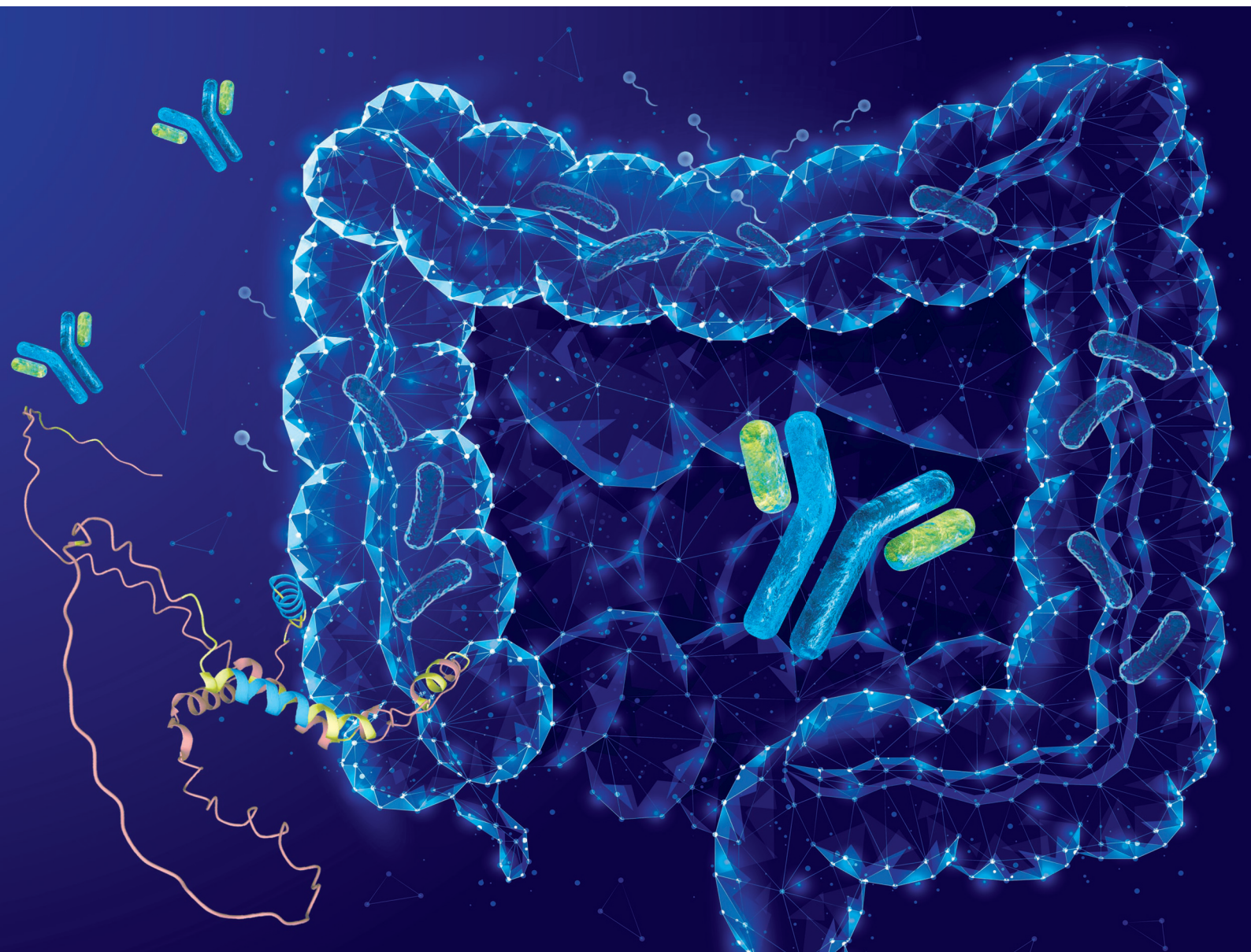


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Cow's milk α_{S1} -casein is more sensitizing than goat's milk α_{S1} -casein in a mouse model†

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The aim of this study was to compare the sensitization of α_{S1} -CN in cow and goat's milk in a mouse model. Fifty mice were divided into control group, adjuvant control group, cow's milk α_{S1} -CN sensitized group, goat's milk α_{S1} -CN sensitized group and cross sensitized group. Cow's and goat's milk α_{S1} -CN were used to establish a mouse sensitization model. The results showed that cow's milk α_{S1} -CN had higher allergenicity than goat's milk α_{S1} -CN, as can be seen in significantly increased s-IgE and Th2 cell-related inflammatory factors, the proportion of Th2, and the expression of Th2 cell-related transcription factors. Furthermore, the sensitization of cow's milk α_{S1} -CN damaged the intestinal barrier of mice, caused the leakage of LPS, activated the TLR4-NF κ B pathway, and thus resulted in the increase of IFN- γ . In addition, mice allergic to cow's milk α_{S1} -CN were less sensitized to goat's milk α_{S1} -CN.

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1. Introduction

Food allergy is an immune mediated and reproducible adverse reaction. According to the mechanism, food allergy can be classified into immunoglobulin E (IgE) mediated, non-immunoglobulin E (non-IgE) mediated or mixed (IgE and non-IgE mediated).¹ IgE mediated food allergy is characterized by the T helper 2 (Th2) cell polarization, leading to the production of specific-IgE (s-IgE). Binding of s-IgE and the allergen to the receptor on mast cells provokes mast cell degranulation and release of mediators, such as histamine, leukotrienes, tumor necrosis factor, prostaglandins, and some cytokines and chemokines. These mediators result in clinical symptoms involving anaphylactic shock, asthma, urticaria and so on.^{2–4}

Although breastfeeding has been highly recommended by the WHO, there were still a certain number of babies relying on infant formula for their growth and development due to the unavailability of breastmilk. As cow's milk based infant formula was the dominant one in the market, cow's milk protein has become the first protein exposed to most of the infants who cannot obtain breastfeeding. However, there are still a certain number of infants allergic to cow's milk based

infant formula. Thus, cow's milk allergy (CMA) is considered as the most common food allergy in early life of infants.⁵ The incidence of CMA varied with age, which was 2–6% in early life and 0.1–0.5% in adulthood.^{6–8} A previous study has shown that α -lactalbumin (α -LA, Bos d4), β -lactoglobulin (β -LG, Bos d5) and casein (CN, Bos d8) are the dominant allergy proteins most frequently recognized by IgE in cow's milk.⁹ Of these allergy proteins, caseins account for approximately 80% of the total protein, and are considered as the major allergy protein group in cow's milk. Among the four subgroups of caseins (α_{S1} -, α_{S2} -, β - and κ -CN) in cow's milk, α_{S1} -CN was reported to have the strongest allergenic activity.¹⁰ According to a previous study, α_{S1} -casein can be recognized by serum IgE in approximately 50% of the patients with cow's milk allergy.¹¹ And there are 7 active epitopes in α_{S1} -CN, which were 1–18, 16–35, 67–85, 91–110, 136–155, 152–169 and 166–183, respectively.¹² Currently, there is no perfect treatment for CMA and the most effective way is to avoid cow's milk protein intake. However, reducing milk based product consumption may significantly influence the nutrient intake for infants.¹³ In recent years, hydrolyzed formula has been used as a milk source for infants with milk protein allergy; however, the taste, nutritional value, and biological function of milk proteins are decreased in partially and deeply hydrolyzed formulas.¹⁴ In addition, clinical studies have shown that infants consuming deeply hydrolyzed milk formula have slow growth, reduced plasma protein concentrations and imbalances in urinary amino acid concentrations.¹⁵ Therefore, it is urgent to find a low allergenic milk protein substitute for cow's milk.

