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ORIGINAL ARTICLE

Isolation and characterization of an exopolysaccharide-producing *Leuconostoc citreum* strain from artisanal cheese

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Significance and Impact of the Study: Some LAB strains are known to use extracellular glycoside-hydrolase enzymes for synthesizing a diversity of exopolysaccharides (EPS) with potential application as natural additives to foods. Previous studies have identified an EPS-producing *Leuconostoc citreum* strain with immunomodulatory properties. This work provides a better understanding of EPS produced by this strain and the potential application of the strain in food fermentation and/or as a probiotic culture.

Keywords
biopolymers, exopolysaccharide, fermented foods, lactic acid bacteria, probiotics.

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Abstract

High molar mass exopolysaccharides (EPS) produced from sucrose by lactic acid bacteria (LAB) are of great interest as natural additives to use in foods, medical and pharmaceutical industry. This study aimed to identify the EPS produced by *Leuconostoc citreum* L3C1E7 isolated from Pico cheese and characterize the strain for technological and probiotic potential. Purified EPS was isolated from the culture of *L. citreum* L3C1E7 by ethanol precipitation, with a yield of 520 mg ml^{-1}. The EPS-producing strain had a mucoid phenotype and average molecular weight of $5{\cdot}88 \times 10^6$ Da. The structural characterization of the purified EPS was determined by ^1H, ^{13}C and two-dimensional NMR spectroscopy. EPS was composed of alternating α-(1→6)-linked and α-(1→3)-linked D-glucopyranyl units, suggesting the existence of an alternan. The strain was slow acidifying, produced diacetyl and displayed high esterase/lipase and aminopeptidase activities, which promote the desirable flavours in dairy products. Moreover, *L. citreum* showed moderate resistance to the adverse conditions of the gastrointestinal (GI) tract and high adhesion to GI cells. This work provides a better understanding of EPS produced by *L. citreum* and the potential application of EPS-producing strain in food and/or as a probiotic culture.

Introduction

Bacterial exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) have found their most valuable application in the improvement of the rheology, texture, stability and sensory properties of fermented foods (Ko *et al.* 2016; Mende *et al.* 2016).

Lactic acid bacteria produce a wide diversity of EPS, with unique chemical compositions and structures, comprising homopolysaccharides and heteropolysaccharides (Sanlibaba and Çakmak 2016). Among the homopolysaccharides, glucans and fructans are most frequently found, and are mainly produced by the genus *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Weissella* (Sanlibaba and Çakmak 2016; Zannini *et al.* 2016). Extracellular enzymes synthesize a variety of α-glucans such as dextrans from sucrose (Leemhuis *et al.*, 2013).

Lactic acid bacteria present in traditional food environments are important sources of unexploited new bacterial strains. These strains must be genetically stable, in particular their plasmids when present (Sewaki *et al.* 2001). Artisanal Pico cheese is produced in the Azores (Pico,

Portugal) and is made from raw cow's milk without the addition of starter cultures (Riquelme *et al.* 2015). Thus, the microbial fermentation is entirely spontaneous and carried out by the indigenous microbiota present in the raw milk and the production environment. Previously, an EPS-producer strain was isolated from this artisanal cheese and identified as *Leuconostoc citreum* (Domingos-Lopes *et al.* 2017b). This strain (L3C1E7) was found to have immunomodulatory properties *in vivo* (Domingos-Lopes *et al.* 2017a). The aim of the present study was to isolate and identify the EPS produced by this strain. The presence and the stability of plasmids were studied to assess the stability of EPS phenotype. In addition, the strain was evaluated for its technological properties and probiotic potential, assessed by conventional *in vitro* tests. These characterizations might help to identify potential applications of this EPS-producer for the food industry.

Results and discussion

Plasmid profile

The plasmid profile of *L. citreum* L3C1E7 is shown in Fig. 1a. This strain harbours seven plasmids, ranging from 1 to 80 kb. After subcultivation for 21 days all the plasmids were present (Fig. 1a), and the strain preserved the mucoid phenotype (Fig. 1b).

The enzymes involved in the synthesis of exopolysaccharides are encoded in specific gene clusters located predominantly on plasmids (Caggianiello *et al.* 2016). In L3C1E7 strain, all plasmids were present after continuous subculturing. This is a noteworthy advantage as plasmids are usually unstable and the encoded characteristics can be lost on repeated subculture.

