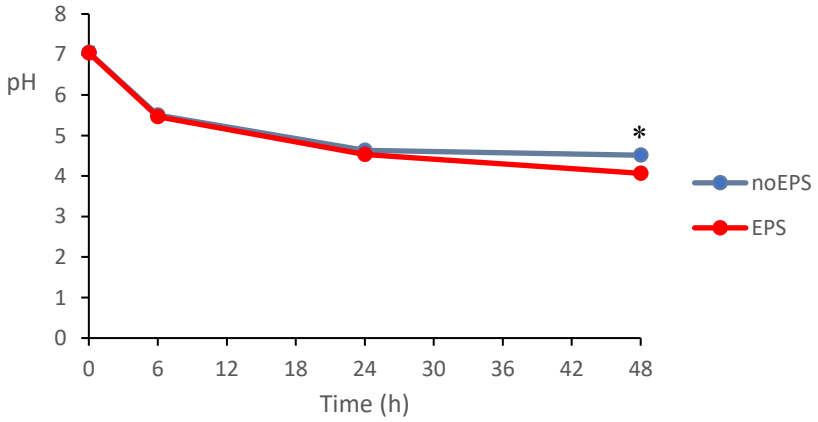

Figure 4. Effect of EPS on pH of fecal slurry over 48h incubation at 37 °C. * indicates significant difference at $p < 0.05$.

The results of the present work support existing evidence that certain EPS exhibit selective prebiotic effects, stimulating beneficial microbes while suppressing pathogens in the human gut [51,52]. For example, EPS produced by *Lacticaseibacillus paracasei* and *Lactobacillus*

sanfranciscensis exhibit similar bifidogenic properties while reducing pathogenic bacteria (*Escherichia-Shigella, Klebsiella* and *Fusobacterium*) [51,52]. In contrast, some EPS, such as those from *Lactobacillus rhamnosus* RW-9595M, show minimal modulation of infant microbiota [53], underscoring the structure-dependent variability in prebiotic activity. In the present study, results indicate that EPS of *Ln. mesenteroides* SJC113 can selectively promote *Bifidobacterium* while inhibiting *Enterobacteriaceae*, which strengthens its potential as a targeted prebiotic. These observations emphasize the intricate relationship between EPS structure and modulation of the infant gut microbiota and reinforce the potential of EPS produced by this strain as an effective prebiotic. However, the translational relevance of these *in vitro* observations remains to be validated in more complex systems, such as *in vivo* infant models or human trials. While this study provides insights into the prebiotic potential of EPS from *Ln. mesenteroides* SJC113, several limitations must be acknowledged. First, the observed antioxidant activity of this EPS was modest compared to other reported EPS, suggesting that its primary benefits may lie in microbial modulation rather than direct free radical scavenging. Secondly, like all *in vitro* models, the fecal fermentation system cannot fully replicate the physiological complexity of the infant gut, where host factors (e.g. immune responses, mucosal interactions and nutrition) significantly influence microbial dynamics. Although selective plate counting allows targeted analysis of key bacterial groups, this approach may miss subtle shifts in the community or functional changes that can only be revealed by metagenomic or metabolomic methods. Finally, the present experiments were based on three individual donor samples, which avoids the use of artificially pooled stool samples, but may limit the generalizability of the results given the large inter-individual variability in the composition of the gut microbiota of infants. Future work should investigate the inter-individual variability and long-term effects to better assess the therapeutic potential of this EPS in infant feeding.

5. Conclusions

This study comprehensively characterized the multifunctional properties of the EPS produced by *Ln. mesenteroides* SJC113, revealing its promising prebiotic potential. The EPS exhibited moderate but consistent antioxidant activity, effectively scavenging both DPPH and hydroxyl radicals in dose-dependent assays. More notably, the EPS displayed a pronounced cholesterol-lowering effect, reducing cholesterol levels in the culture medium by 40%. The EPS

also exhibited noteworthy prebiotic potential, demonstrating dual functionality in microbial adhesion and growth promotion. While *Ln. mesenteroides* SJC113 displayed strong intrinsic mucin-binding capacity, its EPS further enhanced the adhesion of the probiotic *L. rhamnosus* GG to mucin—a critical trait for gut colonization and host interaction. Beyond adhesion, the EPS served as an effective fermentable substrate, consistently stimulating the growth of several LAB strains. In gut-mimicking conditions, the EPS selectively modulated bacterial populations, significantly increasing LAB and *Bifidobacterium* spp. while suppressing *Enterobacteriaceae,* thereby validating its prebiotic efficacy. These multifunctional properties position the EPS-producing *Ln. mesenteroides* SJC113 as a versatile candidate for food and pharmaceutical applications, offering a novel strategy to engineer functional foods and targeted therapies for gut health enhancement. Future research should focus on elucidating the molecular mechanisms – in particular mucus adhesion and cholesterol binding of EPS – using advanced models (e.g. mucus-secreting cell lines, omics approaches) and validating its efficacy using *in vivo* models to fully exploit the potential of this EPS for human health.

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Chapter 5: Exopolysaccharide (EPS)-producing *Streptococcus thermophilus*: Functional and Probiotic Potential

1. Introduction

Streptococcus thermophilus is a lactic acid bacterium (LAB) of significant industrial importance, particularly within the dairy industry. It is widely employed as a starter culture in the production of fermented milk products, including yogurt and cheese. Its rapid acidifying capacity during milk fermentation is essential for the coagulation of milk proteins, a critical step in dairy processing. Recognized globally for its technological roles, *S. thermophilus* holds Generally Recognized as Safe (GRAS) status, confirming its long-established safety for consumption [1,2].

Beyond its primary function in acidification, certain strains of *S. thermophilus* are capable of producing exopolysaccharides (EPS)—high-molecular-weight polymers composed of repeating sugar units, synthesized and secreted by the bacterium. EPS play a crucial role in determining the texture, viscosity, and overall sensory quality of fermented dairy products [3]. The increasing societal demand for natural and functional food ingredients has further elevated the importance of microbial EPS, particularly those derived from well-characterized and safe organisms such as *S. thermophilus* [1]. This Gram-positive, thermophilic bacterium grows optimally at 35–42 °C and is classified as a facultative anaerobe, primarily producing lactic acid via fermentation [4]. In yogurt production, *S. thermophilus* exhibits a well-documented symbiotic relationship with *Lactobacillus delbrueckii* subsp. *bulgaricus*, in which it provides essential metabolites such as folic and formic acids that support purine synthesis by its partner organism [5].

In addition to acidification, *S. thermophilus* contributes to various desirable qualities in dairy products through the production of metabolites including EPS, aromatic compounds, and vitamins such as folic acid. These metabolites enhance texture, viscosity, flavor, and water-holding capacity, all of which are vital for consumer acceptance [1].

S. thermophilus is also considered a probiotic candidate due to its ability to survive gastrointestinal transit and contribute to gut health [6]. It assists in lactose digestion, making dairy products more tolerable for lactose-intolerant individuals, and has been associated with potential health benefits such as anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory activities [6]. It is also suggested its role in lowering serum cholesterol levels [7]. The dual functionality of *S. thermophilus*—as an industrial starter culture and a potential probiotic—underscores the significance of its metabolic products, particularly EPS.

EPS production in *S. thermophilus* is a strain-dependent trait. While some strains produce large quantities of EPS, others synthesize little to none, highlighting the need for targeted screening and selection of strains for specific technological applications [8]. Two main forms of EPS are produced: capsular EPS, which remain attached to the cell surface, and free EPS, which are secreted into the surrounding medium. Free EPS can further be categorized based on their impact on product texture—ropy EPS create a stringy, viscous texture, while non-ropy EPS still contribute to viscosity without the stringiness [9,10].

The composition and concentration of the growth medium, especially the type of carbon source (e.g., glucose, lactose, sucrose), significantly influence both bacterial growth and EPS production [11]. This highlights the importance of optimizing fermentation conditions to enhance EPS yield and tailor its functional properties for specific applications. Additionally, some EPS possess bioactive properties which can enhance the functional and preservative qualities of food [5,12].

In this study, four *S. thermophilus* strains (GM1 to GM4) were isolated from raw goat milk collected in the Azores, Portugal. Preliminary analysis revealed their ability to produce ropy EPS [13]. This work aims to characterize these strains, with a focus on their technological properties for dairy fermentation, safety, and probiotic potential. Additionally, the EPS produced by strain GM4 (which lacks virulence factors) was purified and characterized to assess its potential applications in food.

2. Materials and Methods

2.1. Materials

The following materials were used: MRS broth and agar (Biokar Diagnostics, Allonne, France), Skim milk (VWR Chemicals, Leuven, Belgium), PCA (Biokar Diagnostics, France), M17 (Biokar, France), MSE (Biokar, France), Rogosa (Biokar, France), Muller-Hinton agar (AES, France), Tributyrin agar (Merck, Germany), Potato dextrose agar (PDA, HiMedia), Tryptose Blood Agar Base (Merck Germany), DNAse-Test agar (Sigma-Aldrich, USA), glucose (Sigma, Saint-Quentin-Fallavier, France), lactose (Scharlau, Barcelona, Spain), fructose (Sigma, Burlington, MA, USA), sucrose (Sigma, Germany), HCl (PanReac, Germany), TCA (PanReac AppliChem ITW reagents, Darmstadt, Germany), Peptone (Fluka, USA), Yeast

extract (AES, Bruz, France), Gelatine (Biolife, Milan, Italy), Bacteriological agar (AES, Bruz, France), APIZYM kit (Cat. No. 25200, Bio-Mérieux, Marcy-l'Étoile, France), DNAse type I (Amresco, Solon, OH, USA), pronase E (Amresco, Solon, USA), DPPH (Sigma, Germany), brilliant green (Sigma, India), ascorbic acid (Riedel-de Haën, Seelze, Germany), cholesterol (Sigma-Aldrich, USA), methanol (Fisher Chemical, Loughborough, UK), PBS (Sigma, USA), Fe_2SO_4 (Riedel-de Haën, Berlin, Germany), H_2O_2 (Panreac Química SAU, Barcelona, Spain), dialysis membrane (10 kDa, Cat. No. 11405929, Spectrum Laboratories Inc., Piscataway, NJ, USA), antimicrobial susceptibility discs (Oxoid, Thermo Scientific, Basingstoke, UK), UltraClean® Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA), Sybergreen (Cat. No. S33102, Invitrogen, Carlsbad, USA).

2.2. Bacterial Isolation and Identification

The strains *S. thermophilus* GM1, GM2, GM3 and GM4 were isolated from goat milk (The Azores, Portugal). Briefly, goat milk samples were collected from their disinfected mammary glands, taking care to discard the first three jets. The samples were 10-fold serially diluted with peptone water, spread onto de Man Rogosa Sharpe (MRS) agar and incubated at 37 °C for 72 h. Pure cultures were obtained through ≥3 subcultures under identical conditions. Single colonies were then aerobically grown in MRS broth (37 °C, 24 h). Cells (1 mL aliquots) were pelleted by centrifugation (14,000 × *g*, 2 min), and genomic DNA was extracted from the pellet using the Microbial DNA Isolation Kit (UltraClean®) following manufacturer instructions. The isolates (4) were identified via sequencing of the 16S rRNA gene, as described by Pires et al. [14]. PCR-amplified fragments were sequenced (STAB Vida, Portugal) and analyzed via NCBI BLAST (http://www.ncbi.nlm.nih.gov/). The 16S rDNA sequences were deposited in the GenBank under accession numbers OQ540791, OQ540792, OQ540793 and OQ540794, respectively. Stock cultures were kept at −80 °C in 30% (*v/v*) glycerol and propagated twice in MRS broth with 1% (*v/v*) of inoculum at 37 °C for 24 h.