Similar to cow's milk, goat's milk casein was also the major potential allergen protein group. However, α_{S1} -CN accounts for

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only 5% of the total casein in goat's milk, which is much lower compared to that of cow's milk (38% of the total casein).¹⁶ Furthermore, the amino acid sequence of goat's milk α_{S1} -CN was different from that of cow's milk, which has 88.32% homology between the two species. And the sequence of mouse α_{S1} -CN has 21.70% and 23.05% homology between cow's milk and goat's milk (data from Uniprot: <https://www.uniprot.org>). In addition, the theoretical isoelectric point and stability index of α_{S1} -CN were different between cow and goat species, which may lead to the differences in their sensitization. A previous study had compared the differences in the sensitization of milk proteins between cow and goat species. For instance, cow's milk proteins were observed to increase specific IgE and lymphocyte proliferation index significantly as compared to goat's milk proteins in a murine model.¹⁷ In clinical trials, goat's milk induced higher levels of IL-10 by peripheral blood mononuclear cells than cow's milk, which means that goat's milk can prevent the development of allergies.¹⁸ However, the sensitization of specific milk protein α_{S1} -CN between cow's milk and goat's milk has not been discussed yet. In addition, there is no formal evidence whether goat's milk can be used as a substitute milk source for children sensitized by cow's milk.

The use of mice is recognized as a robust model to help in identifying any potential sensitizers. FAO/WHO suggested that the potential allergenicity of the food protein be ranked against well-known strong and weak food allergens in the animal models.^{19,20} Therefore, the aim of this study is to compare the sensitization between cow's milk and goat's milk α_{S1} casein through a sensitization mouse model. The result of this study may provide theoretical basis for goat's milk to be a substitute milk source for CMA patients.

2. Materials and methods

2.1 α_{S1} -CN extraction

Raw milk was centrifuged at 1500g for 20 min to obtain skim milk. Then, the whole caseins were extracted using isoelectric precipitation. Briefly, the pH was adjusted to 4.6 (4.1 for goat's milk) using 1 M HCl. After precipitation for 30 min, the solution was centrifuged at 3000g for 15 min. The precipitate was washed with deionized water 3 times. To obtain α -CN, the whole caseins were dissolved in 6.6 M urea (pH 7.0). After complete dissolution, the urea solution was diluted to 3.3 M (3.0 M for goat's milk). Then the pH was adjusted to 4.6 (4.1 for goat's milk), and the solution was centrifuged at 3000g for 15 min. The precipitate was washed with deionized water 3 times. After the precipitate was dissolved, the urea was removed by dialysis and finally α -CN was obtained.²¹ As α_{S2} -CN is more sensitive to Ca^{2+} than α_{S1} -CN,²² the α -CN was dissolved (pH 7.0) and 3 mM CaCl_2 was added. After complete precipitation for 30 min, the solution was centrifuged at 3000g for 15 min. Then, the supernatant was added with 20 mM (60 mM for goat's milk) CaCl_2 , and centrifuged at 3000g for 15 min after complete precipitation. The precipitate was α_{S1} -CN, which was dried and preserved at -80°C . The purity and

molecular weight of α_{S1} -CN were analyzed by SDS-PAGE and LC-MS.

2.2 SDS-PAGE

Purified samples were assessed by SDS-PAGE, according to the published methods with minor modifications.²³ Samples were prepared in loading buffer (Bio-Rad, California, USA) with 5% β -mercaptoethanol (Sangon Biotech, Shanghai, China) and boiled for 5 min. The 1.0 mm polyacrylamide gels consisted of a 2.5% stacking gel and a 12% separating gel (Bio-Rad, California, USA). Samples were run on the gel for 1.5 h at 120 V, and then stained using the colloidal Coomassie Blue G 250 method.²⁴

2.3 LC-MS

LC-MS was carried out according to previous studies.^{25,26} 1 mg sample was dissolved in 1 mL distilled water, and the solution was centrifuged at 10 000g for 10 min. The supernatant was analyzed using a Waters Maldi Synapt MS (Waters, Massachusetts, USA), and LC separation was done on a Waters Acquity UPLC (Waters, Massachusetts, USA) equipped with a Waters Acquity UPLC BEH C18 column (2.1 mm $\times$ 150 mm, 1.7 μm) at 45 $^\circ\text{C}$. The mobile phases were solutions of 100% acetonitrile (A) and 0.1% formic acid (B). The flow rates of elution were set at 0.3 mL min^{-1} . The Maldi Synapt Q-TOF MS was in positive ionization mode with a cone voltage of 30 Volts and collision energy of 6 eVolts. The data were analyzed using MassLynx V4.1.

2.4 Animals and treatments

The study was conducted after obtaining approval from the Jiangnan University Institutional Animal Ethics Committee. 50 female Balb/C mice (3–4 weeks old, 10–12 g) were purchased from Charles River Labs (Zhejiang, China). The animal experiment batch number was JN. No. 20201130b0800120 [351]. The mice were randomly divided into five groups and housed in individually ventilated cages (five mice were placed in each cage), provided with standard chow and water. The room temperature was $23 \pm 2^\circ\text{C}$ with 12-hour light/dark cycles. After being acclimatized to these conditions, the mice were sensitized intragastrically with protein and cholera toxin (CT, Sigma, USA) weekly for 5 weeks and challenged at week 6. The animal treatment was referred to Tanarro *et al.*²⁷ The intestinal tissue of 6 mice was used for mRNA extraction, and the other 4 mice were used for sectioning, so a total of 10 mice were required for each group. The feeding and treatment schedules were as follows (Fig. 1):

Group 1 (Con): sensitized with normal saline, challenged with normal saline.

Group 2 (CT): sensitized with CT (0.5 μg per g bw), challenged with normal saline.

Group 3 (C-C): sensitized with cow's α_{S1} -CN (100 μg per g bw) and CT (0.5 μg per g bw), challenged with 20 mg cow's α_{S1} -CN.

Group 4 (G-G): sensitized with goat's α_{S1} -CN (100 μg per g bw) and CT (0.5 μg per g bw), challenged with 20 mg goat's α_{S1} -CN.

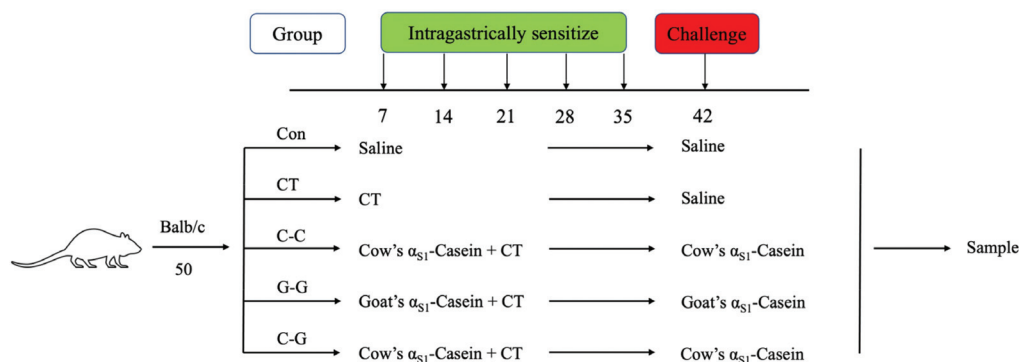


Fig. 1 Immunization protocol of different α_{S1} -casein sensitization models. Five groups of mice were sensitized on days 7, 14, 21, 28, 35, and 42 and challenged on day 42.

Group 5 (C-G): sensitized with cow's α_{S1} -CN (100 μg per g bw) and CT (0.5 μg per g bw), challenged with 20 mg goat's α_{S1} -CN.

After 6 weeks of feeding, mice were sacrificed, and spleen, mesenteric lymph nodes, intestine, and blood were collected for further analysis.