Purification and characterization of EPS

The L3C1E7 strain produced mucoid colonies (Fig. 2a) from sucrose, but not from other carbohydrates such as glucose, fructose and lactose (data not shown). The physiological role of this EPS-producing bacterium was not clear since this strain was isolated from cheese, a food environment lacking sucrose.

The EPS production of *L. citreum* (1%) in MRS with 5% (w/v) sucrose was 515·8 mg l^{-1}. The protein content was 0·01%. The average yield of the dried EPS obtained after the primary purification (purity 81%) was 410 ± 10 mg l^{-1}. The EPS gave a negative result for endotoxins and the estimated molecular mass was $5{\cdot}879 \times 10^6$ Da, in agreement to other studies (Miao *et al.* 2014).

The purified EPS exhibited a dextranase resistance rate of 91·4%, comparable to the high levels (89%) reported by Miao *et al.* (2014). According to Bounaix *et al.* (2009) the dextranase resistance ranged from 4·3 to 37·4% for classic dextrans, from 37·9 to 82% for dextrans containing α-1,2 linkages and from 64·0 to 97·8% for glucans with high percentage of α-1,3 linkages.

Nuclear magnetic resonance (NMR) spectroscopy analysis

The EPS was further analyzed using ^{1}H and ^{13}C NMR spectroscopy (Fig. 2). To identify the monomers present in the EPS, a hydrolyzed sample was dissolved in D_2O. The EPS was entirely composed of glucose units, although the hydrolysis was not complete leading to the presence of small di or trisaccharides with variable structure that account for the remaining signals. The ^{1}H spectrum showed two broad signals (each one displaying a shoulder peak) in the anomeric region, centred at 5·30 and 4·98 ppm with an intensity ratio of 1/1·4, respectively. The presence of the shoulders and the broadness of the peaks indicate that the structure of this EPS does not contain a defined repetitive unit, but two major types of glyosidic bonds that may occur at different lengths within the EPS chain. In the ^{13}C spectrum, signals from the anomeric carbons appear between 98 and 100 ppm, which corresponded to the anomeric carbon involved in the α-1,3 and α-1,6 linkages (Bounaix *et al.* (2009).

In agreement with both spectra, the EPS structure was an irregularly branched form of dextran with the signal at 5·30 ppm belonging to the anomeric signal of glucose involved in α-1,3 linkages and the signal at 4·98 ppm to α-1,6 linkages. Under this hypothesis, six different glucose units are possible (Fig. 3a). A glucose unit can have its anomeric signal involved in an α-1,6 or α-1,3 bond and for each of these, it can be linked to another glucose unit by positions 3, 6 or both.

Despite the extensive signal overlap, the use of increasing mixing times in the HSQC-TOCSY experiments allowed us to partially assign the ^{1}H and ^{13}C signals of the detected glucose units in a sequential way (Fig. 3b). For assignment convenience purposes, the major signal at 5·30 ppm was designated as A and its shoulder as A' while the signal at 4·98 ppm and its shoulder was designated B and B', respectively. The HMBC spectrum revealed clear connections between signals A1 and B3 and between signals B1 and A6 (Fig. 3b).

The detection of only two distinct sets of anomeric signals in the EPS strongly suggests the occurrence of only two types of glycosidic bonds. Having observed HMBC connections between signals A1 and B3 and between signals B1 and A6, we can conclude that A and A' are involved in α-1,3 bonds while B and B' are involved in

