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Molecular basis of the adaption of the anchovy isolate *Lactobacillus sakei* CRL1756 to salted environments through a proteomic approach

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ABSTRACT

Osmotic stress triggered by high salt concentrations results in the injury of starters during food processing. The adaption of the anchovy isolate *Lactobacillus sakei* CRL1756 to salted environments in the presence of osmoprotectants was analyzed by using a proteomic approach. Glycine–betaine (GB) pre-treated cells exposed to 1.7 M NaCl + 3 mM GB showed improved adaption achieving growth after a long lag phase while non-proliferative cells in the salted medium without GB was observed. Comparative proteomic analyses of the *Lactobacillus* cells grown in MRS + GB exposed or not to 10% salt revealed eighteen significantly regulated proteins. Under hypertonic stress, decreased synthesis of four glycolytic enzymes (Fba, Pgk, Gpm5, Tpi) and induction of other two (MleS, Pox2) related to an alternative energetic pathway, were registered. Proteins related to general stress response and nucleotide metabolism were up-regulated. Noticeably, the induction of DyP-type peroxidase, involved in iron transport, detoxification and oxidative stress, was observed for the first time in lactic acid bacteria under osmotic constraint. Our results demonstrate that GB played a significant role in protecting *L. sakei* CRL1756 against salt stress. Indeed, GB commonly found in marine fish may be used by the starter culture cells ensuring their robustness in salted-anchovy based products.

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1. Introduction

Lactobacillus sakei is a species widely used as a starter culture for sausage fermentation and has been applied as biopreservative culture for fresh and processed meat and fish (Vignolo, Saavedra, Sesma, & Raya, 2012). The prevalence of *L. sakei* in such a variety of habitats points out its potential to adapt and/or to compete in different ecosystems. The genome sequence of *L. sakei* 23K has revealed specialized metabolic capacities that reflect its adaption to meat environment exhibiting notable differences in physiological and biochemical properties compared to other lactobacilli (Chaillou et al., 2005). Many genes appeared to be responsible for *L. sakei* robustness during the rigor of food processing, particularly in the presence of changing environmental (redox, water activity — a_w, temperatures and pH) conditions (Champomier-Vergés, Chaillou, & Zagorec, 2002; Marceau, Zagorec, Chaillou, Mera, & Champomier-Verges, 2004).

It is known that to promote their survival, bacteria have evolved quick and complex responses under growth restrictive conditions such as starvation, high temperatures, salinity and changes in pH. Because the osmolarity of the environmental medium is one of the most variable parameters, much attention has been paid in recent years to understand the adaption mechanisms of bacteria to this stressful condition (Parente et al., 2010; Xie, Chou, Cutler, & Weimer, 2004). A sudden increase in the osmolarity of the environment results in the movement of water from the cell to the outside, which causes a detrimental loss of cell turgor pressure changing the intracellular solute concentration and cell volume. One of the most powerful adaptive strategies that bacterial cells have developed for their acclimation to osmotic stress involves intracellular accumulation of small organic compounds, the so-called compatible solutes, which equilibrate cellular osmotic pressure (Wood et al., 2001), this being regarded as largely protein synthesis independent mechanism. These osmolytes such as glycine–betaine (GB), proline, trehalose, and glycerol may also protect native proteins from denaturation and favor the formation of native protein oligomers, some of these behaving as "chemical chaperones" by promoting the correct refolding of unfolded proteins in vitro and in the cell (Diamant, Eliahu, Rosenthal, & Goloubinoff, 2001). Such compatible solutes occur widely in food and other environments where they may protect bacterial cells from deleterious effects of hyperosmolarity. In lactic acid bacteria (LAB), GB was reported to serve as the major effective accumulating osmoprotectant from an exogenous supply, its transport appearing to be activated by exposure of the cells to high osmotic pressure (Diamant et al., 2001). However, in order to survive in high salt concentrations for extended periods of time, cells must adapt to the new conditions. Such adaption, also known as acclimation, is a long-term process along which a program of gene expression is initiated and manifested as an increased or decreased amount of a set of proteins synthesized in

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response to stress (Duché, Trémoulet, Glaser, & Labadie, 2002). Proteomics together with bioinformatics constitutes a complement of the huge amount of DNA information from genome sequencing projects providing new approaches for bacterial functional genomics (Champomier-Vergés, Zagorec, & Fadda, 2010). In particular, LAB adaption to different stress challenges has been illuminated by this approach (Champomier-Vergés et al., 2002; De Angelis, Bini, Pallini, Cocconcelli, & Gobbetti, 2001; Fadda et al., 2010).

