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'Ropy' phenotype, exopolysaccharides and metabolism: Study on food isolated potential probiotics LAB

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ABSTRACT

Lactic acid bacteria are fully recognized for their industrial applications among which the production and release of exopolysaccharides. In the present investigation, we screened fifteen *Lactobacilli* in order to find ropy strains, quantify exopolysaccharides and detect proteins specifically associated with the ropy-exopolysaccharide production. The highest ropy-exopolysaccharide producer (*L. helveticus* 6E8), was grown in stimulating and basal condition (10% and 2% lactose) and subjected to comparative proteomic analysis. The levels of 4 proteins were found significantly increased in the membrane fraction under stimulating conditions: a specific exopolysaccharide biosynthetic protein, a stress-induced protein, a protein involved in secretion and an ATP-synthase subunit. Conversely, several enzymes involved in anabolism and protein synthesis were decreased. These results suggest a general shift from growth to exopolysaccharide-mediated protection from the hyperosmotic environment. Due to the great interest in exopolysaccharides with novel features, the identification of these proteins could have implications for future improvements of industrial strains.

1. Introduction

The ability to produce extracellular polysaccharides is widespread throughout the microbial world, from some algae, fungi and yeasts to prokaryotes (Hidalgo-Cantabrana et al., 2012). Bacterial extracellular polysaccharides form a layer surrounding cells and confer protection due to their excellent water-binding properties. They provide a barrier from desiccation, extreme temperatures, osmotic pressure and salinity (Berecka et al., 2013). Other physiological roles of exopolysaccharides (EPS) include biofilm formation, chelation of essential elements, cellular recognition, resistance to the harsh conditions of stomach and bile acid exposure and inhibition of phagocytosis by predators (Broadbent et al., 2003). Although EPS production may be a high-energy-requiring endergonic pathway, its benefits are significantly higher than its costs, considering the improved growth and survival of microorganisms conferred by EPS under stress conditions (Berecka et al., 2013).

Lactic acid bacteria (LAB) are able to produce extracellular polysaccharides tightly associated to the cell surface by covalent bonds, forming a thick shell (capsule) named capsular polysaccharides (CPS). On the other hand, LAB can also produce polysaccharides loosely bound to the cell by non-covalent interactions or released into the surrounding environment. The latter are most frequently referred to as EPS (Hidalgo-Cantabrana et al., 2012). However, the distinction between EPS and CPS appears somewhat speculative because the abundance and localization of polysaccharides is strongly dependent on the growth conditions (Kleerebezem et al., 2010).

Depending on their chemical composition, EPS from LAB are classified into two main groups: homoexopolysaccharides (Homo-EPS) and heteroexopolysaccharides (Hetero-EPS). Homo-EPS secreted by *Lactobacillus* sp. are composed of a single type of monosaccharide unit, usually D-glucose or D-fructose, named glucans and fructans, respectively. Hetero-EPS consist of multiple copies of an oligosaccharide, usually composed of glucose, galactose, xylose, mannose, arabinose, rhamnose and occasionally of amino-sugars (e.g. N-acetylgalactosamine), polyols and glucuronic acid (Pessione, 2012). Hetero-EPS have greater variability in structure, are often highly branched and are generally produced in lower concentrations than Homo-EPS (Badel et al., 2011).

Different EPS produced by LAB exhibit diverse physical characteristics resulting from their unique chemical composition, structure, radius of gyration etc. The nomenclature used to describe the different phenotypes is confusing, and terms such as, “mucoid”, “slimy” and

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"ropy" have been indistinctly used. However, not all mucoid or slime-producing strains are ropy. The mucoid colonies have a glistening and slimy appearance on agar plates, but are not able to produce strands when extended with an inoculation loop, whereas ropy colonies form a long filament by this method (Ruas-Madiedo and de los Reyes-Gavilán, 2005). From a structural point of view, HePS for example, may be ropy or mucoid (Zannini et al., 2016). When utilized in fermented milk products, ropy strains confer a smoother consistency and higher viscosity than non-ropy strains (Broadbent et al., 2003).

EPS-synthesizing LAB are appreciated in the food industry (yogurt, drinking yogurt, cheese, fermented cream and milk-based desserts) since EPS synthesis *in situ* contributes to viscosity, texture, stability (prevention of syneresis in yogurt) mouth-feel, and taste perception. Furthermore, ropy-EPS producing LAB can confer improved creaminess to novel foods such as chemically modified starches, low-fat dairy and gluten-free products, as well as some health promoting properties (Caggianiello et al., 2016; Papagianni, 2012; Ruffing and Chen, 2006; Van Kranenburg et al., 1999). Taking advantage of EPS produced naturally by LAB thus addresses an increasing demand for products with low fat, low sugar, low cost and with as few food additives as possible (Duboc and Mollet, 2001).

Furthermore, it has been suggested that most health-promoting benefits related to the gut microbiota are actually due to the biological activities of EPS produced by probiotic species, such as LAB (Ruas-Madiedo et al., 2002). Adhesion to the intestinal mucosa is one of the main criteria for the selection of probiotic strains (Ruas-Madiedo et al., 2006) and *in vitro* studies suggest that EPS could increase adhesion of LAB to GI tract (Patel and Prajapat, 2013). EPS can also interact with the immune system as demonstrated by Lee and co-workers (Lee et al., 2016) who reported that immunomodulatory ability is different among EPS positive and EPS negative strains belonging to the same species. Moreover, EPS originating from different lactobacilli have shown specific bifidogenic activity (Patel and Prajapat, 2013; Ryan et al., 2015), thus acting as prebiotics. There is also rapidly growing evidence of important probiotic characteristics of EPS produced by LAB, such as anti-microbial and antiviral activities against pathogens (de Jesus Raposo et al., 2014) and antioxidant effects (Leung et al., 2009). Additionally, it was shown that purified EPS from *L. rhamnosus* decreased pro-inflammatory cytokines (TNF-, IL-6, IL-12) (Kšonžeková et al., 2016), whereas EPS from other LAB species exhibited immune-stimulation properties such as macrophage activation (Surayot et al., 2014). Finally, dietary intervention with EPS-producing LAB has been associated with significant reductions in serum cholesterol and triglycerides (London et al., 2014). Specifically, 'ropy' EPS resulted in lowest serum cholesterol and highest HDL/total cholesterol ratio compared to 'non-ropy' EPS in a rat model (Nakajima et al., 1992). The above studies and several others suggest a possible cardio-protective role, although the mechanism is still unclear (Ryan et al., 2015). Therefore, there is great interest in identifying and developing new LAB strains that produce EPS with novel properties and in increased quantities.

