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Proteomic and genetics insights on the response of the bacteriocinogenic *Lactobacillus sakei* CRL1862 during biofilm formation on stainless steel surface at 10 ºC

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Abstract

Some lactic acid bacteria have the ability to form biofilms on food-industry surfaces and this property could be used to control food pathogens colonization. *Lactobacillus sakei* CR1862 was selected considering its bacteriocinogenic nature and ability to adhere to abiotic surfaces at low temperatures. In this study, the proteome of *L. sakei* CRL1862 grown either under biofilm on stainless steel surface and planktonic modes of growth at 10 °C, was investigated. Using two-dimensional gel electrophoresis, 29 out of 43 statistically significant spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Ten proteins resulted up-regulated whereas 16 were down-regulated during biofilm formation. Differentially expressed proteins were found to belong to carbohydrate, nucleotide, aminoacid and lipid metabolisms as well as translation, peptide hydrolysis, cell envelope/cell wall biosynthesis, adaption to atypical conditions and protein secretion. Some proteins related to carbohydrate and nucleotide metabolisms, translation and peptide degradation were overexpressed whereas those associated to stress conditions were synthesized in lower amounts. It seems that conditions for biofilm development would not imply a stressful environment for *L. sakei* CRL1862 cells, directing its growth strategy towards glycolytic flux regulation and reinforcing protein synthesis. In addition, *L. sakei* CRL1862 showed to harbor nine out of ten assayed genes involved in biofilm formation and protein anchoring. By applying qRT-PCR analysis, four of these genes showed to be up regulated, *srtA2* being the most remarkable. The results of this study contribute to the knowledge of the physiology of *L. sakei* CRL1862 growing in biofilm on a characteristic food contact surface. The use of this strain as green biocid preventing *L. monocytogenes* post-processing contamination on industrial surfaces may be considered.

Keywords: Biofilm, Stainless steel surface, *L. sakei*, Proteomics, Adhesion genes

1. Introduction

Biofilms are complex microbial communities which adhere to a wide range of surfaces, being embedded in a self-produced matrix of extracellular substance composed of various types of biopolymers (Abee et al., 2011). Microbial adhesion to surfaces and the consequent biofilm formation have been documented in many different environments (Donlan and Costerton, 2002; Simões et al., 2010). Bacterial biofilms confer survival advantages to microbes that are part of it because they are protected from environmental stresses, making their removal quite a challenge. In the food industry, biofilms create a persistent source of product contamination adhering to almost any type of surfaces and leading to hygiene problems and economic losses (Bridier et al., 2015; Dominguez-Manzano et al., 2012; Ferreira et al., 2014).

The increasing evidences of the negative environmental effects has led to the exploration of biocides generated *in situ* which are generally considered as eco-friendly and free of chemical residues (Ashraf et al., 2014). Natural compounds from bacteria or plants with GRAS (Generally Recognized As Safe) status as well as bacteriophages were evaluated for their potential to eliminate biofilms (Donlan et al., 2009; Neyret et al., 2014). Accordingly, protective biofilms have been proposed as barrier microbiota against the establishment of food-borne pathogens such as *Listeria monocytogenes*. *L. monocytogenes* is an opportunistic pathogen with biofilmogenic ability that causes serious illness with a high mortality rate and is frequently isolated from food and food-processing environments (Newell et al., 2010). The use of lactic acid bacteria (LAB) as a biotechnological tool to control pathogens biofilms on industrial surfaces involves the production of antimicrobial peptides and the ability to form biofilms. In fact, the use of LAB as biosanitizer to prevent the attachment of *Listeria monocytogenes* onto abiotic surfaces was reported (García-Almendárez et al., 2008; Ndahetuye et al., 2012; Pérez Ibarreche et al., 2016).

Several studies with various food-related bacteria have shown that biofilm cells show physiological differences with respect to their planktonic counterparts, presenting a modified protein expression (Giaouris et al., 2013; Khemiri et al., 2016; Planchon et al., 2009; Trémoulet et al., 2002). It is widely accepeted that the development of multicellular aggregates is initiated by bacteria sensing certain surface-associated molecules that trigger the shift from a planktonic to a biofilm lifestyle mode (Abee et al., 2011; Di Cagno et al., 2011; Lebeer et al., 2007). Bacteria developed highly complex mechanisms to produce, secrete, detect and respond to diverse extracellular signaling molecules that accumulate in the environment proportionally to cell density. Cell-to-cell communication named Quorum Sensing (QS)

allows bacteria to sense the accomplishment of a minimal number of cells (quorum) to initiate a concerted response; QS is mediated by oligopeptides signals (autoinducers, AI) that are modified, processed and exported by an ABC transporter system (Di Cagno et al., 2011). Among LAB, it has been shown that the *lux*S-mediated quorum sensing and autoinducer AI-2 signal molecules are involved in biofilm formation and were described for lactobacilli strains and *Streptococcus mutans* (Lebeer et al., 2007; Tannock et al., 2005; Yoshida et al., 2005). The other major QS system is the peptide-mediated accessory gene regulator (Agr) system, consisting in an operon containing four genes (*agr*BDCA). An *agr*-like two-component regulatory system encoding a cyclic thiolactone autoinducing peptide designated as *lam* system (Sturme et al., 2005) and the *ftsH* gene was found to regulate adhesion to abiotic surfaces and cell surface properties in *Lactobacillus plantarum* WCFS1 (Bove et al., 2012). The meat borne *Lactobacillus sakei* 23K genome showed the presence of genes involved in cell surface components for adhesion and cellular aggregation; the candidates for biofilm formation are genes coding for proteins with an LPXTG anchor motif and WXL C-terminal domain (Chaillou et al., 2005).

Advances in the "Omics" technologies in the last two decades have been of key importance to study biofilm formation of bacteria. The ability of proteomics approaches to elucidate the biofilm phenotype was demonstrated (Khemiri et al., 2016). Particularly for LAB, the proteomic approach has already succeeded in understanding the regulatory mechanisms controlling the adaption to a range of environmental stresses by the combination of protein data with genetic information, however only a few studies were reported on the proteomic profile of LAB grown under planktonic and biofilm conditions on biotic/abiotic surfaces (De Angelis et al., 2015; Jiang et al., 2015) and even less on food processing surfaces or industrial equipments. Based on previously reported feasibility of *Lactobacillus sakei* CRL1862 to form biofilm and control *L. monocytogenes* adhesion on industrial materials (Castellano et al., 2012; Fontana et al., 2015; Pérez Ibarreche et al., 2014; 2016), the cellular mechanisms asociated for the biofilm lifestyle of this LAB were investigated using a proteomics approach and complemented with a genetic insight.

2. Materials and Methods

2.1 Bacterial strain and growth conditions

L. sakei CRL1862 previously known as *L. sakei/curvatus* ACU-1, was isolated from traditional fermented sausages (Castro et al., 2011) and has been deposited in the culture collection of the Reference

Centre for Lactic Acid Bacteria (CERELA-CONICET) (nº CRL1862). This strain is able to produce bacteriocin, a peptide with inhibitory activity against bacteria from the genus *Listeria* (Castro et al., 2011). It was grown in MRS (De Man, Rogosa and Sharpe; Oxoid, Argentina) at 30 °C for 24 h from a single colony and used for further inoculation. A stock culture was stored at -80 °C in milk yeast extract medium (10% w/v skim milk, 0.5% w/v yeast extract) containing 10% (v/v) glycerol as cryo-protectant.

2.2 Biofilm development on stainless steel surface at 10 ºC

Stainless steel (SS) coupons (2.5 cm x 2.5 cm x 1.0 mm thickness, type AISI 304, mechanically polished, Nº 4 grade) were used for biofilm development as previously described by Perez Ibarreche et al. (2014). After a cleaning treatment of SS coupons (70), *L. sakei* CRL1862 biofilm was developed in a glass tray containing 500 mL of sterile MRS broth inoculated with 5% (v/v) of an active culture and incubated under static conditions at 10 °C during ten days. The growth media was then removed from the glass container, coupons rinsed once with PBS pH 7.2 and cell count was carried out using BioTimer Assay method as previously described by Pérez Ibarreche et al. (2014).

