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Effects of synergistic fermentation of tea bee pollen with bacteria and enzymes on growth and intestinal health of *Apis cerana cerana*

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ABSTRACT

While the health benefits of lactic acid bacteria (LAB)-fermented feed on farmed animals are well-established, its potential benefits for honeybees, specifically *Apis cerana cerana*, remain largely unexplored. The present study aimed to optimize an enzymatic hydrolysis process for tea bee pollen, employing a complex enzyme comprising acid cellulase and pectinase, followed by fermentation with *Limosilactobacillus reuteri* LP4. *A. c. cerana* workers were subsequently fed tea bee pollen processed with this optimized method. Under the optimal processing condition of fermented tea bee pollen, the pH value was 4.41, the protein content was 27.75 %, and the viable count of LAB was 2.31×10^9 CFU/g. No molds and yeasts as well as pathogens were detected. Compared to the unfermented pollen, honey bee workers administrated with fermented tea pollen with L. *reuteri* LP4 showed significantly increased survival rate by 24.34 % on day 15. Moreover, the relative abundances of *Lactobacillus* and *Bifidobacterium* were elevated, while those of *Enterococcus* and *Bacteroides* were diminished. Concurrently, the relative expression levels of immune-related genes including *Abaecin, PPO, Defensin*, and *Vg* were significantly upregulated. These findings provide a scientific foundation for application of fermented feeds to enhance the health of *A. c. cerana* populations and contribute to the sustainable development of apiculture in China.

Introduction

Honeybees are essential for crop pollination, with pollinated crops constituting approximately one-third of the global human food supply (Khalifa et al., 2021). *Apis cerana*, particularly the eastern subspecies *Apis cerana*, is native to China and prized for its robust mite resistance and environmental adaptability *Apis cerana* is prized for its robust mite resistance and environmental adaptability and *Apis cerana cerana* is native to China (Koetz, 2013; Grindrod and Martin, 2023). However, *A. c. cerana* production faces significant challenges, including declining population numbers and diversity (Teichroew et al., 2017). Antibiotics have been widely used to prevent and manage of bee-related diseases, such as American foulbrood and European foulbrood (Forsgren et al., 2018). However, excessive antibiotic exposure can compromise larval health through reducing the quantity and relative abundance of gut bacteria, thereby impairing bee survival (Jasny, 2017; Duan et al., 2021). A recent study has shown that feed supplemented with probiotics, as an alternative to antibiotics, can increase animal performance and improve gastrointestinal health by regulating microbiota (Wang et al., 2024). Similarly, pollen combined with probiotics have the potential to enhance gastrointestinal health, bolster immune function, and promote overall health in bees (Smriti et al., 2024).

Limosilactobacillus reuteri is a well-studied LAB species with probiotic properties that confers health benefits including induction of anti-inflammatory cytokines and modulation of the intestinal microbiota through production of antimicrobial substances, such as reuterin (Abuqwider et al., 2022). A strain of L. *reuteri* FLRE5K1, isolated from the kidneys of healthy mice, has been reported to reduce the incidence of melanoma from 70 % to 30 % and significantly increase the lifespan of mice afflicted with the disease (Luo et al., 2020). Oral administration of L. *reuteri* can restore compromised intestinal microbiota and alleviate hypersensitive diarrhea, mast cell activation, and serum

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immunoglobulin E synthesis in hypersensitive mice (Huang et al., 2017). Furthermore, L. *reuteri* supplementation has been shown to modulate *Lactobacillus* abundance in *Drosophila melanogaster* and extend their lifespan (Lee et al., 2023).

Pollen serves as a crucial nutritional resource for growth and development of bees (Filipiak et al., 2017). A study has reported the protein content of 27.3 %, 29 %, 14.3 %, the total amino acid content of 22.45 %, 28.77 % and 12.86 % in bee pollens of rape, tea, and buckwheat, respectively, tea pollen contains high nutritional value (Yang et al., 2013). Modification of the pollen wall structure can significantly improve the ability of intracellular bioactive substances to be digested and absorbed by the body (Wu et al., 2021). In contrast to physical treatments such as ultrasound, freeze-thaw, and their combined treatments, enzymatic hydrolysis can greatly enhance protein production via facilitating the release of proteins from both the inside and outside of the pollen. Proteins extracted with enzymatic hydrolysis exhibit enhanced solubility, emulsification, and gelation properties as a result of partial hydrolysis of proteins by proteases (Xue and Li, 2023).

Fermentation enhances the bioactivity of bee pollen, with the extent of these beneficial effects dependent on both the pollen's botanical origin and the specific fermentation method employed, whether spontaneous or facilitated by the addition of specific bacteria (Kaškonienė et al., 2020). Microbial fermentation also markedly increases the contents of primary metabolites in bee pollen, including 74 amino acids and their derivatives, 42 polyunsaturated fatty acids, and 66 organic acids, compared to unfermented bee pollen (Zhang et al., 2022). Additionally, it stimulates the production of secondary metabolites, such as 38 phenolic acids, 80 flavone aglycones, and 22 phenolamides (Zhang et al., 2022). The synergistic treatment of feed raw materials with the combination of microbes and enzyme has shown to be a necessary supplemental measure for fermented feed or enzyme-treated feed to improve the nutritional value and palatability of feed (Li et al., 2021). The synergistic fermentation of probiotics and enzymes exhibits the synergistic action of enzymes and probiotics, culminating in the completion of substrate fermentation and hydrolysis compared with probiotics or enzymes alone (Li et al., 2020). Furthermore, the release of small molecules such as sugars from cellulose and hemicellulos via enzymatic degradation can benefit the growth and metabolism of probiotics in the feed (Bao et al., 2022). For example, in our previous study, enzymatic hydrolysis has shown to facilitate the propagation of probiotic *Lactobacillus plantarum* in fermented medicinal herb *Codonoposis bulleyana* Forrest ex Deiels with the significant rise in the viable counts about 3 lg CFU/g after fermentation (Ye et al., 2019). The above studies highlight expanding the understanding of the synergistic fermentation of pollen with LAB and enzymes and its application in honey bee production. The intestinal microbiota plays a crucial role in promoting host health, particularly in bees, through modulating immunological and metabolic pathways, aiding in food digestion and detoxification, and providing defense against pathogens and parasites (Kamada et al., 2013; Motta et al., 2022). A balanced gut microbiota can positively impact digestion, nutrient absorption, and weight gain in bees (Zheng et al., 2017). Currently, research on the effects of microbial-enzymatic treated bee pollen with *Limosilactobacillus* on honey bees is limited. The present study aimed to investigate the impact of tea bee pollen fermented with L. *reuteri* LP4, following enzymatic digestion with acidic cellulase and pectinase complex, on the growth and gut health of *A. c. cerana* workers. The findings of this study may provide a theoretical foundation for utilizing *Limosilactobacillus*-fermented pollen to improve the health of *A. c. cerana* populations.

Materials and methods

Reagents

Acid cellulase (400,000 U/g) and pectinase (30,000 U/g) were purchased from Wanbang Industrial Co., Ltd. (Henan, China). 3,5-dinitrosalicylic acid (95 %), potassium sodium tartrate (99 %), anhydrous sodium acetate (99 %), ammonium citrate (98.5 %), peptone and phenol (99 %) were obtained from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Sodium hydroxide (≥ 95 %) and sodium sulfite (≥ 97 %) were obtained from Tianjin Fengchuan Chemical Reagent Technology Co., Ltd. (Tianjin, China). Beef extract, magnesium sulfate anhydrous (> 98 %) and manganous sulfate (> 99 %) were purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Yeast extract was obtained from Oxoid Company, UK. Tween-80, d-glucose (> 99 %), and agar were obtained from BioFroxx Company, Germany. Potassium dihydrogen phosphate (≥ 99.5 %) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Potato dextrose Agar (PDA) was purchased from Guangdong Huankai Biotechnology Co., Ltd. (Guangdong, China). Xylose lysine desoxycholate agar (XLD), Lauryl sulfate tryptose broth (LST) and *Shigella* broth were purchased from Qingdao Haibo Biotechnology Co., Ltd. (Qingdao, China). Total RNA extraction kit and Fecal Genomic DNA Extraction Kit were purchased from Tiangen Biochemical Technology Co., Ltd. (Beijing, China).

