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Modulating Exopolysaccharide Production in Lactic Acid Bacteria and Bifidobacteria: Insights from Physiological, Evolutionary, and Co-Culture Strategies

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Abstract

Lactic acid bacteria (LAB) and *Bifidobacterium* make exopolysaccharides (EPS), which positively affect the physicochemical and sensory properties of fermented food. The isolated EPS is also useful for improving viscosity, stability, and food textures, and also finds applications in the medical field. Thus, there is an increasing research focus on enhancing EPS production by these bacteria. Altering the growth media composition, by varying carbon and mineral sources, is a tested approach for such a purpose. Cultivation conditions like temperature, pH, and shaking also significantly influence EPS production in a strain-specific manner. Given the plausible role of EPS in stress tolerance, elevating EPS yield by exposure to certain stressors, such as bile, has been achieved. Advanced strategies such as evolutionary engineering and cross-kingdom ecological interactions of LAB, especially with yeast, also appear to be promising techniques for enhancing bacterial EPS yield and quality. This review elucidates recent research on all the above-mentioned ways of enhancing EPS production and the possible utility of such bacteria in industrial applications.

Keywords: Adaptive Laboratory Evolution, Biomaterial, EPS Biosynthesis, Food Fermentation, Food Texture, *Lactobacilli*, Stress Response

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INTRODUCTION

Lactic acid bacteria (LAB) and *Bifidobacterium* spp. belong to the generally recognized as safe (GRAS) category of microbes. They are associated with numerous habitats, including fermented foods and the human GI tract. Exopolysaccharides (EPS) are essential products secreted by these bacteria into the extracellular environment. EPS plays a key role in helping bacteria adapt to numerous environmental stimuli, including serving as a physical barrier for protection against bacteriophages, extreme pH, and inhibitory chemicals. Additionally, the water-holding ability of EPS helps bacteria from desiccation. EPS is an essential component of biofilms and thus aids in intra- and inter-kingdom communication.[1]

Foods fermented with these bacteria, such as dairy products, often contain EPS produced *in situ*. Bacterial EPS are also exogenously added to processed foods as emulsifiers and thickening agents.[2] Thus, the human gut receives bacterial EPS via such foods. These bacteria are important components of the human gut microbiome and also produce EPS in the gut. EPS can provide a substrate for fermentation by a portion of the microbiota in the distal colon, acting as a prebiotic and supporting the growth of beneficial bacteria.[3] In addition to its involvement in gut microbial communications, EPS influences intestinal adhesion of microbes, provides a substrate for producing bioactive short-chain fatty acids, imparts immunomodulatory effects, and thus affects the overall gut microbiome and host health.[4]

Bacterial EPS plays a pivotal role in food applications due to its remarkable properties that enhance rheology, texture, mouthfeel, and overall sensory experience. Specific EPS-producing strains of LAB, such as those belonging to the *Lactobacillus* genus, are widely employed in yogurt production to improve the viscosity and texture of the yogurt.[5] Similarly, in cheese-making, EPS enhances the overall quality and texture, elevating the product's appeal.[6] Its water-holding ability also makes EPS useful as a biomaterial in wound-healing applications[7] and as a hydrating agent in the cosmetic industry.[8]

Bacterial EPS can be secreted in two forms: slime polysaccharides, loosely attached or expelled into the environment surrounding the bacteria, and capsular polysaccharides, which form a capsule around the bacteria by adhering to the cell wall.[9] Based on their structures, EPS can be classified as homopolysaccharides (HoPS) comprising a single type of monosaccharide, such as glucose and fructose, and heteropolysaccharides (HePS), composed of different monosaccharides. Some of the well-known producers of HoPS, such as fructan, β-D-glucan, and dextran, are *Lactobacillus* and *Leuconostoc* spp.[10] On the other hand, HePS are more commonly produced by *Lactococcus* and *Lactobacillus* spp.[11] Many LAB strains that combine these polysaccharides are also known.[12] Bacterial production of HoPS is generally higher than that of HePS because the simpler pathway requires fewer cellular resources.

The bioactivity and physicochemical properties of bacterial EPS depend on their structural properties, including molecular weight, monosaccharide composition, branching, linkages, and chain length.[13] For example, EPS with low molecular weight and negative charge often have higher immuno-stimulating properties compared to high molecular weight EPS.[14] High antioxidant activity was also observed in EPS with a low molecular weight.[15] Other factors, such as specific functional groups and glycosidic linkages, are also thought to affect the anticancer and anti-inflammatory activity of EPS produced by *Lactobacillus* spp.[16] The different monosaccharides are seen to have different impacts on the bioactivity of the EPS. EPS rich in glucose and galactose have been reported to have strong immunomodulatory activities; those with mannose possess anti-inflammatory activities, and those composed of rhamnose and glucose have shown antimicrobial activity.[17]