2.3 Planktonic growth of *L. sakei* CRL1862 at 10 ºC

Overnight culture of *L. sakei* CRL1862 was used to inoculate (5% v/v) fresh MRS. After incubation for 16-18 h at 30 °C, cells were reinoculated in fresh MRS and incubated at 10 °C during 10 days. Samples were taken periodically and cell viability was determined in MRS agar (30 °C for 48 h).

2.4 *L. sakei* CRL1862 cells recovery for proteomic analysis

Cells under planktonic mode of growth were collected after 6 days of incubation at 10 ºC, during the stationary phase (~ 9 log CFU/mL). One hundred mL of planktonic culture was collected, harvested by centrifugation (10,000 *g*, 10 min at 4 °C) and rinsed (three times) with 0.1M Tris-HCl pH 7.5 buffer; cell pellet was then subjected to protein extraction. On the other hand, *L. sakei* CRL1862 cells grown under sessile conditions were collected at six days of incubation at 10 ºC. Coupons were removed and rinsed (two times) with 500 mL of PBS pH 7.2 to release loosely attached cells and transferred to a flask containing glass beads (225 g, 4 mm diameter) and 500 mL of 0.1M Tris-HCl, pH 7.5 buffer. The flask was then shaken for 10 min to detach sessile cells from coupons, collected by centrifugation (10,000 *g*, 10 min at 4 °C) and used for protein extraction.The number of viable cells in biofilm was estimated by

plating onto MRS agar (48 h; 30 °C). Three independent cultures (biological replicates) were undertaken for both, planktonic and sessile conditions.

2.5 Proteomic analyses

2.5.1 Protein extraction

Total protein extraction was carried out for planktonic- and biofilm-derived samples from the three independent cultures. Both bacterial cell pellets were separately placed in vials containing the cells, glass beads and 0.1M Tris-HCl, pH 7.5 (1w:1w:2v). Cells were broken using a BeadBeater (MiniBeadBeater-16, BioSpec, Bartlesville, OK) with 10 cycles of 1 min, allowing to stand 1 min on ice between each cycle. Unbroken cells and cell debris were removed by centrifugation (10,000 *g*, 10 min at 4 °C). Protein concentration of cell free supernatants was determined according to Bradford assay using bovine serum albumin as a standard. Protein samples (aliquots containing 400 μg protein) were stored at -70 °C for further use.

2.5.2 Two-dimensional gel electrophoresis (2DE)

Equal amounts of protein (400 μg) were used for each electrophoretic run. Sample preparation and 2DE gels were carried out as described by Grosu-Tudor et al. (2016). Isoelectrofocusing was performed using 18-cm isoelectrofocusing strips in the pI range of 4-7 (GE Healthcare, Uppsåla, Sweden), rehydrated with the buffer containing the sample and focused until 53,500 Vhs using the IPGphor 3 iso-electric focusing unit (GE Healthcare). For the second dimension, 12.5% homogenous SDS-PAGE gels were used using an EttanDALTsix electrophoresis unit (GE Healthcare). Gels were stained with Bio-Safe™ Coomassie blue Stain (Bio-Rad Labs, Richmond, CA, USA), distained with distilled water and digitalized using an Image Scanner III LabScan 6.0 (GE Healthcare, Uppsåla, Sweden). Two technical replicates for each biological sample were performed.

2.5.3 Image acquisition and data analysis

Digitalized images (600 dpi) of stained gels were aligned using the software Prodigy SameSpots version 1.0.3400.25570 (Nonlinear Dynamics Group, UK). A spot was considered significant when its resulting normalized volume showed more than 1.4 fold variation with respect to the control (planktonic cells) at the level of $p \leq 0.05$.

2.5.4 Protein identification by peptide mass fingerprinting

Stained proteins displaying significant and reproducible intensities that differed among both assayed growth conditions were excised from the gels with a sterile 1000 µL pipette tip or a sterile stainless scalpel blade for individual in-gel digestion using trypsin. Briefly, spots were washed with 30 µL of water for 30 min, washed in acetonitrile (50%), reduced with 10 mM dithiothreitol at 56 °C for 45 min, alkylated with 55 mM iodoacetamide for 30 min, washed in acetonitrile (100%) and vacuum dried (SpeedVac®, Thermo Fisher Scientific, Waltham, MA, USA). Gel pieces were rehydrated with a digestion buffer (50 mM NH4HCO3 buffer) containing 50 µL of trypsin (6.7 ng/µL; Promega, Madison, WI, USA) and incubated overnight at 37 °C. The digestion buffer containing the peptides was acidified with formic acid, desalted and concentrated using C8 microcolumns (POROS R2®, Applied Biosystems, Foster City, CA, USA), as described by Almeida et al. (2010). Peptides were subsequently ionized using α-cyano-4-hydroxycinnamic acid as matrix. Mass spectrometric analysis of the peptide solutions from trypsinized spots was carried out on a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, USA) according to Grosu-Tudor et al. (2016). Mass spectrometry analysis was performed at CEQUIBIEM (Facultad de Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina). MASCOT search engine (Matrix Science Inc., Boston, MA; http://www.matrixsciende.com/search-form-select.htmL) was used to identify proteins from peptide mass fingerprint data. All searches were performed against the database for *L. sakei* 23K from the annotated genome (http://migale.jouy.inra.fr/sakei/genome-server).

2.6 Search for genes involved in biofilm formation

Genes related to aggregation, adhesion and biofilm formation phenotypes of *Lactobacillus* species were investigated with primers designed using Primer 3 plus software (Table S1). DNA extraction was carried out from pure cultures of *L. sakei* CRL1862 grown in MRS broth for 16 h at 30°C (Pospiech and Neumann, 1995). PCR amplifications were performed by using a MyCycler thermocycler (Bio-Rad, Hercules, CA, USA) in a final volume of 50 µL containing 1x PCR buffer, 100 µM dNTPs, 1 µM of each primer, 1.5 mM $MgCl_2$, 1.25 U of GoTaq polymerase (Promega, Wisconsin, USA) and 10 ng/µL of DNA. Amplification consisted of an initial denaturalization at 95 ºC for 5 min, 30 cycles at 95 ºC for 1 min, annealing according to each primer pair (Table S1) for 2 min, extension at 72 ºC for 2 min and a final

extension step at 72 ºC during 10 min. PCR products were separated in agarose gels (1.5% w/v) stained with GelRed (Biotium, USA) and DNA fragments size was estimated using a 100 pb DNA ladder (Promega, Wisconsin, USA). PCR products were purified with a Wizard Genomic DNA Purification kit (Promega, Wisconsin, USA). Sequencing reactions were carried out in an automatic DNA sequencer (Applied Biosystems model 3130, Foster City, CA, USA). The sequences were analyzed using MEGA 5 (Tamura et al., 2011) and compared against those deposited in the GenBank (NCBI). Two replicates were performed.

2.7 Evaluation of gene expression by quantitative real-time PCR

2.7.1 RNA isolation and cDNA synthesis

For RNA isolation, cells of *L. sakei* CRL1862 grown under planktonic and sessile conditions were harvested as above mentioned after 6 days of incubation at 10 ºC. Total RNA extractions were performed using the RNeasy kit (Roche, Basel, Switzerland). RNA concentrations were calculated by measuring absorbance at 260 nm and RNA integrity was evaluated by electrophoresis on agarose 0.8% gel. To eliminate residual DNA in RNA samples, a treatment with Turbo DNase kit (Ambion, Thermo Fisher Scientific, MA, USA) was performed. cDNA was synthesized from total RNA (15 ng/µL) with SuperScript III reverse transcriptase kit (Invitrogen, Thermo Fisher Scientific, MA, USA) following protocol described by the manufacturer. Finally, cDNA aliquots were stored at -80 °C.

