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Article

Allergenic risk assessment of porcine myoglobin expressed by engineered *Komagataella Phaffii*

Yongli Ye[a,b,1], Jiadi Sun[a,b,1], Jiayuan Xu[a,b,1], Peipei Li[a,b], Lina Sheng[a,b], Yuan Qian[c], Jian Ji[a,b], Xiaomin Han[d], Xinrui Zhao[c], Jingwen Zhou[c], Xiulan Sun[a,b,*]

[a] *School of Food Science and Technology, International Joint Laboratory on Food Safety, Synergetic Innovation Center of Food Safety and Quality Control, Jiangnan University, Wuxi 214122, China*
[b] *Yixing Institute of Food and Biotechnology Co., Ltd., Yixing 214200, China*
[c] *Science Center for Future Foods, National Engineering Laboratory for Cereal Fermentation Technology (NELCF), Engineering Research Center of Ministry of Education on Food Synthetic Biotechnology, Jiangnan University, Wuxi 214122, China*
[d] *NHC Key Laboratory of Food Safety Risk Assessment, China National Center for Food Safety Risk Assessment, Beijing 100021, China*

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ABSTRACT

Myoglobin produced by fermentation using engineered *Komagataella phaffii* is an important color additive in meat analogue products, but its allergenicity is poorly understood. Here, we initially searched the Allergen Online database and did not find any allergic or cross-reactive proteins in porcine myoglobin (PM). *In vitro* simulated digestion demonstrated that PM did not exhibit notable acid-base resistance or anti-digestion capabilities. However, sensitization was observed in BALB/c mice, including a significant increase in specific antibodies and biomarkers for allergic reactions, as well as alterations in gut microbiome and serum metabolome. Interestingly, the intensity of sensitization exhibited a negative correlation with the purity of PM. 60% and 88% purities showed weaker sensitization compared to the ovalbumin control group. These allergic reactions were likely due to the non-myoglobin protein portion, highlighting the importance of purification processes and the urgent need to assess the allergenicity of this portion.

1. Introduction

Myoglobin, a protein with a heme prosthetic group in muscles, impacts the sensory color of meat. It is responsible for the red or pink color commonly associated with a slurry taste and metallic texture [1–4]. However, the lack of this color in cultured meat has led to a need for improvement in texture and flavor [5,6]. To achieve a meat-like color and texture, coloring agents are added as food additives. However, many synthetic colors have been found to have toxic effects on humans, making their usage strictly regulated [7–9]. Unlike synthetic colors, myoglobin is a natural coloring agent found in traditional meat products. When meat is cooked, myoglobin releases heme, which catalyzes reactions that convert amino acids, nucleotides, vitamins, and sugars in meat into numerous flavor compounds, giving meat its unique flavor [10]. Obtaining large quantities of myoglobin directly from natural raw materials remains a challenge. The rapid development of synthetic biology provides a viable strategy for the efficient synthesis of myoglobin to enhance the flavor and achieve a more natural coloration in cultured meat [11,12].

Currently, there have been reports on the successful expression of natural pigment proteins, such as hemoglobin, bovine myoglobin, and soybean hemoglobin, using synthetic biology techniques [13,14]. However, these synthetic heme proteins lack sufficient safety toxicology evaluations; a solid safety evaluation system and regulatory procedures are urgently needed. Safety is typically assessed through theoretical analysis and traditional protein experience, such as literature searches, sequence databases, bioinformatics search strategies, and *in vitro* pepsin digestibility studies [14–17]. The synthesis, expression, and secretion processes of these proteins may generate potential risk factors due to the culture environment, microbial growth, and other factors that are not present in traditional protein processing and preparation process. Therefore, synthetic proteins and their products should undergo thorough safety eval-

* Corresponding author.
E-mail address: sxlzzz@jiangnan.edu.cn (X. Sun).
[1] These authors contributed equally to this work.

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uations before they are marketed and promoted to enhance consumer confidence and support the sustainable development of the alternative protein industry.

Our research team successfully achieved constitutive secretion of recombinant porcine myoglobin (PM) using *Komagataella PHaffii* as the host strain [18]. To prepare food-grade PM, an ultrafiltration anion-exchange (gravity-flow column) was used as the purification process. The purity of the purified PM was determined to be 88.04%, with a recovery rate of 66.05%. Following acute toxicity tests, the results confirmed its non-toxicity; however, the potential allergenicity of the protein remains unknown. To evaluate the sensitization potential of synthetic PM, the sensitization was studied by animal models, along with bioinformatics analysis and *in vitro* pepsin digestibility assays. Based on the evaluation results, we conducted a comprehensive analysis of the safety of PMM in the food system, combining it with *in vitro* evaluation methods, which will provide guidance for optimizing the production and purification process, as well as for controlling safety risks.

2. Materials and methods

2.1. Allergen sequence similarity analysis

2.1.1. AOL database search

AOL (http://www.allergenonline.org/) provides a database of peer-reviewed allergen lists and searchable sequences for identifying proteins that may pose a potential risk for allergen cross-reactivity. It can be used to evaluate the safety of proteins that may be introduced into foods through food processing methods and genetic engineering. The analysis presented in this manuscript utilized the 21st version of the database. The FASTA 36 tool was employed to retrieve the PM sequence, which is shown in Table S1. Two comparison methods were utilized: overall full-length FASTA36 detection (with a threshold E of 1.0 and a 50% similarity) and the 80 amino acid window search (with a screening standard of consistency higher than 35%).

2.1.2. NCBI database search

BLAST is a sequence similarity retrieval tool provided by NCBI that can retrieve substances from the entire NCBI DNA database that shares certain similarities with the target sequence. In this study, BLAST (v2.13.0) was used to perform a similarity search (with a threshold of 0.05) for poring myoglobin sequences. This search was conducted to assess the presence of similar proteins in other organisms, to screen for toxic or sensitizing substances in those similar proteins, and to ensure that this search included sequences that may not be in the AOL database.

2.1.3. In vitro *simulated digestion*

In vitro simulated digestion of PM was conducted using a previously described method with minor modifications [19]. Briefly, the bile acid content was quantified, and the activities of pepsin and trypsin were measured. Subsequently, the *in vitro* simulated digestion of PM was performed, including the gastric digestive phase, intestinal digestive phase, and termination of digestion. Additional detailed steps can be found in the supplementary information.

2.2. Animal experiment design

Female BALB/c mice (SPF grade), weighing 18 g to 22 g, and aged 4–6 weeks, were obtained from Beijing VitalRiver Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed in a controlled environment with a temperature of 25 ± 1 ˚C and humidity maintained at 55 ± 5%, and a 12 h light/12 h dark cycles. Mice were allowed to drink and eat *ad libitum*. All experimental procedures were approved by the Animal Care and Use Committee of the Zhejiang Academy of the Center of Safety Evaluation, the Zhejiang Academy of Medical Sciences (No. KY-2021-009).

