J Ind Microbiol Biotechnol (2012) 39:191–206
DOI 10.1007/s10295-011-1015-7

ORIGINAL PAPER

Transcriptome analysis of probiotic *Lactobacillus casei* Zhang during fermentation in soymilk

Ji-Cheng Wang · Wen-Yi Zhang · Zhi Zhong · Ai-Bin Wei · Qiu-Hua Bao · Yong Zhang · Tian-Song Sun · Andrew Postnikoff · He Meng · He-Ping Zhang

Received: 11 February 2011 / Accepted: 25 June 2011 / Published online: 22 July 2011

Abstract *Lactobacillus casei* Zhang is a widely recognized probiotic bacterium, which is being commercially used in China. To study the gene expression dynamics of *L. casei* Zhang during fermentation in soymilk, a whole genome microarray was used to screen for differentially expressed genes when grown to the lag phase, the late logarithmic phase, and the stationary phase. Comparisons of different transcripts next to each other revealed 162 and 63 significantly induced genes in the late logarithmic phase and stationary phase, of which the expression was at least threefold up-regulated and down-regulated, respectively. Approximately 38.4% of the up-regulated genes were associated with amino acid transport and metabolism notably for histidine and lysine biosynthesis, followed by genes/gene clusters involved in carbohydrate transport and metabolism, lipid transport and metabolism, and inorganic ion transport and metabolism. The analysis results suggest a complex stimulatory effect of soymilk-based ecosystem on the *L. casei* Zhang growth. On the other hand, it provides the very first insight into the molecular mechanism of *L. casei* strain for how it will adapt to the protein-rich environment.

Ji-Cheng Wang and Wen-Yi Zhang contributed equally to this work.

J.-C. Wang · W.-Y. Zhang · Z. Zhong · A.-B. Wei · Q.-H. Bao · Y. Zhang · T.-S. Sun · A. Postnikoff · H.-P. Zhang (✉)
Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, School of Food Science and Engineering, Inner Mongolia Agricultural University, 010018 Huhhot, China
e-mail: hepingdd@vip.sina.com

H. Meng (✉)
School of Agriculture and Biology, Shanghai Jiao Tong University, 200240 Shanghai, China
e-mail: menghe@sjtu.edu.cn

Keywords *Lactobacillus casei* Zhang · Soymilk · Microarray · Amino acid · Growth

Introduction

Soybean is one of the most important oilseeds in the world. For thousands of years, food products based on soy have been popular in the Far East. These foods were reported to be beneficial to the consumers due to their hypolipidemic, anticholesterolemic, and counteratherogenic properties and to their reduced allergenicity [16, 50]. Consequently, soymilk offered a growing appeal for growing segments of consumers with certain dietary and health concerns. However, soymilk alone might not be enough to satisfy the manufacturing demand or serve as functional foods [47]. Nowadays, much research has been turned to developing fermented soymilk with incorporating commercially used probiotics [12, 26, 45] because of the supposed health-promoting effects associated with these organisms.

Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" according to the Food and Agricultural Organisation (FAO) [38]. Generally, the viability of 10^6 and 10^7–10^8 CFU/ml in the final product until the time of consumption have been accepted as the minimum and satisfactory levels, respectively [43]. The actual viability of probiotic microorganisms that reach the gastrointestinal tract depend on various compositional and process factors, including tolerance ability of the probiotics toward stress, food matrix, and manufacturing conditions [7, 9, 10]. Among those mentioned, food matrix is recognized as the most important factor that impacts the viability of probiotics. Although the performance of probiotics in food vehicles such as soymilk has been investigated, little is known about their real actions at the molecular mechanism level.

Lactobacillus casei Zhang is a probiotic bacterium isolated from koumiss in Inner Mongolia of China [56], which has been extensively studied for its physiological, biochemical, and genetic properties [20, 25, 54, 57, 58, 61]. In vitro tests indicated that it had high tolerance to simulated gastric, intestine juices, and bile salts, similar to commercial probiotic strain *L. casei* Shirota [20]. Recently, we investigated the fermentation characteristics of *L. casei* Zhang and found that it grew much better in the soymilk than in the bovine milk [55]. In the present study, gene expression dynamics of *L. casei* Zhang during fermentation in soymilk was investigated in an attempt to reveal the mechanisms involved in growth stimulation for probiotics.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus casei Zhang was isolated from home-made koumiss in Inner Mongolia of China [56], and a direct vat set culture containing 2×10^{11} CFU/g of *L. casei* Zhang was produced by Inner Mongolia Puze Bio-tech Company of China. Nonfat soymilk powder (Wandefu, Shandong, China) was blended with water at 50°C to a total solids content of 6.0% (w/w). The hydrated solutions was supplemented with 1.5% (w/w) of glucose, and heated at 85°C for 30 min. Subsequently, the samples were cooled to the incubation temperature (37°C), inoculated with 1×10^7 of *L. casei* Zhang. The growth curve was constructed according to the pH value and viable counts were determined at 0, 2, 4, 6, 8, 10, 12 14, and 16 h during fermentation. All analyses were performed in triplicate.

Determination of sugar and organic acids

One gram of sample was dissolved in 10 ml of 0.5 mol/l sulfuric acid and centrifugated at 10,000 × *g* for 10 min. The supernatant was collected and filtrated with a 0.45-μm filter. The sugar content was determined using the method described by Quattrucci et al. [34]. Ten microliters of supernatant was injected into the Agilent 1100 series HPLC (Agilent, Palo Alto, CA, USA) for analysis. Mobile phase was water to acetonitrile (20/80, v/v), with the flow rate set at 0.7 ml/min, a UV detector set at 210 nm and ZORBAX carbohydrate NH_2 column (5 μm, 4.6 × 250 mm, Agilent, Palo Alto, CA, USA) operated at 40°C. The contents of lactic acid and acetic acid were determined as described by Zhang et al. [60]. The mobile phase was 10 mmol/l phosphate buffered solution (pH = 2.5)/methanol (5/95, v/v), with a flow rate of 0.5 ml/min, the UV detector set at 210 nm and ZORBAX SB-Aq column (5 μm, 4.6 × 150 mm, Agilent, Palo Alto, CA, USA) operated at 35°C.

RNA isolation and purification

Samples were obtained from two biological replicates at the lag phase (2 h, pH = 6.4), the late logarithmic phase (9.5 h, pH = 5.2), and the stationary phase (14.5 h, pH = 4.5), followed by centrifugation at 200 × *g* for 10 min at 4°C. The supernatant was removed to a fresh tube and centrifugated at 12,000 × *g* for 5 min at 4°C. The cell pellets were grounded into fine powder in the presence of liquid nitrogen and then resuspended in 1 ml Trizol by vortexing. After repeated centrifugation at 12,000 × *g* for 5 min at 4°C, the supernatant was removed to a fresh tube and incubated for 5 min at room temperature. Next, 0.2 ml chloroform was added, followed by vortexing 15 s and incubated for 3 min. The mixtures were centrifugated at 12,000 × *g* for 15 min at 4°C, the upper aqueous phase was transferred into a fresh tube, mixed with 0.5 ml isopropanol, and incubated at room temperature for 10 min and RNA was precipitated at 12,000 × *g* for 10 min at 4°C. After washing in 75% ethanol, the RNA pellet was dried and dissolved in 20 μl of water. The isolated total RNA was purified using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Quality and concentration of RNA samples were determined by using Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

Microarray design

In situ custom oligonucleotide microarrays (60-mer) of *L. casei* Zhang were generated by Agilent Technologies (Agilent, Shanghai, China). Based on the predicated open reading frames (ORFs) of *L. casei* Zhang [25, 61, 62], oligonucleotide probes for 2,906 genes were designed and contained 6,208 probes, of which 396 oligonucleotides were controls.

cDNA synthesis, labeling, and hybridization

A total of 2 μg of total RNA was reverse transcribed into cDNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent, Palo Alto, CA, USA). Synthesized cDNA was transcribed into cRNA and labeled with either cyanine 3 or cyanine 5-labeled nucleotide (Perkin-Elmer, Wellesley, MA, USA). Labeled cRNA was purified with QIAGEN RNeasy Mini kit (Qiagen, Valencia, CA, USA). Microarray hybridizations were carried out on labeled cRNAs using the Gene Expression Hybridization Kit (Agilent, Palo Alto, CA, USA). Arrays were incubated at 65°C for 17 h in Agilent's microarray hybridization chambers and subsequently washed using a Gene Expression Wash Buffer Kit (Agilent, Palo Alto, CA, USA). Arrays were scanned with the Agilent G2565BA MicroArrayScanner System (Agilent, Palo Alto, CA, USA).