2.7.2 Quantitative real-time PCR

Primers for quantitative real-time PCR (qRT-PCR) were designed to have a length of about 20 bases, a G/C content over 50% and a Tm of about 60 °C using Primer 3 plus software (Table S2). The *fusA* (elongation factor G) and *ileS* (isoleucyl-tRNAsynthetase) genes were used as reference genes, using the primers sequences described by Rimaux et al. (2012). Real-time PCR reactions were carried out in 20 µL final volume containing 10 µL of IQ SYBR Green Supermix, 3 µL of cDNA dilution, 0.75 µL of each primer at 10 µM and 5.5 µL of RNase-free water. Amplifications were performed in an iQ5 Real-time PCR Detection (Biorad, Hercules, CA) with the following conditions: 50 ºC for 2 min, 95 ºC for 10 min and 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. An additional step was performed to establish a melting curve. All samples and primer set combinations were analyzed in triplicate. Non-template control (NTC) for each primer set was included. The cycle threshold (C_T) value in this study was automatically

determined by the instrument. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

2.8 Statistics

Infostat Statistical Software (Universidad Nacional de Córdoba, Córdoba, Argentina) was used for data analysis. One-way analysis of variance (ANOVA) with the post hoc Tukey's test was used to evaluate significant differences among samples. Significant differences were considered with $p \leq 0.05$.

3. Results

3.1 Growth of *L. sakei* CRL1862 under planktonic and sessile mode of growth at 10 ºC

The optimal growth temperature of LAB is 30 ºC, although, in this research 10 ºC was selected due to the importance of low temperatures in meat processing and storage, which are normally below 10 °C according to the different processing technologies. The growth of *L. sakei* CRL1862 in biofilm was carried out on stainless steel (SS) coupons, an abiotic substratum frequently used in food processing equipment. *L. sakei* CRL1862 under planktonic growth at 10 ºC (Fig. 1a), showed 9 log CFU/mL at 4 days; this cell density being maintained during 10 days of incubation. The maximum level of adhesion of CRL1862 strain on SS surface was 7.5 log CFU/cm^2 at 6 days of incubation at 10 ºC (Fig. 1b). Within this context, 6 days was the time chosen for sessile and planktonic cell collection for proteomic and gene expression analyses.

3.2 Differential protein expression of *L. sakei* CRL1862 under sessile and planktonic lifestyles

A proteomic approach was conducted to unravel functions involved in the response of *L. sakei* CRL1862 during the growth under biofilm lifestyle at 10 ºC for 6 days, corresponding to mature biofilm. This condition was compared with *L. sakei* CRL1862 cells growing under planktonic mode, at 10 ºC for 6 days (stationary phase of growth). Cells were subjected to protein extraction and 2DE to identify those proteins differentially expressed in both lifestyle conditions (biofilm versus planktonic). The genome data of *L. sakei* 23K (Chaillou et al., 2005) were used to assign genes encoding the significantly expressed proteins. Seventy-six proteins spots were differentially expressed and 43 resulted up- or down-regulated more than 1.4-fold ($p \leq 0.05$) which were submited to Maldi Tof-MS-MS analyses. Twenty nine out of the 43 statistically significant spots, ranging in their expression level from 3.64 to 1.40-fold were successfully

identified (Fig. 2; Table 1). Several spots were identified as the same protein (spots 70=96, 31=9, 34=61 and 24=40), they could represent isoforms of the same protein or products from post-translational modifications. Ten proteins were up-regulated by *L. sakei* CRL1862 biofilm on SS at 10 ºC, whereas 16 proteins were down-regulated (Fig. 2; Table 1). The proteins whose expression were differentially regulated in biofilm-derived samples, could be classified as belonging to different functional categories such as carbohydrate, amino acids, lipid and nucleotide metabolisms, translation, protein degradation/secretion, adaption to atypical conditions, cell wall biosynthesis and cell envelope and cellular processes. The distribution of identified proteins according to its functional categories is depicted in Fig. 3; most important up-regulated proteins included those related to carbohydrate metabolism (32%), translation (24%) and nucleotide metabolism (12%).

3.3 Identification and expression of genes responsible for biofilm formation in *L. sakei* CRL1862

The presence of ten genes selected among those related to biofilm formation in *L. sakei* 23K and phylogenetically close to LAB, was investigated in *L. sakei* CRL1862. Primers were designed in this study from deposited sequences using Primer 3 Website and bands were confirmed by sequencing and identities between 87 and 100% with sequences deposited in GenBank were found (Table S1). Bands with varying intensity for 9 out of 10 assayed genes are shown in Fig. 4. Results showed no amplification for *luxS* gene indicating it was absent in *L. sakei* CRL1862, whereas genes coding for hypothetical cell surface protein precursors in *L. sakei* 23K (*lsa 0194*, *lsa0313, lsa0534*, *lsa1159* and *lsa1165*) potentially involved in biofilm formation and cellular aggregation were detected in *L. sakei* CRL1862, obtaining amplified products in the expected size range. The *ftsH* gene, strongly connected to cell division and cell envelope-associated functions in *L. sakei* 23K and *L. plantarum* WCFS1 respectively, was also detected in *L. sakei* CRL1862 (Fig. 4). In addition, *srtA1* and *srtA2* genes coding for sortases responsible for protein anchoring to *L. sakei* 23K cell wall were also detected in *L. sakei* CRL1862. These enzymes displaying on the cell surface of Gram-positive bacteria, involve a diverse collection of proteins that allow bacteria to efficiently interact with the environment. Moreover, a weak band was obtained for the *lamA* gene, this is a two-component regulator system with homology to the staphylococcal *agr* QS system harboured by *L. plantarum* WCFS1.

In order to confirm the role of detected genes in biofilm formation by *L. sakei* CRL1862, their expression levels were evaluated in planktonic and sessile cells by using qRT-PCR; those genes showing

higher homology by PCR were selected for expression analyses (Table S2). The growth of *L. sakei* CRL1862 under planktonic mode during 6 days at 10 °C was considered as the calibration condition; a relative expression level higher than 1.5-fold means that the gene was up-regulated. The analyses revealed that four genes (*lsa0194*, *lsa0534*, *srtA1* and *srtA2*) related to biofilm development increased more than 2-fold the relative expression level under sessile mode of growth (Fig. 5). The transcriptional response of *srtA2* gene was the most remarkable (3.58 ± 0.25 fold expression) during *L. sakei* CRL1862 biofilm. The expression level of *ftsH* gene was similar (0.93 ± 0.28) in both growth conditions while *lamA* gene did not show expression in *L. sakei* CRL1862 cells under the assayed conditions.

4. Discussion

In this study, growth kinetics of *L. sakei* CRL1862 under planktonic and biofilm conditions was in agreement with the reported ability of some LAB to growth and produce antimicrobial substances and biofilms at low temperatures (Castellano et al., 2010; Pérez Ibarreche et al., 2014). Bacteriocinogenic and biofilmogenic abilities of *L. sakei* CRL1862 at low temperatures in both modes of life is technologically of paramount importance in view to be used as a green biocide in the meat industry (Ashraf et al., 2014; Condell et al., 2012).

Differential protein expression of *L. sakei* CRL1862 growing in biofilm

The comparative proteomic analysis performed between planktonic and sessile *L. sakei* CRL1862 cells, showed that the relative abundance of 76 protein spots was affected. In fact, marked differences in the expression of proteins related to metabolic functions have been previously revealed between planktonic and biofilm bacterial cells (Sauer, 2003).

Specifically, differential expression of **glycolytic enzymes** under biofilm conditions of LAB have already been described (De Angelis et al., 2015; Hendrickson et al., 2012; Jiang et al., 2015). Compared to planktonic cells, *L. sakei* CRL1862 grown on biofilm showed an increased expression of some enzymes involved in carbohydrate metabolism such as pyruvate kinase (spot 31, Pyk), glyceraldehyde 3-phosphate dehydrogenase (spot 70/96; GapA), 6-phosphofructokinase (spot 77; Pfk) and 6-phosphogluconate dehydrogenase (spot 86*;* gndA) (Table 1). It should be mentioned spots 9 and 31, both identified as pyruvate kinase showed opposite trends. The differences in expression may be ascribed to post translational modifications or artifacts of MS-MS identification. Also, they could correspond to isoenzymes that are known to perform essentially the same biological function but these can have minor

differences in their aminoacid sequence, pI, regulatory properties or temporal/ spatial expression (Muñoz and Ponce, 2003). In fact, two pyruvate kinase isoenzymes are found in LAB, Pyk type I activated by fructose 1,6-diphosphate and Pyk type II activated by adenosine 5'-monophosphate or ribose 5-phosphate (Bourniquel et al 2002).

