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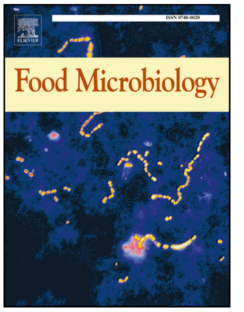

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Lactobacillus fermentum: Could the EPS production ability be responsible for the functional properties?

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ABSTRACT

Exopolysaccharides (EPS) production is a characteristic that has been widely described for many lactic acid bacteria (LAB) of different genera and species, but little is known about the relationship between the functional properties of the producing bacteria and EPS synthesis. Although many studies were addressed towards the application of EPS-producing LAB in the manufacture of several dairy products (fermented milk, cheese) due to their interesting technological properties (increased hardness, water holding capacity, viscosity, etc.), there are not many reports about the functional properties of the EPS extract itself, especially for the genus *Lactobacillus*. The aim of the present revision is to focus on the species *Lactobacillus fermentum* with reported functional properties, with particular emphasis on those strains capable of producing EPS, and try to establish if there is any linkage between this property and their functional/probiotic roles, considering the most recent bibliography.

Keywords: *Lactobacillus fermentum*; exopolysaccharides; functionality, probiotic.

1. INTRODUCTION

The genus *Lactobacillus* is a common member of the small intestine of humans and other mammals (Stearns et al., 2011; Xiao et al., 2015) and it seems to play an important role in maintaining the normal intestinal homeostasis. From a functional point of view, the presence of this genus within the intestinal microbiota has been linked to a healthy *status* of the host and, in general, low levels have been associated with specific health problems as IBS (Irritable Bowel Syndrome; Liu et al. 2017). Therefore, its addition as a probiotic supplement could help to balance an aberrant intestinal microbiota (Staudacher et al., 2017). The term "probiotics" refers to "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). This term was recently discussed, giving more precision and consistency for its correct application (Reid et al., 2019). Some strains with demonstrated probiotic properties were successfully included in diverse functional foods, and, although the mechanisms by which they exert their action are still uncertain, the health-promoting effects of these strains were related to the biological activities of these biopolymers. In fact, diverse scientific reports indicated that EPS from diverse *Lactobacillus* species can contribute to human health by means of numerous demonstrated effects: prebiotic role, modulation of the immune system, antioxidant, antitumor, antiulcer or cholesterol-lowering activities, etc. (Ruas-Madiedo et al., 2002; Castro-Bravo et al., 2018).

Recently, the probiotic potential of some strains of *Lactobacillus fermentum* has been intensively studied, demonstrating several health benefits. *L. fermentum* is an obligate heterofermentative lactic acid bacteria (LAB) commonly found in fermented vegetables (Di Cagno et al., 2008; Offonry and Achi, 1998; Pulido et al., 2012; Sánchez et al., 2000; Seseña and Palop, 2007). Besides, it is part of the infant gut (Ahrne et al., 2005; Kirtzalidou et al., 2011), vaginal (Pavlova et al., 2002) and human breast milk microbiota (López-Huertas, 2015). Furthermore, this species was frequently isolated from the fecal microbiota of healthy elderly people (Park et al., 2015; Silvi et al., 2003). Being generally recognized as safe (GRAS, as all LAB), strains of *L. fermentum* have been widely applied in various food fermentations (cocoa and Iberian dry-fermented sausages, for example; Lefeber et al., 2011; Ruiz-Moyano et al., 2011) and, although it is mainly associated with the industry of fermented vegetables (grains, purées, etc.), it is also naturally present as secondary flora in diverse varieties of cheeses (de Souza et al., 2018). As other LAB, some *L. fermentum* are able to produce exocellular polymers or exopolysaccharides (EPS) which can be secreted to the medium (slime EPS) or remained attached to the cell wall (capsular polysaccharides or CPS). These molecules are frequently used by the food industry, for example, by the application of EPS-producing strains in the manufacture of fermented milk and different types of cheese for improving their textural and organoleptic properties. Some EPS are able to reduce syneresis in yogurts due to their high water-holding properties (Ale et al., 2016b) and they can be used in low-fat dairy products allowing the design of healthy products with acceptable sensory characteristics (Ryan et al., 2015).

