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Allergenicity evaluation of peptides from milk and yogurt after gastrointestinal digestion based on epitopes†

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As we know, milk and yogurt have good nutritional value and it is reported that some peptides can induce tolerance to alleviate or eliminate cow's milk allergy (CMA). However, there is a lack of detailed information on the peptides after digestion which could induce tolerance. In this study, the distribution pattern of digested proteins was detected during gastrointestinal digestion in infants and adults by Tricine-SDS-PAGE and RP-HPLC, and the digestive products were transported by the model of Caco-2 cells and the sequence of peptides was identified by LC-MS/MS. Residual allergenicity was evaluated by indirect ELISA during gastrointestinal digestion and the released peptides were aligned with T cell/IgE epitopes and biological functions by prediction software and previous information. These results indicated that the major allergens in yogurt were more easily digested with stronger transport capacity and had lower IgE-binding capacity. We obtained 113 peptides from the major allergens in the digested products and 38 of these peptides existed in all the digested products, among which 13 peptides had specific biological functions, such as ACE-inhibitory, antimicrobial and DPP-IV inhibitory properties. Although there was no obvious difference in the number of epitopes between fresh milk and yogurt, the difference in the properties and content of specific peptides might be the key factor for the difference in allergenicity. Most importantly, 11 peptides that contained T cell epitopes but not IgE epitopes might induce immune tolerance in CMA, which should be confirmed further.

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1. Introduction

Milk proteins have high nutritional value and are essential in nutritional supplements, especially for infants and children. However, cow's milk allergy (CMA) is the most common food allergy, seriously affecting quality of life. The prevalence of CMA in infants has reached 2.69% in China.¹ The main allergens of cow's milk are casein (α_{s1} -casein, α_{s2} -casein, β -casein and κ -casein), β -lactoglobulin (BLG) and α -lactalbumin (ALA). There are many dairy products on sale, including pure milk, skim milk, and yogurt. Among them, yogurt is given attention

because it could prolong the shelf life of milk and improve its flavor.² It also has many health benefits and is an excellent source of proteins, vitamins, and minerals. Moreover, it contains beneficial lactic acid bacteria that may affect the metabolism, and the balance of endogenous flora in yogurts produces protease and peptidase to hydrolyze part of milk proteins, thereby promoting digestion.³

Gastrointestinal digestion is a necessary process for food allergens, and the allergenicity of the digested products is the key to triggering food allergy. However, it is very difficult to study the gastrointestinal digestion of different food allergens *in vivo*, so simulated digestion *in vitro* provides an effective tool.⁴ Simulated digestion *in vitro* is an important method to understand the degradation and absorption of food proteins in the gastrointestinal tract. And an experiment *in vitro* helps to study digestion from different aspects with high reproducibility. Yogurts could degrade proteins and destroy allergen epitopes to reduce allergy during fermentation and induce tolerance in patients with CMA. Clinical studies have found that yogurts can induce tolerance in most children with CMA.^{5,6} Shoda *et al.* showed that regular consumption of yogurts in infancy could prevent the development of food allergies, based

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on a birth cohort study.⁷ Likewise, fresh milk and yogurt release peptides during digestion that have many beneficial effects. Bioactive peptides are encrypted in the amino acid sequence of milk proteins, which are released and activated by enzymatic hydrolysis and gastrointestinal digestion. And some peptides have immunomodulatory ability and may play a role in immune tolerance. Therefore, it is important to assess the allergenicity of food allergens by considering the changes in proteins and peptides during gastrointestinal digestion.

However, detailed information on the released peptides and epitopes in digested fresh milk and yogurt with complex matrices has not been systematically studied. In our study, we compared the gastrointestinal digestion of fresh milk and yogurt in infants and adults, then explored the degradation of proteins and the change in allergenicity during the digestion process. Meanwhile, the Caco-2 cells model was used to evaluate the transport capacity of the digestive products. The biological activity and epitopes of the released peptides in digestion were further analyzed for the first time by bioinformatics. The study aimed to find target peptides with specific biological and allergenic properties that help us in the development of hypoallergenic dairy products with improved health function and immune tolerance.

2. Materials and methods

2.1 Reagents

Fresh milk and yogurt were obtained from the local supermarket. A pool of 10 sera obtained from patients with CMA was used in indirect enzyme-linked immunosorbent assay (ELISA) experiments and information on the allergic patients is listed in Table 1. 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Sigma Chemical Co. (St Louis, Mo, USA). Pepsin from porcine gastric mucosa and trypsin from porcine pancreas were obtained from Sigma-Aldrich Chemical Co. (St Louis, Mo, USA). Methyl alcohol (Merck, Germany) was used in reversed-phase high-performance liquid chromatography (RP-HPLC). High-glucose Dulbecco's modified Eagle's medium (DMEM),

penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Cellmax Co., Ltd (Beijing, China). L-Glutamine was obtained from Solarbio Co., Ltd (Beijing, China). All other reagents were analytical grade.

2.2 The experiment of simulated gastrointestinal digestion *in vitro*

Fresh milk and yogurt were digested according to the method of Minekus *et al.* with some modifications.⁸ This experiment was performed according to simulated gastric digestion in infants and adults at different pH, followed by gastrointestinal digestion at pH 7.0. Briefly, simulated gastric fluids (SGF, pH 2.0, 3.0, 4.0, 5.0) of infants containing pepsin (500 U mL⁻¹ in the final mixture) and 30 mg mL⁻¹ protein in the samples were mixed at a ratio of 10 : 10 (v : v). SGF with pH 1.0, 2.0, 3.0, 4.0 of adults containing pepsin (4000 U mL⁻¹ in the final mixture) and 30 mg mL⁻¹ protein in samples were mixed at a ratio of 10 : 10 (v : v). All digestions were performed at 37 °C in a horizontal shaker. 2 mL samples were withdrawn at 10 min, 30 min, 60 min, 90 min, and 120 min during gastric digestion and the reaction was quickly stopped with 200 mM NaHCO₃ until the pH was 7.0.

The simulated intestinal fluid (SIF, pH 7.0) of infants contained 1 mL of fresh bile salt solution (0.05 M), 5 µL of CaCl₂ (0.3 M), 8.795 mL of intestinal electrolyte stock solution and 0.2 mL of trypsin (20 U mL⁻¹ in the final mixture). SIF (pH 7.0) of adults was mixed with 4 mL of fresh bile salt solution (0.05 M), 20 µL of CaCl₂ (0.3 M), 8.795 mL of intestinal electrolyte stock solution and 2 mL of trypsin (200 U mL⁻¹ in the final mixture). After 60 min of gastric digestion, the digestive samples were withdrawn and mixed at a ratio of 10 : 10 (v : v) with SIF for intestinal digestion. And 2 mL samples were withdrawn at 10 min, 30 min, 60 min, 90 min, 120 min, and 150 min for detection. The reaction was stopped in a boiling water bath at 100 °C for 5 min. The digestive products were analyzed by Tricine-SDS-PAGE and RP-HPLC at different time points of digestion for evaluation of digestive stability.

2.3 Tricine-SDS-PAGE analysis

Digestive samples were taken for Tricine-SDS-PAGE analysis.⁹ Firstly, 4% stacking gel, 10% interlayer gel and 16.5% compact gel were used. Protein samples (10 µg) were electrophoresed with constant voltage. The electrophoresis conditions were run at 30 V for 1 h, and then run for 1.5 h at 100 V. After electrophoresis, the gels were immersed in a fixative solution for 30 min. Then the gels were stained with Coomassie blue for 20 min and destained with a solution containing 5% methanol and 7.5% acetic acid.

2.4 RP-HPLC

RP-HPLC was used to measure the digestive samples. A liquid chromatograph (Shimadzu, Japan) was used. The samples (25 µL, 1.5 mg mL⁻¹) were placed onto an LC-20AT HPLC column (symmetry C18, 5 µm particle size, 250 × 4.6 mm, Shimadzu) at a flow rate of 1.0 mL min⁻¹. The column temperature was 45 °C. Solvent A was ultrapure water containing

Table 1 Information about cow's milk allergic patients

Patient no.	Sex	Age (year)	Milk-related clinical symptoms	Milk S-IgE (kU _A L ⁻¹)
1	Female	10	AB	3.5
2	Female	13	P	1.0
3	Female	48	AR	3.2
4	Male	1	AS	2.9
5	Male	4	ND*	2.7
6	Male	4	U	5.9
7	Male	9	SU	56.8
8	Male	11	ND*	18
9	Male	12	L	43.7
10	Male	36	AR	3.7

AB, asthmatic bronchopneumonia; P, pneumonia; AR, allergic rhinitis; AS, asthma; ND*, not done; U, urticaria; SU, serum urticaria; L, leukocytosis.

0.1% trifluoroacetic acid. For solvent B, which contained 0.1% (v/v) TFA in acetonitrile, multistep linear gradient elution was continuous from 5% at 0 min to 60% at 70 min. Ultraviolet detection was performed at 220 nm.

2.5 Indirect ELISA

The IgE-binding ability of the samples at different time points of digestion was identified by indirect ELISA. This method was as previously described with some modifications.¹⁰ Briefly, 1 µg mL⁻¹ samples with different digestive times in 0.05 M carbonate buffer (pH 9.6) were used to coat a 96-well microtiter plate (100 µL per well) at 4 °C overnight. The plates were washed three times. Each well was blocked for 1 h at 37 °C with 3% gelatin in PBS. Subsequently, 100 µL of the pooled sera (1 : 600) from patients with CMA were added to each well and incubated for 1 h at 37 °C. The plates were washed, and 100 µL of the diluted 1 : 5000 antisera (biotinylated-labeled anti-human IgE) was added to the wells and incubated at 37 °C for 1 h. Then avidin was added as an additional step before the chromogenic substrate. After washing again, 100 µL of the substrate solution (TMB) was added to each well. The chromogenic reaction was terminated by adding 50 µL of 2 M sulfuric acid per well. Absorbance was determined at 450 nm.

2.6 The transport capacity of digestive products by the Caco-2 cells line model

2.6.1 Establishment of Caco-2 cells line model. Caco-2 cells (2 × 10⁵ cells per well, 30–40 passages) in the logarithmic phase were seeded into the upper chamber of the 12-well transwell culture plate for 21 days.¹¹ The trans-epithelial resistance (TEER) values were measured on days 11, 13, 15, 17, 19 and 21 (until the TEER reached 500 Ω cm²) by a previous approach.¹² TEER was used as a measure of the integrity of the cell monolayer.

2.6.2 Viability of Caco-2 cells treated with the digestive products. Caco-2 cells were seeded at a density of 2 × 10⁵ cells per mL (100 µL per well) in a 96-well culture plate. After 12 h of incubation at 37 °C (until 80% confluence was reached), the culture medium was aspirated and replaced with DMEM medium (1% FBS, 90 µL) supplemented with various concentrations of the digestive products (500, 1000, 1500 and 2000 µg mL⁻¹) and then incubated for 24 h. Subsequently, cell viability was measured using a cell counting kit-8 (CCK-8) assay (Beyotime Co., Ltd, Shanghai, China).