Natural production of EPS by LAB is relatively low (50–400 mg/L) compared to other EPS, like xanthan (25 g/L), produced by non-dairy bacteria. Nevertheless, some LAB-produced EPS are very effective biothickeners (even in low amounts) when produced *in situ* (Boels et al., 2003). The yield and quality of microbial EPS are greatly affected by the microbial growth phases: some EPS are synthesized throughout bacterial growth, whereas others are only produced during late logarithmic or early stationary phase (Sutherland, 2001). Furthermore, an increase in polysaccharide production is possible by manipulating the culture conditions. For example, a high C:N ratio usually favors EPS production (Sutherland, 2001) as well as low temperature (Vera Pingitore et al., 2016) Aeration rate, culture pH, osmolarity and detergents, have all been found to influence EPS production (Kumar et al., 2007).

As far as we know, previous studies did not explore the effect of different carbon sources on the ropy phenotype and quantity of EPS in *Lactobacillus* strains in correlation with comparative proteomic profile analysis. The objective of this work was: i) to find a quick and reliable test for ropy phenotype detection and quantification, ii) to identify *Lactobacillus* strains that produce the highest yield of ropy EPS, iii) to find optimal carbon substrate supporting high ropy-EPS production in view of potential industrial applications, iv) to examine the question whether ropy phenotype is associated with increased EPS production, and v) to investigate which metabolic pathways are activated during EPS synthesis by identifying the proteins that are increased in abundance under conditions stimulating ropy-EPS synthesis.

2. Materials and methods

2.1. Bacterial strains

In this study, 15 LAB strains, isolated from food matrices, were considered. The collection was composed of 6 *Lactobacillus helveticus*, 5 *Lactobacillus rhamnosus* and 4 *Lactobacillus fermentum* strains. All the strains (collected at the beginning of the stationary phase) were stored in MRS broth at −20 °C in 0.5 mL aliquots with 0.5 mL of 40% (v/v) glycerol.

2.2. Culture conditions

All the cultures were grown in semi-defined medium (SDM) according to Kimmel and Roberts (Kimmel and Roberts, 1998), supplemented with 10% (w/v) of one of the following sugars: lactose, sucrose, glucose or fructose. For all the cultures, 50 ml screw cap tubes were incubated for 72 h (unless otherwise stated), at 30 °C without shaking to guarantee a microaerophilic environment.

2.3. EPS production

2.3.1. Determination of ropy phenotype

Cultures were grown in SDM supplemented with 10% (w/v) of either lactose, sucrose, glucose or fructose. After growth, biomass was separated by centrifugation (4000 x *g*, 20 min, 4 °C) and supernatants were collected. The ropy phenotype was assessed by insertion of an inoculation loop into the cell pellet and visual inspection of filament formation when the loop was lifted. This method is a modification of that previously described by Ruas-Madiedo and de los Reyes-Gavilan (Ruas-Madiedo and de los Reyes-Gavilán, 2005).

2.3.2. Measurement of supernatant turbidity

Two RP + (positive ropy phenotype) (*L. helveticus* 6E8 and 1F4) and two RP- (negative ropy phenotype) (*L. helveticus* 2D5 and 5D10) strains were grown in SDM supplemented with either 2% or 10% (w/v) lactose, in order to correlate the supernatant turbidity to the presence of RP phenotype. Among all sugars, lactose was chosen since it was the best carbon source for all the tested strains. After growth, biomass was separated by centrifugation (4000 x *g*, 20 min, 4 °C) and supernatants were collected. Supernatant turbidities were assessed by measuring the optical density at 600 nm (OD600) (Spectrophotometer Ultrospec 2000, Pharmacia Biotech).

2.3.3. Extraction of EPS fraction

For EPS quantification four RP + strains (*L. helveticus* 6E8, 1F4, 1F8 and *L. rhamnosus* 21E1) and one RP- strain (*L. helveticus* 2D5), as control, were grown in SDM supplemented with 10% (w/v) of either lactose, sucrose, glucose or fructose. Cell-free culture supernatant was obtained by centrifugation (4000 x *g*, 20 min, 4 °C). Two volumes of 95% (v/v) cold ethanol (4 °C) were added to the supernatant. The tubes were stored for 24 h at 4 °C. The precipitated polysaccharides were collected by centrifugation (4000 x *g*, 20 min, 4 °C) and the pellets were dissolved in warm ddH_2O. The precipitation step was repeated once and, finally, EPS precipitates were dissolved in warm ddH_2O and stored

at −20 °C for further analysis.

2.3.4. Quantification of EPS

The EPS content was determined by the phenol-sulfuric-acid method (de Jesus Raposo et al., 2014) using glucose as the standard. ddH_2O water was added to 20 µl of EPS solution, to a final volume of 2 mL. To each 2 mL sample, 50 µl of 80% (w/v) phenol and 5 mL of sulfuric acid (H_2SO_4) were added quickly. The sample was incubated at room temperature for 45 min and the optical density at 490 nm was measured.

2.4. Proteomic experiments

L. helveticus 6E8 was chosen for this analysis since it produced the highest EPS amounts in lactose medium. Bacteria were grown in SDM with either 2% or 10% (w/v) lactose, until exponential phase.

2.4.1. Preparation of soluble protein extracts

Cells were harvested by centrifugation (4000 x *g*, 20 min, 4 °C) in a Thermo Scientific SL16 (TX-400 rotor) multispeed centrifuge and washed twice in 45 mL of 0.85% (w/v) NaCl. The biomass was re-suspended in 3 mL of 50 mM Tris-HCl pH 7.3, 1 mM EDTA and sonicated in an Ultrasonic Liquid Processor XL2020 Microsonix Sonicator for a total of 30 min at 20 KHz with intervals of 20 s. while keeping the cells on ice. To remove unbroken cells, the sonicated solution was centrifuged (4000 x *g*, 20 min, 4 °C) and the supernatant was collected. The supernatant was centrifuged (100,000 x *g*, 1 h, 4 °C) in a Beckman L8-60 M (Type 60 rotor) centrifuge and the supernatant, corresponding to the soluble cytosolic fraction, was collected, while the pellet was kept as the membrane fraction. Urea (8 M final concentration) was added, and the sample was vortexed until urea was dissolved. DTT (10 mM final concentration) was added and the sample was heated in a water bath (55 °C for 15 min), vortexing twice throughout. Finally, samples were frozen and stored at −80 °C until further analysis.

