Inside the adaptation process of *Lactobacillus delbrueckii* subsp. *lactis* to bile

Patricia Burns^{a,b}, Borja Sánchez^b, Gabriel Vinderola^{a,b}, Patricia Ruas-Madiedo^b, Lorena Ruiz^b,
Abelardo Margolles^b, Jorge Reinheimer^a and Clara G. de los Reyes-Gavilán^{b*}

^a Instituto de Lactología Industrial (INLAIN, UNL-CONICET), Facultad de Ingeniería
Química, Universidad Nacional del Litoral, 1° de Mayo 3250, Santa Fe (3000), Argentina.

^b Departamento de Microbiología y Bioquímica de Productos Lácteos, Instituto de Productos
Lácteos de Asturias (IPLA, CSIC), Carretera de Infiesto s/n, Villaviciosa (33300), Spain.

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*** Corresponding author**

Clara G. de los Reyes-Gavilán. Instituto de Productos Lácteos de Asturias (IPLA, CSIC),
Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain. Tel. 34 985893335, Fax 34
985892233, Email greyes_gavilan@ipla.csic.es

ABSTRACT

Progressive adaptation to bile might render some lactobacilli able to withstand physiological bile salt concentrations. In this work, the adaptation to bile was evaluated on previously isolated dairy strains of *Lactobacillus delbrueckii* subsp. *lactis* 200 and *L. delbrueckii* subsp. *lactis* 200+, a strain derived thereof with stable bile-resistant phenotype. The adaptation to bile was obtained by comparing cytosolic proteomes of both strains grown in the presence or absence of bile. Proteomics were complemented with physiological studies on both strains focusing on glycolytic end-products, the ability to adhere to the human intestinal epithelial cell line HT29-MTX and survival to simulated gastrointestinal conditions. Protein pattern comparison of strains grown with and without bile allowed us to identify 9 different proteins whose production was regulated by bile in both strains, and 17 proteins that showed differences in their levels between the parental and the bile resistant derivative. These included general stress response chaperones, proteins involved in transcription and translation, in peptidoglycan/exopolysaccharide biosynthesis, in the lipid and nucleotide metabolism and several glycolytic and pyruvate catabolism enzymes. Differences in the level of metabolic end products of the sugar catabolism were found between the strains 200 and 200+. A decrease in the adhesion of both strains to the intestinal cell line was detected in the presence of bile. In simulated gastric and intestinal juices, a protective effect was exerted by milk improving the survival of both microorganisms. These results indicate that bile tolerance in *L. delbrueckii* subsp. *lactis* involves several mechanisms responding to the deleterious impact of bile salts on bacterial physiology.

1. Introduction

Lactobacillus species such as *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum* occur naturally in the human intestine and, for this reason, they are being preferentially isolated and characterized for the development of commercial cultures to be used as probiotics in foods intended for human consumption (Vizoso Pinto et al., 2006). Non-intestinal *Lactobacillus delbrueckii* strains are important organisms for food fermentation. In particular, they are essential to the dairy industry for the production of yoghurt (*L. delbrueckii* subsp. *bulgaricus*) and hard cheeses (*L. delbrueckii* subsp. *lactis*) (Giraffa et al., 1998). Several results have been reported concerning the ability of strains of this species to adhere to intestinal epithelial cells, their capacity to survive the transit through the gastrointestinal tract (GIT) and the occurrence in human faeces after oral consumption, all these properties being strain dependent. Tannock (2003) and Wall et al. (2007) reported that *L. delbrueckii* subsp. *bulgaricus* and *lactis* are rarely found among the human gut microbiota due to their low capacity to survive the harsh conditions found during the GIT transit. However, there is evidence from other authors that a substantial proportion of *L. delbrueckii* can survive the GIT transit (Elli et al., 2006; Lick et al., 2001; Marteau et al. 1997; Mater et al., 2005). Immunomodulating capacity after oral administration of intact cells or cells included in fermented products has been reported (Adolfsson et al., 2004; Guarner et al., 2005; Perdígón et al., 2000; Vinderola et al., 2007). Even for microorganisms not surviving the GIT transit, the health benefits ascribed to starter cultures such as *L. delbrueckii* or *Streptococcus thermophilus*, would primarily come from providing a source of enzymes which are important for improving the digestion of nutrients in the gut, such as β -galactosidase (Gilliland, 1998), or can be related to their high proteolytic capacity which releases functional bioactive peptides from milk proteins (Hebert et al., 2008;

Korhonen and Pihlanto 2006) or to cell-wall rests derived from digested cells (Tsuruta et al, 2009).

It has been shown that some Gram-positive bacteria can develop an adaptive response when subjected to moderate stress conditions. In a previous work we reported on the isolation of bile-resistant derivatives from non-intestinal lactobacilli (Burns et al., 2008). The process of acquisition of permanent resistance to bile conferred the capacity to grow under physiological bile salt concentrations, which might be a valuable and natural tool for increasing the survival of non-intestinal dairy lactobacilli in the GIT ecosystem, thus enhancing their probiotic potential. In depth proteomic and genomic characterization of bile-stress response has been carried out in *L. plantarum* (Bron et al., 2004; Bron et al., 2006), *Lactobacillus reuteri* (Lee et al., 2008 ; Whitehead et al., 2008) and *L. acidophilus* (Pfeiler et al., 2007). Moreover, proteomic studies have been performed on *L. delbrueckii* subsp. *bulgaricus* during its growth in MRS broth, or under stress conditions imposed by lactic acid (Lim et al., 2000), heat (Gouesbet et al., 2002) or spray-drying (Silva et al., 2005). Nevertheless, a proteomic approach for a better understanding of bile tolerance in *L. delbrueckii* has not been performed yet. Taking all these data into consideration, the aim of the present work was to study the bile tolerance phenomenon in *L. delbrueckii* subsp. *lactis* 200, a highly autoaggregative strain isolated from natural whey starters in Argentina, and its bile-resistant derivative strain *L. delbrueckii* subsp. *lactis* 200+, a strain showing stable bile-resistant phenotype (Burns et al., 2008). The effect of bile on these two strains was evaluated by using four different approaches: i) Soluble proteome during progressive bile exposure, ii) levels of glycolytic end products, lactate and acetate, iii) adhesion to human intestinal cell lines during progressive bile exposure, and iv) survival in simulated GIT conditions. To the