2.6.3 Detection of transport ability of digestive products. The transport ability of the digestive products was detected by intact Caco-2 cell monolayers. The wells were rinsed with Hank's solution three times. Then the Hank's solution was replaced with digestive products (1500 µg mL⁻¹) at their optimal concentrations in the upper chamber, and 1.5 mL of Hank's solution was added to the lower chamber. After 4 h of transport, the solutions from the lower chamber were taken out to measure the concentrations of the peptides. The content of peptides was detected with a PierceTM Quantitative Colorimetric Peptide kit (Thermo ScientificTM, USA), and the

transfer rate of the sample was calculated using the following formula:

$$\text{transfer rate (\%)} = \frac{\text{sample amount in the lower chamber}}{\text{initial amount of sample}} \times 100\%$$

2.6.4 Detection of cytokines. After four hours of transport, the medium was taken from the upper chamber. The supernatant was collected by centrifugation to determine the levels of IL33, IL25 and thymic stromal lymphopoietin (TSLP) according to the instructions of the ELISA kits (Neobioscience Co., Ltd, Shenzhen, China).

2.7 LC-MS/MS

Peptides in the digestive products were identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS). This detailed method used was as previously described with some modifications.¹³ The samples were separated using the HPLC liquid system Easy nLC at nanoliter flow rates. Buffer A was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile (84% in acetonitrile). The chromatographic column was equilibrated with 95% of liquid A, and the sample was loaded from an autosampler to the loading column (Thermo Scientific Acclaim PepMap100, 100 µm × 2 cm, nanoViper C18), and then passed through the analytical column (Thermo scientific EASY column, 10 cm, ID75 µm, 3 µm). Then samples were chromatographically separated and analyzed by mass spectrometry using a Q-Exactive mass spectrometer. The detection method was positive ion, and the primary mass spectrometry resolution was 70 000 at 200 *m/z*. The dynamic exclusion time was 60 s. The secondary MS resolution was 17 500 at 200 *m/z*. The final data was queried in MaxQuant software, and the amino acid composition of the final peptides was confirmed.

2.8 Bioinformatics analysis

2.8.1 The prediction of T cell epitopes. IEDB (Immune Epitope Database) was used as the predictive tool to predict allergen peptides that could bind to HLA class II molecules.¹⁴ A combination of consensus and the NetMHCIIpan method has been used to predict T cell epitopes of allergens that can bind to MHC II molecules in IEDB, and the NetMHCIIpan method has been recognized as the most accurate epitope prediction method.¹⁵ 27 HLA molecules were used to predict T cell epitopes,¹⁶ and the epitope length was set to 15 amino acids. Then the predicted T cell epitopes were compared with the digested peptides of major allergens.

2.8.2 Evaluation of the ability to induce IL-4. The predictive tool (<https://webs.iitd.edu.in/raghava/il4pred/>) of IL-4 was used to calculate the ability of epitopes to induce IL-4 secretion by Th2 cells.¹⁷ A hybrid method was chosen, which included the selection of a support vector machine (SVM) and motif. The SVM threshold was chosen as 0.2. The digested peptides of major allergens were analyzed for their ability to induce IL4 by this predictive tool.

2.8.3 The biological activity of peptides. The biological activity of the peptides was queried through the database

(<https://mbpdb.nws.oregonstate.edu/>).¹⁸ The database can be used to search for specific functions of peptides.

2.9 Statistical analysis

The experimental data were expressed as the means $\pm$ standard deviation (SD) for three times. Statistical analysis was carried out by one-way ANOVA, followed by a Student's *t*-test using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). A *p*-value lower than 0.05 was deemed a significant difference.

3. Results and discussion

3.1 Digestive stability analysis

To qualitatively detect protein degradation during digestion *in vitro*, we used Tricine-SDS-PAGE to observe changes in proteins at different times of fresh milk and yogurt digestion. These results are shown in Fig. 1 and 2. In Fig. 1, the protein profiles of fresh milk (A) and yogurt (B) showed the simulated

gastric digestion (pH 2, 3, 4, 5) in infants at different times. In Fig. 1A, a small amount of protein was degraded during the gastric digestion of fresh milk, and the protein band below 5 kDa appeared only at pH 2. In Fig. 1B, during the gastric digestion of yogurt, more low molecular weight bands appeared than from the digestion of fresh milk, which indicated that more intact proteins in yogurt were degraded. However, the protein bands demonstrated that there were still intact proteins that could not be digested during the gastric digestion stage. From Fig. 1C and D it was shown that protein bands of fresh milk (C) and yogurt (D) came from the simulated gastrointestinal digestion in infants after 60 min of gastric digestion (pH 2, 3, 4, 5). During the gastrointestinal digestion of fresh milk, the band of casein almost disappeared and the bands of BLG and ALA were also obviously degraded. This indicated that casein was almost completely digested into small peptides, and ALA and BLG were also degraded into peptides. During the gastrointestinal digestion of yogurt, the bands of the main allergens almost completely disappeared, and bands less than 10 kDa appeared. This demonstrated that

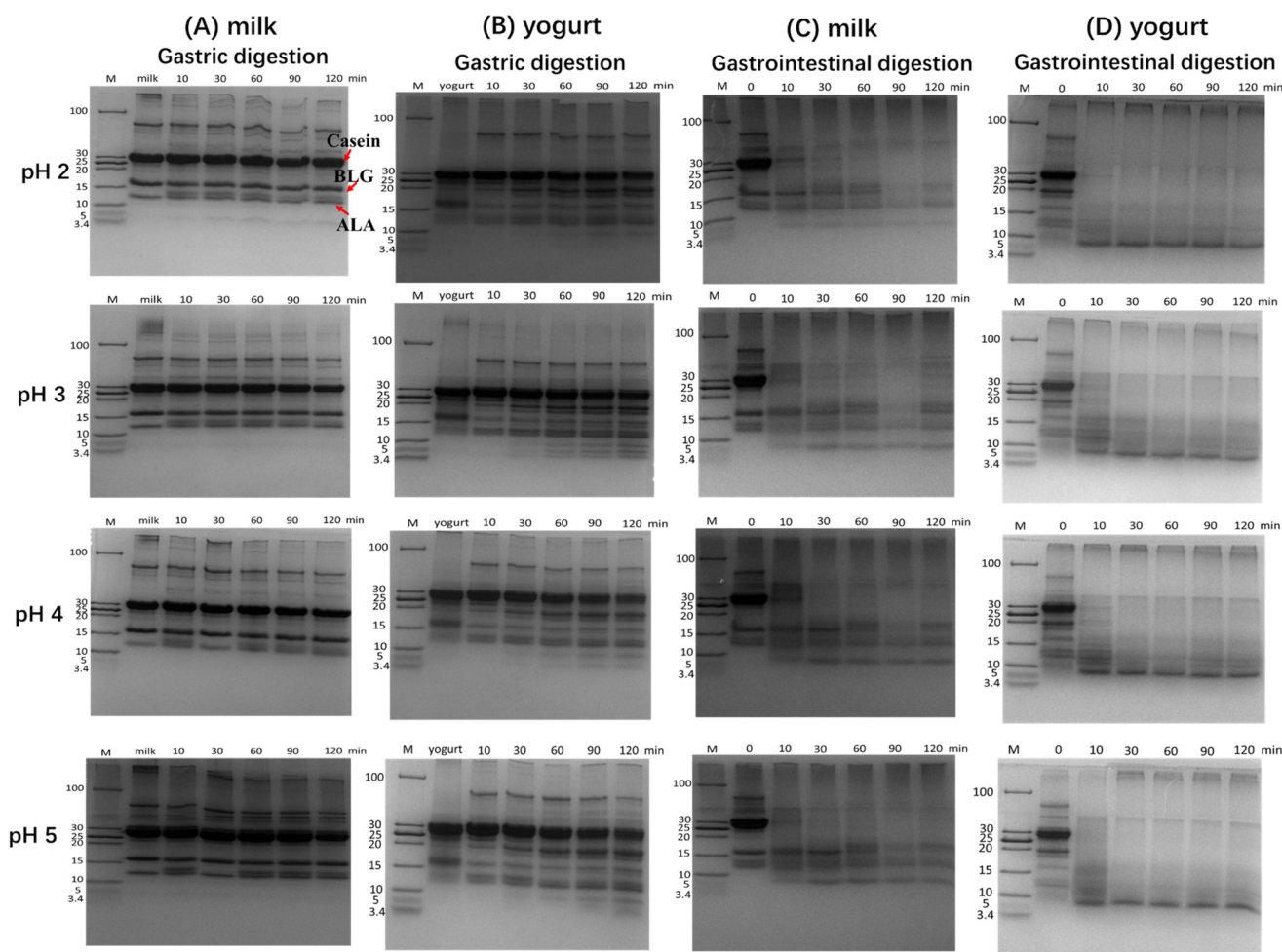


Fig. 1 Tricine-SDS-PAGE protein profiles of fresh milk (A) and yogurt (B) show simulated gastric digestion (pH 2, 3, 4, 5) in infants at different times. Tricine-SDS-PAGE protein profiles of fresh milk (C) and yogurt (D) show simulated gastrointestinal digestion in infants after 60 min of gastric digestion (pH 2, 3, 4, 5). Lane M: molecular mass marker.