2.4.2. Preparation of membrane-enriched protein extract

After cell sonication and clarification, the 100,000 x *g* pellet containing the membrane fraction was re-suspended in 100 µl of 50 mM Tris-HCl, pH 7.3, 1 mM EDTA. 10% (w/v) SDS (1% final concentration), DTT (10 mM final concentration) and Urea (8 M final concentration) were added and the sample was heated in a water bath (55 °C for 15 min), vortexing twice throughout.

2.4.3. Protein quantification

Protein concentration in both cytosol and membrane fractions was determined by the Bradford assay, prior to addition of denaturants (Pierce, Rockford, IL, USA), using BSA (Sigma) as the standard.

2.4.4. Processing of cytosolic and membrane fractions for mass spectrometry analysis

2.4.4.1. Cytosolic sample. 200 µg of proteins dissolved in 8 M urea, 10 mM DTT, 25 mM Tris-HCl pH 8.0 were loaded on 10 kDa cut-off spin filters (Amicon®Ultra-0.5 mL, Millipore), centrifuged at 11,000 x *g* for 10 min (final volume 50 µl) and the flow-through was discarded. The sample was washed once with 400 µl of 8 M urea, 10 mM DTT, 25 mM Tris-HCl pH 8.0 and concentrated to 50 µl.

2.4.4.2. Membrane samples. 50 µg proteins dissolved in 1% (w/v) SDS, 10 mM DTT, 25 mM Tris-HCl pH 8.0 were diluted to 450 µl with 8 M urea, 10 mM DTT, 25 mM Tris-HCl pH 8.0, then loaded on 30 kDa cut-off spin filters (Amicon®Ultra-0.5 mL, Millipore), centrifuged at 11,000 x *g* for 10 min (final volume 50 µl) and the flow-through was discarded. The sample was washed 4 times with 400 µl of 8 M urea, 10 mM DTT, 25 mM Tris-HCl pH 8.0 and concentrated to 50 µl, in order to remove SDS.

Both the cytosolic and membrane samples were alkylated with iodoacetamide and then digested with trypsin (1:10, trypsin: protein ratio) (Promega Corp., Madison, WI, USA) overnight at 37 °C inside the spin-filters, as previously described (Wiśniewski et al., 2008). The peptides were recovered in the flow-through fraction after centrifugation of the spin-filters. The filtered peptides were desalted by loading 25 µg of peptides on C18 Stage tips (Rappsilber et al., 2007). Two µg of the eluted peptides were analyzed by mass spectrometry (MS).

2.4.5. LC MS/MS analysis

MS analyses were performed using a Q Exactive mass spectrometer (Thermo-Fisher Scientific) coupled on-line to a nanoflow UHPLC instrument (Ultimate 3000 Dionex, Thermo-Fisher Scientific). Eluted peptides were separated over a 100 min gradient run at a flow rate of 0.3 µl/min on a reverse phase 50-cm-long C18 column (75 µm ID, 2 µm, 100 Å, PepMap®RSLC, Thermo-Fisher Scientific). The survey scans (300–2000 *m/z*, target value 3E6 charges, maximum ion injection times 120 ms) were acquired and followed by higher energy collisional dissociation (HCD) based fragmentation (normalized collision energy 28). A resolution of 70,000 was used for survey scans and up to 12 dynamically chosen most abundant precursor ions were fragmented (isolation window 2.0 *m/z*). The MS/MS scans were acquired at a resolution of 17,500 (target value 1E5 charges, maximum ion injection times 90 ms).

2.4.6. MS data analysis

Mass spectra data were processed using the MaxQuant computational platform, version 1.5.3.12 (Cox and Mann, 2008) Peak lists were searched against the *Lactobacillus helveticus* Uniprot FASTA sequence database containing a total of 24,377 entries, including both reviewed and unreviewed sequences. The search included cysteine carbamidomethylation as a fixed modification and oxidation of methionine as variable modifications. Peptides with minimum of seven amino-acid length were considered and the required FDR was set to 1% at the peptide and protein level. Protein identification required at least 3 unique or razor (*i.e.* that can be assigned to more than one protein) peptides per protein group. Protein quantification in MaxQuant was performed using the label free quantification (LFQ) algorithm (Cox et al., 2014). LFQ in MaxQuant uses only common peptides for pairwise ratio determination for each protein and calculates a median ratio to protect against outliers. It then determines all pair-wise protein ratios and requires a minimal number of two peptide ratios for a given protein ratio to be considered valid. Statistical analysis of the data was performed using the Perseus software package within MaxQuant, as follows. The relative abundance of proteins (LFQ) in the membrane and cytosol fractions was compared for cells grown in 10% lactose vs. 2% lactose, prepared in two separate experiments. Only those proteins were included in the comparison that gave sufficient MS signal intensities for reliable LFQ in all 4 samples (2 biological duplicates for each of the 2 conditions) and where the Student's *t*-test p-values in comparisons of LFQ intensities under the 2 conditions (10% vs 2% lactose) were < 0.1. These selection criteria resulted in the exclusion of proteins for which only partial LFQ intensity data were available - i.e. if the signal intensity for a protein was insufficient in one of the duplicates, the protein was excluded. However, this ensured that only high-confidence hits were included in the final list of proteins that underwent a quantitative change under the two conditions.

3. Results and Discussion

3.1. Selection of Lactobacillus strains showing ropy phenotype: effect of different sugars

The first aim of this study was to identify *Lactobacillus* strains with the RP + phenotype and to find appropriate conditions that lead to ropy phenotype induction.

Screening for ropy phenotype was performed on food-isolated

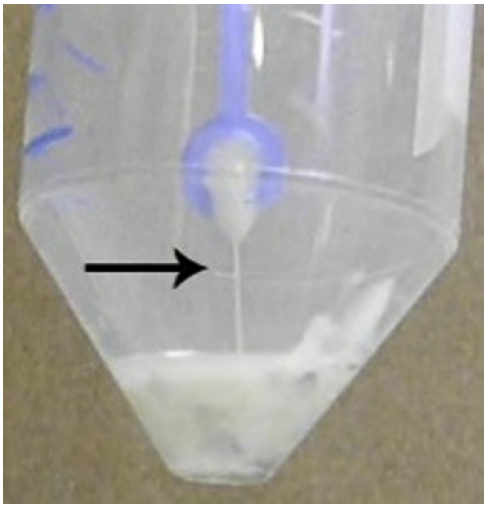

Fig. 1. Detection of ropy Lactobacillus strains in the pellet of a liquid culture containing 10% sugars (lactose, sucrose, glucose or fructose): RP + strains formed a 5 mm filament while RP- strains failed to form any filament at all. The arrow indicates a filament formed by a RP + strain.