Recently, LAB population present in Argentinean fresh anchovies was described and species from the genera *Leuconostoc* and *Lactobacillus* have been identified as predominant microorganisms (Belfiore, 2010; Belfiore, Björkroth, Vihavainen, Raya, & Vignolo, 2010). In particular, the strain *L. sakei* CRL1756 exhibits a high tolerance to NaCl (10–15%) and displays some metabolic activities of technological importance such as the ability to degrade anchovy proteins with the concomitant production of flavor compounds (peptides and free amino acids) as well as the inability to produce biogenic amines (Belfiore, 2010). Although certain osmotolerant characteristics of the *L. sakei* species has already been reported (Marceau et al., 2004), this study was conducted to gain further insight in the osmoadaptive strategy of the anchovy isolate *L. sakei* CRL1756 subjected to salted conditions mimicking that of anchovy fermentation. The role of different osmoprotectants and the proteins affected by the presence of high NaCl concentration were evaluated applying physiologic and comparative proteomic approaches.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The strain *L. sakei* CRL1756 was isolated from fresh anchovies (Belfiore, 2010). A single colony grown in MRS broth (Oxoid, Bioartis, Argentina) at 30 °C for 24 h was used for further inoculation and stored at −80 °C in the same liquid media containing 30% (v/v) glycerol.

2.2. Osmoprotectant selection and NaCl adaption

Overnight culture of *L. sakei* CRL1756 was used to inoculate (OD_{560}: 0.1) fresh MRS with and without the putative osmoprotectant: glycine–betaine (GB), carnitine or proline (Sigma-Aldrich, Bs As, Argentina). Osmoprotectants were tested in the range of 1.5 to 3.0 mM. After incubation for 16–18 h at 30 °C, the pre-adapted *L. sakei* cells were reinoculated at an initial OD_{560}: 0.1 in fresh MRS broth and in MRS broth plus the aforementioned compatible solutes supplemented or not with 10% of NaCl and incubated at 30 °C during 144 h (6 days). Samples were taken periodically and OD_{560}, pH and cell viability in MRS agar (30 °C for 48 h) were determined.

2.3. Extraction of cell-free proteins

For two dimensional electrophoresis (2DE) *L. sakei* CRL1756 was grown overnight in MRS + GB (3 mM) at 30 °C and then used to inoculate 250 ml of MRS + GB (3 mM) supplemented or not with 10% NaCl at initial optical density OD_{560}:0.1; cultures were then incubated until the mid-exponential growth phase was reached (OD_{560}: 0.6) (3.5 h for MRS + GB + 0% NaCl and 72 h for MRS + GB + 10% NaCl). Cells were harvested by centrifugation (7000 *g*, 10 min at 18–20 °C) and washed twice in 0.1 M Tris–HCl buffer, pH 7.5 for 15 min. Bacterial pellets were resuspended in 5 ml of 0.1 M Tris–HCl buffer pH 7.5 and cells were broken by passage through a French press at 1000 psi (SLM Instruments, Inc., Haverhill, MA, USA). Unbroken cells and cell debris were removed by centrifugation (14,000 *g*, 10 min at 4 °C) and cell membrane fraction was discarded from the solution by ultracentrifugation at 50,000 *g* for 30 min at 4 °C (Beckman, MA, USA). The protein concentration was determined by the Bio-Rad protein assay (BioRad, Richmond, CA) and aliquots of 400 μg were stored at −80 °C until the isoelectrofocusing assay.

2.4. Two-dimensional electrophoresis (2DE)

Sample preparation and 2DE gels were carried out according to Fadda et al. (2010) with some modifications. Briefly, samples containing 400 μg of intracellular proteins of *L. sakei* CRL1756 (100 μl) were treated with 1 μl of bezonase (Novagen®) at 37 °C for 30 min and 3 volumes of cold acetone were added to discard NaCl interference. After incubation at −20 °C for 16 h, samples were centrifuged (3500 rpm, 10 min), the supernatant was carefully removed and the protein pellets were air-dried. Proteins were then solubilized in 40 μl of solubilization mixture, centrifuged (3500 rpm, 10 min) and loaded onto immobilized pH gradient strips (pH 4.0 to 7.0, 18 cm, GE Health Care, Sweden). Gels were passively rehydrated for 20 h. The isoelectrofocusing assay was performed using IPGphor (GE, HealthCare, Sweden) at 53,500 V/h. The second dimension was performed by SDS-PAGE on gels containing 12.5% (w/v) polyacrylamide and carried out in a Bio-Rad Protean II xi cell (Biorad, Richmond, CA). Proteins were resolved overnight at a constant current of 10 mA/gel at 4 °C. Gels were stained with Biosafe colloidal Coomassie blue (BioRad, Richmond, CA, USA) and digitalized.

2.5. Image acquisition and data analysis

Digitalized images (300 dpi) of stained gels were aligned using the software Prodigy SameSpots version 1.0.3400.25570 (Nonlinear Dynamics Group, UK). Briefly, prominent spots were used to manually assign vectors to each gel image and the automatic vectors feature of the software was used to add additional vectors, which were manually verified. These vectors were used to warp the images and align the spot position to a common reference gel. Spot detection performed according to this reference gel was edited and artifacts removed. To correct the variability due to staining and to reflect the quantitative variation in intensity of protein spots, the spot volumes were normalized as a percentage of the total volume in all spots in the gel. A spot was considered significant when its resulting normalized volume showed more than 1.49 fold variation with respect to the control (MRS + GB 0% NaCl) at the level of $p<0.05$. At least 3 biologically independent and 2 technical replicates for both assayed conditions were performed.

