Comparative proteomic analyses for elucidating metabolic changes during EPS production under different fermentation temperatures by *Lactobacillus plantarum* Q823".

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Abstract:

Exopolysaccharide (EPS)-producing bacteria are of growing interest in industrial processes, mainly concerning food. Lactic acid bacteria are widely appreciated for their GRAS (generally recognized as safe) status and their ascertained or putative probiotic features. Detailed investigation on what happens at metabolic level during EPS production are scarce in the literature. The facultative heterofermenter *Lactobacillus plantarum* Q823 was studied in order to compare growth and EPS

production at 30°C and 37°C. A higher growth rate was observed at 37°C, whereas, a significantly higher (ten fold increase) EPS amount was produced at 30°C. To understand the molecular mechanisms leading to the different EPS production in the two conditions, a comparative proteomic experiment was performed. The results of the in-gel proteomics revealed that: i) at 37°C a higher abundance of proteins involved in carbon catabolism and nucleic acid biosynthesis together with a significant amount of stress proteins was observed; ii) at 30°C the production of an atypical manganese-containing non-heme catalase (pseudocatalase) was increased, in agreement with previous data reporting that growth-rates of catalase negative *Lactobacillus plantarum* strains were greater than that of catalase positive strains. Taken together, all these findings provide further insights about the metabolic pathways stimulated during EPS production, and the mechanism that triggers EPS biosynthesis.

1. Introduction

Lactic acid bacteria (LAB), naturally present in the human intestine as commensal microbiota, are considered to be safe (GRAS status) and they are the main group of microorganisms used by the food and pharmaceutical industries as starter cultures, biocontrol agents and probiotics (Pessione, 2012).

LAB have also been proposed as microbial cell factories to produce different metabolites of industrial interest such as bacteriocins, sweeteners, nutraceuticals (like selenoproteins), exopolysaccharides (EPS) (Mazzoli et al., 2014). Among the phenotypic features of LAB, the ability to produce extracellular sugar polymers is one of the most promising and widespread. These extracellular polymers include capsular polysaccharides, which form a cohesive layer or capsule covalently linked to the cell surface, and EPS, which form a slime layer loosely attached to the cell surface or secreted into the environment (Ruas-Madiedo and de los Reyes-Gavilán, 2005). A number of LAB strains have been reported to produce hetero- or homo-exopolysaccharides and these EPS have been widely used as viscosifying and bioflocculating agents thus playing an important role in the rheology, texture, and mouthfeel of fermented food products (Badel et al., 2011; Vijayendra and Shamala, 2014).

Apart from EPS application in the food industry as thickeners, stabilizing, gelling, and emulsifying agents (Mazzoli et al., 2014), the ability to biosynthesize these molecules is also essential in the definition of the probiotic status of a strain. Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit to the host (FAO/WHO, 2001). However, to persist and then exert their probiotic potential, they should be able of adhering to the intestinal mucosa or to the extracellular matrix (Selle and Klaenhammer, 2013) by means of specific adhesive proteins (adhesines), and/or teichoic/lipoteichoic acids and/or EPS. Actually, it was demonstrated that the presence of β-D-glucan enhances the *in vitro* adhesive potential of the probiotic *Lactobacillus plantarum* WCFS1 towards human intestinal epithelial cells (Russo et al., 2012). Furthermore, EPS can enhance the immune system and reinforce the innate mucosal barrier (Liu et al., 2011; Zivkovic et al., 2015). On the side of the bacterial cells, EPS play a role in

the protection against desiccation, toxic compounds, osmotic stress, and allow adhesion to solid surfaces and biofilm formation as well (Pessione, 2012).

The particular interest for the food industry in EPS-producing LAB, lies in the fact that LAB, possessing the GRAS status, are considered food-grade bacteria, thus they can be easily included in foodstuffs during its manufacturing. The only limit is that they should be resistant to the starter competitive weapons and, similarly, they have to be harmless for starters (i.e. not to be bacteriocin producing-strains). The use of EPS-producing sourdough starters meets the strict requirements of the modern baking biotechnology for clean labels and consumer demands for a reduced use of additives (Di Cagno et al., 2006).