The sensitization of mice was carried out following the methods described by Sun et al. [20] and our previous research [21], with slight modifications. Fifty quarantine-qualified female BALB/c mice were randomly divided into five groups (n = 10) using weight-balanced zone design using SPSS software: Tris–HCl group, ovalbumin (OVA) group, PM60 (60% purity of PM) group, PM88 (88% purity of PM) group, and porcine hemoglobin (PH) group. All groups of mice were provided with a normal diet for 7 days and were given unrestricted access to food and water to make them adapt to the experimental environment. After 7 days, mice in each group were orally administered the corresponding test sample at a dosage of 250 mg/kg·bw for a total of 5 times on days 0, 7, 14, 21, and 28 of the experiment. The Tris–HCl group was given an equivalent volume of 0.1 mol/L Tris–HCl (pH 7.4). Prior to each sensitization and challenge, the body weight of each mouse was measured. After the intraperitoneal injection challenge, the mice were placed in alcohol-sterilized cages, and their reactions were observed within 60 min.

2.3. Determination of serum biochemical indicators of IgE and IgG

With reference to the reported method [22,23], OVA specimmunoglobulin G (IgG) in mouse serum was determined using indirect-enzyme-linked immuno sorbent assay (ELISA). The detailed steps are as follows. Dissolved or diluted each test sample with 0.1 mol/L carbonate buffer to a concentration of 300 μg/mL. Added 100 μL/well to a 96-well ELISA plate and incubated the plate overnight at 4 ˚C. Discarded the liquid from the wells, added 100 μL of blocking solution to each well, and incubated each well for 2 h at 37 ˚C. Removed the liquid from the wells and washed the plate 3 times with 250 μL of phosphate buffered solution (PBST) per well. Added mouse serum corresponding to the negative control group, diluted 20 times with PBST, to OVA and PH-coated wells, and added the corresponding mouse serum to the test sample group. For the negative control group, added PBST diluted 20 times to PM60 and PM88 coated wells, and added mouse serum to the PM60 group and PM88 group. Each mouse was measured twice in parallel. Added 100 μL of PBST to two wells as blank controls, then incubated the plate for 1.5 h at 37 ˚C, discarded the liquid and washed the plate three times with 250 μL of PBST per well. Added 100 μL of horseradish peroxidase-labeled goat anti-mouse IgE or IgG working solution to each well, incubated each well for 1 h at 37 ˚C, and then wash the plate four times with 250 μL of PBST per well. Then added 100 μL of tetramethyl benzidine solution to each well and incubated each well at 37 ˚C for 20 min, followed by the addition of 50 μL of 2 mol/L sulfuric acid. Measured the absorbance at 450 nm per well within 10 min. Additionally, commercial ELISA kits were used to detect serum OVA-specific immunoglobulin E (IgE), histamine (HIS), and mouse monocyte chemoattractant protein-1 (mMCP-1).

2.4. Mouse spleen lymphocyte isolation

The collected spleen tissues were transferred to an ultra-clean bench, washed twice with pre-cooled Hank's solution in an ice bath, and placed in a 12-well plate. Then, 1 mL of RPMI 1640 medium (containing 10% foetal bovine serum, 0.5% double antibody, the same below) was added. To obtain a cell suspension, the bottom surface of the core barrel of a 5 mL syringe was pressed. The grinding time for each mouse spleen was approximately 1 min. The cell strainer was wetted with RPMI 1640 medium, and the suspension was transferred to a 15 mL centrifuge tube through the strainer. The mixture was centrifuged at 500× *g* for 5 min, and the supernatant was discarded. The cell precipitate was then suspended five times with pre-cooled erythrocyte lysate, lysed on ice bath for 3 min, and centrifuged at 4 °C for 5 min at 400× *g*. The red supernatant was discarded, and the cells were suspended with 10 mL of Hank's solution. Next, the mixture was centrifuged at 500× *g* for 3 min at 4 ˚C, and the supernatant was discarded. The precipitates were then suspended with 500 μL of RPMI 1640 medium and transferred to a 48-well

plate to obtain a splenic lymphocyte (SLC) suspension. The plate was temporarily stored in a 5% CO_2 cell culture box at 37 °C. Afterwards, 50 μL of each SLC sample was absorbed, and 450 μL of PBS was added. After complete mixing, the concentration of lymphocytes in the suspension was counted using a Bayer 2120 five-classification hematology analyzer (Bayer AG, Leverkusen, Germany), and each sample was adjusted to a concentration of 1×10^7/mL using RPMI 1640 culture medium.

2.5. T cell typing analysis

The detection of Th1/Th2 cells is mainly carried out according to the kit instructions (Supplementary Information). Added 100 μL of flow cytometry staining buffer to suspend peripheral T cells (Treg) and detected $Foxp^{3+}CD^{25+}$ cells in CD^{4+} cells using flow cytometry.

2.6. Allergen re-stimulation of spleen lymphocytes in vitro

The OVA solution, PM60, PM88, and PH solution were each diluted to 1 mg/mL with RPMI 1640 medium. In the OVA re-stimulation experiment, the spleen cell suspensions from mice in both the Tris–HCl and OVA groups were added to a 24-well culture plate (400 μL/well, 4×10^6/well) after adjusting the concentration. A mixture of 55 μL of diluted OVA solution and 645 μL of RPMI 1640 culture medium were added to each well, resulting in a final concentration of 50 μg/mL of OVA. The same process was followed for the PM60, PM88, and PH re-stimulation experiments. The cells in each group were then cultured in a 5% CO_2 cell culture incubator at 37 ˚C for 24 h. The culture medium was collected in EP tubes and centrifuged. Subsequently, commercial ELISA kits were used to determine the levels of IL-4, IL-5, and IFN-γ in the supernatant.

2.7. Serum metabolite extraction and derivatization processing

The metabolite extraction from serum and liver tissue was carried out according to the standard operation of the Fiehn laboratory [24,25]. Detailed steps can refer to the supplementary information. Mass spectrometry data was collected using ChromaTOF software from LECO (St. Joseph, MI, US). The data was then exported in “mzML” format and converted to “abf” format using ABF converter software (http://www.reifycs.com/AbfConverter/index.html). MS DIAL software was combined with Fiehn and NIST mass spectrometry databases for baseline calibration, peak alignment, automatic deconvolution, and metabolite comparison and identification [26].

2.8. Detection and data processing of intestinal microflora in mice

To purify DNA from collected mouse fecal samples, the Zymo-BIOMICS DNA Microprep kit was used following the manufacturer’s instructions. The 16S rDNA (V4 region) of the samples was amplified using specific primers (515F, 5′-GTGYCAGCMGCCGCGGTAA-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′). The resulting amplification product was mixed with 6× loading buffer and electrophoresed on a 2% agarose gel. Target bands were recovered using the Zymoclean Gel Recovery kit, and quantification was performed using a Qubit 2.0 fluorometer. For sequencing, HiSeq Rapid SBS Kit v2 with PE250 sequencing was used according to the provided instructions.