The backbone structure affects the water solubility of EPS, mainly due to the quantity and distribution of various linkages. Additionally, biological modifications by enzymes can affect solubility by adding or eliminating functional groups such as acetyl, pyruvyl, succinyl, and glyceryl groups.[18] Furthermore, *in vitro* chemical modifications, such as acetylation and sulfonation, have been observed to enhance the solubility of

the polysaccharide.[19] Similarly, the rheological property of EPS is also influenced by its glycosidic linkages, functional groups, molecular weight, and sugar content.[20]

The interplay of various factors, such as environmental, nutritional, and genetic aspects, can govern bacterial EPS structural characteristics and biosynthesis. The optimization of media, including the carbon and nitrogen sources, along with other parameters, such as temperature and pH, leads to modulation in the yield and structural properties of the EPS. Given the critical interactions of various factors involved, a thorough understanding of the mechanisms behind EPS biosynthesis is essential. Increased EPS production has been found to improve the texture and rheological characteristics of fermented foods in the food industry and stimulate the development of bioactive compounds. Boosting EPS production by optimizing the culture conditions would expand their functionality for industrial use. This mini-review examines the various ways in which the properties and yield of EPS produced by *Lactobacillus* and *Bifidobacterium* spp. could be improved. Since genetically engineered bacteria are not allowed in foods, studies using such strategies for improving EPS production have not been covered in this article.

Effect of Media Composition

The chemical composition of the culture media is one of the obvious factors that impact the yield and the quality of the EPS. The carbon source is the most influential media component affecting bacterial EPS production. A fundamental reason is that homopolysaccharides, such as glucan and fructan, are synthesised from sucrose, while heteropolysaccharides are primarily synthesised from glucose.[21] Some studies showing the varying EPS yields with different sugars in the medium, such as glucose, fructose, mannose, sucrose, galactose, cellobiose, and lactose, have been carried out in various genera of LAB and *Bifidobacterium* spp., such as *Lacticaseibacillus, Lactiplantibacillus, Lactobacillus, Lactococcus, Limosilactobacillus, Weissella, Leuconostoc* and *Bifidobacterium* (Table). One common finding across all such studies is that there is no universal rule regarding which sugar, when included in the medium, leads to higher EPS production in all *Lactobacillus* spp. Such a conclusion also emerges from the fact that the most variable genomic regions in *Lactobacillus* spp. are those involved in carbohydrate utilization, involving genes for sugar transport, energy metabolism and biosynthesis of structural components.[22] Thus, it is imperative that for any given strain, the sugar giving the highest EPS yield be experimentally determined. Indeed, such an approach can itself result novel mechanistic insights into EPS biosynthesis. For example, while glucans are derived from sucrose, Mayer et al.[23] found glucan production in *Lactobacillus johnsonii* FI9785 in the absence of sucrose. Further studies using genetic tools led to the discovery of a novel bactoprenol glycosyltransferase and flippase-dependent EPS biosynthesis pathway.

In addition to the EPS yield, the carbon source also influences the properties of EPS made by the bacteria. Polak-Berecka et al.[24] showed that varying carbon sources in the media can not only affect the chain length and branching of the EPS produced by *Lactobacillus rhamnosus* E/N but also influence the viscosity of the polymers. Esmaeilnejad-Moghadam et al.[25] found that fermentation of milk permeate by *Leuconostoc mesenteroides* NRRL B-512F leads to the production of dextran with a lower molecular weight and higher solubility than that made upon fermentation of MRS broth. This effect of permeate was attributed to the presence of lactose, which inhibits the dextransucrase enzymes, resulting in shorter dextran chains.

Apart from sugars, most other media components are not the direct precursors for EPS biosynthesis. However, since they affect other biochemical and physiological processes in bacteria, their presence in the media can influence EPS production. This is especially applicable to HePS, which are made via a Wzy-dependent pathway that relies on several expensive cellular resources such as sugar nucleotides. Obviously, any media components that support the biosynthesis of sugar nucleotides can result in higher EPS production. In *Lactobacillus helveticus* ATCC 15807, the provision of adenine in the growth media significantly stimulated the synthesis of EPS.[26] The adenine could help in generating ATP, sparing the nucleotides for EPS biosynthesis. Wa et al.[27] found that the inclusion of amino acids, such as histidine, glutamate, and isoleucine, in the

Table. Studies on optimization of media composition and cultivation conditions for improving the EPS production by lactic acid bacteria and bifidobacteria