best of our knowledge, this work constitutes the first proteomic analysis of the species *L. delbrueckii* under bile stress conditions.

2. Materials and Methods

2.1. Strains and culture conditions

The strains used in this study were *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+. The bile resistant derivative was obtained and preliminary characterized in a previous work (Burns et al., 2008). Strains were grown in MRS broth (BioKar Diagnostics, Beauvais, France) and incubated at 37°C for 18 h. After centrifugation, bacteria were resuspended in MRS broth containing 20% (v/v) glycerol and kept at -80°C until use. As standard procedure, the strains were grown overnight directly from the stocks in MRS broth (Difco, Detroit, Michigan, USA) and these cultures were employed to inoculate (2% v/v) fresh MRS broth (Difco) and then incubated for a variable period of time depending on the experiment. For bacterial counting, decimal serial dilutions were made in quarter-strength Ringer's solution (Merck, Darmstadt, Germany) which were pour-plated on MRS- agar (Biokar) and incubated for 48 h at 37°C.

2.2. Growth kinetics in the presence of bile salts

The ability to grow in the presence of different concentrations of bile salts was determined in order to obtain a subinhibitory concentration of bile for the parental strain allowing the growth of both the parental strain and its bile-resistant derivative. This concentration was used to obtain the cytosolic proteome of both microorganisms during

progressive bile exposure. Overnight cultures at stationary phase (18 h) of *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative 200+ were inoculated (2% v/v) into MRS broth containing 0%, 0.2% or 0.5% (w/v) bovine bile salts (Sigma Chemical Co., St. Louis, MO, USA) and incubated in a water bath at 37°C. Cell growth was measured at 600 nm at different time intervals. Two independent cultures were made for each condition.

2.3. Extraction of cell-free proteins for 2D electrophoresis

In order to accomplish proteomic experiments, *L. delbrueckii* subsp. *lactis* 200 and 200+ were grown at 37°C in MRS without bile and in MRS in the presence of a subinhibitory concentration of bile (0.2%). Cells were harvested at mid-exponential phase. Cultures were centrifuged (8,000 g, 4°C, 10 min) and washed twice in 0.1 M Tris-HCl buffer pH 7.5. Pellets obtained from 50 ml cultures were resuspended in 5 ml of 1 M Tris-HCl buffer pH 7.5, and lysed using a Cell Disrupter (Constant Systems Ltd., Daventry, United Kingdom) at 2.05×10^8 Pa. Unbroken cells and cell debris were removed by centrifugation (4,500 g, 4°C, 15 min) and membrane vesicles were discarded by ultracentrifugation (50,000 g, 4°C, 20 min). The protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

2.4. 2D Electrophoresis conditions and proteins identification

Lactobacillus proteins were analyzed by 2D electrophoresis as previously described (Anglade et al., 2000; Marceau et al., 2004). Firstly, nucleic acids were removed from the protein preparations (500 µg) by a single incubation step of 30 min at 37°C in the presence of

134 50 U Benzonase (Merck KGaA) and 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. Proteins were then precipitated
135 using a methanol-chloroform protocol (Wessel and Flugge, 1984), and solubilised in 40 μl of
136 a mixture containing 6.5 M deionised urea, 2.17 M thiourea (GE HealthCare, Biosciences,
137 Uppsala, Sweden), 65 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
138 (CHAPS; Sigma), 100 mM dithiothreitol (DTT), and 25 mM Tris-HCl buffer pH 8.8
139 (Amersham Biosciences, Buckinghamshire, United Kingdom). The volume was adjusted to
140 380 μl with a solution containing 6.5 M deionised urea, 2.17 M thiourea, 65 mM CHAPS, 100
141 mM DTT, 0.5% pH 4.0 to 7.0 carrier ampholytes (Bio-Rad Laboratories, Hercules, CA), and
142 2 μg bromophenol blue. This solution was used to rehydrate 24-cm pH 4.0 to 7.0 linear
143 immobilized pharmalyte gradient strips (GE HealthCare), which were subsequently focused at
144 60,000 V x h in a IPGphor (GE HealthCare). Strips were then sequentially equilibrated in a
145 buffer [1 M Tris-HCl pH 6.8, containing 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS
146 (Merck)] supplemented with 0.83% (w/v) DTT in the first equilibration step (15 min) and
147 with 7.5% (wt/vol) iodoacetamide in the second one (15 min). Proteins were resolved by
148 SDS-PAGE using a final polyacrylamide concentration of 12.5 % (w/v), in an Ettan Dalt Six
149 System (GE HealthCare) (constant current of 10 mA/gel, 4°C). Proteins were visualized with
150 a Bio-Safe Coomassie (Bio-Rad) staining according to the manufacturer's instructions. Spot
151 detection and volume quantification were performed with ImageMaster Platinum software
152 (version 5.00, GE Healthcare). At least three independent gels from three independent
153 extractions, each one coming from a different replicate, were performed for each growth
154 condition. The effect of bile salts on the production of proteins was statistically determined by
155 analysis of variance (ANOVA) of the normalized spot volume using either the bile salt
156 concentration as a factor with two categories, 0 and 0.2%, and the type of strain with two
157 categories, parental and derivative, ($P < 0.05$). This quantification of the variations in spot

intensity was named variation factor (VF) and was fixed at 1.8 based on the former statistical analysis.

