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Exopolysaccharides of Lactic Acid Bacteria for Food and Colon Health Applications

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1. Introduction

Lactic acid bacteria (**LAB**) are used in many fermented foods, particularly fermented dairy products such as cheese, buttermilk, and fermented milks. LAB produce lactic acid, carbon dioxide, and diacetyl/acetoin that contribute to the flavor, texture, and shelf life of fermented foods. Some LAB produce exopolysaccharide (EPS), and generally, EPS play a major role as natural texturizer in the industrial production of yoghurt, cheese, and milk-based desserts. Recently, EPS produced by LAB have received increasing attention, mainly because of their health benefits. In particular, immune stimulation, antimutagenicity, and the antitumor activity of fermented dairy products prepared with EPS-producing LAB or EPS themselves have been investigated [1-4].

EPS are polysaccharides secreted from the cell, or produced on the outer cell by extracellular enzymes. EPS from LAB are divided into two classes, homo- and hetero-EPS. Homo-EPS are composed of one type of monosaccharide, whereas hetero-EPS consist of regular repeating units of 3-8 different carbohydrate moieties synthesized from intracellular sugar nucleotide precursors [5]. The biosynthesis of homo-EPS and hetero-EPS are different. Homo-EPS are made from sucrose using glucansucrase or levansucrase [6-7], and the synthesis of hetero-EPS involves four major steps, sugar transportation, sugar nucleotide synthesis, repeating unit synthesis, and polymerization of the repeating units [8]. The major physiological function of EPS is believed to be biological defenses against various stresses such as phage attack, toxic metal ions, and desiccation [9], and it is very unlikely that bacteria use EPS as an energy source. However, some potentially probiotic LAB strains have been reported to degrade EPS produced by the other LAB strains [10-11].

The term "probiotic" was first proposed by Fuller [12], and its definition was further refined to "Live microorganisms which when consumed in adequate amounts as part of food confer a health benefit on the host" [13]. Probiotic LAB thus represent a class of live food

ingredients that exert a beneficial effect on the health of the host. Beneficial microorganisms in the intestine are enhanced by “prebiotics,” which are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacterial species already resident in the colon, and thus improving host health” [14].

Most of the current prebiotics are low molecular weight except for inulin. As long carbohydrate chains are metabolized more slowly than the short ones, and polysaccharides thus exert prebiotic effects in more distal colonic regions compared to oligosaccharides, which are more rapidly digested in the proximal colon [15]. Therefore, EPS produced by LAB can be used as prebiotics. This chapter reviews the physicochemical properties, genetics, and bioactivities of the EPS produced by LAB.

2. Chemical composition of EPS

2.1. Homo-EPS

Some LAB can produce EPS that are either secreted to the environment or attached to the cell surface forming capsules. EPS are classified into two groups: homo-EPS, consisting of a single type of monosaccharide (α -D-glucans, β -D-glucans, fructans, and others represented by polygalactan) and hetero-EPS, composed of different types of monosaccharides, mainly D-glucose, D-galactose, L-rhamnose, and their derivatives [16].

The differences arise between the homopolysaccharides mainly because of the features of their primary structure such as the pattern of main chain bonds, molecular weight, and branch structure. Two important groups of homo-EPS are produced by LAB; (i) α -glucans, mainly composed of α -1,6- and α -1,3-linked glucose residues, namely dextrans, produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc mesenteroides* subsp. *dextranicum* and mutans produced by *Streptococcus mutans* and *Streptococcus sobrinus*; and (ii) fructans, mainly composed of β -2,6-linked fructose molecules, such as levan produced by *Streptococcus salivarius* [17].

The formation of dextran from sucrose has been recorded for *Leuc. mesenteroides* subsp. *mesenteroides*. However, the ability to form dextran is often lost when serial transfers are made in media with increasing salt concentrations. Nevertheless, non-dextran-producing strains of *Leuconostoc* sp. can revert to dextran production when they are inoculated into medium containing tomato or orange juice [18]. In the 1950s, the use of a cell-free enzyme solution permitted dextran synthesis under controlled conditions yielding a polymer of greater purity. A common feature of all dextrans is the preponderance of α -1,6-linkages with branch points at positions 2, 3, or 4 [17]. Some strains of *Leuconostoc amelibiosum* [19] and *Lactobacillus curvatus* [20] are reported to be dextran-producing strains.

Mutan is the glucan synthesized by various serotypes of *Str. mutans*, and differs from dextran in that it contains a high percentage of α -1,3 linkages. Differences in solubility result

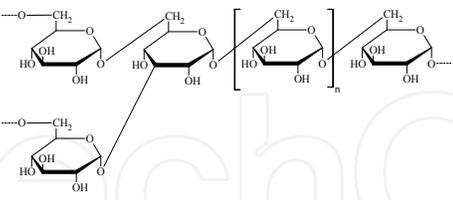
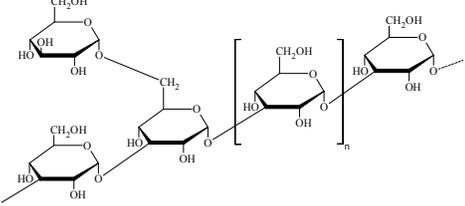
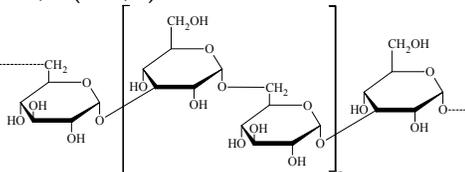
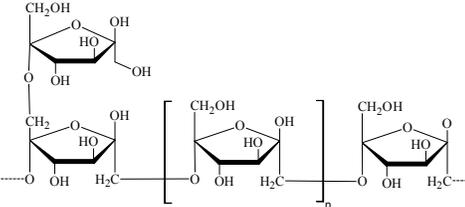
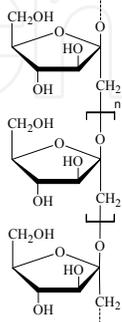
Homo-EPS	Main linkage (branching linkage)	Organism
Glucans Dextran	 <p>α-1,6 (α-1,3)</p>	<i>Leuc. mesenteroides</i> subsp. <i>mesenteroides</i> , <i>Leuc. mesenteroides</i> subsp. <i>dextranicum</i> , <i>Leuc. amelibiosum</i> , <i>Lb. curvatus</i>
Mutan	 <p>α-1,3 (α-1,6)</p>	<i>Str. mutans</i>
Alternan	 <p>α-1,3 and α-1,6</p>	<i>Leuc. mesenteroides</i>
Fructans Levan	 <p>β-2,6 (β-2,1)</p>	<i>Leuc. mesenteroides</i> , <i>Lb. reuteri</i>
Inulin	 <p>β-2,1 (β-2,6)</p>	<i>Str. mutans</i>

