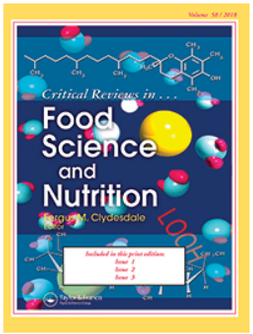

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Exopolysaccharides produced by *Lactobacillus* sp.: Biosynthesis and applications

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ABSTRACT

Lactobacillus sp. synthesize exopolysaccharides (EPS), including both homo- and heteropolysaccharides, which play an important role in the production of fermented foods, and especially in the dairy industry, improving the gustatory and rheological properties of the finished products. These polymers are generated by starter cultures *in situ* in fermented foods, and so they are treated as natural thickening agents. As some *Lactobacillus* strains are generally recognized as safe and have been shown to exhibit probiotic activity, EPS from those bacteria can be used as functional food ingredients, conferring both health and economic benefits to the consumers. However, their industrial applications are hindered by the low yield of EPS from *Lactobacillus* and high costs of their purification. This review focuses on the latest reports concerning the biosynthesis and properties of *Lactobacillus* EPS.

List of abbreviations: EPS: exopolysaccharides; HoPS: homopolysaccharides; HePS: heteropolysaccharides; Glc: glucose; Gal: galactose; Rha: rhamnose; Galp: galactopyranose; Glcp: glucopyranose; Rhap: rhamnopyranoside; GlcA: glucosamine; GlcNac: N-acetylglucosamine; Galp2Ac: 2-O-acetylgalactopyranose; GTF: glycosylotransferases; PEP-PTS: PhosphoEnolPyruvate-sugar PhosphoTransferase System; PEP: phosphoenolpyruvate; C55-P carrier: undecaprenyl phosphate carrier; PGM: phosphoglucomutase; PMI: phosphomannose isomerase; PMM: phosphomannomutase; GMP: GDP-mannose pyrophosphorylase; TGP: dTDP-glucose pyrophosphorylase; UGP: UDP-glucose pyrophosphorylase; TRS: dTDP-rhamnose synthetic enzyme system; UGE: UDP-galactose 4-epimerase; UDP-Glc: UDP-glucose, sugar nucleotides; dTDP-Glc: dTDP-glucose, sugar nucleotides; GDP-Man: GDP-mannose, sugar nucleotides; GDP-Fuc: GDP-fructose, sugar nucleotides; UDP-Gal: UDP-galactose; dTDP-Rha: dTDP-rhamnose

KEYWORDS

Lactobacillus sp.; exopolysaccharides; biosynthesis; functionality; perspectives

Introduction

Lactobacillus sp. are used in the food industry around the globe, being generally recognized as safe (GRAS; Widyastuti and Rohmatussolihat, 2014), and with probiotic properties exhibited by many strains, such as *Lb. rhamnosus* IMC 501®, *Lb. rhamnosus* GG, *Lb. paracasei* IMC 502®, *Lb. plantarum* 299V, *Lb. casei* Shirota YIT9029, *Lb. casei* DN-114 001, *Lb. johnsonii* NCC 533, *Lb. acidophilus* LB, and *Lb. reuteri* DSM 17938 (Liu et al., 2011; Coman et al., 2013; Rubio et al., 2013; Liévin-Le Moal and Servin, 2014). These *Lactobacillus* species confer health benefits to the human gastrointestinal system and play a significant role in the production of fermented foods, including dairy products, vegetables, and meat. They also synthesize many substances, such as bacteriocins (lantibiotics), organic acids (e.g., lactic and acetic) and fatty acids, aromatic compounds, and hydrogen peroxide, which are used in diverse industrial applications. Among others, *Lactobacillus* sp. produce exopolysaccharides (EPS), which have attracted the interest of researchers around the world (Górska et al., 2007; Gajewska and Błaszczyk, 2010).

EPS are high molecular weight, long-chain, linear, or branched biopolymers consisting of frequently repeating saccharide units or saccharide derivatives linked by α- and β-glycoside bonds. EPS are secreted to the external environment in the form of slime (slime EPS) or adhere to the cell bacterial surface forming a capsule (capsular EPS). For many years now, EPS have been used in the food industry to improve the texture, sensory qualities, nutritional properties, and stability of fermented products (Vuyst et al., 2001; Ruas-Madiedo et al., 2002; Górska et al., 2007). They are also widely used in the cosmetic and pharmaceutical industries as bioflocculants, bioabsorbents, and heavy metal absorbers. EPS have been reported to be produced by many animals, plants, fungi, and bacteria, with the global market dominated by plant and alga EPS, such as starch, galactomannan, pectins, carrageenan, and alginate (Badel et al., 2011; Freitas et al., 2011; Trabelsi et al., 2015). In 2008, this market was estimated to be worth over USD 4 billion, with bacterial EPS contributing only 6% despite their rising popularity. Bacterial EPS exhibit unique rheological properties, high purity and quality, and can be produced in high amounts.

Research has also shown that EPS from some *Lactobacillus* strains may confer health benefits and can be used in the prevention of human diseases thanks to their anticancer, antiulcer, immunomodulatory, antiviral, antioxidant, bifidogenic, and cholesterol-lowering properties (Freitas et al., 2011; Polak-Berecka et al., 2013a; El Ghany et al., 2014; Wang et al., 2015). It has also been proven in numerous studies that some

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lactobacilli EPS have toxic metal ions and mutagen binding properties (Tsuda et al., 2008; Bhakta et al., 2012; Polak-Berecka et al., 2014a). In turn, Lee et al. (2013) found that EPS produced by an *Lb. brevis* strain isolated from the brown alga *Ecklonia cava* exhibited antiradiation properties.

EPS from *Lactobacillus* sp.

EPS are natural, nontoxic, and biodegradable polymers, which play an important role in a variety of biological mechanism. Although not all functions of EPS in bacterial cells have been elucidated, it is known that they provide protection from adverse environmental effects and biological factors (the immune response), facilitate colonization by forming structures stabilizing the biofilm, and promote cellular signal transduction (Górska et al., 2007; Öner, 2013). EPS-producing *Lactobacillus* strains exhibit greater resistance to environmental stress. Some studies also indicate that EPS production enhances the probiotic potential of bacteria (Patel and Prajapati, 2013). Table 1 shows selected EPS functions in bacterial cells.

As compared to some other bacteria, *Lactobacillus* sp. are not very efficient producers of EPSs. For instance, around the globe over 2,000 tons of xanthan (used mostly as a thickening agent) are produced annually using the soil bacteria *Xanthomonas campestris*. Interest in EPS from *Lactobacillus* strains is largely attributable to the fact that these bacteria are deemed safe and confer health benefits (Liu et al., 2011; Badel et al., 2011). So far, approximately 30 EPS-producing Lactobacillus species have been identified, with the best-known ones being *Lb. casei, Lb. acidophilus, Lb. brevis, Lb. curvatus, Lb. delbrueckii* subsp. *bulgaricus, Lb. helveticus, Lb. rhamnosus, Lb. plantarum*, and *Lb. johnsonii*. These bacteria are cultured on mineral-rich media such as MRS (de Man, Rogosa, Shrp), milk, or milk derivatives at 30 or 37°C, depending on the strain

Table 1. Selected functions of EPS in bacterial cells (Wingender et al., 1999; Nwodo et al., 2012; Samson et al., 2013).

