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Exopolysaccharide from *Lactobacillus fermentum* Lf2 and its functional characterization as a yogurt additive

Elisa C Ale[1], Marcos J Perezlindo[1], Patricia Burns[1], Eduardo Tabacman[2], Jorge A Reinheimer[1] and Ana G Binetti[1]*

[1] Facultad de Ingeniería Química (UNL), Instituto de Lactología Industrial (UNL – CONICET), Santiago del Estero 2829, 3000 Santa Fe, Argentina
[2] Somalogic, Inc., 2945 Wilderness Pl., Boulder, CO 80301, USA

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Lactobacillus fermentum Lf2 is a strain which is able to produce high levels (approximately 1 g/l) of crude exopolysaccharide (EPS) when it is grown in optimised conditions. The aim of this work was to characterize the functional aspects of this EPS extract, focusing on its application as a dairy food additive. Our findings are consistent with an EPS extract that acts as moderate immunomodulator, modifying s-IgA and IL-6 levels in the small intestine when added to yogurt and milk, respectively. Furthermore, this EPS extract, in a dose feasible to use as a food additive, provides protection against *Salmonella* infection in a murine model, thus representing a mode of action to elicit positive health benefits. Besides, it contributes to the rheological characteristics of yogurt, and could function as a food additive with both technological and functional roles, making possible the production of a new functional yogurt with improved texture.

Keywords: Exopolysaccharide, lactic acid bacteria, IgA, cytokines, *Salmonella*.

Many food-grade microorganisms, including lactic acid bacteria (LAB), are known to synthesise exopolysaccharides (EPS) which have been widely used in the dairy industry, mainly when produced *in situ* during fermentation, to improve attributes such as thickening properties, syneresis reduction and rheology augmentation (Folkenberg et al. 2006). Apart from the technological relevance, there is increasing evidence that suggests a role of EPS from LAB and bifidobacteria in the interaction between the producing bacteria and the host, exerting an impact on human health. It was suggested that hetero-EPS exhibits immune stimulating (Fanning et al. 2012; Salazar et al. 2014), antitumor and cholesterol-lowering activities (Ruas-Madiedo et al. 2002a; Wang et al. 2014). Additionally, they may act as protective agents against pathogens (Nagai et al. 2011; Fanning et al. 2012; Maruo et al. 2012) and stomach ulcers (Rodríguez et al. 2009), as prebiotic factors (Salazar et al. 2008, 2011) or by improving the intestinal functional barrier (Del Piano et al. 2014). Despite these apparent beneficial aspects for health, the low yield of hetero-EPS from LAB (80 to 600 mg/l for *L. lactis* subsp. *cremoris*; Cerning, 1995; Ruas-Madiedo et al. 2002b) prevents their commercial use, in contrast to homo-EPS (10–25 g/l; Becker et al. 1998).

Lactobacillus fermentum Lf2 is a strain which was isolated as a contaminant culture in cheese manufacture. The particular interest in this strain resides in the high levels (approximately 1000 mg/l) of EPS when grown in well-defined conditions. The strains of this bacterial species which have been studied in relation to the EPS production are *L. fermentum* TDS030603 (Fukuda et al. 2010) and an isolate from Burkina Faso fermented milk (Savadogo et al. 2004). To our knowledge, at this moment there are no studies related to the functional properties of EPS from *L. fermentum* or based on the application of EPS as food additives in dairy products. This fact, combined with the increasing demand for natural and functional food ingredients, were the main factors to address the isolation and functional characterization (immunological role and protection against *Salmonella* Thyphimurium) of the EPS produced by *L. fermentum* Lf2, focusing on its final application as a food additive in a functional yogurt.

Materials and methods

Organisms and growth conditions

L. fermentum Lf2 was isolated as a non-starter culture in Cremoso cheese, and stored at −80 °C (INLAIN Collection)

*For correspondence; e-mail: anabinetti@fiq.unl.edu.ar

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in MRS (de Man, Rogosa and Sharpe, Biokar, Beauvais, France) broth plus 150 ml/l glycerol. It was routinely grown in MRS broth at 37 °C and was identified by sequencing of its 16SrDNA (Edwards et al. 1989).

For yogurt production, the commercial strains *Streptococcus thermophilus* SC42 and *Lactobacillus delbrueckii* subsp. *lactis* 254 were used as starter cultures. They were selected based on their inability (visual test) to produce EPS in milk. They were routinely grown in 100 g/l reconstituted skimmed milk (RSM) at 43 °C and stored at −80 °C in the same medium.