Table 1. Homo EPS produced by LAB

from the proportions of different types of linkages; water-soluble glucans are rich in α -1,6 linkages, while water-insoluble glucans are rich in α -1,3 linkages [17]. Ingestion of mutan has been linked with dental caries, as insoluble mutans can adhere to teeth, thus helping microorganisms adhere to the surface of teeth.

Alternan has alternate α -1,6 and α -1,3 linkages, and this structure is thought to be responsible for its distinctive physical properties including high solubility and low viscosity. These characteristics provide this glucan with a potential commercial application as a low viscosity texturizer in foods. *Leuc. mesenteroides* NRRL B-1355 was first reported to be an alternan-producing strain [21]

Levan is an EPS produced from sucrose. It is fructan composed of β -2,6-linked fructose molecules with some β -2,1-linked branches. Incidentally, inulin is a fructan composed of β -2,1-linked fructose molecules with some β -2,6-linked branches. *Str. salivarius*, *Leuc. mesenteroides*, and *Lactobacillus reuteri* are known to be levan-producing LAB [22-23]. In addition, the EPS produced by *Lactobacillus sanfranciscensis* TMW 1.392 has been reported to be fructan [11].

2.2. Hetero-EPS

The chemical composition of hetero-EPS shows wide variability. Hetero-EPS are polymerized repeating units mainly composed of D-glucose, D-galactose, and L-rhamnose. The composition of the monosaccharide subunits and the structure of the repeating units are considered not to be species-specific, except in case of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*. This species, isolated from kefir grain, a fermented dairy food from the North Caucasus region, produces large amounts of polysaccharides [24]. Hetero-EPS-producing strains of *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus delbrueckii*, and *Lactobacillus helveticus*, among others have been identified (Table 2) [25-49]. Heterofermentative LAB such as *Leuc. dextranicum* are well known homo-EPS producers, while homofermentative LAB are well-studied hetero-EPS producers. Heterofermentative in addition to homofermentative LAB can produce EPS. *Lactobacillus fermentum* is an EPS-producing heterofermentative LAB for which the EPS structure has been determined [50]. Figueroa et al. reported that *Lactobacillus brevis* and *Lactobacillus buchneri* showed ropiness on glucose- or sucrose-containing media, although they did not investigate whether such ropiness derived from hetero-EPS or from other slimy substances [51].

The quantities of hetero-EPS produced by LAB vary greatly. EPS production is 50-350 mg/l for *Str. thermophilus*, 80-600 mg/l for *Lc. lactis* subsp. *cremoris*, 60-150 mg/l for *Lb. delbrueckii* subsp. *bulgaricus*, 50-60 mg/l for *Lactobacillus casei* [52], and approximately 140 mg/l for *Lactobacillus plantarum* [45, 53]. The highest recorded yields of hetero-EPS are 2775 mg/l for *Lactobacillus rhamnosus* RW-9595M [54] and 2500 mg/l for *Lb. kefiranofaciens* WT-2B [55]. However, the quantities of EPS produced by LAB are much lower than the yields from other industrially important microorganisms such as *Xanthomonas campestris*, which produces 30-50 g/l xanthan gum [56]. Even so, amounts of EPS produced by LAB are sufficient to exploit for in situ applications. LAB are 'generally recognized as safe' (GRAS) microorganisms, and

LAB strain culture would be a useful method to produce EPS for food applications if the LAB could be grown in edible and safe culture media such as whey, and if fermentation conditions were optimized to obtain a high yield.

Fermentation conditions using undefined media have been improved to maximize yields. However, a chemically defined medium containing a carbohydrate source, mineral salts, amino acids, vitamins, and nucleic acid bases is more suitable for investigating the influence of different nutrients on LAB growth and EPS biosynthesis. The total yield of EPS produced by LAB depends on the composition of the medium (carbon and nitrogen sources) and the growth conditions, i.e., temperature, pH, and incubation time.

Under conditions of higher temperatures and slower growth, the production of the polymer per cell in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was greater in milk [57]. Another study investigated the optimum culture conditions for EPS production by *Lb. delbrueckii* subsp. *bulgaricus* RR in semidefined medium [58], and determined the optimum temperature and pH conditions for EPS production to be 36°C - 39°C and pH 4.5 - 5.5. The optimal temperature for EPS production was approximately 40°C for thermophilic LAB strains, and around 25°C for mesophilic LAB. Gamar et al. [59] reported increased slime production at lower incubation temperatures, and an increase in the final EPS concentration in *Lb. rhamnosus* following incubation at 25°C instead of 30°C. The effects of temperature on EPS production in whey were investigated in *Lb. plantarum* [53], and the yield was found to be higher at 25°C than at either 30°C or 37°C. Moreover, an inverse relationship was observed between EPS production per cell and the growth temperature for *Lactobacillus sake* [49], i.e. the lower the temperature, the higher the EPS production per cell. However, the growth rate in the exponential phase decreased at low temperatures. Therefore, the temperature for the maximal production of EPS is based on a balance of cell density and EPS production per cell. Maximal EPS production by *Lb. sake* was obtained under anaerobic conditions at 20°C, although EPS production per cell was higher at 10°C. Therefore, it is possible that severe environmental conditions trigger EPS production as a protective mechanism.