Using FLASH 1.2.8 software, pairs of sequences were stitched together, with the sequence of each sample separated from the original data based on the barcode, and the barcode sequence truncated. To filter out sequences that are too short, too long, or contain ambiguous bases, the quality of the sequences was assessed using QIIME 1.9.1 software. The specific screening requirements were as follows. (1) Remove sequences with an average quality lower than 25. (2) Remove sequences less than 200 bp in length. (3) Remove sequences with more than 2 ambiguous bases (N).

To remove chimeras, the UCHIME algorithm from the Usearch software (http://drive.com/uparse/, v10.0.240) was utilized. Operational taxonomic unit (OTU) clustering was then performed at 97% concordance according to the UPARSE algorithm. The UCLUST classification and SILVA database (https://www.arb-silva.de/) were employed to analyze each sequence and obtain the final valid gut microbiota data.

2.9. Statistical analysis

GraphPad Prism 8.4.3 (GraphPad Software, USA) was used for the *t*-test and the two-stage step-by-step method of Benjamini, Krieger, and Yekuteili. Raw data for the serum metabolome were obtained by normalizing each metabolite peak in all samples using the SERRF (QC sample-based random forest) normalization method, with the QC sample peak height serving as the benchmark. A p value of <0.05 or a FDR-corrected $p(q)$ of <0.05 was considered statistically significant and presented as mean ± standard deviation (SD). The analysis of microorganisms, including community composition and diversity analysis, was performed using R 3.6.1, and the LefSe tool was utilized for LefSe analysis. The gut microbiota function was predicted based on the microbial functional gene profile in Kyoto Encyclopedia of Genes and Genomes (KEGG). After Pareto scaling and log-transformation, the serum metabolomic data were analyzed using principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA).

3. Results

3.1. PM is not potentially sensitizing or cross-reactive based on database searches

Previous work has achieved efficient expression of PM in the *K. phaffii* system through the optimization of both the host and signal peptide expression, as well as the development of an optimal expression system [18]. To assess the allergenicity of PM, searches for allergen similarity were conducted using AOL data and the NCBI database. The search in the AOL database resulted in the identification of three types of allergen proteins, and the information regarding these allergens and their similarity to PM is shown in Fig. 1a. Based on their consistency and E value, there is no evidence of cross-reactivity between PM and these three identified allergen proteins. Additionally, a search of the NCBI database using the BLAST tool resulted in the identification of 3,009 proteins that are similar to PM. However, upon using the FASTA 36 detection method for the analysis of 80 amino acids in PM, no allergen proteins that meet the established standards were found (Fig. 1b**)**. These proteins mainly consist of globin and various proteins composed of globin, such as hemoglobin, myoglobin, and cytoglobin. No toxic or allergic proteins have been reported among these findings.

3.2. PM exhibits digestive stable and acid resistant

To investigate the stability and acid resistance of PM, *in vitro* simulated digestion test was performed. Most of the protein bands in PM crude extracts expressed by *K. phaffii* (60% purity, PM60) and the PM purified solution expressed by *K. phaffii* (88% purity, PM88) were degraded after digestion (Fig. 1c and d). In the PM88 digestion group, there was an additional protein band (protein A) that was not present in the undigested PM88 group. This protein is presumed to be a digested and degraded substance from the components in PM88. Protein B bands were found in the undigested PM88 group and in the same position in the blank control group, but not in the undigested PM60 group. Therefore, we hypothesize that protein B is a less abundant protein component of PM60 that was purified and concentrated together with PM.

3.3. PM induces an allergic stress response in mice

To further investigate the potential sensitization of PM ingestion, a sensitization stress test was performed on a BALB/c mice model. Based

a

Type of allergen protein	E value	Similarity (%)	Consistency (%)
collagen alpha-1(I) chain [Salmo salar]	0.14	56.9	17.6
collagen alpha-1(I) chain-like [Salmo]	0.66	57.7	18.0
allergen protein [Liposcelis bostrychophila]	0.71	66.2	18.5

b

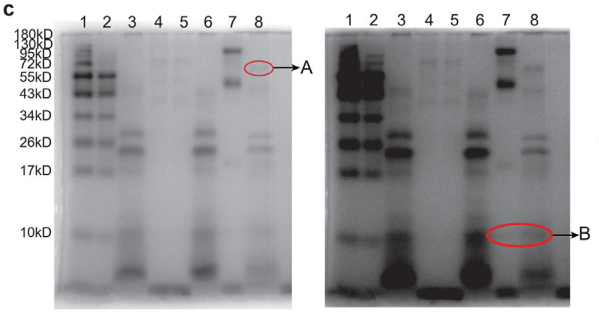
80mer Sliding Window Search Results

Database	AllergenOnline Database v21 (February 14, 2021)
Input Query	>DNA1 ATGGGTTTGTCTGATGGTGAATGGCAATTGGTTTTAAATGTTTGGGGTAAAGTTGAAGCTGATGTTG CAGGTCATGGTCAAGAAGTTTTGATCAGATTGTTTAAAGGTCATCCAGAAACTTTGGAAAAGTTCGA TAAGTTTAAACATTTGAAGTCTGAAGATGAAATGAAGGCTTCAGAAGATTTGAAGAAACATGGTAACA CTGTTTTGACAGCTTTGGGTGGTATTTTGAAAAAGAAAGGTCATCATGAAGCAGAATTGACTCCATTA GCTCAATCTCATGCAACAAAGCATAAGATCCCTGTTAAGTATTTGGAATTCATTTCTGAAGCAATCATC CAAGTTTTACAATCAAAACATCCTGGTGACTTTGGTGCTGATGCACAAGGTGCTATGTCAAAGGCAT TGGAATTGTTTAGAAACGATATGGCTGCAAAGTACAAGGAATTAGGTTTTCAAGGTTAA
Length	465
Number of 80 mers	386
Number of Sequences with hits	0

No Matches of Greater than 35% Identify Found

AllergenOnline Database v21 (February 14, 2021)

c



d

Digestive enzyme species	Corresponding to the enzyme activity
Porcine pepsin	2267 ± 174.2 U/mg
The pig pancreatin	4.063 ± 0.551 U/mg
Bile acid	0.727 ± 0.046 μmol/mg

Fig. 1. Bioinformatics search and digestive stability of PM. (a) Full-length FASTA36 assay results for PM. Similarity and consistency are two indicators that reflect the degree of similarity between two proteins. Similarity can be used as a retrieval criterion to appropriately expand the screening range, while consistency is a key indicator used to judge whether two proteins can cross-react. When the identity is greater than 70%, it indicates cross-reactivity between the two proteins, whereas when the identity is less than 50%, there is basically no cross-reactivity between the two proteins[19]. The E value is a calculated value that reflects the similarity between the query protein and its corresponding counterpart. This value is negatively proportional to the similarity of the two proteins and can be used as a supplementary judgment index for consistency. When the E value is less than 10^{-7}, it indicates cross- reaction between the two proteins. (b) 80mer sliding window search results of PM. Results showed that PM has no sequence similarity to known allergens. (c) Simulated stomach and intestinal digestion experiments. Results reveal no anti-digestive properties of PM. Gel electrophoresis of each group of protein samples (1: 6 μL marker; 2: 3.5 μL marker; 3: blank control; 4: enzyme-free control; 5: PM60 undigested group; 6: PM60 digested group; 7: PM88 undigested group; 8: PM88 digested group). (d) Digestive enzyme activity test results.