Honey bee rearing and tea bee pollen

In the present study, four hundred healthy, one-day-old worker bees of *A. c. cerana* were sourced from four distinct colonies within the apiary of Yunnan Agricultural University (Kunming, Yunnan Province, 102°45′30.5″E, 25°8′5.8″N). Prior to the experiment, these colonies were not administered with antibiotics, treated with any medication, or exposed to any other chemicals throughout the experimental period. The feeding experiments were conducted from October to December 2022, during which the worker bees were maintained outside the hive. The bee pollen sample of *Camellia sinensis* var. *assamica* was monoflorl and obtained from Pu-erh, Yunnan Province of China, where is one of the major areas for cultivation of *Camellia* s. var. *assamica* trees. The tea pollen was identified based on microscopic observation by vice researcher Cheng Xu, Sericultural and Apicultural Research Institute of Yunnan Academy of Agricultural Sciences in China.

Optimization of the process for synergistic fermentation of tea pollen with bacteria and enzymes

The synergistic fermentation of tea pollen was conducted with two-step process. First, the complex enzymatic hydrolysis of tea bee pollen including acid cellulase and pectinase was optimized with the content of reducing sugar as the evaluating parameter. The concentration of reducing sugar in pollen was determined based on 3,5-dinitrosalicylic acid (DNS) method. The glucose standard solutions (0, 100, 200, 300, 400, 500, and 600 μL of 10 mg/mL stock solution) and ultrapure water were mixed up to total volume of 2.0 mL followed by the addition of 1.5 mL of DNS reagent (6.3 g/l of 3,5-dinitrosalicylic acid, 20.96 g/l of sodium hydroxide, 185 g/l of potassium sodium tartrate, 5 g/l of phenol, and 5 g/l of sodium sulfite). The mixture was placed in a boiling water bath for 5 min and cooled in ice water to stop the reaction. Subsequently, ultrapure water was added into the mixture to adjust the volume to 10 mL and the mixture was thoroughly mixed. The absorbance was measured at 540 nm, with the absorbance value at 0 μL glucose standard solution being used as the correction value. A standard curve was plotted with glucose concentration on the x-axis and the corrected absorbance values at 540 nm on the y-axis. The glucose standard curve was shown in Fig. S1. For the single-factor experiments with acidic cellulase and pectinase, the effects of material-to-liquid ratio, enzyme dosage, pH, temperature, and reaction time on hydrolysis were examined individually. In the composite enzyme hydrolysis trials, the influence of enzyme additive ratio, material-to-liquid ratio, pH, and total enzyme dosage on hydrolysis efficiency were further investigated. In all experiments, 2 g of tea bee pollen served as the substrate and was subjected to water bath treatment under specific conditions. The samples were subsequently

dried at 80 °C, and hydrolysis efficiency was evaluated by measuring reducing sugar content. Based on the single-factor experiments using composite enzymes, an $L_9(3^4)$ orthogonal test program was designed to optimize the enzymatic digestion of tea bee pollen. The factors and their levels for the orthogonal test are presented in Table S1.

The L. *reuteri* LP4 strain was isolated from the gut of adult worker bees of *A. c. cerana* by our research team (Lei et al., 2020) and deposited at the China General Microbiological Culture Collection Center (CGMCC No 16,043). The strain was maintained with Man, Rogosa, and Sharp (MRS) broth (10.0 g/l of peptone, 4.0 g/l of yeast extract, 1.0 ml/l of Tween-80, 5.0 g/L of anhydrous sodium acetate, 0.2 g/l of magnesium sulfate anhydrous, 5 g/L of beef extract, 20.0 g/l of d-glucose, 2.0 g/l of potassium dihydrogen phosphate, 2.0 g/l of ammonium citrate, 0.05 g/l of manganous sulfate) at 37℃ for 24 h in an anaerobic jar (Anaero-Jar TM 2.5l, Oxoid Ltd, Basingstoke, UK), including a gas-generating package (AnaeroPack, Mitsubishi Gas Chemical Co., Tokyo, Japan) prior to experiments. The fermentation process of tea bee pollen using L. *reuteri* LP4 was optimized, and single-factor experiments were conducted to evaluate the impact of inoculum dose, temperature, and fermentation time on viable LAB count. In each trial, 5 g of enzymatically hydrolyzed tea bee pollen, prepared under optimal conditions and subjected to a 30-minute water bath at 80 °C, was used as the substrate. The samples were then anaerobically fermented, and the viable LAB count was determined using the plate counting method. Based on the results of the single-factor experiments, an $L_9(3^3)$ orthogonal test protocol was designed to optimize the fermentation conditions of tea pollen. This protocol considered three factors: inoculum dose, temperature, and time. The factors and levels of the orthogonal experimental design are shown in Table S2.

Physicochemical property tests of fermented tea bee pollen

Determination of protein content and pH value

The "Determination of crude protein in feeds-Kjeldahl method" (GB/T 6432–2018) was followed to determine the protein content. One gram of tea bee pollen was mixed with 9 mL of physiological saline, and the pH of the resulting suspension was measured using a pH meter (PB-10, Sartorius Scientific Instruments Beijing Co., Ltd., Germany).

Microbiological indicator testing

One gram of fermented tea bee pollen was mixed with 9 mL of sterile physiological saline for gradient dilution. Subsequently, 100 μL of the diluted solution was pipetted and evenly spread onto the selective solid culture media. PDA was incubated at 28 °C for 48 h to detect molds and yeasts. XLD used for detecting *Salmonella* and LST added with 1.5 % agar used for detecting *Escherichia coli*, were incubated at 37 °C for 48 h. When detecting *Shigella*, after incubation in *Shigella* broth with novobiocin at 41.5 °C under anaerobic conditions for 24 h, the enriched *Shigella* culture was streaked onto XLD Agar medium and incubated at 37 °C for 24 h to detect the presence of *Shigella*. MRS broth added with 1.5 % agar was incubated anaerobically at 37 °C for 48 h to determine LAB viable counts.

Impact of L. reuteri LP4 fermentation on the morphological structure of tea bee pollen

Firstly, conductive adhesive was adhered to the specimen stage. Subsequently, freeze-dried powders of tea bee pollen, enzymatically digested tea bee pollen, and fermented tea bee pollen were shaken onto the conductive adhesive. Finally, loosely adhered samples were removed using a washout ball, and the pollen morphology was observed and recorded under a scanning electron microscope (FlexSEM100, Hitachi, Ltd., Japan).

Impact of L. reuteri LP4 on the growth performance of worker bees of A. c. cerana

In this study, transparent acrylic boxes measuring 10 cm×10 cm×10 cm, topped with gauze, were utilized as miniature beehives. Worker bees were selected from four colonies at the Yunnan Agricultural University apiary, and 100 one-day-old worker bees from each colony were collected and randomly assigned to control group and treatment group. Twenty worker bees each group were designated to monitor survival rate and growth performance, while the remaining 30 bees were reserved for subsequent sampling. All worker bees were placed in the mini-hives after grouping and were reared separately, provided with a 30 % sucrose (m/m) solution, and fed either fermented bee pollen or unfermented bee pollen. Two gram of sucrose solution and 200 mg of pollen per day were placed in feed boxes and offered to bees, fresh prepared pollen were replaced at regular intervals every day. Two additional mini-hives, devoid of bees, were established to account for weight loss of sucrose solution and pollen solution due to natural evaporation. All mini-hives were maintained under conditions of 30 °C and 75 % humidity for 15 days, during which the weight of the mini-hives, the weight of the food containers, and the number of surviving bees in each box were recorded daily.

