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Proteomic analysis of the probiotic *Lactobacillus reuteri* CRL1098 reveals novel tolerance biomarkers to bile acid-induced stress

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ABSTRACT

Lactobacillus (*L.*) *reuteri* CRL1098 is a probiotic bacterium with a proven hypocholesterolemic effect, moderate immune stimulant effect and ability to produce cobalamin. The CRL1098 strain survives the passage through the gastrointestinal tract where the exposure to bile acids (BA) causes deleterious effects. In order to characterize the molecular mechanisms through which *L. reuteri* CRL1098 adapts to bile, its proteomic response was evaluated in the presence of conjugated (glycodeoxycholic acid-GDCA-) and free (deoxycholic acid-DCA-) bile acids (BA). Cell growth inhibition was observed only in the presence of DCA. Two-dimensional gel electrophoresis coupled to mass spectrometry allowed us to identify 25 protein spots differentially expressed in response to both BA. The main functional categories assigned to the proteins were metabolism of nucleotides and glycerolipids, transcription and translation, pH homeostasis and stress-responses. Remarkably, cytosine triphosphate(CTP) synthetase, enzyme related to the repair of oxidative DNA, was over-expressed in the presence of GDCA and significantly repressed by DCA; also three proteins related to protein transcription and translation were over expressed in the presence of the conjugated BA and one, was repressed by the free BA. This differential expression could explain the delayed growth of the cells challenged with the free BA and the unaffected growth in the presence of GDCA. Moreover, some general stress proteins were triggered in the presence of both BA. In addition, the bile salt hydrolase (BSH) enzyme regulation in response to BA was analyzed using real time-PCR to determine its contribution to cell tolerance. An up-regulation of the *bsh* gene in response to BA was observed, suggesting that this enzyme could be a specific biomarker of bile adaption in *L. reuteri* CRL1098. The present work proposes that BA induce a complex physiological response in *L. reuteri* and provide new insights into the mechanisms involved in BA tolerance.

1. Introduction

The successful development of functional foods depends on the careful selection of probiotic strains, whose bile resistance is an important selection criterion. Bile is secreted into the intestine, during the digestion process, where it plays a major role in lipid emulsification and absorption. It is an aqueous solution mainly composed of conjugated bile acids (BA) (approximately 50%), phospholipids, cholesterol and biliverdin (Begley, Gahan, & Hill, 2005). The concentration of bile usually varies between 0.2 and 2% after food ingestion, being higher after an intake of lipids (Begley et al., 2005). The hydrolysis of conjugated BA releases free BA and taurine or glycine. This phenomenon takes place due to the bile salt

Abbreviations: BA, bile acids; GDCA, glycodeoxycholic acid; DCA, deoxycholic acid; HSB, bile salt hydrolase; LAB, lactic acid bacteria; *L.*, *Lactobacillus*; *B.*, *Bifidobacterium*; *Ent*, *Enterococcus*; *Lac.*, *Lactococcus*; Ct, threshold cycle.

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hydrolase (BSH) activity, an enzyme present exclusively in certain species of the intestinal microbiota (Chae, Valeriano, Kim, & Kang, 2013; Jayashree, Pooja, Pushpanathan, Rajendhran, & Gunasekaran, 2014; Joyce, MacSharry, Casey, Kinsella, Murphy, Shanahan, Hill and Gahan, 2014). Some researchers have suggested a possible relationship between BSH activity and BA resistance in many lactic acid bacteria (LAB) (Bustos, Saavedra, Font de Valdez, Raya, & Taranto, 2012; Joyce, Shanahan, Hill, & Gahan, 2014). In addition, BA are biological detergents that could disrupt the lipid bilayer structure of bacterial cellular membranes, induce protein misfolding, and cause oxidative damage to DNA and RNA and intracellular acidification(Begley et al., 2005; Taranto et al., 2006; Bustos et al., 2012; Lebeer, Vanderleyden, & De Keersmaecker, 2010).

Lactobacillus (*L.*) *reuteri* is the most widely distributed *Lactobacillus* species in mammals and usually proposed for the design of functional food. *L. reuteri* CRL1098 is a probiotic bacterium with a proven hypocholesterolemic effect (Taranto, Medici, Perdigon, Ruiz Holgado, & Valdez, 2000) and ability to produce corrinoids with cobalamin activity (Molina, Médici, de Valdez, & Taranto, 2012; Taranto, Vera, Hugenholtz, De Valdez, & Sesma, 2003). Its ability to survive the passage of the intestinal tract, and hence resisting the toxic effect of BA, is a key factor specifically related to its probiotic function. The mechanisms of tolerance to bile stress are not fully understood and little is known about the protein expression profiles of *L. reuteri* strains in response to stress produced by BA. The aim of this study is to characterize, by means of two-dimensional gel electrophoresis (2DE) and mass spectrometry, the intracellular proteins differentially expressed by *L. reuteri* CRL1098 when this bacterium adapts to conjugated- and free-BA. In addition, the molecular regulation of the BSH enzyme in response to BA was evaluated to determine its contribution to cell tolerance. These data will improve our understanding of *L. reuteri* adaption to the intestinal tract passage considering the importance of evaluating resistance/response to bile in a probiotic strain as a prelude to its successful application in functional foods.

2. Materials and methods

2.1. Bacterial strain and growth conditions

L. reuteri CRL1098 was grown on 200 mL MRS broth in the absence of BA (control culture) or in the presence of 1 mM of glycodeoxycholic acid (GDCA) or 1 mM deoxycholic acid (DCA) (both BA from Sigma Aldrich, St. Louis, MO, USA), equivalent to 0.05% (w/w) of GDCA and 0.04% (w/w) DCA.