For in vivo trials, a human isolate of *Salmonella enteritidis* serovar Typhimurium was kindly provided by the INEI-ANLIS 'Dr Carlos Malbrán' Institute (Buenos Aires, Argentina). The strain was maintained at −80 °C in BHI (Brain-Heart Infusion, Britania, Buenos Aires, Argentina) broth with 150 ml/l glycerol. Overnight cultures (16 h, 37 °C, aerobiosis) obtained immediately after thawing were diluted and used to infect animals.

Fermentations and EPS isolation

Fermentations were performed in a 2-l fermentor (Sartorius Biostat A plus)® in a semi-defined broth (SDM; Kimmel & Roberts, 1998). *L. fermentum* Lf2 was inoculated from an overnight culture (1 ml/l) and incubations were made at 30 °C for 72 h, with an agitation rate of 5 ***g*** and sparging with CO_2 at 0·2 l/min. The pH was kept automatically at 6·0 with sterile 8 M NaOH. After incubation, bacteria were removed by centrifugation (19 630 ***g***, 30 min, 5 °C) and EPS was precipitated at 4 °C for 48 h by adding 2 volumes of chilled absolute ethanol (Cicarelli, Buenos Aires, Argentina). The precipitate was collected by centrifugation (4053 ***g***, 30 min, 5 °C), dissolved in ultrapure water and dialyzed against distilled water (12–14 kDa MWCO membranes, Sigma- Aldrich) for 3 d, at 4 °C with a daily change of water, and finally freeze-dried (Chris Alpha 1–4 LD Plus, Tokyo, Japan) to obtain the EPS fraction. The total protein concentration of EPS extract was determined with the Bio-Rad (Hercules, CA, USA) protein assay (Bradford, 1976).

Functional properties of the EPS extract

Manufacture of yogurts. Yogurt was produced in cylindrical bottles with 100 ml RSM inoculated with *S. thermophilus* SC42 (10^6 cfu/ml) and *L. delbrueckii* subsp. *lactis* 254 (10^5 cfu/ml), added with 0 (control) and 300 mg/l of EPS extract, with incubation at 43 °C until a final pH value of 4·6, and immediately cooled-down and stored overnight at 4 °C. The dose of EPS added was selected based on previous assays performed by our group, demonstrating that yogurt with 300 mg/l of EPS extract showed rheological and sensory differences when compared with control yogurts.

Animals and feeding procedures. For in vivo trials, 6-week-old male BALB/c mice weighing 19–21 g were obtained from the random-bred colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias (UNL, Esperanza, Santa Fe, Argentina). All the procedures were approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias (FCV-UNL, Esperanza, Santa Fe, Argentina), and were made following the recommendations of the INLAIN animal facility (Zacarías et al. 2014).

Immunostimulatory ability: determination of s-IgA and cytokines in small and large intestines. Thirty two mice were clustered in 4 groups (8 mice/group) and received, during 15 consecutive days and by gavage, 300 µl/d of any of the following samples: (a) RSM (M), (b) RSM + 300 mg EPS/l (equivalent to 4·5 mg/kg/d) (M-EPS), (c) yogurt (Y), (d) yogurt + 300 mg EPS/l (Y-EPS). All animals simultaneously and *ad libitum* received sterile tap water and a conventional balanced diet (Cooperación, Buenos Aires, Argentina).

After the feeding period, animals were anaesthetised and sacrificed by cervical dislocation. Liver was removed and homogenized in 5 ml of sterile PBS and pour plated onto ABRV agar (37 °C for 24 h in aerobiosis) in order to evaluate translocation of enterobacteria to liver. The small intestine was recovered and flushed with 5 ml of cold PBS buffer containing 10 g/l of a protease inhibitor cocktail (Sigma-Aldrich). This fluid was recovered, centrifuged (10 000 ***g***, 10 min, 4 °C) and the supernatant was stored at −80 °C for secretory IgA quantification by ELISA (Martins et al. 2009). Portions of the distal small intestine (jejunum and ileum) and whole large intestine were removed for homogenate preparations. The tissues were kept frozen (−80 °C) immediately after extractions and then resuspended (1 ml/100 mg of tissue) in extraction buffer (100 ml PBS, 0·293 g EDTA and 50 µl Tween 20) containing a cocktail of protease inhibitor (10 ml/l), homogenized (Ultra-turrax T8, IKA Labortechnik, Staufen, Germany) and centrifuged (9600 ***g***, 10 min, 4 °C). Cytokines IL-10, IL-6 and IFN-γ were determined in the supernatants using the corresponding mouse ELISA Set (BD OptEIA, BD, Biosciences PharMingen, San Diego, CA, USA).

