Proteomic comparison of the probiotic bacterium *Lactobacillus casei* Zhang cultivated in milk and soy milk

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ABSTRACT

Soy milk is regarded as a substitute for milk and has become popular in varied diets throughout the world. It has been shown that a newly characterized probiotic bacterium (*Lactobacillus casei* Zhang) actually grows faster in soy milk than in bovine milk. To elucidate the mechanism involved, we carried out a proteomic analysis to characterize bacterial proteins that varied upon growth in soy milk and bovine milk at 3 different growth phases, and compare their expression under these conditions. A total of 104 differentially expressed spots were identified from different phases using a peptide mass fingerprinting assay. Functional analysis revealed that a major part of these identified proteins is associated with transport and metabolism of carbohydrates, nucleotides, and amino acids as well. The results from our proteomic analysis were clarified by real-time quantitative PCR assay, which showed that *Lb. casei* Zhang loci involved in purine and pyrimidine biosynthesis were transcriptionally enhanced during growth in soy milk at lag phase (pH 6.4), whereas the loci involved in carbohydrate metabolism were upregulated in bovine milk. Particularly, our results showed that L-glutamine might play an important role in the growth of *Lb. casei* Zhang in soy milk and bovine milk, perhaps by contributing to purine, pyrimidine, and amino sugar metabolism.

Key words: *Lactobacillus casei* Zhang, growth in milk and soy milk, proteomic analysis, 2-dimensional electrophoresis

INTRODUCTION

Yogurt consumption exerts a beneficial effect on human health all over the world. In general, yogurt is a bovine milk product fermented by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, which contains 12

to 14 g/100 g of total milk solids; and is soft, friable, and custard like, with a clean and distinct acid flavor (Lee et al., 1990). However, the demand for bovine milk (hereinafter called milk) substitutes is fast growing due to possible allergenic problems and a desire for vegetarian alternatives, among other factors (Farnworth et al., 2007). Soy milk has drawn more attention recently, and is used for production of many yogurt-like products.

Soy milk is an aqueous extract of whole soybean that is rich in high-quality proteins but lacking in quantities of SFA (Villares et al., 2011). In fact, it must be a healthier protein source than milk because it does not contain cholesterol and lactose. Soy milk constitutes sufficient nutrients to support growth of lactic acid bacteria (**LAB**) and fermentation helps to improve the quality of soy milk by removing the unfavorable bean flavor and reduces indigestible α -D-galactosyl oligosaccharide contents (Mital and Steinkraus, 1975). Previously, strains of bifidobacteria and lactobacilli have been widely used in soy milk fermentation (Scalabrini et al., 1998; Hou et al., 2000; Otieno et al., 2007).

Lactobacillus casei Zhang, which was originally isolated from a traditional homemade koumiss in Inner Mongolia (China), has been considered as a new probiotic bacterium (Zhang et al., 2006; Zhang et al., 2007; Ya et al., 2008; Wu et al., 2009b; Zhong et al., 2012). Our laboratory study of fermentation properties of the strain indicated that *Lb. casei* Zhang grows faster in soy milk than in milk at inoculation rates ranging from 2×10^6 to 2×10^7 cfu/g (Wang et al., 2009). The different growth conditions of milk and soy milk may induce different responses of LAB at the protein level. Previously, 2-dimensional electrophoresis (**2-DE**) has been applied to determine the protein variation of several LAB strains during growth in milk during fermentation. Protein syntheses of *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2772 during the first 30 min of fermentation in milk using 2-DE revealed that a change from glucose to lactose as carbon source imposes only little need for synthesis of stress or glycolytic enzymes, as sufficient proteins are present in the stationary phase of Man, Rogosa, and Sharpe (**MRS**) medium-grown cells

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(Rechinger et al., 2000). Furthermore, proteomic profile comparison of the strain growing in the synthetic medium M17 and skim milk showed that 55 proteins expressed differently in *Strep. thermophilus* LMG18311 (Derzelle et al., 2005). The strong upregulation of the pyruvate formate lyase was found as a formate supplier for anabolic purposes. However, only few proteomic studies have been performed on *Lb. casei* so far and most of them were done on bacteria grown in rich laboratory medium (MRS medium).

In this study, we compared the protein profile of *Lb. casei* Zhang during growth in milk and soy milk by carrying out proteomic analysis via 2-DE, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). We present the first report on the proteomic analysis of LAB fermentation in soy milk, and compare the change of its protein profile with that from milk fermentation in 3 phases. Our study provides new insight into the molecular basis of *Lb. casei* Zhang in utilization of varied protein resources.

MATERIALS AND METHODS

Strain, Medium, and Growth Conditions

The strain *Lb. casei* Zhang was isolated from traditional homemade koumiss in Inner Mongolia of China (Wu et al., 2009a). For proteomic analysis, a direct vat-set culture containing 2×10^7 cfu/g of *Lb. casei* Zhang was used to inoculate 6 L of reconstituted skim milk (10 g/100 g of milk powder; NZMP Ltd., Wellington, New Zealand) and reconstituted skim soy milk (Wan-defu Food Co. Ltd., Shandong, China). Each of the hydrated solutions was supplemented with 1.5 g/100 g of glucose. Fermentation was carried out at 37°C, and the pH value and viable counts were determined during fermentation. The viable counts of *Lb. casei* Zhang during growth were enumerated as described previously (Tharmaraj and Shah, 2003). All analyses were performed in triplicate.

Sugar utilization and organic acid production during fermentation were also analyzed using HPLC. One gram of sample was dissolved in 10 mL of 0.5 M sulfuric acid and centrifuged at $10,000 \times g$ for 10 min. The supernatant was collected and filtrated with a 0.45- μ m filter. The sugar content was determined using a previously described method (Quattrucci et al., 1997). Ten microliters of supernatant was injected into the HPLC apparatus (Agilent 1100; Agilent Technologies Inc., Santa Clara, CA) for analysis. The mobile phase was 20:80 water to acetonitrile (vol/vol), the flow rate was set at 0.7 mL/min with a UV detector set at 210 nm, and a Zorbax carbohydrate NH₂ column (4.6 $\times$ 250

mm, 5- μ m film thickness; Agilent Technologies Inc.) was operated at 40°C. The contents of lactic acid and acetic acid were determined by the method described by Zhang et al. (2008). The mobile phase was 5:95 10 mM phosphate buffered solution (pH 2.5) to methanol (vol/vol), with a flow rate of 0.5 mL/min, the UV detector was set at 210 nm, and a Zorbax SB-Aq column (4.6 $\times$ 150 mm, 5- μ m film thickness; Agilent Technologies Inc.) was operated at 35°C.

Culture Collection

Cultures were stopped in the lag, exponential, and stationary phase during growth in milk and soy milk by cooling to 4°C. The corresponding pH values were 6.4, 5.2, and 4.5 after incubation for 2, 14, and 18 h in milk, as well as 2, 9.5, and 14.5 h in soy milk.

Different protocols were used to collect bacteria cultures in milk and soy milk. The unset milk and soy milk at pH 6.4 were pumped through a filter, and then the filtrate was centrifuged at $3,000 \times g$ for 7 min at 4°C to collect the bacteria. The cell pellet was washed successively with 1,200 mL of 0.5 M ice-cold EDTA, 600 mL of 0.5 mM ice-cold EDTA, and 30 mL/100 mL of ice-cold alcohol, as well as 300 mL of 30 mL/100 mL of ice-cold alcohol. Finally, after centrifugation at $3,000 \times g$ for 7 min at 4°C, the harvested bacteria of the lag phase were rapidly put in liquid nitrogen and stored at -80°C. The semisolid or solid fermented milk and soy milk at pH 5.2 and 4.5 were centrifuged at $200 \times g$ for 10 min at 4°C. The supernatant was pumped through a filter and then centrifuged at $3,000 \times g$ for 10 min at 4°C to collect the bacteria. The cells were rapidly put in liquid nitrogen and stored at -80°C for protein extraction.

Preparation of Whole-Cell Extracts

The bacteria pellet was suspended in lysis buffer containing 8 M urea, 2 M thiourea, 0.5 g/100 mL of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 2 g/100 mL of Pharmalyte (pH 3–10), 1 mM PMSF, and 1% dithiothreitol (DTT); and 50 μ g/mL of DNase was added. The solution was maintained at 4°C for 15 min. Then cell disruption was performed with a sonicator (Ultraschallprozessor UP200S; Dr. Hielscher GmbH, Stuttgart, Germany; ultrasonic: 30 s, pulse duration: 30 s, power output: 60 W) while ice-cooling the sample. The cell debris was collected by centrifugation at $14,000 \times g$ for 30 min at 4°C, and the supernatant was frozen at -80°C. The concentration of each protein sample was determined with the Bradford assay.