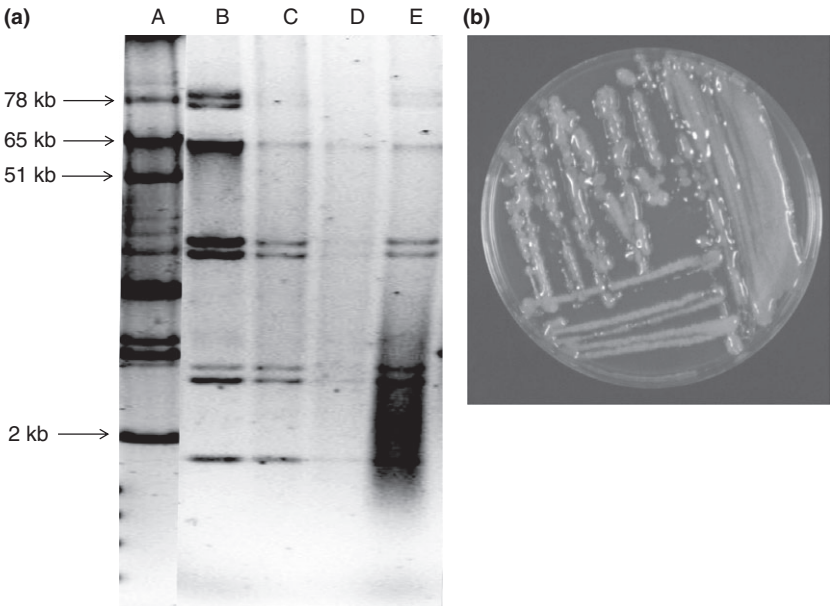

Figure 1 (a) Plasmid profile. Lines A) *Lactococcus lactis* biovar *diacetylactose* DRC3; (B) *Leuconostoc citreum* L3C1E7; (C) *L. citreum* 2nd day; (D) *L. citreum* 12th day; (E) *L. citreum* 20th day. (b) EPS colonial morphology of *L. citreum* L3C1E7 culture in MRS agar with sucrose (mucoid phenotype).

α-1,6 bonds. As can be seen from Fig. 3 the main difference between spin-systems A and A' is in the chemical shift at position 6 leading to the conclusion that the position 6 of A is involved in a α-1,6 bond (scheme 5 in Fig. 3). The same reasoning applied to the B and B' spin-systems and difference at position 3, leads us to conclude that B corresponds to scheme 1 while B' corresponds to scheme 2 in Fig. 3.

The very low ratio of intensities between signals A and A' in relation to B and B' (1/1·4) indicates that there is an unusual amount of α-1,3 *vs* α-1,6 bonds in this dextran. This could be a consequence of a highly branched polymer, most likely alternan, consisting of alternating sequences of α-1,6 and α-1,3 linkages, in a ratio of 1·4/1. Miao *et al.* (2014) described a similar structure of an EPS produced by *L. citreum* isolated from fermented pickles. The EPS was described as alternating α-1,3 and α -1,6 linkages, in a ratio of 4/5, and was similar to alternans produced by *Leuconostoc* strains reported by Misaki *et al.* (1980) and Bounaix *et al.* (2009). The unusual high ratio of α-1,3/α-1,6 bonds found in the present study is only produced by few *L. citreum* strains (Bounaix *et al.* 2009; Miao *et al.* 2014). Alternans are known to exhibit endodextranase resistance and are more soluble in water than the usual dextrans (Bounaix *et al.* 2009). Therefore, the use of this EPS-producing strain could be a suitable alternative to the use of food additives (Huang *et al.* 2015; Caggianiello *et al.* 2016). Moreover, most dextrans display a wide range of biological functions, such as immunological, anti-cancer and prebiotic properties (Caggianiello *et al.* 2016). Recently, Matsuzaki *et al.* (2018) reported the immunostimulatory activity of an EPS produced by a *Leuconostoc mesenteroides* strain (NTM048) isolated from green peas. The EPS (94%) consisted of α-1,6 glucan containing 4% of α-1,3 glucose branches (Matsuzaki *et al.* 2017). The strain of *L. citreum* in the present study produced a higher branched glucan (approx. 42% of α-1,3 glucose branches). It is possible that this EPS was associated to the immune-stimulating activity of the *L. citreum* strain reported previously (Domingos-Lopes *et al.* 2017a).

Technological characterization

The characterization of technological properties is required for the potential utilization of the EPS-producer in novel food products. The results for the acidifying activity of L3C1E7 strain in skim milk are presented in Table 1. This strain showed a strong pH reduction (from 6·15 to 3·47), although the acidification progressed slowly (48 h). As reported by Alegria *et al.* (2013) *Leuconostoc* spp. do not grow well in milk when on its own, since they lack extracellular caseinolytic activity which is essential for reaching high cell densities. This can explain the slow pH reduction in the first 6–12 h. However, the low pH observed in milk after 48 h is remarkable when compared to other *L. citreum* strains isolated from Spanish traditional cheeses (Alegria *et al.* 2013).

Assessing the ability to tolerate salt and acid stresses would facilitate the selection of strains suitable for food manufacture (Morandi and Brasca 2012). In accordance with our results, this strain shows high pH, salt and temperature tolerance (Table 1). Similar results have been reported for *Leuconostoc* strains isolated from artisanal cheeses (Nieto-Arribas *et al.* 2010).