Individual spots were excised from 2D gels. Proteins were identified by peptide mass fingerprinting after trypsinolysis and MALDI-TOF-MS at the Proteomic Unit of Centro Nacional de Investigaciones Cardiovasculares (CNIC; Madrid, Spain). Protein identification was performed against the two genomes of *L. delbrueckii* subsp. *bulgaricus* available at the NCBI site. Annotations were made according to the cluster of orthologous genes (COG) functional groups. Enzyme Database BRENDA (<http://www.brenda-enzymes.info>) was used to identify the metabolic pathway of some of the proteins.

2.5. Metabolic activity in buffered resting cells

In order to detect possible changes that the adaptation to bile could have promoted in the catabolism of carbohydrates of the bile adapted *L. delbrueckii* subsp. *lactis* 200+ strain with respect to the parental strain, glucose consumption and organic acids formation were measured in buffered suspensions of resting cells (Ruas-Madiedo et al., 2005) which were carried out in three independent replicates. In short, parental strain and its bile resistant-derivative were grown in MRS without bile until mid-exponential phase (OD_{600nm} of about 1.2) and cells were harvested by centrifugation (10,000 g, 15 min, 5°C). Pellets were washed twice with 33 mM potassium phosphate buffer pH 7.0 to eliminate residual components of the culture medium. Cells were then resuspended in 10 ml of 33 mM potassium phosphate buffer pH 5.6 containing 25 mM glucose and were incubated with constant mild stirring at 37°C for 4 h. Finally, cells were removed by centrifugation and the supernatants were collected for HPLC analysis.

A HPLC chromatographic system composed of an Alliance 2690 module injector, a Photodiode Array PDA 996 detector, a 410 refractive index (RI) detector and the Empower software (Waters, Milford, MA, USA) was used. Sample separation (50 µl vol injection) was carried out in an ICSep ION-300 ion-exchange column (Transgenomic, San Jose, CA, USA) using 8.5 mM H₂SO₄ as mobile phase at 65°C and with a flow rate of 0.4 ml/min. The glucose was detected by using the RI detector and the organic acids by using the PDA detector set at 210 nm. For quantification, the regression equations ($R^2 \geq 0.99$) were calculated using different concentrations of the corresponding standards purchased from Sigma. Results were expressed as mM and the ratio lactic to acetic acids (L/A) was calculated.

2.6. Adhesion assay to epithelial intestinal cell lines

The adhesion capability of the strains 200 and 200+ during progressive bile salt exposure was assessed against the epithelial intestinal cell line HT29-MTX that is able to constitutively produce mucin (Lesuffleur et al., 1993) and was kindly supplied by Dr. T. Lesuffleur (INSERM U843 Paris, France). The cell line was maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum and a mixture of antibiotics to give a final concentration of 50 IU/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamicin and 1.25 µg/ml amphotericin B. All media and reagents were purchased from Sigma and incubations took place at 37°C, 5% CO₂ in a SL Waterjacked CO₂ Incubator (Sheldon Mfg. Inc., Cornelius, Oregon, USA). Culture media were changed every two days and the cell lines were weekly trypsinised with a trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA) (Sigma) following standard procedures. For experiments, 1×10^5 cells/ml were

seed in 24-well plates and incubated to confluence for 13 ± 1 day. The cell line was used between passes 26 and 28.

Bacterial suspensions were obtained from cultures in stationary phase (24 h) of the parental strain and its bile-resistant derivative in MRS with different concentrations of bile salts. The bile-resistant derivative was grown in MRS with 0%, 0.3% and 0.5% (w/v) bovine bile, whereas the parental strain was grown with 0%, 0.1% and 0.2% (w/v). These different concentrations of bile for parental and derivative strains were chosen looking for bile concentrations with a similar inhibitory capacity for both strains, as deduced from the relative decreases on growth rates. Bacteria were harvested from cultures by centrifugation (8,000 g, 10 min, 5°C), washed twice with PBS and resuspended in DMEM without antibiotics at a concentration of about 1×10^8 CFU/ml. HT29-MTX monolayers were washed twice with Dubelco's PBS buffer (Sigma) to remove the antibiotics and then bacterial suspensions were added in a ratio of epithelial cells/bacteria of 1:10. Plates were incubated for 1 h at 37°C, 5% CO₂ in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany). After the incubation period, supernatant was removed and wells were washed three times with Dubelco's PBS buffer to remove the non-attached bacteria. Finally, the monolayers were trypsinised and counts to determine the number of adhered bacteria were performed in MRS-agar. Results were expressed as the percentage of bacteria adhered with respect to the amount of bacteria added.