on allergic symptom scoring (Fig. 2a), mice in the PH group and the Tris–HCl group did not display any obvious abnormal reactions within 60 min. However, mice in the OVA, PM60, and PM88 groups exhibited grade 1–2 allergic symptoms (Fig. 2a). Nevertheless, there was no significant difference in body weight between these groups ($p > 0.05$) (Fig. 2b and c). Compared to the PM88 group, a higher proportion of mice in the PM60 group displayed grade 2 allergic symptoms. This suggests that PM60 and PM88 have a certain sensitization effect on BALB/c mice as indicated preliminarily (Fig. 2d).

3.4. PM significantly increases serum levels of specific antibodies and biomarkers of allergic reactions

Allergic reactions were manifested through a significant increase in serum levels of specific antibodies and allergy biomarkers, including HIS and mMCP-1 [22,27]. The levels of spec-IgG, IgE, HIS, and mMCP-1 significantly increased in the serum of mice in the OVA, PM60, and PM88 groups compared to the Tris–HCl group ($p < 0.05$). Moreover, the levels of spec-IgG, HIS, and mMCP-1 in the PM60 group were significantly higher than those in the PM88 group ($p < 0.05$) (Fig. 2f–i). However, these indices did not show any significant differences between the PH and Tris–HCl groups ($p > 0.05$) (Fig. 2h and i). These results indicate that significant allergic reactions were observed in the OVA group, PM60, and PM88 group mice, while no considerable allergic reactions were observed in the PH group mice. It is important to note that the PM88 group mice exhibited a lower severity of allergic reaction compared to the PM60 group.

3.5. Low-purity PM induces Th2-type immune responses

The splenic lymphocytes from different groups of mice were analyzed for determining Th1/Th2 cell types and clarifying the immune response to PM sensitization. In comparison to the Tris–HCl group, the percentage of Th1 cells notably decreased in the OVA group (Fig. 2j). The proportions of Th2 cells in both pure PM groups showed a tendency to decrease, but there was no significant difference when compared with the PH group (Fig. 2k). The Th2 cell proportions in the OVA and PM60 groups were significantly higher than those in the Tris–HCl group (Fig. 2j), whereas no significant difference was found between the PM88 and PH groups ($p > 0.05$). The percentage of Treg cells among splenic lymphocytes significantly increased in the OVA-positive group ($p < 0.05$), while there was no significant difference among the PM60, PM88, and PH groups ($p > 0.05$) (Fig. 2l).

In comparison to the Tris–HCl group, the OVA group mice exhibited higher levels of IL-4 and IL5 ($p < 0.05$) and lower levels of IFN-γ ($p < 0.05$) secreted by lymphocytes in response to OVA restimulation. The PM60 group showed significantly lower levels of IL-4 and IFN-γ secreted by lymphocytes ($p < 0.05$), while the level of IL-5 was not significantly different ($p > 0.05$). Conversely, the secretion level of IL-4 in lymphocytes of mice in the PM88 group significantly increased ($p < 0.05$). There was a significant decrease in the secretion of IFN-γ in lymphocytes of mice in both the PM60 and PM88 groups ($p < 0.05$, $p < 0.05$) (Fig. 2m–o). These findings were consistent with the observed changes in the ratio of Th1 and Th2 cells in the OVA, PM60, and PM88 groups. Therefore, these results suggest that the PM60 group displayed a specific Th2-type immune response induced by certain components in PM60, which may be similar to the components present in PM88.

3.6. Purity of PM has no significant effect on intestinal microbiota

To access the impact of PM on intestinal microbiota, a differential analysis of intestinal microbiota was conducted. In the α-analysis, the indices of the PH group did not significantly differ from those of the Tris–HCl group. However, the mice in the OVA, PM60, and PM88 groups exhibited significantly lower indices of intestinal microbiota ($p < 0.05$) (Fig. 3a–c). Additionally, the PM60 group had significantly lower Chao1 and Ace indices compared to the OVA and PM88 groups ($p < 0.05$). Furthermore, the Shannon index was significantly lower in the OVA