2-DE and Image Analysis

Before the first isoelectric focusing, immobilized pH gradient (IPG) strips (18 cm, with a nonlinear range of pH 4–7; Amersham Pharmacia Biotech Inc., Piscataway, NJ) were rehydrated in rehydration buffer with 8 M urea, 2 M thiourea, 0.5 g/100 mL of CHAPS, 1% DTT, 0.52 mL/mL of IPG buffer, and 0.02% bromophenol blue for 12 h. After rehydration, 200 µg of protein sample mixed in 170 µL of rehydration buffer was applied to IPG strips by cup loading. Isoelectric focusing was carried out in a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech Inc.) at 20°C for 50 kV·h. Prior to the 2-DE, IPG strips were equilibrated with 2 steps: reduction with DTT and carboxymethylation with iodoacetamide. The equilibrated strips were run on 12 g/100 g SDS acrylamide gels (Bio-Rad Laboratories Inc., Hercules, CA). Gels were silver stained and scanned with a PowerLook 1000 scanner (UMAX Technologies Inc., Fremont, CA); PDQuest version 7.3.0 (Bio-Rad Laboratories Inc.) was used for image analysis. Comparison of spot volumes was performed after background subtraction, normalization, and spot match. The relative volume of each spot was obtained from its spot intensity in pixel units and normalized to the sum of the intensities of all the spots of the gel to calculate the percent by volume. Proteins displaying at least 2.0-fold percent by volume variations in different phases were considered in the present work for statistical analysis (Student's *t*-test). Significant differentially expressed protein spots ($P < 0.05$) were considered reliable for further analysis.

Peptide Extraction

Protein spots of interest were excised from 2-DE gels and washed 3 times with sterilized deionized water. Following the destaining, gel pieces were saturated with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate in a 1:1 ratio 3 times, for 15 min each time. Gels were then dehydrated with 30 µL of acetonitrile (100% acetonitrile) for 5 min, and completely dried in a SpeedVac (SPD 121P; Thermo Scientific, Waltham, MA). Finally, proteins were digested in 8 µL of trypsin solution (0.05 mg/mL; Promega Corp., Madison, WI) by incubation at 37°C for 16 h. The supernatant containing peptides was used for MALDI-TOF-MS analysis.

Protein Identification

Equal volumes of the resulting peptides and α -cyano-4-hydroxycinnamic acid were mixed and spotted onto

MALDI templates. The MALDI-TOF-MS and tandem TOF (TOF/TOF) mass spectrometry were carried out on a 4700 Proteomics Analyzer (Applied Biosystems Inc., Foster City, CA). Peptide mass maps were acquired in positive reflection mode, averaging 1,000 laser shots per MALDI-TOF spectrum and 3,000 shots per TOF/TOF spectrum. The 4,700 calibration mixtures (Applied Biosystems Inc.) were used to calibrate the spectrum to a mass tolerance within 0.1 Da. Parent mass peaks with a mass range of 700 to 4,000 Da were picked out for analysis. Peptide tolerance and tandem mass spectrometry (MS/MS) tolerance were both 0.1 Da. Mass spectrometry spectra were processed by using GPS Explorer software (version 3.5; Applied Biosystems Inc.). *Lactobacillus casei* Zhang sequences in the National Center for Biotechnology Information (NCBI) database (GenBank accession number: CP001084; <http://www.ncbi.nlm.nih.gov/genbank/>) were set as database searches (Zhang et al., 2010). Searches were performed to allow for carbamidomethylation, oxidation, and a maximum of 1 missed trypsin cleavage. Protein hits were considered to be identified if the protein score confidence interval percentage for MS and ion score confidence interval percentage for MS/MS analysis were greater than 95% (significance level of $P < 0.05$). If more than 1 protein was identified in 1 spot, the single protein member with the highest protein score (top rank) was singled out from the multiprotein family. The molecular weight and isoelectric point values of most proteins were consistent with the gel regions from which the spots were excised. All identified proteins were distributed over clusters of orthologous groups (COG; <http://www.ncbi.nlm.nih.gov/COG>) and were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg>) database (Tatusov et al., 1997).

Verification of Gene Expression

The total RNA of *Lb. casei* Zhang grown in milk and soy milk at different stages were extracted from cells using FastPrep-24 Instrum and FastRNA Pro Blue Kit (MP Biomedicals LLC, Solon, OH), and then purified using a PrimeScript RT reagent kit (TaKaRa, Otsu, Shiga, Japan). For reverse transcription to cDNA, 10 µL of total purified RNA (100 ng) was added to a 20-µL reaction mixture containing 5 µL of 1× PrimeScript buffer, 1 µL of RT enzyme mix, and 2 µL of RT primer mix. Real-time quantitative PCR (RT-qPCR) was performed using a TransStart Green qPCR SuperMix Kit (TransGen Biotech Co. Ltd., Beijing, China) with 2 µL of cDNA template, 10 µL of 2× TransStart Green qPCR SuperMix, 0.4 µM concentration of each primer,

and 0.4 μL of passive reference dye, and subjected to 40 cycles of amplification at 95°C for 5 s, 58°C for 20 s, and 72°C for 30 s by using a StepOnePlus Real-Time PCR System (Applied Biosystems Inc.). The primer pairs were designed based on genomic data of *Lb. casei* Zhang (Table 4). The GAPDH was used as an internal control in PCR amplification. Melting curves for each PCR were carefully monitored to avoid nonspecific amplifications. Gene expression quantization was transformed by using the comparative cycle threshold formula normalized with the expression of GAPDH (Livak and Schmittgen, 2001). As a negative control for all RT-qPCR experiments, cDNA was replaced by water. Individual RT-qPCR reactions were carried out in triplicate for each gene.

RESULTS AND DISCUSSION

Growth Comparison of *Lb. casei* Zhang Cultivated in Milk and Soy Milk

Strain *Lb. casei* Zhang was cultured in bovine milk and soy milk with initial pH 6.47 and 6.50 for 18 h to compare growth patterns and determine changes in pH. Based on colony counting, *Lb. casei* Zhang grew much faster in soy milk than in milk (Figure 1; Supplementary Table S1, available online at <http://dx.doi.org/10.3168/jds.2013-6927>). Bacterial growth reaches the stationary phase after incubation for 16 h in milk and 14 h in soy milk. The number of colony-forming units of *Lb. casei* Zhang from 18-h cultures in milk and soy milk were $10^{8.24 \pm 0.09}$ and $10^{9.29 \pm 0.02}$ cfu/mL, respectively, suggesting that bacteria grow better in soy milk than milk. Moreover, it took *Lb. casei* Zhang 6 and 4 h to grow from the lag phase to exponential phase in milk and soy milk, respectively, indicating that the growth of *Lb. casei* Zhang was slower in milk than in soy milk. Similarly, the faster growth of *Strep. thermophilus* NCDO 1489 in a soy beverage than in milk has been reported (Chumchuere and Robinson, 1999).

The buffering capacity of milk products is an important physicochemical characteristic that corresponds to the ability of the product to be acidified or alkalinized (Salaün et al., 2005). We found previously that, at different inoculation rates, environmental pH decreased faster in the soy milk samples than the milk samples during fermentation, probably due to the lower pH buffering capacity of soy milk than milk (Wang et al., 2009). Similar results were observed in this study; however, the final pH value of both milk and soy milk was similar (Figure 1; Supplementary Table S1; <http://dx.doi.org/10.3168/jds.2013-6927>).

To carry out proteomics analysis, we collected cultures in the lag, exponential, and stationary phases of

milk and soy milk, in which the pH decreased to 6.4, 5.2, and 4.5, respectively. The corresponding viable counts of *Lb. casei* Zhang grown in milk were 7.05 ± 0.03 cfu/mL (2 h), 8.04 ± 0.04 cfu/mL (14 h), and 8.24 ± 0.09 cfu/mL (18 h), whereas the counts were 7.12 ± 0.04 cfu/mL (2 h), 8.27 ± 0.01 cfu/mL (9.5 h), and 9.29 ± 0.02 cfu/mL (14.5 h) in soy milk (Supplementary Table S1; <http://dx.doi.org/10.3168/jds.2013-6927>).

Sugar Assimilation and Organic Acid Production

Previous sugar utilization tests indicated that *Lb. casei* Zhang can metabolize ribose, glucose, mannose, fructose, sucrose, maltose, and sorbitol, but not (or barely use) arabinose, xylose, melibiose, raffinose, and lactose (Wu et al., 2009a). Therefore, the same amount of glucose was added to milk and soy milk samples before fermentation. Both sugar assimilation and organic acid production were detected in the study during the fermentation of milk and soy milk with *Lb. casei* Zhang at the lag (pH 6.4), exponential (pH 5.2), and stationary phase (pH 4.5; Supplementary Tables S1 and S2; <http://dx.doi.org/10.3168/jds.2013-6927>).

The HPLC analyses showed that the glucose concentration in milk and soy milk decreased from 75.33 ± 0.45 and 83.47 ± 1.06 mM (lag phase, pH 6.4) to 56.65 ± 0.59 and 60.15 ± 0.40 mM (stationary phase, pH 4.5) after 18 or 14.5 h of fermentation, respectively, confirming that *Lb. casei* Zhang mainly used glucose sugar to support their growth in milk and soy milk (Supplementary Table S2; <http://dx.doi.org/10.3168/jds.2013-6927>). In soy milk, fructose was also a primary sugar for *Lb. casei* Zhang, as the concentration decreased from 0.59 ± 0.04 mM (lag phase, pH 6.4) to

Figure 1. The trend of growth (solid curves) and pH (dashed curves) of *Lactobacillus casei* Zhang cultured in milk (○) and soy milk (■) at 37°C for 18 h. Error bars indicate standard errors.

