Transcriptome analysis of *Lactobacillus paracasei* SMN-LBK under ethanol stress

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ABSTRACT

Lactobacillus paracasei SMN-LBK (serial number: CCTCC M 2017429) is an ethanol-resistant lactic acid bacteria (LAB) in kumiss. However, the anti-ethanol stress mechanism of *L. paracasei* SMN-LBK remains unclear. Hence, we performed a transcriptome analysis between *L. paracasei* SX10 (*L. paracasei* SMN-LBK under 10% ethanol stress strain, abbreviated as SX10) and *L. paracasei* SMN-LBK (abbreviated as S10) by RNA sequencing. We performed real-time quantitative PCR (RT-qPCR) to verify the accuracy of the transcription data. The transcriptome data revealed that 315 genes exhibited upregulated expression, and 332 genes were downregulated in the SX10 compared with the S10 group. The *PFK*, *LDH*, *GPDH*, and *GK* genes were upregulated, with a $\log_2$ -fold change of 1.10, 0.30, 0.56, and 1.512, respectively. A gene ontology enrichment analysis revealed significant enrichment of ribosomes, ribonucleoprotein complex, non-membrane-bounded organelles, and intracellular non-membrane-bound organelles. Analysis using the Kyoto Encyclopedia of Genes and Genomes database revealed differential genes associated with ribosome function, pyruvate metabolism, biosynthesis of amino acids, fatty acid biosynthesis, fatty acid metabolism, ATP-binding cassette (ABC) transporter, glycolysis, and glycerophospholipid metabolism. The RT-qPCR results were consistent with the transcriptome results. *Lactococcus lactis* NZ9000 is a typical host bacterium. We performed *PFK* and *GK* overexpression to verify the function of the *L. paracasei* SX10 resistance gene in *Lactococcus lactis* NZ9000. Using sodium dodecyl sulfate (SDS)-PAGE electrophoresis, these resistance genes were successfully expressed in *Lactococcus lactis* NZ9000. The survival rate and key enzyme activity of the recombinant strains were deter-

mined under ethanol stress. The survival rate of *Lactococcus lactis* NZ9000-pNZ8148-*PFK* and *Lactococcus lactis* NZ9000-pNZ8148-*GK* under 10% ethanol stress were 3.43- and 3.80-fold higher compared with the *Lactococcus lactis* NZ9000-pNZ8148 control, respectively. These results indicate that *PFK* and *GK* are important for the ethanol tolerance of LAB and can increase the ethanol tolerance of *Lactococcus lactis* NZ9000. Hence, *PFK* and *GK* were identified as key genes of *L. paracasei* SX10 with a high ethanol tolerance. Our results provide novel insight for further studies to perform a systematic analysis of the differentially expressed genes and to determine their potential functions in the ethanol tolerance mechanism of LAB.

Key words: *Lactobacillus paracasei*, ethanol stress, transcriptome analysis

INTRODUCTION

Kumiss is a lactic acid alcoholic beverage traditionally produced using a natural mixed starter consisting of lactic acid bacteria (LAB) and yeast (Danova et al., 2005). Kumiss is an extremely important dairy product for herdsmen in the Xinjiang province in China. Additionally, kumiss is considered an extremely important medicinal and health care product derived from LAB (Wang et al., 2015), as it functions as a muscle relaxant, accelerates blood circulation, promotes digestion, and improves immune function (Danova et al., 2005). Guarner and Schaafsma (1998) found that kumiss displayed both probiotic and antibiotic functions. In the intestinal environment, kumiss can reduce the number of intestinal pathogenic bacteria without affecting the growth of normal microbial community, and it can also reduce the cholesterol level in the blood. In addition, kumiss was thought to be effective because it contained a large number of beneficial microorganisms, of which LAB were among the most important (Liu et al., 2008).

Lactic acid bacteria play a probiotic role as well as producing a unique flavor in kumiss (Tuo et al., 2011). Although LAB have been associated with multiple advantages, the process of natural fermentation is al-

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ways associated with a variety of physical chemical and nutritional factors (e.g., acid, osmotic, oxidative, and ethanol stressors; Spano and Massa, 2006; Li et al., 2015). Among these forms of stress, ethanol stress has been found to be a main inhibitor of the fermentation process in kumiss (Danova et al., 2005), with the accumulation of ethanol possibly inhibiting the cell growth and probiotic effects of LAB. Consequently, LAB with a higher ethanol tolerance were required to improve the quality and flavor of dairy products (e.g., kumiss and milk beer).