The effects of alterations to the nitrogen and carbon sources used in EPS production have also been investigated. According to early reports, neither LAB growth nor EPS production was specifically linked to the presence of casein or whey proteins in the growth medium. Garcia et al. [57] reported that EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 increased during the early growth phase in the presence of hydrolyzed casein in milk, while the addition of hydrolyzed casein to MRS medium did not increase EPS production. This strain produced 25 mg/l EPS when grown on fructose in a defined medium, and 80 mg/l EPS when grown on glucose [60]. The optimum Bacto-casitone concentration for EPS production by *Lb. delbrueckii* subsp. *bulgaricus* RR was investigated in semidefined medium [58]. In this study, there was a significant relationship between the Bacto-casitone concentration and EPS production; the higher the casitone concentration, the higher the EPS yield that was obtained. For *Lb. plantarum* grown in whey, yeast extract was a more effective nitrogen source for EPS production than soy peptide, tryptone, peptone, and Lab-Lemco powder,

and glucose was a more effective carbon source than galactose, sucrose, maltose, fructose, and raffinose [53]. EPS production by *Lb. casei* CG11 was investigated in basal minimum medium containing galactose, glucose, lactose, sucrose, maltose, and melibiose; glucose was the most efficient carbon source, and lactose and galactose were the least efficient ones [61]. EPS production by *Lb. rhamnosus* C83 was investigated in a chemically defined medium containing different carbon sources (glucose, fructose, mannose, and maltose) at different concentrations. Mannose at 40 g/l was by far the most efficient carbon source. Furthermore, increased Mg, Mn and Fe concentrations stimulated EPS production in synthetic media [59]. In addition, Macedo et al. [54] reported about the importance of salts in culture media and the strong positive effect of salts and amino acids on *Lb. rhamnosus* RW-9595M growth and EPS production. The addition of salts and amino acids largely increased EPS production (to 2775 mg/l) in whey permeate supplemented with yeast extract, although the addition of amino acids alone had no effect on EPS production.

It has been shown that an optimal ratio between the carbon and nitrogen is absolutely necessary to achieve high EPS yields [62]. The production of EPS by *Str. thermophilus* LY03 is modulated by both the absolute quantities and the ratio of carbon to nitrogen (C/N ratio). The carbon source is converted into lactic acid to produce energy as well as to synthesize the cell wall and EPS, and nitrogen is necessary for the synthesis of essential cell components. Therefore, a higher C/N ratio and sufficient quantities of both carbon and nitrogen increase EPS production.

3. EPS biosynthesis by LAB

3.1. Homo EPS biosynthesis

Homo EPS are synthesized outside the cell by specific glycosyltransferase (GTF) or fructosyltransferase (FTF) enzymes (commonly named glucansucrases or fructan-sucrases). Homo-EPS producing LAB also use extracellular GTF enzymes to synthesize high-molecular mass α -glucans from sucrose. This process uses sucrose as a specific substrate, and the energy required for the process comes from sucrose hydrolysis. There is no energy requirement for EPS-production other than for enzyme biosynthesis because EPS synthesis by GTF or FTF does not involve active transport processes or the use of activated carbohydrate precursors. Therefore, large amounts of sucrose can easily be converted to EPS. *Lb. sanfranciscensis* produces up to 40 g/l levan and 25 g/l 1-kestose during growth in the presence of 160 g/l sucrose [63].

Glucan synthesis reactions catalysed by GTF can be written as follows (Fig. 1):

sucrose + H₂O → glucose + fructose

sucrose + acceptor carbohydrate → oligosaccharide + fructose

sucrose + glucan (n) → glucan (n+1) + fructose

Although GTF enzymes have a high degrees of similarity, lactobacilli produce a broad spectrum of glucans, including polymers with α -1,6 linkages (dextran), α -1,3 linkages (mutan), and both α -1,6 and α -1,4 linkages (alternan). The relative molecular weight of

glucans from lactobacilli range from 1×10^6 Da to 5×10^7 Da [6]. In addition, GTF enzymes are not saturated by their substrate, and transfer reactions exceed the sucrose hydrolysis under sucrose concentrations above 100 mM [64].

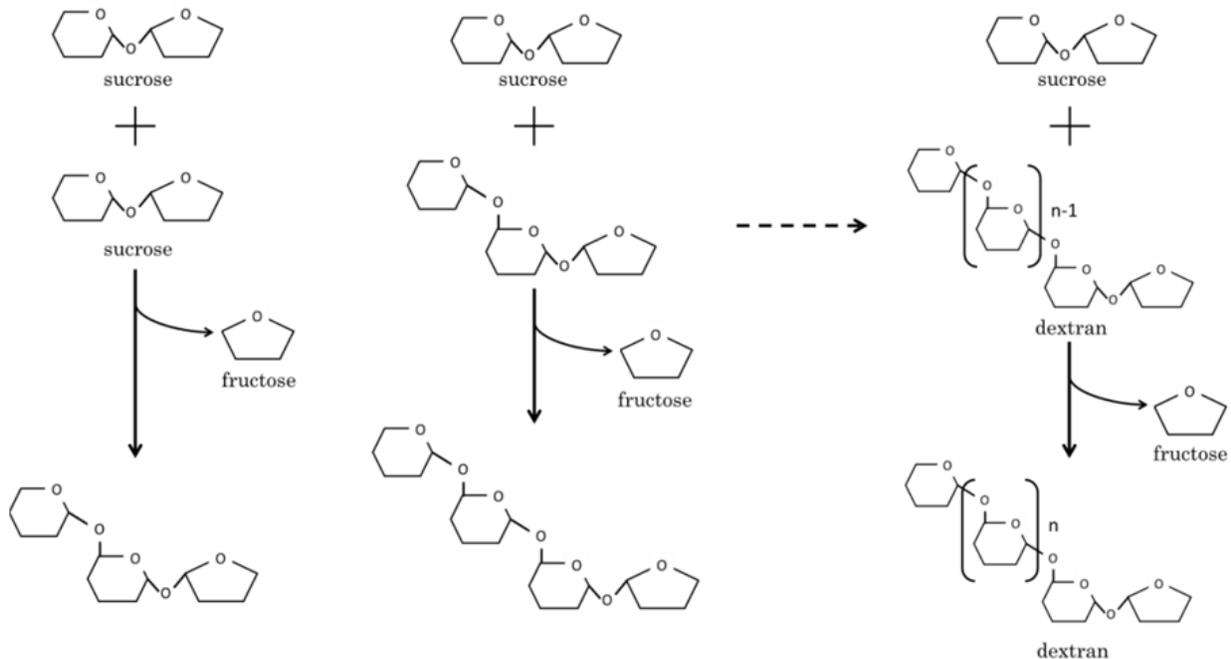
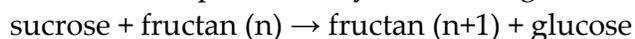
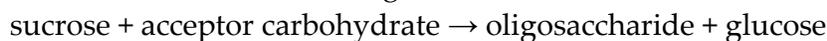
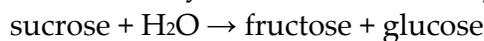