2.3. Tecnological characterization S. thermophilus strains

2.3.1 Production of EPS with different sugar substrates

The bacterial cultures were grown for 48 h at 37 °C in modified MRS broth where glucose was replaced by 10% (w/v) of either of the following sugars: lactose, sucrose, glucose or fructose [15]. After the incubation period, the tubes were centrifuged (4000 x *g*, 20 min, 4°C). The EPS was extracted from the supernatant with two volumes of 95% (v/v) cold ethanol. The precipitation step was repeated twice, before EPS precipitates were dissolved in warm ddH_2O. The total sugar content of EPS was evaluated by the phenol sulfuric acid method [16], using glucose as standard.

2.3.2. Milk acidification

The strains were inoculated into MRS broth and incubated at 37 °C for 24 h. After this incubation, a 1% inoculum was prepared in skim milk and incubated again at 37 °C for 24 h. The pH was measured at 0, 6 and 24 h.

2.3.3. Carbohydrate fermentation

To assess the ability to ferment different carbohydrates, a 1% solution of carbohydrates was tested: glucose, fructose, galactose, ribose, arabinose, sucrose, lactose, maltose, raffinose, starch, mannitol, xylose, trehalose, rhamnose, dextrin, sorbitol, glycerol and inulin. A suspension of each strain was prepared in 10 mL of API 50 CHL suspension medium (10 g/L polypeptone, 5 g/L yeast extract, 0.1% Tween 80, 2 g/L dipotassium phosphate, 5 g/L sodium acetate, 2 g/L diammonium citrate, 0.2 g/L magnesium sulfate, 0.05 g/L manganese sulfate, 0.17 g/L bromcresol purple) and adjust the optical density to 2 on the McFarland scale. The bacterial suspension (400 µL) was then added to 100 µL of each sugar, covered with mineral oil and incubated at 37°C for 48 h. The fermentation was evaluated by the acidification of the medium, which was visible by the color change of the indicator (from blue to yellow).

2.3.4. Enzymatic activity

The APIZYM kit was used as a semi-quantitative method to detect the enzymatic activities of 19 enzymes: alkaline phosphatase, esterase (C4), esterase-lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI phosphohydrolase, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, N-acetyl-*β*-glucosaminidase, α-mannosidase and α-fucosidase [17]. The experimental procedure was made according to the manufacturer's instructions. Results were expressed as nmol of hydrolyzed substrate, determined from reaction intensity measurements using a quantitative scale ranging from 0 (no activity) to 5 (≥40 nmol hydrolyzed substrate).

2.3.5. Proteolytic activity

Extracellular proteolytic activity was determined by plating the isolates on Plate Count Agar (PCA) supplemented with 10% (w/v) Skim milk. The isolates were then incubated at 37°C and after 72 h the plates were flooded with 1% HCl. Proteolytic activity was indicated by a clear zone around the colonies [18]. *Staphylococcus aureus* subsp. *aureus* ATCC 25923 was used as a positive control.

2.3.6. Lipolytic activity

To evaluate the lipolytic activity, the LAB were inoculated on Tributyrin agar and incubated at 37°C for 72 h. After incubation, the presence of colonies surrounded by a clear halo indicated a positive result [19]. *S. aureus* ATCC29423 was used as a positive control.

2.4. Safety evaluation of Streptococcus thermophilus strains

2.4.1. Hemolytic activity

The strains were inoculated onto blood agar medium prepared from Tryptose Blood Agar Base with the addition of sheep's blood. The plates were incubated at 37 °C for 48 h. After

this time, the blood agar plates were analyzed and classified as follows: β-hemolysis, when a translucent halo appeared around the colonies; α-hemolysis, corresponding to a greenish halo around the colonies; and γ- hemolysis, indicating the absence of a halo around the colonies [20]. *S. aureus* ATCC29423 was used as a positive control.

2.4.2. DNase activity

DNAse enzyme production was analyzed using DNAse-Test agar. After incubation for 48 h at 37°C, the results were recorded. A positive reaction was indicated by the appearance of a pink halo around the colonies [21]. *S. aureus* ATCC29423 was used as a positive control.

2.4.3. Gelatinase activity

The gelatinase activity was determined according to the methodology of Terzić-Vidojević *et al.* [22]. A culture medium was prepared containing 5 g/L peptone, 3 g/L yeast extract, 30 g/L gelatine, 17 g/L agar (pH 7.0) After incubation at 37°C for 48 h, the inoculated plates were flooded with a saturated ammonium sulphate solution. In this test, the appearance of a transparent halo around the colonies is considered an indicator of gelatin degradation by gelatinase, indicating a positive reaction. *S. aureus* ATCC29423 was used as a positive control.

2.4.4. Antibiotic susceptibility

Antibiotic resistance was assessed using the Kirby-Bauer method (agar diffusion). First, 100 µL LAB (probably the bacterial lactic acid cultures from the previous steps) were inoculated into 5 mL MRS broth and incubated at 37 °C for 24-48 h. After this incubation time, the optical density (OD) was adjusted to a 0.5 McFarland standard. Subsequently, 400 µL of this suspension was transferred to a new tube containing 5 mL of MRS broth. The contents of each tube were then evenly distributed onto two plates of Muller-Hinton agar. The plates were allowed to stand for approximately 25 min to allow the agar to absorb some of the liquid. Excess liquid was discarded, and the plates were allowed to dry thoroughly in a laminar flow cabinet for 1 h. The antibiotic discs were then applied to the agar surface using sterile forceps. Ten

antibiotics were tested: ampicillin (10 µg), oxacillin (1 µg), vancomycin (30 µg), gentamicin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), erythromycin (15 µg), clindamycin (2 µg), and kanamycin (30 µg). The discs were positioned so that the inhibition zones did not overlap, six discs per plate. The discs were incubated at 37 °C for 24-48 h. After incubation, the zones of inhibition, including the disc diameter, were measured using a ruler. *S. aureus* ATCC 25923 was used as positive control. The reference values from CLSI [23] were used to interpret the results.

2.4.5. Virulence genes

PCR was used to detect genes related to virulence (*gelE, hyl, asa1, esp*, *cylA, efaA, ace*), antibiotic resistance (*vanA, vanB*), and biogenic amine production (*hdc1, hdc2, tdc, odc*) as described by Ribeiro*, et al.* [24]. Genomic DNA was extracted from pure cultures as described in section 2.2. PCR was performed using a Thermocycler TProfessional Basic 96 (Biometra, Göttingen, Germany) with primers and expected fragment sizes detailed in Supplementary Table 1. Amplified products were resolved on a 2% agarose gel in 0.5×TAE buffer and stained with SybrGreen (15 µg/mL). Positive controls included genomic DNA from reference *E. faecalis* and *E. faecium* strains (IITAA collection) [14].

2.5 Potential probiotic properties of S. thermophilus GM4

2.5.1. Gastrointestinal resistance

Two tests were performed to evaluate resistance to gastrointestinal conditions: Resistance to pH 2.5 (to evaluate acidic gastric conditions) and resistance to bile acids and pancreatin (resistance to intestinal conditions) [14]. The isolates were inoculated in MRS broth and incubated at 37 °C for 24 h. After this time, the culture media were centrifuged in Falcon tubes at 4000 × *g* for 20 min at 20 °C (Centrifuge 5804R, Eppendorf, Germany). The pellet was washed with PBS buffer (pH 7.2) and centrifuged under the same conditions. The bacterial pellet was then diluted in 1 mL PBS to obtain the bacterial suspension. One hundred µL of this suspension was placed in two tubes: one tube contained 9.9 mL of a PBS solution with a pH of 2.5 (gastric conditions) and the other tube contained 9.9 mL of a PBS solution (pH 7.2) with 0.3% (w/v) bile salts and 0.1% pancreatin. The tubes were incubated at 37 °C for 1, 2 and 3 h,

reflecting the time the food spends in the stomach and intestine. After the indicated times, viable bacteria were counted by decimal dilution in peptone water and plating on MRS agar.

2.5.2. Mucin adhesion

The mucin-binding ability of *S. thermophilus* GM4 was evaluated using a 96-well plate method as described by Jurášková et al [25]. Porcine mucin (0.5 mg/mL in PBS) was used to coat wells overnight at 4 °C, followed by PBS washes to remove unbound mucin. Bacterial cells (10^8 CFU/mL in PBS) were added to the wells and incubated at 37 °C for 90 min. Non-adherent cells were removed by repeated PBS washes, and adherent cells were released using 0.05% Triton X-100. Viable counts were determined by plating on MRS agar and incubating at 37 °C for 48 h. The probiotic *Lacticaseibacillus rhamnosus* GG (ATCC 53103) was also tested for comparison.

2.5.3. Auto-agregation

Auto-aggregation was measured according to the method described by Todorov and Dicks [26]. The strain was inoculated in MRS broth and incubated at 30 °C for 24 h. After this time, the cultures were centrifuged at 4000 × *g* for 20 min at 20 °C. The bacterial pellet was washed with PBS and centrifuged again under the same conditions. PBS was added to the pellet and the initial absorbance ($Abs_{initial}$) was measured at 600 nm. After 60 min, the cultures were centrifuged again at 300 × *g* for 2 min at 20°C and the final absorbance (Abs_{final}) was measured. This test was performed in quadruplicate. Auto-aggregation was determined using the following equation:

$$\text{Auto} - \text{agregation}\ (\%) = \frac{(\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}})}{\text{Abs}_{\text{initial}}} \times\ 100$$

2.5.4. Co-agregation

The ability of *S. thermophilus* to co-aggregate with pathogenic bacteria was analyzed according to the methodology described by Chen*, et al.* [27]. The following pathogenic bacteria

were used for this test: *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 13932, *Staphylococcus aureus* ATCC 25923 and *Salmonella enterica* subs. *enterica* serovar Typhimurium ATCC 14028. Co-aggregation was determined using the following equation:

$$\text{Co} - \text{aggregation (\%)} = \frac{\left[\left(A_x + A_y\right)/\left(2 - A_{x+y}\right)\right]}{A_x + A_y} \times 100$$

A_x and A_y represent the absorbance (600 nm) of the two bacteria cell suspensions and A_{x+y} means the absorbance of mixed bacteria cell suspensions.

