

Insights into the gut microbiota in companion animals

Edited by

Baichuan Deng, Geert Janssens and Zhenya Zhai

Coordinated by

Jia Xu

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Insights into the gut microbiota in companion animals

Topic editors

Baichuan Deng — South China Agricultural University, China

Geert Janssens — Ghent University, Belgium

Zhenya Zhai — Jiangxi Academy of Sciences, China

Topic coordinator

Jia Xu — Jinhua Polytechnic, China

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Renuka Dahiya,
University at Buffalo, United States
Zhicheng Peng,
University of Pennsylvania, United States

*CORRESPONDENCE

Guangyu Li
✉ tcslgy@126.com

[†]These authors have contributed equally to this work

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Impact of *Pediococcus acidilactici* GLP06 supplementation on gut microbes and metabolites in adult beagles: a comparative analysis

Mengdi Zhao^{1,2†}, Yuanyuan Zhang^{1†}, Yueyao Li¹, Keyuan Liu¹, Kun Bao¹ and Guangyu Li^{1*}

¹College of Animal Science and Technology, Qingdao Agricultural University, Qingdao, China,

²College of Animal Science and Technology, Jilin Agriculture University, Changchun, China

There is growing interest in the potential health benefits of probiotics for both humans and animals. The study aimed to investigate the effects of feeding the canine-derived probiotic *Pediococcus acidilactici* GLP06 to adult beagles by analysing the microbiome and metabolome. Twenty-four healthy adult beagles were randomly assigned to four groups. The CK group received a standard diet, while the three probiotic groups, the LG group (2×10^8 CFU/day/dog), MG group (2×10^9 CFU/day/dog), and HG group (2×10^{10} CFU/day/dog), received the standard diet supplemented with varying amounts of probiotics. The results show that, compared to the CK group, total antioxidant capacity was significantly increased in the MG and HG groups ($p < 0.05$), and superoxide dismutase and catalase were significantly increased in the HG group ($p < 0.05$). Compared to the CK group, malondialdehyde and blood urea nitrogen content were significantly decreased in the MG and HG groups ($p < 0.05$). Additionally, secretory immunoglobulin A activity was significantly increased in the HG group compared to the CK and LG groups ($p < 0.05$), and immunoglobulin G activity was significantly increased in the HG group compared to the CK, LG, and MG groups ($p < 0.05$). In addition, compared with the CK group, the abundance of *Faecalitalea* and *Collinsella* increased in the LG group, and the relative abundance of *Tyzzereella* and *Parasutterella* increased in the MG group. The α diversity and the relative abundances of beneficial bacteria (*Faecalibacterium*, *Lachnospiraceae_NK4A1316*, and *Ruminococcaceae_UCG-005*) were higher in the HG group than in the CK group. Furthermore, acetic acid content was significantly increased in the HG group compared to the CK, LG, and MG groups ($p < 0.05$). Butyric acid, isobutyric acid, and the total SCFA content were significantly increased in the HG group compared to the CK group ($p < 0.05$). Moreover, metabolome analysis revealed 111 upregulated and 171 downregulated metabolites in the HG group. In conclusion, this study presents evidence that supplementing with *P. acidilactici* GLP06 can have a positive impact on antioxidant activity, immunoproteins, SCFAs, and gut microbiota in adult beagles. These findings highlight the potential of probiotics as a dietary intervention to enhance gut health and overall wellbeing in companion animals.

KEYWORDS

probiotic, gut microbiota, short-chain fatty acids, metabolome, beagle

1 Introduction

In recent years, the pet food industry has experienced significant growth, driven by the increasing popularity of pet ownership and the rising demand for pet-related products and services (Samant et al., 2021). Pets are now regarded as beloved family members rather than just animals (Guo et al., 2022). Consequently, there has been a significant increase in the demand for pet products, such as natural pet food ingredients, functional pet food, and prescription pet food (Di Cerbo et al., 2017; Jian et al., 2022). Probiotics, as one of the principal products in functional foods, have been used in human and animal husbandry and have also attracted the focus of the pet industry (Grześkowiak et al., 2015).

“Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” is the definition of probiotics (Hill et al., 2014). Previous studies have shown that probiotics can modulate immune function, interact with the host gut microbiota, enhance the integrity of the intestinal barrier, and produce metabolites such as short-chain fatty acids (SCFAs), extracellular polysaccharides, and bacteriocins (van Baarlen et al., 2013; Aoudia et al., 2016; La Fata et al., 2018; Sanders et al., 2019). Lactic acid bacteria (LAB) have been reported to be among the safest probiotics, including *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Bifidobacterium*, and *Streptococcaceae* (Sanders et al., 2019). *Pediococcus acidilactici*, which belongs to the genus *Pediococcus*, has been reported to have probiotic potential, including the alleviation of anxiety, maintenance of intestinal homeostasis, protection of the intestinal tract, and antioxidant properties (Ruiz-Moyano et al., 2011; Liu et al., 2020; Bai et al., 2021; Tian et al., 2021).

Weaned piglets fed *P. acidilactici* FT28 had better apparent total tract digestibility (ATTD), blood biochemistry, and antioxidant status (Dowarah et al., 2018). *P. acidilactici* has also been reported to improve constipation in mice (Qiao et al., 2021). In addition, a study reported positive effects of canine-derived probiotics on faecal SCFAs and cell-mediated immune responses in healthy dogs (Kumar et al., 2017). On the other hand, host-derived microorganisms are preferred as probiotics compared to non-host-source microorganisms. This is because they are very familiar with the intestinal tract environment, are more adherent and persistent, and host-derived microorganisms have evolved to be more adapted to the gastrointestinal environment of the host gut (Lee et al., 2017; Dowarah et al., 2018; Johnson et al., 2023). Therefore, there is a need to explore the impact of host-derived probiotics on pet health.

However, the effectiveness of *P. acidilactici* GLP06, which was isolated from the gastrointestinal tract of healthy canines, on gastrointestinal health and metabolism has not been reported. We hypothesized that supplementing with canine-derived *P. acidilactici* GLP06 would improve faecal scores and ATTD and have a positive effect on the gastrointestinal environment of canines. The aim of this study was to assess the impact of supplementing probiotic GLP06 on ATTD, nitrogen (N) metabolism, serum antioxidants, immune protein activities, gut microbiota, SCFAs, and metabolism in adult beagles.

2 Materials and methods

The laboratory animals needed for this study were approved by the Laboratory Animal Ethics Committee of Qingdao Agricultural University (grant No. DWKJ202307043; Qingdao, China).

2.1 Experimental strain

P. acidilactici GLP06, used in this study, was isolated from the gastrointestinal tract of healthy beagles and completed the probiotic potential and safety evaluation in our laboratory (Zhao et al., 2023). It was kept in the China Centre for Type Culture Collection (CCTCC; Wuhan, China) under the accession number CCTCC No. M2023200.

In this experiment, *P. acidilactici* GLP06 was cultured in Man, Rogosa, and Sharpe (MRS) broth (Solarbio, China) and passaged three times. The cells were then inoculated into MRS broth at a concentration of 2.0% (v/v) for 20 h. Afterwards, they were centrifuged at $9391 \times g$ (10,000 rpm in Centrifuge 5,430 Eppendorf, Germany) for 10 min at 4°C. After removing the supernatant, the cells were resuspended in PBS (0.1 mol/L, pH=7.2), and the bacterial concentration was adjusted to 1×10^{10} CFU/mL.

2.2 Experimental design and feeding management

The study included 24 adult beagles with an average age of 3.71 ± 1.09 years and a body weight of 17.24 ± 2.66 kg. The study enrolled participants with body condition scores (BCS) of 6.2 ± 0.85 (Laflamme, 1997). They were randomly assigned to four groups, with six individuals per group (three females and three males in each group). The no-probiotic-added group (CK) received a standard diet, whereas the three probiotic groups (low-dose GLP06 group [LG] with 2×10^8 CFU/day/dog, medium-dose GLP06 group [MG] with 2×10^9 CFU/day/dog, and high-dose GLP06 group [HG] with 2×10^{10} CFU/day/dog) received the standard diet supplemented with different amounts of probiotics. The study lasted for 5 weeks, with the first week being an adaptation period. During the second week, the beagles were gavaged with 2 mL of probiotics per day according to the experimental design, while the CK group received the same volume of PBS through gavage.

To achieve clinical effects, probiotic concentrations should be at least 1×10^6 CFU/mL in the small bowel and 1×10^8 CFU/g in the colon (Minelli and Benini, 2008). Kumar et al. (2017) administered a dose of canine-derived *Lactobacillus johnsonii* CPN23 at $2\text{--}3 \times 10^8$ CFU/day/dog to adult dogs, which positively affected hindgut fermentation metabolites and cell-mediated immune responses. On the other hand, there are reports of clinical conditions where probiotics are effective in treating antibiotic-associated diarrhoea only at higher doses ($>1 \times 10^{10}$ CFU/day/dog; Ouwehand, 2017). Similarly, researchers supplemented adult beagles with *Weissella cibaria* JW15 at levels of 1.5×10^{10} CFU/day/dog and 1.5×10^{11} CFU/day/dog, which improved lipid parameters and improved outcomes in adult dogs (Sun et al., 2019). Combining the references and the cost of probiotics (the higher the dosage administered, the higher the cost), this experiment was designed with a minimum dose of 2×10^8 CFU/day/dog, and the maximum dose was set at 2×10^{10} CFU/day/dog.

Prior to the start of the trial, the beagles received vaccinations and were regularly dewormed. All kennels were located in the same environmentally controlled room ($21.0 \pm 1.0^\circ\text{C}$) with a 12-h light and a 12-h dark cycle. Each dog was housed in a separate cage. The cages were disinfected once a week with the compound hydrogen peroxide solution (Aladdin, China). Although animals were housed and fed individually, they were allowed to exercise and play outside of their

cages (with people and toys) in the animal room for several hours, at least three times a week. Based on the maintenance energy requirements of adult dogs (National Research Council, 2006), data from previous feeding records, and an energy estimate from the diet, provide enough food to maintain body weight. Maintaining each animal's body weight requires weekly, or even more frequent, adjustments to the amount of feed. Dogs had free access to fresh water at all times.

The respiration rate, temperature, and pulse were recorded weekly (Xu et al., 2019). Meanwhile, the faecal scores were recorded daily. The faeces of the beagles were evaluated for sensory characteristics using the Waltham® faeces scoring system (WFS; Supplementary Figure S1; Fournier et al., 2021). The following scale was used during faeces consistency observations: 1 = crumbles with little pressure; 1.5 = hard and dry, stool cracks when pressed; 2.0 = well formed, does not leave a mark when picked up; 2.5 = well formed with a slightly moist surface, leaves a mark when picked up; 3.0 = moist, beginning to loose form, leaving a definite mark when picked up; 3.5 = very moist, still with some definite form; 4.0 = most or all form is lost, no real shape; 4.5 = liquid stool with slight consistency; 5.0 = entire liquid stool.

2.3 Ration composition and nutrient levels

The standard diet for the experiment was formulated according to the National Research Council (2006), and the composition and nutritional levels of the standard diets are presented in Table 1.

2.4 Sample collection

On the morning of day 28 of the experiment, fresh faeces (within 15 min) were collected in frozen tubes (Axygen, United States) and immediately stored at -80°C for microbiological analyses, SCFA content, and non-targeted metabolite assays. Additionally, 10 mL of venous blood was collected from the forelimbs of the beagles. Serum was collected by centrifugation at 4°C, 464× g (1,800 rpm, Centrifuge 5,702, Eppendorf, Germany) for 10 min (Kostanjšek et al., 2022; Zentrichová et al., 2023).

The total faeces were collected from the 25th to the 28th day of the experiment, weighed, and frozen at -20°C. Similarly, the total urine was collected from days 25 to 28 and recorded as the total volume. The urine samples were filtered through filter paper and stored at -20°C until the tests were analysed. After the experiment, all the faeces collected from each beagle were mixed thoroughly. Two hundred grams of faeces were weighed and dried at 65°C for 72 h until a constant weight was achieved. The faeces were then crushed (Retsch BB50, DEU), passed through a 40-mesh sieve, and stored for testing.

2.5 Indicators and methods of measurement

2.5.1 Apparent total tract digestibility and nitrogen metabolism

Dry matter (DM; AOAC 934.01) and crude ash (ASH; AOAC 942.05) determinations of the samples were conducted following the AOAC method (Horwitz and Latimer, 2006). The N content was

TABLE 1 Composition and nutrient levels of the diets (air-dry basis, %).

Ingredients	Content	Nutrient levels ^b	Content
Extrusion corn	24	Crude protein	27.22
Extruded soybean	8	Ether extract	9.72
Corn germ meal	30	Crude ash	8.63
Fish meal	5	Carbohydrate	54.43
Meat and bone meal	3	Crude fibre	3.30
Chicken meal	12	GE/(MJ/kg)	19.65
Duck meal	12	ME/(MJ/kg)	16.99
Spray-dried blood cells	0.8	DE/(MJ/kg)	16.71
Chicken oil	2	Ca	0.86
CaHPO ₄	0.8	TP	0.57
Lys	0.9	Lys	1.34
Met	0.5	Met	0.80
Premix ^a	1	Cys	0.18
Total	100.00	Arg	0.90

^aOne kilogram of premix contained the following: vitamin A 625,000 IU, vitamin D₃ 100,000 IU, vitamin E 6,000 IU, vitamin K₃ 200 mg, vitamin B₁ 1,250 mg, vitamin B₂ 900 mg, vitamin B₆ 750 mg, vitamin B₁₂ 2.25 mg, biotin 10 mg, folic acid 150 mg, nicotinic acid 2,500 mg, calcium pantothenate 1,750 mg, vitamin C 10,050 mg, choline 240,000 mg, Fe (FeSO₄) 9,600 mg, Cu (CuSO₄) 1,800 mg, Zn (ZnSO₄) 7,800 mg, Mn (MnSO₄) 4,800 mg, KI 144 mg, Se (Na₂SeO₃) 30 mg.

^bGE, DE, and ME were calculated values, whereas the others were measured values.

^cGE, gross energy; DE, digestive energy; ME, metabolic energy.

determined using the Automatic Kjeldahl Nitrogen Determination (FOSS 8400, DK; Etheridge et al., 1998). The crude protein (CP) content was obtained by calculating the N content multiplied by 6.25. The ether extract (EE) content was determined by the Soxhlet fat extraction method (Haineng SOX606, China; Nielsen, 2010). The calcium (Ca) content was assessed using the ethylenediaminetetraacetic acid disodium salt (EDTA) titration method (Belyea et al., 1976). The total phosphorus (TP) content was determined using the ammonium molybdate method (AOAC 995.11). The crude fat (CF; AOAC 962.09) contents were measured using an Automatic Fibre Tester (ANKOM A2000i, United States), while the amino acid contents were analysed using a fully automatic amino acid analyser (Hitachi L-8800, Japan).

Feed energy was calculated, and the formulas for the ATTD and nitrogen metabolism-related indices for each nutrient are shown in Supplementary Material 1.

2.5.2 Serum biochemical indicators

The detection kit (Nanjing Jiancheng Bioengineering Institute, China) was used to measure the total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) contents in the serum of beagles. Simultaneously, enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Meimian, China) were selected to detect immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA). Because *P. acidilactici* GLP06 was administered via gavage and expected to primarily act at the mucosal level, sIgA was analysed in faeces. The operating procedures strictly

followed the kit instructions, and data were measured using an enzyme marker (Tecan, Switzerland).

2.5.3 16S rRNA sequencing

Genomic DNA from faecal samples was extracted using the E.Z.N.A.[®] Stool DNA Kit (Omega Bio-Tek, United States). Then, the V3–V4 region of the bacterial 16S rRNA gene was amplified using universal primers. The PCR products were then visualised on a 1.0% agarose gel (TSJ001, Tsingke, China), and the nucleic acids were purified using an Agencourt AMPure XP kit (Beckman Coulter, United States). A library was constructed using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, United States). Finally, the library was sequenced using a NovaSeq 6,000 SP Reagent Kit v1.5 (Illumina, United States). The similarity threshold for OTU clustering was set at 97% (Stackebrandt and Goebel, 1994). One dog in the CK group and one in the HG group failed the faecal sample quality control, resulting in only five beagles in each of the CK and HG groups for which results were available.

2.5.4 Short-chain fatty acids

Determination of fatty acids in beagle faeces by LC–MS/MS. Briefly, 30–40 mg of frozen faecal samples were placed into a 1.5-ml centrifuge tube. Then, 1 mL of 50% acetonitrile (ACN, Fisher Chemical, USA) was added, followed by 2–3 metal grinding beads. The sample was processed in an E6618 tissue grinder (Beyotime, China) for 1 min at 60 Hz and then centrifuged at $15,871 \times g$ (13,000 rpm in a Centrifuge 5,430 Eppendorf, Germany) for 10 min at 4°C. One hundred microliters of supernatant was taken and diluted proportionally to 10 mg of sample per 1.8 mL of 50% ACN solution. The mixture was vortexed and shaken (Scilogex, United States) for 30 s and then centrifuged at $15,871 \times g$ for 30 s. Twenty microliters of the supernatant was aspirated, and 10 μ L of 200 mM 3-nitrophenylhydrazine-HCl (3-NPH-HCl, Sigma-Aldrich, United States) was added separately. Ten microliters of 200 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide-HCl (EDC-HCl, Sigma-Aldrich, United States), 80 μ L of 50% ACN, 50 μ L of 7% pyridine (Sigma-Aldrich, United States), and 1 μ L of isotope internal standard solution (Toronto Research Chemicals, Canada) were vortexed and shaken for 3 min, derivatised in a constant temperature water bath at 40°C for 30 min and centrifuged at $15,871 \times g$ for 1 min at 4°C. Finally, 20 μ L of the reaction solution after derivatisation was added to 280 μ L of 50% ACN, vortexed for 30 s, centrifuged at $15,871 \times g$ for 10 min at 4°C, aspirated into the injection vial, and then subjected to LC–MS/MS analysis (LC-30 HPLC, SCIEX QTRAP 5500 mass spectrometry, Phenomenex: Kinetex C18, 2.6 μ m 100 $\times$ 3.00 mm, column temperature: 40°C, flow rate: 0.7 mL/min).

2.5.5 Untargeted metabolomics

50 mg of faecal samples were loaded into 1.5 mL EP tubes with 600 μ L of pre-cooled MeOH (Fisher Chemical, United States): ACN (Thermo Fisher Scientific, United States): H₂O (Thermo Fisher Scientific, United States) solution containing internal standards (v:v:v = 2:2:1). Two steel beads were added, and the tissue grinder was ground for 120 s at 60 Hz. Ultrasonication (PS-60AL, Leidebang, China) was carried out for 10 min, and then the sample was kept at –20°C for 1 h. Centrifugation was performed for 15 min at $15,871 \times g$ and 4°C, and 200 μ L of the sample was freeze-dried using a CentriVap (Labconco, United States). Next, 200 μ L of ACN:H₂O solution

(v:v = 1:1) was added to resolubilise the mixture. It was then shaken for 30 s, sonicated for 10 min, and incubated at 20°C for 2 h before being centrifuged at $15,871 \times g$ at 4°C for 15 min. Finally, 150 μ L of the supernatant was aspirated for liquid chromatography–tandem mass spectrometry (LC-30, Shimadzu, and TripleTOF 5,600+, SCIEX; Fu et al., 2023). The cardinal criteria used for screening differentially abundant metabolites were $p < 0.05$, VIP > 1 , and fold change < 0.67 or > 1.5 . The screening criteria for the chord plot included a correlation coefficient $|r| > 0.8$ and $p < 0.05$.

2.6 Data analysis

Data were expressed as the mean $\pm$ standard error of the mean (SEM), visualised utilising GraphPad Prism (8.3.0), one-way analysis of variance (ANOVA) of SPSS (version 25.0), and Dunnett's multiple comparison test for statistical analysis, with significant differences between groups ($p < 0.05$).

3 Results

3.1 Physiological indices and faecal scores of beagles

The body temperature, respiration rate, and pulse rate of beagles were all within the normal range of 36.9–37.6°C, 19.3–24.6 beats/min, and 84.8–95.2 breaths/min, respectively, and did not differ significantly between treatments ($p > 0.05$; Figures 1A–D).

Regarding faecal scores, there were no significant differences between the groups during the initial 2-week supplementation period (0–14 days). However, after the third and fourth weeks (15–28 days), the faecal scores of beagles in the probiotic-supplemented group significantly differed from those in the CK group ($p < 0.05$; Figure 1E). There were no significant differences found between the four groups in the average daily feed intake of the beagles ($p > 0.05$; Figure 1F).

3.2 Apparent total tract digestibility and nitrogen metabolism of beagles

Analysis of the ATTD of DM, CP, EE, ASH, or carbohydrate to probiotics in beagle dogs showed that the probiotic-fed group was similar to the CK group ($p > 0.05$; Table 2). Similarly, there were no significant differences in nitrogen intake, faecal nitrogen, urinary nitrogen, retained nitrogen, net protein utilisation (NPU), or biological value (BV) between the groups supplemented with probiotics and the CK group ($p > 0.05$; Supplementary Table S1).

3.3 Serum antioxidants and immune proteins

Compared to the CK group, the HG group increased the activities of T-AOC, SOD, CAT, sIgA, and IgG ($p < 0.05$; Figures 2A,B,D,I,J) and decreased the levels of MDA and BUN ($p < 0.05$; Figures 2E,H). However, probiotic treatment did not affect the activities of GSH-Px, AST, and ALT ($p > 0.05$; Figures 2C,F,G).

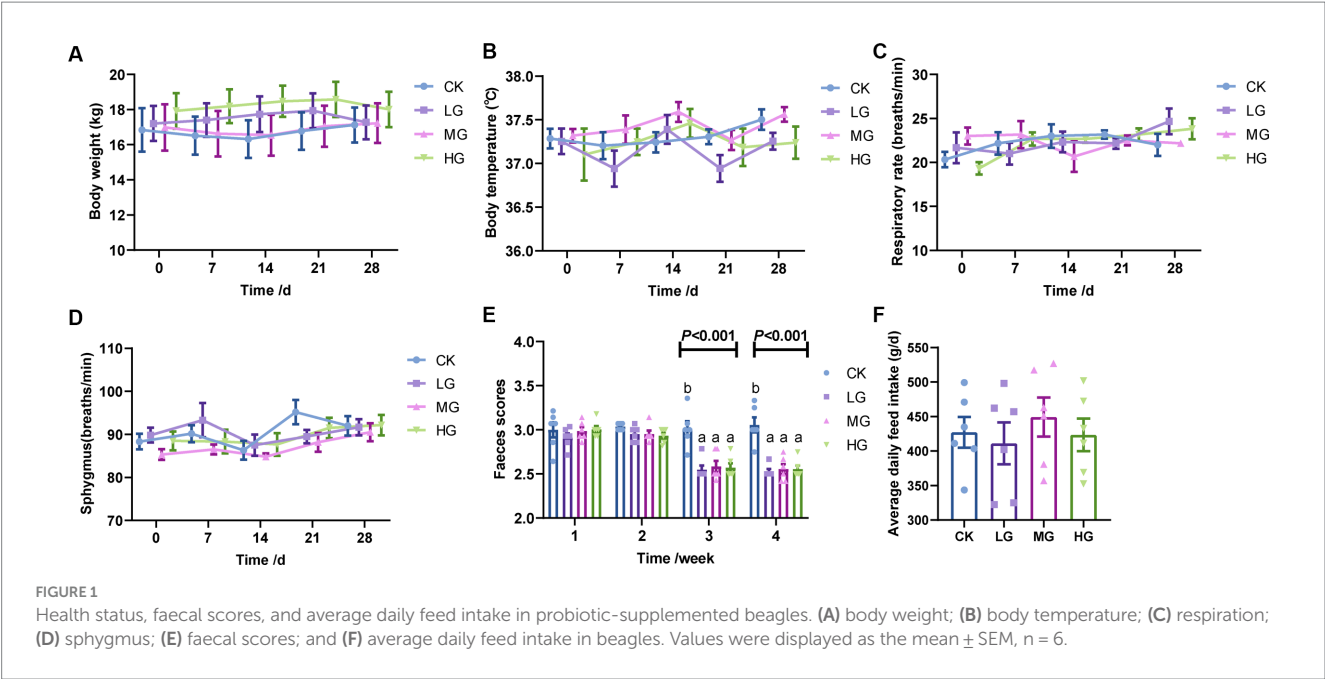


TABLE 2 Effect of supplemented different concentrations of probiotic GLP06 on the ATTD of adult beagles (%).

Items	Dry matter	Crude protein	Ether extract	Crude ash	Carbohydrate
CK	78.46	79.34	94.98	36.09	81.79
LG	79.43	80.82	94.94	38.97	82.39
MG	77.51	77.92	93.61	32.48	81.56
HG	80.63	80.52	95.34	43.67	83.93
SEM	0.72	0.90	0.35	1.90	0.56
p-value	0.48	0.69	0.32	0.20	0.47

Values were displayed as the mean ± SEM, n = 6.

3.4 Short-chain fatty acids

To analyse the effect of *P. acidilactici* GLP06 on the secretion of SCFAs, this study measured the content of SCFAs in faeces. Notably, the acetic acid and butyric acid contents of beagles in the HG group were significantly higher than those in the CK and LG groups ($p < 0.05$; Table 3). Furthermore, the total SCFA content in the HG group was significantly higher compared to the CK group ($p < 0.05$), but there was no significant difference compared to the LG and MG groups ($p > 0.05$). In addition, compared to the CK group, the HG group showed a significant increase in isobutyric acid ($p < 0.05$). There were no significant differences in the contents of propionic acid, 2-methylbutyric acid, isovaleric acid, and total branched-chain fatty acids (BCFAs) of the beagles in four groups ($p > 0.05$).

3.5 Gut microbial diversity and composition

The sequencing coverage of the groups of samples was good, the amount of sequencing data was large enough, the species were more dispersed, and the sampling was more adequate (Supplementary Figure S2). Analysis of α -diversity showed that the

observed species and Shannon indices were considerably higher in the MG and HG groups than in the CK group ($p < 0.05$; Figures 3B,D). The Simpson index was significantly higher in the HG group than in the CK group ($p < 0.05$), but the Simpson index was not significantly different in the MG and LG groups than in the CK group ($p > 0.05$; Figure 3C). However, the Chao index and PD-whole-tree in the LG, MG, and HG groups were similar to those of the CK group ($p > 0.05$; Figures 3A,E). Although there was no significant difference, there seemed to be a trend of improvement in the HG group. In addition, the relative abundance of the four groups at the phylum level is shown in Figure 3F, and the abundance of *Actinobacteria* was markedly increased in the LG and HG groups compared to the CK group ($p < 0.05$; Figure 3G). At the genus level, as shown in Figure 3H, the abundance of *Faecalitalea* and *Collinsella* increased in the LG group compared with the CK group (Figure 3I). The relative abundances of *Tyzzterella* and *Parasutterella* increased in the MG group (Figure 3J). Additionally, *Faecalibacterium*-, *Lachnospiraceae*_NK4A136-, and *Ruminococcaceae*_UCG-005-relative abundances increased in the HG group (Figure 3K; Supplementary Figure S3). Additionally, the principal component analysis showed that PCA1 (35.08%) component MG group differed significantly from the LG group ($p < 0.05$; Figure 3L) and PCA2 (12.05%) component MG and HG groups differed significantly from the CK group ($p < 0.05$). Principal

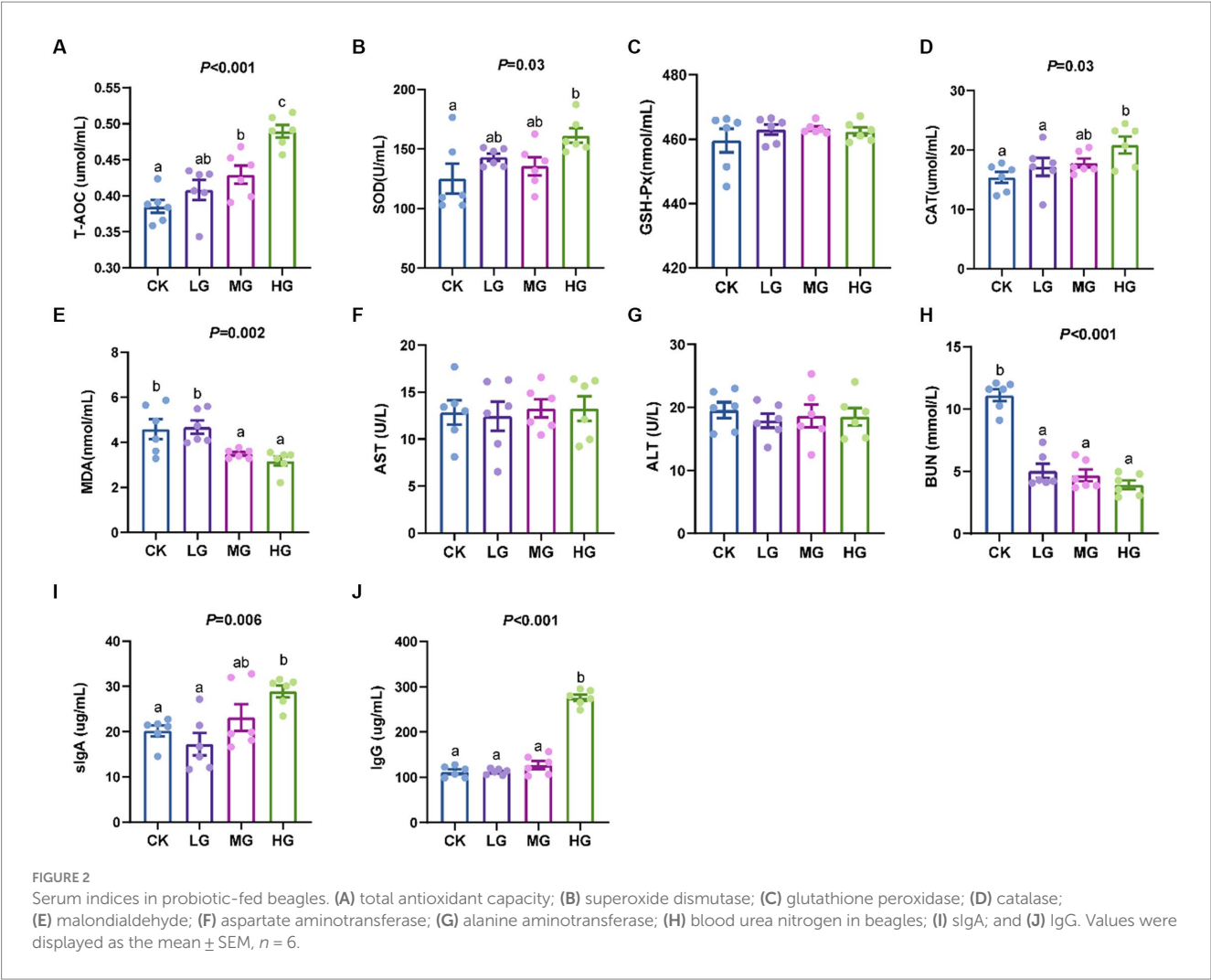


TABLE 3 Effect of supplemented different concentrations of probiotic GLP06 on short-chain fatty acids in adult beagles (mg/g DM faeces).

Items	Acetic acid	Propionic acid	Butyric acid	Total SCFA ¹	Isobutyric acid	2-Methylbutyric acid	Isovaleric acid	Total BCFA ²
CK	6.06 ^a	4.01	0.84 ^a	10.90 ^a	0.40 ^a	0.29	0.48	1.17
LG	7.09 ^a	4.99	0.91 ^a	12.99 ^{ab}	0.43 ^a	0.29	0.58	1.31
MG	7.41 ^a	4.80	1.13 ^{ab}	13.35 ^{ab}	0.53 ^{ab}	0.39	0.72	1.64
HG	9.23 ^b	5.23	1.49 ^b	15.96 ^b	0.70 ^b	0.48	0.75	1.93
SEM	0.37	0.20	0.09	0.62	0.04	0.03	0.05	0.12
P-value	0.012	0.149	0.048	0.026	0.048	0.093	0.212	0.109

^aTotal SCFA content = acetate acid + propionate acid + butyrate acid content.
^bTotal BCFA content = isobutyric acid + 2-methylbutyric acid + isovaleric acid content. Values were displayed as the mean $\pm$ SEM, $n = 6$.

co-ordinates analysis showed that PCoA1 (33.37%) component LG, MG, and HG groups differed significantly compared to the CK group ($p < 0.05$; Figure 3M), and PCoA2 (16.83%) component HG and LG groups of the CK group differed significantly ($p < 0.05$).

3.6 Prediction of metabolomic function

The between-group differences in the PCA score plots show a less pronounced separation (Figure 4A). However, the OPLS-DA model

revealed more significant differences between groups (Figure 4B). In this study, a total of 2,139 metabolites were detected in the HG vs. CK group. Of these, 111 metabolites were upregulated differentially and 171 were downregulated (Figures 4C,E; Supplementary Table S2). The chord diagram illustrates that the metabolites are mainly associated with lipids, lipid-like molecules, organic acids and derivatives, organ heterocyclic compounds, phenylpropanoids and polyketides, and organic oxygen compounds (Figure 4D). Metabolites with similar characteristics were grouped together, and the variation in metabolites between the HG and CK groups is shown in Figure 4E. KEGG

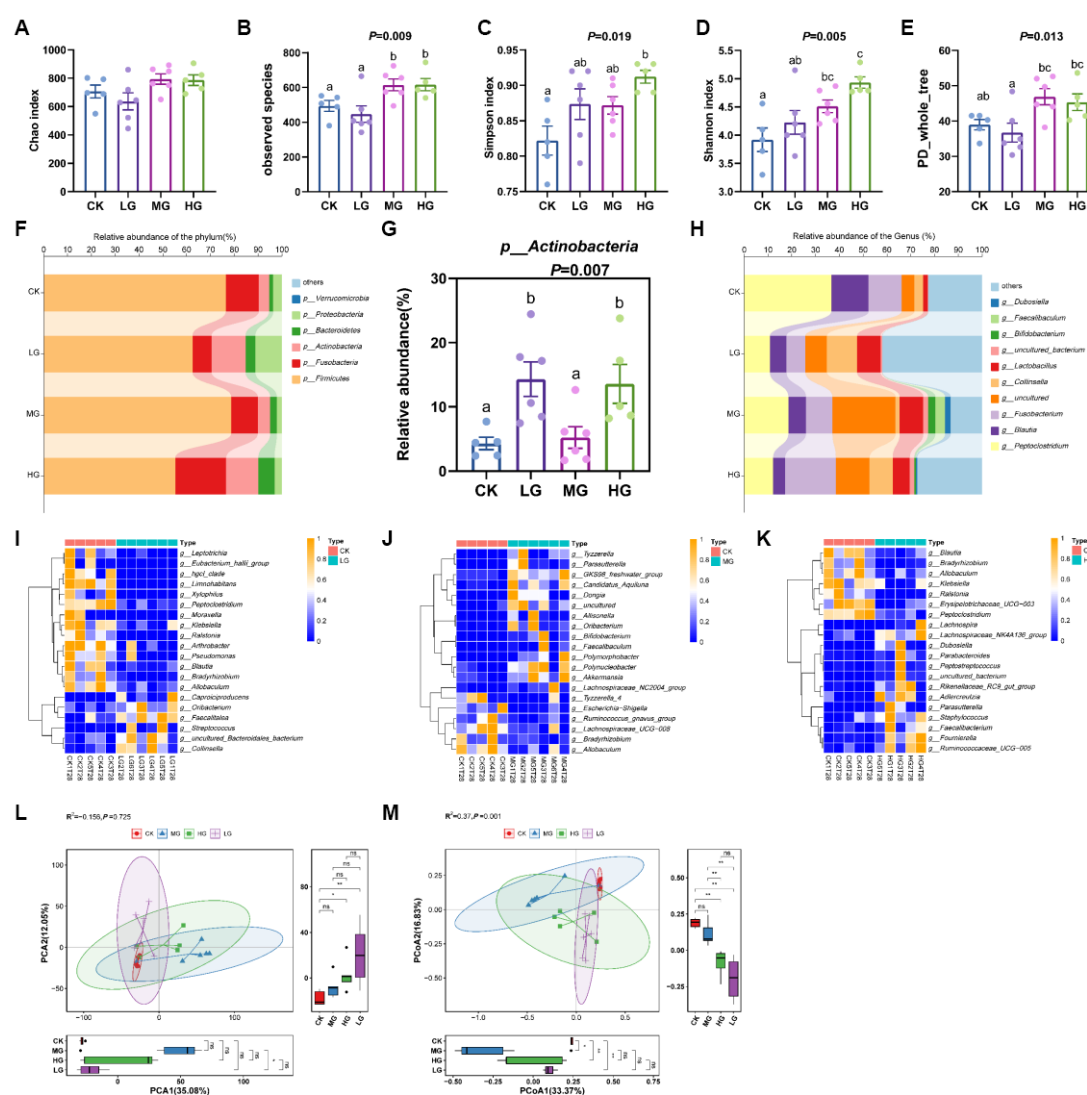


FIGURE 3
Gut microbiota of beagles supplemented with different concentrations of the probiotic GLP06. (A) Chao index; (B) observed species; (C) Simpson; (D) Shannon; (E) PD-whole-tree; (F) relative abundance of the phylum; (G) relative abundance of *p_Actinobacteria*; (H) relative abundance of the genus; (I) genus abundance between CK and LG groups; (J) genus abundance between CK and MG groups; (K) genus abundance between CK and HG groups using the Wilcoxon test; (L) principal component analysis; and (M) PCOA. Values were displayed as the mean $\pm$ SEM, $n = 5$ or $n = 6$.

pathway analyses revealed that probiotic GLP06 mainly influences the serotonergic synapse (prostaglandin B₂, PGB₂, and prostaglandin D₂, PGD₂), retinol metabolism (retinyl ester, RE, and all-*trans*-4-oxoretinoic acid), and phenylalanine metabolism (3-phenylpropionic acid) pathways (Figures 4F,G).

3.7 Correlation between metabolites and microbial genus

The correlation between metabolic differentiators and the microbial genus level showed that PGD₂ was positively correlated with *Fusobacterium* (Figure 5A, $p < 0.05$) and had a negative correlation with *Peptoclostridium* ($p < 0.05$). Additionally, retinyl ester showed negative correlations with *Peptoclostridium* ($p < 0.05$), and PGB₂

displayed negative correlations with *Allobaculum*, *Blautia*, and *Peptoclostridium* ($p < 0.05$ and $p < 0.01$).

The correlation between short-chain fatty acids and the microbial genus level is depicted in Figure 5B. *Collinsella* was positively correlated with BCFAs, 2-methylbutyric acid, and isovaleric acid ($p < 0.05$). *Allobaculum* and *Blautia* were negatively correlated with total SCFAs and acetic acid ($p < 0.05$). Conversely, *Peptoclostridium* was negatively correlated with all SCFAs ($p < 0.05$ and $p < 0.01$).

4 Discussion

Previous studies have demonstrated that *P. acidilactici* can improve host gut microbiota, stimulate various non-specific immunities, and inhibit the growth of pathogenic bacteria in the gut

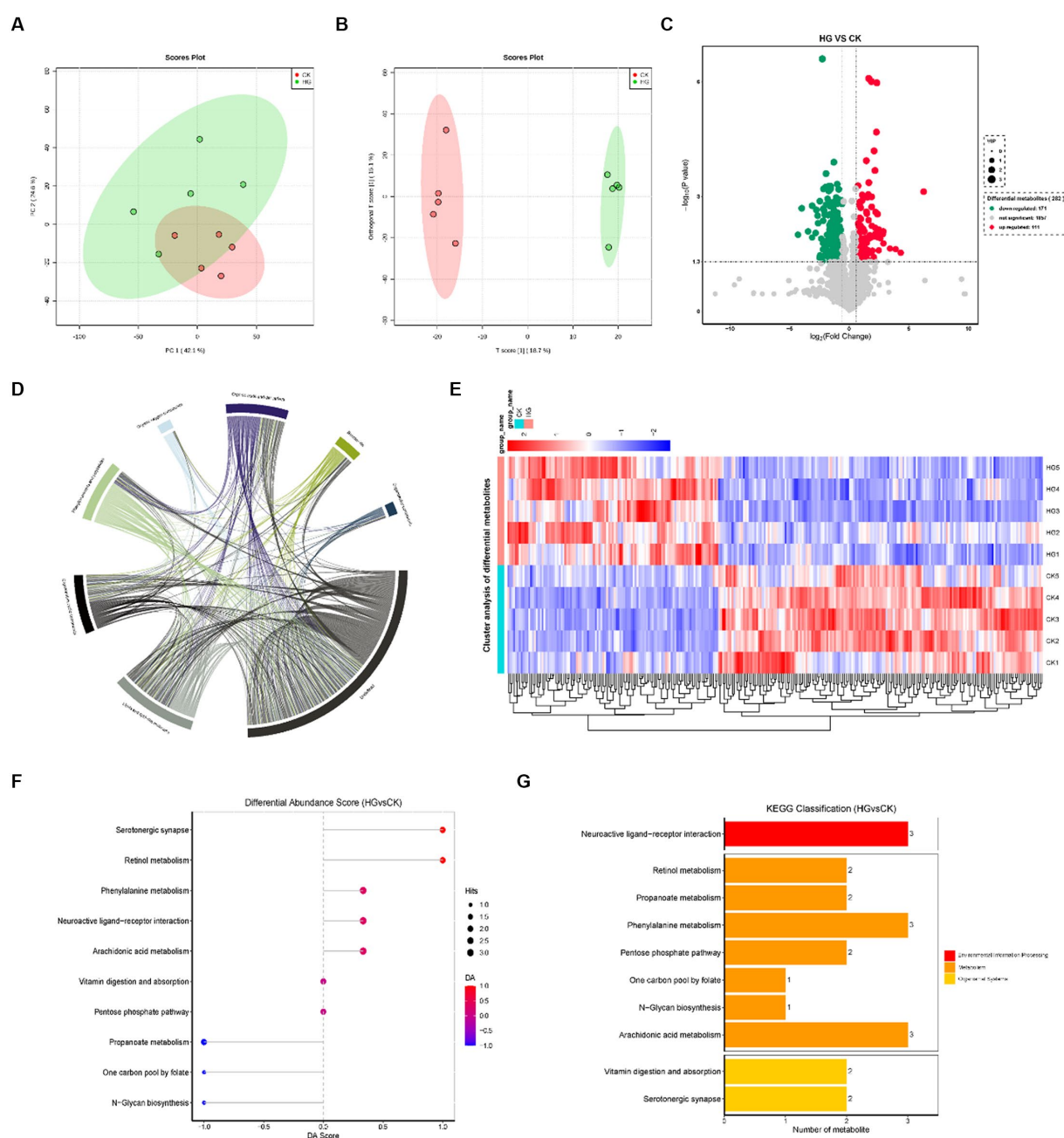


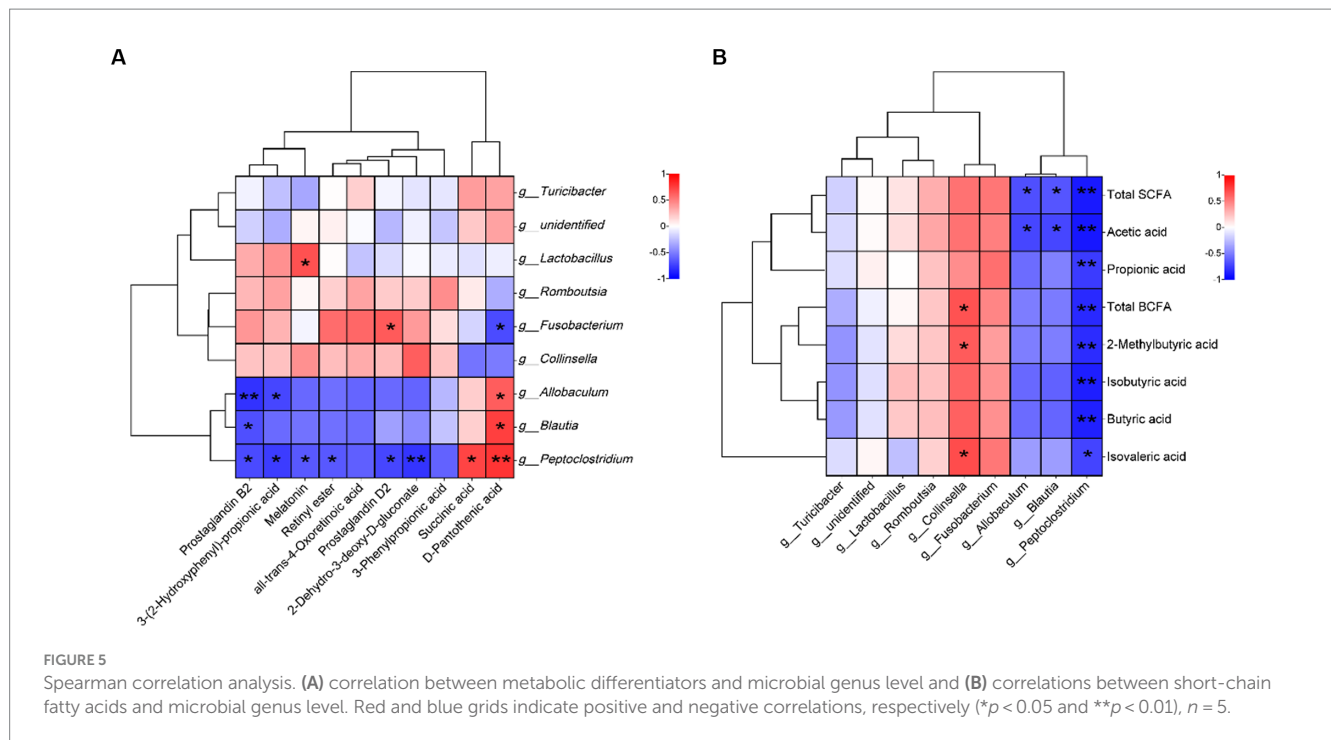
FIGURE 4

Faeces untargeted metabolome in beagles supplemented with different concentrations of probiotic GLP06. (A) principal component analysis; (B) orthogonal partial least squares discrimination analysis; (C) volcano plot; (D) chord diagram; (E) clustering heatmap; (F) differential abundance score of KEGG metabolic pathways; and (G) KEGG metabolic pathway classification histogram, $n = 5$.

intestinal tract (Ferguson et al., 2010; Fernandez et al., 2016; Kim et al., 2018; Zhang et al., 2022). Regarding *P. acidilactici* GLP06, our previous study has shown that canine-derived *P. acidilactici* GLP06 has probiotic potential and antioxidant capacity and is safe and free of drug-resistance genes (Zhao et al., 2023). In this study, neither ATTD nor nitrogen metabolism was significantly altered in beagles fed with *P. acidilactici* GLP06. A previous study found that feeding probiotics did not alter the ATTD of beagles (de Lima et al., 2020). However, previous studies have reported that feeding probiotics GBI-30 (10^9 CFU/mL) had a positive effect on the ATTD of beagles compared

to the CK group. However, this effect was eliminated by decreasing the concentration of the probiotic (Panasevich et al., 2021). This discrepancy could be attributed to differences in probiotic strains and concentrations, diet composition, and nutritional levels, but further trials are needed to verify this. In addition, in the present study, faecal scores were reduced (less moisture, harder) in the probiotic-fed group after 14 days but were within the desirable range (2.5–3.0 on a 5-point scale) both before and after feeding (Lee et al., 2022).

SOD, CAT, and GPX are the first line of antioxidant defence of the organism and play an indispensable role in the overall antioxidant



defence (Ighodaro and Akinloye, 2018). Meanwhile, MDA is a marker for lipid peroxidation and oxidative stress (Del Rio et al., 2005). *P. acidilactici* has been reported to have the ability to increase the antioxidant resistance of the host organism (Hoseinifar et al., 2017). In this study, beagles fed *P. acidilactici* had higher serum levels of T-AOC, SOD, and CAT but lower levels of MDA, confirming that feeding *P. acidilactici* GLP06 improved the resistance to oxidative stress in beagles. The significance of this finding is that it may be essential to alleviate the stress response in pets during transport or environmental changes. Environmental stressors can stimulate cells to produce reactive oxygen species, which disrupt the antioxidant defence system and induce the onset of an inflammatory response (Medzhitov, 2008; Herzog et al., 2014). Liver function (AST and ALT) and kidney function (BUN) indices of beagles were also examined in this study, and the results demonstrated that *P. acidilactici* GLP06 had no adverse effects on liver and kidney function. Another important finding is that oral administration of GLP06 significantly reduced serum levels of BUN in mice, similar to a previous report. Researchers fed *P. acidilactici* NJB421 to mice with ochratoxin A-induced intoxication and found that it alleviated ochratoxin A-induced oxidative stress and liver injury and significantly reduced BUN levels (Tang et al., 2023).

Probiotics modulate the innate and adaptive immune systems of the host, which is crucial for stimulating the production of intestinal antibodies, especially IgA (Yan and Polk, 2011). Symbiotic bacteria provide intestinal immunity by regulating IgA secretion, and IgA deficiency appears to be associated with chronic enteropathy in dogs (Littler et al., 2006; Fagarasan, 2008). IgG is a necessary glycoprotein for protecting against invading pathogens and has anti-inflammatory and immunomodulatory functions (Lux et al., 2010). In this study, feeding *P. acidilactici* GLP06 increased the concentrations of serum IgG and faecal sIgA in beagles. According to these data, we can infer that *P. acidilactici* GLP06 perhaps promotes the immune system's

ability to better fine-tune the microbial balance in the gut of beagles (Rollenske et al., 2021). Previous studies have demonstrated that supplementation with *P. acidilactici* ZPA017 increased IgA and IgG concentrations in weaned piglets (Liu et al., 2020). In addition, feeding a blend of probiotics significantly increased serum IgG and faecal sIgA levels in elderly canines, promoting a shift towards a younger gut microbiota (Xu et al., 2019).

SCFAs are crucial for gut integrity and can regulate metabolic health by modulating gastrointestinal pH, fuelling epithelial cells, and participating in different host signalling mechanisms (Blaak et al., 2020). The fermentation of dietary fibre by the intestinal phylum (*Firmicutes* and *Bacteroidetes*) produces SCFAs, with acetic, propionic, and butyric acids accounting for over 95% of the total, along with BCFAs (isobutyric, 2-methylbutyric, and isovaleric acids, among others), which, although present in low abundance, also have biological effects (Krautkramer et al., 2021). In this study, acetic acid, butyric acid, and isobutyric acid levels were significantly higher in the oral high-dose probiotic group. Butyric acid is a preferred energy source for colonic epithelial cells. It also helps maintain intestinal barrier function and regulates immunity, oxidative stress, and anti-inflammation (Bedford and Gong, 2018; Fu et al., 2019). The metabolites of *P. acidilactici* GLP06 are predominantly acetic acid with only small amounts of butyric acid, and the significant increase in butyric acid may be the enhancement of butyrate production in the gut through cross-feeding of acetic acid with another commensal microbiota (den Besten et al., 2013). However, there is a minimal metabolic exchange between propionate and acetate, which may explain why no significant changes in propionate were observed in this study.

In this study, the primary phyla of the canine gut microbiota were *Firmicutes*, *Fusobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*, which is consistent with previous reports (Hayasaka et al., 2021). Additionally, the α -diversity (observed species, Simpson

and Shannon indices) of the gut microbiota of beagles was significantly higher in the HG group than in the CK group. The observed species and Shannon indices were increased in the MG group. These results indicate that feeding GLP06 is not only safe for beagles but also improves the homeostasis of the gastrointestinal environment. It is reported that dogs with enteritis develop ecological dysregulation, characterised by decreased bacterial diversity and abundance (Minamoto et al., 2015, 2019). Moreover, compared to the CK group, the abundance of *Faecalitalea* and *Collinsella* increased in the LG group, and the relative abundance of *Tyzzellerella* and *Parasutterella* increased in the MG group. *Faecalitalea* and *Collinsella* have been reported as producers of butyrate (Zhou et al., 2021). In this study, an increase in butyric acid content was also observed in the LG group of beagles compared to the CK group. It is reported that *Parasutterella* are all asaccharolytic and producers of succinate (Ju et al., 2019). Succinic acid is one of the key metabolites produced by gut microbes and plays an important role in the cross-feeding of SCFA (Fischbach and Sonnenburg, 2011). This may explain the different levels of acetic acid and butyric acid content in the MG group compared to the control group. Furthermore, the relative abundance of *Actinobacteria*, *Faecalibacterium*, *Lachnospiraceae_NK4A1316*, and *Ruminococcaceae_UCG-005* was significantly higher in the HG group than in the CK group. *Actinobacteria* can produce SCFAs and play a beneficial role in maintaining the intestinal barrier (Binda et al., 2018). *Lachnospiraceae_NK4A1316* is a member of the *Lachnospiraceae* family, *Firmicutes* phylum. This family ferments dietary polysaccharides to produce SCFAs and is negatively correlated with various metabolic and chronic diseases (Truax et al., 2018; Hu et al., 2019). Moreover, *Ruminococcaceae_UCG-005* can maintain intestinal health by metabolising butyrate and other SCFAs (Gu et al., 2022). The bacteria have the metabolic capability to produce SCFAs, which coincides with the increased SCFA content in the HG group of beagles. These results suggest that *P. acidilactici* GLP06 can enhance beagle SCFA content and improve gastrointestinal health by regulating gut microbes.