Overexpressed Pyk, GapA and Pfk enzymes, are involved in the glycolytic pathway, whereas 6-phosphogluconate dehydrogenase is related to the pentose-phosphate pathway (Table 1; Fig. 6). The enzyme 6-phosphogluconate dehydrogenase, overexpressed 1.48-fold in sessile cells, catalyzes the production of pentose phosphates and reduces the redox potential by means of NADPH generation, achieving a redox balance in the cell. Similarly to our findings, Mukherjee and Chatterji (2008) reported the lower production of 6-phospho-gluconate dehydrogenase in a biofilm-defective mutant of *Mycobacterium smegmatis* compared to the biofilmogenic wild-type strain; the potential implicance of this enzyme in biofilm formation was proposed. In many microorganisms including several LAB, the glycolytic variation is mainly related with 6-phosphofructokinase and pyruvate kinase, both enzymes are encoded by a *pfk-pyk* operon (McLeod et al., 2010). It is likely that the higher expression of these key enzymes by *L. sakei* CRL1862 during biofilm growth on SS at 10 ºC enhance the carbon flux towards glycolysis to produce energy and sustain growth rather than the enhancement of other carbohydrate metabolic pathways. In fact, pyruvate oxidase (spot 16; Pox), L-lactate dehydrogenase (spot 34=61; Ldh), glucose-6-phosphatedehydrogenase (spot 62; G6pd) and phosphoglyceratemutase (spot 74; Gpm1), involved in the pentose phosphate pathway, were found at lower level in sessile CRL1862 cells. The decreased expression of Gpm1, Ldh and Pox proteins responsible for the final production of lactic and acetic acids, would suggest a lower acid production by sessile cells. Similarly, the decreased regulation of G6pd as responsible for glucose-6-phosphate to 6-phospho-gluconate conversion, constitutes a rate-limiting step of the oxidative pentose-phosphate pathway. It seems that during biofilm growth, *L. sakei* CRL1862 increases the expression of enzymes involved in the regulation of the glycolytic flux but decrease those leading to adverse environmental conditions such as high acid production. In agreement with this study, a higher expression of proteins associated with different carbohydrates metabolism by plant *L. plantarum* strains was reported, which was attributed to energy metabolism adaption to different growth conditions, including biofilm mode of growth (De Angelis et al., 2015; Koistinen et al., 2007).

In addition, CRL1862 sessile cells exhibited a decreased expression of glycerol-3-phosphate dehydrogenase (GPDH) which belongs to the **glycerol/glycerolipid pathway** associated to the membrane

properties by the conversion of glycerol-3-phosphate to phosphatidic acid, which leads to the synthesis of membrane phospholipids. In agreement, a decreased expression of GPDH by *Streptococcus epidermidis* biofilm under microaerobiosis was also reported (Uribe-Alvarez et al., 2016).

Proteins involved in **translation** such as 50S ribosomal protein L6 (spot 87; RplF) was up regulated in sessile cells. Also aspartyl-tRNAsynthetase (spot 10; AspS) that catalyzes the specific charging of this amino acid residue on tRNAs during translation process, was overexpressed by *L. sakei* CRL1862 sessile cells; although other synthetases such as arginyl-, alanyl- and glycyl-tRNA were down-regulated. Even though, overexpression of other aminoacyl-tRNA synthetases was also reported in sessile cells of *Listeria monocytogenes* in the presence of enterocin AS-48 (Caballero Gómez et al., 2013). Likewise, up-regulation of these synthetases was reported for *L. plantarum* and *L. sakei* strains grown under food-like conditions (Belfiore et al., 2013; Fadda et al., 2010; Koistinen et al., 2007; Siragusa et al., 2014). Overexpression of aminoacyl-tRNA synthetases and the structural constituent of ribosome, 50S ribosomal protein L6 (RplF) was also found during growth at 42 ºC of the S-layer producer *L. acidophilus* IBB801 (Grosu-Tudor et al., 2016). RplF protein is able to react with several ribosomal and extra-ribosomal components serving to the organization and stabilization of rRNA tertiary structure (Maguire and Zimmermann, 2001). The trigger factor (spot 45; *tig*) that displays chaperone activity protecting newly synthesized proteins from misfolding and coupled to the translation process, was underexpressed by CRL1862 sessile cells. A similar result was found during biofilm formation by the oral *Strep. mutans* (Svensäter et al., 2001), while a higher expression by *L. plantarum* DB200 during biofilm formation on Caco2 cells was reported (De Angelis et al., 2015). The highly variable translational proteins expression patterns make impossible to predict the behavior of these proteins during *L. sakei* CRL 1862 sessile life style.

When compared to planktonic, CRL1862 cells grown in biofilm showed increase in the relative abundance of bifunctional DNA-directed RNA polymerase subunit beta-beta, *rpoBC* (spot 55) implicated in the **metabolism of nucleotides** (spot 55-). RpoBC, that catalyses the polymerization of ribonucleotides into a sequence complementary to the template DNA, was overexpressed by sessile cells of *L. sakei* CRL1862. DNA was described as a structural component of the extracellular matrix, having an important role in bacterial biofilm formation (Tetz et al., 2009). The extracellular DNA (eDNA), as a result of active secretion or controlled cell lysis, stimulate bacterial adhesion, ensuring the structural stability and contributing to biofilm resistance to antimicrobial agents. Indeed, as reported by Pérez Ibarreche et al.

(2014), *L. sakei* CRL1862 biofilms formed on abiotic surfaces, exhibited cells surrounded in a cloud of evidently visible material associated with filamentous structures suggesting the presence of eDNA. Thereby, up regulation of RpoBC may be related to the enhanced DNA synthesis as a constitutent of the biofilm matrix. Similarly, the expression of rpoBC protein was increased during early-and late-exponential-phase of growth by plant *L. plantarum* strains (Koistinen et al., 2007). On the opposite, two enzymes implicated in nucleotide metabolism and in other functions such as DNA repair and SOS response and DNA synthesis were found in lower amounts in sessile *L. sakei* CRL1862. These were the excinuclease ABC subunit B (spot 26) constituent of the UvrABC repair system that catalyzes the recognition / processing of DNA lesions and the DNA-directed DNA polymerase III subunit B (spot 36) a multichain enzyme responsible for replicative synthesis in bacteria which also exhibits 3' to 5' exonuclease activity.

On the other hand, the relative abundance of glutamate-ammonia ligase (GlnA), **related to aminoacid metabolism**, was increased at the highest level by *L. sakei* CRL1862 under biofilm conditions. Since the acetylated derivative of glucosamine (N-acetylglucosamine) is involved in the synthesis of cell-wall peptidoglycan (Barreteau et al., 2008), the positive regulation of this protein seems to be closely related to the production of new cells. In this sense, peptidoglycan pathway was reported to be up-regulated during biofilm formation by *Staphylococcus aureus* and oral *Strep. gordonii* (Loo et al., 2000; Resch et al., 2006). Moreover, *Strep. pneumoniae* mutants for glutamine synthesis genes exhibited deficiency to form biofilm and a important decrease in the adhesion to human pharyngeal epithelial cells, suggesting a role of this enzyme in host colonization (Kloosterman et al., 2006).

During biofilm growth, *L. sakei* CRL1862 also showed an increased expression of cysteine aminopeptidase C2 (PepC) involved in **peptide degradation**. This enzyme displays N-terminal hydrolytic activity on cysteine residues, suggesting the need of CRL1862 cells for amino acids and short peptides to growth and adapt to biofilm lifestyle. *L. sakei* species appears to be genetically homogenous with regard to its peptidolytic system, peptidases being reported to be nearly ubiquitous (Freiding et al., 2011). The role of peptidases during biofilm formation by oral microorganisms was reported by Wickström et al. (2009); this activity may enable *Strep. gordonii* to hydrolyse host proteins such as collagen and fibrinogen (or food debris) to obtain nutrients during oral colonization (Chaudhuri et al., 2008). Therefore, the up-regulation of this peptidase could provide advantages for the *L. sakei* CRL1862 growth under biofilm mode of life on meat environments.