2.7. Survival to simulated GIT digestion

The survival potential of the parental strain 200 and its bile resistant derivative 200+ to the passage through the GIT was studied by an *in vitro* model that has been modified from

those previously described (Fernández et al., 2003; Zárate et al., 2000). The following components were used: (i) simulated gastric juice containing 125 mM NaCl, 7 mM KCl, 45 mM NaHCO₃ and 3 g/l pepsin (Sigma) pH 2.0 adjusted with HCl, (ii) simulated duodenal juice containing 1% (w/v) bovine bile (Sigma) pH 8.0 adjusted with 10 N NaOH, and (iii) simulated ileal juice containing 0.3% (w/v) bovine bile, 0.1% (w/v) pancreatin (Sigma) pH 8.0 adjusted with 10 N NaOH. To simulate the GIT transit conditions, 10 ml of cultures (parental and bile-resistant derivative) grown in MRS for 24 h were harvested by centrifugation (10,000 g, 15 min, 5°C). Pellets were washed twice with physiological salt solution (0.85% w/v NaCl) and cells were resuspended in 1 ml of the same saline solution. For each strain, 100 µl of the concentrated suspensions were centrifuged and resuspended either in 1 ml of simulated gastric juice or in 1 ml of gastric juice containing 20% (w/v) of skimmed milk. Bacterial suspensions were then incubated for 90 min at 37°C with mild stirring (200 rpm). Afterwards, cells were harvested (10,000 g, 10 min), resuspended in the duodenal juice and incubated for 10 min at 37°C in an anaerobic chamber (Mac 500, Don Whitley Scientific, West Yorkshire, UK) under a 10% H₂, 10% CO₂, and 80% N₂ atmosphere. After this step, cells were again centrifuged, resuspended in the simulated ileal juice and incubated for 90 min at 37°C in anaerobic conditions. Viable counts were obtained from the initial cultures and after simulation of each condition tested.

2.8. Statistical analysis

Data were statistically analysed by means of one-way ANOVA tests using the SPSS 11.0 software for Windows (SPSS Inc., Chicago IL.). For glucose consumption and organic acids formation by resting cells as well as for the survival to GIT and adhesion experiments,

independent ANOVA tests were performed employing the strain as factor, with two categories: parental and derivative. For adhesion results within each strain, independent ANOVA tests were performed using the bile salt concentration as factor. The differences between the three concentrations of bile salt tested within each strain were assessed by the mean comparison analysis LSD (least-significant difference, $P < 0.05$).

3. Results

3.1. Growth kinetics in the presence of physiological bile salt concentrations

Figure 1 shows the growth of *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+ in the presence of different bile salt concentrations. The growth of both strains was strongly inhibited in the presence of 0.5% bile salts. Thus, a concentration of 0.2% (w/v) bile salts was chosen for further studies since it allowed a moderate growth of the parental strain and a more evident growth of the bile resistant derivative. Samples for proteomic studies were taken when the different cultures reached OD at 600 nm of 0.6. The time necessary to reach this OD varied between 2 and 10 h depending on the strain and the presence or absence of bile.

3.2. Proteomic study of the adaptation to bile of strains 200 and 200+.

We used a proteomic approach to characterize the adaptation to bile salts in strains 200 and 200+. Our study focused on the cytosolic proteins in a pI range of 4.0 to 7.0. In these conditions the estimated coverage of the theoretical proteome of *L. delbrueckii* subsp. *lactis* was 52.1%, as extrapolated from the *in silico* proteomic data obtained from the two *L.*

delbrueckii subsp. *bulgaricus* genomes available at NCBI (data not shown) (<http://www.ncbi.nlm.nih.gov/sites/genome>). The comparison of proteomes from strain 200 and strain 200+ in the absence of bile allowed us to identify constitutive changes leading to a stable bile-resistance phenotype in the strain 200+.

Figure 2 shows 2D gels of proteins from cell-free extracts of strain 200 and its bile-resistant derivative 200+, grown in the presence and absence of bile salts. 52 spots showed statistical differences in their production and they were identified. For further analysis, we focused only on spots which were considered to be up- or down- regulated when their variation factor (VF) was greater than 1.8 or lower than 0.55, respectively. According to this arbitrary criterion, the production of 35 proteins was modified by bile (Table 1).

~~With regard to the transient response,~~ Strains 200 and 200+ displayed 9 proteins that were up- or down- regulated by the presence of bile salts in both strains. The production of three enzymes of the glycolytic pathway was affected (Table 1; Figure 3); in both strains phosphoglycerate mutase (Figure 2; spot 393) and glyceraldehyde-3P-dehydrogenase (Figure 2: spot 266) ~~which successive bioactivity catalyses the interconversion of glyceraldehyde 3-phosphate to glyceraldehyde 1, 3-bisphosphate~~ were overproduced, whereas fructose-biphosphate aldolase (Figure 2: spot 410) was under-produced. Apart from carbohydrate metabolism, other proteins involved in different functional categories such as transcription and translation, lipids metabolism, and peptidoglycan and EPS biosynthesis changed their production levels in both microorganisms following bile salt exposure (Table 1; Figure 2), as it is the case of ~~for example the expression of~~ the protein EF-Tu, that was found to be down-regulated ~~as a response to~~ in the presence of bile salts (Table 1; Figure 2: spot 215). Finally, it is worth mentioning the changes in the expression of proteins involved in general stress response. In our study, chaperones HtpO and GroES (Figure 2: spots 190 and 478,

respectively), as well as protease subunit of ATP dependent Clp protease (Figure 2: spot 427), were highly overproduced in the presence of ~~response to~~ bile salts, both in the parental and in the bile resistant derivative.