Process	Functional relevance
Adhesion	Initial step in colonization to biotic and abiotic surface
Aggregation of bacterial cells and formation of biofilms	Intermediation in upkeep of the mechanical stability of biofilms
	Immobilization of mixed bacterial populations Generation of a meium for commnication processes
Protective barrier	Resistance to nonspecific and specific host defenses (immune response, protection against free radical generation)
	Resistance to certain biocides and antibiotics
	Resistance to bacteriophages (a bacterial surface receptor is masked by EPS)
Sorption of exogenous organic compounds	Scavenging and accumulation of nutrients from the environment
	Sorption of xenobiotics (detoxification)
Sorption of inorganic ions	Accumulation of toxic metal ions (detoxification)
	Promotion of polysaccharide gel formation
Retention of water	Prevention of desiccation under water-deficient conditions
Nutrient source	EPS as source of carbon, nitrogen and phosphorus
Interaction with enzymes	Accumulation/retentions and stabilization of secreted enzymes

Table 2. EPS producers and efficiency of their production according to the culture medium

Microorganism	Media	Yield (mg/L)	References
Lb. sakei 0-1	SDM	1580	Ruas-Madiedo and de los Reyes-Gavilán, 2005
Lb. reuteri LB121	MRS with sucrose	9800	
Lb. rhamnosus R	BMM	495	Badel et al., 2011
Lb. rhamnosus 9595	WM	2775	
Lb. rhamnosus 9595M	BMM	1000	
Lb. kefiranofaciens CIDCA 83118	kefir	202	Hamet et al., 2015
Lb. paracasei CIDCA 8339	kefir	145	
Lb. delb. bulgaricus	MRS	263	Badel et al., 2011
Lb. delb. bulgaricus	milk	110	

BMM, Basal Minimal Medium; MRS, de Man, Rogosa, Sharpe medium; SDM, semi-defined medium;WM, whey medium

(Badel et al., 2011). Some EPS producers are listed in Table 2, which provides data on their yields on various culture media.

Phenotypes of EPS-producing bacteria are often described as "slimy," "mucoid," or "ropy." However, this is not always the case, as EPS may also attach to the cell surface forming a capsule (Fig. 1). Because of the great diversity of EPS from *Lactobacillus* sp., no correlation between EPS concentration in culture medium and "slimy" phenotypes of bacteria has been identified to date (Ruas-Madiedo and de los Reyes-Gavilán, 2005). EPS differ not only in terms of their chemical structure, but also spatial arrangement, and reactivity with various proteins (Welman and Maddox, 2003).

In terms of their composition, *Lactobacillus* EPS can be divided into homopolysaccharides (HoPS) and heteropolysaccharides (HePS; Fig. 2). Although homopolysaccharides contain one type of monosaccharide only (glucose or fructose), they differ in terms of glycosidic bonds, branching, chain length, molecular weight, and polymer structure (Monsan et al., 2001; Badel et al., 2011; Freitas et al., 2011). Glucose-containing HoPS include α-(dextran, mutan, reuteran) and β-glucans, whereas fructose-containing HoPS include fructans (inulin-type fructans, levan; Ruas-Madiedo and de los Reyes-Gavilán, 2005; Öner, 2013).

α-D-glucans

Dextran. Dextran is produced, among others, by *Lb. fermentum, Lb. sakei, Lb. hilgardii, Lb. parabuchneri*, and *Lb. curvatus*. Dextrans are predominantly linked by α-1,6-glycosidic bonds and ramified at the carbon atom in position 3 or, less frequently, in positions 2 and 4 (Sarwat et al., 2008; Minervini et al., 2010; Badel et al., 2011). They are used in the pharmaceutical industry (plasma substitute), food industry (thickening agent for jelly and ice-cream), and chemical industry (emulsifier and matrices). Cross-linked dextran marketed as Sephadex® is widely used as a medium in gel chromatography (Sarwat et al., 2008).

Mutan. This polymer is made of glucose residues mostly linked by α-1,3-glycosidic bonds. It is insoluble in water and contributes to microbial adhesion (Górska et al., 2007).

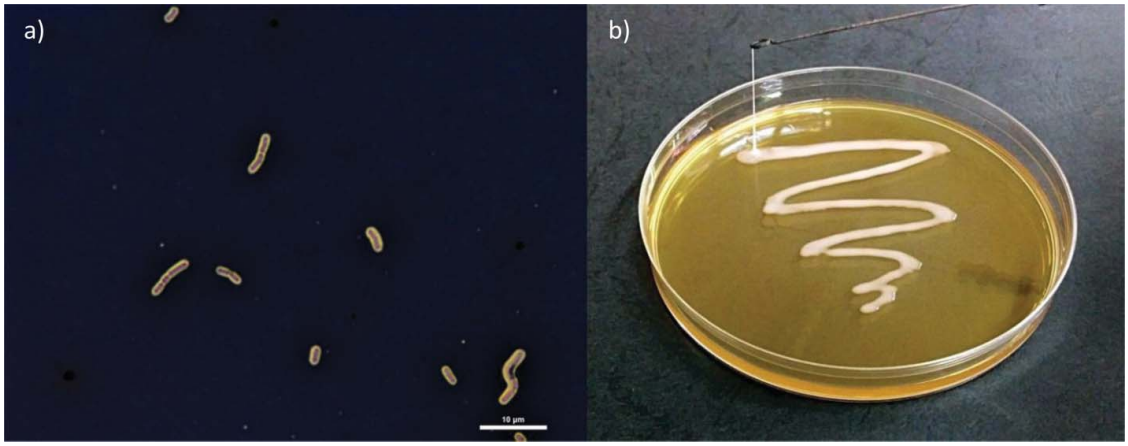

Figure 1. (a) Encapsulated *Lb. casei* ŁOCK 0901 stained using Maneval's capsule staining method (×1000); (b) macroscopic appearance of the "ropy" strand formed by the cellular mass of a EPS-producing *L. acidophilus* ŁOCK 0935 strain growing on the surface of de Man, Rogosa, and Sharpe (MRS) agar plates.

According to Krajl et al. (2004), it is produced by *Lb. reuteri* ML1 and *Lb. reuteri* GTFML1.

Reuteran. This EPS is produced by the probiotic bacteria *Lb. reuteri* 121. Its molecules contain both α-(1,4)- and α-(1,6)-glycosidic linkages (58 and 42%, respectively). It is used in the food industry, for example, in sourdough (Krajl et al., 2005; Meng et al., 2015).

β-D-glucans

β-D-glucans consist of glucose residues linked by β-1,3-glycosidic bonds and are synthesized by *Lb. suebicus* CUPV221 and *Lactobacillus* spp. G-77. β-D-glucans are thought to modify the organism's immune response (Garai-Ibabe et al., 2010; Badel et al., 2011).