One of the most representative probiotic strains of this species is *L. fermentum* CECT 5716, isolated from human breast milk, whose immunomodulatory, anti-inflammatory, and anti-infectious properties were demonstrated (Olivares et al., 2007; Pérez-Cano et al., 2010). Recently, this strain was proposed as an alternative for the prevention of vascular disorders caused by lupus erythematosus (Toral et al., 2019). In other cases, a high production of exopolysaccharides (EPS) was reported but, until now, the functional properties of these EPS^+ strains were not effectively associated with the EPS synthesis, contrary to what was reported for some *Bifidobacterium* strains (Fanning et al., 2012; Hidalgo-Cantabrana et al., 2014; López et al., 2012; Salazar et al., 2014). Besides, it has been reported that EPS produced by probiotic strains are able to interact with the intestinal microbiota and can be used as carbon fermentable sources (prebiotic role) by some beneficial commensal bacteria, as short chain fatty acid (SCFA) producers, even when the *in vivo* synthesis of EPS has not been proven yet. However, as not all EPS can improve the technological properties of fermented foods (Hassan, 2008), not all EPS are able to promote health benefits (Amrouche et al., 2006), being their chemical structure and molecular weight (MW) relevant characteristics to define their health-promoting functions (Hidalgo Cantabrana et al., 2012). For this reason, the structural characterization (MW, presence of charged residues, type of branches, etc.) of these macromolecules is essential when EPS (as an ingredient) or EPS-producing strains are included in a food matrix. In this direction, the deep knowledge about the EPS molecule nature would allow us to infer the techno-functional properties of the final product. The repeating unit structures of EPS (or CPS) from only three *L. fermentum* (Table 1) were described so far.

In particular, the EPS extract from *L. fermentum* Lf2, a strain which was isolated by our group as non-starter culture from Argentinean regional Tybo cheese, has demonstrated interesting functional properties (immunomodulation capacity, protection against *Salmonella* and prebiotic/symbiotic roles) when added as a food ingredient in dairy matrices (Ale et al., 2016a; Ale et al., 2016b; Ale et al., 2019). It represents, according to our knowledge, the first documented EPS from *L. fermentum* with functional properties demonstrated *in vivo*.

This review discusses the recently reported functional (probiotic) properties of *L. fermentum* and the capacity of different strains to produce EPS, focusing on the immunomodulatory properties and antagonistic effects against bacterial pathogens. Despite the increasing evidence that highlights the health-promoting properties of EPS and their active role when interact with the gut microbiota and the intestinal receptors, it would be interesting to demonstrate the linkage between these molecules and the probiotic characteristics of the producing strains; this way, new expectations would arise on the application of EPS-producing probiotic strains. The most relevant and recent reports about *L. fermentum* strains with functional properties, indicating their origins and proven ability to produce EPS, were summarized in Table 2.

2. *Lactobacillus fermentum*: insights into its health promoting properties

One of the strains that has been widely studied regarding its functional properties was *L. fermentum* CECT 5716, which was isolated from human milk (Cárdenas et al., 2015). This strain presented immunomodulatory, anti-inflammatory, and anti-infective (against *Saphylococcus* *aureus*) activities. Pérez-Cano et al. (2010) also demonstrated that it enhanced natural and acquired immune responses, as evidenced by the activation of NK and T cell subsets and the expansion of Treg cells, as well as the induction of several cytokines. Recently, it was suggested as an alternative approach to prevent systemic lupus erythematosus (SLE) (*in vivo* model) and its associated vascular damage, since reductions in lupus disease activity, blood pressure and splenomegaly were observed in treated mice. Also, CECT 5716-treated mice evidenced an increase in the levels of *Bifidobacterium* in the gut microbiota, which was accompanied by a reduction of plasma lipopolysaccharide (LPS) levels, indicating a possible improvement of the gut barrier integrity (Toral et al., 2019). This strain was sensitive to all antibiotics proposed by the European Food Safety Authority (EFSA) standards, and no transmissible genes of antibiotic resistance were detected in its genome, characteristics that allowed its application in food products. All these studies made it possible to incorporate it as a probiotic under the commercial name LC40 in the infant milk Peques Heriditium, commercialized by Puleva (Spain). It was also added as a food complement (Lactanza Hereditum) to restore the balance of the mammary flora and to reduce the rate of mastitis. Furthermore, it was recently (2017) incorporated in two new infant formulas of the Chinese market from Herds company. However, after over a year in the assessment process, the EFSA (2018) rejected the application submitted by Biosearch Life under Article 14 of Regulation (EC) No 1924/2006, considering that no conclusions could be drawn from the informed results. This report argued that a cause and effect relationship had not been well established between the consumption of CECT 5716 and the reduction of *Staphylococcus* load in breast milk (that would decrease the risk of mastitis).

A recent patent (US20180050072A1) described a composition with *L. fermentum* GMNL-296 that improved the infection symptoms of *Clostridium difficile*, the main pathogenic bacterium that causes diarrheas in patients under antibiotic treatments. Moreover, another approach studied the curative effect of *L. fermentum* L23 (isolated from human vagina) after vaginal administration in female BALB/c mice infected with *Gardnerella vaginalis* (Daniele et al., 2014). The results indicated that this strain was able to inhibit the pathogen at concentrations normally used in commercial formulas ($2x10^7$ CFU/mice), suggesting that L23 could be used as a potential biotherapeutic agent for the treatment of this urogenital infection. According to our experience with *in vivo* trials and, although authors offered an interesting approach with promising results, it seems that no scientifically valid results could be obtained from only 4 animals/group when this type of effect is intended to be verified.