0.29 ± 0.01 mM (stationary phase, pH 4.5), contributing to the pH decreasing in soy milk medium. On the other hand, stachyose, raffinose, and sucrose were used much less, in contrast to *Bifidobacterium* RBL 00079, which was found to assimilate raffinose and stachyose (Farnworth et al., 2007). In milk, the lactose level also slightly decreased from 151.9 ± 0.41 mM (2 h, pH 6.4) to 141.0 ± 0.25 mM (18 h, pH 4.5). Accordingly, the galactose level in milk increased from 1.27 ± 0.01 mM (2 h, pH 6.4) to 16.89 ± 0.14 mM (18 h, pH 4.5).

Fermentation of *Lb. casei* Zhang to pH 4.5 was mainly caused by the accumulation of lactic acid to 87.25 ± 2.60 mM (18 h) and 50.59 ± 11.47 mM (14.5 h) concentration in milk and soy milk, respectively. We found that acetic acid was accumulated, however, at a lower level in both milk and soy milk (Supplementary Table S3; <http://dx.doi.org/10.3168/jds.2013-6927>). Consistently, Scalabrini et al. (1998) observed that selected *Bifidobacterium* strains produced more lactic acid than acetic acid during fermentation of soy milk. Although more lactic acid in milk was produced than in soy milk, the apparent pH in soy milk decreased more than in milk probably due to the higher buffering capacity of milk.

Differential Protein Profiles of *Lb. casei* Zhang Cultivated in Milk and Soy Milk

To investigate the different growths of *Lb. casei* Zhang in milk and soy milk, protein expression profiles were analyzed from bacteria cultured at 3 growth phases (Figures 2, 3, and 4): lag phase (pH 6.4), exponential phase (pH 5.2), and stationary phase (pH 4.5). Under these conditions, as many as 948 ± 21 (lag phase), $1,048 \pm 36$ (exponential phase), and 991 ± 18 (stationary phase) protein spots were detected and quantified from milk samples, and 987 ± 23 (lag phase), $1,054 \pm 31$ (exponential phase), and 998 ± 20 (stationary phase) from soy milk samples. These observations confirmed our previous results obtained from *Lb. casei* Zhang cultured in MRS medium (Wu et al., 2009b, 2011).

A total of 144 protein spots were shown on the 2-DE gel to be differently expressed, with statistical significance, between milk and soy milk at varied stages. Of those protein spots, 104 spots were characterized using MALDI-TOF-MS (Tables 1, 2, and 3; Supplementary Figure S1; <http://dx.doi.org/10.3168/jds.2013-6927>) and MALDI-TOF/TOF (Supplementary Table S4).

Thirty-four, 64, and 46 protein spots had changed expression levels upon growth of *Lb. casei* Zhang in milk and soy milk between the 3 different phases, respectively. (1) At the lag growth phase, among the 34 differentially expressed protein spots, 24 proteins were upregulated over 2-fold more in the soy milk sample

compared with the milk sample, whereas 5 proteins were downregulated by 2-fold more. In addition, 2 spots were detected only in the milk sample, and 3 spots only in the soy milk sample. (2) At the exponential growth phase, among the 64 differentially expressed proteins, 10 spots were downregulated over 2-fold more in the milk sample compared with the soy milk sample, whereas 34 spots were upregulated by 2-fold more. Furthermore, 12 spots were found only in the milk sample and 8 spots only in the soy milk sample. (3) At the stationary growth phase, among the 46 differentially expressed proteins, 33 spots were upregulated over 2-fold more in the soy milk sample compared with the milk sample; meanwhile, 4 spots were downregulated by 2-fold more. Furthermore, 3 spots were found only in the milk sample and 6 spots only in the soy milk sample. Interestingly, the number of differential expressed proteins found in the soy milk sample was more than in the milk sample from all 3 fermentation conditions.

The COG and KEGG analyses indicate that these differentially expressed proteins can be grouped according to their cellular functions (summarized in Figure 5). Mainly, the identified proteins are associated with carbohydrate metabolism and energy production (15.4 and 12.5%, respectively), nucleotide transport and metabolism (16.3%), as well as amino acid transport and metabolism (15.4%), suggesting a dramatic shift of central metabolism during the growth of *Lb. casei* Zhang in milk and soy milk.

Differential Expression of Proteins Required for Carbohydrate Metabolism and Energy Production

We classified the enzymes characterized from our proteomic analysis, which are required for carbohydrate metabolism and energy production. (1) Enzymes required for glycolysis: as a facultatively heterofermentative *Lactobacillus*, *Lb. casei* Zhang ferments sugars, mainly via glycolysis (i.e., the Embden-Meyerhof pathway, or **EMP**), thus generating pyruvate, which is subsequently reduced to lactate by lactate dehydrogenase (Kandler, 1983). Consistently, we found that the expression of a key glycolytic enzyme, 6-phosphofructokinase (spot 7303, LCAZH_1351), which catalyzes D-fructose-6-phosphate to D-fructose-1, 6-diphosphate, increased over 2-fold in *Lb. casei* Zhang cultured in soy milk compared with that in milk at the lag phase (pH 6.4). In addition, expression of L-lactate dehydrogenase (spot 7302, LCAZH_2572) and 3-phosphoglycerate kinase (spot 6507 and 7503, LCAZH_0911) was activated 2.10-, 2.21-, and 2.95-fold more in *Lb. casei* Zhang cultured in soy milk than in milk at the exponential phase (pH 5.2) and stationary (pH 4.5), for the 3 spots, respectively. Taken together, these results indicated

Figure 2. Two-dimensional electrophoresis gels from comparison of the whole cytoplasm proteins of *Lactobacillus casei* Zhang grown in milk and soy milk at the lag phase (pH 6.4) at 37°C (pH gradient 4–7). A: milk; B: soy milk. The identified proteins are marked by arrows and numbers and listed in Table 1. Mr = molecular weight; pI = isoelectric point.

Figure 3. Two-dimensional electrophoresis gels from comparison of the whole cytoplasm proteins of *Lactobacillus casei* Zhang grown in milk and soy milk at the exponential phase (pH 5.2) at 37°C (pH gradient 4–7). A: milk; B: soy milk. The identified proteins are marked by arrows and numbers and listed in Table 2. Mr = molecular weight; pI = isoelectric point.

Figure 4. Two-dimensional electrophoresis gels from comparison of the whole cytoplasm proteins of *Lactobacillus casei* Zhang grown in milk and soy milk at the stationary phase (pH 4.5) at 37°C (pH gradient 4–7). A: milk; B: soy milk. The identified proteins are marked by arrows and numbers and listed as in Table 3. *Mr* = molecular weight; *pI* = isoelectric point.

Table 1. List of different expression proteins identified on 2-dimensional electrophoresis (2-DE) gels of *Lactobacillus casei* Zhang cultured in milk and soy milk at the lag phase (pH 6.4)

No.	Spot ¹	Protein name ²	GenBank accession number	Protein MW ³	Protein pI ⁴	Peptide count	Protein score	Protein score CI %	Group ⁵	COG ⁶
1	2813	Phosphoenolpyruvate-protein kinase (PTS EI component in bacteria)	LCAZH_1750	63,426.5	5	10	150	100	1	Carbohydrate transport and metabolism
2	5114	Unidentified							1	
3	7805	Methionine synthase II (cobalamin independent)	LCAZH_0538	86,616.88	5.71	28	361	100	2	Amino acid transport and metabolism
4	2712	Phosphoenolpyruvate-protein kinase (PTS EI component in bacteria)	LCAZH_1750	63,426.5	5	26	340	100	2	Carbohydrate transport and metabolism
5	4301	Tagatose-6-phosphate kinase	LCAZH_1336	32,188.64	5.2	5	76.7	99.9	2	Carbohydrate transport and metabolism
6	1408	Conjugated bile salt hydrolase-related amidase	LCAZH_0447	37,425.5	4.9	16	244	100	2	Cell envelope biogenesis, outer membrane
7	1304	Dihydroorotate dehydrogenase	LCAZH_1724	34,132.18	4.89	8	123	100	2	Nucleotide transport and metabolism
8	7303	6-Phosphofructokinase	LCAZH_1351	34,208.55	5.74	18	286	100	3	Carbohydrate transport and metabolism
9	4805	Phosphoketolase	LCAZH_0200	89,562.84	5.32	19	244	100	3	Carbohydrate transport and metabolism
10	7806	DNA gyrase, B subunit	LCAZH_0007	72,379.26	5.78	16	188	100	3	DNA replication, recombination, and repair
11	5612	IMP dehydrogenase/GMP reductase	LCAZH_0241	52,665.47	5.49	15	173	100	3	Nucleotide transport and metabolism
12	2302	Aspartate carbamoyltransferase, catalytic chain	LCAZH_1444	34,844.05	5.09	6	77.8	99.9	3	Nucleotide transport and metabolism
13	103	Peroxiredoxin	LCAZH_2473	20,570.06	4.77	4	84.8	100	3	Posttranslational modification, protein turnover, chaperones
14	8603	CTP synthase (UTP-ammonia lyase)	LCAZH_2536	59,857.76	6	17	242	100	3	Nucleotide transport and metabolism
15	8104	Dihydrodipicolinate reductase	LCAZH_0104	27,285.08	5.89	8	110	100	3	Amino acid transport and metabolism
16	8104	Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase	LCAZH_1745	26,994.97	5.89	10	145	100	3	Nucleotide transport and metabolism
17	6303	Dihydrodipicolinate synthase/ N-acetylneuraminate lyase	LCAZH_0105	32,089.6	5.51	4	76.7	99.9	3	Amino acid transport and metabolism
18	2305	Diaminopimelate epimerase	LCAZH_0110	35,974.29	5.01	5	71.9	99.9	3	Amino acid transport and metabolism
19	5802	Phosphoketolase	LCAZH_0200	89,562.84	5.32	6	66.4	99.9	3	Carbohydrate transport and metabolism
20	1605	Uncharacterized NAD(FAD)-dependent dehydrogenase	LCAZH_0305	49,194.62	4.86	6	84.4	99.9	3	General function prediction only