2.6. Protein identification by peptide mass fingerprinting

Stained proteins displaying significant and reproducible intensities that differed among the assayed growth conditions (at least 1.5 fold variation, $p<0.05$) were excised from the gels and submitted to tryptic digestion; peptides being 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 157 (Applied Biosystems, Foster City, USA). Mass data acquisitions were piloted by 4000 Series Explorer. Software v3.0 with batched-processing and automatic switching between MS and MS/MS modes was applied. All MS survey scans were acquired over the mass range 800–3500 m/z in the reflectron positive-ion mode and accumulated from 2000 laser shots with acceleration of 20 kV. Mass spectrometry analysis was performed at CEQUIBIEM (Centro de Estudios Químicos y Biológicos de Espectrometría de Masa), Facultad de Ciencias Exactas y Naturales, UBA, 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* 23 K from the annotated genome (http://migale.jouy.inra.fr/sakei/genome-server).

3. Results and discussion

3.1. Growth of L. sakei CRL1756 under high salinity conditions. Effect of osmoprotectants

The studied strain was cultured at 30 °C in MRS broth containing or not 10% (1.7 M) NaCl. Growth rates were significantly affected by NaCl decreasing from 0.331 h^{-1} in the absence of NaCl to less than 0.015 h^{-1} in the presence of 10% of salt (Fig. 1). Similar results were observed with *L. sakei* 23 K and *Oenococcus oeni* IOEB 8413 grown in chemically defined medium with ~1.5 and 0.8 M NaCl, respectively (LeMarrec, Bon, & Lonvaud-Funel, 2007; Marceau, Zagorec, & Champomier-Vergés, 2003) and with *Lactobacillus rhamnosus* HN001 (DR20) grown in a 0 to 0.7 M NaCl-supplemented rich medium (Prasad, McJarrow, & Gopal, 2003). Pre-growth of *L. sakei* CRL1756 in the presence of increasing NaCl concentrations did not substantially improve its growth in 10% NaCl (data not shown) in contrast to *Lactobacillus acidophilus* LA1-1 whose exposure to sublethal NaCl concentration conferred it protection to lethal osmolarities (Kim, Perl, Park, Tandianus, & Dunn, 2001).

A direct relationship between the presence of osmoprotectant solutes and the ability of LAB to survive osmotic stress was established (Baliarda et al., 2003; Obis, Guillot, & Mistou, 2001). Thus, three different osmoprotective agents were assayed in this study. GB, carnitine or proline was added to MRS + 10% NaCl; only 3 mM GB showed a significant osmoprotectant effect to *L. sakei* CRL1756 reducing the lag phase from 72 to 30 h (Fig. 1). Carnitine was less effective in the recovery of cell growth (>48 h of lag phase) while proline did not relieve growth inhibition at the assayed concentrations. It has also been shown that the nature of compatible solute differs according to the genus studied. For instance, although growth restoration by GB against a high external osmolarity was reported for many LAB (Obis et al., 2001), it was unable to relieve growth inhibition of *O. oeni* under hyperosmotic stress (LeMarrec et al., 2007). Moreover, the uptake rate for GB by *Lactobacillus plantarum* ATCC14917 increased instantaneously upon the osmolarity increase and was more pronounced when cells were pre-grown at high osmolarity; thus, transport systems were not induced but were activated by exposure of cells to high osmotic pressure (Glaasker, Konings, & Poolman, 1996). In the case of *L. sakei* CRL1756, the long lag phase observed for GB pre-adapted cells in the presence of 10% NaCl may suggest the induction of transport systems for compatible solutes. Possibly the transporters responsible for allowing the passage of GB into *L. sakei* CRL1756 cells, were overexpressed under the studied conditions, although they were not detected in the proteomic study (see below) since only cytoplasmic proteome was analyzed. On the other hand, the ability to efficiently accumulate osmo- and cryo-protective compounds such as betaine and carnitine was reported as key factors for the acclimation to cold and salt of *L. sakei* 23 K; the accumulation of these compounds being driven by three ABC uptake systems together with a Na^+-dependent symporter (Chaillou et al., 2005). The different efficiency between GB and carnitine for restoring growth of *L. sakei* CRL1756 under salting conditions may be explained by the different affinity of transporters responsible for GB and carnitine uptakes. In this sense, Glaasker, Heuberger, Konings, and Poolman (1998) reported that the transporter for glycine–betaine of *L. plantarum* ATCC 14917 (QacT), an ATP-dependent uptake complex, exhibited higher affinity for glycine–betaine and carnitine than for proline. Regarding proline behavior, the absence of specific transporters for proline, as reported for *L. sakei* 23 K (Chaillou et al., 2005), can explain the inability of this osmolyte to restore cell growth in the studied strain.