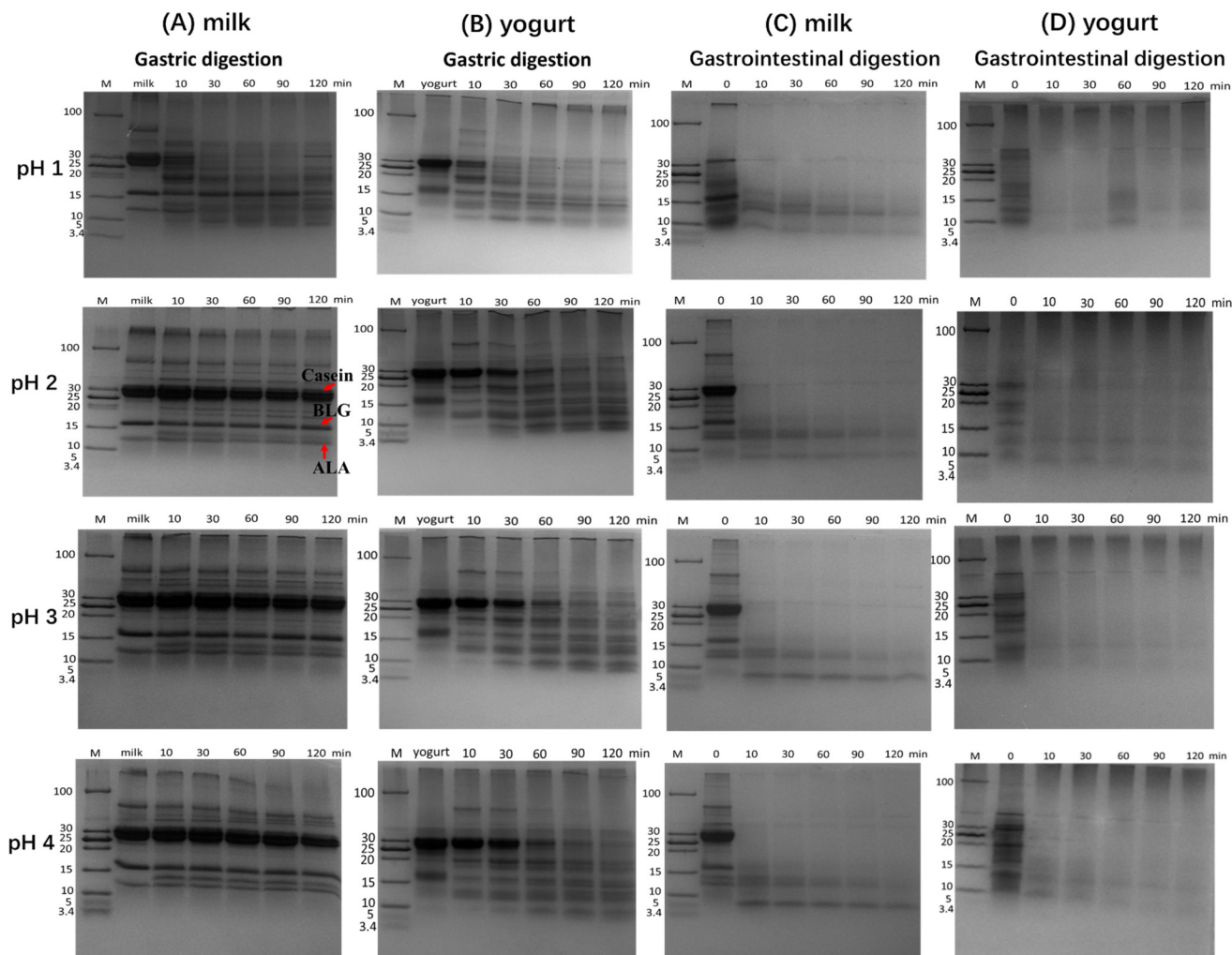


Fig. 2 Tricine-SDS-PAGE protein profiles of fresh milk (A) and yogurt (B) show simulated gastric digestion (pH 1, 2, 3, 4) in adults at different times. Tricine-SDS-PAGE protein profiles of fresh milk (C) and yogurt (D) show simulated gastrointestinal digestion in adults after 60 min of gastric digestion (pH 1, 2, 3, 4). Lane M: molecular mass marker.

the major allergens were almost completely degraded into peptides of less than 10 kDa. These results indicated that yogurt was more digestible than fresh milk in both the gastric and gastrointestinal intestinal digestion stages. And the degradation extent was stronger with a decrease in pH. Fig. 2 shows the simulated gastrointestinal digestion in adults. In Fig. 2A, proteins were gradually degraded during the gastric digestion of fresh milk, and casein was almost completely degraded at pH 1. From Fig. 2B, we can see that a large number of allergen proteins were degraded into peptide fragments by observing protein bands during the gastric digestion of yogurt. Clearly, yogurt was more digestible than cow's milk during gastric digestion. In Fig. 2C and D, the electrophoretic bands were almost below 15 kDa in the simulated intestinal digestion stage of adults, which indicated that proteins in fresh milk were degraded into peptides below 15 kDa. The electrophoretic bands below 15 kDa in the yogurt were further digested compared to fresh milk. By observing the electrophoretic bands, as

expected, adults digested more than infants because of differences in digestion conditions, such as the increase in enzyme activity. LABs in yogurts could produce proteases to hydrolyze milk proteins and produce acid during fermentation to lower the pH, thereby promoting digestion.^{3,19} Several studies have reported that BLG is highly resistant to pepsin hydrolysis and is sensitive to trypsin.^{20,21} In our study, the same result was also found in the electrophoretic bands of BLG.

In Fig. 3, RP-HPLC profiles of fresh milk (A and B) and yogurt (C and D) displayed the simulated gastric digestion in infants at 10 min, 30 min, 60 min, 90 min and 120 min. The RP-HPLC profiles of fresh milk (E and F) and yogurt (G and H) indicated the simulated gastrointestinal digestion (0, 10 min, 30 min, 60 min, 90 min and 120 min) in infants after 60 min of gastric digestion. Fresh milk had a large number of intact proteins during the gastric digestion stage from the peak profile. In yogurt (Fig. 3C and D), some small peaks appeared compared to Fig. 3A and B, indicating that more allergen pro-

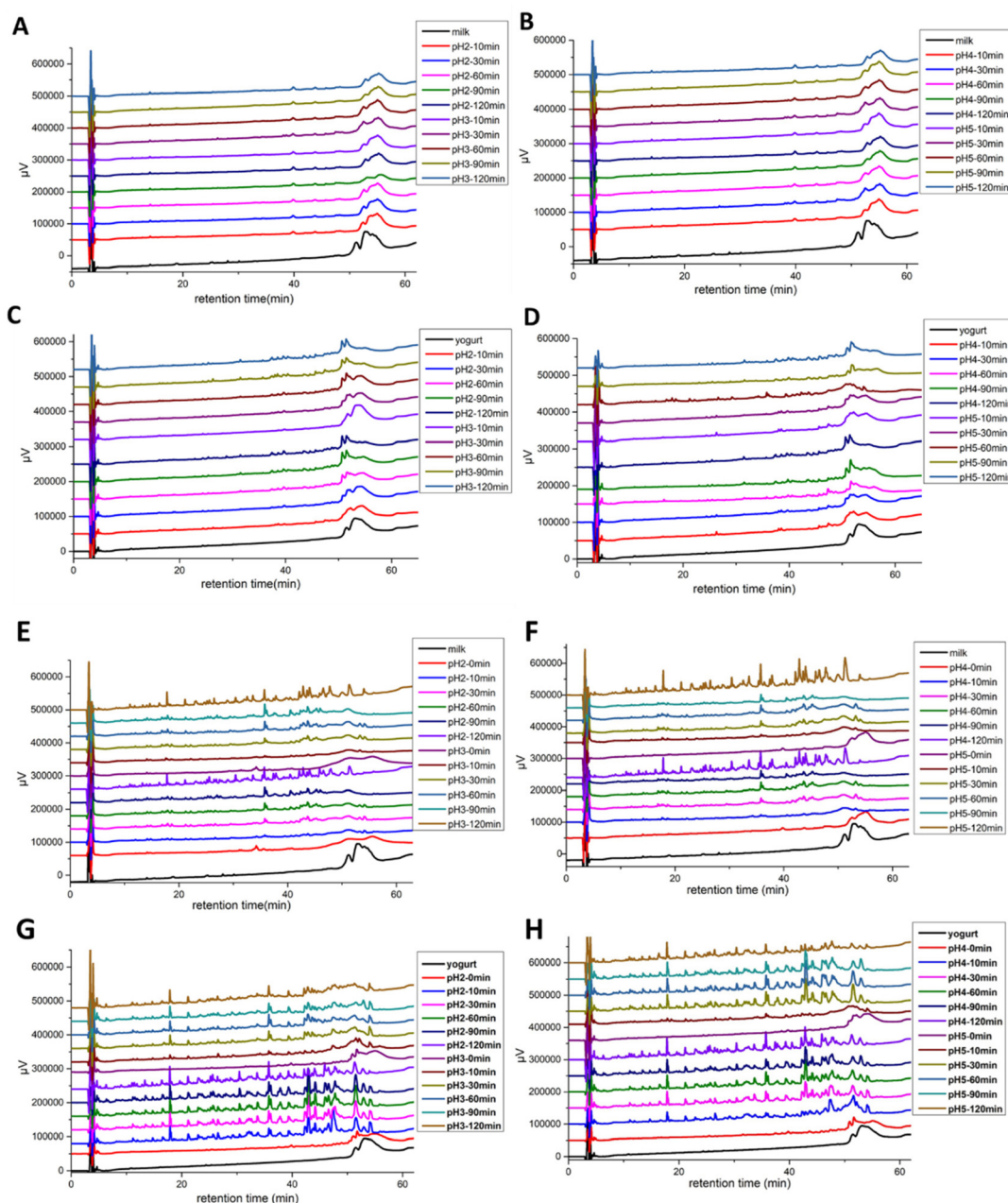


Fig. 3 Reverse phase-HPLC profiles of fresh milk (A: pH 2, 3; B: pH 4, 5) and yogurt (C: pH 2, 3; D: pH 4, 5) show simulated gastric digestion in infants at 10 min, 30 min, 60 min, 90 min and 120 min. Reverse phase-HPLC profiles of fresh milk (E: pH 2, 3; F: pH 4, 5) and yogurt (G: pH 2, 3; H: pH 4, 5) show simulated gastrointestinal digestion (0 min, 10 min, 30 min, 60 min, 90 min and 120 min) in infants after 60 min of gastric digestion.

teins were degraded. During the gastrointestinal digestion of fresh milk, with a decrease in pH, more small peaks gradually appeared. Similarly, more small peaks appeared during the gastrointestinal digestion of yogurt, demonstrating that most intact proteins were degraded into peptides. Fig. 4 shows the RP-HPLC profiles of gastric/gastrointestinal digestion in adults. During the gastric digestion stage of fresh milk (Fig. 4A and B), it was obvious that the peak of casein gradually disappeared at pH 1 and a large number of small peaks appeared. There were still peaks of undigested proteins under pH 2, 3, 4

conditions, which meant that $\text{pH} < 2$ had a strong digestive ability. In Fig. 4C and D, the peaks of proteins from yogurt gradually disappeared and a large number of small peaks appeared during the gastric digestion stage. Likewise, this demonstrated that yogurt was more digestible than fresh milk. In the intestinal digestion stage, the peaks of allergen proteins in fresh milk and yogurt gradually disappeared from 10 min of digestion, and a large number of small peaks appeared. This showed that protein has been digested into small peptides in intestinal digestion in adults. These results were consistent