2.5 Semi-quantification of specific IgE and IgG1

After sacrifice, α_{S1} -CN specific IgE and IgG1 of sera were measured by enzyme-linked immunosorbent assay (ELISA).²⁸ 96 well plates (Corning, New York, USA) were coated overnight at 4 °C with cow's α_{S1} -CN or goat's α_{S1} -CN (10 $\mu\text{g mL}^{-1}$ for IgE and 1 $\mu\text{g mL}^{-1}$ for IgG1). After washing, wells were blocked with 2% bovine serum albumin for 2 h at 37 °C. After washing, mouse sera (5 times dilution for IgE and 40 times dilution for IgG1) were incubated for 2 h at 37 °C. After washing, goat anti mouse IgE-HRP (1:500) or IgG1-HRP (1:10 000) (Thermo, Massachusetts, USA) was incubated for 2 h at 37 °C. After washing, plates were incubated with TMB substrate solution (Biosharp, Anhui, China) for 20 min at 37 °C and stopped with 1 M H_2SO_4 . Absorbance was measured at 450 nm using a Multiskan Skyhigh Full Wavelength Microplate (Thermo, Massachusetts, USA). The final result is expressed in OD value.

2.6 Assessment of Th1 and Th2 cells by flow cytometry

Mesenteric lymph nodes (MLNs) were harvested from mice and placed on nylon mesh cell strainers. The MLNs were moistened by RBMI 1640 medium (Thermo, Massachusetts, USA) and slowly ground using a syringe plug. After the MLNs were fully ground, the medium was transferred to 50 mL sterile tubes and centrifuged at 500g for 10 min. After washing, the cells were stimulated using Leuko Act Cktl With GolgiPlug (BD, New Jersey, USA) for 4 h. After washing, cells were incubated with CD4 FITC antibodies for 20 min at 4 °C. After washing 3 times, the cells were fixed using Cytofix/Cytoperm Soln Kit (BD, New Jersey, USA) for 20 min. After washing 3 times, cells were incubated with IL-4 PE and IFN- γ APC antibodies (BD, New Jersey, USA) for 20 min. After washing, cells

were analyzed using BD FACSAriaII (BD, New Jersey, USA), and data were analyzed using FlowJo 10.

2.7 RT-qPCR

Total RNA from the small intestines and spleens was extracted using FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, Nanjing, China). Then the total RNA sample (500 ng μL^{-1} ; 260:280 = 1.8–2.2) was reverse-transcribed to cDNA using HiScript® II Q Select RT SuperMix for qPCR (Vazyme, Nanjing, China). After cDNA synthesis, the mRNA expression levels were assayed by RT-qPCR using ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme, Nanjing, China) and Roche LightCycler 96 (Roche, Basel, Switzerland). The list of the primers used in this study is summarized in Table S1.† The relative levels of gene expression were calculated by reference to the β -actin in each sample.

2.8 Western bolt analysis

Western bolt was performed as previously described.²⁹ The spleen and small intestine samples were homogenized in RIPA lysis buffer with 1 mmol L^{-1} PMSF (Beyotime, Shanghai, China). The concentration of proteins was measured using BCA Kit (Thermo, Massachusetts, USA). The proteins were separated using SDS-PAGE and transferred to a NC membrane according to the standard scheme. Next, the NC membrane was incubated with specific primary antibodies (Abcam, Shanghai, China) and HRP-conjugated secondary antibodies (Thermo, Massachusetts, USA). The bands were analyzed using a Tanon 5200 Multi (Tanon, Shanghai, China) and ImageJ software. The expression ratios were normalized to the expression ratio to β -actin.

2.9 Histological analysis

For the histopathological analysis, the small intestine samples were trimmed into suitable size and fixed in 10% neutral formalin. Next, the samples were dehydrated in graded alcohol, cleared in xylene and embedded in paraffin. Then, the paraffin was sectioned at 5 μm . Finally, tissue sections were stained with Hematoxylin & Eosin (H&E) or toluidine blue (TB).

2.10 DNA isolation and 16S rRNA gene amplification and sequencing

DNA from fecal samples was extracted using TIANamp Stool DNA Kit (Tiangen, Beijing, China) according to instructions. And the concentration of DNA was measured using Equalbit dsDNA HS Assay Kit (Vazyme, Nanjing, China). The V3–V4 region of the 16S rRNA gene was amplified using universal primers (341F and 806R). Then the libraries were purified by magnetic beads, and the concentration is detected using a Nano Drop (Thermo, Massachusetts, USA) and agarose gel electrophoresis. The libraries were sequenced at Illumina MiSeq/Novaseq (Illumina, San Diego, USA).

2.11 Bioinformatic analysis

VSEARCH (1.9.6) was used for sequence clustering (sequence similarity was set at 97%), and the reference database was Silva 138. Then, the RDP Classifier (Ribosomal Database Program) Bayesian algorithm was used to carry out taxonomic analysis on the representative OTU sequences, and the community composition of each sample was counted at different species classification levels. Proportional representation of taxonomic groups was provided by an OTU table and taxa summary generation within each sample. Alpha diversity, heatmap and Linear Discriminant Analysis Effect Size (LEfSe) were performed online (<https://www.microbiomeanalyst.ca>). Beta diversity was measured using R (version 3.5.0) software.

2.12 Statistical analysis

Statistical analysis was preformed using SPSS 25 and GraphPad Prism 8. Data were presented as means with SD. The *p*-values of less than 0.05 were considered statistically significant. For the analysis of the differences in the biochemical measures between the five groups, one-way ANOVA (Analysis of Variance) with the Tukey *post-hoc* test was used.

3. Results

3.1 α_{S1} -casein extracts

After purification, cow and goat α_{S1} -CN was measured by SDS-PAGE and LC-MALDITOF-MS. The SDS-PAGE result

showed that both cow and goat α_{S1} -CN have one main band with a purity of 94.25% and 90.1% (Fig. 2A and B). In addition, it should be noted that the molecular masses of both cow's milk and goat's milk α_{S1} -CN on SDS-PAGE exceeded the theoretically calculated masses, and this was consistent with the results of other studies and the reason may be that the high phosphorylation of α_{S1} -CN not only resulted in higher molecular weight and but also led to the reduction of SDS binding and slow migration.³⁰ Furthermore, LC-MS analyses were preformed to confirm the purity of the isolated α_{S1} -CN (Fig. S1†). The peak of α_{S1} -CN was found in both cow's milk and goat's milk with purity of more than 90%.

3.2 Differences in allergenicity of α_{S1} -casein between cow's milk and goat's milk

In order to know whether these two different proteins affect the growth and development of mice with and without oral administration, we measured the weight of mice every week. As shown in Fig. 3(A), there was no significant difference in body weight between different groups after 6 weeks of oral administration. We counted the number of mice scratching in 20–40 min after the oral challenge.³¹ As shown in Fig. 3 (B), mice sensitized with cow's α_{S1} -CN (C–C group) showed stronger reactions after challenge. Mice sensitized with goat's α_{S1} -CN (G–G group) had relatively low scratching. Mice of the Con, CT, and C–G groups showed no symptoms of hypersensitivity after challenge. The level of β -hexosaminidase (β -Hex) in serum is an indicator of mast cell degranulation¹ and it had significantly higher concentration in the C–C group than in the Con group and CT group, as shown in Fig. 3(C). Although G–G and C–G groups also increased, their concentrations were significantly lower than that in the C–C group (Fig. 3C).

