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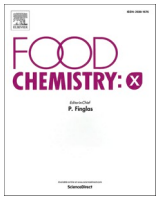

Allergenicity evaluation of fermented milk prepared by co-fermentation of *Lactobacillus plantarum* 7-2 and commercial starters after *in vitro* digestive

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ABSTRACT

Milk allergy is one of the most common food allergies, in which α_S-casein is the major milk allergen. Under optimized conditions, mixed starter (containing *Lactobacillus plantarum* 7-2 and commercial starter) effectively degraded α_S-casein of skimmed milk and reduced the pressure of stomach digestion. The fermented milk prepared by mixed starter was determined by ELISA, the antigenicity of α_S-casein was reduced by 77.53%. Compared with the fermented milk prepared by commercial starter, label-free quantitative proteomics demonstrated that the mixed starter more efficiently degraded the epitopes of major milk allergens and influenced the digestion pattern of the fermented milk. Therefore, *L. plantarum* 7-2 shows positive potential in reducing the antigenicity of α_S-casein and others. In addition, this study predicted that the new epitopes produced in the fermentation process could induce immunity using molecular simulation.

1. Introduction

Cow milk and dairy products are crucial components of daily life, providing essential protein and other nutrients for human, particularly for infants and children. In fact, dairy products may cause reproducible adverse cow milk allergy (CMA), despite their high nutritional value (Høst & Bahna, 2019). CMA is an immune-mediated response to cow milk protein, affecting various systems in the whole body including gastrointestinal digestion, skin, respiration and others. The prevalence of CMA in the general population is about 1 %–3 %, with the majority of affected individuals being infants and children (Bahna, 2002). As individuals grow older, it is commonly observed that the clinical symptoms of CMA in the general population tend to alleviate or even disappear. Besides, United Nations Food and Agriculture Organization (FAO) and World Health Organization (WHO) have officially recognized cow milk as one of the eight major food allergens, alongside nuts, crustaceans, peanuts, fish, wheat, eggs, and sesame (Baumert et al., 2022). Of note, adverse reactions to other foods often occur in infants with CMA, so it is an increasingly serious problem (Høst & Bahna, 2019).

Oral immunotherapy (OIT) has been recognized as an effective treatment for CMA, (Keet et al., 2012; Yeung et al., 2012). Although OIT can effectively induce desensitization in a majority of patients, it is essential to acknowledge that it may entail certain side effects and cannot completely replace the allergen avoidance strategy. Hence, the prevention and management of CMA primarily revolve around consuming dairy products low /without milk proteins, or the elimination of dairy products. The modern dairy industry has explored various processing methods to mitigate the impact of CMA, including physical, chemical, biological, and combined treatments (Aguiar, Veloso, Fernando, & Franco, 2017; Pereira et al., 2018). However, many of these methods have adverse effects on nutrition, safety, and cost (Pi et al., 2019). Enzymatic hydrolysis is one of the commonly used biological treatment to reduce CMA by breaking down allergenic epitopes, but the process of hydrolysis is accompanied by the production of bitter peptides (Clegg, Lim, & Manson, 1974; Cui, Sun, Zhou, Cheng, & Guo, 2021). Notably, fermentation of cow milk with lactic acid bacteria (LAB) is a more popular biological approach to reduce CMA. This method effectively reduces the antigenicity and allergenicity of dairy products, while simultaneously improves the physicochemical properties, flavor and nutritional value (Bu, Luo, Chen, Liu, & Zhu, 2013; Bu, Luo, Zhang, & Chen, 2010; El-Ghaish et al., 2011; Jedrychowski, 1999; Pereira et al., 2018).

Lactobacillus delbrueckii subsp. *bulgaricus* CRL 656 could reduce allergic reactions by hydrolyzing the three major epitopes of β-Lg ("VYVEELKPTPEGDLEILLQK", "YLLFCMENSAEPEQSLACQCLVR" and "LSFNPTQLEEQCHI"), while *L. delbrueckii* subsp. *bulgaricus* CRL 454 could also hydrolyze an additional fourth epitope ("CLVRTPEVDDEALEKFDKAL") (Pescuma et al., 2011, 2015). This indicated that the