Detection of changes in the intestinal microbiota of worker bees of *A. c cerana* by RT-qPCR

Three whole bees were pooled into a single biological sample, and this process was repeated three times for each group. The extracted DNA samples were stored at −80 °C after using the Fecal Genomic DNA Extraction Kit. DNA integrity was assessed by electrophoretic analysis on a 1 % agarose gel under the following conditions: 180 V, 400 mA, and 30 min of continuous electrophoresis. The results were visualized using a gel imaging system. DNA concentration and purity were determined using a NanoPhotometer Ultra-Micro Spectrophotometer (Thermo Fisher Scientific, USA). Specific primers of 16S rRNA for *Lactobacillus, Bifidobacterium, Enterococcus*, and *Bacteroides* and universal primers of bacteria 16S rRNA used in this study are listed in Table S5. Universal primers were used as housekeeping, and relative expression was analyzed as described by Singh et al. (Singh et al., 2019). Real-time fluorescence quantitative RT-PCR (Bio-Rad, USA) was performed with pre-denaturation (95 °C for 30 s) and melting (95 °C for 5 s) cycles followed by 40 cycles of annealing (60 °C for 30 s).

Detection of relative expression of intestinal immunity genes of worker bees of A. c. cerana by RT-qPCR

In each replicate, three whole bees were pooled into one biological sample, and this process was repeated three times. Total RNA was extracted from the honeybee intestines using a total RNA extraction kit and stored at −80 °C. RNA integrity was assessed by 1 % agarose gel electrophoresis, following the conditions described in section 2.6. Electrophoresis results were visualized using a gel imaging system, and RNA concentration and purity were determined using a NanoPhotometer ultra-micro spectrophotometer (Thermo Fisher Scientific, USA). The reverse transcription reaction was configured on an ice plate, gently mixed, incubated at 42 °C for 3 min, and stored at 4 °C. The immunity-related genes *Abaecin, Defensin, PPO*, and *Vg* were selected for real-time fluorescence quantitative real-time RT-PCR analysis, with *ACTB* serving as the internal reference gene. Primers were synthesized by Shanghai Jierui Technology Co., Ltd., and the gene and primer sequences are listed in Table S6. Real-time RT-PCR for gene expression was conducted as described in section 2.6.

Statistical analysis

The data were analyzed using SPSS 27 software. Student *t*-test was

employed to compare differences between two groups, while one-way variance analysis with Duncan's multiple range test was used to compare differences among multiple groups. The survival rate of the unfermented control and fermented pollen groups was analyzed with the Log-Rank test in GraphPad Prism 8.0 software. Results were presented as mean ± standard deviation, with $P< 0.05$ indicating a significant difference and $P< 0.01$ indicating a highly significant difference.

Results

Optimal process for synergistic fermentation of pollen by bacteria and enzymes

Reducing sugar contents at 29.52 % and 31.21 % in pollen treated with acidic cellulase and pectinase were achieved at material-liquid ratio of 1:0.8 and 1:1.4, respectively, with subsequent declines. Appropriate enzyme dosage, pH, temperature, and time were also found to positively impact reducing sugar content and the optimal conditions were determined. When the dosage of acidic cellulase and pectinase was 0.9 % and 0.7 % respectively, the reducing sugar content in pollen reached 25.95 % and 30.89 %, respectively. When the pH for acidic cellulase and pectinase was 5.5, the reducing sugar content in pollen was 34.92 % and 35.94 % respectively. When the temperature for acidic cellulase and pectinase was 35 °C, the reducing sugar content was 29.64 % and 35.62 % respectively. When the time was 150 min, the reducing sugar content were 30.07 % and 31.21 % respectively (Fig. S2).

Total enzyme dosage of 0.9 % maximized reducing sugar content (36.33 %), while pH 5.5 was found to be optimal for sugar release, with a subsequent decline (Fig. S3). The optimal conditions for enzymatic hydrolysis were determined to be a 1:2 enzyme ratio, a 1:1.1 material-liquid ratio, a total enzyme dosage of 0.9 %, and a pH of 5.5. Orthogonal experiments were conducted to investigate the effects of four factors: composite enzyme composition ratio (A), material-liquid ratio (B), pH value (C), and total enzyme dosage (D). As shown in Table S3, pH was identified as the primary influencing factor, followed by composition ratio, solid-to-liquid ratio, and total enzyme dosage. The theoretical optimal combination for composite enzyme hydrolysis was determined to be $A_1B_1C_2D_2$, while the actual optimal process was $A_1B_2C_2D_2$. Validation test results indicated that $A_1B_1C_2D_2$ (38.75 %) yielded a higher reducing sugar content than $A_1B_2C_2D_2$ (37.70 %), leading to the selection of $A_1B_1C_2D_2$ as the optimal hydrolysis process. This optimal process involved an acidic cellulase: pectinase composition ratio of 1:1, a material-liquid ratio of 1:0.8, a pH value of 5.5, and a total enzyme dosage of 0.9 %.

The viable LAB count increased with increasing inoculum dose, temperature, and time, reaching maxima of 9.44 lg CFU/g at an inoculum dose of 1×10^7 CFU/g, 9.35 lg CFU/g at a temperature of 37 °C, and 9.45 lg CFU/g at a fermentation time of 32 h. Further increases in these factors led to a decline in viable counts (Fig. S4). In summary, the optimal conditions for the fermentation of tea pollen by L. *reuteri* LP4 were determined to be an inoculum size of 1×10^7 CFU/g, a fermentation temperature of 37 °C, and a fermentation time of 32 h. A three-factor, three-level orthogonal test was conducted to investigate the effects of inoculum amount (A), fermentation temperature (B), and fermentation time (C). As shown in Table S4, fermentation temperature emerged as the primary influencing factor, followed by inoculum quantity, with fermentation time exhibiting the least influence. Although the theoretically optimal combination $A_1B_1C_3$ (9.36 lg CFU/g) yielded a slightly higher number of live lactic acid bacteria than the process combination $A_1B_1C_1$ (9.34 lg CFU/g), the former required an additional 16 h of fermentation, and the difference in the number of live bacteria was less than one order of magnitude. Considering cost-effectiveness, the final fermentation conditions were selected as follows: an inoculum of 5×10^6 CFU/g, a fermentation temperature of 35 °C, and a fermentation time of 24 h.

Physicochemical properties of fermented tea bee pollen

pH and protein content of L. reuteri LP4-fermented tea bee pollen

The effects of L. *reuteri* LP4 fermentation on the protein content and pH of tea bee pollen are presented in Table 1. A highly significant decrease in pH was observed in the fermented tea bee pollen ($P< 0.01$), dropping from 6.02 to 4.41, compared to the unfermented control. However, no significant change in protein content was detected ($P> 0.05$).

Microbiological hygiene index of fermented tea pollen

The microbiological testing results for L. *reuteri* LP4-fermented tea pollen, as presented in Table 2, indicated the absence of *E. coli, Shigella, Salmonella*, molds, and yeasts. The LAB content was 2.31×10^9 CFU/g.