Fructans

Levan. This fructose homopolymer contains β-2,6-glycosidic linkages and is synthesized by *Lb. reuteri* 121 and *Lb. sanfranciscensis* LTH2590. Some levan molecules have β-2,1-linked branches. This compound is also produced by caries-associated bacteria; however, recent research has focused on its role in plant pathogenesis as well as its anticancer, anticholesterol, and prebiotic properties (Tieking and Gänze, 2005; Szwengiel et al., 2009; Badel et al., 2011).

Inulin-type fructan. This polysaccharide has a structure that is antagonistic with respect to levan; it is composed of fructose residues linked by β-2,1-glycosidic bonds and ramified at position β-2,6. It is synthesized by *Lb. reuteri* LB121 (Harutoshi, 2013).

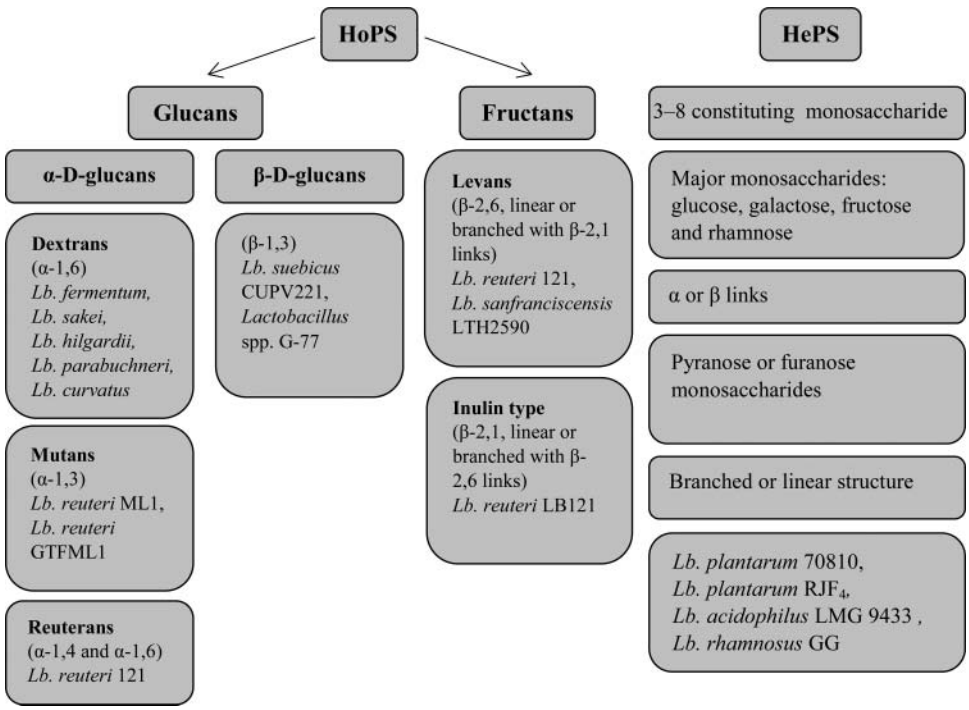

Figure 2. Classification and characterization of EPS produced by *Lactobacillus* sp.

In contrast, heteropolysaccharides consist of three to eight saccharide repeat units, mostly glucose, galactose, fructose, and rhamnose. Some HePS also incorporate N-acetylglucosamines, N-acetylgalactosamines, glucuronic acid, as well as nonsugar substituents, such as phosphate or glycerol. Monomers may also occur as α or β anomers in pyranose or furanose configuration (Ruas-Madiedo and de los Reyes-Gavilán, 2005; Öner, 2013). The heteropolysaccharides produced by *Lactobacillus* vary greatly in their structure. For instance, *Lb. plantarum* 70810 isolated from Chinese sauerkraut synthesizes a HePS composed of glucose, mannose, and galactose at molar ratios of 18.21:78.76:303 and 12.92:30.89:56.19 (Wang et al., 2015), whereas EPS from *Lb. plantarum* RJF_4 contains only glucose and mannose (Dilna et al., 2015). Some HePS incorporate glucuronic acid (*Lb. acidophilus* LMG 9433; Robijn et al., 1996) and N-acetylglucosamine (*Lb. rhamnosus* GG isolated from the human gastrointestinal system; Landersjo et al., 2002).

Sample structures of EPS isolated from cultures of *Lactobacillus* strains are given in Table 3.

Being macromolecules, EPS have high molecular weights, with HePS ranging from 4×10^4 to 6×10^6 Da, and HoPS reaching even larger sizes. There are reports that the size of EPS molecule may depend on the carbon source in the culture medium. For example, Polak-Berecka et al. (2015) showed that molecular masses for dextran synthesized by *Lb. rhamnosus* E/N cultivated on five carbon sources: glucose, galactose, sucrose, maltose, or lactose were in the range 548.7, 3901, 11130, 195, and 7027 kDa, respectively.

Bacterial homopolysaccharides are produced in much greater amounts than heteropolysaccharides. The yield of HoPS is several g/L of culture medium whereas that of HePS is only 50–200 mg/L. According to the literature, the overall EPS yield of *Lactobacillus* depends on the sources of carbon and nitrogen in the medium and on culture conditions, such as pH, temperature, water activity, oxygen concentration, incubation time, etc. (van Geel Schutten et al., 1998; Ruas-Madiedo and de los Reyes-Gavilán, 2005; Badel et al., 2011). The study of Polak-Berecka et al. (2014b) showed that culture conditions have a clear impact on EPS production by *Lb. rhamnosus* E/N. The optimal temperature, pH, carbon source, and nitrogen source conditions for EPS production were 37°C, pH 5.0, galactose, and yeast extract, respectively. Their study showed that the scale up from flask to fermentor can increase EPS biosynthesis by 175.8% in commercial production processes. Seesuriyachan et al. (2010) studied the EPS produced by *Lb. confusus* 1499 in liquid and solid state fermentation using coconut water and sugarcane juice as renewable wastes. They gained higher concentrations of EPS in solid-state fermentation when nitrogen sources were reduced 5-fold from the original medium. The study of Polak-Berecka et al. (2014a, b) indicated that limiting of the concentration of a nitrogen source in the growth medium for *Lb. rhamnosus* E/N

Table 3. Examples of chemical structures of homo- and heteropolysaccharide produced by *Lactobacillus* sp.