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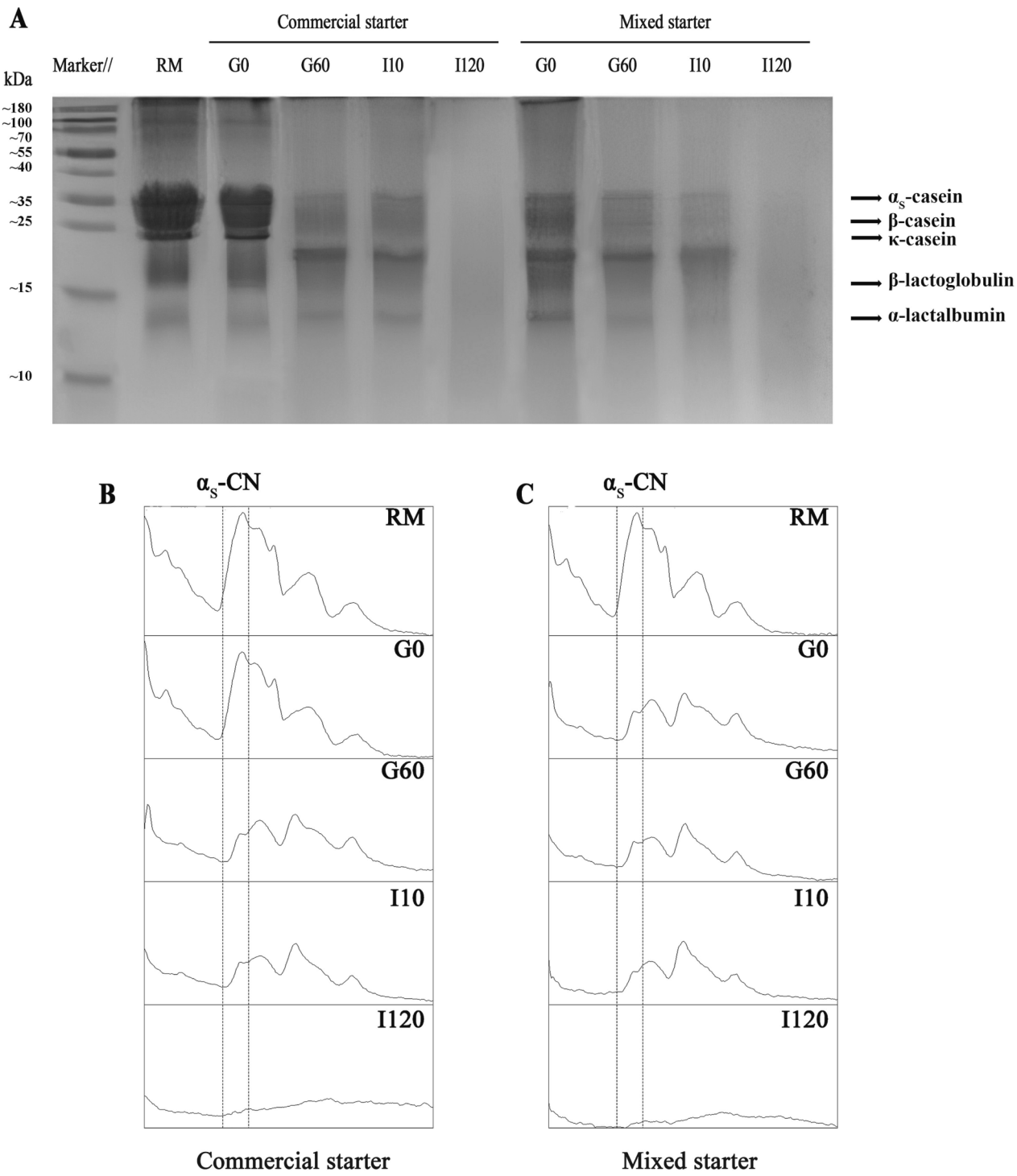

Fig 1. The hydrolysis of milk proteins by different starters (A) and Grey-level profiles of α_S-CN at different stages: B is the sample prepared by commercial starter and C is the sample prepared by mixed starter.

hydrolysis of allergenic epitopes by LAB was specific. *L. fermentum* IFO3956 reduced the immunogenicity of the protein by cleaving the peptide bond within the α_{S1}- casein (CN) epitope (El-Ghaish, Rabesona, Choiset, Sitohy, Haertlé, & Chobert, 2011). *L. paracasei* IN-10 was considered to potentially contribute to reducing the immunoreactivity of β-CN, since the hydrolytic properties of "RINKKIEKFQSEEQQTE-DELQDKIH" and "TVMFPPQSVLSLSKVLPV" (Hadji Sfaxi, El-Ghaish, Ahmadova, Rabesona, Haertlé, & Chobert, 2012).

In addition, the increase of food allergy has aroused great interest in digesting allergenic proteins (Moreno, 2007), and it has been found that digestion can also change the allergenic properties of milk proteins (Høst & Bahna, 2019). Zheng et al. (2014) investigated the effects of fermentation with *L. delbrueckii* subsp. *bulgaricus* strain S-5 and simulated *in vitro* gastrointestinal digestion on the antigenicity of four major allergens in cow milk. Fermentation reduced the antigenicity of three allergens apart from α_S-CN and is more effective for α-lactalbumin (α-La). Additionally, simulated gastrointestinal digestion could effectively reduce the antigenicity of four proteins in fermented milk, with α-La being particularly susceptible to simulated gastric treatment. Moreover, the fermentation of *L. casei* 1134 significantly reduced the