Morphological structure of fermented tea bee pollen

Scanning electron microscopy revealed that untreated tea pollen retained its original morphological structure, with a clearly visible germination pore. Following enzymatic hydrolysis and fermentation, the surface of the tea pollen exhibited an irregular, porous structure, characterized by widened germination furrows. The pollen wall was disrupted with mechanical grinding, consequently resulting in the release of their contents (Fig. 1). These findings suggest that the combined processes of enzymatic hydrolysis, fermentation, and grinding significantly alter the morphological structure of tea pollen, thereby facilitating the release and utilization of its contents.

Effects of L. reuteri LP4-fermented tea bee pollen on sugar and pollen intake of A. c. cerana worker bees

Worker bees in fermented tea bee pollen group consumed significantly more sucrose on days 3, 8, and 15 ($P< 0.05$) than those in unfermented control group. Sucrose intake in the unfermented control group increased and subsequently decreased between days 3–8 and 9–13 ($P> 0.05$), whereas worker bees in fermented tea bee pollen group maintained a higher sucrose intake between days 3–12, 14, and 15 compared to other feeding days ($P< 0.05$). Worker bees in fermented tea bee pollen group exhibited a trend of increased sucrose intake compared to unfermented control group (Fig. 2A).

For pollen intake, worker bees in fermented tea bee pollen consumed significantly more pollen on days 7, 8, and 13 ($P< 0.05$) compared to the unfermented control group. Worker bees in unfermented control group exhibited no significant variation in pollen intake over the 15-day period, whereas those in fermented tea bee pollen group experienced a significant increase in pollen intake from days 4 to 8 ($P< 0.05$). Administration with fermented tea bee pollen demonstrated an upward trend in pollen intake relative to unfermented control pollen (Fig. 2B).

Effects of L. reuteri LP4-fermented tea pollen on weight and survival rate of A. c. cerana worker bees

The effect of L. *reuteri* LP4-fermented tea bee pollen on the body weight of *A. c. cerana* worker bees is presented in Table 3. A significant increase in body weight was observed from days 1–5 to Days 6–10 in both fermented and unfermented groups during the 15-day feeding period. Subsequently, the weight stabilized on days 6–15. While worker bees in fermented pollen group showed a trend towards increased body

Table 1
Effects of L. *reuteri* LP4 fermentation on protein content and pH value of tea pollen.

Projects	Unfermented pollen	Fermented pollen
pH	6.02 ± 0.02	4.41 ± 0.02**
Protein content (%)	27.61 ± 0.44	27.75 ± 0.53

Note: ** denotes extremely significant differences between groups ($P< 0.01$).

Table 2
Microbiological hygiene index of fermented tea pollen.

Projects	Detection value
E. coli	—
Shigella	—
Salmonella	—
Molds and yeasts	—
Lactobacillus	2.31×10^{9} CFU/g

Note: "— "indicates not detected.

weight compared to unfermented control group, this difference was not statistically significant ($P> 0.05$).

At 15 days post-feeding, the survival rate of the fermented pollen group was significantly higher than that of unfermented control group with the increase by 24.34 % (Fig. 3). These results indicate that L. *reuteri* LP4-fermented tea bee pollen can significantly improve worker bees' survival rates.

Effects of L. reuteri LP4-fermented pollen on the gut microbiota composition of A. c. cerana worker bees

The abundance of *Lactobacillus, Bifidobacterium, Enterococcus*, and *Bacteroides* relative to total bacteria was measured by RT-qPCR, the results demonstrated that L. *reuteri* LP4-fermented bee pollen significantly increased ($P< 0.01$) the relative abundance of *Lactobacillus* and *Bifidobacterium* in the intestinal tracts of worker bees. This effect was observed after 5, 10, and 15 days of feeding. Additionally, the levels of these two bacterial groups in the intestinal tracts of bees in unfermented control groups increased on after 5 and 10 days ($P< 0.05$) compared to the initial day but decreased ($P< 0.05$) on day 15. In addition, fermented bee pollen effectively reduced the relative abundance of *Enterococcus* and *Bacteroides* in the honeybee gut. A significant reduction ($P< 0.05$) in *Enterococcus* was observed after 5 and 15 days of feeding, with a highly significant reduction ($P< 0.01$) on Day 10. Similarly, a significant reduction ($P< 0.05$) in *Bacteroides* was observed after 5 and 10 days of feeding. In the unfermented control group, both *Enterococcus* and *Bacteroides* levels decreased ($P< 0.05$), following a similar trend to the experimental group (Fig. 4). In conclusion, LP4-fermented bee pollen significantly enhanced the relative abundance of beneficial flora (*Lactobacillus* and *Bifidobacterium*) and reduced the relative abundance of potentially harmful flora (*Enterococcus* and *Bacteroides*) in the intestinal tracts of honeybees.

Effects of L. reuteri LP4 fermented tea bee pollen on the intestinal immune genes of A. c. cerana worker bees

The study demonstrated that feeding L. *reuteri* LP4-fermented bee pollen significantly upregulated the relative expression of *PPO, Defensin*, and *Vg* mRNA in the intestinal tracts of honeybees at various time points (days 5, 10, and 15) compared to unfermented control. After 5 and 10 days of feeding, the relative expression of *Abaecin* mRNA was significantly increased ($P< 0.01$) in workers bees gut fed fermented pollen compared to unfermented control group. After 15 days of feeding, the relative expression of *Abaecin* mRNA was increased without significance ($P> 0.05$). Specifically, *Abaecin* mRNA expression in fermented pollen group was significantly increased ($P< 0.01$) after 5 and 10 days of feeding. However, the relative expression of *Abaecin* mRNA decreased significantly on day 15 compared to Day 10. Within the unfermented control group, *Abaecin* mRNA increased significantly ($P< 0.05$) on day 10, but the change was not significant ($P> 0.05$) on days 5 and 15, and the expression was significantly lower ($P< 0.05$) on day 15 compared to day 10. Similarly, the expression patterns of intestinal *PPO, Defensin*, and *Vg* mRNA in honeybees from the test group exhibited a trend of significant increase ($P< 0.05$ or $P< 0.01$) in the early period (days 5 and 10) and a decrease ($P< 0.05$) on day 15 compared to day 10, although the expression remained significantly higher than the basal level. In unfermented control group, the relative expression of these genes was significantly increased on day 10, whereas the change was not significant compared to days 5 and 15 (Fig. 5). In conclusion, feeding L. *reuteri* LP4-fermented bee pollen significantly upregulated the expression of several immune-related genes in the intestinal tracts of honeybees, thereby enhancing their intestinal immunity.

Discussion

The pollen wall, a complex and rigid structure, protects the bioactive components within pollen. However, it also limits the absorption and biological activity of these beneficial components (Xu et al., 2009; Zhang et al., 2017). The pollen wall consists of two layers: the exine, primarily composed of sporopollenin, and the intine, containing dense substances such as cellulose and pectin (Ma et al., 2021). In order to enhance nutrients release, it is essential to disrupt the pollen wall and facilitate the following fermentation of bee bread. This study employed a complex enzymatic system consisting of acidic cellulase and pectinase to enzymatically hydrolyze tea pollen, followed by fermentation of the hydrolyzed pollen. A previous study demonstrated that deep enzymatic hydrolysis disrupted the pollen wall (Li et al., 2024). This finding aligned with the results of the present study, where the pollen walls exhibited irregular destruction after enzymatic treatment (Fig. 1B). Microbial fermentation is a promising strategy to improve the nutritional value of bee pollen through facilitating wall-breaking (Zhang et al., 2022). LAB are capable of synthesizing exopolysaccharides (EPS), a natural macromolecular bioactive substance, during the fermentation process (Sørensen et al., 2022). EPS is known as a natural bio-thickener that improves the rheological properties of fermented foods (Jurášková et al., 2022). Fig. 1C may depict the result of LAB metabolites, including EPS, adhering to the pollen surface.