One of the traditional and simplest phenotypic features tested during *in vitro* characterization is the ability to coaggregate with pathogens, considering that co-aggregation decreases the accessibility of the harmful microorganisms to the intestinal epithelium (Castro-Bravo et al., 2018). In this direction, Carmo et al. (2016) proposed *L. fermentum* ATCC 23271 as a potential probiotic candidate to complement candidiasis treatment associated with genital infections. Another strain of this species, *L. fermentum* TCUESC01, isolated from the fermentation of fine cocoa, presented inhibitory activity against *S. aureus* CCMB262 biofilm production (Melo et al., 2016). Furthermore, Ramos et al. (2013) screened a total of 234 LAB isolates from Brazilian food products for their ability to survive at pH 2.0. Fifty-one of them survived and, among them, *L. fermentum* CH58 exhibited antagonistic activity towards the pathogens *Listeria monocytogenes* and *S. aureus*. Finally, Veljovic et al. (2017) studied two strains, *L. fermentum* BGHI14 (isolated from newborn feces of a breast-fed infant) and *L. helveticus* BGRA43 (from human intestinal tract), which significantly reduced the adhesion of *E. coli* ATCC25922 to Caco-2 cells, individually and combined. In this work, a mixed probiotic culture composed of three thermophilic LAB (the both mentioned strains and *Streptococcus thermophilus* BGVLJ1-44) was used for an interesting *in vivo* farm test. This combination resulted in improved microbiota diversity in neonatal piglets (DGGE analysis). Importantly, the number of *Enterobacteriaceae* in fecal samples collected from probiotic treated sows was reduced in comparison to the untreated ones.

Apart from the reported inhibitory effects against pathogenic bacteria, *L. fermentum* was also associated with diverse damage preventive effects. For example, by using a model of chemically (dextran sulfate sodium) induced colitis in mice, Chen et al. (2018a) demonstrated that *L. fermentum* HY01(isolated from traditional fermented yak yoghurt) exerted a preventive effect by down-regulating the concentration of diverse pro-inflammatory factors. In a similar way, *L. fermentum* L-Suo (also isolated form yak yoghurt in China) was able to prevent mice from activated-carbon-induced constipation (Suo et al., 2014). A recent study of the same group reported the impact of *L. fermentum* on liver injury in a murine model (Chen et al., 2018b). In this work, the administration of a strain of *L. fermentum* (whose identity was not indicated) during 14 days to mice with CCl_4–induced liver disease decreased liver damage. Since the assays comprised both live and heat-killed cells, authors hypothesized that the protection would be linked to some components of the strain, whose nature was not indicated.

The strain *L. fermentum* IM-12, an isolate from human fecal microbiota, also presented immunomodulatory effects since it strongly suppressed IL-6 expression in macrophages and exhibited anti-inflammatory effects in mice with carrageenan-induced paw oedema (CIE) or TNBS-induced colitis (TIC) (Lim et al., 2017). Another strain, *L. fermentum* L930BB (isolated from human bioptic samples of colonic and ileal mucosa) presented down-regulation of pro-inflammatory cytokines (IL-6 and IL-12), together with the upregulation of anti-inflammatory IL-10 (Čitar et al., 2015).

Other *L. fermentum* strains were related to the improvement of the general oxidative *status*, such as *L. fermentum* CRL1446 (Abeijón Mukdsi et al., 2012). This strain, together with *L. fermentum* CRL574, *L. fermentum* EFL2 and *L. fermentum* EFL3, were able to produced conjugated linoleic acid (CLA) (Terán et al., 2015). Some studies have also indicated possible anticancer properties (*L. fermentum* HM3, Shokryazdan et al., 2017).

Finally, *L. fermentum* was related to longevity improvement. This was the case of *L. fermentum* JDFM216, which was isolated from a Korean infant feces sample, and had the ability to enhance the longevity and immune response of a *Caenorhabditis elegans* host (Jang et al., 2017; Park et al., 2018). An association between *L. fermentum* and longevity was previously reported from two interesting studies where *L. fermentum* and *Bifidobacterium longum* were the most representative species detected in the gut microbiome from healthy elderly subjects from Fermo, Italy (Silvi et al., 2003) and three Korean counties (Park et al., 2015), suggesting an important role of this species in maintaining the normal intestinal homeostasis in elderly people.

3. EPS-producing *L. fermentum* and functional properties

There are many strains of the species *L. fermentum* with demonstrated probiotic properties (*in vivo* and *in vitro*) and, at the same time, with the capacity to produce EPS. Table 2 lists the most relevant *L. fermentum* strains with demonstrated functional properties with/without proven EPS synthesis. Even though there is not clear evidence about the mechanisms that link these molecules with these probiotic effects, in some cases, a direct relationship among them and the functionality could be suggested. Nevertheless, in most cases, valid *in vivo* assays are necessary to associate the functional response with the ability to synthetize EPS.