Table 1 Primers used for real-time quantitative PCR

Locus	Primer	Sequence (5'–3')	Amplicon size
LCAZH_0910	gap-1	GGCTATCGGTTTGGTTATCC	164
	gap-2	TGTTTTCGGTGTGCTTCTTG	164
LCAZH_2242	PrtM-1	CCAACATATTCTAACTAGCGACG	147
	PrtM-2	TTTGATTCAAAACTAATCTTCCC	147
LCAZH_2241	PrtP-1	CGTTGACCTTCATTTTGGAT	176
	PrtP-2	TTGATGTCTGCGTATTGGCT	176
LCAZH_2078	fabH-1	CACCAAAAAGCTGAAAGCA	179
	fabH-2	TCAACGAACCAACGGATAA	179
LCAZH_0514	metC-1	ATTGTCGATAACACGTTCCTCAC	165
	metC-2	CAAGAAGCCAATCTTTTCACTGA	165
LCAZH_0535	pdxK-1	TGACACCGTATGTTTTGCCTAC	152
	pdxK-2	ATAACCAAGGTAAATGCCATCA	152
LCAZH_0538	metE-1	CATTCCCCACGGATTCCTT	169
	metE-2	CTTGTTTAGCCGCTGCCAC	169
LCAZH_0619	hsp-1	TGAAGTTTTGAATCGTCGTAAT	172
	hsp-2	CTTGGTCTCCTTAACATCAGTT	172
LCAZH_2378	mntH-1	AGGTCAAAACTCCACCATCA	148
	mntH-2	AGCCGTCAACATAACACAGA	148
LCAZH_0682	mleS-1	GTCACCACGGCAGAGAAGC	159
	mleS-2	AAGCAAACCAGCCAACACA	159

Data acquisition and processing

Feature extraction and image analysis software (Agilent, Palo Alto, CA, USA) was used to locate and delineate every spot in the array to integrate each spot's intensity and to normalize data. Systematic differences between the biological duplicates were confirmed by comparing the errors between replicates to the within-array errors between duplicate spots. To identify genes that responded during growth, spot data points with feature of absent or marginal were masked and the remaining data were averaged for each biological duplicates. Subsequently, biological replicates were treated as duplicate measurements and their averages were compared between different growth phases next to each other (exponential phase vs. lag phase, stationary phase vs. exponential phase). A threefold change was used as thresholds for selection of regulated genes. All regulated genes were distributed over clusters of orthologous genes (COGs) and were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [49].

Real-time quantitative PCR

Real-time quantitative PCR (RT–PCR) was used to confirm microarray results. Primers were designed using Primer 5.0 software and were listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative changes in gene expression [27]. The same RNA samples employed for microarray hybridization were used for RT–PCR. cDNA was synthesized by using the PrimeScript™ RT reagent Kit (Takara, Tokyo, Japan). Reverse transcriptions were performed with 0.2 μl of total RNA (1 μg/μl), 0.5 μl PrimeScript™ RT Enzyme Mix I, 2 μl 5 × PrimeScript™ Buffer, 0.5 μl Random 6 mers (50 μM), and 6.8 μl RNase Free ddH_2O. The RT reaction condition was as follows: 37°C for 15 min, 85°C for 5 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, LCAZH_0910) was selected as an internal control for normalizing the amount of RNA added to the reaction of reverse transcription. RT–PCR was performed in the Mastercycler ep realplex system (Eppendorf, Hamburg, Germany) using the following thermo-cycler program: initial denaturation at 95°C for 2 min, followed by 35 cycles of amplification at 95°C for 30 s, and at 55.8°C for 30 s. The reaction mixture contained 1 μl cDNA template, 12.5 μl SYBR® *Premix Ex Taq*™ (2×), 0.2 μl forward primer (10 pM), 0.2 μl reverse primer (10 pM), and 11.1 μl ddH_2O. As a negative control for all RT–PCR experiments, cDNA was replaced by water. Individual RT–PCR reactions were carried out in triplicate for each gene.

Microarray data submission

Microarray platform and data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo)

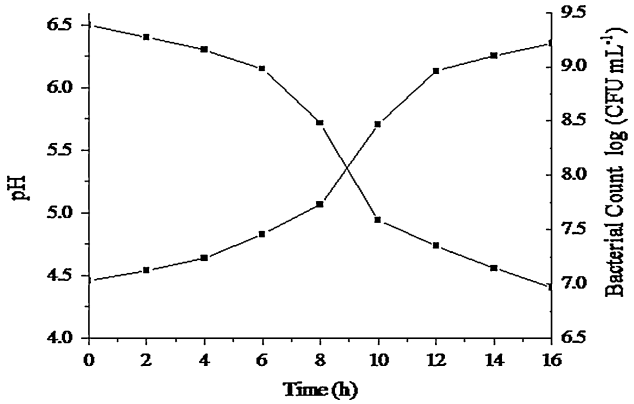

Fig. 1 Growth of *Lactobacillus casei* Zhang in soymilk incubated at 37°C

Table 2 Changes of lactic acid and acetic acid in the soymilk fermented with *L. casei* Zhang during fermentation at 37°C

pH value	Content (mmol l^{-1})[a]	
	Lactic acid	Acetic acid
6.4	10.83 ± 0.17	13.9 ± 4.17
5.2	29.97 ± 1.07	16.96 ± 0.94
4.5	50.59 ± 11.47	17.41 ± 0.67

[a] Data are means of triplicate determinations ± standard deviation

under accession numbers GPL4318 (platform) and GSE23652 (series).

Results

Growth and fermentation characteristics of *L. casei* Zhang in soymilk

To understand the culture conditions of *L. casei* Zhang, the growth and fermentation characteristics in soymilk were assessed. The growth curve based on viable counts is shown in Fig. 1. Bacteria started to grow at the time of 2 h and they grew from the lag phase into the logarithmic phase at a time of nearly 3 h. The viable counts of *L. casei* Zhang increased above 10^9 CFU/ml at the stationary phase. During fermentation, gradual decreases in pH were observed in the fermented soymilk, which might be interpreted as lactic acid and acetic acid production by *L. casei* Zhang cells via consumption of sugars presented in the soymilk (Tables 2, 3).

Expression profiles of *L. casei* Zhang during fermentation in soymilk

Using the whole-genome microarray, the effect of soymilk-based ecosystem on gene expression of *L. casei* Zhang during growth was investigated. We focused our research on

Table 3 Changes of sugar content in the soymilk fermented with *L. casei* Zhang during fermentation at 37°C

pH value	Content (mmol l^{-1})[a]			
	Stachyose	Raffinose	Sucrose	Fructose
6.4	5.26 ± 0.03	1.58 ± 0.04	15.76 ± 0.04	0.59 ± 0.04
5.2	5.18 ± 0.03	1.46 ± 0.06	15.38 ± 0.07	0.41 ± 0.02
4.5	5.25 ± 0.01	1.52 ± 0.07	15.66 ± 0.09	0.29 ± 0.01

[a] Data are means of triplicate determinations ± standard deviation

different growth phases next to each other and made a comparative analysis. A total of 162 genes were found to be significantly differentially (>3 fold) expressed in exponential phase compared to lag phase, and 63 genes were found in the stationary phase compared to the exponential phase, which accounts for approximately 8% of the genome of *L. casei* Zhang (Tables 4, 5).

Based on the functional category of differentially expressed genes during growth in soymilk (Fig. 2), 135 genes between exponential phase and lag phase can be well identified. Among these genes, most of the up-regulated genes belonged to amino acid transport and metabolism and lipid transport and metabolism, whereas most of those down-regulated belonged to carbohydrate transport and metabolism, followed by genes included in replication, recombination and repair, amino acid transport and metabolism. In the stationary phase compared to the exponential phase, 49 genes were identified, among which the most up-regulated or down-regulated genes belonged to amino acid transport and metabolism. It is obviously from the expression data that genes related to amino acid transport and metabolism are highly regulated during growth in soymilk.

Validation of gene expression pattern by using RT–PCR

Of the nine targeted genes (LCAZH_2078, LCAZH_2241, LCAZH_2378, LCAZH_2242, LCAZH_0619, LCAZH_0514, LCAZH_0538, LCAZH_0682, and LCAZH_0535) selected for RT–PCR measurements, the changes of expression were all confirmed. As shown in Fig. 3, there was a strong positive correlation ($r = 0.98$) between the fold changes for gene regulation predicated from the two platforms.