Cells were harvested at the early-exponential phase ($OD_{560\ nm}$ ~ 0.9) by centrifugation at 10,000 ×*g* for 10 min at 4 °C. The cells were then washed with 100 mM Tris–HCl buffer, pH 7.5 and the resulting cell pellets were stored at −20 °C until lysis. Cells were re-suspended in 1 ml lysis buffer (100 mM Tris–HCl, pH 6.8), and disrupted with a French press (SLM Instruments, Inc., Haverhill, MA, USA) at 1200 psi. Unbroken cells and cell debris were removed by centrifugation (10,000 ×*g* for 10 min at 4 °C) and membrane vesicles by ultracentrifugation (Beckman, MA, USA) at 50,000 ×*g* for 30 min at 4 °C. The protein concentration was estimated using the Bradford method (Bradford, 1976) using a commercial kit and according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Aliquots of 400 μg of intracellular proteins were stored at −80 °C, until further analysis.

2.2. Proteomic analyses

2.2.1. 2D electrophoresis (2DE)

Samples preparation and 2DE gels were carried out according to Belfiore, Fadda, Raya, and Vignolo (2013), with several modifications. Two-dimensional electrophoresis was performed in the p*I* range of 4–7, proposed as the pH range where LAB's proteome is predominantly distributed (Savijoki et al., 2005). Isoelectrofocusing strips of 18 cm and a linear pH range 4.0 to 7.0 (GEHealthcare, Uppsala, Sweden) were rehydrated and then focused until 53,500 Vhs using the IPGphor 3 (GE Healthcare, Uppsala, Sweden) iso-electric focusing unit. The IPG strips were equilibrated as was previously described (Almeida et al., 2010). The second dimension was carried out on an Ettan Dalt Six electrophoresis unit (GE Healthcare, Uppsala, Sweden); using homogenous 12.5% sodium dodecyl sulfate-polyacrylamide gels. Proteins were resolved overnight at a constant current of 12 mA/gel at 4 °C. Gels were stained with colloidal Coomassie blue, Biosafe™ (BIORAD Hercules, CA, USA) distained with distilled water and digitized with an Image Scanner III LabScan 6.0 (GE Healthcare, Uppsala, Sweden).

2.2.2. Image acquisition and data analysis

Digitalized images (300 dpi) of stained gels were aligned using the Same Spots software version 1.0.3400.25570 (Nonlinear Dynamics, Newcastle, UK) and the data analysis was performed as was previously described by Belfiore et al. (2013). The analysis was performed comparing proteins expressed in the presence of BA (GDCA and DCA) with those present in the control conditions (with no BA addition). A protein was considered differentially expressed if the mean normalized spot volume varied at least 1.5-fold between compared spots. The effect was confirmed by analysis of variance at a significance level of $p < 0.05$. At least three biological replicates for each growth condition were performed which were run twice (technical duplicates) to obtained at least six gels for each condition.

2.2.3. Protein identification by mass spectrometry

Selected spots were excised from the gels using a scalpel, distained as previously described (Almeida et al., 2010) and submitted to tryptic digestion and then to mass spectrometry analyses according to Shevchenko, Tomas, Havli, Olsen, and Mann (2006). Briefly, peptides were ionized using α-cyano-4-hydroxycinnamic acid as matrix. Mass spectrometric analysis of the peptide solutions from trypsin digested spots was carried out in a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, CA, 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*, Faculty of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina). Mascot (Matrix Science Inc., Boston, MA; http://www.matrixscience.com/search form select.html) software was used for protein identification. All searches were performed against the database for *L. reuteri* DSM20016 from the annotated genome (http://www.ncbi.nlm.nih.gov/nuccore/NC_009513.1).

2.3. Gene regulation analyses

2.3.1. RNA isolation

RNA isolation from *L. reuteri* CRL1098 was performed using the methodology described by Raya, Bardowski, Andersen, Ehrlich, and Chopin (1998) with some modifications. *L. reuteri* CRL1098 was inoculated in MRS broth with or without the addition of 1 mM of GDCA as previously described. The cells were incubated until mid-exponential phase ($OD_{560\ nm}$ ~ 0.9) and harvested by centrifugation at 3500 ×*g* for 10 min followed by cell pellet suspension in ice-cold TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). The cell suspension was transferred to a microcentrifuge tube containing 0.8 g of zirconium beads, 0.18 g of macaloid, 50 μl of 10% (v/v) SDS, and 500 μl of water-saturated phenol–chloroform (1:1). Cells were mechanically disrupted in a Mini-Beadbeater-8TM Cell Disrupter using 7 treatments of 1 min interspaced by 1 min in ice. Subsequently, the RNA was purified

from the upper aqueous phase of the cell extract by phenol–chloroform extraction, precipitated with absolute ethanol, washed with 70% ethanol and resuspended in MilliQ water. The RNA obtained was stored in aliquots at −70 °C until further use.

