

Proteomic analysis of the response to NaCl stress of *Lactobacillus bulgaricus*

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Abstract *Lactobacillus bulgaricus* is commonly used in dairy products as a starter culture. Its viability during freeze-drying is of commercial interest. Here a significant ($p < 0.05$) improvement in survival rate of *L. bulgaricus* ATCC 11842 was achieved during freeze-drying when it was prestressed with 2 % (w/v) NaCl for 2 h in the late growth phase. To understand the mechanism of this stress-related viability improvement in *L. bulgaricus*, protein synthesis was analyzed by 2D difference gel electrophoresis. Nine protein spots were significantly altered by NaCl and were subsequently identified by peptide mass fingerprinting. The functions of the proteins included stress-related protein synthesis, amino acid biosynthesis, nucleotide biosynthesis, sugar metabolism, transport systems, and vitamin biosynthesis. These findings provide a considerable background regarding the NaCl stress response of *L. bulgaricus*.

Keywords 2D-Difference gel electrophoresis · Freeze-drying · *Lactobacillus bulgaricus* · MALDI-TOF/TOF-MS · Proteomics · Sodium chloride stress

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Introduction

Freeze-dried cultures have many advantages, including that they have less volume, are convenient for transportation and storage, and are easy to use compared to traditional liquid cultures. However, freeze-drying can damage cells, including loss of viability, decrease in metabolic activity, and changes in cell morphology, which can affect the physiology and characterization of lactic acid bacteria (LAB), their fermentation efficiency and other beneficial functions (De Giulio et al. 2005).

Moderate, sub-lethal stress can improve the survival rate and activity of freeze-dried LAB. The stress factors could include heat (50 °C) or salt (0.6 M NaCl) for *Lactobacillus rhamnosus* (Prasad et al. 2003) and cold stress (from 26 °C, 8 h to 15 °C) for *Lactobacillus acidophilus* (Wang et al. 2005). The stress responses of LAB exposed to unfavorable conditions include the synthesis of stress proteins or modification of their membrane compositions. These changes enhance the resistance of LAB to these effects and of some additional stress factors (Lorca and Font de Valdez 2001). The development of cross-resistance to stress factors can be used to enhance desirable technological properties, such as resistance to lyophilization. Koch et al. (2007) reported that NaCl at 2.5 % (w/v) significantly enhanced ($p < 0.05$) the survival of freeze-dried *L. delbrueckii* subsp. *lactis* group, and cultures grown with NaCl at 2.5 % (w/v) gave high yields of viable cells in broths before and

after freeze-drying. The authors contended that the very large increased survival after lyophilization by osmotically-stressed cells might be partly due to the accumulation of compatible solutes during growth. However, they did not investigate other mechanisms and little is known about the protein expression changes of *L. bulgaricus* that arise in response to NaCl stress. In this paper, two-dimensional difference gel electrophoresis (2D-DIGE) of whole cell proteins coupled to MALDI-TOF/MS was used to identify nine *L. bulgaricus* proteins differentially expressed under NaCl stress. These data will improve our understanding and knowledge of the molecular mechanisms of *L. bulgaricus* exposed to NaCl stress.

Materials and methods

Bacterial strain and culture conditions

Lactobacillus bulgaricus ATCC 11842 was used. It was kept as frozen stock in 40 % (v/v) glycerol at -80°C . For experimental use, the strain was cultured in MRS broth at 37°C for 14 h to 10^8 c.f.u. ml^{-1} , using three successive subcultures (1 % v/v inoculation each).

NaCl stress

NaCl was sterilized by UV radiation for 30 min and was slowly added to the late growth phase (13.5 h) of 5 l MRS cultures of *L. bulgaricus* at 37°C with stirring at 100 rpm to give final concentrations of 2, 3.5, and 5 % (w/v), with 8×10^8 c.f.u. ml^{-1} for each culture. *L. bulgaricus* was further incubated for 2–6 h at the same temperature. The control sample was processed without adding NaCl. All of the tests were repeated three times.