Health benefits have been claimed for EPS from LAB because of their prebiotic effect (Russo et al., 2012), antioxidant features (Li et al., 2014; Zhang et al., 2013) and anti-biofilm properties (Rendueles et al., 2013). Moreover, immunostimulating (Liu et al., 2011), putative antitumoral (Wang et al., 2014) inducing autophagic cell death of tumor cells (Kim et al, 2010), and blood cholesterol lowering activities (Patten and Laws, 2015) were also reported.

EPS production yield by most LAB species is low, variable and depend on several factors (Rimada and Abraham, 2003). As an example, a high C:N ratio usually favours EPS production (Kumar et al, 2007), as well as the type of the carbon source (sucrose appears to be an inducer) (Rimada and Abraham, 2003). Phosphate-limited cultures as well as manganese enriched cultures strongly stimulate EPS production in *Klebsiella* spp. and *Lactobacillus casei* respectively (Kumar et al, 2007). Furthermore the oxygen availability, culture pH, osmolarity, viscosity and detergents, have all been found to influence EPS production (Kumar et al, 2007).

As far as optimum temperature for EPS production is concerned, a great variability is observed among species and strains. In *Streptococcus thermophylus* maximum EPS production is achieved between 32°C and 42°C (Vaningelgem et al, 2004). *Azotobacter beijerinckii* produced maximum EPS at 30°C whereas *B. subtilis* produced maximum EPS at 37°C (Chug et al, 2016). However, with a different *Bacillus* strain (CMG1403) maximum yield of EPS was obtained at 30°C

(Muhammadi and Hafzal, 2014). In *L. plantarum* EP56 inverse correlation between growth temperature and EPS synthesis was demonstrated with maximum yield at 18°C and poor production at the optimum growth temperature of the strain, 37°C (Tallon et al., 2003). At present, although the importance of environmental factors on the EPS production yields is widely demonstrated, little is known about the metabolic pathways stimulated during EPS production, and finally, the mechanism that triggers EPS biosynthesis is still poorly understood.

In previous studies, the probiotic properties of *Lactobacillus plantarum* Q823, a strain isolated from Andean traditional food, were established, including resistance to gastrointestinal tract stresses and adhesion to human intestinal epithelial cells (Vera Pingitore et al., 2016). When grown in solid media, this strain displays a “ropy” phenotype indicating EPS-biosynthesis capabilities.

Proteomics is a well-established approach allowing to analyze protein profiles during environmental changes (not detectable with genome investigations) and thus supplying a picture of what happens at metabolic level in different experimental conditions. Comparative proteomics, in particular, is a strategy intended to detect modifications occurring in the protein pattern under a certain stimulus.

The aim of the present work was to study the proteomic profile of the potential starter/probiotic strain *L. plantarum* Q823, grown in different experimental conditions, favoring (or not) EPS production. As far as we know, this is the first proteomic investigation during EPS production in LAB.

2. Materials and Methods

2.1. Bacterial strain and growth conditions

Lactobacillus plantarum Q823 was isolated from Quinoa (*Chenopodium quinoa)* seeds and conserved into the Culture Collection of Centro de Referencia para Lactobacillus (CERELA, Tucumán, Argentina). *L. plantarum* Q823 was stored at -20°C in MRS medium supplemented with 20% (v/v) glycerol. The cultures were performed in MRS broth (Difco, USA) with initial pH adjusted to 5.65±0.1 at either 30°C or 37°C. For both growth temperatures, the strain was pre-grown for 12 h and

then inoculated (3%) in 250 mL flasks containing 150 mL of fresh culture medium. The growth of the strain was monitored by 600 nm Optical Density (OD_{600}) and pH measurement. Both supernatants, for exopolysaccharide determination, and cells, for proteomic analysis, were harvested (3000 x g, 20 min, 4°C) at the end of the exponential phase. Three biological replicates for each growth condition were performed.

2.2. Isolation of bacterial EPS

After 24 h growth, the cells of *L. plantarum* Q823 were harvested (3000 x g, 20 min, 4°C) and discarded. The supernatants were incubated with 10% (w/v) trichloroacetic acid under shaking at 4°C for 1 h. The mixture was centrifuged (12500 x g, 30 min, 4°C) and the pellets, containing the extracellular proteins, were discarded. Cold ethanol was added to the supernatants in a 2:1 ratio and the mixtures were incubated at 4°C for 24 hours. The samples were centrifuged again (12500 x g, 30 min, 4°C) and pellets were dried and resuspended in 5 ml deionized water. Hundred microliters of each sample were dialyzed by using 3 kDa cut-off centrifugal filters (Millipore Centricon®, Ireland) and resuspended in milliQ water to remove the impurities. The samples were stored at -20°C.