3.1. Inhibition of pathogenic bacteria

Regarding gastric health, *L. fermentum* UCO-979C is a human isolate able to form biofilms on abiotic and biotic surfaces and to synthesize EPS with demonstrated anti-*Helicobacter pylori* activity, since it reduced its adhesion to human gut (*in vitro* model; García et al., 2017; García-Castillo et al., 2018; Salas-Jara et al., 2016). This strain was able to modulate the cytokine response of gastric epithelial cells and macrophages after *H. pylori* infection, reducing the production of inflammatory cytokines and chemokines, and increasing the levels of immunoregulatory cytokines. Besides, this strain maintained the anti-*H. pylori* inhibitory activity when encapsulated (Vega-

Sagardía et al., 2018). However, there are no reported studies beyond the simple indication about the ability of this strain to produce EPS.

An interesting study was reported by Sharma et al. (2018), who recently evaluated the antibacterial activity against *P. aeruginosa* PAO1 of eighty lactic acid bacteria isolated from neonatal fecal samples. Among these, only four LAB produced simultaneously bacteriocins and EPS, but only one *L. fermentum* strain was found to maximally attenuate the *P. aeruginosa* PAO1 biofilm. This strain (with sequential accession number KT998657, NCBI database) was also able to reduce the biomass of *P. aeruginosa* PAO1 as a result of pre-coating of the abiotic surface with the produced postbiotics (combination of bacteriocin and EPS), suggesting a synbiotic association. In this particular case, a functional response associated to the EPS synthesis could be proposed, and this hypothesis could be demonstrated by additional studies, for example, including an isogenic strain without the ability to produce EPS as negative control.

Another example about antimicrobial properties is *L. fermentum* AI2 (Shah et al., 2016; Patel et al., 2012), specifically against two clinical pathogenic strains, *E. coli* NG 502121 and *S. aureus* AY 507047 in co-cultured assays. In this case, authors observed a significant reduction in the growth of both pathogens when co-cultured with AI2 and proposed a future study focused on the presence of other metabolites with antimicrobial activities apart from the organic acids, possibly the EPS from this strain.

From the complete high quality genome sequence of *L. fermentum* 3872, a number of EPS production-related genes were found (Lehri et al., 2017a). In particular, *epsH* (Locus tag: N573_008790) was predicted to participate in biofilm formation and may also contribute to the protection against colitis. This evidence represents the first indication, at molecular level, of a clear relationship between the synthesis of EPS and the functional potential of *L. fermentum*, although this exopolysaccharide has not been studied yet. In fact, this strain was part of a patented consortium (*L. fermentum* RCM B-2793D (3872), *L. crispatus* VKM B-2727D, *L. gasseri* VKM B-2728D, *L. plantarum* VKM B-273D) which has probiotic properties that could be used for the design of bacterial preparations and novel functional products. The complete consortium evidenced higher antagonistic activity against pathogenic and opportunistic microorganisms than the individual strains (Abramov et al., 2014). Due to its anti-*Campylobacter* activity, authors suggested that *L. fermentum* 3872 could be potentially used for prophylaxis of such *C. jejuni* induced diseases as traveler's diarrhea, inflammatory bowel disease and irritable bowel syndrome (Lehri et al., 2017b).

Recently, de Albuquerque et al. (2018) studied nine wild *Lactobacillus* strains isolated from fruit processing byproducts (*L. plantarum* 53, *L. fermentum* 56, *L. fermentum* 60, *L. paracasei* 106, *L. fermentum* 250, *L. fermentum* 263, *L. fermentum* 139, *L. fermentum* 141, and *L. fermentum* 296). They were tested *in vitro* for a series of safety (antibiotic resistance), functional and technological properties. All of them were positive for EPS production and were able to co-aggregate with *L. monocytogenes* and *E. coli* and antagonize pathogenic bacteria. Overall, *L. fermentum* 139 (47.4 mg/L EPS), *L. fermentum* 263 (55.1 mg/L EPS), and *L. fermentum* 296 (55.6 mg/L EPS) showed the best performance. In a similar study, Owusu-Kwarteng et al. (2015) screened a total of 176 *L. fermentum* strains isolated from West African fermented millet dough regarding their rate of acidification, EPS production and amylase and antimicrobial activities. Among them, four EPS-producing *L. fermentum* strains (identified as10–9, 4–20, 0–17 and 4–30), which were acid and bile resistant, showed inhibitory activities towards *L. monocytogenes* NCTC 10527 and *S. aureus* ATCC 1448 (agar well diffusion method).

In a totally different approach, Adebayo-Tayo and Popoola (2017) used the EPS produced by *L. fermentum* LPF6 for the biosynthesis of silver nanoparticles which had antibacterial activity against pathogens (*Bacillus* sp., *Streptococcus pyogenes*, *S. aureus*, *Klebsiella* sp. and *Pseudomonas aeruginosa*). This particular case represents one of the few examples where the functional capacity of the EPS from *L. fermentum* could effectively be demonstrated, since it was used as an extract. It would be interesting to prove this same functional ability for the EPS-producing strain.