Cell preparation for freeze-drying

Freeze-drying was performed as previously reported (Li et al. 2012a, b). Cells were concentrated by centrifugation ($11,000 \times g$, 15 min at 4°C) and pellets were re-suspended in three-fold their weight of sterilized cryoprotectant, which was composed of 12 % (w/v) skimmed milk, 5 % (w/v) sucrose, and 5 % (v/v) glycerol at 115°C for 15 min. Freeze-drying was performed in a freeze-drier. Vials, 5 ml,

were filled to 2 ml. The samples were frozen at -80°C for 12 h before lyophilization. Freezing progressed at $5^{\circ}\text{C min}^{-1}$ until the samples were at -40°C . After freezing, the vacuum was reduced to 13.3 Pa, and the shelf temperature was raised to -20°C . Secondary drying was performed in a step-wise manner to 30°C for a total of 16 h. The vials were sealed at 13.3 Pa and were analyzed the same day. Freeze-dried cultures were used to calculate survival rate of the cell after freeze-drying.

Rehydration was performed within 2 h after freeze-drying by adding membrane filtered water at ambient temperature to the same volume as before freeze-drying. Cell viability was determined by tenfold serial dilutions and by spotting 10 μl onto MRS agar plates. The plates were incubated at 37°C for 48 h, and 10–30 c.f.u. in three replicates were counted. Survival is reported as the ratio between cell counts before freeze-drying and after freeze-drying and given as percentage values.

Protein extraction

Proteins before and after salt exposure were prepared as described by Shi et al. (2011), with a few modifications. The samples in each group were washed three times in PBS and placed into a 1 ml DIGE lysis buffer (pH 8.8) containing 7 M urea, 2 M thiourea, 65 mM Tris, 4 % (w/v) CHAPS, 0.2 % (v/v) IPG buffer and a protease inhibitor cocktail (Merck Millipore) and were homogenized with a Dounce homogenizer. The suspension was disrupted by ultrasonication (80 W, 10 s, five repeats with 15 s intervals between repeats). All procedures were carried out on ice. The mixture was centrifuged at $15,000 \times g$ for 45 min at 4°C . The protein content in the supernatants was determined by the Bradford assay. The samples were stored at -80°C until proteomic analysis.

Protein labeling with dyes

Three protein samples (50 μg each), group A, B and C, were labeled with 400 pmol Cy3 or Cy5 minimal dye (GE Healthcare), according to the experimental design (Table 1). Dye swap among groups A, B and C protein samples was designed to avoid artefacts due to preferential labeling. A pooled sample consisting of equal amounts of all of the samples and were labeled

Table 1 Experimental design for 2-D DIGE

Gel No.	Cy2 labeled	Cy3 labeled	Cy5 labeled
1	Pool	Group A1	Group B2
2	Pool	Group B1	Group A2
3	Pool	Group A3	Group B3

Pool Internal Standard, the pooled sample consisting of equal amounts of all samples

with Cy2 for use as the mixed internal standard to control for gel-to-gel variation. After incubating on ice for 30 min in the dark, the labeling reaction was quenched with 1 μ l 10 mM L-lysine.

2D-Difference gel electrophoresis (2D DIGE) and gel image analysis

2D-DIGE and gel image analysis were performed as described by Shi et al. (2011). For each gel, Cy2-, Cy3-, and Cy5-labeled proteins (50 μ g each) were mixed and an equal volume of rehydration buffer [8 M urea, 4 % (w/v) CHAPS, 130 mM DTE, and 2 % (v/v), Pharmalyte pH 3–10] was added. Isoelectric focusing (IEF) was carried out on non-linear IPG strips (13 cm long, pH 3–10, GE Healthcare) with an Ettan IPGphor apparatus (GE Healthcare) for a total of 40 kV/h at 20 °C and a maximum current setting of 50 μ A per strip. After IEF, individual strips were incubated in equilibration buffer [50 mM Tris/HCl, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS] supplemented with 1 % (w/v) DTT followed by 2.5 % (w/v) iodoacetamide. The proteins were then resolved in 12.5 % SDS-PAGE gels at 15 mA for 15 min and then at 30 mA at 20 °C until the Bromophenol blue dye front ran off the bottom of the gel. To facilitate MS analysis, 500 μ g unlabeled pool protein sample of each group was run in parallel on a preparative gel and stained by Deep Purple staining (GE Healthcare), according to the manufacturer's instructions.

