Analysis of the complete genome sequence of *Lactobacillus delbrueckii* ssp. *bulgaricus* with post-acidification capacity and its influence on yogurt in storage

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ABSTRACT

In recent years, yogurt has been one of the most popular fermented dairy products and is sold worldwide. In this study, pH and titrated acid changes of 4 strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* fermented milk during storage were detected. The difference between *L. bulgaricus* KLDS1.1011 and KLDS1.0207 was significant, with the latter exhibiting reduced acidity levels. Therefore, we determined the complete genome sequence of the 2 strains. Then the expression of specific genes and common genes related to glucose metabolism and proteolysis of *L. bulgaricus* KLDS1.1011 and KLDS1.0207 were detected by quantitative real-time reverse-transcription PCR. Analysis indicated that the key enzymes in glycometabolism and proteolysis of *L. bulgaricus* KLDS1.1011 were significantly different than those of *L. bulgaricus* KLDS1.0207. The contents of lactose and glucose decreased during storage of *L. bulgaricus* fermented milk, as determined by HPLC, and the contents of lactic acid and galactose increased, with *L. bulgaricus* KLDS1.1011 increasing less. With skim milk as a raw material, *L. bulgaricus* KLDS1.1011, KLDS1.0207, and *Streptococcus thermophilus* S1 were used as fermentation strains to yield yogurt at 42°C, and sensory evaluation was compared with yogurt fermented by commercial starter cultures. Yogurt from *L. bulgaricus* KLDS1.1011 was the highest-rated. Therefore, the study may provide guidelines for the development of yogurt starters.

Key words: *Lactobacillus delbrueckii* ssp. *bulgaricus*, fermentation milk, post-acidification, glycometabolism, proteolysis

INTRODUCTION

Streptococcus thermophilus and *Lactobacillus delbrueckii* ssp. *bulgaricus* ferment milk in yogurt production (Farag et al., 2021). Lactic acid bacteria (LAB) ferment milk to produce lactic acid, which causes milk to solidify. This process prevents the original milk from spoilage and has good viscous texture characteristics, smooth taste, unique flavor, and specific probiotic characteristics (Robinson, 2002). Yogurt starter strains have a high diversity of strains, with notable differences in genome and technological properties (growth characteristics; capacity to produce acid, exopolysaccharide, and flavor compounds; protein hydrolyzation activity; and the symbiosis of strains; El-Sharoud et al., 2012). Fermented milks may then be stored at 4 to 10°C, during which time *L. bulgaricus* mainly produces lactic acid; a large amount of lactic acid will make the yogurt taste sour. This process is called post-acidification. The rapid and effective use of carbon sources is the main factor in rapid acid production and pH decrease. The post-acidification differences shown by *Lactobacillus acidophilus* NCDO 1748 and *Lactobacillus bifidus* NCDO 11863 are directly related to their lactose metabolism. In yogurt, about 400 mg/100 g, or about 40%, of the total lactic acid was the D (–) isomer (Alm, 1982).

Lactobacillus delbrueckii ssp. *bulgaricus* is a bacteria that causes post-acidification of yogurt because of acid resistance (Wang et al., 2013). *Lactobacillus delbrueckii* ssp. *bulgaricus* has a higher protein hydrolyzation activity, which indicates that the proteolytic enzymes of *L. bulgaricus* could participate in metabolism during

Received July 13, 2021.

Accepted October 12, 2021.

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the late stage of milk fermentation (El-Zahar et al., 2003). The best approach to limiting post-acidification and still producing desirable yogurt flavor is to regulate the growth and maintenance of *L. bulgaricus* by controlling its energy metabolism (Oliveira et al., 2010). F₀F₁-ATP synthase (H⁺-ATP synthase) is one of the critical enzymes in the energy metabolism of LAB, which makes anaerobic bacteria excrete protons through ATP hydrolysis and regulate and maintain normal pH in cells (Chuah and Mao, 2020). Lactic acid bacteria produce first pyruvate through the glucose degradation pathway and then lactic acid through the action of lactate dehydrogenase (Even et al., 1999). Pyruvate is an essential intermediate product of glycometabolism, and lactate dehydrogenase is crucial for lactic acid production. It is generally believed that the D-lactic acid content is high in yogurt during storage. The yogurt shows a serious post-acidification phenomenon, which indicates that D-lactate dehydrogenase significantly influences the post-acidification of yogurt. Le Bras and Garel (1991) concluded that the D-lactate dehydrogenase of *L. bulgaricus* was structurally similar to other LAB but had no homology with L-lactate dehydrogenase. The mechanism of *L. bulgaricus* affecting post-acidification of yogurt in glucose metabolism was further elucidated.

Lactic acid bacteria cannot synthesize some amino acids because they lack essential amino acid metabolic pathways (Fernández and Zúñiga, 2006). Therefore, LAB need to obtain the corresponding active substances from the external environment. A proteolysis system can hydrolyze casein to supply the peptides and amino acids needed for physiological metabolism. At the same time, the metabolism of some amino acids can be further converted into aldehydes and alcohols, which play an important role in the formation of flavor substances in fermented dairy products (Liu et al., 2010). The casein hydrolysis process can be divided into 3 stages. First, casein in the dairy product is hydrolyzed into peptide substances by extracellular protease. Then the Opp system transfers peptides into the cell. Finally, free amino acids are formed by intracellular peptidases (dipeptidase, carboxypeptidase, and endopeptidase) entering the metabolic pathway or synthesizing proteins for bacterial use. The Opp system is an integral part of the proteolytic system, consisting of a membrane-anchored oligopeptide binding protein (OppA), 2 proteins responsible for the transmembrane transport (OppB and OppC), and 2 ATP binding proteins (OppD and OppF; Simov and Ivanov, 2005). Therefore, this work analyzes the glycometabolism and proteolysis genes of *L. bulgaricus* and then evaluates the storage quality of fermentation milk, aiming to provide a theoretical

basis for developing yogurt with better taste and better storage quality.