Differential expression of genes in carbon metabolism and energy production

Lactobacillus casei Zhang has been shown to use a variety of carbohydrates such as glucose, galactose, ribose, mannose and fructose, and could weakly metabolize aesculin, inositol, and dextrin [56]. In the present study, glucose was added to the soymilk before fermentation. However, *L. casei* Zhang might utilize sucrose, fructose, and galactose present in the

Table 4 Genes differentially expressed in the logarithmic phase compared to lag phase

Function group and ORF	Gene	Description	Expression ratio
Genes up-regulated			
Carbohydrate transport and metabolism			
LCAZH_0404	*levC*	LevC protein	3.11
LCAZH_0435	*levE*	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIB	3.18
LCAZH_0436	*levF*	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	3.12
LCAZH_0596	*galK*	Galactokinase	6.72
LCAZH_2842	*manM*	Mannose-specific PTS system component IIC	3.86
LCAZH_2843	*manL*	Phosphotransferase system, mannose/fructose-specific component IIA	3.75
Amino acid transport and metabolism			
LCAZH_0201	*oppA*	ABC-type oligopeptide transport system, periplasmic component	17.44
LCAZH_0289	*livA*	ABC-type branched-chain amino acid transport system, periplasmic component	3.79
LCAZH_0290	*livB*	Branched-chain amino acid ABC-type transport system, permease component	4.48
LCAZH_0291	*livC*	Predicted amino acid ABC-type transport system, permease component	3.59
LCAZH_0338	*pepT-2*	Peptidase T	21.05
LCAZH_0339	*oppA*	ABC-type oligopeptide transport system, periplasmic component	28.19
LCAZH_0499	*pepN*	Aminopeptidase N	3.21
LCAZH_0519	*brnQ*	Branched-chain amino acid permease	46.76
LCAZH_0537	*metF*	5,10-methylenetetrahydrofolate reductase	5.31
LCAZH_0538	*metE*	Methionine synthase II (cobalamin-independent)	5.46
LCAZH_0552	*sstT*	Na+/H+ -dicarboxylate symporter	3.19
LCAZH_1414	*hisC*	Histidinol-phosphate/aromatic aminotransferase and cobyric acid decarboxylase	8.35
LCAZH_1415	*hisE*	Phosphoribosyl-ATP pyrophosphohydrolase	8.52
LCAZH_1416	*hisI*	Phosphoribosyl-ATP pyrophosphohydrolase	6.63
LCAZH_1417	*hisF*	Imidazoleglycerol-phosphate synthase	8.4
LCAZH_1418	*hisA*	Phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide (ProFAR) isomerase	8.24
LCAZH_1419	*hisH*	Glutamine amidotransferase	10.36
LCAZH_1420	*hisB*	Imidazoleglycerol-phosphate dehydratase	10.76
LCAZH_1421	*hisD*	Histidinol dehydrogenase	11.13
LCAZH_1422	*hisG*	ATP phosphoribosyltransferase	9.41
LCAZH_1423	*hisX*	ATP phosphoribosyltransferase for histidine biosynthesis	11.42
LCAZH_1641	*pepX*	X-prolyl dipeptidyl aminopeptidase	4.61
LCAZH_1957	*glnP*	ABC-type amino acid transport system, permease component	3.55
LCAZH_1958	*glnM*	ABC-type amino acid transport system, permease component	3.73
LCAZH_1959	*glnH1*	ABC-type amino acid transport/signal transduction system, periplasmic component/domain	3.43
LCAZH_1960	*glnQ*	ABC-type polar amino acid transport system, ATPase component	3.3
LCAZH_1980	*ilvE*	Branched-chain amino acid aminotransferase/4-amino-4-deoxychorismate lyase	7.3
LCAZH_2022	*oppF*	ABC-type oligopeptide transport system, ATPase component	5
LCAZH_2023	*oppD*	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component	4.96
LCAZH_2025	*oppB*	ABC-type dipeptide/oligopeptide/nickel transport system, permease component	6.49
LCAZH_2026	*oppA*	ABC-type oligopeptide transport system, periplasmic component	3.06
LCAZH_2302	*pepC1*	Aminopeptidase C	5.76
LCAZH_2303	*pepC2*	Aminopeptidase C	3.17
LCAZH_2518	*gltD*	NADPH-dependent glutamate synthase beta chain related oxidoreductase	21.32
LCAZH_2519	*gltB*	Glutamate synthase domain 3	19.44

Table 4 continued

Function group and ORF	Gene	Description	Expression ratio
Lipid transport and metabolism			
LCAZH_2068	*accA*	Acetyl-CoA carboxylase alpha subunit	3.8
LCAZH_2069	*accD*	Acetyl-CoA carboxylase beta subunit	5.73
LCAZH_2070	*accC2*	Biotin carboxylase	7.84
LCAZH_2071	*fabA*	3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase	14.09
LCAZH_2072	*accB*	Biotin carboxyl carrier protein	13.41
LCAZH_2073	*fabF*	3-oxoacyl-(acyl-carrier-protein) synthase	17.14
LCAZH_2074	*fabG*	3-oxoacyl-acyl carrier protein reductase	19.31
LCAZH_2075	*fabD*	(acyl-carrier-protein) S-malonyltransferase	19.67
LCAZH_2076	*fabK*	Dioxygenase	9.01
LCAZH_2077	*acpP*	Acyl carrier protein	10.8
LCAZH_2078	*fabH*	3-oxoacyl-(acyl-carrier-protein) synthase III	6.71
LCAZH_2079	*marR*	Transcriptional regulator	6.51
LCAZH_2080	*fabZ1*	3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase	9.09
Energy production and conversion			
LCAZH_0682	*mleS*	Malolactic enzyme	3.67
LCAZH_0683	*mleP2*	Malate permease	4.47
LCAZH_1303	*ldh*	Malate/lactate dehydrogenase	3.13
LCAZH_1413	*ydgI*	Nitroreductase	3.09
LCAZH_2031	*yrjC*	Iron-binding oxidase subunit	3.28
LCAZH_2905	*ypjH*	Glycerol dehydrogenase	19.97
Posttranslational modification, protein turnover, chaperones			
LCAZH_1048	*hflC*	Membrane protease subunit, stomatin/prohibitin family	6.61
LCAZH_2241	*PrtP*	Subtilisin-like serine protease	23.02
LCAZH_2242	*PrtM*	Parvulin-like peptidyl-prolyl isomerase	3
Defense mechanisms			
LCAZH_0466	*pbpE*	Beta-lactamase class C-related penicillin binding protein	7.66
LCAZH_1216	*yvfR*	ABC-type multidrug transport system, ATPase component	5.72
LCAZH_1217	*yvfR*	ABC-type multidrug transport system, ATPase component	7.63
LCAZH_2155	*cydC*	ABC-type multidrug transport system, ATPase and permease component	3.23
Cell wall/membrane/envelope biogenesis			
LCAZH_0467	*ykfB*	L-alanine-DL-glutamate epimerase related enzyme of enolase superfamily	8.27
LCAZH_0597	*galE4*	UDP-glucose 4-epimerase	5.03
Replication, recombination and repair			
LCAZH_2542	*mutT*	NUDIX family hydrolase	4.94
Transcription			
LCAZH_0599	*galR*	Transcription regulator of beta-galactosidase gene	3.18
LCAZH_1273	*cspC*	Cold shock protein	3.48
LCAZH_2079	*marR*	Transcriptional regulator	6.51
LCAZH_2847	–	Transcriptional regulator, xre family	3.34
LCAZH_2848	–	Transcriptional regulator	4.36
Signal transduction mechanisms			
LCAZH_1214	*rrp6*	DNA-binding response regulator, CitB family (Rec-wHTH domains)	3.02
LCAZH_1215	*hpk6*	Signal transduction histidine kinase	4.44
Unknown function			
LCAZH_0229	–	Predicted membrane protein	3.49
LCAZH_0417	–	NAD/NADP octopine/nopaline dehydrogenas	3.66
LCAZH_0525	–	Conserved hypothetical protein	9.09
LCAZH_0534	–	Predicted membrane protein	3.56

