



Proteolytic activities of combined fermentation with *Lactobacillus helveticus* KLDS 1.8701 and *Lactobacillus plantarum* KLDS 1.0386 reduce antigenic response to cow milk proteins

Lina Zhao,^{1,2} Qinggang Xie,³ Fengyi Shi,^{1,2} Shengnan Liang,^{1,2} Qingxue Chen,^{1,2} Smith Etareri Evvie,^{1,2,4,5} Ji Qiu,^{1,2} Bailliang Li,^{1,2*} and Guicheng Huo^{1,2*}

¹College of Food Science, Northeast Agricultural University, Harbin, 150030, China

²Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin, 150030, China

³Heilongjiang Feihe Dairy Co., Ltd., Qiqihaer 164800, China

⁴Department of Food Science and Human Nutrition, University of Benin, Benin City 300001, Nigeria

⁵Department of Animal Science, University of Benin, Benin City 300001, Nigeria

ABSTRACT

Cow milk protein is one of the leading food allergens. This study aimed to develop an effective method for reducing milk sensitization by evaluating antigenicity of fermented skim milk protein using *Lactobacillus helveticus* KLDS 1.8701, *Lactobacillus plantarum* KLDS 1.0386, and a combination of both strains. The proteolytic systems of strains in terms of genotype and phenotype are characterized by complete genome sequence, and evaluation the antigenicity of skim milk proteins was determined by ELISA and liquid chromatography with tandem mass spectrometry. Our results showed that the genomes encoded a variety of peptidase genes. For fermented skim milk, the degree of hydrolysis of the combined strains was higher than that of individual strain. Electrophoresis showed that the band color density of α -casein (α -CN) by fermentation of the combined strains was reduced when compared with control group. The fermentation process of the combined strains inhibited α -CN, β -lactoglobulin, and α -lactalbumin antigenicity by 69.13, 36.10, and 20.92, respectively. Major allergic epitopes of α -CN and β -lactoglobulin were cleaved by abundant proteases of combined strains. In all, this study showed that the fermentation process involving both *L. helveticus* and *L. plantarum* strains could reduce cow milk protein allergenicity through the combination of cell-envelope proteinase and peptidase on α -CN.

Key words: skim milk protein, fermentation, complete genome sequence, antigenicity, linear epitope

INTRODUCTION

Cow milk protein is an important dietary component widely used in food industries due to its rich source of essential AA and bioactive peptides (Kim et al., 2007). However, it is considered one of the 8 allergens by the Food and Agriculture Organization of the United Nations and the World Health Organization; the implicated components are casein and whey proteins (Villa et al., 2018). Cow milk proteins can induce immune hypersensitivity, called cow milk protein allergy (CMPA), a complex disorder in which the main symptoms are atopic dermatitis, eczema, asthma, vomiting, and recurrent diarrhea (Warren et al., 2018). The prevalence of CMPA is approximately 3.6% in Morocco, with increasing incidence (Azdad et al., 2017). Therefore, it is pertinent that research efforts be concerted toward developing effective protocols to reduce CMPA.

In recent years, it has been argued that fermentation can reduce allergen activities and increase the protein's probiotic properties (Yao et al., 2015; Roggero et al., 2020). Lactic acid bacteria (LAB) possess a complex proteolytic system composed of cell-envelope proteinase (CEP), peptidases, and peptide transport systems. (Savijoki et al., 2006). During fermentation, these proteases can degrade protein into short peptides that provide a nitrogen source for the growth of stains in milk (Hebert et al., 2000). At the same time, antigenicity is altered by destroying or masking the allergic linear epitopes (Bu et al., 2010; Ahmadova et al., 2013). Currently, several studies have demonstrated that some LAB have good proteolytic systems and can effectively decrease the antigenicity of the proteins (Abd El-Salam and El-Shibiny, 2021). Ahmadova et al. (2013) found

Received April 27, 2021.

Accepted June 20, 2021.

*Corresponding authors: 15846092362@163.com and guichenghuo@126.com

that the IgE binding ability of hydrolyzed proteins by *Lactobacillus helveticus* A75 was lower than unfermented protein (Ahmadova et al., 2013). Bu et al. (2010) assessed fermentation on whey protein by mixtures of *L. helveticus* and *Streptococcus thermophilus* and found that α -LA and β -LG antigenicity were decreased using indirect competitive ELISA. Wróblewska et al. (2020) evaluated the immunoreactivity of fermented cow milk proteins by *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, and *S. thermophilus* in vitro and in vivo, which was reduced effectively. These suggest that cow milk proteins fermented by LAB appeared to be promising in reducing or eliminating antigenicity of the proteins. Considering that *L. helveticus* is rich in CEP genes and *L. plantarum* is rich in peptidase genes and transport systems, their combined strains maybe degrade protein well. There were few reports about the antigenicity of the combined fermentation of *L. helveticus* and *L. plantarum*, and the capability and mechanism of different LAB to reduce antigenicity are still unclear due to their different proteolytic systems.