MATERIALS AND METHODS

Bacterial Strains

Lactobacillus delbrueckii ssp. *bulgaricus* KLDS1.0207 and KLDS1.1011 and *Strep. thermophilus* S1 were stored in the Key Laboratory of Dairy Science of Northeast Agricultural University (Harbin, China). *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 and KLDS1.1011 were cultured in de Man, Rogosa, and Sharpe (MRS) broth, and *Strep. thermophilus* S1 was cultured in M17 broth (Qingdao Hope Bio-Technology Co. Ltd.). Strains were grown in MRS broth at 37°C under static conditions and collected by centrifugation ($6,000 \times g$ at 4°C for 5 min), and *L. bulgaricus* KLDS1.0207 and KLDS1.1011 were added to sterilized skim milk at 4% respectively. Milk was kept in the constant-temperature incubator until curd at 42°C. Then the fermented milks were stored at 4°C for determination of pH, quantitative real-time reverse-transcription-PCR, and determination of protein hydrolysis activity.

pH and Titrated Acid in Milk Fermented by a Single Strain and Mixed Strains Assay

Fresh skim milk was put in a 65°C water bath, and white sugar was added at 5% by volume. The temperature was raised to 95°C for 10 min to sterilize when the milk was mixed, and then the milk was taken out and placed at room temperature for cooling. *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.1011, *L. bulgaricus* KLDS1.0207, *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1, *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1 (1:1) and Chr. Hansen strains (*L. bulgaricus*, *Strep. thermophilus*, *Bifidobacterium*, *L. acidophilus*; 5.6×10^6 cfu/mL) were added into milk respectively and fermented at 42°C for 5 h. The fermented milk was placed at 4°C for 12 h for ripening and stored at 4°C for 21 d. We measured pH using a pH meter, and the content of titration acidity was measured according to GB 5009.239-2016 (National Standard for Determination of Food Acidity) according to Hang et al. (2020).

Whole-Genome Sequencing

The genomes of *L. bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011 were sequenced and compared. Illumina HiSeq (2× 100 bp paired-end library) and Illumina MiSeq (2× 250 bp paired-end library) plat-

form combinatorial sequencing technology were used to sequence the genomes of 2 strains (Chin et al., 2013). Canu (v. 2.1.1, Celera Assembler) and Unicycler (SPAdes 3.13.0, Center for Algorithmic Biotechnology) were used to assemble the samples, respectively, to obtain the preliminary assembly results. The preliminary results were compared, the 3 assembly sequences and mutually verified with the known sequences of *L. bulgaricus*, such as *L. bulgaricus* ATCC11842, to obtain the final assembly results. The complete genome sequences of *L. bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011 were submitted to the GenBank database, and the accession numbers are CP032451.1 and CP041280.1, respectively. Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (<http://www.ncbi.nlm.nih.gov/books/NBK174280>). The CGView Server (<http://cgview.ca/>) was used to draw a circular genome map (Wu et al., 2011).

COG and KEGG Annotations

The protein sequence in the genome was annotated with Clusters of Orthologous Groups (COG) through the Web MGA website (Wu et al., 2011; <http://weizhong-lab.ucsd.edu/webMGA/>). OrthoMCL v. 1.4 software (VEuPathDB) was used to perform homologous cluster analysis on *L. bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011 sequences (Farrer, 2017) and to obtain the unique genes, respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) online annotations were used for protein-coding genes in the unique genome, using the bi-directional best hit method on the KAAS website (Moriya et al., 2007; <https://www.genome.jp/tools/kaas/>). All biological pathway information related to genes was stored in the PATHWAY database (<https://www.kegg.jp/kegg/pathway.html>), including lactose metabolism and proteolytic pathways. Each gene's biological pathways were obtained through KEGG orthology, and KEGG pathway analysis was performed (Kanehisa et al., 2016).

Real-Time Reverse-Transcription PCR Assay

Bacterial RNA in fermented milk was extracted using Trizol reagent (Invitrogen; Lim et al., 2020). According to the manufacturer's protocol, the RNA extracted from yogurt was reversed transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa). Expression of target genes was determined using the QuantStudio 3 Real-Time PCR system (Applied Biosystems) with TB Green Premix Ex TaqII (TaKaRa). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of

target genes, with 16S rRNA serving as an endogenous gene (Marco and Kleerebezem, 2008). The sequences of primers are listed in Supplemental Table S1 (<https://mfr.osf.io/render?url=https%3A%2F%2Fosf.io%2Ffc7495%2Fdownload>).

