Title: Exopolysaccharide-producing lactic acid bacteria isolated from traditional Algerian dairy products and their application for skim-milk fermentations

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Abstract

Exopolysaccharide (EPS)-producing lactic acid bacteria are used in the dairy industry to obtain products with appropriate sensorial properties. Specific EPS act as stabilizers, thickeners, and/or fat-replacers, and are considered as natural biopolymers. The autochthonous microbiota of foods provides a rich reservoir of biodiversity to find biopolymers for novel applications. In this study, a collection of LAB isolated from homemade Algerian dairy products was screened for a rational selection of EPS-producing strains. Six isolates, identified as *Lactobacillus plantarum*, presented a stable mucoid/ropy phenotype after several sub-cultivations and they were used to ferment skimmed milk. Those fermented with strains LBIO1 and LBIO28 had higher viscosity, a smooth/ creamy texture and presented lower syneresis. The kinetics of growth and EPS-production, followed for 48 h, showed that both strains had similar behaviour, the molar mass (about 2×10^6 Da) and radius of gyration (about 80 nm) of the polymers being also similar. However, LBIO28 was able to increase the apparent viscosity of the fermented milk to a higher extent than LBIO1, which could be related with the earlier accumulation of EPS in the first strain in relation to the gelation point. Thus, these two ropy EPS-producing strains are suitable for different applications in dairy fermentations.

1. Introduction

From ancient times it has been known that fermentation is a natural process to preserve and maintain raw materials for longer periods, lactic acid bacteria (LAB) being one of the main microbial groups involved in this transformation. In fact, the long history shared between LAB and humans favoured the adaptation of these bacteria to food processing (Douillard & de Vos, 2014). In Algeria traditional fermented dairy products, following artisanal practices, have been consumed for a long time in relation to ethnic Berber traditions. Cheeses (“Klila”, “Bouhezza”, “Kamaria”, and “Jben”), fermented milks (“Lben”, “Raib” or “Rayeb”) and some fat-based products (“Smen” and butter) are mostly homemade in the rural areas of Algeria (Bendimerad, Kihal, & Berthier, 2012; Mechai, Debabza, & Kirane, 2014; Medjoudj, Aouar, Zidoune, & Hayaloglu, 2018).

LAB producing exopolysaccharides (EPS) are chosen for their capability to enhance the physical properties of dairy products. These surface carbohydrate polymers can be released into the environment (as “slime”), or be more tightly attached to the extracellular envelope forming a capsule (Castro-Bravo, Wells, Margolles, & Ruas-Madiedo, 2018). In dairy manufacturing the benefits of using EPS-producing LAB is well known; polymers synthesized *in situ* during milk fermentations act as effective stabilizers and/or thickeners, as well as fat-replacers improving the viscosity, texture and structure of the fermented products and reducing syneresis (Mende, Rohm, & Jaros, 2016). The increasing demand by consumers for natural ingredients allows the food industry to favor a reduced cost application of these biopolymers, the use of EPS-producing LAB cultures being the most affordable strategy and, a current practice in dairy food manufacturing (Duboc, & Mollet, 2001). Therefore, the screening for new EPS-producing strains is a very valuable tool to maintain biodiversity and to find new

biopolymers of interest, for both industry and research purposes (Zeidan et al., 2017). Fermented foods obtained from the spontaneous activity of the autochthonous microbiota, as well as autochthonous LAB present in raw vegetable or animal feedstock, (Motato et al., 2017; Tamang, Watanabe, & Holzapfel, 2016) still remain a natural and rich reservoir of biodiversity to search for “novel” strains with technological and functional properties. The aim of the present study was to screen a large number of LAB isolates from traditional Algerian dairy products to search for those able to produce EPS with the optimal technological aptitude to be applied in the dairy industry.

2. Material and Methods

2.1. Sampling and isolation of LAB

Samples of traditional Algerian dairy products (spontaneous fermentations under ambient temperature) and of raw milk from different animals and geographical locations (see supplementary Fig. S1) were collected over three years (2015-2017, Table 1). Products (10 g) were homogenised with 90 ml of buffered peptone water (Biokar Diagnostics, Beauvais, France), before serial dilutions were made that were surface plated on agar-MRS, agar-M17-lactose and agar-MSE (all media from Biokar). After incubation at 30°C for 48 h, several colonies of different morphology were selected and purified by successive streaking on an appropriate medium. Those Gram-positive, catalase-negative and non-spore forming bacteria were selected.

2.2. Screening of EPS-producing LAB

This collection of LAB was screened to search for EPS-producing phenotype using different methods previously described (Ruas-Madiedo & de los Reyes-Gavilán, 2005): i) detection of “ropy” phenotype (formation of an unbreakable strand when the colony is touched with an inoculation loop) and “mucoide non-ropy” phenotype

(glistening, smooth and slimy appearance without occurrence of filament); ii) detection of ropy/ mucoid colonies growing in media supplemented with 50 g/L of sucrose (for homopolysaccharide production); iii) ruthenium-red staining; iv) viscosity of liquid cultures using “exopolysaccharide selection medium” (ESM: 90 g skimmed milk, 3.5 g yeast extract, 3.5 g peptone and 10 g glucose per litre). Nineteen EPS-producing bacteria were obtained which were successively cultivated (at least 5 times) to select those that maintained a stable phenotype. Finally, 10 EPS-producing LAB with stable phenotype were sent to IPLA-CSIC (Spain) for further characterisation (supplementary Fig. S2). From those, 7 isolates were selected according to their different origin (animal and/or geographical location, supplementary Fig. S2) and stored at -80°C in MRS with 30% glycerol.