No.	Bacteria	Type of EPS	Experimental variables	Optimum conditions	References
1	*L. rhamnosus* C83	HePS	Carbon sources and temperatures (20-37 °C)	40 g/L mannose and 20 g/L glucose + fructose; 25 °C	75
2	*L. delbrueckii* subsp. *bulgaricus* CNRZ 1187 and CNRZ 416	HePS	Glucose concentrations	10 g/L glucose	76
3	*L. rhamnosus* RW-9595M	Not specified	Nitrogen sources, amino acids and salts	Whey permeate with yeast extract supplemented with salts and amino acids	28
4	*L. helveticus* ATCC 15807	HoPS	Carbon sources, vitamins, nucleotide bases and pH (4.5 & 6.2)	Lactose, biotin & thiamine, and adenine at pH 4.5	26
5	*L. delbrueckii* subsp. *bulgaricus* B3 & G12, and *S. thermophilus* W22	Not specified	pH (4.0-7.0), temperatures) (30- 45 °C) and cultivation time (5-48 h)	pH of 6.2 at 45 °C for B3 & G12 and pH of 6.8 at 45 °C for W22	43
6	*L. pentosus* LPS26	HoPS	pH (5.0 and 6.0) and temperatures (20-30 °C)	pH 6.0 at 20 °C	42
7	*L. lactis* subsp. *cremosris* (JFR1)	HoPS	pH (5.5-6.5)	pH 5.5	40
8	*L. fermentum* F6	HoPS	Carbon sources, nitrogen sources, pH (5.0-7.0), and temperatures (25-42 °C)	2% (W/V) of glucose, 0.5% (W/V) of whey protein concentrate, pH 6.5 at 37 °C in skim milk	38
9	*B. longum* subsp. *infantis* CCUG 52486 and Bifiinfantis NCIMB 702205	Not specified	Nitrogen sources and temperatures (25-42 °C)	1.5% of casein hydrolysate at 37 °C	77
10	*S. thermophilus* BN1	Not specified	Temperatures (37 and 42 °C)	37 °C	41
11	*L. plantarum* NTMI05	HePS	Carbon, organic, and inorganic nitrogen sources	RSM method - 20 g/L glucose, 25 g/L yeast extract and 2 g/L ammonium sulphate	78
12	*W. confusa* OF126	HoPS	Sucrose concentrations, pH (6.0-8.0), temperature (20-40 °C) and cultivation time (12-96 h)	RSM method - 24 g/L sucrose; pH of 7.0; 30 °C; and 48.5 h	79
13	*S. thermophilus* 1275	Not specified	pH (4.5-6) and temperatures (30-42 °C)	pH of 5.5 at 40 °C	39
14	*Weissella cibaria* 10 M	Dextran	Temperatures (6-30 °C)	A cold shift from 30 °C to 6 °C	44

Table. Cont...

No.	Bacteria	Type of EPS	Experimental variables	Optimum conditions	References
15	*L. mesenteroides* NRRL B-512F	Dextran	Sucrose concentration, permeate powder, and yeast extract	20 g/L sucrose, 15 g/L milk permeate, and 15 g/L yeast extract	25
16	*L. lactis* AV1	Dextran	Carbon sources and temperatures (20-37 °C)	0.8% sucrose, and 20 °C	45
17	*W. confusa* XG-3	Not specified	Carbon sources, pH (2.0-12) and inorganic sources	80.1 g/L sucrose, pH of 5.8, and 3.7 g/L sodium acetate	80
18	*L. rhamnosus* LOCK 0943, LOCK 0935, and OM-1.	Not specified	Carbon and nitrogen sources	20 g/L fructose, glucose and sucrose for LOCK 0935; 20 g/L fructose for LOCK 0943; and 20 g/L sucrose for OM-1; no significant difference found in nitrogen sources	81
19	*L. plantarum* MF460, MF556	Not specified	Carbon sources, nitrogen sources, pH (5.0-7.0), temperature (20-37 °C), cultivation time (48-120 h), and mineral salts	Glucose; bacto-peptone; pH 6.0; 30 °C after 48 h for MF460 and addition of calcium carbonate for MF556	31
20	*L. mesenteroides* SF3	Dextran	Sucrose concentrations (10%-35%), temperatures (25-37 °C), and cultivation time (8-24 h)	RSM method - 10% sucrose; 25 °C; and 16 h	82
21	*L. plantarum* K25	HoPS	Inorganic salt (0-100 mg/L)	20 mg/L of $CaCl_2$	32
22	*Leuconostoc citreum* BD1707	Levan	Sucrose concentrations (50-250 g/L), pH (4.5-8.5), temperatures (15-35 °C), and Cultivation time (0-96 h)	RSM method- 172 g/L sucrose; 26 °C; and 112 h	83
23	*S. thermophilus* 937	HoPS	Amino acids	1 mM histidine, isoleucine and glutamate each	27
24	*L. paracasei* 2333 and *L. rhamnosus* 1019	HePS	Carbon sources	Maltose and lactose, respectively, for each strain	84
25	*L. plantarum* RO30	Not specified	Carbon sources, organic, and inorganic nitrogen source	OFAT method - 20 g/L of sucrose, 25 g/L of beef extract and 4 g/L of ammonium sulfate	
				RSM method - 40 g/L of sucrose, 25 g/L of beef extract, 5.5 of pH, at 30 °C, for 72 h	36

defined media resulted in a 2-fold increase in EPS production in *Streptococcus thermophilus* 937. This phenomenon was shown to be driven by increased activities of enzymes involved in the synthesis of sugar nucleotides, along with upregulation of the *eps* gene cluster (Figure). A similar effect of amino acids on stimulating the EPS production has been reported earlier in *L. rhamnosus* RW-