In this study, significant upregulation of the serotonergic synapse, retinol metabolism, and phenylalanine metabolism pathways was observed in the HG group. Prostaglandins are a class of lipids produced through the enzymatic metabolism of arachidonic acid, including PGB₂ and PGD₂. A previous study reported that PGD₂ increased hydrogen peroxide production and antioxidant enzyme expression in mice (Loupp et al., 2015). Notably, among the altered metabolites, retinyl ester is one of the most abundant forms of retinol in the body, including palmitic, oleic, stearic, and linoleic acid (LA; O'Byrne and Blaner, 2013). It is important to note that LA is known as an essential fatty acid in dogs and plays a vital role in the skin barrier and the prevention of skin diseases (Watson et al., 2018). In addition, retinol is enzymatically activated into retinoic acid (RA) through a two-step oxidation process. It is well known that RA is an active metabolite of vitamin A and essential for immune cell development, differentiation, apoptosis, and function (Larange and Cheroutre, 2016; Erkelens and Mebius, 2017). Another important finding is that 3-phenylpropionic acid was upregulated in the phenylalanine metabolism pathway. A recent study found that *B. fragilis*-derived 3-phenylpropionic acid enhanced the host intestinal epithelial barrier by activating intestinal epithelial AhR signalling (Hu et al., 2023). In this study, the probiotic GLP06 further enhanced the protective effect of the intestinal barrier on the host by targeting and modulating the gut microbiota and increasing the levels of metabolites.

One limitation of the study that should be noted is that we only studied the gastrointestinal environment of healthy beagles, and our findings cannot yet be generalised to puppies or older dogs with gastrointestinal problems or those who are prone to diarrhoea.

5 Conclusion

Despite these limitations, our study demonstrates that supplementing with *P. acidilactici* GLP06 can have a positive impact on faecal scores, serum antioxidant activity, immunoproteins, SCFAs, and gut microbiota in adult beagles, leading to improved gut homeostasis. These findings underscore the potential of *P. acidilactici* GLP06 as a dietary intervention to enhance gut health in companion animals. In the future, further research is needed to clarify the underlying mechanisms and optimise probiotic formulations for specific gastrointestinal conditions in more vulnerable populations, such as puppies or senior dogs.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary material](#).

Ethics statement

The animal study was approved by the Laboratory Animal Ethics Committee of Qingdao Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MZ: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. YZ: Data curation, Software, Visualization, Writing – original draft. YL: Data curation, Software, Visualization, Writing – original draft. KL: Formal analysis, Methodology, Writing – review & editing. KB: Formal analysis, Methodology, Writing – review & editing. GL: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1369402/full#supplementary-material>

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EDITED BY

Xiaolun Sun,
University of Arkansas, United States

REVIEWED BY

Li Zhang,
Mississippi State University, United States
Rishi Drolia,
Old Dominion University, United States

*CORRESPONDENCE

Jinping Deng
✉ dengjinpj@scau.edu.cn
Baichuan Deng
✉ dengbaichuan@scau.edu.cn
Sufang Han
✉ sfhan@scau.edu.cn

[†]These authors have contributed equally to this work

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Protective effect of chicken yolk antibody Y against *Campylobacter jejuni* induced diarrhea in cats

Ziyang Li[†], Jiayi Yan[†], Zhaowei Bian, Jie Zhang, Yuwen Liu, Jinping Deng*, Baichuan Deng* and Sufang Han*

Guangdong Provincial Key Laboratory of Animal Nutrition Control, College of Animal Science, South China Agricultural University, Guangzhou, China

Campylobacter jejuni (*C. jejuni*) is a common pathogen that often causes diarrhea, loss of appetite, and even enteritis in domestic cats, affecting their growth and development, especially in kittens under 6 months of age. Oral passive immunization with chicken yolk antibody Y has been proved effective for the treatment of gastrointestinal pathogen infections due to its high specificity. In this study, *C. jejuni* was isolated from diarrheal cat feces, and the specific egg yolk antibody Y against *C. jejuni* was demonstrated to effectively inhibit its proliferation *in vitro* experiments. To evaluate the effect of anti-*C. jejuni* IgY, the mouse *C. jejuni* infection model was established and it was found that IgY could alleviate *C. jejuni*-induced clinical symptoms. Consistent with these results, the reduction of pro-inflammatory factors and intestinal colonization by *C. jejuni* in the IgY-treated groups, especially in the high dose group. We then evaluated the protective effect of IgY on young Ragdoll cats infected with *C. jejuni*. This specific antibody reduced the rate of feline diarrhea, protected the growth of young cats, inhibited systemic inflammatory hyperactivation, and increased fecal short-chain fatty acid concentrations. Notably, IgY may have a protective role by changing intestinal amino acid metabolism and affecting *C. jejuni* chemotaxis. Collectively, specific IgY is a promising therapeutic strategy for *C. jejuni*-induced cat diarrhea.

KEYWORDS

Campylobacter jejuni, IgY, inflammation, metabolism, cats

1 Introduction

Pets provide physical and mental health benefits, emotional support, and increased social interaction for their owners (McNicholas et al., 2005). In urban areas of China, the pet market has been steadily growing in recent years, with more than 100 million cats and dogs. By 2022, the number of pet cats has surpassed the number of pet dogs.¹

Bacterial infections can cause diarrhea, especially in multi-cat households (German et al., 2015). Diarrhea can interfere with the intestinal absorption of nutrients, and recurrent

¹ <https://www.36kr.com/p/2155618644196871>

episodes can lead to developmental delays in young animals (Stern et al., 1980). At the same time, diarrhea facilitates the spread of enteric pathogens into the environment, which can infect new hosts, and host transmission can increase the virulence of certain bacteria (Wiles et al., 2005; Butler et al., 2006). *Campylobacter jejuni* (*C. jejuni*) is a potential threat to diarrhea in kittens. Many researchers have found a high prevalence of *C. jejuni* in cats (Gruffyddjones, 1980; Acke et al., 2009; Bojanić et al., 2017; Torkan et al., 2018). Infection with *C. jejuni* can lead to chronic diarrhea and even enteritis in cats (Fleming, 1983). Although the infected cats may not show serious clinical signs, they can still act as a reservoir of *C. jejuni* and infect other cats and humans.

However, there are no very effective strategies for dealing with *C. jejuni* infections other than antibiotics. By collecting fecal samples from pets to isolate *C. jejuni* and perform drug susceptibility testing, it has been discovered that pets harbor *C. jejuni* strains that possess resistance genes for several antibiotics, including gentamicin, kanamycin, streptomycin, and tetracycline (Montgomery et al., 2018; Joseph et al., 2020; Moser et al., 2020; Francois Watkins et al., 2021). The unregulated use of antibiotics can contribute to the development of superbugs that can be transmitted from pets to their owners, causing more difficult-to-treat infections and threatening human health. Therefore, it is important to explore new treatment options for human and pet health.

Passive immunity refers to the specific immunity acquired by the body passively receiving antibodies, sensitizing lymphocytes or their products (Brambell, 2010; Kovacs-Nolan and Mine, 2012). It is a more attractive approach because of its rapid delivery and immediate protection. Eggs are a rich source of nutrients and contain large amounts of antibodies. More importantly, chickens are natural hosts for *C. jejuni*. It is noteworthy that *C. jejuni* is not easily detectable in chicks up to 3 weeks of age, which may be related to antibodies in the egg yolk (Kim et al., 2000). This protective effect probably is likely a result of the passive immune effect of the antibodies provided by the hen through the egg yolk. Three types of antibodies are found in chickens: IgY, IgA, and IgM. During yolk formation, IgY is transferred from serum to yolk, providing passive immunity to chicks (Gerrie et al., 1973; Kovacs-Nolan and Mine, 2012). Since its discovery, IgY has been widely used in diagnostics (Grzywa et al., 2013; Bentes et al., 2015; Jinxin He et al., 2015) and food safety testing (Li et al., 2017; Dou et al., 2022). One of its most valuable and promising areas is its use in passive immunization for the prevention and treatment of human and animal diseases (Pereira et al., 2019; Grzywa et al., 2023). IgY is highly effective against pathogenic infections such as *Salmonella* (Li et al., 2016), *E. coli* (Ma et al., 2020), and *Norovirus* (Dai et al., 2013). In addition, IgY is advantageous due to its high yield (Wang et al., 2023), short preparation cycle, high affinity, and relatively humane preparation process (Karthikeyan et al., 2022). However, there have been few studies investigating the therapeutic and prophylactic effects of IgY in cats infected with *C. jejuni*.

In this study, we isolated a strain of *C. jejuni* from the feces of cats with diarrhea, inactivated it as an antigen, and immunized hens to obtain specific antibodies. We established the mouse *C. jejuni*-induced diarrhea model and found the specific IgY could alleviate *C. jejuni*-induced clinical symptoms. And we demonstrated its protective effect on young Ragdoll cats with *C. jejuni*-induced diarrhea. These findings suggest that the specific IgY is a promising therapeutic strategy for *C. jejuni*-induced diarrhea in cats.

2 Materials and methods

2.1 Preparation of IgY

Fecal samples were collected from cases of feline diarrhea and used to isolate a strain of *C. jejuni*, which was then identified by biochemical assays and 16S ribosomal DNA sequencing. Bacteria were cultured on Columbia blood agar containing 5% sterile sheep blood and maintained at 42°C under microaerophilic conditions. Anti-*C. jejuni* IgY was produced and purified by Carmel Biotechnology Co Ltd. (Tianjin, China) following the methods previously described by Thibodeau et al. (2017).

2.2 Growth inhibition test

The *C. jejuni* (1.0×10^5 CFU/mL) was grown in Mueller-Hinton Broth including a range of density (2.5, 5, and 10 mg/mL) of sterilized anti-*C. jejuni* IgY. Nonspecific IgY (10 mg/mL) (Beyotime Biotechnology Co., Ltd., Shanghai) was performed as the negative control. These mixtures were incubated under microaerobic conditions. Absorbance values were read at 600 nm every 2 h with three replicates. The growth inhibition curve was generated by plotting OD₆₀₀ against time.

2.3 Mice and cats infection model

2.3.1 Mice infection model

Thirty-two pathogen-free male C57BL/6J mice (3 weeks of age, 10.38 ± 0.28 g) were acquired from Spearfish (Beijing) Biotechnology Co. Mice were housed in a controlled room with the same humidity and temperature with a 12 h light–dark cycle. All experimental procedures were authorized by the Animal Care and Use Committee before animal experiments and were performed according to the guidelines of the Laboratory Animal Center of South China Agricultural University (Approval number: 2022F233).

Weaned 3 weeks-old C57BL/6J were acclimatized on a standard diet for 3 days. The *C. jejuni* infection model was established according to Natasa Giallourou et al. (Baumler et al., 2018). Briefly, before infection, four antibiotics were added to the drinking water to scavenge the intestinal flora and overcome the colonization resistance, and were also fed zinc-deficient diets (zinc content less than or equal to 1 ppm) for 14 days, which were purchased from Nantong Trofi Feeds Science and Technology Co Ltd. (Jiangsu). Mice were given antibiotic-free water and fasted for 4 h the day before infection. Each infected mouse was inoculated with approximately 1×10^9 CFU *C. jejuni* by oral gavage of 100 µL of freshly prepared broth for 3 consecutive days. After the challenge, all mice were randomly divided into four groups according to receive different treatments for 5 days: the control (CON) group received 100 µL of sterile PBS buffer by gavage ($n=8$), high-dose (HD) group received 10 mg/mL specific IgY ($n=8$), middle-dose group (MD) received 5 mg/mL specific IgY ($n=8$), and low-dose (LD) group received 2.5 mg/mL specific IgY ($n=8$). We applied a standardized clinical scoring system to the mice to evaluate the appearance of the mice and the consistency of the feces maximum 12 points, addressing the occurrence of blood in feces (0 points: no blood; 2 points: microscopic detection of blood; 4 points: overt blood visible), diarrhea (0: formed feces; 2: pasty feces; 4: liquid feces), and the clinical aspect (0: normal; 2: ruffled fur, less locomotion; 4: isolation, severely compromised locomotion, pre-final aspect) (Heimesaat et al., 2014).

2.3.2 Cats infection model

Twelve Ragdoll cats, half male and half female (4 months old, 3.475 ± 0.16 kg), were purchased from the Ramical Dogs and Cats Scientific Experimental Base (Guangzhou). Cats were housed in a negative pressure laboratory at a temperature of 26°C. Each cat was housed individually in a cage (108 cm*70 cm*76 cm) to avoid cross-infection and was cleaned daily to maintain cleanliness. All cats were vaccinated and dewormed one month prior to the experiment. No antibiotics or other medications were administered that might interfere with the results. During the acclimatization period, the cats had access to fresh food and drink clean water. All procedures were authorized by the Animal Care and Use Committee and were carried out according to the guidelines of the Laboratory Animal Centre of South China Agricultural University (Approval number: 2021a030).

After a 7 days acclimatization period, 4 months-old Ragdoll cats were divided into two groups according to sex and weight. The presence of *C. jejuni* was excluded before the experiment by collecting fresh fecal samples from the Ragdoll cats and extracting total bacterial DNA. Commercial cat food and filtered water were provided during the experiment. Fasting was enforced for 12 h before the infection. Each cat participant received an oral instillation of 1 mL of freshly prepared bacterial solution containing approximately 1×10^{10} CFU *C. jejuni* for 5 consecutive days. Subsequently, cats in the C.j group were orally instilled with 1 mL of sterile PBS daily, and cats in the C.j + IgY group received a daily instilled of 1 mL of 25 mg/mL specific IgY for 7 consecutive days. The dose of specific IgY for administration to Ragdoll cats was determined based on dose conversion methods for different animals (Saadh et al., 2020).

The fecal samples were scored on a 1 to 5 scale (1-liquid diarrhea to 5-dry hard pellets) (Carciofi et al., 2008). A score of 3.5 to 4.0 was considered ideal.

2.4 Blood collection and analysis

Blood was collected on days 0 (end of acclimation period), 5 (after modeling), and 13 (end of experiment). Cats were fasted overnight and 3 mL of blood was collected from each cat, allowed to stand for 30 min, centrifuged, and the supernatant transferred to a centrifuge tube and stored at -80°C for further analysis.

Serum albumin (ALB), total protein (TP), globulin (GLOB), and albumin/globulin ratios (A/G) were measured using a commercial kit on an automated blood biochemistry analyzer (Chemray 800, Shenzhen Redu Life Technology, Shenzhen, China). Myeloperoxidase (MPO), procalcitonin (PCT), C-reactive protein (CRP), interleukin-6 (IL-6), interferon- γ (IFN- γ), tumor necrosis factor (TNF- α) and interleukin-1 β (IL-1 β) were assessed using a commercial feline ELISA kits (MEIMIAN, Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China). Nitric oxide (NO) concentration was measured by the commercial kits (Solarbio, Solarbio Biotechnology Co., Ltd., Beijing, China).

2.5 Collection and analysis of fresh feces and cecal contents

2.5.1 Short chain fatty acids

At the end of the experiment, fresh feces from Ragdoll cats and cecal contents from mice were collected to extract SCFAs, which were quantified by gas chromatography–mass spectrometry (GC-MS;

Shimadzu, Tokyo, Japan) according to the method described in our previous work (Kang Yang and Jian, 2021).

2.5.2 Relative loads of *C. jejuni* in cecum contents

The relative load of *C. jejuni* in the samples was determined using qPCR by extracting total bacterial DNA from mouse cecal contents and the bacterial universal primer was used as an endogenous reference. The primers are listed in Table 1.

2.6 Intestinal inflammatory factors

The relative mRNA expression of IL-6 and IL-1 β in mouse jejunum and colon was examined by qPCR. The relative expression of genes was determined by $2^{-\Delta\Delta C_t}$ and the β -actin gene was used as an endogenous reference. Primers are listed in Table 1. Metalloproteinase-9 (MMP-9) was analyzed in mouse cecum using ELISA kits (MEIMIAN, Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China).

2.7 Fecal untargeted metabolomics analysis

Pretreatment of fresh fecal samples from cats as described in our previous work (Jian et al., 2022). The Thermo Fisher Scientific UPLC-Orbitrap-MS/MS system (Q-Exactive Focus, United States) was used for untargeted metabolomic analysis of fecal samples. Raw data were processed using Compound Discoverer 3.3 software by comparing the mzCloud and mzValut online databases to identify metabolites. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was performed using SIMCA-P14.1 software. KEGG pathway analysis of differential metabolites was performed using MetaboAnalyst 5.0.

2.8 Statistical analysis

All data were analyzed using SPSS 26.0 and graphs were generated using GraphPad Prism 8.0 software. *p* values were determined by unpaired Student's *t*-test. Significant differences and tendencies were indicated by $p < 0.05$ and $p < 0.10$, respectively. The OPLS-DA model was used to calculate variable importance in the projection (VIP) values. Metabolites with $\text{VIP} > 1$ and $p < 0.05$ were considered differential metabolites. These differential metabolites were functionally annotated using the KEGG database and further mapped to the KEGG pathway database using MetaboAnalyst 5.0.

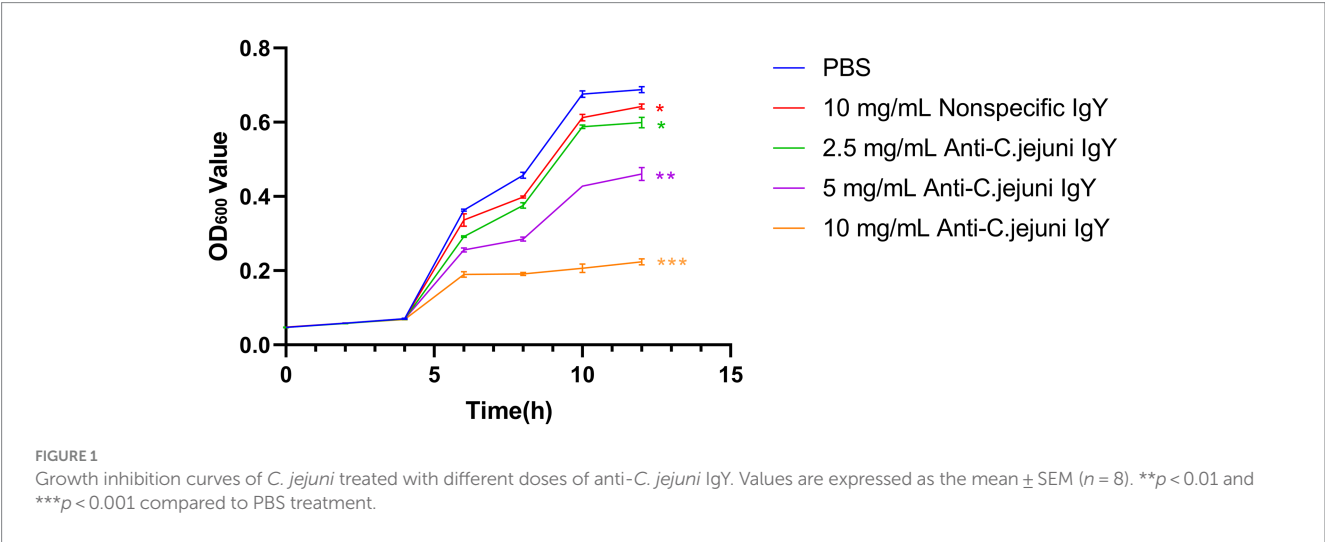
3 Results

3.1 Specific IgY inhibited the proliferation of *C. jejuni*

To assess the bacteriostatic activity of specific IgY *in vitro*, growth inhibition curves were plotted (Figure 1). The results showed that the anti-*C. jejuni* IgY suppressed the growth of *C. jejuni* in a dose-dependent manner, with the inhibitory effect increasing as the concentrations of IgY increased.

TABLE 1 Primer sequences for qPCR.

Gene name	Forward sequence	Reverse sequence
IL-6	ATAGTCCTTCCTACCCCAATTTC	CTGACCACAGTGAGGAATGTCCAC
IL-1 β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
β -actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTC
<i>C.jejuni</i>	TCCATCATATCTTGGGCGCT	AATTTGCTTTGAAAGCATTT
Universal bacterial	GTGSTGCAYGGYYGTCGTCA	ACGTCRTCCMCNCTTCCTC



3.2 Protective effects of IgY against *C. jejuni* infection in mice

3.2.1 IgY alleviated *C. jejuni*-induced clinical symptoms

To investigate whether oral administration of anti-*C. jejuni* IgY could alleviate the negative effects of *C. jejuni* on mice, we recorded the changes in body weight of mice on day 0 (before modeling), day 3 (after modeling), and day 8 (end of the experiment) (Figure 2A). After 3 days of gavage, the mice lost an average of 7% of their body weight. On day 8, the mice in the IgY-treated groups stopped losing body weight, while the mice in the CON group continued to lose weight. The magnitude of body weight loss in the CON group was significantly higher than that in the IgY-treated group (Figure 2B).

The standardized clinical scoring system was used to evaluate the clinical scores of the mice. On day 8, the IgY-treated groups showed significantly lower clinical scores compared to day 3 and the CON group (Figure 2C). In addition, the feces of mice treated with anti-*C. jejuni* IgY were well-formed by the 8th day (Figure 2D). These results showed that specific IgY could effectively improve the clinical symptoms caused by *C. jejuni*, with the most significant effect observed in the high-dose group.

3.2.2 IgY relieved inflammation of the jejunum and colon

To investigate the mitigating effect of IgY on *C. jejuni*-induced intestinal inflammation, we assessed the expression of intestinal inflammatory cytokine mRNAs, the NO content of colonic tissues, and the concentration of inflammatory markers in cecal contents. The relative

mRNA expression of the proinflammatory factor IL-6 in the colon of mice was significantly lower in the HD and MD groups than in the control group, and there was a trend towards lower colonic IL-6 mRNA expression in mice in the HD group compared with mice in the LD group (Figure 3A). And compared with the control group, the relative expression of IL-1 β mRNA was significantly lower in the HD group and also tended to be lower in the MD group, while the relative expression of IL-1 β mRNA in the colon of mice in both the HD and MD groups was highly significantly lower than that in the LD group (Figure 3B). Quantification of the relative expression of jejunal IL-6 mRNA showed similar results: the relative expression of jejunal IL-6 was significantly lower in both the HD and MD groups than in the control group, and the expression of jejunal IL-6 was significantly lower in the HD group than in the LD group (Figure 3C). For the relative expression of jejunal IL-1 β mRNA, it was significantly lower in the HD and MD groups than in the control group, and there was also a trend toward a decrease in the LD group (Figure 3D). In addition, we quantified MMP-9 in cecal contents and NO in colonic tissue and found that specific IgY treatment reduced both MMP-9 and NO levels compared to controls (Figures 3E,F). Thus, IgY treatment groups, especially high-dose IgY treatment, attenuated pro-inflammatory mediator responses in the intestines of mice infected with *C. jejuni*.

3.2.3 IgY inhibited *C. jejuni* colonization and increased SCFAs concentration

To determine whether the protective effect of IgY in mice was due to the rate of bacterial clearance, the relative number of *C. jejuni* in the cecal contents of mice at the end of the experiment was measured. The number of *C. jejuni* in the cecal contents of mice in the HD and MD groups was

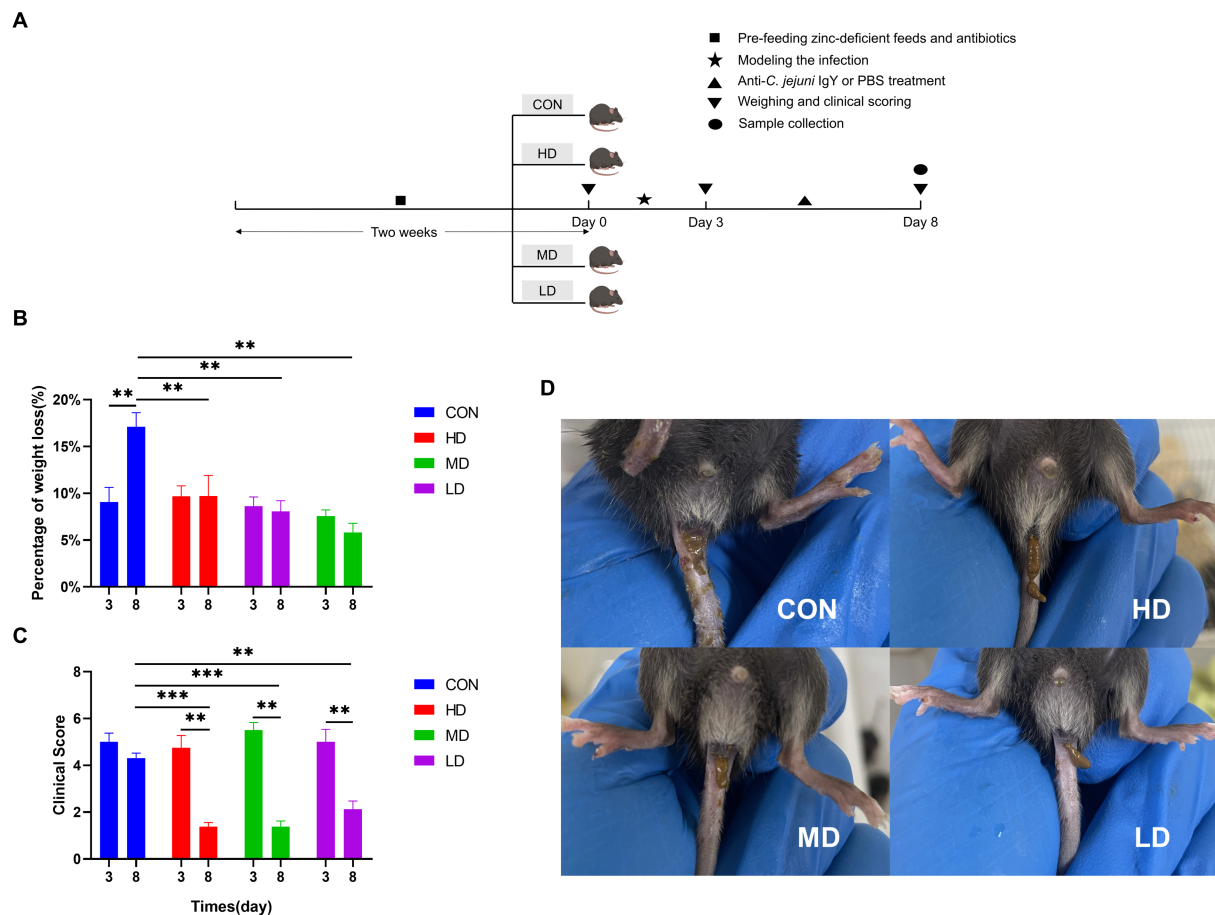


FIGURE 2

IgY blocks weight loss and improves clinical performance in mice. (A) Timeline of the mouse experiments. (B) Mouse body weights were recorded on day 0, day 3, and day 8. (C) Scoring of mice using a standardized clinical scoring system. (D) Images of mouse feces on day 8. Data are presented as mean $\pm$ SEM ($n = 8$), * $p < 0.05$ and ** $p < 0.01$, and the symbol (#) represents difference tendency ($p < 0.1$).

significantly lower than that in the control group, and there was also a trend towards less in the LD group. Furthermore, the relative number of *C. jejuni* in mouse cecal contents was reduced in a dose-dependently manner in the IgY-treated groups (Figure 4A).

In terms of SCFAs, the content of acetic acid in the cecal contents of IgY-treated mice was significantly higher than in the control group and was highest in the HD group (Figure 4B). The cecal contents of the HD group also had the highest levels of butyric acid, significantly higher than those of the control and LD groups. Additionally, there was also a tendency towards increased butyric acid content in the HD group compared to the MD group (Figure 4C). Propionic acid and valeric acid were not detected. These results suggest that high doses of anti-*C. jejuni* IgY improved the efficiency of bacterial clearance and positively influenced the production of microbial metabolites.

3.3 Protective effect of IgY against *C. jejuni* infection in ragdoll cats

3.3.1 IgY improved the clinical presentation in ragdoll cats

To investigate whether oral administration of anti-*C. jejuni* IgY mitigates the negative effects of *C. jejuni* on Ragdoll cats, we recorded

body weights and applied a standardized fecal score system at various stages of the experimental process (Figure 5A). Throughout the experiment, Ragdoll cats in the C.j group consistently experienced weight loss, resulting in a significantly lower final body weight in comparison to their initial weight. In contrast, the Ragdoll cats in the C.j + IgY group showed a decrease after modeling (on day 5), and returned to the initial level at the end of the experiment (on day 12), which was significantly higher than that of the C.j group (Figure 5B). Regarding fecal scores, both groups of Ragdoll cats experienced vomiting and diarrhea on day 5. At the end of the experiment, Ragdoll cats in the IgY group exhibited well-formed stools, whereas the cats in the C.j group continued to experience soft stools and diarrhea (Figure 5C).

3.3.2 Effect of IgY on serum biochemical and inflammation-related parameters

To assess the effect of IgY on liver function in *C. jejuni*-infected Ragdoll cats, the total protein, albumin, globulin, and the AL/GI ratio in serum were determined. These parameters are indicative of liver function and immune response. There was no significant difference in serum albumin levels between the two groups (Figure 6A), while serum total protein (Figure 6B) and globulin (Figure 6C) were significantly higher in the C.j group than in the C.j + IgY group. The

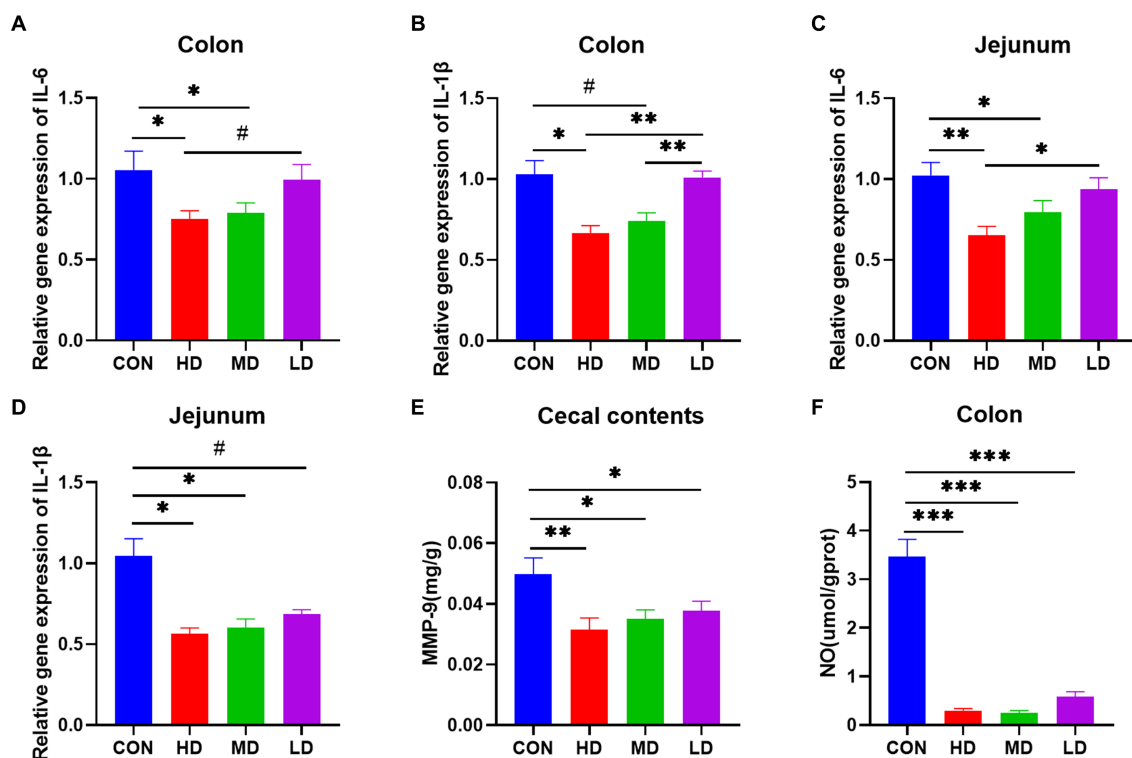


FIGURE 3

Relative mRNA expression of inflammatory genes, inflammatory markers in cecal contents, and colonic NO are decreased in the colon and jejunum of IgY-treated mice. (A) IL-6 mRNA expression in the colon. (B) IL-1β mRNA expression in the colon. (C) IL-6 mRNA expression in the jejunum. (D) IL-1β mRNA expression in the jejunum. (E) MMP-9 content of cecal contents. (F) Nitric oxide content in colon tissues. Data are presented as mean ± SEM (n = 8), * $p < 0.05$ and ** $p < 0.01$, and the symbol (#) represents difference tendency ($p < 0.1$).

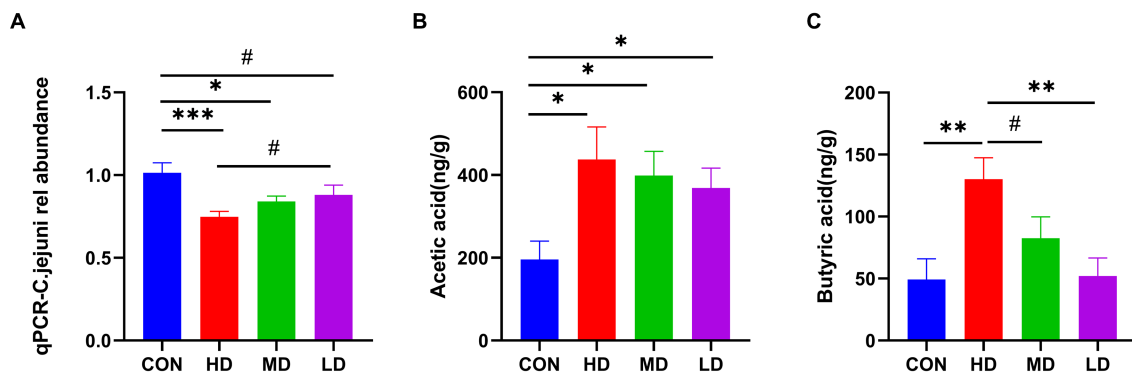


FIGURE 4

IgY reduces *C. jejuni* load and increases the fatty acid concentration in mouse cecal contents. (A) Relative load of *C. jejuni*. (B) The levels of acetic acid. (C) Butyric acid in mice cecal contents. Data are presented as mean ± SEM (n = 8), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, and the symbol (#) represents difference tendency ($p < 0.1$).

serum AI/GI ratio was significantly lower in the C. j group than in the C. j + IgY group (Figure 6D).

C. jejuni causes hyper-immunity and elicits a systemic inflammatory response. We then investigated the effect of anti-*C. jejuni* IgY on systemic inflammation in cats. The fecal marker of inflammation, MPO, was significantly reduced after specific IgY treatment (Figure 7A). Serum levels of PCT and CRP, two markers of bacterial infection, were significantly lower in cats in the C.j + IgY

group (Figures 7B,C). In addition, the pro-inflammatory cytokine IL-6 (Figure 7D) was significantly lower in the serum of cats in the C.j + IgY group than in the C.j group, and IFN-γ (Figure 7E) also tended to be lower. This was accompanied by lower levels of TNF-α and IL-1β (Figures 7F,G). It was also observed that serum NO levels were significantly decreased after specific IgY treatment (Figure 7H). These results suggest that IgY treatment can attenuate the systemic inflammatory response.

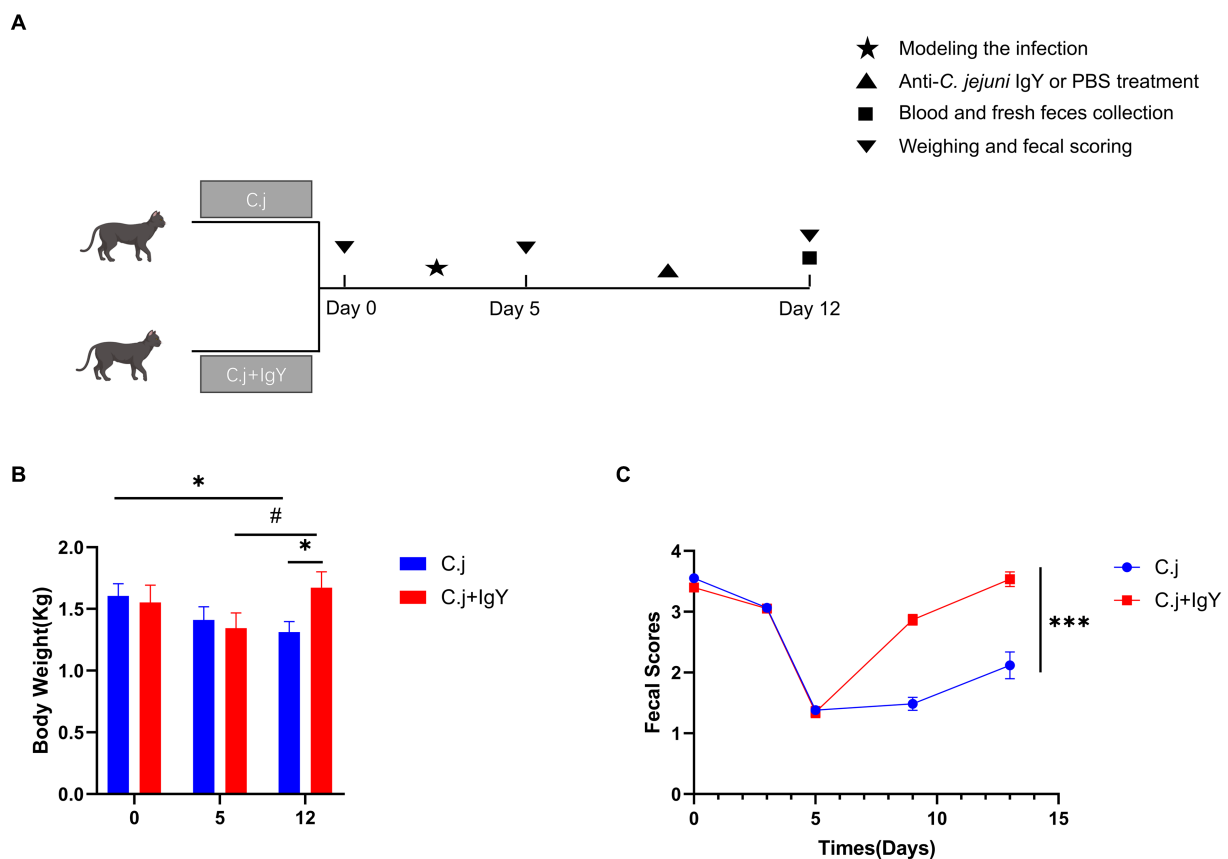


FIGURE 5

Effects of IgY on clinical signs in *C. jejuni*-infected cats. (A) Timeline and time points for collecting cat samples. Changes in (B) body weight and (C) fecal scores of cats in the C. j group and the C. j + IgY group. Fecal samples were scored on a scale of 1 to 5 (1 – liquid diarrhea to 5 – dry hard pellets). A score of 3.5 to 4.0 was considered ideal. Data are presented as mean $\pm$ SEM ($n = 6$), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, and the symbol (#) represents difference tendency ($p < 0.1$).

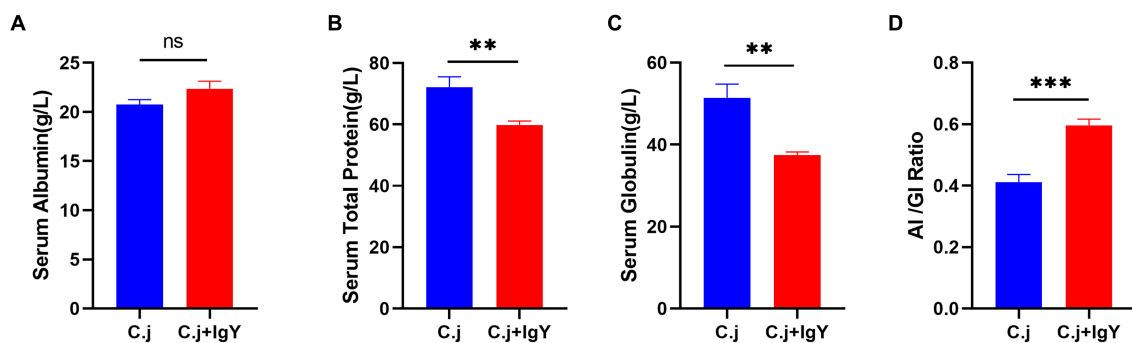


FIGURE 6

Effect of anti-*C. jejuni* IgY on serum biochemical parameters in cats. The levels of (A) Serum albumin, (B) Serum total protein, (C) Serum globulin, and (D) AI/GI Ratio (Albumin to globulin ratio). Data are presented as mean $\pm$ SEM ($n = 6$), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, ns, not significant, and the symbol (#) represents the difference tendency ($p < 0.1$).

3.3.3 IgY increased SCFAs levels in ragdoll cats

SCFAs are an important source of energy for colonic epithelial cells. Measurement of the content of SCFAs in the fresh feces of Ragdoll cats showed that the levels of acetic acid (Figure 8A), propionic acid (Figure 8B), and butyric acid (Figure 8C) were all significantly higher in the C. j + IgY group. The level of valeric acid (Figure 8D) also tended to be elevated.

3.3.4 Effect of anti-*C. jejuni* IgY on the untargeted metabolomics of cat feces

The OPLS-DA score plots showed the separation between the C. j and C. j + IgY groups indicating significant differences in fecal metabolites between the two groups (Figure 9A). Further analysis identified 233 differential metabolites, of which 202 were

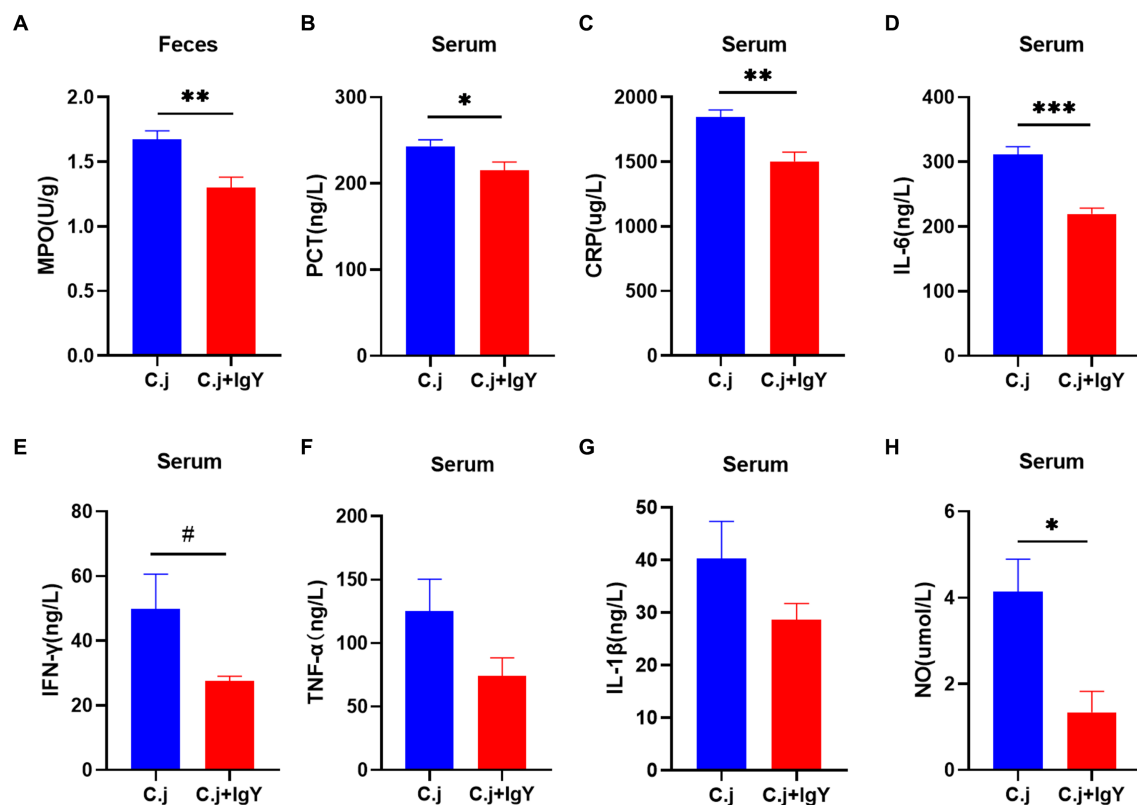


FIGURE 7

Anti-*C. jejuni* IgY attenuated inflammation caused by *C. jejuni* infection. The levels of (A) MPO, (B) PCT, (C) CRP, (D) IL-6, (E) IFN-γ, (F) TNF-α, (G) IL-1β, and (H) NO in serum. Data are presented as mean ± SEM ($n = 6$), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, and the symbol (#) represents difference tendency ($p < 0.1$).

upregulated and 31 were downregulated (Figure 9B). The KEGG enrichment analysis revealed that the differential metabolites were primarily enriched in arginine and proline metabolism, alanine, aspartate and glutamate metabolism, and bacterial chemotaxis (Figure 9C). These amino acids are essential for the metabolism of *C. jejuni*, and bacterial chemotaxis is associated with its colonization and proliferation.

4 Discussion

Domestic cats are important hosts for *C. jejuni*, which can lead to subclinical infections and even the development of enteritis (Acke, 2018). After infection, *C. jejuni* rapidly colonizes the intestine, translocates through the intestinal mucus, and can invade tissues, causing inflammation (Backert et al., 2013) and some highly virulent strains can cause severe diarrhea with bloody stools (Lee et al., 2003).

To evaluate the effectiveness of anti-*C. jejuni* IgY in alleviating symptoms caused by *C. jejuni* infection, we established mouse and cat infection models. The infected animals showed obvious signs of infection such as persistent diarrhea, weight loss, and frequent vomiting in cats, suggesting that our model is suitable for assessing the effectiveness of IgY against *C. jejuni* infection.

Anti-*C. jejuni* IgY effectively alleviated clinical signs of *C. jejuni* infection, including weight gain and well-formed feces.

Campylobacter jejuni infection triggers the immune system, leading to an increase in pro-inflammatory factors such as IL-1β, IL-6, and IFN-γ. Overexpression of these cytokines can be detrimental to health (Haitao et al., 2018). *C. jejuni* infection upregulates the expression of IL-6 and IL-1β in the colon and jejunum of mice, as well as the levels of IL-6 and IFN-γ in the serum of cats. IL-1β induces signal transduction and kinase activation, leading to substrate phosphorylation and enhanced DNA-binding activity of the nuclear factor, which in turn induces the expression of MMP-9 (Shaista et al., 2002). Schnbeck et al. (1998) found that MMP-9 can cleave IL-1 at the site of action of the IL-1β converting enzyme, resulting in the conversion of the inactive precursor pIL-1β (33 kD) to the active IL-1β (17/28 kD). The positive feedback regulation of MMP-9 with IL-1β exacerbates the inflammatory response. Our study found that IgY treatment reduced the levels of these inflammatory factors to varying degrees. CRP and PCT are markers of bacterial infection (Liliana et al., 2004). CRP is an acute response protein produced by the liver that can identify bacterial or viral infections (Coster and Wasserman, 2020). In viral infections, the level of CRP is typically not elevated. However, in bacterial

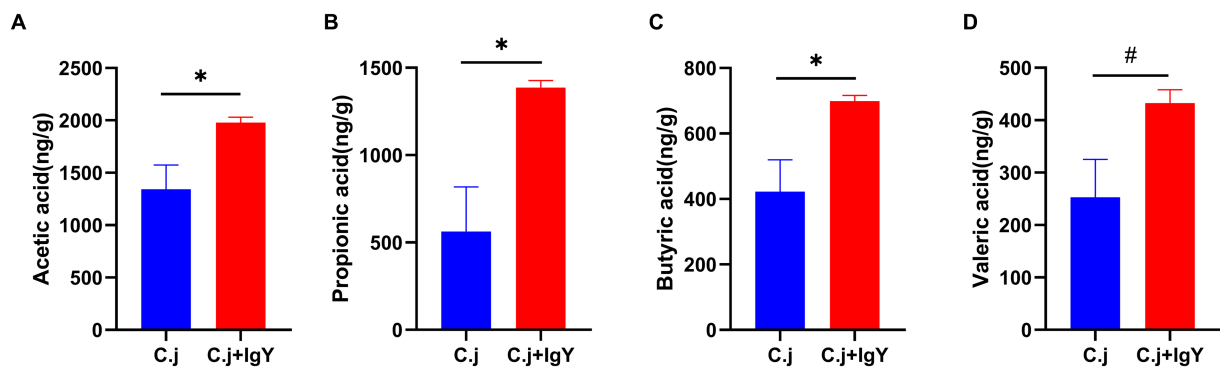


FIGURE 8

Effect of Anti-*C. jejuni* IgY on SCFAs in cat feces. The levels of (A) Acetic acid, (B) Propionic acid, (C) Butyric acid, and (D) Valeric acid in cat feces. Data are presented as mean $\pm$ SEM ($n=6$), * $p<0.05$, ** $p<0.01$, and *** $p<0.001$, and the symbol (#) represents difference tendency ($p<0.1$).

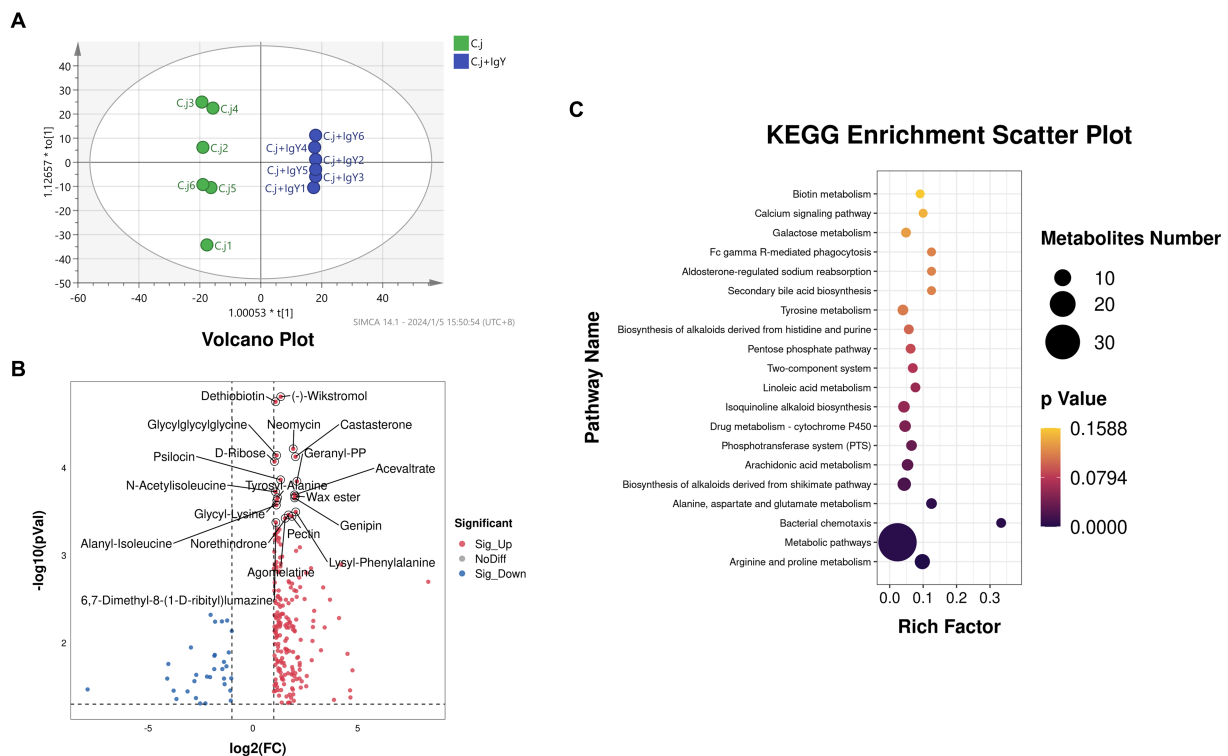


FIGURE 9

Effect of anti-*C. jejuni* IgY on the untargeted metabolomics of cat feces. (A) Score plots for the orthogonal partial least squares discriminant analysis (OPLS-DA) model between the two groups. (B) Volcano plot of differential metabolites in the feces (C.j + IgY vs. C.j). (C) Bubble diagram of metabolic pathway analysis for two groups of differential metabolites.

infections, it begin to rise 4–6 h after infection and decreases once the infection is under control. PCT is another marker of bacterial infection, which is normally confined mainly to the parathyroid glands has low serum levels, and responds more rapidly to bacterial infections, especially gram-negative bacterial infections (Bassetti et al., 2018). Our studies showed that anti-*C. jejuni* IgY treatment was effective in reducing serum

levels of CRP and PCT in cats. MPO is a reliable biomarker of inflammatory bowel disease (Hansberry et al., 2017). *C. jejuni* infection leads to intestinal inflammation, causing neutrophils to recruit to the intestine and release MPO, and fecal MPO levels are positively correlated with the degree of intestinal inflammation (Krawisz et al., 1984). Cats treated with anti-*C. jejuni* IgY showed a significant decrease in fecal

myeloperoxidase levels, accompanied by a decrease in serum nitric oxide levels. These data suggest that treatment with specific IgY can effectively inhibit the inflammatory response induced by *C. jejuni*.