3.2. Damage preventive properties and immunomodulatory effects

Some strains were related to the ability to participate, by unknown mechanisms, in kidney health. In this direction, Sönmez et al. (2018) attempted to relate the oxalate-degradation rate of different *L. fermentum* strains with the EPS production. Although no significant correlation was found, the high-EPS-producing *L. fermentum* IP5 presented high oxalate-degrading activity when compared with the low-EPS-producing strains. Authors hypothesized that EPS plays an important role in oxalate-degrading activity, protecting strains from the toxic effects of oxalate. They concluded that the dietary supplementation with *L. fermentum* IP5 could function as an interesting strategy to prevent oxalate stone disease. Further *in vitro* and *in vivo* studies could help to identify and characterize the strain's bacterial cell wall components (EPS) that participate in the oxalate-degradation mechanisms.

Other EPS-producing *L. fermentum* strains were used to make fermented milk, such as *L. fermentum* J20 and J28 (Santiago-López et al., 2018). In this case, the Th1/Th17 response was evaluated in a murine model of inflammation induced with dextran sulfate sodium (DSS). The authors suggested as a preliminary hypothesis that this milk possibly reduced the inflammatory response (decreasing the Th17 response) at week 6 because of the metabolites (EPS) or cell components present in the product.

An interesting study was done for the strain *L. fermentum* FTDC 8312 which exerted a cholesterol lowering effect in hypercholesterolemic mice. This property may be attributed to the gut microbiota modulation (Lye et al., 2017) that was evidenced by an increase in the members of genera *Akkermansia* and *Oscillospira*. From its genome sequence, five putative *eps* genes were found from the best matched strain, *L. fermentum* MTCC 25067 (TDS030603), one of the most studied EPS^+ *L. fermentum*, but a correlation among the EPS production and its functionality has not been established yet.

Finally, in a recent work, the antioxidant activity (*in vitro* and *in vivo* assays) of the cell-bond exopolysaccharide (c-EPS) from *L. fermentum* S1 was reported (Wang et al., 2019). This CPS extract exhibited strong antioxidant activity *in vitro* and could improve the total antioxidant capacity in *Caenorhabditis elegans*, indicating that the CPS could be a potential effective antioxidant to be applied in functional foods or drugs.

3.3. *L. fermentum* Lf2: EPS as a functional food ingredient

L. fermentum Lf2 is deposited into the INLAIN (Instituto de Lactología Industrial, UNL-CONICET) collection and has been widely studied regarding its ability to synthesize EPS. It produces, under controlled conditions (pH, temperature, time, culture media) 1 g/L EPS, approximately, amount significantly higher than the reported for other LAB (Ale et al., 2016b). Its draft genome sequence was recently published (Harris et al., 2018) and three potential EPS gene clusters were identified in different genomic regions (Figure 1). The first one is composed of eight genes that code for a priming glycosyltransferase, a transcriptional regulator, a CPS synthesis protein, transposases (already described in this species; Dan et al., 2009) and enzymes that are known to regulate the synthesis of these molecules (tyrosine phosphatase and tyrosine kinase). The second cluster (fifteen genes) presents genes that code for a flippase, a chain length determinant protein, several glycosyltransferases, a repeating unit polymerase and another priming glycosyltransferase, different from the first one. And the third cluster only has five genes that code for glycosyltransferases and an EPS polymerization protein. Further studies are needed to understand the role of each gene in the EPS synthesis of this strain.

Additionally, its EPS yield could be doubled, reaching 2 g/L by the modification of the composition of the semi-defined medium used (SDM; Kimmel and Roberts, 1998) and the conditions of growth (Ale et al., 2019a). Among all the components of the broth, the proportions of the nitrogen sources and the type of carbon source (sucrose showed better performance than glucose) were the factors with the highest influence on EPS production, together with pH. Besides, this extract presented interesting functional and technological properties, since it was able to protect mice against *Salmonella* infection when resuspended in milk, it enhanced IgA levels in the intestinal fluid when provided in yogurt, and decreased the levels of the pro-inflammatory cytokine IL-6 in small intestine of mice when administrated in milk (Ale et al., 2016a). Regarding the technological properties, the EPS extract provided yogurt with increased hardness and consistency at concentrations feasible to be applied (300 and 600 mg/L). Furthermore, yogurt samples did not present important sensory defects and enhanced syneresis as well (Ale et al., 2016b).

The total EPS extract is mainly composed by three polysaccharides: a high molecular mass β-glucan whose repeating unit is a trisaccharide (1.23×10^6 Da) (Vitlic et al., 2019), and a combination of two novel medium molecular weight heteropolysaccharides (weight average mass of 8.8×10^4 Da) composed of glucose and galactose. The first one has a main chain of β-1,6-linked galactofuranoses which is non-stoichiometrically 2-*O*-glucosylated, while the second polysaccharide is a heteroglycan with four monosaccharides in the repeating unit (unpublished). In this recent study, the immunomodulatory effect of the higher molecular weight β-glucan on peripheral blood mononuclear cells was analyzed. The exposure of these cells to an aqueous EPS solution for 24 h increased the proliferation and production of TNF-α (proinflammatory cytokine) compared to the controls. However, when the treated cells (from which the EPS was removed) have subsequently been exposed to bacterial LPS (lipopolysaccharide), very low levels of TNF-α were observed. This would indicate that this fraction of EPS would provide immunotolerance to cells, being this ability important for the development of therapies for the treatment of diseases such as ulcerative colitis and Crohn's disease (associated with the excessive release of inflammatory mediators).

