

Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins

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Abstract

BACKGROUND: The main whey proteins α -lactalbumin (α -LA) and β -lactoglobulin (β -LG) are considered as the major allergens in cow's milk. Microbial fermentation can produce some proteolytic enzymes, which can induce the degradation of milk protein allergens. In this study, the effects of fermentation by lactic acid bacteria on the antigenicity of α -LA and β -LG were investigated using indirect competitive ELISA. Meanwhile, the proteolysis of milk proteins was detected by TNBS assay and SDS-PAGE electrophoresis.

RESULTS: Fermentation by lactic acid bacteria could significantly reduce the antigenicity of α -LA and β -LG in skim milk. Combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus* were the most effective in reducing the antigenicity of both whey proteins. In addition, α -LA and β -LG antigenicity decreased to a lower value at 6 h of fermentation and at 0.5 d of cold storage by fermentation with the combined strains. The results of TNBS assay and SDS-PAGE electrophoresis showed that lactic acid bacteria strains used in this study hydrolysed whey proteins only to a limited extent.

CONCLUSION: The fermentation with lactic acid bacteria is an effective way to reduce whey proteins antigenicity.

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Keywords: cow's milk allergy; whey proteins; antigenicity; ELISA; fermentation; lactic acid bacteria

INTRODUCTION

Food allergy can cause serious health problems and has become a major public health concern around the world. Milk and milk products are considered as one of the most common allergens. Cow milk protein allergy (CMPA) is reported to be the most prevalent for infants or young children with an incidence of 2% to 6%.¹ Milk proteins are the first exogenous proteins consumed in large quantities by children.² All cow milk proteins may be potential allergens, of which the main allergens were found to be β -lactoglobulin (β -LG) and α -lactalbumin (α -LA), and they can cause immunologically mediated adverse reactions.^{3–5} Therefore, the reduction or elimination of these major milk allergens by effective methods and technologies will be essential to individuals who are allergic to milk.

Fermentation is a traditional processing technology in the food industry. Fermented foods exert a positive influence on human health or physiology, mainly due to their ability to release bioactive peptides from major food proteins by microbial enzymatic hydrolysis. It has been shown that lactic acid bacteria possess a complex proteolytic system composed of proteinases, peptidases and transport systems.^{6,7} During the fermentation by lactic acid bacteria, the hydrolysis of milk proteins may have important effects on milk digestibility and the production of bioactive peptides. Moreover, the proteolysis can destroy some epitopes and consequently decrease milk allergenicity.^{8,9} In addition, the dietary consumption of probiotics and fermented foods, such as yogurt, can alleviate some symptoms of atopy and reduce the development of allergies through the mechanism of immune regulation. It has been indicated that fermented foods containing lactic acid bacteria can enhance the expression and secretion of

both Type I and Type II interferons at the systemic level, which may explain the immunoregulatory properties of lactic acid bacteria.⁸

It has been demonstrated that fermentation can induce the degradation of some food allergens. Song *et al.*¹⁰ found that natural and induced fermentation significantly reduced IgE immunoreactivity up to 89% in soybean meal. It was shown that by immunoblotting with sera from allergic patients, wheat sourdough fermentation caused the disappearance of some IgE-binding proteins due to proteolytic activity by selected sourdough lactic acid bacteria.¹¹ Barkholt *et al.*¹² showed that fermentation with three lactic acid bacteria and *R. microsporus* reduced the antigenicity to 10% of the antigenicity of the unfermented pea flour. In addition, it was also observed that the antigenicity of whey proteins in sterilized cow's milk was reduced by over 99% as compared with raw milk after the fermentation with selected lactic acid bacteria.¹³ Kleber *et al.*¹⁴ indicated that lactic acid fermentation attenuated β -LG antigenicity in skim milk and sweet whey, but did not eliminate antigenicity.

The fermentation by different lactic acid bacteria could reduce the antigenicity of milk proteins. Besides, the reduction of milk protein antigenicity depends on the types of lactic acid

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bacteria and fermentation conditions. However, the influences of fermentation conditions, such as fermentation time on the antigenicity of whey proteins have not been studied systematically. In addition, there has been little research investigating the effect of synergistic action of different lactic acid bacteria on the antigenicity of milk proteins during fermentation process. In this study, three strains of lactic acid bacteria were selected to ferment fresh cow milk. The objectives of this study were to investigate the effects of different lactic acid bacteria, ferment time and cold storage time on the antigenicity of whey proteins and to explore the possible mechanism by which fermentation could reduce the antigenicity of whey proteins.