Attempts have been made to improve the ethanol tolerance of *Lactococcus lactis* NZ9700 via genetically directed transformation (Díez et al., 2017). The heterologous expression of the *omrA* gene from *Oenococcus oeni* has been found to protect *Escherichia coli* bacteria from ethanol toxicity (Bourdineaud et al., 2004). A fingerprinting analysis using fluorescent differential display-PCR and an investigation of changes in gene expression during the tolerance process of *O. oeni* were carried out. The gene expression pattern reflected the mechanisms involved in tolerance to environmental conditions, among which ethanol stress was included (Sico et al., 2009).

In our previous study, we exposed *Lactobacillus paracasei* SMN-LBK cells to a stepwise increase in ethanol concentration to obtain *L. paracasei* SX10 with a higher ethanol tolerance (Jin et al., 2017). We primarily focused on the effects of key enzyme activity, cell membrane integrity, and structural changes under ethanol stress (Zhu, 2015). However, the molecular mechanism underlying LAB ethanol tolerance is highly complex, and multiple genes, proteins, metabolites, and bio-processes might be involved. Understanding the mechanism of LAB ethanol tolerance is of great significance for the food industry in China. Therefore, we used *L. paracasei* SMN-LBK (CCTCC M 2017429; Wuhan University, Wuhan, China) as a model strain, which was treated with 10% ethanol for 3 h. We used transcriptomics as a primary method, combined with real-time quantitative PCR (RT-q PCR) and overexpression technology using the nisin-controlled gene expression (NICE) system to discover key tolerance genes and related pathways. We also analyzed the ethanol stress response mechanism of *L. paracasei* SMN-LBK. Our results provide a foundation for understanding the ethanol tolerance mechanism of LAB.

MATERIALS AND METHODS

LAB Strain and Media

Lactobacillus paracasei SMN-LBK (CCTCC M 2017429; hereafter abbreviated **S10**) was isolated from

kumiss collected from local herding families living in the Tarbagatay area of Xinjiang, China. The S10 was treated with ethanol stress at concentrations of 5% (vol/vol), 8% (vol/vol), 9% (vol/vol), and 10% (vol/vol). We selected the strain *L. paracasei* SMN-LBK tolerant to 10% ethanol as a highly tolerant strain for expansion culture. This highly tolerant strain *L. paracasei* SX10 was abbreviated **SX10**.

Lactococcus lactis NZ9000 is an MG1363 derivative that contains the pepN::nisRK integration gene. It is also a standard host commonly used in NICE systems. Plasmid pNZ8148 is resistant to chloramphenicol (Cm) and uses *nisA* as the promoter of the inducible expression vector. The standard host strain *Lactococcus lactis* NZ9000 and plasmid pNZ8148 were kindly gifted by the Jiangnan University Biological Collection Center (Jiangsu, China).

The *L. paracasei* SMN-LBK strains were inoculated in de Man-Rogosa-Sharpe (MRS) broth and incubated at 37°C. The *L. paracasei* SX10 strains were inoculated in MRS broth supplemented with 10% ethanol and incubated at 37°C. *Lactococcus lactis* NZ9000 was statically inoculated in M17 broth (Oxoid, Waltham, MA) with 0.5% glucose (**GM17**) at 30°C. To accelerate clonal selection, we added 10 µg/mL chloramphenicol to the medium.

RNA Isolation

To harvest the cells, we centrifuged the bacterial strains at $5,000 \times g$ for 5 min at 4°C. Cells were quickly frozen in liquid nitrogen and ground the cells into a powder using a mortar filled with liquid nitrogen. The bacterial powder was suspended in 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA) and manipulated according to the manufacturer's instructions. The RNA samples were resuspended in RNase-free water (Thermo Fisher Scientific, Waltham, MA), and RNA degradation and contamination were monitored on 1% agarose gels. We used a Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) to measure the RNA concentration; RNA was used only when the optical density 260/280 was greater than 1.8. We used an RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA) to evaluate RNA integrity. Finally, total extracted RNA was stored at -80°C until further use.

RNA Sequencing and Clustering

A total of 3 µg of RNA from each sample was used as the input material for the RNA sample preparations. Based on the manufacturer's recommendations, we used the an NEBNext Ultra Directional Library Prep Kit

for Illumina (New England Biolabs, Ipswich, MA) to generate sequencing libraries, and added index codes to the attribute sequence per sample. Following a cluster generation, the library preparations were sequenced on an Illumina HiSeq platform (Illumina, San Diego, CA), and paired-end reads were generated.

Mapping Reads to the Reference Genome

Raw reads in the FASTQ format were first processed through in-house Perl scripts. In this step, we obtained clean reads by removing the reads containing adapters, reads containing poly-N, and low-quality reads from the raw data. The reference genome and gene model annotation files can be directly downloaded from the genome website (https://www.ncbi.nlm.nih.gov/genome/913?genome_assembly_id=392065). Both the building index of reference genome and alignment of the clean reads to the reference genome used Bowtie2-2.2.3. (Langmead and Salzberg, 2012).