Previously, we reported the complete genome sequence of the *L. helveticus* KLDS 1.8701 strain and unraveled the presence of genes coding such as 2 CEP (*PrtH₁*, *PrtH₄*; Supplemental Figure S1; <https://figshare.com/s/c88f02afea5bbccb0876>). Although *L. plantarum* KLDS 1.0386 does not carry CEP genes, it is rich in 21 peptidase genes and transport systems. In the current study, we evaluated the degradation of α -CN, α -LA, and β -LG and compared antigenicity in fermented milk through *L. helveticus*, *L. plantarum*, and their mixtures. Furthermore, we characterized the effects of fermentation on allergic epitopes of milk proteins. The purpose was to evaluate the possibility of a method for developing hypoallergenic milk products based on proteolytic systems.

MATERIALS AND METHODS

Materials, Chemicals, and Microorganisms

Lakeland Dairies Co-operative Society Ltd. provided skim milk powder used in this study. The protein content was 34.1%, the fat content was 0.3%, and the lactose content was 54.6%. All other chemical reagents were analytical grade. Three different strains used in this study were isolated from different fermented dairy products and preserved at the Key Laboratory of Dairy Science (KLDS), Northeast Agricultural University, China. The *L. helveticus* KLDS 1.8701 was isolated from traditional dairy products in Sinkiang Province, China. The *L. plantarum* KLDS 1.0386 was isolated from Inner Mongolia traditional fermented dairy products.

Inoculum Preparation and Whole Genome Sequencing

The strains were incubated in the Modified de Man Rogosa and Sharpe broth at 37°C for 24 h and grown for 2 successive transfers. The activated cultures were subcultured for another 16 h. All cells were collected by centrifugation at $2,000 \times g$ for 10 min at 4°C and resuspended in sterile PBS to contain 10^{-9} cfu/mL. The strains were mixed at the ratio of 1:1 (vol/vol).

The total DNA of *L. plantarum* KLDS 1.0386 was extracted by the DNeasy Tissue kit (Qiagen). The whole genome sequencing was performed using a combined strategy of Illumina Hiseq 2500 sequencing and Pacific Biosciences (PacBio) RSII sequencing technologies, then assembled (Qi et al., 2021). Genome annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline (<http://www.ncbi.nlm.nih.gov/books/NBK174280>). Functional annotation of protein coding genes was performed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>).

Fermentation

Skim milk powder was resuspended with deionized water at 12 g/100 mL (wt/vol) and sterilized in a heated water bath at 90 to 95°C for 5 min to reduce the pathogenic bacteria. The reconstituted skim milk (RSM) was prechilled immediately and inoculated with the culture in a ratio of 2% (vol/vol) at 37°C to pH 4.5 ± 0.1 (SD). After fermentation, it was stored at -20°C until analysis.

Degree of Hydrolysis Analysis

The evaluation of the degree of hydrolysis (DH) of the fermented RSM was measured by the ortho-phthalaldehyde reagent (Nielsen et al., 2001). It was prepared as follows: Na-tetraborate decahydrate, Na-dodecyl-sulfate, and dithiothreitol were added in deionized water solution, and ortho-phthalaldehyde was added in 100% ethanol solution. The arginine solution (200 $\mu\text{g/mL}$) was used as standard. The ortho-phthalaldehyde reagent (3 mL) was mixed with 400 μL of deionized water (blank), Arg solution (standard), and sample, respectively. Then, the absorbance value was measured at 340 nm to calculate the DH% as follows:

$$\text{DH}\% = \frac{h}{h_{\text{tot}}} \times 100\%,$$

where h_{tot} is the total number of peptide bonds ($h_{tot} = 8.3$) and h is the number of hydrolyzed peptide bonds (Ambrosi et al., 2016).

Electrophoresis

The RSM before and after fermentation was analyzed for the degradation of protein by SDS-PAGE (Laemmli, 1970). Samples (5 mg/mL) were mixed with sample loading buffer (4:1, vol/vol). The Hepes-Tris precast PAGE Gel contained 12% separating gel and 4% stacking gel. The loading volume of the sample was 10 μ L. The running voltage was set at 80 V for 30 min and then 120 V for 1 h. Gels were stained with Coomassie Brilliant Blue (R-250) and decoloration without the dye (Oliveira et al., 2018).