Table 4 continued

Function group and ORF	Gene	Description	Expression ratio
LCAZH_0536	–	Conserved hypothetical protein	3.81
LCAZH_0588	–	Hypothetical protein	5.89
LCAZH_0947	–	ABC-type uncharacterized transport system, ATPase component	4.1
LCAZH_1024	*pheB*	ACT domain-containing protein	3.26
LCAZH_1049	–	Conserved hypothetical protein	4.92
LCAZH_1213	–	Conserved hypothetical protein	17.04
LCAZH_1304	–	Conserved hypothetical protein	3.42
LCAZH_1376	*hly*	Predicted membrane protein, hemolysin III related protein	4.31
LCAZH_1721	–	Conserved hypothetical protein	7.97
LCAZH_1722	–	Conserved hypothetical protein	7.88
LCAZH_2081	–	Conserved hypothetical protein	7.92
LCAZH_2547	–	Predicted membrane protein	112.94
Genes down-regulated			
Carbohydrate transport and metabolism			
LCAZH_0334	–	PTS system, IIa component	0.29
LCAZH_0357	*rbsC*	Ribose/xylose/arabinose/galactoside ABC-type transport system, permease component	0.32
LCAZH_0503	–	Sugar phosphate isomerase/epimerase	0.28
LCAZH_0550	*treA*	Alpha, alpha-phosphotrehalase	0.16
LCAZH_0551	*pts4ABC*	Beta-glucoside-specific PTS system IIABC component	0.2
LCAZH_1109	–	Putative glucose uptake permease	0.31
LCAZH_1335	*fruA*	Fusion of IIA, IIB and IIC component of mannitol/fructose-specific phosphotransferase system	0.24
LCAZH_1336	*fruK*	Tagatose-6-phosphate kinase	0.17
LCAZH_2777	*ugpA*	ABC-type sugar transport system, permease component	0.33
Amino acid transport and metabolism			
LCAZH_0500	–	Amino acid transporter	0.04
LCAZH_0511	*cysK1*	Cysteine synthase	0.3
LCAZH_1642	*glnA*	Glutamine synthetase	0.18
LCAZH_2223	*asnA1*	L-asparaginase	0.05
LCAZH_2873	*ansB*	Aspartate ammonia-lyase	0.1
Replication, recombination, and repair			
LCAZH_0114	–	ADP-ribose pyrophosphatase	0.26
LCAZH_0463	*tag1*	3-methyladenine DNA glycosylase	0.22
LCAZH_0899	*uvrB*	Helicase subunit of the DNA excision repair complex	0.24
LCAZH_0900	*uvrA1*	Excinuclease ATPase subunit	0.18
LCAZH_1027	–	Nucleotidyltransferase/DNA polymerase for DNA repair	0.22
LCAZH_2258	–	Ribonucleotide reductase, alpha subunit	0.2
LCAZH_2619	*recA*	RecA/RadA recombinase	0.33
Defense mechanisms			
LCAZH_1890	*msbA*	ABC-type transport system, ATPase component	0.33
LCAZH_1891	–	Conserved hypothetical protein	0.27
LCAZH_2433		ABC-type antimicrobial peptide transport system, permease component	0.15
LCAZH_2434		ABC-type antimicrobial peptide transport system, ATPase component	0.21
Nucleotide transport and metabolism			
LCAZH_0853	*guaC*	IMP dehydrogenase/GMP reductase	0.33
LCAZH_1193	*dukA*	Deoxynucleoside kinase	0.11
LCAZH_1458	*nrdE*	Ribonucleotide reductase, alpha subunit	0.22
LCAZH_1459	*nrdF*	Ribonucleotide reductase, beta subunit	0.24

Table 4 continued

Function group and ORF	Gene	Description	Expression ratio
Inorganic ion transport and metabolism			
LCAZH_0576	*amtB*	Ammonia permease	0.1
LCAZH_0645	*pacL3*	Cation transport ATPase	0.18
LCAZH_1274	*pstF*	ABC-type phosphate transport system, periplasmic component	0.08
LCAZH_2388	*mtsB*	ABC-type Mn^{2+}/Zn^{2+} transport system, permease component	0.23
Posttranslational modification, protein turnover, chaperones			
LCAZH_1457	*nrdH*	Glutaredoxin-related protein	0.17
LCAZH_1512	*msrA3*	Conserved domain frequently associated with peptide methionine sulfoxide reductase	0.18
LCAZH_2811	*hsp3*	Molecular chaperone (small heat shock protein)	0.19
Transcription			
LCAZH_0549	*treR*	Transcriptional regulator	0.32
LCAZH_1195		Transcriptional regulator	0.33
LCAZH_1337	–	Lactose transport regulator	0.15
Signal transduction mechanisms			
LCAZH_2879	–	Signal transduction histidine kinase regulating citrate/malate metabolism	0.31
Energy production and conversion			
LCAZH_2390	*aldA*	NAD-dependent aldehyde dehydrogenase	0.31
Coenzyme transport and metabolism			
LCAZH_1192	–	Nicotinamide mononucleotide transporter	0.08
Cell wall/membrane/envelope biogenesis			
LCAZH_0973	*glmS*	Glucosamine 6-phosphate synthetase, amidotransferase and phosphosugar isomerase domains	0.23
Unknown function			
LCAZH_0224	–	Conserved hypothetical protein	0.2
LCAZH_0225	–	Conserved hypothetical protein	0.14
LCAZH_0226	–	Conserved hypothetical protein	0.15
LCAZH_0521	–	Conserved hypothetical protein	0.27
LCAZH_0523	–	Conserved hypothetical protein	0.24
LCAZH_0524	–	Conserved hypothetical protein	0.26
LCAZH_0626	–	Conserved hypothetical protein	0.33
LCAZH_0685	–	Conserved hypothetical protein	0.28
LCAZH_0886	–	Conserved hypothetical protein	0.31
LCAZH_0959	–	Conserved hypothetical protein	0.31
LCAZH_1023	–	Conserved hypothetical protein	0.31
LCAZH_1511	–	Conserved hypothetical protein	0.3
LCAZH_1621	–	Conserved hypothetical protein	0.2
LCAZH_1848	–	Hypothetical protein of possible phage origin	0.33
LCAZH_1891	–	Conserved hypothetical protein	0.27
LCAZH_1894	–	Conserved hypothetical protein	0.29
LCAZH_2038	–	Conserved hypothetical protein	0.25
LCAZH_2039	–	Conserved hypothetical protein	0.29
LCAZH_2257	–	Conserved hypothetical protein	0.2
LCAZH_2332	–	Conserved hypothetical protein	0.29
LCAZH_2435	–	Conserved hypothetical protein	0.23
LCAZH_2687	–	Conserved hypothetical protein	0.33
LCAZH_2688	–	Conserved hypothetical protein	0.31
LCAZH_2689	–	Conserved hypothetical protein	0.22
LCAZH_2812	–	Conserved hypothetical protein	0.12
LCAZH_2898	–	Conserved hypothetical protein	0.2

Table 5 Genes differentially expressed in the stationary phase compared to logarithmic phase

Function group and ORF	Gene	Description	Expression ratio
Genes up-regulated			
Carbohydrate transport and metabolism			
LCAZH_1336	*fruK*	Tagatose-6-phosphate kinase	3.12
LCAZH_2382	–	Permease of the major facilitator superfamily	4.25
Amino acid transport and metabolism			
LCAZH_0104	*dapB*	Dihydrodipicolinate reductase	9.24
LCAZH_0105	*dapA*	Dihydrodipicolinate synthase/N-acetylneuraminate lyase	9.28
LCAZH_0106	*dapE*	Metal-dependent amidase/aminoacylase/carboxypeptidase	10.06
LCAZH_0107	*dapD*	Tetrahydrodipicolinate N-succinyltransferase	5.08
LCAZH_0108	*lysA*	Diaminopimelate decarboxylase	8.16
LCAZH_0418	*glnQ*	Putative amino acid ABC transporter, ATP-binding protein	5.64
LCAZH_0419	*atmA*	Putative amino acid ABC transporter, periplasmic amino acid-binding protein	5.58
LCAZH_0420	–	Putative amino acid ABC transporter, permease protein	5.25
LCAZH_0421	*tcyL*	Putative amino acid ABC transporter, permease protein	5.23
LCAZH_0514	*metC*	Cystathionine beta-lyase/cystathionine gamma-synthase	5.76
LCAZH_0515	–	ABC-type amino acid transport/signal transduction system, periplasmic component/domain	6.26
LCAZH_0516	–	ABC-type amino acid transport system, permease component	6.25
LCAZH_0537	*metF*	5,10-methylenetetrahydrofolate reductase	5.01
LCAZH_0538	*metE*	Methionine synthase II (cobalamin-independent)	4.92
LCAZH_0708	–	Methionine synthase II (cobalamin-independent)	4.5
LCAZH_0803	–	Putative L-aspartate transport protein	3.23
LCAZH_2850	–	ABC-type amino acid transport/signal transduction system, periplasmic component/domain	6.98
LCAZH_2851	–	ABC-type polar amino acid transport system, ATPase component	7.21
Inorganic ion transport and metabolism			
LCAZH_0131	*cadA*	Cation transport ATPase	6.89
LCAZH_0577		Predicted iron-dependent peroxidase	3.43
LCAZH_1165	–	ABC-type metal ion transport system, periplasmic component/surface antigen	3.83
LCAZH_2378	*mntH2*	Mn^{2+} and Fe^{2+} transporter of the NRAMP family	3.62
LCAZH_2810	*cadA*	Cation transport ATPase	8.91
Posttranslational modification, protein turnover, chaperones			
LCAZH_0619	–	Molecular chaperone (small heat shock protein)	3.48
LCAZH_2473	*ahpC*	Peroxiredoxin	4.53
Transcription			
LCAZH_1337		Lactose transport regulator	3.67
LCAZH_2386	–	Predicted transcriptional regulator	3.08
Secondary metabolites biosynthesis, transport and catabolism			
LCAZH_2383	*sufI*	Putative multicopper oxidase	4.11
Signal transduction mechanisms			
LCAZH_0709	*luxS*	Autoinducer AI2 synthesis LuxS-like protein	3.41
Coenzyme transport and metabolism			
LCAZH_0535	*pdxK*	Pyridoxal/pyridoxine/pyridoxamine kinase	3.6
Unknown function			
LCAZH_0389	–	Fructan hydrolase	3.31
LCAZH_0417	–	NAD/NADP octopine/nopaline dehydrogenas	5.28
LCAZH_0431	–	Uncharacterized NAD(FAD)-dependent dehydrogenase	6.27