Contents of Lactose, Galactose, Glucose, and Lactic Acid Assay

Fermented milk was treated using the Carrez method (Sun et al., 2012). First, 5 g of milk fermented by a single strain was dissolved in 25 g of water. Then 2.5 mL of the Carrez I solution and the Carrez II solution was added into the mixture, and water was added to make up a weight of 50 g. Then, the solution was placed on a magnetic stirrer and stirred for 30 min and centrifuged for 15 min ($4,000 \times g$) to obtain the supernatant, and the proteins and bacteria were removed by filtering with a 0.22- μ m microporous membrane. Finally, the fermented milks were stored at -20°C . The contents of lactose, galactose, glucose, and lactic acid were determined by HPLC. Samples of 1.5 mL of the fermented milk were taken at 0 d, 6 d, 12 d, and 18 d and centrifuged for 10 min ($9,000 \times g$), then the supernatant was filtered with a 0.22- μ m microporous membrane. The filtrate was placed in the fermentation milk bottle for analysis, and 3 parallel sets were used in each group. For HPLC analysis an HPX-87H column (Waters Corp.) was used, with an RID2414 refractive index detector (Waters Corp.). The mobile phase was 5 mmol/L H_2SO_4 , and the flow rate was 0.6 mL/min. The temperature was 65°C , and the injection volume was 2 μ L. The time of detection for each fermentation milk was 20 min. Standard samples were prepared with mobile phase as solvent at 5 different concentration levels (4, 6, 8, 10, and 12 mg/mL of lactose; 1, 2, 3, 4, and 5 mg/mL of galactose; 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL of glucose; and 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL of lactic acid). The standard samples were filtered through a 0.22- μ m microporous membrane and placed in sample bottles. The standard curve can be plotted using the peak area as the abscissa and the content of the standard sample (mg/mL) as the ordinate (Ohlsson et al., 2017).

Capacity of Strain to Hydrolyze Protein Assay

The capacity of each strain to hydrolyze protein was determined via the α -phthaldialdehyde (OPA) method (Rohm et al., 1996). First, 80 mg of OPA was dissolved in 2 mL of methanol, and then 50 mL of sodium tetraborate (100 mmol/L), 5 mL of SDS (20%, wt/wt), and 200 μ L of β -mercaptoethanol were added. Finally,

distilled water was added to make up the final volume of 100 mL to prepare the OPA solution. Three milliliters of a single-strain fermented milk stored at low temperature was added, with 6 mL of trichloroacetic acid (0.75 mol/L) and 0.6 mL of distilled water. The mixture was evenly mixed and rested at room temperature for 10 min. The supernatant was centrifuged ($6,000 \times g$) to collect supernatant and filtered for later use. Five L-phenylalanine standard solutions with different concentrations were prepared (1, 2, 4, 6, and 32 mmol/L), and 0.3 mL of L-phenylalanine standard solution at each of the 5 concentrations were taken respectively and were added into 6 mL of OPA to mix evenly. A standard curve can be plotted using the concentration of leucine as the abscissa and absorbance as the ordinate. Five milliliters of the supernatant of the fermented milk was taken, and 46 μ L of OPA was added to it. After the mixture was evenly mixed, it was allowed to react at room temperature for 2 min, and its absorbance was measured at the wavelength of 340 nm with a UV-Vis spectrophotometer (Macy China Instruments Inc.).

Determination of Yogurt Texture and Water-Holding Capacity

Quality of yogurt was evaluated following the method described by Saleh et al., using a texture analyzer (TA-XT Plus, Texture Technologies; Saleh et al., 2020) with parameters as follows: distance of the probe A/BE-d35 into the fermentation milk: 30 mm; disk diameter: 35 mm; fixture: TA-BT-KI; trigger point load: 7 g; deformation rate: 50%; cycles: 2; speed in the pretest: 2 mm/s; speed in the test: 1 mm/s; return speed: 1mm/s. Each fermentation milk was repeated 5 times and then the results were averaged. Twenty people with professional sensory evaluation knowledge were selected to evaluate the ripened yogurt, and the yogurt was scored according to the scorecard system by which the USDA scored Swiss yogurt (Bodyfelt and Potter, 2008). The evaluation was carried out from 6 aspects of taste, smell, texture, appearance, level of popularity, and whey separation to further verify the effects of 2 strains of *L. bulgaricus* with significant post-acidification differences on the overall flavor of yogurt (Supplemental Table S2; <https://mfr.osf.io/render?url=https%3A%2F%2Fosf.io%2F67495%2Fdownload>). Water-holding capacity of the fermented milks was determined by the method of Yekta and Ansari (2019), with a few modifications. Briefly, the fermentation milks (10 g) were centrifuged at $6,000 \times g$ for 10 min at 4°C to separate the supernatant (whey) and drained gel (precipitate). The following equation was used to calculate water-holding capacity:

$$\text{WHC}(\%) = \frac{\text{Weight of precipitate (g)}}{\text{Weight of fermented milk (g)}} \times 100.$$

Statistical Analysis

Data listed in this paper are the average of at least 3 samples, and the experimental results are presented as average $\pm$ standard deviation. One-way ANOVA and independent sample *t*-test were analyzed using SPSS version 16.0 (Pallant, 2013; SPSS Inc.). Data were processed and plotted using Origin version 8.0 (OriginLab). In all results, $P < 0.05$ was considered to be significant.

RESULTS

Analysis of PH and Titrated Acid Changes

Skim milk was fermented by 4 strains of *L. bulgaricus*, and the pH and titrated acid changes during the fermented milk's storage period were determined. The results showed that pH decreased significantly and acidity increased significantly from 0 to 9 d of storage for the 4 *L. bulgaricus* strains, and the post-acidification capacities of the milk fermented by the 4 strains were different. The results indicated that 0 to 9 d was the critical period for *L. bulgaricus* to cause post-acidification in yogurt, mainly because the living bacteria in fermented milk continue to use lactose to produce acid. The order of post-acidification capacity was as follows: *L. bulgaricus* KLDS1.0207 > KLDS1.0205 > KLDS1.1001 > KLDS1.1011. The pH and titrated acid levels of *L. bulgaricus* KLDS1.0207 changed the most during the storage period, and *L. bulgaricus* KLDS1.1011 changed the least. The pH decreased to 3.92 and 4.3, respectively (Figure 1A), and the titratable acidity increased by 60°T and 15°T, respectively, after 21 d (Figure 1B). However, the pH and titrated acid changes of *L. bulgaricus* KLDS1.0205 were similar to those of *L. bulgaricus* KLDS1.1001. The results showed that *L. bulgaricus* KLDS1.0207 had higher post-acidification capacity than *L. bulgaricus* KLDS1.011.