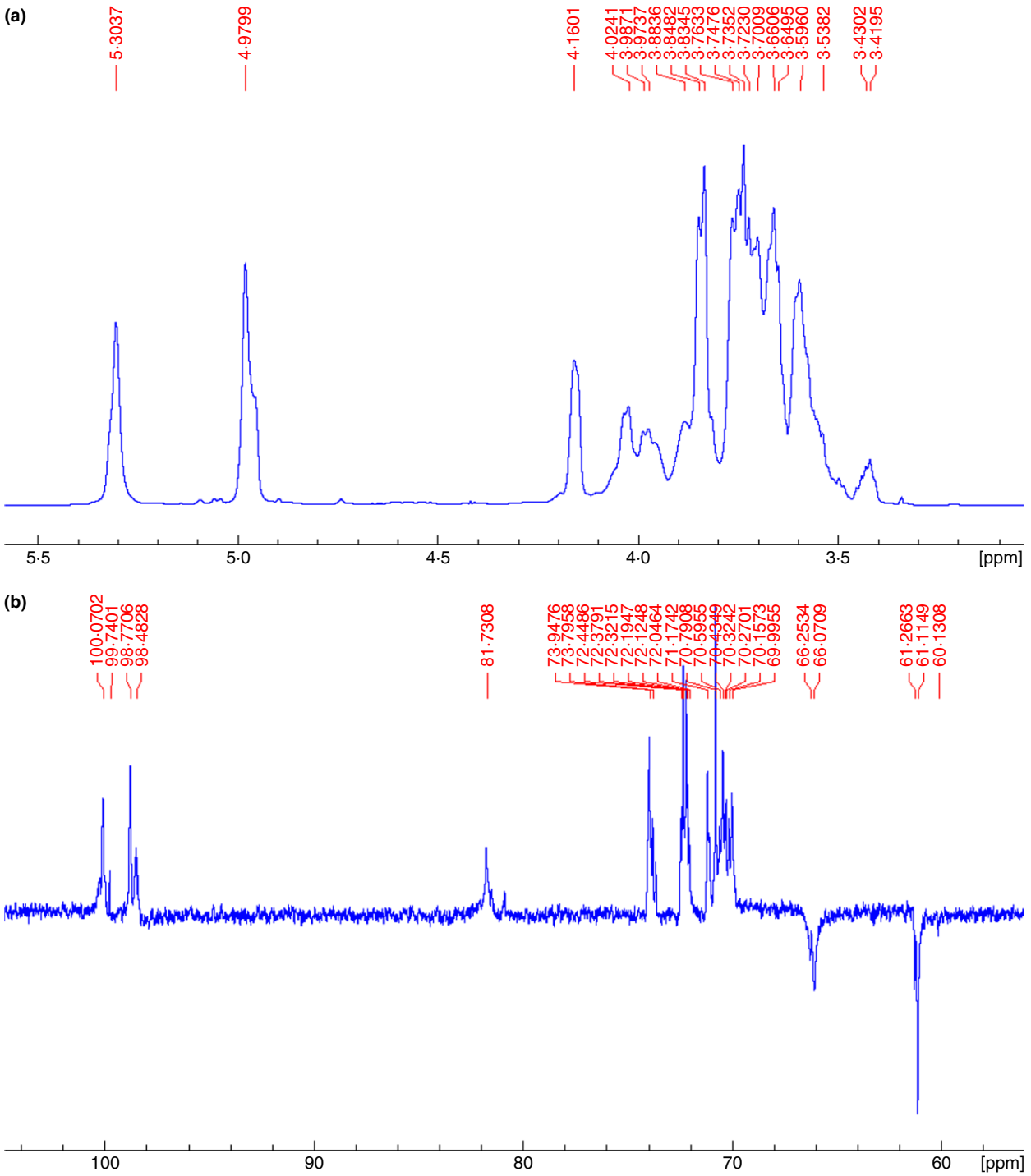

Figure 2 ^{1}H (a) and ^{13}C (b) NMR spectra of exopolysaccharide from *Leuconostoc citreum* L3C1E7. [Colour figure can be viewed at wileyonlinelibrary.com]

The sensory properties of fermented foods are strongly dependent on the production of diacetyl, volatile compounds and free amino acids. This strain tested negative for proteolytic and lipolytic activities (results not shown), but produced diacetyl (Table 1). The production of diacetyl is linked to the utilization of citrate and is a favourable trait in aroma and flavour development. This is not surprising, as *Leuconostoc* are known to metabolize the citrate of milk to produce diacetyl (Hemme and Foucaud-Scheunemann 2004).