Figure 1. The dextran synthesis by GTF (dextran sucrose).

The GTF enzymes of streptococci are generally produced constitutively. In contrast, the GTF enzymes of *Leuconostoc* species are specifically induced by sucrose. For example, GTF expression in *Leuc. mesenteroides* is low in the presence of carbon sources other than sucrose and is increased by the addition of sucrose [5]. GTF expression during sucrose fermentation is 10-15-fold higher than that measured during glucose fermentation in *Leuc. mesenteroides* Lcc4. In fed-batch fermentation with both glucose and sucrose, GTF activity was similar to that obtained with sucrose alone. These results show that GTF expression is low in the presence of glucose alone, and that GTF activity is significantly induced by sucrose. A sucrose concentration of 20 g/l is sufficient to ensure the induction of enzyme synthesis, and higher concentrations (up to 60 g/l) do not lead to a further increase in enzyme synthesis [65].

The fructan synthesis reaction catalyzed by FTF can be written as follows:



Fructans generally have a relative molecular weight exceeding 5×10^6 Da. Similar to GTFs, FTFs are not saturated by their substrate, namely, sucrose, and transfer reactions exceed the rate of sucrose hydrolysis for sucrose concentrations above 200 mM [5]. FTFs such as Lev, Inu, and LevS from lactobacilli exhibit pH optima of between 5.0 and 5.5. The optimum temperature for enzymes from the thermophilic *Lb. reuteri* is higher (50°C) than that of the *Lb. sanfranciscensis* enzyme (35°C – 40°C) [5].

3.2. Hetero EPS biosynthesis

Hetero EPS are not synthesized by extracellular enzymes, but are instead synthesized by a complex sequence of interactions involving intracellular enzymes. EPS are made by polymerization of repeating units, and these repeating units are built by a series of addition of sugar nucleotides at the cytoplasmic membrane. Sugars are the starting materials for the synthesis sequence. LAB strains can utilize various monosaccharides and disaccharides as energy sources, via some well-studied sugar uptake systems include primary transport systems, direct coupling of sugar translocation to ATP hydrolysis via a transport-specific ATPase; secondary sugar transport systems, coupling of sugar transport to the transport of ions or other solutes, both as symport and antiport transport systems; and group translocation systems, coupling of sugar transport to phosphorylation via the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS; Fig. 2) [8]. Polysaccharides must be hydrolyzed before uptake. For example, starch is hydrolyzed by α -amylase, and the reaction products are subsequently hydrolyzed by the enzymes described above.

Lc. lactis strains possess a lactose-specific PEP-PTS sugar transport system that imports extracellular lactose, resulting in increased intracellular lactose-6-phosphate. Lactose-6-phosphate is then hydrolyzed, and the galactose-6-phosphate moiety is metabolized by the tagatose-6P pathway (Fig. 2).

Lb. delbrueckii subsp. *bulgaricus* and *Str. thermophilus* are generally galactose-negative and take up lactose via a lactose/galactose antiport transport system. The glucose moiety of imported lactose is fermented by these strains, while the galactose moiety is excreted via the lactose/galactose antiport system.

After the addition of a hetero-EPS repeating unit, the unit is exported through the cell membrane and becomes polymerized into the final hetero-EPS. Hence, several enzymes and proteins are involved in the biosynthesis and secretion of heterotype EPS, and the enzymes and proteins involved in these processes may not be unique to hetero-EPS anabolism.

Sugars taken into the cell are converted into sugar nucleotides. Intracellular monosaccharides are converted to sugar nucleotide substrates for polymerization reactions, including UDP (uridine diphosphate), dNTP (thymidine diphosphate), and GDP (guanosine diphosphate). Such polymerization reactions are catalyzed by glycosyl pyrophosphorylases.

$\text{Glu-1P (Gal-1P) + UTP} \rightarrow \text{UDP-Glu (UDP-Gal) + pyrophosphate}$

UDP-glucose is then converted to UDP-galactose by epimerases such as UDP-glucose-4-epimerase. This reaction is reversible.