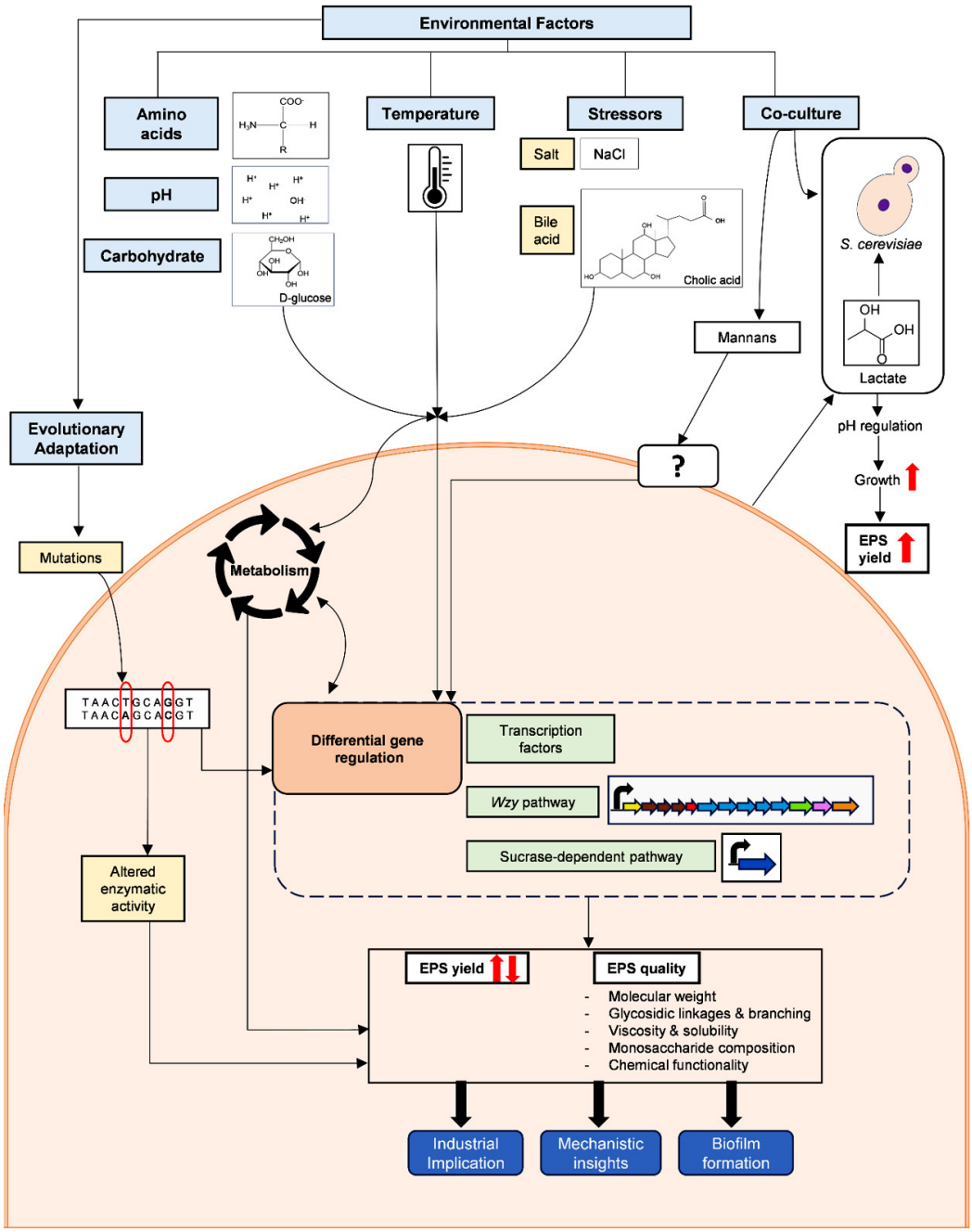

Figure. Mechanism of the influence of various environmental factors on the EPS production by *Lactobacillus* and *Bifidobacterium* spp.