The highest IgE and IgG1 level was detected in the C–C group, which was significantly higher than that of Con, CT, even G–G and C–G groups. It is worth noting that the IgE in G–G and C–G groups also increased slightly, but there was no significant difference. Furthermore, serum specific IgE and IgG1 levels also varied largely between individuals within each

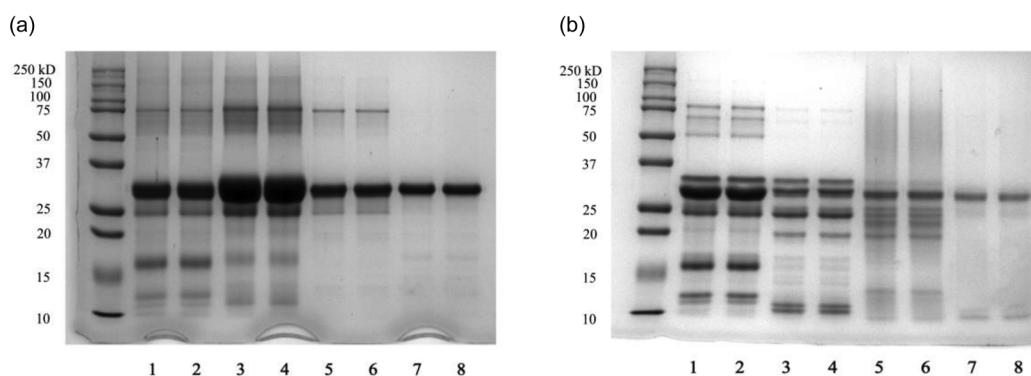


Fig. 2 SDS-PAGE characterization of purified α_{S1} -casein. (A) Cow's milk α_{S1} -casein; (B) goat's milk α_{S1} -casein; lanes 1 and 2, skimmed milk; lanes 3 and 4, casein; lanes 5 and 6, α -casein; lanes 7 and 8, α_{S1} -casein.

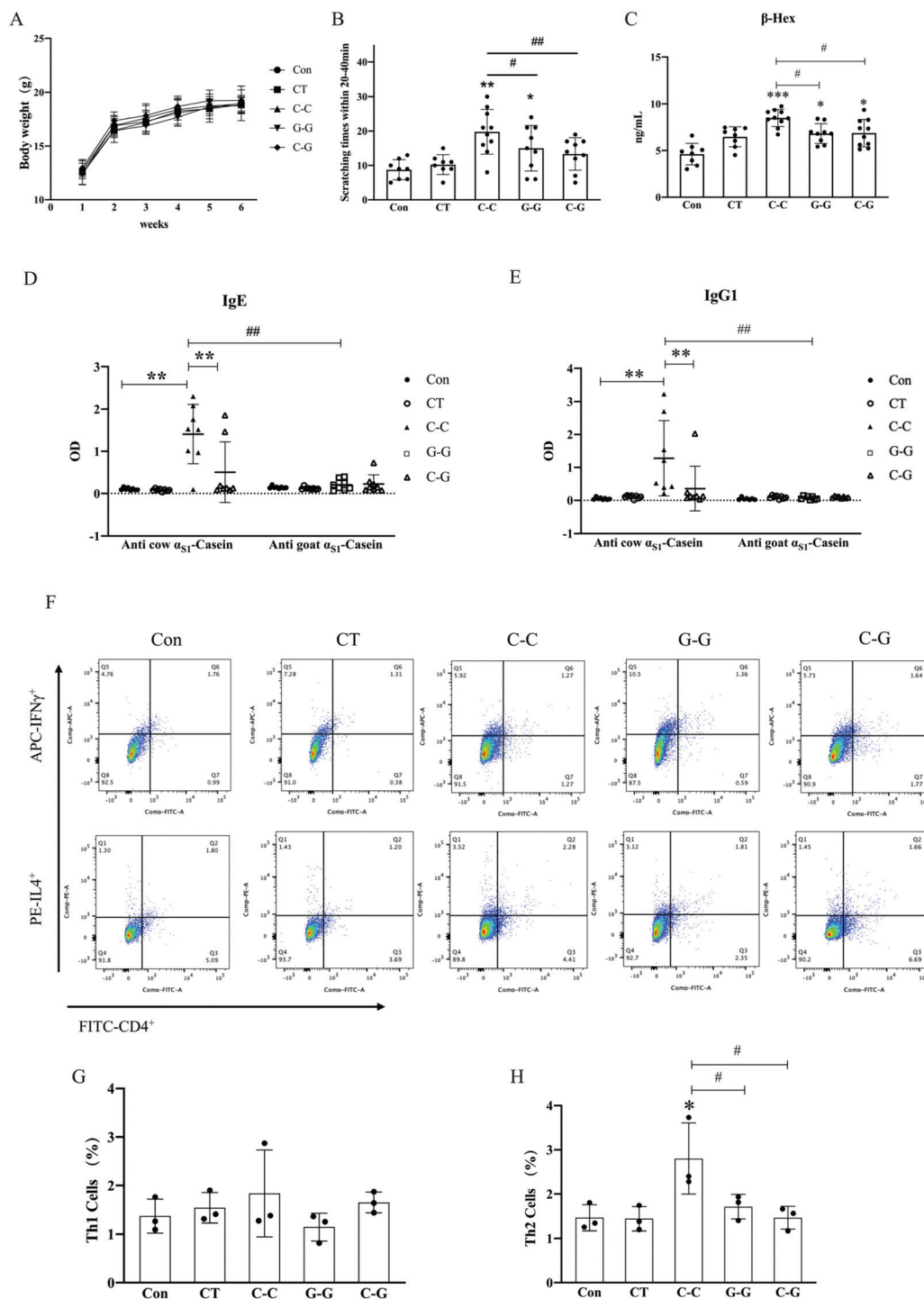


Fig. 3 Variable effect of oral challenge of different species of α_{S1} -CN in mice. (A) Body weight change in mice; (B) the number of times the mice scratched within 20–40 minutes after the oral challenge; (C) β -hexosaminidase levels in serum. (D) Specific IgE levels in serum. (E) Specific IgG1 levels in serum. (F) IFN γ^+ and IL-4 $^+$ within CD4 $^+$ T cells in mesenteric lymph nodes. (G) CD4 $^+$ IFN γ^+ Th1 cells in mesenteric lymph nodes. (H) CD4 $^+$ IL-4 $^+$ Th2 cells in mesenteric lymph nodes. Values indicate the mean $\pm$ SD. * indicates significant difference from the Con group ($P < 0.05$). # indicates significant difference from the C–C group ($P < 0.05$).

experimental group (Fig. 3D and E). Like the trend of other apparent indicators, the population of Th2 cells in the C–C group significantly increased in the mesenteric lymph nodes (MLN) compared with the other four groups. Administration of

goat's α_{S1} -CN didn't increase the population of Th2 cells. On the other hand, the population of mesenteric lymph node Th1 cells has no significant differences in all groups (Fig. 3F–H).

3.3 Immune cell-related inflammatory cytokines and transcription factors

To further evaluate the effect of cow and goat α_{S1} -CN on the immune response, the levels of cytokines in the serum were measured. IL-4 and IL-5 production was significantly enhanced in the C-C group, which was much higher than that of the C-G group and G-G group (Fig. 4A and B). IFN- γ also significantly increased in the C-C group compared with the Con group (Fig. 4C). However, there was no significant change in IL-10 between all groups (Fig. 4D).

Next, we tested Th1 and Th2 cell differentiation related proteins, to see the differences in the influence of cow and goat α_{S1} -CN on the transcription factors of T lymphocytes. T-box transcription factor (T-bet), which is related to Th1 cell differentiation, was not significantly different in each group (Fig. 4E and H), whereas GATA binding protein 3 (GATA3), which is related to Th2 cell differentiation, was significantly increased in the C-C group but not in G-G and C-G groups compared to the Con group (Fig. 3F and I). Forkhead box protein P3 (Foxp3), which is a crucial regulator of regulatory T (Treg) cell gene expression,³² showed no significant differences between groups (Fig. 4G).