2.3. Identification, characterisation and selection of EPS-producing LAB

Identification of the 7 selected strains was approached by partially sequencing the gene coding for the 16S rRNA. Genomic DNA was extracted with the GenElute kit (Sigma-Aldrich) according to the manufacturer's recommendations, but adding an additional step of enzymatic lysis with lysozyme (45 mg/mL) (Merck, Darmstadt, Germany), proteinase K and mutanolysin (5U, Sigma-Aldrich, St. Louis, MO, USA). The PCR was performed using a pair of primers pA-Forward (5'AGA GTT TGA TCC TGG CTC AG 3') and pH-reverse (5'AAG GAG GTG ATC CAG CG CA3'). Amplicon's sequencing was made at Macrogen (Seoul, Republic of Korea) and the BLAST tool was used for comparison. The sequences of the 7 strains, all belonging to genus *Lactobacillus plantarum*, were held in the Genbank database (accession numbers collected in Table 2).

The 7 lactobacilli were used to ferment skimmed milk in order to select those with the best technological properties. Skimmed milk (Difco, Thermo-Fisher Scientific,

Spain) was reconstituted (11% w/v) and supplemented 1% (w/w) with pancreatic casein hydrolysate (Sigma-Aldrich) to obtain SMC (skimmed milk-casein). The SMC was pasteurised in a water bath at 90°C for 5 min, kept at room temperature overnight, and pasteurised again before cooling-down to the incubation temperature. Standardised cultures were made as follows: stocks (-80°C) were seeded on the surface of agar-MRS (grown at 30°C for 48 h), a single colony was picked to inoculate fresh MRS broth (cultivated overnight) and this culture was used to inoculate (1%) fresh MRS broth (grown for 24 h). These cultures were washed once with PBS and used to inoculate (2%) pasteurised SMC. Fermentations were carried out in triplicate in a thermostatic bath (Julabo TW20 GmbH, Germany) at 30°C for 24 h and the acidification curves were followed in real-time using the pHmeter ORION™ Versa Star™ (Thermo Scientific Inc., USA). The initial and final counts of the strains growing in SMC, as well as several final qualitative physical attributes (syneresis, ropiness, viscosity, coagulum firmness and texture) were determined (Table 2). Following this approach, 2 ropy strains (LBIO1 and LBIO28), selected as those able to confer good sensorial attributes to the fermented SMC, and 1 non-ropy strain (LBIO14), unable to confer them, were chosen for further experiments.

2.4. Milk fermentations with selected EPS-producing LAB

Standardised and washed cultures of strains LBIO1, LBIO14 and LBIO28 were used to inoculate (5%) SMC, carrying out fermentations (in triplicate) at 30°C for 24 or 48 h according to the type of experiment.

2.4.1. Kinetic of growth and EPS production

SMC (400 ml) was inoculated, divided into 8 tubes (50 ml each) and incubated for 48 h at 30°C in a water bath. Sampling points (0, 14, 16, 20, 24, 36 and 48 h) were taken to enumerate the bacteria using 5 g of fermented SMC [homogenised in 45 mL of

sterile 2% (w/v) sodium citrate before serial dilutions were made in Ringer ¼ solution]; the remaining sample was used to measure the pH (pHmeter Crison, Hach Lange S.L.U., Barcelona, Spain) before being frozen for the isolation of EPS. Additionally, after the 36-h fermentation one sample was frozen for quantification of organic acids.

Frozen samples collected at 0, 14, 20, 24 and 48 h were used for the isolation of EPS (Burns et al., 2011). In short, 40 g of fermented SMC was mixed with 8 ml of 60% TCA and vigorously stirred for 45 min at room temperature. After centrifugation (10,000xg, 4°C, 30 min), the pH of the supernatants was increased to 5.0±0.5, before being intensively dialysed against ultra-pure water (4°C, for 3 days, daily change of water) using cellulose membranes of 12-14 kDa MWCO (Sigma). Dialyzed samples were finally freeze-dried in a Freezemobile 12EL lyophilizer (VirTis, Gardiner NY, USA). The lyophilised powder was weighed to obtain the EPS-crude yield and its protein content determined using the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Thermo-Fisher, Rockford, IL, USA).