Table 5 continued

Function group and ORF	Gene	Description	Expression ratio
LCAZH_0536	–	Conserved hypothetical protein	3.85
LCAZH_0616	–	Conserved hypothetical protein	7.43
LCAZH_1862	–	Conserved hypothetical protein	3.21
LCAZH_2030	–	Conserved hypothetical protein	3.21
LCAZH_2034	–	Conserved hypothetical protein	3.65
LCAZH_2376	–	Conserved hypothetical protein	4.77
LCAZH_2381	–	Conserved hypothetical protein	8.27
LCAZH_2384	–	Hypothetical protein	9.32
LCAZH_2385	–	Integral membrane protein	8.07
LCAZH_2553	–	ABC-type uncharacterized transport system, ATPase component	3.33
LCAZH_2554	–	ABC-type uncharacterized transport system, permease component	3.35
Genes down-regulated			
Amino acid transport and metabolism			
LCAZH_0290	*livB*	Branched-chain amino acid ABC-type transport system, permease component	0.29
LCAZH_0291	*livC*	Predicted amino acid ABC-type transport system, permease component	0.3
LCAZH_0293	*livF*	ABC-type branched-chain amino acid transport system, ATPase component	0.3
LCAZH_0552	*sstT*	Na^+/H^+-dicarboxylate symporter	0.24
Posttranslational modification, protein turnover, chaperones			
LCAZH_1048	–	Membrane protease subunit, stomatin/prohibitin family	0.33
LCAZH_2241	*PrtP*	Subtilisin-like serine protease	0.17
Defense mechanisms			
LCAZH_1927	–	ABC-type antimicrobial peptide transport system, permease component	0.25
LCAZH_1928	–	ABC-type antimicrobial peptide transport system, ATPase component	0.31
LCAZH_2155	–	ABC-type multidrug transport system, ATPase and permease component	0.32
Energy production and conversion			
LCAZH_0682	*mleS*	Malolactic enzyme	0.17
LCAZH_0683	*mleP2*	Malate permease	0.18
Inorganic ion transport and metabolism			
LCAZH_2415	*phnE*	ABC-type phosphate/phosphonate transport system, permease component	0.26
Secondary metabolites biosynthesis, transport, and catabolism			
LCAZH_0737	*dltA*	D-alanine-activating enzyme	0.2
Unknown function			
LCAZH_0372	–	Conserved hypothetical protein	0.28
LCAZH_0373	–	Conserved hypothetical protein	0.24
LCAZH_1452	–	Predicted oxidoreductase	0.31
LCAZH_1530	–	Conserved hypothetical protein	0.28

soymilk as well, which could be the reason for differential expressions of various genes involved in sugar utilization.

Several sugar transporters were differentially expressed in *L. casei* Zhang during growth in soymilk. Five genes (*manL*, LCAZH_2843; *manM*, LCAZH_2842; *levE*, LCAZH_0435; *levF*, LCAZH_0436; *levC*, LCAZH_0404) predicted to code for mannose phosphotransferase transport system (PTS) were distinguished, which was up-regulated in late logarithmic phase. Members of the mannose PTS are known to be transporters for glucose and fructose in lactic acid bacteria (LAB) [41]. Other carbohydrate transporters were repressed such as the fructose PTS (*fruA*, LCAZH_1335; *rbsC*, LCAZH_0357), the glucose PTS (*pts4ABC*, LCAZH_0551), an uncharacterized PTS (LCAZH_0334), and an ABC-type sugar transport system (LCAZH_2777). Genes differentially expressed also included those in galactose catabolism (*galR*, LCAZH_0599; *galK*, LCAZH_0596), pyruvate metabolism (*mleS*, LCAZH_0682; *mleP2*, LCAZH_0683; *ldh*, LCAZH_1303), and glycerolipid metabolism (*ypjH*, LCAZH_2905).

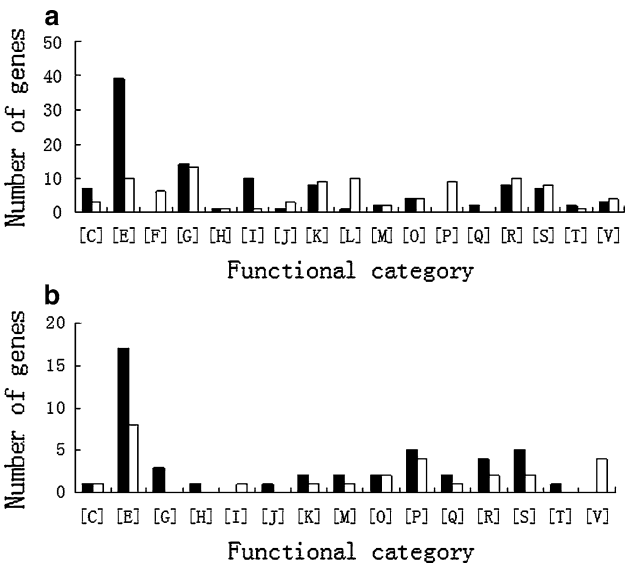

Fig. 2 Clusters of orthologous (COG) classification of differentially expressed genes in exponential phase compared to lag phase (**a**), and in stationary phase compared to exponential phase (**b**). Up-regulated genes (*black bars*) and down-regulated genes (*white bars*) are shown. Functional categories: [C], Energy production and conversion; [E], Amino acid transport and metabolism; [F] Nucleotide transport and metabolism; [G], Carbohydrate transport and metabolism; [H], Coenzyme transport and metabolism; [I], Lipid transport and metabolism; [J], Translation, ribosomal structure, and biogenesis; [K], Transcription; [L], Replication, recombination and repair; [M], Cell wall/membrane/envelope biogenesis; [O], Posttranslational modification, protein turnover, chaperones; [P], Inorganic ion transport and metabolism; [Q], Secondary metabolites biosynthesis, transport and catabolism; [R], General function prediction only; [S], Function unknown; [T], Signal transduction mechanisms; [V], Defense mechanisms

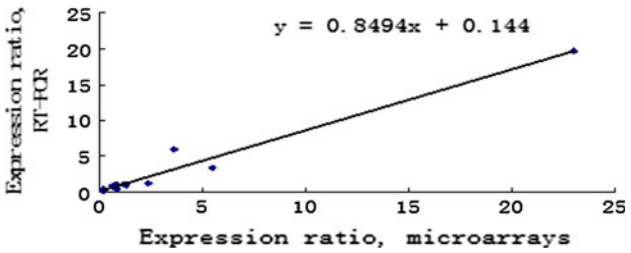

Fig. 3 Correlation of expression ratios from microarray profiling and RT–PCR. The best-fit linear regression curve is shown with the calculated equation

Differential expression of genes in proteolytic enzyme system

In dairy LAB, the proteolytic enzyme system generally consists of cell surface-associated proteinases, transport systems (Opp and Dtp), and peptidases. Once proteins were broken down by the proteinases, the transport systems would translocate peptides or amino acids to the cytoplasm where the peptides were then degraded by the peptidases [42]. In the late logarithmic phase, the expression of *PrtP* (LCAZH_2241), encoding a putative proteinase was highly induced while *PrtR* (LCAZH_0497 and LCAZH_0498) that encodes another type of proteinase was clearly repressed. In addition, *PrtM* (LCAZH_2242), a proteinase maturase protein was up-regulated. Differential expression pattern of these four genes indicate that *PrtM* is more likely required for maturation of *PrtP* enzyme [21].

Four genes distributed in one Opp operon were up-regulated, namely LCAZH_2026 (*OppA*, a gene coding for substrate-binding proteins), LCAZH_2025 and LCAZH_2023 (*OppB* and *OppC*, genes coding for membrane proteins), and LCAZH_2022 (*OppF*, a gene coding for ATP-binding proteins). Besides, expression of two *OppA* genes (LCAZH_0201 and LCAZH_0339) without operon increased. In fact, these two genes showed relatively higher induction levels than the operon encoded *OppA* gene, suggesting that different *OppA* genes might have different specificities. Additionally, five peptidases were significantly up-regulated, including three aminopeptidase proteins (*pepC1,* LCAZH_2302; *pepC2*, LCAZH_2303; *pepN*, LCAZH_0499; *pepX*, LCAZH_1641) and a tripeptidase protein (*pepT-2*, LCAZH_0338).

Differential expression of genes in amino acid uptake and biosynthesis

Genome analysis revealed that *L. casei* Zhang was able to synthesize all amino acids except for leucine (Leu), isoleucine (Ile), and valine (Val), and it did encode a vast number of amino acid transporters. Differential expression of genes for uptake branched-chain amino acids (BCAAs) was observed. Among them, *brnQ* (LCAZH_0519), *livC* (LCAZH_ 0291), *livB* (LCAZH_0290), and *livA* (LCAZH_0289) were highly expressed in the late logarithmic phase, two of which were under expressed in the stationary phase. Interestingly, a glutamate transporter operon (*glnQHMP*, LCAZH_1957-LCAZH_1960) and several uncharacterized transporters responsible for amino acid transport encoded by LCAZH_2851, LCAZH_2850, LCAZH_0516, LCAZH_0515, LCAZH_0421, LCAZH_0419, and LCAZH_0418 were mainly induced in the late logarithmic phase and the stationary phase, respectively.