Genome Analysis

Genome Composition. The genome of *L. bulgaricus* KLDS1.0207 consists of a circular chromosome (1,869,179 bp) with a G+C content of 39.72% and no plasmids. A total of 2,059 genes were predicted in the genome, with a total length of 1,608,390 bp. The total length of coding regions accounted for 86.05% of the whole genome, and the average length of coding

Figure 1. pH (A) and titration acidity curves (B) of single-strain fermented milks during storage. Milks were fermented with starter strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*: KLDS1.0207, KLDS1.0205, KLDS1.1011, and KLDS1.1001. Different lowercase letters indicate significant differences across time ($P < 0.05$). Error bars represent SD.

genes is 781 bp. In addition, we found 96 tRNA, with a total length of 7,250 bp, accounting for 0.3879% of the genome. The genome of *L. bulgaricus* KLDS1.1011 consists of a circular chromosome (1,887,491 bp) with a G+C content of 39.83%. A total of 2,098 genes were predicted in the genome, with a total length of 1,622,760 bp. The total length of coding regions accounted for 85.97% of the whole genome. The average length of coding genes was 773 bp. Moreover, the total length of 95 tRNA of 7,187 bp accounts for 0.3808% of the genome. The genomes of 2 strains have 27 rRNAz [9 (5s), 9 (16s), and 9 (23s)] gene operons without sRNA (Figure 2).

Analysis of COG and KEGG Pathways. The COG annotation was performed on the protein-coding genes with biological functions in the genome of *L.*

bulgaricus KLDS1.0207 and *L. bulgaricus* KLDS1.1011 through the WebMGA website. *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 has a total of 1,426 genes with biological function annotation classification, which are 2, 53, 23, 171, 82, 107, 54, 44, 175, 86, 80, 91, 4, 45, 59, 13, 105, 56, 64, 12, 47, 1, and 52 genes, annotated into COG classifications B to X, respectively, with strains annotated to carbohydrate transport and metabolism (7.50%), amino transport and metabolism (11.99%), and unknown functional protein (3.92%). *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.1011 has a total of 1,258 protein-coding genes annotated in the COG database, with 40, 20, 134, 83, 75, 46, 44, 172, 75, 76, 80, 4, 45, 59, 13, 94, 54, 55, 13, 33, 1, and 42 genes annotated into COG classifications c to x, respectively, of which carbohydrate transport and

Figure 2. Circular genome map of *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011. From the outer circle to the inner circle, the first and second circles represent genes on the positive and negative strands, respectively, including coding sequences, transfer (t)RNA, rRNA, and other genes; the third circle represents GC content; the fourth circle represents GC skew, where green means GC > 0, purple means GC < 0, and the junction of green and purple is the start point and end point of replication, respectively.

metabolism (5.96%), amino transport and metabolism (10.65%), and unknown functional proteins (4.29%) have a large number of genes (Figure 3A).

The specific genes of *L. bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011 were annotated in the KEGG pathway; the distribution of genes related to acid production is shown in Figure 3B. *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011 have 15 and 7 specific genes in the carbohydrate metabolism system, respectively. *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 and KLDS1.1011 have 12 and 7 specific genes involved in the proteolysis amino acid metabolism pathway, respectively. In addition, 6 genes specific to membrane trans-

port pathways were found in *L. bulgaricus* KLDS1.0207 and 13 specific genes in *L. bulgaricus* KLDS1.1011. These specific genes were annotated to the pathway and require further preliminary screening and determination.

Expression of Specific and Common Genes of Strains Related to Acid Production

The amount of gene expression of *L. bulgaricus* KLDS1.1011 in yogurt during the storage period is shown in Figure 4A. After 6-d storage, the gene expression of FG480_01235, FG480_03790, FG480_09835, FG480_03785, FG480_03780, and FG480_00935 were

Figure 3. (A) Clusters of Orthologous Groups functional classification of protein-coding genes in the *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011 genomes. Function: A = RNA processing and modification; B = chromatin structure and dynamics; C = energy production and transformation; D = cell cycle control, cell analysis, chromosome partitioning; E = amino acid transport and metabolism; F = nucleic acid transport and metabolism; G = carbohydrate transport and metabolism; H = coenzyme transport and metabolism; I = lipid metabolism and transport; J = translation, ribosomal structure, and synthesis; K = transcription; L = replication, recombination, and repair; M = cell wall, cell membrane, and cell envelope; N = cell movement; O = post-translational modification, protein conversion, chaperone protein; P = inorganic ion transport and metabolism; Q = secondary metabolite synthesis, transport, and catabolism; R = general function prediction; S = unknown function; T = signal transduction mechanism; U = intracellular transport and secretion; V = defense system; W = extracellular structure; X = phage, transposon. (B) Kyoto Encyclopedia of Genes and Genomes pathway classification of specific genes in *L. bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011 genomes.