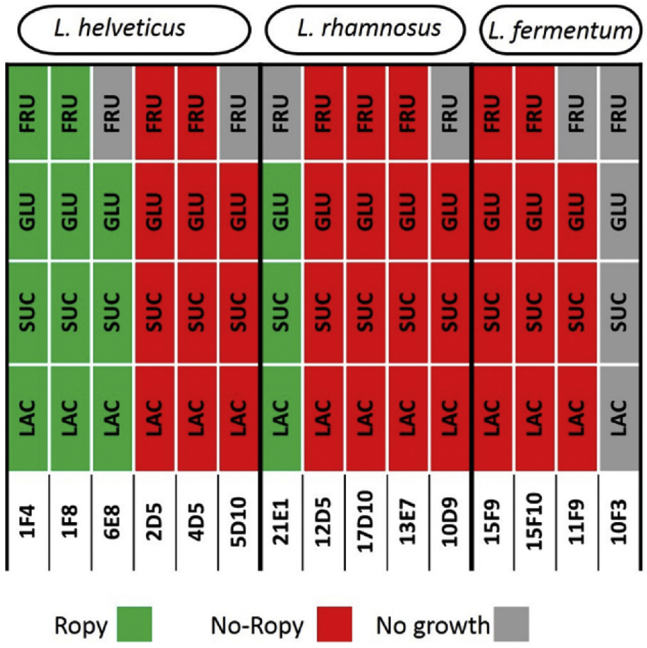

Fig. 2. Expression of ropy phenotype in three different Lactobacillus species, (*Lactobacillus helveticus, L. rhamnosus, L. fermentum)* grown in four different energy sources. The shown results were obtained consistently in three different experiments, performed in duplicates.
RP + strains are marked in . These strains produced a filament of ≥5 mm.
RP- strains are marked in , meaning no filament was observed.
Grey blocks indicate no bacterial growth.

(mainly cheese) Lactobacilli belonging to our culture collection. Four different media containing glucose, fructose, sucrose or lactose as the main carbon source were investigated. Media supplemented with 10% sugars were used on the basis of other studies that found higher EPS production in 10% compared to 5% sugars (Van Geel-Schutten et al., 1998).

The standard screening procedure for ropy phenotype detection is the "modified loop method", which involves gently picking-up colonies grown on agar media with an inoculation loop and observing the presence or absence of a filament (Yamamoto et al., 1995). However, by using this approach, it is difficult to visually detect filaments when biomass is scarce (i.e. small colonies with few cells). We encountered these issues in initial trials and therefore decided to grow cells from individual colonies in liquid medium (containing the same high sugar concentrations previously reported) and then use the inoculation loop method to test cell pellets after centrifugation of the culture. As shown in Fig. 1, when utilizing this method, some strains form at least 5 mm long filaments. These were classified as RP+, while other strains do not form any filament and were classified as RP-. Moreover, identifying RP + strains grown in high-sugar liquid media is much easier, also because growth in suspension can stimulate EPS synthesis (Van Geel-Schutten et al., 1998). This method has also the advantage of being more rapid than other procedures, such as measuring the viscosity and high-resistance-to-flow, and/or colony staining with ruthenium red (Ruas-Madiedo and de los Reyes-Gavilán, 2005). Therefore, this procedure proved to be suitable for optimal reliability and sensitivity of the detection.

We applied the above method to 15 Lactobacillus strains from three different species: *L. helveticus, L. rhamnosus and L. fermentum*. The phenotype screen resulted in the identification of four RP + strains (*L. helveticus* 1F4, *L. helveticus* 6E8, *L. helveticus* 1F8, *L. rhamnosus* 21E1) and ten RP- strains (Fig. 2). Both *L. helveticus* and *L. rhamnosus* are generally good (hetero)-EPS producers, as reported in the literature (Macedo et al., 2002; Staaf et al., 1996; Van Calsteren et al., 2002; Yamamoto et al., 1995). None of the *L. fermentum* strains was ropy, and one of them failed to grow in all tested conditions, possibly because of its sensitivity to osmotic stress induced by the high sugar content of the culture media.

Five of the tested strains (without correlation to RP + or RP-) failed to grow in fructose containing media, which could indicate that fructose alone is not an appropriate energy source for about one third of these cheese-isolated strains. This observation is not surprising in view of the fact that cellular uptake of fructose requires the presence of a specific transporter (apart from enzymes for downstream fructose metabolism) (Wu et al., 2014), a feature that is probably selected during a long evolution on vegetables.

3.2. Effect of medium composition on induction of the ropy phenotype

It was noticeable that if a strain showed RP + in one sugar substrate, it showed RP + in all other sugar substrates tested, indicating that the specific chemical composition of the main carbon/energy source is not a critical determinant for induction of ropy phenotype in these strains. In contrast, Zivkovic et al. (Ryan et al., 2015) reported that the ropyness of *Lactobacillus paraplantarum* BGCG11 (a strain producing high molecular weight EPS) is negatively affected when fructose is the only sugar source in the growth medium. One explanation for this is that in the presence of fructose alone, there is no available glucose-1-phosphate for the beginning of dTDP-L-rhamnose biosynthesis (Zivkovic et al., 2015). Similarly, it has been noted that using fructose as the sole sugar source negatively affects production of high molecular weight EPS without altering production of low molecular weight EPS both in *L. delbrueckii* subsp. *bulgaricus* NCFM 2772 (Grobben et al., 1997) and in *L. pentosus* LPS26 (Sánchez et al., 2006). These data, together with the absence of effect of fructose on RP+/RP-, suggest that the strains described in this study may produce low-molecular weight EPS.