2.3. Quantitative determination of EPS

The carbohydrate concentration of dialyzed samples was determined using the phenol-sulfuric acid method (Dubois et al., 1956). Glucose at different concentrations (10 to 50 mg/L) was used as the standard to obtain a calibration curve. The obtained values, expressed in mg equivalent of glucose per liter of growth medium (mg/L), were reported as mean values ± standard deviation. Not-inoculated MRS culture medium was used as negative control for EPS measurements. All the reported values were calculated by subtracting MRS medium component to the values obtained from fermented broths. Tukey's test was applied for the pairwise comparison of the results obtained. The differences between mean values were considered significant when $p<0.05$.

2.4. Proteome analyses: Comparative proteome analyses of in toto proteins by 2-DE

2.4.1. Protein sample preparation

Fifty mg of cells were treated in each protein preparation. The cells were collected by centrifugation (3000 x g, 20 min, 4°C) and washed three times with NaCl 0.85% (w/v). The obtained pellets were resuspended in 3 mL 50 mM Tris-HCl pH 7.3, 1 mM EDTA and disrupted twice by sonication as previously described (Pessione et al., 2010), to recover the highest amount of proteins. After clarification (4000xg, 20 min, 4°C), supernatants were supplemented with 10 µL/mL Nuclease mix (GE Healthcare, USA) and after 30 min incubation at room temperature they were centrifuged (100000xg, 1h, 4°C) in a Beckman L8-60M ultra-centrifuge (Type 60 rotor). The obtained supernatants were then dialyzed against four volumes of ddH_2O and precipitated with methanol/chloroform according to the method described by Wessel and Flugge (1984). The obtained pellets were then dissolved in rehydration solution consisting of 6.5 M urea, 2.2 M thiourea, 4% (w/v) CHAPS, 5 mM Tris-HCl pH 8.8, 0.5% IPG buffer 4-7 (GE-Healthchare, USA), 100 mM DTT. The protein concentration was evaluated by the 2-D Quant-Kit (GE Healthcare, USA).

2.4.2. Two-dimensional electrophoresis

Isoelectrofocusing (IEF) was performed using 13 cm IPG strips (GE Healthcare) with a linear gradient ranging from 3 to 10: 200 µg of proteins were loaded by in gel rehydration method. IEF was performed by using IPGphor (GE Healthcare, USA) at 20°C, with 83000 Vhrs. After IEF, the strips were incubated at room temperature in 6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 8.6, supplemented at first with 2% w/v DTT for 15 minutes and subsequently with 4.5% w/v iodoacetamide for 15 min. They were then loaded at the top of 1.0 mm vertical second dimension gels. SDS-PAGE was performed on 11.5% T and 3.3% C acrylamide (Biorad Acrylamide, USA) homogeneous gels. The running buffer was 2.5 mM Tris, 192 mM glycine, 0.1% SDS. The running conditions were 11°C,

600V constant voltage, 20 mA/gel, 60W for 15 min and 11°C, 600V constant voltage, 40 mA/gel, 80W for about 2.5 h. Molecular weight markers were from the Low Mr Electrophoresis Calibration Kit (GE Healthcare, USA). The gels were automatically stained using the Processor Plus (Amersham Biosciences, USA) with freshly prepared Neuhoff stain (Colloidal Coomassie Blue) (Neuhoff et al., 1988). They were digitized with the Personal Densitometer SI (Amersham Biosciences, USA) and then stored after dehydration in a GD 2000 Vacuum Gel Dryer System (GE Healthcare, USA).

2.4.3. Image and statistical analyses

Image analysis was performed with the Progenesis PG 220 software (Non Linear Dynamics, UK). Spot detection was automatically performed using the 2005 detection software algorithm and manually verified. After the establishment of some user seeds, matching was automatically performed and manually checked.

Two analytical replicates of all the three biological replicates were performed. A spot was considered significant when it was present in both the technical replicates of at least two out of three biological replicates. Spots showing at least 1.3-fold change between 30°C and 37°C were analyzed by mass spectrometry (MS).