MATERIALS AND METHODS

Materials

Fresh milk from infection-free cows was provided by Beijing SanYuan Foods Co., Ltd. The content of protein, lipid and total dry material in the fresh milk was 31.2 g kg^{-1} , 38 g kg^{-1} and 124.5 g kg^{-1} respectively.

The antigen proteins used for sensitization tests and enzyme-linked immunosorbent assay (ELISA) were α -LA from bovine milk (L5385; purity $\geq 85\%$) and β -LG from bovine milk (L3908; purity, 90%) purchased from Sigma Chemical Company (St Louis, MO, USA). The first antibody from rabbits was prepared by our own laboratory and the second antibody, a conjugate of goat anti-rabbit immunoglobulin with peroxidase, was also purchased from Sigma. All other chemical agents used were of analytical grade.

Preparation of polyclonal antibodies

Antibodies were obtained from New Zealand white rabbits sensitised with bovine α -LA (Sigma, L5385) and β -LG (Sigma, L3908), respectively, according to the immunisation protocol described in our previous study.¹⁵ For the first immunisation, rabbits were injected intramuscularly with $1.0 \text{ mg } \alpha$ -LA or β -LG per kg weight that was dissolved in 9 g L^{-1} sterile sodium chloride and emulsified with an equal amount of Freund's complete adjuvant. Two weeks later the rabbits were subjected to a subcutaneous injection with the same dose of immunising antigen in the presence of incomplete Freund's adjuvant. The next two immunisations were made with the same dose of immunising antigen without adjuvant every 2 weeks.

Blood samples were drawn from the immunised rabbit marginal vein 2 days before the subsequent scheduled immunisation and the production and titer of antibodies were controlled by the indirect ELISA method. Ten days after the fourth sensitisation, blood samples were obtained from the rabbit hearts. Blood samples were incubated at room temperature for 1 h and left overnight at 4°C , followed by centrifuging at $3000 \times g$ for 20 min to get sera. The sera were then stored at -20°C until the following analysis on the antigenicity of α -LA and β -LG by the indirect competitive ELISA.

The culture of microorganisms

Lactobacillus bulgaricus (strain S-5) was obtained from the College of Food Science and Nutritional Engineering, China Agricultural University (Beijing, China); *L. helveticus* (strain B1) and *S. thermophilus* (strain 9Y) were obtained from Jilin Academy of Agricultural Sciences (Changchun, China). The three strains were cultured overnight in MRS-broth or M17-broth and incubated at 37°C or 42°C . Then they were subcultured in the same culture

medium as above for two to three generations in order to activate them.

Fermentation of milk

The milk samples were defatted and then heated in a water bath at 90°C for 5 min to remove undesirable microbial growth, similar to the industrial process of making fermented milk products. After heat treatment, the milk was rapidly cooled to 45°C , and then inoculated with 20 mL L^{-1} of lactic acid bacteria strains and cultured at 37°C for *L. bulgaricus* and *L. helveticus* or 42°C for *S. thermophilus* and combined strains, respectively. In another trial, the milk was fermented with lactic acid bacteria until the coagulum was formed and the fermented milk was then stored at 4°C . Samples were taken from the substrates with certain hours intervals during the fermentation or during the cold storage at 4°C , followed by mixing and centrifuging at $6000 \times g$ for 15 min, and finally the resulting whey was portioned and kept frozen (-20°C) until analysis. Unfermented heated milk served as controls.