Sandwich ELISA for Antigenicity Analysis

The antigenicity of α -LA, β -LG, and α_{S1} -CN in the fermented RSM was determined using sandwich ELISA according to the manual provided by manufacturer (MEI MIAN). The RSM before and after fermentation was diluted with PBS to adjust the concentration of protein. Then, the sample and standard solution were added to the Microelisa strip plate accurately and gently mixed. The plate was closed with a membrane, incubated for 30 min at 37°C, and then washed 5 times with PBS solution. The horseradish peroxidase-conjugate reagent was added to each well except the blank well and incubated for 30 min at 37°C. The wells were rewashed 5 times. The plate had chromogen solution added to each well and incubated for 10 min. Finally, the stop solution was added to each well to stop the reaction. The absorbance was recorded at 450 nm within 15 min and converted to concentration equivalents. The inhibition rate (%) of antigenicity was calculated using the following formula:

$$\text{Inhibition rate(\% of antigenicity)} = \left(1 - \frac{C_1}{C_0}\right) \times 100\%,$$

where C_1 represents the protein concentration of fermented skim milk and C_0 represents the protein concentration of unfermented skim milk.

Liquid Chromatography with Tandem Mass Spectrometry

To evaluate the potential of fermentation in decreasing the antigenicity of some milk proteins, the peptides released after the fermentation of RSM proteins were

analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) to understand the proteolysis sites and the destruction of antigen epitopes.

Peptide Extraction

The fermented RSM was centrifuged at $4,500 \times g$ for 10 min at 4°C. Then, the supernatant was centrifuged again by the ultrafiltration centrifuge tube (10 kDa, Millipore) at $5,500 \times g$ for 20 min at 4°C to extract peptides (Jiang et al., 2018). The peptides were de-salted by C18 stage tips (Thermo Scientific). The tip was activated with 0.2% trifluoroacetic acid solution. According to the manufacturer's manual, peptide mixtures (200 μ L) were loaded into the tip and eluted with 80% acetonitrile/0.2% trifluoroacetic acid solution.

Peptides Identification by LC-MS/MS

The obtained peptides were analyzed using LC-MS/MS. The easy nano-LC 1000 system (Thermo Fisher Scientific) equipped with nanocolumn Pepmap C18 (100 μ m $\times$ 2 cm, 5 μ m, 100 Å) was performed to separate the peptides. Furthermore, a Q Exactive Plus (Thermo Fisher Scientific) equipped with analytical column Pepmap C18 (75 μ m $\times$ 100 cm, 3 μ m, 100 Å) was performed to identify the peptides. Briefly, the flow was set at 300 nL/min for 90 min (Sun et al., 2019). The eluting buffer was composed of 0.1% formic acid and 80% acetonitrile. The full MS scan range was 350 to 1,800 m/z , and the maximum ion injection time was 50 ms. After each full scan, the 20 fragment maps were chosen and determined.

All MS/MS raw data were analyzed using Proteome Discoverer 2.2. software (Thermo Scientific). The MS/MS spectra were searched with SEQUEST engine against protein database of UniProt-bovine reviewed-20191010-yes.fasta (<http://www.uniprot.org>). Criteria for protein identification were input as follows: enzyme: none; maximum missed cleavage sites: 2; minimum peptide length: 6; maximum peptide length: 144; precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.02 Da.

Statistical Analysis

Data are presented as the mean $\pm$ standard deviation. Statistical analyses were performed by SPSS 22.0 software (SPSS Inc.) using one-way ANOVA and Duncan's multiple comparison test. The graphs were generated using GraphPad Prism 8.0 software (GraphPad Inc.). In all results, $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Gene Analysis of Proteolytic System

The genome of *L. plantarum* KLDS 1.0386 consists of a circular chromosome (3,025,306 bp) with guanine + cytosine content of 44.82% and 6 circular plasmids (Figure 1A). A total of 3,210 genes were predicted, and a large number of gene functions were annotated in the KEGG database. Among them, 146 and 151 genes were associated with AA metabolism and membrane transport, respectively (Figure 1B). That suggested that *L. plantarum* KLDS 1.0386 may have an advantage in protein and AA utilization. The genome of *L. plantarum* KLDS 1.0386 harbors rich transport systems through bioinformatics analysis, including a complete oligopeptide permease operator *oppABCD* (J4Q33_04590-J4Q33_04610) and 4 oligopeptide junction genes of *oppA* (J4Q33_00080, J4Q33_02805, J4Q33_00840 and J4Q33_00845; Table 1). *Lactobacillus plantarum* KLDS 1.0386 also encodes a *dtgT* transporter (J4Q33_02100) that is primarily responsible for transporting hydrophilic dipeptides and tripeptides. In addition, the genome of *L. plantarum* KLDS 1.0386 is rich in genes encoding intracellular peptidases, including aminopeptidase (*pepC*, *pepM*, *pepN*), tri/dipeptidases (*pepD*, *pepT*, *pepV*), proline peptidase (*pepR*, *pepI*, *pepQ*, *pepP*) and endopeptidase (*pepO*, *pepF*, *pepE*; Table 2). Several peptidases also have multiple copy coding genes, such as *pepD* (*pepD*₁, *pepD*₂, *pepD*₃, and *pepD*₄), which may contribute to their ability to metabolize various peptides. These peptidases seemed to be essential for bacterial growth or survival (Qi et al., 2021). However, *L. plantarum* alone cannot utilize proteins. This may be related to the fact that *L. plantarum* does not encode corresponding CEP (Kleerebezem et al., 2003). It is suggested that the rich CEP of *L. helveticus* can promote the utilization of protein by *L. plantarum*. Previously, we reported that *L. helveticus* KLDS 1.8701 strain unraveled the presence of genes coding such as CEP (*prtH*₁, *prtH*₄) (Supplemental Figure S2; <https://figshare.com/s/c88f02afea5bbccb0876>). Subsequently, the hydrolysis potential of combined fermentation was evaluated.