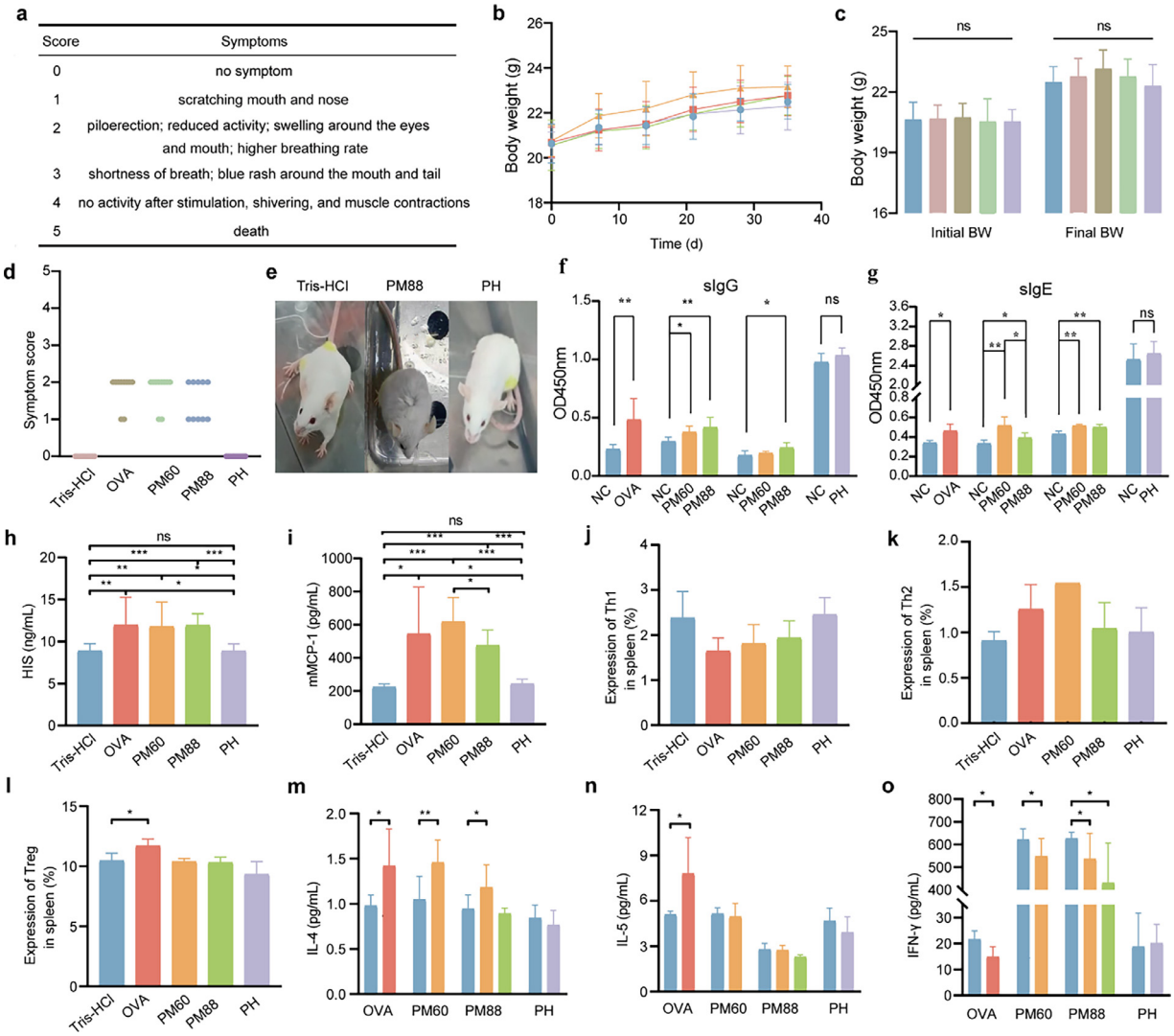


Fig. 2. Relationship between sensitization and purity of PM extract. (a) Body weight changes of mice during sensitization. (b-c) Comparison of the body weights of the five groups before and after sensitization (ns: $p > 0.05$). (d) Allergic symptom score. (e) Piloerection occurred in mice of the PM88 group. (f-g), Spec-IgG and spec-IgE levels in the serum of each group of mice corresponding to the tested samples (ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$). (h) HIS levels in the serum of mice (ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$). (i) mMCP-1 levels in the serum of mice (ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$). (j) Proportion of Th1 cells in mice spleen lymphocytes. (k) Proportion of Th2 cells in mouse spleen lymphocytes. (l) Proportion of Treg in mice spleen lymphocytes (*: $p < 0.05$; ***: $p < 0.001$). (m–o) Levels of IL-4, IL-6, and IFN-γ induced by OVA, PM60, PM88, and PH, respectively (*: $p < 0.05$; **: $p < 0.01$).

and PM60 groups compared to PM88 group. The OPLS-DA score plot between the OVA, PM60, PM88, and Tris–HCl groups indicated that the samples could be categorized into four distinct clusters (Fig. 3d). Subsequently, cluster analysis was performed on the intestinal microbiota of each mouse group using the Bray-Curtis distance. The results demonstrated that the composition of the gut microbiota in the PM60 group was more closely aligned with that of the OVA positive group, while the PM88 group exhibited similarities with the Tris–HCl group (Fig. 3e).

At the phylum and class level, the composition of the microbiome in different groups was analyzed (Fig. 3f,g). At the phylum level, the microbial community was mainly composed of *Firmicutes, Bacteroidota*, and *Proteobacteria*, with these phyla representing over 80% of the microbiota. The PM60 group of mice exhibited a decrease in the relative abundance of *Firmicutes* compared to the Tris–HCl group, while the relative abundance of *Bacteroidota* increased compared to the OVA group. Additionally, at both the phylum and class levels, the community composition of the intestinal microbiota in the PM60 group was similar to that of mice in the OVA group. Furthermore, a comparative analysis of the gut microorganisms among the four groups of mice was also performed at the genus level (Fig. 3h). At the genus level, the intestinal microbiota community composition of mice in the PM60 group was similar to that of the Tris–HCl group. In contrast, at the phylum and class levels, the community composition of the intestinal microbiota in the PM88 group did not differ significantly from that of the Tris–HCl group.

Three groups of mice with OVA, PM60, and PM88 exhibited altered the metabolic pathway of microbial KEGG at three levels after the metabolism of intestinal microbiota compared to the Tris–HCl group. In the level 2 metabolic pathway analysis, there was a significant enhancement observed in starch and sucrose metabolism, other glycan degradation, mino sugar and nucleotide sugar metabolism, mino sugar and nucleotide sugar metabolism, and nitrogen metabolism in the PM60 and PM88 groups, although the changes were weaker (Fig. 4a). In the level 3 metabolic pathway, in the OVA group showed significant enhancements in glycan biosynthesis and metabolism, metabolism of cofactors and vitamins, cell growth and death, and transport and catabolism when compared to the Tris–HCl group. However, the changes in these metabolic pathways were comparatively smaller in the PM60 and PM88 groups (Fig. 4b).