2.4.4. Protein identification by MS

The protein spots were excised from the dried gels and rehydrated with MilliQ water. They were washed twice with 50% v/v ACN in a 25mM NH_4CO_3 and once in 100% v/v ACN and vacuum-dried. The proteins were in-gel digested with sequencing-grade, modified porcine trypsin (Promega, USA) and added to a MALDI target plate as described by Hewitson et al., (2008). Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, USA) in reflectron mode. MS spectra were acquired over a mass range of m/z 800–4000 and monoisotopic masses were obtained from centroid of raw, unsmoothed data. Finally, the mass spectra were internally calibrated using the tryptic autoproteolysis products at m/z 842.509 and 2211.104.

CID-MS/MS was performed on the 20 strongest peaks with an S/N greater than 40. A source 1 collision energy of 1 kV was used for CID-MS/MS, with air as the collision gas. The precursor mass window was set to a relative resolution of 50, and the metastable suppressor was enabled. Default calibration was used for the MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points across a peak and a polynomial order of 4), the peak detection used a minimum S/N of 5, a local noise window of 50 m/z, and a minimum peak width of 2.9 bins. S/N 20 and 30 filters were used to generate peak lists from the MS and MS/MS spectra, respectively. The mass spectral data from the protein spots were submitted to a database search using a locally running copy of the MASCOT programme (Matrix Science, version 2.1).

Batch-acquired MS/MS data were submitted to an MS/MS ion search through the Applied Biosystem GPS explorer software interface (version 3.6) with MASCOT.

The search parameters allowed a maximum of one missed cleavage, the carbamidomethylation of cysteine, the possible oxidation of methionine, peptide tolerance of 100 ppm and an MS/MS tolerance of 0.1 Da. The spectra were searched against a recent version of the NCBI non-redundant protein database.

The significance threshold for peptide identification was set at $p<0.05$; protein identification required that each protein contained at least one peptide with an expect e-value <0.05.

3. Results

3.1. Growth of *L. plantarum* Q823 and quantitative determination of EPS production. After 24 h of incubation of *L. plantarum* Q823 the final biomass values reached at 30°C (OD_{600nm}= 11.8±0.7) and at 37°C (OD_{600nm}=11.35±09) were not statistically different (Figure 1). However, the specific growth rate (μ), was higher at 37°C ($\mu_{37°C}$=0.27±0.02 h^{-1}) than 30°C ($\mu_{30°C}$= 0.23±0.03 h^{-1}). Moreover, there was an inverse relationship between EPS production and growth temperature. The EPS

production of *L. plantarum* Q823 was significantly higher at 30°C (133.23 mg/L) than 37°C (13.18 mg/L) (Table 1).

3.2. Proteomic profiles of *L. plantarum* Q823 at different temperatures. As displayed in figure 2, the *in toto* proteomic patterns of *L. plantarum* Q823 grown at 30°C and 37°C were overall similar (a total 226 ± 10 well-resolved protein spots was detected in each growth condition). Image analysis revealed that 19 protein spots showed different intensities ($p<0.05$) between the two tested conditions: 12 were more expressed at 37°C and 7 at 30°C. These spots were all identified by MALDI TOF/TOF mass spectrometry exception made for spot 3 which did not give a significant protein identification score. Considering that spots 8 and 9 were both identified as glucose-6-P isomerase, 17 proteins were found to be differentially expressed between the two tested conditions: 10 proteins were expressed to a higher level at 37°C and 7 proteins were up-regulated at 30°C. The identified proteins and the sequenced peptides leading to their identification are summarized in Table 2.

The identified proteins fell into different functional families. The proteins showing different abundance in the two conditions were divided in two groups (Table 2). The first group includes proteins that are overexpressed at 37°C: Molecular chaperon GroEL, Glucose-6-P-isomerase, Glyceraldheyde-3-P-dehydrogenase, Phosphoribosylaminoimidazole carboxylase (ATPase subunit), Oxidoreductase, Orotate phosphoribosyltransferase, Putative elongation factor Tu, Alkaline shock protein, ATP-binding subunit ClpB and Co-chaperone GrpE. The second group includes proteins that are over-expressed at 30°C: DNA-directed RNA polymerase subunit alpha, cystathionine beta-lyase, Elongation factor Ts, cell division initiation protein DivIVA, D-lactate dehydrogenase, cell division protein FtsA and Catalase.