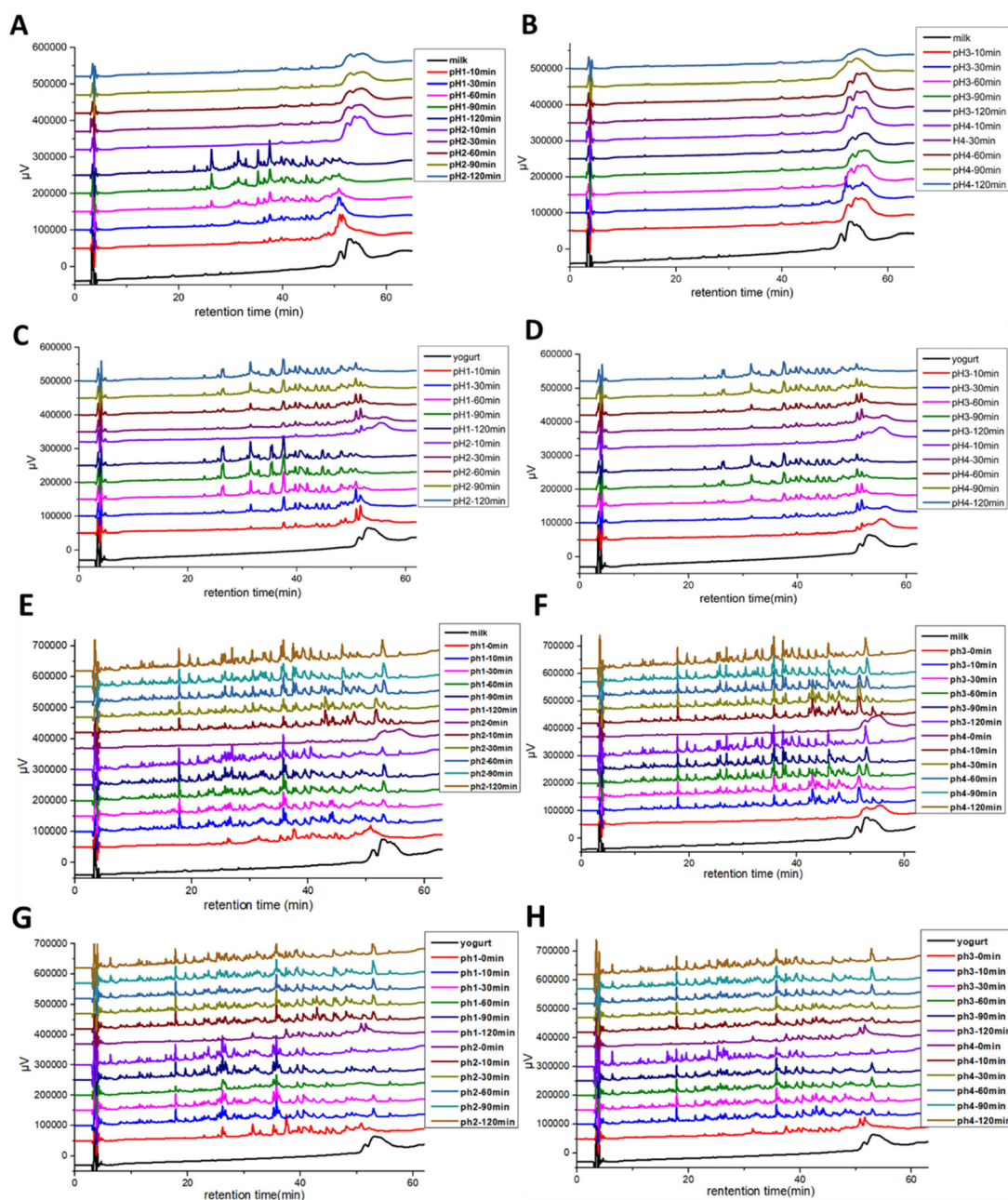


Fig. 4 Reverse phase-HPLC profiles of fresh milk (A: pH 1, 2; B: pH 3, 4) and yogurt (C: pH 1, 2; D: pH 3, 4) show simulated gastric digestion in adults at 10 min, 30 min, 60 min, 90 min and 120 min. Reverse phase-HPLC profiles of fresh milk (E: pH 1, 2; F: pH 3, 4) and yogurt (G: pH 1, 2; H: pH 3, 4) show simulated gastrointestinal digestion (0 min, 10 min, 30 min, 60 min, 90 min and 120 min) in adults after 60 min of gastric digestion.

with the electrophoresis results. In brief, these results showed that yogurt was more easily digestible than fresh milk. It might be predigested by proteases during fermentation, and fermentation affects the structure of milk proteins, which would change the digestibility of proteins. Adolfsson *et al.* revealed that proteins in yogurt were partially predigested by proteases during fermentation.²² Levi *et al.* indicated that processing affected the structure of milk proteins.²³ At low-pH conditions, proteins were more easily digested. Similarly, Gan *et al.* also

found that the enzymatic hydrolysis of milk proteins was affected by pH.²⁴ In gastrointestinal digestion in infants, most proteins were not digested until the intestinal digestion stage. However, in adults, a large amount of casein, especially in yogurt, has been fully digested in the early stages of gastric digestion. Compared with infants, adults had a complete digestive barrier, and proteins were digested faster and degraded into smaller peptides due to high enzymatic activity. Previous studies found similar results.²⁵

3.2 Analysis of IgE-binding ability

The fragments of digested proteins need to have intact IgE-binding epitopes to react with IgE antibodies, thereby triggering allergic reactions. Therefore, it is very important to detect the binding capacity of IgE in the digested products. The linear IgE-binding epitopes appear to be more relevant in the context of food allergy, since conformational epitopes are readily digested in the gastrointestinal tract.²⁶

In Fig. 5, there is a significant change in IgE-binding ability during gastrointestinal digestion by indirect ELISA. In Fig. 5A, the IgE-binding capacity of fresh milk and yogurt was also

compared, and that of yogurt was significantly reduced. The results showed that fermentation destroyed the epitopes of proteins and reduced the IgE-binding ability. In Fig. 5(A–D), IgE-binding capacity was significantly reduced in gastrointestinal digestion in infants. However, no regular reduction in IgE-binding capacity was found as pH decreased. The previous results indicated that the digestibility was strong at low pH. IgE-binding ability is determined by the structure of proteins and the characteristics of the peptides.²⁷ Likewise, in Fig. 5(E–H), the IgE-binding capacity of gastrointestinal digestion was also significantly reduced in adults. The IgE-binding capacity of adults was lower than the digested product of infants. This

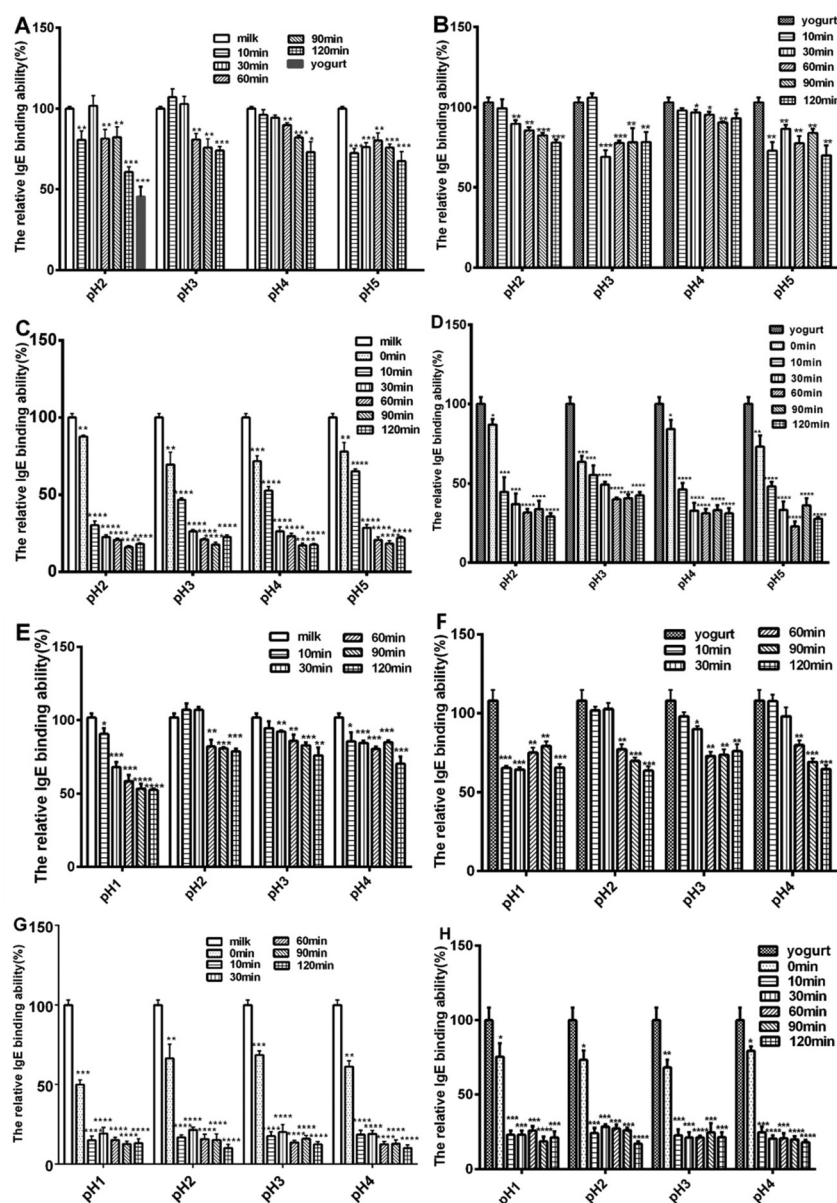


Fig. 5 The IgE-binding ability of gastrointestinal digestion was defined by indirect ELISA. Fresh milk (A) and yogurt (B) show simulated gastric digestion (pH 2, 3, 4, 5) in infants at different times. Fresh milk (C) and yogurt (D) show gastrointestinal digestion in infants after 60 min of gastric digestion. Fresh milk (E) and yogurt (F) show simulated gastric digestion (pH 1, 2, 3, 4) in adults. Fresh milk (G) and yogurt (H) show gastrointestinal digestion in adults after 60 min of gastric digestion (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$).

may be due to the production of different peptides during digestion in adults and infants. Digestion in infants might contain more IgE-binding epitopes due to low enzymatic activity. Resistance to gastrointestinal digestion is one of the potential factors for the allergenicity of food proteins, which allows the intestinal absorption of some allergic epitopes and sensitizes the mucosal immune system.²⁸ In our study, it was clear that the immature gastrointestinal tract of infants had lower enzymatic activity and generated more allergen epitopes, which made them more susceptible to allergy than adults. Moreover, fermented yogurts are known to degrade milk proteins in the intestine and are included in infant formulas to induce oral tolerance.²⁹ Minjing Yao *et al.* indicated that yogurt could significantly reduce the antigenicity and human IgE-binding ability of α -LA, β -LG, α -casein and β -casein in reconstituted milk.³⁰

3.3 Transport capacity of digestive products

The assessment of allergen transport through the intestine may be an important parameter for assessing allergic risk.³¹ Caco-2 cells have been used to determine the permeability of the intestinal mucosa to allergen proteins.³² It has also been proven to be the best model for studying intestinal allergen absorption.³³ Therefore, the transport capacity of digestive products was assessed by the Caco-2 cells model in our study.

The viability of Caco-2 cells was tested with various concentrations of digestive products by CCK-8 assay. The cell survival rate was higher than 95% with a concentration of 1500 $\mu\text{g mL}^{-1}$ (ESI Fig. 1†). So the optimal concentration (1500 $\mu\text{g mL}^{-1}$) was used for further experiments. Fig. 6 shows the transport rate in gastrointestinal digestive products. We compare the transport rate in infants under different pH conditions in Fig. 6A. The transport rate of fresh milk digestion under pH 2 conditions was the lowest, and the others showed no significant change. In Fig. 6B, the transport rate in adults

of gastrointestinal digestive products is shown. The transport rate of the digestive product of fresh milk was lowest at pH 1 and 3 and highest at pH 4. For the digestive products of yogurt, there was no significant change in the transport rate, but it was significantly higher than in the digestive products of fresh milk. These results suggested that the transport rate might be affected by multiple factors and did not exhibit regular changes. The digestive products under different conditions contained many peptides with different properties and the transport rate might be different.³⁴ Peyron *et al.* indicated that the degree of hydrolysis and the released peptides sequence of allergen proteins would determine their ability and reactivity to cross the intestinal barrier.³⁵

Typical cytokines (IL-33, TSLP and IL-25) produced by epithelial cells could activate dendritic cells to promote adaptive Th2 cell immunity.³⁶ TSLP is commonly expressed on the surfaces of the epithelial cells of intestinal barriers and is a promoter of Th2-type allergic reactions.^{37,38} Cytokines IL33 and IL25 were not detected in our results. Fig. 7 shows the level of TSLP in Caco-2 cells. From Fig. 7A, the TSLP level of fresh milk was significantly higher than that of yogurt in the gastrointestinal digestive products of infants. From Fig. 7B, TSLP was significantly increased at pH 1 and 2 in fresh milk, and TSLP levels decreased in the other groups without significant change in the digestive products of adults. These results showed that the digested products of yogurt induced lower levels of TSLP in Caco-2 cells compared with fresh milk. These results indicated that the digested products of fresh milk might be conducive to the development of Th2. Roth *et al.* found that BLG induced Caco-2 cells to produce specific IgE antibodies, Th2 cytokines and TSLP.³⁹