Quantification of the Level of Gene Expression

The numbers of reads mapped to each gene were counted using HTSeq version 0.6.1 (New South Wales, Australia). Then we calculated the fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) of each gene based on the length of the gene and read counts mapped to this gene. The FPKM considers the effect of sequencing depth and gene length for the read count simultaneously, and is currently the most commonly used method to estimate the level of gene expression (Cole et al., 2009).

Differential Expression Analysis

The DESeq R package (1.18.0) was applied for the differential expression analysis (Wang et al., 2010). The DESeq package is a statistical program that uses a model based on negative binomial distribution to determine differential expression in digital gene expression data (Anders and Huber, 2012). We adopted the method of Benjamini and Hochberg (2000) to control the false discovery rate to adjust the resulting *P*-values. Genes with an adjusted *P*-value < 0.05 found by DESeq and $\log_2|\text{foldchange}| > 1$ were considered differentially expressed.

Functional Enrichment of Differentially Expressed Genes

The Goseq R package was used to perform a gene ontology (GO) enrichment analysis of differentially expressed genes, corrected for gene length bias (Young et

al., 2010). We considered differentially expressed genes with GO terms having a corrected *P*-value < 0.05 to be significantly enriched. The GO enrichment analysis was performed based on the Wallenius noncentral hypergeometric distribution. The Kyoto Encyclopedia of Genes and Genomes database (KEGG) is a resource used mainly to understand the high-level functions and effectiveness of the biological system (e.g., the cell, the organism, and the ecosystem) from molecular-level information, especially large-scale molecular data sets generated through genome sequencing and other high-throughput experimental techniques (<https://www.genome.jp/kegg/>; Kanehisa et al., 2007). We used KOBAS software to test the statistical enrichment of the differentially expressed genes in KEGG pathways (Mao et al., 2005). The KEGG analysis used the pathway in the KEGG database as a unit, and applied the hypergeometric distribution to identify the pathway that was significantly enriched in the differentially expressed genes compared with the entire genomic background.

RT-qPCR Validation

We performed RT-qPCR to confirm the RNA sequencing (RNA-seq) results. Four genes were selected for validation using RT-qPCR. The primer pairs were designed using Primer 5 software (version 5.0, Palo Alto, CA); these are listed in Table 1. We chose *ACTB* as a reference in these experiments. All RT-qPCR experiments were conducted using Ultra SYBR Mixture (Low ROX; CWBio, Beijing, China) and a Roche LightCycler 480 real-time fluorescence qPCR system (Roche, Basel, Switzerland). According to the instructions of the Roche LightCycler 480 system, we applied the following amplification procedure: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C for and 1 min at 60°C. We observed the formation of single peaks and excluded the possibility of primer dimers and nonspecific products. At the end of each PCR reaction, a melt curve (15 s at 95°C, 60 s at 60°C, 15 s at 95°C, and 60 s at 60°C) was generated. All reactions were performed in triplicate. Finally, the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) was used to calculate the relative gene expression level.

Plasmid and Strain Construction

Construction of the expression plasmids is illustrated in Figure 1. The *PFK* and *GK* genes from *L. paracasei* SX10 genomic DNA were amplified by PCR using primers 5'-CATGCCATGGTGATTATACCTGACTT-3' and 5'-GCTCTAGATTAAACCCTCCATCTTGCG-3'; 5'-CATGCCATGGTGACAAGTGATCAGAAAGTAC-3'; and 5'-GCTCTAGATCAGGCTTTCTTC-

Table 1. List of primers used in the real-time quantitative PCR analysis

Gene ¹	Primers 5'-3'(forward)	Primers 5'-3'(reverse)
<i>PFK</i>	AATGGTAAAGGGGTGAAT	AAAAGTCGGCGATGTAAT
<i>GPDH</i>	CAATCAACGGGAAACCAC	AATCCAGCGAATCTTAGTC
<i>LDH</i>	ATCAAACCTCAAGGGTGCG	GATGTAGATGTCGTTCAAGC
<i>GK</i>	CGAGGTTGTGAACGCTTAT	CCGTCTGTCGCTTGGTGA
<i>ACTB</i>	CTCCATCCTGGCCTCGCTGT	GCTGTACCTTCACCGTTCC

¹*ACTB* was used as the internal reference gene to calculate the level of gene expression. The *PFK* gene encodes phosphofructokinase; the *GPDH* gene encodes glycerol-3-phosphate dehydrogenase; the *LDH* gene encodes L-lactate dehydrogenase; the *GK* gene encodes glycerol kinase.