2.6. Functional properties of S. thermophilus GM4

2.6.1. *β*-galactosidase activity

The β-galactosidase activity of strain GM4 was assessed based on Miller's method, with slight adjustments [28]. The strain was cultured overnight in MRS broth at 37 °C, and its optical density was measured at 600 nm. For the assay, 20 µL of bacterial culture was mixed with 80 µL of freshly prepared permeabilization solution (containing 100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/mL CTAB, 0.4 mg/mL sodium deoxycholate, and 5.4 µL/mL β-mercaptoethanol added just before use). Then, 600 µL of substrate solution (1 mg/mL ONPG in phosphate buffer, pH 7.0, with 2.7 µL/mL *β*-mercaptoethanol added freshly) was added. The mixture was incubated at 37 °C for around 30 min or until a yellow color developed. The reaction was stopped with 700 µL of 1 M Na_2CO_3 and centrifuged at 16,100 × g for 5 min (Eppendorf model 5415D). The absorbance of the supernatant was read at 420 nm to calculate *β*-galactosidase activity.

$$\beta - \text{galactosidase activity (Miller units)} = 1000 \times \frac{\text{Abs420}}{(\text{Abs600} \times 0.2 \times \text{reaction time})}$$

2.6.2. Cholesterol lowering ability

The cholesterol-reducing ability of GM4 was evaluated in vitro using MRS broth supplemented with 0.5 mg/mL cholesterol [29]. The medium was inoculated with 1% log-phase bacterial culture and incubated at 37 °C for 24 h. The remaining cholesterol in the medium was measured enzymatically (CHOD-PAP method, NS Biotec, Egypt), and the reduction percentage was calculated as:

$$\text{Cholesterol reducing ability (\%)} = 100 \times \frac{\text{Cholesterol}_{initial} - Cholesterol_{final}}{Cholesterol_{initial}}$$

2.6.3. DPPH Scavenging activity

The antioxidant activity was determined using the DPPH method according to Aarti and Khusro [30], with some modifications. Overnight cultures were centrifuged and resuspended in dipotassium hydrogen phosphate buffer. The cells were added to 1 mL of a 0.2 mM DPPH solution and incubated for 30 min in the dark at room temperature. A methanol-treated sample served as a blank, and DPPH without bacteria was the control. Ascorbic acid was used as positive control. The absorbance was measured at 517 nm to determine the scavenging ability.

$$\text{DPPH scavenging activity (\%)} = \left[1 - \left(\frac{\text{A}_{\text{sample}} - \text{A}_{\text{blank}}}{\text{A}_{\text{control}}}\right)\right] \times 100$$

2.6.4. Hydroxyl scavenging activity

To evaluate the degradation of hydroxyl radicals, the method of He *et al.* [31] was adapted. The reaction mixture contained 1 mL 0.5 mM Brilliant Green, 2 mL 0.5 mM $FeSO_4$ and 1.5 mL H_2O_2. The bacterial cells were centrifuged and added to the mixture, which was incubated for 15 min at room temperature. The absorbance was then measured at 624 nm. Ascorbic acid was used as positive control.

$$\text{Hydroxyl radical scavenging activity (\%)} = \left[\frac{\text{A}_1 - \text{A}_0}{\text{A} - \text{A}_0}\right] \times 100$$

2.6.6. Production of EPS in skim milk

EPS production was investigated in skim milk supplemented with 10% lactose. A 1% bacterial inoculum was grown in MRS broth overnight at 37 °C and then centrifuged at 3500 × *g* for 30 min at 4 °C. The pellet was resuspended in skim milk with 10% lactose or without addition of this sugar (control), and incubated at 37 °C for 48 h. During fermentation, the pH, titrable acidity and viable counts in MRS (CFU/mL) were measured (at 0h, 2h, 4h, 6h, 24h, 48h). To extract EPS, 25 mL of the fermented medium was diluted 1:1 with MilliQ water. The casein was precipitated with 4 mL of 0.2 M TCA and removed by centrifugation (3500 × g, 30

min, 4 °C). The supernatant was adjusted to a pH of 6.8 with 4 M NaOH, then boiled for 30 min and centrifuged again to remove the whey proteins. An equal volume of cold ethanol was added to the supernatant to precipitate EPS at 4 °C overnight. The resulting pellet was resuspended in 10 mL of MilliQ water, sonicated for 1 h and vortexed until uniform. The solution was dialyzed against MilliQ water for 7 days at 4 °C (the water was changed twice daily) and then stored at -20 °C for further analysis. The EPS concentration was determined using the phenol-sulfuric acid method [16].

2.7. Purification and characterization of EPS from S. thermophilus GM4

2.7.1. Purification of EPS

EPS was produced using a modified method described by Domingos-Lopes *et al.* [32], with modifications. *S. thermophilus* GM4 (1% inoculum in 50 mL MRS broth) was grown overnight at 37 °C, then transferred to 1 L of modified MRS broth supplemented with 10% (w/v) of test sugars (glucose, fructose, sucrose, or lactose) for 48 h fermentation at 37 °C. Cells were removed by centrifugation (9,000 × *g*, 5 min, 4°C), and EPS was precipitated from the supernatant using two volumes of cold ethanol (4°C, 48 h). After recovery centrifugation (10,000 × g, 20 min, 4°C), the pellet was dissolved in Milli-Q water, dialyzed (3 days, 4 °C; water changed twice daily), and freeze-dried. The entire process was repeated four times to ensure sufficient material for EPS yield quantification, which was performed using the phenol-sulfuric acid method.

For further purification to analyze the EPS composition and linkage, the crude EPS was dissolved at a concentration of 5 mg/mL in a buffer solution (50 mM Tris–HCl, 10 mM $MgSO_4.7H_2O$, pH 7.5) and treated with DNAse type-I (2.5 µg/mL) for 6 h at 37 °C to remove any DNA. Subsequently, Pronase E (50 µg/mL in 50 mM Tris–HCl, 2% EDTA, pH 7.5) was added, and the mixture was incubated for 18 h at 37 °C to degrade any proteins present. This purified EPS was used for detailed compositional and linkage analysis.

2.7.2. EPS purity

EPS moisture and ash content was determined using thermogravimetric analysis (Setaram Setsys Evolution 1750 TGA) under a nitrogen atmosphere (200 mL/min) [33]. Total

sugar content was quantified by the phenol sulphuric acid method [16] and protein was assayed by the method of Bradford [34].

2.7.3. Carbohydrate Composition

Samples were analyzed for their neutral sugar content after acid hydrolysis, derivatization to alditol acetates, and analysis of individual neutral sugars and glycosamines by gas chromatography with flame ionization detector (GC-FID) and coupled to mass spectrometry detector (GC-MS). To identify uronic acids, hydrolyzed exopolysaccharides were analyzed using high-performance anion exchange chromatography with pulsed amperometric detection (HPAECPAD) [33]. The total sugars were determined by the sum of the amount of the individual sugars, considering that the hydrolysis of a glycosidic linkage results in an addition of a water molecule into the sugar structure.

Neutral Sugar Analysis by GC-FID

EPS (1 mg) was hydrolyzed in 2 M TFA (120 °C, 1 h). After cooling and TFA evaporation (speedvac), the hydrolysate was reconstituted in water (1 mg/mL). A 60 µL aliquot was mixed with 80 µL internal standard (2-deoxyglucose, 0.1 g/L) and 100 µL of 25% NH_3. Samples were reduced with $NaBH_4$ (15% in 3 M NH_3; 1 h, 30°C) and acetylated with acetic anhydride/1-methylimidazole (30 min, 30°C) after acidification. Alditol acetates were extracted into dichloromethane, washed repeatedly with water, and dried under vacuum with anhydrous acetone.

A separate hydrolysis using 1M H_2SO_4 was also tested. Briefly, 1-2 mg of each EPS was weighed into 10 mL tubes, and 200 µL of 72% H_2SO_4 was added. After 3 h incubation at room temperature with occasional stirring, 2.2 mL of distilled water was added to achieve a 1 M concentration, and the mixture was incubated for 1 h at 100 °C. The tubes were cooled, and 0.5 mL was removed for subsequent uronic acid analysis. Hydrolysis continued for another 1.5 h at 100 °C, and the neutral sugars were analyzed by GC-FID after reduction and acetylation, as described above.

Alditol acetates from both methods were dissolved in 25 µL of anhydrous acetone and analyzed by GC-FID (Perkin Elmer–Clarus 400) equipped with a 30 m DB-225 column (i.d. 0.25 mm, film thickness 0.15 µm, J&W Scientific, Folsom, CA, USA). The oven temperature was held at

220 °C for 7 min, then increased to 240 °C at 20 °C/min and held for 1 min. The injector and detector temperatures were 220 °C and 240 °C, respectively, with an H_2 carrier gas flow rate of 1.7 mL/min.

Alditol acetates were also analyzed by GCqMS (GC-2010 Plus, Shimadzu, Japan) using a non-polar ZB-5HT INFERNO column (30 m × 0.25 mm × 0.10 μm). A 0.5 μL sample was injected in split mode at 250 °C. The oven temperature program was: 140 °C to 180 °C at 5 °C/min, hold 1 min; 180 °C to 250 °C at 5 °C/min; 250 °C to 350 °C at 100 °C/min. The He carrier gas flow rate was 0.86 mL/min. The mass spectrometer operated in electron impact (EI) mode at 70 eV, scanning *m/z* 50–400 in full-scan mode.

Uronic Acid Analysis

Uronic acids were determined by the *m*-phenylphenol colorimetric method, using galacturonic acid (GalA, 10 to 100 μg/mL) as standard. To 100 μL of diluted sample hydrolyzed with 1 M H_2SO_4 (1:4) were added 1 mL of 200 mM boric acid in 96%(w/w) H_2SO_4. After vortex for 5-7 s, the test tubes were heated at 100 °C, during 10 min. After cooling, 20 μL of *m*-phenylphenol were added, the test tubes were vortexed, and 300 μL were transferred to well plates in duplicate, performing a reaction time of 15-30 min before the absorbance was read at 520 nm.

Carbohydrate analysis by HPAEC-PAD

HPAEC-PAD analysis was performed as described by Circuncisão et al. [35] on a Dionex ICS-6000 system equipped with an AS-AP autosampler, an SP pump, a DC chromatography oven, and an electrochemical detector fitted with a permanent gold working electrode and an AgCl reference electrode. The system was controlled by Chromeleon 7.3 software (Thermoscientific Dionex), and detection was achieved using integrated amperometry with the standard quadruple waveform for carbohydrates. Separation was achieved using a Dionex CarboPac PA100 analytical column (250 × 4 mm) protected by a corresponding guard column (50 × 4 mm), maintained at 30 °C, with a constant flow rate of 1.0 mL/min. The eluents consisted of MilliQ water (eluent A), 500 mM NaOH (eluent B), and 500 mM sodium acetate (eluent C). Prior to analysis, EPS samples (1-2 mg) were hydrolyzed in 2 M TFA for 1 h at 120 °C in sealed tubes.

The hydrolysate was evaporated to dryness, reconstituted in MilliQ water to a final concentration of 1 mg/mL, and filtered through a 0.2 μm nylon membrane. A 25 μL aliquot of

the prepared sample was injected and eluted using a multi-step gradient: initial conditions were held at 100% A for 40 min, followed by a linear gradient to 82:3:15 (A:B:C) over 5 min, which was held isocratically for a further 10 min. This was followed by a 5-min step to 0:75:25, a 10-min wash with 100% B, and finally a 15-min re-equilibration to initial conditions. Standards of neutral sugars, aldobiouronic acid from arabic gum, and uronic acids were used to confirm or characterize the carbohydrate composition of EPS.

2.7.4. Methylation Analysis

Glycosidic linkage composition of EPS was analyzed by methylation [36]. EPS (1–2 mg) was dissolved in 1 mL of anhydrous dimethyl sulfoxide and then NaOH (40 mg) was added under argon. The samples were methylated by addition of 80 µL of CH_3I and stirring (20 min). $CHCl_3$/MeOH (1:1, v/v, 3 mL) was added, and the solution was dialyzed using membranes with 12 kDa cut-off against 3 lots of 1 L of 50% EtOH. The dialysate was evaporated to dryness and the material was remethylated using the same procedure. The methylated polysaccharide was hydrolyzed with 2 M TFA for 1h at 120 °C. After evaporation of TFA, the hydrolyzed methylated sugars were reduced with sodium borodeuteride ($NaBD_4$) and acetylated as described for the neutral sugar analysis by GC-FID.