Degree of Hydrolysis Analysis

The DH of the fermented RSM was displayed in Figure 2. In this study, the proteolytic capacity of *L. plantarum* KLDS 1.0386 was lower than *L. helveticus* KLDS 1.8701. The DH was 2.93% and 9.43%, respectively. However, compared with *L. plantarum* KLDS 1.0386, the DH of the combined strains of the *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386

was improved in the corresponding fermented samples (DH = 10.34%; $P < 0.05$). The finding was consistent with a previous study in which the *L. plantarum* I91 promoted its growth in the hydrolytic product of the *L. helveticus* 138 (Bergamini, 2013). This may be because *L. helveticus* hydrolyzes proteins into small peptides, and *L. plantarum* uses abundant peptidases and transport systems for further hydrolysis. A few proteases were generated during LAB fermentation, including CEP and peptidases, which can hydrolyze proteins

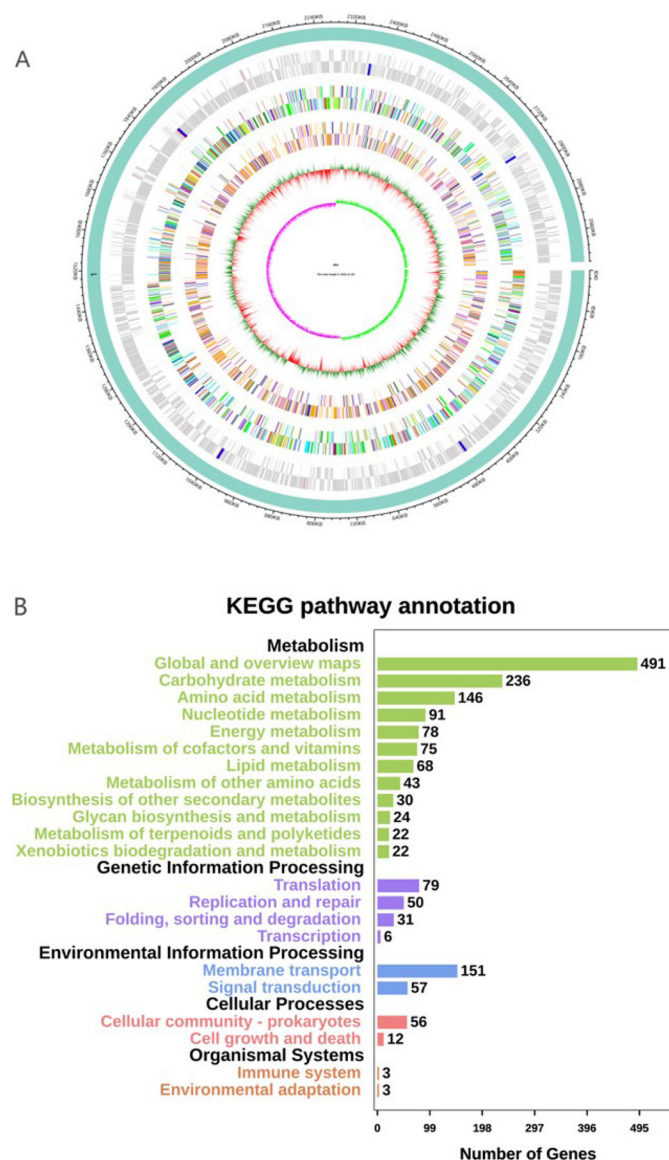


Figure 1. Circular genome map of *Lactobacillus plantarum* KLDS 1.0386 (A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional categories in the complete genome (B). From inside to outside, circle (1, 2) shows the locations of genes, including GC skew ($G - C / (G + C)$), GC content, genes, and chromosome, respectively. C = cytosine; G = guanine.

Table 1. Peptide transport systems in *Lactobacillus plantarum* KLDS 1.0386

Locus tag	Function	Gene
J4Q33_00080	Oligopeptide-binding protein	<i>oppA</i>
J4Q33_02805	Oligopeptide-binding protein	<i>oppA</i>
J4Q33_00840	Dipeptide-binding protein	<i>oppA3</i>
J4Q33_00845	Dipeptide-binding protein	<i>oppA4</i>
J4Q33_04590	Dipeptide-binding protein	<i>oppA5</i>
J4Q33_04595	Oligopeptide transport system permease protein	<i>oppB</i>
J4Q33_04600	Dipeptide transport system permease protein	<i>oppC</i>
J4Q33_04605	Oligopeptide transport ATP-binding	<i>oppD</i>
J4Q33_04610	Oligopeptide transport ATP-binding protein	<i>oppF</i>
J4Q33_02100	Dipeptide/tripeptide transporter	<i>dtpT</i>

into peptides (Bu et al., 2010). As described in some previous studies, most of *L. plantarum* possessed poor protease activity. For example, *L. plantarum* L3-4 and 70810 had inefficient protein degradation properties to the tested samples (Rui et al., 2019), and *L. plantarum* WCFS1 was absent of genes that code CEP (Kleerebezem et al., 2003).