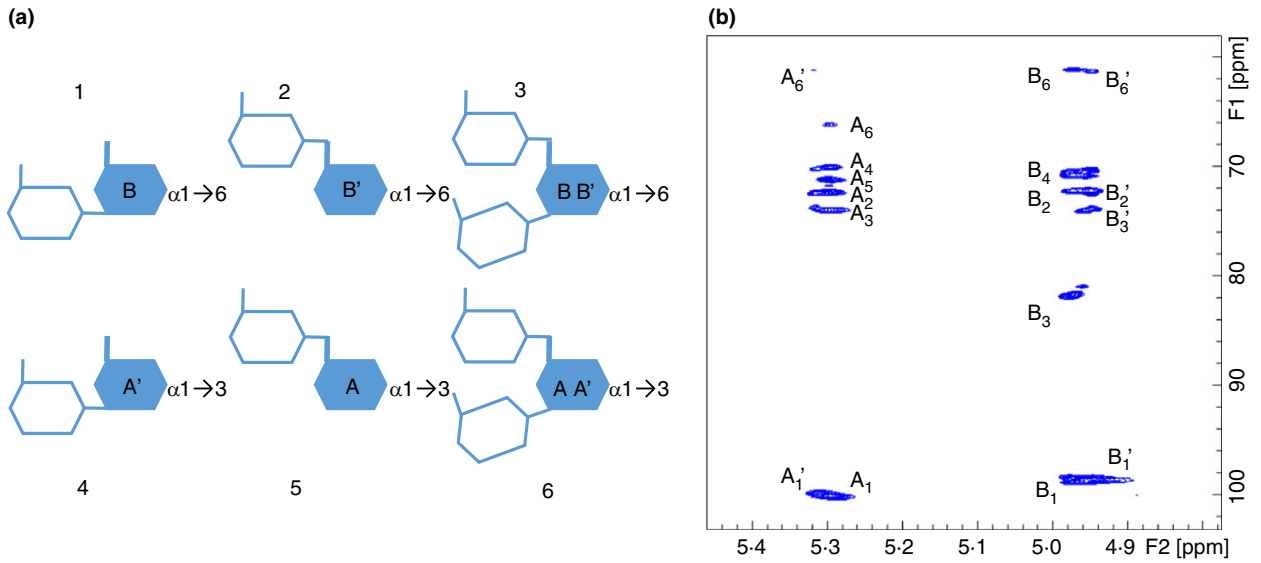

Figure 3 (a) Possible glyosidic linking patterns of glucose units in dextran. (b) Section of the ^{1}H-^{13}C HSQC-TOCSY spectrum with sequential signal assignment A and A′ correspond to the glucose units responsible for the main anomeric signal at 5·61 ppm and its shoulder, respectively; while B and B′ correspond to the main signal at 5·27 ppm and its shoulder, respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

Although enzyme activities were not detected (Table 1), the high esterase/lipase activities displayed by this strain are noteworthy. This is an uncommon trait found in this species, although high variation in technological characteristics has been reported (Bonomo and Salzano 2013; Heperkan *et al.* 2014). In addition, this strain showed high acid phosphatase, valine and cysteine arylamidase activities that are known to contribute positively to the development of the desirable flavours in dairy products (Floros *et al.* 2012; Alegria *et al.* 2016). These enzymes were also reported to have a de-bittering effect during cheese ripening and may play a critical role in flavour development of fermented dairy products (Herreros *et al.* 2003).

Probiotic assessment

Tolerance to acidic conditions in the stomach and bile concentrations in the small intestine is essential to probiotic strain selection. As shown in Fig. 4a, the strain presented a satisfactory survival, since it resisted to low pH (2·5 for 1 h), bile salts and pancreatin (3 h). High variability of resistance to acidity, bile salts and pancreatin has been reported by various authors (Argyri *et al.* 2013; Lee *et al.* 2016; Manini *et al.* 2016; Silva *et al.* 2017). Different strains of *Leuconostoc* have been shown variable probiotic potential not related to EPS production (Manini *et al.* 2016; Silva *et al.* 2017). These differences are not only the result of different species and strains tested, but are also the consequence of the use of different methodologies (Manini *et al.* 2016).

The capacity to adhere to the intestinal mucosa is also an important property for probiotic strains. The strain was able to adhere to Caco-2 and HT-29 cells with the efficiency of 4% ($5{\cdot}8 \times 10^{6}$ CFU per cm^{2}) and 1% ($2{\cdot}1 \times 10^{4}$ CFU per cm^{2}), respectively (Fig. 4b). This high adherence to Caco-2 and HT-29 cells was similar to the probiotic strain *Lactobacillus rhamnosus* GG reported by Lee *et al.* (2016) and suggests that *L. citreum* may be a good GI tract colonizer.