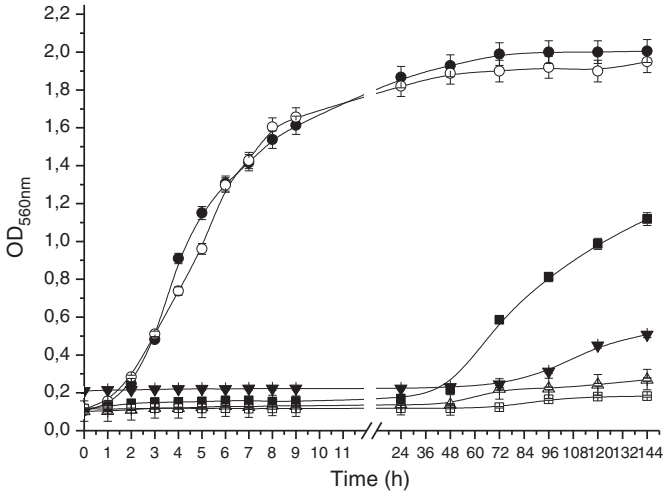


Fig. 1. Growth of *L. sakei* CRL1756 (pre-adapted with 3 mM of each osmoprotectant) at 30 °C in MRS (•); MRS + 10% NaCl (□); MRS + GB (○); MRS + GB + 10% NaCl (■), MRS + proline + 10% NaCl (Δ) and MRS + carnitine + 10% NaCl (▼).

3.2. Proteomic patterns of L. sakei CRL1756 grown in the presence of GB with or without 10% NaCl

To unravel *L. sakei* CRL1756 functions involved in the response to osmotic constraint in the presence of GB, a proteomic approach was applied. After the GB pre-adaption, cells grown in MRS + GB with and without 10% NaCl were harvested at mid-log phase of growth (Fig. 1) and their intracellular proteins submitted to 2DE to identify those putatively involved in the salt response. The genome data of *L. sakei* 23 K (Chaillou et al., 2005) were used to assign genes encoding proteins experimentally obtained by comparison of their peptide mass fingerprinting. The number of spots detected on 2DE gels prepared with proteins from cells exposed to 10% NaCl was 29% lower than that without NaCl, this fact was reflected in the total protein concentration since salt stressed cells showed 30% lower protein than cells grown in control conditions (data not shown). Two-dimensional protein patterns revealed a total of 50 spots differentially expressed under the assayed conditions. Eighteen out of 21 statistically significant spots, considered up- or down-regulated by a factor greater than 1.5 in the presence of NaCl, were identified by MaldiTof–MS (Fig. 3). The differential expression levels ranged from 8.8 to 1.4 fold variations ($p<0.05$). Seven proteins (spots 1, 2, 9, 11, 12, 14 and 15) were only detected in the presence of salt and 4 proteins (spots 7, 19, 22 and 86) were overexpressed with variable intensities (Fig. 2). On the other hand, 7 spots (3, 5, 6, 8, 16, 17 and 18) were synthesized in lower amounts by *L. sakei* cells growing under salt stress (Fig. 2). Identified proteins belonged to different functional categories involved in general stress response, carbohydrate metabolism, lipid and nucleotide metabolism, adaption to atypical conditions as well as inorganic ions transport and detoxification processes (Fig. 2; Table 1). The increased production of stress proteins such as chaperones and proteases is a widely distributed mechanism of cell protection (Koponen et al., 2012). In this study, a set of proteins associated with stress-responsive pathways were induced and overexpressed. Hsp20 (spot 1) and ClpB (spot 2) were detected among the most abundant (8.3- and 4.4-fold change, respectively) in the presence of 10% NaCl (Table 1). In addition, the overexpression of ClpL ATPase, putative specificity factor for CLpP protease (spot 7); GrpE (spot 19) and DnaK (spot 86) occurred during growth of *L. sakei* CRL1756 under osmotic stress. In particular, ClpL and ClpB from Clp proteolytic complex exhibiting both chaperone and proteolytic activities are widely conserved in bacteria; in association with the proteolytic core, the ClpATPases are responsible for cellular quality control systems by refolding or degrading damaged proteins in stressed and non-stressed cells (Frees, Savijoki, Varmanen, & Inhmer, 2007). The induction of *clpB* gene has been previously reported during growth of *Bifidobacterium breve* in the presence of 0.7 M NaCl (Ventura, Kenny, Zhang, Fitzgerald, & van Sinderen, 2005). As regards the chaperone DnaK overexpressed by *L. sakei* CRL1756 in this study, overexpression of this protein was first described in *Escherichia coli* under high osmolarity suggesting a role in desplasmolysis promotion which is

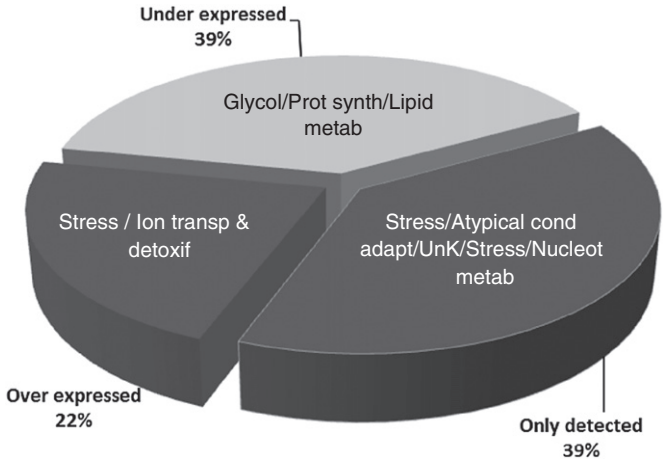


Fig. 2. Expression percentages and cellular functions of *L. sakei* CRL1756 identified proteins significantly modified during its growth in MRS + GB + 10% NaCl. Glycol: glycolysis; Prot synth: protein synthesis; Lipid metab: lipid metabolism; Atypical cond adapt: Atypical conditions adaption; UnK: unknown function; Nucleot metab: Nucleotides/nucleic acids metabolism; Ion transp & detoxif: inorganic ions transport and detoxification process.