2.3.2. cDNA construction and real-time PCR assays

RNA samples were treated with the RNase-free DNase I set (Tecnolab, Buenos Aires, Argentina) according to the manufacturer's recommendations. The absence of DNA in the samples was confirmed by PCR. For reverse transcriptase PCR (RT-PCR) analysis, cDNA was synthesized from RNA by using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The resulting cDNA samples were used for Real-Time PCR amplification. Specific primers for *bsh* gene and housekeeping genes primers were designed to have melting temperatures of 60 to 65 °C and amplicon sizes around 130 bp. *RecA* primers were used as internal control gene to normalize the amount of RNA added; the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen, 2008) where $\Delta\Delta CT = (CT_{bsh} - CT_{recA})_{BA} - (CT_{bsh} - CT_{recA})_{Control}$. Cells growing in MRS broth without BA addition were used as reference condition. The data obtained were submitted to ANOVA, followed by comparison with a control using Dunnett's test using the software Graph Pad Prism. The experiments were performed in IQ iCycler (Biorad, Hercules, CA, USA) detection system, using the doublestranded DNA intercalating fluorescent agent SYBR green for product detection. Each well contained SYBR green Master Mix, primers and a template. Control PCRs were included to detect background contamination (no-template control) and remaining chromosomal DNA (RT reactions in which Superscript II was omitted). PCR specificity and product detection were checked post-amplification by examining the dissociation curves (melting curve) of the PCR products.

3. Results and discussion

3.1. Physiological response of L. reuteri CRL1098 to bile acids

Probiotics as natural members of intestinal microbiota must tolerate high stressful conditions prevailing throughout the gastrointestinal tract (GIT) such as different concentrations of chloride acid, pancreatic enzymes or bile, among others. The growth of *L. reuteri* CRL1098 in MRS containing 1 mM of DCA or 1 mM GDCA was compared with bacterial growth in BA-free medium. The results demonstrated that even when the DCA affected *L. reuteri* CRL1098 cell viability, this strain showed considerable survival in the presence of free BA compared to the growth in the presence of GDCA or under control conditions (Fig. 1). Previous studies showed that bile salt hydrolase (BSH) present in CRL1098 strain is more efficient in hydrolyzing glycoconjugated BA, being more active against GDCA (Taranto, Sesma, & Font de Valdez, 1999). Moreover, it was demonstrated that the free acid, DCA is the most deleterious BA and affects critical functions in *L. reuteri* CRL1098. In fact, this free BA has been described to cause permeabilization of cells, glucose uptake halting, and severe distortion of the cell envelope and depolarization of the cytoplasmic membrane (Bustos, Raya, Bru, Font de Valdez, & Taranto, 2011).

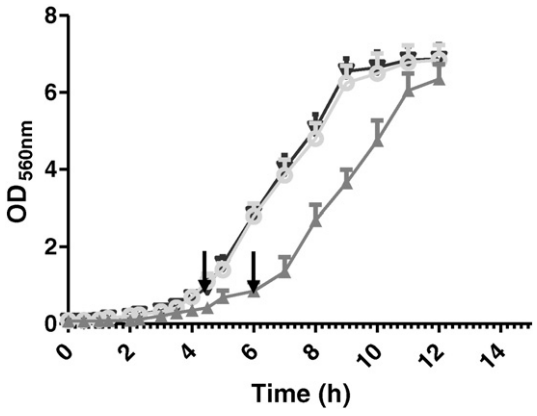


Fig. 1. Growth of *L. reuteri* CRL1098 in presence of GDCA (○, light gray), DCA (△, dark gray) and without bile acids (control) (▽, black).The arrows indicate the growth phase point when cells were harvested for proteomic and real time experiments. Error bars represent standard deviations of triplicate experiments.

3.2. Proteomic response of L. reuteri CRL1098 to bile acids

To unravel functions involved in the response of *L. reuteri* CRL 1098 to BA stress, a proteomic approach was conducted to evaluate differences in protein expression after exposure of CRL1098 to each BA. Representative gels obtained are depicted in Fig. 2. The differential expression levels ranged from 5.1 to 1.5 fold variations ($p < 0.05$). *L. reuteri* CRL1098 proteome from cells growing in the presence of DCA revealed that 28 spots clearly displayed different levels of expression, including 13 proteins significantly over-expressed and 15 under-expressed. In the presence of GDCA, 22 proteins showed a different expression profile; where 14 and 8 spots were up- and down regulated, respectively. From the 50 differentially expressed proteins in all analyzed conditions, 25 were successfully identified (Table 1); from these, 14 were modulated by DCA, while 11 proteins were affected by GDCA. As shown in Fig. 3, identified BA-responsive proteins were assigned to different functional categories, namely nucleotide (7 proteins) and glycerolipid metabolism (6 proteins), transcription and translation (4 proteins), pH homeostasis and stress-responses (3 proteins), amino acid biosynthesis (2 proteins), sugar metabolism (1 protein), as well as two proteins with unassigned functions.

3.2.1. Metabolism of carbohydrates and nucleotides

A D-isomer specific 2-hydroxyacid dehydrogenase NAD-binding protein, related to carbohydrate metabolism was under-expressed in presence of DCA (spot no. 33). This enzyme, responsible for pyruvate production from D-lactate, is involved in NADH production in the cell which could be affected under this stress effector.

A number of proteins related to purine and pyrimidine metabolism showed to be differentially expressed under the studied conditions. The inosine/uridine-preferring nucleoside hydrolase is an enzyme that catalyzes the hydrolysis of the commonly purine and pyrimidine nucleosides into ribose and its associated base, but has a preference for inosine and uridine as substrates. Interestingly, we found two isoenzymes Lreu_1658 (spot nos. 7 and 28) and Lreu_0102 (spot no. 10) differentially expressed in the conditions assayed. The isoenzymes identified presented different masses and pIs. Such differences may be explained by one of the spots being a proteolytic fragment or carrying a post-translational modification. The Lreu_1658 was up-regulated in the presence of the GDCA and DCA while the remaining isoenzyme (Lreu_0102) was significantly repressed after DCA exposure. This is partly consistent with the works of Sanchez et al. (2007) that reported a lower activity of this enzyme in the bile resistant mutant *B. longum* NCIMB8809 4549dOx. For this reason, no clear-cut conclusion could be drawn about the link of this enzyme to BA resistance, although it could be speculated that it is indeed involved. The enzyme cytosine triphosphate (CTP) synthetase (spot nos. 8 and 20) is the responsible for the de novo synthesis of CTP from uridine triphosphate (UTP) which is essential for cell growth. We detected a different expression pattern of this enzyme in *L. reuteri* CRL1098, according to the BA assayed. In fact, in the presence of GDCA it was over-expressed and significantly repressed by DCA. This enzyme could be linked to the repair of oxidative DNA injuries caused by BA and its down regulation could explain the retarded growth of the cells challenged with the free BA. Accordingly, when the probiotic *L. rhamnosus* GG strain was exposed to bile, the growth rate was significantly decreased and the abundance of CTP synthetase (*pyrG*) was also reduced at the proteomic and trancriptomic levels (Koskenniemi et al., 2011). On the other hand,

