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Effect of culturing conditions on the expression of key enzymes in the proteolytic system of *Lactobacillus bulgaricus*[*]

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Abstract: The proteolytic system of *Lactobacillus bulgaricus* breaks down milk proteins into peptides and amino acids, which are essential for the growth of the bacteria. The aim of this study was to determine the expressions of seven key genes in the proteolytic system under different culturing conditions (different phases, initial pH values, temperatures, and nitrogen sources) using real-time polymerase chain reaction (RT-PCR). The transcriptions of the seven genes were reduced by 30-fold on average in the stationary phase compared with the exponential growth phase. The transcriptions of the seven genes were reduced by 62.5-, 15.0-, and 59.0-fold in the strains KLDS 08006, KLDS 08007, and KLDS 08012, respectively, indicating that the expressions of the seven genes were significantly different among strains. In addition, the expressions of the seven genes were repressed in the MRS medium containing casein peptone. The effect of peptone supply on *PepX* transcription was the weakest compared with the other six genes, and the impact on *OppD* transcription was the strongest. Moreover, the expressions of the seven genes were significantly different among different strains ($P<0.05$). All these results indicated that the culturing conditions affected the expression of the proteolytic system genes in *Lactobacillus bulgaricus* at the transcription level.

Key words: Gene expression, Proteolytic system, *Lactobacillus bulgaricus*, Proteinase
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1 Introduction

Lactic acid bacteria (LAB) have been used for centuries as a starter in dairy fermentations (Chen *et al*., 2014). The degradation of milk proteins through proteolysis by LAB plays an important role in generating peptides and amino acids for bacterial growth (Kunji *et al*., 1996; Savijoki *et al*., 2006). The proteolytic system of LAB consists of extracellular peptidases, peptide transport system, and intracellular peptidases. (1) Proteolysis of casein (milk proteins) by LAB is initiated by a cell-wall bound proteinase (Prt), which degrades the extracellular casein into peptides (Roland, 1999; Chen *et al*., 2003). The only cell-wall bound proteinase in *Lactobacillus delbrueckii* subsp. *bulgaricus* is *PrtB* (Gilbert *et al*., 1996; Stefanitsi and Garel, 1997). (2) The peptides are subsequently transported by several peptide transport systems into cells; oligopeptides are transported by the oligopeptide transport system (Opp) and the di/tri-peptides are transported by a proton motive force-driven transporter DtpT or an adenosine triphosphate (ATP)-driven Dpp system (Doeven *et al*., 2005; Picon *et al*., 2010). In *Lactobacillus delbrueckii* subsp. *bulgaricus*, the peptide transport system is Opp and Dpp. (3) After peptides are taken up into the cells, they are degraded into amino acids by concerted actions of intracellular peptidases including aminopeptidases that cleave

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amino acids from the N-terminus of the peptides, endopeptidases that cleave the internal peptide bonds, and di- and tri-peptidases that degrade di- and tri-peptides, respectively (Liu *et al*., 2010). In particular, caseins are rich in proline and LAB have numerous proline peptidases for degrading proline-rich peptides (Stucky *et al*., 1995; Christensen *et al*., 1999).

Lactobacillus bulgaricus can decrease bitterness and produce characteristic flavor to acidified milk, which makes this bacterium an important component of starter cultures for the dairy industry (Griffiths and Tellez, 2013). Milk proteins are the most primary source of bioactive peptides that can be produced through hydrolysis by *Lactobacillus helveticus*. As a result of proteolysis, fermented milk has a higher content of peptides and free amino acids, especially histidine, valine, proline, and serine, than non-fermented milk (Matar *et al*., 2003). LAB obtain all the necessary free amino acids required for growth in milk by their complicated proteolytic system. In a more recent study, the expressions of six transcriptional units, including *PepD*, *PepN*, *PepC*, *PepX*, *PrtP*, and *Opp-PepO1*, were found to be repressed by 5- to 150-fold with the addition of casein hydrolysate containing 20% amino acids and 80% peptides to the growth medium and the expression was released only when cells encountered nitrogen-limiting conditions (Guédon *et al*., 2001). Environmental factors are also known to influence the expression of certain peptidase genes. For example, the *PepR* expression of *Lactobacillus sakei* was repressed during aerobic growth but induced 20-fold under anaerobic conditions (Champomier-Vergès *et al*., 2002).