Moreover, proteins related to **cell wall/envelope biosynthesis**, such as MurE, involved in the biosynthesis of D-alanyl-lipoteichoic acid, was underexpressed by CRL1862 sessile cells. In addition, D-alanine-poly(phosphoribitol) ligase subunit 1 *(dltA)*, that promotes the addition of meso-diaminopimelic acid to the nucleotide precursor UDP-N-acetylmuramoyl-L-alanyl-D-glutamate in the bacterial cell-wall peptidoglycan biosynthesis also were under-regulated indicating a decreased cell wall/envelope activity by CRL1862 sessile cells.

Some proteins coupled to translation, as mentioned above, and related to **stress conditions,** such as the trigger factor (spot 45; *tig*) was underexpressed in CRL1862 biofilm. Similarly, the Iron/Sulphur ABC transporter (spot 47; SufB homolog), involved in bacterial adaption to atypical conditions, or the preprotein translocase subunit SecA (spot 59;SecA), with **secretion function** through the cell membrane and carbamate kinase (ArcC, spot 72) involved in **arginine metabolism**, respectively, were synthetized in lower amounts by sessile *L. sakei* CRL1862 cells. These results indicate that biofilm mode of growth, would not represent a stressful condition for *L. sakei* CRL1862 cells. In consequenece this condition would not promote a stress-induced response in view that proteins related to adaption to atypical conditions and stress resulted under regulated.

Identification and expression of genes responsible for biofilm formation in *L. sakei* CRL1862

When LAB genes involved in biofilm formation were screened, the presence of nine out of ten genes involved in biofilm was evidenced in *L. sakei* CRL1862 (*lsa*0534, *lsa*0313, *lsa*1165, *lsa*1159, *lsa*0194, *ftsH*, *srtA1*, *srtA2*, *lamA*). The protein LSA0534 containing the LPXTG motif responsible for the covalent anchor to the cell wall was found to be similar to BAP (Biofilm Associated Protein), a *S. aureus* surface protein present in sessile cells (Chaillou et al., 2005). In addition, LSA0313/LSA0194 are homologs to lipoteichoic acid binding cell wall protein while LSA1165/LSA1159 proteins are homologs to a lipoteichoic acid binding cell wall and autoaggregation-promoting factors from intestinal lactobacilli from the S-layer-like family (Ventura et al., 2002). The presence of these genes in *L. sakei* CRL1862 may assist this strain to colonize SS surface lacking homology to other lactobacilli. Sortase enzymes function as transpeptidase anchoring surface proteins to the peptidoglycan and are found in all Gram-positive bacteria, including technological and health relevant LAB. Among them, SrtA is mostly present in Firmicutes, having a housekeeping role in the cell, capable of anchoring a large number of functionally distinct proteins to the cell wall by recognizing the LPXTG sequence (Spirig et al., 2011). *L. sakei* CRL1862 was shown to harbor *srtA1* and *srtA2* genes as was reported for *L. sakei* 23K (Chaillou et al.,

2005). In agreement, genes coding housekeeping sortase enzymes (*srtA*) have been detected in the genomes of a handful of LAB, such as probiotic strains (Call and Klaenhammer, 2013). The inactivation of the *srtA* gene in *Listeria monocytogenes* was reported to inhibit anchoring of surface proteins and affects its virulence (Bierne et al., 2002). The presence of *ftsH* gene in CRL1862 strain, as reported for *L. sakei* 23K, has been discovered in various other bacteria and were associated with stress resistance. FtsH protein is an ATP-dependent zinc metalloprotease anchoring with its ATPase and Zn^{+2} metalloprotease domains located in the cytoplasm. Additionally, the *lamA* (*lam* from *Lactobacillus agr*-like module) gene encoding a cyclic thiolactone autoinducing peptide involved in adherence regulation, was also detected in *L. sakei* CRL1862. In *L. plantarum* WCFS1, *lamA* gene is encoded from the *lamABCD* operon which is a two-component regulatory system homologous to the *agr* system of *S. aureus* and this gene is related to regulation of expression of genes encoding surface polysaccharides, cell membrane proteins and sugar utilization (Sturme et al., 2005). On the opposite, *lux*S gene that is responsible for the production of AI-2 signal molecules was absent in *L. sakei* CRL1862. Although a number of studies have been confirmed the role of LuxS enzyme during biofilm growth of different LAB species (Lebeer et al., 2007; Moslehi-Jenabian et al., 2011; Tannock et al., 2005; Yoshida et al., 2005), database analysis showed that *lux*S was absent in *L. sakei* (National Center for Biotechnology Information, NCBI). Moreover, our results are in agreement with studies of QS molecules in meat and LAB isolated from meat products which reported that LAB species different of *L. sakei* were responsible of AI-2 production (Blana et al., 2011; Blana and Nychas, 2014).

Finally, to elucidate the relationships among the expression of genes specifically related to biofilm formation under planktonic and sessile modes of growth, a complementary approach using qRT PCR revealed that four out of five genes exhibited more than 2-fold relative expression in *L. sakei* CRL1862 under biofilm growth, confirming their role in biofilm formation on SS surface. Specifically, *srtA2* was the highest regulated gene at the transcriptional level in CRL1862 sessile cells. As reported by Call and Klaenhammer (2013), both SrtA enzymes are required by probiotic LAB to produce the binding to colonic epithelial cell line, while inactivation of other sortases class did not affectthe adhesion. In summary, most of the genes related with adherence, autoaggregation-promoting factors and anchoring to the peptidoglycan in *L. sakei* species was evidenced and up-regulated in CRL1862 strain. This fact could explain its ability to form biofilm showing, in some cases, a dissimilar proteome expression compared to

other highly efficient biofilmogenic species which have a more complete genetic machinery, such as *Pseudomonas* or *Streptococcus* (Ahn et al., 2008; Sauer et al., 2002; Yoshida et al., 2015).

5. Conclusion

The results from this study contribute to extend the knowledge of the physiology of *L. sakei* CRL1862 growing in a biofilm mode of life on a typical food contact surface at lower temperature. Its adaptive strategy was mainly directed towards regulating glycolytic flux and reinforcing protein synthesis. In addition, several cell surface proteins involved in biofilm formation were up regulated at the transcription level, confirming their functionality during biofilm lifestyle. *L. sakei* CRL1862, is a technological interesting food associated strain with a great potential as meat starter and bioprotective culture that based on its biofilmogenic and bacteriocinogenic ability may be applied as an environmentally-friendly sanitation agent to reduce or inhibit *Listeria monocytogenes* biofilms on abiotic surfaces used in meat processing facilities. Thus, this study provides a valuable biotechnological tool for biofilm control to avoid post-processing contamination of RTE (Ready To Eat) foods with the final aims to preserve consumer's health.