The EPS-crude powder was analysed by means of size exclusion chromatography coupled with multiangle laser light scattering (SEC-MALLS) to quantify the EPS amount, its molar mass and the radius of gyration. For that, 5 mg of lyophilized powder was dissolved overnight in 1 ml of 0.1 M NaNO₃ and centrifuged. Two columns TSK-Gel (G3000 PW_{XL} + G5000 PW_{XL}) placed in series and protected with a TSK-Gel guard column (Supelco-Sigma) were used for SEC-MALLS. Separation was carried out with 0.1 M NaNO₃ at 40°C, at flow rate of 0.45 ml/min, using an HPLC system composed of the Alliance 2690 module injector, the Photodiode Array (PDA) 996 detector, the refractive index (RI) 2414 detector and the Empower software (Waters, Milford, MA, USA), coupled with the MALLS detector Dawn Heleos II (Wyatt Europe GmbH, Dembach, Germany) and its software Astra 3.5. After

separation, peaks giving a signal in the RI detector but not in the PDA detector (set at 280 nm, which denotes the presence of protein) corresponded with the EPS. Then, the EPS amount was quantified from the signal of the RI detector using linear regression equations from different concentrations of dextran standards (Fluka-Sigma), whereas the weighted average molar mass (M_w) and the weighted average radius of gyration (R_w) were directly given by the MALLS detector (Burns et al., 2011).

2.4.2. Organic acid production

The concentration (g/100g) of lactic, formic, and acetic acids present in the fermented SMC was quantified by ion-exchange HPLC, as previously reported, using the ICsep ION-300 ion-exchange column (Transgenomic, San Jose, CA, USA) with 8.5 mM H_2SO_4 (as the mobile phase) at 65°C, with a flow rate of 0.4 mL/min (Salazar et al., 2009). The organic acids were detected with the PDA detector (set at 210 nm) and, for quantification, regression equations from different concentrations of the corresponding standards (Fluka-Sigma) were used.

2.4.3. Apparent viscosity of stirred fermented milks

The apparent viscosity of stirred fermented SMC was determined by means of the Posthumus funnel method (Salazar et al., 2009). For that, 500 ml of SMC were inoculated (separately) with the three strains and fermented at 30°C to reach a final pH of 4.6; then, the fermented SMC were immediately cooled down to 14°C and stored at 4°C for 24 h. Afterwards, the samples were stirred in a standardised way (20 times with a special spoon) to obtain the stirred-fermented SMC. A Posthumus funnel, placed in a cold chamber (at 4°C), with an opening size (diameter x length) of 8.0 x 6.0 mm was filled with approximately 450 ml of the stirred fermented SMC; the time (s) needed to pass the mark inside the funnel was the measure of the apparent viscosity of the sample.

2.5. Statistical analyses

A correspondence factorial analysis (Teil, 1975) of the qualitative data obtained for the six ropy EPS-producing strains was performed using the free software statistical computing R version 3.3. For that, the qualitative data of the fermented SMC attributes were categorized in numeric values (from 0 to 3, as indicated in Table 2) to select the best performing strains ($p < 0.05$). Quantitative data were analysed by means of one-way ANOVA to determine differences among strains (LBIO1, LBIO14 and LBIO28). The mean comparison test SNK (Student–Newman–Keuls) was applied to find which the differing ($p < 0.05$) strains were.

3. Results and discussion

3.1. Selection of EPS-producing strains with stable phenotype

In this work, we report the search for LAB with an EPS-production phenotype using traditional Algerian dairy products as the source of isolation. Among the 584 Gram-positive, non-spore forming, catalase negative isolates, nineteen had a ropy or a mucoid character, of which ten kept a stable phenotype after sub-cultivations (Table 1, supplementary Fig. S2). A final selection of six ropy/mucoid strains, together with one strain in which this character was absent, were identified as *L. plantarum* (Table 2). This versatile species can be found in many natural fermented foods, including dairy products, as well as different human niches (Hamet, Piermaria, & Abraham, 2015; Ribeiro, Stanton, Yang, Ross, & Silva, 2018). It could also be used as an adjunct culture for cheese manufacturing (Ayyash, Abu-Jdayil, Hamed, & Shaker, 2018; Wang, Fang, Wu, Min, & Yang, 2018), or for other vegetable-based fermented foods (Russo et al., 2016). Besides, strains belonging to this species might present health-promoting properties and they could be used for the formulation of functional dairy foods (Linares et al., 2017).