Antigenicity analysis by the indirect competitive ELISA

The antigenicity of α -LA and β -LG in fermented milk was estimated by the indirect competitive ELISA. Microtitre plates with 96 wells (flat-bottomed; Costar; Corning, New York, USA) were coated with $100 \mu\text{L}$ per well of antigen diluted in 50 mmol L^{-1} carbonate buffer (pH 9.8) at the concentration determined earlier in the indirect ELISA method ($2 \mu\text{g mL}^{-1}$ for α -LA and $0.5 \mu\text{g mL}^{-1}$ for β -LG), and incubated overnight at 4°C . Sample solutions and standard antigen (α -LA and β -LG) were respectively incubated overnight at 4°C in test tubes with an equivalent volume of rabbit anti- α -LA serum (1:128 000) or anti- β -LG serum (1:6400) diluted in 10 mmol L^{-1} phosphate-buffered saline (PBS, pH 7.4) containing 10 g L^{-1} bovine serum albumin (BSA) and 1 g L^{-1} Tween 20 (PBS-BSA-T). The plates were washed four times the next day with 10 mmol L^{-1} PBS (pH 7.4) containing 0.5 g L^{-1} Tween 20 (PBS-T). This washing procedure was repeated after each analytical step. After washing the plates, the wells were filled with $100 \mu\text{L}$ per well of PBS-BSA-T to block residual free binding sites and incubated at 37°C for 1 h. The plates were washed and then $100 \mu\text{L}$ per well of reactive mixtures of samples or standard antigen and polyclonal rabbit antibodies (IgG) were added and incubated at 37°C for 1 h. After washing the plates, the wells were filled with $100 \mu\text{L}$ per well of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Sigma A 6154, 1:10 000) in PBS-BSA-T. After incubation at 37°C for 1 h, the plates were washed again and then $100 \mu\text{L}$ per well of 3,3',5,5'-tetramethylenbenzidine (TMB; Amresco, Ohio, USA) substrate solutions were immediately added. The plates were incubated at 37°C for 15 min. Finally, $50 \mu\text{L}$ per well of 2 mol L^{-1} H_2SO_4 were added to stop the reaction. Absorptions were determined at dual wavelengths of 450 nm and 630 nm by Multiskan MK3 ELISA plate reader (Thermo Labsystems, Franklin, MA, USA).

The antigenicity was calculated from standard curves of α -LA and β -LG, and a linear logarithmic correlation was observed in the range of $0.05\text{--}200 \mu\text{g mL}^{-1}$ for α -LA and in the range of $0.01\text{--}400 \mu\text{g mL}^{-1}$ for β -LG. All analyses were carried out in triplicate and the averaged values were converted to concentration equivalents in milligrams per millilitre.

Inhibition rate (%) of the antigenicity of α -LA and β -LG by lactic acid fermentation was calculated as follows: inhibition rate (%) = $[(B_0 - B)/B_0] \times 100$, where B is the antigenicity of α -LA or β -LG in fermented milk and B_0 is the antigenicity of α -LA or β -LG in unfermented heated milk.

Determination of free amino group content

The content of free amino groups in fermented milk was determined according to the trinitrobenzene sulfonic acid (TNBS) method.¹⁶ The absorbance was measured at 420 nm, and was transformed into mmol of leucine L⁻¹ using a calibration curve within the range of 0.25–3.5 mmol L⁻¹.

SDS-PAGE analysis of fermented milk

The fermented milk was performed on 120 g L⁻¹ acrylamide plates according to Laemmli.¹⁷ The resolving gel was prepared in 1.5 mol L⁻¹ Tris-HCl buffer at pH 6.8 and the stacking gel comprising 40 g L⁻¹ acrylamide was diluted by 0.5 mol L⁻¹ Tris-HCl buffer (pH 8.8). Samples were prepared in denaturing buffer with β -mercaptoethanol and heated at 100 °C for 3 min before electrophoresis.

The gels were run in a Tris-glycine buffer (pH 8.3) and the electrophoresis was carried out at a constant voltage of 80 V for 1 h in the stacking gel, and then 100 V for 1.5 h in the resolving gel. After electrophoresis, proteins in gels were stained with Coomassie Brilliant Blue R-250.

Statistical analysis

The results were statistically analysed by one-way analysis of variance (ANOVA) using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA). The significance of the dates was assessed by Duncan's multiple-range test. A *P* value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Changes in whey proteins antigenicity by different lactobacillus fermentation

Besides lactic acid bacteria, proteases from other microorganisms, animals or plants were also used to hydrolyse milk proteins and decrease their antigenicity.^{3,18,19} However, some bitter peptides were produced during the hydrolysis process, which greatly affected the development and application of the hypoallergenic milk products.^{20,21} The hydrolysis of lactic acid bacteria during the fermentation not only reduced the antigenicity of milk proteins, but also made the hydrolysate taste good and generated many bioactive substances. In the present study, we selected two traditional starters (*L. bulgaricus* and *S. thermophilus*) and one functional probiotic (*L. helveticus*) to ferment the milk.