In a recent work (Ale et al., 2019b), other functional properties of the EPS extract from this strain were studied when incorporated as an ingredient in yogurt, individually or combined with a probiotic autochthonous strain, *Bifidobacterium animalis* subsp. *lactis* INL1 (Zacarías et al., 2011; 2014; Burns et al. 2017). From an *in vitro* assay it was found that the EPS in its purified form caused an increase in the levels of TNF-α, while this EPS extract in its purified and crude forms produced an increase in the regulatory cytokine IL-10. Besides, it was observed (*in vivo* assay) that the EPS-treated group presented increased concentrations of total SCFA in faeces, especially acetic and butyric acids, result that could be related to the increase of the levels of the *Clostridium coccoides* cluster (SCFA-producing group) during time, suggesting a prebiotic role. Furthermore, a possible bifidogenic role was observed for the group treated with the combination of *B. animalis* subsp. *lactis* INL1 and EPS (synbiotic effect), reflected in the increased levels of the genus *Bifidobacterium* along time, fact that was not observed when the probiotic was administered solely.

All these characteristics make this extract attractive for the formulation of novel functional products. Thus, *L. fermentum* Lf2 represents, to our knowledge, the most studied EPS producing *L. fermentum* strain. Analysis related with the functional properties of this strain and the possible linkage between the potential health effects and its EPS are currently being addressed. Preliminary evidence from a chronic colitis murine model (unpublished) seems to associate the protection of animals from the symptoms with the EPS synthesis.

4. EPS-producing *L. fermentum* and technological studies

Other *L. fermentum* strains were studied regarding their ability to produce EPS without focusing on their functional properties. An extensively studied strain was *L. fermentum* MTCC 25067, with a yield of 100 mg/L of EPS in its purified form when grew in MRS broth (Leo et al., 2007; Fukuda et al., 2010). Although the chemical structures of the EPS obtained in MRS and in a chemically defined medium supplemented with glucose, galactose, lactose or sucrose were similar, their viscosities appeared to differ probably because of the differences in their molecular mass distributions. The genome of MTCC 25067 was completely sequenced (Aryantini et al., 2017) and the structure of the EPS was revised (Gerwig et al., 2013), indicating that the repeating unit is a branched tetrasaccharide composed of glucose and galactose. Other authors reported a similar study for the strain *L. fermentum* TC21, isolated from 'Tom Chua', a Hue traditional fermented shrimp. They observed that, when the medium was supplemented with lactose and beef extract, the EPS yield was the highest (405.7 mg/L). Recently, Luyen et al. (2018) reported the effects of carbohydrate sources in various concentrations and the conditions of growth on EPS synthesis of *L. fermentum* MC3. The results showed that adding different sugars to the culture medium significantly increased the EPS production. The highest yield was obtained for glucose, reaching 178.2 mg/L. These reports are in accordance with our observations for *L. fermentum* Lf2, since the results obtained highlighted the importance of the carbon and nitrogen sources on EPS yield, parameters that should be considered for making the industrial application of EPS feasible.

Another case is represented by *L. fermentum* 222, an isolate from a spontaneous Ghanaian cocoa bean fermentation, which demonstrated to be an interesting starter culture for this process (Illeghems et al., 2015). The genome sequence of this strain contained a 14-kb EPS biosynthesis gene cluster, including a gene that codes for a dextransucrase, indicating that it would produce homo-EPS (dextran type); however, no other information related to the EPS synthesis was described in the bibliography.

5. CONCLUSIONS

Considering all the recent literature revised, it is quite clear that the species *L. fermentum* should be considered of great importance when the search for novel probiotics is addressed. Different strains displayed encouraging therapeutic potential in terms of protection against pathogens, immunomodulation, anti-oxidation and promotion of a functional digestive tract. Moreover, although these activities were not always clearly related to the production of EPS, there is increasing evidence (still preliminary) that associates the health-promoting properties of these strains with the synthesis of these metabolites. In this sense, assessment of the tolerance of EPS to the gastrointestinal digestion and demonstration of EPS biosynthesis under *in vivo* conditions will be also required when EPS are proposed as food ingredients. It is also necessary to highlight the limitation of the application of these polymers due to the general scarce yields and the use of expensive culture media. Additionally, as for all proposed probiotic strains, it is always critical to verify the resistance to the different technological barriers (spray or freeze-drying, freezing, acidity, etc.) involved in the process when *L. fermentum* strains are incorporated into a food matrix.

From our point of view, it is evident that certain EPS structures play central roles in the bioactivity of the producing strain and, in order to understand the mechanisms by which probiotic bacteria exert health benefits, the EPS structure seems to have an important impact. Even though these properties were already assessed *in vivo* (only in some cases) and *in vitro*, human trials should be carried out to validate the functional properties of the EPS-producing strains or the EPS as a functional ingredient itself.