$\text{UDP-glucose} \leftrightarrow \text{UDP-galactose}$

Glycosidic linkages are formed on membranes in the cytoplasm. A sugar moiety is transferred to C55-polyprenyl phosphate, a carrier lipid and component of the membrane, by priming glycosyl transferases. This transfer triggers the addition of a repeating unit to the hetero-EPS molecule. Disruption of the priming glycosyl transferase gene generates non-EPS-producing mutants [66]. Thus, priming glycosyl transferases are thought to be crucial

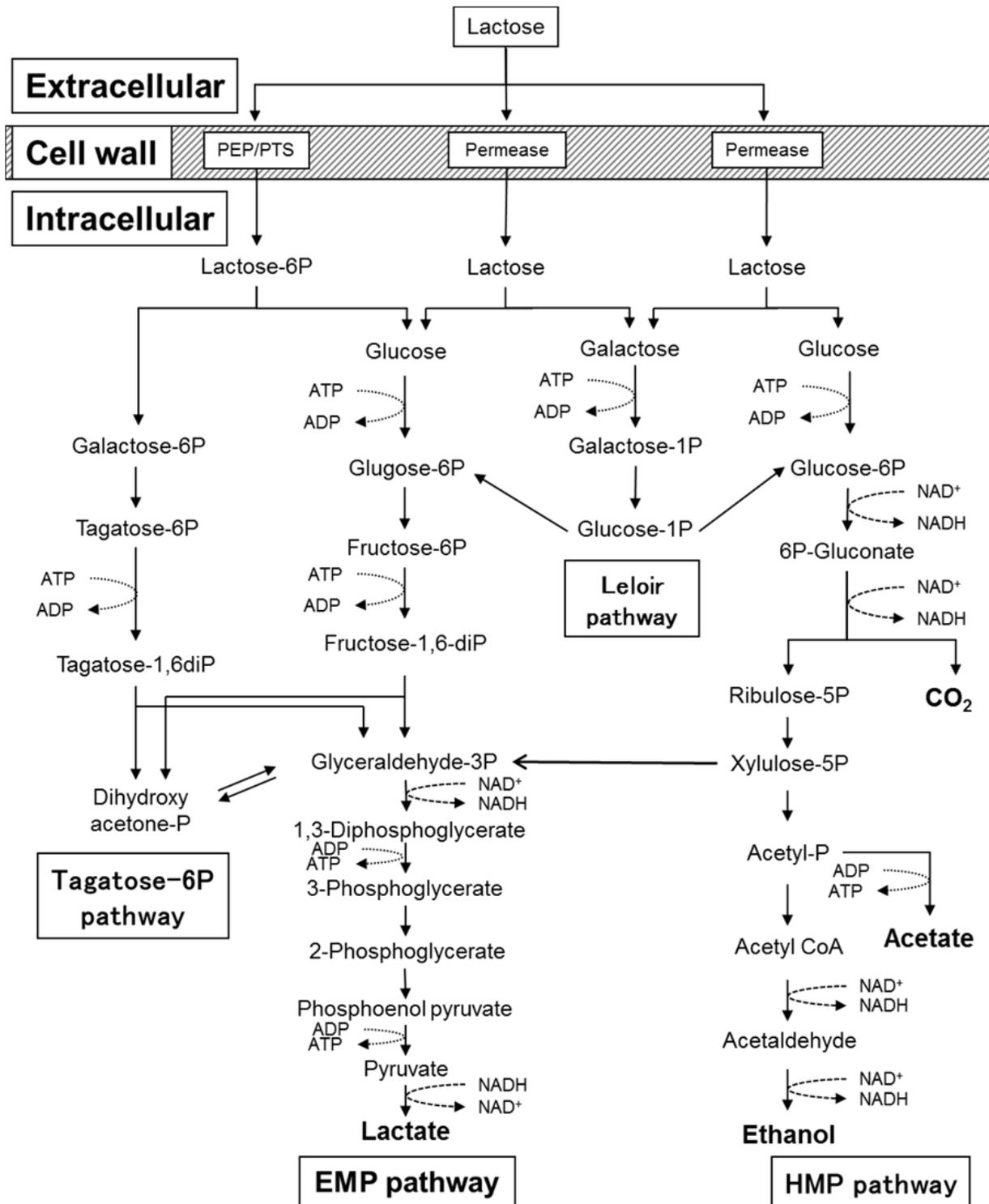


Figure 2. Pathway of lactose fermentation in lactic acid bacteria.

for EPS biosynthesis. The addition of the repeating unit is completed by the action of glycosyl transferase on the sugar residue attached to C55-polyprenyl phosphate. Therefore, the type and number of glycosyl transferases available determine the range of repeating units in hetero-EPS. C55-polyprenyl phosphate is also involved in bacterial cell wall biosynthesis, and therefore, cell wall biosynthesis and EPS synthesis compete for this

substrate. The repeating unit is exported through the bacterial membrane, and is polymerized to become a hetero-EPS (Fig. 3).