4. Discussion

As referred in the introduction chapter, EPS are multifunctional compounds that have found interesting applications in both the food and pharmaceutical

industry. Moreover, EPS are important when testing the probiotic potential of a strain because of their involvement in both *in vivo* adhesion to human mucosa (Russo et al., 2012), in immunomodulation (Liu et al., 2011) and in anti-infective (anti-biofilm) properties (Rendueles et al., 2013). Among the main factors affecting EPS production (e.g. carbohydrate source, pH, temperature, growth phase harvesting) temperature showed to be crucial (Tallon et al., 2003).

L. plantarum Q823, object of the present investigation, shows a mucoid ("ropy") phenotype clearly related to EPS production on MRS agar plates, (one of the most common media used for testing lactobacilli EPS production).

In view of possible industrial or probiotic use of this strain, we compared *L. plantarum* Q 823 EPS production at 30°C (LAB optimal temperature for industrial processes) and at 37°C (human body temperature for probiotic application of LAB).

The experimental data obtained on *L. plantarum* Q823 revealed that significant EPS biosynthesis (ten-fold increase) can be obtained at 30°C whereas a higher growth rate (37°C) correlated with lower EPS production. This is in agreement with the results reported by Degees et al. (2001) who observed enhanced EPS production at low temperatures in *Lactobacillus sakei* and by Minervini et al. (2010) who found that EPS synthesis by *Lactobacillus curvatus* DPPMA10 was higher at 30°C than 37°C. Similar results were also obtained by Tallon and co-workers (2003) on a different *L. plantarum* strain (*L. plantarum* EP56). This behavior can be partly explained by LAB metabolic necessity during the rapid logarithmic growth (observed at 37°C), to direct all carbon nutrients towards glycolysis and substrate-level ATP production, rather than EPS synthesis. Furthermore, some authors demonstrated that at lower growth rate, cells exhibit higher isoprenoid glycosyl lipid carriers, whereas at a higher growth rate, these precursors are used for cell-wall synthesis and result less available for EPS production (Sutherland, 1972; Tallon et al., 2003). The experimental evidences of EPS synthesis at different growth temperatures suggest that *L. plantarum* Q823 cannot be fully performant at 37°C *in vivo,* at least for what concerns EPS involvement in adhesion and immunostimulation. On the other hand, this feature

seems quite regular among *Lactobacilli,* exception made for *L. delbrueckii* subsp *bulgaricus* which increases EPS production with increased temperature. Unfortunately, no truly probiotic traits have been demonstrated for the latter (Kumar et al, 2007).

To better elucidate the metabolic aspects underlying EPS overproduction at 30°C, a comparative proteomic analysis (30°C versus 37°C) was performed. At optimal growth rate (37°C) a higher abundance of enzymes involved in glycolysis was observed. This is in agreement with the higher energy requirement of a fast replication. LAB do not have respiratory metabolism due to their lack of heme biosynthetic pathways, hence glycolysis constitutes the main route for synthesizing ATP by substrate level phosphorylation (Pessione et al., 2010). *L. plantarum* is a facultative heterofermenter generally degrading hexoses by the Embden Mayeroff route. On the other hand, orotate phosphoribosyl transferase, an enzyme involved in pyrimidine biosynthesis, and Phosphoribosylaminoimidazole carboxylase, involved in purine anabolism, are also present in increased amounts at 37°C confirming the need of building blocks for DNA replication. The highly abundant Elongation factor Tu, involved in the translation process, can account for the need of newly synthesized proteins (membrane carriers, cell wall components, etc.). All the other highly abundant proteins in cells grown at 37°C are connected with stress. GroEL, GrpE co-chaperone, Alkaline shock protein, and ATP-binding subunit of ATP-dependent Clp protease are all proteins involved in repair of unfolded proteins or rather in proteolytic degradation of irreversibly damaged proteins.