Three possible reasons can account for increased EPS biosynthesis in high-sugar media: i) osmotic stress; ii) unlimited supply of sugar building blocks; iii) high energy availability. It is well known that EPS are produced as a defense against environmental stressors (Berecka et al., 2013). Furthermore, mono- and di-saccharides are a convenient source of building blocks for EPS biosynthesis and energy generation (Sutherland, 2001). This factor is important, as Hetero-EPS biosynthesis is a highly energy-demanding process, where at least two glucose molecules are catabolized through glycolysis to provide enough energy for

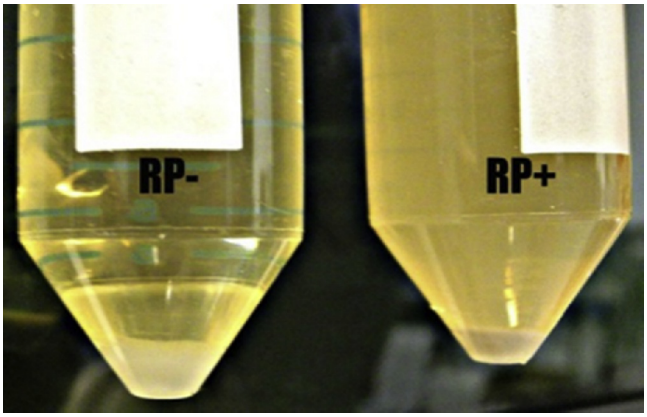

Fig. 3. Cell-free supernatant turbidity as a potential indicator of RP + EPS production (on the left: clear supernatant typical of RP- strain; on the right: opaque supernatant typical RP + strain).

the incorporation of one glucose molecule into Hetero-EPS (Welman and Maddox, 2003). This consideration may also explain the superior ability of lactose to induce the ropy phenotype observed in this study.

3.3. Use of supernatant turbidity as a measure of ropy EPS

The loop filament test for assessing the presence of ropy EPS has the advantage of simplicity; however, it is a subjective test based on visual observation and is not quantitative. Therefore, it was of interest to find an alternative method that would overcome these drawbacks. In initial experiments, we observed that the cell-free supernatant turbidity varied according to RP + and RP- strains. In particular, RP + strains were turbid in all sugars tested, while RP- strains had clear supernatants (Fig. 3).

To explore this further, we analyzed supernatants from three RP + strains (6E8, 1F4, 1F8) and one RP- (2D5) belonging to the same species (*L. helveticus)*, in 2% and 10% lactose After a suitable incubation period, optical density (OD_{600}) of the culture supernatants was compared and normalized to the cell density of the culture. As shown in Table 1, the turbidity of the RP- strains was significantly lower compared to the RP + strains, both in 10% and 2% lactose. Also, the turbidity of the EPS from RP + strains increased in 10% compared to 2% lactose, whereas no differences could be observed in RP- strains (Table 1). Similar observations were made by Miao et al., in *Lactobacillus reuteri* (Miao et al., 2015), but, to our knowledge, the phenomenon has not been further investigated.

This result also correlated with the observation that solutions of EPS isolated from RP + strains were much more turbid than EPS from RP- strains (Fig. 4b). Interestingly, some turbidity was detected even for RP + strains in non-stimulating conditions (2% lactose). Conceivably. these strains produce ropy-type EPS even in 2% lactose, however, if a threshold EPS level is required for the ropy phenotype, these levels may be insufficient to generate ropy colonies (Table 1) (Kumar et al., 2007; Van Geel-Schutten et al., 1998).

Table 1
Supernatant turbidity (OD600) in selected RP- (2D5) vs. RP + strains (6E8, 1F4 and 1F8), after growth on 2% and 10% lactose. Results shown are average ODs of the supernatant normalized to OD of the cell resuspension.

		OD600 (supernatant/culture)	
L. helveticus strains	Ropy phenotype	2% lactose	10% lactose
2D5	–	0.055 ± 0.005	0.044 ± 0.002
6E8	+	0.198 ± 0.029	0.869 ± 0.07
1F4	+	0.226 ± 0.015	0.851 ± 0.032
1F8	+	0.194 ± 0.024	0.728 ± 0.042

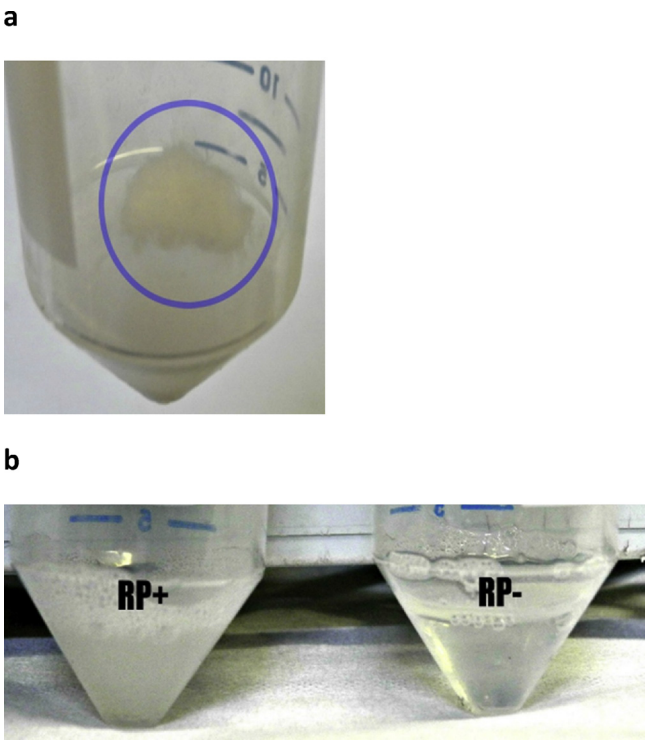

Fig. 4. a. Pellet formed after ethanol precipitation and containing EPS and possibly other hydrophilic, polymeric substances such as water-soluble proteins and proteoglycans. Low-molecular compounds such as mono- and di-saccharides and amino acids are ethanol-soluble and remain in the supernatant. **b**. Differences in opacity of ethanol precipitates dissolved in ddH2O of cell supernatants from a RP + strain (on the left) and from a RP- strain (on the right).

Additional observations of differences in properties of RP + and RP- strains (data not shown) include: 1) cell pellets of RP + strains were always smaller than those of RP- strains, 2) cell pellets of RP + strains were without exception much 'looser' in consistency and not as compact as those from RP- strains. One explanation for this feature may be that the RP- strains possess a polysaccharide capsule covalently linked to the cell surface, conferring higher volume and hardness to the cell pellet, whereas the RP + strains may produce loosely bound EPS that render the external medium opaque, but leave the pellet soft and smaller. According with Patil et al. (Patil et al., 2015) secreted EPS, but not capsular polysaccharides, are associated with the ropy phenotype. The latter give a glossy aspect to the colonies without producing any filaments.