ensured by a net osmo-dependent K^+ influx (Meury & Kohiyama, 1991). Moreover, DnaK and ClpE up-regulation was also reported to be triggered in *Lactococcus lactis* as a response to osmotic stress (Zhang, Zhang, Zhu, Mao, & Li, 2010). It has also been shown that DnaK and GrpE proteins were up-regulated under other stress challenges such as acidic conditions while they were also induced during heat adaption in *L. plantarum* DPC2739 (De Angelis et al., 2004). Moreover, the protein from the Hsp20 family regulated by *L. sakei* CRL1756 was reported to be induced by *Debaryomyces hansenii* upon exposure to 8–12% NaCl (Gori et al., 2007) and by *B. breve* in response to osmotic stress and severe heat shock regimens (Ventura, Canchaya, Zhang, Fitzgerald, & van Sinderen, 2007). Although the control of stress protein expression is variable among bacteria, the highly conserved Hsps are important for protection against environmental stress producing tolerance to high temperatures, high salt and heavy metal concentrations (Ron, 2006).

Interestingly, two proteins involved in the adaption to atypical conditions (putative ferritin-like DNA-binding protein, LSA1782) and related to inorganic ion transport and detoxification processes (iron-containing dye-decolorizing peroxidase-DyP-type peroxidase-, LSA1831) were overexpressed by *L. sakei* CRL1756 in the presence of 10% NaCl (Table 1). The DNA-binding ferritin-like protein is a member of the bacterial Dps (DNA-binding Protein from Starved cells) family that can form DNA-Dps cocrystals to protect DNA from damaging environments while the DyP-type peroxidase is involved in the regulation of iron transport proteinases as well as in hydrogen peroxide detoxification process (Horsburgh, Clements, Crossley, Ingham, & Foster, 2001; Zhao et al., 2002). DyP proteins have wide substrate specificity, lack homology to most other peroxidases and have the ability to function under lower pH conditions. The proteins consist of an N-terminal domain and a C-terminal domain and its heme iron is penta-coordinated. A conserved Asp most likely acts as a proton donor/acceptor and takes the place of the catalytic histidine used by plant peroxidases. This Asp substitution helps to explain why the DyP family is active at low pH (Sugano, 2009). DyP-type peroxidase, LSA1831, composed of 318 amino acids with a molecular mass of 36 kDa and pI of approximately 4.6, was involved in peroxide reducing mechanisms for protein activity restoration after oxidation damage (Chaillou et al., 2005). Our study describes for the first time the potential involvement of NaCl in the induction of the DyP-type peroxidase in a *L. sakei* strain. In fact, sodium chloride is known as a potent pro-oxidant that could act as an oxidant agent inducing proteins involved in adaption to atypical conditions. Recently, it was shown that environmental salinity determines the specificity and need for a DyP-type peroxidase secretion in *Bacillus subtilis* (Van der Ploegl et al., 2011). The other protein induced by *L. sakei* under hypertonic stress is the ferritin-like-DNA-binding protein (LSA1782). Although lactobacilli are generally considered as not requiring iron, the heme-dependent catalase for *L. sakei* indicates that iron or heme acquisition is correlated with bacterial resistance to oxidative stress (Hertel, Schmidt, Fisher, Oellers, & Hammes, 1998). The protective activity of Dps protein family was suggested to be induced by oxidative and nutritional stress and exerted by three intrinsic properties of the protein as DNA binding, iron sequestration, and its ferroxidase activity, thus avoiding oxidative damage (Calhoun & Kwon, 2010; Zhao et al., 2002). The induction of DNA-binding ferritin like protein from Dps family in the presence of glutathione was reported in *Lac. lactis* and *E. coli* as a response to osmotic challenge (Weber, Kögl, & Jung, 2006; Zhang et al., 2010). Interestingly, McLeod, Zagorec, Champomier-Vergés, Naterstad, and Axelsson (2010) reported that *L. sakei* MF1053 isolated from fermented fish exhibited the highest expression of the ferritin-like DNA-binding protein when grown in glucose and ribose as carbohydrate source. In the presence of exogenous heme sources some LAB have been shown to be genetically equipped for energetically favorable aerobic respiration metabolism which leads to less oxidative and acid stress during growth (Lechardeur et al., 2011). Since meat and fish muscles are iron-rich substrates and reactive oxygen species may be produced by enzymatic or spontaneous processes, it can be hypothesized that the induction of ferritin-like DNA-binding protein by *L. sakei* CRL 1756 could occur during growth on these raw materials to cope with oxidative stress provoked during salt curing process.