Further characterisation of the seven *L. plantarum* isolates from Algerian dairy products was focused at evaluating their technological aptitude to improve the viscosity, texture and structure of fermented skimmed milk (Table 2). In spite of the presence of an available nitrogen source (pancreatic hydrolysed casein) in the SMC, only 3 (LBIO1, LBIO10 and LBIO33) out of 7 strains were able to reach a final pH below or equal to 4.6 after 24 h at 30°C, those remaining having pH values of between 4.79 and 4.95 (Figure 1A). The acidification rate was slow for all strains, which is agreement with data previously reported, supporting the fact that *L. plantarum* is a nonstarter adjunct culture (Briggiler-Marcó et al., 2007). All strains were able to grow in milk, increasing by at least one logarithmic unit the number of cells after 24 hours of fermentation. The physical properties of the seven acidified SMC gels were different; gels obtained with strains LBIO1, LBIO2 and LBIO28 had high viscosity (ropiness), smooth/ creamy texture and all, except LBIO2, presented higher syneresis. The remaining strains, including the non-ropy/mucoid control (LBIO14), gave SMC gels with a granulated structure, low viscosity and high whey-release from the milk coagulum (Table 2). After the correspondence factorial analysis performed with this qualitative data, the strains showing the best performance (LBIO1 and LBIO28) clustered together ($p < 0.05$) and separately from the other strains (Figure 1B). These results suggest that these two strains synthesize EPS which are able to confer desirable physical attributes to the fermented milk (Hamet et al., 2015). The physical-chemical properties of the polymers, such as composition, structure, presence of charges, molar mass, as well their interaction with milk constituents are the key factors involved in this improvement (Han et al., 2016; Ruas-Madiedo, Hugenholtz, & Zoon, 2002a). Therefore, strains LBIO1 and LBIO28 were selected to study the EPS-production during SMC fermentations, using the strain LBIO14 as a negative control.

3.2. Characterization of milks fermented with selected EPS-producing strains

New batch fermentations were carried out with monocultures of the three strains increasing the inoculum rate by 5% following incubation for 48 h (supplementary Fig. S3 and Table S1). The final pH after 48 h decreased to below 4.0 and the number of bacteria reached the maximum value for all strains around 36 h, slightly declining afterwards. The ropy LBIO28 showed (from 20 to 48 h, $p < 0.05$) pH values lower than LBIO1 and LBIO14, whereas there were no statistical differences in the counts among the three strains ($p \geq 0.05$). Finally, the three strains produced lactic acid as a major organic acid during milk fermentation (Table 3), thus showing homolactic fermentation corresponding with this facultative heterofermentative lactobacilli (Kleerebezem et al., 2003).

Regarding EPS production, the EPS-crude (lyophilized powder) yield obtained for strain LBIO14 was always lower ($p < 0.05$) than that of LBIO1 and LBIO28 and remained constant during the fermentation period, suggesting that EPS was not synthesised in appreciable amounts by this strain in SMC (Figure 2, statistical analysis in supplementary Table S1). The weight of lyophilised powder increased with the fermentation time for the two ropy strains, and the highest production was obtained for LBIO28 at all sampling points. On the contrary, the amount of protein present in the lyophilised powder remained, in general, constant during the fermentations made with the three strains, suggesting that this protein comes from milk and it was co-precipitated with the EPS. Some authors have used the weight of the lyophilized powder as a rough indication of the EPS yield, but the presence of other non-EPS molecules don't make this parameter very accurate (Ruas-Madiedo & de los Reyes-Gavilán, 2005). Therefore, in order to detect and quantify the carbohydrate polymer in this lyophilized powder a

SEC-MALLS analysis was performed. The chromatogram profile of the unfermented (0 h) SMC and LBIO14-fermented milk (after 48 h) was very similar (Figure 3), which corresponded with the absence of yield-increase in the last sample (shown in Figure 2), supporting the lack of EPS production. On the contrary, the RI detector indicates the presence of a high molar mass peak (shadow bar) in the EPS-crude obtained from milks fermented with LBIO1 and LBIO28, which does not give a signal in the PDA detector (set at 280 nm for protein detection), allowing us to identify this peak as the EPS present in the lyophilised powder (Figure 3). The elution time of this peak was similar at the 4 sampling points (14, 20, 24 and 48 h), collected in three replicated fermentations carried out with each strain; this indicates that the molar mass of the EPS remained constant during fermentation. Then, the average molar mass (M_w) and the average radius of gyration (R_w), given by the MALLS detector, were calculated from data obtained in the twelve samples analysed for each strain. The EPS synthesised for strains LBIO1 and LBIO28 had similar molar masses (around 2×10^6 Da) and radius of gyration (around 80 nm) (Figure 3). These values correspond with high molar mass polymers reported in literature for EPS synthesised by lactobacilli (Burns et al., 2011; Miao et al., 2015) including also *L. plantarum* (Das & Goyal, 2014). However, polymers with a smaller molar mass have been described as well for this species (Gangoiti et al., 2017; Wang, Zhao, Tian, Yang, & Yang, 2015) and other species (Shao et al., 2015). So, we cannot discard the presence of a second fraction of smaller molar mass in the three strains under study (between 10^4 - 10^5 Da, corresponding with an elution time 34-38 min observed with dextran standards of these molar masses). However, the high signal obtained with the PDA detector at this elution range indicates the presence of protein in the same peak (Figure 3), thus not allowing us to obtain a conclusive result about the presence of a smaller molar mass EPS-fraction. In this

regard, there has also been reported the presence of more than one EPS-fraction differing in their molar masses in the polymer produced by a given lactobacilli strain (Hamet et al., 2015; Salazar et al., 2009; Tallon, Bressollier, & Urdaci, 2003).