Microorganism	Structure	References
Lactobacillus helveticus LB-H-3A	β-D-Glcp 1 ↓ 2 β-D-Galp-(1→4)-β-D-Glcp 1 ↓ 6 →4)-β-D-Galp-(1→4)-β-D-Glcp-(1→4)- α -D-Glcp-(1→	Yang et al. (2000)
Lactobacillus plantarum C88	β-D-Galp 1 ↓ 6 →4)-α-D-Galp2Ac-(1→2)-α-D-Glcp-(1→3)-β-D-Glcp-(1→ 1 ↓ 3 β-D-Glcp	Fontana et al. (2015)
Lactobacillus spp. G-77	→6-α-D-Glcp-(1→6)-α-D-Glcp-(1→ 2 ↑ 1 α-D-Glcp	Dueñas-Chasco et al. (1998)
Lactobacillus paracasei	α-D-Galp 1 ↓ 6 →6)-α-D-Galp-(1→3)-β-L-Rhap-(1→4)-α-D-Glcp-(1→4)-β-D-GlcpNAc-(1→ 3 ↓ 1 α-L-Rhap	Van Casteran et al. (2000)

Glc – glucose; Gal – galactose; Rha – rhamnose; Galp – galactopyranose; Glcp – glucopyranose; Rhap – rhamnopyranoside; GlcA – glucosamine; GlcNAc – N-acetylglucosamine; Galp2Ac – 2-O-acetylgalacto-pyranose

does not decrease EPS yield. In turn, Li et al. (2014) showed that soybean peptone was most effective nitrogen source than other tested nitrogen sources for EPS production of *Lb. helveticus* MB2-1. The study of Seesuriyachan et al. (2012) showed that calcium ions at a level of 3 mM can enhance the rate of EPS production by *Lb. confosus* 1498 at pH 5.8. They also observed that under optimized conditions with NaCl 4.97%, sucrose 136.5 g/L and 40.79-hour incubation, the EPS yield was 259% higher than the yield produced with modified MRS medium (120 g/L sucrose) without NaCl. In turn, Liu et al. (2015), who studied EPS synthesized by *Lb. acidophilus* ATCC (mutated by diethyl sulfate), reported that the three important parameters (Dipotassium hydrogen phosphate, Tween 80 and Trisodium citrate) had significant effect on EPS yield. The experiments confirmed that the EPS yield from *Lb. acidophilus* mutant strains reached 5.12 ± 0.73 g/L under the optimized fermentation and extraction conditions, which was 3.8 times higher than that of the control (1.05 ± 0.06 g/L). These reports lead to conclusion that the composition of the medium and culture conditions should be adjusted to a given strain. Within the genus *Lactobacillus*, one of the highest EPS yields is exhibited by the probiotic strain *Lb. rhamnosus* 9595, which produces approx. 2.7 g EPS per L of medium enriched with yeast extract, salts, and vitamins (Table 2). These EPS are mostly composed of rhamnose and, to a lesser extent, of glucose and galactose (Badel et al., 2011). In turn, on MRS agar with sucrose *Lb. reuteri* synthesizes EPS with a yield of almost 10 g/L of medium (Ruas-Madiedo and de los Reyes-Gavilán, 2005). It is assumed that thermophilic *Lactobacillus* produce more EPS than mesophilic one (van de Velde et al., 2015).

The applications of *Lactobacillus* EPS are limited due to the high costs of production and isolation from the culture medium. In response to this problem, researchers continue to seek strains with more efficient biosynthesis capacity, manipulate fermentation conditions, genetically engineer bacterial metabolism, and search for more cost-effective substrates. These efforts considerably increase the likelihood of the use of *Lactobacillus* EPS on an industrial scale (Nwodo et al., 2012; Patel and Prajapati, 2013).

Ways of synthesis

Biosynthesis of homopolysaccharides

EPS biosynthesis is very complex and occurs at different bacterial growth phases, depending on the microorganism and environmental conditions. *Lactobacillus* sp. synthesized EPS mainly during the exponential growth phase. Polak-Berecka et al. (2013a) have studied in this regard EPS produced by *Lb. rhamnosus* E/N. In this case EPS production started with the growth of bacteria and continued even in stationary phase. However, the amount of EPS was decreasing at the end of fermentation suggesting that EPS is not accumulated in the culture and may be used as an alternative carbon source.

Very little is known about the mechanisms of EPS production and intensive research efforts aimed at their elucidation are under way (Madhuri and Prabhakar, 2014). Homopolysaccharides are synthesized by extracellular glycansucrases belonging to the class of glycosyltransferases (GTF, E.C. 2.4.x.y) They catalyze the hydrolysis of sucrose, with the resulting monosaccharide residues attached to a glycan acceptor chain (De Vuyst et al., 2007). GTF enzymes

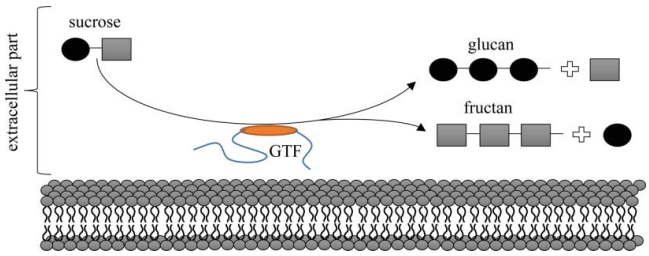

Figure 3. Diagram of glucans and fructans synthesis (based on Badel et al., 2011; Ryan et al., 2015) glucose; fructose; catalytic domain; attachment of monosaccharides supplied from the sucrose hydrolysis to the glycan acceptor chain (glucans and fructans); GTF, glycosylotransferase. © 2010 Elsevier. Published in [short citation] under CC BY 4.0 license. Available from: doi:10.1016/j.biotechadv.2010.08.011

can be divided into transglucosidases (E.C. 2.4.1.y) and transfructosidases (E.C. 2.4.1.y or 2.y), depending on their products. The former include glucan-synthesizing dextransucrases, mutansucrases, and reuteransucrases (E.C. 2.4.1.5) encoded by the *gtf* genes (Badel et al., 2011). Glucan synthesis may be represented by means of the following formulas (Harutoshi, 2013):

$$\text{sucrose} + \text{H}_2\text{O} \rightarrow \text{glucose} + \text{fructose}$$

$$\text{sucrose} + \text{carbohydrate acceptor} \rightarrow \text{oligosaccharide} + \text{fructose}$$

$$\text{sucrose} + \text{glucan(n)} \rightarrow \text{glucan} + \text{fructose}$$

In turn, fructan-catalyzing transfructosidases include levansucrases (E.C. 2.4.1.10) and inulosucrases (E.C. 2.4.1.9) encoded by *ftf* genes. These enzymes are induced by chemical or physical stress (high/low temperature, bacteriostatic agents) (Badel et al., 2011). Fructan synthesis may be represented by means of the following formulas (Harutoshi, 2013):

$$\text{sucrose} + \text{H}_2\text{O} \rightarrow \text{glucose} + \text{fructose}$$

$$\text{sucrose} + \text{carbohydrate acceptor} \rightarrow \text{oligosaccharide} + \text{fructose}$$

$$\text{sucrose} + \text{fructan(n)} \rightarrow \text{fructan(n+1)} + \text{glucose}$$

One glycosyltransferase is responsible for the synthesis of one EPS, so two enzymes from two different *gtf* genes will generate two different EPS. For instance, dextran produced by *Lb. fermentum* Kg3 (gene *gtf* Kg3) is 89% linked by α-(1,6) linkages, whereas dextran produced by *Lb. reuteri* 180 (gene *gtf* 180) is linked only by 51% α-(1,6) linkages (Badel et al., 2011). Figure 3 presents a scheme of homopolysaccharide biosynthesis.