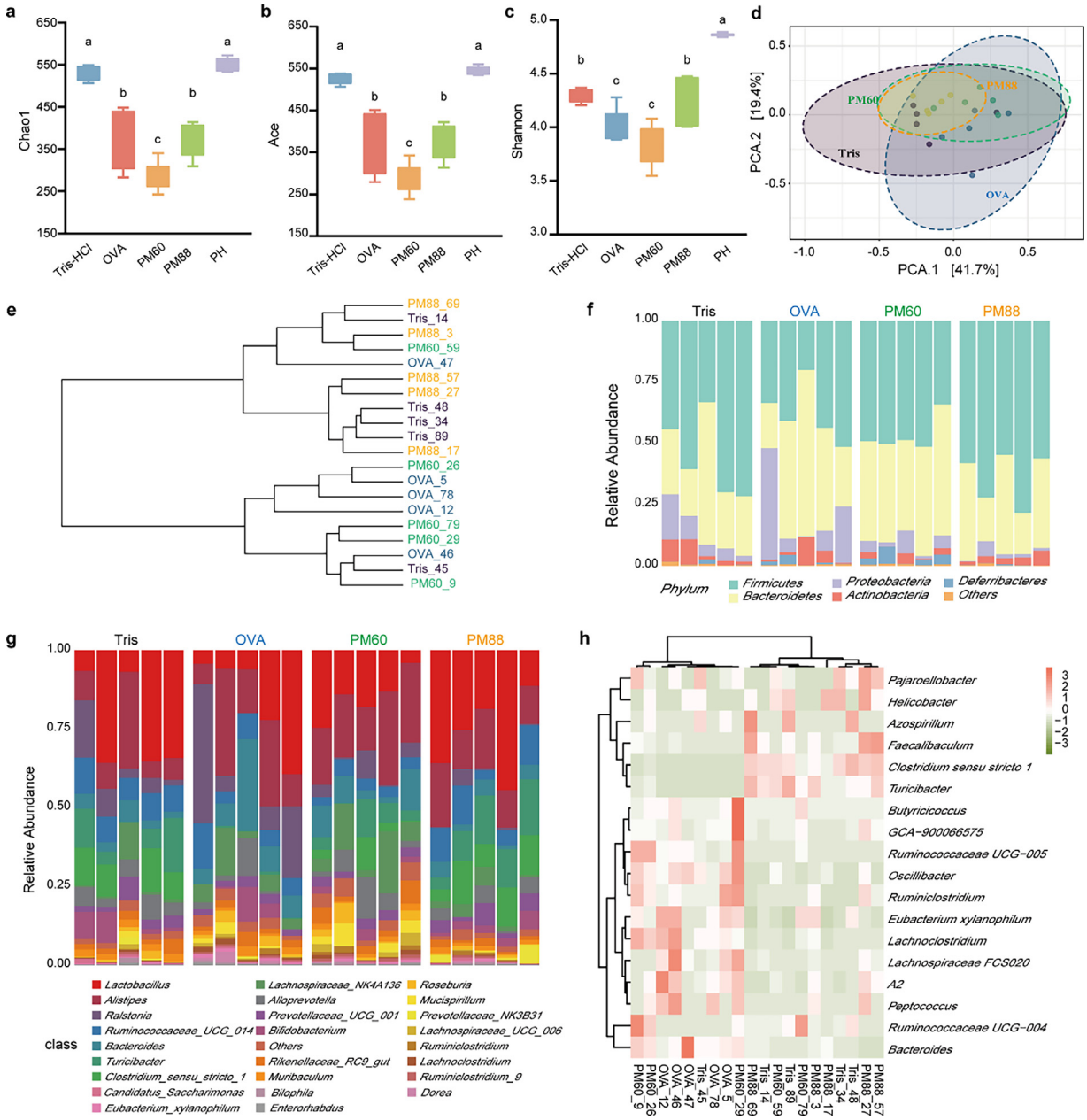


Fig. 3. Effect of PM88 on intestinal microorganisms. (a-c) Chao1 index, Ace index, and Shannon index in all groups of mice. Differences indicated by lowercase letters denote significant differences at the *t*-test 95% level). (d-e) β-diversity analysis and cluster tree analysis. (f-h) Analysis of Phylum, Class level, and differential microbiological analysis.

3.7. PM and OVA have different effects on serum metabolism

To exclude the interference of other substances in the extract and to clearly investigate the influence of PM on the metabolic physiological process, we further analyzed the serum metabolites of mice in the groups of Tris–HCl, OVA, and PM88. OPLS-DA model showed that the changes in serum metabolic profiles of mice in the OVA and PM88 groups were greater than those in the Tris–HCl group. However, the point clusters in the PM88 and OVA groups were significantly separated (Fig. 4c). The volcano plot results further showed that, compared with the OVA group, levels of metabolites such as oleamide, trans-4-hydroxyproline, and glutamyl-valine were upregulated in the PM88 group. Conversely, the levels of glycine, ketohexose, and 2-deoxythritol were down-regulated (Fig. 4d). This indicated that there were significant differences in the serum metabolic profile between the PM88 group and the OVA group.

Differential metabolites were screened based on parameters of variable importance of the projection values (VIP) >1, p <0.05, and a fold change (FC) >2, and the top 20 differential metabolites were shown in Fig. 4e. The levels of ketohexose, 2-deoxythritol, and galactonic acid in the PM88 group were similar to those in the Tris–HCl group, while they were elevated in the OVA group. However, compared with the Tris–HCl group and OVA group, the levels of 5 metabolites in the PM88 group were significantly up-regulated, such as cholic acid, isothreonic acid, trans-4-hydroxyproline, noradrenaline, and 12 metabolites were significantly down-regulated, including oxalic acid, 3-hydroxybutyric acid, capric acid, and glycine. Surprisingly, the levels of these metabolites in the OVA group were reversed from those in the PM88 group, suggesting that OVA treatment has a lesser effect on serum metabolite levels than PM88. Combined with the animal phenotype and biochemical results, we hypothesized that gavage and intraperitoneal injection of PM88 may have other non-allergenic effects on serum metabolism in mice.