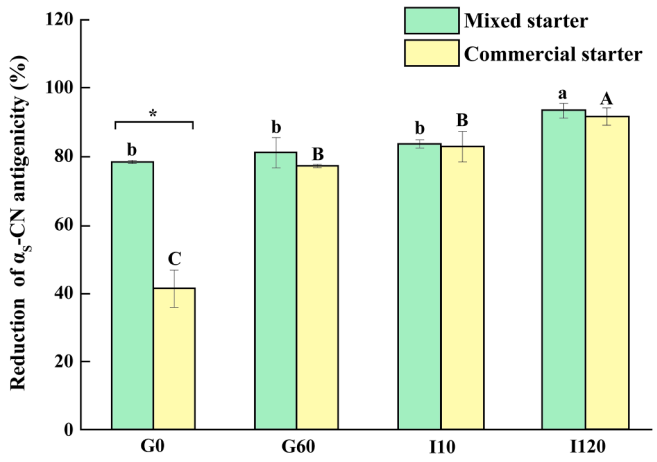

Fig 2. Reduction of α_S-CN antigenicity at different stages. Different letters represent statistical significance in the same fermented milk at different stages ($p < 0.05$). "*" represent statistical significance between different samples at the same stage ($p < 0.05$).

antigenicity and allergenicity of the four proteins, and the allergenicity of milk proteins was further reduced during simulated gastrointestinal digestion (Yao, Xu, Luo, Shi, & Li, 2015).

However, antigenic epitopes may also escape or resist digestion of proteins and pass through the intestinal lumen in intact form (Untersmayr & Jensen-Jarolim, 2006). The persistence of food epitopes when gastric digestion is blocked has been identified as a risk factor for food allergy induction. In the case of IgE-mediated allergies, the gastric mucosa, as the target organ of specific IgE reactions, plays a direct role in food-induced inflammation (Hogan & Rothenberg, 2006; Rothenberg, 2004). Individuals with immature or compromised intestinal barriers are more prone to experiencing non-IgE-mediated allergies.

The present work attempts to simulate the digestion process *in vitro* to investigate the different outcomes of fermented milk. Initially, the digestive fate of fermented milk was evaluated. Subsequently, Label-free quantitative proteomics approach of the breakdown of six major allergens (α-La, β-Lg, α_{S1}-, α_{S2}-, β- and κ-CN) in cow milk was performed. The changes of peptides were observed along the digestion time, and the determined peptides were mapped to the allergens sequence to clarify the enzymatic hydrolysis pattern of proteins that depend on the digestion time and residual allergenic epitopes.

2. Materials and methods

2.1. Starters

Commercial starter (*L. delbrueckii* subsp. *Bulgaricus* CICC 6047 and *Streptococcus thermophilus* CICC 6038, with a total viable count ratio of 1:1) is purchased from China Center of Industrial Culture Collection (Beijing, China). Mixed starter is a mixture of commercial starter and *L. plantarum* 7-2 with a total viable count ratio of 1:1, and *L.plantarum* 7-2 (A strain of bacteria that can reduce the antigenicity of α_S-CN(Mu et al., 2021)) was deposited in China Center for Type Culture Collection (CCTCC No. M 2020806 7-2)(Wuhan, China).

2.2. Fermented milk

The preparation of fermented milk was based on prior studies with some modifications (Mu et al., 2021). The 11 % (w/v) skimmed raw milk was prepared in sterilized flasks (100 mL/250 mL) and treated in boiling water bath for 30 min. After the milk was naturally cooled to room temperature in a sterile environment, 4 % starter (with a total viable count of approximately 2×10^8 CFU) was put into the skimmed raw milk and incubated at 37 ℃ for 18 h without shaking.

2.3. In vitro digestion experiments

In vitro simulated digestion of fermented milk was conducted, following the method described by Zhang et al. (2022). The fermented milk and simulated gastric fluid (SGF) were incubated at 37 ℃ for 10 min respectively, then mixed the two by equal volume, and SGF was collected at 0 min (G0) and 60 min (G60) of digestion, respectively. The rest of gastric fluid was adjusted to pH 6.5 and used as the initial sample for simulated intestinal digestion. The initial samples and the simulated intestinal fluid (SIF) were incubated at 37 ℃ for 10 min respectively, then mixed the two by equal volume, and the intestinal digestion samples were collected at 10 min (I10) and 120 min (I120) of digestion, respectively. The four collected digests were incubated in boiling water bath for 5 min to inactivate digestive enzymes, and the pH values were adjusted to 7.0 and stored at −20 ℃ for subsequent analysis.

2.4. Digestibility of samples in vitro

The digested product was mixed with trichloroacetic acid (65.4 %, w/v) solution in equal volume and incubated above 10 min at room temperature to precipitate the protein. The nitrogen (N) content of was determined by Kjeldahl method (K9860 Automatic Kjeldahl system, Hanon, China) and the digestibility of protein was calculated by the following formula (Jain, Jain, & Jain, 2020):

$$\text{N content}(\%) = \frac{(m_s - m_0) \times 6.38}{m_t} \times 100$$

m_s: N content in the supernatant of digested product;
m_0: N content in the supernatant of undigested sample;
m_t: total protein content in undigested samples.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and grey-level analysis

The relative molecular masses of the digestion products were analyzed by SDS-PAGE with some modifications (Wang, Feng, Jin, Murad, Mu, & Wu, 2022). The sample was diluted 10 times with deionized water, the same volume of 2× sample buffer was added, and was treat in the boiling water bath for 5 min. The sample solution of 10 μL was separated by 5 % stacked gel (80 V constant pressure) and 15 % acrylamide separation gel (120 V constant pressure). The sample gels were stained with Coomassie Blue Staining Solution (Beyotime Biotechnology, Shanghai, China). The gels were then decolorized with Coomassie Blue Staining Destaining Solution (Beyotime Biotechnology, Shanghai, China). Azure C300 Gel Imaging System (Azure Biosystems, California, USA) is used for protein band imaging. The grey-level of α_S-CN was analyzed using Image J analysis software (National Institutes of Health, Maryland, USA).