Protective capacity of EPS against an infection with S. enteritidis *serovar Typhimurium*. A completely randomized design was chosen for this study including 64 animals which were clustered in 2 groups (32 animals/group). Each mouse received, during 15 consecutive days and by gavage, 300 µl/d of any of the following samples: (a) 600 mg/l of EPS in RSM (equivalent to 9 mg/kg/d) and (b) RSM (control group). This dose was selected based on a previous assay with 10 animals/group, where 4·5 and 9·0 mg/kg/d of EPS extract were administered, evidencing that the lower dose (4·5 mg/kg) caused no significant effect on the survival of treated mice. After the feeding period, all animals were challenged with a single infective dose of a fresh culture (16 h incubation, 37 °C, aerobiosis) of *S. enteritidis* serovar Typhimurium (10^6 cfu/mouse) according to a previous study of the optimal dose able to infect 40–50% of mice.

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The body weight and number of surviving animals were registered daily for 25 d post-infection and results were expressed as the percentage of animals that survived the infection.

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used to carry out the statistical analysis of the data. *T*-test was performed to compare control groups with treated groups, and the Mann-Whitney *U* test was applied when the distribution was not normal (Shapiro-Wilk test). Data were considered significantly different when $P < 0{\cdot}05$.

The proportions of survivors at the end of the challenge with *Salmonella* Typhimurium were compared with Barnard's exact test using the software R (R Core Team, 2015), as implemented in the 'Exact' library. The difference in death risk between the treated and control groups was estimated with Cox's model of Proportional Hazards, calculated with the 'survival' R library (Calhoun, 2015; Therneau, 2015). The *P*-value of the estimation of the risk ratio in this model was estimated using a permutation test of group membership (100 000 repetitions), while confidence intervals were estimated using bootstrap (100 000 repetitions).

Results

Fermentation, EPS isolation and manufacture of yogurt

At controlled pH (6·0), 30 °C, and in SDM broth, the best EPS production (1090·9 mg/l) was reached after 72 h of fermentation, corresponding with the late stationary phase of growth of *L. fermentum* Lf2. It is important to highlight that the EPS extract had 0·9% protein (Bradford method), a value lower than the limit recommended for EPS when added to food matrices (<3%, De Vuyst et al. 2003). The EPS that was released in SDM at pH 6·0, after its acid hydrolysis in 2 mol/l TFA (120 °C for 2 h, Ruas-Madiedo et al. 2002a) consisted of glucose and galactose, in a relation 2 : 1 approximately (data not shown).

Functional properties of the EPS extract

Immunostimulatory ability: determination of s-IgA and cytokines in small and large intestines. The chosen dose of EPS extract was appropriate, since no translocation was observed in mice from the groups M-EPS and Y-EPS (data not shown). The effects of EPS administration on small intestine s-IgA are shown in Table 1. The levels of s-IgA were increased when the exopolysaccharide extract was added in yogurt (Y-EPS $P < 0{\cdot}05$) relative to the yogurt control group. When the EPS was administered in milk, the stimulation of immune response mediated by IgA was markedly lower (approximately 2·6 times) than that observed in yogurt, evidencing only a slight and non-significant ($P > 0{\cdot}05$) enhancement.

Table 1. Effects of EPS extract from *L. fermentum* Lf2 on the small intestine fluid IgA and small and large intestines tissues cytokines IFN-γ, IL-10 and IL-6, when suspended (4·5 mg/kg body weight) in yogurt (Y-EPS) or milk (M-EPS) after 15 d of administration, compared to control mice (Y and M, respectively)

	Small intestine	Large intestine
IFN-γ		
Y	821 ± 243	2628 ± 280
Y-EPS	724 ± 109	3053 ± 124
M	634 ± 176	2672 ± 476
M-EPS	589 ± 95	2597 ± 325
IL10		
Y	6833 ± 1932	16 404 ± 798
Y-EPS	3594 ± 927	14 875 ± 1567
M	3010 ± 827	14 873 ± 1649
M-EPS	4993 ± 1028	15 096 ± 1648
IL6		
Y	4409 ± 535	8440 ± 647
M-EPS	4632 ± 485	6690 ± 1089
M	5656 ± 699	6079 ± 1303
M-EPS	2853 ± 715*	5642 ± 686
IgA		
Y	447 ± 40	
Y-EPS	959 ± 189*	
M	202 ± 25	
M-EPS	328 ± 68	

Asterisks indicate statistically significant ($P < 0{\cdot}05$) difference from the corresponding control value. Results are expressed as mean values ±SEM

The cytokines measured in the small and large intestine tissues at the end of the administration were comparable to their respective controls (Table 1), with the exception of IL-6 from small intestine in mice that received the EPS extract in milk (M-EPS), in which the lowest production ($P < 0{\cdot}05$) of this cytokine was detected.