Because of the important role of the proteolytic system of LAB, numerous studies have been carried out to characterize the proteins and determine the factors that influence their expression. The operon of the putative (Opp) of *Lactobacillus delbrueckii* subsp. *bulgaricus* B14 was cloned and characterized (Peltoniemi *et al*., 2002). Other peptidases and transport proteins of the proteolytic system were also characterized (Mayo *et al*., 1991; Tynkkynen *et al*., 1993; Kunji *et al*., 1995; Savijoki and Palva, 2000). Expressions of *PrtP* (proteinase precursor), *PrtM* (maturase), several peptidases and transport proteins of the proteolytic system during the growth in skim milk were investigated (Azcarate-Peril *et al*., 2009; Wu *et al*., 2011). The gene expression dynamics of the *PrtP* and *PrtM* of *Lactobacillus casei* Zhang during fermentation in soymilk was also studied, and real-time polymerase chain reaction (PCR) was used to analyze the differentially expressed genes at the lag phase, the late logarithmic phase, and the stationary phase (Wang *et al*., 2012). Furthermore, the expressions of *Opp*, *DtpT*, and *PepT* in the presence of different peptides were investigated, and it was found that the growth phase and the peptide source significantly affected the gene transcription (Guédon *et al*., 2001; Vermeulen *et al*., 2005).

In this study, we used the *Lactobacillus bulgaricus* to explore the expressions of seven genes in the proteolytic system at different growth phases, initial pH values, culturing temperatures, and nitrogen sources by real-time PCR. Our research suggested that the gene expressions were significantly different under different conditions. These results are valuable for the effective use of the strains.

2 Materials and methods

2.1 Strains and growth conditions

The *Lactobacillus bulgaricus* strains used in this study are listed in Table 1. They were from the Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin, China. The strains were previously isolated from naturally fermented milk and koumiss made by herdsman families in the Tibet Autonomous Region, the Xinjiang Uygur Autonomous Region, and Gansu and Qinghai Provinces of China. The strains that had high growth and glucose catabolism rates showed serious post acidification, and the strains that had lower tolerance to acid showed weaker post acidification. The different *Lactobacillus bulgaricus* strains were available as frozen (−80 °C) stock cultures. They were revived by growth at 42 °C in normal deMan, Rogosa, and Sharpe medium (MRS; composition per liter: 20 g glucose·H_2O, 10 g peptone, 10 g beef extract, 5 g yeast extract, 2.0 g $KH_2PO_4 \cdot 3H_2O$, 5.0 g CH_3COONa, 2.0 g $(NH_4)_2HC_6H_5O_7$, 0.58 g $MgSO_4 \cdot 7H_2O$, 0.25 g $MnSO_4 \cdot H_2O$, 1 ml Tween 80, and 15 g agar for solid media). The revived strains were stored at 4 °C until required for experiments. The growth and proteolytic activity of LAB are affected by some factors, for example, strains species, type and concentration of

cultivation nitrogen, time, temperature, and initial pH. So the conditions were selected to determine the impact on key enzyme gene expression. In the MRS medium containing extra peptone, 10 g/L casein peptone was added. The initial pH of MRS was adjusted to 5.6 and 6.5, and the culture temperature was set up to 30, 37, 42, and 45 °C, respectively, for culturing strains in the experiment.

Table 1 *Lactobacillus bulgaricus* strains used in this study

No.	Strain name	Strain No.
1	*Lactobacillus delbrueckii* subsp. *bulgaricus*	KLDS 08006 (1.0207)*
2	*Lactobacillus delbrueckii* subsp. *bulgaricus*	KLDS 08007 (1.0501)
3	*Lactobacillus delbrueckii* subsp. *bulgaricus*	KLDS 08012 (1.1007)

* The number of the strain is the preservation number of Dairy Industry Culture Collection of Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin, China

2.2 Proteolytic activity in reconstituted skim milk (RSM)

Proteolytic activity by LAB was measured every 2 h from 0 to 12 h by the *o*-phthaldialdehyde (OPA) test (Church *et al*., 1983). The increase in optical density at 340 nm (OD_{340}) relative to the control was determined using an ultraviolet spectrophotometer (UV1601, Shimadzu, Japan). All the bacterial strains were grown overnight at 37 °C and pH 6.2 (OD=0.70). Cells (2%, v/v) were inoculated into 12% (0.12 g/ml) RSM and incubated at their optimum culture condition. The 2-ml sample of each incubated RSM was mixed with 4 ml of 0.75 mol/L trichloroacetic acid (TCA). The cells were collected from the growth medium by centrifugation at 8000*g* for 10 min at 4 °C. The supernatant aliquot of this mixture was added to 3 ml of OPA reagent and incubated at room temperature for 5 min until the OD_{340} was read in the spectrophotometer, and 3 ml aliquots of the samples at each time point were selected.