Figure 4. Relative expression of specific genes of *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.1011 (A) and *L. bulgaricus* KLDS1.0207 (B). Different lowercase letters indicate significant difference between storage times ($P < 0.05$). Error bars represent SD.

significantly lower than at 12 h ($P < 0.05$). The amount of gene expression of *L. bulgaricus* KLDS1.0207 in yogurt during storage is shown in Figure 4B. After 6 d of storage, the amount of gene expression of D4Z81_03920, D4Z81_09055, D4Z81_01110, and D4Z81_00970 had no significant difference compared with that at 12 h ($P > 0.05$), but the amount of expression of the D4Z81_06780 gene was higher than at 12 h ($P < 0.05$).

The expression of genes of key enzymes in lactose metabolism of *L. bulgaricus* KLDS1.1011 and KLDS1.0207 were different during storage, as shown in Figure 5. Both strains had 1 *LacZ*-coding gene, and we observed 1 *ldhL*-coding gene in *L. bulgaricus* KLDS1.1011 and 2 in *L. bulgaricus* KLDS1.0207 (Table 1). The *LacZ* and *ldhL* gene expression levels of *L. bulgaricus* KLDS1.0207 were significantly higher at 6 d of storage than at 12 h ($P < 0.05$; Figure 5A). However, the amount of *ldhL*

gene expression of *L. bulgaricus* KLDS1.1011 was significantly lower than that at 12 h ($P < 0.05$), and the amount of *LacZ* gene expression had no significant difference at 6 d storage compared with 12 h ($P > 0.05$; Figure 5B). The gene expression levels of *L. bulgaricus* KLDS1.0207 were higher than those of *L. bulgaricus* KLDS1.1011 after 12 h and 6 d of storage.

The gene expression of key enzymes of *L. bulgaricus* KLDS1.1011 and KLDS1.0207 in proteolysis (*pepC*, *oppD*, *pepF*, *pepQ*, *pepX*, and *pepT*) were different during storage. Both strains had all 6 genes (Table 1). The amount of *oppD*, *pepC*, *pepF*, and *pepX* gene expression by *L. bulgaricus* KLDS1.0207 was significantly decreased ($P < 0.05$) but the amount of *pepT* gene significantly increased after 6-d storage compared with 12 h ($P < 0.05$), and the amount of *pepQ* gene expression had no significant difference compared with 12 h ($P >$

Figure 5. Relative expression of enzyme genes of *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 (A) and *L. bulgaricus* KLDS1.1011 (B) related to glycometabolism. Different lowercase letters indicate significant difference between storage times ($P < 0.05$). Error bars represent SD.

Figure 6. Relative expression of proteolytic enzyme-related genes of *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 (A) and *L. bulgaricus* KLDS1.1011 (B). Different lowercase letters indicate significant differences between storage times ($P < 0.05$). Error bars represent SD.

0.05; Figure 6A). The amount of *oppD*, *pepQ*, *pepC*, and *pepX* gene expression of *L. bulgaricus* KLDS1.1011 was significantly decreased ($P < 0.05$) after 6 d of storage compared with 12 h. On the contrary, the amount of *pepT* gene expression was significantly increased after 6-d storage compared with 12 h ($P < 0.05$), whereas *pepF* gene expression had no significant difference compared with 12 h. (Figure 6B).

Glycometabolism and Protein Hydrolyzation Capacity

The variation of components of milk fermented by *L. bulgaricus* KLDS1.0207 and KLDS1.1011 during storage is shown in Figure 7, and the differences between strains with different capacity post-acidification can be observed. The content of lactose and glucose with *L. bulgaricus* KLDS1.0207 decreased, but the content of galactose and lactic acid increased. The content of lactose decreased significantly from 0 to 12 d ($P < 0.05$). However, we found no significant difference from 12 to 18 d ($P > 0.05$). No significant difference was detectable for the content of glucose from 12 to 18 d ($P > 0.05$), and the contents of galactose and lactic acid increased

significantly from 0 to 12 d ($P < 0.05$). Lactic acid is critical to glycometabolism of *L. bulgaricus*, and it is also a factor influencing post-acidification capacity. The lactic acid content increased by 11.06 mg/mL during storage, demonstrating that *L. bulgaricus* KLDS1.0207 had a greater acid-producing capacity (Figure 7A). The contents of lactose and glucose with *L. bulgaricus* KLDS1.1011 decreased significantly, but the contents of galactose and lactic acid increased significantly from 0 to 12 d ($P < 0.05$). The content of lactose decreased by 15.18 mg/mL, glucose content decreased by 2.04 mg/mL, the content of galactose increased by 4.11 mg/mL, and lactic acid content increased by 7.04 mg/mL during storage (Figure 7B). Overall, *L. bulgaricus* KLDS1.0207, with high post-acidification capacity, consumed 9.05 mg/mL more lactose than *L. bulgaricus* KLDS1.1011, with low post-acidification capacity, and produced 2.01 mg/mL more lactic acid than *L. bulgaricus* KLDS1.1011 during storage.