Gut microbiota-derived metabolites are crucial for the integrity of the intestinal barrier. SCFAs produced by intestinal microorganisms through carbohydrate fermentation play an important role in various physiological processes in the body (Koh et al., 2016). *C. jejuni* colonizes the gut and disrupts the host's normal intestinal flora, creating competition for nutrients and space between foreign pathogens and the host's intestinal commensals. This effect was particularly evident in the antibiotic-scavenged intestines of the mice. Our study found that the relative load of *C. jejuni* decreased significantly in the number of *C. jejuni* in the HD and MD groups, and the concentration of SCFAs increased with the use of anti-*C. jejuni* IgY. This suggests that anti-*C. jejuni* IgY enhances the excretion of *C. jejuni* and limits its colonization of the intestine, thereby improving the metabolism of intestinal microorganisms.

To further investigate how IgY confers protection, we conducted an untargeted metabolomics study of Ragdoll cat feces. We identified 233 differential metabolites, of which 202 were up-regulated and 31 were down-regulated. Mapping these metabolites to the KEGG metabolic network revealed that IgY mainly affects amino acid metabolism and bacterial chemotaxis. *C. jejuni* cannot use sugar as a carbon source because it lacks the glycolytic enzyme phosphofructokinase (Parkhill et al., 2000). However, it can utilize aspartic acid, proline, and glutamic acid, as demonstrated in early *in vitro* experiments (Leach et al., 1997; Edward Guccione et al., 2010; Leon-Kempis et al., 2010), Parsons et al. quantified the chicken gut and found high levels of amino acids on which *C. jejuni* depends (Parsons et al., 1983). The acquisition of nutrients is a key factor in bacterial colonization and pathogenesis. *C. jejuni* can detect the concentration of certain components around the intestinal mucosa through chemotactic sensors, the combination of motility and chemotactic cascade responses facilitates *C. jejuni* colonization at suitable sites for growth (Korolik, 2019). Changes in these amino acid-related pathways and the effect of specific IgY on bacterial chemotaxis may be important in the clearance of *C. jejuni* from the gut.

Our study showed that specific IgY had potent inhibitory activity *in vitro* and effectively ameliorated the inflammatory response and intestinal structural damage induced by *C. jejuni*. Anti-*C. jejuni* IgY reduced the bacterial load of cecal contents and gut colonization through mechanisms that may involve amino acid metabolism and bacterial chemotaxis. The results suggest that anti-*C. jejuni* IgY can help protect gut health and modulate inflammatory responses. In conclusion, anti-*C. jejuni* IgY could potentially offer passive immune protection against pathogenic bacterial infections.

The study also had several limitations. Although clinical signs such as diarrhea and weight loss were observed in both the mouse and Ragdoll cat infection models, significant bloody stools were not observed. This may be due to the low virulence of the isolated *C. jejuni*. It is unclear whether this antibody has similar effects on different subspecies or serotypes of *C. jejuni*, and further in-depth studies are necessary.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee; the Laboratory Animal Center at the South China Agricultural University.

Author contributions

ZL: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. JY: Conceptualization, Investigation, Methodology, Visualization, Writing – review & editing. ZB: Conceptualization, Data curation, Investigation, Supervision, Writing – review & editing. JZ: Data curation, Investigation, Software, Supervision, Writing – review & editing. YL: Data curation, Investigation, Validation, Writing – review & editing. JD: Writing – review & editing, Conceptualization, Funding acquisition. BD: Conceptualization, Supervision, Writing – review & editing. SH: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing, Data curation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1378029/full#supplementary-material>

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Dipak Kumar Sahoo,
Iowa State University, United States
Andrew Knight,
Griffith University, Australia

*CORRESPONDENCE

Adronie Verbrugghe
✉ averbrug@uoguelph.ca

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Comparison of the fecal microbiota of adult healthy dogs fed a plant-based (vegan) or an animal-based diet

Brooklynn D. Liversidge¹, Diego E. Gomez¹, Sarah A. S. Dodd²,
Jennifer L. MacNicol³, Jennifer L. Adolphe^{4,5}, Shauna L. Blois¹
and Adronie Verbrugghe^{1*}

¹Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada, ²Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada, ³Department of Animal Biosciences, Ontario Agricultural College, University of Guelph, Guelph, ON, Canada, ⁴Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, ⁵Petcarean Pet Nutrition, Chilliwack, BC, Canada

Purpose: Pet guardians are increasingly seeking vegan dog foods. However, research on the impact of these diets on gastrointestinal (GI) physiology and health is limited. In humans, vegan diets modify the GI microbiota, increasing beneficial digestive microorganisms. This study aimed to examine the canine fecal microbiota in response to a vegan diet compared to an animal-based diet.

Methods: Sixty-one client-owned healthy adult dogs completed a randomized, double-blinded longitudinal study. Dogs were randomly assigned into two groups that were fed either a commercial extruded animal-based diet (MEAT, $n = 30$) or an experimental extruded vegan diet (PLANT, $n = 31$) for 12 weeks. Fecal collections occurred at the start of the experimental period and after 3 months of exclusively feeding either diet. Bacterial DNA was extracted from the feces, and the V4 region of the 16S rRNA gene was amplified using PCR and sequenced on Illumina MiSeq. Beta-diversity was measured using Jaccard and Bray–Curtis distances, and the PERMANOVA was used to assess for differences in fecal microbiota within and between groups. Alpha-diversity indices for richness, evenness, and diversity, as well as relative abundance, were calculated and compared between groups.

Results: Beta-diversity differences occurred between diet groups at exit time-point with differences on Bray–Curtis distances at the family and genus levels ($p = 0.007$ and $p = 0.001$, respectively), and for the Jaccard distance at the family and genus level ($p = 0.006$ and $p = 0.011$, respectively). Significant differences in alpha-diversity occurred when comparing the PLANT to the MEAT group at the exit time-point with the PLANT group having a lower evenness ($p = 0.012$), but no significant differences in richness ($p = 0.188$), or diversity ($p = 0.06$). At exit-timepoint, compared to the MEAT group, the relative abundance of *Fusobacterium*, *Bacteroides*, and *Campylobacter* was lower in the PLANT group. The relative abundance of *Fusobacterium* decreased over time in the PLANT group, while no change was observed in the MEAT group.

Conclusion: These results indicate that vegan diets may change the canine gut microbiota. Future studies are warranted to confirm our results and determine long-term effects of vegan diets on the canine gut microbiome.

KEYWORDS

canine, alternative diets, companion animal nutrition, microbiome, plant-based ingredients, vegan

Introduction

To most pet guardians, dogs are viewed as members of the family. This has resulted in pet nutrition trends to closely following popular trends in human nutrition (Buff et al., 2014; Knight and Leitsberger, 2016; Dodd et al., 2018). Consumption of an entirely plant-based (vegan) diet that avoids consuming any ingredients that come from an animal has increased in popularity in human nutrition because of the potential positive impacts on the environment and overall health (Berschneider, 2002; Michel, 2006; Dodd et al., 2018; Eisen and Brown, 2022; Feigin et al., 2023; Knight, 2023). As more pet guardians switch to a vegan diet, the potential for moral conflicts has developed when it comes to feeding their pets because most commercial diets are formulated using animal-based ingredients (Rothgerber, 2013; Dodd et al., 2018). A survey conducted in 2019, indicated that three quarters of vegan pet guardians would reportedly feed a plant-based diet to their pets if one was available to meet their needs (Dodd et al., 2019). A third of pet guardians who did not already feed a plant-based diet expressed interest in doing so, and a fifth of those pet guardians who expressed interest stated that further knowledge about plant-based diets is needed before they will feed such diet to their pets (Dodd et al., 2019). The gap in required research to determine the feasibility of vegan diets for dogs pertains to the long-term nutritional suitability with regards to the ability of these diets to meet all nutritional requirements (Berschneider, 2002; Michel, 2006; Remillard, 2008; Dodd et al., 2018, 2019; Knight et al., 2022).

The limited research regarding nutritional adequacy of vegan diets, poses questions for both veterinarians and pet guardians on their long-term sustainability. Commercial vegan diets for dogs have been voiced by veterinary nutritionists as potentially being insufficient in meeting the maintenance requirements stated by the National Research Council, The Association of American Feed Control Officials (2019), and the European Pet Food Industry Federation (McMillan et al., 2006; Beloshapka et al., 2016; Dodd et al., 2021). These concerns are based on lower concentrations of nutrients and poor bioavailability of nutrients in plant-based ingredients used in vegan diet formulations (Dodd et al., 2021). Digestibility studies however, on individual plant ingredients, mildly cooked human-grade, and whole plant-based diets have demonstrated that plant-based ingredients if processed correctly have the potential to demonstrate similar nutrient digestibility when compared to conventional animal-based ingredients (Kendall and Holme, 1982; Bednar et al., 2000; Liversidge et al., 2023). This is likely due to coevolution of dogs and humans which has led to dogs becoming closer related biologically to humans rather than to their wolf ancestors

(Buff et al., 2014). Unlike their wolf ancestors, the canine gut microbiota shows to have greater ability to adapt to either a high-protein or high-carbohydrate diet and contains a diverse number of bacteria used for fermentation of these nutrients (Alessandri et al., 2019). Specifically, the gut microbiota of dogs has adapted to show an increase in gut microbiota used for fermentation of plant material and reduction of gut microbiota used in the fermentation of protein and fats from animal ingredients (Coelho et al., 2018; Alessandri et al., 2019; Liu et al., 2021). Thus, if a vegan diet that was formulated with highly digestible plant-ingredients was consumed, the canine gut microbiota has the ability to shift making it possible for dogs to meet daily nutrient requirements with consumption of a plant-based diet.

In humans, diet has a large impact on the richness and diversity of microorganisms present within the gastrointestinal (GI) tract (Bosch et al., 2009; Kamboj and Nanda, 2018; Alvarenga et al., 2019). Individuals following a vegan diet compared to an omnivorous diet showed differences in the diversity and abundance of several taxa of the GI microbiota (Honda and Littman, 2012; Singh et al., 2017). Specifically, the gut microbiota has an increased abundance of bacterial communities that aid in fermentation and absorption of plant-materials and a decreased abundance of communities that breakdown animal-derived protein and fat sources (Winglee and Fodor, 2015; Zhenjiang and Knight, 2015; Singh et al., 2017). Vegetarian – devote of meat products but including dairy and eggs – or vegan diet consumption has been seen to shift gut microbiota in a state of dysbiosis to aid in reducing inflammation and treating conditions such as inflammatory bowel disease, obesity, and type-2 diabetes in humans (Li et al., 2020; Santhiravel et al., 2022; Sahoo et al., 2023). This shift in the GI microbiota appears to be helpful to properly digest and utilize plant-based ingredients and is essential for plant-based diets to provide beneficial effects. Some researchers have investigated fecal microbial effects of single plant-based ingredients in dogs (Carciofi et al., 2010; Bazolli et al., 2015; Hankel et al., 2020); however, currently, there is no research conducted on the effects of vegan diets on the gut microbiota in dogs.

Following thousands of years of domestication, the GI microbiota of dogs and humans have developed structural and functional similarities (Coelho et al., 2018). Based on these resemblances, we hypothesized that similar alterations in microbial taxonomy seen in humans consuming a vegan diet would occur in dogs fed a diet consisting of only plant-based ingredients, in combination with mineral or synthetic additives, but devote of meat-based ingredients. Therefore, the objective of this study was to examine the canine fecal microbial composition and

structure in response to an experimental vegan diet compared to a commercially available animal-based diet.

Materials and methods

All experimental procedures for this study were approved by the University of Guelph Animal Care Committee (AUP#4192) and the Research Ethics Board (Research Ethics Approval Number 19-02-036), and were in accordance with institutional, provincial, and national guidelines for the care and use of animals and humans participating in research.

Animals and experimental design

This study was conducted as part of a larger randomized, double-blinded longitudinal study in client-owned healthy adult dogs, which occurred between July 2019 and November 2020 (Dodd et al., 2023). Recruitment of trial participants was conducted through an eSurvey designed on the Qualtrics (Provo, UT, USA) platform to collect data regarding suitability for study enrollment. Details regarding this recruitment survey were published previously (Dodd et al., 2023; Liversidge et al., 2023). The survey was advertised locally around the University of Guelph campus and surrounding community, as well as shared virtually on social media to local dog-related groups. Dogs were excluded from enrollment consideration if they were reproductively intact, had a body weight (BW) less than 5 kg, had an owner reported body condition scores (BCS) greater than 5 (WSAVA, 2013), were fed homemade or raw pet food, were housed outdoor without supervision, had access to unmonitored food sources, had current medical problems, or had any known dietary allergies. Dogs in households without children or other animals were prioritized. Recruitment resulted in a total of 87 dogs scheduled for enrollment appointments (Supplementary Figure 1).

The enrollment appointment included discussion of the study procedures, collection of a signed informed consent from participants and a wellness examination of the dogs conducted by a licensed veterinarian. The wellness examination included a medical and dietary history, physical examination, and a bodyweight measurement. Blood was collected for complete blood count and serum biochemistry. Dogs were approved for inclusion in the trial if they were confirmed to be spayed/neutered, aged 3 years or older, had a BCS between 4 and 7 on a 9-point scale (Laflamme, 1997), and were deemed healthy based on a physical examination and routine blood work. Seventy-six dogs met the inclusion criteria and started the 4-week adaptation period during which all dogs received the same commercial extruded animal-based diet (MEAT) (Supplementary Figure 1). Eleven dogs did not continue the study after the adaptation period due to not eating the diet, GI abnormalities, excessive weight gain, or COVID-related pet guardian dropouts.

The remaining 65 dogs were randomly assigned into two diet groups; continuing with the animal-based diet (control group; MEAT, $n = 31$) or being fed an experimental extruded vegan diet (PLANT, $n = 34$) (Supplementary Figure 1). Diets were fed for 12 weeks, maintaining current energy intake, as determined based

on diet history information. Four dogs were excluded during the experimental period (MEAT, $n = 1$; PLANT, $n = 3$) due to pet guardian personal reasons or dog health concerns unrelated to diet including development of GI ulcers after administrations of non-steroidal anti-inflammatory drugs and development of a urinary tract infection. Fecal collections occurred at the end of the 4-week adaptation period (baseline time-point) and after 3 months of exclusively feeding either the MEAT or PLANT diet (exit time-point). Owners were instructed to collect fecal samples immediately after voiding and as close to their appointment as possible, freeze and deliver the samples to the research team in a provided container stored in a Styrofoam cooler box. Upon arrival, fecal samples were immediately stored frozen at -20°C until the microbiome analyses could be completed.

Due to the COVID-19 pandemic, the trial was paused for 4 months from March 2020 until July 2020. During this period dogs were maintained on the experimental diets, either PLANT or MEAT depending on which phase of the trial they were in (adaptation or feeding trial) to allow for immediate resumption of data collection when restrictions were lifted. This resulted in some variation in trial duration for dogs participating in the study, with the adaptation period for five dogs lasting more than 4 weeks (PLANT, $n = 2$; MEAT, $n = 3$), the experimental period for two dogs lasting more than 12 weeks (PLANT, $n = 1$; MEAT, $n = 1$), and for three dogs both the adaptation period and the experimental period lasting more than 4 and 12 weeks, respectively (PLANT, $n = 2$; MEAT, $n = 1$). During this period, frequent communication between the research team members and the pet guardians was maintained. This was performed to aid as a reminder to stay consistent with trial protocol during the extended 4 months.

Diets

Both diets, MEAT and PLANT, used in this study were formulated to meet or exceed nutrient recommendations according to The Association of American Feed Control Officials (2019) nutrient profile for canine adult maintenance. The MEAT diet was a commercial extruded dog food (Petcurean Go! Solutions Skin + Coat Care Chicken Recipe, PPN Ltd., Chilliwack, BC, Canada). The PLANT diet was an experimental extruded dog food, excluding all animal-based ingredients, formulated to be isoenergetic and as similar as possible in macronutrient and micronutrient profiles to the MEAT diet (Supplementary Table 1). Proximate analysis and mineral analysis were performed on both diets post-manufacturing at a commercial laboratory (Bureau Veritas, Mississauga, ON, Canada). Details regarding the diets used were published previously (Dodd et al., 2023; Liversidge et al., 2023). Pet guardians and researchers were blinded to the identity of the diets being fed to study participants throughout the duration of the testing period and remained blinded until after all data were analyzed.

Food quantity was calculated based on the dogs' current dietary intake to match calories and maintain current BW. A gram scale was provided to each household to precisely measure the recommended quantity of food per day. Pet guardians were given a list of plant-based treats without added micronutrients. An acceptable treat dose was calculated for each dog to avoid exceeding 10% of their daily energy intake from sources other than the

experimental PLANT or MEAT diets. Pet guardians were instructed not to feed their dogs any other food items as well as to record food and treat intake in a daily food diary for the duration of the study.

Sample preparation and DNA extractions

Whole fecal samples were thawed in their original containers in a refrigerator (4°C) the night before DNA extractions. Bacterial DNA was extracted using a commercial stool extraction kit (E.Z.N.A. Stool DNA Kit, Omega Bio-Tek Inc., Doraville, GA, USA) according to the manufacturer's instructions. Extracted DNA samples were stored in a -80°C freezer until further analysis.

Polymerase chain reaction

To test the quantity of extracted DNA a spectrophotometer (Nanodrop 1000 Spectrophotometer, Nanodrop Technologies Inc., Thermo Fisher Scientific, Waltham, MA, USA) was used on all extracted DNA samples. The DNA extractions were thawed in rounds of 27 samples and diluted to a range of 30–100 ng/ml. The V4 region of the 16S rRNA gene was amplified using polymerase chain reaction (PCR) with the forward primer 515F(5'-GTGCCAGCMGCCGCGGTAA-3') and reverse primer 806R(5'-GGACTCTACHVGGGTWTCTAAT-3') (Walters et al., 2016), KAPA HiFi ReadyMix (Kapa Biosystems, Wilmington, MA, USA), and PCR grade water. The PCR products were purified with Mag Bind RXNPure plus (Omega Biotek Inc.). To prepare the purified PCR products for Illumina MiSeq sequencing, the purified PCR products were amplified using Illumina adapters N716-N729 and S513-S522, and then purified again. All finalized PCR products were evaluated using gel electrophoresis and DNA was measured using spectrophotometry to ensure the concentration of DNA was greater than 15 ng/μl before Illumina sequencing.

DNA sequencing

Bridge amplification was completed on an Illumina MiSeq system (Illumina, San Diego, CA, USA) at the University of Guelph Agri-Food Labs using terminator nucleotides that were incorporated into the amplified PCR products with the removal of the terminator group (Tal et al., 2020; Chui et al., 2023).

Sequence processing

Following DNA sequencing of fecal samples, Mothur v1.48.0 was used for sequence processing according to the software standard operating procedures (Schloss et al., 2009; Kozich et al., 2013). Assembly of paired end reads was performed using the make.contigs command to extract the sequences and create its reverse complement to join the reads into contigs. Next multiple filtrations were conducted using a variety of screen.seqs commands to remove sequences greater than 250 base pairs (bp) in length and those with any ambiguous base calls or runs of homopolymers greater than 8 bp. Alignment of sequences to the Silva v132 16S

rRNA reference database was completed, with removal of sequences that did not align with the correct region (Quast et al., 2013). With the use of chimera.vsearch command all identified chimeras were removed to further remove sequencing errors. Sequences were clustered into operational taxonomic units using 97% sequences similarity and taxonomy assigned using the Ribosomal Database Project classifier (v14) (Cole et al., 2014).

Statistical analysis

To examine community membership and structure, Beta diversity indexes were assessed using the Jaccard and Bray–Curtis distances and PERMANOVA tests were used to assess for the effect of time, diet, and its interaction on both distances in R (R Core Team, Vienna, Austria). Visual similarities and clustering of each diet was plotted with principal coordinate analysis (PCoA) using the package ggplot2. Evenness [how even the abundance of species is within a community (Hagerty et al., 2020)], richness [total number of different species present in the community (Hagerty et al., 2020)] and diversity (evenness and richness) were calculated using Shannon diversity (Shannon, 1948), and Chao (Chao, 1984) and Simpson diversity (Simpson, 1949) indices, respectively. Normality of the data distribution was assessed using the Shapiro–Wilk test. The Wilcoxon tests were used to compare alpha-diversity indexes between diet groups (PLANT and MEAT) and between time-points (baseline and exit). Relative abundance (%) was calculated for the different taxonomic levels for each diet. The relative abundance was calculated and comparison between and within groups was conducted among phyla with a relative abundance >1% (10 most abundant) phyla, >0.01% for family, and >0.001% for genus. Differences were evaluated using nonparametric Wilcoxon tests, with *p*-values adjusted for multiple comparisons (Benjamini and Hochberg, 1995). Relative abundances are presented as median with range (minimum and maximum). A *p* < 0.05 for all comparisons was considered statistically significant. Labeled scatter plots were created for each significantly different dependent variable to identify potential outliers.

Results

Of the total 61 client owned dogs completing the study, 47 were included for fecal microbiota analysis (27 consuming PLANT and 20 consuming MEAT). Sample size was reduced due to a lack of fecal samples submitted by the pet owners on the day of sample collection (*n* = 5), poor DNA yield after extraction (*n* = 7), or a repeated inability to obtain adequate sequence numbers (*n* = 2).

As mentioned previously, the COVID-19 pandemic added variation in trial duration for 10 dogs participating in the study. Examination of labeled scatter plots revealed these dogs were not identified as outliers and results were not significantly different from the dogs consuming the experimental diet for the intended 12 weeks.

The dogs were aged 3 years or older (mean age being 4.734 ± 0.347), both neutered male (*n* = 22) and spayed female (*n* = 25) of various purebred or crossbreeds. All dogs enrolled in this study had BCS between 4 and 7 on a 9-point-scale with BW

ranging between 5 and 50 kg (baseline BW: 24.12 ± 1.48 ; exit BW: 24.13 ± 1.53). All dogs in the study tolerated their diets well, with none showing signs of malnutrition or illness during the baseline or trial periods.

Fecal microbiota analysis

Fecal analyses resulted in a total of approximately 14,673,953 sequences. Following quality control filtering a total of 11,725,298 sequences remained, with a median of 117,591 sequences per sample (range: 88,435–209,427). To standardize sequence numbers used for analysis, subsampling was completed based on the smallest number of sequences from a sample. The coverage was then assessed using Good's coverage value.

Beta-diversity

Comparison between diet groups at entrance time-point revealed no significant differences on Bray–Curtis and Jaccard distance at the phylum, family, or genus levels. Similarly, in both the PLANT and MEAT groups over time (baseline – exit), no significant differences in Bray–Curtis and Jaccard distance at the phylum, family, or genus levels were found. Comparison between diet groups at the exit time-point revealed differences on Bray–Curtis distances at the family and genus level ($p = 0.007$ and $p = 0.001$, respectively) (Supplementary Table 2) and for the Jaccard distance ($p = 0.006$ and $p = 0.011$, respectively) (Supplementary Table 3) but differences at the phylum level were not identified (Supplementary Tables 2, 3). Principal coordinate analyses plots obtained from Jaccard and Bray–Curtis distances showed no apparent clustering of samples based on diet groups and time-points (Figure 1).

Alpha-diversity indices

Alpha-diversity indices for richness, evenness, and diversity between diet group and time-point are described in Table 1. There were no differences in alpha-diversity indices when comparing the PLANT to MEAT group at baseline. Comparing the baseline to the exit time-point of the MEAT group, MEAT had greater richness (Chao index, $p = 0.028$) but not evenness ($p = 0.839$) or diversity ($p = 0.081$) at the exit time-point. Similarly, when comparing the baseline to exit time-points of the PLANT group, the exit samples had lower evenness than the baseline samples ($p = 0.036$) but no differences in richness ($p = 0.387$) or diversity index ($p = 0.368$). When comparing the PLANT to the MEAT group at the exit time-point, the PLANT group had lower evenness ($p = 0.012$) but no differences in richness ($p = 0.188$) or diversity ($p = 0.060$).

Relative abundance

Median relative abundance was examined across all taxa between diet groups and time-points. After performing a Benjamini–Hochberg adjustment, significant changes in relative

bacterial abundance between diet groups and time were present at the phylum level (Table 2); however, almost all changes to the family and genus level were no longer present (Supplementary Tables 4, 5, respectively). In the MEAT group over time, on the family level only Streptococcaceae ($p = 0.02$) was significant and on the genus level only Bifidobacterium ($p = 0.03$) and Streptococcus ($p = 0.03$) was significant (Supplementary Tables 4, 5, respectively). The most abundant phyla in both diet groups were Firmicutes, Actinobacteria, Fusobacteria, and Bacteroidetes. The most abundant families in both diet groups were Peptostreptococcaceae, Lachnospiraceae, Fusobacteriaceae, and Erysipelotrichaceae. The most abundant genera in both diet groups were Peptacetobacter, Blautia, Fusobacterium, and Collinsella. The relative abundance of three of the predominant phyla differed between diet groups at the exit time-point, with Firmicutes being higher in the dogs fed the PLANT diet ($p = 0.021$), while Fusobacteria, Bacteroidetes, and Campilobacterota were lower in dogs fed the PLANT diet ($p = 0.013$, $p = 0.012$, and $p = 0.001$, respectively) (Figure 2). The relative abundance of Fusobacteria ($p < 0.001$) decreased from baseline to exit ($p = 0.001$) in dogs fed the PLANT diet (Figure 3). No differences were observed overtime in the MEAT diet group. No other differences were noted in relative abundance across all other taxa between diet groups and time-points.

Discussion

The present study revealed that feeding an entirely plant-based or vegan diet for 3 months had minor effects on the gut microbiota in dogs when compared to a conventional animal-based diet. A recent study conducted a gene catalog comparison between dog, human, pig, and mouse to determine which animal the dog's GI microbiota was most closely related to (Coelho et al., 2018). The distribution of genes at the phylum level of this study showed that the canine GI microbiota has a higher taxonomic and functional overlap with the human GI microbiota (Coelho et al., 2018). These structural and functional similarities imply that human research findings could predict results in dogs and vice versa (Coelho et al., 2018). Based on this similarity to the human gut microbiota, it was expected that similar fecal microbiota changes consisting of increased abundance of microbiota that aid in carbohydrate fermentation and decreased abundance of microbiota related to fermentation of protein and fat would occur in dogs fed an entirely plant-based diet as those seen in humans consuming a vegan diet compared to an omnivorous diet. However, in the current canine study the bacterial fecal microbiota was minimally affected. Despite the lack of change in beta-diversity comparisons, there were a few taxonomic changes on the phylum level between the plant-based and animal-based diet groups, but no significant differences were found at the family or genus level.

In humans, a healthy adult intestinal microbiota is characterized by the dominance of Bacteroidetes and Firmicutes phyla (Simpson and Campbell, 2015; Bamberger et al., 2018). Variability between these two bacteria is shown to be heavily affected by diet, specifically between omnivorous, vegetarian, or vegan diets. In one study comparing Indian participants, whose diets mainly contained plant-based foods, with Chinese

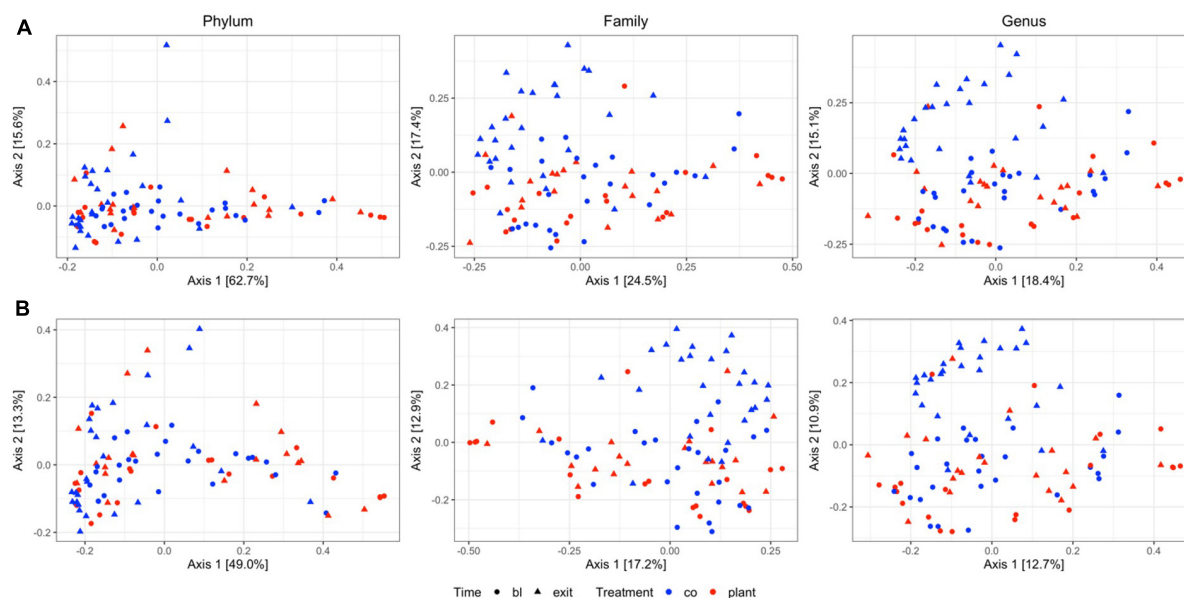


FIGURE 1

Two-dimensional principal coordinate analyses plots of (A) community structure (Bray–Curtis distances) and (B) community membership (Jaccard distances) of 47 healthy adult client-owned dogs fed an experimental plant-based (PLANT, $n = 27$) or commercial animal-based (MEAT, $n = 20$) extruded diet in a 12-week randomized, double-blinded longitudinal study. Samples are colored by treatment (CO, MEAT diet; plant, PLANT diet), and time-points are shaped by time (circle, baseline; triangle, exit). Figure panels in each column are grouped to the taxonomic level.

participants, whose diets contain mainly animal-based fats and proteins, researchers found the population of Bacteroidetes of Indian participants was nearly four times greater than in the Chinese individuals (Jain et al., 2018). An additional study involving European individuals identified a higher relative abundance of Bacteroidetes in vegans and vegetarians than in omnivores (Losasso et al., 2018). These findings agree with previous research showing that the relative abundance of Bacteroidetes in Italian children, typically consuming an omnivorous diet high in animal protein and fat, was less than half of that seen in Burkina Faso children who consume a diet rich in plant-based starch, fiber, and plant protein (De Filippo et al., 2010). These studies indicate that in humans a lower consumption of animal products is associated with a greater abundance of Bacteroidetes. In contrast, Lin et al. (2013) reported that children from the United States following a Western diet had a higher abundance of Bacteroidetes than children from Bangladesh following a Mediterranean diet. Like humans, the dominance of Bacteroidetes and Firmicutes was confirmed in healthy dogs (Deng and Swanson, 2015). However, the abundance of Bacteroidetes behaved differently in the present study in response to a plant-based diet. Dogs fed on the control animal-based diet had a higher abundance of Bacteroidetes than dogs on the experimental plant-based diet at the exit time-point. These distinct results in humans and dogs may be due to the many exogenous factors affecting the microbiota within the GI tract (Wu et al., 2011; Deng and Swanson, 2015; Li et al., 2017). For instance, results reported by Lin et al. (2013) indicated an abundance of Bacteroidetes between children consuming a Western diet compared to a Mediterranean diet; the researchers found both age and geographical differences as the potential explanation for their unexpected results (Lin et al., 2013). In dogs, similar significant associations between canine alpha-diversity indexes

and geographical regions have been observed (Jha et al., 2020). Moreover, the same study observed differences in alpha-diversity indexes between rural and suburban dogs (Jha et al., 2020). In the current study, these exogenous factors may have played a role in affecting the GI microbiota, as client-owned dogs of various ages came from different household environments.

The balance between Bacteroidetes and Firmicutes is affected by each other where a decrease in Firmicutes levels usually occurs in favor of increasing Bacteroidetes levels (Tomova et al., 2019). The ratio between Bacteroidetes and Firmicutes in humans is related to a predisposition to disease development (Ley et al., 2006; Verdum et al., 2013). For example, increases in Firmicutes abundance are observed in obesity and GI diseases such as inflammatory bowel disease (Ley et al., 2006; Verdum et al., 2013). Humans consuming a vegan diet have a lower abundance of Firmicutes compared to omnivores; this finding is linked to positive health benefits, including weight loss, decreased inflammation, and improvements to the immune system that lower the risk of developing chronic diseases (Ley et al., 2006; Kim et al., 2013; Kumar et al., 2014; Singh et al., 2017). In the current study, however, dogs fed the experimental plant-based diet had a higher abundance of Firmicutes than the control animal-based diet. In human literature, these changes are attributed to endogenous factors that can affect the gut microbiota within the GI tract (Wu et al., 2011; Deng and Swanson, 2015; Li et al., 2017). In humans, the nutrient profiles between an omnivore diet and a plant-based diet are typically very different, with a vegan diet in humans expressing lower total energy and protein intake but increased fat and fiber intake when compared to an omnivorous diet (Clarys et al., 2014). In the current study, the experimental plant-based diet was formulated to have a similar nutrient profile to the control animal-based diet, despite different ingredients. Potentially, the changes in human

TABLE 1 Alpha-diversity indices (Chao, Richness; Shannon, evenness; and Inverse Simpson's, diversity) observed in the feces of 47 healthy adult client-owned dogs fed an experimental plant-based (PLANT, $n = 27$) or commercial animal-based (MEAT, $n = 20$) extruded diet in a 12-week randomized, double-blinded longitudinal study.

Alpha-diversity Indexes	Diet group and time-point		p-Value
Chao: richness	PLANT ¹ baseline	MEAT ² baseline	0.57
	460.53 [264.75–1,254.56]	427.33 [201.26–854.91]	
	PLANT ¹ exit	MEAT ² exit	0.12
	488.81 [295.23–1,288.89]	616.14 [342.52–1,498.64]	
	PLANT ¹ baseline	PLANT ¹ exit	0.39
	460.53 [264.75–1,254.56]	488.81 [295.23–1,288.89]	
	MEAT ² baseline	MEAT ² exit	0.03 ^a
	427.33 [201.26–854.91]	616.14 [342.52–1,498.64]	
Shannon: evenness	PLANT ¹ baseline	MEAT ² baseline	0.80
	0.54 [0.34–0.63]	0.51 [0.38–0.62]	
	PLANT ¹ exit	MEAT ² exit	0.01 ^a
	0.47 [0.28–0.61]	0.53 [0.36–0.60]	
	PLANT ¹ baseline	PLANT ¹ exit	0.04 ^a
	0.54 [0.34–0.63]	0.47 [0.28–0.61]	
	MEAT ² baseline	MEAT ² exit	0.84
	0.51 [0.38–0.62]	0.53 [0.36–0.60]	
Inverse Simpson's: diversity	PLANT ¹ baseline	MEAT ² baseline	0.45
	11.20 [2.73–22.67]	9.13 [3.12–19.88]	
	PLANT ¹ exit	MEAT ² exit	0.06
	8.74 [2.48–19.66]	12.98 [3.41–24.91]	
	PLANT ¹ baseline	PLANT ¹ exit	0.37
	11.20 [2.73–22.67]	8.74 [2.48–19.66]	
	MEAT ² baseline	MEAT ² exit	0.08
	9.13 [3.12–19.88]	12.98 [3.41–24.91]	

Comparisons were made between the baseline and exit time-points for each diet group. As data was presented as non-parametric alpha-diversity indices between diet group at each timepoint are presented as median and interquartile range [minimum and maximum].

¹ PLANT, plant-based diet.

² MEAT, animal-based diet.

^a Coefficient of correlation significant at $p < 0.05$.

fecal microbiota when comparing individuals consuming a vegan diet to individuals consuming an omnivorous diet may be in response to nutrient differences in the diets, not in response to ingredient changes. Thus, due to the nutrient profiles being as close as possible between the PLANT and MEAT diet this may be the reason that the canine gut microbiota in the current study was shown to be opposite of what is reported in human literature with dogs in the PLANT group expressing higher abundance of Firmicutes and lower abundance Bacteroidetes.

In humans, differences in *Fusobacteria* abundance have been documented when comparing individuals consuming a vegan diet to individuals consuming an omnivorous diet, with a greater abundance of *Fusobacteria* associated with an omnivorous diet (Losno et al., 2021). Similarly, the abundance of *Fusobacterium* (*Fusobacteria* phylum) was increased in dogs fed entirely animal-based raw food compared to dogs consuming omnivorous extruded diets (Pilla and Suchodolski, 2020). *Fusobacteria* is more abundant in other carnivorous species such as cats and wolves (Zhang and Chen, 2010; Bermingham et al., 2013). In the current study, relative abundance of *Fusobacteria* decreased from the baseline to exit time-point in dogs fed the PLANT diet. Furthermore, dogs fed the ANIMAL diet showed a higher abundance than those fed PLANT at the exit time-point. These results complement previous research found in humans (Losno et al., 2021) and other predator species (seals, tigers, polar bears, etc.) supporting that high abundance of *Fusobacteria* is associated with digestion of meat (Jiang et al., 2020).

The current study had several limitations including a smaller sample size, the use of client-owned animals, sample collection times, and the ability to extrapolate the study results to other vegan diets for dogs. Due to the relatively small sample size, one should be careful extrapolating of the results to a greater population. Nonetheless, the current study had a larger sample size than previously published gut microbiota research in client-owned dogs (Beloshapka et al., 2016; Herstad et al., 2017; Sanches et al., 2020; Onozawa et al., 2022). With use of client-owned animals we are reliant completely on their dedication to the trial protocol. Pet guardians were provided with a small Styrofoam cooler and waste collection bags and asked to collect feces immediately after defecation and freeze using the provided resources. With the use of client-owned animals, despite instructions to freeze fecal samples immediately after defecation, there was no supervision by the research team to ensure the fecal collection and storage was performed properly by pet guardians prior to delivery of the samples. However, the authors do not expect this to influence the results based on previous research in companion animals showing limited changes in the microbiota from short term (2 weeks) exposure to ambient temperature and time (Weese and Jalali, 2014; Tal et al., 2020; Chui et al., 2023). A further limitation factor with respect to data collection and the research variables assessed in the study was the frequency of sampling. Fecal collections from the dogs were only done at baseline (after the adaption period where all dogs were fed the MEAT diet) and exit (after feeding either the PLANT or MEAT diet exclusively for 3 months) timepoints. Based on recent research, the gut microbiota in dogs is very resilient meaning microbiota shifts and stabilization following a dietary intervention can be seen in just 2 weeks (Lin et al., 2022). In the current study changes may have occurred soon after introducing the MEAT or PLANT diet however, the pattern of change was indeterminable due to the data collection timepoints. This reduces the ability to interpret the significance of some of the changes. More frequent measurements to document the temporal trends and patterns in variables would have been beneficial. Furthermore, extrapolation of findings from this trial to all vegan dog foods should be made with caution. The PLANT diet used in the current trial was formulated to exceed minimum AAFCO nutrient recommendations (The Association of American Feed Control Officials, 2019) and also postproduction nutrient analysis occurred

TABLE 2 Median relative abundance of predominant taxonomic classifications on the phylum level of bacteria from the feces of 47 healthy adult client-owned dogs fed an experimental plant-based (PLANT, $n = 27$) or commercial animal-based (MEAT, $n = 20$) extruded diet in a 12-week randomized, double-blinded longitudinal study.

Phylum	PLANT ¹ baseline	MEAT ² baseline	p -Value	FDR ³
Firmicutes	71.77 [34.16–91.01]	70.31 [27.40–91.92]	0.94	0.94
Actinobacteria	8.33 [0.65–15.97]	6.06 [0.54–23.99]	0.27	0.80
Fusobacteria	6.11 [0.27–60.63]	6.73 [0.21–32.09]	0.91	0.94
Bacteroidetes	4.31 [0.23–20.60]	6.87 [0.14–39.08]	0.40	0.84
Proteobacteria	1.06 [0.44–36.22]	1.77 [0.02–15.45]	0.67	0.87
Campilobacterota	0.04 [0–4.58]	0.10 [5e ⁻⁴ –2.69]	0.15	0.69
Deinococcus-Thermus	0 [0–0.01]	0 [0–0.01]	0.55	0.84
Cyanobacteria/Chloroplast	0.01 [9e ⁻⁴ –0.48]	6.2e ⁻³ [0–0.06]	0.09	0.69
Verrucomicrobia	2.7e ⁻³ [0–0.08]	2e ⁻³ [0–0.07]	0.56	0.84
Phylum	PLANT ¹ exit	MEAT ² exit	p -Value	FDR ³
Firmicutes	78.09 [32.30–96.33]	63.08 [31.16–86.41]	0.01	0.02 ^{a,b}
Actinobacteria	10.35 [0.54–67.34]	10.70 [1.19–39.61]	0.64	0.80
Fusobacteria	0.72 [0.02–46.99]	3.93 [0.02–64.33]	0.00	0.01 ^{a,c}
Bacteroidetes	1.72 [0.04–25.98]	6.13 [0.21–38.29]	0.00	0.01 ^{a,c}
Proteobacteria	0.74 [0.04–4.62]	0.87 [0.05–16.49]	0.36	0.65
Campilobacterota	6.6e ⁻³ [0–5.01]	0.16 [3.9e ⁻³ –4.27]	0.00	0.001 ^{a,c}
Deinococcus-Thermus	0 [0–2.7e ⁻³]	0 [0–1.7e ⁻³]	0.55	0.80
Cyanobacteria/Chloroplast	0.01 [0–0.92]	9.35e ⁻³ [0–0.26]	0.75	0.80
Verrucomicrobia	0.04 [0–0.49]	7.75e ⁻³ [0–0.12]	0.80	0.80
Phylum	PLANT ¹ baseline	PLANT ¹ exit	p -Value	FDR ³
Firmicutes	71.77 [34.17–91.02]	78.09 [32.30–96.33]	0.04	0.15
Actinobacteria	8.33 [0.6515–15.9701]	10.35 [0.54–67.34]	0.12	0.21
Fusobacteria	6.11 [0.2737–60.6256]	0.72 [0.02–46.97]	0.001	0.001 ^{a,d}
Bacteroidetes	4.31 [0.23–20.60]	1.72 [0.04–25.98]	0.05	0.15
Proteobacteria	1.06 [0.44–36.22]	0.74 [0.04–4.62]	0.12	0.21
Campilobacterota	0.04 [0–4.58]	0.01 [0–5.01]	0.27	0.35
Deinococcus-Thermus	0 [0–0.01]	0 [0–2.7e ⁻³]	0.41	0.46
Cyanobacteria/Chloroplast	0.01 [9e ⁻⁴ –0.483]	0.01 [0–0.92]	0.68	0.68
Verrucomicrobia	2.7e ⁻³ [0–0.08]	0.04 [0–0.49]	0.19	0.29
Phylum	MEAT ² baseline	MEAT ² exit	p -Value	FDR ³
Firmicutes	70.31 [27.40–91.92]	63.08 [31.16–86.41]	0.40	0.72
Actinobacteria	6.06 [0.54–23.99]	10.70 [1.19–39.61]	0.09	0.71
Fusobacteria	6.73 [0.21–32.09]	3.93 [0.02–64.34]	0.62	0.72
Bacteroidetes	6.87 [0.14–39.08]	6.13 [0.21–38.29]	0.64	0.72
Proteobacteria	1.77 [0.02–15.45]	0.87 [0.05–16.49]	0.23	0.71
Campilobacterota	0.10 [5e ⁻⁴ –2.69]	0.16 [3.9e ⁻³ –4.27]	0.74	0.74
Deinococcus-Thermus	0 [0–0.01]	0 [0–1.7e ⁻³]	0.47	0.72
Cyanobacteria/Chloroplast	6.2e ⁻³ [0–0.06]	0.01 [0–0.26]	0.52	0.72
Verrucomicrobia	2e ⁻³ [0–0.07]	0.01 [0–0.12]	0.24	0.71

Comparisons were made between the baseline and exit time-points for each diet group. As data was presented as non-parametric alpha-diversity indices between diet group at each timepoint are presented as median and interquartile range [minimum and maximum].

¹ PLANT, plant-based diet.

² MEAT, animal-based diet.

³ FDR, false discovery rate.

^a Coefficient of correlation significant at $p < 0.05$.

^b Denotes higher relative abundance in the PLANT group compared to the MEAT group.

^c Denotes lower relative abundance in the PLANT group compared to the MEAT group.

^d Denotes a decrease in relative abundance over time.

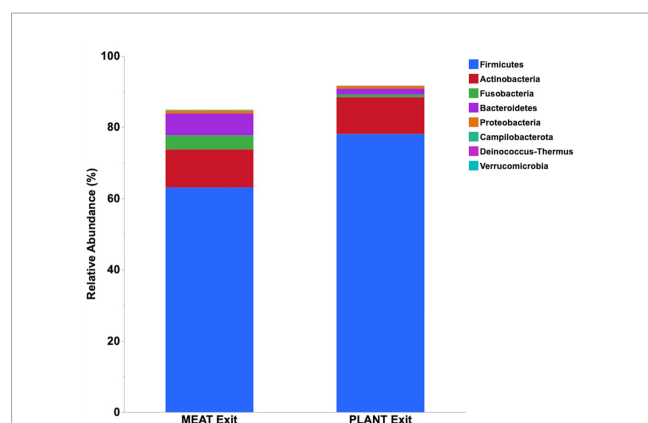


FIGURE 2

Comparison of median relative abundance of the main phyla identified in feces 47 healthy adult client-owned dogs fed an experimental plant-based (PLANT, $n = 27$) or commercial animal-based (MEAT, $n = 20$) extruded diet in a 12-week randomized, double-blinded longitudinal study. Comparisons were made after the dogs were exclusively fed either the experimental PLANT diet or commercial MEAT diet for 3 months.

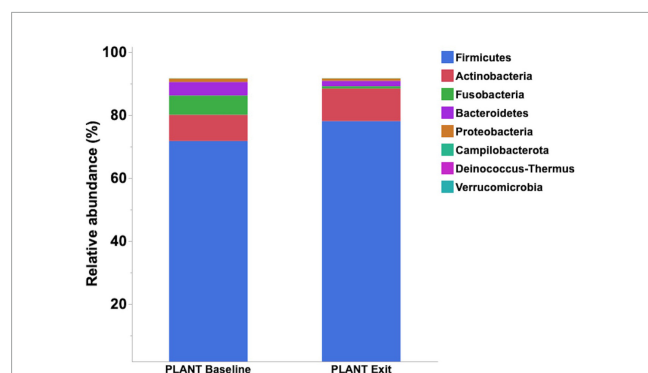


FIGURE 3

Comparison of median relative abundance of the main phyla identified in feces of 47 healthy adult client-owned dogs fed an experimental plant-based (PLANT, $n = 27$) or commercial animal-based (MEAT, $n = 20$) extruded diet in a 12-week randomized, double-blinded longitudinal study. Comparisons were made after the dogs were exclusively fed either the experimental PLANT diet or commercial MEAT diet for 3 months.

to ensure nutrient content was on target. Other commercially available vegan diets may not provide the same nutrient profile which could affect the gut microbiota composition. Lastly, COVID-19 lockdowns introduced some variability into the duration of the diet trial. Dogs were in various stages of the study (adaptation or experimental) when data collection was forcibly paused during the global pandemic. This limitation was unacceptable however, was taken into account during statistical analysis and the authors feel that the variation in trial duration did not influence the results.

Conclusion

The current study showed that feeding an entirely plant-based or vegan diet for 12 weeks to healthy dogs has some potential to change the composition of the canine fecal microbiota, but these

changes were not as dramatic or as distinct as those reported in humans. Future microbiota research investigating vegan dog food should consider the effects of different nutrient profiles as well as length of feeding. Moreover, further research is warranted to assess the metabolic function of the microbiota through fecal metabolomics and metagenomics in response to vegan dog foods, in addition to the fecal microbial composition and structural effects examined in the present study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Borealis (the Universities Repository), <https://doi.org/10.5683/SP3/VOELA6>.

Ethics statement

The animal studies were approved by the University of Guelph Animal Care Committee (AUP#4192) and the Research Ethics Board (Research Ethics Approval Number 19-02-036), and were in accordance with institutional, provincial, and national guidelines for the care and use of animals and humans participating in research. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BL: Writing – original draft. DG: Writing – review & editing. SD: Writing – review & editing. JM: Writing – review & editing. JA: Writing – review & editing. SB: Writing – review & editing. AV: Writing – review & editing.

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Conflict of interest

SD discloses they are the owner/operator of Dodd Veterinary Services (Dodd Veterinary Services, East Garafraxa, ON, Canada), providing veterinary nutritional consultation services to pet owners and within the pet food industry, and hold scientific advisory positions with pet food companies. AV discloses they are the Royal Canin Veterinary Diets Endowed Chair in Canine and Feline Clinical Nutrition at the University of Guelph Ontario Veterinary College, have financial support from companies within the pet food industry and hold scientific advisory positions with pet food companies. JA was an employee of Petcurean Pet Nutrition Limited Partnership at the time of the study and is now employed by Archer-Daniels-Midland Co. (ADM).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1367493/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Flow chart of recruitment and enrollment of healthy adult client-owned dogs in a randomized, double-blinded longitudinal study investigation comparisons in fecal microbiota of an experimental plant-based (PLANT) versus a commercial animal-based (MEAT) extruded diet.

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Chi-Hsuan Sung,
Texas A&M University, United States
Christopher McMullen,
Feedlot Health Management Services Ltd.,
Canada

*CORRESPONDENCE

Moez Rhimi
✉ moez.rhimi@inrae.fr

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Gut microbiota in cats with inflammatory bowel disease and low-grade intestinal T-cell lymphoma

Amandine Drut^{1,2}, Héra Mkaouar¹, Aicha Kriaa¹,
Vincent Mariaule¹, Nizar Akermi¹, Tristan Méric^{1,2},
Odile Sénécat², Emmanuelle Maguin¹, Juan Hernandez^{1,2} and
Moez Rhimi^{1*}

¹MIHA Team, INRAE, AgroParisTech, Micalis Institute, Université Paris-Saclay, Jouy-en-Josas, France,

²Oniris VetAgroBio Nantes, Université de Nantes, Nantes, France

In cats and humans, several physiological and environmental factors have been shown to alter the gut microbiota of healthy individuals. Cats share several diseases with humans such as inflammatory bowel diseases and low-grade intestinal T-cell lymphoma. The physiopathology of these chronic enteropathies is poorly understood but may involve disequilibrium of the gut microbiota composition and disruption of normal microbiome activity profiles. These disorders are increasingly diagnosed in the feline species due to improved medicalization and easier access to endoscopy in veterinary practice. This review addresses the current data on the gut microbiota of cats in health and in chronic enteropathies. Such functional analysis will help the advancement of innovative diagnostic tools and targeted therapeutic strategies.

KEYWORDS

cat, inflammatory bowel diseases, low-grade intestinal T-cell lymphoma, gut microbiota, chronic enteropathies

1 Introduction

Domestic cats, residing in 29% of North American households in 2022, hold a distinguished place as beloved pets (Scott Nolen, 2022). Their owners maintain elevated expectations for their healthcare. In feline medicine, chronic enteropathies (CE), comprising Inflammatory Bowel Diseases (IBD) and Low-Grade Intestinal T-Cell Lymphoma (LGITL), are major concerns with increasingly high incidence (Marsilio, 2021). Both incurable diseases display challenging diagnosis and available treatments have significant adverse effects (Marsilio, 2021). Moreover, LGITL is thought to evolve toward higher-grade lymphoma despite appropriate treatment in some cats (Wright et al., 2019). Therefore, there is still a need to understand the pathophysiology of feline CE to set out new therapeutic strategies. Many spontaneous feline diseases have their counterpart in human medicine, which highlights their interest in comparative pathology. Recently, LGITL has been described as an interesting model of indolent T-cell lymphoproliferative disorder of the gastrointestinal tract (ITLPD-GI) in humans (Freiche et al., 2021). Advances in this biomedical field are likely to benefit to feline patients but also to provide new insights into human diseases.

Like humans, the digestive tract of cats hosts a myriad of microorganisms whose links with physiology and health have been established. In humans, the gut microbiota interacts with the immune system and has been identified as a major player in the pathogenesis of IBD, comprising Crohn’s disease (CD) and ulcerative colitis (UC), and various gastrointestinal neoplasms including gastric mucosa-associated lymphoid tissue lymphoma (Nakamura et al., 1998; Nishida et al., 2018). Pet cats share the domestic environment of their owners and are therefore exposed to the same environmental factors. Additionally, they are in daily contact with their owners and share resting and cooking areas, which may promote the fecal-oral transmission of commensals and enteropathogenic bacteria in both directions, as suspected with *Clostridioides difficile* (Alves et al., 2022). Cat ownership has been shown to induce bacterial fluxes in humans and influence owners’ gut microbiota composition and function (Du et al., 2021).

The domestic cat (*Felis silvestris catus*) is a strict carnivore but can also digest, absorb and metabolize dietary carbohydrates. Despite this major difference with the human dietary regimen, feline models remain relevant for the study of the gut microbiota and intestinal health in humans, regarding the wide variation in meat consumption in human populations and the possible association between excessive intake of red meat or overprocessed meat and colorectal neoplasms (Farvid et al., 2021). The special dietary requirements of cats impact the profile of their core gut microbiome (Ganz et al., 2022). Several recent studies have focused on the composition of the feline gut microbiota in health and CE (Garraway et al., 2018; Marsilio et al., 2019; Ganz et al., 2022). Therefore, we aim in this review to discuss the links between gut microbiota, intestinal inflammation and the development of IBD and LGITL in cats, in order to highlight the gaps in knowledge and propose future research perspectives.