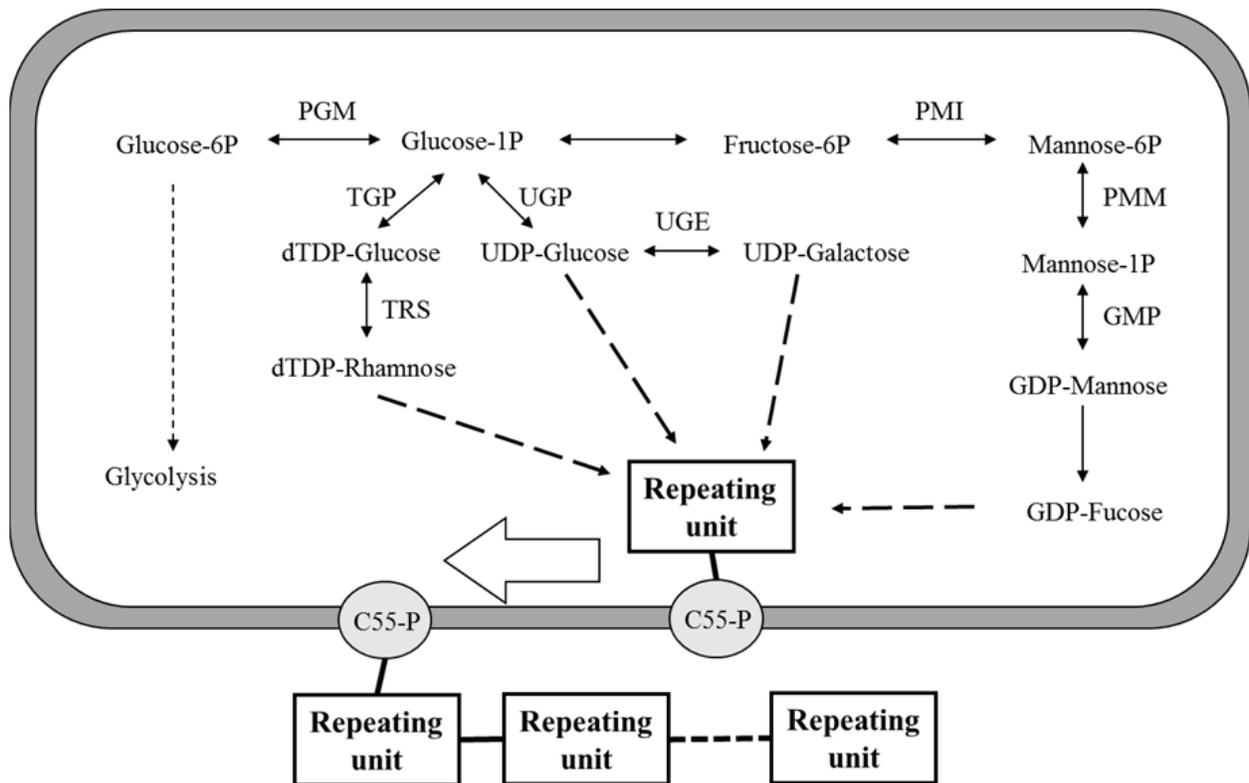


Figure 3. Outline of biosynthesis of hetero EPS.

PGM: α -phosphoglucomutase, UGP: UDP-glucose pyrophosphorylase

UGE: UDP-galactose 4-epimerase, TGP: dTDP-glucose pyrophosphorylase

TRS: dTDP-rhamnose synthetic enzyme system, PMI: phosphomannoisomerase

PMM: phosphomannomutase, GMP: GDP-mannose pyrophosphorylase

3.3. Instability of EPS production

The instability of hetero-EPS production has been reviewed by de Vuyst et al. [8]. Briefly, a loss in the ability to produce slime may be caused by repeated subculture of bacterial strains or incubation at high temperatures. The loss of plasmids from ropy mesophilic LAB strains is generally the reason for loss of slime production. On the other hand, thermophilic LAB, namely, *Lb. delbrueckii* subsp. *bulgaricus* and *Str. thermophilus*, have been shown to lack plasmids encoding components required for slime production. These species can usually recover the ability to produce slime following loss due to culture conditions. Thus, genetic instability could be a consequence of the actions of mobile genetic elements such as insertion sequences. Recently, the EPS gene cluster in *Lb. fermentum* TDS030603 was reported to be located in chromosomal DNA [67].

Priming glycosyl transferases are thought to be crucial for EPS biosynthesis and disruption of the priming glycosyl transferase gene generates non-EPS-producing

mutants. Tsuda et al. generated the EPS-producing mutant strain 301102S from the non-EPS-producing *Lb. plantarum* 301102 following exposure to the mutagens acridine orange and novobiocin [4]. The activities of α -phosphoglucomutase (PGM), UDP-glucose pyrophosphorylase (UGP), and UDP-galactose 4-epimerase (UGE) were measured in parental and mutant strains by using the method of Mozzi [68], and were found to be almost the same for both [Tsuda & Miyamoto, unpublished data]. Next, priming glycosyl transferase genes in parental and mutant strains were amplified with the thermal cycler. Primers were designed to amplify a priming glycosyl transferase gene referring to complete *Lb. plantarum* WCFS1 genome sequenced [69]. PCR products were subjected to restriction digestion, which allowed identification of putative priming glycosyl transferase gene. PCR products were also applied to single strand conformation polymorphism (SSCP) analysis for detecting point mutations. However, both parental and mutant strains had the same priming glycosyl transferase gene sequence, and similar levels of activities of the PGM, UGP, and UGE enzymes. Thus, although priming glycosyl transferases are essential, other factors may also be necessary for EPS production, and a mutation affecting EPS production may occur in another gene. Morona et al. reported that an autophosphorylating protein-tyrosine kinase is essential for encapsulation in *Streptococcus pneumoniae* [70]. A point mutation in the gene encoding the autophosphorylating protein-tyrosine kinase affecting the ATP-binding domain resulted in loss of EPS production.

4. Polysaccharides and oligosaccharides for colon health

EPS produced by LAB have various functional roles in human or animal health including immunomodulatory properties, antiviral activity, antioxidant activity, and antihypertensive activity [1, 55, 71, 72], and have also been used as food additives for texture improvement. These properties have been extensively reviewed [8, 9, 56, 73, 74]. Besides these properties, prebiotics based on LAB and oligosaccharides have other health benefits. Prebiotics are usually non-digestible oligosaccharides that selectively stimulate the growth and activity of a limited number of bacterial species in the colon, such as bifidobacteria and lactobacilli, and therefore, improve host health. Detrimental bacteria may form substances such as ammonia, hydrogen sulfide, indoles, and amines that are noxious to the host. However, beneficial bacteria such as bifidobacteria and lactobacilli inhibit the proliferation of detrimental bacteria, and their cell components stimulate the host immune system [75]. Gastrointestinal microflora consist of approximately 10^{14} colony forming units (cfu)/g of various types of both detrimental and beneficial bacteria, and the numbers and composition vary greatly along the gastrointestinal tract. The balance of the gastrointestinal micro flora influences different aspects of host health such as bowel movement, tympanites flatulence, and the absorption of nutrients. Many factors may upset this balance, including stress, consumption of antibiotics, infection, food poisoning, and the natural ageing process. To redress this balance, the growth and activities of beneficial bacteria may be enhanced by specific ingredients in foods.