The antigenicity of whey proteins was determined after fermentation for 12 h by each single strain or combined strains, and the results were presented in Table 1. Since the preheat treatment for commercial fermented milk is usually at 90 °C for 5 min, in this research prior to fermentation all milk samples were heated at 90 °C for 5 min to prevent undesirable microbial growth. It can be seen from Table 1 that, after the fermentation with lactic acid bacteria, the antigenicity of whey proteins significantly decreased, with an inhibition rate of 53–87% for α -LA and 86–95% for β -LG as compared with unfermented milk (*P* < 0.05). The reduction of the antigenicity suggested that in the fermentation process some epitopes of milk proteins were destroyed due to the hydrolysis by proteolytic enzymes. Kleber *et al.*¹⁴ studied the effect of different lactic acid bacteria on the antigenicity of β -LG in skim milk by a treatment at 90 °C for 40 min and found that the antigenicity of β -LG decreased by 84–98% compared with unfermented skim milk. Jedrychowski and Wroblewska¹³ claimed that after the fermentation with lactic acid bacteria the antigenicity

Table 1. Effect of fermentation by different lactic acid bacteria on the antigenicity of α -LA and β -LG

Sample	Antigenicity of α -LA (mg mL ⁻¹)	Inhibition rate (%) [*]	Antigenicity of β -LG (mg mL ⁻¹)	Inhibition rate (%) [*]
Unfermented milk	1.69 ± 0.20 ^a	–	1.72 ± 0.19 ^a	–
<i>L. bulgaricus</i>	0.80 ± 0.03 ^b	53	0.18 ± 0.01 ^{bc}	89
<i>L. helveticus</i>	0.49 ± 0.03 ^c	71	0.09 ± 0.01 ^c	95
<i>S. thermophilus</i>	0.87 ± 0.07 ^b	49	0.24 ± 0.01 ^b	86
<i>L. bulgaricus</i> + <i>S. thermophilus</i>	0.25 ± 0.04 ^d	86	0.12 ± 0.02 ^{bc}	93
<i>L. helveticus</i> + <i>S. thermophilus</i>	0.22 ± 0.00 ^d	87	0.08 ± 0.01 ^c	95

^{*} Inhibition rate is compared to unfermented milk.

^{a–d} Means ± SD (standard deviation) for three experiments within a column sharing common lower-case letters were not significantly different (*P* > 0.05).

of sterilised milk (110 °C, 10 min) was reduced by over 99%. These results suggested that the preheating temperature and time of fermented milk might have a significant impact on the changes in the antigenicity of whey proteins.

It can be seen from Table 1 that the inhibition rate of β -LG antigenicity in the fermented milk was higher than that of α -LA. This result may be due to the stronger hydrolysis capacity of some protease from lactic acid bacteria on β -LG as compared with α -LA. In addition, thermal stability of β -LG is poorer than that of α -LA, and hence, preheating treatment before fermentation can lead to the denaturation of β -LG and the unfolding of the conformational structure, thus making β -LG easier to hydrolyse. However, Tzvetkova *et al.*²² studied the hydrolysis of 21 kinds of lactic acid bacteria from Balkan yogurt on milk protein and found that the hydrolysis capacity of lactic acid bacteria on the α -LA was higher than β -LG by electrophoresis and RP-HPLC analysis. This difference may be explained by the fact that there is a great diversity in the hydrolysis ability and the specificity of the proteinases from different lactic acid bacteria strains.^{23,24}

In addition, the fermentation with combined strains of *L. helveticus* and *S. thermophilus* was the most effective way to reduce the antigenicity of α -LA and β -LG compared with *L. helveticus* alone and *S. thermophilus* alone in this study. It can be seen from Table 1 that α -LA antigenicity was reduced by 87% with combined strains fermentation of *L. helveticus* and *S. thermophilus*; however, α -LA antigenicity was inhibited by 71% and 49% with *L. helveticus* alone and *S. thermophilus* alone, respectively. On the whole, the combination of different lactobacilli strains with *S. thermophilus* at a ratio of 1 : 1 resulted in more reduction of the antigenicity of α -LA and β -LG than the single strains, suggesting that the combined strains may have synergistic actions on the reduction of whey proteins antigenicity.

Effects of fermentation time on whey proteins antigenicity and hydrolysis degree

Some proteases were released during lactic acid fermentation and the amount and characteristics of these proteases were different. In order to estimate the degree of hydrolysis of milk protein and investigate protease activity of lactic acid bacteria during fermentation, the content of free amino groups in fermented milk was determined by TNBS assay. Figure 1 displayed the changes

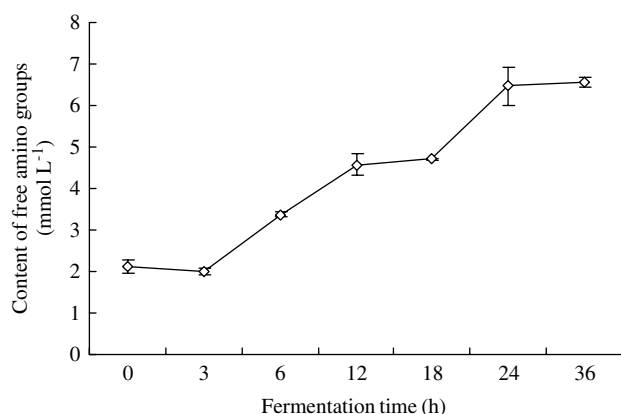


Figure 1. Changes in content of free amino groups in fermented milk during fermentation by combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus*.