The production of 17 proteins was significantly and permanently modified ~~after the adaptation process, represented by~~ in the bile resistant strain 200+. Two enzymes of the sugar catabolism were clearly over-produced in the bile resistant derivative: pyruvate kinase and phosphoglycerate mutase (Table 1; Figure 2: spots 84 and 393, respectively). Another enzyme of the pyruvate metabolism, the pyruvate oxidase (Figure 2: spot 79), was down-regulated (Table 1). Acquisition of permanent bile salt resistance ~~Bile adaptation~~ also promoted a pronounced up-regulation of glycosyltransferase, as well as of glycerol-3-phosphate dehydrogenase. Both enzymes may have an impact on the surface properties of the bile resistant ~~adapted~~ strain. The ~~adaptation process~~ bile promoted in the strain 200+ the down-regulation of the chaperone HtpO, and the up-regulation of the protease and ATPase subunits of ATP dependent Clp protease (Figure 2: spots 228 and 427).

A schematic representation of the proteins affected by the bile salts ~~response and adaptation~~ is shown in Fig. 4. Four categories were considered: energy metabolism, stress response, lipid metabolism and cell-envelope.

3.3 Acquisition of stable bile resistant phenotype ~~Bile adaptation~~ modifies glycolytic flux

Bile induced notable changes in several enzymatic activities involved in the central metabolism. This prompted us to measure the production of end-metabolites of the glycolytic pathway. HPLC analysis of the cell-free supernatants from resting cells incubated in a glucose containing buffer showed higher glucose consumption and more lactic acid formation in the bile resistant ~~adapted~~ strain 200+, whereas the acetic acid production was reduced (Table 2).

Therefore, the ratio lactic acid/acetic acid was significantly increased as a consequence of the acquired bile salt resistance.

3.4. Bile salts affect adhesion capacity

Bile salts induced changes in the production of some enzymes related to the cell envelope structure, so we hypothesized that bile may be influencing the surface properties of the cells. Therefore, we compared the adhesion to the intestinal cell line HT29-MTX of the strains 200 and 200+ at different bile concentrations. Parental and ~~adapted~~ bile resistant strains displayed a high adhesion capacity to the intestinal cell line HT29-MTX although this ability decreased in the strain ~~adapted~~ resistant to bile with respect to the parental microorganism. Notably, a significant decrease of cell adhesion capacity occurred when both parental and ~~adapted~~ bile resistant derivative strains were previously grown in the presence of bile salts (Figure 5).

3.5. Survival to simulated GIT conditions.

Finally, we tested whether the survival of both strains to simulated GIT conditions was affected. The influence of milk on this process was also investigated. When *L. delbrueckii* subsp. *lactis* 200 and 200+ were submitted to *in vitro* simulated GIT digestion, their viability decreased by about 5-6 log units, with some cells remaining still viable at the end of the sequential passage through the different solutions (Figure 6). No significant differences between either strain were found at the end of the process, although significantly higher viability of the parental strain was found after the gastric juice challenge. The presence of 20% (v/v) milk improved the survival of both microorganisms through the simulated GIT and no differences between the strains 200 and 200+ were found.

4. Discussion

In vitro data on bile resistance, adhesion and survival to simulated GIT conditions are performance indicators for probiotic strains intended to be used in human foods. Bile salts are able to disrupt the lipid bilayer structure of cellular membranes in Gram positive bacteria, which confers upon them a strong antimicrobial activity. Microorganisms surviving bile should undergo transient or permanent changes allowing them to overcome the deleterious action of these compounds. In the present study proteins involved in the adaptation of *L. delbrueckii* subsp. *lactis* to bile have been identified. Proteomics were complemented with physiological studies focusing on glycolytic end-products, the ability to adhere to the epithelial cell line HT29-MTX and the survival to simulated GIT conditions. The main results obtained are discussed next.

4.1 Impact of ~~transient response~~ and adaptation and acquisition of stable bile resistant phenotype on the sugar metabolism

Changes occurred in the production of enzymes from the glycolysis and pyruvate metabolism in *L. delbrueckii* subsp. *lactis* as a response to bile suggest an activation of the central glycolysis pathway favouring the formation of lactate, as indicated before for *L. acidophilus* in transcriptomic analyses (Pfeiler et al., 2007). As a direct consequence, ATP production and NAD⁺ regeneration may increase, which may provide the cells with enough energy for facing the higher energy consumption and the stress imposed by the adaptation to bile salts. Lee et al. (2008) recently showed the up-regulation in *L. reuteri* of five stress-related proteins involved in sugar metabolism, which was consistent with similar findings found in *Enterococcus faecalis* by Giard et al. (2001). Among other enzymes, Lee et al.

(2008) observed a bile salt-associated up-regulation of mannitol-1-phosphate dehydrogenase, which catalyzes the conversion of fructose-phosphate to manitol-phosphate, favouring the generation of the electron acceptor NAD⁺ as observed in our study.

The analysis of the production of end metabolic products from the carbohydrate catabolism (lactate and acetate) by the strain 200 and its bile resistant derivative 200+ showed an enhanced glucose consumption, and lactic acid formation to the detriment of acetic acid. This supported the hypothesis of global activation of the glycolytic pathway as a consequence of bile adaptation in *L. delbrueckii* subsp. *lactis*. Several authors have previously reported an activation of the glycolytic pathway in lactic acid bacteria and bifidobacteria, with enhanced production of energy rich intermediates under bile and acid stress conditions (Lee et al., 2008; Len et al., 2004a; Len et al., 2004b; Sánchez et al., 2005; Wilkins et al., 2001).