A varied expression of proteins from other cell processes such as from the carbohydrate metabolism in *L. sakei* CRL1756 occurred in the presence of 10% NaCl (Table 1; Fig. 3). Differential under-regulation for triosephosphate isomerase (LSA0606, spot 6), phosphoglycerate mutase (LSA1855, spot 8), phosphoglycerate kinase (LSA0605, spot 16) and fructose bisphosphate aldolase (LSA1527, spot 17) involved in the glycolytic pathway was observed in the presence of salt (Fig. 4). The decreased expression of these enzymes was reported for *L. rhamnosus* HN001 (DR20) in response to heat and osmotic stress (Prasad et al., 2003) and in *L. sakei* 23 K during its adaption to cold temperatures and NaCl (Marceau et al., 2004). These results suggest that the main pathway for lactobacilli carbohydrate metabolism is generally impaired when they are exposed to osmotic stress. In addition, comparing to *L. sakei* strains from different sources the induction of the glycolytic enzymes Fba (LSA1527) and Gpm5 (LSA1855) was reported in a lower extent in most of the strains, however, the strain from fermented fish (MF1058) exhibited the lowest expression for the glycolytic enzyme (Pgk, LSA0605) compared to the rest of the strains. It is likely that *L. sakei* from fish origin has a more efficient down-regulation mechanism for the glycolytic pathway (McLeod et al., 2010). Conversely, a high synthesis rate of glycolytic enzymes was reported for *Lac. lactis* subjected to heat treatments and to osmotic stress in the presence or absence of glutathione (Zhang et al., 2010). It is well known that LAB may change their metabolism in response to various conditions, resulting in a different end-product pattern than with glucose fermentation under normal conditions. Pyruvate oxidase (LSA1830, spot 15) and malate dehydrogenase (LSA0441, spot 9) induction in *L. sakei* CRL1756 exposed to osmotic stress may be an alternative way of utilizing pyruvate than reduction to lactate (Table 1). The overexpression of Pox2 (LSA1830) might create a pool of acetyl phosphate which could be converted into acetate and ATP guaranteeing *L. sakei* survival. Pyruvate oxidase has been suggested to be involved in the aerobic metabolism of *L. plantarum*, which forms significant amounts of acetic acid (Sedewitz, Schleifer, & Götz, 1984). On the other hand, overexpression of cytosolic malate dehydrogenase may also enhance metabolic flux toward organic acids in *E. coli* (Liang et al., 2011). From the results obtained, it can be hypothesized that *L. sakei* CRL1756 induced an alternative energetic pathway accumulating organic acids other than lactic acid to counteract the down-regulation of glycolytic enzymes under osmotic stress (Fig. 4).

Table 1
Main identified proteins of *L. sakei* CRL1756 significantly modified during its growth in MRS + GB + 10% NaCl.

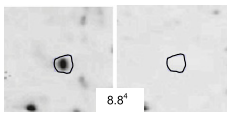
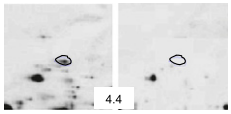
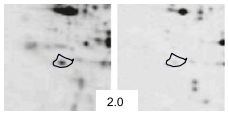
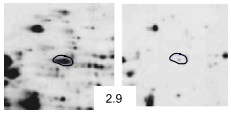
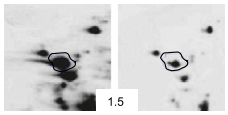
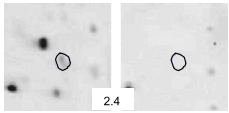
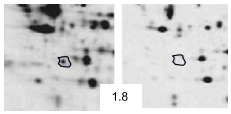
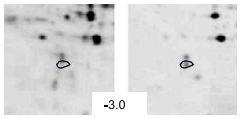
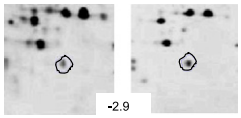
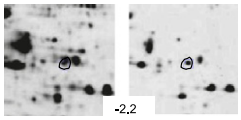
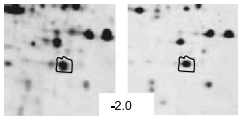
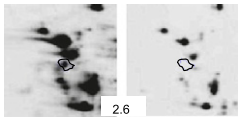
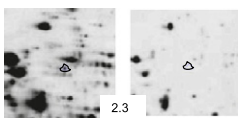
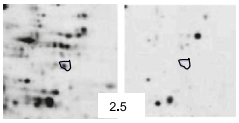
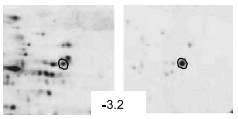
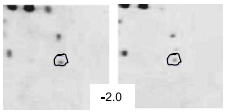
Spot no./protein	Function	Proteomic change Salt/control
1. Hsp20 (LSA0050[1] *lsa50*[2]; 16844[3])	Stress response	
2. ClpB (LSA1040; *clpB*; 96937)	Stress response	
19. Chaperone GrpE (LSA1237; *grpE*; 22520)	Stress response	
7. ClpL ATPase protein (LSA0207; *clpl*; 78400)	Stress response	
86. Chaperone DnaK, (LSA1236; *dnaK*; 66250)	Stress response	
14. Ferritin-type DNA-binding protein (LSA1782; *lsa1782*; 18040)	Atypical conditions adaptation	
22. Dyp-type peroxidase (LSA1831; *lsa1831*; 35719)	Inorganic ions transport and detoxification	
6. Triosephosphate isomerase (LSA0606; *tpi*; 27480)	Glycolysis	
8. Phosphoglycerate mutase (LSA1855; *gpm5*; 25836)	Glycolysis	
16. Phosphoglycerate kinase (LSA0605; *pgk*; 42700)	Glycolysis	
17. Fructose bisphosphate aldolase (LSA1527; *fba*; 30979)	Glycolysis	
9. Malate dehydrogenase (LSA0441; *mleS*; 59331)	Carbohydrates metab and related molecules	
15. Pyruvate oxidase (LSA1830; *pox2*; 66727)	Carbohydrates metab and related molecules	