Electrophoresis

The SDS-PAGE profiles of the fermented and un-fermented RSM are shown in Figure 3. The primary milk proteins were separated on different lanes. Compared with RSM protein, the heated RSM sample bands showed no notable change (lane 3, Figure 3), but fermented samples were faded in different degrees, including α -CN and β -LG. There are differences in the abilities of the different strains to hydrolyze proteins. We found α -CN was likely to be degraded by most

LAB, but whey protein was not susceptible to them. In detail, the combined strains of the *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386 preferred to degrade α_{S1} -CN (lane 6, Figure 3), where the band color density was reduced. This might be because *L. helveticus* and *L. plantarum* can utilize protein cooperatively (Man et al., 2012; Bergamini, 2013). However, the *L. helveticus* KLDS 1.8701 (lane 4, Figure 3) displayed weak proteolytic activity for α_{S1} -CN; it may depend on its P_T-type CEP, which are sensitive to other caseins but not to α_{S1} -CN (Ahmadova et al., 2013). In addition, *L. plantarum* KLDS 1.0386 (lane 5, Figure 3) possessed less changes for the band color density, and this result was in consonance with the low DH. It may be because the strain did not bring genes of the coding CEP (Kleerebezem et al., 2003). Although the ability of LAB to degrade proteins was limited, it was significant to reduce antigenic epitopes. However, the specific antigenicity changes need to be further studied.

Table 2. Intracellular peptidases in *Lactobacillus plantarum* KLDS 1.0386

Peptidase	Locus tag	Function	Gene
Aminopeptidase	J4Q33_02305	Aminopeptidase C	<i>pepC1</i>
	J4Q33_01095	Methionine aminopeptidase	<i>pepM</i>
	J4Q33_03435	Aminopeptidase N	<i>pepN</i>
Tri/dipeptidases	J4Q33_14185	Aminopeptidase C	<i>pepC2</i>
	J4Q33_01450	Dipeptidase A	<i>pepD2</i>
	J4Q33_00975	Dipeptidase	<i>pepD1</i>
	J4Q33_03530	Dipeptidase A	<i>pepD3</i>
	J4Q33_06385	Dipeptidase A	<i>pepD4</i>
	J4Q33_07150	Tripeptide aminopeptidase	<i>pepT</i>
	J4Q33_04880	Putative dipeptidase	<i>pepV</i>
	J4Q33_03110	Proline iminopeptidase	<i>pepR</i>
Proline peptidase	J4Q33_00355	Proline peptidase	<i>pepI</i>
	J4Q33_09020	Xaa-Pro dipeptidase	<i>pepQ</i>
	J4Q33_02600	Xaa-Pro dipeptidase	<i>pepQ</i>
	J4Q33_11730	Proline iminopeptidase	<i>pepR2</i>
	J4Q33_03125	Xaa-Pro dipeptidyl-peptidase	<i>pepXP</i>
	J4Q33_05875	Xaa-Pro aminopeptidase	<i>pepP</i>
	J4Q33_13550	Neutral endopeptidase	<i>pepO</i>
Endopeptidase	J4Q33_08740	Oligoendopeptidase F	<i>pepF1</i>
	J4Q33_10465	Oligoendopeptidase F	<i>pepF2</i>
	J4Q33_05780	Dipeptidase E	<i>pepE</i>