Through single-factor and orthogonal experiments, the optimal conditions for enzymatic hydrolysis were determined as follows: a 1:1 ratio of acidic cellulase to pectinase, a total enzyme addition of 0.9 %, a

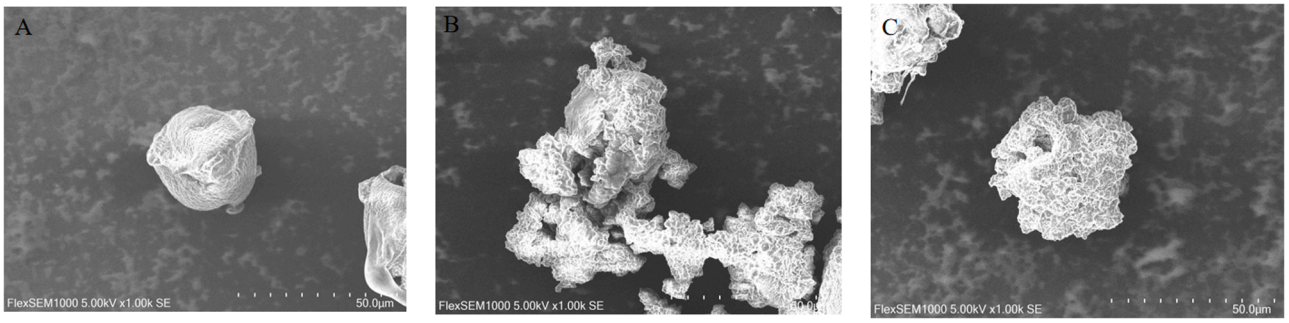

Fig. 1. Scanning electron microscopic observation of pollen morphology after different treatments. (A) untreated tea pollen, magnification factor×700. (B) enzymatic hydrolyzed tea pollen, magnification factor×1000. (C) fermented tea pollen, Magnification factor×1000.

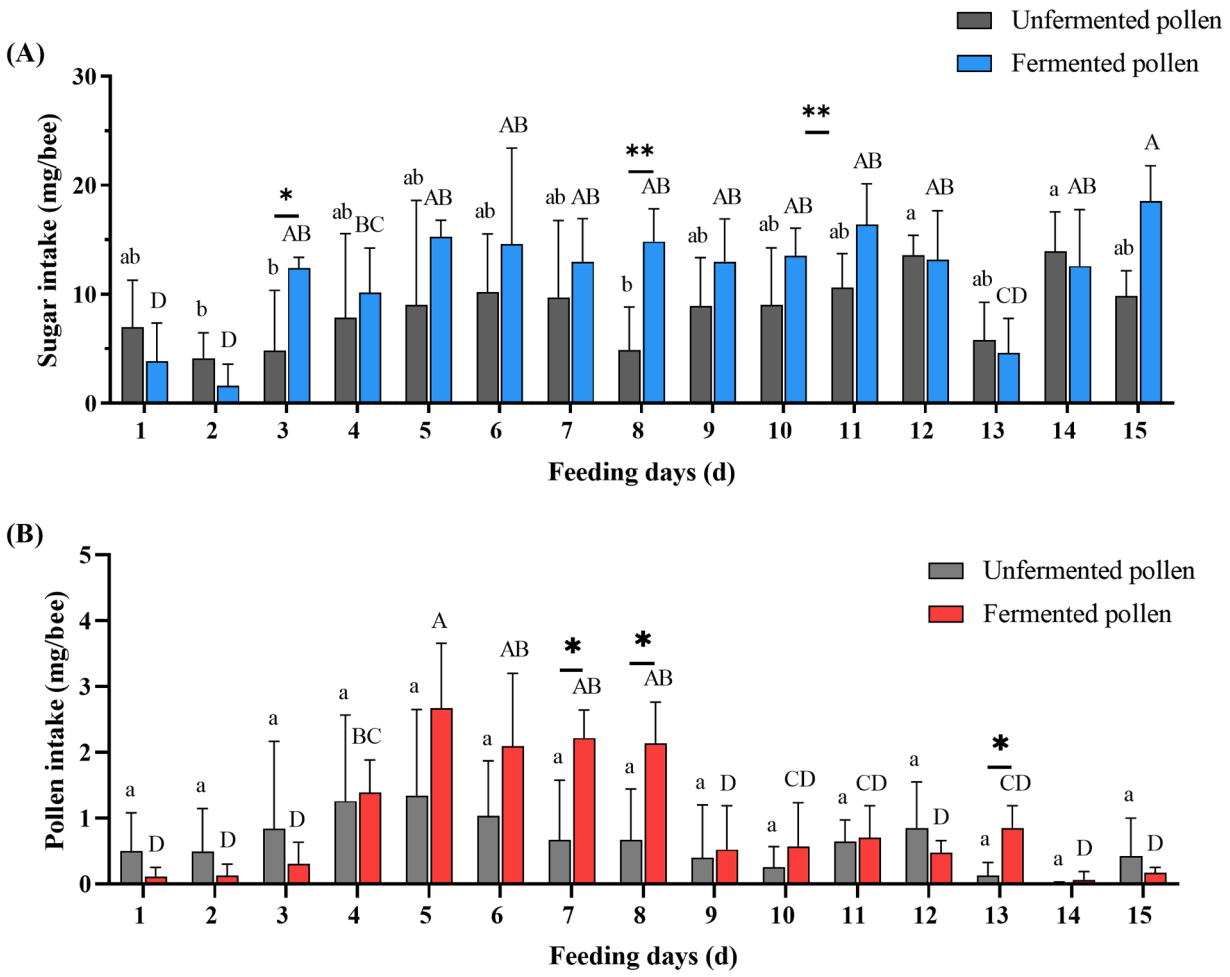

Fig. 2. Effect of fermented tea pollen with L. *reuteri* LP4 on the sugar and pollen intake of worker honeybees of *A. c. cerana* (n=4). (A) Sugar intake. (B) Pollen intake. Different letters among different feeding days at the same treatment denoted significance ($P< 0.05$). Different uppercase letters indicate significant differences between different feeding days within the fermented pollen groups ($P< 0.05$). Different lowercase letters indicate significant differences between different feeding days within the unfermented pollen groups ($P< 0.05$). *: indicates significant differences between groups ($P< 0.05$); **: indicates highly significant differences between groups ($P< 0.01$).

Table 3
Effect of fermented tea pollen with L. *reuteri* LP4 on the body weight of worker honeybees of *A. c. cerana.*

Weight (mg/bee)	Unfermented pollen	Fermented pollen	*P*-value
d 1–5	89.68 ± 8.74^{b}	87.96 ± 6.70^{b}	0.7659
d 6–10	109.65 ± 9.55^{a}	113.42 ± 9.66^{a}	0.5989
d 11–15	109.40 ± 10.51^{a}	115.37 ± 11.45^{a}	0.4719
Average daily weight gain (mg/bee/day)	1.04 ± 0.99	2.26 ± 0.52	0.0713

Note: different letters on the shoulder of the same column of data indicate significant differences ($P < 0.05$).

material-liquid ratio of 1:0.8, a pH of 5.5, and a reaction time of 150 min. Under these conditions, the reducing sugar content of bee pollen reached 37.75 %. Additionally, enzymatic digestion of tea bee pollen led to an increased release of nutrient compounds such as carbohydrates compared to untreated samples (Xie et al., 2022). These findings aligned with the results of the present study, which demonstrated an increase in reducing sugar content of pollen after co-enzymatic hydrolysis using acidic cellulase and pectinase. Acidic cellulase and pectinase may degrade cellulose and pectin in the tea bee pollen wall, releasing reducing sugars. Concurrently, they increase the pore diameter of the

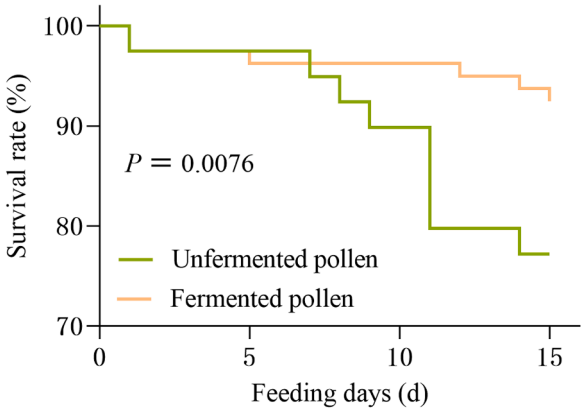

Fig. 3. Effect of fermented tea pollen with L. *reuteri* LP4 on the survival of worker bees (n=80).

germ pore, further releasing internal reducing sugars. This process enhanced the reducing sugar content of tea bee pollen following enzymatic hydrolysis, providing a favorable environment for the growth of inoculated bacteria in subsequent fermentation experiments and for digestion by *A. c. cerana* workers after fermentation.