Protection capacity of the EPS against an infection with S. enteritidis *serovar Typhimurium*. The survival proportion of mice that received EPS was 31%. Significant differences (unilateral *P*-value of 0·016, exact Barnard test of proportions for a 95% confidence level) were observed when compared with the control group which evidenced 9% survival (Fig. 1 shows the graphics of survival applying the Kaplan-Meier estimation of distribution for this EPS dose). The estimated power of this test was 87·3% (assuming 95% confidence level and, true proportions equal to those observed, for 2 groups of 32 mice/group), which is the probability of detecting a significant difference when the difference does exist. The difference ratio in the instantaneous death risk among groups was estimated by the Cox Proportional Hazards model. The instantaneous death risk for the EPS group calculated by permutation test was 0·63 of that for the control group (unilateral *P*-value of 0·053, 95% confidence level: [0·35, 1·09]), indicating that no significant difference was detected among groups when this statistical treatment was applied.

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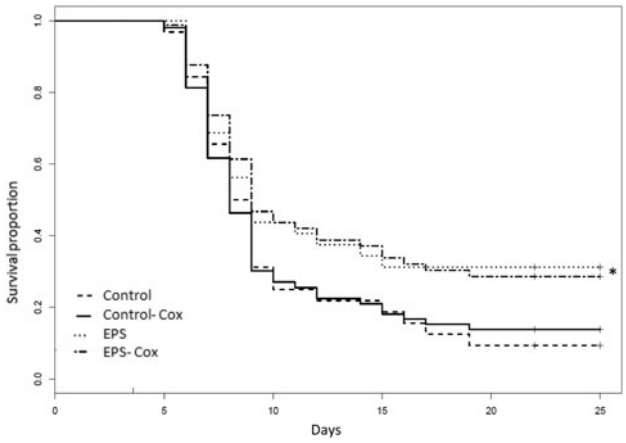


Fig. 1. Effect of the EPS extract from *L. fermentum* Lf2 on the survival rate of mice infected with *Salmonella enteritidis* serovar Typhimurium applying the Kaplan-Meier estimation of distribution. BALB/c mice (32 animals/group) were administered orally with sterile RSM (Control) or EPS suspended in sterile RSM (9 mg/kg body weight). Mice were infected with *S.* Typhimurium (10^6 cfu) and the survival rates were observed for 25 d. The curves corresponding with the estimated Cox Proportional Hazards model are specified (Control-Cox and EPS-Cox). Asterisk indicates a statistically significant difference ($P < 0{\cdot}05$, exact Barnard test of proportions) from the control group.

Discussion

Our study was addressed to the functional characterization of milk and yogurt supplemented with an EPS extract (300 and 600 mg/l) from *L. fermentum* Lf2. In previous experiments, these concentrations of EPS were suitable to produce beneficial rheological and sensory changes in yogurt, improving the consistency without unpleasant defects, when compared with controls (unpublished).

This study is the first to report the effect on the s-IgA and cytokines (IL-6, IL-10 and INF-γ) in small and large intestines following the administration, at a dose of 4·5 mg/kg of an exopolysaccharide produced by *L. fermentum* when used as food additive in milk and yogurt. It seems that the EPS extract when suspended in milk showed a mild in vivo immune suppressive profile by reducing the proinflammatory cytokine IL-6 in small intestine. Similar results were reported by Salazar et al. (2014) for an EPS-producing *B. animalis* subsp. *lactis* strain in healthy rats, evidencing an immune protective profile by a suppression of IL-6 and a promotion of the synthesis of the regulatory cytokine TGF-β. When yogurt was used as matrix, we observed an increased ($P < 0{\cdot}05$) level of s-IgA for the EPS extract, again in small intestine. As expected (Gill, 1998; Simon et al. 2003), live microorganisms delivered via yogurt seemed to improve the gut mucosal immune system by increasing the level of secretory IgA, since the effect of this food matrix was significantly higher than those detected in milk. In the gut, secretory IgA is the main immunoglobulin whose key role is to exert an immune exclusion by intimate cooperation with the innate non-specific protection mechanisms (Brandtzaeg et al. 1987). Various authors described antibody production at the systemic level after the oral administration of exopolysaccharides or their producing strains (Willers et al. 1995; Vinderola et al. 2006; Fanning et al. 2012; Salazar et al. 2014). In general, these studies refer to administration in higher doses than those evaluated in the present work, which are more representative of a real concentration of any EPS in a food matrix. For example, Vinderola et al. (2006) have reported the administration of 100 mg/kg of kefiran (a dose 22 times higher than the used in our study), evidencing an immunomodulation (mediated by IgA, Il-2, IL-6, IL-10, TNF-α) at the level of large intestine and blood, enhancing the IgA production at both the small and large intestine level.