GCCTTGTG-3'. We inserted the NcoI and XbaI sites into the amplified gene at the same time; underlining shows the amplified sites in the primer sequences. We used NcoI (Thermo Fisher) and XbaI (Thermo Fisher) to digest the target fragment and ligated it into plasmid pNZ8148. The plasmids containing *PFK* and *GK* were termed pNZ8148-PFK and pNZ8148-GK, respectively. We transformed recombinant plasmids into *Lactococcus lactis* NZ9000 by electroporation and named the transformants *Lactococcus lactis* NZ9000-pNZ8148-PFK and *Lactococcus lactis* NZ9000-pNZ8148-GK, respectively. At the same time, we transformed the empty plasmid pNZ8148 into *Lactococcus lactis* NZ9000 and named it *Lactococcus lactis* NZ9000-pNZ8148.

PFK and GK Expression in *Lactococcus lactis* NZ9000 and SDS-PAGE Analysis

To research the expression levels of *PFK* and *GK* in *Lactococcus lactis* NZ9000, nisin (Sigma-Aldrich, St. Louis, MO) was used to express the protein; the specific steps are as follows. We cultured *Lactococcus lactis* NZ9000-pNZ8148-PFK, *Lactococcus lactis* NZ9000-pNZ8148-GK, and *Lactococcus lactis* NZ9000-pNZ8148 in GM17 medium supplemented with 10 µg/mL chloramphenicol at 30°C. Cultures were stimulated with 10 ng/mL nisin at an optical density at 600 nm of 0.5 and cultured for 12 h. We centrifuged for 5 min (12,000 × g) to harvest the cells at 4°C. Cell pellets were washed twice in ice-cold 1× PBS (pH 7.2) and resuspended in PBS (Thermo Fisher). Ultrasound was used to break the cells for 30 cycles of 5 s at -2°C (VCX 500, Sonics and Materials Inc., Newtown, CT). We centrifuged (12,000 × g) for 5 min at 4°C to remove cell debris, to obtain cell extracts. Bovine serum albumin (Thermo Fisher) was used as the standard protein, and the protein concentration was determined according to the Bradford method. For protein analysis of samples, the cell extracts were mixed with 5-fold concentrated SDS-PAGE loading buffer after heating at 100°C for 5 min. Finally, we performed SDS-PAGE on a 12% (wt/vol) gel, with 20 µL of sample per well.

Determination of Growth Performance

Lactococcus lactis NZ9000-pNZ8148-PFK, *Lactococcus lactis* NZ9000-pNZ8148-GK, and *Lactococcus lactis* NZ9000-pNZ8148 strains were inoculated for 3 h in GM17 liquid medium, which contained 10 µg/mL chloramphenicol and 0%, 2%, 4%, 6%, 8%, and 10% (vol/vol) ethanol, respectively. The bacterial solution of each sample was mixed with 9 mL of 0.85% (wt/vol) sterile physiological saline. The decimal dilutions of the bacterial solutions (10⁻¹ to 10⁻⁸) were plated in triplicate on GM17 agar medium containing 10 µg/mL chloramphenicol. We counted the numbers of different ethanol-stressed colonies of the recombinant strains and calculated the average number of colonies without ethanol stress as a 100% survival rate. The specific survival rate of the strains under ethanol stress was calculated using the following formula:

$$\text{Survival rate of strains} = \frac{\text{Number of viable bacteria after ethanol stress}}{\text{Number of viable bacteria without ethanol stress}} \times 100\%.$$

Statistical Analysis

Statistical analyses of RT-qPCR validation and determination of growth performance were performed using SPSS Statistics 19 software (IBM Corp., Armonk, NY). The RT-qPCR validation and determination of growth performance were analyzed by an independent samples *t*-test. All experiments were performed in triplicate, and data were presented as the mean ± standard deviation.

RESULTS

Level of Gene Expression

As shown in Table 2, gene expression levels in S10 and SX10 were lowest in the FPKM range of 1 to 3, and the expression level of each gene ranged from 2.43 to 3.07%. Expression of each gene was highest for FPKM >60, and the expression level ranged from 49.26