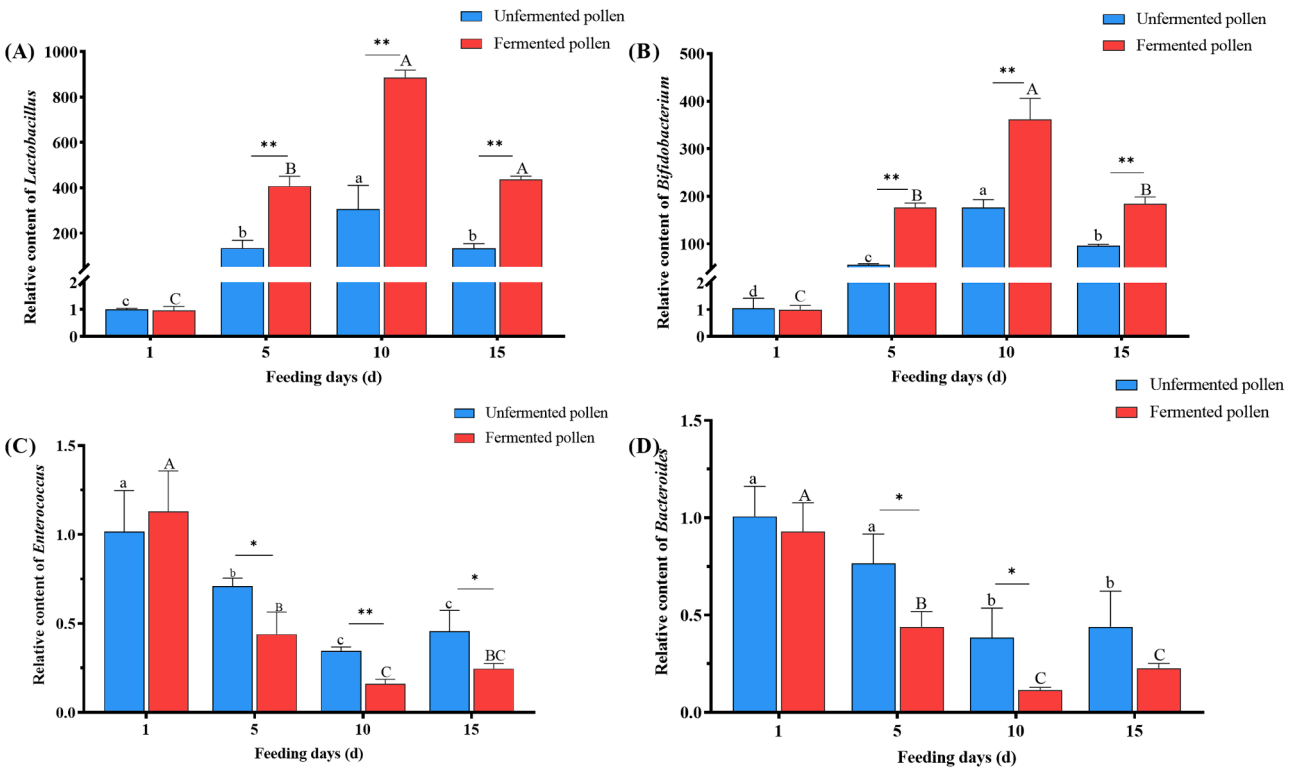

Fig. 4. Effect of fermented tea pollen with L. *reuteri* LP4 on the intestinal flora of honey bees (n=3). (A) Relative content of *Lactobacillus* in bee intestines after feeding L. *reuteri* LP4 fermented tea pollen. (B) Relative content of *Bifidobacterium* in bee intestines after feeding L. *reuteri* LP4 fermented tea pollen. (C) Relative content of *Enterococcus* in bee intestines after feeding L. *reuteri* LP4 fermented tea pollen. (D) Relative content of *Bacteroides* in bee intestines after feeding L. *reuteri* LP4 fermented tea pollen. Different uppercase letters indicate significant differences between different feeding days within the fermented pollen groups ($P< 0.05$). Different lowercase letters indicate significant differences between different feeding days within the unfermented pollen groups ($P< 0.05$). *: indicates significant differences between groups ($P< 0.05$); **: indicates highly significant differences between groups ($P< 0.01$).

The optimal fermentation conditions for L. *reuteri* LP4 to ferment tea pollen were determined to be a material-to-liquid ratio (m/v) of 1:1, an inoculum amount of 5×10^6 CFU/g, a fermentation temperature of 35 °C, and a fermentation time of 24 h. Under these conditions, the viable count reached 2.31×10^9 CFU/g. Previous studies have investigated the fermentation of pollen using LAB isolated from natural bee bread, resulting in artificial bee bread with good sensory evaluation, high viable bacterial counts, and biological efficacy. Additionally, research on *Aspergillus niger* fermentation of artemisinin polysaccharides identified optimal conditions of 5 % *A. niger* inoculum, 36 °C, 2 days, and 180 rpm shaker speed, resulting in a high extraction rate of artemisinin polysaccharides (17.04 %) (Tao et al., 2022). The honeysuckle beverage supplemented with 8 % sucrose was fermented with of *Lactiplantibacillus plantarum* and L*actobacillus acidophilus* (1:1) at 3 % inoculation and at 37 °C for 24 h and evaluated for sensory property including four aspects: color, aroma, shape, and texture. The sensory score was 87.30 ± 0.17 (out of a maximum of 100 points), and the viable bacterial count reached 9.84 ± 0.02 lg CFU/mL (Ran et al., 2024). The low viability of probiotic strains including L. *acidophilus* and *Lactobacillus paracasei* in fermented pollen was reported (Fuenmayor et al., 2011). In a recent study, high Andean bee pollen was fermented with a probiotic starter of L. *acidophilus* NCFM at 42 for 35 h and the viable counts of the starter reached to 3.15×10^6 CFU/g from initial inoculum with 2.15×10^5 CFU/g (Mora-Adames et al., 2021). Relative to this study, our present study showed the substantial rise in the viable counts of probiotic L. *reuteri* LP4 from 5×10^6 to 2.31×10^9 CFU/g in tea bee pollen after fermentation at 35 °C for 24 h. These promising results may attribute that the enzymatic hydrolysis processing prior to fermentation causes the release of intracellular nutrients and reducing sugars, providing the abundant substrates for probiotic propagation. Therefore, our results highlight the significance of microbial-enzymatic synergism in the development of bee pollen products.