Once the EPS peak was identified, the area of the peak obtained with the RI detector was used to quantify the amount [% relative abundance = 100 (μg of the EPS-peak/ μg the total peaks)] of EPS produced by LBIO1 and LBIO28 during the fermentation period (Salazar et al., 2009). These data were used to analyse the kinetic of EPS production showing that EPS synthesis was coupled with growth in both strains (Figure 4, supplementary Table S1) and both reached a similar amount of EPS (12.8 ± 2.00 and 11.8 ± 2.01 μg for LBIO1 and LIO28) after 48 h of fermentation. It has been reported that EPS synthesised by LAB are primary metabolites (Abid et al., 2018), but this characteristic is dependent on the strain and on the optimal or sub-optimal growth conditions (Vera Pingitore, Pessione, Fontana, Mazzoli, & Pessione, 2016), as well as on the composition of the medium, the growth conditions and the genetics of the producing strain (Mozzi et al., 2006). Regarding EPS synthesised by *L. plantarum*, variable yields have been reported, for example, *L. plantarum* WLPL04 isolated from human breast milk produced 426 mg/L EPS in MRS after 24 h (Jiang et al., 2016); *L. plantarum* KF5 isolated from Tibet kefir produced 95.68 mg/L under optimized conditions in a whey-based medium (Wang et al., 2010); or *L. plantarum* NTMI05 and NTMI20 isolated from milk synthesised 197 mg/L and 187 mg/L, respectively, in MRS (Imran et al., 2016). It is worth mentioning again that, behind the fermentation conditions, the method used for isolation and quantification of EPS also has a strong influence on polymer yield, which makes comparing the strains reported in literature difficult (Notararigo et al., 2013; Ruas-Madiedo, & de los Reyes-Gavilán 2005).

The accumulation of EPS in a dairy product is pivotal for the improvement of viscosity and texture, although other characteristics of the polymer also count (Ruas-Madiedo et al., 2002a). The moment of EPS production in relation to the gelation point (at 30°C the micelles of casein initiate the aggregation at about pH 5.2) is also relevant for the interaction of the EPS with the casein network and for the final structure of the dairy product (Ruas-Madiedo, & Zoon, 2003). The strain LBIO28 started the production of EPS earlier than LBIO1 (supplementary Table S1); at the gelation point (after 20 h fermentation, corresponding with middle-exponential phase) the amount of polymer accumulated in milks fermented with LBIO28 was 43.6%, slightly higher than the 32.3% produced by LBIO1 (Figure 4). This fact could affect the final rheological and structural properties of the fermented products. Indeed, the apparent viscosity determined by the Posthumus funnel method of milks fermented with both ropy strains was significantly different ($p<0.05$) and strongly differed from that obtained with the non-ropy LBIO14 strain (Figure 5A). Similarly, this last strain produced a lumpy, non-homogeneous fermented milk, whereas the milk fermented with the LBIO28 strain presented a smooth and homogeneous structure (Figure 5B). Differences in Posthumus viscosity among milks fermented with ropy and non-ropy EPS producing strains have been previously reported (Ruas-Madiedo, Tuinier, Kanning, & Zoon, 2002b). Additionally, differences in the apparent viscosity of EPS in aqueous solution, tested at the same concentration, but having different physical-chemical characteristics, were reported to have a strong influence on the viscosity and texture formation of the final fermented dairy product (De Vuyst et al., 2003).

In conclusion, a rational selection procedure was followed to identify novel LAB strains, obtained from natural sources, suitable for application in the manufacturing of

dairy products as adjunct cultures, due to their capability to synthesise EPS with appropriate technological aptitude. The strains *L. plantarum* LBIO1 and LBIO28 were able to produce polymers in skimmed milk, supplemented with an available nitrogen source, conferring optimal viscosity and texture to the fermented product. Although the amount, molar mass and radius of gyration of EPS produced by both strains was rather similar, the strain LBIO28 was able to significantly increase the apparent viscosity of the fermented milk to a greater extent than LBIO1. This could be related with the differences in the kinetic of EPS production and the amount of EPS accumulated around the gelation point detected between both strains. Therefore, these strains could have a range of different applications depending on the physical characteristics demanded for the final fermented product which, of course, must be always accepted by the end consumer after the corresponding sensorial evaluation.

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Figure Legends

Fig. 1. Acidification of SMC (11% skim milk supplemented with 1% pancreatic casein hydrolysate) by six “ropy” EPS-producing *Lactobacillus plantarum* strains and one non-ropy strain, selected according to their different (animal and geographical location, see Table 2) origin, incubated at 30°C for 24 h (A). Correspondence analysis (conceptually similar to principal component analysis) of the qualitative data from Table 2 showing three clusters of strains (B).

Fig. 2. Yield of lyophilised powder (or crude EPS, A) and amount of protein (B) obtained following the EPS- purification procedure in different sampling points during the SMC (11% skim milk supplemented with 1% pancreatic casein hydrolysate) fermentation (at 30°C for 48 h) by the “ropy” EPS-producing *Lactobacillus plantarum* LBIO1 (grey bar/symbol) and LBIO28 (white bar/symbol) and by the non-ropy strain LBIO14 (black bar/symbol). The statistical differences (ANOVA and SNK mean test) among the three strains are showed in supplementary Table S1.