Apparently, these results, exception made for GrpE, a stress protein specifically acting as a thermosensor and hence probably related to the growth temperature (Grimshaw et al., 2003), suggest that a too fast replication rate causes stress, situation that is partly limited at lower growth rate and higher EPS synthesis. Actually, at 30°C, during EPS best production, stress proteins are present in lower abundance. This result is new since it is generally believed that EPS are produced under stress conditions (Pessione, 2012) and, in a Gram

negative model, a proteomic study revealed induction of stress proteins during EPS synthesis (Gallo et al., 2012). However, it has been observed that during bile salt exposure, a very stressing condition for LAB, enzymes for EPS biosynthesis are present in low abundance (Koskenniemi et al, 2011). Interestingly, in the present study a non-heme catalase enzyme was biosynthesized only by *L. plantarum* Q823 grown at 30°C. *L. plantarum* is one of the first described LAB able to produce a weak catalase activity (Dacre and Sharp, 1956). This enzyme is an atypical manganese-containing catalase (pseudocatalase) whose crystal structure has been solved in 2001 (Barynin et al., 2001). The enzyme proved to be useful to protect the bacterial cell from hydrogen peroxide derived from oxygen reduction. In 1975, growth rates of catalase-negative and catalase-positive strains of *L. plantarum* were compared and the rates of the catalase negative strains were greater than that of the catalase positive strains (Yousten et al., 1975). This is exactly what we observe during growth at 30°C and 37°C: the strain is genetically catalase positive but when the pseudocatalase enzyme is not synthesized (37°C) growth rate is higher. In which way EPS biosynthesis could be connected with oxygen reduction is still unclear. D-lactate dehydrogenase production in high abundance together with significant amounts of cystationine beta lyase allow a good balance between acid-alkali production since the former produces lactic acid and the latter ammonia. Once again, this condition is stress protecting.

5. Conclusion

The present investigation represents a further step towards the identification of bacterial biomarkers for each particular probiotic feature. In this respect, significant lower production of EPS by *L. plantarum* Q823 was caused by growth at higher temperature (37°C). This renders this strain not so suitable for probiotic applications in terms of adhesion, although other factors, like adhesive surface proteins, can contribute to its persistence in the human gut (Koskenniemi et al, 2011**,** Pessione et al., 2015). This aspect can be investigated in the near future by means of extracellular and surface proteomics. Conversely, a good EPS

biosynthesis was observed at 30°C condition suitable for industrial processes since it allows energy saving. An inverse correlation between growth rate and EPS synthesis has been demonstrated: this is an expected result since energy requirements during fast growth induce active carbon catabolism generally preventing most biosynthetic pathways. In this case, fructose, rather than being diverted from glycolysis to EPS production continues to be metabolized through the Embden-Meyeroff pathway. As far as we know, this is the first proteomic investigation on LAB during EPS production. Curiously, no stress related proteins were found during EPS synthesis for this bacterial model, in spite of the general assessment that EPS production is induced by stress. Clearly, each bacterial strain has its own behavior. Furthermore, the present results did not highlight specific induction of enzymes involved in EPS biosynthesis at 30˚C degrees, when a ten-fold enhance of EPS was observed. These experimental data suggest that the high abundance of EPS at this temperature may be due to enhanced catalytic activity of EPS biosynthetic enzymes rather than to increased transcription of encoding genes, as previously observed by Degees et al., (2001).

Finally, taken together all these findings underline the importance of strain typing, both to avoid easy generalizations and to screen the correct strains to be employed as probiotics. The good EPS production observed at 30°C opens new possibilities for production of EPS to be used in innovative processes not only restricted to the food industry.

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Figure Captions

Fig. 1. Growth kinetic of *L. plantarum* Q823 at 30°C and 37°C. OD_{600nm} and pH trends are reported.

Fig. 2. Representative 2-DE gel pictures (pH range: 4–7) of *in toto* proteins expressed by *Lactobacillus plantarum* Q823 grown at 30ºC (Gel A) and 37ºC (Gel B). The differentially expressed spots are indicated.

Table 1. Effect of temperature on EPS production by *L. plantarum* Q823 grown in MRS broth

Temperature of incubation	Growth medium	EPS recoverd		
		Average (mg/L)	SD	P value
30°C	MRS (pH 5.65±0.1)	133.23	±66.21	0.0113*
37°C	MRS (pH 5.65±0.1)	13.18	±7.63	