In conclusion, a *L. citreum* strain was isolated from a traditional cheese and produced a glucan type EPS from sucrose. According NMR results, the EPS consisted of a highly branched polymer with α-1,6 and α-1,3 linkages, in a ratio of 1·4/1, analogous to alternan. The strain showed high pH, salt and temperature tolerance and presented a slow acidification rate in milk. The strain also produced diacetyl and specific enzymes important for the development of desirable flavours in dairy products. In addition, *L. citreum* strain showed moderate resistance to the adverse conditions of the GI tract and high adhesion to GI cells. These results revealed the potential of this strain to be used as a novel probiotic in the food industry.

Materials and methods

Bacterial strain and plasmid isolation

The strain *L. citreum* L3C1E7 was isolated from Pico cheese produced in the Azores (Pico, Portugal). The strain was routinely propagated in MRS broth or agar (Difco Laboratories, Detroit, MI).

Table 1 Technological characterization of the EPS-producing strain (acidifying activity, growth at non-optimal pH, salt and temperature, diacetyl production and enzymatic profile).

Acidifying activity		pH*		Salt*		Temperature*			Enzymatic profile (nmol of hydrolysed substrate)			
Time	pH	pH	Growth	NaCl	Growth	Temp.	Growth	Diacetyl Production †	0	10–20	30	40
0 h	6·15	4·4	+	4%	+	10°C	+	+	α-galactosidase	Leu. arylamidase	α- glucosidase	Alkal. phosphatase
2 h	6·10								β-galactosidase	Nap-Phosp.Hydr		Esterase/Lipase C4,C6,C14
6 h	6·01	9·6	+	6·5%	+	45°C	+		β-glucuronidase			Val-, Cys-arylamidase
12 h	5·73								N-acet.glucosam.			Trypsin/α-Chymotrypsin
24 h	4·68			18%	–				α-mannosidase			Acid phosphatase
48 h	3·47								α-fucosidase			β-glucosidase

*+: Positive growth, – : Negative growth,
†+: Fast production.

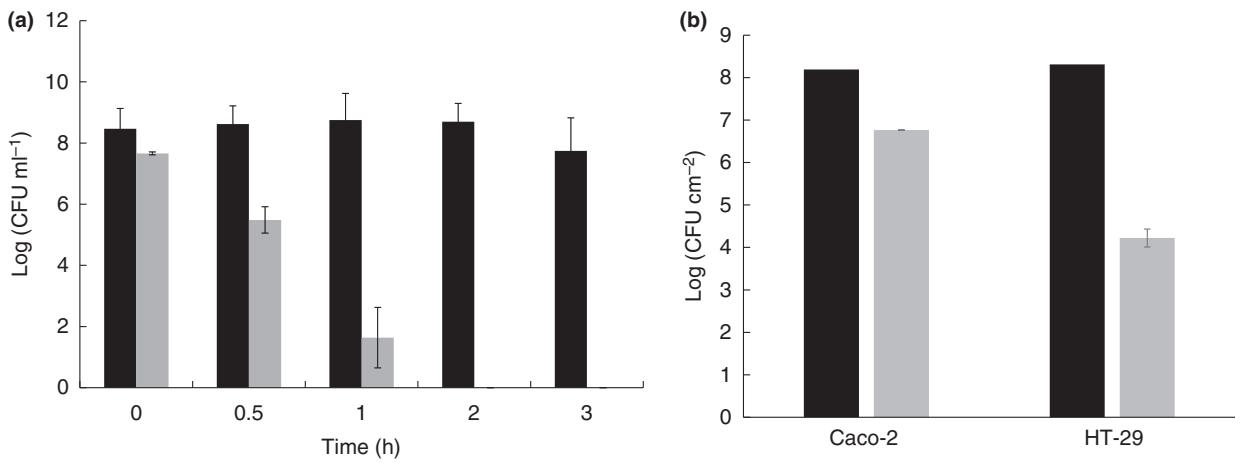

Figure 4 (a) Resistance of *Leuconostoc citreum* to low pH (2·5), bile salts (0·3%, w/v) and pancreatin (0·1%, w/v), after 0, 0·5, 1, 2 and 3 h. The black bars indicate tolerance to bile salts and pancreatin and grey bars indicate tolerance to low pH. Error bars represents SEM from four different experiments. (b) Adhesion of *L. citreum* to Caco-2 and HT-29 cell line. The black and grey bars indicate applied and adherent cells, respectively. Error bars represents SEM from three independent experiments. (a) (■) Bile salts + pancreatin; (■) pH (2·5); (b) (■) Applied cells; (■) Adherent cells.