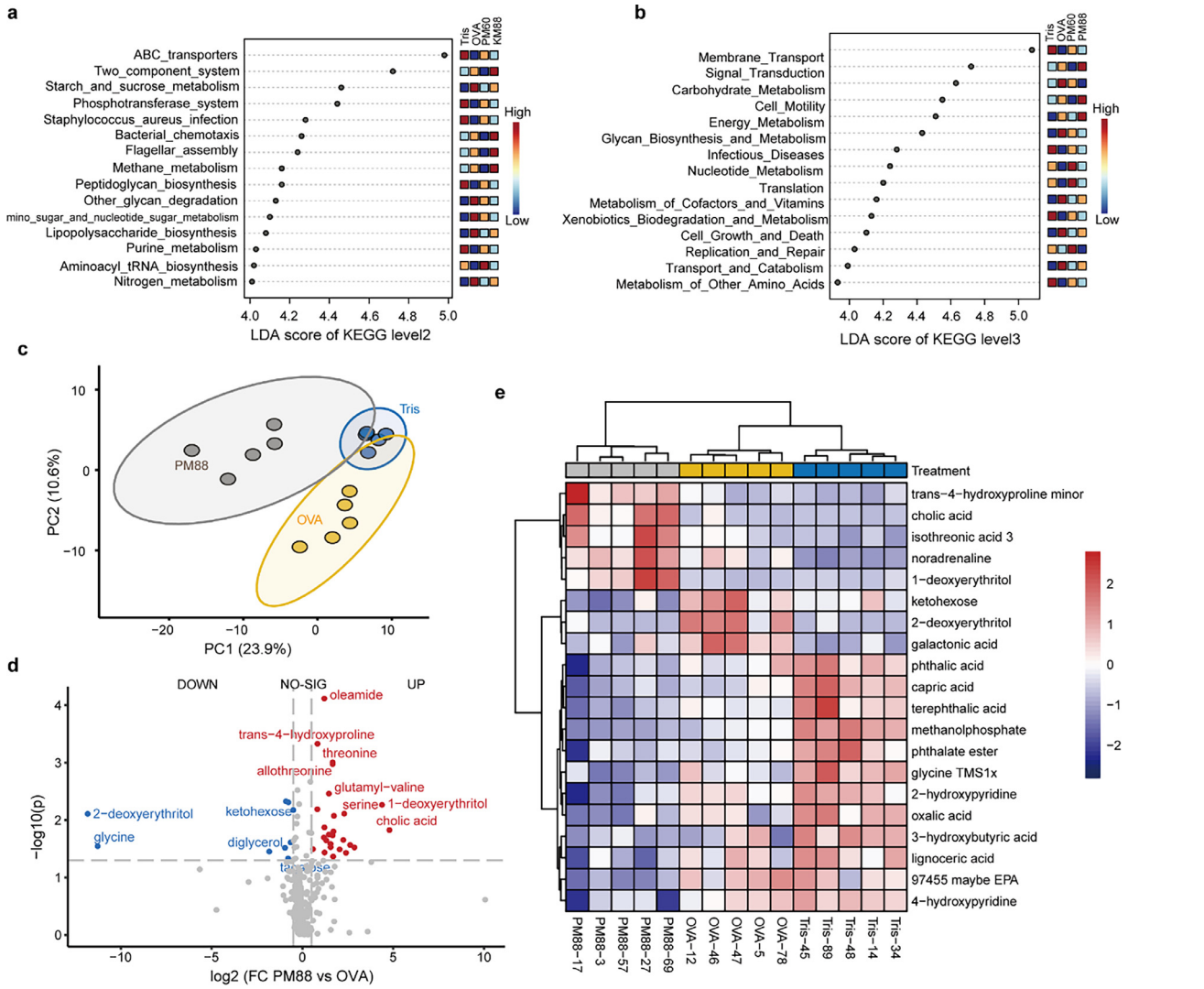


Fig. 4. Analysis of serum metabolomics. (a) Changes and LDA analysis of predicted KEGG pathways at level 2. (b) Changes and LDA analysis at level 3. (c-e) Changes of metabolites presented through OPLS-DA plot, volcano plot (Tris–HCl *vs* PM88), and heatmap of differential metabolites.

3.8. *Identification of intestinal flora and metabolites associated with allergy*

Correlation analysis, specifically Spearman's correlation test, was used to analyze the correlation between metabolites and the characteristic indexes of allergy, as well as changes in intestinal microbiota. In Fig. 5, metabolites showed significantly negative correlation with sIgE levels, including glycolic acid, aspartic acid, ribitol, oxoproline, trans-4-hydroxyproline, methylhexose, and glutamic acid ($p < 0.05$). Additionally, 5-methoxytryptamine, trans-4-hydroxyproline, and methylhexose were significantly negatively correlated with sIgG levels ($p < 0.01$). On the other hand, Th1 level showed significant positive correlations with hexadecylglycerol and noradrexose ($p < 0.01$), as well as negative correlations with 2-hydroxypyridine and terephthalic acid ($p < 0.05$). There was no significant correlation observed between hexadecylglycerol and Th2 level. However, a significant negative correlation was found between hexadecylglycerol and mMCP-1 and Treg, while phenylalanine and tyrosine levels showed a significant positive correlation with HIS ($p < 0.05$). Among the differential metabolites, at least 5 were found to be associated with *Lactobacillus, Negativibacillus, Ruminococcaceae UCG-005, Ruminococcaceae UCG-014, Alistipes*, and *Oscillibacter*. The abundances of *Ruminiclostridium 5* and *Ruminococcus 1* also showed significant positive or negative correlations ($p < 0.05$). Furthermore, at least 18 different metabolites were significantly correlated with *Faecalibaculum, Turcibacter*, and *Helicobacter* ($p < 0.05$), with the correlation being mainly positive.

4. Discussion

Genetic engineering encompasses a wide range of species, making it difficult to generalize about allergenicity. However, the potential allergenic risk of genetically engineered expressed proteins must not be overlooked. Therefore, we performed a comprehensive assessment to determine the allergenicity of the PM expressed by our team in the previous period. We did not search the Web of Science database for studies on PM sensitization. Additionally, based on the results of informatics searches and *in vitro* digestion tests, it was determined that PM expressed by *K. phaffii* did not have the potential for sensitization (as stated in Refs. 28–30). Furthermore, our previous research, which utilized the BALB/c mice model for allergic evaluation [21], revealed that there was a certain level of allergic reaction. The intensity of the allergic reaction was found to be negatively correlated with purity. The PM60 group exhibited a significant Th2-type immune response, while the PM88 group did not exhibit a significant trend of Th2-type immune response. When compared to the Tris–HCl group, the OVA and PM88 groups exhibited no-

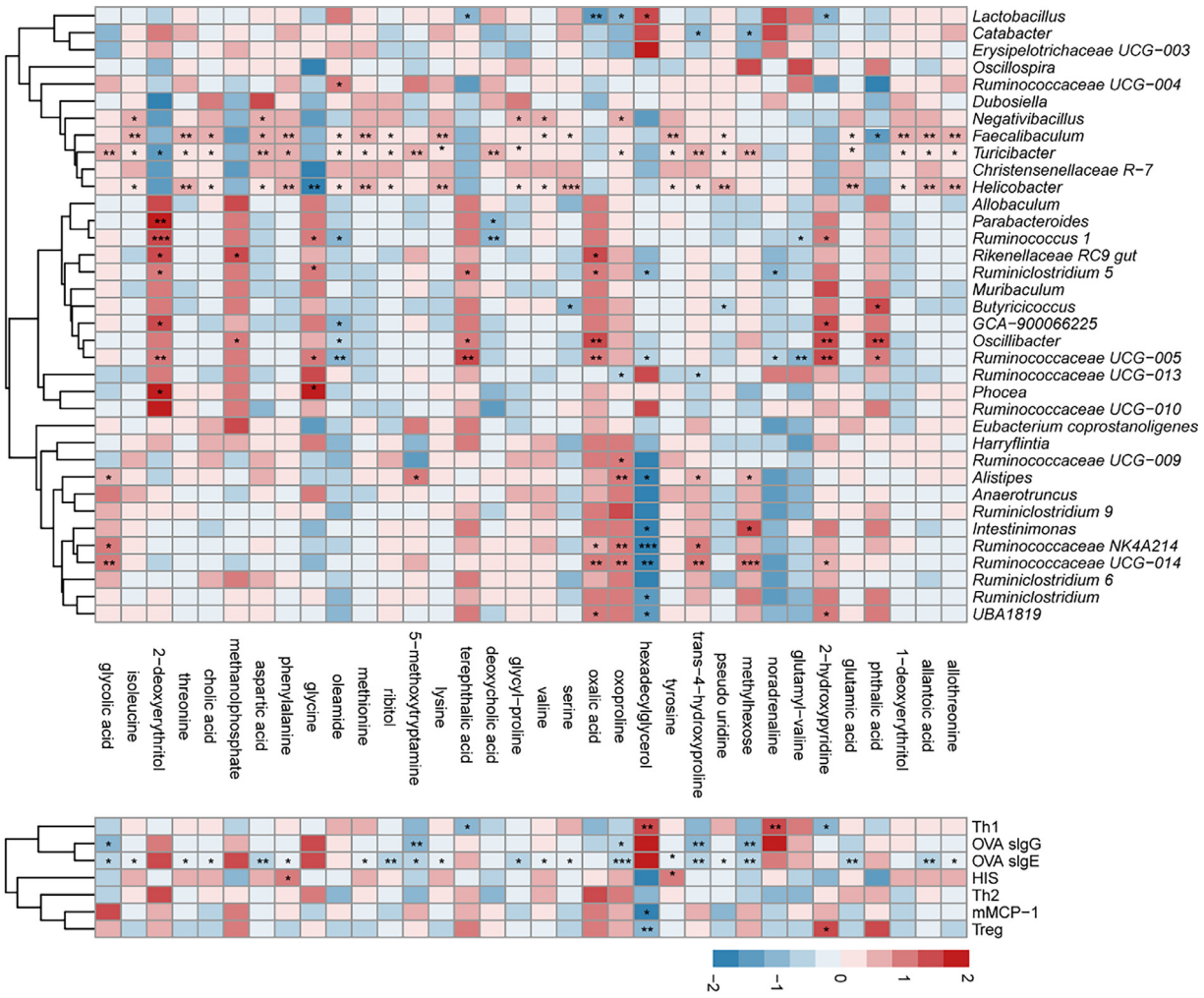


Fig. 5. Correlation analysis of immune factors, microbiota, and metabolomics that determines the non-allergenicity of PM.

ticeable changes in the serum metabolic profile. Moreover, the changes in the serum metabolic profile of the PM88 group were significantly different from those observed in the OVA group.