Interestingly, IFN- γ , an inflammatory factor for Th1 cell differentiation, was significantly increased in the C-C group. However, transcription factors related to Th1 cells and the population of Th1 cells did not change in the C-C group. Therefore, we believed that there might be other factors inducing the increase of IFN- γ .

3.4 Sensitization of cow's milk α_{S1} -CN leads to activation of the TLR4/NF- κ B pathway

Our data showed that the mRNA levels of Toll-like receptor 4 (TLR4) in the C-C group were significantly higher than that of G-G and C-G groups (Fig. 5A). It is reported that TNF receptor associated factor 6 (TRAF6) and myeloid differentiation factor 88 (Myd88) are major signal transducers in the TLR4/NF- κ B (nuclear factor kappa-B) pathway.³³ Consistent with TLR4 mRNA expression, the C-C group expressed higher levels of TRAF6 and Myd88 mRNA expression compared with the Con and CT groups. G-G and C-G groups reduced the levels of TRAF6 and Myd88 mRNA expression, which was basically the same as that in the Con group (Fig. 5B and C). In addition, our data showed increased NF- κ B mRNA levels in the C-C group (Fig. 5D), consistent with the trend for IFN- γ .

3.5 Characterization of fecal microbiota in mice with α_{S1} -CN allergy

A total of 338 574 high-quality sequence reads were generated for analysis, and clustered into 256 OTUs (97% nucleotide identity). Ordination and classification independent analyses of the fecal bacterial community structure indicated that Chao1 index has increased in C-C and G-G groups but there is no significant difference. However, allergic mice (C-C group) had significantly higher Shannon index than Con groups (Fig. 6A and B). In addition, PCoA results showed that the G-G

group was closer to the Con group than the C-C group (Fig. 6C), suggesting that allergen immunization strongly influenced the fecal microbiota composition.

At the phylum level, *Firmicutes* was enriched in C-C group mice. The *Bacteroidetes* to *Firmicutes* ratio decreased in the mice with cow's milk α_{S1} -CN allergy. The most significant difference was 21.57% increase in the relative abundance of *Firmicutes* and 20.57% reduction in *Bacteroidetes* found in the C-C group compared with the Con group. With respect to comparison between the G-G and Con groups, the most significant differences were 16.41% increase of *Firmicutes* and 17.23% reduction of *Bacteroidetes* in the G-G group (Fig. 6E).

To further identify the bacterial taxa in the feces of sensitized mice, Linear discriminant analysis Effect Size (LEfSe) was used to evaluate the effect of size on each differentially abundant taxon. The result showed that *Saccharimonadaceae* was enriched in the Con group, *Lachnospiraceae*, *Oscillospiraceae*, and *Butyricicoccaceae* were significantly enriched in the C-C group and *Ruminococcaceae* was enriched in the G-G group (Fig. 6F). It was surprising to find that Gram-negative bacteria in the fecal microbiota of the sensitized mice didn't increase, but short-chain fatty acid-producing bacteria increased.

3.6 Cow's α_{S1} -CN allergies cause damage to the intestinal barrier

Following our observation that the change of fecal microbiome was not the cause of the increase in LPS, we examined the effect of milk allergy on the integrity of the mouse intestinal barrier to determine whether the increase of LPS was caused by intestinal barrier impairment. The challenge of cow's milk α_{S1} -CN led to histopathological alterations in intestinal tissues. The jejunum stained with H&E (hematoxylin-eosin) revealed marked structure looseness, vascular congestion and edema of the lamina propria in the C-C group. In addition, the villi in the C-C group had obvious swelling and sloughing of enterocytes (arrow area) with a remarkable inflammatory process compared with the Con group. On the other hand, there was no obvious damage in the jejunum and only minor signs of edema and congestion in the G-G group and C-G group (Fig. 7H). In addition, the toluidine blue (TB) staining result showed that the C-C group has an increased number of intact mast cells and degranulated mast cells in the jejunum (arrow area) compared to the Con group; however, no difference was found between G-G, C-G and Con groups (Fig. 7I).

Furthermore, we examined the intestinal barrier related mRNA expression in the colon. As shown in Fig. 7, we observed that the level of tight junction proteins, Occludin, Claudin1 and Claudin3 significantly decreased in the colon of the C-C group compared with the Con group, whereas Claudin2 significantly increased. Although zonula occludens-1 (ZO-1) and zonula occludens-2 (ZO-2) decreased in the C-C group the decrease was not significant. In sum, there was no obvious damage in colon in the G-G group and C-G group.

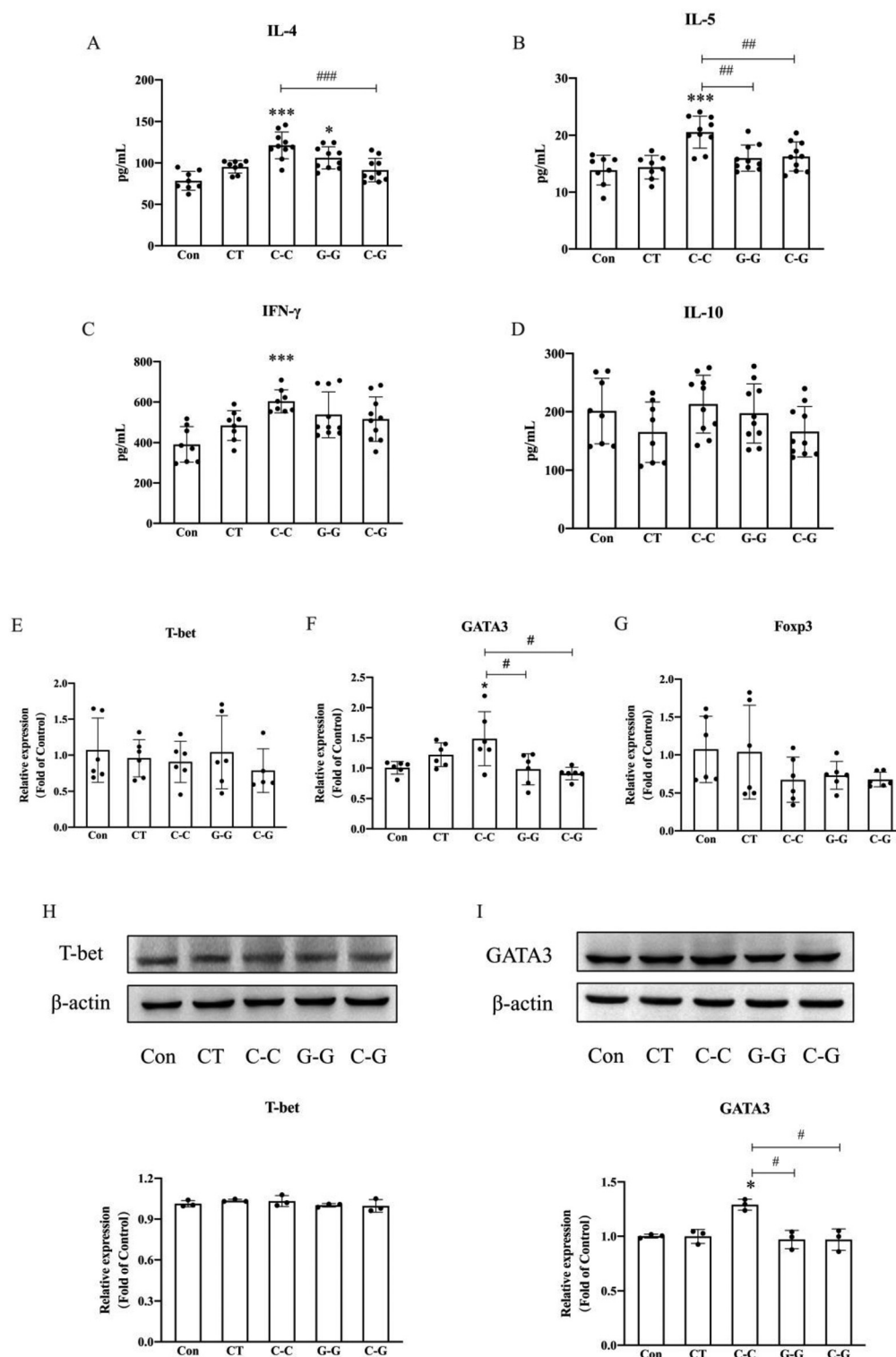


Fig. 4 Cytokines and the expression of Th1/Th2 transcription factors after challenge. (A–D) The content of IL-4, IL-5, IFN- γ , and IL-10 in serum; (E–G) relative expression level of T-bet, GATA3, and Foxp3 mRNA in the spleen; (H and I) relative expression level of T-bet and GATA3 protein in the spleen. Values indicate the mean $\pm$ SD. * indicates significant difference from the Con group ($P < 0.05$). # indicates significant difference from the C–C group ($P < 0.05$).