The partially methylated alditol acetates were separated and analyzed by gas chromatography quadrupole mass spectrometry (GCqMS, GC-2010 Plus, Shimadzu, Japan) using a non-polar column HT5 (50 m × 0.22 mm × 0.10 µm, Trajan, Australia), with He carrier gas (1.75 mL/min) and injection of 0.5 µL in split mode (at 250 °C). The GC oven temperature program was set to start at 110 °C, raised to 170 °C at 7.5 °C/min, holding for 4.93 min, raised to 174 °C at 0.20 °C /min, raised to 250 °C at 15 °C/min, holding for 5 min. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 70–370 *m/z*, in a full scan acquisition mode. Chromatogram peaks were identified comparing all mass spectra with a laboratory made database and the Complex Carbohydrate Research Center Spectral database (https://glygen.ccrc.uga.edu/ccrc/specdb/ms/pmaa/pframe.html, accesed on 6 August 2025).

2.7.5. Molecular weight distribution and homogeneity

The molecular weight (Mw), number-average weight (Mn) and polydispersity index (Mw/Mn) of EPS were estimated using two PL aquagel-OH MIXED 8 µm 300x7.5 mm

columns protected by a PL aquagel-OH Guard 8 µm 50x7.5 mm pre-column on a Malvern Panalytical system (UK) on a OMNISEC v11.41 software. The columns and the refractive index detector were maintained at 40 °C during the analysis and the autosampler was maintained at 35 °C. The eluents (water and 0.1 M $NaNO_3$) were pumped at a flow rate of 0.9 mL/min. The columns were calibrated with pullulans (Polymer Laboratories, UK) in the range 5.8–380 kDa (calibration curve: logM= 74.262 - 11.199x + $0.626x^2$ - $0.012166x^3$, R^2=0.998). EPS was dissolved in 0.1 M $NaNO_3$ aqueous solution at 25 °C for 60 min to reach a 2-4 mg/mL concentration and filtered through a 0.22 µm PTFE filter [37].

2.7.6. Dextranase resistance

The EPS purified from GM4 was tested for dextranase resistance using 1,6-*α*-D-glucan 6-glucanohydrolase from *Chaetomium erraticum* (D0443, Sigma), as reported by Domingos-Lopes *et al* [32].

2.7.7. DPPH Scavenging activity

The DPPH radical scavenging ability of EPS was evaluated following the method of Hu*, et al.* [38]. The EPS was dissolved in distilled water at concentrations of 1, 2, and 3 mg/mL. For the assay, 2 mL of 0.1 mM DPPH solution was mixed with 2 mL of the EPS solution (denoted as A_1) and incubated in the dark for 30 min. To account for background absorbance, the DPPH solution was replaced with ethanol, and 2 mL of the EPS solution was added to measure A_2. As a control, 2 mL of distilled water was mixed with 2 mL of DPPH solution (denoted as A_0). Ascorbic acid (1 mg/mL) was used as a positive control. The DPPH scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = \left[1 - \left(\frac{A_1 - A_2}{A_0}\right)\right] \times 100$$

2.8.4. Hydroxyl scavenging activity

The hydroxyl radical scavenging capacity of EPS was determined using a modified version of the Fenton reaction [39]. The reaction mixture included 100 µL of 0.435 mM

Brilliant Green, 200 µL of 0.5 mM $FeSO_4$, 150 µL of 3.0% H_2O_2, and 100 µL of EPS solution. The mixture was incubated at room temperature for 20 min, then centrifuged at 5000 × g for 5 min before measuring absorbance at 624 nm. The scavenging activity was calculated using the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = \left[\frac{A_s - A_0}{A - A_0}\right] \times 100$$

As is the absorbance of the sample-containing mixture, A_0 is the absorbance of the mixture with distilled water instead of the sample, A is the absorbance of the complete Fenton reaction system without any sample. Ascorbic acid (1 mg/mL) was used as a positive control.

2.9. Statistical analysis

All experiments were conducted in triplicate, with results presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test to identify significant differences ($p < 0.05$) between treatment groups. All statistical analyses were performed using SPSS software (version 30; SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Tecnological characterization S. thermophilus strains

3.1.1. Production of EPS with different sugar sources

Figure 1 presents EPS production by *S. thermophilus* strains isolated from goat milk using different sugar sources. While the strains generally exhibited high EPS yields in glucose, sucrose, and lactose, their ability to utilize fructose varied. Two strains (GM1 and GM2) produced EPS across all tested substrates, including fructose. However, the failure of strains GM3 and GM4 to produce EPS in fructose indicates that sugar source may play a role in EPS production for some strains. These divergent results imply that while sugar type is not restrictive for the ropy phenotype, strain-specific metabolic differences likely influence substrate preferences.

The highest EPS yields (4.6–9.2 mg/mL) were observed in 10% sucrose, followed by glucose (4.5–8.5 mg/mL), lactose (4.3–6.2 mg/mL), and fructose (4.9–5.0 mg/mL). Strain GM2 achieved its maximum EPS production (9.2 mg/mL) in sucrose-supplemented MRS medium, highlighting sucrose as the most effective carbon source for this strain. In contrast, GM4 produced higher EPS levels in glucose, with comparable yields in lactose and sucrose.

These findings are consistent with prior studies indicating that sucrose is the preferred carbon source for EPS production by *S. thermophilus*, followed by glucose and lactose [4]. The strains under study produced higher EPS content than *S. thermophilus* ASCC 1275 (0.430 mg/mL in M17 medium supplemented with 1% sucrose) [40], and *S. thermophilus* ST1 (0.1358 mg/mL in skim milk containing 2% sucrose) [10]. However, some strains exhibit divergent preferences, with lactose leading to higher yields than sucrose [41,42], as *S. thermophilus* LY03, which produced higher EPS with lactose than glucose [43], and *S. thermophilus* JM905, which favored lactose as a carbon source [44].

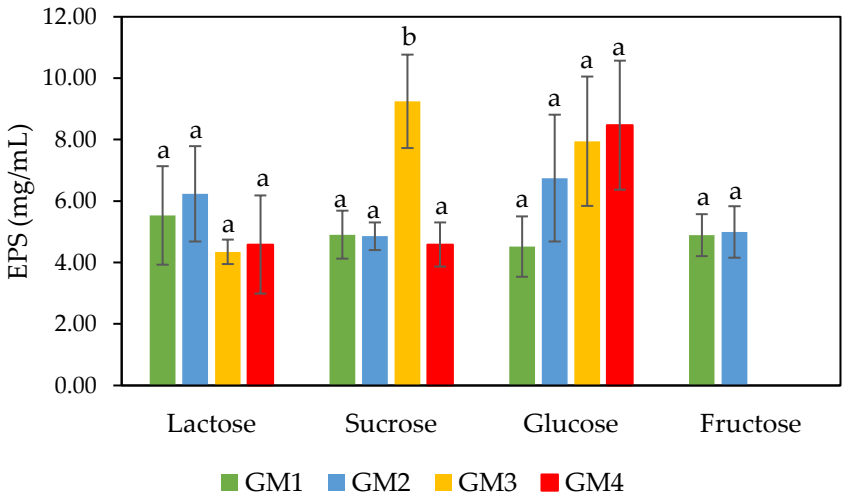
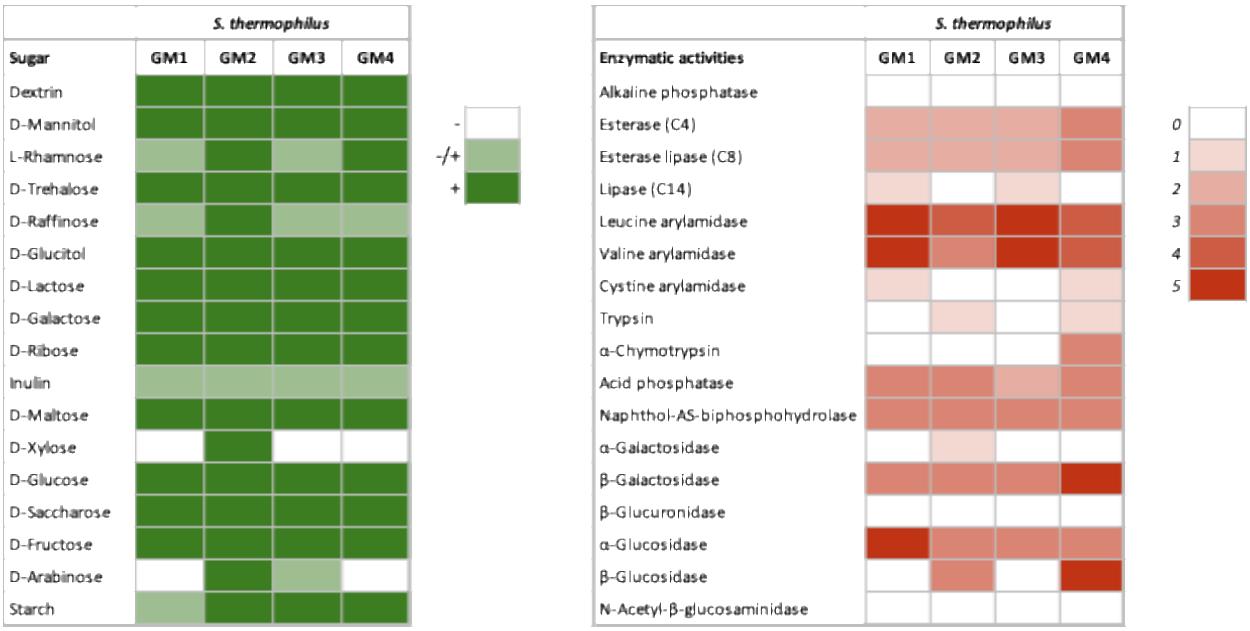


Figure 1. EPS production by *S. thermophilus* GM1, GM2, GM3 and GM4 in mMRS supplemented by different sugar sources: lactose, sucrose, glucose and fructose. Within each sugar, different letters indicate a significant difference between strains ($p < 0.05$).

Genomic and biochemical analyses confirm that *S. thermophilus* can utilize various sugars for EPS synthesis, though molecular mass and yield vary depending on the carbon source [45]. The optimal substrate for EPS production is strain-dependent, with sucrose and lactose often outperforming other sugars, though exceptions exist [41,42]

3.1.2. Carbohydrate fermentation

A broad fermentation profile was observed in all four strains of *S. thermophilus* (Figure 2). They showed the ability to ferment lactose, galactose, ribose, maltose, glucose, sucrose, fructose, raffinose, sorbitol (glucitol), mannitol, dextrin, inulin and starch. This broad substrate utilization indicates their metabolic versatility in a dairy context. The ability of *S. thermophilus* strains to utilize carbohydrates is of great importance in the fermentation of dairy products, as it has a direct effect on the rate of acidification of the milk. Various studies indicate that the utilization of glucose, lactose and fructose by *S. thermophilus* is consistently observed, while the utilization of sucrose, galactose and maltose has different profiles [2,46]. Gal-positive strains are of technological importance primarily because of their ability to ferment lactose completely. This results in a lower amount of galactose present, which cannot serve as a carbon source for spoilage or pathogenic bacteria [47,48].