3.4 Epitope analysis and biological functions of peptides

Milk proteins are the main source of exogenous bioactive peptides and are often used as a suitable model for digestion

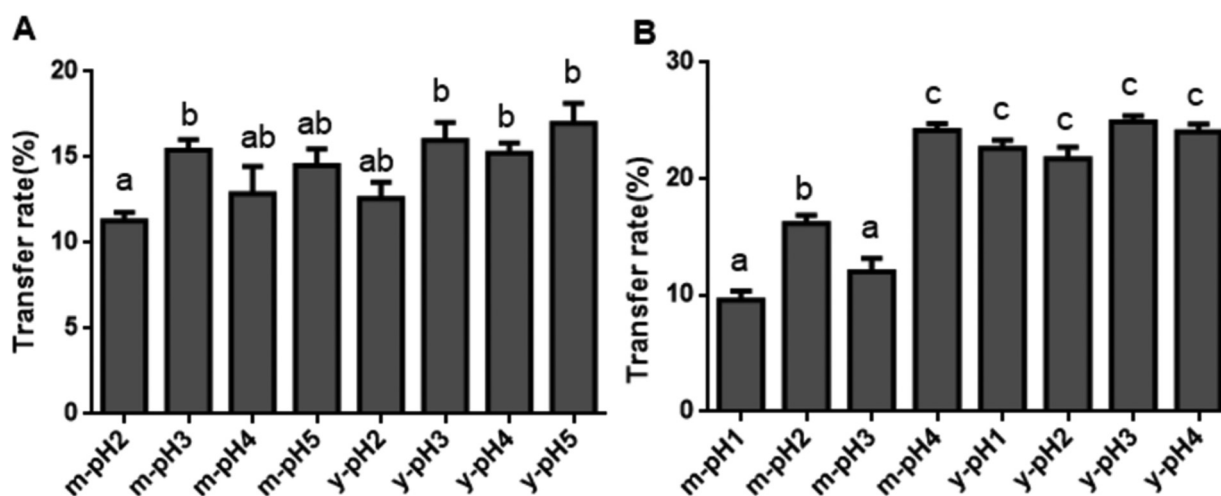


Fig. 6 The transport rate of gastrointestinal digestive products (A: infants; B: adults) was determined by the Caco-2 cells model for 4 h in different pH conditions of gastric digestion. m-pH 1, 2, 3, 4, 5: fresh milk in pH 1, 2, 3, 4, 5 conditions; y-pH 1, 2, 3, 4, 5: yogurt in pH 1, 2, 3, 4, 5 conditions. Different letters represent significant differences ($P < 0.05$).

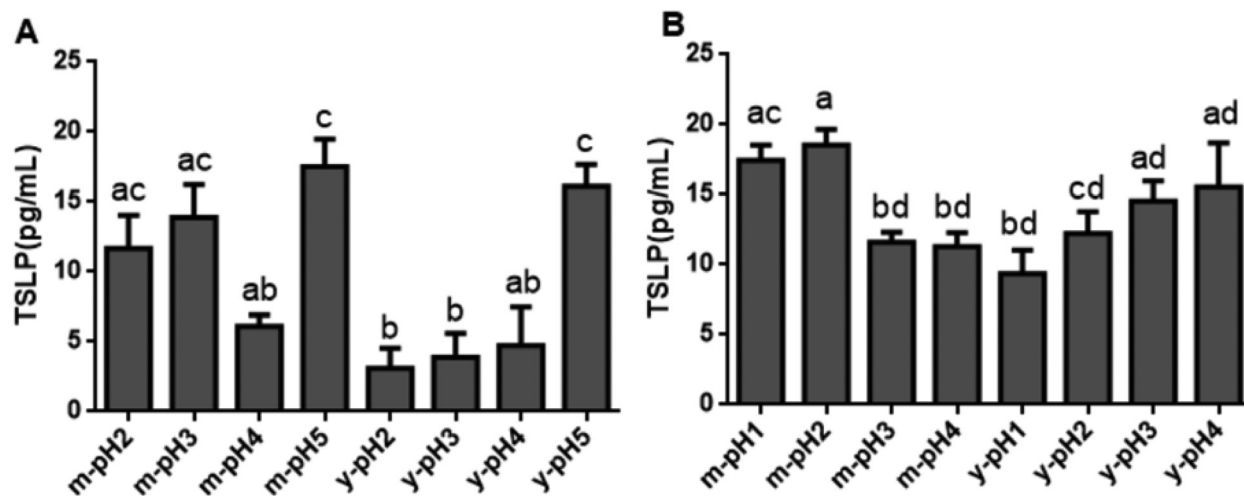


Fig. 7 The levels of TSLP in the supernatant of the upper chamber of the Caco-2 cells model were detected by ELISA in which the digestive products (A: infants; B: adults) were transported for 4 hours. m-pH 1, 2, 3, 4, 5: fresh milk in pH 1, 2, 3, 4, 5 conditions; y-pH 1, 2, 3, 4, 5: yogurt in pH 1, 2, 3, 4, 5 conditions. Different letters represent significant differences ($P < 0.05$).

experiments. Yogurts are widely used around the world to produce a variety of food products with different health benefits. Fermented milk could produce protease and peptidase to hydrolyze part of the milk proteins, thereby promoting digestion.^{3,19} In our study, peptides after digestion of fresh milk and yogurt were detected by LC-MS/MS. In Fig. 8, we list the detected peptides of major allergen proteins (BLG, ALA, casein). Meanwhile, the identification and analysis of the epitopes were carried out on the peptides of major allergens through the databases in Tables 2 and 3. The biological activities of the peptides were queried through the database (<https://mbpdb.nws.oregonstate.edu/>).¹⁸ The peptides released by allergen proteins would determine their ability and reactivity across the gut barrier.³⁵ The specific cleavage sites of trypsin are also marked in Fig. 8, indicating that proteins were easily cleaved by trypsin. It was found that some amino acid sequences of proteins were not detected, and it may be that the entry of the protease is restricted due to steric hindrance, or the concentrations of peptides are too low to be detected.²⁵

In our study, 113 peptides of major allergens were detected after digestion, of which 34% had specific biological functions. Resistance to digestion is a prerequisite to exerting the biological activity of milk-derived peptides.⁴⁰ These active peptides are released during digestion and absorbed by intestinal epithelial cells and have beneficial effects on the gut. The digested yogurt contained 95 peptides, of which 7%, 6% and 4% of peptides have an angiotensin-converting enzyme (ACE)-inhibitory, antimicrobial, and dipeptidyl peptidase-IV (DPP-IV)-inhibitory properties, respectively. And there were 102 peptides in the digested fresh milk, of which 9%, 8% and 5% of peptides possessed ACE-inhibitory, antimicrobial and DPP-IV inhibitory activities. There were also other biological functions, including stimulating proliferation, promoting

calcium uptake, and antioxidation. Peptides existed in all the digested products and are listed in Table 4; therefore, these were highly resistant to gastrointestinal digestion. Among them, 13 peptides had specific biological functions, such as ACE-inhibitory, antimicrobial and DPP-IV inhibitory properties. These peptides were very likely to exist in the gut and exert biological functions.

IL4 has been reported to play a key role in various biological activities.¹⁷ IL4 promotes the proliferation and differentiation of antigen-presenting cells and is the main cytokine that guides the transition of T cells to the Th2 phenotype.⁴¹ IL4 is also a Th2-type cytokine in CMA. The induction of IL4 towards Th2 by allergen epitopes is complex, depending on many factors, including the cytokine milieu, MHC haplotypes, co-stimulatory molecules and the peptide. The IL4pred tool (<https://webs.iitd.edu.in/raghava/il4pred/>) was used to calculate the ability of epitopes to induce IL4 secretion by Th2 cells.¹⁷ Tables 2 and 3 list the peptides that can induce the production of IL4 by Th2 cells. A high score indicates the strong ability of the peptide to induce IL-4 production. After an allergen enters the human body, peptides are processed by antigen-presenting cells and then combined with MHC II molecules. The MHC II-T cell epitope complex interacts with CD4⁺ T cells to synthesize and secrete cytokines such as IL4, which will play a key role in stimulating the production of IgE.¹⁷ Bioinformatics with the development of databases is an initial screening method that is widely used for the prediction of food allergen epitopes, and its advantages include high throughput, rapid analysis and low cost. IEDB has been used to predict T cell epitopes (<https://tools.iiedb.org/mhcii/>), and the NetMHCIIpan method has been recognized as the most accurate epitope prediction method.⁴² T cell epitopes usually consist of 12–20 amino acids, and T cell epitopes of fewer than 12 amino acids could not effectively stimulate CD4⁺ T cells.⁴³