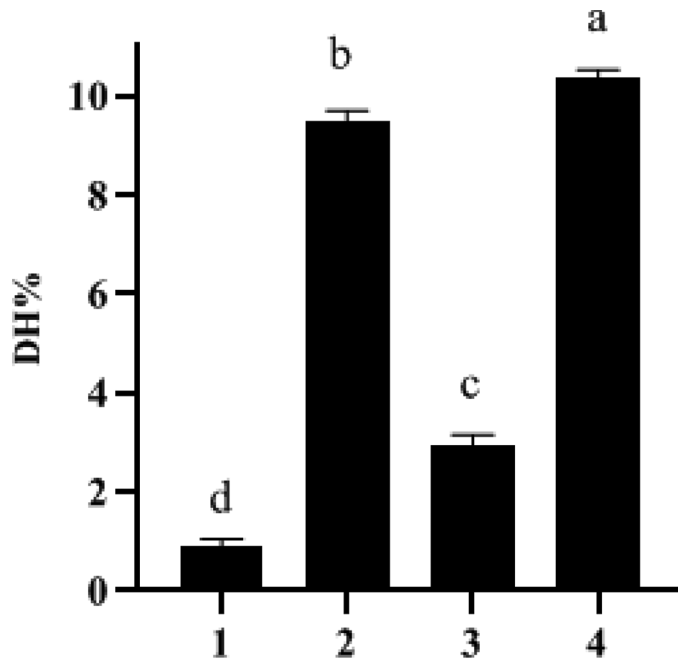


Figure 2. Degree of hydrolysis (DH) of the fermented reconstituted skim milk (RSM). 1 = heated RSM; 2 = *Lactobacillus helveticus* KLDS 1.8701; 3 = *Lactobacillus plantarum* KLDS 1.0386; 4 = *L. helveticus* KLDS 1.8701 + *L. plantarum* KLDS 1.0386. Different letters (a–d) indicate significant differences ($P < 0.05$) among groups.

Sandwich ELISA for Antigenicity Analysis

The antigenicity of the fermented RSM was shown in Figure 4 by a sandwich ELISA experiment. However, compared with the unfermented RSM, the antigenicity of α -CN, β -LG, and α -LA was reduced in different degrees and inhibited by 39.03 to 69.13%, 24.52 to 36.10%, and 12.70 to 20.92%, respectively. Compared with *L. helveticus* KLDS 1.8701 fermentation alone, the inhibition rate of the α -CN showed the largest effect with 69.13% by the combined strains of the *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386 in the corresponding fermented samples ($P < 0.05$). Simultaneously, the inhibition rate of antigenicity of the α -CN of *L. plantarum* KLDS 1.0386 was significantly higher than that of *L. helveticus* KLDS 1.8701 ($P < 0.05$), but the effect was slightly lower. This might be caused by hydrolyzing allergenic epitopes. This suggested that peptidases of *L. plantarum* KLDS 1.0386 hydrolyze α -CN more easily than proteases of *L. helveticus* KLDS 1.8701. This is the main reason for the decrease of milk protein antigenicity. This result was supported by the DH and SDS-PAGE profiles. However, the effect of the inhibition of whey seemed to vary slightly. The antigenicity of β -LG and α -LA was slightly reduced to 36.10% and 20.92%, respectively, after a combined strain treat-

ment. However, the effects of the other treatments on the antigenicity were slightly lower. During the thermal processing, the antigenicity was changed due to the destruction of the protein conformation (Davis et al., 2001). After sterilization, the antigenicity of α -LA and α -CN decreased slightly, whereas the antigenicity of β -LG increased slightly. Our observation agrees with a previous study (Yao et al., 2015). Modification of milk proteins during LAB fermentation may be related to the destruction of allergic epitopes by proteases, the change of conformation epitopes by acid, and sterilization by heating so that allergic epitopes can be inaccessible to IgE binding (Meinlschmidt et al., 2016; Rui et al., 2019). We identified the peptides fragments using the LC-MS/MS technique to assess antigen reduction levels and enzyme-specific sites in the following steps.

Peptides Identification by LC-MS/MS

Peptides were released by hydrolysis of LAB proteases, and the sequence is indicated by arrows (Figures 5, 6, and 7). As reported in previous studies, the main allergenic epitopes of the α -CN and β -LG have been marked in red (Sélo et al., 1999; Järvinen, et al., 2001; Busse et al., 2002; Ruiter et al., 2006; Cong et al., 2013). The results showed that fermented RSM of the combined strains (*L. helveticus* KLDS 1.8701 + *L. plantarum* KLDS 1.0386) could decrease protein immunoreactivity, especially α -CN (Figure 7). The coverage rate of proteolytic peptides of α_{S1} -CN was 73%. A total of 59 characteristic peptides were obtained, and 6 epitopes

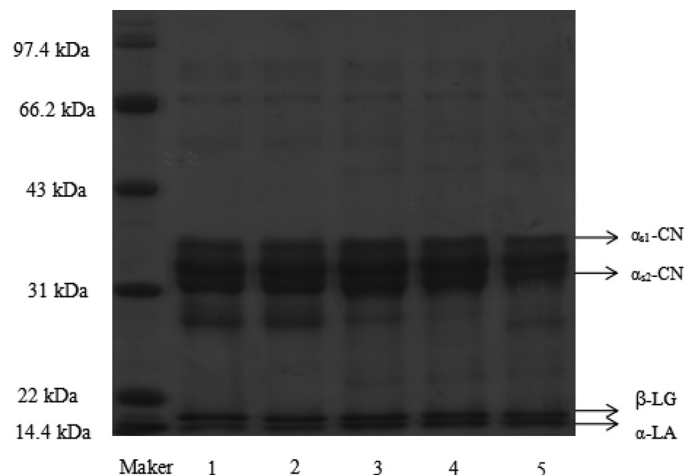


Figure 3. The SDS-PAGE profiles of reconstituted skim milk (RSM) and the fermented RSM. Lane 1 = RSM; lane 2 = heated RSM; lane 3 = *Lactobacillus helveticus* KLDS 1.8701; lane 4 = *Lactobacillus plantarum* KLDS 1.0386; lane 5 = *L. helveticus* KLDS 1.8701 + *L. plantarum* KLDS 1.0386.