9595M.[28] While the exact mechanism of how amino acids upregulate the EPS gene cluster is not known, studies from other bacteria can provide some insights. Trouillon et al.[29] found that certain amino acids caused differential expression of as many as 32 transcription factors in *Escherichia coli*. Provided that HePS biosynthesis in LAB is controlled by various transcriptional regulators, including those belonging to LytR,[30] the impact of amino acids on their expression is highly plausible and needs to be investigated.

Since milk is the traditional medium for cultivating LAB and is naturally rich in calcium, the influence of calcium on the physiology of LAB has drawn significant scientific interest. Studies suggest varying effects of calcium on the EPS production by different *Lactobacillus* spp. strains. Midik et al.[31] found that calcium carbonate marginally affects EPS production only in a few LAB strains. In the case of *Lactiplantibacillus plantarum* K25, calcium chloride significantly enhanced EPS production while also affecting structural features such as molecular weight and monosaccharide composition, along with causing the upregulation of EPS biosynthesis genes.[32] The total content of rhamnose in EPS was observed to be increased in the presence of calcium, possibly because of upregulation of *cps4F* (capsular polysaccharide biosynthesis gene) and *rfbD* (encodes rhamnose pathway enzyme). Such an influence of calcium could be driven via transcriptional regulators, which were found to be differentially regulated by calcium. The modulation of EPS biosynthesis by calcium is also intertwined with its effect on the related phenomenon in probiotics, such as biofilm formation, adhesion to eukaryotic cells, and possible regulation of mucus-binding proteins.[33,34]

While varying individual media components and studying the influence on EPS generation and their properties provide useful mechanistic insights into EPS biosynthesis, industries are ultimately interested in getting the maximum EPS yield from LAB. Thus, varying all possible media components for identifying the best combination giving the highest EPS yield has been a common theme of many studies (Table). Kefiran is one such commercially important EPS made primarily by *Lactobacillus kefiranofaciens*. Its production was optimized by testing varying carbon and nitrogen sources, vitamins, minerals, fermentation temperature, and agitation speed.[35] Assessing and optimizing various culture parameters in all possible combinations manually can be a laborious task. Thus, statistical methods such as one-factor-at-a-time experimentation,[36] response surface methodology,[25] and Plackett-Burman design[37] have also been found useful in media optimization for modulating EPS production by *Lactobacillus* spp. strains.

Because of the production of lactic acid, fermentation of the given medium by *Lactobacillus* spp. strains lead to a drop in pH. Such acidification is integral for their growth as it suppresses competitive microbes, but also affects many physiological processes in LAB, including EPS production. However, the direction of such an effect shows genus-wise variation, highlighting possible physiological and metabolic differences. For example, while *Limosilactobacillus fermentum* F6 yielded the highest quantity of EPS at pH 6.5, at lower pH,[38] *L. helveticus* ATCC 15807 depicted higher EPS synthesis at pH 4.5 than at pH 6.2. In the case of *S. thermophilus* ASCC 1275, the expression of genes involved in EPS production was upregulated as the pH decreased to 5.5, which can lead to higher EPS biosynthesis.[39] EPS can increase the viscosity of the cultivation medium and is one of the most crucial determinants of its textural applications. Viscosity appears to be positively correlated to the acidity of the cultivation medium of the EPS-producing LAB.[38,40] In case of *Lactococcus lactis* subsp. *cremoris* JFR1, such an increase in the viscosity of the cultivation medium at low pH was possibly because of complex interactions between EPS and proteins in the medium.[40] Contrastingly, the solution made from purified EPS obtained from a low pH medium had lower viscosity in spite of a higher molecular weight. Thus, it is important to evaluate the impact of EPS on the rheological properties of foods by distinctly considering whether the EPS is produced in situ or added exogenously, as EPS produced during fermentation is due to the adaptive response to the acidification of the medium, while an exogenous source of EPS is obtained under specified culture conditions. Also, by taking into account the specific physicochemical properties and composition of the final matrix.