Fig. 3. Representative chromatograms of SEC-MALLS (size exclusion chromatography- multiangle laser light scattering detection) analysis of the lyophilised powder obtained from SMC (11% skim milk supplemented with 1% pancreatic casein hydrolysate) after 48 h fermentation at 30°C by the “ropy” EPS-producing *Lactobacillus plantarum* LBIO1 and LBIO28 strains and by the non-ropy strain LBIO14; the chromatogram of the lyophilised powder obtained from the unfermented SMC is also showed. A peak of high molar mass (detector LS-90°C of the MALLS) giving also signal in the refraction index (RI) detector, but no in the photodiode array (PDA) detector in the ultraviolet way length (UV, 280 nm), was detected in samples from the

ropy strains LBIO1 and LBIO28 but was absent in the samples from strain LBIO14 or SMC; thus we consider that this peak corresponds with the EPS fraction. The table shows the means and standard deviation (n=12) of the elution time, molar mass and radius of gyration of the EPS fraction corresponding with 4 sampling points (14, 20, 24 and 48 h) of the three independent replicates of SMC fermentations carried out with the two “ropy” EPS-producing strains; the data were analysed together since no statistical variations for these parameters were detected along the fermentation time (see supplementary Table S1).

Fig. 4. Kinetics of EPS production (grey symbols), counts (black symbols) and pH evolution (white symbols) of two “ropy” EPS-producing *Lactobacillus plantarum* (LBIO1 and LBIO28) strains growing in SMC (11% skim milk supplemented with 1% pancreatic casein hydrolysate) at 30°C for 48 h. The EPS production was calculated using the data of SEC-MALLS analysis and the % of EPS (% of relative abundance showed in Table S1) corresponds with the amount (µg) of the EPS-peak respect the total peaks (µg) present in the sample (see Fig. 3). The statistical differences (ANOVA and SNK mean test) among the three strains are showed in supplementary Table S1.

Fig. 5. Apparent viscosity, determined by Posthumus method, of the stirred fermented SMC (11% skim milk supplemented with 1% pancreatic casein hydrolysate) at 30°C for 24 h by the “ropy” EPS-producing *Lactobacillus plantarum* LBIO1 and LBIO28 strains and by the non-ropy strain LBIO14 (A). Ropy filament (up, photography on the left) and smooth/glistening appearance (bottom, photography on the left) of the SMC fermented by LBIO28 and granular/lumpy appearance (bottom, photography on the right) of the SMC fermented by LBIO14 (B)

Table 1. Characteristics of traditional Algerian fermented dairy products used to isolate nineteen exopolysaccharide (EPS)-producing lactic acid bacteria (LAB), of which, ten maintained a stable phenotype after subsequent cultivations.

Product name	Dairy product type	Number of samples	Animal	Region	Origin	Ripening time	Number of putative EPS-producing LAB (strains with stable phenotype)
"Rayeb" ^a	Fermented milk	6	Bovine	Bordj El Ghadir	Farm	1 day	5 (LBIO10, LBIO28, LBIO33, LBIO41, LBIO43)
		6	Goat	Tebessa	Farm	1 day	
		2	Goat	Touggourt	Household	1 day	0
		1	Goat	Hassi Messaoud	Breeder	15 days	0
		1	Camel	Ain Mlila	Nomadic	10 days	2 (LBIO4)
"Lben"	Stirred fermented milk	8	Bovine	Constantine	Market	1 day	0
"Klila"	Cheese (mild ripened)	2	Bovine	Batna	Household	10 days	5
"Bouhezza"	Cheese (fresh)	3	Bovine	Batna, Khechela	Market	ND	3 (control strain LBIO14) ^d
		1 (spicy) ^b					
"Kamaria"	Cheese (ripened)	1	Goat	Touggourt	Household	> 60 days	4 (LBIO1, LBIO2, LBIO3, LBIO22)
"Zebda"	Fermented butter	1	Bovine	Constantine	Market	2 days	0
"Smen"	Clarified butter ^c	2	Ovine	El Kantra Biskra	Household	60 days	0

^a this product was made at laboratory (fermentation temperature 20-25°C) using milk obtained from different animal/ geographical locations

^b with hot paprika "kalb el serdouk"

^c with Artemisia herba-alba

^d non-EPS phenotype (control) strain

ND: no determined

9 **Table 2.** Preliminary characterisation of different *Lactobacillus plantarum* isolated from traditional Algerian fermented milks growing
10 (inoculated at 2%) in skim milk (reconstituted at 11%) supplemented with 1% of pancreatic casein hydrolysate (SMC) at 30°C for 24 h. The
11 correspondence factorial analysis of the qualitative data is represented in Figure 1.