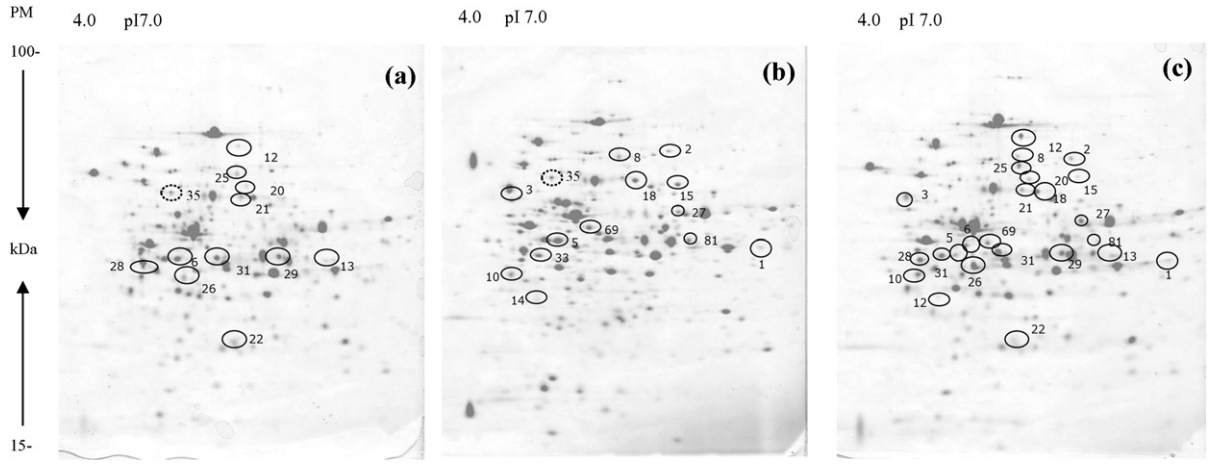


Fig. 2. 2D gels showing the *L. reuteri* CRL1098 proteins expressed in the presence of MRS medium with GDCA (a), DCA (b) and control (c). The successfully identified proteins are numbered and circled. Dotted circle was not identify and could correspond to BSH of *L. reuteri* CRL1098.

a positive regulation of CTP synthetase was observed in *Bifidobacterium* strains exposed to bile stress, denoting that this protein is part of the global response to bile in the genus (Sanchez et al., 2007). Moreover, Budin Verneuil, Pichereau, Auffray, Ehrlich, and Maguin (2007) reported the up-regulation of CTP synthetase when pre-adapted cells of *Lactococcus* (*Lac.*). *lactis* MG1363 were exposed to acid stress while in non-adapted cells no differences were observed denoting a link between the over-expression of the enzyme and survival. On the other hand, down regulation of *pyr* gene transcription has been observed previously at high ethanol concentrations in *Lac. Lactis* (Maligoy, Mercade, Cocaign-Bousquet, & Loubiere, 2008) and decreased production of Pyr proteins has been detected at high CO_2 concentrations in *L. plantarum* (Bringel, Hammann, Kugler, & Arsène-Ploetze, 2008). These results suggest that the down regulation of CTP synthetase constitutes a stress response activated in different stressful conditions that lead to a decreased nucleotide synthesis. A negative regulation of formate-tetrahydrofolate ligase (spot no. 15) was evidenced in *L. reuteri* CRL1098 when growing in presence of the free BA. Under stress conditions, formate–tetrahydrofolate ligase is required for the synthesis of a range of metabolites, including purines, histidine, and formyl tRNA-Met, which stimulates bacterial growth (Lee, Lee, & Choi, 2008). This result may suggest that DCA disturbed cellular balance and nucleotides metabolism, which could lead to the observed delay on *L. reuteri* CRL1098 growth under this condition. Our results differed with those by Lee et al. (2008) in *L. reuteri* ATCC 23272 that reported a slight over-expression of this enzyme in the presence of bile. Also, adenylosuccinate synthetase (spot no. 27), involved in nucleotides and nucleic acids metabolism was under-expressed in the presence of DCA. This could be related to the inhibition of macromolecules synthesis such as DNA, RNA and ATP and explain partially, the lower growth observed in CRL1098 in the presence of this compound. Negative regulation of nucleotide synthesis genes was previously reported in *Lac. lactis* subjected to acid stress (Xie, Chou, Cutler, & Weimer, 2004). On the other hand, in *L. plantarum* WCFS1, the adenylosuccinate synthetase gene was repressed under osmotic stress (Pieterse, Leer, Schuren, & van der Werf, 2005). These findings showed once more that the expression of a key enzyme is affected by a broad range of stress effectors.