Continued

Table 1 (Continued). List of different expression proteins identified on 2-dimensional electrophoresis (2-DE) gels of *Lactobacillus casei* Zhang cultured in milk and soy milk at the lag phase (pH 6.4)

No.	Spot ¹	Protein name ²	GenBank accession number	Protein MW ³	Protein pI ⁴	Peptide count	Protein score	Protein score CI %	Group ⁵	COG ⁶
21	9801	NAD-dependent alcohol-acetaldehyde dehydrogenase and iron-binding alcohol dehydrogenase	LCAZH_0718	94,997.71	6.15	27	338	100	3	Energy production and conversion
22	4701	Phosphomannomutase	LCAZH_0892	63,525.22	5.26	7	107	100	3	Carbohydrate transport and metabolism
23	6807	Exoribonuclease R	LCAZH_0919	89,095.58	5.67	11	150	100	3	Transcription
24	6604	Acetoin/pyruvate dehydrogenase complex, E3 component, dihydrolipoamide dehydrogenase	LCAZH_1302	49,240.59	5.79	12	152	100	3	Energy production and conversion
25	2804	Stress response membrane GTPase	LCAZH_1306	67,925.66	5.11	5	80.4	99.9	3	Signal transduction mechanisms
26	8505	Dihydroorotase	LCAZH_1443	45,021.07	5.85	7	112	100	3	Nucleotide transport and metabolism
27	6105	Pyrimidine operon attenuation protein/uracil phosphoribosyltransferase	LCAZH_1446	20,459.9	5.52	8	125	100	3	Nucleotide transport and metabolism
28	6508	Glutamine synthetase	LCAZH_1642	50,536.62	5.58	23	353	100	3	Amino acid transport and metabolism
29	809	Phosphoribosylformylglycinamide (FGAM) synthase, synthetase domain	LCAZH_1742	79,495.53	4.75	16	257	100	3	Nucleotide transport and metabolism
30	7603	Biotin carboxylase	LCAZH_2070	49,220.25	5.75	5	65.1	99.9	3	Lipid and metabolism
31	7203	Predicted hydrolase of the HAD superfamily	LCAZH_2734	30,452.76	5.66	12	216	100	3	General function prediction only
32	6206	Unidentified							4	
33	8601	Glutamine phosphoribosylpyrophosphate amidotransferase	LCAZH_1741	52,490.03	5.94	6	105	100	4	Nucleotide transport and metabolism
34	4807	Pyruvate formate lyase	LCAZH_1396	85,260.82	5.25	21	276	100	4	Energy production and conversion

¹Spot numbers match those marked in Figure 4.²PTS = phosphotransferase system; EI = enzyme I; IMP = inosine monophosphate; GMP = guanosine monophosphate; CTP = cytidine triphosphate; UTP = uridine triphosphate; FAD = flavin adenine dinucleotide; GTPase = guanosine triphosphatase; HAD = haloacid dehalogenase.³MW = molecular weight.⁴pI = isoelectric point.⁵Protein expression patterns include 1 for spots only existing in milk, 2 for spots upregulated above 2.0-fold in milk compared with soy milk, 3 for spots upregulated above 2.0-fold in soy milk compared with milk, and 4 for spots only existing in soy milk.⁶Clusters of orthologous groups.

Table 2. List of different expression proteins identified on 2-dimensional electrophoresis (2-DE) gels of *Lactobacillus casei* Zhang cultured in milk and soy milk at the exponential phase (pH 5.2)

No.	Spot ¹	Protein name ²	GenBank accession number	Protein MW ³	Protein pI ⁴	Peptide count	Protein score	Protein score CI %	Group ⁵	COG ⁶
1	7812	Methionyl-tRNA synthetase	LCAZH_2568	74,853.52	5.54	6	67.7	99.9	1	Translation, ribosomal structure and biogenesis
2	7814	Methionyl-tRNA synthetase	LCAZH_2568	74,853.52	5.54	10	94.8	100	1	Translation, ribosomal structure, and biogenesis
3	3013	Conserved hypothetical protein	LCAZH_2175	18,031.03	5.04	3	52.3	98.3	1	Function unknown
4	2422	Phosphoribosylaminoimidazole carboxylase (NCAIR synthetase)	LCAZH_1099	40,322.23	4.82	5	61	99.7	1	Nucleotide transport and metabolism
5	7424	S-Adenosylmethionine:tRNA-ribosyltransferase-isomerase (queuine synthetase)	LCAZH_0714	40,448.62	5.65	7	88.1	100	1	Translation, ribosomal structure and biogenesis
6	7815	Glycine/serine hydroxymethyltransferase	LCAZH_1146	44,585.45	5.67	13	150	100	1	Amino acid transport and metabolism
7	7811	Unidentified							1	
8	7813	Unidentified							1	
9	8118	Unidentified							1	
10	2110	Unidentified							1	
11	6114	Unidentified							1	
12	7707	Unidentified							1	
13	8807	Unidentified							2	
14	8903	Methionine synthase II (cobalamin independent)	LCAZH_0538	86,616.88	5.71	16	145	100	2	Amino acid transport and metabolism
15	5721	Phosphomannomutase	LCAZH_0892	63,525.22	5.26	7	116	100	2	Carbohydrate transport and metabolism
16	7704	Oligoendopeptidase F	LCAZH_0270	67,209.68	5.59	13	157	100	2	Amino acid transport and metabolism
17	5801	Threonyl-tRNA synthetase	LCAZH_1692	74,567.54	5.2	24	297	100	2	Translation, ribosomal structure and biogenesis
18	2607	Phosphomannomutase	LCAZH_0972	48,878.75	4.72	16	202	100	2	Carbohydrate transport and metabolism
19	8701	Arginyl-tRNA synthetase	LCAZH_1719	62,265.31	5.79	16	249	100	2	Translation, ribosomal structure and biogenesis
20	6209	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	LCAZH_0631	29,488.92	5.36	8	117	100	2	Coenzyme metabolism
21	4213	Conserved hypothetical protein	LCAZH_1426	31,987.64	5.12	4	50.8	97.6	2	Function unknown
22	8308	Phosphoribosylpyrophosphate synthetase	LCAZH_2889	33,657.71	5.87	7	101	100	2	Nucleotide transport and metabolism
23	3527	D-Alanine-D-alanine ligase-related ATP-grasp enzyme	LCAZH_0156	39,052.62	4.82	6	61.1	99.7	3	Cell envelope biogenesis, outer membrane
24	4310	Acetoin dehydrogenase complex, E1 component, β subunit	LCAZH_1300	35,333.2	5.19	7	79.6	99.9	3	Energy production and conversion
25	4410	Acetoin dehydrogenase complex, E1 component, β subunit	LCAZH_1300	35,333.2	5.19	9	142	100	3	Energy production and conversion
26	3105	Inorganic pyrophosphatase/exopolyphosphatase	LCAZH_1400	33,760.93	4.74	8	78.6	99.9	3	Energy production and conversion
27	4618	NAD-dependent aldehyde dehydrogenase	LCAZH_2249	49,180	5.07	5	55	99.1	3	Amino acid transport and metabolism
28	7103	Nitroreductase	LCAZH_1413	23,060.99	5.44	5	73.4	99.9	3	Function unknown
29	4107	dTDP-4-dehydrorhamnose 3,5-epimerase related enzyme	LCAZH_1992	21,513.76	5.17	5	51.5	98.0	3	Cell envelope biogenesis, outer membrane

Table 2 (Continued). List of different expression proteins identified on 2-dimensional electrophoresis (2-DE) gels of *Lactobacillus casei* Zhang cultured in milk and soy milk at the exponential phase (pH 5.2)

No.	Spot ¹	Protein name ²	GenBank accession number	Protein MW ³	Protein pI ⁴	Peptide count	Protein score	Protein score CI %	Group ⁵	COG ⁶
30	5001	Nucleoside 2-deoxyribosyltransferase	LCAZH_2259	16,125.19	5.19	5	87.9	100	3	Nucleotide transport and metabolism
31	7302	L-2-Hydroxyisocaproate/malate/lactate dehydrogenase	LCAZH_2572	32,664.69	5.49	6	77.4	99.9	3	Energy production and conversion
32	6405	Unidentified							3	
33	6214	Unidentified							3	
34	5411	Unidentified							3	
35	6313	Unidentified							3	
36	3003	Unidentified							3	
37	8313	Unidentified							3	
38	7102	Unidentified							3	
39	4418	Unidentified							3	
40	5701	Unidentified							3	
41	4209	Unidentified							3	
42	5403	Unidentified							3	
43	4713	Unidentified							3	
44	5105	Unidentified							3	
45	3111	Unidentified							3	
46	8409	Unidentified							3	
47	3529	Unidentified							3	
48	4008	Unidentified							3	
49	3307	Unidentified							3	
50	1017	Unidentified							3	
51	8003	Unidentified							3	
52	8502	Dihydroorotase	LCAZH_1443	45,021.07	5.85	7	112	100	3	Nucleotide transport and metabolism
53	5211	Unidentified							3	
54	7413	Carbamoyl phosphate synthase small subunit	LCAZH_1442	39,490.01	5.63	8	113	100	3	Amino acid transport and metabolism
55	2620	Uncharacterized NAD(FAD)-dependent dehydrogenase	LCAZH_0305	49,194.62	4.86	6	84.4	99.9	3	General function prediction only
56	2705	Malolactic enzyme	LCAZH_0682	60,370.92	4.83	20	259	100	3	Energy production and conversion
57	9611	Formyltetrahydrofolate synthetase	LCAZH_1449	59,092.05	6.32	11	125	100	4	Nucleotide transport and metabolism
58	9310	Unidentified							4	
59	9202	Unidentified							4	
60	2109	FA/phospholipid biosynthesis enzyme	LCAZH_1603	36,063.83	6.16	7	87.2	100	4	Lipid and metabolism
61	9105	Endonuclease IV	LCAZH_1510	32,274.45	6.3	5	58.1	99.5	4	DNA replication, recombination, and repair
62	9008	Unidentified							4	
63	5221	Unidentified							4	
64	2301	Unidentified							4	