Strains	Gene bank accession No.	Origin	Fermented SMC						
			Log CFU/ml	Δ Log CFU/ml	Syneresis ^a	Ropiness ^b	Viscosity ^c	Coagulum firmness ^d	Texture ^e
LBIO1	MG822866	“Bouhezza”	9.12±0.01	1.39	+/-	+	++	Semi-hard	Smooth
LBIO2	MG822861	“Bouhezza”	9.11±0.02	1.38	+	+	++	Semi-hard	Smooth
LBIO4	MG822863	“Rayeb” goat	9.10±0.03	1.12	+/-	-	-	Soft	Granulated
LBIO10	MG822867	“Rayeb” cow	8.96±0.17	1.24	+	-	+	Soft	Smooth
LBIO14	MG822868	“Klila”	8.82±0.03	0.95	+	-	-	Very-soft	Granulated
LBIO28	MG822865	“Rayeb” cow	9.16±0.014	1.34	+/-	+	++	Semi-hard	Smooth
LBIO33	MG822864	“Rayeb” cow	9.02±0.014	1.13	-	-	+	Soft	Granulated

12 ^a: + means presence of whey on top of the gel, +/- means few of whey on top of the gel, and – means absence
13 ^b: + means occurrence of the “ropy” strand, and – means absence
14 ^c: ++ means very high resistance (more time is needed) of the stirred fermented milk to flow, + means high resistance of the stirred fermented milk to flow, and – means low
15 resistance
16 ^d: semi-hard means that is necessary to shake the coagulum more vigorously to be broken than the soft one
17 ^e: smooth means creamy appearance and granulated has a lumpy appearance
18

Table 3. Production of organic acids (g/100g) of skim milk (supplemented with 1% pancreatic casein hydrolysate) fermented for 36 h with the EPS-producing *L. plantarum* LBIO1 and LBIO28 strains and the non-EPS producer LBIO14 strain. One-way ANOVA test was used to compare values among strains (** p< 0.01) and, when necessary, the SNK mean comparison test was performed, in this case those strains that does not share a common letter are significantly different (p<0.05),

	Mean $\pm$ SD (n=6)		
	Lactic acid	Formic acid	Acetic acid
LBIO1	280.1 $\pm$ 15.4 ^b	0.52 $\pm$ 0.14	14.2 $\pm$ 3.7
LBIO14	252.7 $\pm$ 14.9 ^a	0.43 $\pm$ 0.11	15.1 $\pm$ 2.0
LBIO28	277.3 $\pm$ 4.94 ^b	0.54 $\pm$ 0.10	15.6 $\pm$ 3.4
	**		

Fig. 1

(A)

(B)

Fig. 2

Fig 3

Mean $\pm$ SD (n=12)			
	Time (min)	Mw (10^6 Da)	Rw (nm)
EPS-LBIO1	26.5 $\pm$ 0.3	2.13 $\pm$ 0.9	76.3 $\pm$ 3.0
EPS-LBIO28	26.3 $\pm$ 0.2	2.04 $\pm$ 0.5	83.7 $\pm$ 2.9

Fig. 4

Figure S1. Geographical location of the sampling of the dairy products used in this study for the isolation of EPS-producing lactic acid bacteria.

Figure S2. Strategy followed for the selection of EPS-producing lactic acid bacteria (LAB) **(A)**. Ropy and mucoid phenotypes of EPS-producing LAB growing in the surface of agar-MRS **(B)**; ropy strand formed in ESM (exopolysaccharide selection medium) fermented with an EPS-producing LAB **(C)**; EPS-producing strain (white colony: the presence of the polymer prevents the red staining) growing in the surface of agar-MRS supplemented with 0.08 g/L red ruthenium **(D)**.

Figure S3. Comparison of pH evolution (white-dotted symbols) and counts (grey-continuous symbols) of the two EPS-producing strains LBIO 1 (diamond) and LBIO28 (square) and the control, non-EPS producing strain LBIP14 (circle).

Table S1. ANOVA and SNK-test performed to compare the data (from 3 replicates) obtained for the three strains under study in each fermentation point.