* t test, significant differences $p<0.05$

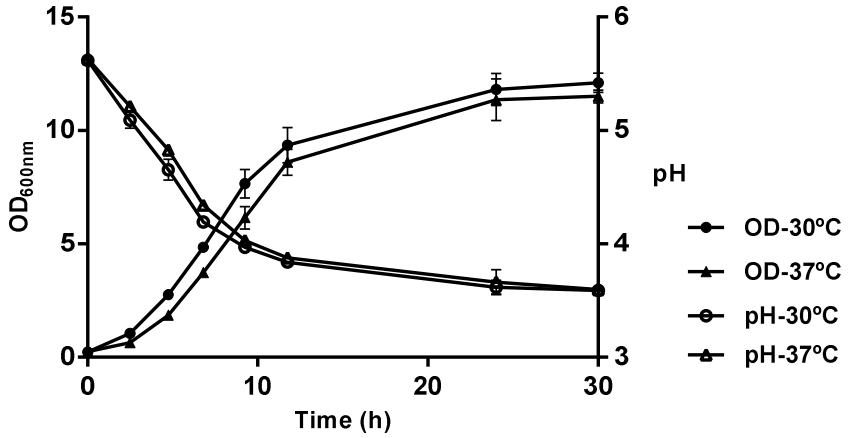
Table 2. Differentially abundant proteins (identified by MALTI-TOF/MS) found in *L. plantarum* Q823 grown at 37°C and 30°C respectively.

Spot n°	Increased expression (fold-change)†	Protein	Identified peptides	Score	Mw(Da)/pI	Sequence coverage
1	37°C (1.40)	Alkaline shock protein	MEATATSLKPEVVFDDTVLAK IASNTAQEVEGVLSLQGNLIDDI SNR DMLTTEEWR DMLTTEEWR Oxidation (M)	526	16056/4.77	38%
2	37°C (9.10)	Putative elongation factor Tu	STVTGLEMFR TLDLGEAGDNVGALLR VGDEVEIVGLHEDVLK	333	29575/4.82	15%
4	37°C (2.42)	orotate phosphoribosyltran sferase	SPIYTDNR QHIAHGIAAIIK QADYIDDEELASLHTWR	293	22691/5.64	17%
5	37°C (13.80)	Oxidoreductase	AVLNGEQIVSASAVAALR FSPEEFEEPGIR LDYVHVSLNNYDR LPLVGVGGVR	319	41736/5.76	14%
6	37°C (4.86)	Phosphoribosylami noimidazole carboxylase, ATPase subunit	VVGALNDQQQLQNFAER VPQGADALEITQDR SGDTTAFFPTVENR	227	42863/4.84	11%
7	37°C (1.31)	Glyceraldheyde-3-P-dehydrogenase	VYAEPQAQNIPWVK TIVYNVNDDILTADDR VGVVDGSLTELVAILDK TVAWYDNEYGFTCQMVR	507	36644/5.30	18%
8	37°C (1.32)	Glucose-6-P-isomerase	GWLNLPTDYDKEEFAR IQDDSDVLVVIGIGGSYLGAR SGTTTEPSIAFR QEADAEGYETFVIPDDVGGR NEAYQYAAYR	551	49816/4.96	17%
9	37°C (1.65)	Glucose-6-P-isomerase	GWLNLPTDYDKEEFAR IQDDSDVLVVIGIGGSYLGAR SGTTTEPSIAFR QEADAEGYETFVIPDDVGGR NEAYQYAAYR	487	49816/4.96	17%
10	37°C (1.97)	Molecular chaperon GroEL	NVVLEQSYGSPTITNDGVTIAK AIELDDHFENMGAK NVTAGANPVGIR VGHDGVITIEESR GVDTSLDVVEGMQFDR GVDTSLDVVEGMQFDR IEDALNATR AAVEEGFVAGGGTALINVIK	873	57402/4.69	19%
11	37°C (3.32)	ATP-dependent Clp	FLTQPGELVR	319	96511/5.19	7%