In conclusion, the following remarks could be summarized:

- An increasing number of *L. fermentum* strains have demonstrated functional potential by *in vitro* and *in vivo* assays.
- A high number of *L. fermentum* strains can produce EPS.
- It is necessary to know whether, or not, the bacterial EPS from *L. fermentum* are responsible for the probiotic properties of the producing strains by means of adequate *in vivo* assays, for example, including isogenic EPS^- strains.
- The chemical characterization of these macromolecules is essential when EPS (as ingredients) or the EPS-producing strains are included in a food matrix, since recent studies strongly associated these characteristics with their rheological and health-promoting properties.
- It is crucial to demonstrate the functionality of *L. fermentum* strains and their EPS with human trials.

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Conflict of Interest

The authors declare that no conflicts of interest exist.

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Table 1. Repeating unit structures of EPS (or CPS, indicated) from different *L. fermentum* strains.

***L. fermentum* strain**	**EPS repeating unit**	**Reference**
Lf2	→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→ 2 ↑ 1 β-D-Glc*p*	Vitlic et al. (2019)
MTC 25067	→3)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→ 2 ↑ 1 α-D-Gal*p* 6 ↑ 1 α-D-Glc*p*	Gerwig et al. (2013)
S1 (CPS)	→3-α-D-Gal*p*-(1→2)-β-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→4)-β-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→ 3 ↓ 1 α-D-Man*p*	Wang et al. (2019)

Figure captions

Figure 1. Preliminary EPS gene cluster of *L. fermentum* Lf2. Gene annotation was based on BLASTx (NCBI database) and results with ≥90 % identity were considered.

Table 2. Summary of the different *L. fermentum* strains regarding their origins, functional properties and proven EPS production abilities. The EPS producing strains are listed first (gray shaded).

L. fermentum strain	Origin	Functional properties	EPS production	Reference
AI2	Dhokla batter	It was able to inhibit the growth of *E. coli* and *S. aureus*	+	Shah et al. (2016); Patel et al. (2012)
FTDC 8312	School of Industrial Technology, University Sains Malaysia (Penang, Malaysia)	Reduction in serum total cholesterol levels, decrease in serum low-density lipoprotein cholesterol levels, increase in serum high-density lipoprotein cholesterol levels, and a decreased ratio of apoB100:apoA1 when compared to the control. The administration of FTDC 8312 also altered the gut microbiota population such as an increase in the members of genera *Akkermansia, Oscillospira, Lactobacillus and Desulfovibrio*	+ (eps-related genes)	Lye et al. (2017)
IP5	İzmir Tulum cheese	High oxalate-degrading activity. Dietary supplementation with the probiotic strain *L. fermentum* IP5 could be a promising strategy for the prevention of oxalate stone disease	+	Sönmez et al. (2018)
Isolate from fecal samples (accession Nº KT998657)	Neonatal fecal samples	Attenuation of *P. aeruginosa* PAO1 biofilm. It produces EPS and bacteriocin	+	Sharma et al. (2018)
J20 and J28	Mexican Cocido cheese	Decreased inflammatory response in a murine model	+	Santiago-López et al. (2018); Heredia-Castro et

				al. (2015)
Lf2	Argentine cheese	High EPS production. EPS provided prevention against *Salmonella* infection and presented immunomodulatory effects *in vivo* and *in vitro*	+	Ale et al. (2019; 2016a;b); Vitlic et al. (2018)
LPF6	Fermented food	Its EPS was applied in the synthesis of silver nanoparticles with antibacterial activities	+	Adebayo-Tayo et al. (2017)
MC3	Fermented bamboo shoots	High EPS yield, interesting for further research	+	Luyen et al. (2018)
MTCC 25067	Fermented milk	High EPS yield, interesting for further research	+	Gerwig et al. (2013); Fukuda et al. (2010); Leo et al. (2007)
RCM B-2793D (3872)	Milk of a healthy woman	Antagonistic activity against pathogenic and opportunistic microorganisms. When it is part of a consortium of strains, it has probiotic action and it is used for production of bacterial preparations and lactic acid products of functional nutrition. The consortium has a higher antagonistic activity against pathogenic and opportunistic microorganisms compared to individual strains of lactobacilli	+ (eps-related genes)	Lehri et al. (2017) Abramov et al. (2014)
S1	Fuyuan pickle	Potential natural antioxidant for application in functional foods	+ (CPS)	Wang et al. (2019)
TC21	Tom chua in Hue, Vietnam	High EPS yield, interesting for further research	+	Luyen et al. (2016)