Table 2. Number and percent of genes in different expression level intervals¹

FPKM interval	S10-1	S10-2	S10-3	SX10-1	SX10-2	SX10-3
0-1	583 (18.86)	584 (18.89)	598 (19.34)	591 (19.11)	592 (19.15)	583 (18.86)
1-3	95 (3.07)	101 (3.27)	95 (3.07)	84 (2.72)	79 (2.55)	75 (2.43)
3-15	339 (10.96)	342 (11.06)	334 (10.80)	356 (11.51)	341 (11.03)	338 (10.93)
15-60	512 (16.56)	528 (17.08)	518 (16.75)	538 (17.40)	542 (17.53)	543 (17.56)
>60	1,563 (50.55)	1,537 (49.71)	1,547 (50.03)	1,523 (49.26)	1,538 (49.74)	1,553 (50.23)

¹FPKM = fragments per kilobase of transcript sequence per million base pairs sequenced. S10-1, S10-2, S10-3 were 3 parallel samples of the *Lactobacillus paracasei* SMN-LBK (S10). SX10-1, SX10-2, and SX10-3 were 3 parallel samples of *L. paracasei* SMN-LBK under 10% ethanol stress strain (SX10). Values in parentheses represent the percentage of genes contained in each FPKM interval.

to 50.55%. The direct expression of a gene expression level is the abundance of its transcripts; the higher the degree of transcript abundance-FPKM, the higher the gene expression level. Our results indicated that most of the genes were expressed at high levels.

Analysis of Differential Gene Expression

To understand and interpret the RNA-seq results, the differential expression patterns of the transcripts were analyzed between the tolerant strain, *L. paracasei* SX10, and wild-type strain, *L. paracasei* SMN-LBK (S10). From the SX10 and S10 libraries, differential expression analysis identified 647 genes with significant changes in the transcriptional expression level ($P < 0.05$). The different expression patterns between *L. paracasei* SX10 and *L. paracasei* S10 revealed 332 downregulated differential genes and 315 upregulated differential genes (Figure 2). Of these, *PFK*, *LDH*, *GPDH*, and *GK* were upregulated, with a $\log_2$ -fold change of 1.10, 0.30, 0.56, and 1.512, respectively (Table 3). Because the $\log_2[\text{foldchange}] > 1$ and $P\text{-value} < 0.05$ of the *PFK* and *GK* genes were highly expressed, we further verified the effect of these 2 genes on the ethanol tolerance of *L. paracasei* SX10.

GO Annotation Analysis of Differentially Expressed Genes

To clarify changes in biological processes under ethanol stress in *L. paracasei* SX10 and *L. paracasei* S10, the up- and downregulated genes identified via RNA-seq were subjected to a GO term enrichment analysis. The GO-based enrichment analysis was carried out using a threshold value $P < 0.05$ to evaluate the major biological functions of the differentially expressed genes, which were further classified into 3 main categories: (1) cellular component, (2) molecular function, and (3) biological process. The cellular component category contained the majority of GO annotations (40%) followed by the biological process (26.66%) and molecular function (26.66%) categories. The major subcategories

of the analysis of all the transcripts between *L. paracasei* SX10 and *L. paracasei* S10 under 10% ethanol stress are presented in Figure 3. The GO annotations revealed that the significant enrichment items focused on the classification of cellular components, which included the ribosomes, ribonucleoprotein complex, non-membrane-bounded organelles, and intracellular non-membrane-bounded organelles.

KEGG Analysis of the Differentially Expressed Genes

To further identify significant changes in the biochemical pathways during ethanol stress, all differentially expressed genes were further mapped using the KEGG database (Dong et al., 2017). Based on the KEGG analysis, 20 pathways were enriched, including those related to ribosomes, pyruvate metabolism, biosynthesis of amino acids, fatty acid biosynthesis, fatty acid metabolism, ATP-binding cassette (ABC) transporter, glycolysis, and glycerophospholipid metabolism (Figure 4).

Verification of the Differentially Expressed Genes by RT-qPCR

To further verify the level of gene expression obtained from the Illumina RNA-seq platform, we extracted RNA samples that were of good quality with complete 5S, 16S, and 23S bands, which ensured that RT-qPCR could be optimally performed (Figure 5). We selected 4 genes that responded to ethanol stress to perform RT-qPCR, including the genes encoding phosphofructokinase (*PFK*), L-lactate dehydrogenase (*LDH*), glycerol-3-phosphate dehydrogenase (*GPDH*), and glycerol kinase (*GK*). The experimental results show a strong correlation with the RNA-seq data (Figure 6).

PFK and GK Overexpression

Lactococcus lactis is a gram-positive bacterium that has important functions in the gastrointestinal tract of animals and humans, and has been classified as a

Table 3. Differential changes in gene expression between *Lactobacillus paracasei* SMN-LBK (S10) and *L. paracasei* SMN-LBK under 10% ethanol stress (SX10)

Gene ¹	Expression	P-value ²	Log ₂ -fold change ³
<i>PFK</i>	Upregulated	5.34E-05	1.10
<i>LDH</i>	Upregulated	1.18E-08	0.30
<i>GPDH</i>	Upregulated	0.025326	0.56
<i>GK</i>	Upregulated	7.37E-16	1.512

¹*PFK* = phosphofructokinase; *LDH* = lactate dehydrogenase; *GPDH* = glycerol-3-phosphate dehydrogenase; *GK* = glycerol kinase.