2.6. Double antibody sandwich enzyme-linked immunosorbent assay (ELISA)

The antigenic changes of α_S-CN in fermented milk at different stages of digestion were determined using double antibody sandwich ELISA. The ELISA kits were purchased from Wuhan Cloud-Clone Technology Co (Wuhan, China), and the method was performed as the instructions. The antigenicity of α_S-CN in unfermented skimmed raw milk was used as a control, and reduction of digestive products' antigenicity were calculated by the following formula:

$$\text{Reduction of antigenicity}\ (\%) = \left(1 - \frac{C_s}{C_0}\right) \times 100$$

C_s: antigen concentration of digested product;
C_0: antigen concentration of unfermented skimmed raw milk.

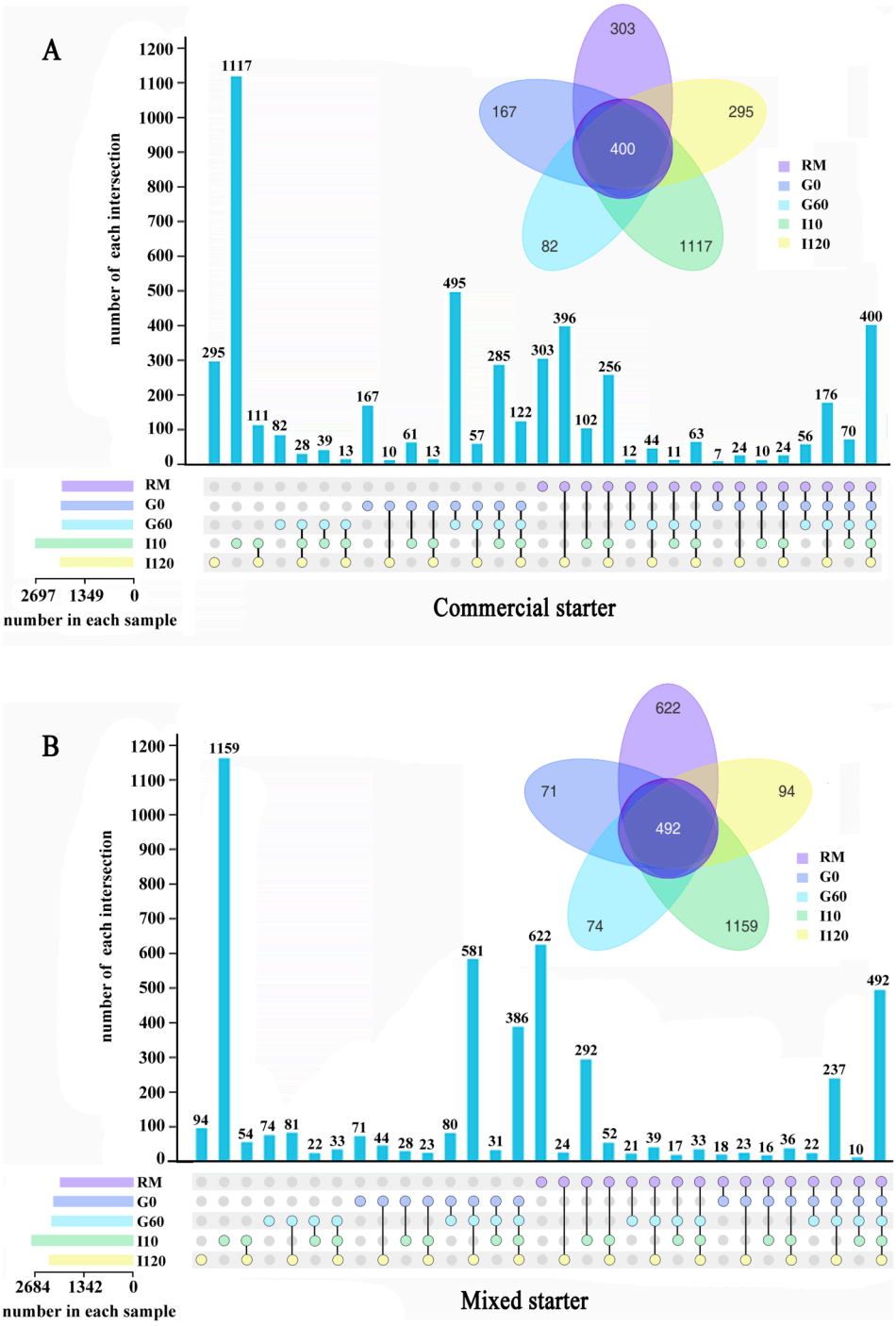

Fig 3. Intersection diagram of the peptides identified at different stages. A is the sample prepared by commercial starter and B is the sample prepared by mixed starter. The number on the bar chart indicates the number of peptides.