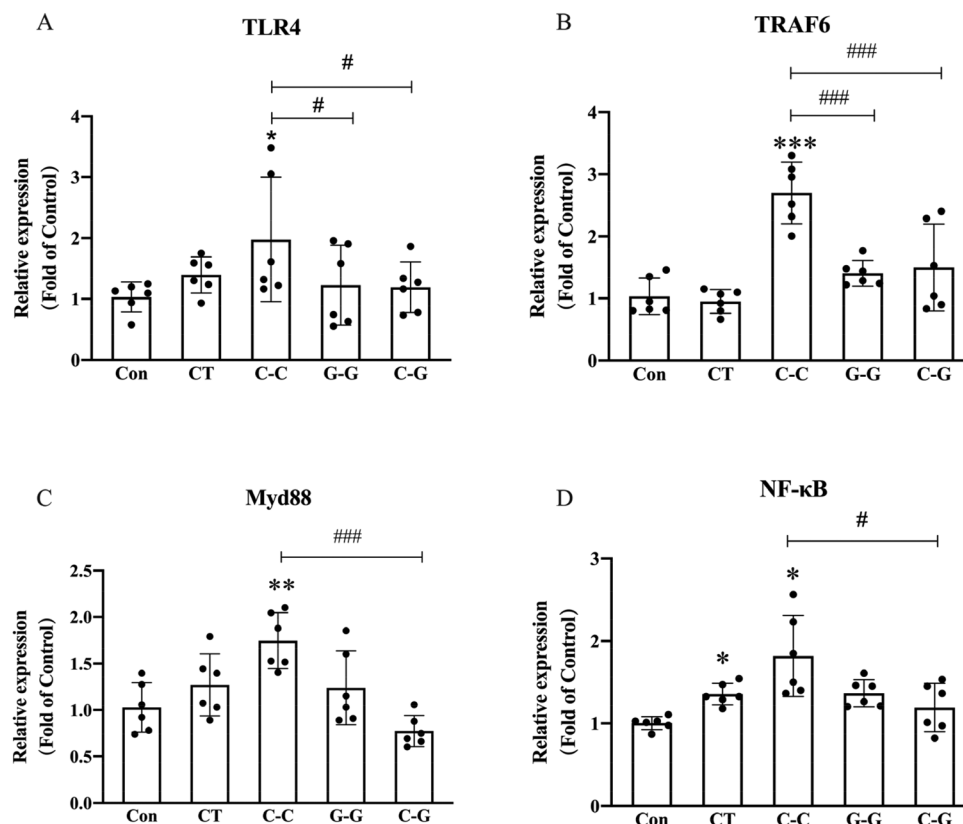


Fig. 5 Effect of α_{S1} -CN sensitization on the TLR4/NF- κ B pathway in mice. (A) Relative expression level of TLR4 mRNA in the spleen; (B) relative expression level of TRAF6 mRNA in the spleen; (C) relative expression level of Myd88 mRNA in the spleen; (D) relative expression level of NF- κ B mRNA in the spleen. Values indicate the mean $\pm$ SD. * indicates significant difference from the Con group ($P < 0.05$). # indicates significant difference from the C-C group ($P < 0.05$).

4. Discussion

There have been constant disputes about the sensitization of goat's milk and cow's milk and whether goat's milk is less allergenic than cow's milk is still one of the biggest issues. Some studies have shown that the sensitization and immunogenicity of allergy patients reduced when switching the milk source from cow's milk to goat's milk.³⁴ But there are also many studies showing that there is no difference in sensitization between cow's milk and goat's milk.³⁵ The main reason for these disputes is that goat's milk contains low quantity of α_{S1} -CN, which is recognized as one of the important allergens in milk. However, there is no research showing the difference in the allergenicity of cow's milk α_{S1} -CN and goat's milk α_{S1} -CN. So, the aim of the present study was to test the differences in sensitization of α_{S1} -CN in cow's milk and goat's milk to mice. According to our knowledge, this is the first study to apply a multi-level approach to analyze allergy responses to α_{S1} -CN between cow and goat in mice.

There are multiple immune cells in the mesenteric lymph nodes, such as T lymphocytes, B lymphocytes and dendritic cells, which can produce an immune system response under the induction of allergen proteins, so they are important induction and effect sites for allergic reactions.³⁶ As expected,

we also found that mice with cow's milk α_{S1} -CN allergy had higher levels of CD4⁺ IL-4⁺ Th2 cells, which indicated that Th1/Th2 balance is altered in MLN of C-C group mice but not in G-G and C-G groups. Th2 cell cytokines and transcription factor are central to the pathogenesis of allergic diseases. GATA3 is a T cell specific transcription factor, which regulates the development of T cells, promotes Th2 cell differentiation and stimulates the secretion of IL-4, IL-5, and IL-6 cytokines from activated Th2 cells.³⁷ The increase of IL-4, IL-5, IL-6 and GATA3, in C-C group mice was consistent with many previous studies,³⁸ suggesting that allergy induced the changes in Th2 cytokines and transcription factor.

The transition to Th2 determines allergic reactions, while Th1 cytokines inhibit these reactions. The balance of Th1/Th2 is crucial in the development of allergies. Transcription factors and inflammatory factors are key molecules that determine the balance of Th1/Th2.¹ Therefore, GATA3 and T-bet play an important role in the transition of Th1 and Th2.³⁹ We found that the proportion of Th1 cells did not change, and there was no significant difference in T-bet content in all groups. However, IFN- γ , as a predominant Th1 cytokine, was significantly increased in the most sensitized C-C group, which is different from previous studies.⁴⁰ There are multiple signaling pathways that can control the expression of IFN- γ , such as