¹Spot numbers match those marked in Figure 4.²tRNA = transfer RNA; dTDP = deoxythymidine diphosphate; FAD = flavin adenine dinucleotide.³MW = molecular weight.⁴pI = isoelectric point.⁵Protein expression patterns include 1 for spots only existing in milk, 2 for spots upregulated above 2.0-fold in milk compared with soy milk, 3 for spots upregulated above 2.0-fold in soy milk compared with milk, 4 for spots only existing in soy milk.⁶Clusters of orthologous groups.

Table 3. List of different expression proteins identified on 2-dimensional electrophoresis (2-DE) gels of *Lactobacillus casei* Zhang cultured in milk and soy milk at the stationary phase (pH 4.5)

No.	Spot ¹	Protein name ²	GenBank accession number	Protein MW ³	Protein pI ⁴	Peptide count	Protein score	Protein score CI %	Group ⁵	COG ⁶
1	6121	Unidentified							1	
2	7210	Unidentified							1	
3	6217	Unidentified							1	
4	7201	Ribose 5-phosphate isomerase	LCAZH_2674	25,002.33	5.71	9	152	100	2	Carbohydrate transport and metabolism
5	6703	5'-Nucleotidase/2',3'-cyclic phosphodiesterase-related esterase	LCAZH_1347	56,821.91	5.45	13	210	100	2	Nucleotide transport and metabolism
6	5207	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	LCAZH_0631	29,488.92	5.36	6	93.4	100	2	Coenzyme metabolism
7	4307	Unidentified							2	
8	4807	Stress response membrane GTPase	LCAZH_1306	67,925.66	5.11	12	131	100	3	Signal transduction mechanisms
9	1704	Malolactic enzyme	LCAZH_0682	60,370.92	4.83	20	259	100	3	Energy production and conversion
10	2601	Uncharacterized NAD(FAD)-dependent dehydrogenase	LCAZH_0305	49,194.62	4.86	6	84.4	99.9	3	General function prediction only
11	8503	Dihydroorotase	LCAZH_1443	45,021.07	5.85	6	64.4	99.8	3	Nucleotide transport and metabolism
12	7609	Glycine/serine hydroxymethyltransferase	LCAZH_1146	44,585.45	5.67	13	150	100	3	Amino acid transport and metabolism
13	7501	Carbamoyl phosphate synthase small subunit	LCAZH_1442	39,490.01	5.63	8	113	100	3	Amino acid transport and metabolism
14	9901	NAD-dependent alcohol-acetaldehyde dehydrogenase and iron-binding alcohol dehydrogenase	LCAZH_0718	94,997.71	6.15	19	220	100	3	Energy production and conversion
15	2512	3-Oxoacyl-(acyl-carrier-protein) synthase	LCAZH_2073	42,093.2	4.9	5	61.2	99.7	3	Lipid and metabolism secondary metabolites biosynthesis, transport, and catabolism
16	3614	Peptidase T	LCAZH_0338	45,224.81	5.09	11	137	100	3	Amino acid transport and metabolism
17	5810	Glucosamine-6-phosphate amidotransferase domains	LCAZH_0973	65,828.24	5.36	25	355	100	3	Cell envelope biogenesis, outer membrane
18	6106	Pyrimidine operon attenuation protein/uracil phosphoribosyltransferase	LCAZH_1446	20,459.9	5.52	13	192	100	3	Nucleotide transport and metabolism
19	7615	Histidyl-tRNA synthetase	LCAZH_1514	47,535.14	5.73	10	66.9	99.9	3	Translation, ribosomal structure and biogenesis
20	2105	Peptidyl-prolyl <i>cis-trans</i> isomerase (rotamase)-cyclophilin family	LCAZH_0772	21,335.65	4.91	8	120	100	3	Posttranslational modification, protein turnover, chaperones
21	705	FKBP-type peptidyl-prolyl <i>cis-trans</i> isomerase (trigger factor)	LCAZH_1325	49,349.73	4.53	9	96.9	100	3	Posttranslational modification, protein turnover, chaperones
22	2108	Copper resistance protein	LCAZH_0848	22,247.45	4.85	6	90.3	100	3	Inorganic ion transport and metabolism
23	7504	Predicted nucleotide-utilizing enzyme, CinA	LCAZH_0864	44,228.72	5.71	11	134	100	3	General function prediction only
24	6212	ABC-type multidrug transport system, ATPase component	LCAZH_1217	32,161.27	5.55	10	129	100	3	Defense mechanisms
25	2703	Dipeptidase	LCAZH_1917	53,756.64	5.03	9	94.4	100	3	Amino acid transport and metabolism
26	3801	Phosphoenolpyruvate-protein kinase (PTS EI component in bacteria)	LCAZH_1750	63,426.5	5	8	51.7	98.1	3	Carbohydrate transport and metabolism
27	2106	DNA-binding response regulator, OmpR family (Rec-WTH domains)	LCAZH_0243	25,511.37	4.9	6	78.9	99.9	3	Signal transduction mechanisms

Continued

Table 3 (Continued). List of different expression proteins identified on 2-dimensional electrophoresis (2-DE) gels of *Lactobacillus casei* Zhang cultured in milk and soy milk at the stationary phase (pH 4.5)

No.	Spot ¹	Protein name ²	GenBank accession number	Protein MW ³	Protein pI ⁴	Peptide count	Protein score	Protein score CI %	Group ⁵	COG ⁶
28	3805	Phosphoenolpyruvate-protein kinase (PTS EI component in bacteria)	LCAZH_1750	63,426.5	5	19	179	100	3	Carbohydrate transport and metabolism
29	2102	Predicted phosphatase	LCAZH_0697	24,595.47	4.85	7	79	99.9	3	General function prediction only
30	1701	Unidentified							3	
31	6507	3-Phosphoglycerate kinase	LCAZH_0911	42,193.28	5.64	14	173	100	3	Carbohydrate transport and metabolism
32	9008	PTS, IIB component	LCAZH_0319	17,161	6.41	4	56.6	99.3	3	Carbohydrate transport and metabolism
33	6608	Glutamine synthetase	LCAZH_1642	50,536.62	5.58	19	219	100	3	Amino acid transport and metabolism
34	3510	Alcohol-acetaldehyde dehydrogenase	LCAZH_2084	43,189.02	5.08	11	175	100	3	Energy production and conversion
35	3106	ATP-dependent protease HslVU (ClpYQ), peptidase subunit	LCAZH_1390	18,731.57	5.01	7	116	100	3	Posttranslational modification, protein turnover, chaperones
36	5805	Glucosamine-6-phosphate amidotransferase domains	LCAZH_0973	65,828.24	5.36	25	347	100	3	Cell envelope biogenesis, outer membrane
37	6813	Methionyl-tRNA synthetase	LCAZH_2568	74,853.52	5.54	19	205	100	3	Translation, ribosomal structure and biogenesis
38	7503	3-Phosphoglycerate kinase	LCAZH_0911	42,193.28	5.64	9	133	100	3	Carbohydrate transport and metabolism
39	7505	Selenocysteine synthase (seryl-tRNA ^{Ser} selenium transferase)	LCAZH_2717	39,636.58	5.77	8	134	100	3	Amino acid transport and metabolism
40	4504	3-Carboxymuconate cyclase	LCAZH_0850	37,599.93	5.17	11	170	100	3	Carbohydrate transport and metabolism
41	2909	Aminopeptidase	LCAZH_0499	94,360.88	4.95	12	103	100	4	Amino acid transport and metabolism
42	2502	ATP-dependent Clp protease, ATP-binding subunit ClpX	LCAZH_1326	46,009.94	4.82	6	79.6	99.9	4	Posttranslational modification, protein turnover, chaperones
43	9001	Acetyltransferase, GNAT family	LCAZH_1758	16,209.5	6.07	4	55.6	99.2	4	Transcription general function prediction only
44	7803	Acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II	LCAZH_2418	56,053.59	6.36	6	52.4	98.3	4	Lipid and metabolism
45	3605	Imidazolonepropionase-related amidohydrolase	LCAZH_2545	42,336.8	4.96	11	150	100	4	Secondary metabolites biosynthesis, transport, and catabolism
46	2103	Translation elongation factor P	LCAZH_1632	20,901.51	4.8	3	48.2	95.7	4	Translation, ribosomal structure and biogenesis

¹Spot numbers match those marked in Figure 4.²GTPase = guanosine triphosphatase; FAD = flavin adenine dinucleotide; tRNA = transfer RNA; FKBP = FK506-binding protein; ABC = ATP-binding cassette; PTS = phosphotransferase system; EI = enzyme I; OmpR = outer membrane protein R; HslVU = heat shock protein; Clp = caseinolytic protease.³MW = molecular weight.⁴pI = isoelectric point.⁵Protein expression patterns include 1 for spots only existing in milk, 2 for spots upregulated above 2.0-fold in milk compared with soy milk, 3 for spots upregulated above 2.0-fold in soy milk compared with milk, 4 for spots only existing in soy milk.⁶Clusters of orthologous groups.