3.2.2. Metabolism of glycerolipids

The expression of many proteins involved in glycerolipids metabolism was affected in the presence of both BA; among these proteins, we can highlight the diol/glycerol dehydratase reactivating factor (spot no. 2) — catalyzing 3HPA to glycerol in *L. reuteri* CRL1098 — and three isoenzymes of iron-containing alcohol dehydrogenase (IAD). In fact, Lreu_1840 (spot nos. 6 and 5), Lreu_0030 (nos. 13 and 18) and Lreu_1734 (spot no. 1), identified as IAD isozymes, were differentially expressed in CRL1098 strain in the presence of both BA. These enzymes are involved in different steps of the glycerolipids pathway. In presence of GDCA and DCA the isoenzyme Lreu_1840, responsible for glycerol production was over-expressed. In contrast, the spots identified as Lreu_0030 involved in 1-propanol synthesis had a decreased expression level in the presence of GDCA and DCA. Interestingly, the isoenzyme Lreu_1734 involved in the 1-propanol to propanal conversion had a significant down-regulation (five-fold respect to the control) in presence of DCA. This reaction is strongly necessary to ensure the NAD^+ supply to the cell and could partially explain the higher toxicity of the free BA and the delayed adaption of cells to the presence of DCA.

3.2.3. Protein transcription and translation

During translation, the elongation factors (EFs) are responsible for the delivery of aminoacyl-tRNA to the ribosome, and it has been proposed as a chaperone for unfolded and denatured proteins in *E. coli* (Caldas et al., 2000). The EF Ts (spot no. 26), the EF G (spot no. 21) and the threonyl-tRNA synthetase (spot no. 25) were over-expressed when *L. reuteri* CRL1098 was challenged with 1 mM (0.05% w/w) of GDCA; while 50S ribosomal protein L29P (spot no. 81) was down shifted by DCA effect. On the contrary, Lee et al. (2008) reported that EF Ts was repressed in *L. reuteri* ATCC23272 in the presence of bile. This divergence could be partially explained by the bile composition used (a mix of two different free BA) and the greatest sensibility to bile observed in the ATCC23272 strain compared to *L. reuteri* CRL1098. These results suggest that the over-expression of translation related proteins observed in presence of GDCA can be associated to the adaption of CRL1098 strain to the presence of this BA. Sanchez et al. (2005) reported that EFTu and P were slightly over-expressed and repressed respectively, in *B. longum*. EF Tu was also induced during acid stress of *Streptococcus mutans* and *Propionibacterium freudenreichii*, and EF P was induced in *Campylobacter jejuni* challenged with BA (Fox, Raftery, Goodchild, & Mendz, 2007; Leverrier et al., 2003; Wilkins, Homer, & Beighton, 2002). On the other hand, EF G was transiently repressed in *Enterococcus* (*Ent.*) *faecalis* V583 in the presence of 1% of bile during 20 and 60 min; however, it was over-expressed after 120 min of exposure (Bøhle et al., 2010). The 50S ribosomal protein was under regulated in CRL1098 strain exposed to DCA. Consistent with this finding, other authors have reported a repression of this protein in strains of *L. reuteri* and *L. casei* subjected to bile stressful conditions (Lee et al., 2008). Additionally, Koskenniemi et al. (2011) observed reduced levels of protein and mRNA in *L. rhamnosus* GG cells in the presence of bile. On the contrary, some 30S ribosomal proteins were induced in bacteria from other genus. In fact, *Listeria monocytogenes* showed over expression of these ribosomal proteins in response to bile treatment (Duché, Trémoulet, Namane, Labadie, & Consortium, 2002). In *Bacillus subtilis*

strain this effect was observed in the presence of several stress conditions (Eymann, Homuth, Scharf, & Hecker, 2002). A putative threonyl-tRNA synthetase was overexpressed by GDCA in the CRL1098 strain. On the contrary, Sanchez et al. (2007) and Belfiore et al. (2013) reported the opposite behavior of two related proteins (tRNA synthetase and arginyl tRNA synthetase, respectively) in a bile resistant mutant of *B. animalis* subsp. *Lactis* challenged with bile and in *L. sakei* CRL1756 exposed to osmotic stress. These results suggest that BA together with other stressful conditions, such as osmotic and acid, exert a modulation in transcription and translation of related proteins.

3.2.4. pH homeostasis and stress proteins

Several proteins involved in pH homeostasis and stress were differentially expressed by the presence of BA. Genes involved in general stress response are highly conserved in bacteria. These include a large number of proteins usually involved in the maturation of new proteins, in membrane-associated proteins, in the refolding or degradation of denatured proteins and in DNA repair (Georgopoulos & Welch, 1993). Bile stress affected a variety of cell systems; therefore it seems reasonable that regulation of homeostasis would be essential under this stressful condition. In fact, the exposure of *L. reuteri* CRL1098 to GDCA produced over-expression of ornithine carbamoyltransferase (spot no. 27). This enzyme, part of the arginine deiminase pathway, contributes to proton motive force (PMF) generation and elevates extracellular pH, via the neutralization of the intracellular compartment, to compensate acid stress (Rollan, Lorca, & Font de Valdez, 2003). Protonated BA, such as GDCA, may exhibit toxicity through intracellular acidification in a similar manner to organic acids (Bustos et al., 2012). As the internal pH decrease, ATP- and PMF-dependent transports becomes less efficient (Jaichumjai, Valyasevi, Assavanig, & Kurdi, 2010), protein activities may decrease, and protein and DNA damage occurs. Accordingly, the over-expression of this protein when *Lac. lactis* MG1363 was exposed to acid stress was reported (Budin Verneuil et al., 2007). We further observed that GDCA triggered the up-regulation of GTP-binding protein Typ A (spot no. 12), which may be involved in the maintenance of a functional membrane protein composition, findings comparable with those reports in *L. reuteri* ATCC 23272 (Lee et al., 2008). Chaperonine GroEL (spot no. 3) was shown to be overproduced in *L. reuteri* CRL1098 in presence of DCA, as previously found in other Gram-positive bacteria. Induction of GroEL expression was reported in *B. longum* (Sanchez et al., 2005), *Ent. faecalis* (Bøhle et al., 2010)and *Lac. lactis* (Champomier-Vergès, Maguin, Mistou, Anglade, & Chich, 2002) in response to bile.