3.4. Relationship between EPS levels and ropy phenotype

To examine the question whether ropy phenotype is associated with increased EPS production, we compared the amounts of EPS produced by RP- and RP + strains. Different methods for extracting (Ruas-Madiedo and de los Reyes-Gavilán, 2005), partially purifying and accurately quantifying (Rimada and Abraham, 2003) secreted EPS are reported in the literature. In general, the more complex is the medium (e.g., milk products), the more steps are needed before the actual quantification (Ruas-Madiedo and de los Reyes-Gavilán, 2005)(Sánchez et al., 2006). However, because of the relative simplicity of the media used here we adopted a standard protocol for EPS separation (Kimmel and Roberts, 1998).

The amounts of EPS were compared in the four RP + Lactobacilli (6E8, 21E1, 1F4, 1F8) and one RP- (2D5) as control, all grown in 10% sugars (lactose, sucrose or glucose) to obtain maximal induction. Fructose was omitted from these experiments, as it failed to support the propagation of some of the *Lactobacillus* strains. We made certain that

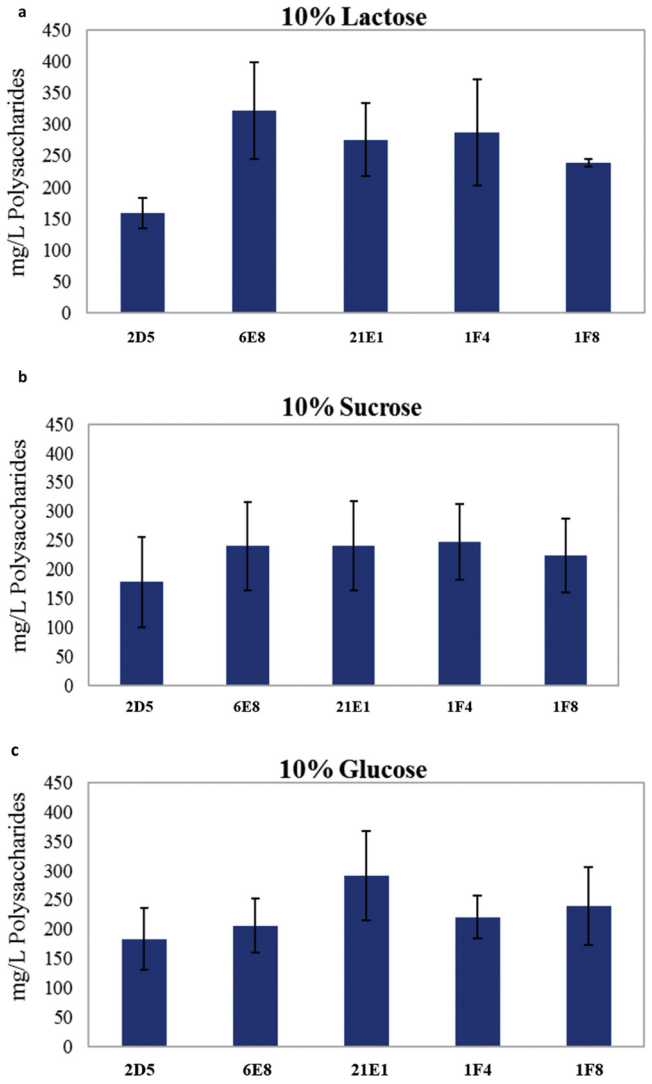

Fig. 5. EPS produced by an RP- strain (2D5) and 4 RP + strains (6E8, 21E1, 1F4, 1F8), grown in media containing lactose (Fig. 5A), sucrose (Fig. 5B) or glucose (Fig. 5C) as energy sources. Results shown are averages of three separate experiments, each performed in duplicate. (The results related to the media containing lactose were statistically significant with p-value < 0,001).

the analyzed sugars were EPS (and not monosaccharides or disaccharides originating from the medium) by performing two ethanol precipitation steps on the cell supernatants according to Kimmel and Roberts (Kimmel and Roberts, 1998) and using the growth medium as background reference in EPS determinations. The precipitation protocol produced a pellet of EPS with mono-disaccharides and other impurities left in the supernatant (Fig. 4a). After dissolving in water, the EPS precipitate of the RP- strain was transparent, compared to those from RP + strains, which were opaque (Fig. 4b). The average EPS levels (quantified by the phenol sulfuric acid assay) were generally higher in all the four RP + strains compared to the RP-strain in all sugars tested (Fig. 5a-c).

The increase was variable, ranging from 24% to 50% in lactose, 18% to 28% in sucrose and 5% to 35% in glucose. However, the statistical analyses revealed that these differences are significant only for specific sugars and strains. The RP + strain *L. helveticus* 6E8 produced the highest average amount of EPS in 10% lactose medium. This was significantly higher (p-value < 0,001) than the amount of EPS produced by the RP- strain, *L.helveticus* 2D5, grown in the same medium (322 ± 76 mg/L and 158 ± 24 mg/L, respectively) (Fig. 5a).

The highest average amount of EPS in RP + strains was produced in lactose (280 ± 56 mg/L), followed by glucose (239 ± 56 mg/L) and sucrose (237 ± 70 mg/L). In contrast, the RP- strain produced 158 ± 24 mg/L EPS in lactose, 183 ± 51 mg/L EPS in glucose and 177 ± 77 mg/L EPS in sucrose. Other authors reported average amounts of LAB-produced EPS ranging between 80–500 mg/L (Badel et al., 2011) and between 25–600 mg/L (Ruas-Madiedo and de los Reyes-Gavilán, 2005) using different sugar substrates. In the present study, a statistically (Poli et al., 2011)significant difference between EPS levels of all the RP + versus RP- was found only in lactose medium. These results indicate that 10% lactose is potentially the preferred sugar source for maximal EPS production in these strains and the optimal substrate for enabling discrimination between RP + and RP-. These findings agree with what was observed with a *L. rhamnosus* strain, which produced the highest amounts of EPS in lactose (219 mg/L) (Poli et al., 2011).