²P-value = significant difference in expression between SX10 and S10.

³2-fold difference for each gene, calculated using the FPKM values (fragments per kilobase of transcript sequence per million base pairs sequenced) between SX10 and S10.

Differentially Expressed Genes (647)

- up regulated: 315
- down regulated: 332

Figure 2. Volcano plot of differentially expressed genes. Genes with significant differences in expression are shown in red (upregulated) or green (downregulated), and genes with no significant differential expression are shown in blue. The horizontal axis represents the change in gene expression in different samples. SX10 = *Lactobacillus paracasei* SMN-LBK under 10% ethanol stress strain; S10 = *L. paracasei* SMN-LBK; padj = adjusted P-value.

“generally regarded as safe” strain. Due to its safety and probiotic effects, *Lactococcus lactis* has been studied as a vector for recombinant protein expression. To verify the role of *PFK* and *GK* in the regulation of LAB against ethanol, *PFK* and *GK* from the tolerant *L. paracasei* SX10 strain were overexpressed in *Lactococcus lactis* NZ9000 using the NICE system. The schematic overview of the construction procedure of the expression plasmids pNZ8148-*PFK* and pNZ8148-*GK* is presented in Figure 1. In the presence of 10 ng/mL nisin, *Lactococcus lactis* NZ9000-pNZ8148-*PFK* and *Lactococcus lactis* NZ9000-pNZ8148-*GK* overexpressed a protein exhibiting a molecular weight of approximately 34 kDa and 55 kDa, respectively, which was absent in the nisin-treated control strain, *Lactococcus lactis* NZ9000-pNZ8148 (Figure 7).

Growth Performance of the Recombinant Strains

To clarify the tolerance of the recombinant strains, *Lactococcus lactis* NZ9000-pNZ8148-*PFK* and *Lactococcus lactis* NZ9000-pNZ8148-*GK*, to ethanol stress, we counted the number of different ethanol-stressed colonies for the recombinant strains (Supplemental Table S1, <https://doi.org/10.3168/jds.2019-16955>) and determined the survival rate of the recombinant strain in response to different percent volumes of ethanol. We found that a 10^{-5} dilution concentration was suitable for counting the survival rate. The survival rates of *Lactococcus lactis* NZ9000-pNZ8148-*PFK* under 2%, 4%, 6%, 8%, and 10% ethanol stress were respectively 1.20-, 1.21-, 1.37-, 2.14-, and 3.43-fold higher than the control strain (strains cultured in medium without ethanol) *Lactococcus lactis* NZ9000-pNZ8148. The survival rate of *Lactococcus lactis* NZ9000-pNZ8148-*GK* was 1.18-, 1.25-, 1.31-, 2.13-, and 3.80-fold higher than the control strain *Lactococcus lactis* NZ9000-pNZ8148, under 2%, 4%, 6%, 8%, and 10% ethanol stress, respectively. These results show that the survival rates of the recombinant *Lactococcus lactis* NZ9000-pNZ8148-*PFK* and *Lactococcus lactis* NZ9000-pNZ8148-*GK* strains were significantly higher than that of *Lactococcus lactis* NZ9000-pNZ8148. Thus, *PFK* and *GK* played an important role in the ethanol tolerance mechanism of LAB (Figure 8).

DISCUSSION

Lactic acid bacteria have been widely used in the food and pharmaceutical chemical industries (Dan et al., 2017; Sauer et al., 2017); however, LAB are confronted with various environmental stresses during the process of production and marketing (Schott et al.,

2017). Therefore, it is important for LAB to exhibit good stress tolerance, to cope with unfavorable environmental conditions and exert beneficial functions (G-Alegria et al., 2010; Xu et al., 2012). Ethanol stress is widespread during the fermentation of lactic acid alcoholic beverages (Chao et al., 2013), and it can dramatically impair the growth of *L. paracasei* SMN-LBK. Therefore, if changes in gene expression are not compensated by post-transcriptional mechanisms, protein synthesis is likely to be affected in response to ethanol stress.