Regarding the amino acid biosynthesis, the histidine (His) and lysine (Lys) pathways were massively induced in the late logarithmic phase and the stationary phase, respectively, while the over-expression of *gltB* (LCAZH_2519) *gltD* (LCAZH_2518) indicated that glutamate (Glu) biosynthesis may also be modified. In contrast with expression patterns of genes involved in His, Lys, and Glu biosynthesis, we observed a concomitant increase of *metE* (LCAZH_2518) and *metF* (LCAZH_0537), which is necessary for methionine (Met) conversion. Another gene (*metC*,

LCAZH_0514) involved in Met biosynthesis was found to be up-regulated in the stationary phase.

Differential expression of genes in lipid metabolism

The membrane lipid composition of *L. casei* consists of saturated:unsaturated membrane fatty acids (FA) and cyclopropane content [14]. In the genome of *L. casei* Zhang, genes involved in FA (*fab*) biosynthesis are located in a 13-gene cluster, consisting of an acetyl-CoA carboxylase alpha subunit (*accA*, LCAZH_2068), an acetyl-CoA carboxylase beta subunit (*accD*, LCAZH_2069), a biotin carboxylase (*accC2*, LCAZH_2070), a 3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase (*fabA*, LCAZH_2071), a biotin carboxyl carrier protein (*accB*, LCAZH_2072), a 3-oxoacyl-(acyl-carrier-protein) synthase (*fabF*, LCAZH_2073), a 3-oxoacyl-acyl carrier protein reductase (fabG, LCAZH_2074), a S-malonyltransferase (*fabD*, LCAZH_2075), a dioxygenase (*fabK*, LCAZH_2076), an acyl carrier protein (*acpP*, LCAZH_2077), a 3-oxoacyl-(acyl-carrier-protein) synthase III (*fabH*, LCAZH_2078), a transcriptional regulator (*marR*, LCAZH_2079), and a 3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratase (*fabZ1*, LCAZH_2080). The gene cluster is structurally unique among all sequenced LAB by virtue of its inclusion of the gene for cyclopropane synthase. During the growth of *L. casei* Zhang, the transcription of the whole gene cluster increased in the late logarithmic phase. Note that one gene (LCAZH_2067) immediately upstreamed the gene cluster, previously identified possibly related to FA biosynthesis, was not affected at the transcription level, indicating it serves an alterative role.

Differential expression of genes related to inorganic ion transport and metabolism

The transcription of three inorganic ion transporters, namely an ammonia permease (*mtsB*, LCAZH_2388), an ABC-type phosphate transport system, periplasmic component (*pacL3*, LCAZH_0645), and an ABC-type Mn^{2+}/Zn^{2+} transport system permease component (*mtsB*, LCAZH_2388), as well as a cation transport ATPase (*pstF*, LCAZH_1274), was down-regulated in the late logarithmic phase of growth. When bacterial cells went in the stationary phase, five genes potentially involved in ion transport were differentially expressed, four of which were significantly up-regulated. All together, our data established that at the late growth stage, *L. casei* Zhang induced several systems to increase its intracellular ion concentration.

Differential expression of genes related to stress response

Several of the genes altered during *L. casei* Zhang growth in soymilk are involved in stress adaptation. Of 17 two-component regulatory systems (2CRSs) encoded by *L. casei* Zhang, one (*rrp6*, LCAZH_1214; *hpk6*, LCAZH_1215) showed high levels of expression in the late logarithmic phase. In addition, the up-regulation of a molecular chaperone encoded by LCAZH_0619 in the stationary phase could be related to stress. Five genes involved in oxidative stress response were down-regulated in the late logarithmic phase, including genes coding for RecA/RadA recombinase (*recA*, LCAZH_2619) [13], excinuclease ATPase subunit (*uvrA1*, LCAZH_0900) [4], helicase subunit of the DNA excision repair complex (*uvrB*, LCAZH_0899), molecular chaperone (*hsp3*, LCAZH_2811), and peptide methionine sulfoxide reductase (*msrA3*, LCAZH_1512) [46].

Discussion

Soymilk is one of the right vehicles for the delivery of the probiotics. Physiological factors involved with growth and survival of probiotic strains in soymilk have been described so far in terms of nutritional exchanges, in turn, limited molecular information can be obtained. In this study, microarray techniques were used to determine genes differentially expressed when *L. casei* Zhang was grown in soymilk. To facilitate further studies and provide information about the activities and metabolic processes of the cells under various conditions for industrial applications, glucose was added as the primary carbon source of carbohydrate available.

Carbon metabolism and energy production

Transcriptomic data indicated that *L. casei* Zhang diversified its carbon metabolism in late stages during growth in soymilk. It can be seen from the microarray data, although glucose was the primary carbohydrate in soymilk, few genes involved in glucose metabolism were induced in the late logarithmic phase and the stationary phase, except for two glucose-specific phosphotransferase system (PTS) transporters (LCAZH_2843 and LCAZH_2842). On the contrary, several genes related to fructose utilization (LCAZH_0435, LCAZH_0436, LCAZH_0404, LCAZH_1335, and LCAZH_1336), galactose catabolism (LCAZH_0599 and LCAZH_0596) as well as in other sugar utilization (LCAZH_2777, LCAZH_0551, and LCAZH_0357) were significantly up-regulated. According to Redon et al. [37], switching from using glucose to alternative carbon sources

could be explained by the decreasing glucose content in the growth media. At the opposite, some genes involved in sugar transportation (LCAZH_2777, LCAZH_1335, LCAZH_1109, LCAZH_0551, LCAZH_0357, and LCAZH_0334) and metabolism (LCAZH_1336 and LCAZH_0550) were down-regulated in the late logarithmic phase. It is possible that during growth in soymilk, synthesis of enzymes involved in a rapidly metabolizable carbon source reduces the expression of genes involved in the utilization of other carbon sources [28]. This phenomenon, termed carbon catabolite repression (CCR), is well understood in *Escherichia coli* and has been confirmed in many LAB species [18].

Another set of genes affected by carbon sources in the presence of soymilk encoded six genes (LCAZH_2905, LCAZH_2031, LCAZH_1413, LCAZH_1303, LCAZH_0683, and LCAZH_0682) relevant to energy production. Given that *L. casei* is a facultatively hetero-fermentative bacterium, the major production of energy is likely through the Embden-Meyerhof pathway, leading to a homo-lactic fermentation profile. The up-regulation of lactate dehydrogenase (LCAZH_1303) may point to accumulation of lactate in growth medium [40]. Interestingly, both genes for malolactic enzyme (LCAZH_0682) and malate/lactate antiporter (LCAZH_0683) were among the highly expressed genes. In malolactic fermentation (MLF), L-malate is decarboxylated by the malolactic enzyme to produce L-lactate and CO_2, and then the malate/lactate antiporter serves as a transporter for lactate efflux [33, 39]. This process contributes to alkalinization of the cytoplasm and allows for energy production, and thus may link to cell survival in acidic conditions as reported by Sheng and Marquis [44].

Nitrogen uptake and biosynthesis

The proteolytic enzyme system plays an important role in the development of LAB during growth in a protein-rich environment [22]. For most LAB species, including *L. casei*, the degradation of milk proteins depends on the activities of cell-surface associated proteins [19]. As expected, growth in soymilk in the late logarithmic phase raised the expression of genes for a proteinase (LCAZH_2241) and a proteinase maturase protein (LCAZH_2242), but repressed the expression of genes for *PrtR* (LCAZH_0497 and LCAZH_0498), another type of proteinase previously identified in *L. rhamnosus* [32], probably coinciding with fast decreased pH value in the medium. These results and the patterns of *PrtR* expression were in correlation with proteolytic activities obtained for the whole cells of *L. rhamnosus* BGT10 [32]. Soymilk-grown cells also showed high expression levels of genes for oligopeptide transport and intracellular peptidases. Genetic studies have shown that in the whole process of protein breakdown to peptides and subsequently to free amino acids, transport of peptides by the oligopeptide transport system is a crucial step for *Lactococcus lactis* grown in milk [23]. Of the five peptidases, *pepT-2* (LCAZH_0338) was reported to have rather strict substrate size specificity, which hydrolyses tripeptides with a wide range of amino acids including substrates comprised of hydrophobic/uncharged, aromatic, basic, acidic, and sulfur-containing residues [6]. It was more strongly induced than that of the others, likely reflecting the abundance of such small peptides in soymilk.