3.2.5. Proteins with no described function

Spot no. 22 was identified in *L. reuteri* DSM 20016 as a microcompartment Lreu_1748 protein that seems to be identical to a propanediol utilization protein PduB. This carboxysome shell protein may be involved in the formation of the polyhedral organelles involved in propanediol degradation in some Gram positive and Gram negative bacteria (Yeates, Thompson, & Bobik, 2011). On the other hand, spot no. 14 was firstly described as a hypothetical protein (Lreu_1196), however is 100% identical to an exonuclease SbcC of *L. reuteri* DSM 20016. In *E. coli* this protein is involved in DNA mismatch repair, a highly conserved biological pathway that plays a key role in maintaining genomic stability.

3.3. Expression and regulation of bile salt hydrolase gene (bsh)

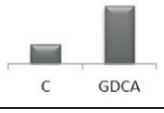
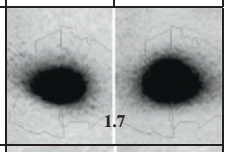
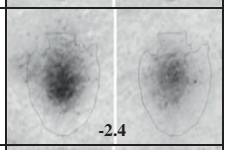
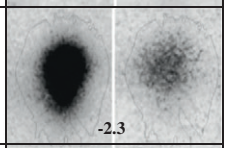
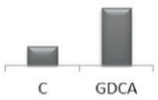
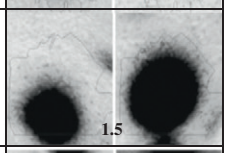
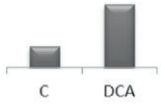
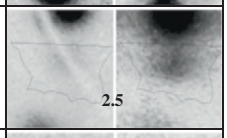
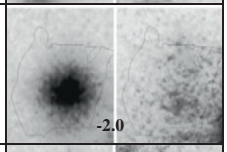
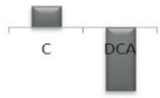
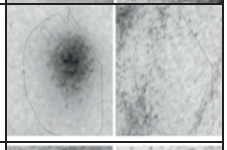
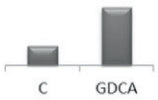
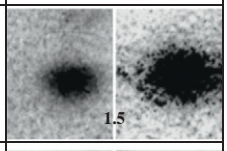
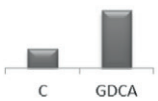
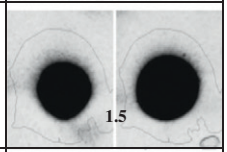
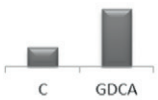
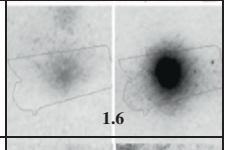
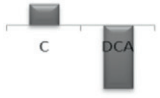
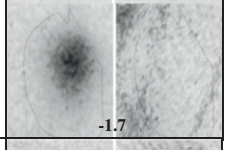
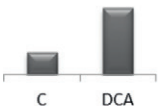
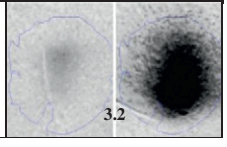
Bile salt hydrolases (BSH) are enzymes that catalyze the BA deconjugation, a mechanism that can be considered as part of a cell detoxification strategy and could play a role in bile tolerance in some Gram-positive bacteria(Begley, Hill, & Gahan, 2006; Grill, Cayuela, Antoine, & Schneider, 2000). The genome of *L. reuteri* CRL1098 contains one gene that encodes for BSH and also, displayed high BSH activity against BA, especially GDCA (Bustos et al., 2012; Taranto et al., 1999). The BSH regulation at genomic, transcriptomic or proteomic levels strongly fluctuates according to the studied microorganism (Begley et al., 2005). Even though, none spot was identified as the BSH enzyme, in the presence of GDCA. We characterized the HSB of *L. reuteri* CRL1098 as a polypeptide of 325 amino acids with a calculated mass of 36,098.1 Da, and predicted pI of approximately 4.81 (unpublished data). The spot no. 36 (Fig. 2), coinciding in pI and mass with the characterized CRL1098 BSH, was over-expressed (1.5 fold) in the presence of GDCA, however this spot was not successfully identified by Maldi Tof MS-MS. Studies with Gram-positive bacteria on bile-induced regulation reported that no changes either at the transcriptome or at the proteome level were observed (Bøhle et al., 2010; Lee et al., 2008; Solheim, Aakra, Vebo, Snipen, & Nes, 2007). In a proteomic study, a down-regulation in the expression level of BSH enzyme in a bile resistant strain of *L. plantarum* was observed, while no changes were found in the sensitive strains (Hamon et al., 2011). In an in vivo proteomic experiment, the exposition of *B. longum* NCC2705 to rabbit large intestine conditions induced the production of BSH (Yuan et al., 2007). As can be observed, BSH regulation is one of the complex mechanisms of bile resistance and strongly varying according to the studied microorganism.