2 Gut microbiota in healthy cats

The cat’s gut microbiota is teeming with a variety of microorganisms including bacteria, fungi, viruses, and protozoa. Bacteria overwhelmingly dominate the cats’ microbiota, comprising roughly 98% of the total population and playing crucial roles in maintaining host health (Pilla and Suchodolski, 2021). The role of the fungal population, or mycobiota, and its contribution to health and

disease in the feline gut remains elusive (Tay et al., 2022), as well as the implication of protozoa and viruses in the microbiota community.

When examining the bacterial composition, several studies of the feline gut microbiota have identified five phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* (Ritchie et al., 2008, 2010; Tun et al., 2012; Ganz et al., 2022). Two additional studies recovered only four of these bacterial phyla and failed to detect *Fusobacteria* and *Proteobacteria*, respectively (Desai et al., 2009; Handl et al., 2011). The five bacterial phyla identified in cats are shared with the core microbiome of humans and pet dogs (Arumugam et al., 2011; You and Kim, 2021). In cats, two studies have identified the *Bacteroidetes*/*Chlorobi* group as prominent (Tun et al., 2012; Ganz et al., 2022), with *Bacteroides* species such as *B. vulgatus*, *B. thetaiotaomicron*, *B. fragilis* and *Porphyromonas gingivalis* being the most common and widespread (Tun et al., 2012). *Firmicutes* also prevail (Ritchie et al., 2008, 2010; Desai et al., 2009; Handl et al., 2011), with *Clostridia* being the most abundant class, along with *Erysipelotrichia*, *Negativicutes* (Ganz et al., 2022), *Bacilli*, *Mollicutes*, and *Lactobacillales* (Tun et al., 2012). *Lactobacillales* comprise the most important probiotic bacteria of the cat, dog and human gut microbiome, and has great beneficial effects on their intestinal health (Strompfov et al., 2017; Fusi et al., 2019; Rastogi and Singh, 2022). In the widest study aimed at establishing a comprehensive dataset for the “core microbiota” in 161 North American domestic cats, the taxonomic analysis identified 30 major genera belonging to the five predominant phyla listed above (Ganz et al., 2022). Detected genera were noted in over 55% of the healthy cat population and comprised *Prevotella*, *Bacteroides*, *Collinsella*, *Catenibacterium*, *Blautia*, *Faecalibacterium* and *Megasphaera* which displayed the highest relative abundance. Of these genera, several feline gut microorganisms were associated with short-chain fatty acid (SCFAs; e.g., acetate, butyrate, and propionate) synthesis, such as *Faecalibacterium* spp. (Ganz et al., 2022). SCFAs represent the main metabolites produced by gut microbiota fermentation of dietary fibers and non-digestible carbohydrates. They serve as key energy sources for the host epithelium, improve motility, and exert immunomodulatory and anti-inflammatory effects (Yao et al., 2022).

Discrepancies between studies are noticeable and may be partly attributable to various methodologies (Table 1). Most studies rely on sequence-based methods whose results may differ depending on the choice of bacterial targets, such as 16S rRNA and groEL (or *cpn60*) gene sequences. Both genes have been shown to be relevant barcodes for bacteria, with groEL providing a more robust target for

TABLE 1 Methodology of studies assessing the gut microbiota composition in healthy adult cats.

Study	Animals	Samples	Microbiota analysis method
Desai et al. (2009)	9 pet cats	Feces	Cpn60 gene sequencing
Ganz et al. (2022)	161 pet cats	Feces	16S rRNA gene sequencing Universal Primers 505F/816R
Handl et al. (2011)	12 pet cats	Feces	16S rRNA gene pyrosequencing Universal primers 28F/519R
Ritchie et al. (2008)	5 colony cats	Intestinal fragments	16S rRNA gene sequencing Primers 342F/786R
Ritchie et al. (2010)	27 pet cats	Feces	16S rRNA gene sequencing Universal primers 341F/786R + Group-specific primers for <i>Bifidobacterium</i> and <i>Lactobacillus</i> spp.
Tun et al. (2012)	5 pet cats	Feces	Shotgun 454-pyrosequencing

species-level identification (Links et al., 2012). Within the numerous studies using 16S rRNA gene sequencing, various amplicon primers, sequencing technologies and bioinformatics tools are also susceptible to impact the assessment of microbial composition (Clooney et al., 2016; O'Sullivan et al., 2021). Of note, group-specific primers were more effective than universal bacterial primers for detection of *Bifidobacterium* and *Lactobacillus* spp. in the feces of healthy pet cats (Ritchie et al., 2010). More advanced techniques such as shotgun metagenomic sequencing are better able to detect less abundant taxa in bacterial communities, and the genera missed by sequence-based methods may carry biologically relevant function (Durazzi et al., 2021). The shotgun metagenomic approach has only been performed in five client-owned healthy cats, thus the representativeness of these results for the overall pet cat population is questionable (Tun et al., 2012). A dysbiosis index has been established to evaluate the balance of intestinal microbiota by measuring the fecal abundances of *Bacteroides*, *Bifidobacterium*, *Clostridium hiranonis*, *Escherichia coli*, *Faecalibacterium*, *Streptococcus*, and *Turicibacter* by quantitative PCR techniques (Sung et al., 2022). It provides a targeted assessment of these bacterial groups that have been determined to be frequently altered in chronic enteropathies or after antibiotic therapies. Thus it depicts the extent of intestinal dysbiosis, and the abundance of *Clostridium hiranonis* also predicts the ability of the intestinal microbiota to convert primary bile acids (Sung et al., 2022). It is now available in routine clinical practice and is also frequently used in the research setting, although it fails to provide in-depth description of the composition of the fecal microbiota. Besides biases introduced by methodological differences, batch effect correction is rarely addressed in the analysis of the gut microbiota from healthy cats. Regarding sample collection and storage, most studies assessed the fecal bacterial community and some surveys were based on the collection and mailing of samples by owners, with risks of inconsistent technical lead times (Ganz et al., 2022). There is a need to reinforce the robustness of the data produced by defining pre-analytical and analytical standards. The feline species lacks a reference gut microbiome gene catalog established by whole genome metagenomics studies, as described in humans and pet dogs (Coelho et al., 2018; Kim et al., 2021). This is a major shortcoming for taxonomic assignment and functional characterization of the healthy feline gut microbiota. It is worth noting that gut microbes are far from being inert and are constantly evolving in response to the different changes in environment and physiology. While such a community plays an essential role in cats' health, it still can be influenced by many factors including age, dietary habits and exposure to xenobiotics. In this regard, several studies assessed the effect of age on the gut microbiota composition and provided inconsistent results, likely caused by different methodologies, including bacterial culture, 16S rRNA gene sequencing and shotgun sequencing technologies conducted on the feces of colony or client-owned cats (Deusch et al., 2015; Masuoka et al., 2017; Bermingham et al., 2018; Li et al., 2022). For example, the shifts in abundances of *Bifidobacteria* and *Lactobacillus* varied, but both bacterial genera were not found to be predominant in the feline gut microbiota, unlike dogs and humans (Masuoka et al., 2017; Bermingham et al., 2018). Furthermore, the impact of macronutrient composition of pet foods on gut microbiota is a current area of interest. Regarding the effects of dietary complex carbohydrates, two studies conducted on four experimental and 10 privately-owned healthy adult cats respectively, failed to identify significant shifts in

microbial communities when adding dietary fiber to the diet (i.e., cellulose, fructo-oligosaccharides (FOS), pectin, and inulin) and showed high inter-individual variability in the response to nutritional modifications (Barry et al., 2012; Garcia-Mazcorro et al., 2017). In a separate study involving eight experimental cats, adding dietary supplements of short-chain FOS, galacto-oligosaccharides (GOS) or a combination of both prebiotics resulted in increased levels of cultured *Bifidobacterium* spp. (Kanakupt et al., 2011), which is known to be associated with gut health (O'Callaghan and van Sinderen, 2016). Cats consuming the diet enriched with mixed short-chain FOS and GOS showed significantly greater fecal concentrations of butyrate and valerate, and a trend toward greater fecal concentrations of acetate (Kanakupt et al., 2011). Of note, *Bifidobacteria* have been shown to produce acetate, thus being involved in the SCFAs-mediated favorable effects on host gastrointestinal health (Fukuda et al., 2011). This experimental dietary intervention mimics the supplementation with prebiotic fibers that may be implemented in pet cats suffering from gastrointestinal signs. On the other hand, feeding 12 experimental cats with a raw meat-based diet was found to raise the levels of *Clostridium* and *Fusobacterium* in their feces (Butowski et al., 2019). Of note, *Fusobacterium* has been incriminated in the development of cancer in cats and humans (Kostic et al., 2012; Garraway et al., 2018). Addition of plant-based fiber to this high-protein diet induced the predominance of *Prevotella* (similarly to a control industrial pet food) and a group of unclassified *Peptostreptococcaceae* (Butowski et al., 2019). Available data report that *Peptostreptococcaceae* have been involved in the development of various human infections within the digestive tract (Tannock et al., 2012; Wei et al., 2019). However, this bacterial family has been shown to be prominent in eight experimental healthy cats, particularly when fed canned diets, and does not seem to exhibit deleterious effects (Bermingham et al., 2018). Most of the aforementioned dietary interventions have been conducted on small numbers of experimental animals and relied on sequence-based methodologies to assess the gut microbiota. Further studies should be carried out in larger populations of pet cats, and involve the use of whole-genome shotgun sequencing with functional analyses to ensure the consistency and the relevance of these findings. Recent evidence has also shown that lifestyle influences the feline gut mycobiota composition, with indoor cats displaying more *Ascomycota*, while outdoor cats show more *Basidiomycota*. Nevertheless, the presence of the genus *Peniophorella* has been observed in both indoor and outdoor cats and stands out as the dominant component of the mycobiota (Tay et al., 2022). Understanding the influence of different factors on microbial communities would be a prerequisite to better decipher the molecular mechanisms involved in the symbiosis that marks holobiont health (Masuoka et al., 2017; Whittemore et al., 2019; Pilla and Suchodolski, 2021). The dysbiosis index has been shown to remain stable over 2 months in 17 indoor adult pet cats exempt from any living or dietary changes throughout the follow-up period, whereas it showed large shifts in eight client-owned cats receiving antibiotics and in 68 client-owned cats with IBD (Sung et al., 2024). Similar prospective studies including monitoring of the dysbiosis index and deeper assessment of the gut microbiota composition and metabolic activities during nutritional interventions would be desirable to document the impact of physiological factors, that are thought to be minimal when compared with dysbiotic alterations associated with pathological states.

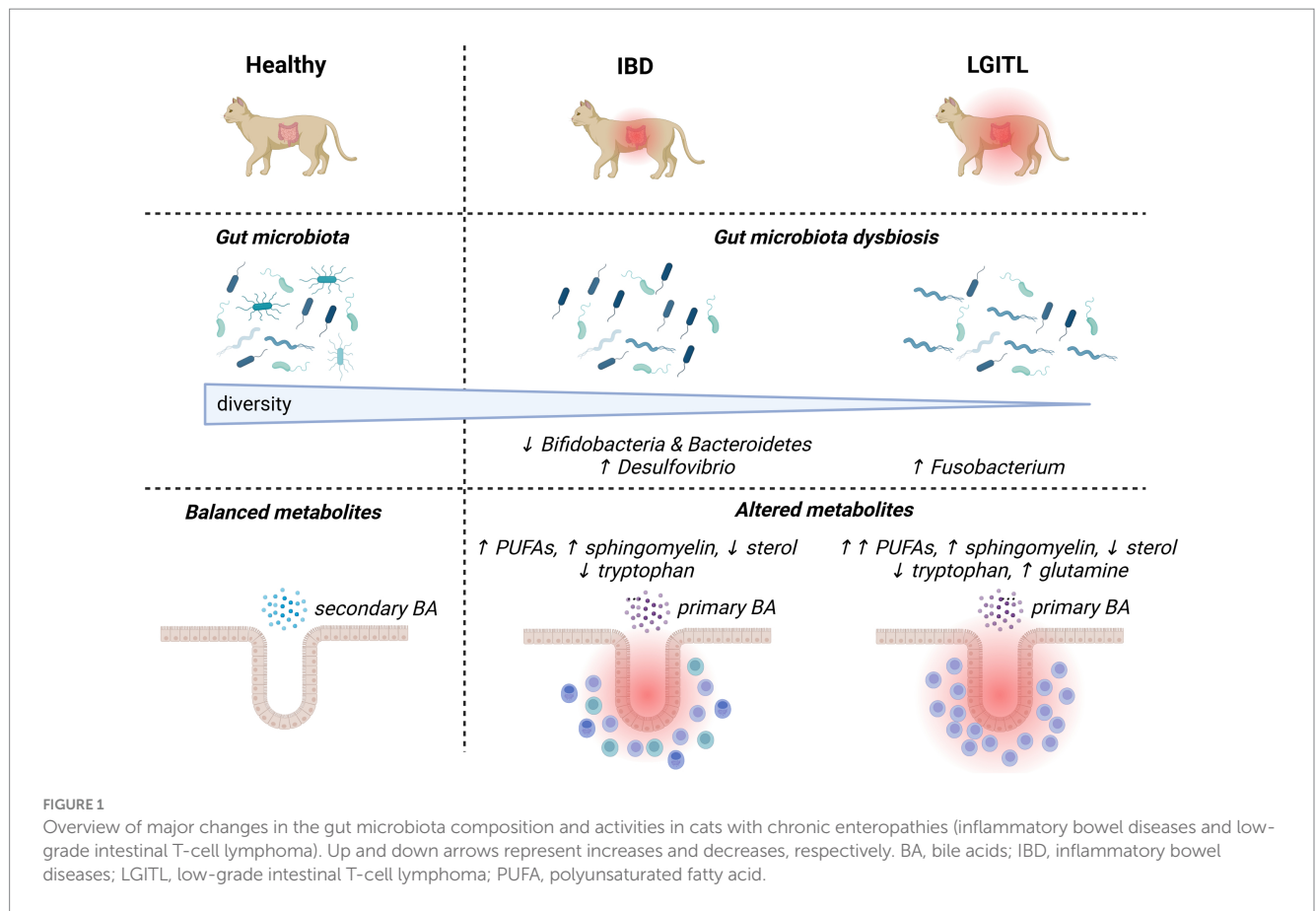
3 Gut microbiota in feline spontaneous IBD and LGITL

Chronic inflammation is thought to arise in genetically susceptible individuals, due to complex interactions between the intestinal immune system, exposome factors (mainly dietary components) and the microbiome (Marsilio, 2021). In populations of pet cats with chronic gastrointestinal signs (i.e., vomiting and/or diarrhea of at least 2–3 weeks' duration) that did not benefit from exhaustive characterization, bacterial diversity of the fecal microbiota was shown to be decreased when compared with healthy individuals (Suchodolski et al., 2015; Kathrani et al., 2022). The fecal microbiota of 15 partially characterized colony cats with chronic diarrhea, in whom only extraintestinal and infectious/parasitic disorders were ruled out, was predominantly composed of bacteria belonging to the phyla Firmicutes (with prevailing classes *Bacilli* and *Clostridia*), Bacteroidetes (with prevailing classes *Bacteroidia* and *Flavobacteria*), Fusobacteria, Proteobacteria, Tenericutes and Actinobacteria, in decreasing order (Ramadan et al., 2014). Of note, Tenericutes are not major components of the healthy feline gut microbiota and are predominantly commensals or obligate parasites in humans and domestic animals. They are primarily known for their Mollicutes clade, housing opportunistically pathogenic genera like *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* (Trachtenberg, 2005). Alterations in microbial communities from the phylum Firmicutes have been further described in comparison to healthy cats: pet cats with chronic gastrointestinal signs exhibited greater abundance of bacteria from the class *Erysipelotrichia* and the genera *Lactobacillus* and *Clostridium*, and a decrease in the genus *Faecalibacterium* (Suchodolski et al., 2015; Kathrani et al., 2022). In humans, bacteria from the *Erysipelotrichiaceae* family have been correlated with gastrointestinal inflammation and metabolic disorders (Labbé et al., 2014). *Faecalibacterium* spp. are also commonly decreased in fecal and mucosal samples from human patients with IBD. For instance, *Faecalibacterium prausnitzii*, a strain that has been shown to produce butyrate and exert anti-inflammatory properties, is commonly described as a general health biomarker (Cao et al., 2014). Functionally, qualitative changes in the fecal microbiota of cats with chronic gastrointestinal signs were associated with significant alterations in bacterial gene contents referring to the metabolism of carbohydrates, vitamins, amino acids and xenobiotics (Suchodolski et al., 2015). All these results should be treated with caution, because a more comprehensive characterization of these studied feline populations would be highly desirable, with exhaustive exclusion of extraintestinal illnesses, infectious or parasitic diseases and focal gastrointestinal disorders, with final demonstration of inflammatory or low-grade lymphomatous intestinal infiltration.

Regarding the fecal microbiota of privately-owned cats with histologically confirmed IBD, dysbiotic alterations associated with the disease (Figure 1) are less documented than in humans and dogs. In feline IBD, overall bacterial diversity was shown to be decreased in comparison to healthy cats, but specific patterns of dysbiosis could not be identified in these 13 privately-owned diseased cats (Marsilio et al., 2019). Increased populations of mucosa-associated bacteria, assessed by a more targeted fluorescence *in situ* hybridization (FISH) approach, have been associated with clinical disease activity and duodenal inflammation evaluated by histopathology and cytokine mRNA profiles (Janeczko et al., 2008). Another molecular-based enumeration study using FISH in feline feces showed increased

numbers of sulfate-reducing bacteria from the genus *Desulfovibrio* spp., and decreased numbers of bacteria from the genera *Bifidobacteria* spp. and *Bacteroidetes* spp. in colony cats with IBD when compared with healthy colony cats (Inness et al., 2007). However, studies using advanced techniques for in-depth assessment of the gut microbiota in sufficiently large populations of cats with IBD are still lacking. From a functional standpoint, untargeted metabolomic analysis found that metabolic changes identified in cats with IBD were similar to humans and other animal models with IBD. They comprised increased fecal amino-acids consistent with malabsorption, increased fecal arachidonate, omega-3 fatty acids and simple sphingolipids possibly accompanying intestinal inflammation, and decreased fecal indole derivatives attributed to intestinal dysbiosis (Marsilio et al., 2021).

A pathogenic theory that currently prevails in cats is that of a continuum between IBD and LGITL, given the common coexistence of inflammatory and lymphomatous infiltrations, and frequent previous history of IBD in animals with LGITL (Marsilio et al., 2023). Cats with LGITL have been shown to exhibit lower fecal bacterial diversity than healthy cats, and a trend toward lower alpha-diversity than cats with IBD based on 16S rRNA gene sequencing (Figure 1) (Marsilio et al., 2019). This study comparing feces from 14 pet cats with LGITL with feces from 38 privately-owned healthy cats and 13 pet cats with IBD failed to identify significant differences in microbial communities between groups, probably due to low numbers of animals (Marsilio et al., 2019). Targeted bacterial quantification based on FISH in endoscopic or laparoscopic gastrointestinal biopsies identified increased numbers of mucosa-associated *Fusobacterium* spp. and *Bacteroides* spp. in the ileum and increased numbers of mucosa-associated *Fusobacterium* spp. in the colon of 14 pet cats with histologically confirmed LGITL when compared with 14 pet cats with histologic IBD (Garraway et al., 2018). The abundance of *Fusobacteria* spp. in adherent mucus showed the highest correlation with the number of CD11b⁺ myeloid cells and with the up-regulation of NF- κ B expression in the gastrointestinal mucosa of cats with LGITL, when compared with cats with IBD (Garraway et al., 2018). A more recent study based on 16S rRNA gene sequencing also identified increased abundance of bacteria from the *Fusobacteriaceae* family in seven pet cats with histologically diagnosed LGITL compared with 13 pet cats with histologic IBD (Benvenuti et al., 2024). Taken together, these findings open the door to a possible implication of *Fusobacterium* spp. in the carcinogenesis of LGITL. Of note, *Fusobacterium* has also been incriminated in carcinogenesis in humans with colorectal neoplasia (Kostic et al., 2012). Functional alterations related to dysbiosis were found to be more severe in 11 pet cats with LGITL than in 11 pet cats with IBD, and fecal concentrations of polyunsaturated fatty acids were shown to discriminate between both types of feline CE (Marsilio et al., 2021). Lower fecal concentrations of indolelactate, a microbial indole catabolite of tryptophan, were also recovered in 31 cats with LGITL when compared with 44 cats with IBD (Barko et al., 2023). These data being scarce, further studies are required to determine whether some characteristics of the fecal microbiota and its metabolic activities may predict the diagnosis of feline IBD or LGITL, and improve therapeutic interventions. In humans, the ITPD-GI is a recently described entity recognized as a low-grade, clonal, T-cell lymphoproliferative neoplasm arising in the digestive tract (Sanguedolce et al., 2021). It is a rare disease with a protracted clinical course, whose diagnosis is particularly challenging due to heterogeneous histological and



molecular features. The relationship between IBD and ITPD-GI in humans remains unclear and no data are available regarding the implication of gut microbiota in the development of ITPD-GI. LGITL being a frequent disorder in cats, studying its pathogenesis may provide new insights into the pathogenesis of human ITPD-GI.

As a whole, the state of knowledge concerning alterations in the intestinal microbiota of cats with CE remains insufficient. Studies are frequently underpowered with small populations of spontaneously ill privately-owned cats (Table 2). Thus, cats with histologically confirmed IBD and LGITL are sometimes grouped together into a broader group of cats with CE to characterize the gut microbiota composition in comparison with healthy cats. A 16S rRNA gene sequencing approach identified a distinct pattern of dysbiosis in 27 pet cats with CE, with increased abundances of facultative anaerobes from the *Enterobacteriaceae* and *Streptococcaceae* families, and decreased abundances of obligate anaerobic members of the phyla Firmicutes (*Ruminococcaceae* and *Turicibacteraceae* families), Actinobacteria (*Bifidobacterium* genus) and Bacteroidetes (*Bacteroides plebius*) (Marsilio et al., 2019). A similar study conducted in 16 pet cats with histologically confirmed CE recovered increased abundances of the phylum Proteobacteria, the orders Enterobacterales and Lactobacillales, the family *Enterobacteriaceae* and the genus *Escherichia Shigella*, and decreased abundances of the phylum Bacteroidetes and the order Peptococcales when compared with 14 privately-owned healthy pet cats (Miller et al., 2023). Some of these shifts are similar to those identified by more robust studies conducted in humans with IBD, such as decreased abundances of obligate anaerobes from the Firmicutes and Bacteroidetes phyla (Frank et al.,

2007), and increased abundance of facultative anaerobes from the *Enterobacteriaceae* family (Khorsand et al., 2022). Regarding more targeted molecular methods, the dysbiosis index based on the quantification of the abundances of seven select bacterial groups was able to discriminate between groups of 68 cats with CE and 80 healthy pet cats, with 76% of cats with CE presenting increased dysbiosis index (Sung et al., 2022). This biomarker has the advantage of being accessible for the diagnosis of dysbiosis in routine clinical practice. However, it provides partial assessment of the gut microbiota composition and does not bring better insights into the interactions between gut microbiota and feline CE. Metabolic alterations resembling those described in human IBD and relating to tryptophan, arachidonic acid, glutathione and lipids have also been underlined in pet cats with CE (Marsilio et al., 2021; Barko et al., 2023; Miller et al., 2023; Sung et al., 2023). Additional studies are mandatory to relate gut microbiota compositions and metabolic alterations.

4 Modulation of the gut microbiota in cats with CE

Interventional dietary trials (unspecified composition) in 15 experimental cats with chronic diarrhea showed significant correlations between improvement of fecal consistency (assessed by fecal score) and fecal bacterial abundance of the phyla Actinobacteria (i.e., genus *Slackia* and *Collinsella*), Proteobacteria (i.e., *Campylobacter upsaliensis*, the genus *Raoultella*, and unidentified genus of the family Succinivibrionaceae), and Firmicutes (unidentified genus of the family

TABLE 2 Methodology of studies assessing the gut microbiota composition in cats with suspected or confirmed chronic enteropathies.

Study	Animals	Samples	Microbiota analysis method
Benvenuti et al. (2024)	13 pet cats with IBD; 7 pet cats with LGITL	Feces	16S rRNA gene sequencing Primers for V3-V4 regions (341F/805R)
Garraway et al. (2018)	14 pet cats with IBD; 14 pet cats with LGITL	Intestinal biopsies	Fluorescence <i>in situ</i> hybridization Universal bacterial probe Specific probes (<i>Clostridium</i> spp., <i>Bacteroides</i> /Prevotella group, <i>Fusobacterium</i> spp., Enterobacteriaceae, <i>Helicobacter</i> spp., <i>Faecalibacterium</i> spp.)
Inness et al. (2007)	11 colony cats with IBD; 34 healthy colony cats	Feces	Fluorescence <i>in situ</i> hybridization Specific probes (<i>Bifidobacterium</i> spp., <i>Bacteroides</i> spp., <i>C. histolyticum</i> subgp., <i>Lactobacillus</i> - <i>Enterococcus</i> subgp., <i>Desulfovibrio</i> spp.)
Janeczko et al. (2008)	17 pet cats with IBD; 10 healthy colony cats	Intestinal biopsies	Fluorescence <i>in situ</i> hybridization Universal bacterial probe Specific probes (<i>Clostridium</i> spp., <i>Bacteroides</i> /Prevotella group, Enterobacteriaceae, <i>E. coli</i> , <i>Streptococcus</i> spp., <i>Helicobacter</i> spp.)
Kathrani et al. (2022)	42 pet cats with suspected or confirmed CE; 14 healthy pet cats	Feces	16S rRNA gene sequencing Primers for V3-V4 regions
Marsilio et al. (2019)	13 pet cats with IBD; 14 pet cats with LGITL; 38 healthy pet cats	Feces	16S rRNA gene sequencing Primers for the V4 region (515F/806R)
Miller et al. (2023)	6 pet cats with IBD; 6 pet cats with LGITL; 6 pet cats with uncharacterized CE; 14 healthy pet cats	Feces	16S rRNA gene sequencing Primers for V3-V4 regions
Ramadan et al. (2014)	15 colony cats with uncharacterized chronic diarrhea	Feces	16S rRNA gene pyrosequencing Primers for the V1-V2 region
Suchodolski et al. (2015)	29 pet cats with uncharacterized chronic diarrhea; 21 healthy pet cats	Feces	16S rRNA gene sequencing Primers for the V4 region (515F/806R)
Sung et al. (2022)	68 pet cats with IBD or LGITL; 80 healthy pet cats	Feces	Quantitative polymerase chain reaction Total bacteria + Specific groups (<i>Bacteroides</i> , <i>Bifidobacterium</i> , <i>E. coli</i> , <i>Faecalibacterium</i> , <i>Fusobacterium</i> , <i>Streptococcus</i> , <i>Turicibacter</i> , <i>Blautia</i> , <i>Clostridium hiranonis</i>)

CE, chronic enteropathy; IBD, histologically confirmed inflammatory bowel disease; LGITL, histologically confirmed low-grade intestinal T-cell lymphoma.

Lachnospiraceae) determined by 16S rRNA 454-pyrosequencing (Ramadan et al., 2014). Feeding a hydrolyzed protein diet to 36 client-owned cats with suspected or histologically confirmed CE induced a decrease in alpha-diversity and an increase in the abundance of *Bifidobacterium* assessed with a 16S rRNA sequencing approach (Kathrani et al., 2022). Various *Bifidobacteria* strains enhance intestinal barrier functions and regulate cytokine network, reducing therefore intestinal inflammation (Caviglia et al., 2020). Cats that did not respond to a hydrolyzed protein regimen showed higher baseline alpha-diversity and increased abundance of Oscillobacter and Desulfovibrionaceae than responders (Kathrani et al., 2022).

Data regarding the use of probiotics in cats with CE are scarce. In a trial conducted on eight pet cats from the same household suffering from chronic diarrhea, administration of *Bacillus licheniformis*-fermented products improved fecal scores and decreased feline chronic enteropathy activity indexes in some individuals (Lee et al., 2022). Changes in the bacterial composition of the gut microbiota were also described, with a decrease in the abundance of *Clostridium perfringens* and an increase in the abundance of *Blautia* spp., *Ruminococcus torques* and *Ruminococcus gnavus* (Lee et al., 2022). Oral fecal microbiota transplant capsules were prospectively administered to 46 pet cats with chronic gastrointestinal signs, leading to partial stool donor bacterial engraftment (Rojas et al., 2023). The fecal microbiota of responders tended to become more similar to the fecal microbiota of healthy cats (Rojas et al., 2023). All these data

determined by 16S rRNA sequencing techniques must be interpreted cautiously because the underlying disorders were not fully investigated and the evolution of gastrointestinal inflammation was not assessed in these populations of cats with suspected CE.

5 Conclusion and future prospects

Chronic enteropathies are of increasing interest in companion animals, including in cats, given their challenging diagnosis and multifactorial nature. Recent evidence has questioned the role of the dysbiotic microbiota in such diseases. Still, only a few data are available at this point and it remains a field in its infancy. Characterization of the feline gut microbiota mainly relies on sequence-based approaches and lack more comprehensive evaluation of its composition and activities with deeper shotgun metagenomics studies. Thus, reference data regarding the gut microbiota of healthy cats are lacking. Studies of gut microbiota alterations in cats spontaneously suffering from CE rarely include a sufficient number of histologically confirmed cases of IBD or LGITL. Regarding therapeutic perspectives, microbiota modulation trials are frequently conducted on small populations of experimental animals and fail to sufficiently document beneficial effects on the pathological process of CE. Accordingly, it is now imperative to bear a clear picture of the relevance of the different contributing factors in the development of

feline CE and to gain a better mechanistic understanding of microbiota-host interactions in order to open the way to a more individualized medicine.

Author Contributions

AD: Validation, Writing – original draft, Writing – review & editing. HM: Writing – original draft, Writing – review & editing, Formal Analysis. AK: Writing – original draft, Writing – review & editing, Formal Analysis. VM: Writing – review & editing, Visualization. NA: Writing – review & editing. TM: Writing – review & editing. OS: Writing – review & editing. EM: Writing – review & editing, Conceptualization. JH: Writing – review & editing, Funding acquisition, Writing – original draft. MR: Conceptualization, Writing – review & editing, Funding acquisition, Investigation, Validation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Yun Ji,
China Agricultural University, China
Xiaohua Teng,
Northeast Agricultural University, China

*CORRESPONDENCE

Guangyu Li
✉ tcslgy@126.com

[†]These authors have contributed equally to this work

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Genomic analysis and functional properties of *Lactobacillus johnsonii* GJ231 isolated from healthy beagles

Mengdi Zhao^{1,2†}, Yueyao Li^{1†}, Yuanyuan Zhang¹ and Guangyu Li^{1*}

¹College of Animal Science and Technology, Qingdao Agricultural University, Qingdao, China,

²College of Animal Science and Technology, Jilin Agricultural University, Changchun, China

Probiotics are one of the management tools to improve the host's healthy microbiota. The positive effects of probiotics on host health are species-specific, so probiotics isolated from host's own gut may be most beneficial. Many of the metabolites (e.g., short-chain fatty acids, bacteriocins, and hydrogen peroxide) produced by *Lactobacillus johnsonii* have specific inhibitory profiles against invading pathogens. In this study, we isolated *L. johnsonii* GJ231 from the intestinal tract of healthy female beagles. The genome size of 1.763 M encoded a total of 1,691 predicted genes. Many carbohydrate-active enzymes responsible for carbohydrate degradation and the production of short-chain fatty acids were also predicted. The metabolic profile of short-chain fatty acids in *L. johnsonii* GJ231 was determined using LC-MS/MS. The bacteriocin-producing gene bacteriocin (lactacin F) in *L. johnsonii* GJ231 was also predicted. *In vitro*, experiments demonstrated that GJ231 can thrive in weak acids, 0.3% bile salts, and artificial gastrointestinal fluid models. It was tolerant of to high temperatures up to 70°C, was non-hemolytic, inhibited pathogenic bacteria, and had a high antioxidant capacity. *In vivo* safety experiments conducted in mice revealed that oral administration of GJ231 not only had no toxic side effect but also increased their antioxidant capacity. In conclusion, combining the above test results, which collectively demonstrate that canine-derived *L. johnsonii* GJ231 was a non-pathogenic, acid-tolerant and bile-salt-tolerant probiotic strain that inhibits pathogenic bacteria and improves host antioxidant function. This may make it a promising candidate for the development of innovative functional foods for pets.

KEYWORDS

Lactobacillus johnsonii, beagle, whole genome sequencing, probiotic, functional food

1 Introduction

Dogs and cats have been companions to humans for thousands of years (Grześkowiak et al., 2015). Nowadays, pet owners consider their pets as family members, so the health of pets is a major concern (Redfern et al., 2017). The health of companion animals depends on their gut microbiota (Grześkowiak et al., 2015). Pet owners and veterinarians have typically used antibiotics to treat or prevent animal diseases. However, the use of antibiotics can lead to gut microbiota disruption, and overuse can lead to a rapid increase in antibiotic residue and resistance (Ma et al., 2021; Shao et al., 2021). Close contact between pets and their owners can lead to the transfer of bacterial resistance, resulting in harm to both parties as a result

(Scarborough et al., 2021; El-Razik et al., 2023). Therefore, there is a considerable need to develop new products to replace antibiotics.

In recent years, there has been a growing interest in the use of probiotics as an alternative to antibiotics for the prevention and treatment of bacterial infections, in particular *Lactobacillus* and *Bidobacterium* (Wan et al., 2019). Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Probiotics such as lactic acid bacteria (LAB) can inhibit the adhesion and activity of pathogenic bacteria, modulate immunity, improve intestinal barrier integrity, and produce organic acids as well as antimicrobial compounds (La Fata et al., 2018; Sanders et al., 2019). *Lactobacillus johnsonii*, a type of LAB, has been shown to improve antioxidant capacity, alleviate colitis, and improve hypercholesterolemia (Kim et al., 2006; Jia et al., 2022; Yoon et al., 2023).

Probiotic candidates isolated from host's own gut may be most effective, as the co-evolution of gut microbes with the host can better prepare the strain to colonize and thrive in the host's gastrointestinal tract (Garcia-Gutierrez et al., 2019; Johnson et al., 2023). A previous study reported that *L. johnsonii* strains were specific to their hosts (Buhnik-Rosenblau et al., 2012). Whole-genome sequencing, aided by the rapid advancement of sequencing technology, is becoming essential for studying microorganisms, presenting a new technique for investigating the probiotic potential and possible pathogenic features of microbes (Tyagi et al., 2022). In this study, we isolated *L. johnsonii* strain GJ231 from the gastrointestinal tract of healthy beagles. A comprehensive analysis of the probiotic potential and safety of strain GJ231 was conducted using whole-genome sequencing, along with *in vivo* and *in vitro* tests, demonstrating the strain's potential for use in companion animals.

2 Materials and methods

2.1 Sample collection

Animals were acquired from the Jimo Pet Base of Qingdao Agricultural University. Six well-grown and healthy 6-month-old female beagles with an average weight of 5.45 ± 0.36 kg were selected. All test animals were not given probiotics or antibiotics in the past 2 months. A cotton swab was dipped into a small amount of saline and used for sampling. Samples were promptly transported to the laboratory.

2.2 Strain isolation and identification

Experimental samples were subjected to gradient dilution (10^{-3} – 10^{-7}), and 50 μ L of each dilution was coated onto Man, Rogosa, and Sharpe (MRS) agar plates (Haibo, China). The plates were incubated inverted at 37°C for 48–72 h. Single colonies with lysogenic rings were selected and transferred to MRS broth. After 2–3 passages, glycerol (25.0%) was added as a preservative, and cultures were stored for future use. Bacterial DNA was extracted with a Bacterial Genome Kit (Tiangen, China). Subsequently, polymerase chain reaction (PCR) was performed using the primers 16S-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1492R (5'-TACGGCTACCTTGTTCAGACTT-3') (Wang et al., 2018) and

the resulting products were sent to Qingke Biotech (Beijing, China) for sequencing. The sequencing results were compared with other sequence data at National Center for Biotechnology Information, and a phylogenetic tree (Neighbor Joining) was reconstructed using Mega 7.0 (Sudhir Kumar, King Abdulaziz University, Saudi Arabia) (Kumar et al., 2016).

L. johnsonii GJ231 used in this study has been conserved at the China Center for Type Culture Collection (CCTCC M2023981).

2.3 Probiotic potential

2.3.1 Growth and acid production curves of strains

GJ231 cultured for 18–20 h was inoculated into MRS broth at 37°C with an inoculum amount of 2.5% (v/v) and then incubated for 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 36, or 48 h, respectively. The optical density at 600 nm (OD_{600}) was measured at each time point, while the pH value was determined at different time points using a pH detector (Shanghai INESA, China).

2.3.2 Antimicrobial ability

Cultures in the exhibiting stationary phase after 18 h of incubation (1×10^8 CFU/mL) and the bacterial suspension (BS) was centrifuged at 4°C, $10,621 \times g$ (10,000 rpm in Centrifuge 5430R) for 10 min. The cell-free supernatant (CFS) was collected, and the remaining bacterial pellet (BP) was resuspended in the same volume of PBS. Similarly, cultures of pathogenic bacteria (*Escherichia coli* ATCC 25922; *Staphylococcus aureus* ATCC 25923; *Salmonella enterica* subsp. *Enterica* serovar Typhimurium ATCC 14028; *Pseudomonas aeruginosa* ATCC 27853; *Listeria monocytogenes* ATCC 19115) in the stationary phase of growth were adjusted to a concentration approximately 1×10^7 CFU/mL. A two-layer method was adopted using Oxford cups on Tryptone Soy Agar (TSA) (Solarbio, China), with 100 μ L of CFS, BS, BP, or CFS_{7.0} (CFS was adjusted to pH 7.0) added in each well. The wells were then incubated at 37°C for 24 h, and the inhibition zone diameter (IZD) was determined.

2.3.3 High temperature tolerance

The method described by Zhao et al. (2023) was followed with minor modifications. Briefly, GJ231 bacterial solution cultured for 18–20 h was placed into a water bath heated to 37, 50, 60, 70, or 80°C for 5 min, respectively. Then was quickly transferred to ice for 30–60 s. The number of bacteria in 100 μ L of the solution was then calculated by plate counting. The survival rate was calculated using the following formula:

$$\text{Survivability (\%)} = T_{\text{treatment}}^1 / T_{\text{initial}}^1 \times 100\% \quad (1)$$

Where T_{initial}^1 and $T_{\text{treatment}}^1$ are the number of bacteria (log CFU/mL) before and after treatment, respectively.

2.3.4 Resistance to gastrointestinal environmental

The method described by Gilliland and Walker (1990) was followed with minor modifications. Briefly, GJ231 bacterial suspension cultured for 18–20 h were centrifuged at 4°C, $10,621 \times g$ for 10 min and the supernatant was discarded. The

bacterial cells were washed three times with PBS (pH 7.0). The cells were then resuspended in 0.1 and 0.3% (w/v) bile salt solutions, and the 0, 1, 2, and 4 h incubations were taken for counting using dilution plate counting method. Similarly, bacterial cultures from GJ231 were cultured until reaching the stationary phase, and samples were taken at 1.5 h or 2 h intervals after resuspension in gastric or intestinal fluids. The remaining steps were the same as those for pre-bile salt tolerance treatment. For the treatment with gastrointestinal fluid, samples were initially resuspended in gastric fluid for 1.5 h, followed by resuspension in intestinal fluid for 2 h before being sampled and counted. All cultures were incubated at 37°C, and survival rates were calculated using Equation 1.

2.3.5 Antioxidant test

Ascorbic acid was used as a positive reference and a DPPH free radical scavenging capacity assay kit (Solarbio, China) was operated. The absorbance was measured at 515 nm (Cheng et al., 2021). Calculated using Equation 2.

$$\text{scavenging activity}\% = \left[\frac{A_{\text{blank}}^1 - (A_{\text{sample}}^1 - A_{\text{control}}^1)}{A_{\text{blank}}^1} \right] \times 100\% \quad (2)$$

(2) A_{sample}^1 is the absorbance of the sample; A_{control}^1 is the absorbance of the mixture to sample and anhydrous ethanol; A_{blank}^1 is the absorbance of the mixture of the extract solution and working solution.

A working solution was made by mixing 7 mM ABTS and 2.45 mM potassium persulfate and after incubation for 12 h away from light. Add 20 μL of sample to 200 μL of working solution and mix well. Next, the reaction was carried out for 20 min at room temperature and protected from light, and the absorbance was measured at 734 nm. As a positive control, ascorbic acid was utilized. Calculated using Equation 3.

$$\text{scavenging activity}\% = \left(1 - \frac{A_{\text{sample}}^2 - A_{\text{control}}^2}{A_{\text{blank}}^2} \right) \times 100\% \quad (3)$$

(3) A_{sample}^2 is the absorbance of a mixture of sample and working solution; A_{control}^2 is the absorbance of the mixture of deionized water and sample; A_{blank}^2 is the absorbance of the mixture of deionized water and working solution.

O₂- scavenging capacity was determined using the Superoxide Anion Scavenging Capacity Kit (Solarbio, China). The OD value was measured at 560 nm. Calculated using Equation 4 (Zhao et al., 2023).

$$\text{scavenging activity}\% = \left(\frac{A_{\text{blank}}^3 - A_{\text{sample}}^3}{A_{\text{blank}}^3} \right) \times 100\% \quad (4)$$

(4) A_{sample}^3 is the absorbance of a mixture of sample and working solution; A_{blank}^3 is the absorbance of the mixture of deionized water and working solution.

2.3.6 Autoaggregation activity

Cultures during the stationary phase were washed three times with PBS (pH 7.0), and the OD₆₀₀ (A_0) of the bacterial solution was determined. The bacterial solution was vortexed for 25–30 s and then incubated at 37°C for 8 h. The OD₆₀₀ (A_1) was then determined. Calculated using Equation 5.

$$\text{Autoaggregation activity (AAG, \%)} = (1 - A_1 / A_0) \times 100\% \quad (5)$$

2.3.7 Cell surface hydrophobicity

GJ231 was cultured at 37°C for 18–20 h and then centrifuged at 4°C at $10,621 \times g$ for 10 min. Cells were washed three times with PBS (pH 7.0) and resuspended until the OD₆₀₀ was approximately 0.25 ± 0.05 (A_2). An equal volume of xylene and chloroform (1,1, v/v) was then added, and samples were vortexed for 120 s before incubating at 37°C for 3 h. The OD₆₀₀ of the aqueous phase was then measured (A_3). Calculated using Equation 6.

$$\text{Cell Surface Hydrophobicity (CSH, \%)} = (1 - A_3 / A_2) \times 100\% \quad (6)$$

2.3.8 Hemolytic activity

A culture of GJ231 was streaked on the blood agar base (Oxoid, Germany) containing 5.0% (v/v) sheep blood, which were incubated at 37°C for 20–24 h (Nataraj et al., 2023). Blank blood plates without inoculation were used as a negative control (data not shown), and *Staphylococcus aureus* (ATCC 25923) was used as a positive control.

2.4 Complete genome sequencing analysis

GJ231 bacterial pellet and genomic DNA were extracted using the SDS extraction method (Wang et al., 2023c). Libraries were prepared using an SQK-LSK110 kit (Oxford, United Kingdom) following the manufacturer's instruction manual using 1.0 g DNA. After sample purification and quality control, small fragment libraries were generated using a Universal Plus DNA Library Prep Kit for MGI V2 (Vazyme, China). The libraries underwent quality checks and were sequenced using the Nanopore PromethION and Illumina NovaSeq 6,000 platforms. Reads were assembled using Unicycler (Version: 0.5.0) software, plasmids were identified using PlasFlow software, and the assembled genome was used for coding gene prediction using Prokka (Version: 1.14.6) software (Seemann, 2014). Functional elements, genomic functions, database annotation, and websites were listed in Supplementary Table S1. Thresholds for carbohydrate annotation were set at an *E*-value of $<1e-18$ and coverage of $>35.0\%$. Pathogen–host interaction thresholds were set at an *E*-value of $<1e-5$, and virulence factor annotation was performed using the VFDB database with a coverage of $>40.0\%$ (Wang et al., 2023b).

Potential gene clusters for natural product biosynthesis in GJ231 were predicted using the antiSMASH database. In addition, the bacteriocin production gene cluster was predicted using the Bagel4 database. The amino acid sequences of bacteriocin lactacin F (*lafA* and *lafX*), which have the highest sequence similarity, were submitted to

the SWISS-MODEL web server to generate protein models (Wang et al., 2024).

2.5 Safety evaluation *in vivo*

Forty Kunming white mice (from Jinan Pengyue Experimental Animal Breeding Co., Ltd.) were randomly divided into two groups, with an equal number of males and females in each group. The oral administration experiment began after 7 d of acclimatization with unrestricted access to food and water, and a room temperature of 25°C. The control group (CK) was administered 0.1 mL of saline daily via oral gavage for 28 d, while the GJ231 group was administered an equal volume of GJ231 bacterial solution (1×10^9 CFU/mL) daily via oral gavage for 28 d. Throughout the study, mice were monitored daily for diarrhea, and their body weights and food intake were recorded weekly. Before the end of the experiment, the mice were fasted for 12 h, rendered unconscious with 1.0% (w/v) sodium pentobarbital (50 mg/kg), and blood was collected from the aorta. Blood samples were centrifuged at $825 \times g$ (3,000 rpm, using a centrifuge 5,702 Eppendorf, Germany) at 4°C for 15 min. The serum was collected and assayed for biochemical markers using a kit (Nanjing, China). Subsequently, the hearts, livers, spleens, kidneys, and thymuses of the mice were observed and collected. The organs of 10 mice in each group were randomly selected and added to 10 mL of sterile saline (1.0 g/mL). The organs were homogenized and crushed, and 0.1 mL of the homogenate was spread evenly on MRS plates. The plates were then incubated at 37°C for 48 h for observation.

2.6 Short chain fatty acids

Short-chain fatty acids in the supernatant of strain GJ231 were determined using LC–MS/MS. Briefly, 30 to 40 mg of frozen faecal samples were placed into a 1.5 mL centrifuge tube. Then, 1 mL of 50% acetonitrile (ACN, Fisher Chemical, United States) was added, followed by 2 to 3 metal grinding beads. The sample was processed in an E6618 tissue grinder (Beyotime, China) for 1 min at 60 Hz and then centrifuged at $15871 \times g$ (13,000 rpm in a Centrifuge 5,430 Eppendorf, Germany) for 10 min at 4°C. One hundred microliters of supernatant was taken and diluted proportionally to 10 mg of sample per 1.8 mL of 50% ACN solution. The mixture was vortexed and shaken (Scilogex, USA) for 30 s and then centrifuged at $15871 \times g$ for 30 s. Twenty

microliters of the supernatant was aspirated, and 10 μ L of 200 mM 3-nitrophenylhydrazine-HCl (3-NPH-HCl, Sigma Aldrich, United States) was added separately. Ten microlitres of 200 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide-HCl (EDC-HCl, Sigma Aldrich, United States), 80 μ L of 50% ACN, 50 μ L of 7% pyridine (Sigma–Aldrich, United States), and 1 μ L of isotope internal standard solution (Toronto Research Chemicals, Canada) were vortexed and shaken for 3 min, derivatized in a constant-temperature water bath at 40°C for 30 min and centrifuged at $15871 \times g$ for 1 min at 4°C. Finally, 20 μ L of the reaction solution after derivatization was added to 280 μ L of 50% ACN, vortexed for 30 s, centrifuged at $15871 \times g$ for 10 min at 4°C, aspirated into the injection vial, and then subjected to LC–MS/MS analysis (LC-30 HPLC, SCIEX QTRAP 5500 mass spectrometry, Phenomenex: Kinetex C18, 2.6 μ m 100 $\times$ 3.00 mm, column temperature: 40°C, flow rate: 0.7 mL/min).

2.7 Statistical analysis

Data were expressed as the mean $\pm$ standard error of mean (SEM). Statistical significance was determined using t-tests and one-way analysis of variance in GraphPad Prism 8.3.0, with statistical significance set at a level of $p < 0.05$.

2.8 Data availability

The whole-genome sequence data has been deposited under accession number PRJNA1028141 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1028141>) (17-Oct-2023).

3 Results

3.1 Identification of GJ231

The results of physiological and biochemical experiments on strain GJ231 were shown in Table 1. Strain GJ231 could utilize glucose, galactose, maltose, fructose, raffinose, rhamnose, xylose and sucrose. The colony morphology of strain GJ231 on MRS agar plates was raised, creamy white, and spherical or ovoid (Figure 1A). After Gram staining, bacteria appeared under a microscope as purple, rod-shaped cells with rounded ends, indicating that they are Gram-positive (Figure 1B). By

TABLE 1 Physiological and biochemical characteristics of *Lactobacillus johnsonii* GJ231.

Items	Results ^a	Items	Results ^a	Items	Results ^a
Aesculin	–	Mannitol	–	Salicin	–
Glucose	+	Sorbitol	–	Rhamnose	+
Cellobiose	–	Raffinose	+	Xylose	+
Galactose	+	Hydrogen sulfide	–	Mobility	–
Maltose	+	1% sodium equine	–	Sucrose	+
Fructose	+	Methyl Red test	–	Inulin	–
L-arabinose	–	Voges-Prokauer test	–	Gelaune liquefaction	–

^a+: positive; –: negative.

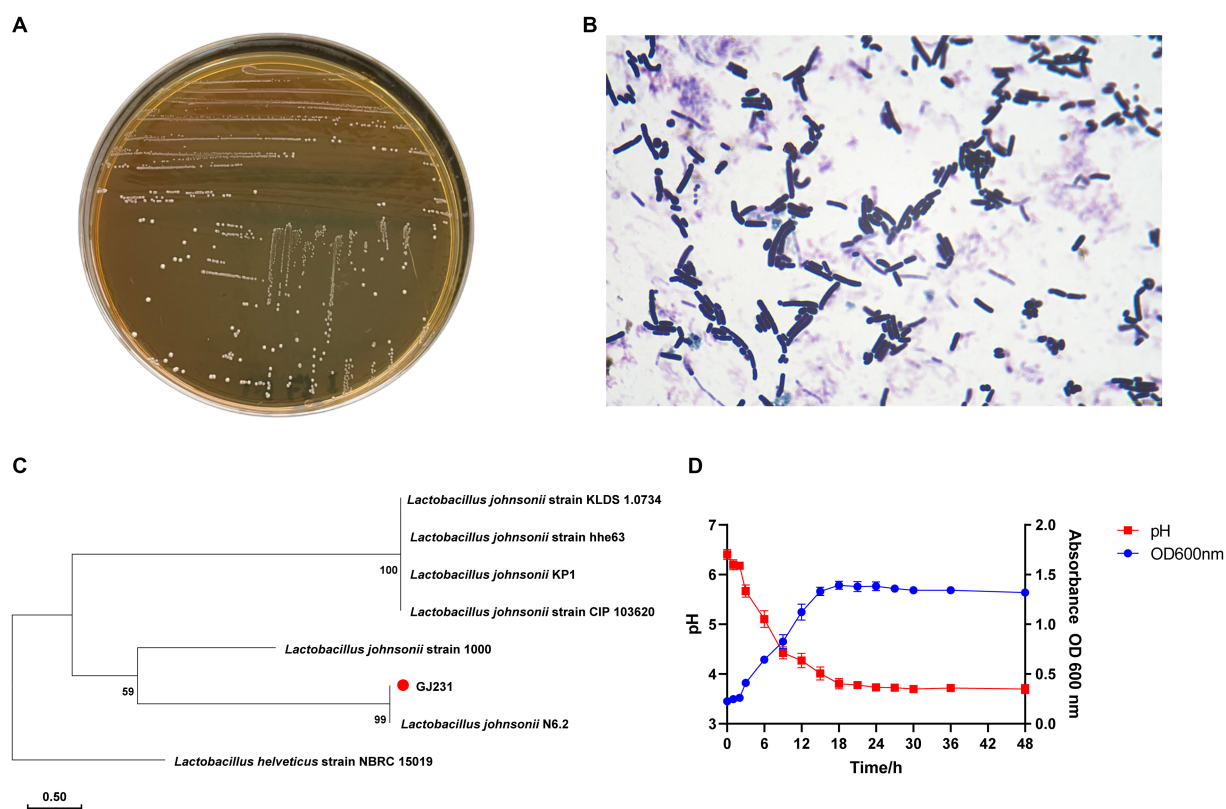


FIGURE 1 Morphology, staining and phylogenetic tree of strain GJ231. **(A)** Morphology of strain GJ231, **(B)** gram staining results of strain GJ231, **(C)** neighbor-joining phylogenetic tree of strain GJ231 (*Lactobacillus helveticus* as an exogenous species) and **(D)** growth and acid-producing curve.

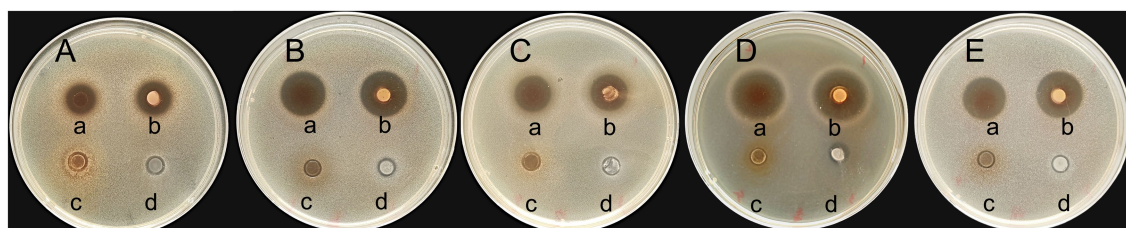


FIGURE 2 The inhibitory effects of GJ231 against pathogenic indicator bacteria. **(A)** *Escherichia coli*; **(B)** *Staphylococcus aureus*; **(C)** *Salmonella*; **(D)** *Listeria monocytogenes*; and **(E)** *Pseudomonas aeruginosa*. In each TSA agar, (a) added the cell-free supernatant of GJ231; (b) added the bacterial suspension of GJ231; (c) added the cell-free supernatant (pH = 7.0) of GJ231; (d) added the bacterial pellet.

reconstructing an evolutionary tree, we determined that GJ231 shared a nucleotide similarity of 99% with *L. johnsonii* N6-2 (Figure 1C). We therefore concluded that GJ231 represented *L. johnsonii*.