2.7. Analysis of peptides in the digestion products by label-free

To analyze the epitopes of the digestion products, label-free quantitative proteomics were performed at Shanghai Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). The bands of the allergen proteins stained by Coomassie were excised from SDS-PAGE gels (the bands were cut to 0.5 mm^3–1 mm^3). The bands were twice washed with distilled water (ThermoFisher Scientific, Shanghai, China), and then were washed with 25 mM Triethylammonium bicarbonate buffer (Sigma-Aldrich, Shanghai, China) in 50 % (v/v) acetonitrile at 37 ℃ over 30 min. After removing the supernatant, the bands were dried. The dried products were reduced with 25 mM buffer containing 50 mM dithiothreitol (Sigma-Aldrich, Shanghai, China) for 1 h at 56 ℃. The supernatant was removed, and the products were incubated with 25 mM buffer containing 100 mM iodoacetamide (Sigma-Aldrich, Shanghai, China) for 40 min at room temperature to alkylate. After washing, the modified products were dried and then incubated overnight at 37 ℃ with 0.01 mg/mL mass spectrometry grade modified Trypsin (Promega Corporation, Beijing, China). The supernatant was collected, washed with 0.5 % trifluoroacetic acid and 50 % acetonitrile, and completely dried for peptide quantification (Pierce Quantitative Colorimetric Peptide Assay, ThermoFisher Scientific, Shanghai, China) and label-free analysis. The separation and analysis of the samples were done by an Easy-nLC1200 coupled to a Q-Exactive mass spectrometer

Table 1
Identified peptides that exactly matched the liner epitopes of milk protein antigens in IEDB.

Antigen	Epitope ID	Epitope	Length
α-lactalbumin(Bos d 4)	558,397	CSEKLDQWLCEKL	13
	558,436	INYWLAHKALCSEKL	15
	2,150,165	FLDDDLTDDIMCVK	14
	161,701	STEYGLFQINNK	12
	558,425	FHTSGYDTQAIVQNN	15
	115,376	LTKCEVFREL	10
β-lactoglobulin(Bos d 5)	36,760	LIVTQTMK	8
	426	AASDISLLDAQSAPLR	16
	115,530	VAGTWYSLAM	10
	72,178	VYVEELKPTPEGDLEILLQK	20
	33,732	KTKIPAVFKI	10
	69,827	VLVLDTDYK	9
	222,188	CLVRTPEVDDEALEKFDK	18
	98,691	ALKALPMHIR	10
	39,349	LSFNPTQLEEQCHI	14
αS1-casein(Bos d 9)	31,145	KHQGLPQEVLNENLLRFF	18
	47,450	PEVFGKEKVN	10
	28,169	IQKEDVPSER	10
	74,687	YLGYLEQLLR	10
	190,579	VPQLEIVPNSAEERL	15
	190,447	IGVNQELAYFYPELF	15
	55,417	RQFYQLDAYPSG	12
	76,634	YYVPLGTQYT	10
	229,820	TDAPSFSDIPNPIGSENSEK	20
αS2-casein(Bos d 10)	115,437	QKALNEINQFYQKFPQYLQY	20
	95,682	PQYLQYLYQG	10
	115,462	QYLQYLYQGPIVLNPWDQVK	20
	115,512	TKKTKLTEEEKNRLNFLKKI	20
	95,237	ALPQYLKTVY	10
	115,384	MKPWIQPKTKVIPYVRYL	18
β-casein(Bos d 11)	53,557	RELEELNVPGEIVE	14
	115,709	DKIHPFAQTQ	10
	115,973	SLVYPFPGPI	10
	115,420	PLTQTPVVVPPFLQPEVMGV	20
	115,906	PVEPFTESQS	10
	115,726	ENLHLPLPLL	10
	116,019	VMFPPQSVLS	10
	115,850	LPVPQKAVPY	10
	42,283	MPIQAFLLYQEP	12
	115,763	GPVRGPFPII	10
	75,481	YQEPVLGPVR	10
κ-casein(Bos d 12)	115,915	QEQNQEQPIR	10
	115,733	ERFFSDKIAK	10
	116,037	YIPIQYVLSR	10
	116,039	YPSYGLNYYQ	10
	115,905	PVALINNQFL	10
	115,793	INNQFLPYPY	10
	115,979	SPAQILQWQV	10
	54,078	RHPHPHLSFM	10

(ThermoFisher Scientific, Shanghai, China), and the whole process was referred to Wang et al. (2018).

2.8. Data analysis

Mass spectrometry data analysis was referenced to Wang et al. (2018). The statistical data was analyzed using SPSS 22.0 (IBM, Illinois, USA) by single factor ANOVA. $p < 0.05$ was regarded as statistically significant.