2.3 Total RNA isolation and cDNA synthesis

The fermented samples, cut into small pieces, were flash frozen in liquid nitrogen to inhibit further transcription and stored at −80 °C until use. Just before isolation, samples were thawed at 4 °C. At no stage was the temperature of the samples allowed to exceed 4 °C. The fermented samples (10 g) were homogenized in 90 ml of 2% (0.02 g/ml) trisodium citrate solution at 4 °C. Cells from 10 ml of suspension were then recovered by centrifugation for 5 min at 6000*g* and at 4 °C (Ulve *et al*., 2008; Genay *et al*., 2009).

RNA was isolated from each sample using the RNeasy Midi Kit (TIANGEN Biotech (Beijing) Co., Ltd., China) according to the manufacturer's instructions. The RNA templates were eluted in 40 ml RNase-free water. The quality and integrity of the total RNA templates were assessed by electrophoresis on 2% (0.02 g/ml) denaturing formaldehyde agarose gel. Complementary DNA (cDNA) was synthesized using the cDNA RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. The reverse transcriptase reaction mixture consisted of 8 μl PrimeScript® Buffer, 2 μl PrimeScript® RT Enzyme Mix I, 2 μl Oligo dT Primer (50 μmol/L), 2 μl Random 6 mers (100 μmol/L), 20 μl Total RNA, and 6 μl RNase Free dH_2O. The total volume was 40 μl. The reverse transcriptase reaction conditions were as follows: 37 °C for 15 min and 85 °C for 5 s.

2.4 Real-time PCR

Primers of corresponding genes as shown in Table 2 were designed based on the published genome sequences of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (GenBank accession number: NC_008054.1) and gene quantification was performed with 16S ribosomal RNA (16S rRNA) as an internal standard. Real-time PCR was performed in a LightCycler instrument (ABI PRISM® 7500 Real-Time PCR System, Applied Biosystems, USA) according to the manufacturer's instructions. Samples were subjected to 30 s denaturation at 95 °C, followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C. The reaction mixture consisted of 10.0 μl SYBR® Premix Ex Taq™ (Perfect Real Time; 2× Conc.; TaKaRa, Dalian, China), 2.0 μl cDNA, 6.8 μl dH_2O, 0.4 μl ROX Reference Dye II (50×), and 0.4 μl of each primer (final concentration, 10 μmol/L). The total volume was 20 μl. The data were analyzed using the ABI PRISM 7500 System Sequence Detection software. The relative gene expression was calculated from three independent experiments and analyzed using the $2^{-\Delta\Delta C_T}$ value method (Livak and Schmittgen, 2001; Niesters, 2001; Schmittgen and Livak, 2008). An application plot and dissociation curves were used for the examination of the amplified products.

Table 2 Oligonucleotide pairs used for determining gene expression of *Lactobacillus bulgaricus*

Protein	Gene	Primer sequence (5' to 3')	Product (bp)	T_m (°C)
16S ribosomal RNA	16S rRNA	Sense: ATCGGAAACTGTCATTCTTG Antisense: CTAATCCTGTTCGCTACCC	157	51.6
Oligopeptide transporter	*OppD*	Sense: CCAGTTTGAACCCTTTGA Antisense: CTTAGGCATCCCTACTTGAT	128	51.3
Cell-envelope proteinase B	*PrtB*	Sense: GTGGCAATTCAAATCTAATC Antisense: GCTGTAATCATCACCCTCAT	107	47.9
Aminopeptidase C	*PepC*	Sense: CAAGCCGACTAAGTGGAAG Antisense: AACAGCCTGGTAAACGAA	109	50.1
Endopeptidase F	*PepF*	Sense: TATCGCAGTGAGGTTCC Antisense: TTCTTCTGGTTGCTGGTGT	254	53.8
Proline peptidase Q	*PepQ*	Sense: ACTGATAAGATGCGGGAAAT Antisense: GCAAAGCCTGGGATGTAG	275	54.8
Proline peptidase X	*PepX*	Sense: TATTCAACGGCAAGTCCC Antisense: TCCCGCCAAAGTAAGGTG	195	54.9
Tripeptidase T	*PepT*	Sense: TACGGACGGGTCACAACT Antisense: GTCAACAGCCTTCCACATT	121	51.7

T_m: melting temperature

2.5 Statistical analysis

Data were expressed as the mean±standard deviation (SD) of triplicates. The statistical significance of the difference between two means was evaluated using the Student's *t*-test. Values of $P<0.05$ were considered significant. Values of $P<0.01$ were considered very significant.