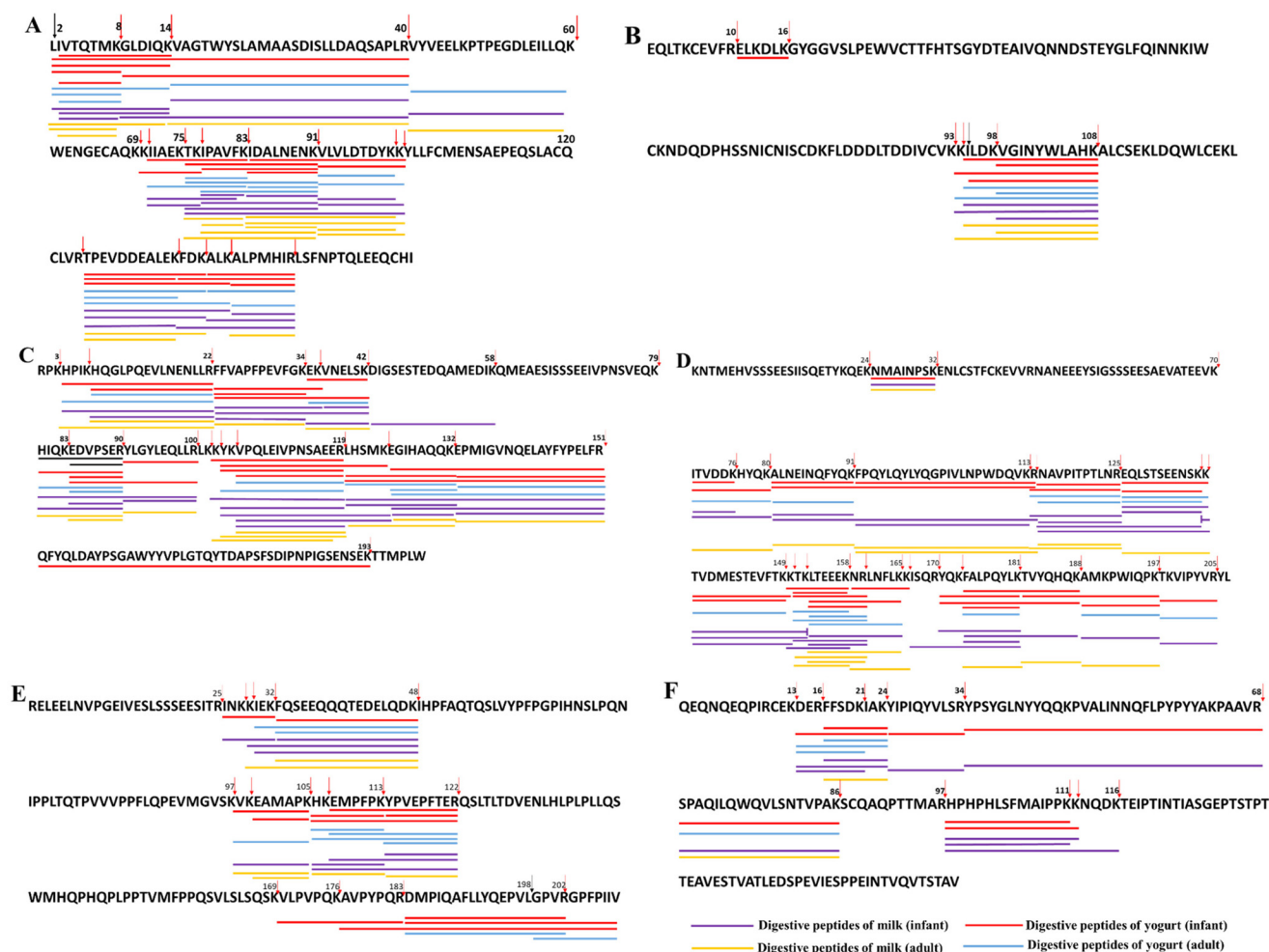


Fig. 8 Identification of peptides of major allergens (A: BLG; B: ALA; C: α_{s1} -casein; D: α_{s2} -casein; E: β -casein; F: κ -casein) after gastrointestinal digestion in infants (pH 3) and adults (pH 1) by LC-MS/MS. The red arrows indicate specific trypsin sites and the black arrows indicate non-specific trypsin sites.

Pascal *et al.* identified four T cell epitope regions of peanut allergen Ara h, and further proved the accuracy of NetMHCIIpan and NetMHCII methods in predicting T cell epitopes through T cell proliferation assay and cytokine profile analysis.⁴⁴ In previous studies, 6 T cell epitopes were predicted by consensus and the NetMHCIIpan method in IEDB combined with other bioinformatics tools, and further confirmed in T cell proliferation and cytokine release.⁴⁵ Allergen-specific T cells epitopes play an important role in allergic reactions and are also considered as safe and effective immunomodulators.⁴⁶ Oral immunotherapy using T-cell epitopes has been reported to ameliorate allergic responses in mouse models of egg and shrimp allergy.⁴⁷ In this study, we performed the prediction of major allergen T cell epitopes in cow's milk in IEDB (ESI Table 1†). Meanwhile, in Tables 5 and 6, we summarize the allergic epitopes and T cell epitopes of major allergens from previous literature. After listing the information, we compared and summarized in Tables 2 and 3 whether the digested

peptides contained allergenic epitopes and T cell epitopes. Tables 2 and 3 also show that some major allergic epitopes are disrupted and some peptides contained IgE epitopes.

Firstly, we analyzed all peptides in the digested samples for epitope analysis. In Tables 2 and 3, the peptides of BLG (AA 76–91), α_{s1} -casein (AA 104–124), and β -casein (AA184–209) had higher IL-4pred scores, and these peptides contained T cell epitopes and IgE epitopes. There was also digestive resistance and this was present in the digestion of both fresh milk and yogurt. Similarly, the peptide of BLG (AA 41–60) had higher IL-4pred scores, and it contained IgE epitopes and was resistant to gastrointestinal digestion. Therefore, these peptides had great potential to enter the body to cause Th2 cell response and induce allergy. Studies have also demonstrated that BLG (AA 76–91, AA 41–60) and α_{s1} -casein (AA 104–124) are IgE epitopes, which can induce CMA.^{48,49} BLG (AA 78–91) and α_{s1} -casein (AA 23–42, AA 106–119, AA 106–124, AA 23–42, AA106–119) had higher IL-4pred scores and contained T cell

Table 2 Identified peptides of BLG and ALA in yogurt and gastrointestinal digestion (infants and adults) of fresh milk or yogurt. “✓” means “contains”. “*” indicates that the peptides and IEDB-predicted T cell epitopes are more than 80% identical. “#” indicates that the peptides contain previously reported T cell epitopes. “H” means that the epitope is hydrolyzed, and “N” means that the epitope is not hydrolyzed

Protein sequence (fragment)	Digestedmilk (infant)	Digestedyogurt (infant)	Digestedmilk (adult)	Digestedyogurt (adult)	Biological activity	IL4 inducer	IgE epitopes	T cell epitopes
BLG								
LIVTQTMK (1-8)		✓	✓	✓	Cytotoxic		H (1-16)	
LIVTQTMKGLDIQK (1-14)		✓	✓	✓			H (1-16)	
LIVTQTMKGLDIQKVAGTWYSLAMAASDISILLDAQSAPLR (1-40)		✓	✓	✓			N (1-16, 21-40)	#
IVTQTMK (2-8)	✓	✓				0.22	H (1-16)	
IVTQTMKGLDIQK (2-14)	✓	✓					H (1-16)	#
GLDIQKVAGTWYSLAMAASDISILL	✓	✓					N (21-40), H (1-16)	*
DAQSAPLR (9-40)								
VAGTWYSLAMAASDISILLDAQSAPLR (15-40)	✓		✓	✓			N (21-40), H (1-16)	#
VYVEELKPTPEGDLLEILLQK (41-60)	✓		✓	✓	Hypocholesterolemic	1.20	N (41-60)	
KIAEKTk (70-77)		✓				0.55	H (76-90, 76-95, 58-77, 67-86)	
IIAEKTK (71-77)	✓						H (76-90, 76-95, 58-77, 67-86)	
IIAEKTKIPAVFK (71-83)	✓	✓		✓		0.49	H (76-90, 76-95, 58-77, 67-86)	
TKIPAVFK (76-83)	✓			✓		0.29	H (76-90, 76-95, 58-77, 67-86)	
TKIPAVFKIDALNENK (76-91)	✓	✓	✓	✓		1.36	N (76-90), H (76-95, 58-77, 67-86)	*
IPAVFK (78-83)	✓		✓		Stimulates proliferation, DPP-IV Inhibitory, antimicrobial, ACE-inhibitory	0.28	H (76-90, 76-95, 67-86)	
IPAVFKIDALNENK (78-91)	✓	✓	✓	✓		1.35	H (76-90, 76-95, 67-86)	*
IDALNENK (84-91)	✓	✓	✓	✓	Stimulates proliferation, antimicrobial	0.29	H (76-90, 76-95, 67-86)	
IDALNENKVLVLDTDYK (84-100)			✓				H (76-90, 76-95, 67-86)	*
IDALNENKVLVLDTDYKK (84-101)	✓	✓	✓	✓			H (76-90, 76-95, 67-86)	*
VLVLDTDYK (92-100)	✓		✓	✓	DPP-IV Inhibitory, antimicrobial		H (76-90, 76-95, 67-86)	
VLVLDTDYKK (92-101)	✓	✓	✓	✓			H (76-95)	
TPEVDDEALEK (125-135)	✓	✓	✓	✓	DPP-IV Inhibitory, antimicrobial	1.32	H (121-140, 127-152)	
TPEVDDEALEKFDK (125-138)	✓	✓	✓	✓			H (121-140, 127-152, 136-150)	
TPEVDDEALEKFDKALK (125-141)	✓	✓		✓		1.27	H (121-140, 127-152, 136-150)	*
FDKALKALPMHIR (136-148)	✓	✓					H (121-140, 127-152, 136-150)	

Table 2 (Contd.)

Protein sequence (fragment)	Digestedmilk (infant)	Digestedyogurt (infant)	Digestedmilk (adult)	Digestedyogurt (adult)	Biological activity	IL4 inducer	IgE epitopes	T cell epitopes
ALKALPMHIR (139–148)	✓	✓		✓	Stimulates proliferation	0.25	H (121–140, 127–152, 136–150)	
ALPMHIR (142–148)	✓	✓	✓	✓	Stimulates proliferation, ACE-inhibitory	0.26	H (127–152, 136–150)	
ALA								
ELKDLK (11–16)		✓				0.28	H (1–16, 1–19, 13–26, 15–34)	
KILDKVGINYWLAHK (94–108)	✓	✓	✓	✓		1.14	H (90–109, 93–102, 105–123)	
ILDKVGINYWLAHK (95–108)	✓	✓	✓	✓	DPP-IV Inhibitory	0.94	H (90–109, 93–102, 105–123)	
LDKVGINYWLAHK (96–108)		✓				1.02	H (90–109, 93–102, 105–123)	
VGINYWLAHK (99–108)	✓	✓	✓	✓	ACE-inhibitory, DPP-IV Inhibitory		H (90–109, 93–102, 105–123)	

epitopes. Therefore, there was great potential to elicit a Th2 cell response. Other anti-digestive peptides with high IL4 scores also have the potential to induce Th2 cell responses. These peptides also provide potential targets for the treatment of CMA. But the experimental evidence for Th2 peptides is limited.

Peptides are an important factor with important implications in immunotherapy and vaccine design. Candreva *et al.* developed a new strategy for the prevention of milk allergy based on a soy-derived peptide that cross-reacts with casein.⁵⁰ The peptide contains T and B cell epitopes of the soy allergen Gly-m-Bd-30K and stimulates T cells without causing IgE cross-linking on basophils and mast cells. Identification of allergen-specific T cell epitopes facilitates the development of immunotherapeutic vaccines targeting T cell epitopes.⁵¹ Some studies have demonstrated that peptides containing T cell epitopes may induce immune tolerance and the absence of IgE epitopes reduces the risk of allergic reactions.^{52–54} Meanwhile, these peptides are not digested by the gastrointestinal tract, which is an important factor in their effect. Immunodominant T-cell epitopes can also induce oral tolerance to pollen allergy in mice and humans.^{55,56} It was found from the results in Tables 2 and 3 that a total of 11 peptides might induce tolerance to CMA. The peptides of BLG (AA 2–14, AA 84–100, AA 84–101, AA 136–148), α_{s1} -casein (AA 84–100, AA 120–151, AA 125–151), β -casein (AA 184–202), and κ -casein (AA 98–111, AA 98–112, AA 98–116) did not have the IgE epitopes, but also contained T cell epitopes and were not the inducers of IL4 according to prediction. This revealed that these peptides had the potential to induce tolerance.