Intestinal microbiota may play an important role in the induction of allergic reactions caused by PM products. We observed significant reductions in the abundances of *Lactobacillus* and *Bifidobacterium* at the class level in the OVA and PM60 groups. However, the variation in *Bifidobacterium* abundance in the PM88 group did not significantly differ from that in the Tris–HCl group (Fig. 3g). It has been suggested that gut microbiota can mediate immune function regulation through protein metabolism, fatty acid metabolism, and other pathways. For instance, microbial metabolites such as short-chain fatty acids and secondary bile acids have been implicated in directly or indirectly influencing the differentiation of Treg cells [31,32]. Research has shown that d-tryptophan produced by *Bifidobacterium* and *Lactobacillus* can inhibit allergic inflammation in the lungs by promoting gut microbial diversity and enhancing the production of Treg cells [33]. However, a decrease in the abundance of *Bifidobacterium* has been observed in the gut microbiota of children with allergies.

Moreover, at the genus level, a significant increase in the abundance of *Lachnospiraceae FCS020* in the OVA group and *Ruminococcaceae UCG-005* in the PM60 group were observed (Fig. 3h). Increased abundances of *Ruminococcaceae* and *Lachnospiraceae* have been associated with triggering host allergic reactions and are linked to egg and milk allergies [34–36]. Particularly, the abundance of *Alistipes* at the class level increased in the OVA, PM 60, and PM88 groups (Fig. 3g). *Alistipes* is a type of bacteria that has shown conflicting results, with some studies suggesting it has a protective effect on diseases like pulmonary fibrosis and colitis, while others have associated it with the development of colorectal cancer and depression [37,38]. At the genus level, the relative abundances of *Lachnoclostridium, Ruminiclostridium*, and *Bacteroides* increased in the OVA and PM60 groups compared to the Tris–HCl and PM88 groups. Certain species of these bacteria are thought to be involved in the development of inflammation, endogenous infections, and various diseases [39–42]. Furthermore, there was an increase in the abundance of *Oscillibacter* in the OVA and PM60 groups (Fig. 3h). *Oscillibacter* is considered as a potential candidate for a new generation of probiotics [43]. However, these results only provide a preliminary indication that the allergic characteristics of PM products may be related to the diversity of intestinal microbiota, and additional parallel trial groups are required to validate and elucidate the underlying mechanisms.

In our study, the results of database searches and *in vitro* simulated digestions showed that PM was not allergenic. However, BALB/c mice showed allergic reactions after being administered the incompletely purified protein in animal tests. Therefore, before novel proteins enter the market, the government needs to improve regulatory policies and systems. In recent years, a series of policies and regulations have been introduced by international organizations and countries to regulate and supervise the safety of alternative proteins. Food and Agriculture Organization of the United Nations (FAO) founded the Cell Culture Meat Safety Committee in 2022, and three meetings have clarified the standard terminology, safe production, and management framework for cell-

cultured meat [44–46]. The European Union issued the New Food Ingredients Regulation (No 2015/2283) in 2015, which stipulated that cell cultures can be used as food ingredients. The U.S. Food and Drug Administration /United States Department of Agriculture (FDA/USDA) discussed the potential hazards of cell-cultured meat and regulatory labeling in 2018. Canada introduced safety evaluation guidelines for new food products in 2006, pioneered "artificial meat" technology in 2016, and established a safety assessment framework in 2018. The Chinese Society of Food Science and Technology released two group standards, "Plant-based Meat Products" and "General Rules for Plant-based Foods", in 2020 and 2021, respectively [47,48]. However, at present, many countries mainly consider the safety of novel proteins based on bioinformatics search, *in vitro* simulated digestion, and acute toxicity. Our results, however, show that it is inaccurate to speculate on the non-allergenicity of alternative protein resources based solely on traditional experience. Therefore, from the perspective of the development of commercial production of novel proteins and the protection of human health, alternative proteins and their products require a sound risk assessment system as well as a regulatory framework.

5. Conclusion

In this study, the sensitization of PM, expressed by genetic engineering in *Komagataella phaffii*, was evaluated using the previously established sensitization evaluation BALB/c mouse model, and *in vitro* sensitization analysis technology. It was found that PM produced by microbial expression itself was not sensitizing. However, the unpurified protein had the potential to sensitize BALB/c mice. Subsequent studies should combine proteomics technology to analyze various impurity components in the protein expressed by *Komagataella phaffii* and identify the causes of sensitization. Furthermore, optimizing the process of production, extraction, and purification is necessary to control the risk of food sensitization.

CRediT authorship contribution statement

Yongli Ye: Investigation, Methodology, Writing –original draft, Writing–review & editing. **Jiadi Sun:** Formal analysis, Writing–review & editing. **Jiayuan Xu:** Investigation, Methodology, Writing–original draft. **Peipei Li:** Methodology, Formal analysis, Writing–original draft, Writing–review & editing. **Lina Sheng:** Investigation, Writing–original draft, Writing–review & editing. **Yuan Qian:** Methodology, Sample preparation, Writing–review & editing. **Jian Ji:** Analysis tools contribution. **Xiaomin Han:** Investigation, Writing–review & editing. **Xinrui Zhao:** Sample preparation, Writing–review & editing. **Jingwen Zhou**: Supervision, Conceptualization, Funding, Writing–review & editing. **Xiulan Sun:** Conceptualization, Supervision, Funding, Writing–review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest in this work.

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Supplementary materials

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Yongli Ye (BRID: 08081.00.15626) is an assistant research fellow at the School of Food Science and Technology, Jiangnan University. Her research interests are focused on exposure risk assessment of foodborne hazard factors and health intervention, development of functional foods, and construction of *in vivo* and *in vitro* biological evaluation models.

Xiulan Sun (BRID: 09351.00.67072) is a second-class professor and assistant dean of School of Food Science and Technology at Jiangnan University, and director of Joint Laboratory of International Cooperation in Food Safety. She has been funded by the National Science Fund for Distinguished Young Scholars. She has focused on the development of efficient analytical methods for foodborne hazard factors, toxicological evaluation and control of biotoxins, toxic intervention of mycotoxins, and activity evaluation of natural compounds for more than 15 years and has several main findings.