Figure 5. Schematic of cellular role categories of theoretical (open bars) and identified proteins on 2-dimensional electrophoresis gel at pH 4 to 7 (black bars) in *Lactobacillus casei* Zhang.

that the EMP is strengthened in *Lb. casei* Zhang to get energy for its growth in soy milk-containing medium. (2) Enzymes involved in pyruvate metabolism: analysis of organic acid production revealed that, in addition to lactate, *Lb. casei* Zhang also generated a small amount of acetate in both milk and soy milk (Supplementary Table S3; <http://dx.doi.org/10.3168/jds.2013-6927>). The aerobic mixed-acid fermentation was probably partly due to the pyruvate dehydrogenase complex, which catalyzes sequential reactions, oxidative decarboxylation of pyruvate, and acetylation of CoA (Soo et al., 2007). Then, acetate is generated from acetyl-CoA through phosphotransacetylase (LCAZH_0944) and acetate kinase (LCAZH_0188 and LCAZH_2132). It was shown that *Lb. casei* L3 metabolized glucose to produce lactate and then acetate, but not ethanol, under both anaerobic and aerobic conditions (de Vries et al., 1970). We observed that the expression of pyruvate dehydrogenase E3 (spot 6604, LCAZH_1302) and the β subunit of pyruvate dehydrogenase complex E1 component (spot 4310 and 4410, LCAZH_1300) was enhanced in soy milk at the lag phase (pH 6.4) and exponential phase (pH 5.2), respectively, which might be required to produce more acetate in soy milk than in milk. Moreover, increasing the acetyl-CoA level should be beneficial for lipid biosynthesis because it is an enzymatic substrate of this process (Hugenholtz and Kleerebezem, 1999). Biotin carboxylase (spot 7603, LCAZH_2070) and 3-oxoacyl-(acyl-carrier-protein) synthase (spot 2512, LCAZH_2073), enzymes that participate in FA biosynthesis probably for membrane lipids (Fozo et al., 2004), were upregulated in soy milk at the lag phase (pH 6.4) and stationary phase (pH 4.5), respectively. We also found that expression of malolactic enzyme (spot 2705 and 1704, LCAZH_0682), which converts pyruvate and L-malate reversely, was upregulated more in *Lb. casei* Zhang cultured in soy

milk at the exponential phase (pH 5.2) and stationary phase (pH 4.5) than in milk, respectively. Meanwhile, expression of NAD-dependent alcohol-acetaldehyde dehydrogenase and iron-binding alcohol dehydrogenase (spot 9801 and 9901, LCAZH_0718) were also stimulated at the exponential phase (pH 5.2) and stationary phase (pH 4.5) of soy milk, which could perhaps induce the conversion of acetyl-CoA and acetaldehyde. Hence, we propose that lactate production in milk could be shifted to a fermentation of mixed acids as well as a few other products in soy milk from glucose metabolism. (3) Enzymes required for the pentose phosphate pathway (PPP): phosphoketolase (spot 4805 and 5802, LCAZH_0200) and 3-carboxymuconate cyclase (spot 4504, LCAZH_0850), which catalyze D-xylulose-5-phosphate to D-glyceraldehyde-3-phosphate and D-glucono-1,5-lactone-6P to 6-phospho-gluconate reactions, were upregulated in *Lb. casei* Zhang grown in soy milk at the lag phase (pH 6.4) and stationary phase (pH 4.5), and ribose 5-phosphate isomerase (spot 7201, LCAZH_2674), which catalyzes the D-ribulose-5P to D-ribose-5P reaction, was upregulated in *Lb. casei* Zhang grown in milk at the stationary phase (pH 4.5). These 3 enzymes participate in the PPP, supplying pentose mainly for nucleotide metabolism (Menezes et al., 1990). (4) Enzymes for the tagatose pathway: the expression of tagatose-6-phosphate kinase (spot 4301, LCAZH_1336), an enzyme in galactose metabolism that converts D-tagatose-6-phosphate to D-tagatose-1,6-diphosphate to enter glycolysis increased over 2-fold in *Lb. casei* Zhang incubated in milk compared with soy milk at the lag phase (pH 6.4), which might be related to the slight decrease in lactose concentration in milk found in sugar fermentation. Three genes (LCAZH_0608, LCAZH_1336, and LCAZH_0323) are expressed as tagatose-6-phosphate kinase in the genome of *Lb. casei* Zhang. Although *Lb. casei* Zhang does not have the genetic basis for lactose metabolism, the strain can ferment lactose weakly. However, the expression of LCAZH_1336 of *Lb. casei* Zhang during growth in soy milk needs further study.

Differential Expression of Proteins Required for Nucleotide Metabolism

Proteomic analysis characterized 15 spots involved in nucleotide metabolism at 3 growth phases; 12 of the total were upregulated in *Lb. casei* Zhang grown in soy milk, whereas 3 increased protein spots in milk, showing the important connection to the growth of *Lb. casei* Zhang in soy milk and milk (Figure 6).

Especially at lag phase, among 9 changed nucleotide enzymes, 8 were expressed at higher levels in soy milk

Figure 6. The partial nucleotide metabolism map of *Lactobacillus casei* Zhang during growth in milk and soy milk at the lag, exponential, and stationary phase (pH 6.4, 5.2, and 4.5, respectively). PPP = pentose phosphate pathway.

Table 4. Primers used for validation of 2-dimensional electrophoresis (2-DE) data by real-time PCR

Locus	Protein ¹	Gene	Primer	Sequence 5' to 3'	Product (bp)
LCAZH_0910	GAPDH	<i>gapdh</i>	Sense Antisense	GTATCGGTCGTTTGGCATT GCACTAACTTCACCAGGGA	139
LCAZH_0721	PPP Glucose-6-phosphate 1-dehydrogenase	<i>zwf</i>	Sense Antisense	GGTCGCAAGTCCGTTATTC CCAAGGGCCTTACCATTCA	111
LCAZH_0850	3-Carboxymuconate cyclase		Sense Antisense	ATTATGATGCGGCAACTGGTC GCGGTTGGATACATAAAGGAA	134
LCAZH_0262	6-Phosphogluconate dehydrogenase	<i>gntZ</i>	Sense Antisense	AGAAGCCACGATTAGTCAG ACAGATAGCCACCTTTTGC	260
LCAZH_2287	Ribose 5-phosphate isomerase	<i>rpiA</i>	Sense Antisense	TGTGACAGTGCCGATAAGG GGTAGCCATTTCGTCGTTTT	237
LCAZH_2549	Phosphoribosylpyrophosphate synthetase	<i>prs</i>	Sense Antisense	TTACCCGTGCCCGAAAAC ACCATCAGCGGACCAACT	365
LCAZH_1642	Amino acid metabolism Glutamine synthetase	<i>glnA</i>	Sense Antisense	AAGTATGCTGATGCCTTAGAG GTAGGAGTTCACAGTTGGGTT	291
LCAZH_2518	NADPH-dependent glutamate synthase subunit β -like oxidoreductase	<i>gltB</i>	Sense Antisense	GCACAGCGATTTCGTCAG AAACAATGCTTTAGCCTCT	149
LCAZH_0200	Phosphoketolase	<i>xpk</i>	Sense Antisense	GGCTTTAACGAAGGCTACACG AAATACCTGGGTCCTGATGGG	223
LCAZH_0973	Amino sugars metabolism Glucosamine 6-phosphate synthetase, amidotransferase and phosphosugar isomerase domains	<i>glmS</i>	Sense Antisense	TGTTGTCCAAGCACCCAC TTAGCAGCCTGCAATCCT	277
LCAZH_1741	Purine metabolism Glutamine phosphoribosylpyrophosphate amidotransferase	<i>purF</i>	Sense Antisense	ACGGGATTGATTTTCAGACGA TGACTTTGAGGGATGCCACTT	246
LCAZH_1737	Phosphoribosylamine-glycine ligase	<i>purD</i>	Sense Antisense	GGGAAAGGCGTCGTTATTG AGTTGATGGGCAGCGGTTA	284
LCAZH_1739	Folate-dependent phosphoribosylglycinamide formyltransferase	<i>purN</i>	Sense Antisense	AATAAAACGGCGGCAGAG TGGCACTTGGGCGATAAT	267
LCAZH_1742	Phosphoribosylformylglycinamide	<i>purL</i>	Sense Antisense	AAGCGTGGAGATGGCAGGTA TCGGTCAACGAACCTCACAGG	282
LCAZH_1740	Phosphoribosylaminoimidazole		Sense Antisense	ACGATTTATTGGCGTCAGG CGTAATGATGTTGCGGGTA	186
LCAZH_1738	AICAR transformylase/IMP cyclohydrolase	<i>purH</i>	Sense Antisense	GTGACCAGCGTTGTAAGAGG TGGCGGCAAATGGATAGA	187
LCAZH_1745	Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase	<i>purC</i>	Sense Antisense	ACACCAATCAAGCCACAGC ACTACTTCAAGCGCAACAT	193
LCAZH_1099	Phosphoribosylaminoimidazole carboxylase	<i>purK2</i>	Sense Antisense	AATGGGACGCTTTATGTG AATCGCCAATCAGGTTTA	224
LCAZH_1747	Phosphoribosylcarboxyaminoimidazole (NCAIR) mutase	<i>purE</i>	Sense Antisense	CCTTATGAGAAAGCGGGTTA ATGTTTCGTGTTTTGATGGG	187
LCAZH_0241	IMP dehydrogenase/GMP reductase	<i>guaB</i>	Sense Antisense	GCTGACGGTGGGATCAAGTA TAACCCCGCTCTGGAATAA	241
LCAZH_1440	Pyrimidine metabolism Dihydroorotate dehydrogenase	<i>pydB</i>	Sense Antisense	CCACTGGCAAACCGCTTCT CCACCCATTCCAATCAACG	127
LCAZH_1441	Carbamoyl phosphate synthase large subunit	<i>pyrAB</i>	Sense Antisense	TTTGCCTTGGATCGTAAC CCTTCATTTGGGTGCCTA	280