4.2 Bile and general stress response

Presence of bile salts induced the production of the chaperones GroES, HtpO and the protease subunit of ATP-dependent Clp protease in both strains, as well as the under-production of HtpO and the over-production of two subunits of the ATP-dependent Clp protease. Van de Guchte et al. (2006) reported the presence in the *L. bulgaricus* genome of a few of the genes that are known to be involved in resistance to several stresses in other bacteria. A general response to stressful factors, such as bile or acid, has been previously demonstrated in other lactic acid bacteria including *E. faecalis*, *Streptococcus mutants*, *L. reuteri*, *L. plantarum* and *L. acidophilus* (Bron et al., 2006; Giard et al., 2001; Lee et al., 2008; Len et al., 2004b; Wilkins et al., 2001) and in bifidobacteria (Sánchez et al., 2005, 2007a, 2007b, 2008; Margolles and Yokota 2009).

Stress proteins such as heat shock proteins (HSPs) play different key roles in living systems. Many HSPs act as chaperones by guiding conformational states for the correct folding, translocation and protein assembly (Horváth et. al., 2008), contributing also to the degradation of proteins damaged by stress (Streit et. al., 2008). In our study, two subunits from the Clp protease were over-produced in the presence of bile. Frees and Ingmer (1999) suggested that Clp proteolytic complexes are essential for survival under adverse conditions. Previous works indicated that Clp proteases were up-regulated by bile in *L. acidophilus* and *L. reuteri* (Pfeiler et al., 2007; Whitehead et al., 2008), supporting an important role for molecular chaperones in counteracting the deleterious effects of bile.

4.3 Impact of bile on enzymes involved on the cell-envelope synthesis and on the surface characteristics of L. delbrueckii subsp. lactis

Bile induced the over-expression of two proteins related with the physiology of the bacterial cell-envelope, glycosyltransferase and glycerol-3-phosphate dehydrogenase. Glycosyltransferases catalyze the transfer of a monosaccharide unit from an activated sugar phosphate to an acceptor molecule and they can be involved in peptidoglycan and exopolysaccharide biosynthesis. Glycerol-3-phosphate dehydrogenase participates in the glycerolipid and glycerophospholipid metabolism, which are important components of cell membranes. Bile salt and other environmental stresses have been found to promote changes in fatty acid cell composition and cellular surface properties in lactobacilli and bifidobacteria (Gomez-Zavaglia et al., 2002; Guerzoni et al., 2001; Ruiz et al., 2007; Taranto et al., 2003). In addition, it is known that bile induced the synthesis of biofilm and EPS and some related genes in enteric bacteria (Hung et al., 2006; Ruas-Madiedo et al., 2009).

In order to know if bile could affect some surface-linked properties of our strains, we complemented proteomic findings by performing adhesion assays of the two strains in absence or presence of bile salts. We found that the presence of bile salts promoted a global decrease of the adhesion capacity to the intestinal cell line HT29-MTX. Bile salts have previously been shown to decrease the ~~adherence~~ adhesion of *Bifidobacterium* to intestinal mucus (Gueimonde et al. 2005). It may be possible that one or several proteic adhesins become down-regulated under bile stress, thus resulting in a decrease of the adhesion of the bacteria tested. In fact, cell surface characteristics depend on a number of properties such as presence of adhesins, exopolysaccharides, hydrophobicity, and rigidity, among other parameters.

4.4 Survival to simulated GIT conditions

After sequential exposure to simulated GIT juices, a reduced percentage of our *L. delbrueckii* subsp. *lactis* strains remained viable and no remarkable differences in survival were found between parental and bile resistant strains at the end of the process. Viability losses were in accordance with those previously reported for other strains of the same species (Vinderola and Reinheimer 2003). Our results also indicated that decreases in viability due to GIT digestion can be attenuated if the strains are added to milk.

Surviving the transit through the GIT is generally considered a key feature of probiotics in order to fulfil their health-promoting effects (WHO/FAO 2002). It has traditionally been considered that yoghurt starters do not survive the GIT, although recent studies seem to confirm the presence of some strains of *S. thermophilus* and/or *L. delbrueckii* subsp. *bulgaricus* in human and animal faeces after the intake of these microorganisms in probiotic preparations (Elli et al., 2006; Lick et al., 2001; Mater et al., 2005).

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438 In short, it could be assumed that both *L. delbrueckii* subsp. *lactis* 200 and 200+ can
439 survive GIT digestion if ingested within a food matrix. The cell response to bile salts,
440 although revealing both cell-wall and membrane adaptations, did not contemplate the
441 synthesis of bile salts catabolising enzymes. The exposure to bile during passage through the
442 small intestine could promote physiological changes in *L. delbrueckii* subsp. *lactis* directed to
443 overcome the deleterious impact of bile salts on bacterial physiology. In spite of this, the
444 adaptation to bile in *L. delbrueckii* subsp. *lactis* did not promote any improvement of their
445 survival to the GIT conditions and adhesion capacity to intestinal eukaryotic cell lines as
446 occurs with other intestinal lactic acid bacteria, probably because of the non-intestinal origin
447 of this species.

448

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FIGURE LEGENDS

Figure 1 - Growth kinetics of *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+. Solid lines with white symbols represent the parental strain and dashed lines with black symbols the bile-resistant derivative. Growth in MRS broth with 0% (squares), 0.2% (triangles) and 0.5% (circles) of bile salts.

Figure 2 – 2D gels of cytoplasmatic extracts from mid-exponential phase cells of *L. delbrueckii* subsp. *lactis* 200 (a, and b) and *L. delbrueckii* subsp. *lactis* 200+ (c, and d) grown without (a, and c) and with (b, and d) bile salts. Spots identified by peptide mass fingerprinting are labelled and their identification is listed in Table 1.