In the present study, the fermentation of tea bee pollen promoted an increase in the body weight of worker honeybees, with bees in fermented pollen group exhibiting a trend of increased weight compared to the unfermented control group. Previous studies have confirmed that fermented feed can improve feed efficiency and enhance animal growth performance (Xu et al., 2020). For instance, the joint fermentation of basic diets with microorganisms and enzymes has been shown to benefit broiler chicken growth performance and nutrient digestibility and increase apparent metabolizable energy and cecal microbial abundance (Li et al., 2022). Additionally, the use of a mixed probiotic comprising *Bacillus licheniformis* (CGMCC 8147), *Saccharomyces cerevisiae* H11, and *Lactobacillus casei* (CGMCC 8149) to ferment feed significantly increased the average daily gain of weaned piglets and significantly reduced the incidence of diarrhea and mortality, thereby benefiting the growth and health of weaned piglets (Jiang et al., 2023). In the present study, it is highly conceivable that the organic acids produced during fermentation of tea bee pollen improved its palatability and facilitated the breakdown of macromolecules, enhancing the digestibility of pollen by worker honeybees. This may contribute to the observed trend of increased weight of bees in fermented pollen group compared to the unfermented control group.

Feeding L. *reuteri* LP4-fermented tea pollen tended to increase the intake of sucrose and pollen by worker honeybees. When newly emerged worker honeybees were fed fresh sunflower pollen, sunflower bee pollen stored by *Apis mellifera*, fresh aloe pollen, and mixed pollen, along with a 50 % sucrose solution as a carbohydrate source, the group fed fresh sunflower pollen consumed the highest amounts of sugar and pollen. The daily sugar consumption per bee ranged from 25 to 50 mg, and the daily pollen consumption per bee ranged from 0.5 to 2 mg (Nicolson et al., 2018), similar to the range of sugar and pollen consumption

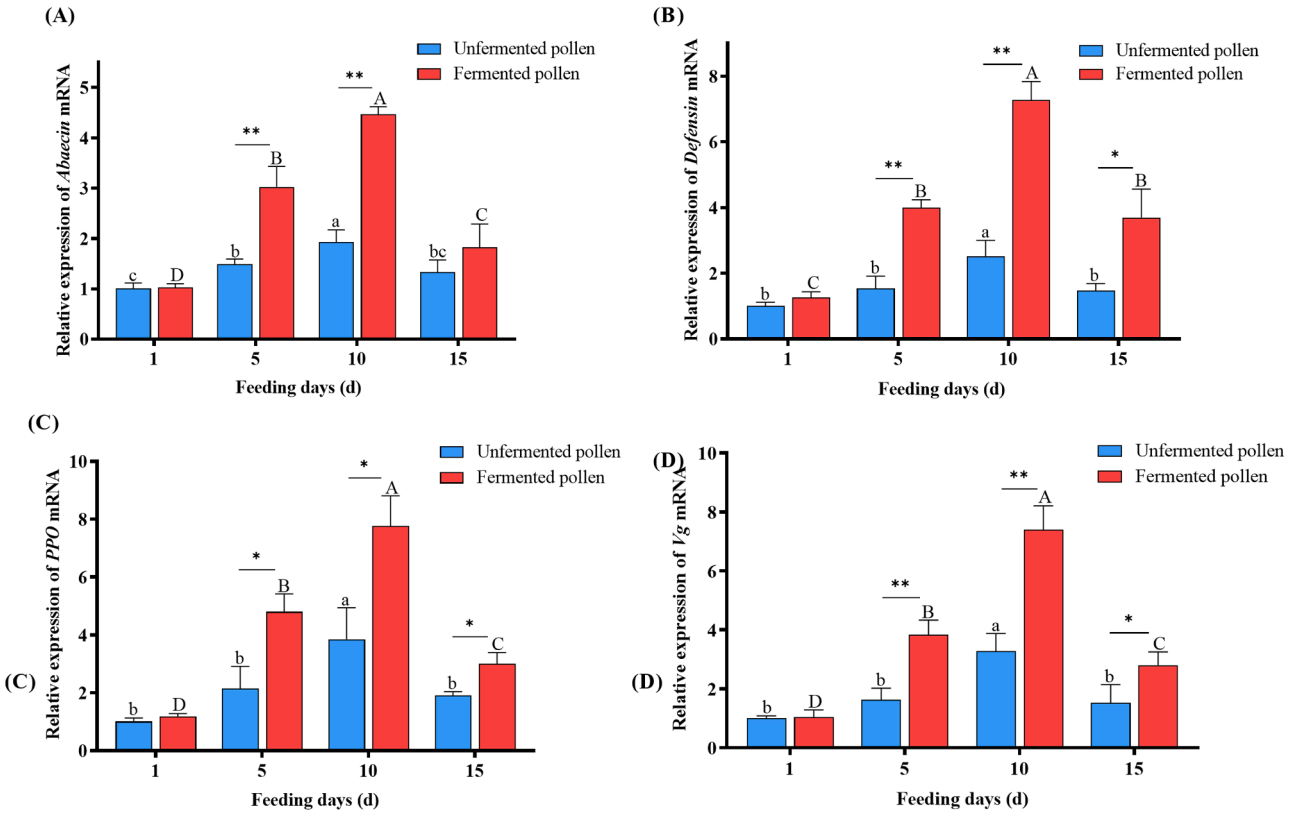

Fig. 5. Effect of bee pollen fermented by L. *reuteri* LP4 on immune-related genes in honey bees (n=3). (A) Relative expression of *Abaecin* mRNA after feeding L. *reuteri* LP4 fermented tea pollen. (B) Relative expression of *Defensin* mRNA after feeding L. *reuteri* LP4 fermented tea pollen. (C) Relative expression of *PPO* mRNA after feeding L. *reuteri* LP4 fermented tea pollen. (D) Relative expression of *Vg* mRNA after feeding L. *reuteri* LP4 fermented tea pollen. Different uppercase letters indicate significant differences between different feeding days within the fermented pollen groups ($P< 0.05$). Different lowercase letters indicate significant differences between different feeding days within the unfermented pollen groups ($P< 0.05$). *: indicates significant differences between groups ($P< 0.05$); **: indicates highly significant differences between groups ($P< 0.01$).

observed in this study. Studies have shown that the gut microbiota of worker *Apis mellifera* significantly affects sugar sensitivity (Zheng et al., 2017). This finding aligned with our results, where the experimental group exhibited significantly higher sugar intake on days 6–10 compared to the unfermented control group. This increased sugar intake may be attributed to accelerated sugar absorption due to the ingestion of L. *reuteri* LP4. Additionally, studies on the feeding and survival of *Apis mellifera* fed different carbon-to-nitrogen ratio nutritional solutions have shown that as the age of the worker honeybees increased, their food intake gradually increased, and they tend to prefer foods with a higher carbon-to-nitrogen ratio (Paoli et al., 2014), this could be the reason for the decrease in protein and amino acid requirements of worker bees with increasing day old in this study.

Our previous study has indicated that the addition of *Lactobacillus helveticus* KM7 to the diet increased the survival rate of worker bees of *A. c. cerana*, the content of *Lactobacillus* in the intestinal tract of honeybees, and the relative expression of the immune-related genes including *Abaecin* and *Defensin*, while concurrently reduced the levels of the detrimental bacterium *Enterococcus* (Lv et al., 2022). These findings are consistent with the results of this study. These results suggest that LAB fermented bee pollen can improve the survival rate of worker bees of *A. c. cerana* through regulating the gut microbiota and the expression of related genes. In our study, the high intake of exogenous LAB by the experimental group may have contributed to maintain gut balance, compete with harmful bacteria for colonization sites, and promot the expression of immunity-related genes in *A. c. cerana*, thereby protecting against detrimental environmental factors and significantly increasing survival rates.