3. Results and discussion

3.1. Digestion of fermented milk in vitro

The hydrolysis of proteins by lactic acid bacteria (LAB) fermentation is mainly dependent on cell envelope proteases (CEPs) and extracellular proteases (EPs), and the specificity of the enzymes directly affects the hydrolytic properties of LAB (El-Ghaish, Rabesona, et al., 2011; Hadji Sfaxi et al., 2012). Fig. S1 illustrates that the protein content in fermented milk decreases with increasing digestion time. During the initial period of digestion (G0), the protein content of commercial fermented milk (CFM) was much higher than that of fermented milk prepared with mixed starter (MFM), indicating that the addition of *L. plantarum* 7-2 enhanced the hydrolysis of milk proteins by the starter.

In addition, at the end of gastric digestion (G60), the protein contents of both fermented milks were similar (about 2.10 mg/mL), and the digestibility of CFM was higher than that of MFM, indicating that the efficiency of *L.plantarum* 7-2 in hydrolyzing milk proteins was similar to that of gastric digestion. At the end of *in vitro* digestion (I120), the content of milk protein in the MFM digestion product (0.98 mg/mL) was slightly lower than that in the CFM digestion product (1.33 mg/mL). In contrast, the fermented milk prepared by mixed starter was more effectively, indicating that the addition of *L.plantarum* 7-2 could promote the digestion of fermented milk.

3.2. SDS-PAGE analysis

SDS-PAGE was used to analyze the degradation of α_S-CN before and after fermentation and during digestion more visually. As shown in Fig. 1, compared with unfermented skimmed milk (RM), the protein bands of both fermented milks (G0) became weaker, especially MFM. This confirms that LAB fermentation effectively degrades milk proteins, and the effect of co-fermentation of multiple LAB was more significant. A similar pattern was reported by Bu et al. in their study on LAB fermentation of milk proteins (Bu et al., 2010). In addition, co-fermentation of multiple probiotics promotes the *in vitro* digestibility of fermented products (Manus, Millette, Uscanga, Salmieri, Maherani, & Lacroix, 2021). In this paper, the protein bands gradually weakened and eventually disappeared as digestion proceeded, these were in agreement with the above results, indicating that the addition of *L. plantarum* 7-2 contributes more than commercial starter to the gastrointestinal digestion of fermented milk.

3.3. Detection of antigenicity by ELISA

ELISA was used to determine the antigenicity of α_S-CN hydrolysate (Fig. 2). The results indicated a decrease in antigenicity throughout the digestion process, but even at the end of digestion, a certain level of antigenicity remained. The results of both fermented milks were compared at different digestion stages. At the early stage of digestion (G0), the antigens in both samples were decreased and the effect of the mixed starter was more significant ($p < 0.05$) at 77.53 %. The results indicated that the presence of *L. plantarum* 7-2 significantly enhanced the degradation efficiency of antigens compared to using a commercial starter alone. There was no significant change in antigenicity of MFM before and after gastric digestion ($p > 0.05$), while CFM exhibited the opposite trend. At the end of intestinal digestion (I120), the antigenicity of both samples further reduced, and the reduction of MFM (93.13 %) was slightly higher than that of CFM (89.47 %).

IgE-mediated cow milk hypersensitivity is initiated by a specific immune response to antigens by microfold cells (Membranous cells, M cells) in the gastrointestinal mucosa (Sakhon, Ross, Gusti, Pham, Vu, & Lo, 2015), and most non-IgE-mediated allergic reactions involve the gastrointestinal tract (Shek, Bardina, Castro, Sampson, & Beyer, 2005). Considering the observed hydrolysis phenomenon during digestion, it can be inferred that the hydrolysis properties of *L. plantarum* 7-2 may be

Fig 4. Schematic diagram of epitopes changes during digestion of α_{S1}-CN(A), α_{S2}-CN(B), β-CN(C) and κ-CN(D) *in vitro*. Color code red represents a high number of amino acids detected in the epitopes found in the sample, while the color code green represents a low number. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

similar to those of pepsin, as it reduces the digestive pressure of stomach, but with insignificant effects on intestinal digestion. In addition, it can be shown that the mixed starter containing *L. plantarum* 7-2 led to more effective cleavage of the antigens, thereby reducing the probability of allergic mechanism involving the gastrointestinal mucosa.

On the other hand, the impact of temperature on the antigenicity of proteins is not to be ignored. The processing of dairy products is inseparable from heat treatment, and heating affects the spatial structure of proteins. At some temperature, more epitopes will be exposed and subjected to the action of proteases within the system, promoting the hydrolysis of epitopes and thereby reducing antigenicity.

3.4. *Identification of peptides in fermented milk hydrolysates by label-free*

Fig. S2 illustrates the changes of polypeptide concentration. After fermentation by mixed starter, the concentration of peptides showed a significant increase ($p < 0.05$), while there was no obvious change ($p > 0.05$) observed after fermentation by commercial starter. At the end of digestion (I120), the peptide concentrations in the digestive products of both fermented milks were similar.