3 Results

3.1 Proteolytic activity of *Lactobacillus bulgaricus*

During fermentation, milk proteins were hydrolyzed by LAB proteinases and peptidases, resulting in an enhanced amount of free amino groups and peptides. Fig. 1 represents the proteolytic activities in RSM of three *Lactobacillus bulgaricus* strains (KLDS 08006, KLDS 08007, and KLDS 08012), which manifested the different activities of α-amino group release as a result of the proteolysis of milk proteins. The amount of liberated amino groups and peptides increased significantly during fermentation from 0 to 12 h for three strains ($P<0.05$). The extent of proteolysis in the KLDS 08007 fermented milk was significantly higher ($P<0.01$) than those of the KLDS 08012 and KLDS 08006 fermented strains in this study at the 12 h of culturing time. On the other hand, KLDS 08007 had the highest proteolytic activity in this study, which reached 0.363 of absorbance. The extent of proteolysis variance among strains appeared to be time-dependent.

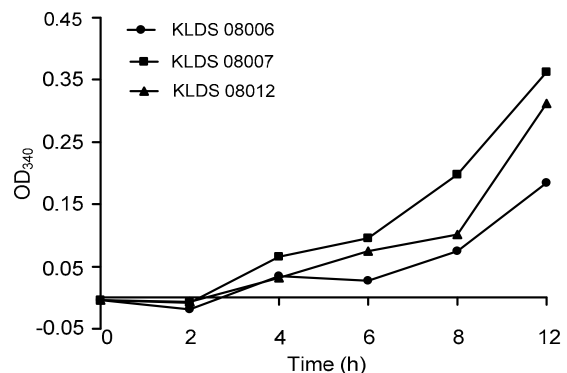

Fig. 1 Extent of proteolysis measured using OPA method during growth of three selected *Lactobacillus bulgaricus* strains in RSM for 12 h at 37 °C

3.2 Transcriptional expression of the target genes in *Lactobacillus bulgaricus* during different growth phases

In order to investigate how the proteinases, peptide transport proteins, and peptidases are regulated for peptide metabolism by *Lactobacillus bulgaricus* during different growth phases, we studied their expressions at the transcriptional level at the exponential growth and stationary phases. Total RNA was isolated at the exponential (6 h) and stationary (18 h) growth phases. Gene expression was determined by quantification of the respective transcripts in the cDNA library using 16S rRNA as the internal control by real-time PCR (Table 3). The gene expression decreased significantly with the growth of the strains. The transcription levels of the seven genes were

reduced by 30-fold on average in the stationary phase compared with the exponential growth phase. Specifically, the transcription levels of the seven genes were reduced by 62.5-, 15.0-, and 59.0-fold in the strains KLDS 08006, KLDS 08007, and KLDS 08012, respectively, indicating that the expressions of the seven genes were significantly different among strains.

The gene expression was significantly increased with the growth of the strains, except the expression of the *PepQ* gene of the KLDS 08012 strain in milk (Table 3). Particularly, the transcription levels of the *PepF* and *PepT* genes were significantly up-regulated compared with the other five genes. Therefore, LAB depend on a proteolytic system to degrade milk proteins for their growth.

3.3 Transcriptional expression of the target genes in *Lactobacillus bulgaricus* cultivated at different initial pH values

Total RNAs of *Lactobacillus bulgaricus* cultivated in normal MRS medium for 12 h at the initial pH of 5.6 and 6.5 were extracted to determine the gene expression by real-time PCR. The results are shown in Fig. 2. The expressions of the seven genes were up-regulated at initial pH 6.5 compared with that at initial pH 5.6. Furthermore, the change of gene expression was significantly different among different strains. In particular, expressions of *OppD*, *PepC*, *PepF*, *PepQ*, and *PepX* most significantly increased in strain KLDS 08006 at the initial pH 6.5; however,