The honeybee gut microbiome is relatively simple but plays a crucial role in maintaining its own gut stability and health, and endocrine signaling pathways associated with feeding behaviour and body weight gain, as well as growth and developmental pathways in honeybees, are strongly influenced by gut microbiota (Motta and Moran, 2024). *Lactobacillus* and *Bifidobacterium* species are essential in the microbiota of honey bees, as *Bifidobacterium* has genes related to carbohydrate utilisation and *Lactobacillus* has a number of phosphatase transfer systems that allow them to derive energy from the utilisation of carbohydrates and occupy a central position in the intestinal community of honey bees (Ellegaard et al., 2015; Kwong and Moran, 2016). This study compared the effects of L. *reuteri* LP4-fermented bee pollen versus unfermented tea bee pollen on the gut microbiota of worker bees. Previous studies have shown that probiotics such as *Bifidobacterium* and *Lactobacillus* in bee food helps maintain a more stable microbial environment in the bee gut (Huang et al., 2018). The results of our study showed that the relative levels of *Lactobacillus* and *Bifidobacterium* in the intestines of bees fed fermented pollen were highly significantly higher than those of the unfermented control group on days 5, 10 and 15, and the relative levels of *Enterococcus* and *Bacteroides* were lower than those of the unfermented control group. A mixture of four strains of *Lactobacillus kunkeei* isolated from the gut of Italian honeybees, when fed to microsporidia-infected bee larvae, significantly reduced the abundance of *Enterobacteriaceae* in the gut and increased the abundance of *Lactobacillus*, effectively reducing larval mortality (Arredondo et al., 2018). Consistent with this, our research results show that the survival rate of worker bees fed fermented tea pollen and the levels of *Lactobacillus* and *Bifidobacterium* in their intestines significantly increased. This may be attributed to the fact that exogenous *Lactobacillus* suppressed *Enterococcus* and *Bacteroidetes* levels through antimicrobial substances while

improving the gut environment and stimulating the immune system, promoting the growth of beneficial bacteria. In this experiment, the bees' intake of fermented pollen was relatively reduced on days 11–15, with fewer *Lactobacillus* competing for colonization in the gut and an increase in the number of other bacteria. This could be the reason why the bees showed higher levels of *Bacteroidetes* and *Enterococcus* in the gut compared to those on day 10.

Antimicrobial peptides (AMPs) are essential components of the immune defense system in numerous organisms, providing protection against infections (Danihlík et al., 2015). Abaecin is a significant broad-spectrum antibacterial proline-enriched cationic peptide among the antimicrobial peptides in honeybees (Luiz et al., 2017). Defensin is a cationic antimicrobial peptide capable of exerting immune control against a wide range of infectious microorganisms, including bacteria, viruses, and fungi. They possess antiviral activity and play a crucial role in defense against enteroviral infections (Gao et al., 2021; Wilson et al., 2016). In the present study, it was found that the mRNA expression of *Abaecin* and *Defensin* in the intestines of bees from the group consuming fermented pollen was highly significantly higher than that of the unfermented control group on day 5 and 10. In fermented pollen group, the relative mRNA expression of *Abaecin* and *Defensin* was significantly increased on day 5, 10 and 15 of feeding compared to day 1 in fermented pollen group. It was shown that feeding L. *reuteri* LP4 fermented bee pollen increased the relative expression of mRNAs for *Abaecin* and *Defensin* to a greater extent in the honeybee gut. The administration of a probiotic formulation including L*actobacillus brevis* B50 dramatically elevated the expression of *Abaecin* mRNA in honey bees (Maruscakova et al., 2020). The study found that feeding *Apis mellifera ligustica* with a mixed probiotic diet containing *Lactobacillus* and *Bifidobacterium* for 12 h significantly increased the expression level of *Abaecin* mRNA compared to the control group (Evans and Lopez, 2004). *Defensin* expression in the gut increased when honeybee larvae were infected with *Ascosphaera apis* (Nie et al., 2020). The regulation of the expression of this antimicrobial peptide is closely related to the intensity of the antigenic stimulus (Nie et al., 2020). In both unfermented control group and fermented pollen groups, the relative mRNA expression levels of *Abaecin* and *Defensin* significantly decreased on day 15 compared to day 10. The dietary needs of bees vary with age development and changes in behavioral responsibilities. Research indicates that foraging bees of *A. cerana*, approximately 15 days old, exhibit decreased consumption of bee pollen (Paoli et al., 2014). It was shown thatforaging bees of *Apis mellifera* around 15 days with reduced bee pollen intake revealed a reduction in *Abaecin* mRNA expression (da Luz et al., 2022). The results of the present study were consistent with previous studies, showing a decreasing trend in immune gene expression after 10 days. This may be attributed to the reduced intake of bee pollen, which lead to a decrease in the number of intestinal microflora and a subsequent inability to stimulate the expression of immune genes.

Insect phenoloxidase (*PPO*), as a group of key proteins in the innate immune system of insects, is mainly found in the haemolymph and are essential in the denfence against pathogens such as fungi, bacteria and viruses (Cerenius et al., 2008; Chen et al., 2020). When the *PPO* gene is disturbed in the houseworm, it leads to an increase in the number of intestinal bacteria and consequently an increase in mortality (Shao et al., 2012), suggesting that *PPO* is a key player in insect immune defense. Vitellogenin (*Vg*) is present in the intestinal cells of honey bee workers (Harwood et al., 2019). *Vg* is widely recognized as an important indicator of the overall health of honey bees, playing an important role in immunity and antioxidant processes, as well as having an impact on longevity extension (Bitondi and Simoes, 2015; Park et al., 2018). In this study, feeding fermented bee pollen significantly increased the expression levels of *PPO* mRNA and *Vg* mRNA in the bee gut, this has a positive impact on the survival and health of bees.

Conclusion

The present study investigated synergistic fermentation of LAB and enzymes in tea bee pollen and its effects on the survival and gut health of worker honey bees. Acid cellulase and pectinase were employed to hydrolyze tea bee pollen, and consequently probiotic L. *reuteri* LP4 derived from the gut of *A. c. cerana* was used to prepare fermented tea bee pollen. The results showed that the protein content of tea pollen fermented by L. *reuteri* LP4 after optimization was 27.75 %, the pH value was 4.41, and no *E. coli, Shigella, Salmonella*, mold, or yeast were detected. The viable count of LAB was 2.31×10^9 CFU/g. Scanning electron microscopy revealed an enlarged germination groove and an irregular porous surface structure in the fermented tea pollen. Compared to the unfermented tea pollen group, worker honey bees receiving L. *reuteri* LP4-fermented tea pollen exhibited increased survival rate. Additionally, L. *reuteri* LP4-fermented tea pollen administration elevated the relative abundances of gut *Lactobacillus* and *Bifidobacterium* were elevated while reduced gut *Enterococcus* and *Bacteroides* concentrations. Furthermore, the relative expression of the immune-related genes *Abaecin, PPO, Defensin*, and *Vg* in the gut of worker honey bees were significantly increased with L. *reuteri* LP4-fermented tea pollen intervention. Overall, the results of this study can provide the theoretical basis for the application of fermented feed to promote the health of *A. c. cerana* populations and the sustainable development of the apiculture industry in China.

CRediT authorship contribution statement

Panpan Xue: Investigation, Formal analysis, Visualization, Writing – original draft. **Le Xu:** Methodology, Formal analysis, Visualization, Writing – review & editing. **Yakai Tian:** Methodology, Writing – review & editing. **Mingkui Lv:** Investigation, Formal analysis, Visualization. **Pingping Fang:** Investigation, Formal analysis, Visualization. **Kun Dong:** Conceptualization, Funding acquisition, Resources. **Qiuye Lin:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Zhenhui Cao:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100343.

Data availability

Data will be made available on request.

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