(K6–20L, L11–25V, N17–36K, L109–120L, Y173–194T, P177–186I) were split (Figure 7B). The coverage rate of hydrolyzed peptides of α_{S2} -CN was 34%. A total of 15 characteristic peptides were obtained, and 3 epitopes (N83–100Y, S143–158K, K165–188K) were destroyed (Figure 7C). Therefore, some major allergic epitopes of α -CN were modified, and allergenicity was reduced. This is consistent with the results of ELISA. Numerous studies have reached similar conclusions. Beganović et al. (2013) opined that *L. helveticus* M92 possessed high proteolytic activity, and the peptides sequence were identified by proteases *prtM*, peptidases (*pepE*, *pepN*, *pepQ*), and the peptide transport system (Beganović et al., 2013). Proteolytic peptides of β -LG covered 34%, and 11 peptides were obtained. The allergic epitopes (L1–16A, V41–60K, V92–100K, D129–138K) were destroyed, and almost all of the allergic epitopes were destroyed. Therefore, the allergenicity of β -LG was reduced (Figure 7A). However, the degradation pep-

tides of α -LA were not detected. It may be that shorter peptides and a small peptide content are difficult to capture and detect by EASY-nLC-orbitrap MS/MS in this study (Sun et al., 2019). However, the degradation effect of the separate fermentation of skim milk by *Lactobacillus helveticus* KLDS 1.8701 and *Lactobacillus plantarum* KLDS 1.0386 on allergy epitopes was slightly less than the compound fermentation of *Lactobacillus helveticus* KLDS 1.8701 and *Lactobacillus plantarum* KLDS 1.0386, as shown in the figures (Figures 5 and 6). This is due to the specificity of the protease (Vukotić et al., 2016). During fermentation, LAB produce large amounts of proteinase that hydrolyze milk proteins into short peptides and AA. This can split a large number of allergic epitopes and reduce the IgE binding capacity of α -CN, β -LG, and α -LA to reduce immunoreactivities of cow milk (Shi et al., 2014). Moreover, the mechanism of reducing the immunoreactivities in dairy products after fermentation were attributed with changes in the Th1/Th2 balance toward a Th1 response, enhancement of the secretion of regulatory cytokines, and reduction in allergy marker levels (Wróblewska et al., 2020). Also, the immunoreactivity of fermented RSM proteins in vivo should be further studied.

CONCLUSIONS

Fermentation reduced RSM protein antigenicity by destroying the linear epitopes. Complete genome data shown that *L. plantarum* KLDS 1.0386 codes a large number of peptidase genes, and specificity in amino-peptidase activities was observed among proteins. A combined strain treatment (*L. helveticus* KLDS 1.8701 + *L. plantarum* KLDS 1.0386) had the highest protein degradation ability, especially α -CN. The results showed that combined fermentation can utilize protein cooperatively to reduce the antigenicity of cow milk protein. This study provided insight into the genetic and phenotypic diversity of *L. helveticus* and *L. plantarum* enzymes in fermented milk and provided a theoretical basis for the application of *L. plantarum* in fermented milk.

ACKNOWLEDGMENTS

We are grateful to the “Hundred, Thousand and Ten Thousand” Science and Technology Major Special Project of Heilongjiang Province: Dairy Products and Meat Processing (No. 2020ZX07B01; Heilongjiang, China), and Chinese nutrition society – Feihe physique nutrition and health research fund (CNS-Feihe2020A37; Heilongjiang, China) for supporting this study. The authors declare no conflicts of interest.