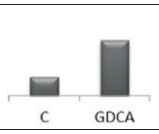
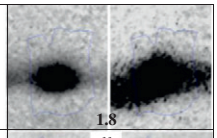
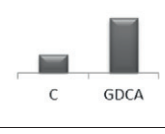
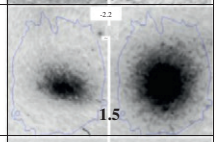
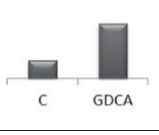
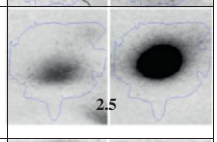
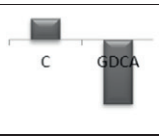
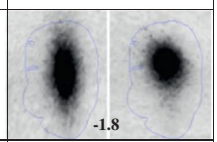
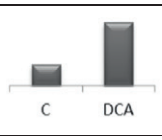
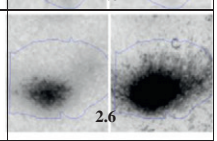
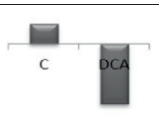
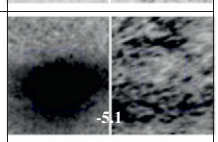
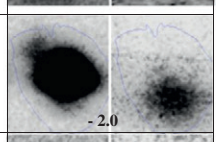
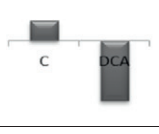
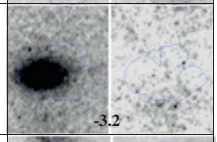
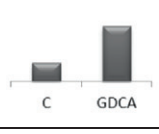
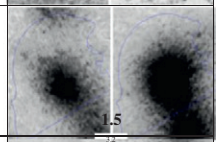
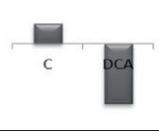
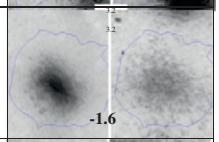
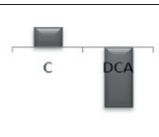
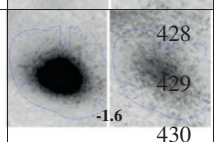
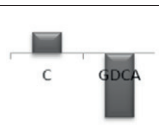
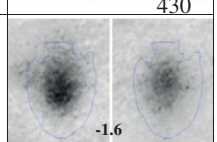
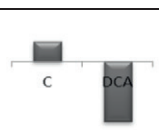
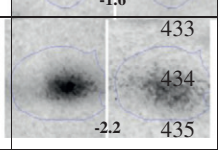
The regulation of *bsh* gene of *L. reuteri* CRL1098 in response to conjugated BA (GDCA) was evaluated using quantitative real time-PCR. Applying the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak, 2008) increases in *bsh* gene expression levels of 1.64 ± 0.07 (Fig. 4) in the presence of GDCA were obtained respect to the Ct obtained in the control conditions. In this sense, Koskenniemi et al. (2011) reported the strong up regulation of a *bsh* gene upon bile exposure in *L. rhamnosus* GG. Also, Duary, Batish, and Grover (2012) using RT-PCR reported the *bsh* expression levels increase in two indigenous *L. plantarum* strains exposed to increasing concentrations of bile. However, the standard *Lactobacillus* strain CSCC5276 did not show appreciable increase in the expression of *bsh* gene in the same conditions. Lambert, Bongers, De Vos, and Kleerebezem (2008) reported that the expression of the four *bsh* genes of *L. plantarum* WCFS1 was not induced as consequence of the exposure to porcine bile. Considering On the base of our RT-PCR (transcription) results we can postulate that the *bsh* gene is indeed regulated by the presence of a conjugated BA in *L. reuteri* CRL1098. Nevertheless, more extensive work is required to confirm the expression pattern of the BSH at the protein level.

4. Conclusions

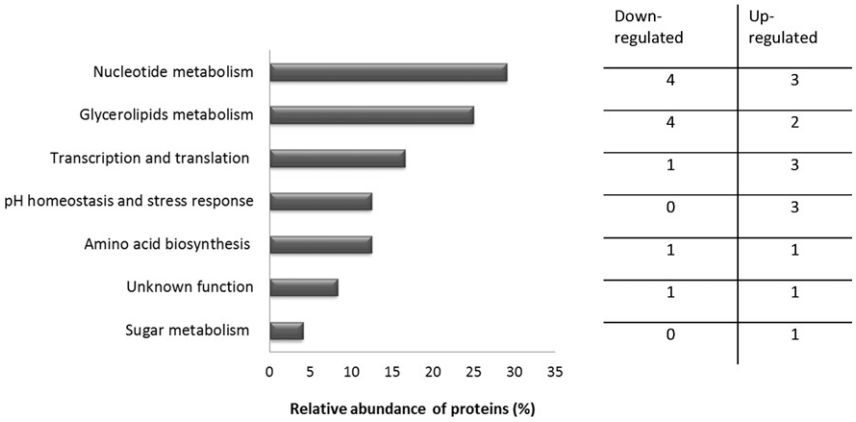
The present work reveals that conjugated (GDCA) and free (DCA) BA induce a deep metabolic reorganization in *L. reuteri* CRL1098 in order to achieve adaption and survival to stressful conditions such as those herein induced. This is reflected by differences in growth parameters as well as by differential expression of many proteins related to different metabolic categories. DCA clearly affected cell viability while in the presence of GDCA growth parameters were similar to the control conditions without BA. Twenty five successfully identified proteins were affected by the presence of BA; among the most important are those involved in nucleotide metabolism such as CTP synthetase, which was over-expressed in GDCA and, significantly repressed by DCA. In addition three proteins related to protein transcription and translation — elongation factors Ts and G — were over expressed in the presence of the conjugated BA, while one, the 50S ribosomal protein L29P, was repressed by the free BA. This pattern of results could explain the delayed growth of the cells challenged with the free BA. Moreover, three proteins related to pH homeostasis and stress response — chaperonin GroEL, GTP-binding protein TypA and ornithine carbamoyltransferase — were over produced in the presence of both BAs. Some of these constitute nonspecific responsive proteins towards different stress effectors such as acid, osmotic or heat among other. Finally, a significant up-regulation of the *bsh* gene in response to BA was observed, although more studies are necessary to confirm the regulation of BSH at the protein level, this

Table 1
Identified cytoplasmic proteins differentially expressed when *L. reuteri* CRL1098 was grown in the presence of conjugated GDCA and free DCA bile acids (BA).