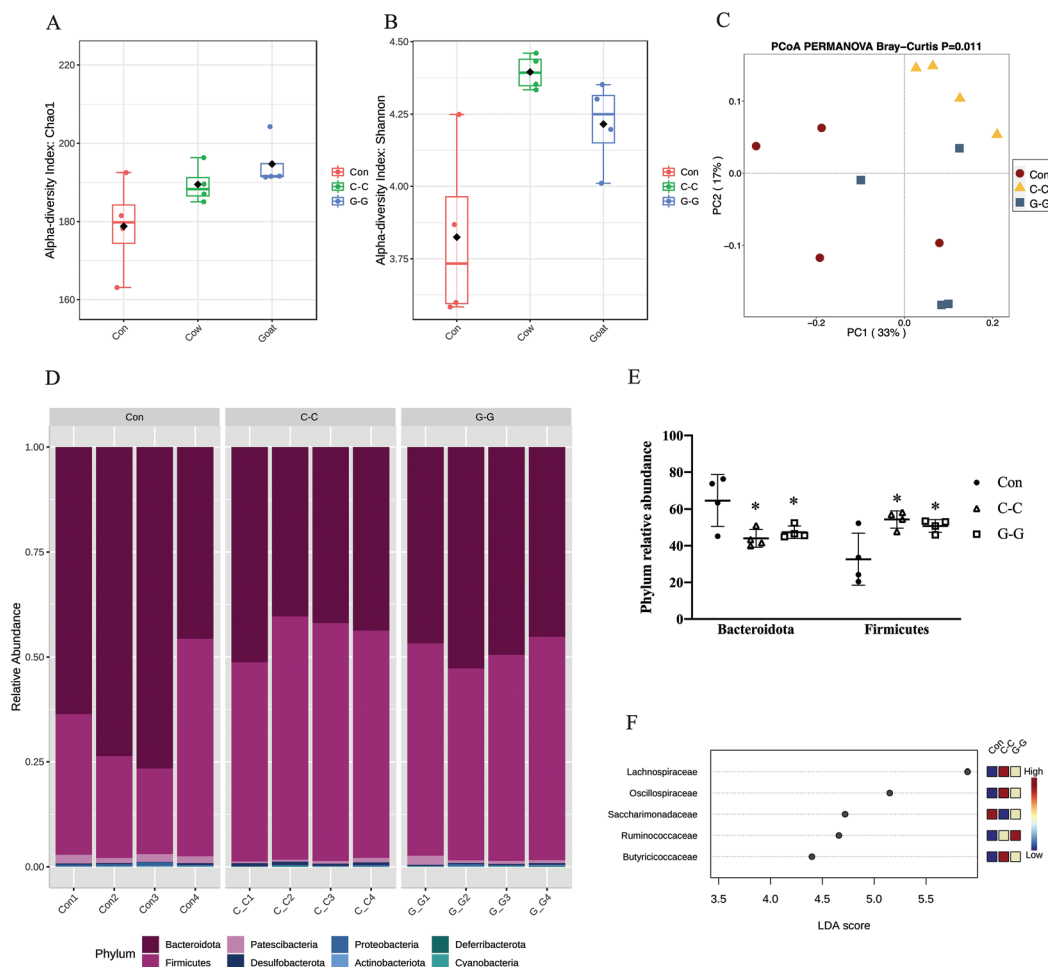


Fig. 6 Significant diversity bacterial community dynamic across different α_{S1} -CN. (A) Chao1 diversity; (B) Shannon diversity; (C) PcoA of microbiota compositions (genus level); (D and E) phylum level differential abundance; (F) LefSe identifies the most differentially abundant taxa between each group. Values indicate the mean $\pm$ SD. * indicates significant difference from the Con group ($P < 0.05$). # indicates significant difference from the C–C group ($P < 0.05$).

AMPK signaling pathway, T-bet signaling pathway and NF- κ B signaling pathway.⁴¹ Although LPS induced the release of IFN- γ by NK cells and the release of pro-inflammatory cytokines, including IFN- γ , IL-1 and IL-6 have been reported, LPS may play roles in allergy, which is not clear.⁴² LPS, also known as endotoxin, is the main component of the outer membrane of the cell wall of Gram-negative bacteria. It is released by lysis of Gram-negative bacteria in the intestine and is a key factor in inducing systemic inflammatory reactions.⁴³ Thus, we would like to measure LPS content in serum. Unfortunately, the serum had been used up for previous experiment. Therefore, we detected the mRNA expression of the TLR4 in the spleen, the receptor of LPS. The results showed that TLR4 was significantly increased in the C–C group, suggesting that the sensitization of cow's milk α_{S1} -CN caused an increase in LPS content in mice. At the same time, the expression of TRAF6 and Myd88 in the downstream of TLR4 also increased, which eventually led to an increase in the expression of NF- κ B. However, there were no changes in the above indicators in the G–G group and

C–G group. Previous studies have shown that NF- κ B is a positive regulator of IFN- γ production.⁴⁴ And in our data, the increased NF- κ B mRNA levels in the C–C group were consistent with the trend for IFN- γ .

These results indicated that the sensitization of cow's milk α_{S1} -CN was stronger than that of goat's milk α_{S1} -CN in the mouse model, which was manifested by more specific antibodies, mast cell lysates, Th2-related inflammatory factors and transcription factors, and the proportion of Th2 cells produced by cow's milk α_{S1} -CN sensitization. In addition, cow's milk α_{S1} -CN hypersensitivity could increase LPS in mice and induce a series of inflammatory reactions. At the same time, the intensity of the goat's milk α_{S1} -CN allergy group was lower than that of the cow's milk α_{S1} -CN allergy group.

Although our data suggested that LPS positively regulated IFN- γ in cow's milk α_{S1} -CN sensitized mice through the TLR4/NF- κ B pathway, it is not clear whether the increase of LPS was caused by the change of the intestinal microbiota or the damage of the intestinal barrier.⁴⁵ We next investigated the