		protease, ATP-binding subunit ClpB	GELHLIGATTLDEYR VLVAEPSVEDTISILR LLHLADHLHER ALAENLFDADDHMVR			
12	37°C (2.13)	co-chaperone GrpE	AQAEIVNMQNR DVLPVLDNLER HGVTEIAAAGEK FDPNIHQAVQTVPVDDDHPAD TVVQVLQR	358	22448/4.84	30%
13	30°C (2.97)	D-lactate dehydrogenase	VIGYDVYR DGAYILNFAR ELNTMTVGVIGTGR DNYHMLNADAFSK VAGAALDTYEYETK	400	37158/4.89	17%
14	30°C (2.56)	cell division initiation protein DivIVA	VLSPDDIHNK GYNIDEVNDFLEQIIK DSLNQSILVAQEAADK LQVMLESQLEVVK	411	26158/4.49	23%
15	30°C (1.85)	Elongation factor Ts	DVAMHVAAINPEYVNR DVAMHVAAINPEYVNR Oxidation (M) WLSEISLDDQEFVK DSDQTVAHFVESK	353	31653/4.91	14%
16	30°C (3.40)	cystathionine beta-lyase	IGQNQYEYSR HFGMTFTAVDTR YLGGHSDVIGGLVVTK IYYPGDPDNPDFSIAK LQNGIKDELIR LSVGVEASDDLLADLER	491	40839/5.52	21%
17	30°C (Nd)	DNA-directed RNA polymerase subunit alpha	FVVEPLER VNYQVENTR MLEMTIEELDLSVR MLEMTIEELDLSVR+oxidation LADLGLSLR	302	34935/4.80	12%
18	30°C (2.82)	cell division protein FtsA	EINNEDVQNVAAAALVQSLPPE R YTYVDQEGGQYITK QHLDEIR ALELPGGIVLTGGVAALPGITDL AAQR HPSFDEALAVIK	457	48352/5.03	18%
19	30°C (Nd)	non-heme pseudocatalase	LTTETGQPWANNEHSQTAGA R LGANFEDLPVNKPVVPVHNYE R YQVDYTTQAGDLYR	310	55294/5.31	11%

[†]Fold change is shown as a ratio (volume of the spot with higher protein expression/volume of the spot with lower protein expression). Nd: the spot was detected only at 30°C.

Figure 1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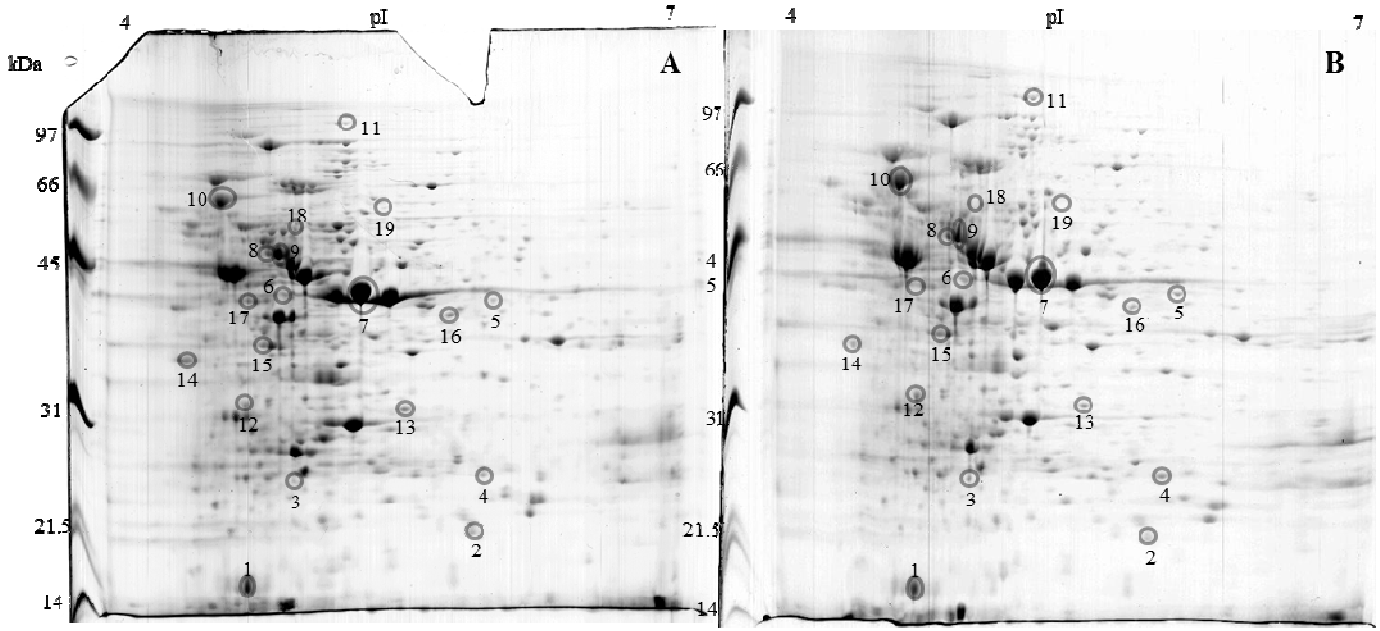
pH-37ºC

Figure 2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