Table 2. Effect of fermentation time on the antigenicity of α -LA and β -LG by combined strains fermentation of *Lactobacillus helveticus* and *Streptococcus thermophilus*

Fermentation time (h)	Antigenicity of α -LA (mg mL ⁻¹)	Inhibition rate (%) [*]	Antigenicity of β -LG (mg mL ⁻¹)	Inhibition rate (%) [*]
0	0.82 ± 0.08 ^a	51	0.32 ± 0.03 ^a	81
3	0.64 ± 0.07 ^b	62	0.26 ± 0.03 ^b	85
6	0.34 ± 0.02 ^c	80	0.11 ± 0.01 ^e	94
12	0.23 ± 0.01 ^d	87	0.09 ± 0.01 ^e	95
18	0.21 ± 0.02 ^d	88	0.12 ± 0.01 ^{de}	93
24	0.22 ± 0.03 ^d	87	0.14 ± 0.00 ^{cd}	92
36	0.20 ± 0.02 ^d	88	0.15 ± 0.01 ^c	91

^{*} Inhibition rate is compared to unfermented milk.

^{a-e} Means ± SD (standard deviation) for three experiments within a column sharing common lower-case letters were not significantly different ($P > 0.05$).

in the content of free amino groups during fermentation process with the combined strains of *L. helveticus* and *S. thermophilus*. It could be observed that the content of free amino groups increased with the increasing fermentation time, which indicated that the hydrolysis degree of milk proteins was gradually enhanced with extending fermentation time.

The effect of fermentation time on the antigenicity of α -LA and β -LG is shown in Table 2. In the process of fermentation, compared with unfermented milk, the antigenicity of α -LA and β -LG was inhibited by 51–88% and 81–95%, respectively. In addition, at 6 h of fermentation by combined strains of *L. helveticus* and *S. thermophilus*, the antigenicity of α -LA and β -LG significantly decreased ($P < 0.05$). This may be related to the result that the milk coagulated at about 6 h of fermentation where the microbial activity was higher and the amount of proteases produced were more, thus causing a significant reduction of the antigenicity of whey proteins.

Changes in the antigenicity of α -LA and β -LG varied with the fermentation time. From 0 h to 18 h, the antigenicity of α -LA gradually decreased, while its antigenicity had only slight changes when the fermentation was at 24 h and 36 h. The decrease of antigenicity may be due to the fact that microbial proteases produced by the hydrolysis split the epitopes on whey proteins.

The antigenicity of β -LG showed a gradual decrease trend from 0 h to 12 h, but its antigenicity had a slight increase from 18 h to 36 h. This small increase in β -LG antigenicity at longer fermentation time was probably because peptidases further cleaved the peptides into smaller peptides and amino acids,⁷ resulting in the exposure of some hidden epitopes or linear epitopes.

As can be seen from Fig. 1 with the extended fermentation time, the hydrolysis degree of milk protein increased gradually. However, neither the antigenicity of α -LA nor that of β -LG gradually decreased with the fermentation time (Table 2), which indicated that there was not a linear correlation between the hydrolysis degree of milk protein and the antigenicity of whey proteins during the fermentation.

Effects of cold storage time on whey proteins antigenicity and hydrolysis degree

In the process of the yogurt production, the cooling treatment after fermentation is widely used in order to make yogurt characteristics (texture, taste, flavour, acidity) achieve the set requirements. Lactic acid bacteria were still very active within 24 h of cold storage after fermentation, whereas with the extension of cold storage time, the microbial growth became slow. In this present study, the effect of cold storage time on the antigenicity of whey proteins by fermentation with the combined strains of *L. helveticus* and *S. thermophilus* was investigated. The changes in free amino group contents in fermented milk during cold storage at 4 °C were also determined. It was observed from Fig. 2 that the content of free amino groups slowly increased without regularity during the whole cold storage. This result may be attributed to the possible case that during cold storage, microorganisms for their own growth in milk take up the free amino acids released from the protein by the hydrolysis.²⁵