(a) (b)

Figure 2. Heatmap of *S. thermophilus* GM1, GM2, GM3 and GM4 showing (**a**) carbohydrate fermentation (results were classified as negative -, slight positive -/+, and positive +); (**b**) enzymatic activities: ranging from 0 (no activity) to 5 (40 nmol hydrolyzed substrate).

3.1.3. Enzymatic activities

The *S. thermophilus* GM1, GM2, GM3 and GM4 exhibited high levels of desirable enzymatic activities, particularly aminopeptidases (leucine and valine arylamidases), *β*-galactosidase and acid phosphatase (Figure 2). The elevated aminopeptidase activity is a well-documented feature of high-quality *S. thermophilus* starter cultures [49]. These enzymes are of crucial importance for the hydrolysis of peptides from casein into free amino acids. They support bacterial growth in milk and play a crucial role in the development of flavor precursors during fermentation [50]. Such enzymatic activity not only accelerates acidification, which is crucial in the production of yoghurt and cheese, but also improves the sensory quality of the end product [49]. Acid phosphatase is also associated with improved acidification and survivability during cold storage, properties that are beneficial in the commercial processing of dairy products [51]. In addition, galactosidase activity is a probiotic characteristic (high in GM4), as evidence suggests that sufficient bacterial lactase production in the intestinal tract can prevent lactose intolerance symptoms in intolerant individuals [52].

All tested strains lacked detectable N-acetyl-β-glucosaminidase and β-glucuronidase activity - a crucial safety advantage. These enzymes pose significant health concerns as β-glucuronidase can release carcinogenic compounds (e.g., polycyclic aromatic hydrocarbons) through hydrolysis of glucuronides [53], while N-acetyl-β-glucosaminidase reduces N-nitro compounds to mutagenic/carcinogenic amines. Their absence minimizes potential risks of intestinal toxicity and carcinogenesis [54,55], making these strains particularly suitable for food applications.

3.1.4. Acidification, proteolytic and lipolytic activities

As shown in Table 1, most *S. thermophilus* strains exhibited limited pH drop after 6 h of incubation. However, *S. thermophilus* GM1 demonstrated significantly greater acidification ($p < 0.05$) compared to other isolates. By 24 h, strains GM1, GM3, and GM4 showed the most pronounced acidification ($\Delta pH > 1.5$), qualifying them as medium acidifiers. This variability in acidification capacity may be attributed to genetic differences among strains. Previous studies have associated rapid acidification phenotypes with the presence of *prtS*, which encodes a cell envelope proteinase crucial for efficient milk acidification [56].

Table 1. Acidification (ΔpH) in milk after 6 and 24 h, proteolytic and lipolytic activities of *S. thermophilus* strains.

***S. thermophilus* strains**	**Acidification**		**Proteolytic Activity**	**Lipolytic Activity**
	ΔpH 6h	**ΔpH 24h**		
GM1	0,37 ± 0,05[a]	1.64 ± 0.04[a]	-	-
GM2	0,05 ± 0,01[b]	1.32 ± 0.14[b]	-	-
GM3	0,05 ± 0,01[b]	1.55 ± 0.06[a]	+	-
GM4	0,01± 0,01[b]	1.63 ± 0.09[a]	+	-

Different letters as superscripts in the same column indicate a significant difference ($p < 0.05$). Positive (+) and negative (-) results.

Among the tested strains, only *S. thermophilus* GM3 and GM4 exhibited proteolytic activity, while none showed lipolytic capacity (Table 1). These findings align with previous reports describing typically weak proteolytic activity in *S. thermophilus*, albeit with strain-dependent variability [45]. Such differences in casein degradation may be attributed to the variable presence of *prtS* gene, despite the conservation of cytoplasmic peptidases across strains [57]. This limited proteolytic activity explains the common industrial practice of co-culturing *S. thermophilus* with *Lactobacillus delbrueckii* subsp. *bulgaricus* to enhance proteolysis through microbial synergy [57]. Proteolytic activity is an essential trait for *S. thermophilus*, enabling growth in milk by releasing amino acids that fulfill the strict nutritional requirements of this auxotrophic bacterium [46], particularly in pure culture [6]. This activity is also the primary driver of desirable flavors, textures, and bioactive peptides in fermented dairy products [4,49]. However, if uncontrolled, this same activity can lead to significant product defects, including the development of bitter off-flavors and a shortened shelf-life due to persistent protein breakdown [19].

The absence of detectable lipolytic activity in all strains presents a technological advantage for dairy applications, as it eliminates risks of fat degradation and associated off-flavor development [19].

3.2 Safety evaluation of S. thermophilus strains

3.2.1. Haemolysis, DNAse and gelatinase activity

All four isolates displayed γ-hemolysis (non-hemolytic activity) and tested negative for both DNase and gelatinase production (Table 2). These results confirm a favorable safety profile, supporting the classification of *S. thermophilus* as a non-pathogenic microorganism under the Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA) [58].

Table 2. Safety assessments of *S. thermophilus* strains, focusing on virulence-associated enzyme production, antibiotic resistance and virulence genes.

Strains	Hemolysis	DNase	Gelatinase	Antibiotic Resistance*	Virulence genes†
GM1	γ	-	-	OX, VAN, KAN	-
GM2	γ	-	-	OX, VAN, KANN, GEN	-
GM3	γ	-	-	OX, TETR, STR, ERY	-
GM4	γ	-	-	Sensitive to all	-

* OX: oxacillin; VAN: vancomycin; KAN: kanamycin; TETR: tetracycline; STR: streptomycin, GEN: gentamicin, ERY: Erythromycin.

† Virulence genes tested: *gelE, hyl, asa1, esp, cylA, efaA, ace, vanA, vanB, hdc1, hdc2, tdc,* and *odc*.

3.2.2. Antibiotic susceptibility

Antibiotic susceptibility profiling (Table 2) was performed according to EFSA guidelines for the assessment of bacteria for resistance to antibiotics of human or veterinary importance [59]. The antibiotics tested included ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol, selected for their relevance as critically or highly important antimicrobials in human and animal medicine [59]. Oxacillin was also tested as this β-lactam antibiotic is widely used to treat infections caused by *S. aureus* [23].

Distinct resistance profiles were observed among the *S. thermophilus* strains. Contrary to previous reports of common resistance to ampicillin, erythromycin, and tetracycline in dairy isolates [60], all the strains tested were susceptible to ampicillin, and resistance to the latter two antibiotics was limited (one strain each). The most frequent resistance was to oxacillin (3/4 strains), with GM2 and GM3 showing the broadest resistance (4 antibiotics each). In contrast, GM4 strain was susceptible to all antibiotics tested. This generally low resistance prevalence aligns with the understanding that significant acquired resistance is uncommon in *S. thermophilus* beyond intrinsic aminoglycoside resistance [61].

Probiotics should not add to the pool of antimicrobial resistance (AMR) genes already present in the gut bacterial population or otherwise increase the spread of AMR.

Although *S. thermophilus* holds GRAS (FDA) and QPS (EFSA) status for food applications, the presence of antibiotic resistance warrants evaluation of potential gene transfer to other bacteria, including pathogens. In this context, strain GM4 emerges as particularly promising for food and probiotic applications due to its complete antibiotic sensitivity profile. However, whole genome sequence analysis is essential to conclusively confirm the absence of transmissible antibiotic resistance genes.

3.2.3. Virulence genes

The absence of all seven tested virulence genes (*gelE, hyl, asa1, esp, cylA, efaA, ace, vanA, vanB, hdc1, hdc2, tdc,* and *odc*, Table 2) in the present study is noteworthy, especially when compared to the high prevalence of these determinants reported in several studies [62,63]. Even among foodborne LAB strains, a partial presence of virulence genes has been reported [64,65].

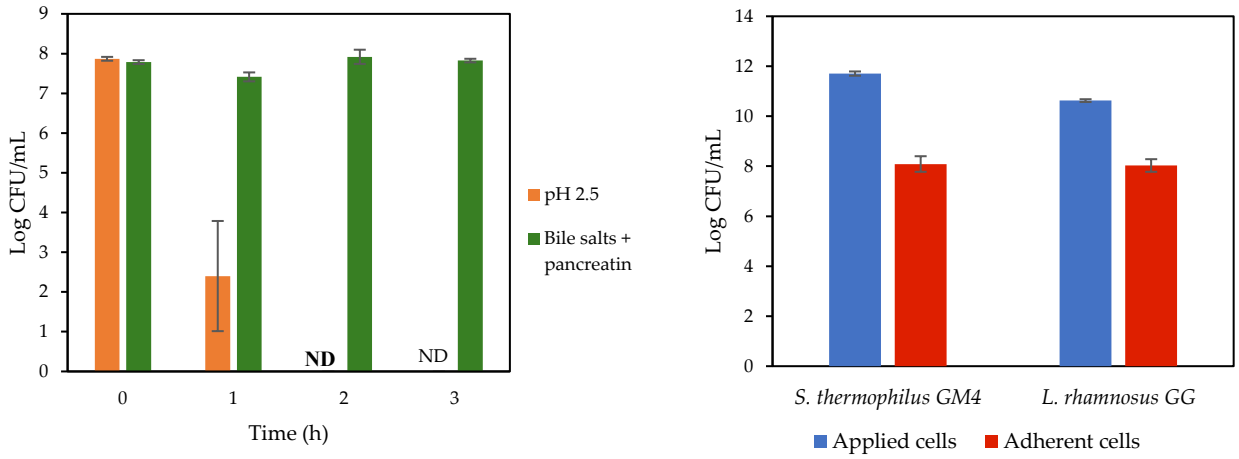
Based on its favorable safety profile – including the absence of virulence factors and antibiotic resistance – *S. thermophilus* GM4 was selected for subsequent probiotic evaluation and EPS characterization. However, its safe application in food may require further validation through animal studies.

3.3. Probiotic evaluation of S. thermophilus GM4

3.3.1. Gastrointestinal resistance and adhesion to mucin

As shown in Figure 3a, *S. thermophilus* GM4 exhibited moderate but consistent survival under simulated gastrointestinal conditions, with detectable viability after exposure to acidic stress (pH 2.5, 1 h) and intestinal challenges (0.3% bile salts + 0.1% pancreatin, 3 h). These survival levels are comparable to those reported for other *S. thermophilus* strains [66].

S. thermophilus GM4 exhibited strong mucin adhesion capability (69.1% binding efficiency), approaching the performance of the probiotic *L. rhamnosus* GG (75.5%) (Figure 3b). This high adhesion capacity, combined with its gastrointestinal stress tolerance, suggests that strain GM4 possesses relevant traits for probiotic applications, though further characterization of its functional properties would be valuable.



(**a**) (**b**)

Figure 3. (**a**) Resistance of *S. thermophilus* GM4 to low pH (2.5), bile salts (0.3%, w/v) and pancreatin (0.1%, w/v), after 0, 1, 2 and 3 h. ND: Not detected. Error bars represent SEM from two experiments; (**b**) Adhesion of *S. thermophilus* GM4 and *L. rhamnosus* GG to mucin. Error bars represent SEM from three experiments.

3.3.2. Auto-aggregation and co-aggregation

S. thermophilus GM4 exhibited strong aggregation properties that enhance its probiotic potential (Table 3). The high auto-aggregation ability (46.8%) is an indicator of cell surface hydrophobicity, a nonspecific interaction that can contribute to bacterial adhesion to cells. This

is an important mechanism that promotes intestinal colonization by favoring biofilm formation and adherence to the mucosa, thus improving persistence in the gastrointestinal tract [26]. This property is crucial also for the exclusion of competitors, as probiotic bacteria can physically occupy niches that would otherwise be available to pathogens [67].