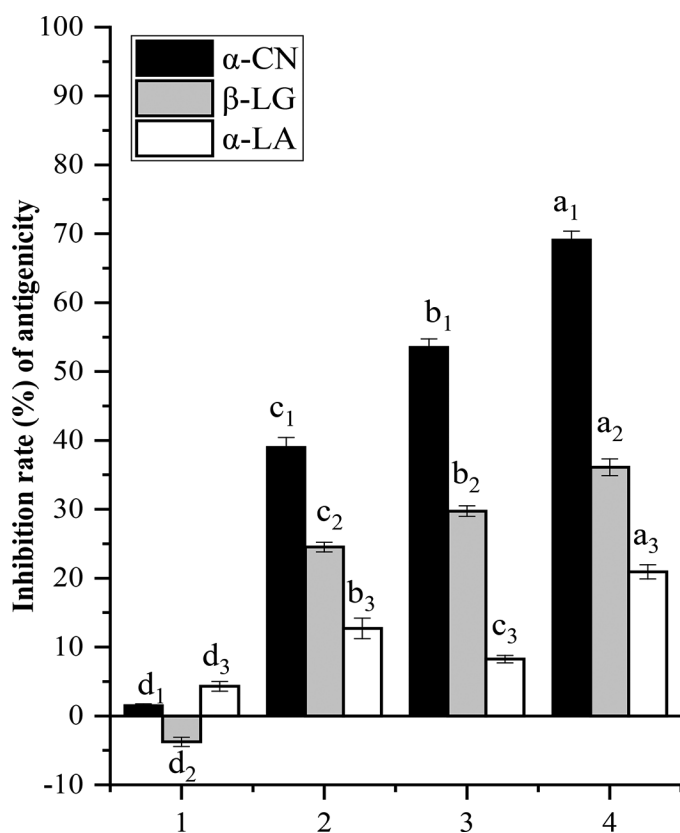


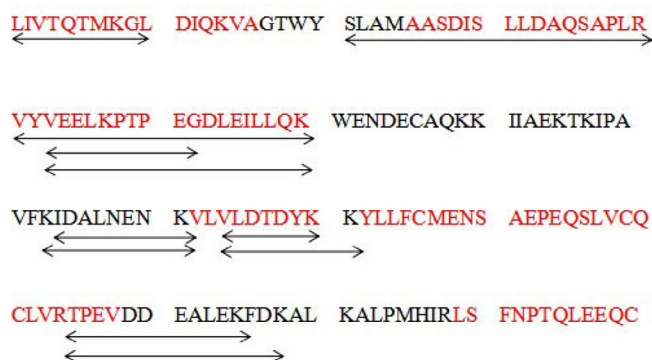
Figure 4. The antigenicity of reconstituted skim milk (RSM) and the fermented RSM. 1 = heated RSM; 2 = *Lactobacillus helveticus* KLDS 1.8701; 3 = *Lactobacillus plantarum* KLDS 1.0386; 4 = *L. helveticus* KLDS 1.8701 + KLDS 1.0386. Different letters (a₁–d₁) indicate significant differences ($P < 0.05$) among groups (α -CN); different letters (a₂–d₂) indicate significant differences ($P < 0.05$) among groups (β -LG); different characters (a₃–d₃) indicate significant differences ($P < 0.05$) among groups (α -LA).



Figure 5. Sequence of β -LG (A), α_{S1} -CN (B), and α_{S2} -CN (C) and peptides released by the proteolytic action of the combined strains of the *Lactobacillus helveticus* KLDS 1.8701, indicated by arrows. The main allergenic epitopes of the α -CN and β -LG have been marked in red (Séio et al., 1999; Järvinen et al., 2001; Busse et al., 2002; Ruiter et al., 2006; Cong et al., 2013).

Figure 6. Sequence of β -LG (A), α_{S1} -CN (B), and α_{S2} -CN (C) and peptides released by the proteolytic action of the *Lactobacillus plantarum* KLDS 1.0386, indicated by arrows. The main allergenic epitopes of the α -CN and β -LG have been marked in red (Séio et al., 1999; Järvinen et al., 2001; Busse et al., 2002; Ruiter et al., 2006; Cong et al., 2013).

A



HI

B



C

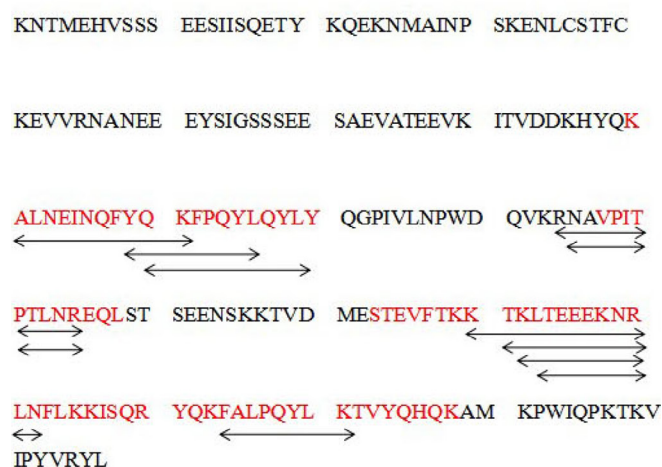


Figure 7. Sequence of β -LG (A), α_{S1} -CN (B), and α_{S2} -CN (C) and peptides released by the proteolytic action of the *Lactobacillus helveticus* KLDS 1.8701 and *Lactobacillus plantarum* KLDS 1.0386, indicated by arrows. The main allergenic epitopes of the α -CN and β -LG have been marked in red (Sélo et al., 1999; Järvinen et al., 2001; Busse et al., 2002; Ruiter et al., 2006; Cong et al., 2013).

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ORCIDS

Smith Etareri Evvie  <https://orcid.org/0000-0002-4209-0978>

Bailiang Li  <https://orcid.org/0000-0002-4115-655X>

Guicheng Huo  <https://orcid.org/0000-0002-2617-9737>