Gel images for analysis were obtained by using a Typhoon 9700 Imager (Amersham Bioscience) and processed with DeCyder 6.5 differential analysis software (Amersham Bioscience). The spots on the gels were collected automatically as 2D-DIGE image pairs, which intrinsically link a sample to its in-gel standard. Matching between gels was performed using the in-gel standard from each image pair. The experimental set-up and relationship between samples were assigned by the DeCyder software. Each

individual Cy3 or Cy5 gel image was assigned an experimental condition and all of the Cy2 images were classified as standards. The gel with the highest spot count was considered the master gel. Statistical analysis was carried out for every matched-spot set comparing the average and standard deviation of protein abundance for a given spot between group A and group B or between group B and group C using Student's *t* test, and the comparison among the three groups was carried out using ANOVA. The procedure was performed using the DeCyder difference in-gel analysis (DIA) and DeCyder biological variation analysis (BVA) software module. Protein spots of interest with significant differences in abundance (more than 1.1-fold, $p < 0.05$) were selected in the stained preparative gels for spot picking.

MALDI-TOF/TOF MS analysis and protein identification

Spots of interest in the preparative gels were manually excised. The gel samples were placed in a tube and were washed twice with 500 μ l and 250 μ l double-distilled water for 15 min. For trypsin digestion, the gel samples were washed twice with 50 mM (w/v) NH_4HCO_3 and covered with 0.7 μ l porcine trypsin solution (Promega) in 50 mM (w/v) NH_4HCO_3 . After incubation overnight at 37 °C, the supernatant was removed into a second tube and 40 μ l 50 mM (w/v) NH_4HCO_3 was added. Gel samples were washed with 40 μ l 50 mM (w/v) NH_4HCO_3 , the supernatant was collected, and both collected supernatants were combined. Then, the collected solution was washed with 70 % (v/v) acrylonitrile (ACN) and dried in a Speed Vac (Vacuum Concentrator, Bachhofer). The peptide mixtures were desalted using ZipTip C-18 RP tips (Millipore), which were wetted with 100 % ACN and equilibrated with 0.1 % (w/v) trifluoroacetic acid (TFA). Peptide samples, which were redissolved in 10 ml 0.5 % (w/v) TFA, were eluted with 50 % (v/v) ACN/0.1 % (w/v) TFA and were dried in a Speed Vac.

The purified peptides were spotted on a MALDI plate and covered with 0.7 ml 2 mg 3,5-dimethoxy-4-hydroxycinnamic acid ml^{-1} with 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ in 60 % (v/v) ACN. All of the samples were then analyzed by an ABI 4800 MALDI-TOF/TOF MS proteomics analyzer (Applied Biosystems, Foster City, CA, USA). Mono-isotopic peak masses were acquired in a mass range of 800–4,000 Da, with a

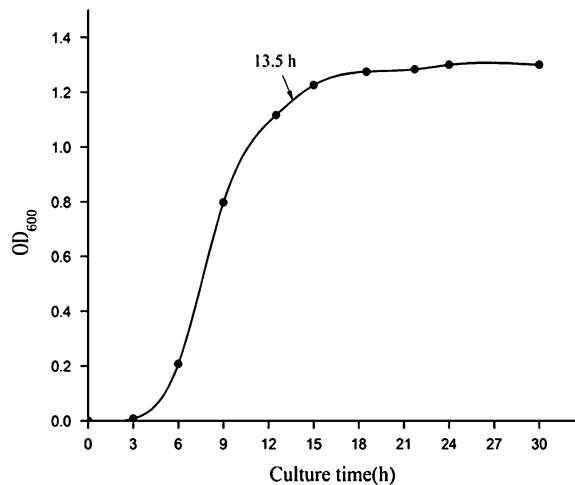


Fig. 1 The growth curve of *Lactobacillus bulgaricus*

signal-to-noise ratio (S/N) of 200. Five of the most intense ion signals, excluding common trypsin autolysis peaks and matrix ion signals, were selected as precursors for MS/MS acquisition. The peptide mass fingerprint (PMF) combined MS/MS data were submitted to MASCOT version 2.1 (Matrix Science, <http://www.matrixscience.com/>) for identification according to the NCBI Bacteria(12539933) database (<http://www.ncbi.nih.gov/>).

The main searching parameters were set as follows: Rattus, trypsin cleavage (one missed cleavage allowed) as a fixed modification, methionine oxidation as a variable modification, peptide mass tolerance set at 100 ppm, and fragment tolerance set at 0.8 Da. The criteria for successfully identified proteins were follows as: ion score confidence interval (C.I %) for PMF and MS/MS data ≥ 95 %, peptide count (hit) ≥ 4 and at least two peptides of distinct sequence were identified in MS/MS analysis.