In addition, Fig. 3 shows the intersection plot of unique peptides identified in the samples at different hydrolysis stages. After successive fermentation and digestion of raw milk, the highest amounts of peptides were found in the hydrolysates of both fermented milks at the initial stage of intestinal digestion (I10), as well as the unique peptides. For CFM (Fig. 3A), a total of 4849 peptides were identified, and the numbers of unique peptides in the five stages (RM-I120) were 303, 167, 82, 1117 and 295, respectively. During the fermentation stage (RM-G0), the number of peptides in the sample increased from 1954 to 1977; In RM, 1187 peptides were hydrolyzed, accompanied by the generation of 1210 new peptides, and out of these hydrolyzed peptides, 303 peptides did not reappear until the end of gastrointestinal digestion. In the gastrointestinal digestion stage (G0-I120) of RM, 571 peptides in RM were completely disappeared, 983 peptides were hydrolyzed and then released again, and 400 peptides existed throughout. For MFM (Fig. 3B), a total of 4715 peptides were identified, which is more than that of CFM. This indicates the digestive fate of fermented milk prepared with different starters varies, which is related to the hydrolytic specificity of the bacterial strains that make up the starters. The numbers of unique peptides in the five stages (RM-I120) were 622, 71, 74, 1159 and 94, respectively. During the fermentation stage (RM-G0), the number of peptides in the sample increased from 1954 to 2098; In RM, 1100 peptides were hydrolyzed, accompanied by the generation of 1244 new peptides, and out of these hydrolyzed peptides, 622 peptides did not reappear until the end of gastrointestinal digestion. The results demonstrate that the changes in peptide profiles of both fermented milks

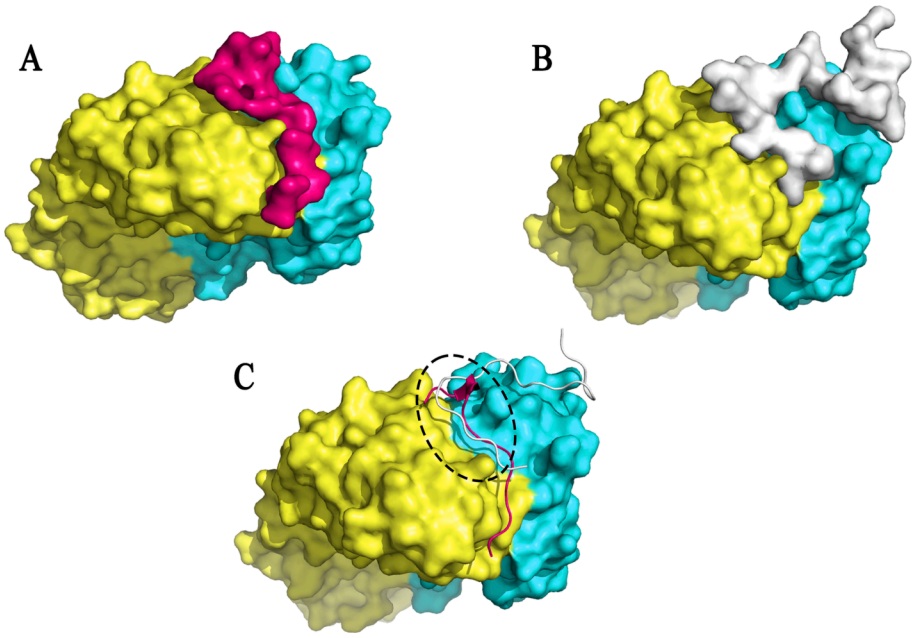

Fig 5. Schematic diagrams of docking between receptor and ligands. Epitope-R is in pink, epitope-V is in gray, receptor light chains are in yellow, and receptor heavy chains are in blue. There is a crossover region between the two docking results (A and B) in the dashed ellipse. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are similar, but the antigenicity changes of both fermented milks vary significantly ($p < 0.05$). This indicates that even though the peptide profiles are similar in both fermented milks, the addition of *L. plantarum* 7-2 more effectively hydrolyzes epitopes and releases non-epitope peptides. In the gastrointestinal digestion stage (G0-I120) of RM, 1018 peptides in RM were completely disappeared, 444 peptides were hydrolyzed and then released again, and 492 peptides existed throughout.

The relationship between different data sets at the same digestion stage was represent by Venn diagram (Fig. S3). The same peptides present between the two data sets were 1674, 1690, 2150, and 1015 at G0, G60, I10 and I120, respectively. The unique peptides peaked in both sets at I120 (Fig. S3D), it can be presumed that the digestive fate of both fermented milks is different, which may be attributed to the compositions of the starters.

3.5. Hydrolysis of liner epitopes of major antigens

By the analysis of the concentration and type of peptides, it can be further explained that the addition of *L. plantarum* 7-2 to the commercial starter alters the hydrolysis characteristics and subsequently affect the digestion process of fermented milk. Of note, the changes of antigenicity of milk proteins are inextricably linked to the concentration and number of peptides. Therefore, the hydrolysis of identified peptides that exactly matched the liner epitopes of milk protein antigens (Table 1) in Immune Epitope Database (IEDB, https://www.iedb.org/) was analyzed. Diagrams of the hydrolysis of epitopes are present in Fig. 4&S3.

In these images, each amino acid residue that was part of an epitope was color-coded according to its frequency of occurrence in the peptide mapping analysis. Comparing the results on the epitopes of the six major antigens at G0, it was observed that the hydrolysis of epitopes by different starters led to different incidences of the amino acid residues. This was attributed to differences in starters, such as the types of LAB present, proteolytic specificity and activity, and the interactions among bacteria (antagonism and synergism). In addition, comparison of the results of the digestion process (G0-I120) revealed that the incidence of residues in the digestive products of different fermented milks was different at the same stage. This was attributed to variations in the substrate of digestion due to differences in starters, resulting in different digestion outcomes.