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Figure captions

Fig. 1. Planktonic (a) and biofilm (b) growth of *L. sakei* CRL1862 in MRS at 10 ºC for 10 days. Arrows indicate the time when cells were harvested for protein and gene expression analyses

Fig. 2. 2DE gels showing *L. sakei* CRL1862 proteins expressed during planktonic (a) and biofilm (b) mode of growth during 6 days at 10 ºC. The successfully identified proteins are numbered and circled

Fig. 3. Relative abundance (%) of differentially expressed proteins in sessile *L. sakei* CRL1862 cells growing at 10 ºC during 6 days, grouped according to their functional categories. OE: overexpressed; UE: underexpressed

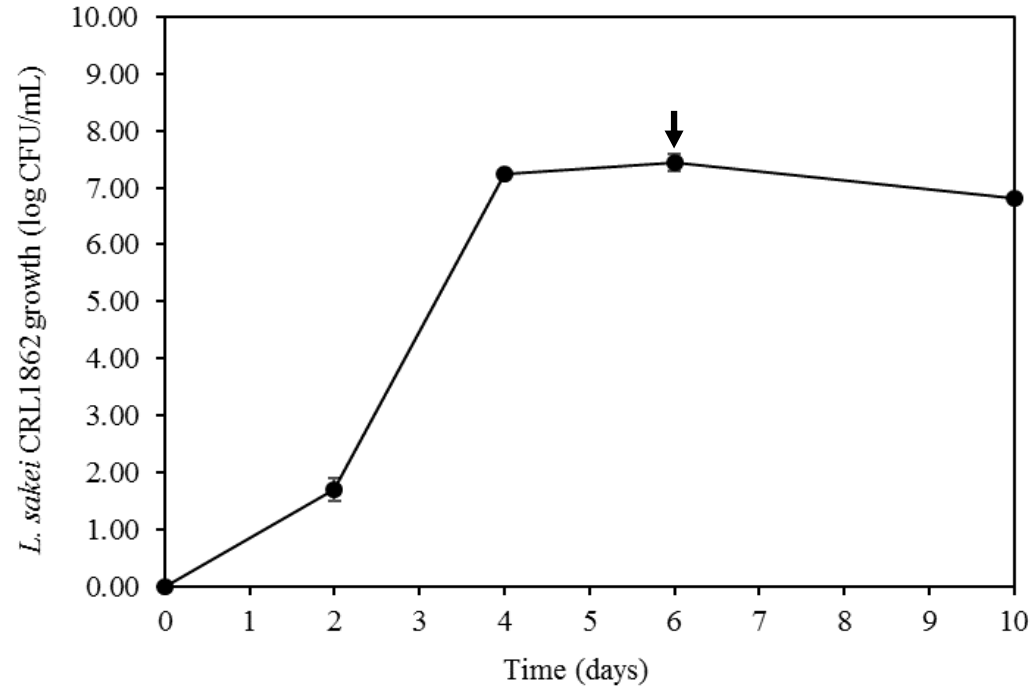
Fig. 4. PCR products detected in *L. sakei* CRL1862 corresponding to genes related to biofilm formation. MW: Molecular weight marker (100 bp). *lsa0194, lsa0313, lsa1159, lsa1165* coding for hypothetical surface proteins; *lsa0534* coding for anhypothetical surface protein with LPxTG motif; *ftsH*: coding for a membrane-integrated ATP-dependent metalloprotease; *srtA1, srtA2* coding for hypothetical proteins associated with anchor cell wall proteins (recognizing LPxTG motive); *lamA* coding for a two-component regulator system homolog to the staphylococcal Agr QS system

Fig. 5. Relative expression levels of *L. sakei* CRL1862 genes involved in biofilm formation. The comparison was carried out between sessile and planktonic cells. The calibrator condition used was planktonic cells grown during 6 days at 10 ºC. Data are the mean values of triplicate measurements ± standard deviations

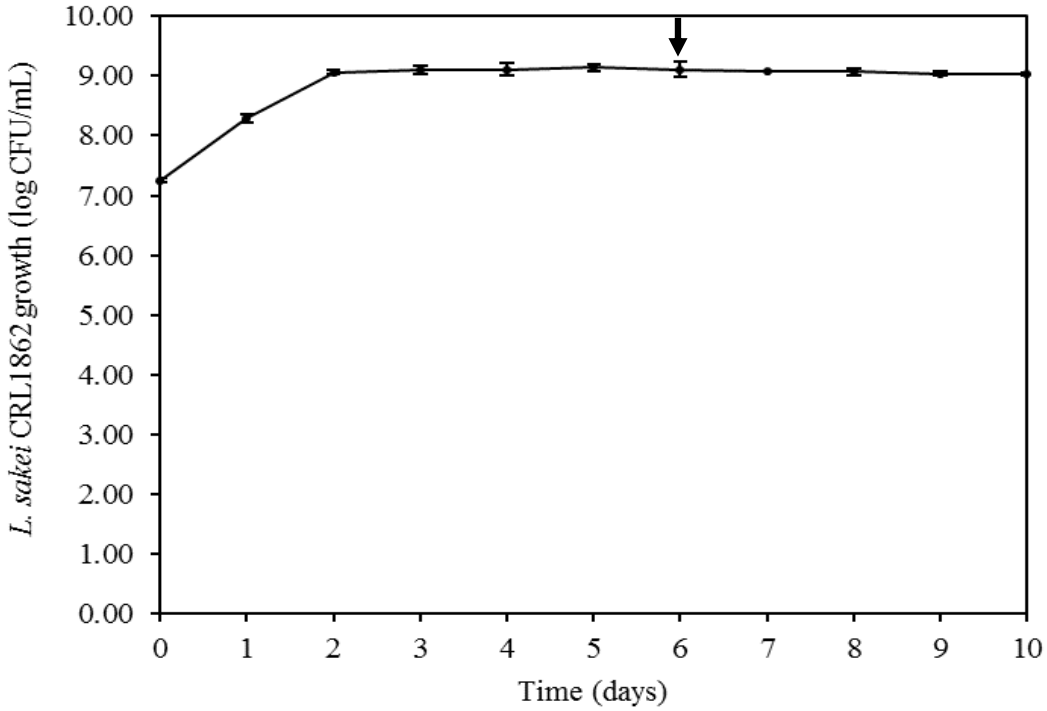
Fig. 6. Pentose phosphate pathway, showing the differentially expressed enzymes in biofilm and planktonic mode of growth of *L. sakei* CRL1862 at 10 ºC during 6 days. Bars represent relative expression of proteins in sessile (B) versus planktonic (P) cells. Boxes highlight enzymes involved in acid production and glutamine biosynthesis