Table 3. Auto-Aggregation (%) and co-Aggregation (%) of GM4 with pathogenic bacteria (*E. coli* ATCC 25922, *L. monocytogenes* ATCC 13932, *S. aureus* ATCC 25923 and *S.* Typhimurium ATCC14028), β-galactosidase activity, cholesterol lowering ability and antioxidant activity (DPPH and hydroxyl scavenging activities). Results are the average (n=3) ± SEM.

Auto-aggregation (%)	46.8 ± 0.9
Co-aggregation (%)	
Escherichia coli	36.0 ± 0.2
Listeria monocytogenes	31.4 ± 0.3
Staphylococcus aureus	30.9 ± 0.3
Salmonella enterica serovar Typhimurium	26.4 ± 0.2

Moreover, GM4 exhibited substantial co-aggregation with the key foodborne pathogens *E. coli, L. monocytogenes,* and *S. aureus* (>30%), along with moderate binding to *S. enterica* (26.4%). This suggests a direct, multi-targeted antagonistic mechanism. Such co-aggregation likely facilitates the physical "capture" and subsequent clearance of pathogens from the gut lumen via peristalsis, effectively reducing their viable population and opportunity to adhere and invade [27].

The observed binding profiles are consistent with established criteria for beneficial pro-biotic microorganisms, suggesting that *S. thermophilus* GM4 may effectively contribute to microbial barrier function in the intestinal ecosystem by 1) enhancing its own ecological ability to colonize and 2) acting as a biological scavenger to neutralise a spectrum of pathogens.

3.4. Functional properties of S. thermophilus GM4

3.4.1. *β*-galactosidase activity

The *β*-galactosidase activity of *S. thermophilus* GM4 was remarkably high at 56.2 ± 2.5 × 10^3 Miller units (Table 4). The *β*-galactosidase activity of GM4 surpassed values reported for

S. thermophilus ST20 and ST28 [4], and MCC0200 [5]. High enzymatic activity is also documented in *Lactobacillus acidophilus* strains [30]. This is a crucial finding, as *β*-galactosidase is essential for lactose hydrolysis, making it highly relevant for individuals with lactose intolerance. *S. thermophilus* sp. are widely recognized for their contribution to lactose digestion in fermented dairy products like yogurt. For instance, the *β*-galactosidase produced by *S. thermophilus* was shown to remain active during transit through the digestive tract, aiding in lactose breakdown [68,69].

Table 4. Functional characterization of the *S. thermophilus* GM4 strain: *β*-galactosidase activity, cholesterol lowering ability, antioxidant activity (DPPH and hydroxyl scavenging activities), EPS yield after fermentation (48h) in skim milk and skim milk supplemented with 10% lactose (Milk+Lac) and dextranase resistance of purified EPS. Results are the average (n=3) ± SEM.

β-galactosidase activity (10^3 Miller units)	56.159 ± 2.512
Cholesterol biding (%)	26.9 ± 3.3
DPPH scavenging activity (%)	22.7 ± 1.2
Hydroxyl scavenging activity (%)	5.7 ± 0.2
EPS yield Milk (g/L)	0.13 ± 0.03
EPS yield Milk+Lac (g/L)	2.58 ± 0.05
Dextranase resistance of EPS (%)	70.3 ± 1.2

3.4.2. Cholesterol lowering ability

S. thermophilus GM4 demonstrated a notable cholesterol-lowering ability (Table 4). This finding aligns with a growing body of research suggesting that certain LAB strains, including *S. thermophilus* strains, can contribute to cholesterol reduction. Several mechanisms are proposed for this effect, including the assimilation of cholesterol by bacterial cells [70]. While some studies on *S. thermophilus* strains report cholesterol removal rates ranging from 2.2% to 6.3% after short incubation periods [71], others, particularly for *Lactobacillus casei* strains, have shown higher reductions, sometimes exceeding 50-60% in simulated gastrointestinal conditions [70].

3.4.3. Antioxidant activity

Regarding antioxidant properties, *S. thermophilus* GM4 exhibited a DPPH scavenging activity of 22.7% (Table 4). This indicates a moderate ability to neutralize free radicals. LAB are known to produce various antioxidant compounds, including glutathione, NADH, and antioxidant enzymes, which contribute to their radical-scavenging capabilities [72,73]. *Lactobacillus* strains have shown DPPH scavenging activities ranging from approximately 11% to 35% [74]. In contrast, *S. thermophilus* GM4 hydroxyl scavenging activity was comparatively lower (5.70%, Table 4). Hydroxyl radicals are highly reactive and pose significant oxidative stress. While some probiotic strains have demonstrated higher hydroxyl radical scavenging activities (e.g., above 35% and even up to 82%) [75], the modest activity observed for GM4 suggests that its primary antioxidant defense might rely on other mechanisms (e.g. EPS production).

3.4.4. EPS production during milk fermentation

S. thermophilus GM4 demonstrated differential EPS yields depending on lactose concentration. In skim milk (lactose content 5%), EPS production remained low (0.13 g/L). However, lactose supplementation (10% w/v) significantly enhanced EPS yield by approximately 20-fold (2.58 g/L, Table 4), suggesting that carbohydrate availability represents a key limiting factor for EPS biosynthesis in dairy fermentations.

3.5. Characterization of EPS from S. thermophilus GM4

3.5.1. Purity and carbohydrate composition of EPS

The EPS from *S. thermophilus* GM4 had 51% of carbohydrates and the protein content was 122 mg/g (12%). This EPS was mainly composed by glucose (Glc, 53 mol%) and mannose (Man, 29 mol%), followed by uronic acids (8 mol%), ribose (Rib, 6 mol%), arabinose (Ara, 2 mol%), galactose (Gal, 2 mol%) and traces of rhamnose (Rha) (Table 5). Minor sugars (e.g. Rha) are likely attributable to trace contamination from glycoproteins or other medium components (yeast extract) co-purified during extraction.

Table 5. Carbohydrate composition of EPS from *S. thermophilus* GM4.

	Sugar composition (%mol)							Total sugars
	Rha	Rib	Ara	Man	Gal	Glc	Uronic acids	(mg/g)
EPS GM4	tr*	6	2	29	2	53	8	509 ± 12

* tr: trace amounts.

The EPS composition aligns with several reports on EPS produced by other *S. thermophilus* strains. For example, the EPS from *S. thermophilus* S-3 was also found to be primarily composed of glucose and mannose, with minor amounts of other sugars such as galactose [76]. Another study on EPS from *S. thermophilus* S-3 demonstrated a composition largely consisting of glucose (59.6%), galactose (21.4%), and mannose (19.0%) [77]. While the exact ratios and presence of minor sugars like ribose, rhamnose, and arabinose can vary between strains the dominance of glucose and mannose appears to be a recurrent theme for *S. thermophilus* and related LAB. Similar structural configurations have been also observed in EPS from *S. thermophilus* ST538 [7].

The analysis of hydrolyzed EPS in HPAEC-PAD confirmed the neutral sugar composition and showed that EPS main uronic acid was glucuronic acid followed by galacturonic acid (Figure 5). This profile closely matched those reported in microbial EPS such as *Flavobacterium* (glucuronic and glucose dominant) [78], *Enterobacter* A47 (approx. 29 mol % glucuronic acid) [79], and *Tetraselmis suecica* EPS containing approx. 20–25 % glucuronic acid plus minor galacturonic acid (approx. 0.1–3 %) [80].

To elucidate the glycosidic linkages of EPS, these were methylated and analyzed as partially methylated alditol acetates (PMAA) by GC-MS (Figure 5). The methodology used only allows to see the glycosidic linkages of neutral sugars, as uronic acids were not carboxyl reduced. The main linkages found in EPS from *S. thermophilus* GM4 (Table 6) were (1→4)-Glc*p* (57.1 mol%), terminally linked Glc*p* (t-Glc*p*, 21.6 mol%), (1→2,6)-Man*p* (7.1 mol%), (1→2)-Man*p* (6.6 mol%), and (1 →4,6)-Glc*p* (5.4 mol%), followed by traces of (1→6)-Man*p* and (1→6)-Glc*p*, (1.1 and 0.9 mol%, respectively).

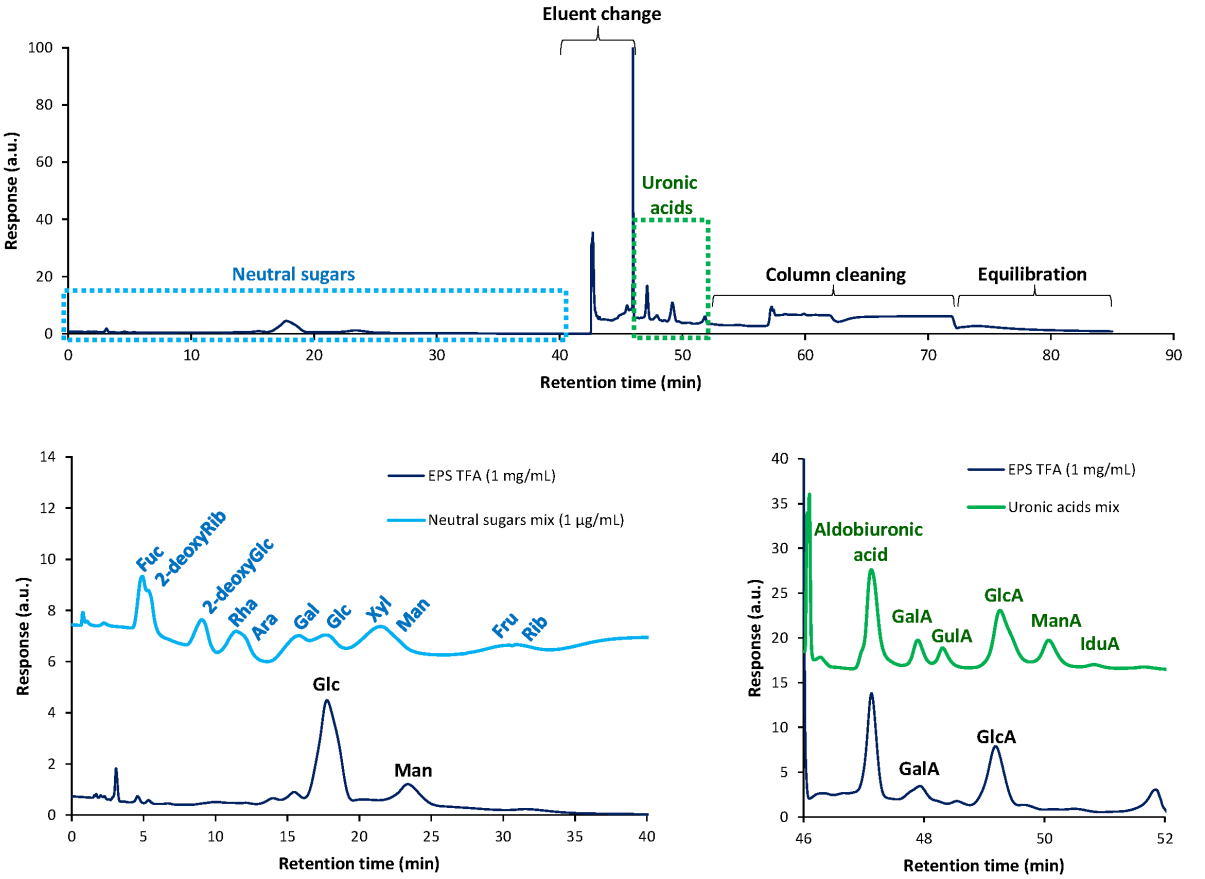


Figure 4. HPAEC-PAD chromatogram of hydrolyzed EPS and comparison with neutral sugars and uronic acids standards.