3.2 Growth and acid production curve

L. johnsonii GJ231 was in the lag period from 0 to 3 h, entered the exponential phase of growth after 3 h and reached the stationary phase at 18 h. In addition, the strain had a good capacity for acid production (Figure 1D).

3.3 Antimicrobial ability

The bacteriostatic activity of GJ231 against five pathogenic bacteria (Figure 2; Supplementary Table S2) was observed, with the most significant bacteriostatic effect observed against *L. monocytogenes*, followed by *P. aeruginosa*, *S. aureus*, *Salmonella*, and *E. coli*. However, there was no significant difference in the inhibitory effects of BS or CFS against the same pathogenic bacteria ($p > 0.05$). When the pH of the CFS was adjusted to pH 7.0, the inhibitory effect disappeared, suggesting that organic acids may play the primary inhibitory role in the CFS of GJ231. Furthermore, the BP of GJ231 had no inhibitory effect on pathogenic bacteria.

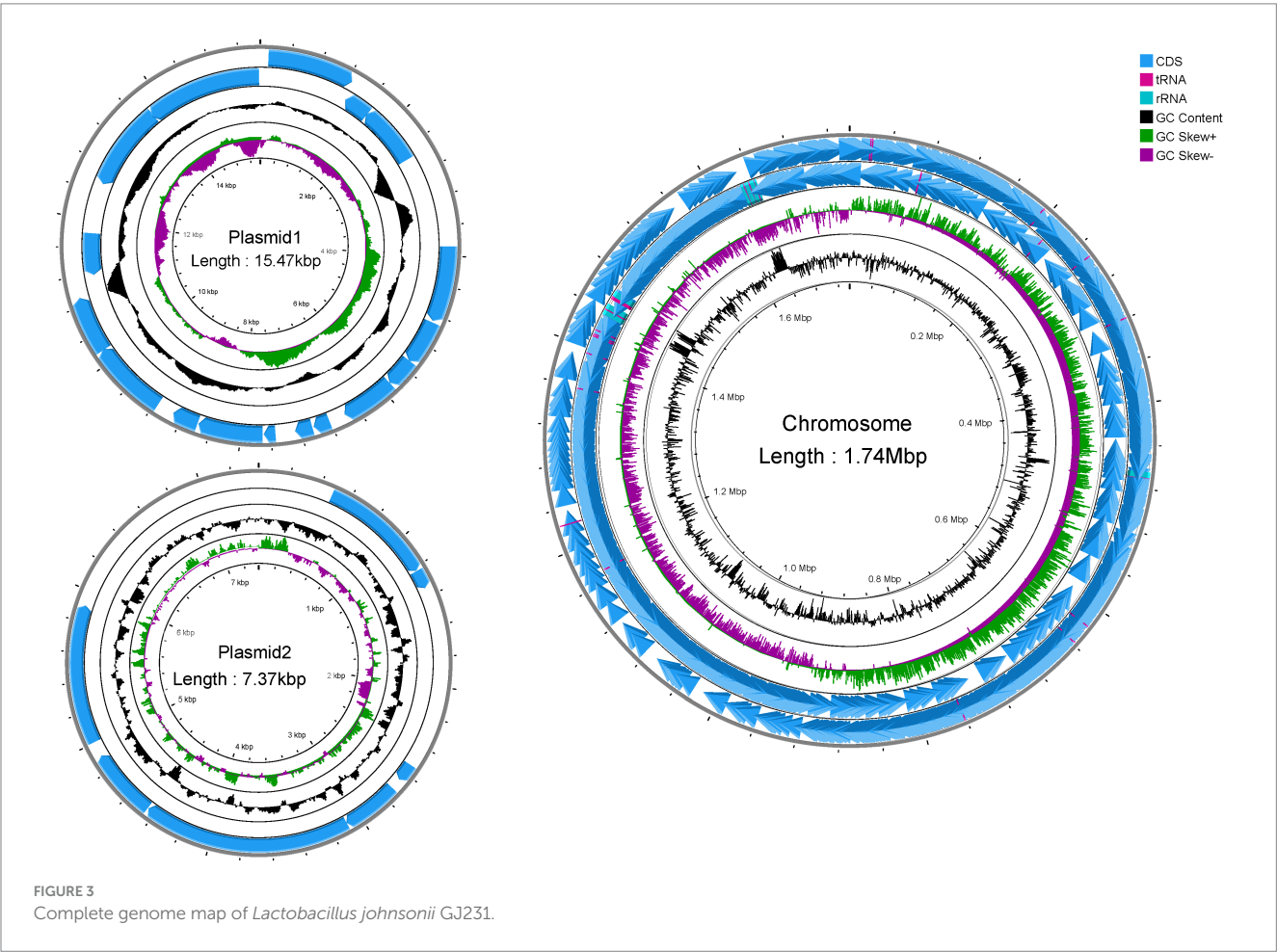
3.4 Complete genome sequencing and bioinformatic analysis

Genome-wide sequencing revealed that GJ231 contained one chromosome with a length of 1,740,019 bp and two plasmids; these

had a G + C content of 34.70, 35.73, and 34.32%, respectively (Table 2; Figure 3). There were 1,691 coding sequences (CDSs) with a total length of 1,553,460 bp. Furthermore, we identified 152 pseudogenes, 7 rRNAs, and 78 tRNAs. Our analysis revealed three genomic islands (Supplementary Table S3), eight prophages

TABLE 2 General genome features of *Lactobacillus johnsonii* GJ231.

Indicator	Number or content
Chromosome (bp)	1,740,019
Plasmid1 (bp)	7,371
Plasmid2 (bp)	15,479
G + C content of chromosome (%)	34.70%
G + C content of plasmid1 (%)	35.73%
G + C content of plasmid2 (%)	34.32%
Coding gene numbers	1, 691
Total length of coding genes (bp)	1,553,460
Pesudogene size (bp)	66,354
Pesudogene number	152
rRNAs (16 S–23 S-5S)	7
tRNA	78
tmRNA	1
misc_rna	34



(Supplementary Table S4), 189 repeated sequences totaling 17,652 bp in length (Supplementary Table S5), 17 insertions (Supplementary Table S6), two cytochromes P450 (Supplementary Table S7), and 83 virulence factors (Supplementary Table S8). In addition, there were 49 carbohydrate-active enzymes, with 26 being glycoside hydrolases and many being glycosyltransferases (Table 3). Moreover, the whole genome of GJ231 contained a 185-bp CRISPR-Cas sequence located on the chromosome (Supplementary Table S9). In addition, three resistance genes, namely *InuA*, *ErmT*, and *ErmB*, were predicted (Table 4).

Figure 4A showed a map of the coding genes in GJ231 annotated in the universal database. A total of 1,674 genes were predicted using the COG database (Figure 4B). Translation and carbohydrate metabolic processes, as well as the phosphoenolpyruvate-dependent glucose phosphotransferase system, were identified as highly active biological processes. We also determined that the plasma membrane, an essential cellular component, plays a crucial role in cellular function, while ATP binding, DNA binding, and metal ion binding were essential molecular functions (Figure 4C). KEGG annotation revealed 1,498 genes classified into 23 functional groups. These groups included global and overview maps (284 genes), carbohydrate metabolism (120 genes), and membrane transport (98 genes), as shown in Figure 4D. The database annotation results indicated that *L. johnsonii* GJ231 has a robust metabolic capacity and can adapt to multiple ecological niches.

We annotated 537 pathogen–host interactions (Figure 5A). Furthermore, we identified 387 membrane transporter proteins, with primary active transporters being the most numerous at 164 (Figure 5B). The three most common structural domains identified were 65 ABC transporters (ABC_tran), 29 Major Facilitator Superfamily (MFS_1) domains, and 24 RecF/RecN/SMC_N terminal domains (SMC_N) (Figure 5C). The amino acid sequence database of non-redundant (NR) proteins was also analyzed, consistent with the results of 16S rRNA gene sequencing (Figure 5D). In addition, genes conferring resistance to acid, bile salts, high temperatures, and oxidative stress were also identified in the genome of GJ231 (Table 5).

We used the antiSMASH database to identify potential gene clusters for natural product biosynthesis in GJ231, and two potential

products, gassericin E and gassericin T, were identified (Figures 6A–C). In addition, several potential bacteriocins were predicted in the GJ231 genome, such as gassericin, lactacin F (*lafA* and *lafx*), pediocin, and bacteriocin helveticin J (Figure 6D). Furthermore, the tertiary structure of the protein lactacin F (*lafA* and *lafx*) was modeled (Figure 6E).

3.5 Evaluation of the probiotic potential

Strain GJ231 tolerated high temperatures well, with over 60.0% survival after treatment at 70°C and approximately 40.0% survival after treatment at 80°C (Figure 7A). The survival rate was still greater than 85.0% with 0.1% bile salt treatment and greater than 80.0% with 0.3% bile salt treatment (Figures 7B,C). Moreover, the survival rate of strain GJ231 after treatment with gastric fluid was almost 80.0%, the attrition rate of strain GJ231 after treatment with intestinal fluid was very low, and the survival rate after co-treatment with gastrointestinal fluid was about 79.0% (Figure 7D). In addition, strain GJ231 exhibited significantly lower DPPH radical scavenging compared with ABTS and O₂⁻ scavenging (Figure 7E; $P < 0.01$). Adhesion of the strain is also an important indicator of *in vitro* probiotic properties. Strain GJ231 exhibited 50.0% autoaggregation activity and greater than 60.0% cell surface hydrophobicity (Figure 7F). Furthermore, GJ231 tested negative for hemolysis (Figure 7Gb), while *Staphylococcus aureus* exhibited β -hemolysis (Figure 7Gb). LC–MS/MS results showed that the metabolite (short-chain fatty acids) was mainly acetic acid at 1304.31 μ g/mL (Table 6).

3.6 Safety evaluation *in vivo*

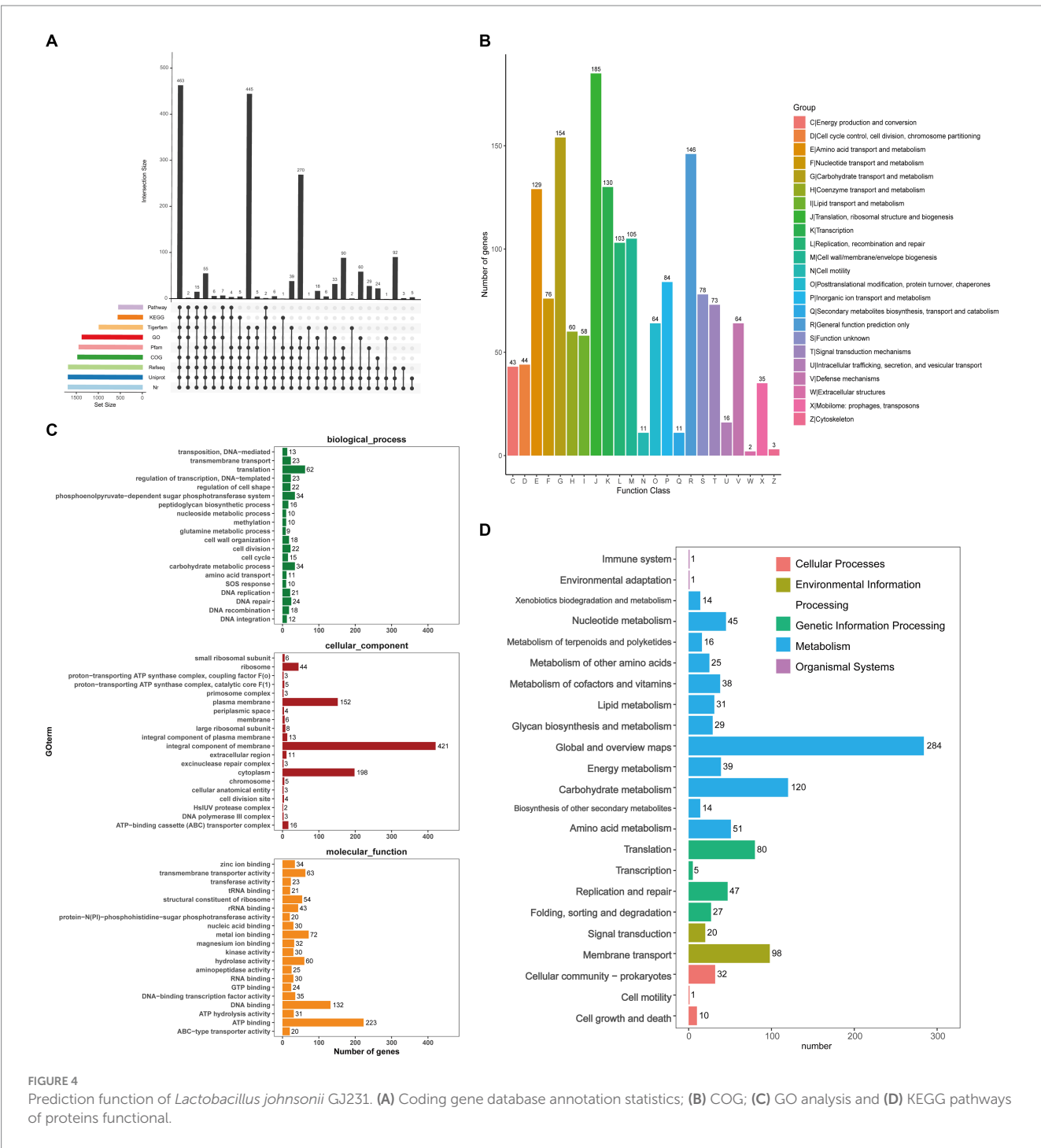
Safety experiments for the oral administration of GJ231 in mice were conducted. Throughout the oral experiment, all mice were active and did not develop diarrhea, die, or exhibit any other signs of illness. No significant pathological changes were observed in the various organs during autopsy. The body weight, average daily gain, heart coefficient, liver coefficient, spleen coefficient, and kidney coefficient of mice orally administered with GJ231 were not significantly different

TABLE 3 CAZymes-encoding genes of *Lactobacillus johnsonii* GJ231.

CAZymes class	Gene counts
Glycoside Hydrolases (GHs)	26
Glycosyl Transferases (GTs)	21
Carbohydrate Esterases (CEs)	1
Carbohydrate-Binding Modules (CBMs)	1
Total	49

TABLE 4 Resistance gene prediction of *Lactobacillus johnsonii* GJ231.

Class	Resistance gene	Identity	Alignment length/ Gene length	Phenotype
Lincosamide	<i>InuA</i>	99.38	100	Lincomycin
Lincosamide Aminoglycosides	<i>ErmT</i>	99.17	98	Lincosamide, Streptogramin_b, Macrolide
Macrolide	<i>ErmB</i>	99.18	100	



from those of the CK group (Figures 8A–F; $P > 0.05$). The thymus coefficient of mice orally administered with GJ231 was significantly higher than that of the CK group (Figure 8G; $P < 0.01$). The liver and kidney function indexes of mice were also measured. There was no significant difference in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the oral GJ231 group compared with those in the CK group (Figures 9A,B; $P > 0.05$). However, the blood urea nitrogen (BUN) and malondialdehyde (MDA) contents were significantly lower than those in the CK group (Figures 9C,D; $P < 0.01$). In addition, the total antioxidant capacity (T-AOC) and superoxide dismutase (SOD) levels in mice orally administered with

the GJ231 group were significantly higher than those in the CK group (Figures 9E,F; $P < 0.01$). However, the catalase (CAT) and Glutathione peroxidase (GSH-PX) levels did not show a significant difference compared with those in the CK group (Figures 9G,H; $P > 0.05$).

4 Discussion

Evaluating probiotic strains by combining phenotypic characterization and whole-genome sequencing analysis provides more comprehensive information about their potential biological

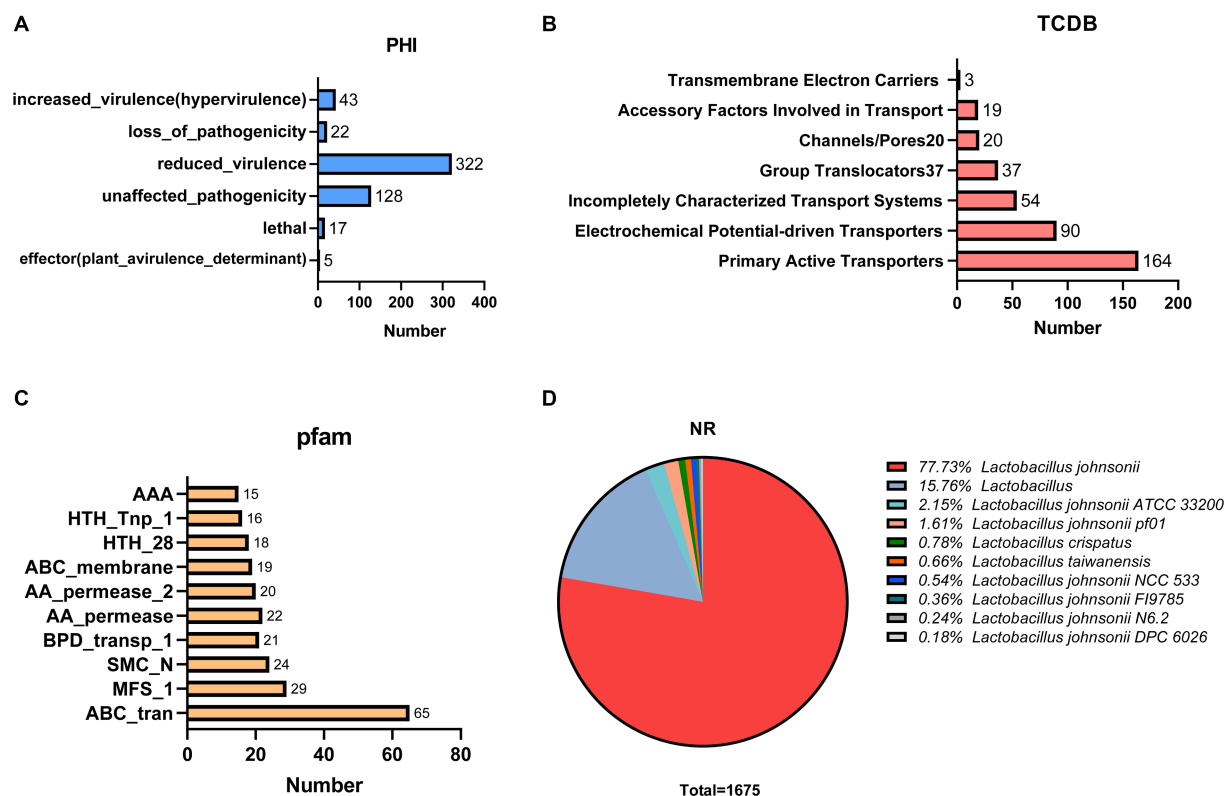


FIGURE 5

Proprietary database annotations of *Lactobacillus johnsonii* GJ231. (A) Pathogen host interactions annotations; (B) TCDB transporter protein; (C) PFAM domain and (D) NR annotations of *Lactobacillus johnsonii* GJ231.

properties than using one method of evaluation alone and is one of the most effective methods for exploring high-quality probiotic resources (Evanovich et al., 2019). In this study, a strain GJ231 from the healthy beagles was evaluated by a combination of whole genome sequencing and *in vitro* and *in vivo* experiments.

Whole-genome sequencing can analyze the complete genetic information of a strain at the gene level, providing a fast and accurate method for evaluating the function and safety of probiotics (Umanets et al., 2023). In this study, the whole-genome sequencing of strain GJ231 revealed that the strain had a genome size of 1.763 M with one chromosome and a G+C content of 34.70%, which was consistent with previous reports (Chen et al., 2023). Compared with *Lactiplantibacillus plantarum* and *Lactobacillus rhamnosus*, *L. johnsonii* has a smaller genome and lower G+C content, indicating a high degree of variation among different species of *Lactobacillus* to facilitate better adaptation to the environment (Jarocki et al., 2018). Understanding how carbohydrates are processed by the gut microbiota can help us investigate the influence of dietary carbohydrates on host health, as carbohydrate-active enzymes play a crucial role in host nutrient metabolism. The GJ231 genome contains 49 CAZymes: 26 glycoside hydrolase (GH, 53.06%) genes, 21 glycosyl transferase (GT, 42.86%) genes, one carbohydrate esterase (CE, 2.04%) gene, and one carbohydrate-binding module (CBM, 2.04%). It is worth noting that GJ231 contains numerous genes encoding GHs and GTs, which catalyze the transfer of sugars to specific receptors and play a crucial role in forming surface structures recognized by the host immune system. GHs catalyze the cleavage of glycosidic bonds, releasing

abundant energy. Both GHs and GTs can help the host to resist the invasion and adhesion of potential pathogenic bacteria and their toxins to the intestinal epithelium (Mazmanian et al., 2008; Becerra et al., 2015). In addition, the presence of CRISPR-Cas was detected in the GJ231 genome, indicating that strain GJ231 may inhibit horizontal transfer of virulence or antibiotic resistance genes and has the capacity to resist foreign genetic elements (phage, plasmids, and insertion sequences) (Kim et al., 2021).

Transporter Classification Database (TCDB) is a database that categorizes membrane transport proteins (Saier et al., 2006). Transporter proteins are essential for bacterial life activities as they are involved in the uptake of nutrients and defense against endogenous and environmental stresses (Piepenbreier et al., 2017). The most prominent transporters in the GJ231 genome were Primary Active Transporters (164, 42.38%) followed by Electrochemical Potential-driven Transporters (90, 23.26%). Furthermore, the most PFAM-annotated proteins in strain GJ231 were ABC transporter proteins (65), which were involved in critical host transport processes and play critical roles in host defense and antibiotic resistance (Feng et al., 2020). The ABC transporter proteins in GJ231 may have similar functions. Moreover, when the GJ231 genome was annotated using the NR Database, the highest number of genes was annotated to *L. johnsonii*, consistent with the results of 16S rRNA gene sequencing.

The bacteriostatic effects of LAB have been widely reported (Adetoye et al., 2018; Mani-López et al., 2022). LAB can inhibit the growth of pathogenic bacteria through metabolites such as organic acids, extracellular polysaccharides, and bacteriocins. By decreasing

TABLE 5 Probiotic genes that render resistance to *Lactobacillus johnsonii* GJ231 in harsh gastric conditions.

Genes detected in GJ231	EC Number	Predicted function
Adenosine triphosphate (ATP) synthase F0 sector subunit a	EC 3.6.3.14	Acid tolerance
ATP synthase F0 sector subunit c	EC 3.6.3.14	
ATP synthase subunit alpha	EC 3.6.3.14	
ATP synthase subunit gamma	EC 3.6.3.14	
ATP synthase subunit beta	EC 3.6.3.14	
ATP synthase subunit epsilon	EC 3.6.3.14	
ATP synthase subunit delta	EC 3.6.3.14	
Na ⁺ /H ⁺ antiporter NhaC		
Na ⁺ /H ⁺ antiporter NhaD		
Na ⁺ /H ⁺ antiporter		
Phosphotransferase system cellobiose-specific component IIA	EC 2.7.1.205	
Phosphotransferase system cellobiose-specific component IIB	EC 2.7.1.205	
Phosphotransferase system cellobiose-specific component IIC		
L-lactate dehydrogenase	EC 1.1.1.27	
L-lactate permease		
ATP-dependent Clp endopeptidase proteolytic subunit ClpP	EC 3.4.21.92	
Glucose-6-phosphate isomerase	EC 5.3.1.9	
Guanosine triphosphate (GTP) pyrophosphokinase	EC 2.7.6.5	
Pyruvate kinase	EC 2.7.1.40	
Glucosamine-6-phosphate deaminase	EC:3.5.99.6	Acid/Bile tolerance
Cytosine triphosphate (CTP) synthase	EC:6.3.4.2	
Manganese-dependent inorganic pyrophosphatase (<i>ppaC</i>)	EC:3.6.1.1	
Choloylglycine hydrolase <i>cbh</i>		
Asp23/Gls24 family envelope stress response protein		Stress response
universal stress protein		
peroxide stress protein YaaA		
RpiR family transcriptional regulator		Bile inducible genes
RpiR family transcriptional regulator YbbH		
Secondary bile acid biosynthesis		

(Continued)

TABLE 5 (Continued)

Genes detected in GJ231	EC Number	Predicted function
Heat-inducible transcriptional repressor <i>HrcA</i>		Temperature
Hsp33 family molecular chaperone HslO		
<i>DnaJ</i>		
<i>DnaK</i> (hsp70)		
Heat shock protein HtpX		
Small heat shock protein IbpA (HSP20)		
Ribosomal 50S subunit-recycling heat shock protein		
Molecular chaperone GrpE (heat shock protein HSP-70)		
Cold shock protein, CspA family		
Cold shock domain-containing protein <i>CspD</i>		
DEAD/DEAH box helicase		
S-ribosylhomocysteine lyase luxS	EC:4.4.1.21	Biofilm formation
Catabolite control protein A <i>ccpA</i>		
Family DNA-binding protein ComEA		
DCMP deaminase <i>comEB</i>	EC:3.5.4.12	
DNA internalization-related competence protein <i>ComEC</i>		
Competence protein ComGC		
Biofilm regulatory protein A		
Biofilm formation stimulator VEG		
Regulator of purF expression and biofilm formation		
peroxide stress protein YaaA		Peroxide
alkyl hydroperoxide reductase subunit F	EC:1.8.1.7	Superoxide
nickel-type superoxide dismutase maturation protease	EC:3.4.21.89	
removal of superoxide radicals	EC:1.8.1.9	

(Continued)

TABLE 5 (Continued)

Genes detected in GJ231	EC Number	Predicted function
Putative oxidoreductase		Hydroxyl radicals
NAD(P)/FAD-dependent oxidoreductase	EC:1.8.1.7	
FAD dependent oxidoreductase	EC:1.3.5.4	
SDR family NAD(P)-dependent oxidoreductase	EC:1.1.1.100	
Pyridine nucleotide-disulphide oxidoreductase	EC:1.8.1.7	
Guanosine 5'-monophosphate oxidoreductase	EC:1.7.1.7	
DsbA family oxidoreductase		
NADPH-dependent oxidoreductase		
Fatty acid repression mutant protein (predicted oxidoreductase)		
PPOX class F420-dependent oxidoreductase		
MocA family oxidoreductase		
Thiol peroxidase		Oxidative stress
Thioredoxin family protein		
Arsenate reductase (thioredoxin)		
Thioredoxin		
Thioredoxin-disulfide reductase	EC:1.8.1.7	
Hsp33 family molecular chaperone <i>HslO</i>		

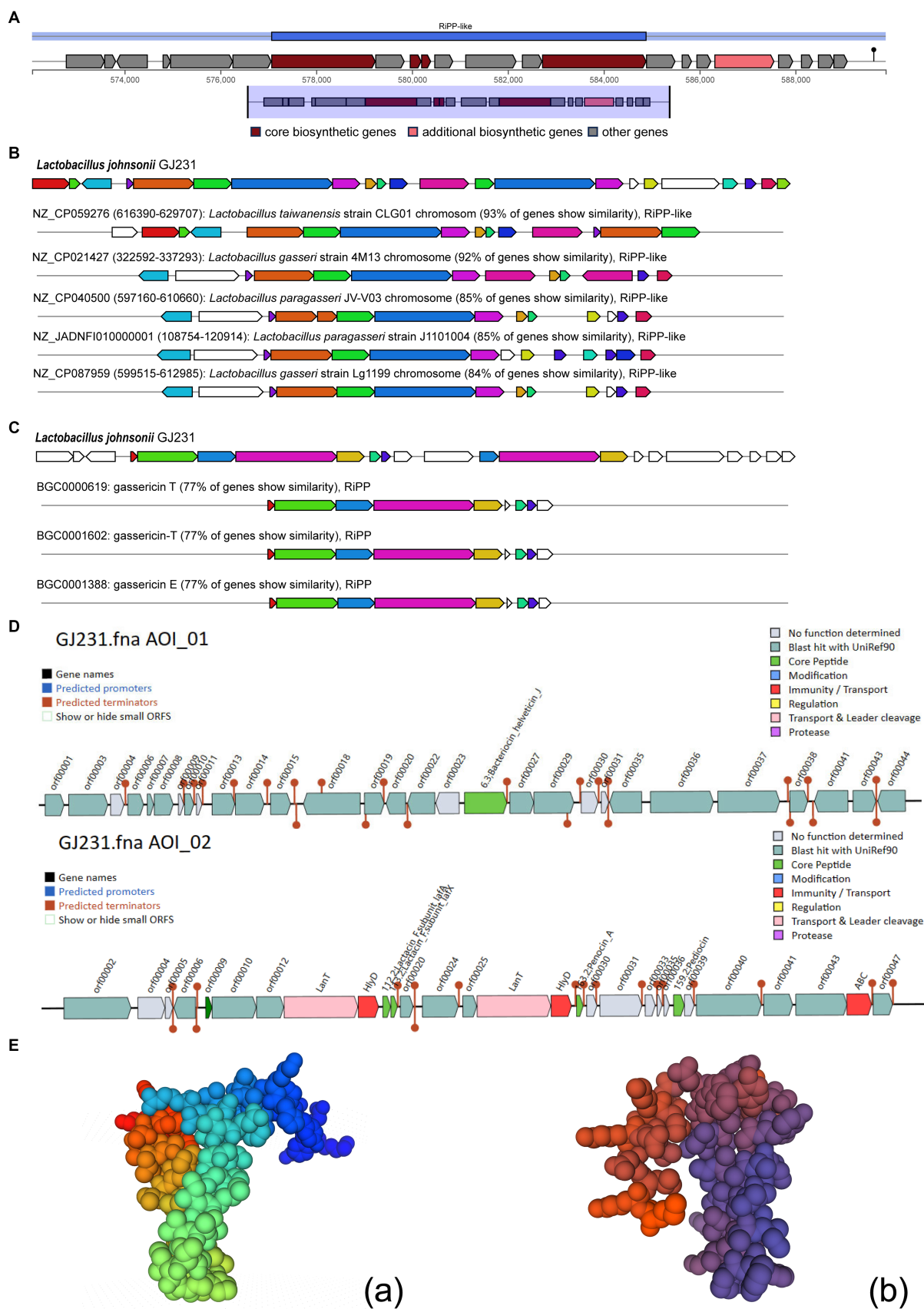


FIGURE 6
Bacteriocin prediction of *Lactobacillus johnsonii* GJ231. **(A)** Region of RiPP-like; **(B)** Cluster Blast; **(C)** Known Cluster Blast; **(D)** bacteriocin prediction by BAGEL 4 and **(E)** Protein tertiary structure prediction of *Lactobacillus johnsonii* GJ231.

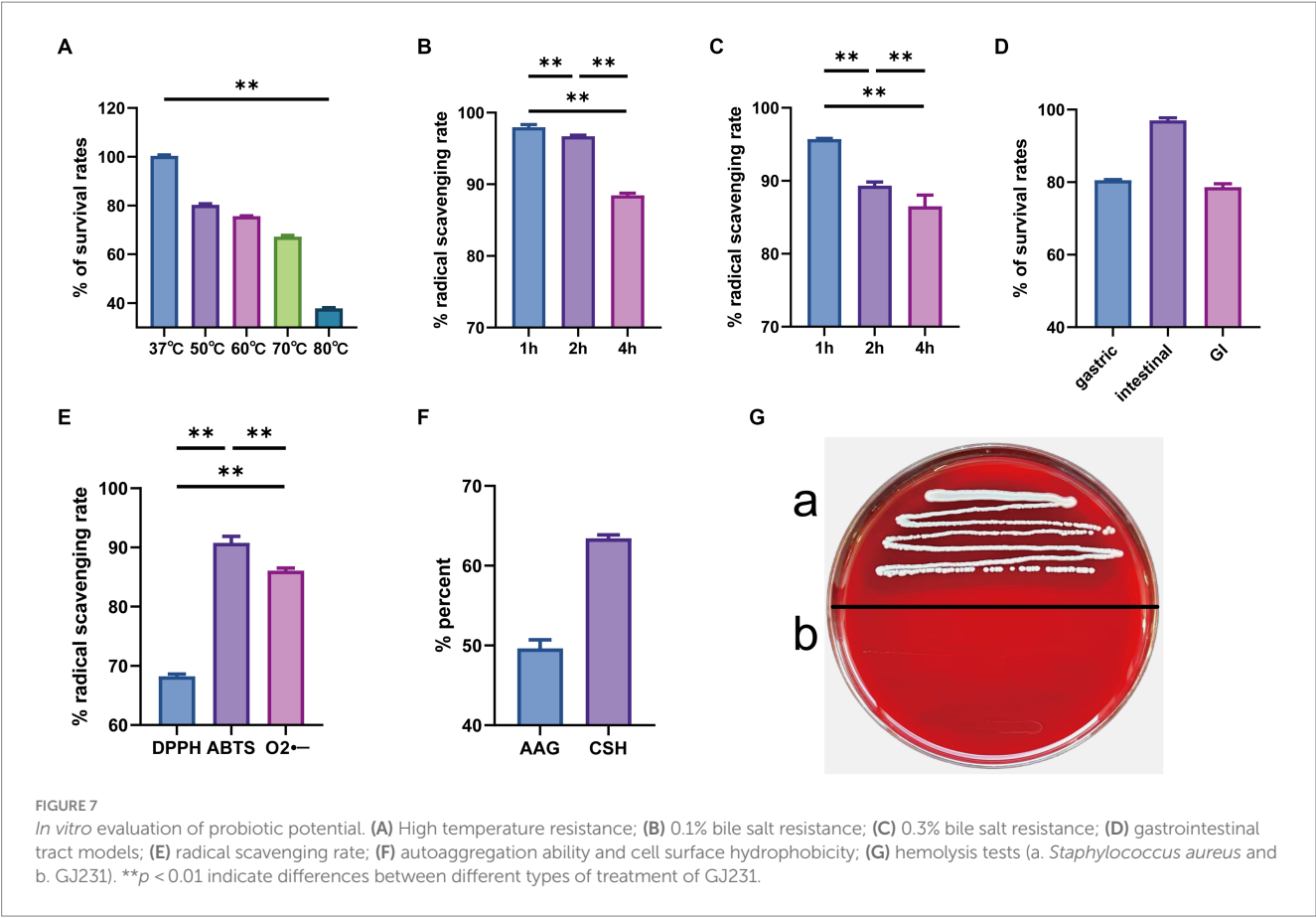


TABLE 6 Short-chain fatty acid content of the supernatant of *Lactobacillus johnsonii* GJ231.

Items	Content (ug/mL)
Acetic acid	1,385.187 ± 135.407
Propionic acid	4.904 ± 0.022
Isobutyric acid	1.674 ± 0.025
Butyric acid	0.775 ± 0.023
Isovaleric acid	1.146 ± 0.164
Valeric acid	0.059 ± 0.019
Caproic acid	0.268 ± 0.018
Enanthic acid	0.0854 ± 0.004
Caprylic acid	0.094 ± 0.017
Pelargonic acid	0.286 ± 0.024
Decanoic acid	0.036 ± 0.008

Values are mean with SD of three replications.

intracellular pH and enabling the accumulation of ionized forms of organic acids in the cytoplasm, organic acids, especially lactic and acetic acids, exercise bactericidal action (van Zyl et al., 2020). The results of this study showed that a BS of GJ231 and the CFS were bacteriostatic against five pathogenic bacteria. We hypothesized that the organic acids were responsible for bacteriostatic activity of the CFS, as the bacteriostatic effect disappeared when the pH was changed. Therefore, we examined the organic acid content in the supernatant of strain GJ231 and the results showed a high content of

acetic acid. It was noteworthy that the GJ231 genome also contained clusters of secondary metabolic genes, such as gassericin E and gassericin T, as well as gene encoding bacteriocins including gassericin, lactacin F, pediocin, and bacteriocin helveticin J. Chen et al. (2023) predicted that bacteriocin helveticin J was present in every bacteriocin-containing strain of *L. johnsonii*. Lactacin F, a two-component class II peptide bacteriocin, has been reported to reduce inflammation in patients with inflammatory bowel disease (Allison et al., 1994; Miri et al., 2022). Bacteriocin is one of the critical factors in the ability of probiotics to inhibit pathogens in the gastrointestinal tract (GIT). The antimicrobial activity of bacteriocins provides bacteriocin-producing probiotic strains with a competitive advantage in the complex GIT environment. These exciting phenomena will be studied more in depth in future research.

Probiotics are widely used in pets to prevent or combat diseases as they improve overall health. With the increasing variety of probiotic strains, the safety evaluation of probiotics has become a popular issue (Ishibashi and Yamazaki, 2001; Wang et al., 2023a). Hemolytic strains may cause sepsis in the host. Therefore, non-hemolytic bacteria are considered essential for exceptional probiotics. GJ231 was non-hemolytic on blood agar plates. In addition, the non-hemolytic enterotoxin (*Nhe*) and hemolysin BL (*Hbl*) genes were not identified in the GJ231 genome, which aligns with the strain's lack of hemolytic activity. A potential risk for antibiotic resistance was the possibility of vertical or horizontal transfer. The GJ231 genome contains three antibiotic resistance genes: *InuA*, *ErmT*, and *ErmB*. Although there was a risk of gene transfer in GJ231, these genes are localized to genomic DNA rather than plasmid DNA, and

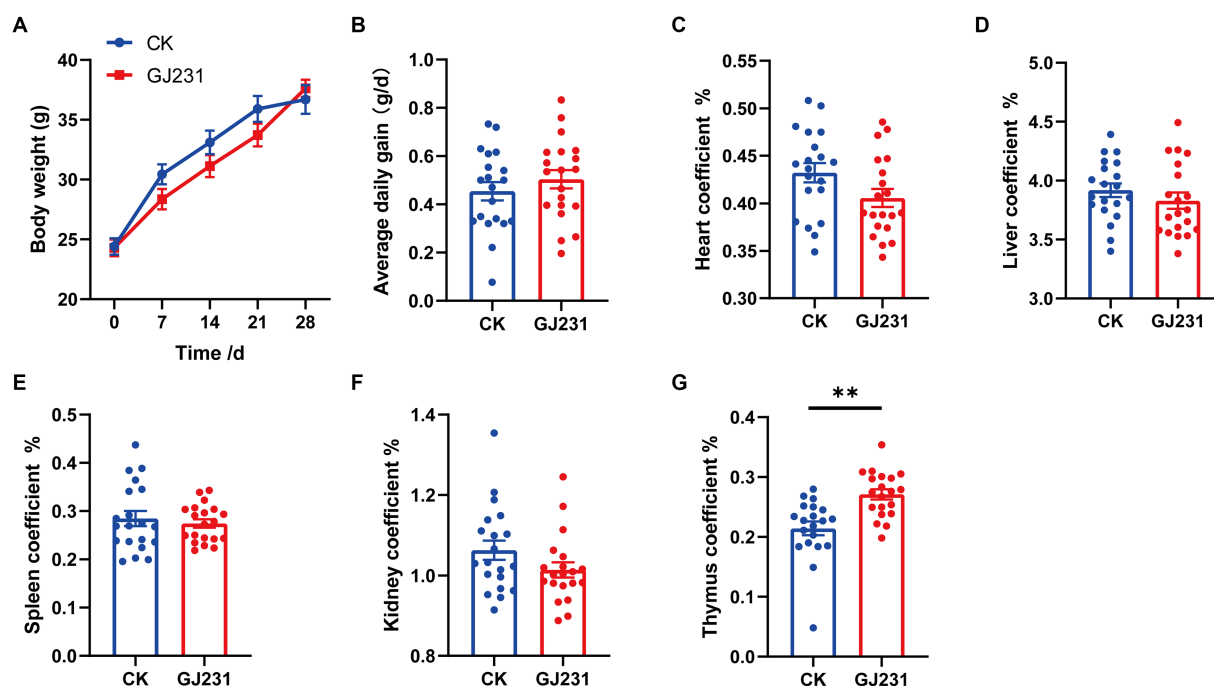


FIGURE 8

Effect of oral administration of *Lactobacillus johnsonii* GJ231 on organ indices in mice. (A) Body weight; (B) average daily gain; (C) heart coefficient; (D) liver coefficient; (E) spleen coefficient; (F) kidney coefficient and (G) thymus coefficient in mice. ** $p < 0.01$ indicate differences between different types of treatment of *Lactobacillus johnsonii* GJ231, $n = 20$.

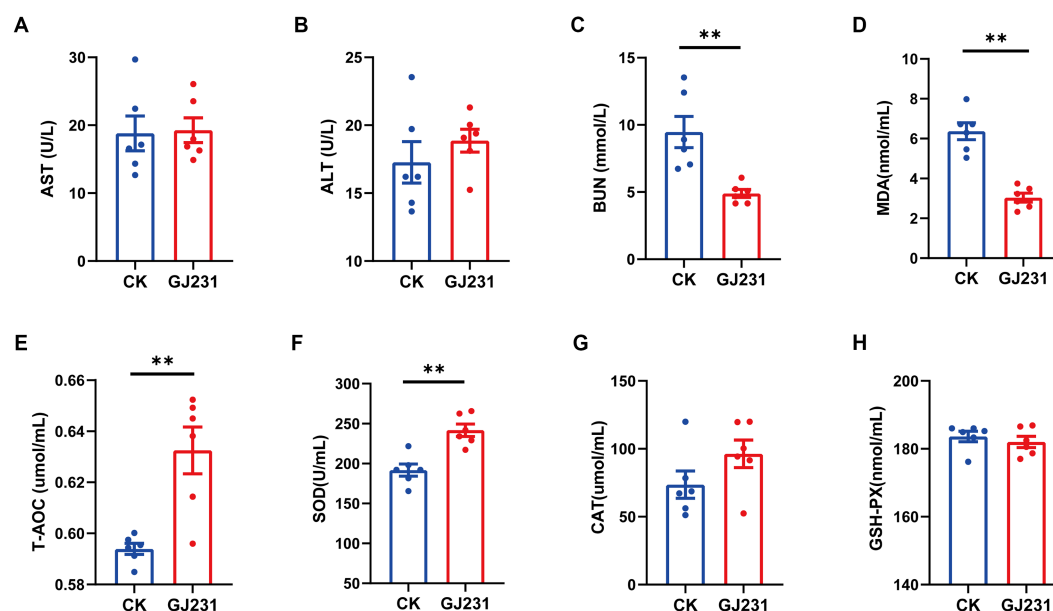


FIGURE 9

Effect of oral administration of *Lactobacillus johnsonii* GJ231 on serum indices in mice. (A) Aspartate amino transferase; (B) alanine aminotransferase; (C) blood urea nitrogen; (D) malondialdehyde; (E) total antioxidant capacity; (F) superoxide dismutase; (G) catalase; and (H) glutathione peroxidase in mice. ** $p < 0.01$ indicate differences between different types of treatment of *Lactobacillus johnsonii* GJ231, $n = 6$.

therefore the risk was reduced. In addition, we annotated GJ231 with CRISPR-Cas, which prevents the horizontal transfer of virulence or antibiotic resistance genes, reducing the risk of gene transfer (Saidumohamed and Ganapathy Bhat, 2021). Analysis of potential

metabolites in strain GJ231 did not reveal the presence of any toxic products.

After ingestion, bacteria are immediately exposed to the dual stresses of weak acids and bile salts. Therefore, acid and bile salt

tolerance are essential criteria for selecting probiotics. Because the strong acid tolerance of LAB is not universal, strain-by-strain testing is required (Kailasapathy and Chin, 2000). Strain GJ231 exhibited both acid tolerance and bile salt tolerance. At weak acids, ATP synthase hydrolyses ATP to produce a transmembrane ionic gradient, which facilitates bacterial tolerance to strong acids (Deckers-Hebestreit and Altendorf, 1996). The GJ231 genome contains genes encoding ATP synthase and sodium antiporter proteins, as well as genes for L-lactate dehydrogenase, L-lactate permease, ATP-dependent *Clp*, and the phosphotransferase system. All of these components have mechanisms for providing resistance to weak acids and can work together to maintain pH homeostasis (Liang et al., 2023). Cholesteryl glycolate hydrolase (CBH), which hydrolyses the amide bond between glycine and bile acids, and inorganic pyrophosphatase, which affects the activity of bile acid coenzymes, were identified in the genome of *L. johnsonii* GJ231. Their presence implied a potential mechanism for the bile salt tolerance of strain GJ231 (Da et al., 1987; Lakshmi Ragavan and Das, 2021). The GJ231 genome also contained a variety of genes related to heat and stress tolerance, which echoed the results of the *in vitro* test. The heat-inducible transcriptional repressor *HrcA* and chaperone proteins (*HslO*, *DnaJ*, *HtpX*, *HSP20*, and *HSP70*) can help maintain the structure of proteins by preventing misfolding (Gladysheva et al., 2022; Chen et al., 2024). In addition, HSP had the ability to modulate the immune response (Huang et al., 2019). Under hyperosmotic conditions, the synergistic action of chaperone proteins (*dnaK* and *dnaJ*) encoded by the GJ231 genome contributes to repairing damage to the cellular macromolecular machinery (Schröder et al., 1993). *In vitro* assays revealed the excellent antioxidant capacity of strain GJ231. GJ231 also contained various oxidative stress-related enzymes scavenging of superoxide, peroxide, and hydroxyl radicals. These enzymes play a vital role in the antioxidant system and are closely related to the strain's resistance to oxidative stress (Ezraty et al., 2017).

In vivo experiments are the primary method used to evaluate the safety of probiotics (Fan et al., 2023). A previous study reported that after administering *L. delbrueckii* subsp. *lactis* CIDCA to mice, no differences in body weight, feed intake, or organ indices were found in the group with CIDCA (de Jesus et al., 2022). The liver is an important organ responsible for metabolism and detoxification (Cui et al., 2023). In this study, the oral administration of GJ231 did not have a significant effect on body weight, food intake, or liver function (AST and ALT) in mice. This demonstrating that oral administration of GJ231 had no toxic effect on mice. However, we found the oral administration of GJ231 an increased thymic index in mice. The development of the thymus as a major lymphoid organ is vital for adaptive immunity (Kernfeld et al., 2018; Chen et al., 2019). SOD is the first line of defense against oxidative damage and plays a crucial role in oxidative stress and T-AOC is an oxidative stress marker (Zhao et al., 2021; Zhou et al., 2023). MDA is a byproduct of lipid peroxidation and has been utilized as a biomarker to indicate oxidative stress in organisms. In this study, the levels of serum T-AOC and SOD were significantly increased, while the levels of serum MDA was notably decreased in mice after administering GJ231. It suggested that the oral administration of GJ231 improved the mice's ability to handle oxidative stress.

Probiotics have been reported to promote health and well-being in healthy dogs, e.g., as an adjunct product for the treatment of

atopic dermatitis in dogs (Marsella, 2009; Kim et al., 2015) and to reduce the incidence of diarrhea (Nixon et al., 2019; Shmalberg et al., 2019). However, some studies have shown that supplemental feeding of *E. faecium* NCIB 10415 (isolated from the feces of healthy dogs) had no positive effect on the health status of dogs (Vahjen and Männer, 2003). Therefore, more studies are needed to discover the negative and positive effects of probiotics (canine or non-canine origin) on healthy and sick dogs to recycle probiotic products in dogs. Therefore, a strain with good probiotic potential (*Lactobacillus johnsonii* GJ231) was obtained in this study; larger studies are needed to explore the beneficial significance of the strain *Lactobacillus johnsonii* GJ231 at a broader level for prescription pet food and functional pet food development and/or improvement of canine intestinal health.

5 Conclusion

We isolated *L. johnsonii* GJ231 from the intestinal tract of healthy beagles. *In vitro* experiments revealed that GJ231 was tolerant of acid, bile salts, and heat. It was also capable of inhibiting the growth of various pathogens, had excellent free-radical scavenging activity, exhibited high levels of adherence, and possessed genes with the potential to biosynthesize a variety of bacteriocins. In conclusion, *L. johnsonii* GJ231 is a promising probiotic candidate for functional pet foods.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was approved by the Laboratory Animal Ethics Committee of Qingdao Agricultural University (Approved No. DWKJ2023121901 Qingdao, China). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MZ: Data curation, Methodology, Visualization, Writing – original draft. YL: Data curation, Methodology, Visualization, Writing – original draft. YZ: Data curation, Methodology, Visualization, Writing – original draft. GL: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Sangnam Oh,
Jeonju University, Republic of Korea
Jeehwan Choe,
Korea National University of Agriculture and
Fisheries, Republic of Korea

*CORRESPONDENCE

Min-Jin Kwak
✉ nea7103@snu.ac.kr
Minho Song
✉ mhsong@cnu.ac.kr
Younghoon Kim
✉ ykeys2584@snu.ac.kr

[†]These authors have contributed equally to
this work

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Gut microbiome and metabolome library construction based on age group using short-read and long-read sequencing techniques in Korean traditional canine species Sapsaree

Seon-Hui Son^{1†}, Min-Geun Kang^{1†}, Anna Kang^{1†}, Yonggu Kang²,
Kimoon Kim², Min-Jin Kwak^{1,3*}, Minho Song^{2*} and
Younghoon Kim^{1*}

¹Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul, Republic of Korea, ²Division of Animal Science and Dairy Science, Chungnam National University, Daejeon, Republic of Korea, ³Emerging Pathogens Institute, University of Florida, Gainesville, FL, United States

This study investigated age-related changes in the gut microbiota and metabolome of Sapsaree dogs through metagenomic and metabolomic analyses. Using Illumina (short-read) and Nanopore (long-read) sequencing technologies, we identified both common and unique bacterial genera in the dogs across different age groups. In metagenomic analysis, Firmicutes were predominant at the family level. At the genus level, *Lactobacillus*, *Streptococcus*, *Romboutsia*, and *Clostridium XI* were the most abundant, and the bacterial genera typically considered beneficial were less prevalent in senior dogs, whereas the genera associated with pathogenicity were more abundant. These findings suggest age-related shifts in gut microbiota composition. Metabolomic analysis showed distinct clustering of metabolites based on the age group, with changes in metabolite profiles correlating with metagenomic findings. Although Illumina and Nanopore methods provided distinctive results, the genera detected by both methods exhibited similar trends across all age groups in Sapsaree dogs. These findings highlight the relationship between ages, metabolite profiles and gut microbiota composition in dogs, suggesting the need for further research to explore this relation in greater depth.

KEYWORDS

microbiome, metabolome, Sapsaree, Illumina sequencing, Nanopore sequencing

1 Introduction

The Sapsaree is a traditional Korean dog (*Canis familiaris*) breed recognized for its long, shaggy coat and drooping ears. In 1992, the Korean government designated the Sapsaree as a “natural monument” to protect it from extinction and preserve its pure bloodline (Gajaweera et al., 2019). Sapsaree has a large body (~60 cm in height), a friendly and gentle nature, and deep loyalty to its host (Cho, 2005). Despite its cultural significance and popularity in Korea, the population of Sapsaree is decreasing owing to limited scientific research on their physiological traits (Kang et al., 2009). Through this study, we aim to investigate the lifestyle

and health of Sapsaree by understanding its gut environment via multiomics approaches, including metagenomic and metabolomic analysis.

Gut microbiota refers to the living microorganisms present in a specific environment, in contrast, the microbiome encompasses the complete set of genomes from all the microorganisms in that environment, including not only the microbial community itself but also the structural components, metabolites, and environmental conditions associated with it (Hou et al., 2022). Gut microbiome plays a crucial role in maintaining host health and preventing animal disease (Kwak et al., 2021). Also, recent studies have revealed that the gut microbiota influences various physiological functions, including immune regulation, nutrient absorption, and metabolic processes (Brown et al., 2023). Research specifically on dogs has shown that alterations in the gut microbiota are associated with a range of conditions, such as obesity, gastrointestinal diseases, and even behavioral changes (Kang et al., 2024; Kielbik and Witkowska-Piłaszewicz, 2024). Understanding the composition and function of the gut microbiota is essential for promoting health and preventing diseases in companion animals (Kwak et al., 2024).

In recent years, next-generation sequencing (NGS) techniques have revolutionized the profiling of microbial communities globally and various studies have been focused on comparison of the efficacy of two types of NGS techniques: the second-generation platform from Illumina (San Diego, CA, United States) and the third-generation platform from Oxford Nanopore Technologies (Oxford, the United Kingdom) (Stevens et al., 2023). MiSeq, a short-read sequencing method using the Illumina platform, provides high accuracy and depth but can be limited in its ability to obtain complete genomic information owing to short-read lengths. By contrast, the Nanopore platform, a long-read sequencing method, can better detect structural variation at the genomic level by providing a longer read length, albeit with a higher error rate (Cha et al., 2023). By comparing these two methods, we aim to evaluate the impact of each sequencing method on analysis of the gut microbiota of Sapsaree.

In this study, we analyze the gut microbiota and gut metabolites of Sapsaree and investigate their correlations to compare the differences between the MiSeq and Nanopore sequencing methods. We also aim to gain a better understanding of the interactions between the gut microbiota and metabolites in Sapsaree to provide valuable foundational information for the health management and disease prevention of companion animals.