It has to be underlined that only a minor correlation between ropy phenotype and the overall concentration of secreted EPS has been detected (Fig. 5). Probably, the difference between RP + and RP- is not determined by the total amount of synthesized EPS but rather by their chemical structure, as previously suggested by Ruas Madiedo et al. (Ruas-Madiedo et al., 2002). At present, the precise structural differences between EPS conferring ropy phenotype or not are not yet fully elucidated. Likely, the total EPS produced by the RP + strains are composed by more branched polysaccharides than those synthesized by RP- strains, which might also explain why they create a more turbid solution (Fig. 3 and 4b), as also discussed below. This speculation is supported by the evidence brought by Xu and co-workers (Xu et al., 2014) which demonstrated a different precipitation behavior in ethanol for polysaccharides with equal molecular weight but different branching degree. In agreement with their results, it is reasonable to hypothesize that at the ethanol concentration used in the present study (66%) the amount of EPS precipitated will depend on the type of EPS (branched or unbranched) produced by each *Lactobacillus* strain. Moreover, the observation that physical properties of EPS such as viscosity are not related to EPS abundance, but rather to molar mass and stiffness of the polysaccharide chains, has been noted previously (Welman et al., 2003)(van Marle and Zoon, 1995) (Ruas-Madiedo et al., 2002). Our results with RP+ *L. helveticus* and *L. rhamnosus* are consistent with this conclusion.

Furthermore, it is known that both *L. helveticus* and *L. rhamnosus* can synthesize Hetero-EPS (Robijn et al., 1995)(Staaf et al., 2000)(Landersj et al., 2002) and the levels synthesized are relatively low (few mg/L). In contrast, Homo-EPS can reach several grams per liter (e.g., *L. reuteri* producing 10 g/L) (Ruas-Madiedo and de los Reyes-Gavilán, 2005). It has been reported that Hetero-EPS yield cannot be higher than 33% of a given substrate (Welman and Maddox, 2003), which may explain the lower EPS levels obtained in the present study. These levels are more consistent with Hetero-EPS rather than Homo-EPS. Thus, our current working hypothesis is that the EPS isolated from our RP + strains is Hetero-EPS, however, future qualitative analysis (that is out of the scope of the present investigation) will have to validate this assumption.

3.5. Changes in L. helveticus 6E8 proteome upon induction of ropy EPS synthesis

Among the strains identified as RP+, *L. helveticus* 6E8 produced the highest levels of EPS, with the highest turbidity and greatest difference between high and low lactose (more than four-fold). Therefore, this strain was chosen for comparative proteomic investigations.

To obtain an overview on the metabolic pathways activated during ropy EPS synthesis, we compared the proteomic profiles of *L. helveticus* 6E8 in 2% lactose (poor induction of ropy phenotype) and 10% lactose (strong induction of ropy phenotype). Both soluble (cytosolic and periplasmic) and insoluble (membrane) fractions were analyzed. A total of 361 proteins were identified, among which 100 proteins were found

Table 2
Membrane proteins that showed a change in abundance in the RP + strain *L. helveticus* 6E8 grown in 10% versus 2% lactose.

UNIPROT ENTRY	PROTEIN NAME	FOLD CHANGE	*p*-value	FUNCTION
W5XEZ0	Protein translocase subunit SecA	5.7	0.01	protein secretion
Q2L369	ATP synthase alpha subunit	4.0	0.05	energy metabolism
U6FQG1	General stress protein, Gls24 family	3.8	0.04	Stress response (starvation, oxidants, hyperosmoticity)
U6FPH7	L-lactate dehydrogenase	3.0	0.07	Fermentation
U6FD26	Exopolysaccharide biosynthesis protein	2.9	0.02	EPS production
W5XH37	ADP-ribosylglycohydrolase	0.4	0.09	ND
U6FMM3	Glutamate–tRNA ligase	0.3	0.08	protein synthesis
U4QGH5	ABC transporter family protein	0.2	0.10	transmembrane transport
W5XD98	30S ribosomal protein S13	0.1	0.06	protein synthesis

[a]The proteins are arranged in descending order of fold increase in 10% over 2% sugar.

in both fractions.

3.6. Changes in the abundance of specific proteins in the membrane fraction

A total of 207 proteins represented by ≥ 1 unique peptide was identified in the membrane fraction (data not shown). The abundance of nine proteins changed (increased/decreased) in 10% vs. 2% lactose (Table 2). Among these proteins, four showed a statistically significant difference (p-value < 0.05), while the rest had a p-value < 0.1. The four proteins for which the statistical difference is most reliable include: Protein translocase subunit SecA (increased by 5.7-fold in 10% lactose; p-value 0.01), exopolysaccharide biosynthesis protein (increased by 2.9-fold in 10% lactose; p-value 0.02), general stress protein, Gls24 family (increased by 3.8-fold in 10% lactose; p-value 0.04) and alpha subunit of ATP synthase (increased by 4.0-fold in 10% lactose; p-value 0.05).

Protein translocase subunit SecA (increased by 5.7-fold) is part of the Sec protein translocase complex, which interacts with the pre-protein conducting channel, identified in *L. helveticus* strain H9. This complex uses ATP to drive the stepwise translocation of polypeptide chains into or across the cell membrane. An increase in this protein indicates a rise in overall secretion of newly synthesized proteins and enhanced insertion of new proteins into the membrane. A possible reason for an increase in newly synthesized proteins on the cell surface is the need for glycosyl polymerases. Although Hetero-EPS repeating units are biosynthesized intracellularly, they are then exported and polymerized on the cell surface by extracellular polymerases (Welman and Maddox, 2003). Moreover, there may be a need of hydrolyzing enzymes for releasing attached EPS from the cell surface.

General stress protein, Gls24 family (increased by 3.8-fold), is a predicted protein deduced from the whole genome sequence of *L. helveticus* strain CIRM-BIA 101. This protein belongs to the Gls24 family, which was described in both *Enterococcus faecalis* and *Lactococcus lactis* as generally induced by a variety of stresses such as starvation and exposure to heavy metals and bile salts (Giard et al., 2000). The most likely stress experienced by *L. helveticus* 6E8 in our experiment is hyperosmosis due to 10% lactose. It is known, as discussed above, that EPS provide protection from a number of environmental stresses, including osmotic stress (Berecka et al., 2013).

Exopolysaccharide biosynthesis protein (increased by 2.9-fold) is also a predicted protein deduced from the whole genome sequence of *L. helveticus* strain MB2-1, which was recently isolated from traditional "Sayram" ropy fermented milk in southern Xinjiang, China. Although the protein itself has not been characterized, the genome sequence of the MB2-1 strain indicated the presence of a 15.20 kb gene cluster involved in EPS biosynthesis, which includes this predicted protein sequence (Li et al., 2015). The structure of the EPS from the MB2-1 strain has been partially characterized and has been claimed to have potential anti-cancer activity (Li et al., 2015). Clearly, multiple genes organized in gene clusters are involved in EPS production in LAB and they code for a variety of enzymes. A gene cluster for the biosynthesis of a long, galactose-rich EPS in *Lactobacillus rhamnosus* GG has been described (Lebeer et al., 2009). At present, it is not known in which step of EPS synthesis the 'exopolysaccharide biosynthesis protein' identified here takes part.