Secondly, comparing the released peptides, it was found that some peptides only existed in the digestion of fresh milk or yogurt. In the digestion of fresh milk, a total of 16 peptides were specific. Among them, BLG (AA 84–100), α_{s1} -casein (AA 80–100), and κ -casein (AA 98–116) contained T cell epitopes and could not induce IL4 that might induce tolerance to CMA. Besides, α_{s1} -casein (AA 43–58, AA 106–124), α_{s2} -casein (AA 167–181) and β -casein (AA 29–48) could induce the release of IL4 and contained T cell epitopes. α_{s2} -Casein (AA 92–113) could induce the release of IL4 and contained T cell and IgE epitopes. α_{s2} -Casein (AA 115–136, AA 115–137) contained IgE epitopes. α_{s1} -Casein (AA 37–42) and α_{s2} -casein (AA 182–188) could induce the release of IL4. So the allergic properties of peptides in fresh milk digestion might induce CMA. Likewise, the properties of specific peptides in the digestion of yogurt might also induce CMA. β -Casein (177–209) contained T cell and IgE epitopes, and could induce the release of IL4. α_{s2} -Casein (AA 171–188) could induce the release of IL4 and contained T cell epitopes. α_{s1} -Casein (AA 152–193) contained T cell epitopes and IgE epitopes. ALA (AA 11–16, AA 96–108), BLG (AA 70–77, AA 78–83), α_{s2} -casein (AA 182–197, AA 138–149) and β -casein (AA 199–209) could induce the release of IL4. In the digestion of yogurt, α_{s1} -casein (AA 84–100) contained T cell epitopes and could not induce the release of IL4 that might induce tolerance to CMA.

Table 3 Identified peptides of casein (α_{s1} -casein, α_{s2} -casein, β -casein, κ -casein) in yogurt and gastrointestinal digestion (infants and adults) of fresh milk or yogurt. “✓” means “contains”. “*” indicates that the peptides and IEDB-predicted T cell epitopes are more than 80% identical. “#” indicates that the peptides contain previously reported T cell epitopes. “H” means that the epitope is hydrolyzed, and “N” means that the epitope is not hydrolyzed

Protein sequence (fragment)	Digestedmilk (infant)	Digestedyogurt (infant)	Digestedmilk (adult)	Digestedyogurt (adult)	Yogurt	Biological activity	IL4 inducer	IgE epitopes	T cell epitopes
α_{s1}-Casein									
HPKHQGLPQEVLENENLLR (4–22)	✓	✓	✓	✓				N (6–20), H (11–35, 16–35, 17–36, 19–30)	
HQGLPQEVLENENLLR (8–22)	✓	✓	✓	✓	✓			H (6–20, 11–35, 16–35, 17–36, 19–30)	
FFVAPFPEVFGK (23–34)	✓	✓	✓		✓	ACE-inhibitory,	0.36	H (28–50, 11–35, 16–35, 17–36, 19–30)	*
FFVAPFPEVFGKEK (23–36)	✓	✓			✓		0.37	H (28–50, 11–35, 16–35, 17–36, 19–30)	*
FFVAPFPEVFGKEKVNELSK (23–42)	✓	✓					1.50	H (28–50, 11–35, 16–35, 17–36, 19–30, 39–48)	*
EKVNELSK (35–42)	✓	✓	✓	✓			0.32	H (28–50, 17–36, 39–48)	
VNELSK (37–42)	✓						0.28	H (28–50, 39–48)	
DIGSESTEDQAMEDIK (43–58)	✓	✓	✓			Promotes calcium uptake	1.09	H (28–50, 39–48)	
HIQKEDVPSEK (80–90)	✓	✓	✓	✓	✓			H (76–90, 73–92, 83–102, 93–98)	*
HIQKEDVPSERYLGYLEQLLR (80–100)	✓							H (76–90, 73–92, 83–102, 93–98)	
EDVPSEK (84–90)	✓	✓	✓	✓	✓			H (76–90, 73–92, 83–102, 93–98)	
EDVPSERYLGYLEQLLR (84–100)	✓	✓	✓			Anxiolytic	0.35	H (73–92, 83–102, 93–98)	*
YLGYLEQLLR (91–100)	✓	✓	✓				0.48	H (109–120)	*
KYKVPQLEIVPNSAEER (103–119)	✓	✓	✓			Promotes calcium uptake	0.58	H (109–120)	*
YKVPQLEIVPNSAEER (104–119)	✓	✓	✓	✓				N (109–120), H (122–132)	*
YKVPQLEIVPNSAEERLHSMK (104–124)	✓	✓					1.39	H (109–120, 122–132)	*
VPQLEIVPNSAEER (106–119)	✓	✓	✓	✓			0.57	H (109–120)	*
VPQLEIVPNSAEERLHSMK (106–124)	✓	✓					1.39	H (109–120, 122–132)	*
LHSMKEGIHAQQK (120–132)	✓	✓	✓	✓	✓		0.41	H (122–132, 126–140, 109–120)	*
LHSMKEGIHAQQKEPMIGVGNQELAYFPPELFR (120–151)	✓	✓			✓			H (122–132, 126–140, 109–120, 139–154, 141–150)	*

Table 3 (Contd.)

Protein sequence (fragment)	Digested milk (infant)	Digested yogurt (infant)	Digested milk (adult)	Digested yogurt (adult)	Yogurt	Biological activity	IL4 inducer	IgE epitopes	T cell epitopes
EGIHAAQK (125–132)	✓		✓	✓			0.30	H (122–132, 126–140)	
EGIHAAQKEPMIGVNQELAYFYPELFR (125–151)	✓	✓		✓	✓			H (122–132, 126–140, 139–154, 141–150)	*, #
EPMIGVNQELAYFYPELFR (132–151)	✓	✓	✓	✓			0.25	H (122–132, 126–140, 139–154, 141–150)	*, #
QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEK (152–193)		✓						N (159–174, 171–185), H (139–154, 173–194, 181–199)	*, #
α _{s2} -Casein									
NMAINPSK (25–32)	✓	✓	✓			ACE-inhibitory	0.29	H (13–32, 31–44)	
ITVDDK (71–76)	✓	✓	✓				0.28	H (67–82)	
ITVDDKHYQK (71–80)	✓	✓	✓	✓			0.28	H (67–82)	
ALNEINQFYQK (81–91)	✓	✓	✓	✓		ACE-inhibitory	0.22	H (67–82, 83–100)	
ALNEINQFYQKFPQYLQYLYQGPIVLPWDQVKR (81–114)	✓	✓						N (83–100, 93–108, 105–114), H (67–82, 106–125)	*
FPQYLQYLYQGPIVLPWDQVK (92–113)	✓		✓				0.33	N (93–108), H (106–125, 105–114)	*
FPQYLQYLYQGPIVLPWDQVKR (92–114)		✓	✓				0.34	N (93–108, 105–114), H (106–125)	*
RNAVPTPTLNR (114–125)	✓	✓	✓	✓			0.38	H (106–125, 105–114, 117–128, 122–141)	
NAVPTPTLNR (115–125)	✓	✓	✓	✓			0.34	H (106–125, 117–128, 122–141)	
NAVPTPTLNRQELSTSENSK (115–136)	✓							N (117–128), H (106–125, 122–141)	
NAVPTPTLNRQELSTSENSKK (115–137)	✓							N (117–128), H (106–125, 122–141)	
EQLSTSENSK (126–136)	✓	✓		✓			0.31	H (117–128, 122–141)	
EQLSTSENSKK (126–137)	✓	✓	✓	✓			0.28	H (117–128, 122–141)	
KTVDMESTEVFTKTK (137–152)	✓							H (122–141, 143–158)	
TVDMESTEVFTK (138–149)		✓		✓			0.20	H (122–141, 143–158)	

Table 3 (Contd.)

Protein sequence (fragment)	Digested milk (infant)	Digested yogurt (infant)	Digested milk (adult)	Digested yogurt (adult)	Yogurt	Biological activity	IL4 inducer	IgE epitopes	T cell epitopes
TVDMESTEVFTKK (138–150)	✓	✓						H (122–141, 143–158)	
TVDMESTEVFTKTK (138–152)	✓							H (122–141, 143–158)	
KTKLTEEEK (150–158)	✓	✓						H (143–158, 157–172, 157–182)	
TKLTEEEK (151–158)		✓	✓	✓				H (143–158, 157–172, 157–182)	
TKLTEEKNR (151–160)	✓	✓	✓	✓				H (143–158, 157–172, 157–182)	
LTEEKNR (153–160)	✓	✓	✓	✓				H (143–158, 157–172, 157–182)	
LTEEKNRLNFKL (153–165)	✓	✓	✓	✓				H (143–158, 157–172, 157–182)	
NRLNFKK (159–166)		✓	✓				0.28	H (157–172, 157–182, 165–188)	
ISQRYQKFALPQYLK (167–181)	✓						0.42	H (157–172, 157–182, 165–188)	*
YQKFALPQYLK (171–181)	✓	✓					0.30	H (157–172, 181–207)	
YQKFALPQYLKTVYQHQQ (171–188)		✓					0.46	H (157–172, 157–182, 165–188, 181–207)	*
FALPQYLK (174–181)	✓	✓	✓	✓	✓	Antioxidant, ACE-inhibitory	0.29	H (157–182, 165–188, 181–207)	
FALPQYLKTVYQHQQ (174–188)	✓	✓					0.44	H (157–182, 165–188, 181–207)	*
TVYQHQQ (182–188)			✓				0.29	H (157–182, 165–188, 181–207)	
TVYQHQQAMKPWIQPK (182–197)		✓					0.24	H (157–182, 165–188, 181–207)	
AMKPWIQPK (189–197)	✓	✓	✓	✓		ACE-inhibitory	0.28	H (181–207, 191–200)	
TKVIPYVR (198–205)	✓	✓		✓	✓		0.24	H (181–207, 191–200)	
β-Casein INKKIEK (26–32)	✓	✓					1.27	H (25–50)	

Table 3 (Contd.)

Protein sequence (fragment)	Digested milk (infant)	Digested yogurt (infant)	Digested milk (adult)	Digested yogurt (adult)	Yogurt	Biological activity	IL4 inducer	IgE epitopes	T cell epitopes
KIEKFQSEEQQTEDELQDK (29–48)	✓		✓				0.30	H (25–50, 45–54)	#
IEKFQSEEQQTEDELQDK (30–48)	✓			✓			0.32	H (25–50, 45–54)	
FQSEEQQTEDELQDK (33–48)	✓	✓	✓	✓		Promotes calcium uptake		H (25–50, 45–54)	
VKEAMAPK (98–105)	✓	✓	✓	✓		Antioxidant	0.27		
EAMAPK (100–105)	✓	✓	✓	✓		Antimicrobial	0.28		
HKEMPPPK (106–113)	✓	✓	✓	✓		Antimicrobial	0.29		
HKEMPPPKYPVEPFTER (106–122)	✓	✓							
EMPPKYPVEPFTER (108–122)	✓	✓		✓			0.25	H (107–120) N (107–120), H (121–140)	
YPVEPFTER (114–122)	✓	✓	✓	✓				H (107–120, 121–140)	
VLPVPQK (170–176)	✓		✓			Antioxidant, antimicrobial, ACE-inhibitory, anti-apoptotic effect		H (154–173, 167–184)	
VLPVPQKAVPYPQR (170–183)	✓	✓				Antimicrobial		H (154–173, 167–184)	
AVPYPQRDMP IQAFLL (177–192)					✓			H (167–184, 185–208)	
AVPYPQRDMP IQAFLLYQEPVLGPVRGPFPIIV (177–209)		✓					0.73	N (185–208), H (167–184)	*
DMPIQAFLLYQEPVLGPVR (184–202)	✓	✓	✓	✓		Anti-inflammatory		H (167–184, 185–208)	*
DMPIQAFLLYQEPVLGPVRGPFPIIV (184–209)	✓	✓			✓		0.87	N (185–208), H (167–184)	*
GPVRGPFPIIV (199–209)				✓			0.35	H (185–208)	
κ-Casein									
DERFFSDK (14–21)	✓			✓			0.36	H (9–26, 16–35, 21–44)	
DERFFSDKIAK (14–24)	✓	✓		✓			0.30	H (9–26, 16–35, 21–44)	
FFSDKIAK (17–24)	✓	✓	✓	✓			0.29	H (9–26, 16–35, 21–44)	
YIPQYVLSR (25–34)	✓	✓			✓	Opioid	0.37	H (9–26, 16–35, 21–44)	
YPSYGLNYYQQKFWALINNQFLPYPPYAKPAVR (35–68)	✓	✓						N (47–68), H (16–35, 21–44, 34–53, 67–78)	*
SPACILQWQVLSNTVPAK (69–86)	✓	✓	✓	✓	✓		0.22	H (67–78)	*
HPHPLSFMAPPK (98–111)	✓	✓						H (95–116, 111–126)	*
HPHPLSFMAPPKK (98–112)	✓	✓						H (95–116, 111–126)	*
HPHPLSFMAPPKKNQDK (98–116)	✓	✓						H (95–116, 111–126)	*