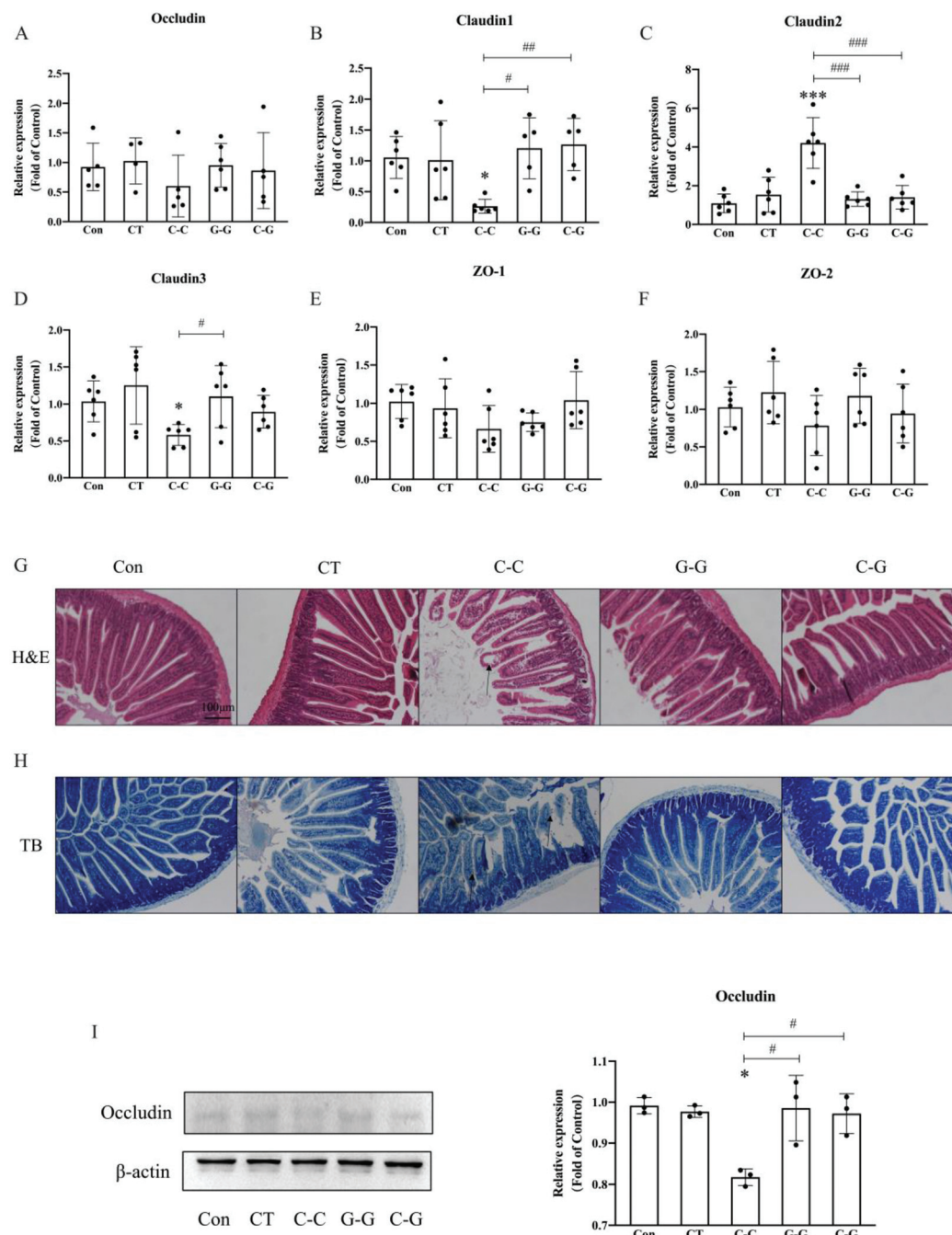


Fig. 7 The expression of intestinal barrier related mRNA and protein in the colon and the histopathology results of jejunum segments. (A–F) Relative expression level of Occludin, Claudin1, Claudin2, Claudin3, ZO-1, and ZO-2 in the colon; (G) relative expression level of Occludin protein in the colon; (H and I) Jejunum stained with H&E and TB. Values indicate the mean $\pm$ SD. * indicates significant difference from the Con group ($P < 0.05$). # indicates significant difference from the C–C group ($P < 0.05$).

mechanism by which allergy regulates LPS production in mice. Here we have made two conjectures: (1) The increase of Gram-negative bacteria in the intestinal microbiota of cow's milk α_{S1} -CN allergic mice resulted in the increase of LPS content; (2) The cow's milk α_{S1} -CN allergy caused intestinal barrier damage in mice, leading to LPS leakage. To verify our conjecture, we tested the fecal microbiota and intestinal barrier of mice. Numerous clinical and epidemiological studies have

shown that the clinical manifestations and development of allergies are related to abnormal intestinal microbiota.⁴⁶ To our surprise, the diversity of intestinal microbiota in the allergic mice was higher than that in the control group, contrary to reasoning based on other enteritis diseases. However, most allergy-related intestinal microbiota showed that the diversity of intestinal microbiota increased, indicating that the diversity of intestinal microbiota was not always positively correlated

with individual health.⁴⁷ In this study, we found that the diversity and distinct taxa in the cow's milk α_{S1} -CN group were increased compared to other two groups. Genera belonging to the *Lachnospiraceae*, *Oscillospiraceae* and *Butyrivibrionaceae* families were enriched in mice with cow's milk α_{S1} -CN allergy, while *Saccharimonadaceae* was enriched in Con and *Ruminococcaceae* was enriched in the G-G group. These findings were consistent with previous studies of the milk allergy induced change in microbiome. A previous study found that *Ruminococcaceae* was higher in the fecal microbiome of CMPA children than in healthy children.⁴⁸ Another study found that the intestinal microbial community structure of infants with CMA was diverse, mainly in the families of *Lachnospiraceae* and *Ruminococcaceae*.⁴⁷ They also reported that *Oscillospiraceae* was significantly different between infants that became tolerant and those that remained allergic.⁴⁷ Although differences in the microbial structure were observed in the allergic mice, no significant changes in Gram-negative bacteria were found, such as *Escherichia coli*. Therefore, the changes of LPS were not caused by changes in the fecal microbiome.

The intestinal epithelial barrier has important functions, such as forming a barrier that separates the luminal contents from the interstitium and protects the intestine from microorganisms including Gram-negative bacteria, greater virulence and antigenic molecules.⁴⁹ Previous studies have shown that the disruption of the intestinal barrier causes the leakage of endotoxin into the circulation, where it binds to the TLR4 receptor and activates the NF- κ B pathway, which in turn induces the production of inflammatory cytokines and induces systemic inflammation.⁵⁰ Occludin was the first identified tight junction specific integral membrane protein.⁵¹ The extracellular portion and transmembrane domain of Occludin regulate paracellular permeability, and the intracellular portion is linked to the actin cytoskeleton by ZO-1. Therefore, the interaction and integrity of Occludin-ZO-1 is an important part of maintaining the paracellular barrier.⁵² Like Occludin, the extracellular portion of claudin is critical for the formation of homophilic and/or heterophilic tight junction protein interactions or the formation of ion selective channels. Intracellular portion is anchored to the cytoskeleton by ZO-1 and ZO-2.⁵³ Claudin1 and Claudin3 could reduce paracellular permeability and protect the intestinal barrier.⁵⁴ Claudin2, however, is an essential protein for the formation of intercellular pores.⁵⁵ Our results showed that the content of Occludin, Claudin1 and Claudin3 in the colon of mice was decreased in the cow's milk α_{S1} -CN allergy group, whereas the content of claudin2 increased, indicating that allergies caused disrupt to the intestinal barrier of mice. Previous experimental and clinical data suggest that IL-4 increased the expression of Claudin2 in colon epithelial cells *via* the phosphoinositide 3-kinase pathway, thereby reducing intestinal barrier permeability.⁵⁶ In addition, mast cell degranulation products also have a serious impact on the mouse intestinal barrier. There are approximately 2–3% of mast cells within the intestinal lamina propria. In the course of intestinal diseases, this amount can be augmented, which is consistent with our sectioning

results.⁵⁷ After mast cell degranulation, they release a powerful array of inflammatory factors, such as Mcpt1 histamine, trypsin, 5-hydroxytryptamine, IL-3, IL-4, IL-5, IL-6, TNF- α and GM-CSF.⁵⁸ Among them, mast cell protease-1 (Mcpt1) and trypsin mediated increase in intestinal permeability is related to the degradation of Occludin.⁵⁹ In our results, hypersensitivity resulted in the degranulation of mast cells and the release of Th2 cell-associated inflammatory factor IL-4 in mice, and severe intestinal barrier impairment, which led to the leakage of LPS and stimulated the increase of IFN- γ in Th1 cells.

5. Conclusion

Our research showed that cow's milk α_{S1} -CN was more sensitized than goat's milk α_{S1} -CN as can be seen in the increased expression of Th2-type inflammatory factors and transcription factors, increased proportion of Th2 cells, higher amount of α_{S1} -CN specific antibodies, and the increase in degranulation of mast cells. At the same time, inflammatory factors and mast cell degranulation products could change the expression of intestinal barrier protein, increase intestinal permeability, and lead to the leakage of LPS, which activated the TLR4-NF κ B pathway, resulted in an increase of IFN- γ , and triggered systemic inflammation. On the other hand, the sensitization to goat's milk α_{S1} -CN was decreased in mice allergic to cow's milk α_{S1} -CN. In sum, goat's milk α_{S1} -CN has significantly lower allergenicity than cow's milk α_{S1} -CN in the mouse model. However, the situation in humans may be different from that of mice. For one thing, humans are more complicated than mice; for another, humans and mice have relatively low homology and their sensitization to goat's milk α_{S1} -CN and cow's milk α_{S1} -CN may be different. Therefore, the lower allergenicity of goat's milk α_{S1} -CN than cow's milk α_{S1} -CN has to be further confirmed by human clinical trials.

Author contributions

Lina Zhang and Peng Zhou: conceptualization and methodology; Kai Zhang and Ruoya Zhou: data curation and writing – original draft preparation; Kui Xie: supervision; Yanmei Hou and Jinjing Zhong: writing – reviewing and editing.

Ethical statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jiangnan University and approved by the Animal Ethics Committee of Jiangnan University.

Conflicts of interest

Kui Xie, Yanmei Hou and Jinjing Zhong are employees of Ausnutria Hyproca Nutrition Co. Ltd a company selling infant

formula. None of the other authors reported a conflict of interest related to the study.

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