Plasmid profile was determined according to the method of O'Sullivan and Klaenhammer (1993). The plasmid DNA was run in 0·7% agarose gel in 1× TE buffer with ethidium bromide, overnight, at 40 V and 4°C. The strain *Lactococcus lactis* biovar *diacetylactose* DRC3 was used as a positive control.

Production and purification of EPS

For EPS production, modified MRS in which glucose was replaced with 50 g l^{-1} of sucrose was used. Stock cultures were kept at −80°C in 50% (v/v) glycerol and propagated twice in MRS broth with 1% (v/v) of inoculum and incubated at 30°C for 48 h, A β-glucan–producing *Lactobacillus paracasei* (National Food Biotechnology Centre, NFBC 338) was used as positive control.

The EPS was isolated and purified twice according to the method of Garcia-Garibay and Marshall (1991). For the primary purification, the strain was propagated twice in MRS to minimize carry-over of interfering polysaccharides from the medium. Following fermentation, the EPS was isolated according to Zang *et al.* (2016). The purified EPS was recovered by freeze-drying (Steris, Lyovac GT 2; Hürth, Germany) and stored at −20°C for further analysis.

For the secondary purification, the EPS-crude powder was treated with DNAse type-I (Sigma, 2·5 μg ml^{-1}) for 6 h, at 37°C, and Pronase E (Sigma, 50 μg ml^{-1}, pH 7·5) for 18 h, at 37°C. Trichloroacetic acid (12%) was added to the mixture, stirred at room temperature for 30 min and centrifuged at 3260***g***, for 30 min. The supernatant was adjusted to pH 4·0–5·0 with NaOH (10 mol l^{-1}) and

dialyzed with 12–14 kDa dialysis tube (MWCO) against ultra-pure water, for 3 days at 4°C.

Characterization of EPS

Total sugar content was quantified by the phenol-sulphuric acid method (Dubois *et al.* (1956). Protein was assayed by the method of Bradford (1976). The susceptibility of the EPS to dextranase was determined using dextranase from *Chaetomium erraticum* (D0443, Sigma), according to Miao *et al.* (2014). Endotoxin detection was performed by using EndoLISA® test.

The molecular weight of the purified freeze-dried EPS was determined by high-performance gel permeation chromatography (Waters 2695), fitted with a refractive index detector (410 nm) and a Shodex OHpak separation column (SB-806 HQ, 8·0 × 300 mm; 13 μm). Injection volume was 10 μl and the mobile phase consisted of 20 mmol l^{-1} phosphate buffer, pH 7·2 (flow rate: 0·5 ml min^{-1}). A calibration curve was obtained by using pullulan (Shodex STANDARD, Showa Denko K.K., Tokyo, Japan). Measurements were carried out on three isolated samples.

Nuclear magnetic resonance (NMR) spectroscopy analysis

For the identification of the monomers, 0·45 g of freeze-dried EPS was dissolved in 2·16 ml of 4 mol l^{-1} trifluoroacetic acid, deuterated for 20 min under N_2 and placed at 80°C for 5 h. After the incubation, the sample was freeze dried and dissolved in 1 ml of D_2O for NMR analyses. All spectra were acquired on a Bruker AVANCE III 800 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 800·33 MHz, equipped with a four channel 5 mm inverse detection probe head with pulse-field gradients along the *Z* axis. Spectra were run at 60°C using standard Bruker pulse programs. ^{1}H and ^{13}C chemical shifts were referenced to the methyl group of acetone designated at 2·22 ppm and 30·89 ppm, respectively (Fulmer *et al.*, 2010). ^{13}C spectra were recorded at 201·24 MHz using the attached proton test sequence. The modulation of peak sign was achieved using a delay of 6·89 ms for the evolution of $^{1}J_{CH}$. Proton decoupling was applied during the acquisition stage using the WALTZ-16 sequence (Shaka *et al.* 1983). In the two-dimensional ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra, a delay of 3·45 ms was used for evolution of $^{1}J_{CH}$, while in the heteronuclear multiple bond connectivity (HMBC) spectra a delay of 73·5 ms was used for the evolution of long range couplings. In the HSQC, proton decoupling was achieved using the GARP4 sequence (Shaka *et al.* 1985). A nonproton decoupled HSQC was acquired to measure ^{1}H-^{13}C one bond coupling constants. A series of ^{13}C-^{1}H HSQC TOCSY spectra were acquired with increasing mixing times of the MLEV sequence (20, 40, 60, 80 and 100 ms).