Table 1 (*continued*)

Spot no./protein	Function	Proteomic change Salt/control
12. Adenylosuccinate synthetase (LSA0063; *purA*; 46823)	Nucleotides/nucleic acids metabolism	
5. Arginyl-tRNAsynthetase (LSA1421; *argS*; 62933)	Translation	
18. Enoyl-(acyl carrier) reductase (LSA0823; *fabI*; 26786)	Lipid metabolism	

[1] accession number; [2] gene; [3] molecular mass (Da); [4] Proteomic fold change.

As shown in Table 1, salt stress also induced adenylosuccinate synthase (*pur*A, spot 12) in *L. sakei* CRL1756, an enzyme involved in nucleotide and nucleic acid synthesis, suggesting the accumulation of nucleotides for further synthesis of DNA, RNA and ATP, ensuring bacterial survival under harmful conditions. Similarly, one protein involved in purine metabolism (PurH) was up-regulated by *Lac. lactis* under 5 M NaCl showing that a carbon shift from glycolysis to purine biosynthesis following osmotic challenge was produced (Zhang et al., 2010). The down-regulation of the gene *fabI* [enoyl-(acyl carrier protein) reductase, spot 18] and *argS* (arginyltRNAsynthetase, spot 5) involved in fatty acid and aminoacyl-tRNA biosynthesis respectively, was observed in this study (Table 1). Accordingly, the genes involved in fatty acid biosynthesis were reported to be repressed in *Lac. lactis* under osmotic stress which may be reflective of the low growth rate observed, since this parameter is commonly correlated with bacterial fatty acid biosynthesis involved in cell membrane structure (Xie et al., 2004).

3.3. Conclusions

The presence of high concentrations of NaCl represents a major stressful condition for *L. sakei* CRL1756; the addition of GB as compatible solute was crucial to overcome this osmotic constraint. The improved tolerance to high salt concentration in the presence of GB was validated by the induction and overexpression of proteins related to different cellular functions. For the first time the induction of a protein involved in inorganic ion transport and detoxification mechanisms (DyP-type peroxidase) was related to high salt concentrations in LAB. In addition, to counteract the decreased synthesis of glycolytic enzymes under hypertonic stress, *L. sakei* CRL1756 was able to induce

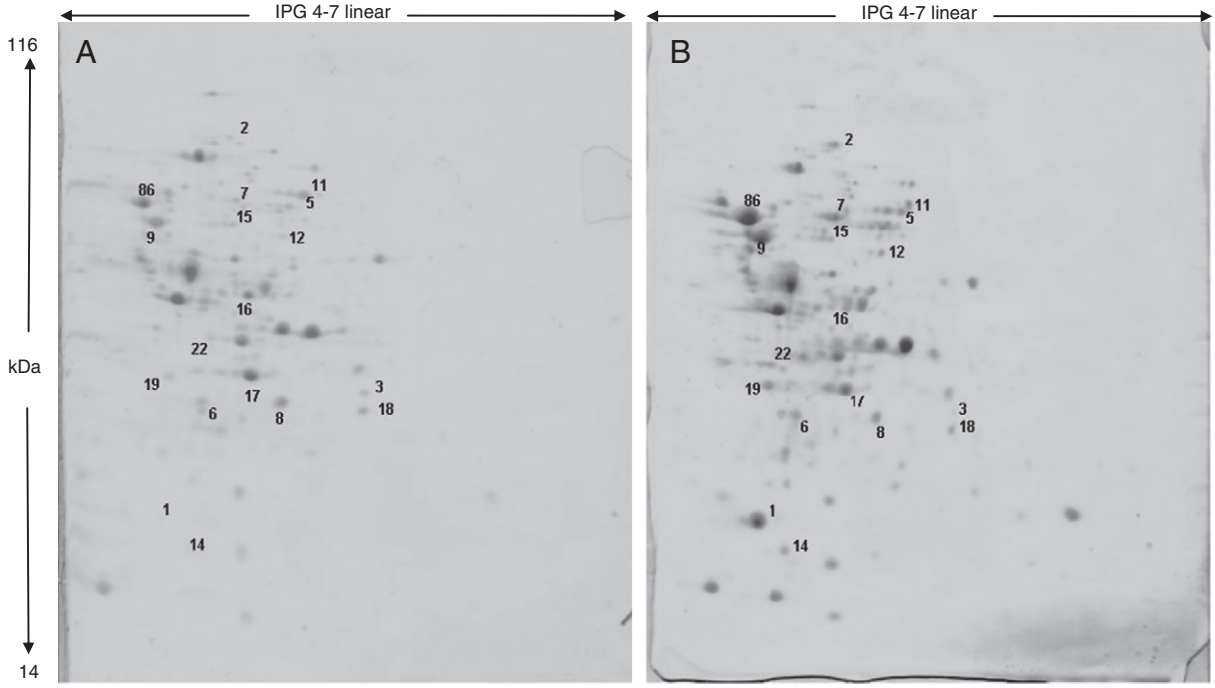


Fig. 3. 2DE images of *L. sakei* CRL1756 cytosolic proteins expressed during growth in MRS + GB in the absence (0%) (A) and the presence of 10% NaCl (B). Numbers indicate identified spots.