Continued

Table 4 (Continued). Primers used for validation of 2-dimensional electrophoresis (2-DE) data by real-time PCR

Locus	Protein ¹	Gene	Primer	Sequence 5' to 3'	Product (bp)
LCAZH_1443	Dihydroorotase	<i>pyrC</i>	Sense	CAACTGGTGCCATCGTCAT	242
			Antisense	CGTAATCGGTGCAAACTGC	
LCAZH_1444	Aspartate carbamoyltransferase catalytic subunit	<i>pyrB</i>	Sense	GGAAACCCCTTGAAGACACCA	233
			Antisense	CGCCAACAATCCCGATATCTC	
LCAZH_1439	Orotidine-5'-phosphate decarboxylase	<i>pyrF</i>	Sense	ATGACGCCCTATATCGCACTG	154
			Antisense	GGTGTAGGGATGTCGTGAAG	

¹PPP = pentose phosphate pathway; AICAR = aminoimidazole carboxamide ribonucleotide; IMP = inosine monophosphate; GMP = guanosine monophosphate.

than in milk, including phosphoribosylformylglycin-
amide synthase (spot 809, LCAZH_1742), phospho-
ribosylaminoimidazolesuccinocarboxamide (SAICAR)
synthase (**PurC**; spot 8104, LCAZH_1745), inosine
monophosphate dehydrogenase (**GuaB**; spot 5612,
LCAZH_0241), belonging to purine metabolism; as-
partate carbamoyltransferase (**PyrB**; spot 2302, LCA-
ZH_1444), cytidine triphosphate synthase (spot 8603,
LCAZH_2536), dihydroorotase (**PyrC**; spot 8505,
LCAZH_1443), pyrimidine operon attenuation protein
(spot 6105, LCAZH_1446), involved in pyrimidine me-
tabolism; and glutamine phosphoribosylpyrophosphate
amidotransferase (**PurF**, spot 8601, LCAZH_1741),
only observed in soy milk samples. On the other hand,
just 1 spot, representing dihydroorotate dehydrogenase
(spot 1304, LCAZH_1724), which participates in py-
rimidine metabolism, was upregulated in milk at the
lag phase (pH 6.4).

Furthermore, expression of nucleoside 2-deoxyribosyl-
transferase (spot 5001, LCAZH_2259), dihydroorotase
(spot 8502 and 8503, LCAZH_1443), and pyrimidine
operon attenuation protein (spot 6106, LCAZH_1446)
increased in soy milk at the exponential phase (pH 5.2)
and stationary phase (pH 4.5), whereas expression of
5'-nucleotidase/2',3'-cyclic phosphodiesterase-related
esterase (spot 6703, LCAZH_1347) and phosphoribo-
sylaminoimidazole carboxylase (NCAIR synthetase;
spot 2422, LCAZH_1099) was enhanced in milk at
the exponential phase (pH 5.2) and stationary phase
(pH 4.5). However, formyltetrahydrofolate synthetase
(spot 9611, LCAZH_1449) was only found in soy milk
samples at the exponential phase (pH 5.2).

Lactobacillus can produce nucleotides through de
novo biosynthesis or acquire them from its surround-
ings (Kilstrup et al., 2005). Milk contains a substantial
amount of the pyrimidine precursor orotate, but is very
poor in purine bases (Beyer et al., 2003). Consistent
with this notion, proteomic analysis indicated that
levels of 7 enzymes involved in the de novo purine bio-
synthetic pathway increased when *Strep. thermophilus*
LMG18311 was cultivated in milk (Herve-Jimenez et
al., 2009). Also, when *Lactococcus lactis* NCDO763 was
cultivated in skim milk microfiltrate (**SMM**) and milk,
it synthesized 11 enzymes that were directly involved
in the de novo synthesis of purine bases to a high level
(Gitton et al., 2005). Thus, the expression of enzymes
that are involved in de novo synthesis of nucleotides
were also important for the growth of *Lb. casei* Zhang
in both milk and soy milk media. The higher expression
of proteins participating in purine and pyrimidine me-
tabolism in *Lb. casei* Zhang during growth in soy milk
than in milk at the lag phase might be connected with
the fast growth of *Lb. casei* Zhang in soy milk.

Differential Expression of Proteins Required for Amino Acid Metabolism

Proteolysis sometimes limits the growth of LAB in milk because the bacteria have multiple amino acid auxotrophies (Guédon et al., 2001). The growth of *Lb. lactis* in milk depends largely on the activity of a cell wall-bound proteinase that is responsible for casein hydrolysis in milk (Guédon et al., 2001). Caseins are the main amino acid source in milk, whereas glycinin (11S) and β -conglycinin (7S) are the major proteins present in soybeans, which comprise around 40 and 30% of the total soy protein, respectively (Nik et al., 2008). Therefore, the hydrolysis of caseins or soy proteins is required to support the growth of *Lb. casei* Zhang in milk and soy milk. In the current study, the expression of 16 enzymes associated with amino acid metabolism, particularly involved in methionine, lysine, and glutamate amino acid metabolism, was differentially induced in *Lb. casei* Zhang during culture in milk and soy milk media.

Proteins in both milk and soy milk lack sufficient amounts of the essential amino acid methionine. Consistently, we found that methionine synthase II (spot 7805 and 8903, LCAZH_0538) in *Lb. casei* Zhang was produced at higher levels during growth in milk at the lag (pH 6.4) and exponential (pH 5.2) phase than during growth in soy milk. We also observed that the methionyl-transfer RNA (**tRNA**) synthetase (spot 7812 and 7814, LCAZH_2568), which forms methionine-charged tRNA, was found in milk samples and was also expressed more (spot 6813) in soy milk than in milk at the stationary phase (pH 4.5). Similarly, proteomic and transcriptomic approaches indicated that the Met-Cys biosynthesis pathway was fully induced in *Strep. thermophilus* LMG18311 at late growth in milk (Herve-Jimenez et al., 2008). Thus, the induction of Met-Cys metabolism must be important for *Lb. casei* Zhang grown in milk. Lysine is abundant in soy milk. However, the expression of 3 enzymes that were involved in lysine synthesis [i.e., dihydrodipicolinate reductase (spot 8104, LCAZH_0104), dihydrodipicolinate synthase (spot 6303, LCAZH_0105), and diaminopimelate epimerase (spot 2305, LCAZH_0110)] was upregulated during growth of *Lb. casei* Zhang in soy milk at the lag phase (pH 6.4). Perhaps, this induction of lysine synthesis is due to consumption of this amino acid in bacterial growth and may be connected with the fast growth of *Lb. casei* Zhang incubated in soy milk at an early stage. Besides, the expression of glutamine synthetase (**GlnA**; spot 6508, LCAZH_1642) and glucosamine-6-phosphate amidotransferase domains (**GlmS**; spot 5805 and 5810, LCAZH_0973) was upregulated more at the stationary phase (pH 4.5) in soy milk than in milk, and NAD-dependent aldehyde dehydrogenase (spot 4618, LCA-

ZH_2249) was upregulated at the exponential phase (pH 5.2) of soy milk. The synthesis of L-glutamine is attached to purine and pyrimidine metabolism, as well as amino sugar metabolism. The results indicated that *Lb. casei* Zhang probably converts L-glutamine to purine and pyrimidine metabolism at an early stage of growth in soy milk and milk, and to amino sugar metabolism at the stationary phase. Moreover, the higher expression level of related enzymes might be related to the faster growth of *Lb. casei* Zhang cultivated in soy milk than in milk. Additionally, Gitton et al. (2005) reported that glutamine synthetase in *Lb. lactis* NCDO763 would play a pivotal role in the adaptation to SMM and milk (Gitton et al., 2005). The expression of glutamine synthetase in *Lb. casei* Zhang during growth in milk and soy milk will be studied in the future.