2 Materials and methods

2.1 Animal care

Fecal samples of Sapsaree were obtained from the Korean Sapsaree Foundation (Gyeongsan-si, Korea) to investigate the age-related metagenomic and metabolomic changes. The dogs were categorized into three age groups ($n = 6$ per group): junior, adult, and senior. The junior Sapsaree group consisted of two males and four females aged 1 year. The adult group consisted of five males and one female aged between 4 and 6 years. The senior group consisted of four males and two females aged between 10 and 12 years. The average weights for the junior, adult, and senior groups were 17.6 ± 2.9 kg, 27.7 ± 2.8 kg, and 23.7 ± 2.8 kg, respectively. All dogs were individually housed indoors

under controlled conditions. They were fed twice daily, with their daily food intake divided into two meals. Junior Sapsaree dogs were fed with puppy feed for large breeds (Equilibrio, FL, United States), while adult and senior Sapsaree dogs were given Proplan Performance feed from Nestle Purina PetCare Company (MO, United States). Food consumption was monitored immediately after feeding, and the amount of remaining food was recorded. The housing environment was maintained at a constant temperature of $21^\circ\text{C} \pm 2^\circ\text{C}$, with a relative humidity of $50\% \pm 20\%$ and a photoperiod of 12 h (08:00 to 20:00). This study was conducted with approval from the Institutional Animal Care and Use Committee at Chungnam National University (Approval No. 202310A-CNU-179).

2.2 Sapsaree fecal sample DNA sequencing using Illumina NextSeq-300

Fecal samples from each group were collected, and genomic DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). The V3–V4 region of the 16S rRNA gene was then amplified using the extracted DNA (V4 amplicon primer set: forward, 515F, 5'-GTGYCAGCMGCCGCGTAA-3'; reverse, 806R, 5'-GGACTACNVGGGTWTCTAAT-3'). NGS was conducted using the Illumina® NextSeq-300 platform (Illumina, Inc., CA, United States). The quality of the obtained sequences was evaluated using NanoPlot (v1.42.0). The raw V3–V4 amplicon sequencing data have been deposited in NCBI's SRA with the data accession number [SRR30105962](https://www.ncbi.nlm.nih.gov/sra/SRR30105962).

2.3 Metagenomic analysis using Nanopore

DNA samples were quantified using a Qubit 4 fluorometer (Q33226, Invitrogen) and the Qubit dsDNA HS Assay Kit (Q32851, Invitrogen). Amplicon sequencing libraries targeting the V1–V9 regions of the 16S rRNA gene were prepared using the 16S Barcoding Kit 24 V14 (SQK-16S114.24) according to the manufacturer's instructions. The libraries were sequenced on the MinION Mk1B platform (Oxford Nanopore Technologies, Cambridge, the United Kingdom). The following primers were used: V1 amplicon primer set—forward, 27F (5'-AGAGTTTGATCMTGGCTCAG-3'); reverse, 1492R (5'-GGTTACCTTGTTACGACTT-3'). The quality of the obtained sequences was evaluated using NanoPlot (v1.42.0). The raw V1–V9 amplicon sequencing data have been deposited in NCBI's SRA with the data accession number [SRR30105961](https://www.ncbi.nlm.nih.gov/sra/SRR30105961).

2.4 Metagenomic analysis

The amplicon sequences were classified using an analysis pipeline (Figure 1B). Fastq files generated from the Illumina® NextSeq 300 platform were preprocessed using Trimmomatic (v0.39) and fastp (v0.23.4). Paired-end sequences with a read length between 200 and 400 bp and a quality score of at least 20 were classified for 16S rRNA taxonomy using Kraken2 (v2.1.3) with the RDP database (v11.5). Similarly, fastq files generated by the Nanopore MinION Mk1B platform were preprocessed using Porechop (v0.2.4) and fastp (v0.23.4). Single-end sequences with a read length between 1,000 and

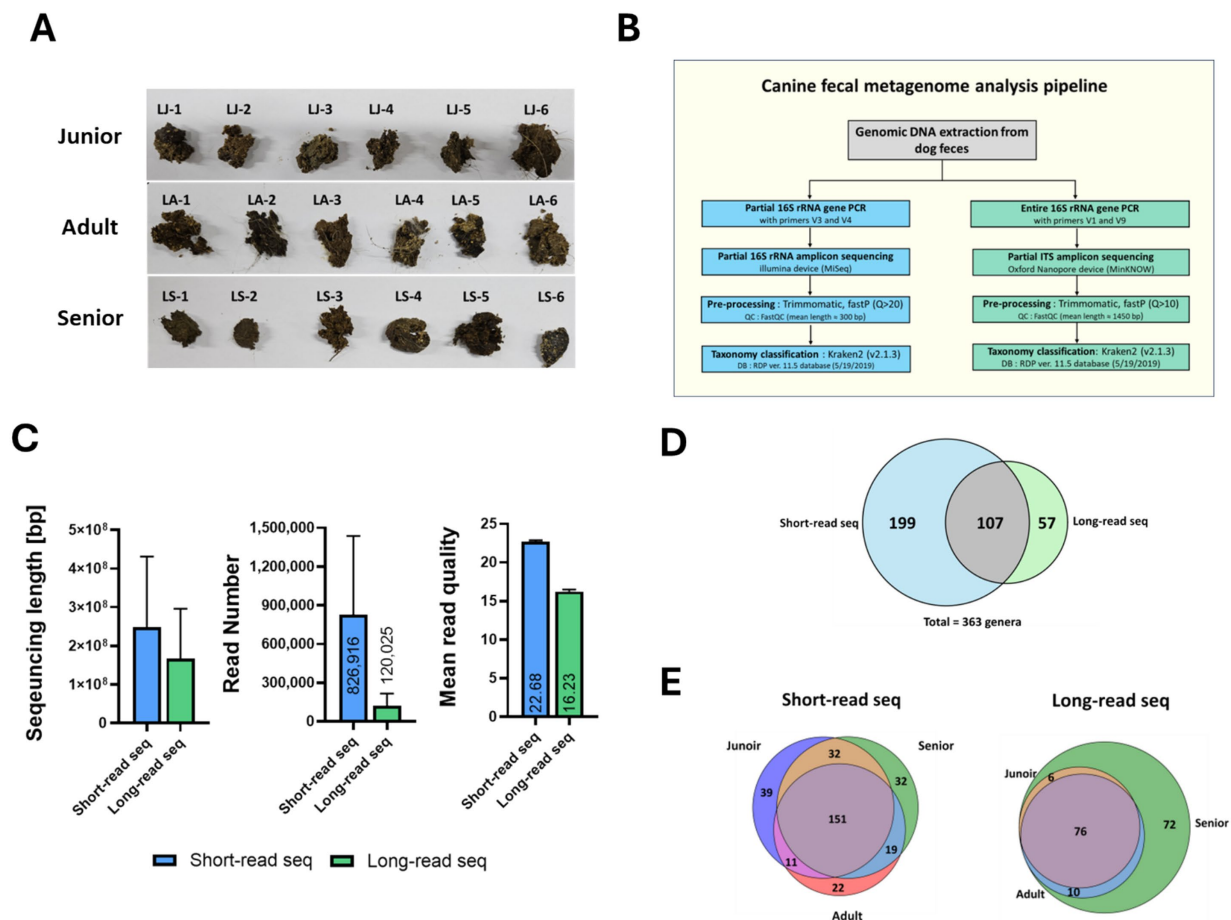


FIGURE 1

Sampling and metagenomic analysis protocol for Sapsaree. (A) Characteristics of fecal samples from Sapsaree dogs. (B) Pipeline schematic of metagenomic analysis of fecal samples. (C) Comparison between short-read and long-read sequencing. (D) Number of the genera analyzed via short- and long-read sequencing techniques. (E) Number of genera according to the age group via short- and long-read sequencing techniques. Junior: <1 year old; adult: 4–6 years old; senior: >10 years old.

2000 bp and a quality score of at least 10 were also classified using Kraken2 (v2.1.3) with the RDP database (v11.5).

For alpha-diversity analysis, the proportions of classified genera were used to calculate the Shannon diversity index ($H = -\sum (P_i \times \ln(P_i))$) and the Simpson diversity index ($\sum n_i (n_i - 1) / (N(N - 1))$) to assess microbial diversity in each sample. For bacterial beta-diversity analysis, Bray–Curtis dissimilarity was calculated based on the abundance data. Results were visualized using principal coordinate analysis (PCoA) plots.

2.5 Metabolomic analysis

For the extraction of metabolites for GC–MS analysis, each fecal sample was weighed and diluted with ice-cold 100% methanol to achieve a final concentration of 30 mg/mL. The samples were then centrifuged at $15,000 \times g$ for 5 min at 4°C. The resulting supernatant was filtered through a 0.2 µm polyvinylidene fluoride syringe filter. A 0.2 mL aliquot of the filtered supernatant was concentrated using a vacuum concentrator and stored at –80°C until derivatization. The extract was derivatized with 30 µL of 20 mg/mL methoxyamine

hydrochloride in pyridine for 90 min at 30°C, followed by 50 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 30 min at 60°C. Fluoranthene was used as an internal standard.

For analysis, a Thermo Trace 1,310 GC system was coupled with a Thermo ISQ LT single quadrupole mass spectrometer (Waltham, MA, United States). Metabolite separation was achieved using a 60 m-long DB-5MS column with an internal diameter of 0.2 mm and a film thickness of 0.25 µm (Agilent, Santa Clara, CA, United States). The sample was injected at 300°C with a split ratio of 1:60 and a helium split flow of 7.5 mL/min. Metabolites were separated using a constant helium flow of 1.5 mL/min and an oven temperature ramp of 5°C/min. The temperature program was started at 50°C (held for 2 min), increased to 180°C (held for 8 min), then increased to 210°C (held for 2.5 min), and finally increased to 325°C (held for 10 min). Mass spectra were acquired over a scan range of 35–650 *m/z* at an acquisition rate of 5 spectra per second using electron impact ionization. The ion-source temperature was maintained at 270°C. Data analysis, including automated peak detection and metabolite identification, was conducted using the Thermo Xcalibur software by matching mass spectra and retention indices of the samples with those in the NIST Mass Spectral Search Program (version 2.0, Gaithersburg,

MD, United States). Metabolite data were normalized based on the intensity of the internal standard, fluoranthene.

2.6 Correlation between the metagenomic and metabolomic analyses

The correlation between the microbiota and metabolites was assessed using the integrative analysis pipelines MetaboAnalyst 6.0¹ and MicrobiomeAnalyst 2.0², with a significance threshold set at $p < 0.05$. Variations in microbial community composition were analyzed at the phylum and genus levels, employing microbiome-metadata correlation analysis. Spearman correlation coefficients were calculated using pairwise correlation analysis to explore the relationships between differential microbiome communities and the presence and concentration of specific metabolites.

2.7 Statistics

If not otherwise specified, all values are presented as the mean $\pm$ standard deviation and were derived from analysis of samples from six Sapsaree dogs per group. Statistical analysis of the experimental data was performed using the GraphPad Prism 9 software. Nonparametric multiple t -tests were conducted to assess statistical significance. Significance levels are indicated by asterisks as follows: * $p < 0.05$.

3 Results

3.1 Experimental scheme of metagenome study

The metagenomes of 18 Sapsaree dogs, categorized by age (<1 year, 1–6 years, and > 7 years), were analyzed (Figure 1A). The sequencing reads obtained using Illumina MiSeq had an average length of 300.0 bp, while those obtained using Nanopore MinION had an average length of 1405.8 bp, demonstrating a significant difference in the read lengths (Figure 1C). The mean read quality score of the raw data was 22.7 for the short reads obtained by MiSeq and 16.2 for the long reads obtained by MinION, with the short reads exhibiting significantly better quality. When these reads were classified using Kraken2 (v2.1.3) based on the RDP 11.5 database, the short reads were annotated to 306 genera. By contrast, the long reads were annotated to 164 genera (Figure 1D). There were 107 genera in common, i.e., identified by both the sequencing analysis platforms. Despite using the same samples, the genera assignments varied depending on the employed 16S rRNA analysis platform. When examining the number of genera annotated from short reads based on age groups, Illumina-derived short reads identified 233 genera in the junior group, 203 genera in the adult group, and 234 genera in the senior group. Some genera were only identified in specific age groups. However, the

age-specific genera were predominantly composed of non-dominant species, with fecal abundance ratios below 0.1% (Figure 1E). When examining the number of genera annotated from long reads based on age group, 164 genera were annotated in the senior group, which included 82 genera from the junior group and 86 genera from the adult group.

3.2 Gut microbiome analyzed via short-read sequencing

Composition of the gut microbiome, at the phylum level, of junior, adult, and senior Sapsaree dogs is shown in Figure 2A. The population of the dominant phylum *Firmicutes* gradually decreased with increasing age of Sapsaree dogs, and the populations of *Actinobacteria* and *Proteobacteria* were significantly increased in the adult and senior groups compared to the junior group. The populations of intestinal genera are depicted in Figure 2B; in the senior group, the smallest bacterial populations corresponded to *Lactobacillus* and *Turicibacter* and largest populations were attributed to *Romboutsia* and *Collinsella*. As shown, the Simpson index was numerically decreased compared to CON group (Figure 2C), and the gut microbiome community of the junior group was significantly separated from those of adult and senior groups (Figure 2D).

3.3 Gut microbiome analyzed via long-read sequencing

Intestinal microbial populations at the phylum and genus levels determined by long-read sequencing are presented in Figures 3A,B. More than 95% of the *Firmicutes* population was found in all age groups. Meanwhile, the populations of *Blautia* and *Lactobacillus* were significantly larger and the populations of *Romboutsia* and *Clostridium* XI were significantly smaller in the junior group compared to the adult and senior groups. There were no differences in alpha-diversity indexes among the groups (Figure 3C); however, the gut microbial community in samples from dogs in the junior group was completely separated from those from dogs in the adult and senior groups (Figure 3D).

3.4 Gut metabolome and its predicted metabolism

The metabolome in intestinal contents from Sapsaree dogs was significantly different in dogs of different age groups (Figure 4A). In the junior group, γ -amino butanoic acid, benzofuran, cadaverine, octadecadienoate, uracil, and D-cellobiose were the dominant fecal components. Conversely, phosphite, trihydroxy benzophenone, monooleoglycerol, α -tocopherol, butylaniline, and androsterone were the dominant components in the adult group, and butyl alcohol, dihydrocaffeic acid, propanoic acid, glyceric acid, glycolic acid, and D-serine were the dominant components in the senior group (Figure 4B). Moreover, comparison analysis using volcano plots demonstrated that the concentrations of cadaverine, uracil, benzofuran, γ -amino butanoic acid, and D-cellobiose were significantly higher in the junior group compared to the adult and

¹ <https://www.metaboanalyst.ca/>

² <https://www.microbiomeanalyst.ca/>

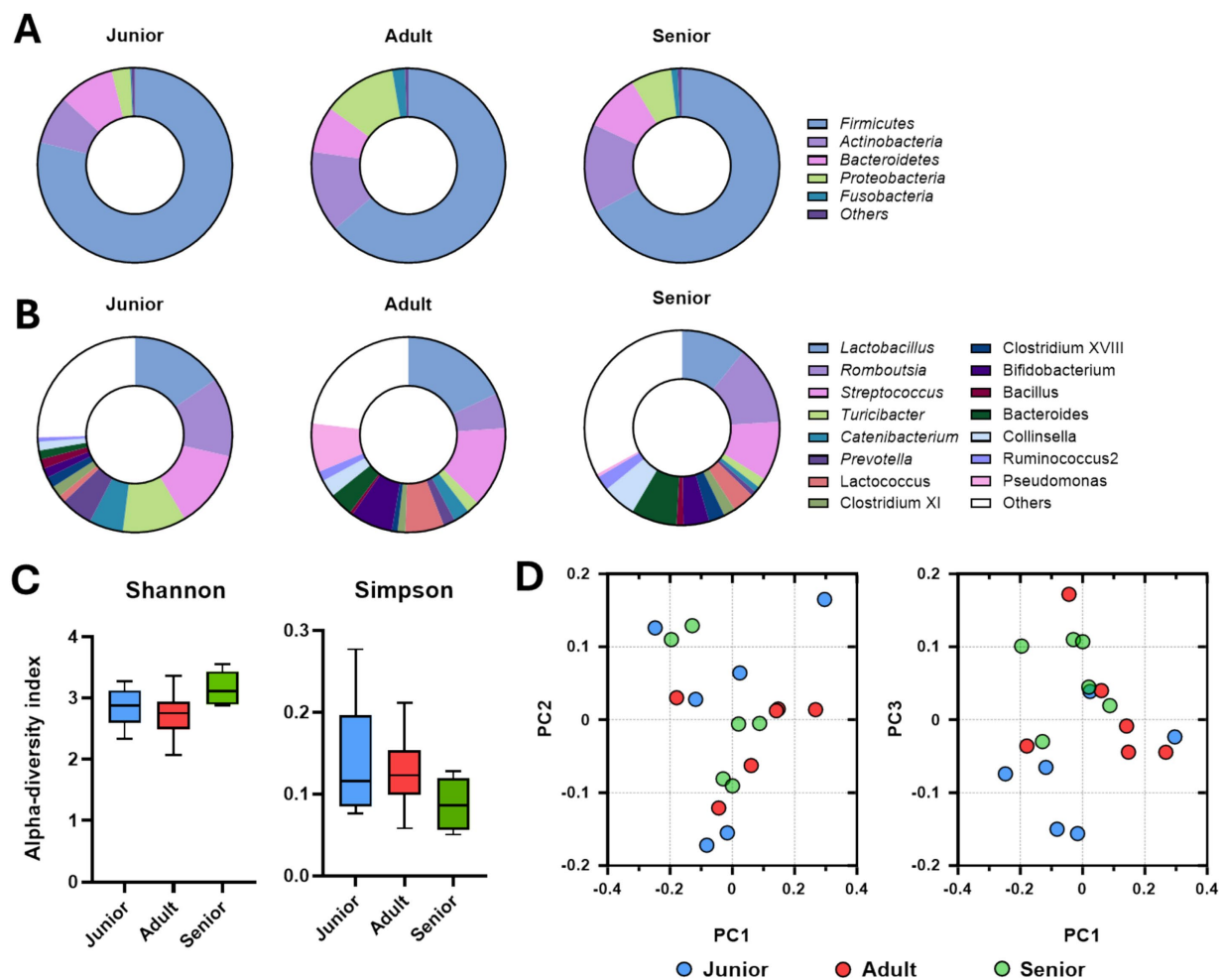


FIGURE 2

Gut microbiome composition determined via Illumina short-read sequencing. (A) Intestinal microbial population at the phylum level according to the age group. (B) Intestinal microbial population at the genus level according to the age group. (C) Alpha-diversity analysis of gut microbiome samples from Sapsaree dogs (Shannon and Simpson indexes). (D) Beta-diversity analysis of gut microbiome samples from Sapsaree dogs. Junior: <1 year old; adult: 4–6 years old; senior: >10 years old.

senior groups and that the concentrations of D-serine, α -tocopherol, and glyceric acid were significantly higher in the senior group compared to the other groups (Figure 4C). Based on gut metabolite composition, we predicted higher glutathione, arginine, proline, glyoxylate, dicarboxylate, glycine, serine, and threonine metabolism in the junior group compared to the other groups. Similarly, we predicted that pentose phosphate pathway activity, starch and sucrose metabolism, pyruvate metabolism, and steroid hormone biosynthesis would be significantly increased in the senior group compared to the other groups (Figure 4D).

3.5 Relationship between gut microbiome and metabolome

Correlation analysis between the gut microbiome and metabolome was performed to investigate the differences between short-read and long-read sequencing. Short-read sequencing results demonstrated that the populations of *Bifidobacterium* and *Lactobacillus* had significant positive correlations with the concentrations of γ -amino

butanoic acid and α -tocopherol and the populations of *Catenibacterium* and *Prevotella* had significant positive correlations with the concentrations of cadaverine and benzofuran (Figure 5). Additionally, the concentration of D-cellobiose showed a positive correlation with the populations of *Collinsella* and *Prevotella*.

Results from long-read sequencing showed that the concentration of *Blautia* was significantly correlated with the concentrations of 2,4,6-tri-butylaniline, α -tocopherol, androsterone, benzofuran, cadaverine, and glyceric acid (Figure 5). In accordance with the short-read sequencing results, long-read sequencing also demonstrated significant correlation between the concentration of γ -amino butanoic acid and the population of *Lactobacillus*. In addition, the concentrations of cadaverine and benzofuran were significantly correlated with the populations of *Faecalibacterium* and *Terrisporobacter*.

4 Discussion

Canine fecal metagenome analysis using Illumina and Nanopore technologies exhibited both common and unique genera. At the genus

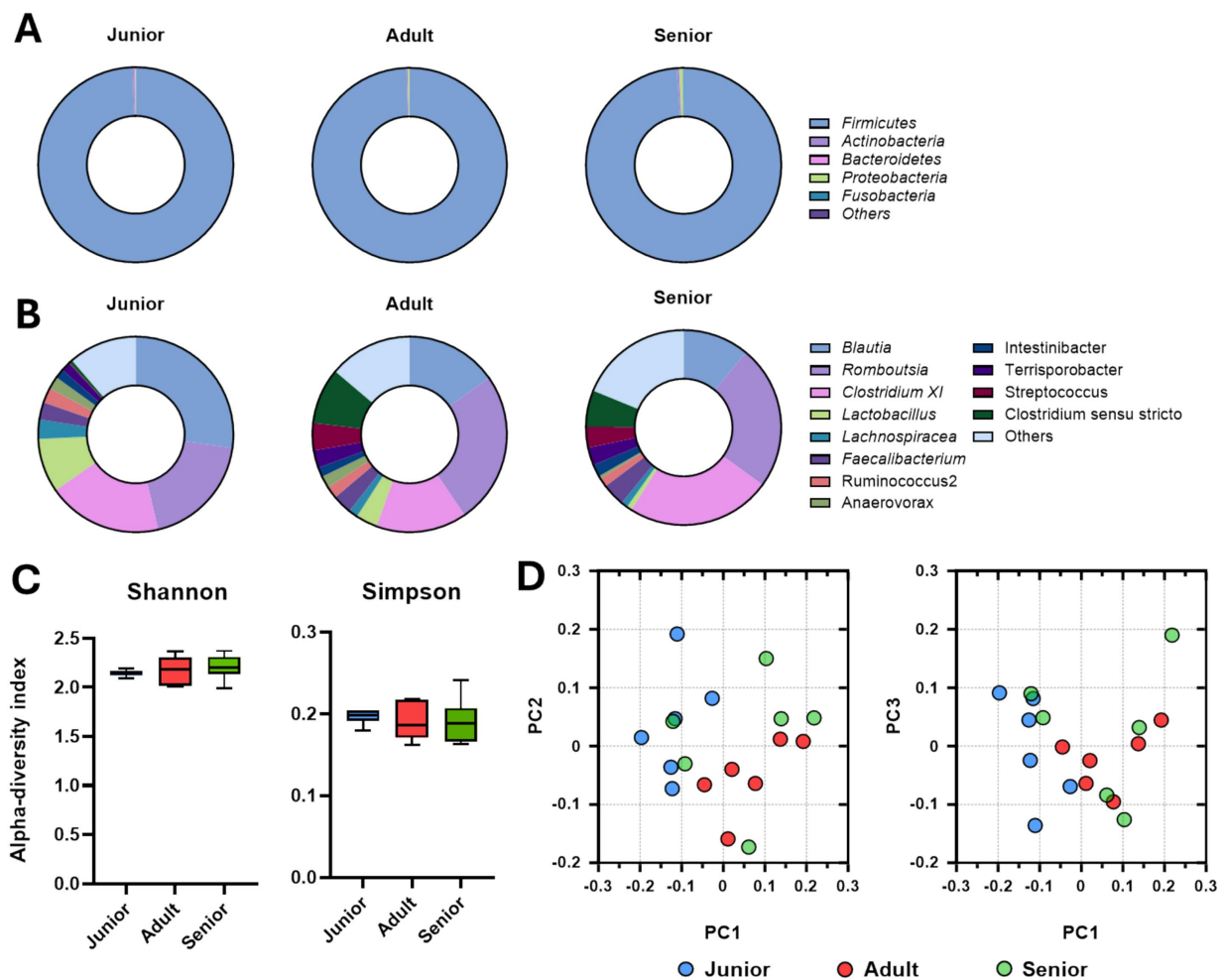


FIGURE 3

Gut microbiome composition determined via Nanopore long-read sequencing. (A) Intestinal microbial population at the phylum level according to the age group. (B) Intestinal microbial population at the genus level according to the age group. (C) Alpha-diversity analysis of gut microbiome samples from Sapsaree dogs (Shannon and Simpson indexes). (D) Beta-diversity analysis of gut microbiome samples from Sapsaree dogs. Junior: <1 year old; adult: 4–6 years old; senior: >10 years old.

level, genera identified by both the technologies included *Lactobacillus*, *Romboutsia*, *Streptococcus*, *Clostridium XI*, and *Ruminococcus*, with these genera showing similar trends in abundance across different age groups.

Specifically, *Lactobacillus* was observed to have a lower relative abundance in senior dogs compared to junior and adult dogs. *Lactobacillus* is widely recognized as a probiotic, known for its role in strengthening the gut epithelial barrier (Di Luccia et al., 2022; Yao et al., 2024) and exhibiting anti-inflammatory effects that can alleviate gastrointestinal disorders such as inflammatory bowel disease (IBD) (Oh et al., 2018; Aghamohammad et al., 2022). Recent studies have also reported the impacts of *Lactobacillus* on the central nervous system, with its effects on the gut–brain axis being related to γ -aminobutyric acid (GABA) (Patterson et al., 2019; Zhong et al., 2023; Tette et al., 2022). GABA is an important neurotransmitter that primarily functions as an inhibitory agent in the central nervous system (Hampe et al., 2018), reducing anxiety and promoting relaxation (Möhler, 2012). GABA is produced through the decarboxylation of glutamate (Rowley et al., 2012), with a significant proportion of its synthesis occurring via the gut

microbiota (Strandwitz, 2018). In this study, we observed a positive correlation with *Lactobacillus* and *Bifidobacterium*, which are known as major GABA producers (Strandwitz et al., 2019), explaining their significant correlation with GABA. The decrease in the proportion of GABA-producing bacteria in senior dogs may be associated with age-related declines in learning and cognitive abilities. Conversely, the heatmap analysis revealed that D-serine levels were higher in senior dogs compared to junior and adult dogs. D-serine acts as a co-agonist for the N-methyl-D-aspartate receptor, assisting in receptor activation upon glutamate binding (Ploux et al., 2021). Unlike GABA, D-serine is primarily synthesized within the body rather than by the gut microbiota (Pollegioni and Sacchi, 2010). Increased D-serine levels can be seen as an adaptive response to maintain neurotransmitter homeostasis in case of decreased GABA levels due to aging-related changes in the gut microbiota. However, as no direct correlations between GABA and D-serine synthesis have been reported, additional research is needed to investigate how GABA levels, which are impacted by gut microbiota composition, affect serine levels in the body.

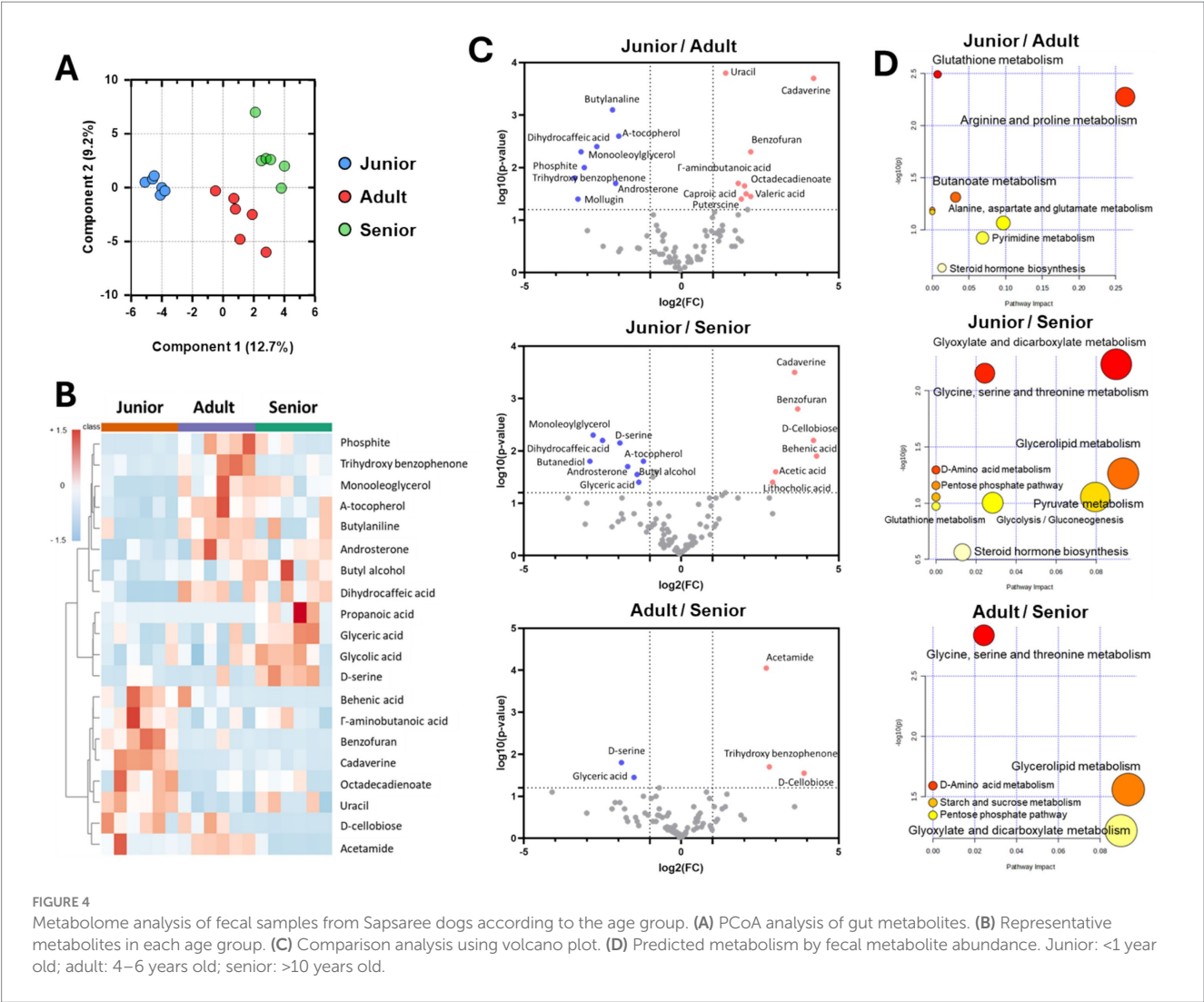


FIGURE 4 Metabolome analysis of fecal samples from Sapsaree dogs according to the age group. (A) PCoA analysis of gut metabolites. (B) Representative metabolites in each age group. (C) Comparison analysis using volcano plot. (D) Predicted metabolism by fecal metabolite abundance. Junior: <1 year old; adult: 4–6 years old; senior: >10 years old.

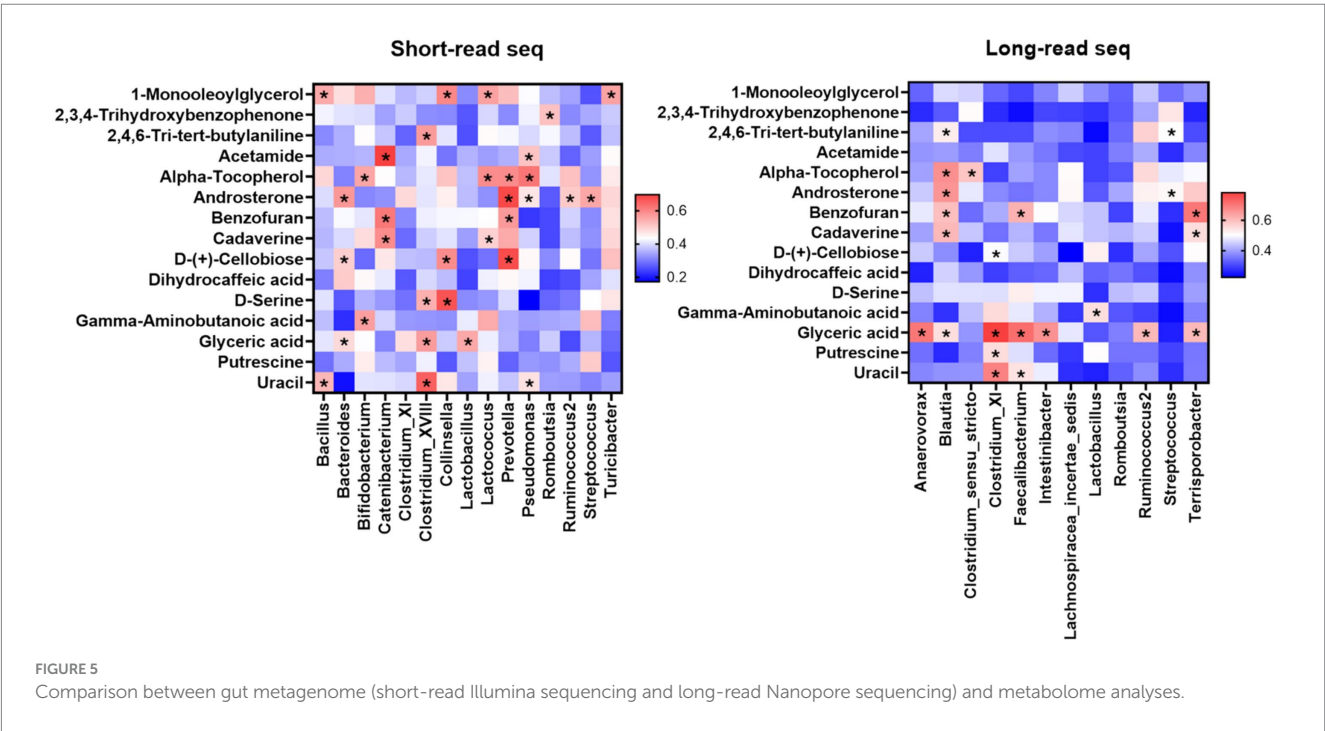


FIGURE 5 Comparison between gut metagenome (short-read Illumina sequencing and long-read Nanopore sequencing) and metabolome analyses.

In addition, the relative abundance of *Clostridium XI* was the highest in senior dogs, followed by juniors and adults. *Clostridium XI* is a cluster that includes several pathogenic species (Ohashi and Fujisawa, 2019) such as *Clostridium difficile*, which produces enterotoxins causing colitis (Savidge et al., 2003), and *Clostridium perfringens*, which produces neurotoxins leading to necrotizing enteritis (Cooper and Songer, 2010). However, the *Clostridium* cluster also includes important commensal bacteria that exhibit probiotic effects by producing short-chain fatty acids such as butyrate (Xu et al., 2020). Additionally, species within *Clostridium XI*, such as *Clostridium sordellii* and *Clostridium hiranonis*, are known to secrete 7 α -hydroxysteroid dehydrogenases, which deconjugate primary bile acids to form secondary bile acids (Guo et al., 2020). This process not only aids in the circulation of bile acids but also helps inhibit infections caused by *Clostridium difficile*, which is part of the same genus (Buffie et al., 2015). To date, prior research has predominantly focused on the pathogenic aspects of *Clostridium*. Therefore, to gain a comprehensive understanding of the role of *Clostridium* in the gut, further investigation of its potential contributions to intestinal health is needed.

The relative abundance of *Collinsella* was specifically measured using Illumina, unlike with Nanopore. The abundance of *Collinsella* is known to be influenced by the host's diet; when the host consumes a high-protein diet, the abundance of *Collinsella* decreases, whereas it increases with a fiber-rich diet (Walker et al., 2011; Candela et al., 2016). *Collinsella* showed the lowest abundance in junior dogs, followed by adults and seniors. Junior dogs require more calories for growth and development compared to adult and senior dogs; therefore, puppy food typically has lower carbohydrate content and higher protein and fat content than adult dog food (Jacuńska et al., 2023). This difference in the composition of food for junior and adult dogs can result in junior dogs having the lowest abundance of *Collinsella*. *Collinsella* has a positive correlation with D-(+)-cellobiose, a metabolite not produced by mammalian digestive enzymes but by bacterial enzymes in the gut (Nishimura et al., 2010). This suggests that increased abundance of *Collinsella* due to a high-carbohydrate diet significantly enhances carbohydrate metabolism in the gut, with *Collinsella* performing this necessary function for the host.

Although not detectable with Illumina, the relative abundance of *Blautia* was measured using Nanopore and found to be the highest in senior dogs, followed by adults and juniors. *Blautia* is a genus with members noted as potential probiotics (Mao et al., 2024; Mao et al., 2023). The lipid metabolism capabilities of *Blautia* have also been reported, including the breakdown of glycosylceramides into ceramides, fatty acids, and sphingoid bases for absorption in the gut (Furuya et al., 2010). Notably, mice fed a diet containing 1% purified glycosylceramides as prebiotics showed an increase in gut *Blautia* abundance, accompanied by a significant reduction in blood sugar levels (Hamajima et al., 2016). *Blautia* thus possesses lipid metabolism abilities and has demonstrated positive effects on blood sugar regulation. Additionally, a significant correlation between *Blautia* abundance and obesity has been shown. When mice were given dietary fiber extracted from corn along with a high-fat diet, there was a notable anti-obesity effect characterized by reduced body weight and tissue weight, and the proportion of *Blautia* in the gut microbiome significantly increased (Yang et al., 2016). The diet of the junior Sapsaree dogs in this study included fiber extracted from corn, which is believed to have contributed to the high relative abundance of *Blautia*. *Blautia* abundance is also influenced by the host's

age. An analysis of stool samples from 367 Japanese individuals aged 0–104 years revealed that adults (21–69 years) had higher abundances of *Blautia* and *Bifidobacterium* in their gut microbiome, with decreased *Blautia* abundance observed with increased age and a correlated decline in microbiome diversity (Vaiserman et al., 2017). This finding is consistent with our results showing the lowest *Blautia* abundance in senior dogs.

In contrast to *Blautia*, the highest abundance of *Faecalibacterium* was found in senior dogs, followed by adults and then juniors. *Faecalibacterium* is one of the butyrate producers, and it has been confirmed that the proportion of butyrate producers (including *Faecalibacterium* and *Roseburia*) is reduced in the gut microbiota of patients with IBD compared to those without IBD (Kowalska-Duplaga et al., 2019). In our study, heatmap analysis of the metabolome of junior dogs, which have the lowest abundance of *Faecalibacterium*, revealed a significant increase in the concentration of octadecadienoate (omega-6), a type of unsaturated fatty acid. A previous study found that the abundance of *Faecalibacterium* decreased and that of *Blautia* increased in the gut microbiome of adult men who consumed omega-3 fatty acid supplements (Noriega et al., 2016). This finding is similar to the pattern of gut microbiota composition observed in junior dogs in our study, suggesting an interaction between unsaturated fatty acids, *Blautia*, and *Faecalibacterium* abundance in the gut. However, there is no direct research on the effects of omega-6 fatty acids on gut microbiota changes, indicating a need for further studies on the correlation between omega-6, other unsaturated fatty acids and gut microbiota composition.

This study highlights the differences in metagenome analysis results using Illumina and Nanopore sequencing technologies. Illumina and Nanopore technologies represent short-read and long-read sequencing methods, respectively. Short-read sequencing can provide read sequences up to 600 bp and is cost effective (Pollard et al., 2018; Heather and Chain, 2016). However, there are limitations of short-read sequencing owing to the preferential amplification of repetitive DNA during the random fragmentation and amplification processes. By contrast, long-read sequencing can provide read sequences over 10 kb, recognize repeat sequences, and detect structural variations, although it has lower per-read accuracy compared to short-read sequencing (Adewale, 2020). Due to the shorter sequence length provided by Illumina's short-read methodology, the results are comparatively easier to annotate, often resulting in an exaggerated detection of microorganism diversity relative to the actual microbial composition (Buetas et al., 2024). Results of this study show that the 16S rRNA metagenome analysis using short reads identified a significantly higher number of genera compared to long reads. The observed differences in gut microbiota analysis between the Illumina and Nanopore methods intuitively demonstrate that each method has its own strengths and limitations, making them complementary to one another. Considering this, it is crucial to interpret data by focusing on genera that are consistently significant for both methods, rather than relying exclusively on a single method. Ultimately, further research comparing short-read and long-read sequencing is necessary.

5 Conclusion

In conclusion, this study revealed age-related changes and correlations between the metagenome and metabolome of the gut

microbiota in Sapsaree dogs. Metabolome analysis displayed clear clustering tendencies based on age groups, indicating a correlation with the metagenome composition. Metagenomic analysis showed that the genera generally considered beneficial were found in the lowest proportion in seniors, whereas those known as pathogens tended to be higher in proportion, suggesting age-related changes in gut microbiota composition and metabolome. Using Illumina and Nanopore technologies for metagenomic analysis, we observed differences in the results obtained using each technology; however, the genera commonly detected by both technologies showed similar trends in abundance across different age groups. This finding underscores the association between aging and gut microbiota composition, highlighting the need for further research in this area.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/SRR30105962>; <https://www.ncbi.nlm.nih.gov/SRR30105961>.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee at Chungnam National University (Approval No. 202310A-CNU-179). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

S-HS: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. M-GK: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AK: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. YKa: Data curation,

Formal analysis, Writing – original draft, Writing – review & editing. KK: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. M-JK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. YKi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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EDITED BY

Senem Kamiloglu,
Bursa Uludağ University, Türkiye

REVIEWED BY

Zhenya Zhai,
Jiangxi Academy of Sciences, China
Weiwei Wang,
South China Agricultural University, China
Fengyang Wu,
Hebei Agricultural University, China

*CORRESPONDENCE

Guo Liu

✉ liuguo@zhku.edu.cn

Xin Mao

✉ andy.mao@chinasweetpet.com

†These authors have contributed equally to this work and share first authorship

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Effects of Chenpi (*Citrus reticulata* cv. *Chachiensis*) on serum antioxidant enzymes, inflammatory factors, and intestinal health in Beagle dogs

Wencan Wang^{1†}, Ling Xu^{1†}, Yan Zhang², Yong Cao³,
Yixue Yang¹, Guo Liu^{3,4*} and Xin Mao^{1*}

¹Chongqing Sweet Pet Products Co., Ltd., Chongqing, China, ²Department of Animal Nutrition and Feed, College of Biological Engineering, Sichuan Water Conservancy Vocational College, Chengdu, China, ³Guangdong Provincial Key Laboratory of Nutraceuticals and Functional Foods, College of Food Science, South China Agricultural University, Guangzhou, China, ⁴College of Light Industry and Food Science, Zhongkai University of Agriculture and Engineering, Guangzhou, China

Ensuring companion animal welfare is a top priority for the pet industry and owners alike. The health of the pets can be directly and effectively improved through diet. Chenpi includes beneficial ingredients with proven anti-inflammatory, antioxidant, and immunomodulatory properties. The present investigation involved feeding snacks infused with Chenpi powder (CPP) to dogs for 42 days to examine the potential health benefits of CPP. The research evidenced a notable increase in serum superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activity in dogs, accompanied by a decrease in malondialdehyde (MDA), interleukin-8 (IL-8), and interferon-gamma (IFN- γ) level. Additionally, CPP increased fecal scores and significantly reduced fecal odors due to inhibition of 3-methylindole, hydrogen sulfide (H₂S), and ammonia nitrogen (NH₄⁺-N), and also raised the levels of fecal secretory immunoglobulin A (SIgA). Analysis of the microbial composition *via* 16S rRNA sequencing showed that CPP increased *Bacteroidota* and decreased *Firmicutes* in the gut flora at the phylum level. Functional prediction study of microbial communities also showed that the CPP group enriched metabolic and genetic information processing pathways. In addition, there were significant correlations between serum indicators and several significantly altered microorganisms. These findings suggest that CPP can potentially enhance the overall health of dogs by reducing fecal odorants, enhancing antioxidant and immunological capabilities, and modulating intestinal flora. This study establishes a solid scientific foundation regarding the application of CPP in functional pet foods.

KEYWORDS

Chenpi, intestinal flora, pet health, functional foods, dog

1 Introduction

The intestinal tract serves as the primary site for nutrient absorption and functions as the body's largest immune organ, playing a crucial role in defending against invading pathogens and other potentially harmful substances (Turner, 2009). Therefore, the intestinal tract is a crucial component of a pet's overall wellbeing, and its condition significantly influences the organism's health. Continuous contact with the external environment exposes the intestinal tract to various stressors, making intestinal ailments one of the most frequent clinical conditions in dogs. As the importance of pets in people's lives grows, pet health, particularly intestinal health, has been an increasing concern for pet owners (Alessandri et al., 2020). Intestinal microorganisms play an indispensable role in maintaining the normal function of the intestinal barrier, stimulating the immune system, and combating pathogenic microbes (Thursby and Juge, 2017). In addition, it has been suggested that disturbances in gut microbial homeostasis can trigger neurological disorders through the brain-gut axis (Cryan and Dinan, 2012). Intestinal health issues in dogs can arise from several sources, including genetics, environment, nutrition, diet, and viral and bacterial infections. However, the primary cause of intestinal diseases is disturbances in the intestinal flora (Blake and Suchodolski, 2016). In dogs and cats, common intestinal diseases, such as inflammatory bowel disease, acute diarrhea, and acute hemorrhagic diarrhea, are associated with an elevated presence of harmful intestinal bacteria (Allenspach et al., 2010; Guard et al., 2019; Janeczko et al., 2008; Suchodolski et al., 2010; Suchodolski et al., 2012b). Therefore, improving intestinal flora may have a preventive effect on developing intestinal diseases in pets.

Chenpi, the peel of citrus fruits, is renowned for its production in Xinhui, China. As a substance with dual medicinal and culinary properties, Chenpi contains many valuable beneficial compounds like flavonoids (Huang R. et al., 2020) and essential oils (Tranchida et al., 2012). These compounds have antioxidant (Chen et al., 2017), anti-inflammatory (Zhao et al., 2019), and immunomodulatory (Kim et al., 2019) effects. It has been shown that citrus peel dietary fiber enhanced serum and liver antioxidant enzyme activities as well as reduced malondialdehyde (MDA) levels in oxidative stress model mice (Fu et al., 2024). Similarly, citrus peel extract (CPE) had an enhancing effect on the activity of serum antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), while decreasing MDA levels in rabbits under heat stress (El-Gindy et al., 2023). Zhao et al. (2022) found that CPE also increased milk total antioxidant capacity (T-AOC) and SOD activity. On the other side, Kono et al. (2022) reported that nobiletin, a polymethoxylated flavone in Chenpi, inhibited NLR family pyrin domain containing 3 (*Nlrp3*), interleukin-1 β (*IL-1 β*), and tumor necrosis factor- α (*TNF- α*) gene expression in bladder mucosa of cystitis mice. Moreover, the hesperetin in citrus peel significantly reduced NF- κ B, TNF- α , and IL-1 β protein levels in the cerebral cortex and hippocampus of LPS-induced mice (Muhammad et al., 2019). Leray et al. (2011) indicated that supplementation with CPE notably reduced *INF- γ* gene expression in peripheral blood mononuclear cells of obese cats and improved obesity-induced inflammatory status. Moreover, studies have demonstrated that citrus peel can alleviate colonic mucosal injury (Han et al., 2023; Kawabata et al., 2018), inhibit central nervous system inflammation and oxidative stress (Omasa

et al., 2023), and prevent liver injury in mice (Chowdhury et al., 2015). Research has shown that nobiletin also has strong anti-tumor effects on lung, stomach, and colon cancer (Manthey and Guthrie, 2002; Rodriguez et al., 2002). The flavonoid extracts of Chenpi also have weight loss and lipid-lowering functions, which can reduce lipid accumulation in HepG2 cells and lower the levels of total cholesterol and triglycerides in the serum of mice (Kim et al., 2003; Su et al., 2019). It is noteworthy that citrus peel has the potential to enhance intestinal health and function. The citrus peel contains plentiful insoluble dietary fiber, which aids in regulating the composition, structure, and activity of intestinal flora, thus promoting the intestinal health of rats and mice (Huang J. Y. et al., 2020; Paturi et al., 2017). *In vivo* studies have also indicated that citrus peel can modify the composition and abundance of mice's intestinal flora (Hu et al., 2021; Qian et al., 2021). Furthermore, citrus peel has been shown to modulate intestinal immunity and enhance the immunological balance of the intestinal mucosa in mice (Chau et al., 2005).

Currently, citrus peels are already being used in livestock feed. However, there is a shortage of research on its potential benefits for pet health. Pets, being domesticated animals, have frequent interaction with humans, and the wellbeing of the pet is interconnected with the physical and emotional wellbeing of the pet owners. Therefore, the present study aims to evaluate the impact of Chenpi powder (CPP) on antioxidant status, immunomodulation, and intestinal health of dogs. The research involves assessing serum antioxidant and immune indicators, fecal odorant content, and examining changes in fecal microorganisms through 16S rRNA sequencing after feeding dogs with CPP-containing snacks. The present study will provide a scientific basis for incorporating CPP into functional foods to enhance pet health.

2 Materials and methods

2.1 Study design

The CPP used in the current study was purchased from Lianfu Food Co., Ltd. (Taizhou, China). Based on previous experimental explorations, the experimental snacks with a CPP concentration of 0.2% were formulated based on the control snacks. The composition of the control snacks and CPP is shown in Tables 1, 2, respectively.

This study included 20 adult and healthy Beagles (10 male and 10 female). The control group (CON) consisted of five male and five female dogs with an average age of 2.35 ± 0.05 years, average body weight of 13.29 ± 0.30 kg and average body condition score (BCS) of 4.6 ± 0.16 . The CPP group consisted of five male and five female dogs with an average age of 2.42 ± 0.06 years, average body weight of 12.92 ± 0.31 kg and average BCS of 4.7 ± 0.21 . The kennel was kept at 25°C with a humidity of 55%, and all dogs were housed individually in cages (1.2 m length $\times$ 1.2 m width $\times$ 1.4 m height), ensuring sufficient light and exercise once a day. All dogs received two meals of maintenance diet (Jiangsu Xietong Inc., Nanjing, China; 5.9% ash, 20.88% protein, 8.4% fat, and 3.5% fiber) per day, 150 g per meal at 9:00 a.m. and 3:00 p.m., and with adequate water intake. Two hours after consuming the maintenance diet, the dogs in the CON and the CPP group were provided 30 g of snacks,

TABLE 1 Composition of the control snacks.

Ingredients	Content (g)
Corn starch	60.00
Calcium carbonate	0.80
Glycerol	10.00
Monoacylglyceride	0.80
Sodium hexametaphosphate	0.50
Sugar	1.00
Chicken meal	2.00
Chicken paste	4.00
Chicken liver meal	3.00
Potassium sorbate	0.20
Calcium propionate	0.08
Water	20.00
Total	102.38

TABLE 2 Composition of the CPP.

Ingredients	Content (μg/g)
Heptamethoxyflavone	33.58
Hesperidin	5,545.56
Quercetin	1.69
Narirutin	60.03
Hesperetin	2.45
Didymin	29.33
Nobiletin	22.65
Sinensetin	144.75

respectively. Prior to the experiment, all dogs were acclimated to the snacks for 7 days. Afterward, serum biochemical analysis was conducted to evaluate the health status of the dogs. This study lasted for 42 days, and all the procedures were approved by the Institutional Animal Care and Use Committee of South China Agriculture University (Permit Number: SCAU-AEC-2010-0416).

2.2 Serum biochemical analysis

Canine blood samples were collected using previously established procedures with some modifications (Wang et al., 2022). Briefly, the blood samples were collected on the morning of day 0 (1 day before the experiment) and day 42 in a fasting state. Firstly, the dogs were secured in a suitable position, and the hair at the blood collection site was removed. Next, the forelimbs were tightly wrapped with tape to constrict the veins, and a syringe was used to extract blood gradually following the sterilization of the skin using 75% alcohol. A volume of 3 ml of blood was obtained and then transferred into sterile EP tubes. The tubes were centrifuged at $1,200 \times g$ for 15 min at 4°C to separate the serum. A portion of the serum was promptly applied for the automatic biochemical assessment (Seamaty, SMT-120VP, Chengdu, China). In contrast, the remaining serum

was stored at −20°C for subsequent determination of serum SOD (Solarbio Science & Technology Co., Ltd., Beijing, China), CAT (Solarbio Science & Technology Co., Ltd., Beijing, China), glutathione peroxidase (GSH-Px) (UpingBio, Shenzhen, China), MDA (Jiancheng Bioengineering Institute, Nanjing, China), and enzyme-linked immunosorbent assay (ELISA) testing.

2.3 Measurement of fecal odor substances

Dogs in the morning fasting state on days 0 and 42, fecal samples were collected and subjected to fecal scoring. After natural defecation, dogs' feces were scored according to the method by Middelbos et al. (2007). The criteria were as follows: 1 = hard, dry pellets; small, hard mass; 2 = hard-formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool; assumes the shape of unformed stool; assumes the shape of the container; and 5 = watery; liquid that can be poured. After scoring, feces not contaminated with urine and hair were collected with a sterile swab, transferred to 15 ml sterile EP tubes, and immediately kept on dry ice (−78.5°C). The fecal content of 3-methylindole was measured by gas chromatography/mass spectrometry (GC/MS; GC2010-QP2010, Shimadzu, Tokyo, Japan). Additionally, the fecal levels of hydrogen sulfide (H₂S) and ammonia nitrogen (NH₄⁺-N) were determined using specific kits (UpingBio, Shenzhen, China).