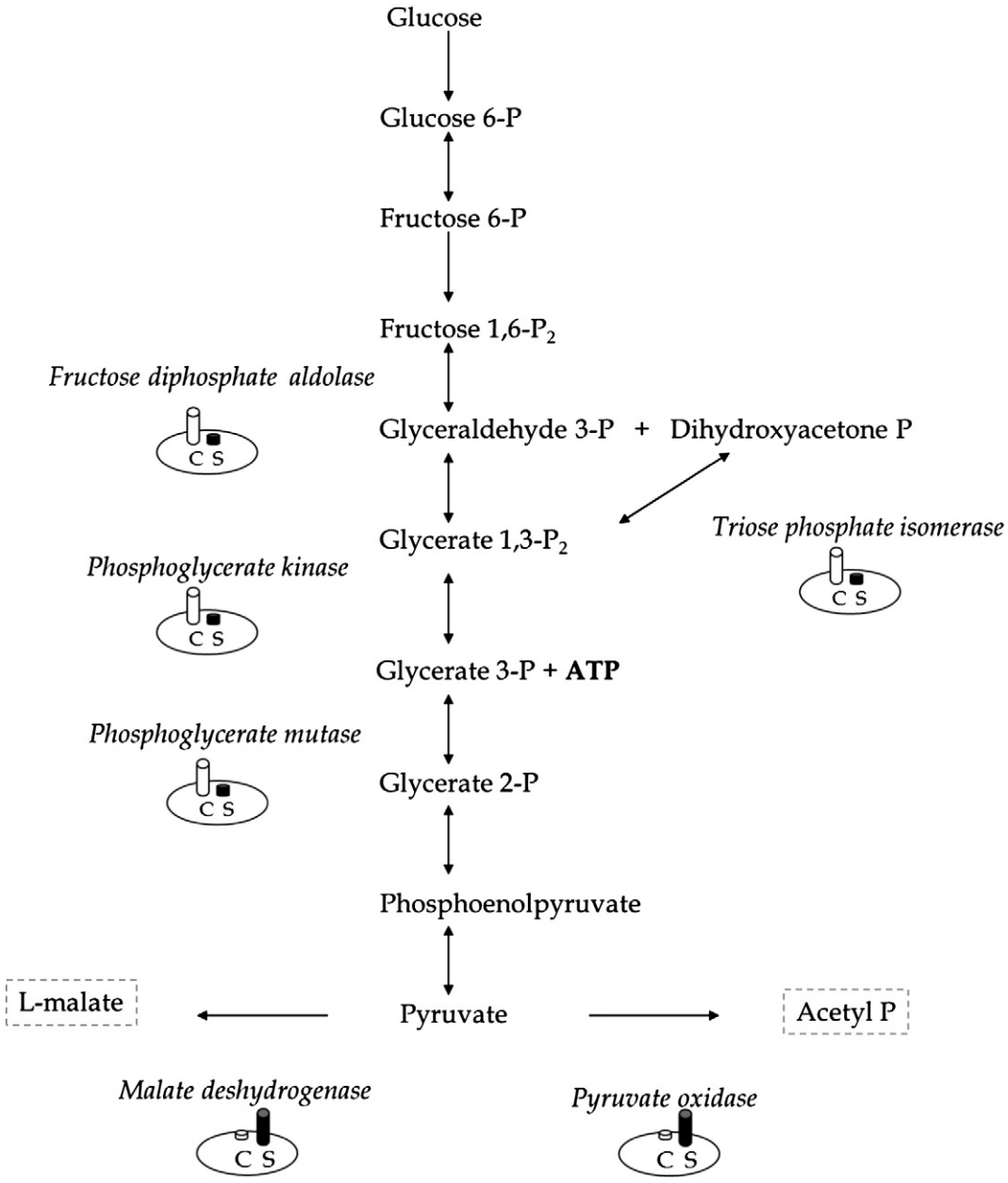


Fig. 4. Carbohydrate metabolism: Proteins regulated by *L. sakei* CRL1756 under osmotic stress. C: control (MRS + GB + 0% NaCl); S: in the presence of 10% NaCl (MRS + GB + 10% NaCl).

the enzymes malate dehydrogenase and pyruvate oxidase as an alternative energetic pathway for cellular metabolism maintenance. The results obtained in this work contribute to the understanding of the complex molecular basis of *L. sakei* response to salt stress. The natural presence of GB in fish guarantees better adaption of *L. sakei* CRL1756 to salted environment ensuring its robustness and stability in view to be used as starter culture for new salted anchovy-based products.

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