Results

Effect of NaCl on survival rate of *Lactobacillus bulgaricus* after freeze drying

The growth curve of *L. bulgaricus* in MRS broth without adding NaCl is shown in Fig. 1. Due to the differences in metabolic activity and protein synthesis, different growth stages of cells could show different

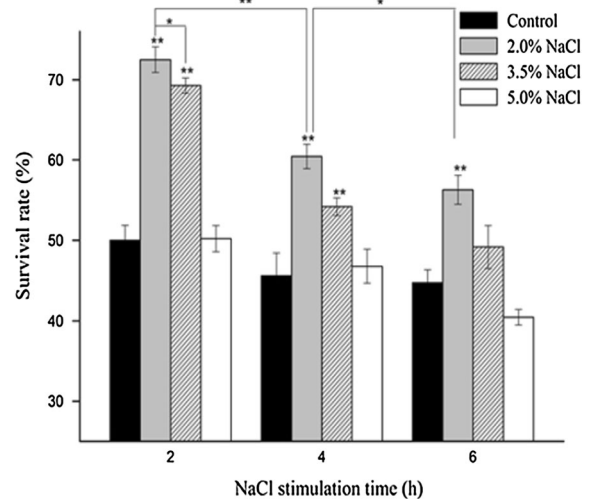


Fig. 2 The survival of freeze-dried cells after NaCl stress * $P < 0.05$, ** $P < 0.01$

resistance to NaCl stress. This study selected culturing for 13.5 h to ensure that *L. bulgaricus* had entered the late growth phase before adding exogenous NaCl.

The different concentrations of NaCl added to MRS broth led to different freeze-drying survival rates of *L. bulgaricus* (Fig. 2). The survival rate for freeze drying at 2–6 h was significantly increased ($p < 0.01$) for 2 % (w/v) and 3.5 % (w/v) NaCl compared to the control. When NaCl was added to cultures at 13.5 h at 37 °C with stirring at 100 rpm, the survival rate of *L. bulgaricus* was increased by 1.45-fold with 2 % (w/v) NaCl and by 1.4-fold with 3.5 % (w/v) NaCl compared to the control. However, no significant change was found for the 5 % (w/v) NaCl stress.

Proteome profiles in the presence of NaCl stress

After 2D-DIGE, using the same amount of proteins of *L. bulgaricus* with and without NaCl stress, protein maps, including $1,568 \pm 8$ spots, were detected in the gel. The maps were clear and the reproducibility was high (Fig. 3). The position of the protein spots of *L. bulgaricus* with and without NaCl stress (Fig. 3a) and nine differentially expressed protein spots (Fig. 3b) in the representative 2D-DIGE images are shown. Nine spots were identified by DeCyder software and were analyzed by mass spectrometry (Supplementary Fig. 1), and these spots showed significant differences between the control and 2 % (w/v) NaCl stress group.