Fig. 1

a



b



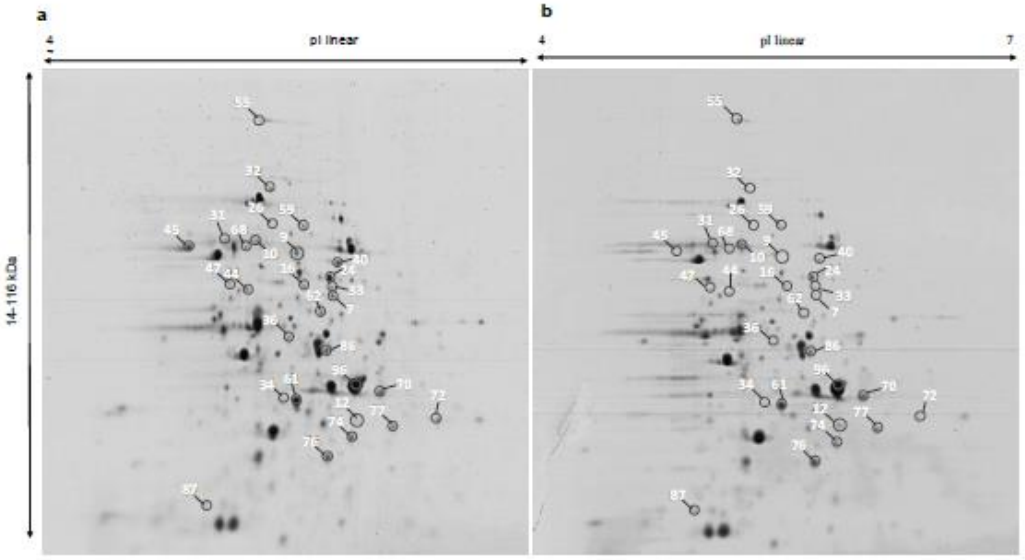


Fig. 2

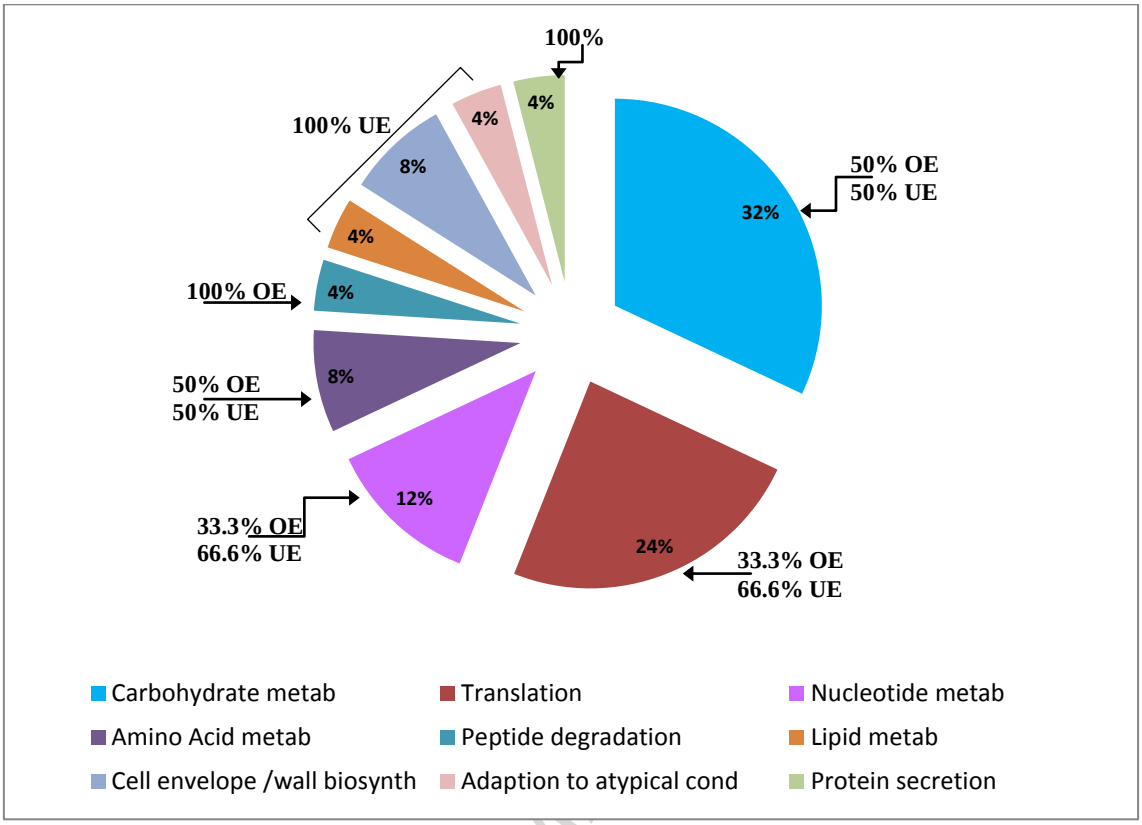


Fig. 3

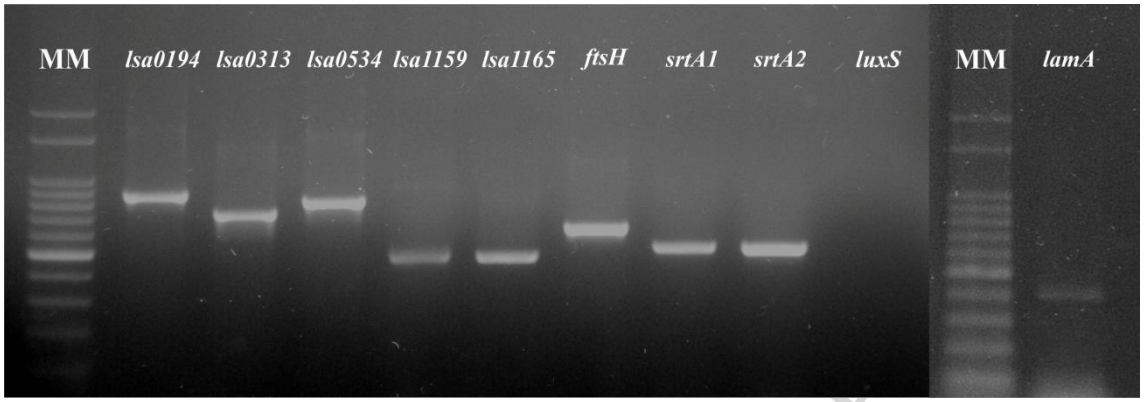


Fig. 4

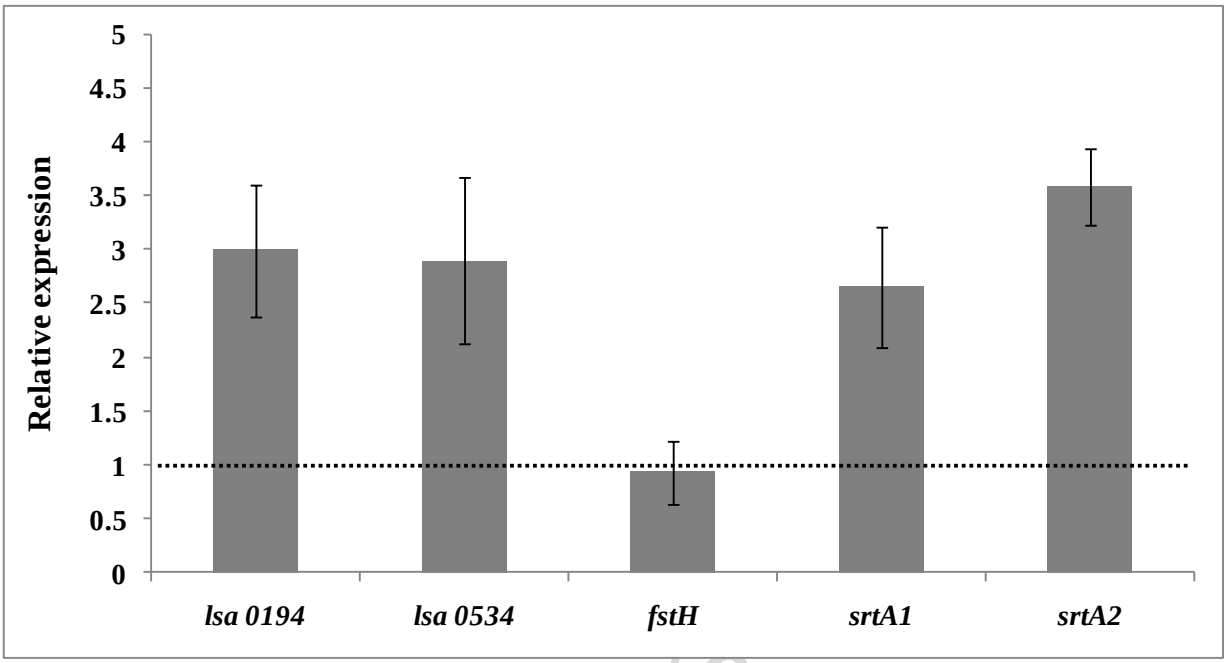


Fig 5

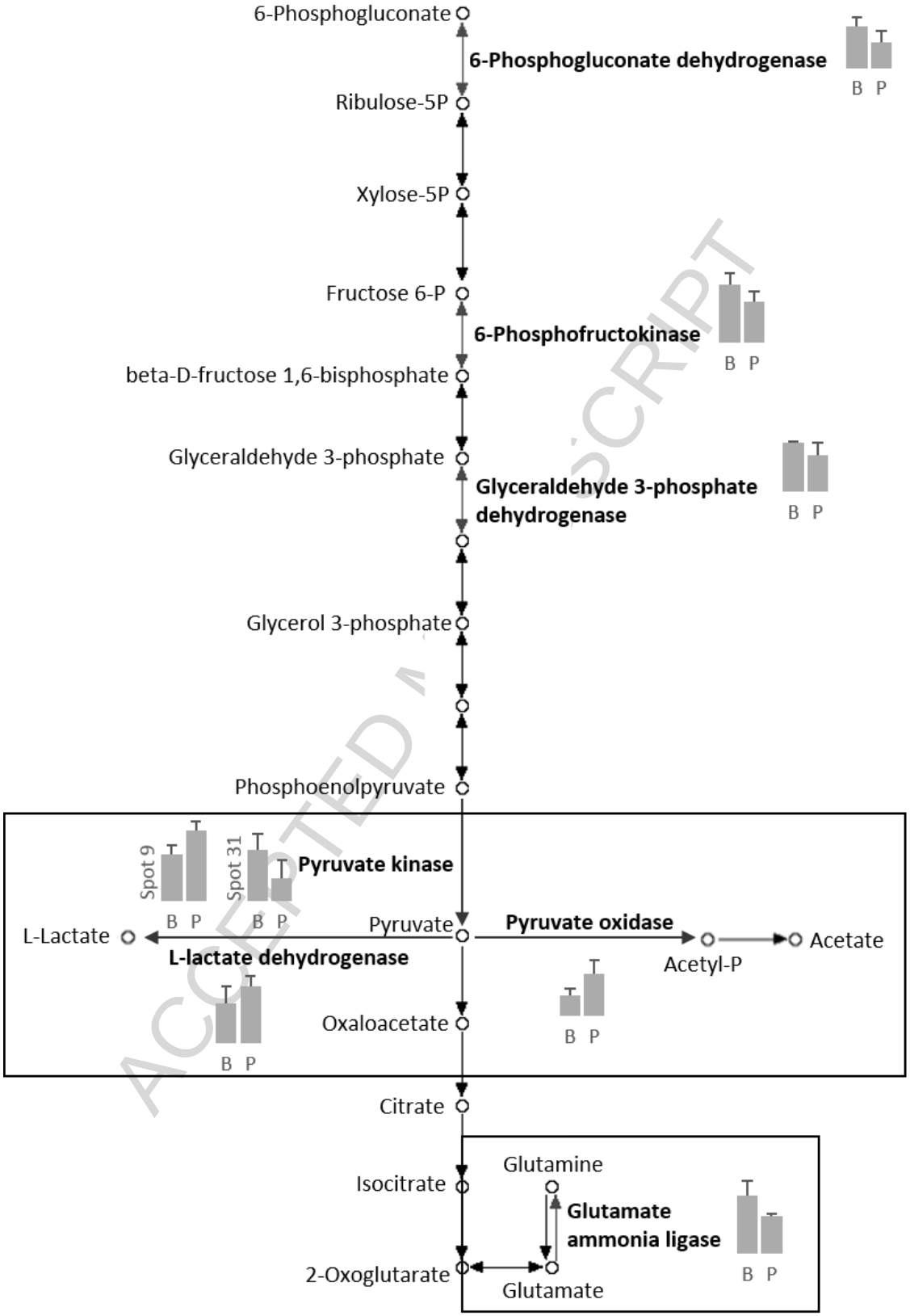


Fig 6.

Table 1. Differentially expressed proteins by sessile versus planktonic *L. sakei* CRL1862 cells at 6 days of growth at 10 ºC.

Function	Spot[a]	Protein name	B/P[b]	Score	MW (Da)[c] Theor/exp[e]	Gen/Protein	AccessionNº	pI [d] Theor/exp[e]
Carbohydrate metabolism	31	Pyruvate kinase	**2.11**	172	62998 /63020;	*pyk*/Pyk	G6CEW3	5.27/5.13
	9		**-3.35**	213	62925/61905		Q38WU7	5.27/5.52
	70	Glyceraldehyde 3-phosphate dehydrogenase	**1.61**	419	35565/35505	*gap*/GapA	Q5NJZ2	5.19/5.96
	96		**1.40**	215	35565/35510			5.19/5.85
	77	6-phosphofructokinase	**1.55**	108	34329/34239	*pfk*/Pfk	Q5QGJ0	5.36/6.05
	86	6-phosphogluconate dehydrogenase	**1.48**	97	52402/51806	*gndA*/GndA	Q38VU5	5.09/5.69
	16	Pyruvate oxidase	**-2.45**	103	62326/62120	*pox2*/Pox	Q38UJ8	4.99/5.56
	74	Phosphoglycerate mutase	**-1.85**	274	25849/25998	*gpm1*/Gpm1	A0A0R1SK80	5.04/5.82
	34	L-lactate dehydrogenase	**-2.01**	86	35402/35202	*ldh*/Ldh	Q38V71	4.99/5.43
	61		**-1.71**	237	35402/35200			4.99/5.50
	62	Glucose-6-phosphate 1-dehydrogenase	**-1.71**	90	56731/56691	*zwf*/G6pd	Q38YP5	5.07/5.65
Lipid metabolism	24	Glycerol-3-phosphate dehydrogenase	**-2.25**	241	66736/62656	*glpD*/GPDH	A0A0R1SKE5	5.13/5.70
	40		**-1.93**	240	66736/66620			5.13/5.75
Translation	68	Glycyl-tRNAsynthetase beta chain	**-1.63**	93	77733/67036	*glyS*	R9GIX6	4.68/5.24
	10	Aspartyl-tRNAsynthetase	**3.16**	100	67216/67110	*aspS*/AspS	Q38XB6	4.93/5.30
	7	Arginyl-tRNAsynthetase	**-3.64**	267	62933/61998	*argS*	Q38VQ9	5.02/5.71
	32	Alanyl-tRNAsynthetase	**-2.10**	215	97208/97099	*alaS*	Q38YN9	4.81/5.39
	87	50S ribosomal protein L6	**1.48**	75	19466/19236	*rplF*/RplF	Q38US6	5.42/5.00
	45	Trigger factor	**-1.88**	126	47862/47685	*tig*	Q38WR8	4.44/4.93
Nucleotides metabolism	55	Bifunctional DNA-directed RNA polymerase subunit beta-beta'	**1.79**	168	133850/115036	*rpoBC*	Q38UQ2	4.92/5.33