We also characterized several peptidases, including oligoendopeptidase F (**PepF**; spot 7704, LCAZH_0270), whose expression increased in milk at pH 5.2; peptidase T (**PepT**; spot 3614, LCAZH_0338) and dipeptidase (PepD3, spot 2703, LCAZH_1917), which were expressed to a higher level in soy milk than in milk at the stationary phase (pH 4.5); and aminopeptidase (**PepN**; spot 2909, LCAZH_0499), which was only expressed when cultured in soy milk at the stationary phase (pH 4.5). As protein substrates were broken down by these proteinases, resulting peptides or amino acids, or both, were transported to the cytoplasm and then degraded by the peptidases (Savijoki et al., 2006). Among these 4 peptidases, PepT was demonstrated to be specific to hydrolyze tripeptides with varied compositions, including hydrophobic/uncharged, aromatic, basic, acidic, and sulfur-containing amino acids. A previous study showed that, in soy milk, expression of this peptidase was induced at a higher level than others, suggesting that its substrates are abundant in soy milk medium (Christensen et al., 1999). In addition, PepN and PepF were upregulated in *Lb. lactis* cultivated in SMM and milk, whereas PepN did not have a higher synthesis in *Strep. thermophilus* grown in milk (Derzelle et al., 2005). It was also mentioned that expression of PepN and peptidase C, which comprise part of the CodY regulon in *Lb. lactis*, is activated by intracellular branched-chain amino acids (BCAA). Thus, PepT and PepN might play an important role in *Lb. casei* Zhang when cultivated in soy milk, whereas PepF might be related to the growth of *Lb. casei* Zhang in milk. Further study will be required.

Differential Expression of Proteins in Other Pathways

We found that expression of 3 stress-response enzymes, [caseinolytic protease X (**ClpX**; spot 2502,

LCAZH_1326), heat shock protein HslVU (spot 3106, LCAZH_1390), and endonuclease IV (Nfo; spot 9105, LCAZH_1510)] increased in soy milk at pH 4.5 and 6.4, probably resulting from the rapid decrease in pH; ClpX is a member of the Clp ATPase family, which functions as molecular chaperones (Frees et al., 2007). As shown in a previous proteomic analysis, heat shock-induced expression of ClpX, ClpQ, and ClpL occurs in *Lb. delbrueckii* ssp. *bulgaricus* (De Angelis and Gobetti, 2011). Identification of the ClpX protein implies that its chaperone activity may be important for growth of *Lb. casei* Zhang in soy milk at pH 4.5. It was shown that oxygen stress specifically stimulates Nfo in *Escherichia coli* K-2 in an OxyR-independent manner (Chan and Weiss, 1987). However, it remains to be investigated why the function of Nfo is required for growth of *Lb. casei* Zhang in soy milk.

The translation process appears to be enhanced in soy milk because expression of methionyl-tRNA synthetase (spot 6813, LCAZH_2568), threonyl-tRNA synthetase (spot 5801, LCAZH_1692), arginyl-tRNA synthetase (spot 8701, LCAZH_1719), and histidyl-tRNA synthetase (spot 7615, LCAZH_1514) was upregulated in soy milk at both the exponential phase (pH 5.2) and stationary phase (pH 4.5), and because translation elongation factor P (spot 2103, LCAZH_1632) was significantly increased in soy milk at pH 4.5. In addition, expression of D-alanine-D-alanine ligase-related ATP-grasp enzyme (spot 3527, LCAZH_0156), deoxythymidine diphosphate-4-dehydrorhamnose 3,5-epimerase-related enzyme (spot 4107, LCAZH_1992), and glucosamine 6-phosphate synthetase (spot 5805 and 5810, LCAZH_0973), which play a role in cell membrane biogenesis, were induced in soy milk at pH 5.2 and 4.5, providing evidence that protection of membrane integrity is important for *Lb. casei* Zhang to resist acid stress.

Verification of Gene Expression by RT-qPCR

To verify results from our proteomic analysis, we carried out RT-qPCR to determine transcription of *Lb. casei* Zhang loci that were implicated to be involved in carbohydrate, amino acid, and nucleotide metabolism (with the primers used for validation listed in Table 4). The KEGG pathway of *Lb. casei* Zhang illustrated that GlnA converts L-glutamate to L-glutamine, which is changed to D-glutamine-6P, 5-phosphoribosylamine, or carbamoyl phosphate by sequential reactions catalyzed by GlnS, PurF, and carbamoyl phosphate synthase (PyrAB), and then used for the metabolism of amino sugars, purines, and pyrimidines, respectively. Therefore, we detected the corresponding genes in the study. As shown in Figure 6, the mRNA levels of the loci involved in glutamine metabolism [i.e.,

glnA (LCAZH_1642), *glnS* (LCAZH_0973), and *gltB* (LCAZH_2518)], the loci involved in de novo purine metabolism [i.e., *purF* (LCAZH_1741), *purL* (LCAZH_1742), *purD* (LCAZH_1737), *purH* (LCAZH_1738), *purN* (LCAZH_1739), *purC* (LCAZH_1745), *purE* (LCAZH_1747), *purK2* (LCAZH_1099), and *guaB* (LCAZH_0241)], and the gene of phosphoribosylaminoimidazole (LCAZH_1740), as well as the loci for pyrimidine metabolism [i.e., *pyrAB* (LCAZH_1441), *pydB* (LCAZH_1440), *pyrC* (LCAZH_1443), *pyrB* (LCAZH_1444), and *pyrF* (LCAZH_1439)] were tested at 3 phases of fermented milk and soy milk.

Except for *purC*, *purE*, *guaB*, and *purK2*, most genes involved in purine metabolism were upregulated more at the lag (pH 6.4) and exponential (pH 5.2) phases of *Lb. casei* Zhang growth in soy milk than in milk. On the other hand, at the stationary phase (pH 4.5), the mRNA levels of *purD*, *purN*, *purL*, and the gene of phosphoribosylaminoimidazole, which encode key enzymes for synthesis of phosphoribosylaminoimidazole from ribosylamine-5P, were downregulated more in soy milk or similar to the expression in milk. The expressions of *pyrAB*, *pyrB*, *pyrC*, *pydB*, and *pyrF* involved in pyrimidine metabolism were also upregulated more in soy milk compared with in milk at the lag phase (pH 6.4). However, the *pydB*, *pyrC*, and *pyrF* loci were expressed similarly in soy milk and milk. These findings indicate that purine and pyrimidine metabolism was also enhanced in *Lb. casei* Zhang during growth in soy milk at the lag and exponential phases at the transcriptional level, probably due to fast growth of *Lb. casei* Zhang in soy milk at the beginning of fermentation.

Consistent with the results of proteomic analysis, expression of the *glnA*, *glnS*, and *gltB* loci increased in soy milk at the stationary phase (pH 4.5) compared with milk. It is possible that the utilization of amino sugars in soy milk was enhanced at the stationary phase when glucose was exhausted and (or) purine and pyrimidine synthesis was reduced in soy milk at the stationary phase (pH 4.5).

Furthermore, the loci encoding the enzymes of the PPP [i.e., *xpk* (LCAZH_0200 phosphoketolase), *zwf* (LCAZH_0721, glucose-6-phosphate 1-dehydrogenase), *gntZ* (LCAZH_0262, 6-phosphogluconate dehydrogenase), *rpiA* (LCAZH_2287, ribose 5-phosphate isomerase), *rpe* (LCAZH_1610, pentose-5-phosphate-3-epimerase), and *prs* (LCAZH_2549, phosphoribosylpyrophosphate synthetase)] and the gene of 3-carboxymuconate cyclase (LCAZH_0850) were investigated. Consistent with proteomic analysis, expression of *xpk*, *zwf*, *rpiA*, *rpe*, and *prs*, but not *gntZ*, were upregulated in milk at the lag phase (pH 6.4). Expression of the gene encoding 3-carboxymuconate cyclase was also upregulated under the same conditions. At the expo-

nential phase (pH 5.2), transcription of these loci was upregulated more in *Lb. casei* Zhang grown in milk compared with in soy milk. However, at the stationary phase (pH 4.5), their mRNA levels remained similar, regardless of the growth conditions. Taken together, our results from both proteomic analysis and mRNA determination suggest that expression of the genes involved in PPP metabolism was differentially regulated in *Lb. casei* Zhang during culture in soy milk and milk.

In addition, it is interesting that most genes encoding enzymes belonging to the EMP metabolism displayed 2-fold higher transcription in *Lb. casei* Zhang cultured in milk than in soy milk at the lag (pH 6.4) and exponential (pH 5.2) phases. On the other hand, transcription of these genes remained similar in milk and soy milk at the stationary phase (pH 4.5; data not shown).

Recently, we published 2 similar studies regarding the transcriptome analysis of probiotic *Lb. casei* Zhang during fermentation in milk and soy milk (Wang et al., 2012a,b). Because the previous data was analyzed in a different way, we cannot correlate it to the proteomic data here. Further discussion about these 2 different data sets will be addressed in future work.

CONCLUSIONS

Our study provides the first insight into the protein profiles of *Lb. casei* grown in milk and soy milk. Through this proteomic analysis, we were able to compare 144 proteins that displayed different expression levels in *Lb. casei* Zhang when cultured in milk and soy milk. We found that complicated control systems might be involved in the regulation of genetic loci required for the metabolism of carbohydrates and energy, nucleotides, and amino acids, which covered over 60% of the total differentially expressed proteins. Thus, this study reveals metabolic processes that are potentially important for bacterial fermentation in milk and soy milk, and that could help us to further understand the molecular basis of specific metabolisms in this new probiotic bacterium in the future.

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