Technological characterization

The strain was tested for the proteolytic and lipolytic activities, diacetyl production and enzyme activity profile using the API-ZYM system (Biomerieux, Craponne, France), as described by Domingos-Lopes *et al.* (2017b). The acidifying activity in milk was tested as described by Floros *et al.* (2012). Briefly, 1 ml of overnight culture was inoculated into 100 ml (1%, v/v) of sterile reconstituted skim milk (Merck, Damstadt, Germany). The pH was measured by a pH meter (WTW Inolab, Weilheim, Germany) after 2, 6, 12, 24 h and 48 h of incubation at 30°C. The strain was also tested for its ability to grow at 10°C and 45°C in MRS broth, salt tolerance (growth with 4, 6·5 and 18% of NaCl in MRS broth) and pH tolerance (growth at pH of 4·4 and 9·6 adjusted in MRS broth). All experiments were performed in triplicate.

Probiotic assessment

Tolerance to low pH and bile acid and pancreatin was determined as reported by Silva *et al.* (2015). Bacterial cells from overnight cultures were harvested (10 000***g***, 5 min, 4°C), washed twice with sterile phosphate-buffered saline (PBS) buffer (pH 7·3), before being re-suspended in 1 ml of PBS. Cells were diluted (1 : 100) in PBS solution adjusted to pH 2·5 (tolerance to low pH), or PBS solution (pH 7·3) containing 0·3% (w/v) of bile salts (LP0055, Oxoid) and 0·1% (w/v) of pancreatin (P1500, Sigma-Aldrich, St Louis, MO). Resistance was assessed in terms of viable colony counts and enumerated in duplicate on MRS agar (Biokar Diagnostics) after incubation at 37°C for 0, 0·5, 1, 2 and 3 h, reflecting the time spent by food in the stomach. Results are expressed as the average of four independent experiments.

Bacterial adherence to differentiated Caco-2 cells (ECACC 09042001) and HT-29 cells (ECACC 91072201) was tested as described by Argyri *et al.* (2013) with minor modifications. Caco-2 cells and HT-29 cells were grown in cell culture flasks or 24 well tissue culture plates. For Caco-2 cells culture, wells were precoated with rat-tail collagen (Collagen, Type I Solution, C3867 Sigma) to support Caco-2 cell monolayers. Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (D6429, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, F2442 Sigma), 1% nonessential amino acids (M7145, Sigma) and 1% gentamicin (G1397, Sigma). HT-29 cells were culture in McCoy's 5A Medium (M4892, Sigma), supplemented with 10% Fetal Bovine

Serum (FBS, F2442 Sigma) and 1% gentamicin (G1397, Sigma). Both cell-lines were incubated at 37°C in a humid atmosphere of 5% CO_2. Caco-2 and HT-29 cells were transferred into 24 well tissue culture plates at a seeding density of 10^4 cells in basal medium. The medium in the wells was replaced with fresh medium every 2–3 days for 15 days, until monolayers formed with no further visible differentiation for Caco-2 cells. For HT-29 cells culture, medium was refreshed every day until 80% confluence. One day before the co-incubation with the LAB, the medium in each well was replaced with prewarmed fresh medium without antibiotics. Prior to experiments, all bacterial cultures were grown overnight until stationary phase in MRS at 37°C, washed twice with PBS and diluted in fresh cell culture medium without antibiotics. Subsequently, approximately 10^8 CFU per ml and 10^6 CFU per ml of *L. citreum* L3C1E7 were added to each well of Caco-2 and HT-29 cells, respectively. Following co-incubation for 2 h at 37°C, cells were washed three times with sterile PBS to remove any nonadherent bacteria. Then, 0·1 ml of trypsin-EDTA solution was added and the mixture was incubated for 10 min at 37°C in 5% CO_2. Then, 0·4 ml of 0·25% Triton X-100 was added and each monolayer was disrupted by repeated pipetting to release the bacteria. In this adhesion assay, total Caco-2 and HT-29 associated bacteria (invasive plus surface adherent) were quantified. The number of associated bacteria was determined by plating appropriate dilutions of the lysate onto agar plates and incubating 48 h at 37°C. All experiments were repeated three times.

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Conflict of Interest

No conflict of interest is declared.

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