Changes in the antigenicity of α -LA and β -LG in fermented milk during cold storage were showed in Table 3. In the early stage of cold storage (0–2 d), it was observed that there was an obvious change in the antigenicity of α -LA and β -LG, and at 0.5 d of the fermentation, the antigenicity decreased to a lower value, which may be related to the relatively higher proteolytic activity of microbial proteases at this time. However, the antigenicity of both proteins had a smaller change in the late stage of cold storage (3–7 d). Although at this stage microbial protease still hydrolysed milk protein, the hydrolysis ability of specific enzymes on α -LA and β -LG may be reduced. In addition, after the long time of cold

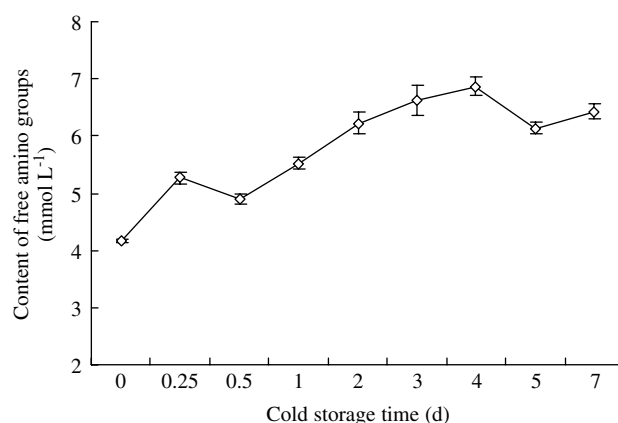


Figure 2. Changes in content of free amino groups in fermented milk during cold storage at 4 °C.

Table 3. Effect of cold storage time on the antigenicity of α -LA and β -LG by combined strains fermentation of *Lactobacillus helveticus* and *Streptococcus thermophilus*

Storage time (days)	Antigenicity of α -LA (mg mL ⁻¹)	Inhibition rate (%) ^a	Antigenicity of β -LG (mg mL ⁻¹)	Inhibition rate (%) ^a
0	0.37 ± 0.02 ^a	78	0.19 ± 0.02 ^{ab}	89
0.25	0.34 ± 0.01 ^b	80	0.20 ± 0.03 ^a	88
0.5	0.21 ± 0.00 ^d	87	0.11 ± 0.01 ^e	94
1	0.23 ± 0.02 ^d	86	0.17 ± 0.02 ^{bc}	90
2	0.28 ± 0.01 ^c	83	0.15 ± 0.02 ^{cd}	91
3	0.27 ± 0.00 ^c	84	0.13 ± 0.01 ^{de}	92
4	0.28 ± 0.02 ^c	83	0.12 ± 0.01 ^e	93
5	0.27 ± 0.01 ^c	84	0.12 ± 0.02 ^e	93
7	0.26 ± 0.03 ^c	85	0.11 ± 0.01 ^e	94

^a Inhibition rate is compared to unfermented milk.

^{a-e} Means ± SD (standard deviation) for three experiments within a column sharing common lower-case letters were not significantly different ($P > 0.05$).

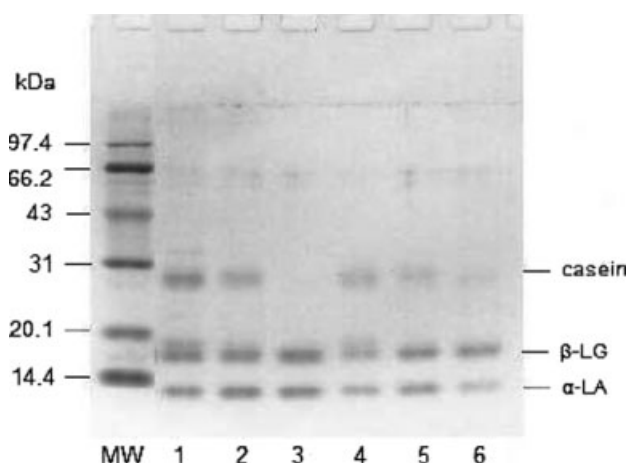


Figure 3. SDS-PAGE analysis of milk proteins by fermentation with different lactic acid bacteria. MW: molecular weight markers; lane 1: unfermented milk; lanes 2–6: fermented milk with different lactic acid bacteria, lane 2: *Lactobacillus bulgaricus*; lane 3: *Lactobacillus helveticus*; lane 4: *Streptococcus thermophilus*; lane 5: *Lactobacillus bulgaricus* + *Streptococcus thermophilus* (1 : 1); lane 6: *Lactobacillus helveticus* + *Streptococcus thermophilus* (1 : 1).

storage the growth and metabolism of lactic acid bacteria slowed down, which also led to the reduction in producing proteases.