	26	Excinuclease ABC subunit B	**-2.19**	110	76468/74461	*uvrB*	Q38YA4	4.87/5.39
	36	DNA-directed DNA polymerase III subunit B	**-1.99**	113	41553/45689	*dnaN*	A0A0R1SSP4	4.86/5.49

Function	Spot[a]	Protein name	B/P[b]	Score	MW (Da) Theor/exp[e]	Gen/Protein	AccessionNº	pI Theor/exp[e]

Amino acid metabolism	12	Glutamate-ammonia ligase	**2.74**	115	50713/30560	*glnA*/GlnA	Q38W08	5.34/5.84
Amino acid metabolism	72	Carbamate kinase	**-1.58**	104	33575/30360	*arcC*	G3ADI5	5.18/6.29
Cell wall biosynthesis	33	UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	**-2.01**	90	56248/63063	*murE*	Q38YZ5	5.17/5.71
Cell envelope and cellular processes	44	D-alanine-poly(phosphoribitol) ligase subunit 1	**-1.89**	99	56287/61589	*dltA*	A0A0R1SNJ9	4.73/5.25
Peptide degradation	76	Cysteine aminopeptidase	**1.56**	101	51174/24854	*pepC2*	Q38UY9	5.09/5.69
Protein secretion	59	Preprotein translocase subunit SecA	**-1.77**	96	89465/74480	*secA*	Q38YD2	4.98/5.55
Adaption to typical conditions	47	ABC-type system involved in Fe-S cluster assembly (sufB homolog)	**-1.86**	118	52790/52710	LSA1109	Q38WM2	4.68/5.14

[a] Spot designations correspond to those of the gels shown in Fig. 2
[b] Relative Fold change: Normalized Volumes of protein spot in biofilm (B) /Normalized Volumes of protein spots in planktonic (P) condition.
[c] Molecular weight theoretical/experimental.
[d] Isoelectric point theoretical/experimental
[e] Differences in experimental and theoretical MW and pI could be ascribed to protein degradation, isoforms or postraslational modifications (PTM).

Highlights

- Proteomic response of *L. sakei* CRL1862 under biofilm growth at 10 ºC was analyzed
- Enzymes involved in sugar/nucleotide metabolism and translation were overexpressed
- Proteins related to stress/adaption to atypical conditions were under produced
- CRL1862 harbors 9 genes related to biofilm; 4 of them were overexpressed (qRT-PCR)
- A contribution to physiology of CRL1862 biofilm on a food contact surface is provided
- CRL1862 could be an eco-friendly sanitation agent against Listeria on abiotic surfaces