Biosynthesis of heteropolysaccharides

In contrast to the extracellular biosynthesis of HoPS, the mechanism of heteropolysaccharide synthesis appears to be extremely complex. It involves many enzymes and other proteins, and still remains to be fully elucidated. It is known that the genes encoding the enzymes and regulatory proteins needed

for EPS synthesis are of plasmid origin in mesophilic *Lactobacillus* strains and of chromosomal origin in the thermophilic strains (de Vuyst and Degeest, 1999; de Vuyst et al., 2001; Laws et al., 2001). The HePS biosynthesis pathway consists of four stages (Laws et al., 2001): (i) sugar transport into cytoplasm; (ii) synthesis of sugar-1-phosphates; (iii) polymerization of sugars; and (iv) exportation of EPS from the cell.

Monosaccharides and disaccharides comprise the main source of carbon for microorganisms. Even though Lactobacillus species depend on lactose for a source of carbon, they can also absorb some other saccharides. The transport of sugar molecules from the environment to the cytoplasm is strictly regulated by several proteins (de Vuyst et al., 2001; Madhuri and Prabhakar, 2014). To date, three different mechanism of saccharide transport into the cytoplasm have been described: (i) a primary transport system in which sugar translocation is coupled to ATP hydrolysis; (ii) a secondary saccharide transport system in which sugar transport is coupled to the transport of ions and other solutes; (iii) a bacterial PEP–PTS (phosphoenolpyruvate-phosphotransferase) system (de Vuyst et al., 2001), which is thought to be the most important process regulating sugar import into bacterial cells (Badel et al., 2011). In this system, two protein groups are responsible for linking sugar residues to the carriers, intermembrane transport, and sugar phosphorylation. The phosphate group is released by pyruvate kinase, converting PEP into pyruvate. The first group of proteins, including enzyme I and HPr, transports phosphate and the carbohydrate-specific permease complex, whereas the other group of proteins regulates the processes of nutrient accumulation (Górska et al., 2007; Badel et al., 2011; Madhuri and Prabhakar, 2014). Sugar phosphorylation yields 6- and 1-phosphates. Polysaccharides are made of sugar 1-phosphates; the 6-phosphates are degraded along the glycolytic pathway, and some are converted to 1-phosphates by phosphoglucomutases (PGMs). β-PGM catalyzes the conversion of β-1-phosphates into 6-phosphates, which are subsequently transformed by α-PGM to α-1-phosphates. Sugar 1-phosphates are the main metabolites used in the synthesis of nucleotide sugar derivatives, such as UDP-glucose and dTDP-glucose, by means of respective pyrophosphorylases. They may arise in the course of different metabolic pathways, depending on substrate composition; for instance, galactose is converted to glucose-1-phosphate via the Leloir pathway (Jolly et al., 2002; Górska et al., 2007; Madhuri and Prabhakar, 2014). The genes responsible for synthesizing the enzymes involved in the production of nucleotide sugar derivatives have been extensively studied; they include *galU, galE, rfbA, rfbB, rfbC*, and *rfbD* (Jolly and Stingele, 2001).

The last step in the synthesis of HePS is polymerization and exportation from the cell. It is known that EPS are formed the linkage of several hundred to several thousand oligosaccharide units. The undecaprenyl phosphate carrier C55-P is attached to the cytoplasmic membrane. The first sugar residue is linked to the undecaprenyl phosphate by a β-glycosidic pyrophosphate bond and subsequent sugars are added to the growing polysaccharide chain (Górska et al., 2007; Madhuri and Prabhakar, 2014). The complete polymer is secreted out of the cell in the form of slime or adheres to the cell wall, forming a capsule (De Vuyst et al., 2001). Fig. 4 shows a scheme of HePS synthesis using the example of lactose conversion.

Each stage of EPS synthesis requires a source of energy, which may be difficult to deliver due to the anaerobic conditions typically preferred by *Lactobacillus* sp. Furthermore, the C55-P carrier participates not only in polysaccharide polymerization, but also in the synthesis of other cell wall polymers, such as teichoic acids, peptidoglycans, and lipopolysaccharides (Górska et al., 2007). It should also be mentioned the dairy industry faces the problem of heteropolysaccharide instability. *Lactobacillus* strains may lose their ability to synthesize EPS as a result of multiple subculturing. Polysaccharides may also be lost in the course of prolonged incubation, especially at high temperature (De Vuyst et al., 2001). The loss of polysaccharide production is generally caused by loss of plasmids from "ropy" mesophilic strains. The thermofilic species like *Lb. delbrueckii* subsp. *bulgaricus* can usually recover the ability to produce EPS lost due to unfavorable culture conditions. In this case, the EPS gene cluster is located in chromosomal DNA, thus genetic instability could be a consequence of the actions of mobile genetic elements such as insertion sequences (Harutoshi, 2013).

Applications for food engineering

EPS from lactic acid bacteria, including *Lactobacillus* sp., are mostly used as thickening, stabilizing, binding, and structure-forming agents thanks to their non-Newtonian behavior and viscosity in aqueous media (Freitas et al., 2011). They play a key role in the production of fermented foods, such as vegetables, meat, and especially dairy products, also improving consistency and taste. Although EPS themselves are tasteless, they augment the gustatory sensations of the consumers (Bernardeau et al., 2008; Hamet et al., 2015; Vinogradov et al., 2015). For many years now, *Lactobacillus* sp. have been found in kefirs (*Lb. kefir, Lb. kefiranofaciens, Lb. brevis*), fermented milk (*Lb. casei, Lb. acidophilus, Lb. rhamnosus, Lb. johnsonii*), yoghurts, and butter (*Lb. delbrueckii subsp. bulgaricus*), Swiss and Italian cheeses (*Lb. delbrueckii subsp. lactis, Lb. helveticus, Lb. casei, Lb. delbrueckii subsp. bulgaricus*). In turn, European fermented sausages are made using *Lb. sakei* and *Lb. curvatus* (Leroy and De Vuyst, 2004). EPS-producing *Lactobacillus* strains have also been isolated from cider (*Lb. suebicus*; Ibarburu et al., 2015).

Knowledge about the physical and chemical properties of EPS and their interactions with other food ingredients is crucial in developing EPS applications. Despite the fact that *Lactobacillus* EPS are promising thickening agents, their production is uneconomical due to low yields. The recent concept of "functional starters" has been characterized as cultures that "can contribute to microbial safety, or offer one or more organoleptic, technological, or nutritional and health advantages to the food." This definition fits *Lactobacillus* because, if added to food, they can produce polymers *in situ*, creating natural products with improved rheological properties (Ruas-Madiedo et al., 2002; Salazar et al., 2009). Slime-forming LAB cultures are commercially available in many European and North American countries. In Europe, ropy thermophilic LAB starter cultures are also used for yoghurt production (De Vuyst et al., 2001).