Speceis	Strain	Glc	Gal	Rha	Fuc	NAc Gal	GlcA	Gly	Reference
<i>Streptococcus thermophilus</i>	CNCMI 733	1	2			1			[25]
	SFi39	1	1						[26]
	SFi12	1	3	2					[26]
	LY03	1	4						[27]
	OR901		5	2					[28]
	MR-1C		5	2	1				[29]
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	NIZO B891	3	2						[30]
	Ropy352	2	3						[31]
	NIZO B39	2	3	2					[32]
	SBT 0495	2	2	1					[33]
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	OLL 1073R-1	1	1.6						[34]
	NCFB 2772	1	2.4						[35]
	Lb18	1	1						[36]
	EU23	1		1					[37]
	rr	1	5	1					[38]
	NCFB 2772	1	7	0.8					[35]
<i>Lb. helveticus</i>	TN-4	1	1						[39]
	766	2	1						[40]
	2091	1	2						[41]
	Lb161	5	2						[42]
<i>Lb. rhamnosus</i>	RW-9595M	2	1	4					[43]
	GG	1	4	1					[44]
<i>Lb. plantarum</i>	EP56	3	1			1			[45]
	EP56	3	1	1					[45]
<i>Lb. pentosus</i>	LPS26	1		2			2		[46]
<i>Lb. paracasei</i>	34-1		3			1		1	[47]
<i>Lb. kefiranofaciens</i>	K ₁	1	1						[48]
<i>Lb. sake</i>	0-1	3		2					[49]

Table 2. Monosaccharide ratio in hetero EPS

Glc: glucose, Gal: galactose, Rha: rhamnose, Fuc: fucose, NAc Glu: N-acetyl glucosamine, NAc Gal: N-acetyl galactosamine, GlcA: glucuronic acid.

Various oligosaccharides have been identified as prebiotics, that can increase the number of *Bifidobacterium* in the host colon. Galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are considered important prebiotics. Other carbohydrates including gluco-oligosaccharides, isomalto-oligosaccharides, lactulose, mannan-oligosaccharides, and nigero-oligosaccharides are also considered prebiotics. Increased numbers of bifidobacteria and/or lactobacilli in the colon have been shown to have beneficial effects, although the specific mixtures of populations of these genera necessary to provide health-promoting effects has not yet been determined. This is because the beneficial effects are likely to be due to improvement in the balance of colonic micro flora. However, differences do exist in the micro flora among individuals. To function most effectively, prebiotics must be resistant to digestive processes in the stomach and small bowel, so that they can come into contact with the bacteria growing in the large intestine.

The food for specified health use (FOSHU) system was introduced in Japan in 1991. FOSHU refers to foods containing ingredients that provide health benefits and have officially approved physiological effects on the human body. FOSHU is intended to be consumed for the maintenance or promotion of health or for special health uses, for example, to control conditions such as blood pressure or blood cholesterol. To be defined as FOSHU, it is important to assess the safety of the food as well as the effectiveness of health promotion, and this assessment must be approved by the Ministry of Health, Labour and Welfare in Japan. At present (2012), 990 foods are recognized as FOSHU, and of these, 86 provide gastrointestinal health benefit. Foods for balancing gastrointestinal micro flora contain galactosylsucrose, soy oligosaccharides, lactulose, GOS, FOS, isomalto-oligosaccharides, raffinose, xylo-oligosaccharides, mannanose, and brewer's yeast cell wall as functional ingredients.

4.1. GOS

GOS are well-known type of prebiotic oligosaccharides found in human milk. The concentration of oligosaccharides is 100 times higher in human breast milk than in bovine milk [76]. Many studies have shown that breast-fed infants have intestinal microflora dominated by bifidobacteria. The reason for this phenomenon is thought to be that the oligosaccharides in breast milk, including GOS, can reach the upper gut without being digested where the bifidobacteria can utilize them. At present, GOS is produced by the enzymatic treatment of lactose by β -galactosidase. GOS produced in this manner usually have degrees of polymerization (DP) between 2 and 10. Furthermore, the type of glycosidic linkage is determined by the reaction conditions: final products usually possess β -1,2, β -1,3, or β -1,4 linkages. GOS is given a caloric value of 2 kcal/g in Japan and Europe for food-labelling purpose.

The effect of GOS on defecation has been studied in healthy volunteers. Defecation frequency was significantly increased, and faeces became significantly softer after the subjects drank a beverage containing 5.0 g of GOS, on a daily basis. Therefore,

consumption of a beverage containing 5.0 g of GOS can improve defecation in individuals with a tendency for constipation [77]. Ishikawa et al. reported that the number of faecal bifidobacteria increased significantly after subjects consumed 2.5 g of GOS/day for 3 weeks [78]. GOS utilization by enterobacteria was further investigated in vitro. The trisaccharide forms of GOS were utilized by *Bifidobacterium*, *Lactobacillus acidophilus*, *Lb. reuteri*, *Bacteroides*, *Clostridium perfringens*, *Klebsiella pneumoniae*, *Enterococcus faecium*, and the tetra-saccharide forms were utilized by *Bifidobacterium adolescentis*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Ent. faecium*. These results suggest that a higher DP of GOS enhanced selectivity, and that the tetrasaccharide forms of GOS are specifically utilized by bifidobacteria. Similarly, *Bifidobacterium lactis* DR10 utilizes trisaccharide and tetra-saccharide forms of GOS, whereas *Lb. rhamnosus* DR20 prefers disaccharides and monosaccharides [79]. Barboza reported that *Bif. breve* and *Bif. longum* subsp. *infantis* can consume GOS with a DP ranging from 3 to 8 [80]. Furthermore, *Bif. longum* subsp. *infantis* preferentially consume GOS with a DP of 4, and *Bif. adolescentis* utilizes GOS with DP of 3. In addition, the structure of GOS influences its utilization by lactobacilli and bifidobacteria [81]. Trisaccharides of 4'-GOS (β -1,4 linkage) and 6'-GOS (β -1,6 linkage) can be used as the sole carbon source. Almost all lactobacilli and bifidobacteria tested preferred to utilize 4'-GOS, while *Lb. acidophilus*, *Lb. reuteri*, and *Lb. casei* could utilize both 4'- and 6'-GOS. GOS are used to stimulate beneficial bacteria, but can also be utilized by bacteroides and clostridia [82]. GOS selectivity may be enhanced by altering the structure and increasing the DP.