Effect of cultivation conditions

In addition to the composition of the culture medium, the conditions under which bacteria are cultivated play a crucial role in the biosynthesis of various metabolites. Cultivation temperature is one such factor that impacts bacterial physiology and influences EPS production in different directions. In *S. thermophilus* and *L. pentosus* LPS26, EPS production was higher when the bacteria were cultivated respectively at 37 °C and 20 °C, which were temperatures lower than the optimal growth temperatures of these bacteria.[41,42] On the other hand, in *Lactobacillus delbrueckii* subsp. *bulgaricus*, cultivation at higher temperatures (45 °C) resulted in an increase in EPS yields.[43] The studies mentioned above indicate that the production of EPS in LAB is influenced by temperature, and this effect is strain-specific. Temperature has a more nuanced effect on in situ EPS production in foods. Dextran is an α-glucan synthesized by some LAB through dextransucrase catalytic activity. *Weissella* spp. naturally produce such dextrans in the sourdough environment, and this phenomenon affects the dough texture and bread quality. Because of the crucial involvement of EPS in possibly dictating consumer acceptance of sourdough bread, the influence of fermentation temperature on the EPS quality of sourdough has been investigated. When the temperature during sourdough fermentation by *Weissella cibaria* 10 M was shifted from 30 °C to 6-25 °C, the expression of dextransucrase involved in dextran biosynthesis was enhanced. Furthermore, sourdough fermentation under low temperatures also supported high dextran production without an excess pH drop (Table).[44] *Leuconostoc* spp. is also a well-known dextran producer, especially in non-dairy environments like fermented plants. Similar to the above observation for *W. cibaria*, cultivation of *Leuconostoc lactis* AV1n at a low temperature of 20 °C resulted in a 10-fold increase in dextran production compared to 37 °C. This effect was plausibly driven by the higher activity of dextransucrase since *dsrLL*, the gene encoding this enzyme, was also found to be upregulated in the presence of sucrose.[45] These examples highlight the fact that cold shift can be used as a strategy for enhancing dextran production in these two bacteria. However, such a phenomenon might be strain-specific, and more studies are required to understand if the production of other EPS types is also affected by such a cold shift.

Bifidobacterium and *Lactobacillus* spp. strains are obligate and facultative anaerobes, respectively. In the case of *Bifidobacterium* spp., specific gases are used to limit the oxygen availability and enhance the growth. Ninomiya et al.[46] found that *Bifidobacterium longum* JBL05 cultivated under 20% CO_2 yields maximum EPS production. This effect is likely mediated via promoting the *Bifidobacterium* spp. growth instead of the direct regulation of EPS production by CO_2. *Lactobacillus* spp. can tolerate oxygen, and several of them can even respire when exogenously provided with heme and menaquinone, since they cannot biosynthesize these electron chain components.[47] Respiration positively affects several important biotechnological properties of *Lactobacillus* spp., including biomass yield, stress tolerance, long-term storage survival, and production of flavoring chemicals.[48] Surprisingly, the effect of respiration on the EPS production of *Lactobacillus* spp. has been scarcely studied. Li et al.[49] found that recombinant *Lactobacillus casei* LC2W overexpressing NADH oxidase produces 75% higher EPS under aerobic cultivation than the wild-type strain. The probable mechanism involves increased availability of oxidized NAD and lesser accumulation of lactate. Such an influence of aerobic metabolism needs to be studied in numerous other EPS-producing LAB strains.

Effect of stressors

Considering that one of the main functions of EPS is to protect bacteria from environmental stress, such stressful conditions significantly affect the yield and characteristics of the EPS produced. LAB frequently encounters osmotic stress in food environments containing salts added as preservatives. An increased salt concentration can impact bacterial survival by causing water loss. Often, the EPS production under such stress acts as a barrier that retains water and protects the cells from dehydration. However, the direction of the effect of salt on EPS synthesis varies across different strains. For instance, in *Lactobacillus sakei* TMW 1.411, high saline stress reduced EPS production. Cultivation at 10 °C without salt resulted in a dextran yield of 6.7 g/L, whereas the addition of 9.5% NaCl drastically

decreased the yield to just 0.5 g/L.[50] This reduction is likely to be because of the extracellular nature of dextransucrase, leading to its inactivation by the high salt content of the medium. In contrast, when *Lactobacillus confusus* TISTR 1498 was cultivated using solid-state fermentation on an agar surface, a medium containing 4.97% NaCl led to an increased yield of EPS compared to its production in the absence of salt.[51] Such an effect might be due to the adaptive response of bacteria to the salt-induced dehydration, considering a biological role of EPS in desiccation prevention. The contrasting effect of salt stress observed in the above studies also has a few important implications. Firstly, the differences could be due to the obvious differences in the species tested, which, based on the current classification, belong to different genera (*Latilactobacillus* and *Weissella*, respectively). Furthermore, the usage of different media (broth and agar, respectively) could confound the effect of salt on EPS production. Lastly and most importantly, such contrasting findings indicate the need to undertake a comprehensive study to understand the effect of such stressors on EPS production by diverse LAB in various media.