For casein (Fig. 4), some epitopes were hydrolyzed after fermentation (G0), and the effect of mixed starter with *L. plantarum* 7-2 was better than that of commercial starter. Mixed starter caused the disappearance of three epitopes (PEVFGKEKVN, YLGYLEQLLR and VPQLEIVPNSAEERL) from α_{S1}-CN (Fig. 4A), one epitope (VMFPPQSVLS) from β-CN (Fig. 4C), and one epitope (ERFFSDKIAK) from κ-CN (Fig. 4D). Notably, "PEVFGKEKVN" never reappeared throughout the whole digestion. On the contrary, commercial starter fermented RM released some epitopes, including one epitope from β-CN that did not exist in the raw milk initially—"RELEELNVPGEIVE" (Fig. 4C). Notably, Chatchatee et al. (2001) have confirmed that the epitope is a major ligand for both IgE and IgG.

To speculate on the allergenic potential of the epitope "RELEELNVPGEIVE" (epitope-R), the fragment was subjected to semi-flexible molecular docking via HPEPDOCK2.0 online server (https://huanglab.phys.hust.edu.cn/hpepdock/, School of Physics, Huazhong University of Science and Technology) (Tao, Zhang, & Huang, 2020). The receptor is a recombinant IgE Fab fragment in complex with β-Lg allergen (PDB ID: 2R56) (Niemi et al., 2007). Since the Fab fragment targets β-Lg, the results of docking with the major epitope "VYVEELKPTPEGDLEILLQK" (epitope-V) of β-Lg were used as a control (Selo et al., 1999). The best mode of interactions between the two epitopes and the Fab fragment are shown in Fig. 5A&B, with docking scores of −183.222 (epitope-R) and-176.591 (epitope-V), respectively, and there is a crossover region between the two docking results (Fig. 5C). Based on the analysis of the interactions between the two epitopes and receptor, it was revealed that the interaction between epitope-V and receptor was stronger (Fig. S5&Table S1). It can be inferred that the epitope-R may bind to the Fab fragment and cause antigenic reaction, but the effect may not be as effective as that of the epitope-V. However, it is important to note that these speculations are based on simulation, so further laboratory experiments are needed to prove it. Besides, some of the epitopes were incomplete after fermentation and digestion, while a portion of the epitopes remained in the digested products, indicating that these products still have the potential to cause immune response.

After intestinal digestion (I120) of MFM (Fig. S4), all the listed α-La epitopes disappeared, while the CFM epitopes appeared repeatedly during the process. Notably, the epitopes of β-Lg were hardly affected by

fermentation and digestion, due to its properties. β-Lg is acid-resistant, allowing it to maintain its structural integrity under acidic conditions and thus be resistant to enzymatic digestion (Kostenko, Bratsikhin, & Shpak, 2019; Lee, Yim, Choi, Ha, & Ko, 2012). However, these epitopes that are not hydrolyzed are absorbed by the intestine and participate in the immune response.

4. Conclusions

In this study, both starters demonstrated the ability to reduce the antigenicity of α_S-CN, and the mixed starter containing *L. plantarum* 7-2 showed better potential. Additionally, the complex and diverse protease system of LAB contributes to the fermentation properties of the starters. Therefore, there were differences in pre-hydrolysis of RM by the two starters, leading to variations in the digestion fate of the fermented milks, including peptide types and concentrations, as well as residual levels of epitopes.

Moreover, Pescuma et al. (2009) confirmed that the extracellular polysaccharides produced by LAB promote the hydrolysis of cow milk protein, resulting in the release of smaller size peptides during gastrointestinal digestion with reduced potential for immune responses. Therefore, further research is needed to investigate if the presence of extracellular polysaccharides from *L. plantarum* 7-2 has the similar effects.

After label-free analysis of the hydrolysis products, the addition of *L. plantarum* 7-2 enabled the mixed starters to eliminate more epitopes of six major antigens. However, the epitopes of β-Lg remained relatively intact, both fermentation and gastrointestinal digestion, indicating that its potential to cause allergies in the organism was not reduced. Obviously, it is necessary to screen for strains that can specifically hydrolyze the epitopes of β-Lg. It is also worth noting that hydrolysis of peptides, both fermentation and digestion, may lead to the production of new epitopes or the reappearance of previously hydrolyzed epitopes. This study also evaluated the allergenic potential of the newly generated epitope using molecular simulations, although no experimental verification was carried out. It provides a workable strategy to evaluate the potential antigenicity of the fragment.

CRediT authorship contribution statement

Yunpeng Xu: Conceptualization, Methodology, Software, Validation, Resources, Formal analysis, Writing - original draft, Writing - review & editing. **Hongxin Wang:** Formal analysis, Investigation, Resources. **Guangqing Mu:** Funding acquisition, Project administration. **Xuemei Zhu:** Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100911.

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