The use of beneficial bacteria or their enzymes in the synthesis of prebiotics may be a good way to produce prebiotics with high specificity. Rabiou reported that five different GOS were produced using β -galactosidase extracted from five different *Bifidobacterium* species, and that each GOS showed an increased growth rate in producer strains, except for *Bif. adolescentis* [83]. The utilization of these GOS by faecal bacteria was investigated using commercial GOS as control. The number of *Bacteroides* was decreased with GOS from bifidobacteria, whereas both GOS extracts and commercial GOS increased the number of bifidobacteria, lactobacilli, and clostridia.

4.2. FOS

FOS is used as a generic term for all β -2,1 linear fructans with a variable DP. Inulin and oligofructose are common forms of FOS that are widely found in nature. Chicory inulin has a DP of 2-60, and the product of its partial enzymatic hydrolysis is oligofructose or FOS with a DP of 2-10.

The effect of FOS intake on intestinal microflora was studied in humans. The number of bifidobacteria in faeces was significantly increased during the FOS intake (1 g/d) period, and a significant increase in stool frequency and a softening effect on stool were observed [84]. FOS increased the level of bifidobacteria in faeces, whereas that of bacteroides, clostridia, and fusobacteria decreased in subjects that were fed FOS (15 g/d) for 15 days [85]. Another study measured the increase in number of *Bifidobacterium* species in faeces by using real-

time PCR [86]. The composition of bifidobacteria in the gut microflora was studied by clone library analysis in ten volunteers. All ten volunteers carried *Bif. longum*, and nine of these also carried *Bif. adolescentis*. The consumption of inulin (10 g/d) increased the number of bifidobacteria in faeces with *Bif. adolescentis* showing the highest increase response among *Bifidobacterium* species. Rossi et al. reported that only 8 of 55 *Bifidobacterium* strains fermented inulin in pure cultures, although inulin increased the number of bifidobacteria in faecal culture [87]. They, therefore, suggested that most bifidobacteria were not able to utilize long fructans in the absence of other intestinal bacteria that can hydrolyze fructans, and that fermentation of oligosaccharides in the colon is the result of a complex metabolic sequence carried out by numerous species.

4.3. Selection of high-efficiency prebiotics

It is not clear which oligosaccharides are the most suitable substrates for the selective growth of specific beneficial species or strains. Several research groups have suggested useful methods to investigate the potential prebiotic activity of oligosaccharides [88-92]. Potential prebiotic activities were determined on the basis of the changes in the growth of beneficial and undesirable bacteria, such as bifidobacteria, lactobacilli, clostridia, and bacteroides. Such methods can evaluate the ability of specific strains to utilize a particular prebiotic, and a comparison of the prebiotic activities of oligosaccharides by using these methods could help in the choice of prebiotics for improving the gastrointestinal microflora on an individual basis. However, it is important to understand that only a limited group of bacteria can be chosen from the gastrointestinal microflora by using these methods, and that polysaccharides and oligo-saccharides are fermented by numerous species in the gastrointestinal tract.

Oligosaccharides produced by beneficial bacteria or their enzymes may enhance the growth of beneficial bacteria. A novel GOS mixture produced using *Bif. longum* NCIBM 41171 galactosidases increased the proportion of bifidobacteria in faeces relative to commercial GOS [93]. In the above-described study, oligosaccharides synthesized by the enzymes from *Bifidobacterium* strains were favored by the producer strains [83]. These studies suggest that the oligosaccharides produced by beneficial bacteria are selectively utilized by the producer strain, because the enzymes required for their degradation are already available. In addition, glycosyltransferases may possess both hydrolytic and transglycosylation activities [94], and glycosidases and glycosyltransferases may coexist in the same strains. Schwab et al. reported the production of novel oligosaccharides [95]. Hetero-oligosaccharides were produced from lactose, mannose, fucose, and N-acetylglucosamine by using crude cell extracts and whole cells of LAB and bifidobacteria. These hetero-oligosaccharides contained mannose, fucose, and N-acetylglucosamine, and could be digested by LAB strains. The prebiotic activities of these oligosaccharides were not investigated; however, a similar approach using probiotic and intestinal beneficial bacteria may lead to the production of highly selective prebiotics.

The dietary fiber, arabinoxylan is the predominant hemicellulose from cereals and exhibits prebiotic activity [96]. The addition of water-unextractable arabinoxylans increased the population of bifidobacteria and bacteroides in a medium inoculated with faecal slurry. Polysaccharides are not usually utilized by microorganisms. Remarkably, however, *Bifidobacterium bifidum* DSM20456 can utilize the EPS produced by *Pediococcus pentosaceus*, *Lb. plantarum*, *Weissella cibaria*, and *Weissella confusa*, and some growth is observed in cas of *Bif. longum*, *Bif. adolescentis*, and *Lb. acidophilus* [97]. For EPS production by LAB, reduced yields were frequently observed after the maximal level had been reached, which might be caused by the enzymes produced by the bacteria [98]. Tsuda and Miyamoto investigated the prebiotic activity of EPS produced by *Lb. plantarum* 301102S [52], a mutant strain derived from *Lb. plantarum* 301102. Oral administration of the parental strain 301102 showed the survivability and proliferation in porcine gastrointestinal tract [99]. The potential prebiotic activities of EPS, GOS, and inulin were measured in 37 LAB strains, and the activity scores of EPS in the strains 301102 and 301102S were highest. This suggests that the EPS produced by the mutant strain is utilized by the same strain 301102S and the parental strain, and that the parental strain has enzymes that can degrade the EPS.

5. Conclusion

Poly- and hetero-oligosaccharides produced by LAB may be potential prebiotics. Studies on the production of polysaccharides and oligosaccharides by enzymes in beneficial microorganisms may lead to the production of highly selective prebiotics, although in vitro evaluation may be difficult because of degradation and utilization of polysaccharides by various microorganisms in the gastrointestinal tract. Administration of synbiotic food containing a combination of a probiotic bacterial strain and the prebiotic sugar produced by that strain could be effective in improving human health.

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6. References

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