EPS produced *in situ* improve sensory characteristics such as mouthfeel, shininess, clean cut, ropiness, and

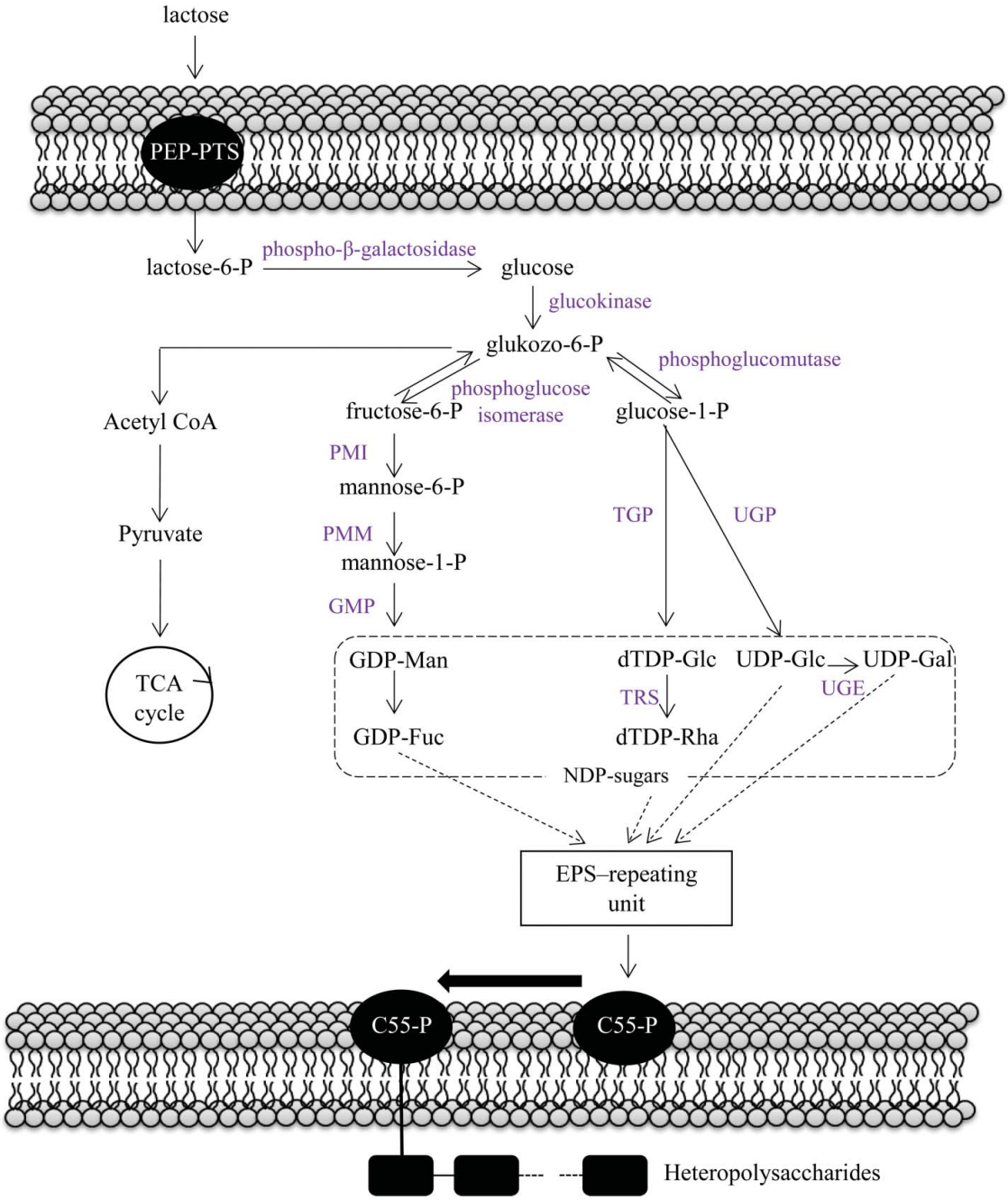

Figure 4. Diagram of the bioconversion of lactose to EPS (based on Welman and Maddox, 2003; Freitas et al., 2011; Harutoshi, 2013). Man, mannose; Glc, glucose; Gal, galactose; Fuc, fructose; Rha, rhamnose; NDP, nucleotide; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-mannose pyrophosphorylase; TGP, dTDP-glucose pyrophosphorylase; UGP, UDP-glucose pyrophosphorylase; TRS, dTDP-rhamnose synthetic enzyme system; UGE, UDP-galactose 4-epimerase; acetyl CoA, acetyl coenzyme A; TCA cycle, Krebs cycle; C55-P, undecaprenyl phosphate carrier; PEP-PTS, PhosphoEnolPyruvate-sugar PhosphoTransferase System

creaminess of final products. EPS production levels *in situ* and their conformational characteristics is dependent on intrinsic and extrinsic factors: availability of sugar nucleotides in EPS structure, complex genetic mechanism of EPS production, incubation temperature and time, nutrient availability (Yang et al., 2014). The factors, such as monosaccharide composition, linkage type, side chains, molecular weight, their interaction with milk constituents (mainly casein and ions) also affects on the rheological functions of lactobacilli EPS in fermented dairy products (Yilmaz et al., 2015). Several reports revealed that *in situ* production of ropy EPS plays an important role in the manufacture of a variety of cultured dairy products like yoghurt, sourdough, cheese. Dertli et al. (2016) reported that using EPS-producing *Streptococcus thermofilus* strains may be produced a fermented functional ice cream without any stabilizer. Other studies carried out by Hamet et al. (2015) showed that EPS synthesized by *Lb. plantarum, Lb. kefiranofaciens*, and *Lb. paracasei* in the process of milk fermentation may potentially replace hydrocolloids. In turn, London et al. (2015)

showed that EPS produced by *Lb. mucosae* DPC 6426 positively influenced the techno-functional properties of the yoghurt throughout storage, compared with the non-EPS control yoghurt.

EPS have ability to confer viscosity of EPS in aques solutions, which is determined by the molecular weight (*M*), the degree of chain stiffness, the radius of gyration (Rg) of the molecule and the presence of ionizable groups that confer the polymer a polyelectrolyte behavior (Ruas-Madiedo and de los Reyes-Gavilán, 2005; Freitas et al., 2011; Miao et al., 2015). Tuinier et al. (1999) formulated the equation, that allows to calculate the intrinsic viscosity (hydrodynamic volume of a single molecule) ($[\eta]_0$) of the EPS, determining its viscosity-intensifying ability:

$$[\eta]_0 = 3.1 \times 10^{24} \times \mathrm{Rg}^3 \times M^{-1} \left(\mathrm{units} : \mathrm{m}^3/\mathrm{kg}\right)$$

Inter alia, Looijesteijn et al. (2000) reported that major factor of EPS viscosity-intensifying ability was the molecular weight. In their study, *Lactococcus lactis* subsp. *cremoris* strains NIZO B40 and NIZO B891 produced EPS with lower molecular weight in a medium with lower glucose content. The chemical composition of those polymers remained unaffected, but the rheological properties of the substrate changed. In turn, Vincent et al. (2001) reduced the chain stiffness of the EPS produced by *Streptococcus macedonicus* Sc136 through cleveage of the side chains, thus reducing the viscosifying capacity of tested biopolymers.