More recently, diverse reports indicate that not only the presence/absence of the polymer, but also the intrinsic characteristics of each EPS, mainly charge and molecular weight, are relevant for their capability to induce an immune response. López et al. (2012) have demonstrated that different bifidobacterial EPS *in vitro* induced a variable cytokine production pattern by human peripheral blood mononuclear cells. In general, it was proposed that EPS having negative charge and/or small molecular weight (lower than 10^6 Da) could act as mild stimulators of immune cells, whereas those polymers non-charged and with a large size could elicit a lower production of any cytokine, evidencing a suppressive profile (Hidalgo-Cantabrana et al. 2014; Salazar et al. 2014; Ryan et al. 2015). Therefore, based on preliminary evidence indicating a relatively low MW (lower than 10^5 Da) of our EPS, we could justify the observed mild stimulation of immune cells when administered to mice.

Our experimental model was performed with standard, naïve (not challenged) healthy BALB/c mice. Thus, this could be the main reason why most cytokines tested were not significantly modified by the ingestion of the EPS extract. In this regard, Fanning et al. (2012) have demonstrated in a naïve murine model that the EPS-producing *Bifidobacterium breve* UCC2003 strain failed to elicit a strong immune response in comparison to its EPS-deficient variant strains, evading the B-cell response. The reason why the two matrices (milk and yogurt) evidenced different responses on s-IgA and IL-6 remains unclear, and would require additional studies to elucidate (by increasing the number of cytokines evaluated, for example).

In general, it is desirable for a pathogen challenge to cause 40–50% mortality in the control group, in order to evaluate the protective effect of the treatment in a moderately hostile model. We based our pathogen dose on our previous studies with the same strain of *S. Typhimurium,* which indicated a dose of 10^6 cfu to allow the survival of 40% of mice. In our assay, only 9% of the control group survived after the challenge, which turned the assay into a very demanding one. The reason for this variability remains unknown, but it could be related to the fact that *Salmonella* infection in mice is considered dynamic and dependent on multiple host variables (Watson & Holden,

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2010). Despite the challenging conditions of our assay, the results indicated that the daily administration of EPS extract, at the dose of 9 mg/kg before a challenge with *Salmonella* provided enhanced protection against the infection. Similar survival rates were observed by Nagai et al. (2011) when evaluating the effect of an EPS from a strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* on the influenza virus infection after 21 d of administration of yogurts. Although they evaluated the capacity of EPS against a virus, the survival of mice ranged from 0% for the control groups to 38·9% for diverse EPS-fractions. Similar results were reported by an EPS producing strain of *Lactococcus lactis* subsp. *cremoris* (Nagai et al. 2011). The role of polysaccharides in oral tolerance and in balancing the immune status associated with some infections or inflammatory disorders has been well described (Hidalgo-Cantabrana et al. 2012). Fanning et al. (2012) demonstrated the effects of an EPS$^+$ *Bifidobacterium breve* strain on the immunostimulation and pathogen protection, since EPS presence was associated to the evasion of adaptive B-cell responses and to the reduction on the colonisation levels of *Citrobacter rodentium* (murine model). These authors proposed that the EPS covers the ephitelial host cell surfaces by formation of a biofilm, leaving *C. rodentium* with fewer receptors on which to attach to successfully.

Conclusions

Our study highlights the relevance of evaluating the functional properties of EPS from *L. fermentum* Lf2 when carried in dairy matrices as additive (and not as an isolated component) and considers a reasonable concentration to be applied in real cases without overestimating its potential. This fraction contributed to functional properties and, considering that it may also enhance the rheological characteristics of the yogurt, it could function as a food additive with both, technological and functional roles, making possible the manufacture of a new functional dairy product.

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