Figure 3 - Schematic representation of the homofermentative pathway and pyruvate metabolism in *L. delbrueckii* according to the KEGG database (<http://www.genome.jp/kegg>). Glk, glucokinase; Gpi, glucose-6-phosphate isomerase; Pfk, 6-phosphofructokinase; Fba, fructose-1,6-bisphosphate aldolase; Gap, glyceraldehyde-3-phosphate dehydrogenase; Tpi, triosephosphate isomerase; Pkg, phosphoglycerate kinase; Gpm, phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase; Ldh2, lactate dehydrogenase; Pox, pyruvate oxydase; Pta, Phosphate acetyltransferase; Ack, acetate kinase; Acp, acylphosphatase; Adh, alcohol-acetaldehyde dehydrogenase. Enzymes whose production changed in response and/or adaptation are highlighted in grey.

Figure 4 – Histogram representation of the number of proteins from *L. delbrueckii* subsp. *lactis* 200 and its bile resistant derivative *L. delbrueckii* subsp. *lactis* 200+ whose production changed by exposure to bile grouped in four categories: energy metabolism and translation (including the following functional COG categories: carbohydrate metabolism, nucleotide metabolism, and translation), stress response (corresponding with folding, sorting and degradation category of COG), lipid metabolism, and exopolysaccharide synthesis (corresponding with peptidoglycan biosynthesis category of COG). COG: Cluster of Orthologous Genes.

Figure 5 - Adhesion of *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+ to the HT29-MTX intestinal cell line. (□) MRS without bile; (■)) MRS + 0.1% or 03% bile for strains 200 and 200+, respectively; (▣) MRS + 0.2% or 0.5% bile for strains 200 and 200+, respectively.

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714 **Figure 6** - Survival of *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative 200+
715 during simulated GIT transit in absence and presence of 20% skimmed milk. Bacterial counts
716 were determined in each step. GJ: gastric juice, DJ; duodenal juice: IJ; ileal juice. *: parental
717 and bile-resistant derivative are statistically different ($p < 0.05$). (□) parental strain, (■) bile-
718 resistant derivative strain

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Table 1. Proteins affected by bile salts exposure in *L. delbrueckii* subsp. *lactis* 200 and its bile resistant derivative *L. delbrueckii* subsp. *lactis* 200+. N = 3 for each growth condition. Bold face letters indicate proteins whose expression changed in the derivative in the absence of bile (S: stable bile resistance phenotype) and in both the parental and the derivative in the presence of bile (A: adaptation).