Functional category	Spot no./protein/ access number[a]	Score	MS-MM/MS[b]	IP[c]	MW[d]	Relative change	Change fold (BA/C)	
							Control	BA
Nucleotide metabolism	20.CTP synthetase (Lreu_023)	163	10-2	5.07	59,589.8			
	8.CTP synthetase (Lreu_023)	236	11-3	5.07	59,589.8			
	10. Inosine/uridine-preferring nucleoside hydrolase (Lreu_0102)	138	6-2	4.48	32,567.0			
	28. Inosine/uridine-preferring nucleoside hydrolase (Lreu_1658)	223	3-3	4.54	34,899.4			
	7. Inosine/uridine-preferring nucleoside hydrolase (Lreu_1658)	122	6-3	4.54	34,899.4			
	15. Formate-tetrahydrofolate ligase(Lreu_0131)	183	11-2	5.5	60,240.9			
	27.Adenylosuccinate synthase(Lreu_0069)	200	14-2	5.36	47,716.5			
Protein transcription and translation	25. Putative threonyl-tRNAsynthetase (Lreu_1863)	109	13-1	5.01	68,754.2			
	26. Elongation factor Ts (Lreu_0685)	103	6-2	4.66	31,956.2			
	21. Elongation factor G (Lreu_1486)	131	10-1	4.52	76,756.9			
	81.50S ribosomal protein L29P(Lreu_1735)	70	8-1	5.18	51,117.5			
pH homeostasis and stress	3. Chaperonin GroEL(Lreu_0354)	162	8-2	4.46	57112.7			

	12. GTP-binding protein TypA (Lreu_0637)	158	8-2	4.92	68,815.7		
	29. Ornithine carbamoyltransferase (Lreu_0425)	85	5-3	5.20	37,553.0		
Fatty acids metabolism	6. Iron-containing alcohol dehydrogenase (IAD) (Lreu_1840)	119	4-2	4.70	40,691.2		
	13. IAD (Lreu_0030)	80	4-1	5.63	42,172.7		
	5. IAD (Lreu_1840)	189	10-2	4.70	40,691.2		
	1. IAD (Lreu_1734)	143	14-2	6.10	40,353.1		
	18. IAD (Lreu_0030)	130	12-1	5.63	42,172.7		
	2. Diol/glycerol dehydratase reactivating factor (Lreu_1744)	116	9-2	5.32	65,682.8		
Metabolism of amino acids	31. Cystathionine gamma-lyase (Lreu_0293)	106	5-1	4.87	41,473.1		
	69. Aminotransferase (Lreu_0744)	223	15-2	4.84	43,099.4		
Sugar metabolism	33. D-isomer specific 2-hydroxyacid dehydrogenase, NAD-binding(Lreu_1631)	136	9-1	4.55	36,559.3		
Unknown function	22.Microcompartment protein (Lreu_1748)	189	6-3	5.07	24,940.6		
	14. Hypothetical protein (Lreu_1196)	197	12-3	4.56	35,190.9		

[a]Accession number in the NCBI database for *L. reuteri* DSM 20016.
[b]No. of peptides obtained by MS and MS/MS respectively.
[c]Calculated isoelectric point.
[d]Molecular mass (Da).



Down-regulated	Up-regulated
4	3
4	2
1	3
0	3
1	1
1	1
0	1

Fig. 3. Relative abundance of *L. reuteri* CRL1098 identified proteins in response to BA. Proteins (25 in total) are grouped according to their functional category. On the right, the number of proteins down or up regulated for each functional category is indicated.

enzyme could be a specific biomarker of bile adaption in *L. reuteri*. Although more detailed validation experiments are needed to confirm these findings, the present work provides novel insights into the mechanisms involved in BA tolerance of *L. reuteri*, being of usefulness for future investigations into the genetic and physiological aspects of this response.

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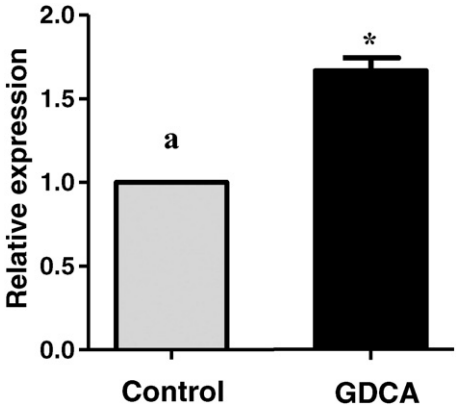

Fig. 4. Relative expression of *bsh* gene in *L. reuteri* CRL1098 cells grown in MRS broth with and without BA. *Rec A* primers were used as internal control gene to normalize the amount of RNA added. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. a Cells growing in MRS broth without BA addition were using as reference condition and an arbitrary value of 1 was assigned. ∗ Standard deviation; GDCA: glycodeoxicholic acid.

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