Although *L. casei* strains were auxotrophic only for three BCAAs (Leu, Ile, and Val) [3], for optimal growth, it might require either uptake of amino acids from growth medium or de novo amino acid biosynthesis. Many genes were found to be likely involved in amino acids accumulation of bacterial cells. Members of BCAA transport system have been characterized in a variety of bacteria [29], but much less is known about the transport system in lactobacilli. The data presented in the present study indicated that active transport of BCAAs across the cytoplasmic membrane of *L. casei* was probably mediated by two distinct systems at the transcriptional level, based on *liv* gene cluster (LCAZH_0291, LCAZH_0290, and LCAZH_1980) and *brnQ* (LCAZH_0519), respectively. In *L. lactis* [11] and *L. delbruckii* [48], the gene *brnQ* has been characterized as a low-affinity BCAA system, which is responsible for a minor fraction of BCAA uptake; while evidence for the importance of *liv* genes in BCAA transport capacity has been described by Matsubara et al. [29]. Notably, the expression of Glu operon (LCAZH_1957-LCAZH_1960), together with two genes involved in Glu biosynthesis (LCAZH_2518 and LCAZH_2519) was simultaneously induced. In bacteria, Glu can be synthesized by the combined actions of glutamine synthetase encoded by *glnA* and glutamate synthase encoded by *gltBD* [59]. Considering their necessity for biosynthesis, the down-regulation of *glnA* (LCAZH_1642) in our study may lead to inactivation of the whole pathway, the internal Glu pool concentration therefore should be raised mainly by the encoded Glu operon. In this way, exogenously supplied amino acids could appear to "derepress" biosynthetic pathways for amino acids of a shared transport system [35]. Moreover, the accumulation of His and Lys was seen, genes fit into the formation of His (LCAZH_1414-LCAZH_1423) and Lys (LCAZH_0104-LCAZH_0108) showed predominant profiles in the late logarithmic phase and the stationary phase, respectively. In view of the fact that both of these amino acids are necessary for bacterial growth during fermentation studies, the switching on of these two biosynthesis pathways in *L. casei* is likely due to the phase effect. Surprisingly, two genes related to Met biosynthesis presented a constitutive up-regulation profile during growth, specifically the gene coding for 5, 10-methylenetetrahydrofolate reductase (LCAZH_0538) and the gene coding

for methionine synthase II (LCAZH_0537). These are key enzymes catalyzing the conversion reaction of homocysteine to Met, reflecting a higher level of Met production in bacterial cells.

Inorganic ion transport and metabolism

Metal ions, the so-called micronutrients, are involved in several functions in the metabolism of LAB so that are of importance for living cells [2]. The main process for regulating the intracellular concentrations of trace metals is generally performed by membrane transport systems [30]. It is obvious that among the genes altered, several are involved in inorganic ion transport. The expression of five (LCAZH_0577, LCAZH_0131, LCAZH_2378, LCAZH_2810, and LCAZH_1165) of ten altered genes was specifically induced in the stationary phase, suggesting their important, physiological roles in ion homeostasis in *L. casei* Zhang during late growth. Intriguingly, the *mntH2* (LCAZH_2378) gene codes for an Mn^{2+} transporter of the NRAMP family and two *cadA* (LCAZH_0131 and LCAZH_2810) genes confer for high resistance to Cd^{2+} were simultaneously up-regulated. It was previously reported that, in some Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, Cd^{2+} competes for transport with Mn^{2+} [24, 51]. We therefore wondered whether the observed modification could be a coordinated response aiming at enhancing the importing of Mn^{2+} into the cells.

Stress response

During growth, gradual decreases of pH in fermented soymilk were observed, which implied the frequent confrontation of LAB cells with acid stress. Generally, the cell surface is recognized as the first shield against an acidic environment. To increase the acid tolerance, bacteria can change the composition of the membrane or cell wall for decreasing the permeability for protons [5, 8]. It was shown that the cariogenic organism *Streptococcus mutans* altered its membrane FA profile in response to acidification of its environment, specifically by increasing the proportions of monounsaturated membrane FAs when grown in low-pH environments [36]. Preventing the ability to increase or produce mono-unsaturated FAs of this species also leads to the acid-sensitive phenotype [15]. In *L. casei* Zhang, the expression of *fab* operon was increased in the late logarithmic phase, indicating that cell membrane alterations are significant for its survival in response to low pH. Up-regulation of genes associated with FA composition has been consistently observed in *L. reuteri* when exposed to acidic environments [53].

The increased transcript level of a molecular chaperone (LCAZH_0619) in the stationary phase could also relate to acid adaptation in *L. casei* Zhang. Molecular chaperones intervene in numerous stresses for various tasks such as protein folding, renaturation, protection of denatured proteins and evacuation of damaged proteins. In fact, many protein members with chaperone activities such as DnaK and GroEL were found to be up-regulated in response to acidity as well as other stress environments [52]. Additionally, a 2CRS of CitAB family was up-regulated in the late logarithmic phase. In *L. acidophilus*, a 2CRS similar to the lisRK system described in *Listeria monocytogenes* has been demonstrated to be involved in acid resistance [1]. In addition, six 2CRS were detected in *L. lactis*, with four of them implicated in cellular responses to stress [31]. However, the CitAB family of 2CRS commonly senses and responds to changes in citrate and/or C_4 dicarboxylate levels [17]. Therefore, it is unclear at present what role it has on the overall fitness of stressed *L. casei* cells.

To conclude, the present study revealed specific transcript changes in *L. casei* during fermentation in soymilk, which to our knowledge has not been reported for this species. The use of transcriptome analysis revealed some stimulatory factors for bacterial growth in soymilk, such as oligopeptides, amino acids, and Mn^{2+}. We believe that this study will underpin new strategies to improve the viability of probiotic microorganisms of fermented soymilks and is of importance for its industrialized production.

Acknowledgments This research was supported by the National Natural Science Foundation of China (Grant No. 30860219), the Hi-tech Research and Development Program of China (863 Program) (Grant No. 2010AA10Z302), the earmarked fund for Modern Agro-industry Technology Research System (Grant No. nycytx-0501), and the Innovation Research Team Development Program of Ministry of Education of China (Grant No. IRT0967). We would like to thank Eric Richard Spaans for the English editing.

References

1. Azcarate-Peril MA, McAuliffe O, Altermann E, Lick S, Russell WM, Klaenhammer TR (2005) Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. Appl Environ Microbiol 71:5794–5804
2. Boyaval P (1989) Lactic acid bacteria and metal ions. Lait 69:87–113
3. Cai H, Thompson R, Budinich MF, Broadbent JR, Steele JL (2009) Genome sequence and comparative genome analysis of *Lactobacillus casei*: insights into their niche-associated evolution. Genome Biol Evol 1:239–257
4. Cappa F, Cattivelli D, Cocconcelli PS (2005) The *uvrA* gene is involved in oxidative and acid stress responses in *Lactobacillus helveticus* CNBL1156. Res Microbiol 156:1039–1047