2.4 ELISA analysis

The content of canine fecal secretory immunoglobulin A (SIgA) (F66008-A), serum IL-1β (F5558-A), TNF-α (F7578-A), IL-8 (F4427-A), and interferon-gamma (IFN-γ) (F66003-A) was measured using the respective ELISA kits (Fankew, Shanghai Kexing Trading Co., Ltd., Shanghai, China).

2.5 Fecal microbiota sequencing analysis

2.5.1 Fecal DNA extraction, amplification, and sequencing

Fecal microbial DNA was extracted using the Stool DNA Kit (Tiangen Biotech, Beijing, China). The variable regions of V3–V4 in 16S rRNA genes were amplified *via* primers pair 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3'). Briefly, PCR amplification was performed by combining 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 μM of primers, and 10 ng of DNA template. The PCR conditions were as follows: pre-denaturation at 98°C for 1 min followed by 30 cycles at 98°C (10 s), 50°C (30 s), and 72°C (30 s). Finally, 72°C was kept for 5 min to obtain the PCR products, and it was purified using the magnetic beads. Based on the concentration of products, an equal amount of sample was fully mixed and the products were electrophoresed to recover the target bands. Sequencing libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). The libraries

were examined using Qubit (Thermo Fisher Scientific, Waltham, MA, USA) and q-PCR, then complied with the high-throughput sequencing using the No-vaSeq6000 PE250 (Novogene Co., Ltd., Beijing, China).

2.5.2 Bioinformatics analysis

The barcode and primer sequences were truncated to get the paired-end reads. These reads were then joined together using the FLASH program (v 1.2.11¹) for each sample, producing raw tags (Magoc and Salzberg, 2011). Next, the reverse primer sequence was matched using Cutadapt, and the remaining sequence was cut off to prevent it from interfering with subsequent analysis (Martin, 2011). The Fastp program (v 0.23.1) was utilized to eliminate the spliced raw tags and generate purified tags (Bokulich et al., 2013). The final effective tags were generated by comparing the tags obtained from the foregoing approach with the species annotation database² for detecting and removing chimeric sequences (Edgar et al., 2011). The denoising process in the QIIME2 software (v 2022.2) utilizing the DADA2 plugin (Wang et al., 2021) resulted in the acquisition of the final amplicon sequence variants (ASVs) and feature table. Following that, species annotation was conducted utilizing the Silva database.

Venn diagrams were generated using the Venn Diagram function in R software (v 4.2.1). Based on the top 10 species concerning the abundance at various taxonomic levels, the SVG function was used to plot relative abundance distribution in Perl as a histogram for each sample. The QIIME2 was utilized to calculate alpha diversity indices, such as Observed_Operational Taxonomic Units (OTUs), Shannon, Chao1, and Simpson. Beta diversity was assessed using the principle coordinate analysis (PCoA) in QIIME2. Moreover, the bacterial composition disparity between the two groups was further investigated utilizing ANOSIM to discern significant differences. The linear discriminant analysis (LDA) effect size (LEfSe) was used to identify the major microbial taxa that differed significantly in the experimental group. Furthermore, the *t*-test (*p*-value < 0.05) was applied for statistical analysis, and the microbial functions were predicted using PICRUSt analysis, which utilized the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.³

2.6 Statistical analysis

The data is presented as mean ± standard error of the mean (SEM). All data was verified for normality and variance homogeneity before statistical analysis. The Student's *t*-test (IBM SPSS, v 21.0) was used to assess the differences in serum antioxidant, immune indicators levels, as well as fecal odorants and SIgA levels between the two groups on day 0 and 42. The non-parametric test was used to analyze the differences in fecal scores. When *p* < 0.05, the differences were regarded as significant.

3 Results

3.1 CPP did not affect physiological indicators

To determine that CPP was not detrimental to the dogs' health, the blood biochemical analyses were performed on dogs fed for 42 days. The results showed that serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), and glutamyl-transferase (GGT) remained within the normal physiologic range (Supplementary Table 1). In addition, the dogs' body weight (Supplementary Figure 1, *p* > 0.05) and food intake (Supplementary Figure 2, *p* > 0.05) exhibited no significant changes throughout the study.

3.2 CPP enhanced serum antioxidant enzyme activity and lowered inflammatory factor levels

The results from biochemical and ELISA studies showed that feeding CPP for 42 days considerably enhanced the activity of canine serum SOD (*p* < 0.05) and GSH-Px (*p* < 0.05) (Figures 1A, C). Additionally, it significantly increased the CAT activity (Figure 1B, *p* < 0.01) compared to the CON group. Furthermore, there was a significant decrease in the serum MDA content in the CPP group (Figure 1D, *p* < 0.05). As shown in Figure 2, the content of IL-8 and IFN-γ decreased significantly after CPP feeding (Figures 2A, B, *p* < 0.05). However, no significant variation was observed in IL-1β and TNF-α between the two groups (Figures 2C, D, *p* > 0.05).

3.3 CPP reduced fecal H₂S, 3-methylindole, NH₄⁺-N content, and increased SIgA level

The fecal score, SIgA level, and the inhibitory effects of CPP on fecal odorants were estimated. The results demonstrated a significant increase in the fecal score of dogs following the administration of CPP for 42 days (Figure 3A, *p* < 0.05). Additionally, the levels of fecal odorants, including H₂S (Figure 3B, *p* < 0.01), 3-methylindole (Figure 3C, *p* < 0.05), and NH₄⁺-N (Figure 3D, *p* < 0.01), were significantly reduced. Moreover, a significant rise in the level of SIgA was observed (Figure 3E, *p* < 0.05).

3.4 CPP modulated the structural composition and abundance of intestinal microbiota

The impact of CPP on the intestinal microbiota of dogs was investigated using 16S rRNA sequencing. The sequencing results revealed that the Q30 value for all samples exceeded 93%. After filtering the raw tags, the proportion of effective tags that could

1 <http://ccb.jhu.edu/software/FLASH/>

2 www.arb-silva.de/

3 <https://www.genome.jp/kegg/pathway.html>, accessed on 10 February 2023.

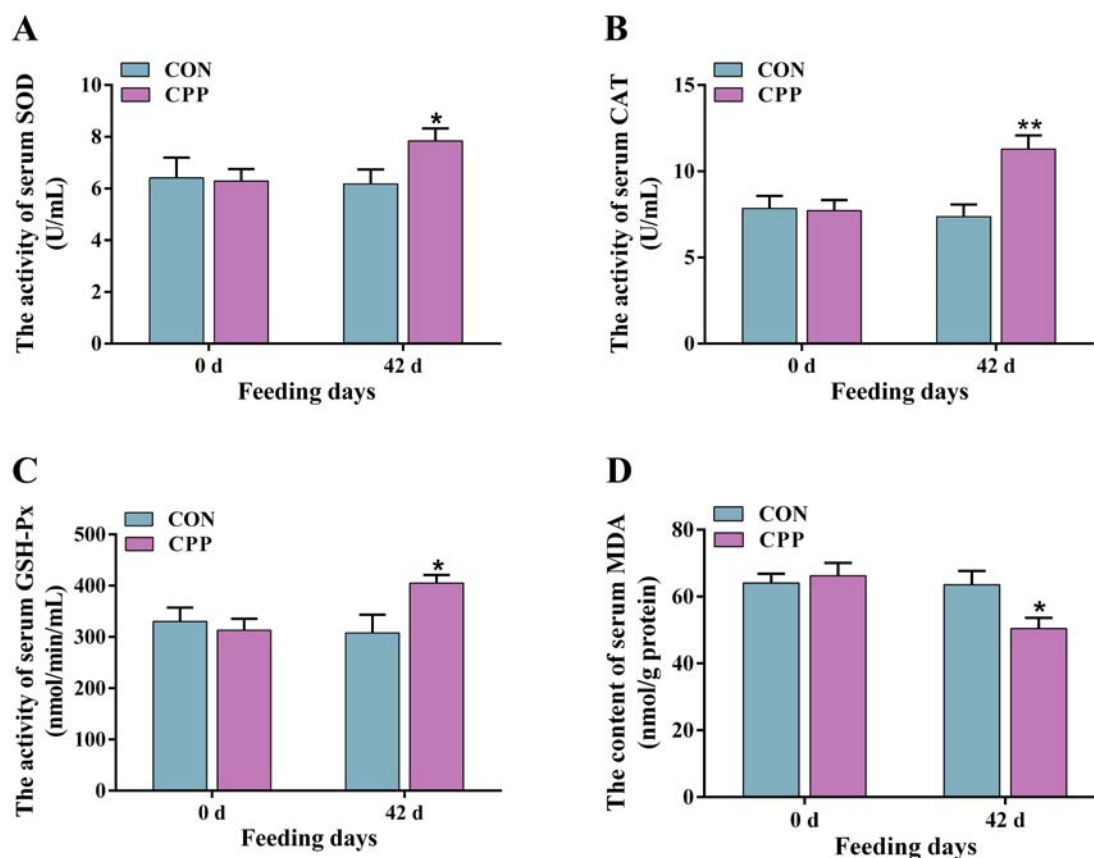


FIGURE 1

The activity changes of serum SOD (A), CAT (B), GSH-Px (C), and MDA (D) level. * $p < 0.05$; ** $p < 0.01$.

be used for subsequent analysis was above 80% in all cases (Supplementary Table 2). This indicates that the sequencing data was reliable. As shown in Supplementary Figure 3, 165 OTUs were shared across two groups. Of these, 249 OTUs were unique to the CPP group and 230 OTUs to the CON group (Supplementary Figure 3A). All samples' rarefaction curves eventually flatten out, suggesting that the number of species does not rise as the number of sequences does, and most of the microorganisms in each sample were detected sufficiently to characterize the sample's flora (Supplementary Figures 3B, C). According to the rank abundance curve, CPP enhanced the species richness of the intestinal flora in dogs (Supplementary Figure 3D).

The microbiota richness of the CPP group was higher than that of the CON group, as shown by the significantly higher Chao1 and Observed_OTUs indexes (Figures 4A, B). In contrast, the Shannon and Simpson indexes did not show significant changes (Figures 4C, D). Additionally, principal coordinate analysis (PCoA) showed the sample distances between the CON and CPP groups and revealed that the CPP group was segregated from the CON group (Figure 5A). Figure 5B illustrates the main genera that changed in abundance between the two groups.

At the phylum level, Firmicutes, Bacteroidota, Fusobacteria, Actinobacteria, and Proteobacteria are the principal bacterial phylum in the dogs' feces. Compared to the CON group, the CPP group had a decreased abundance of Firmicutes and Proteobacteria, with an increased abundance of Bacteroidota and

Actinobacteriota (Figure 6A). This resulted in a 2.6-fold higher Bacteroidota/Firmicutes (B/F) ratio (Supplementary Figure 4). At the family level, the CPP group had a higher relative abundance of Bifidobacteriaceae, Veillonellaceae, Lactobacillaceae, and Prevotellaceae than the CON group. In contrast, the relative abundance of Coriobacteriaceae, Erysipelotrichaceae, and Streptococcaceae was lower (Figure 6B). At the genus level, CPP raised the relative abundance of Prevotella_9, Lactobacillus, Megasphaera, and Bifidobacterium and reduced the relative abundance of Turicibacter, Streptococcus, and Collinsella (Figure 6C).

The microbial taxa that act as biomarkers for each group were found using the LEfSe analysis. As shown in Figure 7, Streptococcaceae, Streptococcus, Clostridia, Collinsella, Coriobacteriaceae, etc. were significantly enriched in the CON group, and Bifidobacterium_animalis, Actinobacteria, Bifidobacterium, Bifidobacteriales, Bifidobacteriaceae, Lactobacillus, and Lactobacillaceae were significantly enriched in the CPP group.

3.5 Predicted functional analysis by PICRUSt

Analysis of significant differences in functions between the two groups revealed that 33 KEGG pathways were altered in the

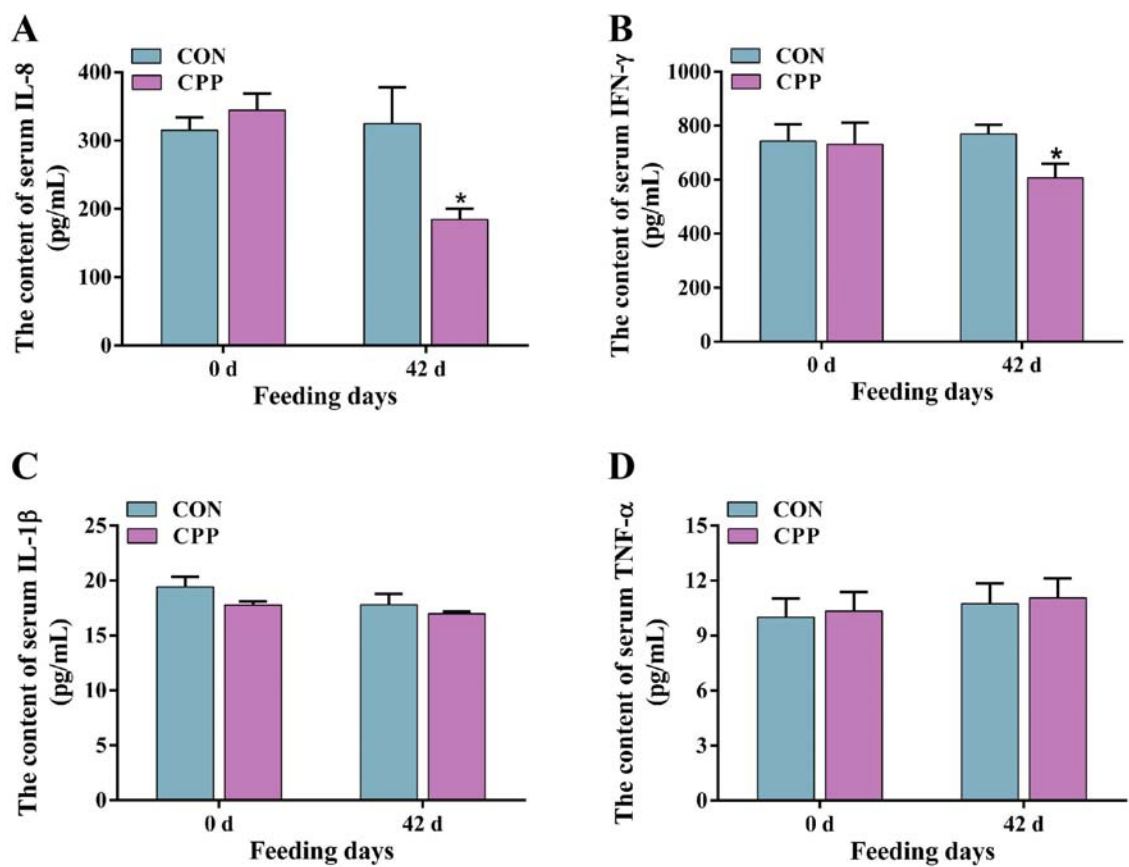


FIGURE 2
The changes in serum IL-8 (A), IFN- γ (B), IL-1 β (C), and TNF- α (D) level. * $p < 0.05$.

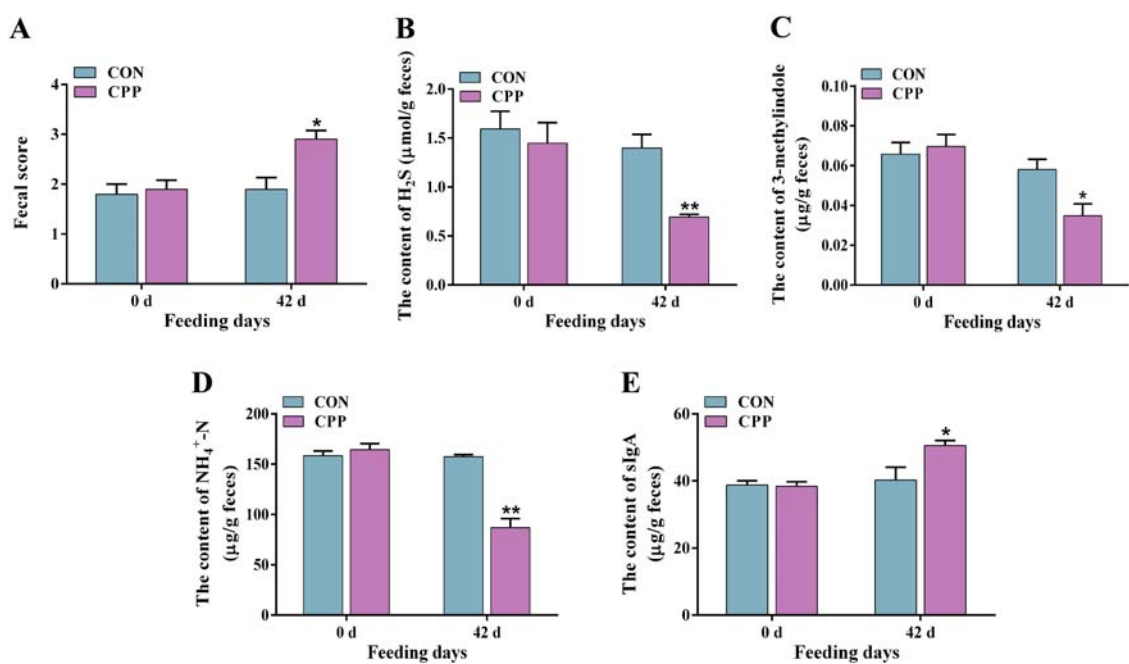


FIGURE 3
The fecal score (A) and content of fecal H₂S (B), 3-methylindole (C), NH₄⁺-N (D), and SlgA (E). * $p < 0.05$; ** $p < 0.01$.

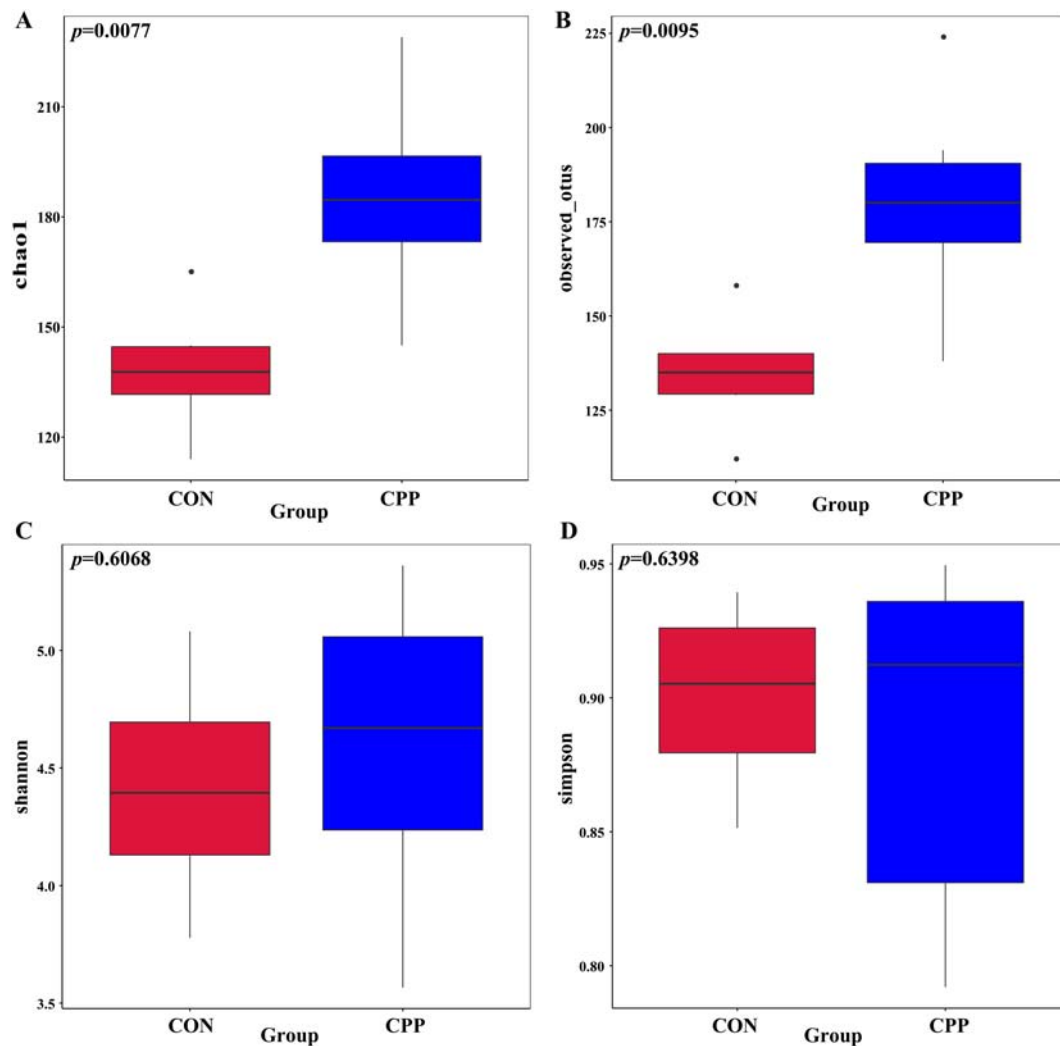


FIGURE 4
The Chao1 (A), Observed_OTUs (B), Shannon (C), and Simpson (D) indexes in α -diversity analysis of intestinal flora.

CPP group at level 2, among which 21 were increased and 12 decreased (Figure 8). The top 20 presumed microbial functions were compared using level 3 of the KEGG pathways (Table 3). Compared to the CON group, the CPP group had significantly higher abundance in the Genetic Information Processing-related pathways, such as “DNA repair and recombination proteins,” “Ribosome,” “Ribosome Biogenesis,” “Aminoacyl-tRNA biosynthesis,” “DNA replication proteins,” “Homologous recombination,” “Translation proteins,” and “Mismatch repair.” Moreover, Metabolism-related pathways were also found, including “Purine metabolism,” “Peptidases,” “Pyrimidine metabolism,” “Amino acid related enzymes,” “Cysteine and methionine metabolism,” and “Peptidoglycan biosynthesis.”

3.6 Correlation analysis between differential genera and serum indicators

Figure 9 demonstrates the results of Spearman’s correlation analysis, which examined the relationship between fecal

differential microbial and serum indicators. The analysis revealed that the CAT showed a significant positive correlation with the *Parasutterella*, *Bifidobacterium*, and *Megasphaera*. On the other hand, it showed a negative correlation with the *Megamonas*, *Peptoclostridium*, *Holdemanella*, *Blautia*, *Ligilactobacillus*, *Catenibacterium*, *Escherichia-Shigella*, and *Streptococcus*. The GSH-Px was significantly positively correlated with the *Parasutterella* and *Lactobacillus* while negatively correlated with the *Peptoclostridium*, *Holdemanella*, *Blautia*, *Catenibacterium*, *X. Ruminococcus_gnavus_group*, *Escherichia-Shigella*, *Collinsella*, and *Streptococcus*. The MDA exhibited a significant negative correlation alone with the *Bifidobacterium*. The levels of IFN- γ and IL-8 were shown to have a strong positive correlation with the presence of *Peptoclostridium*, *Holdemanella*, *Blautia*, *Catenibacterium*, *Escherichia-Shigella*, *Collinsella*, and *Streptococcus*. Conversely, there was a negative correlation between the levels of IFN- γ and IL-8 and the presence of *Parasutterella* and *Lactobacillus*. Moreover, the IFN- γ was also significantly negatively correlated with the *Bifidobacterium*.

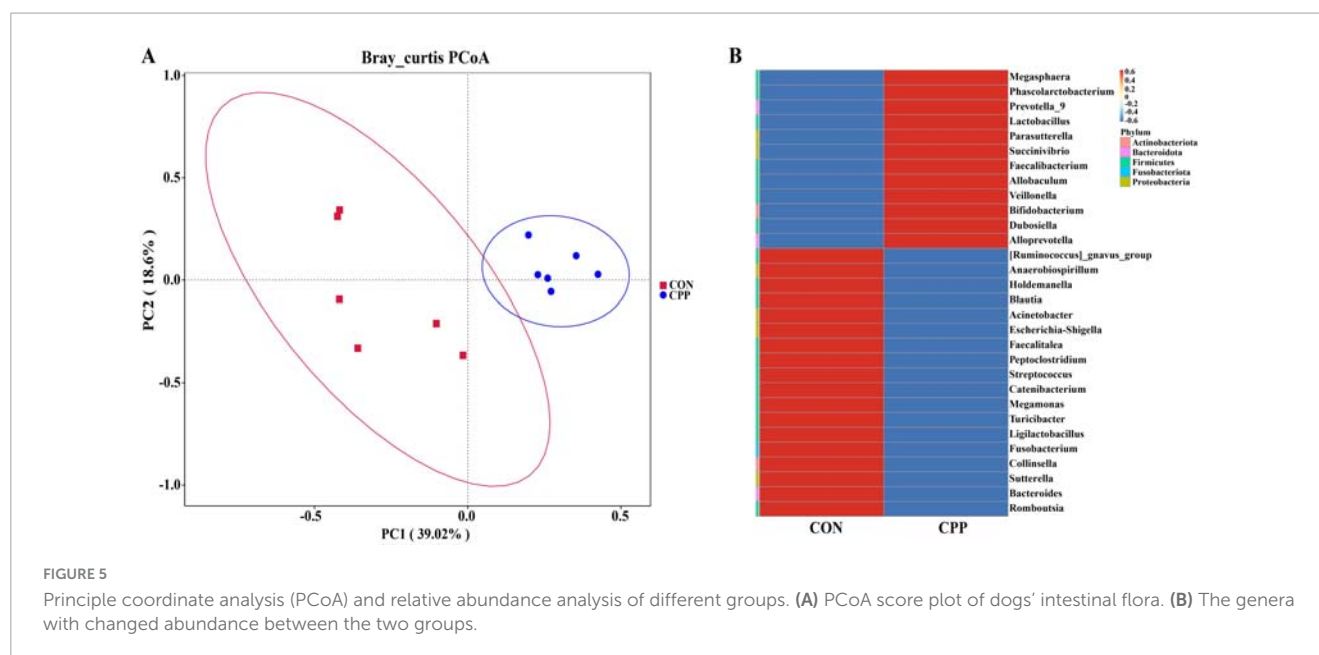


FIGURE 5

Principle coordinate analysis (PCoA) and relative abundance analysis of different groups. (A) PCoA score plot of dogs' intestinal flora. (B) The genera with changed abundance between the two groups.

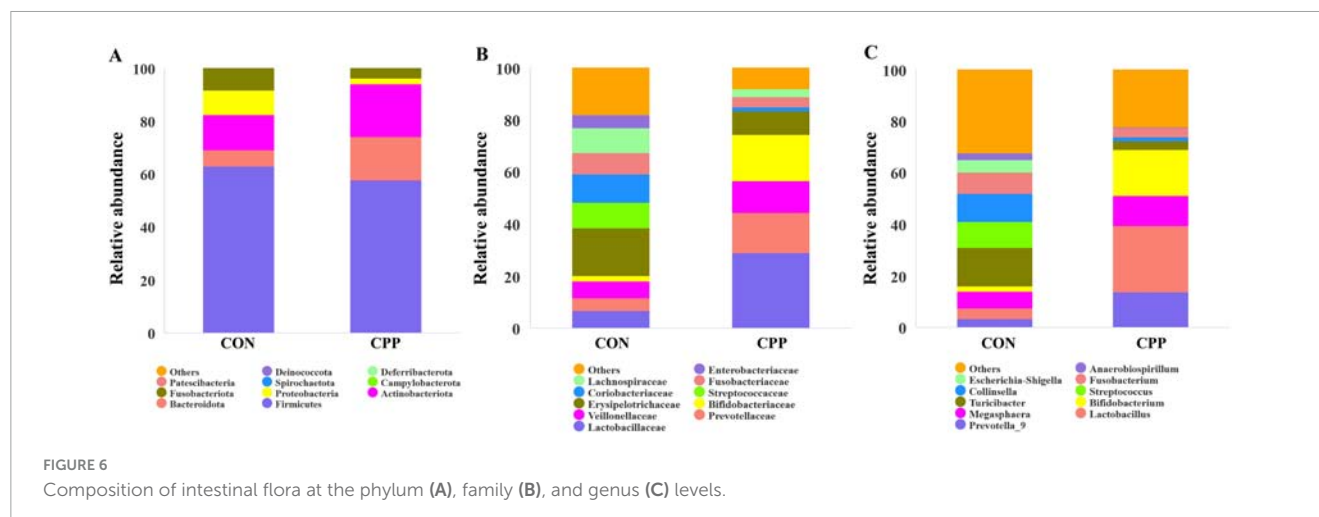


FIGURE 6

Composition of intestinal flora at the phylum (A), family (B), and genus (C) levels.

4 Discussion

This study investigates the effects of CPP on the health parameters and intestinal flora of dogs. The results revealed that CPP enhances dogs' health by increasing serum antioxidant enzyme activity, reducing inflammatory factor levels, and regulating the composition of the intestinal flora. Oxidative stress poses a threat to both humans and animals and is linked to various chronic diseases, including cancer, neurological disorders, and cardiovascular disease (Pizzino et al., 2017). Therefore, the antioxidant properties protect the overall wellbeing of the organism and extend its lifespan. SOD, GSH-Px, and CAT are three primary antioxidant enzymes, while MDA serves as a biomarker of oxidative damage that can be detrimental to health (Tsikas, 2017). Prior research has shown that CPP can increase the levels and activity of serum GSH-Px and CAT while decreasing MDA levels in mice with liver damage (Chowdhury et al., 2015). Additionally, Qian et al. (2021) showed that feeding aqueous solution containing CPP causes a significant decrease in hepatic MDA content and a slight

increase in SOD content in mice, although no significant changes were observed (Hu et al., 2021). Similarly, Chen et al. (2012) found that CPP effectively suppressed the rise in MDA levels in HepG2 cells caused by oxidative stress while simultaneously increasing the activity of CAT and GSH-Px enzymes, as well as the concentration of SOD. Similar to research conducted on other animal species, the current study demonstrated that supplementation of CPP resulted in significant rises in dogs' blood SOD, GSH-Px, and CAT activities while reducing MDA levels. These findings indicate that CPP greatly enhances the antioxidant capacity in dogs.

Pro-inflammatory cytokines regulate intestinal mucosal barrier function and can increase mucosal epithelial cell permeability, consequently inducing inflammation (Katsanos and Papadakis, 2017). Studies conducted on mice with colitis have demonstrated that intact CPP is more efficacious in reducing the expression of pro-inflammatory factors IL-6, CXC motif chemokine ligand 2 (CXCL2), IL-1 β , and TNF- α than a single CPP extract (Kawabata et al., 2018; Tinh et al., 2021). Han et al. observed a significant

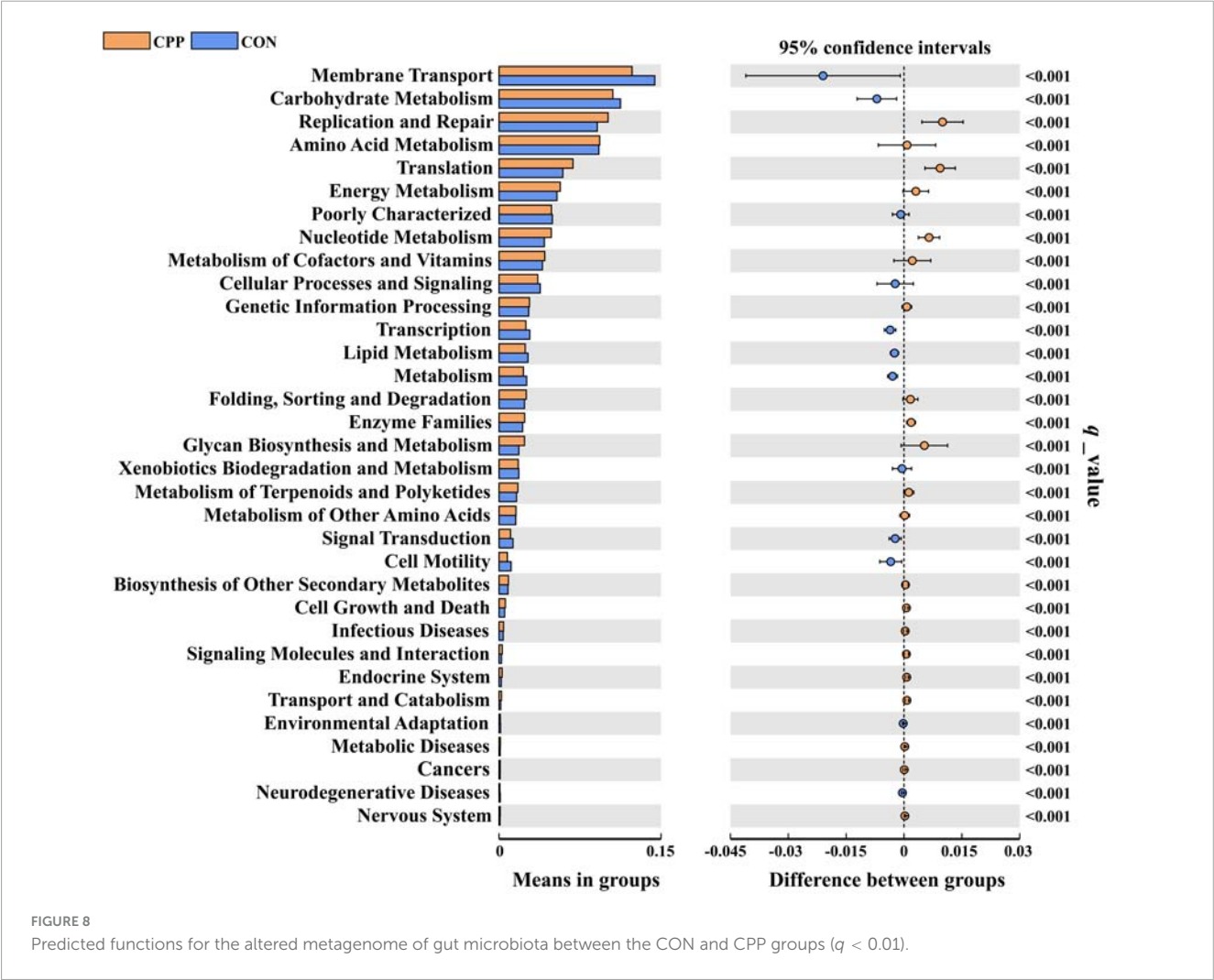
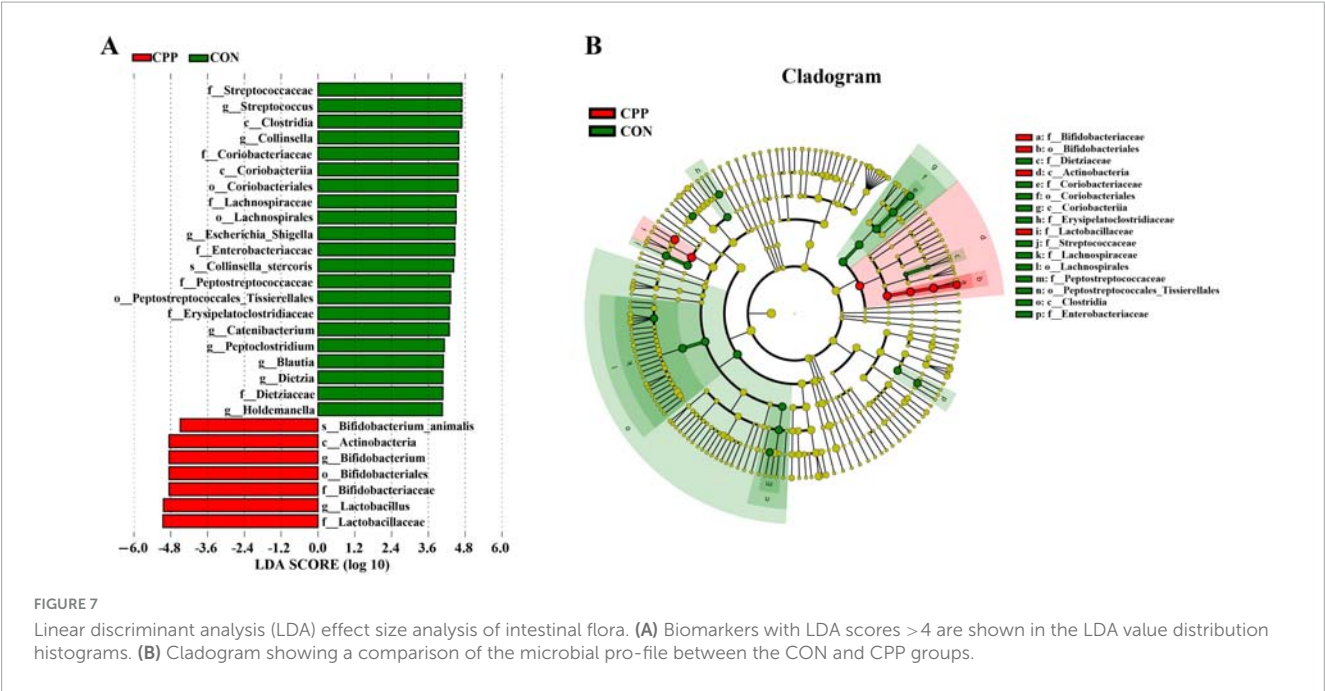
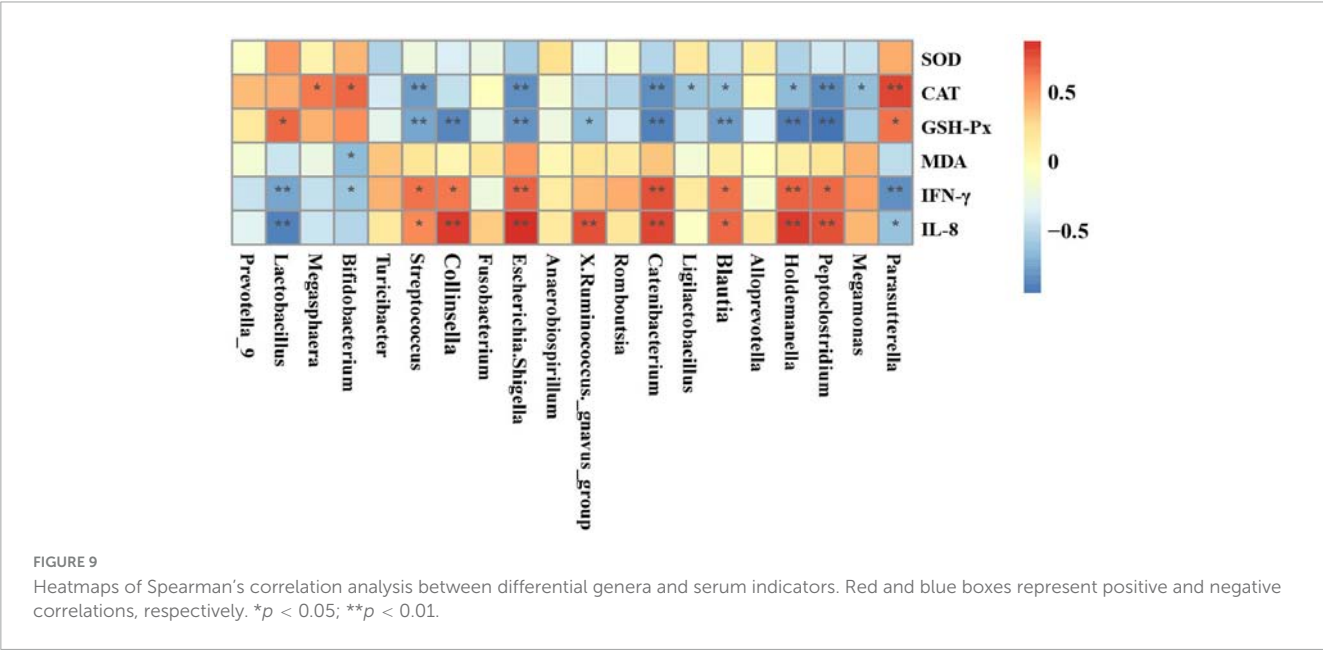


TABLE 3 The selected top 20 most abundant pathways at level 3.

KEGG pathways	Group		q-value
	CON	CPP	
Environmental information processing			
Transporters	0.0776 ± 0.004	0.0653 ± 0.011	0.042
Two-component system	0.0117 ± 0.001	0.0095 ± 0.001	0.019
Translation proteins	0.0094 ± 0.000	0.0099 ± 0.000	0.040
Genetic information processing			
DNA repair and recombination proteins	0.0294 ± 0.001	0.0334 ± 0.001	0.002
Ribosome	0.0242 ± 0.001	0.0295 ± 0.002	0.002
Transcription factors	0.0186 ± 0.001	0.0145 ± 0.001	0.002
Ribosome biogenesis	0.0148 ± 0.001	0.0157 ± 0.000	0.014
Aminoacyl-tRNA biosynthesis	0.0126 ± 0.001	0.0150 ± 0.001	0.005
DNA replication proteins	0.0123 ± 0.000	0.0135 ± 0.001	0.030
Homologous recombination	0.0097 ± 0.000	0.0106 ± 0.000	0.009
Mismatch repair	0.0083 ± 0.000	0.0092 ± 0.001	0.010
Metabolism			
Purine metabolism	0.0229 ± 0.001	0.0266 ± 0.001	0.002
Peptidases	0.0193 ± 0.001	0.0217 ± 0.001	0.008
Pyrimidine metabolism	0.0190 ± 0.000	0.0218 ± 0.002	0.009
Amino acid related enzymes	0.0149 ± 0.001	0.0170 ± 0.000	0.007
Methane metabolism	0.0119 ± 0.001	0.0110 ± 0.000	0.035
Fructose and mannose metabolism	0.0120 ± 0.001	0.0100 ± 0.001	0.009
Pyruvate metabolism	0.0108 ± 0.000	0.0089 ± 0.001	0.002
Cysteine and methionine metabolism	0.0096 ± 0.000	0.0105 ± 0.000	0.009
Peptidoglycan biosynthesis	0.0087 ± 0.000	0.0098 ± 0.001	0.009



inhibition of serum IL-6 and TNF-α content in mice with high-fat-induced intestinal mucosal barrier damage following CPP supplementation (Han et al., 2023). It was found that CPP significantly reduced the serum content of IL-8 and IFN-γ, suggesting a potential contribution of CPP to the enhancement of intestinal mucosal defenses in dogs. However, no significant change

in TNF- α and IL-1 β levels was observed, which differed from other studies and may be attributed to species and diet differences. SIgA, found in high quantities in the mucous membranes of animals' intestines, is crucial in protecting against the infiltration of harmful intestinal pathogens and toxic substances (Mantis et al., 2011). This study showed a significant increase in fecal SIgA content following CPP supplementation. This further underlines the beneficial effect of CPP on the intestinal health of dogs.

Chenpi powder has been proven to affect the composition of intestinal flora (Hu et al., 2021; Qian et al., 2021). However, there is a lack of research data pertaining to dogs. The 16S rRNA sequencing results revealed that the five predominant microbial phyla in dogs' feces were Firmicutes, Bacteroidota, Fusobacteria, Actinobacteria, and Proteobacteria, consistent with findings from other studies (Honneffer et al., 2017; Pilla and Suchodolski, 2020). CPP supplementation increased the B/F ratio compared to the CON group. It has been shown that the B/F ratio significantly decreases under conditions of impaired intestinal health in obese mice (Evans et al., 2014; Long et al., 2019). Conversely, the addition of CPP inhibition to the diet has been discovered to prevent the reduction of fecal B/F ratio in obese mice (Li et al., 2021). Moreover, Qian et al. (2021) also found that CPP increased the B/F ratio in the feces of healthy mice. Hoffman et al. (2017) proposed that a decrease in the B/F ratio leads to the production of inflammatory factors by pathogenic bacteria. These results indicate that CPP supplementation could elevate the B/F ratio, thus improving the intestinal health status of dogs. The α -diversity and β -diversity analyses showed that CPP supplementation affected the abundance of intestinal flora in dogs. Bifidobacteriaceae, Lactobacillaceae, and Prevotellaceae are beneficial bacteria for animals that ferment carbohydrates to produce short-chain fatty acids, which boost the host immune response and provide energy for colonocytes, and is essential for intestinal health (Minamoto et al., 2015; Rivera-Chávez et al., 2016; Shen et al., 2018; Vazquez-Baeza et al., 2016; Zhang et al., 2021). Furthermore, research has shown that Lactobacillus and Bifidobacterium can safeguard the health of the intestines by counteracting the establishment of detrimental bacteria, therefore affecting inflammation (Guandalini, 2011; Kok and Hutkins, 2018; Konstantinov et al., 2008; van Baarlen et al., 2009). Li et al. (2019) found that Lactobacillus and Bifidobacterium also alleviated oxidative stress damage in mice. Ojo et al. (2019) showed that Lactobacillus was negatively correlated with serum MDA levels, reflecting the organism's antioxidant capacity to some extent, and the current results are consistent with the existing studies. It was also found that several beneficial bacteria with increased abundance in the CPP group were positively correlated with antioxidant enzymes and negatively correlated with MDA and pro-inflammatory factors. In contrast, Escherichia-Shigella and Turicibacter were negatively correlated with antioxidant enzymes and positively correlated with MDA and pro-inflammatory factors. This indicates that these CPP-enriched beneficial bacteria may be crucial in enhancing antioxidant activity and boosting the immune system. Also, CPP elevated the prevalence of Veillonellaceae in the dogs' intestinal tract. Research has demonstrated that the abundance of Veillonellaceae significantly decreases in the intestinal tract of dogs with enteritis (Suchodolski et al., 2012a), whereas Veillonellaceae abundance rises in healthy dogs following probiotic supplementation (Garcia-Mazcorro et al., 2017).

The results of KEGG pathway analysis showed that the enrichment of genetic information processing and metabolism-related pathways in the CPP group was significantly higher than that in the CON group, such as DNA repair and recombination proteins, aminoacyl-tRNA biosynthesis, amino acid metabolism, nucleotide metabolism, metabolism of cofactors and vitamins, energy metabolism, and peptidoglycan biosynthesis, suggesting that CPP has a higher level of nutrient metabolism, which is favorable for the reproduction of the intestinal flora.

The studies showed a strong link between the onset of colitis and specific pathogenic bacteria like Escherichia-Shigella and Turicibacter (Peng et al., 2020; Wan et al., 2021). Doulidis et al. (2023) indicated a significant increase in the abundance of intestinal Escherichia-Shigella in dogs with enteritis. Similarly, Díaz-Regañón et al. (2023) found a 16-fold increase in the abundance of Escherichia-Shigella in the intestinal tract of dogs with enteritis compared to healthy dogs. Additionally, an elevated presence of Enterobacteriaceae, which indicates an imbalance in the gut microbiota, was found to negatively correlate with beneficial metabolites in the intestine (Vazquez-Baeza et al., 2016). Several studies have shown a significant increase in intestinal Enterobacteriaceae abundance in dogs with inflammatory bowel disease (Suchodolski et al., 2010; Suchodolski et al., 2012a; Xenoulis et al., 2008). Furthermore, these bacteria could produce endotoxins, leading to systemic inflammation (Boopathi et al., 2023; Deng et al., 2023). Coriobacteriaceae and Fusobacteriaceae produce phenolic compounds posing a serious threat to host health (Saito et al., 2018). Coriobacteriaceae abundance was found to be approximately twice as high in dogs with enteritis as in healthy dogs (Díaz-Regañón et al., 2023). The obtained results of the present study suggested that CPP promoted intestinal and overall health in dogs.

The unpleasant odors from animal feces can be bothersome, impacting mood and sensory experiences. The primary odorous substances in dogs' feces include sulfur compounds, indolic compounds, and ammonia, which result from the metabolic breakdown of food residues (Cho et al., 2015). Research has not yet investigated the decrease in the number of these malodorous compounds by CPP. This study uncovered that CPP significantly reduced the levels of H₂S, NH₄⁺-N, and 3-methylindole in dogs' feces, indicating a favorable impact of CPP on odor reduction. This may be attributed to decreased odor-producing bacteria such as Escherichia-Shigella, Enterobacteriaceae, Streptococcus, and Fusobacteria (Carbonero et al., 2012; Richardson et al., 2013). However, further research is still needed to elucidate the specific regulatory mechanism.

5 Conclusion

In conclusion, this investigation found that CPP enhanced the antioxidant and immunological functions of dogs by augmenting the activity of antioxidant enzymes in the serum and reducing the levels of MDA and inflammatory indicators. Furthermore, CPP increased intestinal SIgA levels and successfully reduced the content of malodorous compounds in the feces, which contributed to improving intestinal immunity and alleviating fecal odor. CPP modulated the composition of the canine intestinal flora and stimulated beneficial bacterial growth, which had a considerable

positive impact on the host's intestinal and organismal health. These findings provide an innovative perspective and a scientific theoretical foundation for developing functional foods promoting pets' health in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA1078076.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of South China Agriculture University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WW: Conceptualization, Data curation, Methodology, Validation, Visualization, Writing – original draft. LX: Data curation, Investigation, Methodology, Validation, Writing – review & editing. YZ: Data curation, Methodology, Writing – review & editing. YC: Methodology, Resources, Writing – review & editing. YY: Conceptualization, Investigation, Writing – review & editing. GL: Data curation, Methodology, Project administration, Supervision, Writing – review & editing. XM: Conceptualization, Project administration, Supervision, Writing – review & editing.

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The reviewer WW declared a shared parent affiliation with the authors GL and YC to the handling editor at the time of review.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1415860/full#supplementary-material>

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Sidharth Prasad Mishra,
University of South Florida, United States
Dan Cheng,
Guangdong Longsee Medical, China

*CORRESPONDENCE

Honglin Yan
✉ honglinyan@swust.edu.cn

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Deciphering of differences in gut microbiota and plasma metabolites profile between non-obese and obese Golden Retrievers dogs

Yazhen Cai, Huayu Tang, Guilin Xiang, Hongyu Yi, Jie Zhong,
Zhaoxi Xie, Qinfeng Hu, Romaisa El Bouhi, Pan Zhou,
Yong Zhang and Honglin Yan*

School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, China

Introduction: Golden Retrievers have a high risk of obesity, which is prevalent in dogs and is associated with inflammation and cancer, impairing the health and life expectancy of companion animals. Microbial and metabolite biomarkers have been proposed for identifying the presence of obesity in humans and rodents. However, the effects of obesity on the microbiome and metabolome of Golden Retrievers remains unknown. Therefore, this study was designed to evaluate the signatures of serum biochemistry indexes, gut microbiota and plasma metabolites in non-obese and obese Golden Retrievers, aiming to recognize potential biomarkers of canine obesity.

Methods: A total of 8 non-obese (Ctrl group) and 8 obese (Obe group) Golden Retrievers were included in the present study to collect blood and feces samples for measurements. The fecal microbiome and plasma metabolome were determined using 16S rRNA amplicon sequencing and liquid chromatography-mass spectrometry, respectively.

Results: Results showed that the alanine aminotransferase activity and total bilirubin concentration, which have been measured using serum biochemistry analysis, were higher in the Obe group than in the Ctrl group ($p < 0.05$). Moreover, there was a significant difference in gut microbiota composition between the two groups ($p < 0.05$). The phyla Proteobacteria, Fusobacteriota, and Bacteroidota as well as genera *Fusobacterium*, *Prevotella*, *Faecalibacterium*, *Escherichia-Shigella*, and *Alloprevotella* were more abundant, while phylum Firmicutes and genera *Peptoclostridium*, *Blautia*, *Turicibacter*, *Allobaculum*, and *Erysipelatoclostridium* were less abundant in the Obe group compared to the Ctrl group ($p < 0.05$). Plasma concentrations of citrulline and 11-dehydrocorticosterone were significantly higher in the Obe group than those in the Ctrl group ($p < 0.05$). Close correlations between serum biochemistry parameters, gut microbiome, and plasma metabolites were observed in the current study.

Conclusion: The obesity-induced shifts in serum biochemistry indexes, gut microbiota, and plasma metabolites profiles suggest that obese Golden Retrievers exhibit a different microbiome and metabolome than non-obese ones, and the certain metabolites like citrulline and 11-dehydrocorticosterone could be considered as potential biomarkers to recognize obese Golden Retrievers.

KEYWORDS

obesity, Golden Retriever, serum biochemistry indexes, plasma metabolome, microbiota

1 Introduction

Obesity has been declared as a major global health problem in both human beings and domesticated animals, and it has been most commonly observed in companion animals (Chandler et al., 2017). Previous studies have showed that approximately 59% of dogs are overweight or obese (Courcier et al., 2010). Obese dogs have been demonstrated to be more prone to suffering from a subclinical inflammatory state than their lean counterparts, characterized by the higher levels of certain inflammatory markers, which are associated with a higher risk of cardiovascular illnesses and cancer (Vecchiato et al., 2023). Thus, accurate diagnosis is crucial for early obesity treatment in dogs, thereby enhancing their welfare and quality of life. The most common tool used by veterinarians to assess the nutritional status of dogs is the body condition score (BCS) system (Broome et al., 2023), which has relatively high accuracy and reproducibility, but it still has a certain degree of subjectivity in determining canine obesity because the evaluation primarily relies on visual examination and palpation (Tesi et al., 2020; Miller et al., 2018). Even among experienced veterinarians, discrepancies in evaluating BCS for the same dog may occur. Therefore, the additional biomarker indicators are needed to identify for providing supplementary references in the diagnosis of canine obesity by the veterinarians using BCS system.