Table 3 Expression of the genes related to peptide transport and hydrolysis in *Lactobacillus bulgaricus* during growth in MRS medium

Growth phase	Strain number	Relative expression						
		OppD	*PrtB*	*PepC*	*PepF*	*PepQ*	*PepX*	*PepT*
Exponential	KLDS 08006	1	1	1	1	1	1	1
	KLDS 08007	1	1	1	1	1	1	1
	KLDS 08012	1	1	1	1	1	1	1
Stationary	KLDS 08006	0.010±0.003**	0.004±0.001**	0.012±0.004**	0.033±0.017**	0.025±0.003**	0.009±0.001**	0.020±0.001**
	KLDS 08007	0.036±0.002**	0.319±0.069**	0.008±0.002**	0.010±0.004**	0.004±0.001**	0.006±0.001**	0.087±0.003**
	KLDS 08012	0.012±0.006**	0.028±0.015**	0.004±0.001**	0.006±0.003**	0.011±0.004**	0.004±0.002**	0.053±0.042**

** $P<0.01$, compared to the control group (exponential phase). Data are expressed as mean±SD of triplicates

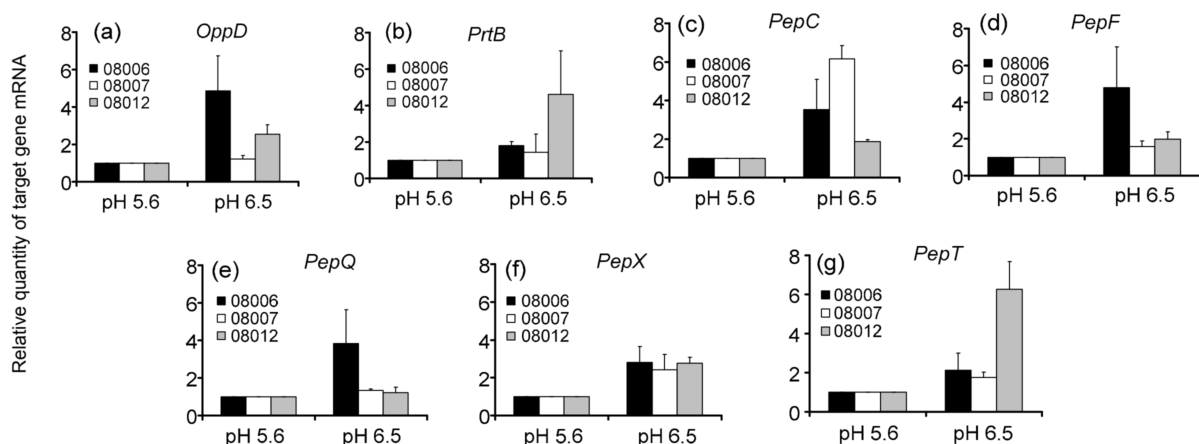

Fig. 2 Transcriptional expressions of the seven genes in *Lactobacillus bulgaricus* cells after cultivation at the initial pH of 5.6 and 6.5 for 12 h by real-time PCR analysis

(a) *OppD*; (b) *PrtB*; (c) *PepC*; (d) *PepF*; (e) *PepQ*; (f) *PepX*; (g) *PepT*. 16S rRNA was used as an internal control. Data are expressed as mean±SD of triplicates

it is the expressions of *PepC* and *PepX* in strain KLDS 08007, and those of *OppD*, *PrtB*, *PepX*, and *PepT* in strain KLDS 08012 that were most significantly increased, compared with the initial pH 5.6.

3.4 Transcriptional expression of the target genes in *Lactobacillus bulgaricus* cultivated at different temperatures

The transcriptional expressions of seven target genes in *Lactobacillus bulgaricus* cultivated in normal MRS medium for 12 h at 30, 37, and 42 °C, respectively, are shown in Fig. 3. The expressions of the key enzymes showed significant differences among the test strains cultured at the 37 °C for 12 h in normal MRS medium. The expressions of seven target genes of KLDS 08006 were the highest at 30 °C, which were significantly lower at 37 and 42 °C than at 30 °C (Fig. 3a). The expression of each target gene in KLDS 08007 and KLDS 08012 increased at first and then decreased, reaching the highest values at 37 °C (Figs. 3b–3c).