Alpha subunit of ATP synthase (increased by 4.0-fold), is predicted to exist in *L. helveticus* on the basis of homology to a similar gene in *Lactobacillus casei*. In general, ATP synthase only play roles in ATP synthesis among organisms that gain energy by electron-transport phosphorylation. This is not the case of LAB; whose main energy supply is the substrate-level phosphorylation. However, because of the low energy gain of the latter, LAB have evolved different systems (lactate extrusion, malic acid and amino acid decarboxylations) coupled with the generation of a transmembrane proton gradient (Pessione, 2012). In this condition, ATP synthase-mediated ATP synthesis can occur, especially when a high number of glucose molecules are converted into lactic acid. The major limiting step for Hetero-EPS synthesis is the cell concentration of activated sugar-nucleotide precursors (to be used as subunits for building the polymeric chain) (Welman and Maddox, 2003). The enhanced abundance of ATP synthase may be a response to an increased need for ATP to produce sugar nucleotides for EPS synthesis. EPS production is an anabolic process that involves up to 70% of the total energy reserve, representing a significant carbon investment for microorganisms (Berecka et al., 2013). We hypothesize that when sugar energy availability increases from 2% to 10%, the limiting step is removed and EPS synthesis can proceed at a maximal rate.

3.7. Changes in the abundance of specific proteins in the soluble fraction

A total of 154 proteins represented by ≥1 unique peptide was identified in the cytosolic fraction (data not shown). Fifteen proteins showed a change in abundance in 10% lactose, all of them showing a decrease relative to 2% lactose, while none showed an increase (Table 3). However, the differences observed were statistically significant (*p*- values < 0.05) only for seven proteins, most of which belong to pathways involved in maintenance functions: conversion of energy intermediates, metabolism of nucleic acids and RNA/protein synthesis.

This may indicate a decreased investment in cell growth, possibly because of osmotic stress, as discussed above, or because of shifting of resources toward increased EPS production. It has been noted previously that EPS synthesis possibly competes with cell-wall polymers and therefore with growth (Kumar et al., 2007). Moreover, it has been speculated that Hetero-EPS synthesis is relatively low because it is ATP-consuming and hence competes with other metabolic pathways that involve several shared housekeeping enzymes (Ryan et al., 2015).

Unexpectedly, two proteins, 30S ribosomal protein S13 and L-lactate dehydrogenase, were found in both the membrane and cytosolic fractions. Possibly, a fraction of these proteins is attached to the membrane, as has been shown for D-lactate dehydrogenase in *E. coli* (Kohn and Kaback, 1972). However, as these are highly abundant proteins considered to be soluble and located primarily in the cytosol, it is more likely that they are adsorbed non-specifically to the membrane fraction. In either case, both proteins were decreased, although not significantly, in both fractions.

At present, microbial EPS might offer to the food, pharmaceutical and cosmetic industries (Selbmann et al., 2003) a valid alternative to

Table 3
Cytosolic proteins that showed a change in abundance in the RP + strain *L. helveticus* 6E8 grown in 10% versus 2% lactose.

UNIPROT ENTRY	PROTEIN NAME	FOLD CHANGE[A]	*p*-value	FUNCTION
W5XFS3	Fumarate reductase	0.8	0.00	Energy metabolism
J4BP80	cytidine triphosphate synthetase	0.7	0.05	Pyrimidine synthesis
F3MM89	Bifunctional purine biosynthesis protein PurH	0.6	0.08	Purine synthesis
W5XEF9	Valine–tRNA ligase	0.6	0.09	protein synthesis
W5XEF7	Glutamate–tRNA ligase	0.6	0.06	protein synthesis
U6FV35	Isoleucine–tRNA ligase	0.5	0.09	protein synthesis
U6FP11	Citrate lyase	0.5	0.01	acetyl-CoA metabolic process, lipid biosynthesis
W5XDV8	ABC transporter ATPase component	0.5	0.08	transmembrane transport
W5XDF2	Cysteine–tRNA ligase	0.5	0.03	protein synthesis
U6FPH7	L-lactate dehydrogenase	0.4	0.07	Fermentation
W5XDQ4	Aspartyl/Glutamyl-tRNA (Asn/Gln) amidotransferase	0.4	0.08	protein synthesis
W5XD98	30S ribosomal protein S13	0.4	0.02	protein synthesis
U6FEX5	Phosphoribosylformylglycinamidine synthase subunit PurL	0.4	0.05	Purine metabolism; IMP biosynthesis
W5XFL3	30S ribosomal protein S2	0.4	0.08	protein synthesis
U4QLN6	tRNA pseudouridine synthase A	0.3	0.03	RNA metabolism

[a]The proteins are arranged in descending order of fold increase in 10% over 2% sugar.

plant and algal products (starch, alginate, arabic gum, carrageenan, agar and guar gum) for their high quality, biocompatibility, unusual structures and independence of seasonal variations (Selbmann et al., 2002). Furthermore, using 'food-grade' bacteria (Konings et al., 1999) such as LAB allows *in situ* EPS secretion i.e. directly in the food matrix (Caggianiello et al., 2016). This project focused on ways of identifying ropy EPS producing *Lactobacillus* strains, maximizing EPS synthesis since the low production yields of EPS in LAB constitute the main bottleneck (Boels et al., 2003).

In the present investigation, a method for the quick detection and partial quantification of ropy EPS as well as a strategy for stimulating maximal EPS biosynthesis has been reported. We also assess the proteomic changes occurring when EPS synthesis is induced. The aim was to unravel the metabolic pathways and the molecular targets for further enhancing EPS yields. Analysis of proteomic changes occurring in *L. helveticus* 6E8 during high EPS biosynthesis showed that, together with an enhanced stress profile, a number of enzymes involved in cell maintenance, replication and protein synthesis were decreased, whereas a subunit of ATP synthase was increased. The higher abundance of ATP synthase, together with the low EPS amount found, strongly support that EPS from *L. helveticus* 6E8 are Hetero-EPS. The overall results could mean that our experimental conditions (high sugar concentrations) cause a general shift of cell resources from growth to protection from the stressful environment by EPS secretion.

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