One of the most important research topic is protein–polysaccharide interactions in milk products. EPS–protein interactions may be of aggregative or segregative nature. Ayala-Hernandez et al. (2008) showed that negatively charged EPS tend to associate or aggregate with the milk protein phase. The studies performed using 3D reconstructions of confocal laser scanning microscopy showed the local phase separation occurring between EPS and milk proteins. In the presence of EPS, the microstructures of EPS containing milk gels depict caseins clustered into protein strands. In turn, the absence of EPS causes finer strands of proteins that are more evenly distribute in milk (van de Velde et al., 2015). The EPS interaction with milk constituents have been studied by researchers around the world. In their investigation of EPS synthesized by *Streptococcus thermophiles* Purwandari et al. (2007) indicated that the amount of EPS is not in itself a critical factor affecting the texture of food products. Consecutive studies showed that *Lb. bulgaricus* synthesizes 60–150 mg of EPS per liter of yoghurt, of critical importance to its consistency is not the amount of EPS, but its structure as well as pH levels enabling interaction with the caseins contained in the yoghurt (Badel et al., 2011). In turn, Ayala-Hernández et al. (2008) demonstrated interactions between the anionic EPS produced *in situ* by *Lactococcus lactis* and whey proteins in a simplified milk system (2–8%) by using scanning electron microscopy. The fermentation process was carried out for 12 hours at 30°C, at pH 4.5. In this regard, EPS synthesized by two strain of *Streptococcus thermofilus*: HC15 and NIZO2104, and three *Lb. delbrueckii* subsp. *bulgaricus* strain: 210R, NCIMB702074 and DGCC291 were tested by Gentés et al. (2013). Their study showed that EPS contributed to gel stiffness, possibly through associative behavior due to electrostatic interactions with caseins, as well as to viscosity. Among the tested strains, stronger gels were formed with the anionic EPS from strain *Lb. delbrueckii* subsp. *bulgaricus* 2104.

Perspectives for *Lactobacilli* EPS

Therapeutical properties

The market of probiotic-containing functional foods, conferring health and economic benefits to consumers, is now developing rapidly (Ruas-Madiedo et al., 2002). Some studies have suggested that the probiotic properties of strains depend on their ability to synthesize EPS and many EPS have been analyzed in terms of their prebiotic potential (Hussein et al., 2015). Russo et al. (2012) reported that *Lb. plantarum* WCFS 1, *Lb. plantarum* WCFS 1β-gal, and *Lb. acidophilus* NCFM secrete prebiotic β-glucans. In turn, Hussein et al. (2015), who studied EPS synthesized by *Lb. delbrueckii* subsp. *bulgaricus, Lb. helveticus*, and *Lb. casei*, found the first one to be the most potent prebiotic. In addition, EPS have also been shown to exhibit immunomodulating, cholesterol-lowering, anti-cancer, anti-ulcer, antiviral activity, and improvement of intestinal adhesion of probiotics (Badel et al., 2011; Nwodo et al., 2012; Polak-Berecka et al., 2014b).

The study of Dilna et al. (2015) into EPS from *Lb. plantarum* RJF_4 showed some EPS to have cholesterol-lowering properties and inhibit α-amylase as well as cancer cell lines. As early as in 1995, a similar study was carried out by Ebina et al. for *Lb. bulgaricus* 878R, whereas Wang et al. (2014) investigated the strain *Lb. plantarum* 70810. London et al. (2015) investigated cholesterol-lowering properties of EPS produced by *Lb. mucosae* DPC 6426 on mice. They have shown that mice fed a high-cholesterol diet, supplemented with *Lb. mucosae* DPC 6426, resulted in significantly lower serum cholesterol and triglyceride levels compared with unsupplemented controls. In turn, Maeda et al. (2004) tested on rats the properties of kefiran synthesized by *Lb. kefiranofaciens*. The use of 100–300 mg kefiran per kg lowered blood pressure, cholesterol level, and blood glucose. It was also found that EPS from *Lb. kefiranofaciens* improved the health of the intestinal mucosa, prevented constipation, and regulated bowel movement (Vindarola et al., 2006). Recent research made by Sarikaya et al. (2016) suggested that EPS synthesized by *Lb. fermentum* LB-69 has anti-biofilm activity and potential prebiotic application. It gives these polymers the new possibility to use as limiting factor for the biofilm formation on medical indwelling devices.

Several studies have shown that EPS synthesized by some bacteria of the genus *Lactobacillus* have bifidogenic activities. One of the first studies were carried out by Polak-Berecka et al. (2013b) on *Lb. rhamnosus* E/N strain. They observed the greater growth of *Bifidobacterium bifidum, B. animalis, B. longum, and B. infantis* on media supplemented with EPS in comparison with the control. Bifidobacterial strains possess extracellular enzymes belonging to glycoside hydrolase families 2, 13, 36, and 42 (e.g. α-galactosidases, ß-galactosidases), which are active towards EPSs. However, the degradation ratio is dependent on primary structure of EPS, because their substituents may be more or less accessible to degradative enzymes

(Polak-Berecka et al., 2013b). The similar studies with similar results were carried out by Sarikaya et al. (2016) on six lactic acid bacterial strains: *Lb. delbrueckii* ssp. *bulgaricus* B-3, *Lb. delbrueckii* ssp. *bulgaricus* A-12, *Lb. fermentum* LB-69, *Lb. paracasei* LB-8, *Lb. plantarum* GD-2, *Lb. rhamnosus* GD-11.

Antioxidant and anticancer activity

There is plenty of well-described evidence of anticancer and antioxidant activity of EPSs synthesized by *Lactobacillus* strains, which is affected by monosaccharide composition, molecular weight, structure of the polymeric backbone and side chain, and number of branching points. Inter alia, the presence of mannose and glucose residues and branching points in repeating unit in EPS structure effects on increasing their anticancer activity (Li et al., 2015). The study El Ghany et al. (2014) confirmed the antioxidant and anticancer properties of *Lb. acidophilus* P185 strain. This study demonstrated that EPS from *Lb. acidophilus* P185 is able to inhibit the proliferation of Ehrlich ascites carcinoma (EAC) cell line with 93–99%. Also, they made *in vivo* study for toxic properties of EPS on solid tumor bearing mice. The use of EPS resulted in reduction of tumor volume. Moreover, this study showed that EPS synthesized by *Lb. acidphilus* P185 has a stronger antitumor effect *in vivo* and *in vitro*, while *Escherichia coli* increasing solid tumor volume in comparision with control. The study El Ghany et al. (2014) also suggested that the therapeutical properties of Lactobacillus sp. may depend on their ability to synthesized EPS. In these studies, the EPS produced by *Lb. acidphilus* P185 showed stronger antioxidant and antitumor activities against EAC cells in comparision with *Lb. acidophilus* P85 itself. A similar study was carried out on the EPS from strain *Lb. acidophilus* LA1 (El Ghany et al. 2015). In turn, Wang et al. (2014) investigated in this regard strain *Lb. plantarum* 70810 from Chinese Paocai. These studies demonstrated that EPS from this strain has antitumor activities on HepG-2, BGC-823 and HT-29 tumor cells. Anticancer properties of EPS synthesized by *Lb. helveticus* MB2-1 were investigated by Li et al. (2015). This study showed that one of three purified EPS fractions (LHEPS-1) significantly inhibited cell proliferation of human colon cancer cells – Caco-2. These studies suggested that specific EPS from the Lactobacillus sp. might be suitable for use as functional foods and antitumor drugs.