3.5 Transcriptional expression of the target genes in *Lactobacillus bulgaricus* cultivated in MRS with different nitrogen sources

To evaluate the role of peptide source on the expression of genes involved in peptide uptake and hydrolysis, MRS medium containing extra peptone made from casein was used in addition to normal MRS. The total RNA was isolated after culturing in the normal MRS medium without additives and the MRS medium containing peptone for 12 h. As shown in Fig. 4, the transcription levels of the seven genes were reduced in the MRS medium containing peptone. The addition of the peptide source decreased the transcription levels of *OppD*, *PrtB*, *PepC*, *PepF*, *PepQ*, *PepX*, and *PepT* in exponentially growing cells on average by 9.1-, 8.0-, 4.4-, 3.8-, 2.9-, 2.5-, and 3.2-fold, respectively. Therefore, the effect of peptone supply on *PepX* transcription was the weakest compared with the other six genes (Fig. 4) and the impact on *OppD* transcription was the strongest. In addition, the expressions of the seven genes showed significant differences among the three strains ($P<0.05$). The expressions of all the seven genes decreased the most in strain KLDS 08012 and the least in strain KLDS 08006.

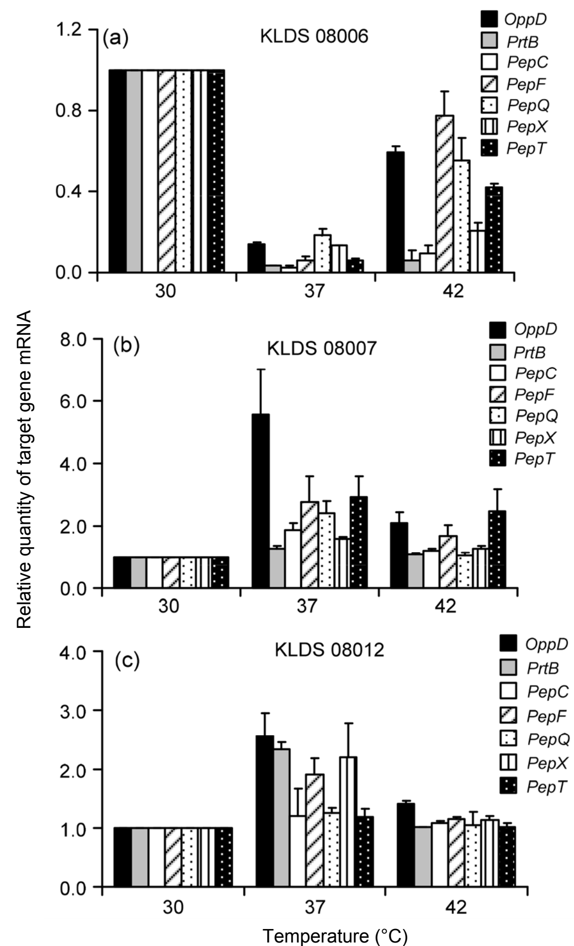

Fig. 3 Transcriptional expressions of the seven genes in *Lactobacillus bulgaricus* after cultivation at 30, 37, and 42 °C for 12 h by real-time PCR analysis
(a) KLDS 08006; (b) KLDS 08007; (c) KLDS 08012. 16S rRNA was used as an internal control. Data are expressed as mean±SD of triplicates

4 Discussion

4.1 Proteolytic activity of *Lactobacillus bulgaricus*

Only 1%–2% of milk proteins undergo proteolysis during milk fermentation and the principal milk protein is casein, but limited degradation of whey proteins may also occur (Szwajkowska *et al*., 2011; Griffiths and Tellez, 2013). Proteolysis leads to higher contents of peptides and free amino acids, especially histidine, valine, proline, and serine, in fermented milks than in non-fermented milk (Matar *et al*., 2003).

Production of amino acids and peptides from the degradation of milk proteins by LAB enzymes and