UCO-979C	Human gastric tissue	Capacity to reduce adhesion of *H. pylori* to human gastric epithelial cells. Anti-inflammatory cytokine profile	+	Garcia-Castillo et al. (2018)
10–9, 4–20, 0–17 and 4–30	Fermented millet dough	Inhibition towards *Listeria monocytogenes* and *Staphylococcus aureus*. Interesting technological and functional properties	+	Owusu-Kwarteng et al. (2015)
222	Fermented Ghanaian cocoa bean	Interesting functional starter culture strain for cocoa bean fermentations	+ (eps-related genes)	Illeghems et al. (2015)
263,139 and 296	Fruit processing byproducts	Performance compatible with probiotic properties and technological features that enable the development of probiotic foods with distinct characteristics	+	de Albuquerque et al. (2018)
ATCC 23271	Human intestine	A potential probiotic candidate, particularly to complement candidiasis treatment	-	Carmo et al. (2016)
BGHI14	Newborn feces of a breast-fed infant	Reduction of adhesion of *E. coli* and improvement of microbiota in piglets in a probiotic formula	-	Veljovic et al. (2017)
CECT 5716	Human breast milk	Immunomodulatory, anti-inflammatory, and anti-infectious properties. It produces organic acids, glutathione, riboflavin, and folates and moderately stimulates the maturation of mouse dendritic cells	-	Cárdenas et al. (2015); Gil-Campos et al. (2012); Maldonado et al. (2012); Olivares et al. (2007, 2006); Pérez-Cano et al. (2010)
CH58	Brazilian food products	Antagonistic activity towards the pathogens *Listeria monocytogenes* and *Staphylococcus aureus*	-	Ramos et al. (2013)

CRL574, EFL2, EFL3	Child feces (CRL574) and cheese (EFL2 and EFL3)	CLA producing strains (conjugated linoleic acid)	-	Terán et al. (2015)
CRL1446	Argentinean goat milk cheese	Enhancement of the oxidative status by increasing the bioavailability of ferulic acid, providing protection against oxidative stress-related disorders. CLA producing strain. Potential food supplement	-	Russo et al. (2016); Terán et al. (2015); Abeijón Mukdsi et al. (2013, 2012)
GMNL - 296	Human intestines	Production of a composition to improve the infection symptoms of *Clostridium difficile*	-	Chen and Tsai (2016)
HM3	Human milk	Inhibitory effects against cancer cells after 72 h of incubation. Selectivity in killing cancer cells when compared to the normal liver cell line. It presented cholesterol-reducing ability and antioxidant activity	-	Shokryazdan et al. (2017)
HY01	Traditional fermented yak yoghurt	Enhancement of intestinal peristalsis and ability to prevent mice from constipation	-	Chen et al. (2018a)
IM-12	Human fecal microbiota	Anti-inflammatory effects in mice with carrageenan-induced paw oedema or TNBS-induced colitis	-	Lim et al. (2017)
JDFM216	Korean infant feces	Ability to enhance the longevity and immune response of *C. elegans* and makes it resistant to *S. aureus* and *E. coli*	-	Park et al. (2018); Jang et al. (2017)
L-Suo	Yak yoghurt in China	Prevention from activated-carbon-induced constipation in mice	-	Suo et al. (2014)
L23	Human vagina	Inhibition of *G. vaginalis*. Potential biotherapeutic agent for the treatment of this	-	Daniele et al. (2014)

		urogenital infection		
L930BB	Human bioptic samples of colonic and ileal mucosa	Immunomodulatory effects and enhancement of epithelial barrier integrity when combined with *B. animalis* subsp. *animalis* IM386	-	Paveljšek et al. (2018); Čitar et al. (2015)
TCUESC01	Fermentation of fine cocoa	Reduction of the thickness of *S. aureus* biofilm	-	Melo et al. (2016)
72, 75, 1-1	Coastal serum	Probiotic potential	-	Cueto-Vigil et al. (2010)

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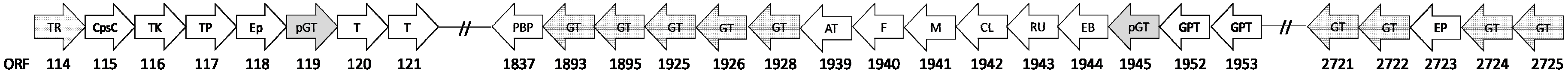

Cluster 1
TR: Transcriptional regulator
CpsC: Capsular polysaccharide biosynthesis protein
TK: Tyrosine kinase
TP: Tyrosine phosphatase
Ep: Epimerase
pGT: priming glycosyltransferase
T: Transposase

Cluster 2
PBP: Polysaccharide biosynthesis protein
GT: Glycosyltransferase
AT: Acyltransferase
F: Flippase
M: Mutase
CL: Chain length determinant protein
RU: Oligosaccharide repeat unit polymerase
EB: Exopolysaccharide biosynthesis protein
pGT: priming glycosyltransferase
GTP: phosphatidylglycerol-membrane-oligosaccharide glycerophosphotransferase

Cluster 3
GT: Glycosyltransferase
EP: Exopolysaccharide polymerization protein

Highlights

- *Lactobacillus fermentum* is a widely studied species regarding its functional properties.
- There is increasing evidence that suggests an active role of exopolysaccharides (EPS) in the functional properties of *L. fermentum*.
- More studies are needed in order to understand the participation of these molecules in the health benefits exerted by EPS-producing lactic acid bacteria.
- Knowledge about the responsible factors for the probiotic properties of lactic acid bacteria will bring new guidelines to study and select new potential probiotic strains.