		Mean $\pm$ SD (n = 3)								
Time	Strains	Fermented milks		Crude EPS		SEC-MALLS analysis (EPS peak)				
		pH	Counts (Log CFU/ml)	Lyophilised powder (mg)	Protein content (mg)	Elution time (min)	Amount (μ g)	Relative abundance (%) ^a	Mw ($\times 10^6$ Da)	Rw (nm)
0 h	LBIO1	6.39 $\pm$ 0.02	8.38 $\pm$ 0.02 b	23.2 $\pm$ 5.1 b	4.7 $\pm$ 1.1					
	LBIO14	6.36 $\pm$ 0.02	8.23 $\pm$ 0.03 a	15.5 $\pm$ 0.6 a	3.3 $\pm$ 0.1	ND	ND	ND	ND	ND
	LBIO28	6.37 $\pm$ 0.02	8.28 $\pm$ 0.03 a	23.9 $\pm$ 3.3 b	4.3 $\pm$ 0.5					
		p=0.198	p=0.001	p=0.048	p=0.127					
14 h	LBIO1	5.66 $\pm$ 0.12 a	8.82 $\pm$ 0.11 a	19.7 $\pm$ 1.7 b	3.8 $\pm$ 0.39 a	26.1 $\pm$ 0.10	0.89 $\pm$ 0.25	0.60 $\pm$ 0.07	3.34 $\pm$ 0.95	78.7 $\pm$ 2.8
	LBIO14	5.85 $\pm$ 0.04 b	8.88 $\pm$ 0.01 ab	12.6 $\pm$ 1.4 a	2.6 $\pm$ 0.48 a	ND	ND	ND	ND	ND
	LBIO28	5.56 $\pm$ 0.08 a	8.99 $\pm$ 0.03 b	26.9 $\pm$ 3.8 c	5.2 $\pm$ 0.97 b	26.2 $\pm$ 0	1.38 $\pm$ 0.42	0.90 $\pm$ 0.23	2.77 $\pm$ 1.05	79.8 $\pm$ 3.2
		p=0.016	p=0.049	p=0.001	p=0.009	p=0.431	p=0.157	p=0.086	p=0.362	p=0.696
16 h	LBIO1	5.45 $\pm$ 1.10	9.03 $\pm$ 0.13							
	LBIO14	5.52 $\pm$ 0.07	8.89 $\pm$ 0.01	NP	NP	NP	NP	NP	NP	NP
	LBIO28	5.30 $\pm$ 0.09	9.00 $\pm$ 0.02							
		p=0.058	p=0.149							
20 h	LBIO1	5.12 $\pm$ 0.13 a	9.14 $\pm$ 0.13	20.1 $\pm$ 0.8 b	3.9 $\pm$ 0.2 ab	26.6 $\pm$ 0.43	4.14 $\pm$ 1.17	2.51 $\pm$ 0.89	2.17 $\pm$ 0.45	77.5 $\pm$ 5.74
	LBIO14	5.36 $\pm$ 0.04 b	9.05 $\pm$ 0.05	12.5 $\pm$ 5.7 a	2.6 $\pm$ 1.0 a	ND	ND	ND	ND	ND
	LBIO28	4.75 $\pm$ 0.10 a	9.15 $\pm$ 0.04	28.2 $\pm$ 2.1 c	4.9 $\pm$ 0.6 b	26.2 $\pm$ 0.03	5.15 $\pm$ 1.09	3.81 $\pm$ 1.15	1.93 $\pm$ 0.16	86.6 $\pm$ 4.84
		p=0.001	p=0.373	p=0.002	p=0.021	p=0.178	p=0.436	p=0.196	p=0.440	p=0.103
24 h	LBIO1	4.69 $\pm$ 0.21 b	9.29 $\pm$ 0.05 b	24.6 $\pm$ 1.2 b	4.4 $\pm$ 0.3 b	26.5 $\pm$ 0.19	8.51 $\pm$ 2.31	5.35 $\pm$ 1.11	1.47 $\pm$ 0.18	73.2 $\pm$ 2.03
	LBIO14	4.88 $\pm$ 0.02 b	9.12 $\pm$ 0.06 a	12.7 $\pm$ 2.4 a	2.6 $\pm$ 0.4 a	ND	ND	ND	ND	ND
	LBIO28	4.26 $\pm$ 0.03 a	9.21 $\pm$ 0.02 ab	29.5 $\pm$ 1.6 c	4.8 $\pm$ 0.4 b	26.3 $\pm$ 0.12	10.38 $\pm$ 2.30	7.30 $\pm$ 1.44	1.80 $\pm$ 0.16	84.7 $\pm$ 5.34
		p=0.011	p=0.031	p=0.000	p=0.001	p=0.132	p=0.377	p=0.135	p=0.1078	p=0.025
36 h	LBIO1	3.90 $\pm$ 0.05 b	9.51 $\pm$ 0.11							
	LBIO14	3.94 $\pm$ 0.03 b	9.32 $\pm$ 0.01	NP	NP	NP	NP	NP	NP	NP
	LBIO28	3.76 $\pm$ 0.02 a	9.52 $\pm$ 0.10							
		p=0.001	p=0.051							
48 h	LBIO1	3.79 $\pm$ 0.02 b	9.23 $\pm$ 0.09	25.8 $\pm$ 2.9 b	4.3 $\pm$ 0.1 b	26.8 $\pm$ 0.21	12.8 $\pm$ 2.00	8.30 $\pm$ 0.23	1.41 $\pm$ 0.17	74.6 $\pm$ 2.64
	LBIO14	3.80 $\pm$ 0.01 b	9.20 $\pm$ 0.02	12.3 $\pm$ 3.2 a	2.5 $\pm$ 0.5 a	ND	ND	ND	ND	ND
	LBIO28	3.69 $\pm$ 0.01 a	9.22 $\pm$ 0.08	34.2 $\pm$ 0.2 c	6.4 $\pm$ 0.2 c	26.5 $\pm$ 0.19	11.8 $\pm$ 2.01	8.04 $\pm$ 2.26	1.66 $\pm$ 0.41	83.8 $\pm$ 5.46
		p=0.000	p=0.860	p=0.000	p=0.000	p=0.231	p=0.553	p=0.851	p=0.067	p=0.059

ND: no detected; NP: no performed

^a: % relative abundance = 100 * (μ g EPS-peak/ μ g of total peaks)