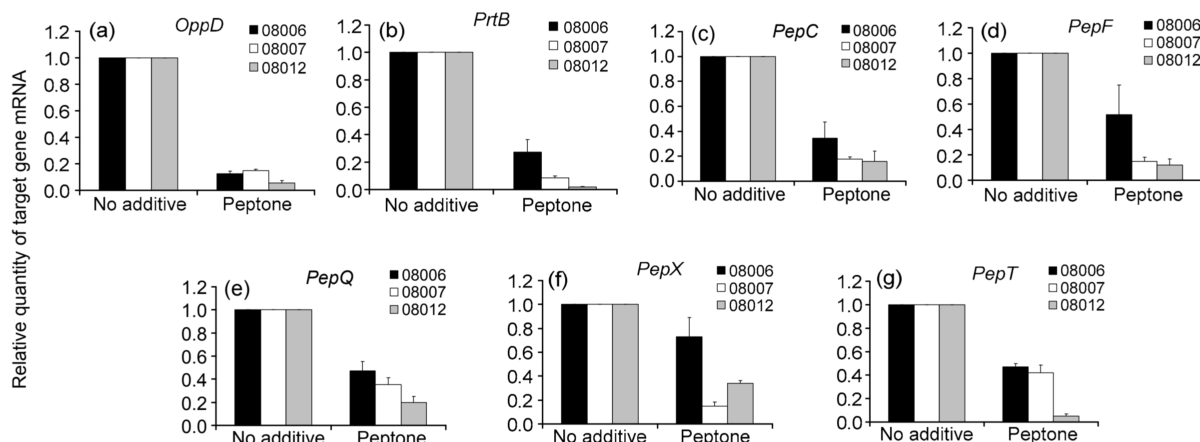

Fig. 4 Transcriptional expressions of the seven genes in *Lactobacillus bulgaricus* cells after cultivation in the normal MRS without additives or the MRS containing peptone for 12 h by real-time PCR analysis
(a) *OppD*; (b) *PrtB*; (c) *PepC*; (d) *PepF*; (e) *PepQ*; (f) *PepX*; (g) *PepT*. 16S rRNA was used as an internal control. Data are expressed as mean±SD of triplicates

utilization of these amino acids are central metabolic activities of LAB (Gobbettl *et al*., 2002). LAB, isolated from milk products, require 4 to 14 amino acids depending on the strain (Chopin, 1993). However, the amount of free amino acids and peptides in milk is very low. The amount of liberated amino groups and peptides increased significantly during fermentation from 0 to 12 h for three strains ($P<0.05$) in this study. These findings were consistent with those reported by Donkor *et al.* (2007).

4.2 Transcriptional expression of the target genes in *Lactobacillus bulgaricus* during different growth phases

Even though many studies have investigated the proteolytic ability of LAB, few reports determine the effect of growth media and different growth phases on their proteolytic system. It is a well-known fact that the proteolytic system of LAB is activated when milk is used as a growth medium (Wakai and Yamamoto, 2012). The results of this study indicated that the expression of key enzymes showed down-regulated trend in general in the MRS medium, but up-regulated trend in RSM in the stable phase compared with that in the logarithmic phase (the control group). These findings are consistent with those reported by Gitton *et al.* (2005) and Liu *et al.* (2012). Smeianov *et al.* (2007) reported that *Lactobacillus helveticus* CNRZ 32 genes encoding cell-envelope proteinases (CEPs), endopeptidases, and oligopeptide transporters were up-regulated when the strain was grown in milk compared with the levels of expression in the MRS medium. The CEP activities of six *L. helveticus* strains on α_{s1}- and β-casein in MRS and milk were evaluated. It was shown that the CEPs of the different strains hydrolyzed intact α_{s1}- and β-casein after growth in milk, but not in MRS (Jensen *et al*., 2009; Griffiths and Tellez, 2013). Hébert *et al.* (1997) showed that the effect of the growth medium on proteinase activity was evident with *Lactobacillus helveticus* CRL581. Smeianov *et al.* (2007) investigated the regulation of genes encoding peptidases of *Lactobacillus helveticus* CNRZ 32, and found that the *PepI* gene was up-regulated during growth in MRS medium, while the genes *PepN*, *PepX*, and *PepR* were up-regulated during growth in skim milk. The same level of expression of *PepC* was observed in skim milk and MRS. The expression of key enzyme genes decreased in the presence of an available nitrogen source and their activities increased in the growth medium of milk.

4.3 Transcriptional expression of the target genes in *Lactobacillus bulgaricus* cultivated under different conditions

Our results showed that the transcriptional expression of the genes in the proteolytic system was affected by the initial pH of the normal MRS medium, culturing temperature, the nitrogen source, and the

growth phase. Moreover, the transcriptional expression of the genes was significantly different among different strains as the needs for amino acids were not the same among different strains (Neviani *et al*., 1995).