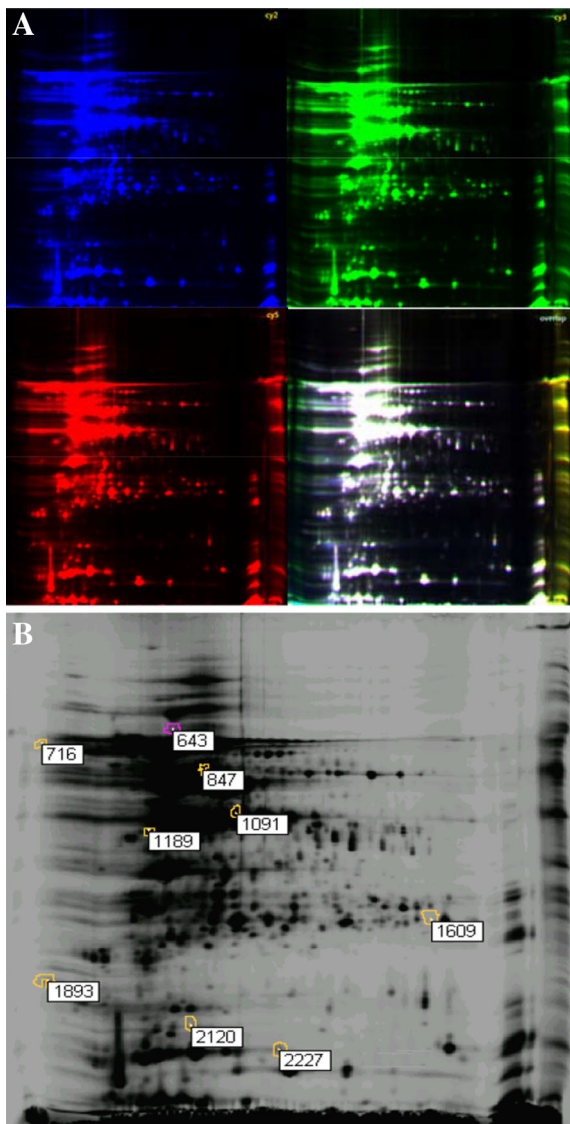


Fig. 3 Representative 2D-DIGE gels illustrating proteins from *L. bulgaricus* with and without NaCl stress. **a** Standard conditions; the internal control samples containing the proteins of *L. bulgaricus* with and without NaCl stress are labeled with Cy2 (blue). Proteins from the control were labeled with Cy3 (green), and proteins from *L. bulgaricus* with NaCl stress were labeled with Cy5 (red). The color picture is the overlapping images. **b** A preparative gel showing nine differentially expressed protein spots

Protein identification by MALDI-TOF/TOF-MS

Peptide mass fingerprints and queries of the database were used to analyze proteomic changes by MALDI-TOF/TOF-MS. Six spots identified were up-regulated

and another three spots were down-regulated in the 2 % (w/v) NaCl stress group compared with the control group (Table 2). The six up-regulated proteins were: elongation factor G, threonine synthase, lactate dehydrogenase, UMP kinase, D-lactate dehydrogenase and oxidase V related flavin-nucleotide-binding protein. The three down-regulated proteins were: molecular chaperone GroEL, inosine-5'-monophosphate dehydrogenase and glucitol/sorbitol-specific transporter subunit.

Discussion

Although the physiological characteristics of cells may change at the middle of its growth phase by salt stress, our aim was to investigate how to increase the freeze-drying survival of cells with a higher c.f.u. values. Therefore, the study was designed to stress cells at the late growth phase and subsequently incubated the cells for 2–6 h. The proteomic analyses allow a more comprehensive view of the response of *L. bulgaricus* to NaCl stress using 2D-DIGE technology, which eliminates gel-to-gel variation and determine subtle changes. Proteins were up- or down-regulated in cultures grown with 2 % (w/v) NaCl. Their functional categories were identified using the protein database (Table 2).

Elongation factor G (EF-G) plays a crucial role in the translocation of transfer RNAs and messenger RNA during translation by the ribosome. EF-G also plays a chaperone-like function in protein folding, protects against thermal denaturation and interacts with unfolded proteins. EF-G was up-regulated in *L. bulgaricus* under NaCl stress. EF-G displayed different behavior in different conditions, such as down-regulation of expression in *L. plantarum* under alkaline stress (Lee et al. 2011). These differential effects of stress indicate important organism-specific differences in responses to environmental challenges and highlight limits in the ability to generalize about adaptive responses to different stress factors.

GroEL is a molecular chaperone that prevents premature folding and promotes the attainment of the correct state in vivo. It may offer additional protection against protein denaturation and produce a more viable and physiologically active product. Prasad et al. (2003) found that GroEL from NaCl-stressed *L. rhamnosus* showed a three-fold increase when

Table 2 2D-DIGE analysis of altered protein spots between the control and stress group

Spots	Protein name	Accession number	Function ^a	Av. Ratio	MW (Da)	PI	Protein score	C.I. %	Peptide count	T-test
643	EF-G	gil104773540	Protein synthesis	1.11	76562.5	4.84	1160	100	23	0.045
716	GroEL	gil104774423	Stress-related protein	-4.16	57296.8	4.86	889	100	23	0.0057
847	TS	gil104774524	Amino acid biosynthesis	1.85	54538.7	5.18	380	100	15	0.043
1091	IMPDH	gil104773462	Nucleotide biosynthesis	-1.12	40197.5	5.42	1160	100	15	0.041
1189	LDH	gil116513302	Sugar metabolism	1.65	36997.9	4.88	954	100	19	0.013
1609	UMPCK	gil422843956	Nucleotide biosynthesis	1.15	25943.4	7.82	743	100	19	0.00017
1893	D-LDH	gil418029749	Sugar metabolism	1.36	37013.9	4.88	324	100	11	0.013
2120	SlrB	gil104773699	Transport system	-1.21	14117.3	5.07	205	100	7	0.045
2227	Pyridoxine 5	gil116513336	Vitamin biosynthesis	1.18	13039.7	5.07	122	100	3	0.048