During storage, the protein hydrolyzation capacities of *L. bulgaricus* KLDS1.0207 and KLDS1.1011 were measured, respectively, and the results are shown in Figure 8. We found that the hydrolysis speed of *L. bulgaricus* KLDS1.0207 was high from 6 to 72 h, with

Table 1. Key enzyme genes for glycometabolism and proteolysis of *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.1.0207 and *L. bulgaricus* KLDS1.1011

Item	Gene	<i>L. bulgaricus</i> KLDS1.1.0207 Locus tag(s)	<i>L. bulgaricus</i> KLDS1.1011 Locus tag(s)	Product
Glucose metabolism	<i>LacZ</i>	D4Z81_09625	FG480_09590	β -Galactosidase
	<i>ldhL</i>	D4Z81_04510, D4Z81_04640	FG480_04550	L-Lactate dehydrogenase
Proteolysis	<i>oppD</i>	D4Z81_03860	FG480_03915	Oligopeptide transport system
	<i>pepC</i>	D4Z81_07205	FG480_07235	ATP binding protein
	<i>pepF</i>	D4Z81_05850	FG480_05910	Bleomycin hydrolase
	<i>pepQ</i>	D4Z81_07810, D4Z81_07820	FG480_07385	Oligoendopeptidase F
	<i>pepX</i>	D4Z81_08460	FG480_08485	Xaa-Pro dipeptidase
	<i>pepT</i>	D4Z81_03460	FG480_03465	X-Pro dipeptidyl-peptidase
				Tripeptide aminopeptidase

Figure 7. Glycometabolism of *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 (A) and *L. bulgaricus* KLDS1.1011 (B). Different lower-case letters indicate significant differences across times ($P < 0.05$). Error bars represent SD.

a significant difference ($P < 0.05$), and low from 72 to 216 h, with no significant differences at different times ($P > 0.05$). The hydrolysis speed of *L. bulgaricus* KLDS1.1011 declined during storage and showed a significant difference at 0 to 144 h ($P < 0.05$). The results showed that the protein hydrolyzation capacity of *L. bulgaricus* KLDS1.0207 was higher than that of KLDS1.1011 from 6 to 216 h, indicating the protein hydrolyzation of *L. bulgaricus* KLDS1.0207, with high post-acidification capacity, was greater than that of *L. bulgaricus* KLDS1.1011, with low post-acidification capacity.

Figure 8. Assay of proteolytic activity (as concentration of free amino nitrogen) of strains *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207 and *L. bulgaricus* KLDS1.1011. Different lowercase letters indicate significant differences within a strain between times ($P < 0.05$). Error bars represent SD.

Analysis of the Quality of Milk Fermented With Different Starters During Storage

The curve of pH (Figure 9A) and titrated acid (Figure 9B) of milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1, *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1, and Chr. Hansen (commercial starter) were determined. The acid-producing capacity of all the milks fermented by 3 different starters increased significantly in 0 to 9 d ($P < 0.05$), and we observed no significant difference ($P > 0.05$) from 9 to 21 d. The maximum titratable acidities of the milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 and Chr. Hansen strains were 85°T and 95°T, respectively. However, the maximum titratable acidity of milk fermented by *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1 was 110°T.

The consistency, solidity, viscosity index, cohesiveness, and water-holding capacity were determined by a texture analyzer (Table 2). Analysis revealed significant difference in the solidity, water-holding capacity, and consistency of all the milk fermented by 3 different starters ($P < 0.05$). The water-holding capacities of milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 and Chr. Hansen strains were similar, at about 36%, and the water-holding capacity of the milk fermented by *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1 was 44.93%. The consistency and solidity of the milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 were the lowest, and the consistency of the milk fermented by *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1 was the highest. The consistency reflects the mobility of yogurt, and mobility is inversely proportional to consistency (Che et al., 2019); our results indicate that the liquidity of the milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 was greatest, followed by the

Table 2. Water-holding capacity and texture of fermented yogurt with different starters¹

Starter strains	Water-holding capacity (%)	Solidity (g)	Consistency (g·s)	Cohesiveness (g)	Viscosity index (g·s)
KLDS1.1011 + <i>Streptococcus thermophilus</i> S1	35.97 ± 0.13 ^a	103.76 ± 4.24 ^a	25.51 ± 1.26 ^a	-11.43 ± 1.75 ^a	-10.29 ± 1.91 ^a
Chr. Hansen ²	36.30 ± 0.11 ^b	111.27 ± 5.27 ^b	33.60 ± 2.13 ^a	-35.25 ± 2.69 ^b	-36.48 ± 6.31 ^b
KLDS1.0207 + <i>Strep. thermophilus</i> S1	44.93 ± 0.19 ^c	107.17 ± 4.25 ^c	34.36 ± 2.32 ^b	-11.35 ± 1.28 ^a	-10.71 ± 3.25 ^a

^{a-c}Different lowercase letters indicate significant difference between different samples ($P < 0.05$).

¹Values shown ± SD.

²Chr. Hansen commercial strains: *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium*, and *Lactobacillus acidophilus*.

Chr. Hansen strains, and the milk fermented by *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1 had the least liquidity. The viscosity index and cohesiveness are closely related to the capacity of producing viscous substances of starters. We found no significant differences in viscosity index and cohesiveness ($P > 0.05$) between the milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 and *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1, but the viscosity index and cohesiveness of the milk fermented by the Chr. Hansen commercial strains were the lowest, significantly different compared with the milk fermented by the other 2 starters ($P < 0.05$).

Twenty people conducted the sensory evaluation. As shown in Figure 10, the scores of taste, smell, and texture, and the popularity of the milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 were the highest; its score for the separation of whey was lower than that of the milk fermented by Chr. Hansen strains but higher than the milk fermented by *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1. The appearance evaluation score of the milk fermented by *L.*

bulgaricus KLDS1.1011 + *Strep. thermophilus* S1 was the lowest and closest to that of the milk fermented by Chr. Hansen strains. The scores for taste, smell, and separation of whey for the milk fermented by *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1 were the lowest, possibly due to its high acid-producing capacity. In addition, its appearance evaluation score was the highest, but the score of milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 was more popular than the milk fermented by *L. bulgaricus* KLDS1.0207 + *Strep. thermophilus* S1. In conclusion, the enjoyment of the milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 was not worse than that of the milk fermented by Chr. Hansen strains, but it had some deficiencies in the separation of whey, which can be solved by adding thickener. Our experiments proved that *L. bulgaricus* KLDS1.1011, with low post-acidification capacity and a stable genetic sequence, had stable characteristics, could reduce the cost, created a product with acceptable taste, resulted in a texture that met consumers' requirements, and had broad market prospects.