Real-time PCR analysis indicated that the gene expression was decreased significantly at the stationary phase and the extent of decrease was significantly different among different strains. Similar observations have been made by Vermeulen *et al*. (2005). The increased gene expression at the logarithmic phase is likely because more amino acids are needed for the fast growth at the logarithmic phase.

Sánchez *et al*. (2007) have observed higher expression of *PepP* in *Bifidobacterium longum* NCIMB 8809 during growth at pH 4.8 than at pH 7.0. However, there are few reports on the acid-induced changes in genes related to the proteolytic system in LAB. Our real-time PCR analysis confirmed the up-regulation of the genes for *OppD*, *PrtB*, *PepC*, *PepF*, *PepQ*, *PepX*, and *PepT* at the initial pH 6.5 compared with that at the initial pH 5.6. It has been observed that the strains grow faster in MRS at the initial pH 6.5 than at the initial pH 5.6. Therefore, the strains need more proteinases to produce more amino acids in order to maintain the fast growth. Wu *et al*. (2011) reported that the gene expression of the *PepP* was suppressed at the lower pH. The expression of all the studied genes demonstrated consistent changes with medium pH at the transcriptional level and the protein level (Argyle *et al*., 1976; Law, 1978).

Milk contains few amino acids and peptides and has low proteolytic activity. Therefore, proteinase activity is a prerequisite for the growth of LAB in milk. It is reported that *Lactobacillus bulgaricus* has stronger proteinase activity than *Lactococcus lactis* (Simova and Beshkova, 2007; Griffiths and Tellez, 2013). We found that the addition of peptides to MRS resulted in the reduced transcription of the seven genes studied in exponentially growing cells, which is consistent with previous reports on the biochemical and genetic studies in dairy *Lactobacilli* such as *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* (Hébert *et al*., 2002) as well as *Lactobacillus lactis* (Guédon *et al*., 2001) that demonstrated sufficient peptides reduced the expression of proteinases. Vermeulen *et al*. (2005) reported that during the stationary phase, *DtpT* and *Opp* transcriptions are reduced regardless of the addition of peptides. Because amino acids and peptides are accumulated during fermentation, externally added peptides no longer affect the expression of peptide transporters during the stationary phase. Therefore, peptide availability strongly affects the expression of the genes in the proteolytic system.

5 Conclusions

In summary, the expressions of seven genes in the proteolytic system of *Lactobacillus bulgaricus* under different conditions were investigated. The gene expression significantly increased with the growth of the strains in RSM, except the *PepQ* gene of the KLDS 08012 strain. On the other hand, the gene expression was down-regulated in the MRS medium. We found that the initial pH of the MRS medium, nitrogen source, and growth phase affected the transcriptional expression of these genes. And the gene expressions of the different strains under different conditions were significantly different. Therefore, these factors must be taken into account for the effective use of this bacterium.

Compliance with ethics guidelines

Jun-cai HOU, Fei LIU, Da-xi REN, Wei-wei HAN, and Yue-ou DU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题 目：培养条件对保加利亚乳杆菌蛋白水解体系关键酶基因表达的影响

目 的：研究不同培养条件（不同生长阶段、初始 pH 值、培养温度和氮源）对保加利亚乳杆菌蛋白水解体系关键蛋白酶基因表达的影响。

创新点：首次证明在脱脂乳中培养保加利亚乳杆菌可以显著增加蛋白酶体系中关键酶基因表达，而在 MRS 培养基中，关键酶基因表达则显著下调。

方 法：应用实时荧光定量聚合酶链反应（qRT-PCR）技术，测定不同生长阶段的 3 株保加利亚乳杆菌（KLDS 08006、KLDS 08007 和 KLDS 08012）中 7 种关键酶基因表达的动态变化规律。

结 论：研究发现保加利亚乳杆菌的初始 pH 值、氮源和培养阶段影响关键酶基因的表达。稳定生长期的 7 个蛋白酶基因表达比指数生长期的表达平均下降 30 倍，其中 KLDS 08006、KLDS 08007 和 KLDS 08012 菌株的 7 个蛋白酶基因表达分别下降 62.5、15.0 和 59.0 倍。菌株间的 7 个蛋白酶基因表达显著不同。研究发现含有酪蛋白胨的 MRS 培养基抑制 7 个蛋白酶基因表达，其中对 *PepX* 基因表达影响最弱，而对 *OppD* 基因表达影响最强。

关键词：基因表达；蛋白水解体系；保加利亚乳杆菌；蛋白酶