As expected, both the GO annotation enrichment and KEGG analysis showed that genes related to ribosomes, the ribonucleoprotein complex, and the biosynthesis of amino acid pathways responded to ethanol stress. Consequently, the differentially expressed genes related to protein synthesis may be associated with significant changes in growth rate (Pearson and Haber, 1980), an inevitable result of the ethanol stress response mechanism. This finding was consistent with

the ethanol response observed in *O. oeni* (Bonomo et al., 2018). Teixeira et al. (2002) observed that *O. oeni* maintained a high level of phospholipid biosynthesis via the relative increase in phosphoethanolamine and sphingomyelin biosynthesis in the presence of ethanol, to improve survival vitality.

We also found that genes in the glycolysis pathway were related to the improvement of LAB ethanol resistance. These genes encoded PFK and LDH, and were upregulated upon ethanol exposure in *L. paracasei* SX10. In addition, PFK catalyzed the production of fructose-1,6-diphosphate by fructose-6-phosphate and consumed ATP, a key regulator of the glycolytic pathway (Shirakihara and Evans, 1988). The upregulation of PFK expression is consistent with the higher energy requirements associated with the survival of *L. paracasei* SX10 under ethanol stress.

Interestingly, PFK was also induced in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* in response to ethanol shock (Alexandre et al., 2001; Diniz et al.,

Figure 3. Gene ontology (GO) enrichment analysis histogram. The ordinate is the enrichment of the GO term, and the horizontal axis is the number of different genes in the term. Different colors are used to differentiate between the categories of differentially expressed genes: biological processes are shown in green, cellular components in orange, and molecular functions in blue, with * indicating a significant enrichment of the GO term. When the corrected *P*-value < 0.05 of a certain GO term, this GO term is a significant enrichment term. FAD binding = flavin adenine dinucleotide binding; SX10 = *Lactobacillus paracasei* SMN-LBK under 10% ethanol stress strain; S10 = *L. paracasei* SMN-LBK.

Figure 4. Kyoto Encyclopedia of Genes and Genomes database (KEGG) enrichment scatter plot. The ordinate indicates the path name, and the abscissa indicates the Rich factor. The size of each point indicates how many differentially expressed genes are in the pathway, and the color of each point corresponds to its *Q*-value range.

Figure 5. RNA electropherogram. Lane 1 is an RNA sample of *Lactobacillus paracasei* SMN-LBK (S10); lanes 2, 3, and 4 are RNA samples of *L. paracasei* SMN-LBK strain exposed to 10% ethanol stress (SX10).

2017). Wu et al. (2010) indicated that the implicated pathways are involved with a complex physiological response of *Lactobacillus casei* Zhang. In an analysis of bile salt stress, the key components of central metabolism (PFK) were found to play an important role in improving bile salt tolerance. In the present study, when we overexpressed the PFK protein, the ethanol tolerance of the recombinant strain was significantly improved. Similarly, *E. coli* lacking the key glycolytic enzyme PFK has been found to be considerably more sensitive to tellurite than is the parental, isogenic, wild-type strain (Valdivia-González et al., 2012). Therefore, we believe that *PFK* plays a crucial role in microbial stress resistance and adaptability in LAB. In particular, *PFK* was found to play an important role in the high ethanol tolerance of *L. paracasei* SX10.

Figure 6. Verification of relative level of differential gene expression by real-time quantitative PCR (RT-qPCR). A–D represent the relative levels of *PFK*, *GK*, *GPDH*, and *LHD* gene expression, respectively. Diagonal lines connect levels determined via RT-qPCR. Vertical bars represent RNA sequencing (RNA-seq) transcriptomic analysis. FPKM = fragments per kilobase of transcript sequence per million base pairs sequenced. Data are expressed as mean $\pm$ SD relative to the control. * $P < 0.05$; ** $P < 0.001$.

Lactate dehydrogenase (LDH) is an important enzyme involved in the redox reaction between lactic acid and pyruvic acid associated with glycolysis and gluconeogenesis (Legrand et al., 1992). He et al. (2012) successfully identified 127 genes in *Zymomonas mobilis* that are differentially expressed in response to ethanol. Ethanol fermentation-related genes (e.g., *adhB*) can improve the efficiency of glucose utilization under ethanol stress. The metabolic engineering of a *Lactobacillus plantarum* double-LDH knockout strain revealed that LDH-deficient strains have low ethanol yield and lower ethanol tolerance. These results were consistent with the high tolerance of the *L. paracasei* SMN-LBK strain to ethanol stress.

The cell membrane is the structure most affected by ethanol, because ethanol stress leads to an increase in membrane permeability, enhances passive proton influx, and eliminates the electrochemical potential gradients maintained across them (Fried and Novick, 1973), eliminates membrane dehydration, and rehydration

(Graça da Silveira et al., 2002). To maintain membrane stability, the UFA content in the plasma membrane has been shown to increase in *S. cerevisiae* under ethanol stress (Kim et al., 2011). The UFA biosynthesis related to *K. marxianus* genes was downregulated under ethanol stress, as shown by RNA-seq (Diniz et al., 2017). Our data have shown that fatty acid biosynthesis and metabolism are related to the ethanol adaption of *L. paracasei* SMN-LBK.