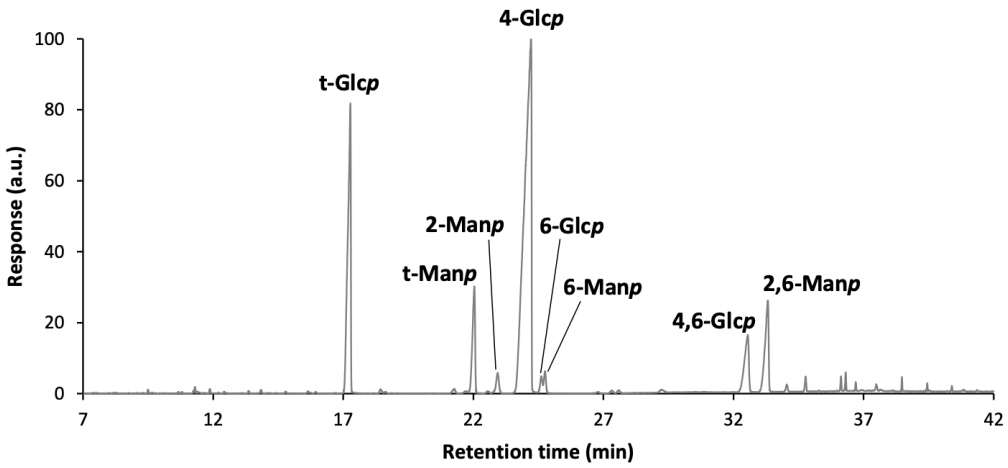


Figure 5. GC-MS chromatogram of partially methylated alditol acetates of EPS from *S. thermophilus* GM4.

Table 6. Glycosidic linkage analysis of EPS from *S. thermophilus* GM4.

Deduced linkages	mol%
t-Glc*p*	21.6
4-Glc*p*	57.1
6-Glc*p*	0.9
4,6-Glc*p*	5.4
Total Glc*p*	**85.0**
2-Man*p*	6.6
6-Man*p*	1.1
2,6-Man*p*	7.1
Total Man*p*	**14.7**

The presence of lower amounts of deduced linkages for Man, in comparison with previous compositional analysis, could indicate their linkage to uronic acids and formation of aldobiuronic disaccharides, along methylated EPS hydrolysis, that are lost during PMAA extraction with dichloromethane, before GC-MS analysis. This is in accordance with HPAEC-PAD analysis, where a peak in the elution time of aldobiuronic can be seen (Figure 4).
This complex structure of GM4 EPS is consistent with patterns observed in other LAB-derived polysaccharides [8,76].

3.5.2 Molecular weight distribution

Size-exclusion chromatography (SEC) analysis of the EPS revealed two distinct peaks: Peak 1 at 17.0 min and Peak 2 at 18.9 min, corresponding to two polymer populations of considerably different molecular mass (Figure 6).

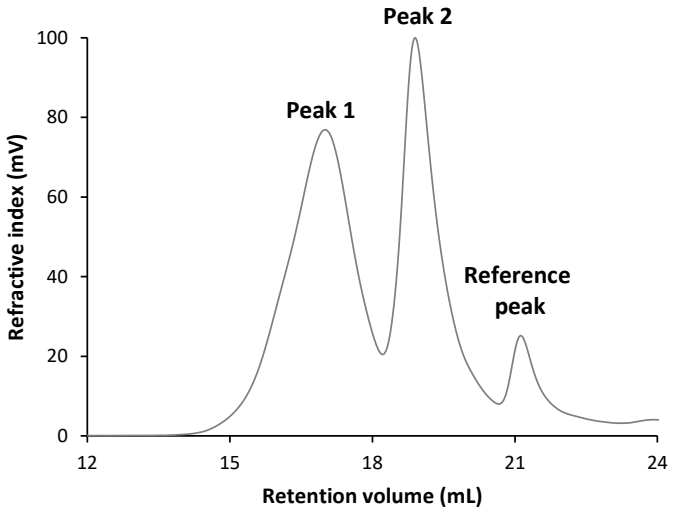


Figure 6. GPC chromatogram of EPS from *S. thermophilus* GM4.

The presence of these two populations of polymers gave average values of Mn = 12.0 kDa and Mw = 112 kDa, when an overall integration of the chromatogram was performed, revealing a highly polydisperse global distribution (Mw/Mn = 9.27, Table 7). The analysis of each peak showed that the peak 1, associated with a higher molar mass fraction, had average values of Mn = 95.1 kDa and Mw = 189 kDa, while Peak 2 had Mn = 5.6 kDa and Mw = 10.1 kDa. The dispersity within each peak (Mw/Mn = 1.99 and 1.80, respectively) suggests broad distributions, indicating that each population presents considerable variation in chain size.

Table 7. The weight and number-average molecular weight (Mw and Mn) and polydispersity index (Mw/Mn) of EPS from *S. thermophilus* GM4.

	Total	**Peak 1**	**Peak 2**
Mn (g/mol)	12027	95102	5614
Mw (g/mol)	111500	188959	10110
Mw/Mn (g/mol)	9.271	1.987	1.801

This heterogeneity aligns with reports for other LAB EPS: *Limosilactobacillus reuteri* FW2 produces a bimodal levan (4.6×10^6 Da and 1.2×10^4 Da fractions) [81], while *Lactiplantibacillus plantarum* EPS exhibits similar size polymorphism under different carbon sources [82]. Such

multimodal distributions appear characteristic of microbial EPS, likely reflecting biological synthesis mechanisms that generate diverse polymer lengths.

The functional consequences of this molecular diversity are potentially significant: higher-MW fractions may contribute to viscosity, film-forming ability, and immunomodulatory action, while lower-MW fractions could influence solubility, diffusivity, or prebiotic activity. Previous reports link EPS Mw with bioactivity such as antioxidant activity, immune stimulation, and rheological behavior [83].

3.5.3. Dextranase resistance

The EPS from GM4 exhibited high dextranase resistance (70.3%, Table 4). This level of resistance is similar to that found in EPS from *L. reuteri* [84] and *Leuconostoc citreum* [32], all of which demonstrate high resistance due to specific 4- and 6-linked sugar residues and high molecular weight. While direct studies on dextranase resistance of *S. thermophilus* EPS are limited, comparative data from other LAB species suggest significant variability in dextranase resistance. For instance, a dextran-type EPS produced by *Leuconostoc mesenteroides* strain demonstrated only 52% resistance to dextranase degradation [85].

3.5.4. Antioxidant activity

The EPS from *S. thermophilus* GM4 demonstrated substantial antioxidant capacity, particularly in DPPH radical scavenging, reaching 52.46% at 3 mg/mL (Table 8). This observation aligns with previous reports on LAB-derived EPS, including *S. thermophilus* strains showing 55.83% DPPH scavenging at 1 mg/mL [86]. While the hydroxyl radical scavenging activity was more modest (12.86% at 3 mg/mL), it remains comparable to values reported for other bacterial EPS [71]. This difference in scavenging efficiency between radical types reflects the varying reactivity of these oxygen species and the specific antioxidant mechanisms involved.

Table 8. Antioxidant activity of EPS produced by *S. thermophilus* GM4.

EPS (mg/mL)	DPPH scavenging activity (%)	Hydroxyl scavenging activity (%)
1	30.10 ± 2.43	2.41 ± 0.40
2	41.32 ± 0.74	7.26 ± 1.02
3	52.46 ± 0.57	12.86 ± 0.67

The antioxidant properties of the EPS produced by GM4 strain are likely the result of the structural features of its polymers, particularly the presence of electron-donating functional groups such as hydroxyl and carboxyl groups, that facilitate free radical neutralization [87]. Furthermore, the consistent dose-dependent response observed in both assays underscores the EPS's potential as a natural antioxidant for food or biomedical applications.

4. Conclusions

This study comprehensively characterizes four EPS-producing *S. thermophilus* strains (GM1–GM4) isolated from goat milk, with GM4 emerging as a particularly promising candidate for functional dairy applications. All isolates exhibited efficient lactose fermentation, broad sugar utilization, and desirable enzymatic profiles (notably aminopeptidase and acid phosphatase activity), while lacking harmful enzymes and virulence traits. GM4 was prioritized for further study due to its absence of antibiotic resistance, confirmed safety profile (no hemolytic, DNase, or gelatinase activity), and probiotic potential—demonstrated by gastrointestinal stress tolerance (pH 2.5, bile salts), moderate antimicrobial activity, strong mucin adhesion (69.1%), and pathogen co-aggregation. Technologically, GM4 showed exceptional β-galactosidase activity (56,158.95 ± 1,450.28 units) and a versatile carbohydrate fermentation profile. Its EPS, a high-molecular-weight heteropolysaccharide rich in glucose, mannose, and uronic acid with branched structure, exhibited notable antioxidant capacity (52.46% DPPH scavenging) and dextranase resistance, underscoring its stability and bioactivity. Collectively, these attributes position *S. thermophilus* GM4 as a multifunctional strain capable of enhancing dairy products through simultaneous technological (texture, lactose

reduction) and functional (probiotic, antioxidant) benefits, meriting further development for food applications.

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Chapter 6 : General discussion and conclusions

EPS produced by LAB have emerged as multifunctional biomolecules of noteworthy scientific and industrial interest. While traditionally valued as starter cultures for fermentation, LAB are now increasingly recognized for their ability to synthesize extracellular polymers that confer both technological and health-related advantages. This thesis systematically investigates EPS from selected LAB strains, focusing on their structural characteristics, technological applications, and health-promoting properties to contribute to this evolving field.

A key contribution of this work is the characterization of the structural and functional diversity of LAB-derived EPS. The high-molecular-weight glucan produced by *Leuconostoc mesenteroides* SJC113, featuring α-(1,6) linkages with α-(1,3) branching, demonstrated resistance to dextranase hydrolysis. This structural profile is critical, as molecular weight and branching directly influence rheological behavior, solubility, and digestibility [1,2]. While similar branching patterns are observed in dextrans from other *Leuconostoc* strains, their yield and enzymatic resistance can vary considerably [3]. In contrast, *Streptococcus thermophilus* GM4 produced two heteropolysaccharides (12.0 kDa and 112 kDa) composed primarily of glucose and mannose, with minor amounts of uronic acids, ribose, arabinose, and galactose. Furthermore, the EPS from *Ln. mesenteroides* SJC113 was mucoid, while that from *S. thermophilus* GM4 was ropy—a texture consistent with strains used in yogurt production [6,7].

Functional evaluations confirmed that these EPS act as active contributors to food texture, stability, and health. Technologically, both polymers enhanced dairy systems by exhibiting antioxidant capacity. In addition, the dextran from *Ln. mesenteroides* SJC113 increased water-holding capacity and improved the rheological profile of fat-free fresh cheese. This finding is highly relevant for the dairy industry, which seeks to reduce fat content without compromising texture and mouthfeel, and provides further experimental support for using LAB-EPS as natural fat replacers [5,6].

In addition to establishing the safety of the strains studied, a key result of this work was the detailed characterisation of their functional and technological properties. These results are crucial for understanding their potential as starter or additional cultures in dairy fermentations and as sources of bioactive exopolysaccharides (EPS).