EF-G elongation factor G, *GroEL* molecular chaperone GroEL, *TS* threonine synthase, *IMPDH* inosine 5'-monophosphate dehydrogenase, *LDH* lactate dehydrogenase, *UMPCK* UMP kinase, *D-LDH* D-lactate dehydrogenase, *SlrB* PTS system glucitol/sorbitol-specific transporter subunit IIA, *Pyridoxine 5* pyridoxine 5'-phosphate oxidase V related flavin-nucleotide-binding protein

^a Protein base from <http://www.uniprot.org/> and <http://www.ncbi.nlm.nih.gov/>

cultures were subjected to 0.6 M NaCl. The expression of GroEL in NaCl stress was the least down-regulated protein (approx. a decrease of 76 %) during 2 % (w/v) NaCl stress in the present study. The differentially expressed protein may be related to differences among strains.

In *L. bulgaricus*, in response to NaCl stress, the highest up-regulation (~1.85-fold) of proteins, threonine synthase, was observed. Threonine synthase is a pyridoxal-phosphate enzyme that catalyzes the transformation of homoserine-phosphate into threonine. Li et al. (2012a, b) found that the content of aspartate in *L. bulgaricus* clearly increased under NaCl stress. Whereas aspartate is the precursor of threonine, some bacteria synthesize threonine by accumulating aspartate in cells and not only allows the cells to re-establish osmotic balance but also to increase tolerance to osmotic stress (Bayles and Wilkinson 2000). Thus, we contend that threonine may play a role as osmoprotectant for bacteria. Mechanisms of adaptation involving accumulation of aspartate or threonine may enhance survival during freeze-drying.

Pyridoxine 5'-phosphate oxidase V-related flavin-nucleotide-binding protein is directly involved in pyridoxal phosphate biosynthesis and results in the formation of pyridoxal phosphate, the active form of

vitamin B6. It also plays a role in oxidative stress responses because the active form of vitamin B6 has been shown to exhibit antioxidant activity (Mooney et al. 2009). Garifzyanov et al. (2012) demonstrated that an increased NaCl concentration will lead to oxidative stress, and oxidative stress tolerance should be regarded as a component of salt tolerance.

Inosine 5'-monophosphate dehydrogenase controls a key metabolic step of the NAD-dependent oxidation of inosine 5'-monophosphate to xanthosine 5'-monophosphate in the synthesis of the guanine nucleotides and plays a key role in the regulation of cell growth and differentiation (Kozhevnikova et al. 2012). This protein was down-regulated, which may indicate that NaCl stress disturbed a cellular balance in the metabolism of nucleotides, and this unbalance may reduce cell growth of *L. bulgaricus*.

NaCl stress has been associated with expression of lactate dehydrogenase, an enzyme involved in energy metabolism and reoxidation of NADH through the reduction of pyruvate. Lee et al. (2008) found a similar result with *L. reuteri* at low pH stress. In addition, NaCl induced a down-regulation of the PTS system glucitol/sorbitol-specific transporter subunit IIA that transports sugar (glucitol/sorbitol) into the cell and catalyzes the phosphorylation of incoming sugar

substrates concomitantly with their translocation across the cell membrane, thereby suggesting a lower glycolytic flux after exposure to NaCl. In contrast, Hamon et al. (2012) observed that a number of glycolytic enzymes were up-regulated after exposure to bile stress. This difference may exist due to differences in strain and test conditions.

In addition, the fatty acid composition is the major way for cells to maintain its membrane fluidity and its changes in the structural and dynamic characteristics affect the functions of proteins associated with lipid environment, and may even influence their subsequent resistance to freeze or drying (Teixeira et al. 1996). In this study, none of the expressed proteins were associated with lipid metabolism.

In conclusion, NaCl stress response of *L. bulgaricus* is a complex process. The changes of several key proteins were observed. These findings provide early insight into the NaCl stress response of *L. bulgaricus* and may facilitate future investigations into the genetic and physiological aspects of this response.

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