We also found that GPDH and GK are related to the adaption of *L. paracasei* SMN-LBK to ethanol. Glycerol kinase catalyzes the conversion of glycerol to glycerol-3-phosphate, and GPDH catalyzes the reversible redox conversion of dihydroxyacetone phosphate to sn-glycerol 3-phosphate (Ou et al., 2006). Furthermore, the elevated level of GPDH cDNA expression on cold treatment suggests that GPDH plays a key role in the cold stress-related response of *Schizothorax richardsonii* (Barat et al., 2012). The prokaryotic expression of ZmGPDH1 in *E. coli* and ZmGPDH1 overexpression

Figure 1. Schematic overview of the construction procedure of the expression plasmids pNZ8148-PFK and pNZ8148-GK. Cm = chloramphenicol resistance marker; MCS = multiple cloning site; repA and repC = replication determinants; PnisA = inducible promoter.

substantially enhanced the tolerance of *Arabidopsis* to salinity or osmotic stress by enhancing glycerol production and maintaining cellular redox homeostasis (Zhao et al., 2019).

Levels of NHO1 (GK) and GPDH expression have been found to be upregulated following flg22 stimulation or heat stress; because the level of glycerol 3-phosphate (G3P) increased, G3P was associated with the flg22-induced defense response and heat stress response in *Porphyra haitanensis* (Lai et al., 2015). Moreover,

the level of GPDH was found to increase in response to salt stress, and overexpressing the GK of *Synechocystis* sp. PCC 6803 may represent a promising feedstock for biodiesel production due to its high lipid productivity and oxidative stress tolerance (Sivaramakrishnan and Incharoensakdi, 2018). In addition, we found that the ethanol tolerance of *Lactococcus lactis* NZ9000 was significantly improved when the GK of *L. paracasei* SX10 was overexpressed. Therefore, we believe that *L. paracasei* SX10 can resist the toxic effects of ethanol by regulating the metabolism of glycerophospholipids and fatty acid synthesis associated with cell membrane maintenance, thereby improving its ethanol resistance. The findings of this study lay a foundation for increasing our understanding of the ethanol tolerance mechanism of LAB. Our research team continues to conduct further research to identify a more comprehensive mechanism.

CONCLUSIONS

As stated, we performed a transcriptomic analysis between *L. paracasei* SX10 subjected to ethanol-induced stress and wild-type *L. paracasei* SMN-LBK to understand the ethanol tolerance mechanism of LAB. *Lactobacillus paracasei* SX10 was found to exhibit high activity under ethanol stress because it first regulated its cell membrane permeability, glycerolipid metabolism, and fatty acid biosynthesis pathways associated with the cell membrane, and increases the activity of

Figure 7. SDS-PAGE analysis of PFK and GK expression in *Lactococcus lactis* NZ9000. Lane 1 = Protein Maker (Beijing, China), which is 14, 25, 30, 40, 50, 70, and 100 kDa from bottom to top; lanes 3, 5, 6, and 7 = *Lactococcus lactis* NZ9000-pNZ8148; lane 4 = *Lactococcus lactis* NZ9000-pNZ8148-PFK; lane 8 = *Lactococcus lactis* NZ9000-pNZ8148-GK.

Figure 8. Effects of different ethanol concentrations on the survival rate of recombinant strains. Data are expressed as the mean $\pm$ SD relative to the control. * $P < 0.05$; ** $P < 0.001$. Blank bars represent *Lactococcus lactis* NZ9000-pNZ8148; diagonally striped bars represent *Lactococcus lactis* NZ9000-pNZ8148-PFK; cross-hatched bars represent *Lactococcus lactis* NZ9000-pNZ8148-GK.

GK and GPDH to enhance its resistance to ethanol toxicity. *Lactobacillus paracasei* SX10 also regulated the enzyme activity of the major central metabolic pathway (i.e., the glycolysis pathway), consistent with the higher energy requirements associated with survival under ethanol stress. In addition, when we overexpressed PFK and GK, the ethanol tolerance of *Lactococcus lactis* NZ9000 significantly improved. Therefore, we believe that the adaption mechanism of LAB under ethanol stress is closely related to lipid metabolism and the central metabolic glycolysis related to cell membrane synthesis. Our research provides a more in-depth theoretical basis for the application of LAB in the food, medicine, and fine chemical industries.

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