Bile and low pH impose another natural stress on commensal and food-originating bacteria in the GI tract and have been studied for their impact on bacteria from numerous facets. *Lactobacillus* and *Bifidobacterium* spp. depict strain-wise variation in the bile and acid resistance, and in general, EPS production is correlated to resistance to these stressors.[52-54] In agreement with these observations, bile salts have also been shown to promote biosynthesis of EPS in LAB such as *Bifidobacterium animalis* subsp. *lactis*, *L. delbrueckii* subsp. *lactis*, and *L. rhamnosus* GG.[55-57] While some of the above studies also suggest upregulation of EPS biosynthesis genes upon bile exposure, Koskenniemi et al.[58] showed, using transcriptomic and proteomic approaches, the downregulation of EPS biosynthetic genes in *L. rhamnosus* GG in the presence of bile. This could be a signalling mechanism wherein the removal of EPS production would be inhibited by gastric bile. Since EPS generally interferes with the intestinal adhesion of *Lactobacillus* spp.,[59] the bile-induced silencing of EPS could allow for the adhesion of *Lactobacillus* spp. to the intestinal epithelial cells. Thus, the response of *Lactobacillus* and *Bifidobacterium* spp. in terms of EPS production to bile appears to be multifaceted and species-specific, and thus needs to be studied in greater detail on a diverse set of bacteria.

Evolutionary modulations in EPS production

Adaptive laboratory evolution (ALE) involves cultivating microbes under specific environmental conditions for extended periods to enhance their fitness under those stressful conditions. This improvement is achieved through the natural selection of the fittest mutants, which exhibit traits beneficial for industrial applications.[60] Changes in environmental conditions can lead to alterations in the genetic makeup of bacterial populations, influencing both the quality and quantity of metabolites produced. *Lactobacillus* and *Bifidobacterium* spp. have been subjected to ALE to improve their industrial robustness, with altered EPS production observed in many such studies.

Considering the role of EPS in the prevention of desiccation, adaptation to such dehydrating conditions can lead to an enhancement in the EPS production. For instance, during efforts to enhance the freeze-drying tolerance and storage stability of *L. mesenteroides* WiKim33 by exposing it to heat and osmotic shock, researchers observed increased EPS production and a remarkable 331% increase in biofilm thickness.[61] Although there is no evident role of EPS in thermo-protection in LAB, evolutionary adaptation to higher temperature has been found to enhance the firmness of milk fermented by *L. delbrueckii* subsp. *bulgaricus*. Since the mutant was unable to acidify the milk, the enhanced firmness was attributed to higher EPS production by the mutant.[62] This suggests the possibility of a metabolic trade-off, wherein under thermal stress, EPS synthesis might be favoured as compared to lactic acid production, underlining the complex metabolic coordination by LAB.

In contrast to the above examples of a positive correlation between stress adaptation and EPS production, evolutionary acid adaptation in *B. longum* does not result in an increase in EPS production. Jiang et al.[63] found that adapting *B. longum* BBMN68 to low pH resulted in a mutant with reduced EPS accumulation compared to the ancestral strain. This reduction was attributed to a

point mutation in the *cpsD* encoding a *galactosyl transferase* involved in EPS biosynthesis, leading to amino acid substitutions. This change is speculated to alter the monosaccharide composition of the EPS. At the same time, under acidic conditions (pH 5), *cpsD* was upregulated in the wild-type strains and downregulated in the acid-adapted strains.[63] Thus, the change in gene expression of the eps cluster might explain the tolerance to acid stress in the adapted strains.

Another evidence of the genomic impact of stress adaptation on EPS biosynthesis has been obtained in *L. rhamnosus* GG. Kwon et al.[64] subjected *L. rhamnosus* GG to freeze-thaw growth for 150 cycles to improve freeze-thaw tolerance. Genome resequencing of the adapted strain revealed a loss-of-function mutation in *wze*, which encodes for a tyrosine kinase involved in EPS regulation. Although EPS production was not analyzed, such mutations likely result in continuous elongation of capsular polysaccharides on the cell.

Apart from these studies, the mechanistic details of evolutionary EPS modulation have not been much studied in *Lactobacillus* and *Bifidobacterium* spp. However, there appears to be a complex interplay between evolutionary adaptation and transient stress response which affects EPS production in these bacteria. Unlike *Lactobacillus* spp., the transcriptional regulators of EPS biosynthesis have not been studied in detail in *Bifidobacterium* spp.[65] The EPS gene clusters in both these bacteria have numerous genes involved in EPS biosynthesis, export, and their regulation. Thus, studies on how all these functions are affected under stress exposure and evolutionary adaptation using a multi-omics approach are required.

In addition to the apparent physiological role of EPS in stress tolerance, the physicochemical properties of EPS can themselves be useful in developing and identifying EPS overproducing mutant strains. Since EPS production prevents bacterial sedimentation in low-viscosity environments, Martin et al.[59] identified slower-sedimenting isolates of *L. rhamnosus* CNCM I-3690 as high-EPS producers. Although these strains exhibited diminished anti-inflammatory effects, reduced adhesion to colonic epithelial cells, and decreased probiotic efficacy *in vivo*, they provide a useful tool for EPS generation for technological applications. Additionally, such studies demonstrate the potential of evolutionary selection using other physicochemical properties of EPS for identifying EPS-overproducing strains.