Similarly, it could be seen from Fig. 2 and Table 3 that there was no definite correlation between the hydrolysis degree of milk proteins and the changes in α -LA and β -LG antigenicity during the cold storage.

SDS-PAGE analysis of whey proteins after fermentation

Changes of whey protein concentration after fermentation were analysed using SDS-PAGE electrophoresis. Figure 3 shows the whey protein pattern after the fermentation by different lactic acid bacteria. It could be seen from Fig. 3 that in different fermented samples the concentration of α -LA and β -LG decreased to different degrees compared with unfermented heated milk. Through the fermentation with combined strains of *L. helveticus* and *S. thermophilus*, α -LA and β -LG concentration had an obvious reduction, possibly because of the high hydrolysis ability of

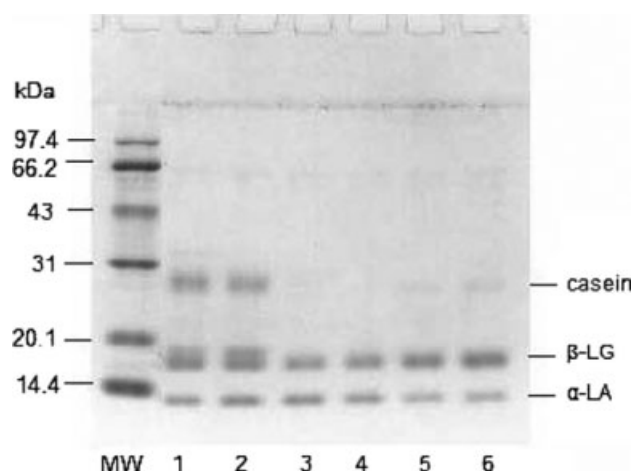


Figure 4. SDS-PAGE analysis of milk proteins at different fermentation time with combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus*. MW: molecular weight markers; lane 1: unfermented milk; lanes 2–6: fermented milk at different fermentation time, lane 2: 0 h; lane 3: 6 h; lane 4: 12 h; lane 5: 24 h; lane 6: 36 h.

L. helveticus and *S. thermophilus* on both proteins. This may also explain the result that the antigenicity of α -LA and β -LG became lower by fermentation with combined strains of *L. helveticus* and *S. thermophilus* (Table 1).

Changes in whey protein concentration at different fermentation time by the combined strains of *L. helveticus* and *S. thermophilus* was observed in Fig. 4. It was seen from the electrophoresis pattern that at 0 h of fermentation the concentration of α -LA or β -LG was not significantly different between the fermented milk and the unfermented milk. However, when fermentation time increased from 6 h to 36 h, the concentration of both whey proteins had a reduction compared with the unfermented milk; nevertheless, the changes were less significant with fermentation time increasing. These results showed that lactic acid bacteria used in this study hydrolysed whey proteins only to a limited extent, although they had significant proteolytic potential against whey proteins.

CONCLUSIONS

Lactic acid bacteria fermentation could significantly decrease the antigenicity of whey proteins in skim milk. Combined strains of *L. helveticus* and *S. thermophilus* were the most effective in reducing the antigenicity of α -LA and β -LG. Synergistic actions were observed between the combined strains. The antigenicity of α -LA and β -LG decreased to a lower value at 6 h of fermentation and at 0.5 d of cold storage. In future research, more strains of lactic acid bacteria will be screened to further reduce the antigenicity of milk proteins. Moreover, it is also interesting to decrease the antigenicity to the minimum level by means of the combination of various methods, such as fermentation and hydrolysis of proteases from animals or plants. These will give some help in the development of hypoallergenic milk products.