5. Chang YY, Cronan JE Jr (1999) Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol Microbiol 33:249–259
6. Christensen JE, Dudley EG, Pederson JA, Steele JL (1999) Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 76:217–246
7. Corcoran BM, Stanton C, Fitzgerald G, Ross RP (2008) Life under stress: the probiotic stress response and how it may be manipulated. Curr Pharm Des 14:1382–1399
8. Cotter PD, Hill C (2003) Surviving the acid test: responses of Gram-positive bacteria to low pH. Microbiol Mol Biol Rev 67:429–453
9. Dave RI, Shah NP (1998) Ingredient supplementation effects on viability of probiotic bacteria in yogurt. J Dairy Sci 81:2804–2816
10. De Vuyst L (2000) Technology aspects related to the application of functional starter cultures. Food Technol Biotechnol 38:105–112
11. den Hengst CD, Groeneveld M, Kuipers OP, Kok J (2006) Identification and functional characterization of the *Lactococcus lactis* CodY-regulated branched-chain amino acid permease BcaP (CtrA). J Bacteriol 188:3280–3289
12. Donkor ON, Shah NP (2008) Production of beta-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in soymilk. J Food Sci 73:M15–M20
13. Duwat P, Ehrlich SD, Gruss A (1995) The *recA* gene of *Lactococcus lactis*: characterization and involvement in oxidative and thermal stress. Mol Microbiol 17:1121–1131
14. Fozo EM, Kajfasz JK, Quivey RG Jr (2004) Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol Lett 238:291–295
15. Fozo EM, Quivey RG Jr (2004) The *fabM* gene product of *Streptococcus mutans* is responsible for the synthesis of monounsaturated fatty acids and is necessary for survival at low pH. J Bacteriol 186:4152–4158
16. Franck P, Moneret Vautrin DA, Dousset B, Kanny G, Nabet P, Guenard-Bilbaut L, Parisot L (2002) The allergenicity of soybean-based products is modified by food technologies. Int Arc Allergy Immunol 128:212–219
17. Gerharz T, Reinelt S, Kaspar S, Scapozza L, Bott M (2003) Identification of basic amino acid residues important for citrate binding by the periplasmic receptor domain of the sensor kinase CitA. Biochemistry 42:5917–5924
18. Gorke B, Stulke J (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol 6:613–624
19. Guedon E, Renault P, Ehrlich SD, Delorme C (2001) Transcriptional pattern of genes coding for the proteolytic system of *Lactococcus lactis* and evidence for coordinated regulation of key enzymes by peptide supply. J Bacteriol 183:3614–3622
20. Guo J, Wang J, Yan L, Chen W, Liu X, Zhang H (2009) In vitro comparison of probiotic properties of *Lactobacillus casei* Zhang, a potential new probiotic, with selected probiotic strains. Lebenson Wiss Technol 42:1640–1646
21. Haandrikman A, Kok JJ, Venema G (1991) Lactococcal proteinase maturation protein PrtM is a lipoprotein. J Bacteriol 173:4517–4525
22. Juillard V, Le Bars D, Kunji ER, Konings WN, Gripon JC, Richard J (1995) Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. Appl Environ Microbiol 61:3024–3030
23. Kunji ER, Hagting A, De Vries CJ, Juillard V, Haandrikman AJ, Poolman B, Konings WN (1995) Transport of beta-casein-derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. J Biol Chem 270:1569–1574
24. Laddaga RA, Bessen R, Silver S (1985) Cadmium-resistant mutant of *Bacillus subtilis* 168 with reduced cadmium transport. J Bacteriol 162:1106–1110
25. Li H, Lu M, Guo H, Li W, Zhang H (2010) Protective effect of sucrose on the membrane properties of *Lactobacillus casei* Zhang subjected to freeze-drying. J Food Protect 73:715–719
26. Lin FM, Chiu CH, Pan TM (2004) Fermentation of a milk-soymilk and Lycium chinense Miller mixture using a new isolate of *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *Bifidobacterium longum*. J Ind Microbiol Biotechnol 31:559–564
27. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–408
28. Luesink EJ, van Herpen RE, Grossiord BP, Kuipers OP, de Vos WM (1998) Transcriptional activation of the glycolytic las operon and catabolite repression of the gal operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. Mol Microbiol 30:789–798
29. Matsubara K, Ohnishi K, Kiritani K (1992) Nucleotide sequences and characterization of liv genes encoding components of the high-affinity branched-chain amino acid transport system in *Salmonella typhimurium*. J Biochem 112:93–101
30. Nies DH, Silver S (1995) Ion efflux systems involved in bacterial metal resistances. J Ind Microbiol 14:186–199
31. O'Connell-Motherway M, van Sinderen D, Morel-Deville F, Fitzgerald GF, Ehrlich SD, Morel P (2000) Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363. Microbiology 146:935–947
32. Pastar I, Tonic I, Golic N, Kojic M, van Kranenburg R, Kleerebezem M, Topisirovic L, Jovanovic G (2003) Identification and genetic characterization of a novel proteinase, PrtR, from the human isolate *Lactobacillus rhamnosus* BGT10. Appl Environ Microbiol 69:5802–5811
33. Poolman B, Molenaar D, Smid EJ, Ubbink T, Abee T, Renault PP, Konings WN (1991) Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. J Bacteriol 173:6030–6037
34. Quattrucci E, Bruschi L, Manzi P, Aromolo R, Panfili G (1997) Nutritional evaluation of typical and reformulated Italian cheeses. J Sci Food Agr 73:46–52
35. Quay SC, Dick TE, Oxender DL (1977) Role of transport systems in amino acid metabolism: leucine toxicity and the branched-chain amino acid transport systems. J Bacteriol 129:1257–1265
36. Quivey RG Jr, Faustoferri R, Monahan K, Marquis R (2000) Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. FEMS Microbiol Lett 189:89–92
37. Redon E, Loubiere P, Cocaign-Bousquet M (2005) Transcriptome analysis of the progressive adaptation of *Lactococcus lactis* to carbon starvation. J Bacteriol 187:3589–3592
38. Reid G (2005) The importance of guidelines in the development and application of probiotics. Curr Pharm Des 11:11–16
39. Renault P, Gaillardin C, Heslot H (1988) Role of malolactic fermentation in lactic acid bacteria. Biochimie 70:375–379
40. Rico J, Yebra MJ, Perez-Martinez G, Deutscher J, Monedero V (2008) Analysis of ldh genes in *Lactobacillus casei* BL23: role on lactic acid production. J Ind Microbiol Biotechnol 35:579–586
41. Saulnier D, Molenaar MD, WMd Vos, Gibson GR, Kolida S (2007) Identification of prebiotic fructooligosaccharide metabolism in *Lactobacillus plantarum* WCFS1 through microarrays. Appl Environ Microbiol 73:1753–1765
42. Savijoki K, Ingmer H, Varmanen P (2006) Proteolytic systems of lactic acid bacteria. Appl Microbiol Biotechnol 71:394–406
43. Shah NP (2000) Probiotic bacteria: selective enumeration and survival in dairy foods. J Dairy Sci 83:894–907
44. Sheng J, Marquis RE (2007) Malolactic fermentation by *Streptococcus mutans*. FEMS Microbiol Lett 272:196–201

45. Shimakawa Y, Matsubara S, Yuki N, Ikeda M, Ishikawa F (2003) Evaluation of *Bifidobacterium breve* strain Yakult-fermented soymilk as a probiotic food. Int J Food Microbiol 81:131–136
46. Singh VK, Moskovitz J (2003) Multiple methionine sulfoxide reductase genes in *Staphylococcus aureus*: expression of activity and roles in tolerance of oxidative stress. Microbiology 149:2739–2747
47. Siro I, Kapolna E, Kapolna B, Lugasi A (2008) Functional food. Product development, marketing and consumer acceptance–a review. Appetite 51:456–467
48. Stucky K, Hagting A, Klein JR, Matern H, Henrich B, Konings WN, Plapp R (1995) Cloning and characterization of *brnQ*, a gene encoding a low-affinity, branched-chain amino acid carrier in *Lactobacillus delbruckii* subsp. *lactis* DSM7290. Mol Gen Genet 249:682–690
49. Tatusov RL, Koonin EV, Lipman DJ (1997) A genomic perspective on protein families. Science 278:631–637
50. Trindade CS, Terzi SC, Trugo LC, Della Modesta RC, Couri S (2001) Development and sensory evaluation of soy milk based yoghurt. Arch Latinoamericanos Nutr 51:100–104
51. Tynecka Z, Gos Z, Zajac J (1981) Reduced cadmium transport determined by a resistance plasmid in *Staphylococcus aureus*. J Bacteriol 147:305–312
52. van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E (2002) Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek 82:187–216
53. Wall T, Bath K, Britton RA, Jonsson H, Versalovic J, Roos S (2007) The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. Appl Environ Microbiol 73:3924–3935
54. Wang H, Dong C, Chen Y, Cui L, Zhang H (2010) A new probiotic cheddar cheese with high ACE-inhibitory activity and γ-Aminobutyric acid content produced with koumiss-derived *Lactobacillus casei* Zhang. Food Technol Biotechnol 48:62–70
55. Wang J, Guo Z, Zhang Q, Yan L, Chen W, Liu XM, Zhang HP (2009) Fermentation characteristics and transit tolerance of probiotic *Lactobacillus casei* Zhang in soymilk and bovine milk during storage. J Dairy Sci 92:2468–2476
56. Wu R, Wang L, Wang J, Li H, Menghe B, Wu J, Guo M, Zhang H (2009) Isolation and preliminary probiotic selection of lactobacilli from koumiss in Inner Mongolia. J Basic Microbiol 49:318–326
57. Wu R, Wang W, Yu D, Zhang W, Li Y, Sun Z, Wu J, Meng H, Zhang H (2009) Proteomic analysis of *Lactobacillus casei* Zhang, a new probiotic bacterium isolated from traditionally home-made koumiss in Inner Mongolia of China. Mol Cell Proteomics 8:2321–2338
58. Ya T, Zhang Q, Chu F, Merritt J, Bilige M, Sun T, Du R, Zhang H (2008) Immunological evaluation of *Lactobacillus casei* Zhang: a newly isolated strain from koumiss in Inner Mongolia, China. BMC Immunol 9:68
59. Yan D (2007) Protection of the glutamate pool concentration in enteric bacteria. Proc Natl Acad Sci USA 104:9475–9480
60. Zhang H, Xu J, Wang J, Menghebilige SunT, Li H, Guo M (2008) A survey on chemical and microbiological composition of kurut, naturally fermented yak milk from Qinghai in China. Food Control 19:578–586
61. Zhang W, Yu D, Sun Z, Chen X, Bao Q, Meng H, Hu S, Zhang H (2008) Complete nucleotide sequence of plasmid plca36 isolated from *Lactobacillus casei* Zhang. Plasmid 60:131–135
62. Zhang W, Yu D, Sun Z, Wu R, Chen X, Chen W, Meng H, Hu S, Zhang H (2010) Complete genome sequence of *Lactobacillus casei* Zhang, a new probiotic strain isolated from traditional homemade koumiss in Inner Mongolia, China. J Bacteriol 192:5268–5269