Effect of co-culture

Lactobacillus spp. produce lactic acid as their primary metabolic end product. While they are relatively adapted to lactate-rich environments, high lactate concentrations can inhibit their growth and reduce yields of valuable products like EPS. This issue has been particularly noted during kefiran polysaccharide production by *L. kefiranofaciens* KPB-167B, a key microbe in kefir grains.[66]

One of the most thoughtful ways this limitation was overcome was by utilizing the microbial diversity of kefir. Kefir grains contain yeasts like *Saccharomyces cerevisiae*, which utilize lactate. Co-culturing *L. kefiranofaciens* JCM6985 with *S. cerevisiae* reduces lactate accumulation, facilitating higher bacterial growth and increased kefiran yields.[67] This strategy was further optimized using fed-batch co-culture techniques that balanced lactate production by *L. kefiranofaciens* JCM6985 with lactate consumption by *S. cerevisiae*, achieving even higher kefiran yields.[68] The cross-taxa induction of EPS is also known to occur in *L. kefiranofaciens* OSU-BDGOA1 by *Kluyveromyces marxianus,* which is another yeast found in kefir grains[69] and in *S. thermophilus* 1275 (EPS-producing strain) by *S. thermophilus* 1303 (a non-EPS-producing strain).[70] The increased yield of EPS may result from the complementary relationship between the microbes in terms of exchange of metabolites, similar to such interactions known in yogurt.[71] Apart from lactate consumption and allowing for the growth of LAB, the stimulation of EPS production by yeast also takes place via a direct mechanism. Specifically, *S. cerevisiae* was found to upregulate the EPS biosynthesis genes in *Lacticaseibacillus paracasei* ATCC 334[72] and *L. rhamnosus* RW-9595M.[73]

In case of *L. paracasei*, this phenomenon was further shown to be driven by recognition of mannans on the yeast surface (Figure). Although such physical interaction of yeast mannans is speculated to occur via *L. paracasei* surface proteins, which were upregulated by mannans,

the detailed mechanism remains to be studied. Intriguingly, EPS obtained from *L. plantarum* ATCC 8014 cocultured with *S. cerevisiae* was found to have lower solubility, water uptake, and DPPH radical scavenging activity.[74] The EPS produced under both conditions was found to have glucose residues with α-(1→6) linkage. The other structural features affected by co-culture, along with the mechanism of such a change in the properties of EPS, have not been studied. It would be interesting to delve deeper into the cross-taxa interactions by reverse genetics and biophysical approaches, can not only provide a detailed understanding of this phenomenon but also provide a means of its application for further modulating EPS production.

CONCLUSION

Researchers are increasingly exploring ways to enhance the production of EPS by LAB and *Bifidobacterium* spp. due to their potential health benefits and applications in food, medicine, and environmental biotechnology. Regardless of the tremendous progress in characterization and production of EPS, much of the underlying regulatory mechanisms and strategies that may be useful for enhancing the total yield remain less explored.

LAB and *Bifidobacterium* spp. have EPS biosynthetic gene clusters which are strain-specific, with carbon sources and amino acids acting as stimuli for their upregulation. The modulation of gene expression affects the quantity of EPS produced while altering its composition, which might lead to modulation in its bioactivity, indicating the requirement of precise nutrient optimization for targeted EPS synthesis. More studies and a better understanding of combining the metabolomic knowledge of the bacterial nutrient sensing regulatory network with its EPS activation clusters are required.

Adaptive laboratory evolution (ALE) has proven effective for improving microbial resilience under stress conditions while amplifying EPS yield and structure. A better understanding of the molecular, transcriptomic and genome levels could help enhance the conditions to maximize the EPS synthesis by the strains. While considerable research has focused on environmental stresses that promote EPS synthesis, evolutionary approaches remain underexplored but hold promise for industrial applications.

Co-culturing strategies have potential for diversifying structural properties and improving EPS yields through synergistic microbial interactions. A synergetic balance is created by co-cultures, such as the lactate synthesized by LAB, and utilized by the yeast. This influences bacterial growth along with EPS production, yielding biopolymers in higher quantities compared to monocultures. The polysaccharide synthesized by such a method would result in a novel polymer with functional characteristics to be employed for various industrial purposes, such as in targeted delivery systems, texture modifications, etc. EPS with refined rheological properties could be utilized for food emulsification and as environmental absorbents.

Future research should focus on optimizing evolutionary strategies while investigating molecular mechanisms underlying co-culture-induced changes in EPS bioactivity. Model systems should be used to experimentally validate the link between the EPS structure and its functional outcomes, which can establish a basis for targeted engineering. Integrating the techniques of media optimization, adaptive evolution, and co-culture systems strategically could not only enhance the quality and quantity of EPS but also expand its applicability across industries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable

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