COG ^a	Spot ^b	Putative function ^c	pI ^d	MM ^e	Cv ^f	Mo ^g	Norm. V ^h 200	Norm. V ^h 200b	Norm. V ^h 200+	Norm. V ^h 200+b	Stable bile resistance	Adaptation	
											VF ⁱ 200+/200	VF ^j 200b/200	VF ^k 200+b/200+
Carbohydrate metabolism	266	Glyceraldehyde 3-phosphate dehydrogenase (A)	5.51	36.7	15	176	1.500±0.880	4.750±0.250	1.250±0.060	3.500±0.630	0.83	3.17	2.80
	84	Pyruvate kinase (S)	5.22	63.1	48	512	0.100±0.100	0.150±0.060	0.430±0.050	0.280±0.050	4.30	1.50	0.65
	410	Fructose-bisphosphate aldolase (A)	4.90	33.2	24	107	0.130±0.070	0.017±0.017	0.100±0.020	0.033±0.030	0.77	0.13	0.33
	322	HPr kinase/phosphorylase	5.32	35.2	45	465	0.013±0.013	0.025±0.025	-	-	na	1.92	na
	326	UTP-glucose-1-phosphate uridylyltransferase	6.42	33.9	26	256	0.005±0.005	0.010±0.010	-	-	na	2.00	na
	123	Acetyl-CoA carboxylase, biotin carboxylase subunit	5.74	51.2	33	296	2.170±1.170	2.500±0.170	1.670±0.670	3.670±0.250	0.77	1.15	2.20
	312	Phosphotransacetylase	5.05	35.6	38	301	0.110±0.020	0.250±0.030	0.110±0.010	0.190±0.010	1.00	2.27	1.73
	79	Pyruvate oxidase (S)	4.96	67.7	30	263	0.200±0.003	0.310±0.030	0.100±0.100	0.380±0.150	0.50	1.55	3.80
	281	D-lactate dehydrogenase	5.08	36.9	29	246	0.880±0.060	0.550±0.560	1.560±0.500	1.630±0.130	1.77	0.63	1.04
	393	Phosphoglycerate mutase (A, S)	5.24	26.1	47	204	0.033±0.025	0.210±0.050	0.120±0.090	0.260±0.060	3.64	6.36	2.17
Lipid metabolism	332	Glycerol-3-phosphate dehydrogenase (S)	5.78	36.6	27	248	0.012±0.012	0.011±0.010	0.027±0.012	0.034±0.006	2.25	0.92	1.26
	226	3-oxoacyl-(acyl-carrier-protein) synthase (A)	5.39	41.7	32	165	0.400±0.020	0.011±0.008	0.570±0.120	0.010±0.008	1.43	0.03	0.02
	360	Enoyl-[acyl-carrier protein] reductase (NADH) (S)	5.23	27.1	29	228	0.180±0.140	-	0.440±0.040	-	2.44	na	na
Peptidoglycan and EPS biosynthesis	261	Glycosyltransferase (S)	4.86	37.2	26	123	0.010±0.010	0.020±0.020	0.160±0.160	0.260±0.140	16	2.00	1.63
Amino acid metabolism	296	Branched-chain amino acid aminotransferase	5.15	38.0	26	89	0.025±0.025	0.100±0.090	-	-	na	4.00	na
	247	Predicted tRNA methyltransferase	5.20	42.4	35	214	0.090±0.040	0.038±0.006	0.060±0.010	0.037±0.003	0.66	0.42	0.62
	438	Peptide methionine sulfoxide reductase	4.69	19.9	32	119	0.056±0.016	0.031±0.031	0.089±0.006	0.106±0.003	1.59	0.55	1.19
Nucleotide metabolism	459	Adenylyl-sulfate reductase (Thioredoxin)	4.28	11.8	26	118	0.210±0.050	0.100±0.100	-	-	na	0.48	na
	38	Adenosylcobalamin-dependent ribonucleoside-triphosphate reductase (S)	5.02	82.5	23	220	0.040±0.040	0.030±0.030	0.520±0.280	0.250±0.240	13	0.75	0.48
	181	Putative pyridine nucleotide-disulphide oxidoreductase (S)	6.13	48.7	49	285	0.430±0.040	0.460±0.060	0.200±0.040	0.240±0.020	0.47	1.07	1.20
	136	Putative pyridine nucleotide-disulphide oxidoreductase (S)	6.12	48.7	53	360	0.060±0.060	0.090±0.010	0.210±0.050	0.130±0.010	3.5	1.50	0.62
	411	Guanylate kinase (S)	5.62	22.9	21	194	0.069±0.050	-	0.013±0.013	-	0.19	na	na
	56	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones (S)	5.53	76.9	23	274	0.064±0.020	0.081±0.004	0.008±0.008	0.010±0.010	0.13	1.26	1.25
Stress	228	ATP-dependent protease Clp, ATPase subunit (S)	5.03	46.5	32	149	0.043±0.043	0.071±0.069	0.160±0.040	0.190±0.040	3.72	1.65	1.19
	427	Protease subunit of ATP-dependent Clp protease (A, S)	4.96	21.3	36	153	0.160±0.020	0.860±0.020	0.300±0.020	0.870±0.080	1.88	5.38	2.90
	190	Heat shock induced protein HtpO (A, S)	5.65	43.7	11	131	0.160±0.060	0.900±0.030	0.050±0.050	0.630±0.050	0.31	5.63	12.6
	478	10 kDa chaperonin GroES (A)	4.84	10.3	54	111	0.360±0.040	0.800±0.040	0.270±0.020	1.020±0.100	0.75	2.22	3.78
	390	Transcription elongation factor (S)	4.64	18.0	51	168	0.110±0.040	0.240±0.006	0.210±0.040	0.280±0.030	1.91	2.18	1.33
Translation	429	Ribosome recycling factor	6.03	20.7	12	147	0.061±0.003	0.022±0.022	-	-	na	0.36	na
	365	Putative dipeptidyl-peptidase	5.21	32.2	31	134	0.038±0.019	0.081±0.050	-	-	na	2.13	na
	215	Elongation factor Tu (A)	4.85	43.3	47	191	0.910±0.090	0.360±0.180	1.270±0.550	0.640±0.550	1.40	0.40	0.50
	117	Asparagine-tRNA ligase (A)	5.03	50.2	26	212	0.075±0.050	0.350±0.100	0.100±0.100	0.280±0.004	1.33	4.67	2.80
	130	Glutamyl-tRNA synthetase	5.49	57.1	25	245	0.120±0.040	0.042±0.042	0.100±0.010	0.067±0.010	0.83	0.35	0.67
	175	Seryl-tRNA synthetase (S)	5.11	49.4	55	472	0.080±0.080	0.070±0.070	0.280±0.280	0.280±0.100	3.50	0.80	1.00
	68	Threonyl-tRNA synthetase (S)	5.05	72.9	23	221	0.380±0.130	-	0.050±0.050	-	0.13	na	na

^a COG: functional category according to the Cluster of Orthologous Genes.

^b Spots numbers refer to the proteins excised from gels and reported in Figure 2.

^c Putative functions were assigned from the Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways (www.genome.jp/kegg).

^d Theoretical isoelectric point expressed in pH.

^e Theoretical molecular masses expressed in kDa.

^f Percentage of sequence coverage.

^g Molecular weight search score.

^h , Normalized relative volumes (percent volumes) for matching spots of parental and derivative strains. Only statistically significant results are presented. Values are means $\pm$ standard deviations: $n \geq 3$ for each strain, - not detected.

ⁱ , Variation factor: ratio between normalized volumes for each protein derived from cells of *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative (200+) cultured in MRS broth without bile salts.

^{j,k} , Variation factor: ratio between normalized volumes for each protein derived from cells of *L. delbrueckii* subsp. *lactis* 200 (j) or its bile-resistant derivative (200+) (k) cultured in MRS broth with 0.2% wt/vol bile salts (b) and the corresponding strain grown without bile salts.

n.a.: not applicable, due to the absence of detectable amount of protein for one of the conditions used for calculation of VF.

Table 2. Glucose consumption and organic acids formation in buffered suspension of resting cells obtained from cultures of *Lactobacillus delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative 200+ grown in MRS until mid-exponential phase (OD₆₀₀ about 1.2). *** $P < 0.001$.

Strain	Glucose consumed	Mean $\pm$ SD (mM)		
		Lactic acid	Acetic acid	Lactic/Acetic
200	2.72 $\pm$ 0.52	4.14 $\pm$ 0.45	0.80 $\pm$ 0.00	5.15 $\pm$ 0.57
200+	6.30 $\pm$ 0.33	10.01 $\pm$ 0.24	0.41 $\pm$ 0.02	24.70 $\pm$ 0.62
	***	***	***	***

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6