Table 4 The biological activity of anti-digestive peptides

Protein	Anti-digestive peptides (fragment)	Biological activity
BLG	LIVTQTMKGLDIQK (1–14)	Stimulates proliferation, antimicrobial
	IVTQTMK (2–8)	
	TKIPAVFKIDALNENK (76–91)	
	IPAVFKIDALNENK (78–91)	
	IDALNENK (84–91)	
ALA	VLVLDTDYKK (92–101)	DPP-IV Inhibitory, antimicrobial
	TPEVDDEALEK (125–135)	
	TPEVDDEALEKFDK (125–138)	
	ALPMHIR (142–148)	
α_{s1} -Casein	KILDKVGINYWLAHK (94–108)	DPP-IV inhibitory ACE-inhibitory, DPP-IV Inhibitory
	ILDKVGINYWLAHK (95–108)	
	VGINYWLAHK (99–108)	
	HPIKHQGLPQEVLENLLR (4–22)	
	HQGLPQEVLENLLR (8–22)	
	EKVNELSK (35–42)	
	HIQKEDVPSER (80–90)	
	EDVPSER (84–90)	
	YKVPQLEIVPNSAEER (104–119)	
	VPQLEIVPNSAEER (106–119)	
α_{s2} -Casein	LHSMKEGIHAQQK (120–132)	Promotes calcium uptake
	EPMIGVNQELAYFYPELFR (132–151)	
	ITVDDKHQYQK (71–80)	
	ALNEINQFYQK (81–91)	
	RNAVPITPTLNR (114–125)	
	NAVPTPTLNR (115–125)	
	EQLSTSEENSKK (126–137)	
	TKLTEEEKNR (151–160)	
	LTEEEKNR (153–160)	
	LTEEEKNRLNFK (153–165)	
β -Casein	FALPQYLK (174–181)	Antioxidant, ACE-inhibitory ACE-inhibitory
	AMKPWIQPK (189–197)	
	FQSEEQQTEDELQDK (33–48)	
	VKEAMAPK (98–105)	
	HKEMPPFK (106–113)	
κ -casein	YPVEPFTER (114–122)	Antioxidant Antimicrobial
	DMPIQAFLLYQEPVLGPVR (184–202)	
	FFSDKIAK (17–24)	Anti-inflammatory
	SPAQILQWQVLSNTVPAK (69–86)	

Under the same digestion conditions (infants or adults), the total number of major allergen peptides in fresh milk and yogurt was not obviously different. In digestion in infants, fresh milk and yogurt released 94 and 87 peptides, respectively, of which 72 peptides were identical. And 22 peptides in fresh milk and 15 peptides in yogurt were specific. By comparing the epitopes of these specific peptides, 22 peptides in fresh milk had 15 allergenic sequences that either contained IgE epitopes or induced IL4 production and had 2 peptides to

induce tolerance. 15 peptides in yogurt had 12 allergenic sequences that either contained IgE epitopes or induced IL4 production and had the peptide of α_{s1} -casein (AA 84–100) to induce tolerance. Moreover, fresh milk and yogurt released 59 and 58 peptides in digestion in adults, respectively. 44 peptides were identical, and 15 and 14 peptides were specific in fresh milk and yogurt, respectively. 15 specific peptides in fresh milk had 12 potential allergic sequences and had 2 peptides to induce tolerance. Similarly, 14 specific peptides in yogurt possessed 13 potential allergic sequences and had the peptide of α_{s1} -casein (AA 125–151) to induce tolerance. Therefore, these specific epitopes might be responsible for the difference in IgE-binding capacity between fresh milk and yogurt digestion. However, this was different to our expectation: peptides released from major allergens in yogurt did not have more tolerogenic peptides or fewer allergenic sequences than fresh milk. The possible reasons were that we only analyzed the peptides of the main allergens, or the content of the peptides was different. In food allergy, destroying IgE epitopes and preserving T cell epitopes are the key to reducing or eliminating allergy. Predicting epitopes in these peptides provided targets for reducing or eliminating CMA.

Research has reported that feeding a fraction of BLG hydrolysates in mice induced tolerance to BLG.⁵⁷ The co-administration of BLG and its digestive products reduced the sensitization ability of the intact protein.²⁰ Meulenbroek *et al.* previously screened the four peptides containing T cell epitopes in human T cell lines and used them in an animal model. Among them, T-cell epitopes of BLG (AA 31–48) could reduce acute allergic skin response and increase the percentages of CD11b⁺ CD103⁺ dendritic cells and CD25⁺ Foxp3⁺ T cells to enhance oral tolerance.⁵⁸ Subsequently, the four peptide mixtures of BLG combined with other substances could further enhance oral tolerance to milk proteins. A mixture of four BLG-peptides combined with a fructo-oligosaccharide and *Bifidobacterium breve* M-16V-enriched diet could reduce the allergic response to whole whey protein.^{59,60} The mixture of peptides in poly-nanoparticles facilitated the capacity of BLG peptides to suppress the allergic response to whole whey protein and was further developed as an adjunct strategy for BLG-peptide based oral tolerance induction.⁶¹ Thus, peptides containing T cell epitopes contribute to the development of oral tolerance and prevent CMA.

In this study, the actually released peptides in digestion were compared with previously reported epitopes and predicted T cell epitopes for the first time, and the prediction of the release ability of IL-4 and the biological activity of the peptides were evaluated. Through these bioinformatic analyses, it is hoped that the role of peptides in gastrointestinal digestion can be more realistically reflected. It also provides new strategies for the prevention and treatment of CMA and contributes to the generation of peptide vaccines. Meanwhile, preliminary screening of peptides was also carried out, which also reduced the working cost for further related basic research.

Table 5 The IgE epitopes previously reported in the literature

Protein	IgE-binding epitopes (amino acid number)	Ref.
BLG	1–16, 56–70, 76–90, 136–150	48
	58–77, 76–95, 121–140	62
	1–16, 31–60, 67–86, 127–152	63
	21–40, 41–60, 102–124, 149–162	49
ALA	1–19, 15–34, 45–64, 60–79, 90–109, 105–123	64
	1–16, 13–26, 47–58, 93–102	63
α_{s1} -Casein	6–20, 11–35, 76–90, 126–140, 171–185	48
	16–35, 28–50, 73–92	62
	17–36, 39–48, 69–78, 83–102, 109–120, 122–132, 139–154, 159–174, 173–194	65
	181–199	66
α_{s2} -Casein	19–30, 93–98, 141–150	67
	31–44, 43–56, 83–100, 93–108, 105–114, 117–128, 143–158, 157–172, 165–188, 191–200	68
	1–20, 13–32, 67–2, 106–25, 122–41, 157–182, 181–207	62
	1–16, 45–54, 55–70, 83–92, 107–120, 135–144, 149–164, 167–184, 185–208	69
B-Casein	25–50, 52–74, 121–140, 154–173	62
	9–26, 21–44, 47–68, 67–78, 95–116, 111–126, 137–148, 149–166	69
K-Casein	16–35, 34–53	62

Table 6 T cell epitopes previously reported in the literature

Protein	T cell epitopes (amino acid number)	Ref.
BLG	17–36, 129–148, 137–156	70
	1–21, 14–29, 30–47, 47–67, 77–97, 97–117, 142–162	71
	13–30, 19–36, 25–42, 31–48	58
ALA	19–36, 25–42, 31–48, 43–60	72
α_{s1} -Casein	17–36, 105–124, 137–156, 145–164, 169–188	70
	43–66, 73–96, 91–114, 133–156	73
	49–66, 55–72, 103–120, 109–126, 127–144, 133–150, 139–156, 145–162, 151–168	74
	1–18, 16–35, 67–85, 91–110, 136–155, 152–169, 166–183	75
α_{s2} -Casein	17–36, 177–196, 185–204	70
β -Casein	25–44, 41–60, 49–68, 65–84, 89–108, 113–132, 153–172, 193–212	70
κ -Casein	1–20, 57–76, 105–124, 153–172	70

4. Conclusion

In this study, we explored the differences in digestive stability and IgE-binding capacity of major allergens from yogurt and fresh milk and found that the major allergens in the yogurt were more easily digested with stronger transport capacity and had lower IgE-binding capacity. By comparing the sequence of peptides, we found there were 16 and 12 peptides with sequence specificity in fresh milk and yogurt, respectively. Meanwhile, under the same digestion conditions (infants or adults), the total number of major allergen peptides in fresh milk and yogurt was not obviously different, but 72 and 44 peptides were identified in infants and adults, respectively. Although there was also no obvious difference in the number of epitopes derived from peptides of digested yogurt and fresh milk, the properties and content of specific peptides might also be the main factor in CMA. Therefore, the destruction of these allergic peptides may be the key to reducing or eliminating allergy. Of note, 11 peptides contained T cell epitopes but not IgE epitopes, which might induce immune tolerance in CMA. Identification of T cell epitopes contributes to understanding allergy mechanisms and is expected to be applied in the development of hypoallergenic dairy products. The study also provides new strategies for the prevention and treatment

of CMA, and contributes to the generation of peptide vaccines. However, further experiments are required.

Abbreviations

ACE	Angiotensin-converting enzyme
ALA	α -Lactalbumin
BLG	β -Lactoglobulin
CCK-8	Cell counting kit-8
CMA	Cow's milk allergy
DMEM	High-glucose Dulbecco's modified Eagle's medium
DPP-IV	Dipeptidyl peptidase-IV
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
IEDB	Immune Epitope Database
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
RP-HPLC	Reversed-phase high-performance liquid chromatography
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SVM	Support vector machine
TMB	3,3',5,5'-Tetramethylbenzidine
TSLP	Thymic stromal lymphopoietin

Author contributions

Meijia Huang was responsible for designing the experiments and writing the manuscript. Hongkai Tan, Ziyi Xiong and Wei Hu conducted the experiments about SDS-PAGE and HPLC. Yong Wu and Xuanyi Meng analyzed the data. Hongbing Chen helped to conduct the experimental design. Xin Li (corresponding author) conducted the experimental design and revised the manuscript.

Conflicts of interest

All authors declare no conflict of interest.

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