Figure 9. pH (A) and titration acidity curves (B) of milk fermented with different starter cultures: *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207, *L. bulgaricus* KLDS1.1011, and Chr. Hansen strains (*L. bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium*, and *Lactobacillus acidophilus*), during storage. Different lowercase letters indicate significant difference between different times ($P < 0.05$). Error bars represent SD.

Figure 10. Sensory evaluation of fermented milk with different starters: *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.0207, *L. bulgaricus* KLDS1.1011, and Chr. Hansen strains (*L. bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium*, and *Lactobacillus acidophilus*).

DISCUSSION

The decomposition of lactose mainly causes production of lactic acid by *L. bulgaricus* and *Strep. thermophilus* during their growth, and the acidity is determined by the choice of strains, the quality of the milk, and the fermentation temperature (Jia et al., 2016; Moonga et al., 2021). The *Strep. thermophilus* and *L. bulgaricus* selected in this experiment had a good symbiotic effect. For milks fermented by a single strain and mixed strains, the pH and acidity of the milk fermented by *L. bulgaricus* KLDS1.1011 were consistently lower than that of the milk fermented by *L. bulgaricus* KLDS1.0207, and the results showed that *L. bulgaricus* KLDS1.1011 had a low post-acidification capacity. The differences in titration acidity and pH of yogurt lie in the different strains, mixing proportions, and acid-producing capacities of different starters (Casarotti et al., 2014).

Lactobacillus delbrueckii ssp. *bulgaricus* cannot metabolize pentose, but lactic acid can be produced by metabolizing lactose, glucose, and fructose through carbohydrate degradation. Lactose metabolism is the key in yogurt's post-acidification, and lactose is transported into the cell through lactose permease and decomposed to galactose and glucose by β -galactosidase (Song et al., 2016). Strains that cannot use galactose are transported out of the cell through the permease, but glucose can be transported into the carbohydrate degradation pathway through systems such as PTS and can be metabolized to produce lactic acid to control the post-acidification of yogurt (Hutkins and Nannen, 1993). Mollet et al. (2003) identified a β -galactosidase-

deficient strain regulated by IS elements, which could control the post-acidification of yogurt because it could not metabolize lactose to produce lactic acid in the milk. A study has shown that the β -galactosidase gene has strong activity at 40°C by mutation, but the activity was weakened or even lost during storage (Benbadis et al., 2005). Therefore, the post-acidification of yogurt can be reduced by reducing the lactose metabolism of *L. bulgaricus*. In this study, the amount of expression of 2 β -galactosidase genes (*LacZ* and *ldhL*) of *L. bulgaricus* KLDS1.0207 increased in 2 periods of yogurt storage. However, the *ldhL* gene of *L. bulgaricus* KLDS1.1011 decreased after 12 h, suggesting that the expression of genes related to the post-acidification capacity of the 2 strains during 2 different storage periods was significantly different. These 2 genes may be the key to the difference in acidification of *L. bulgaricus* KLDS1.1011 and KLDS1.0207 after yogurt storage.

Lactobacillus delbrueckii ssp. *bulgaricus* cannot assimilate inorganic nitrogen sources and needs to meet its growth requirement by producing peptides and releasing free amino acids, and its proteolytic enzymes produce peptides and free amino acids by hydrolyzing casein (Cotter and Hill, 2003). Considerable differences exist in the activity of proteolytic enzymes among different *L. bulgaricus*. Hou et al. (2015) studied the expression of the *PepD*, *PepB*, *PepC*, *PepF*, *PepQ*, *PepX*, and *PepT* genes in 3 strains of *L. bulgaricus* under different conditions. The results showed that the protein supply had the least effect on the *PepX* gene and the strongest effect on *OppD* (Hou et al., 2015). In our study, the sequences of the genes *prtB*, *oppD*, *pepC*, *pepF*, *pepQ*, *pepX*, and *pepT*, which are key in the proteolysis system, were compared between *L. bulgaricus* KLDS1.1011 and KLDS1.0207. The result showed a high similarity of these gene sequences between the 2 strains. At the same time, the gene expression levels in 2 milks fermented by a single strain were analyzed, revealing both that the amount of gene expression of *pepT* increased during the storage period and that the multiple of difference is not large, suggesting that the *pepT* gene was closely related to post-acidification but was not the key gene that caused the difference in post-acidification between the 2 strains. The amounts of expression of *oppD*, *pepC*, *pepF*, *pepQ*, and *pepX* after 12-h storage were significantly different from 6-d storage, and 2 strains had apparent differences in the multiples of difference in different periods, suggesting that these 5 genes were the key genes that cause *L. bulgaricus* KLDS1.1011 to have a lower post-acidification capacity than *L. bulgaricus* KLDS1.0207.