Ln. mesenteroides SJC113 proved to be a strong EPS producer, forming a high molecular weight dextran-type homopolysaccharide resistant to hydrolysis by dextranase. This EPS exhibited remarkable functional activities, including antioxidant capacity and cholesterol

binding capacity, thus contributing to both food quality and health-promoting effects. From a technological perspective, SJC113 showed measurable *β*-galactosidase activity, which is important for lactose hydrolysis and could alleviate symptoms in lactose-intolerant consumers. The strain tolerated elevated concentrations of sodium chloride, suggesting robustness under variable processing conditions. Application trials in fat-free fresh cheese showed that in situ production of EPS significantly improved water holding capacity, rheological behaviour and sensory properties, effectively compensating for the absence of fat and increasing consumer acceptance. These results emphasise the dual role of SJC113, as it simultaneously contributes to a desirable food texture and provides functional bioactivity.

S. thermophilus GM4 proved to be the most promising of the four goat milk isolates analysed. Its broad carbohydrate fermentation profile, which includes lactose, galactose, glucose, fructose, ribose, and maltose, indicates great metabolic versatility and adaptability to different milk substrates. Enzymatic tests showed increased aminopeptidase and acid phosphatase activities, both of which are crucial for proteolysis and flavour development in cheese. Importantly, undesirable enzymatic activities such as *β*-glucuronidase, *α*-chymotrypsin, and N-acetyl-*β*-glucosaminidase were absent or negligible, further enhancing the technological suitability of the strain. GM4 also exhibited high *β*-galactosidase activity, emphasising its importance for lactose digestion and its positioning as a probiotic additive. Functionally, GM4 showed autoaggregation and co-aggregation with pathogens (*E. coli, L. monocytogenes, S. aureus*), properties associated with the exclusion of competition in the gastrointestinal tract. The EPS, a heteropolysaccharide composed mainly of glucose and mannose, showed a remarkable antioxidant capacity and resistance to enzymatic degradation, extending its potential as a bioactive ingredient.

Taken together, these results show that *Ln. mesenteroides* SJC113 and *S. thermophilus* GM4 are not only microbiologically safe, but also technologically robust and functionally versatile. SJC113 is particularly valuable for its dextran-like EPS with techno-functional and health benefits, while GM4 combines efficient lactic fermentation characteristics, proteolytic capacity, probiotic potential, and production of bioactive EPS. Their complementary properties make them strong candidates for the next generation of functional dairy products, combining sensory quality, technological performance, and health-promoting effects.

Safety assessments of the EPS-producing strains further support their potential application. The absence of virulence genes and favorable antibiotic susceptibility profiles of *Ln.*

mesenteroides SJC113 and *S. thermophilus* GM4 are consistent with the long history of safe use of LAB in food fermentation [12], reinforcing their suitability for commercial use.

Beyond the technological roles, this thesis provides compelling evidence for the bioactive potential of LAB-EPS. Both polymers exhibited consistent *in vitro* antioxidant capacity, a property attributed to radical-scavenging functional groups such as hydroxyl and carboxyl moieties [8,9]. Particularly noteworthy is the observed cholesterol-binding activity of the dextran, suggesting a potential mechanism for cardiovascular benefits by binding bile salts and cholesterol to reduce absorption, a phenomenon supported by previous research [10].

This work also shows that the dextran from *Ln. mesenteroides* SJC113 exhibits prebiotic and microbiota-modulating properties. It stimulated the growth of beneficial LAB *in vitro*, promoted mucin adhesion, and altered fecal microbiota composition. These findings support its classification as a fermentable dietary fiber with prebiotic activity, aligning it with established microbiota-modulators like β-glucans [3,11] and underscoring its potential as a dietary component for gut health.

Collectively, these findings position EPS-producing LAB as promising candidates for designing next-generation functional foods. From a technological standpoint, their ability to act as natural stabilizers, viscosity enhancers, and fat replacers offers a clean-label alternative to synthetic additives, addressing current consumer and industry trends [5]. The dairy sector, in particular, can leverage these traits to maintain quality in reduced-fat products [6].

The demonstrated health-promoting attributes, including antioxidant capacity, cholesterol-binding, and prebiotic effects, elevate EPS from mere technological agents to real bioactive compounds. The prebiotic properties are especially significant, given the established role of gut microbiota in influencing metabolic, immune, and neurological health [11]. The ability of EPS to selectively stimulate beneficial microorganisms suggests they may complement or even surpass traditional prebiotics like inulin [3].

At a broader level, this thesis contributes to the recognition of EPS production as a valuable probiotic trait. Beyond traditional criteria, EPS can enhance bacterial survival, facilitate colonization, and deliver direct bioactive benefits, positioning EPS-producing LAB as next-generation probiotics [13,14]. Thus, EPS can be viewed as extracellular effectors that mediate complex interactions between probiotics, the host, and the gut microbiota, expanding the mechanistic understanding of probiotic action.

In conclusion, this thesis has provided new insights into the multifunctional roles of EPS derived from LAB, integrating structural characterization, technological evaluation, and health-related functions. By demonstrating the complementary properties of dextran-type EPS from *Leuconostoc mesenteroides* SJC113 and heteropolysaccharides from *Streptococcus thermophilus* GM4, it underscores the structural and functional diversity of these biomolecules. The results confirm that EPS are not only key contributors to texture and stability in dairy systems but also bioactive compounds with antioxidant, cholesterol-binding, and prebiotic effects.

Taken together, the findings of this thesis support the positioning of EPS-producing LAB as valuable tools for the development of next-generation functional foods. They also highlight the importance of EPS as extracellular effectors of probiotic action, expanding the current understanding of how beneficial microbes contribute to host health. While challenges remain in terms of yield optimization, in vivo validation, and regulatory approval, the prospects for EPS are promising. The growing convergence of food science, microbiology, and human health research provides fertile ground for advancing EPS from a largely academic topic to a cornerstone of functional food innovation. Ultimately, this work contributes to the broader vision of designing sustainable, health-promoting foods that meet both consumer expectations and societal needs.

6.1 Future prospects

The work presented in this thesis on lactic acid bacteria-derived exopolysaccharides (EPS) provides a solid basis for future research and commercial applications. A major focus must be on the optimisation of EPS production on an industrial scale. This includes not only fine-tuning fermentation conditions, but also exploring the genetic and metabolic engineering of superior strains to increase yield and control EPS structure. In addition, the relationship between EPS structure and their specific bioactivities remains a significant knowledge gap. Advanced analytical techniques such as NMR and mass spectrometry, in combination with high-throughput screening, will be crucial to fully capture how different monosaccharide compositions, linkages, and branching patterns influence functional properties such as prebiotic effects and immunomodulatory activity. Finally, while this work has successfully demonstrated the potential of certain EPS strains in in vitro and ex vivo models, the next crucial step is to

validate these health-promoting effects through large-scale human clinical trials. This will provide the necessary evidence for regulatory approval and market acceptance and pave the way for the widespread use of these natural biopolymers in clean label, functional foods.

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Annexes

Annex 1 – Supplementary material of author's contribution

This thesis is divided into six chapters that include a version of four original papers, general introduction at the beginning of the thesis, general conclusions and discussion at the end of the thesis.
To the author knowledge, this thesis contains no material previously published nor written by another person. Author contributions to each chapter will be described below.

Chapter 1 - Introduction
Dominika Jurášková wrote the text, which was revised by the supervisors.

Chapter 2 - Literature Review
Exopolysaccharides Produced by Lactic Acid Bacteria: From Biosynthesis to Health-Promoting Properties
A version of this chapter has been published. Dominika Jurášková wrote the manuscript. The co-authors S. Ribeiro and C.C.G. Silva revised and agreed with the manuscript.
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Chapter 3 - Exopolysaccharide (EPS) Produced by *Leuconostoc mesenteroides* SJC113: Characterization of Functional and Technological Properties and Application in Fat-Free Cheese
A version of this chapter has been published. Dominika Jurášková, prof. Dr. Célia C. Silva, Dr. Susana Riberiro planned the majority of the experimental design and Dominika Jurášková performed the majority of the work and wrote the manuscript. Parts of the experimental work was also performed by Elisabete Coelho and Rita Bastos (EPS Characterization). All co-authors revised the manuscript and agreed with the publication.
Jurášková, D., Ribeiro, S. C., Bastos, R., Coelho, E., Coimbra, M. A., & Silva, C. C. G. (2024). Exopolysaccharide (EPS) Produced by Leuconostoc mesenteroides SJC113: Characterization of Functional and Technological Properties and Application in Fat-Free Cheese. *Macromol*, *4*(3), 680-696.

Chapter 4 - Prebiotic and health-promoting benefits of dextran-type exopolysaccharide produced by *Leuconostoc mesenteroides* SJC113

A version of this chapter has been published. Dominika Jurášková, prof. Dr. Célia C. Silva, Dr. Susana Riberiro planned the majority of the experimental design and Dominika Jurášková performed the work and wrote the manuscript. All co-authors revised the manuscript and agreed with the publication.

Jurášková, D., Ribeiro, S. C., & Silva, C. C. G. (2025). Prebiotic and Health-Promoting Benefits of Dextran-Type Exopolysaccharide Produced by Leuconostoc mesenteroides SJC113. *Foods*, *14*(15), 2635.

Chapter 5 - Exopolysaccharide (EPS)-producing *Streptococcus thermophilus*: Functional and Probiotic Potential

A version of this chapter has been submitted. Prof. Dr. Célia Silva and Dr. Susana Ribeiro planned the majority of the experiment. Dominika Jurášková and Vanessa C. Pires performed the majority of the experimental work. Parts of the experimental work was also performed by Sónia S. Ferreira, Fábio Bernardo and Dimitry Evtyugin (EPS Characterization). Dominika Jurášková wrote the manuscript. All co-authors revised the manuscript and agreed with the publication.

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All co-authors revised the manuscript and agreed with the publication.

Chapter 6 – General discussion and conclusions

Dominika Jurášková wrote the text, which was revised by the supervisors.

Annex 2 – Supplementary material of Chapter 5

Table S1. Primers used for PCR detection of virulence genes.

Target Gene	Primers Sequences	Fragment Size (bp)	References
Gelatinase (*gel*E)	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213	[1]
Hyaluronidase (*hyl*)	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276	[1]
Aggregation substance (*asa*1)	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	[1]
Enteroccocal surface protein (*esp*)	AGATTTCATCTTTGATTCTTG AATTGATTCTTTAGCATCTGG	510	[1]
Cytolisin (*cyl*A)	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	[1]
Endocarditis antigen (*efa*A)	GCCAATTGGGACAGACCTC CGCCTTCTGTTCCTTCTTTGGC	688	[2]
Adhesion of collagen (*ace*)	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	1008	[2]
Vancomycin resistance (*van*A)	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCAGCATT	377	[2]
Vancomycin resistance (*van*B)	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC	529	[2]
Histidine decarboxylase (*hdc*1)	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	367	[3]
Histidine decarboxylase (*hdc*2)	AAYTCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTTRTGNCC	534	[3]
Tyrosine decarboxylase (*tdc*)	GAYATNATNGGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG	924	[3]
Ornithine decarboxylase (*odc*)	GTNTTYAAYGCNGAYAARCANTAYTTTGT ATNGARTTNAGTTCRCAYTTYTCNGG	1446	[3]

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LAB Exopolysaccharides: Production, Characterization and Food Applications

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