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REFERENCES

- 1 Hill DJ and Hosking CS, Cow milk allergy in infancy and early childhood. *Clin Exp Allergy* **26**:243–246 (1996).
- 2 Järvinen K-M and Suomalainen H, Development of cow's milk allergy in breast-fed infants. *Clin Exp Allergy* **31**:978–987 (2001).
- 3 Fritsche R, Role of technology in dairy allergy. *Aust J Dairy Technol* **58**:89–91 (2003).
- 4 Savilahti E, Heyman M, MacDonald T, Navarro J, Stern M, Strobel S, et al, Diagnostic criteria for food allergy with predominantly intestinal symptoms. *JPediatr Gastroenterol Nutr* **14**:108–112 (1992).
- 5 Sharma S, Pravindra K, Betzel C and Singh TP, Structure and function of proteins involved in milk allergies. *J Chromatogr B* **756**:183–187 (2001).
- 6 Bianchi-Salvadori B, Camaschella P and Cislighi S, Rapid enzymatic method for biotyping and control of lactic acid bacteria used in the production of yoghurt and some cheeses. *Int J Food Microbiol* **27**:253–261 (1995).
- 7 Law J and Haandrikman A, Proteolytic enzymes of lactic acid bacteria. *Int Dairy J* **7**:1–11 (1997).
- 8 Cross ML, Stevenson LM and Gill HS, Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria? *Int Immunopharmacol* **1**:891–901 (2001).
- 9 Bertrand-Harb C, Ivanova IV, Dalgarrondo M and Hartllé T, Evolution of β -lactoglobulin and α -lactalbumin content during yoghurt fermentation. *Int Dairy J* **13**:39–45 (2003).
- 10 Song Y-S, Frias J, Martinez-Villaluenga C, Vidal-Valverde C and Gonzalez de Mejia E, Immunoreactivity reduction of soybean meal by fermentation, effect on amino acid composition and antigenicity of commercial soy products. *Food Chem* **108**:571–581 (2008).
- 11 Rizzello CG, De Angelis M, Coda R and Gobbetti M, Use of selected sourdough lactic acid bacteria to hydrolyze wheat and rye proteins responsible for cereal allergy. *Eur Food Res Technol* **223**:405–411 (2006).
- 12 Barkholt V, Jørgensen PB, Sørensen D, Bahrenscheer J, Haikara A, Lemola E, et al, Protein modification of fermentation: effect of fermentation on the potential allergenicity of pea. *Allergy* **53**(Suppl. 46):106–108 (1998).
- 13 Jedrychowski L and Wroblewska B, Reduction of the antigenicity of whey proteins by lactic acid fermentation. *Food Agric Immunol* **11**:91–99 (1999).
- 14 Kleber N, Weyrich U and Hinrichs J, Screening for lactic acid bacteria with potential to reduce antigenic response of β -lactoglobulin in bovine skim milk and sweet whey. *Innovat Food Sci Emerg Technol* **7**:233–238 (2006).
- 15 Bu GH, Lu J, Zheng Z and Luo YK, Influence of Maillard reaction conditions on the antigenicity of bovine α -lactalbumin using response surface methodology. *J Sci Food Agric* **89**:2428–2434 (2009).
- 16 Adler-Nissen J, Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J Agric Food Chem* **27**:1256–1262 (1979).
- 17 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685 (1970).
- 18 El-Agamy EI, The challenge of cow milk protein allergy. *Small Ruminant Res* **68**:64–72 (2007).
- 19 Ena JM, van Beresteijn ECH, Robben AJPM and Schmidt DG, Whey protein antigenicity reduction by fungal proteinases and a pepsin/pancreatic combination. *J Food Sci* **60**:104–116 (1995).
- 20 van Beresteijn ECH, Peeters RA, Kaper J, Meijer RJGM, Robben AJPM and Schmidt DG, Molecular mass distribution, immunological properties and nutritive value of whey protein hydrolysates. *J Food Protect* **57**:619–625 (1994).
- 21 Vegarud GE and Langsrud T, The level of bitterness and solubility of hydrolysates produced by controlled proteolysis of casein. *J Dairy Res* **56**:375–379 (1989).
- 22 Tzvetkova I, Dalgarrondo M, Danova S, Iliev I, Ivanova I, Chobert JM, et al, Hydrolysis of major dairy proteins by lactic acid bacteria from Bulgarian yogurts. *J Food Biochem* **31**:680–702 (2007).
- 23 Ehn B-M, Allmere T, Telemo E, Bengtsson U and Ekstrand B, Modification of IgE binding to β -lactoglobulin by fermentation and proteolysis of cow's milk. *J Agric Food Chem* **53**:3743–3748 (2005).
- 24 Oberg CJ, Broadbent JR, Strickland M and McMahon DJ, Diversity in specificity of the extracellular proteinases in *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp *bulgaricus*. *Lett Appl Microbiol* **34**:455–460 (2002).
- 25 Lu JP, Luo CX and Liu FM, Studies on the proteolytic activity of various lactic acid bacteria fermented milk. *JNE Agric Univ* **30**:68–74 (1999).