Immunological activity

Although a variety of physiological functions of lactobacilli EPS were reported, their immunomodulatory activities are drawing much attention, for example induced phagocytosis, stimulation of the growth of immune cells, secretion of proinflammatory and anti-inflammatory cytokines (Kšonžeková et al., 2016). Many authors have observed the immunological activity of *Lactobacillus* EPS so far; for instance, according to Yasuda et al. (2008) *Lb. casei* activated macrophages. In addition, Ruas-Madiedo et al. (2002) reported increased lymphocyte proliferation as a result of *Lactobacillus* EPS activity. Sasaki et al. (2015) demonstrated that EPS synthesized by *Lb. brevis* KB290 has a critical role in enhancing cell-mediated cytotoxic activity in mouse spleen. The ability to stimulate splenocytes proliferation *in vitro* have also EPS produced by *Lb. rhamnosus* KF5 grown on fermented skim milk (Shao et al. 2014). Immunomodulatory properties of *Lb. plantarum* N14 was studied by Murofushi et al. (2015). They showed that EPS produced by *Lb. plantarum* N14 were able to decrease the production of pro-inflammatory cytokines in porcine intestinal epithelial cells in response to enterotoxigenic *Escherichia coli* (ETEC) challenge. Similar studies was carried out by Kšonžeková et al. (2016) on EPS originated from *Lb. reuteri* strain DSM 17938 and *Lb. reuteri* strain L26 Biocenol^{TM}. These EPS was able to inhibit ETEC adhesion on enterocyte-like IPEC-1 cells, suggesting their role as a prophylactic agent to gastrointestinal infections.

Heavy metal and mutagen binding

Biotechnologically important research topic is metal ions biosorption by EPS. Contamination of metals in the environment and human diet represents a persistent problem that will continue to be a burden on human health. The food and the water we consume are often mainly contaminated with lead, cadmium, arsenic, chromium, and so on (Monachese et al., 2012). These heavy metals are not biodegradable. Their causes bioaccumulation in human organisms, resulting in several health problems such as cancer, kidney failure, metabolic acidosis, oral ulcer, renal failure, and damage in for stomach of the rodent. Therefore, exploration of potentially heavy metal absorbing bacteria and potential application of them for the treatment of metal contaminated water and food is one of the low-cost and promising bioremedial technologies (Bhakta et al., 2012; Sofu et al., 2015).

The adsorption of heavy metal by EPS is a metabolism-independent process, and it is attributed to interaction between metal cations and negative charges of acidic functional groups of EPS, such as including carboxyl, acetate, hydroxyl, amine, phosphate, and, more rarely, sulfate groups. It has been demonstrated that metal sorption involves physicochemical interaction based on physical sorption, ion exchange, complexion, and/or precipitation and its lead to formation of stable complexes (Pal and Paul, 2008; Pérez et al., 2008; Polak-Berecka et al., 2014a). The interaction between bacterial EPS and metal ions has stimulated specific research interest due to its important ecological and practical implications. A number of environmental microorganisms have long been known for their ability to bind metals. For example EPS-producing cyanobacteria have ability to absorb Cu, Mn, Pb, Hg, and *Bacillus firmus* remove Pb, Cu, and Zn. In turn, Mo, Ni, Cr are removed by marine sulphate reducing bacteria (Pal and Paul, 2008; Pérez et al., 2008). Recent study of Hao et al. (2016) showed that complex system cell-EPS-Fe(III)-mineral aggregates formed by Fe(II)-oxidizing bacteria has significant amounts of sorption sites for heavy metal species (e.g., Au^{3+}, Cd^{2+}, Cr^{3+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pd^{2+}, Zn^{2+}).

Many authors have observed that *Lactobacillus* sp. could prove to be an effective tool in reducing heavy metal exposure. In 2007 Halttunen et al. reported that *Lb. rhamnosus* GG (ATCC 53103), *Lb. casei* Shirota and *Lb. fermentum* ME3 have the ability to bind cadmium and lead efficiently and rapidly in water. Removal capability of cadmium and lead was also examined by Bhata et al. (2012). They demonstrated that

Lb. reuteri Cd70-13 and Pb71-1 strains could be applied as efficient probiotic candidates for heavy metals bioaccumulation in food. The study of Polak-Berecka et al. (2014a) showed that EPS isolated from *Lb. rhamnosus* E/N has a cadmium and aluminum biosorption capacity. Fourier transform-infrared spectroscopy analysis made in their research suggests that some functional groups of EPS (—OH, C═O, and COO—) is involved in cadmium and aluminum biosorption. Moreover, their research shows that EPS isolated from *Lb. rhamnosus* E/N is a promising tool for removing heavy metal ions from the gastrointestinal tract by detoxication.

Usage of *Lactobacillus* strains for heavy metal binding is important research topic because previous studies showed that in addition to the absorption of cadmium, aluminum and lead by lactobacilli EPS, some strains have also an arsenic and chromium biosorption capacity (Monachese et al., 2012). Furthermore, there are many studies proving that *Lactobacillus* sp. have also the capacity to bind many toxic compounds like aflatoxins, food-borne mutagens and microcystin-LR from aqueous solution. (Halttunen et al., 2007). The study of Tsuda et al. (2008) showed that EPSs from *Lb. plantarum* mutant strain 301102S are able to bind and inactivate the potent carcinogenic mutagen produced by overcooking meat and fish, 3-amino-1,4-dimethyl-5H-pyrido [4,3-b]indole (Trp-P-1) (Jeon et al., 2011).

The properties of bacterial EPS and their mechanisms of action have not been fully elucidated due to their great diversity in terms of chemical composition, structure, and capacity to bind to proteins, and are still subjected to considerable research efforts. Although bacterial EPS have already found many applications in the textile and cosmetic sectors, medicine, pharmaceutical industry, and environmental protection (reclamation, flocculation, heavy metal absorbers, etc.), the use of EPS from probiotic bacteria for health-promoting purposes would represent a major milestone.

Conclusion

EPS synthesized by *Lactobacillus* sp. are highly diverse and are widely used for their beneficial properties in the food industry, with intensive efforts under way to extend their applications in the medical, cosmetic, and pharmaceutical sectors. However, the factors that hinder their use are the high cost and low yield of production. An appropriate selection of strains and optimization of culture conditions by means of molecular biology and genetic engineering can significantly increase the output of *Lactobacillus* EPS. In order to broaden the application potential of these EPS in industry, it is also essential to establish correlations between the structure of EPS, their physical properties, and health benefits.

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