Enzyme genes involved in post-acidification are (1) the F1F0-ATPase gene cluster; (2) genes related to the

lactose metabolism system and genes related to the pyruvate metabolism system; (3) genes related to basic amino acid metabolism and synthesis; (4) key protease genes in the proteolytic system; (5) genes related to enzymes involved in the biosynthetic metabolic pathway of cell membranes; and (6) genes related to proteins involved in ion transport, among others. *Lactobacillus delbrueckii* ssp. *bulgaricus* KLDS1.1011 had 6 key enzyme genes associated with post-acidification, and the amount of gene expression of the 6 genes decreased after the 12-h and 6-d storage periods, which may relate to the biofilm-associated genes (FG480_03790, FG480_09835, FG480_03785, and FG480_03780), the proteolysis-associated gene (FG480_00935), and the glycometabolism-associated gene (FG480_01235) of *L. bulgaricus* KLDS1.1011. The decline of the gene expression levels of *L. bulgaricus* KLDS1.1011 in the critical stage of post-acidification proved that *L. bulgaricus* KLDS1.1011 had a low post-acidification capacity. Therefore, the 6 *L. bulgaricus* KLDS1.1011 genes were the key genes that reduce its post-acidification capacity. However, *L. bulgaricus* KLDS1.0207 had 5 key enzyme genes related to post-acidification. The amount of expression of the D4Z81_06780 gene after the 6-d storage period was higher than the 12-h storage period, suggesting that the D4Z81_06780 gene was key to enhancing the post-acidification capacity of *L. bulgaricus* KLDS1.0207. D4Z81_06780 is a key enzyme in the glycolysis and gluconeogenesis metabolic system, and the glycolysis pathway is the key for *L. bulgaricus* acid production. The other 4 genes are related to the formation of biofilms and the material transport of strains. Overall, the 11 key enzyme genes of *L. bulgaricus* are associated with post-acidification.

Lactose entered the cells by lactose permease and then was decomposed by β -galactosidase to produce galactose and glucose. After that, glucose entered the glycolytic pathway and was metabolized to produce lactic acid (Daryaei et al., 2010). Therefore, the contents of galactose and glucose in yogurt decreased, and the contents of galactose and lactic acid increased. The contents of lactose and glucose in yogurt were measured by Ohlsson and colleagues under different storage times and temperatures (Ohlsson et al., 2017). The results showed no significant variation for the lactose content at 4°C, consistent with *L. bulgaricus* KLDS1.1011 used in our experiment, whereas for *L. bulgaricus* KLDS1.0207, the content of lactose changed more obviously.

The starter used in milk fermentation is generally mixed and mainly composed of *Strep. thermophilus* and *L. bulgaricus*. The enzyme systems between LAB are different, and the co-cultivation system is considered effective to specific fermentation (Gu et al., 2020). In a study, Wang et al. found that 4 wild *Lactobacillus*

strains could ferment skim milk quickly and provide a unique volatile flavor for the yogurt, and the volatile flavor characteristics of the yogurt were evaluated combined with sensory analysis (Wang et al., 2018). Xu et al. (2015) analyzed 4 different acidifying strains of *L. bulgaricus* and their influence on the quality of yogurt. When the 4 *L. bulgaricus* strains were mixed with *Strep. thermophilus* TA040, the fermentation times of the yogurt starter cultures LS1 and LS2 were shorter than those of the yogurt starter cultures LS3 and LS4, and the milk fermented by LS3 and LS4 had low post-acidification capacity, high viscosity, and strong capacity to produce exopolysaccharides. There are 2 reasons for the increase in viscosity of milk during fermentation: one is the precipitation of casein; the other is the extracellular polysaccharide polymer produced by *L. bulgaricus* (Xu et al., 2015). In our study, *L. bulgaricus* KLDS1.1011 and KLDS1.0207 were used to ferment milk with *Strep. thermophilus* S1, which have good characteristics, respectively, compared with yogurt fermented by Chr. Hansen strains. The results showed no differences between the fermented milks, but the milk fermented by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 had a low post-acidification capacity and a high viscosity, a good texture, and it was more popular than the others and met the requirements of industrial production. Beahkova et al. found better flavor in milk fermented by mixed strains than in milk fermented by a single strain (Beshkova et al., 1998).

CONCLUSIONS

Lactobacillus delbrueckii ssp. *bulgaricus* is an important strain for the post-acidification of yogurt and has good acid resistance. In our study, the 2 *L. bulgaricus* with significant differences in post-acidification were selected. Among them, the strain KLDS1.1011 was relatively weak in pH, acidity, glycometabolism, and proteolysis during storage of fermentation milk. The quality of the fermented milks with mixed strains showed that the fermented milk made by *L. bulgaricus* KLDS1.1011 + *Strep. thermophilus* S1 had the weakest post-acidification, and *L. bulgaricus* was suitable for the development of yogurt starter. Meanwhile, we may obtain even better fermented strains and provide a theoretical basis for developing yogurt starters by combining genomics methods to study strains' mechanisms.

ACKNOWLEDGMENTS

The present research work was financially supported by "Hundred, Thousand and Ten Thousand" Science and Technology Major Special Project of Heilongjiang Province: Dairy Products and Meat Processing

(2019ZX07801; Harbin, China), the National Natural Science Foundation of China (32101919, 32072190, and 31801518; Beijing), the Heilongjiang Postdoctoral Science Foundation (LBH-Z20006; Harbin, China), the Academic Backbone Plan of the Northeast Agricultural University (No. 19YJXG10; Harbin, China), the “Young Talents” Project of Northeast Agricultural University (18QC52; Harbin, China), and the Open Research Fund for Key Laboratory of Dairy Science (Northeast Agricultural University; Harbin, China), Ministry of Education, Heilongjiang Province, China (2020-KLDS-OF-001; Harbin, China). The authors have not stated any conflicts of interest.

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