Serum biochemistry parameters are commonly used to detect the metabolic health of humans and animals. Alanine aminotransferase (ALT) and total bilirubin (TB) serve as significant potential biomarkers in the clinical assessment of liver inflammation-related diseases not only in humans, but also in feline and canine populations (Dirksen et al., 2017; Kozat and Sepehrizadeh, 2017). Furthermore, recent research has demonstrated a positive correlation between ALT levels and body weight in humans, highlighting its potential utility in evaluating metabolic health (Bekkelund and Jorde, 2019).

Although obesity is highly prevalent in dogs, there are higher frequencies of overweight conditions in certain breeds of dogs including Golden Retrievers, Pug, Beagle and English Springer Spanie than in others (Reddy et al., 2019; Pegram et al., 2021). The breed susceptibility of Golden Retriever to obesity is 49.53% according to the clinical study (Mounika et al., 2020), implying that Golden Retrievers should be paid more attentions on their obese status (Pegram et al., 2021; O'Neill et al., 2021). In recent years, increasing evidence has shown that gut microbiota dysbiosis played a causal role in the early onset of obesity and lipid metabolism dysfunction (Yu et al., 2019; Zhang and Gérard, 2022). On the other hand, obese individuals have harbored a distinct gut microbiome from those lean individuals, indicated by reduced microbial diversity and altered taxonomic abundances in the overweight cohort (Lippert et al., 2017). The higher Firmicutes and Bacteroidetes ratio has been related to obesity in some studies, but these observations are inconsistent across studies, and it is unknown which specific microbial taxon is directly related to the development of obesity (Xie et al., 2012). Likewise, overweight Beagle dogs also have exhibited a significantly different gut microbiome from the normal ones,

characterized by higher abundances of genera *Faecalibacterium*, *Phascolarctobacterium*, *Megamonas*, *Bacteroides*, *Mucispirillum* (Kim et al., 2023). These evidences further support the contention that gut microbiota may be involved in the metabolic health of the host. Owing to the close relationship between gut microbiota and host obesity, several attempts have been made to reveal the potential microbial biomarkers for detecting dietary responsiveness in obese individuals with impaired metabolic health. Indeed, some microbial biomarkers have been identified to hold the potential to predict obesity, and interventions based on these microbial biomarkers might be beneficial to weight loss and metabolic risk improvement (Barengolts et al., 2019). It remains unknown the differences in gut microbiota composition between non-obese and obese Golden Retriever dogs, and thus it is necessary to identify microbial biomarkers of obesity in Golden Retrievers.

Gut microbiota dysbiosis in obese individuals has been shown to affect host physiology possibly via modulating the metabolism and excretion of microbial-derived metabolites including lipids and lipid-like metabolites, amino acids, bile acids derivatives, and catabolites of plant bioactive components, which were also proposed to be predictors of overweight or obesity (Machate et al., 2020; Lippert et al., 2017). Metabolomics has been enlightened as a useful tool to evaluate changes in metabolites due to overweight and obesity at the cellular level and body fluid level (Xie et al., 2012). Thus, metabolite profiling with metabolomics might represent an opportunity to methodically establish biomarkers for obesity diagnosis and control that are relatively simple to measure in comparison to traditional approaches like the BCS system (Rauschert et al., 2014).

Therefore, the alterations in composition of the gut microbiota and the plasma metabolites profile may be informative in predicting the obese status of Golden Retrievers. The present study adopted microbiome and metabolomics techniques to test the differences in gut microbiota and plasma metabolites profile between non-obese and obese Golden Retrievers to reveal the potential obesity biomarkers from microbiota and/or metabolites.

2 Materials and methods

The experimental protocols related to animal treatment in the present study were approved by the Institutional Animal Care and Use Committee of Southwest University of Science and Technology (No. L2023029).

2.1 Animals and management

The design of the analytical workflow is shown in Figure 1, which illustrates the enrollment of groups. Before screening, a total of 77 Golden Retrievers applicants were registered by the owners to participate in the study. Dogs were excluded if they had a history of drugs or antibiotics administration within a month. Importantly,

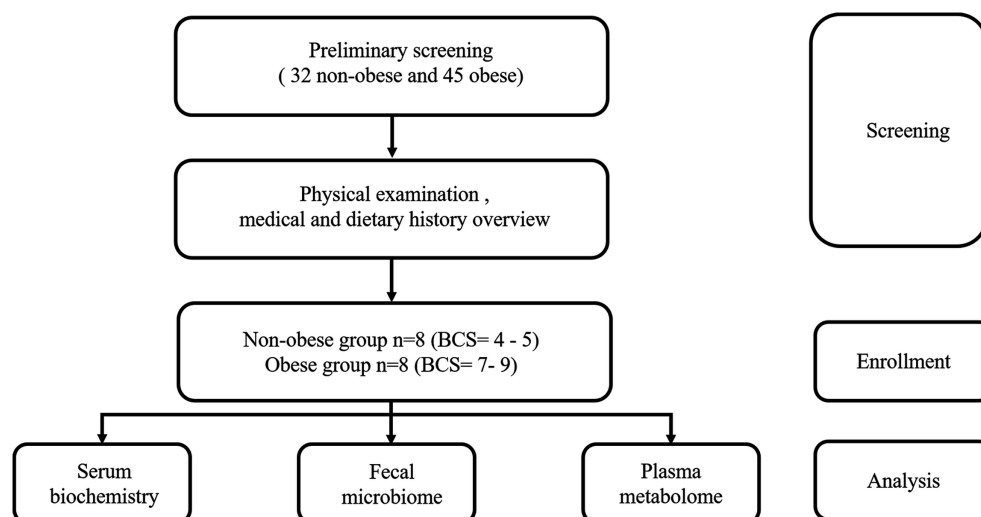


FIGURE 1
Flow diagram of study design.

dietary history was also considered as a crucial screening factor. Only the Golden Retrievers consuming diets with similar nutrient levels and ingredients composition over 3 months were kept for the further screening procedures. All feeds were formulated primarily with duck and chicken meat. In addition, the physical examinations of all Golden Retrievers were performed to exclude those with obvious inflammatory disease and to assess the BCS according to a validated 9-point BCS system. The dogs with a BCS range from 4 to 5 were the non-obese objects in the present study, with a BCS range from 7 to 9 defined as the obese studying objects and their counterparts (Broome et al., 2023). Accordingly, those with BCS at other scores were excluded as well. After screening, 8 non-obese (Ctrl group) and 8 obese (Obe group) adult Golden Retrievers (Males = 11; Females = 5) aged between 1 and 7 years were enrolled in this study. The average body weight of the Ctrl and Obe groups ($n = 8$) were 27.00 ± 9.16 kg and 39.00 ± 10.38 kg, and the average BCS of the Ctrl and Obe groups were 4.9 and 7.2, respectively (Table 1). The dietary history and obesity duration details of these 16 Golden Retrievers were listed in Supplementary Table 1, and the nutrient levels of diets for the dogs were presented in Supplementary Table 2.

2.2 Sample collection

Spontaneous excreted fecal samples were collected by the owner following the procedures under the guidance of veterinarians. After the fecal samples were delivered to the laboratory, the samples were kept at -20°C until DNA extraction and the following analysis. Blood samples were collected by the veterinarians from the forelimb veins into vials with or without anticoagulants. After collection, plasma and serum samples were obtained by centrifuging the blood at $2,500 \times g$ for 15 min. Plasma and serum aliquots were stored at -20°C pending metabolomics analysis and biochemical index measurement, respectively. All of the collection processes were conducted under the supervision of the veterinarians.

2.3 Serum biochemistry analysis

The concentrations of glucose, creatinine, blood urea nitrogen, phosphorus, calcium, total protein, albumin, globulin, TB, and cholesterol, along with the activities of ALT, alkaline phosphatase, glutamyl transferase, amylase, and lipase, were analyzed using a veterinary automatic biochemical analyzer (IDEXX Procyte Dx, IDEXX Laboratories, USA) by the colorimetric method previously used for canine blood biochemistry. Subsequently, the blood urea nitrogen/creatinine ratio and the albumin/globulin ratio were calculated.

2.4 Fecal analysis

Genomic DNA in feces samples of dogs were extracted using the TIANamp Soil DNA Kit DP336 (TIANGEN, China) according to the manufacturer's instructions. The quantity and integrity of isolated DNA were determined on a NanoDrop ND-1000 instrument and evaluated visually by agarose gel electrophoresis, respectively. The V4 region of the bacterial 16S rRNA gene was amplified using 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGG GTWTCTAAT). Sequencing libraries were generated and indexes were added. The 16S rRNA amplicon sequencing was performed by the company (Novogene, China) on the Illumina MiSeq platform through NovaSeq6000 PE25. Software FLASH (Version 1.2.11) and Fastp (Version 0.23.1) were used to process the raw data to exclude low-quality reads. The resulting clean reads were assembled into effective tags, which were assigned to the Operational taxonomic unit (OTUs) based on 97% similarity by Uparse (Version 7.0. 1,001). Taxonomic classification of OTU clusters was performed using the Greengenes database with RDP (Version 2.6). The alpha diversity indexes were calculated by R studio (Version 4.1) accordingly. The Bray-Curtis distance was used to compare the structural difference of the microbiota communities across samples and was visualized by principal coordinates analysis (PCoA) to demonstrate the clustering

TABLE 1 Characteristics of non-obese (Ctrl) and obese (Obe) Golden Retriever dogs included in the present study.

Group	Name	Gender	Age	BW (kg)	BCS (V1)	BCS (V2)	BCS (Ave)	Diet	Calories intake (Kcal)
Ctrl	Dada	Female	1.00	25.00	5.00	5.00	5.00	Diet 4	1686.00
	Fugui	Female	5.50	25.00	4.00	4.00	4.00	Diet 3	1777.00
	Zhaozhao	Female	1.00	23.00	5.00	5.00	5.00	Diet 4	1686.00
	Legou	Male	6.50	29.00	5.00	5.00	5.00	Diet 2	1764.00
	Pipi	Male	1.00	23.00	5.00	4.00	4.50	Diet 1	1715.00
	Niangao	Male	2.00	33.00	5.00	5.00	5.00	Diet 2	1764.00
	Jiuyi	Female	1.00	30.00	5.00	5.00	5.00	Diet 1	1715.00
	Xilin	Male	2.00	35.00	5.00	5.00	5.00	Diet 1	1715.00
Obe	Mocha	Male	5.00	40.00	7.00	7.00	7.00	Diet 1	1715.00
	Dingman	Male	4.00	35.50	7.00	7.00	7.00	Diet 3	1777.00
	Pangguo	Male	3.00	40.00	8.00	7.00	7.50	Diet 3	1777.00
	Rouwan	Male	5.00	47.00	8.00	8.00	8.00	Diet 4	1686.00
	Hanzai	Male	3.00	35.00	7.00	7.00	7.00	Diet 1	1715.00
	Otto	Male	2.00	44.00	8.00	7.00	7.50	Diet 2	1764.00
	Nicol	Female	2.00	33.00	7.00	7.00	7.00	Diet 2	1764.00
	Guolicheng	Male	3.50	33.00	7.00	7.00	7.00	Diet 2	1764.00

Ctrl, control group, of which the body condition score (BCS) ranges from 4 to 5; Obe obese group, of which the BCS ranges from 7 to 9; BCS (V1) (V2), body condition score were determined by the first veterinarian and the second veterinarian respectively; BCS (Ave), the average body condition scores of two veterinarians BW, body weight.

of different samples using a vegan package in R studio (Version 3.4.1). The raw sequencing data can be obtained by searching against the National Center for Biotechnology Information databases with the project number PRJNA1158542.

2.5 Plasma metabolomics analysis

Plasma samples were thawed at room temperature before analysis. 100 μ L of samples were placed into polyethylene tubes, mixed with 400 μ L of 50% methanol buffer, then vortexed for 30 s, and sonicated for 10 min in a 4°C water bath. After that, the samples were vortexed (1 min) and centrifuged (14,000 g, 15 min, 4°C) to collect the supernatant for nontargeted metabolite profiling. LC–MS/MS analysis was performed on an ultra-high performance liquid chromatography system (Vanquish, Thermo Fisher Scientific) coupled to an Orbitrap Exploris 120. To monitor the stability of the analysis, the QC sample was obtained by pooling all the experimental samples with equal volume. The raw data were processed through ProteoWizard and BiotreeDB (Version 3.0). The metabolites were identified by comparing with the internal database mass-to-charge ratio (m/z), retention time, and chromatographic data. The partial least-square discriminant analysis (PLS-DA) was performed using the MetaboAnalyst 6.0 web-based system. The metabolites with $p < 0.05$ were considered significantly different. The significantly distinct metabolites were imported into the MetaboAnalyst 6.0 database for the pathway enrichment analysis.

2.6 Statistical methods

The body condition indexes and serum biochemistry parameters were analyzed using the student *t*-test with SAS software (Version 9.4). The non-parametric Wilcoxon rank-sum test was adopted to analyze the

differences in abundances of microbial taxa at the phylum and genus levels as well as the alpha diversity indices. The Spearman's correlations between the predominant genera and plasma-differed metabolites or serum biochemistry parameters as well as the correlation between plasma- differed metabolites or serum biochemistry indexes were calculated and demonstrated as heatmaps by R studio (Version 4.0.3). The Benjamini–Hochberg method was applied to calculate the false discovery rates (FDR) adjusted *p*-value. Data were expressed as mean $\pm$ SEM, and significance was declared at $p < 0.05$.

3 Results

3.1 Body condition and serum biochemistry indexes

The results related to body condition and serum biochemistry parameters are listed in Table 2. A significant difference in body weight and BCS was observed between the Ctrl and Obe groups ($p < 0.05$). There was a tendency toward higher activity of ALT ($p = 0.09$) in the serum of obese dogs compared to their non-obese counterparts. A higher concentration of total bilirubin was found in the Obe group than that in the Ctrl group ($p < 0.05$). Moreover, there were no significant differences in other indicators between the two groups ($p > 0.05$).

3.2 Gut microbiota composition

An average of 99,790 high-quality sequences were obtained from all samples, with a range of 97,361 to 107,162. These sequences were assigned to 265 OTU as the core OTU. Among them, 157 OTU were found in both groups, while 35 and 73 OTU were found in Ctrl and Obe groups, respectively (Supplementary Figure 2).

TABLE 2 The differences in body condition and serum biochemistry parameters between the Ctrl and Obe group.

Parameters	Ctrl	Obe	p-value
Body Weight (kg)	27.88 ± 1.62	38.44 ± 1.84	<0.001
Body Condition Score	4.81 ± 0.13	7.25 ± 0.13	<0.001
Glucose (mg/dL)	80.88 ± 7.00	84.38 ± 6.28	0.715
Creatinine (mg/dL)	1.04 ± 0.02	1.09 ± 0.05	0.426
Blood urea nitrogen (mg/dL)	17.5 ± 1.46	17.25 ± 1.25	0.899
Blood urea nitrogen/Creatinine	17.13 ± 1.25	15.29 ± 1.10	0.287
Phosphorus (mg/dL)	3.66 ± 0.23	3.56 ± 0.24	0.754
Calcium (mg/dL)	9.74 ± 0.13	9.54 ± 0.10	0.256
Total protein (g/dL)	6.71 ± 0.16	6.64 ± 0.08	0.688
Albumin (g/dL)	3.31 ± 0.03	3.42 ± 0.07	0.218
Globulin (g/dL)	3.36 ± 0.16	3.18 ± 0.05	0.273
Albumin/Globulin (%)	1.00 ± 0.05	1.10 ± 0.05	0.152
Alanine aminotransferase (U/L)	30.75 ± 2.07	39.88 ± 4.57	0.090
Alkaline phosphatase (U/L)	50.00 ± 7.00	44.38 ± 4.84	0.519
Glutamyl transferase (U/L)	0.01 ± 0.01	0.50 ± 0.38	0.302
Total bilirubin (mg/dL)	0.29 ± 0.02	0.35 ± 0.02	0.011
Cholesterol (mg/dL)	219.63 ± 11.02	220.25 ± 19.83	0.210
Amylase (U/L)	745.63 ± 101.6	721.38 ± 82.15	0.855
Lipase (U/L)	448.25 ± 56.98	598.25 ± 75.72	0.136

Ctrl, control group, of which the body condition score (BCS) ranges from 4 to 5; Obe obese group, of which the BCS ranges from 7 to 9.

No significant differences were detected in α diversity indices (Supplementary Figure 1) between Ctrl and Obe groups. The PCoA plot showed that the Ctrl and Obe samples could form two distinct clusters by treatment (Figure 2A). This indicates that the composition of the microbiota of the Ctrl and Obe groups was different.

At the phylum level, Firmicutes, Proteobacteria, Fusobacteriota, Actinobacteriota, and Bacteroidota were predominant taxa in the feces of dogs. Moreover, the presence of phylum Firmicutes was the most dominant taxon in both groups, accounting for 75.12 and 41.21% of the fecal microbiota in the Ctrl and Obe groups, respectively (Figure 2B; Supplementary Table 3). Besides, Proteobacteria, Fusobacteriota, and Bacteroidota were found to be more abundant in the feces of the Obe group compared to the Ctrl group ($p < 0.05$) (Figure 2C; Supplementary Table 3). Firmicutes/Bacteroidota ratio were 1.55 and 16.73%, respectively, in Obe and Ctrl group. At the genus level, the most predominant taxon in feces of the Ctrl and Obe groups was *Peptoclostridium* and *Prevotella*, respectively (Figure 2D; Supplementary Table 4). Compared to the Ctrl group, the abundances of *Fusobacterium*, *Prevotella*, *Faecalibacterium*, *Escherichia-Shigella* and *Alloprevotella* were higher and the abundances of *Peptoclostridium*, *Blautia*, *Turicibacter*, *Allobaculum*, and *Erysipelatoclostridium* were lower in the feces of Obe group ($p < 0.05$) (Figure 2E; Supplementary Table 4).

3.3 Plasma metabolic pathways of non-obese and obese Golden Retrievers

The PLS-DA score plot (Supplementary Figure 4) from the mass spectrometer data showed that the distribution of the quality control samples was clustered tightly, demonstrating the accuracy of the

method for metabolomics data acquisition. In the PLS-DA plot (Figure 3A), Ctrl and Obe samples were clustered separately, indicating that there was a remarkable difference in plasma metabolomics profile between the Ctrl and Obe groups.

A total of 10 significantly different metabolites were identified (Table 3). Among them, citrulline, PA (16:0/16:0), 3-Methoxy-4-Hydroxyphenylglycol sulfate, 11-dehydrocorticosterone, ditryptophenalanine and N-alpha-Acetyl-L-citrulline were found to be more abundant and N-hydroxy-L-isoleucine, pelargonic acid and 8-iso-15-keto-PGE2 were less abundant in the Obe group compared to the Ctrl group ($p < 0.05$).

The different metabolites were checked against the SMPDB database to match the corresponding metabolic pathways and the biological pathways (Figure 3B). The differently enriched metabolites were mainly involved in glycerolipid metabolism, urea cycle, phospholipid biosynthesis, steroidogenesis, aspartate metabolism as well as arginine and proline metabolism.

3.4 The correlation between fecal microbiota, plasma metabolites and serum biochemistry index

The correlation analyses between the altered genera and plasma distinct metabolites, which were identified by 16S rRNA sequencing and LC-MS-based untargeted metabolomics, respectively, were performed and visualized in a heat map (Figure 4). The levels of genera *Peptoclostridium*, *Blautia*, *Allobaculum*, and *Erysipelatoclostridium* were positively related to plasma concentrations of 8-iso-15-keto-PGE2 and N-Hydroxy-L-isoleucine

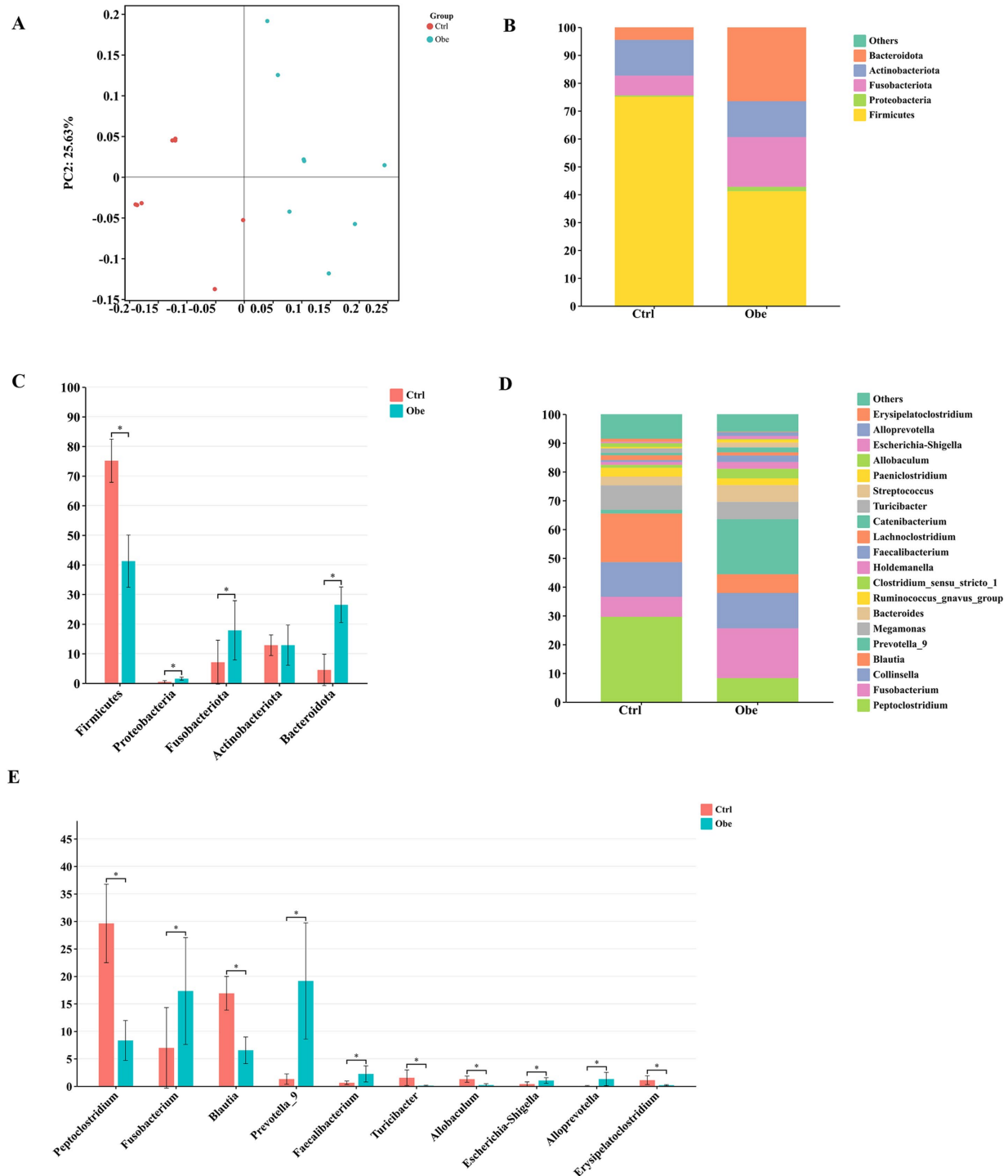


FIGURE 2

The beta diversity and taxonomic distribution of fecal microbiota in the Ctrl and Obe dogs. (A) principal coordinates analysis (PCoA) plot of unweighted UniFrac distances of fecal microbial communities. (B) The stacked plot of dominant taxa at the phylum level. (C) Significantly different phyla between the Ctrl and Obe groups. (D) The stacked plot of dominant taxa at the genus level. (E) Significantly different genera between the Ctrl and Obe groups.

($p < 0.05$), while the abundances of genera *Prevotella* and *Alloprevotella* were negatively related to plasma levels of these two metabolites ($p < 0.05$). The abundance of genus *Escherichia-Shigella* was negatively associated with the plasma content of 8-iso-15-keto-PGE2 ($p < 0.05$). Besides, the levels of genera *Prevotella* and *Collinsella* were in positive connection with the plasma citrulline

level, while the abundance of genus *Blautia* was in negative connection with its level in plasma ($p < 0.05$).

The correlation analyses between plasma metabolites and serum biochemistry indexes as well as the fecal microbiota and serum biochemistry parameters were performed and visualized in heat maps. Plasma concentrations of metabolites 8-iso-15-keto-PGE2 and

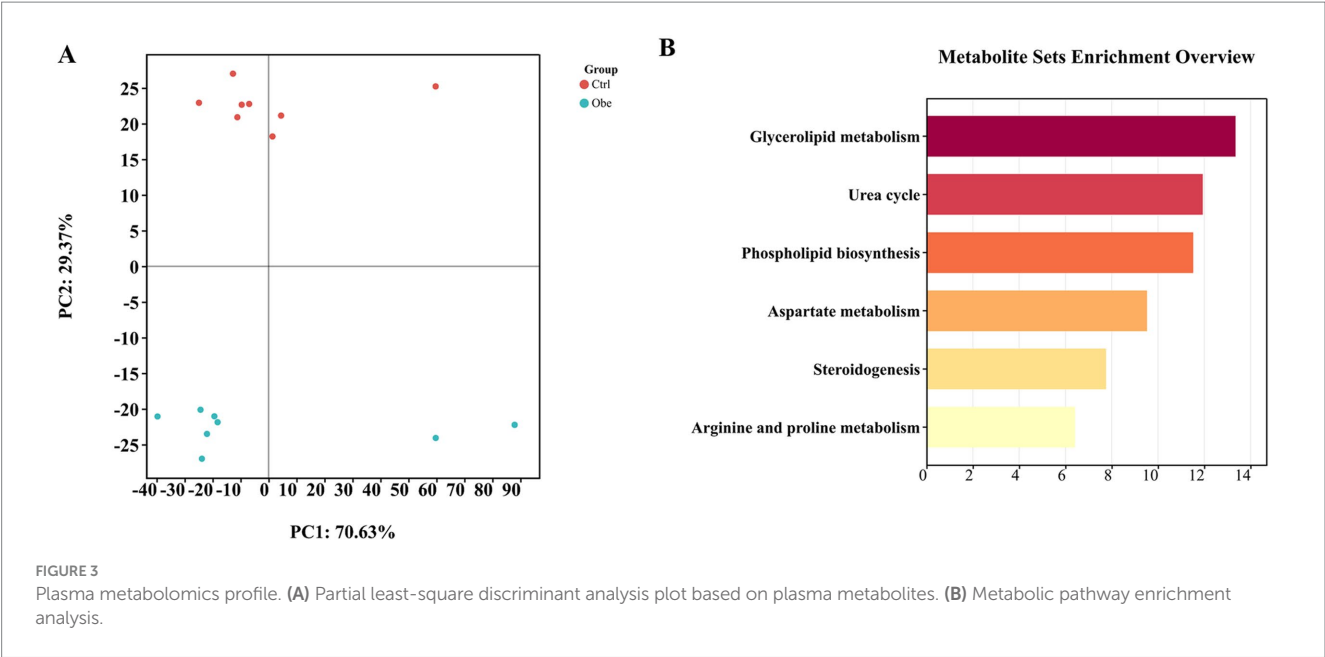


TABLE 3 Significantly different metabolites in plasma between the Ctrl and Obe group.

Parameters	Ctrl	Obe	p-value
Citrulline	0.11 ± 0.01	0.14 ± 0.01	0.038
N-Hydroxy-L-isoleucine	2.06 ± 0.29	1.18 ± 0.18	0.021
Pelargonic acid	2.61 ± 0.14	1.86 ± 0.18	0.006
PA (16:0/16:0)	0.15 ± 0.02	0.25 ± 0.03	0.018
3-Methoxy-4-Hydroxyphenylglycol sulfate	0.79 ± 0.10	1.24 ± 0.15	0.022
11-Dehydrocorticosterone	21.8 ± 3.45	35.97 ± 3.43	0.011
8-is0-15-keto-PGE2	0.21 ± 0.03	0.10 ± 0.02	0.006
Canrenone	12.57 ± 2.00	20.00 ± 2.19	0.025
Ditryptophenaline	19.48 ± 2.87	34.05 ± 3.79	0.008
N-alpha-Acetyl-L-citrulline	0.13 ± 0.01	0.18 ± 0.02	0.045

Ctrl, control group, of which the body condition score (BCS) ranges from 4 to 5; Obe obese group, of which the BCS ranges from 7 to 9.

N-alpha-Acetyl-L-citrulline were negatively related to aminotransferase activity ($p < 0.05$). The content of metabolite 3-Methoxy-4-Hydroxyphenylglycol sulfate was in a positive relation with serum TB level ($p < 0.05$) (Supplementary Figure 3A). Furthermore, the abundances of genera *Escherichia-Shigella*, *Clostridium sensu stricto* 1, and *Catenibacterium* were positively related to serum aminotransferase activity ($p < 0.05$). The abundances of genera *Allobaculum* and *Faecalibacterium* were in a negative and positive relation with the serum concentration of TB, respectively ($p < 0.05$) (Supplementary Figure 3B).

4 Discussion

Obesity has reached pandemic proportions in dogs (Courcier et al., 2010; Butterwick, 2000), in which has significant implications for canine physiology, health and welfare, especially causing gut microbiota dysbiosis and metabolism dysfunction. Previous research

has identified potential microbial and metabolomic biomarkers associated with obesity in humans and rodents (Metwaly et al., 2022; Wang et al., 2021; Giudetti, 2023). However, the similar kinds of biomarkers for diagnosis of canine obesity remain unclear. Accordingly, the present study compares the serum biochemical indices, fecal microbiota and plasma metabolites profile of non-obese and obese Golden Retrievers to preliminarily reveal the potential microbial or metabolic biomarkers for obesity in Golden Retrievers.

Studies in both humans and animals have showed that obesity causes the disruption of the homeostasis of lipid metabolism and increases fat accumulation not only in adipose tissue but also in the liver, resulting in adipose inflammation and hepatic damage (Giudetti, 2023; Richard et al., 2000). Obesity-associated fatty liver has been shown to induce a substantial increase in the activity of ALT, which suggests a potential connection between ALT and obesity. A previous study has demonstrated that obese dogs exhibited higher activities of ALT and gamma-glutamyl transferase (Tribuddharatana et al., 2011). Consistently, a tendency toward higher activity of ALT was found in

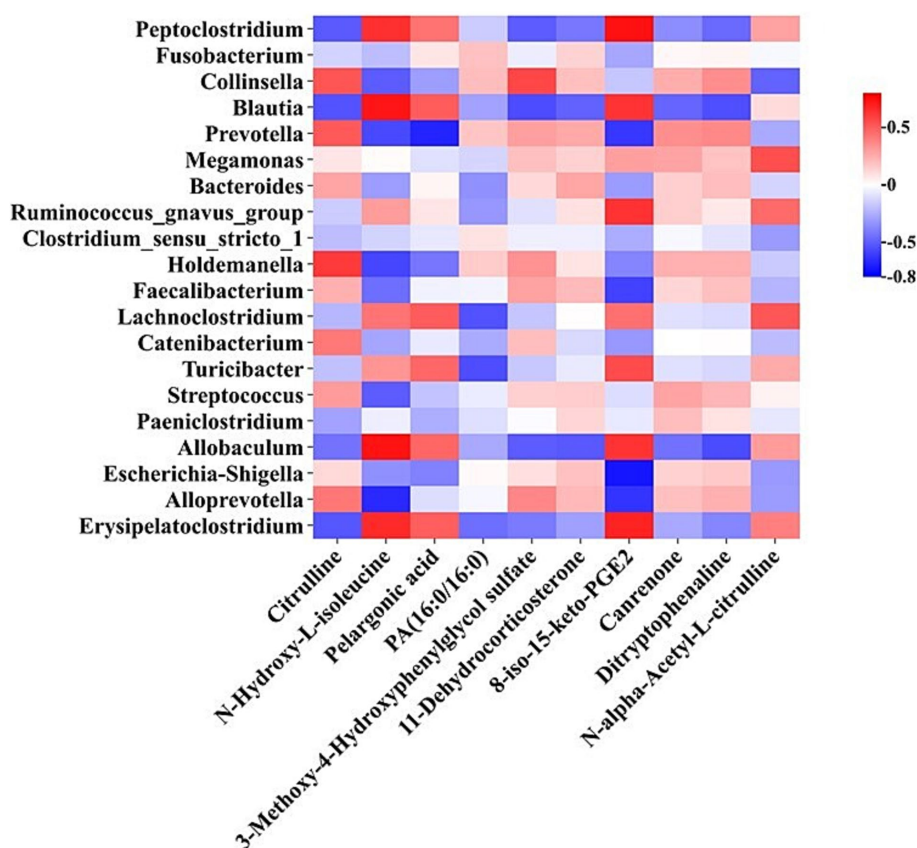


FIGURE 4
The correlation analysis between the altered genera and plasma distinct metabolites.

the obese Golden Retrievers compared to the control dogs. Besides, previous study found that serum TB was associated with an elevated risk of obesity-associated metabolic syndrome (Wei et al., 2021). TB including direct and indirect bilirubin, the degradation products of the heme moiety of hemoglobin and other hemoproteins, is potentially toxic to the liver where it is detoxified (Roy-Chowdhury et al., 2020). Bilirubin has been proposed as a biomarker to monitor hepatic function and of which the high level in blood was the consequences of liver dysfunction according to the previous study (Guerra Ruiz et al., 2021). In the present study, a significantly higher level of bilirubin was found in the Obe group, indicating the potential liver impairment in overweight dogs.

Gut microbiota play a crucial role in host metabolism and physiology, but the pathophysiological mechanisms and clinical outcomes were still unclear. On one hand, the presence of obesity has been associated with the imbalance of gut microbiota, which has been demonstrated to play a causal role in the induction of metabolic dysfunction (Le Chatelier et al., 2013; Stanislowski et al., 2019). On the other hand, several studies observed significant differences in the abundance and biodiversity of gut microbiota between non-obese and obese individual (Pinart et al., 2021; Yun et al., 2017). The Firmicutes/Bacteroidota ratio was considered as a microbial indicator for obesity by some studies, as the higher abundance of phylum Firmicutes and increased ratio of Firmicutes/Bacteroidota were observed in obese individuals or animals than their lean counterparts. However, others hold the different opinion that there is a weak connection between

obesity and the Firmicutes/Bacteroidota ratio (Schwartz et al., 2010). Previous studies showed that the most predominant taxa at the phylum level in dogs were Firmicutes (You and Kim, 2021), which is also the most abundant phylum in the feces of Golden Retrievers. In addition, phylum Firmicutes has been found more abundant in non-obese dogs than the obese ones (Macedo et al., 2022; Park et al., 2015; Kim et al., 2023). Consistent with these findings, non-obese Golden Retrievers exhibited the higher level of phylum Firmicutes in feces than the Obe group, implying that phylum Firmicutes might be negatively correlated with the presence of obesity in dogs, which needs to be further verified in the future study. Genus *Fusobacterium* was found related to the whole-body inflammation state and has been more abundant in the fecal microbiota of children with obesity as well as in the feces of type 2 diabetes subjects (Sedighi et al., 2017; Yuan et al., 2021; Chen et al., 2020; Lee et al., 2018). In agreement with these findings, the increased abundance of genus *Fusobacteria* was observed in the Obe group in the present study. A previous study showed that fat pigs had significantly higher proportions of *Prevotella. copri* (Chen et al., 2021). However, a higher abundance of genus *Prevotella* has been associated with enhanced insulin sensitivity in rodents. These contradictory results might stem from the inter-study difference in the genetic background of studying animals, which has been proved as a determinant factor for the microbiota composition. A previous study on dogs showed that the abundance of *Prevotella. copri* in fecal microbiota elevated with increasing BCS (You and Kim, 2021). Consistently, in the present study, obese Golden Retrievers exhibited

higher levels of genus *Prevotella* in feces. Likewise, another study also demonstrated that high carbohydrate diet-fed obese dogs exhibited higher abundance of *Prevotella. copri* in feces than dogs with normal weight (Li et al., 2017). Previous study compared the microbiota differences between dogs with differed degree of overweight have found that highly overweighted dogs had higher abundance of *Prevotella* in feces compared to their counterparts (Forster et al., 2018). Therefore, the abundance of *Prevotella* might be used as a potential biomarker for the detection of obesity in Golden Retrievers, but its effects on and reasons for its abundance in obese dogs should be examined in the future study. A higher level of *Peptoclostridium* was found in dogs with normal BCS compared to those with high BCS in a previous study on Beagle dogs (Kim et al., 2023). Consistently, in this study, the obese Golden Retrievers exhibited a lower abundance of *Peptoclostridium* in feces. Genus *Blautia* has been reported to exhibit beneficial effects on intestinal health and metabolic disorders (Liu et al., 2021). A previous study showed that oral administration of *Blautia* can ameliorates obesity and type 2 diabetes by increasing the production of short chain fatty acids (SCFAs) and activating SCFAs-related signaling pathways (Patel and Preedy, 2017). In the present study, non-obese Golden Retrievers had higher levels of genus *Blautia* in feces, implying that increasing the colonization of genus *Blautia* might be helpful for weight management in dogs, which needs to be studied in future research. The alterations in abundances of genus *Ruminococcus_gnavus_group* might the unique response of Golden Retrievers to obesity, because overweight had no effects on the abundances of these genera in other dog breeds like Beagle dogs (Kim et al., 2023). Therefore, the verification of the application of the genus as a biomarker indicator of obesity should be further studied.

The metabolite profiling via metabolomics is frequently used to reveal metabolic biomarkers for obesity and associated metabolic syndrome. In the present study, the plasma metabolite profile in obese Golden Retrievers differed from the non-obese dogs, which was consistent with previous findings that the presence of obesity significantly altered the whole-body metabolism in humans and rodents (Lai et al., 2014). Citrulline was produced by the hydrolysis of peptidyl arginine catalyzed by protein arginine deiminases, of which high activity was observed in a host of human diseases including metabolic syndrome and inflammation (Allerton et al., 2018). The conversion process of peptidyl arginine into citrulline also called protein citrullination, has been demonstrated to be an inflammation-dependent process since it is upregulated in various inflammatory states (Makrygiannakis et al., 2006). In this study, the plasma level of citrulline was higher in obese Golden Retrievers than in the control ones, suggesting that the presence of obesity might activate this posttranslational process, namely protein citrullination, in tissues of Golden Retrievers dogs. Besides, a previous study found that chronic administration of 11-dehydrocorticosterone to mice increased the circulating glucocorticoid level and downregulated the expression of genes related to the hypothalamic–pituitary–adrenal axis, leading to insulin resistance, adiposity and an elevation of body weight (Marzolla et al., 2014). In the current study, a higher concentration of 11-dehydrocorticosterone in plasma was observed in obese Golden Retrievers, indicating that the elevated cortisol secretion might contribute to the pathogenesis of obesity in Golden Retrievers (Abraham et al., 2013).

The catabolites of prostaglandin E2 (PGE2), including 15-keto-PGE2 and 8-iso-15-keto-PGE2, have been shown to alleviate hepatic

inflammation in diet-induced obesity mouse model, indicative of lower activities of ALT and aspartate transferase and inhibited macrophage infiltration (Hee et al., 2023). Consistently, lower plasma levels of 8-iso-15-keto-PGE2 in obese dogs and the negative correlation between 8-iso-15-keto-PGE2 and aminotransferase activity were observed in the current study, suggesting that the approaches increasing the levels of catabolites of PGE2 might be effective for the treatment of obesity in Golden Retrievers. N-hydroxy-L-isoleucine is a derivative of L-isoleucine which has been shown to enhance muscle growth, inhibit fat deposition, and exhibit anti-inflammation activity (Patil et al., 2012). Given the beneficial effects of L-isoleucine on the metabolism and inflammation of the host, it can be speculated that N-hydroxy-L-isoleucine might exhibit similar effects as well. In the present study, obese dogs exhibited lower concentrations of N-hydroxy-L-isoleucine, implying that the intake of L-isoleucine and its derivatives might be useful for controlling overweight in dogs.

Overall, obesity significantly altered the concentrations of certain serum biochemistry parameters, which might be associated with the changes in gut microbiota and plasma metabolites profile. The verification of these possible biomarkers for diagnosing obesity in Golden Retrievers and the dietary intervention approaches targeting the microbiome and metabolome for weight management in dogs need to be further studied and explored.

5 Conclusion

In summary, obesity notably modified the levels of specific serum biochemical markers, which may correlate with alterations in gut microbiota and plasma metabolite profiles. Further investigation and exploration are necessary to validate these potential biomarkers for diagnosing obesity in Golden Retrievers, alongside dietary intervention strategies aimed at the microbiome and metabolome to manage weight in dogs.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee of Southwest University of Science and Technology. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YZC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization,

Writing – original draft, Writing – review & editing. HYT: Formal analysis, Methodology, Writing – review & editing. GLX: Formal analysis, Methodology, Writing – review & editing. HYY: Formal analysis, Methodology, Writing – review & editing. JZ: Formal analysis, Methodology, Writing – review & editing. ZXX: Formal analysis, Methodology, Writing – review & editing. QFH: Formal analysis, Methodology, Writing – review & editing. REB: Formal analysis, Methodology, Writing – review & editing. PZ: Writing – review & editing. YZ: Writing – review & editing. HLY: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1514633/full#supplementary-material>

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EDITED BY

Zhenya Zhai,
Jiangxi Academy of Sciences, China

REVIEWED BY

Tsungcheng Tsai,
University of Arkansas, United States
Jiayao Guan,
Chinese Academy of Agricultural Sciences
(CAAS), China
Haiqin Li,
Jiangxi Academy of Agricultural Sciences
(CAAS), China

*CORRESPONDENCE

Xiaoqin Xu
✉ xuxiaoqin21@126.com

[†]These authors have contributed equally to this work

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Establishment and maturation of gut microbiota in White King pigeon squabs: role of pigeon milk

Xiaoqin Xu^{1,2*}, Zihan Wang^{1,3†}, Yi Jian¹, Long Zhang^{1,2}, Caiquan Zhou^{1,2}, Li Liu⁴ and Hui Liu⁵

¹Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), China West Normal University, Nanchong, China, ²Institute of Ecology, China West Normal University, Nanchong, China, ³College of Life Sciences, China West Normal University, Nanchong, China, ⁴Agricultural Technique Promotion Station of Nanchong, Nanchong, China, ⁵Yingshan Fucheng Meat Pigeon Breeding Professional Cooperative, Nanchong, China

Background: Pigeons are significant economic animals in China; however, research regarding the establishment and influencing factors of gut microbiota in squabs remains limited. Understanding how the gut microbiota develops in pigeons, particularly in relation to pigeon milk, is importance in pigeon production. This study aims to elucidate the establishment characteristics of the gut microbiota in White King pigeon squabs and explore the role of pigeon milk in this process.

Methods: This study employed 16S rRNA sequencing technology to investigate the dynamics of microbial composition in feces and pigeon milk at various growth stages of White King pigeon. Functional prediction analysis was performed to assess the metabolic pathways involved, and correlation analysis was used to explore the relationships between microbial communities in different sample types.

Results: The findings revealed a diverse microbiome present in the meconium of newborn pigeons, with a microbial composition that significantly differed from that of other feces groups. In contrast, the microbial composition of feces (FN) from pigeons aged 7 to 21 days exhibited less variability. At the phylum level, the predominant microbial taxa identified in the feces of FN were Firmicutes, Actinobacteriota, and Proteobacteria. At the genus level, the main dominant bacterial groups included *Lactobacillus*, *Limosilactobacillus*, and *Turicibacter*. Functional prediction analysis indicated that the gut microbiota of pigeons primarily participate in metabolic pathways related to carbohydrates, amino acids, lipids, cofactors, and vitamins. Furthermore, the dominant bacteria found in pigeon milk (MN) were identified as probiotics, including *Limosilactobacillus*, *Ligilactobacillus*, *Lactobacillus*, *Bifidobacterium*, and *Aeriscardovia*, which collectively accounted for over 90% of the total abundance. Correlation analysis of the abundance of shared microbes revealed that the association between meconium and feces at the other stages was extremely low. In contrast, the correlation between colostrum and feces at the post-feeding stage were found to be the highest.

Conclusion: This study indicates that prenatal colonization occurs in White King pigeons. Notably, within the first week after birth, the gut microbial composition of young pigeons becomes stable. Furthermore, the colostrum serves as the most significant driver for the establishment of intestinal microbiota in squab post-birth. The findings of this study suggest that microorganisms can be added to artificial pigeon milk based on the predominant microbial composition of colostrum. This approach could facilitate the establishment of gut microbiota in young pigeons, thereby promoting their growth and development and providing production benefits.

KEYWORDS

16S rRNA sequencing, microbiota, pigeon milk, feces, shared microbes

1 Introduction

The gut microbiota of poultry is vast, complex, and diverse. Apajalahti et al. found that approximately 10^9 to 10^{11} microorganisms, belonging to 140 genera and 640 species, reside solely in the chicken cecal feces (Apajalahti et al., 2004). These gut microbes play a crucial role in the host's intestinal energy and material metabolism, extracting energy from indigestible carbohydrate feed and producing essential energy substrates, and directly absorbable nutrients, such as short-chain fatty acids, lactic acid, vitamins, and amino acids (Tannock and Liu, 2020; Vrieze et al., 2010). Additionally, they significantly contribute to the regulation of the immune system by stimulating immune cell proliferation, enhancing humoral immunity, and inhibiting the growth and reproduction of harmful bacteria (Dogi et al., 2008; Li et al., 2024; Samli et al., 2010). The early colonization and establishment of gut microbiota are closely linked to host growth and development, profoundly influencing later growth and production (Aimeric et al., 2018; Rubio, 2019). Furthermore, the effectiveness of subsequent nutritional interventions is often constrained by the already-established gut microbiota homeostasis of the host (Kogut, 2019; Rubio, 2019). Therefore, research on microbial colonization and community establishment is of utmost importance in poultry production.

Early studies have indicated that the establishment of gut microbiota in birds commences postnatally, as the digestive tract of healthy newborn birds is sterile, and microbiota colonize the intestine through external contact. However, numerous recent studies have revealed that prenatal colonization of gut microbiota also occurs in birds, with transmission occurring through various routes, including the environment, eggshell, amniotic fluid, or other mechanisms before birth (Dai et al., 2021; Dietz et al., 2019; Fisayo et al., 2020), with significant changes in the microbiota occurring shortly after birth, particularly during the first few days (Jinmei et al., 2017). In broiler chickens, the period from 0 to 4 days is a rapid colonization phase for the microbiota, and after 10 days, the microbial growth rate slows down, and the microbiota structure tends to stabilize (Jurburg et al., 2019; Ranjitkar et al., 2016). The gut microbiota of laying hens matures relatively slowly, achieving stability during the peak or later stages of egg production (Videnska et al., 2014). Research on three Arctic waterbirds has showed that the period from 0 to 2 days is marked by rapid colonization and growth of gut microbiota, with the gut microbiota structure stabilizing by the third day (Grond et al., 2017). Therefore, there are considerable differences in the establishment of gut microbiota among different species and between various breeds of the same species. However, while much is known about the microbiota of precocial birds, less is understood about the early establishment and development of microbial communities in altricial birds, which are born in a more underdeveloped state and rely heavily on parental care. Pigeons, as altricial birds, offer a unique opportunity to investigate the dynamics of gut microbiota in early life stage. As the fourth major poultry breed in China, pigeons are significant economic animals due to their high production efficiency and minimal environmental pollution. White King pigeons, a dual-purpose breed for both meat and eggs, are widely reared; however, research on the colonization and establishment of gut microbiota in White King pigeons remains scarce.

The establishment of the gut microbiota in birds is a dynamically evolving process influenced by numerous factors, with the impact of food-source microbiota being particularly significant, in addition to genetic factors (Aimeric et al., 2018; Teyssier et al., 2018; Flint et al.,

2015). Pigeons, as altricial birds, are unable to feed independently at birth and rely on pigeon milk regurgitated by their parents for nourishment. After approximately 21 days, they are capable of foraging independently, although they still require parental feeding. This pigeon milk, which is rich in proteins, fatty acids, and minerals (Xie et al., 2019), as well as a diverse array of microbiota (Ding et al., 2020; Shetty et al., 1990), serves as the sole food source for newborn squabs. Consequently, the microbiota present in pigeon milk can directly enter the gastrointestinal tract, thereby influencing the establishment of the early gut microbiota in pigeons.

16S rRNA sequencing is widely used in the analysis of bacterial community composition due to its high sensitivity, cost-effectiveness, and other advantages (Schrieffer et al., 2018). Hence, this study employs 16S rRNA sequencing to investigate the dynamics of microbial composition in feces and pigeon milk at various growth stages of White King pigeon squabs, with a particular focus on analyzing their functional roles. By conducting correlation analyses, we seek to elucidate the contributions of microbial communities in pigeon milk and prenatal colonization to the gut microbial colonization and flora establishment in squabs across these developmental stages.

2 Materials and methods

2.1 Animal model and sampling

Pigeon milk samples from White King pigeons were collected in Nanchong, Sichuan province, China. The experimental parent pigeons, approximately 2 years old, and at the peak of their egg production, maintained a stable egg-laying cycle. The pigeons were housed under uniform conditions: each male-female pair was kept in a cage and fed three times daily, with access to free water and natural light.

Squabs are entirely dependent on pigeon milk produced by their parents for survival during the first 21 days after hatching and cannot feed independently during this period. This period provides an optimal window for investigating the establishment of early gut microbiota in squabs and the impact of pigeon milk on its colonization. Therefore, this study collected samples at four time points during this stage for analysis. Fecal samples from the squabs were collected at four stages: day 0, day 7, day 14, and day 21, designated as F0, F7, F14, and F21, respectively, with six replicates for each group. Notably, the F0 samples presented the first feces from newly hatched squabs that had not yet consumed any food, and were therefore considered as meconium. Meconium differs from other fecal samples as it primarily consists of substances ingested by the squab from the egg before external feeding begins. The samples (FN) from F7, F14, and F21 were obtained from the same cohort of squabs. To minimize the interference of environmental microbiota, all samples in this study were carefully extracted from the cloaca by gently pressing after massaging the abdomen of squabs. These samples were immediately collected in 2 mL sterile cryovials.

Pigeon milk samples were also taken from four stages of the parent pigeon's lactation period: day 0, day 7, day 14, and day 21, and were named as M0, M7, M14, and M21 respectively, with six replicates for each group. All pigeon milk samples are denoted as MN. To reduce slaughter, the pigeon milk was not collected directly from the parent

pigeons after execution, but rather from the aforementioned squabs. As soon as the squabs were fed by their parents, the pigeon milk was expelled from the squabs' crops into their mouths and then collected for immediate freezing in liquid nitrogen for microbial DNA extraction. Among these, the samples in group M0 were collected immediately after the newly hatched squabs were first fed by their parents, categorizing them as the pigeon's colostrum.

2.2 DNA extraction and 16S rRNA gene amplicon sequencing

Microbial genomic DNA was extracted from frozen fecal and milk samples using the OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, United States), in accordance with the manufacturer's instructions. The V3–V4 hypervariable region of the 16S rRNA genes was PCR amplified from the extracted microbial genomic DNA utilizing the forward primer 338F (5'-ACTCCTACG GGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVG GGTWTCTAAT-3'). All PCR reactions were conducted in a total volume of 25 μ L, comprising 5 μ L of 5 $\times$ buffer, 0.25 μ L of Fast Pfu DNA Polymerase (5 U/ μ L), 2 μ L (2.5 mM) of dNTPs, 1 μ L (10 μ M) of each forward and reverse primer, 1 μ L of template DNA, and 14.75 μ L of ddH₂O. The thermal cycling protocol included an initial denaturation step at 98°C for 5 min, followed by 25 cycles of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, concluding with a final extension at 72°C for 5 min. After purification and individual quantification, the amplicons were pooled in equal amounts, and paired-end 2 $\times$ 250 bp sequencing was performed using the Illumina NovaSeq platform.

2.3 Sequence analysis

The sequencing data was analyzed using QIIME 2 software (Bolyen et al., 2018), employing the DADA2 method (Callahan et al., 2016) and utilizing the Silva 138.1 database (Quast et al., 2012) for a comprehensive evaluation. After quality filtering, denoising, merging and chimera removal, the raw data generated representative non-singleton amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin (Bokulich et al., 2018) against the database. Alpha and beta diversity metrics were estimated using the diversity plugin. Microbial functions were predicted by PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2020) upon KEGG¹ databases.

2.4 Statistical analysis

Data analysis was conducted using IBM SPSS Version 19. Analysis of variance (ANOVA) was used to examine differences between groups, while a permutation test was applied to assess the significance of these differences. Pearson's correlation coefficient was used for the

correlation analysis, and a two-tailed test was performed to evaluate the statistical significance of the correlations.

3 Results

3.1 The sequencing data

Following quality control, a total of 3,236,401 valid sequences NCBI accession number: PRJNA1146154 were obtained from 48 samples through 16S rRNA gene sequencing, yielding an average of 67,425 sequences per sample. The average length of the clean tags was 425 bp. After rigorous clustering, we identified a total of 11,646 amplicon sequence variants (ASVs). Among these, we annotated 34 phyla, 82 classes, 182 orders, 300 families, and 612 genera in meconium. Similarly, analysis of pigeon fecal samples from FN revealed 26 phyla, 51 classes, 122 orders, 204 families, and 392 genera. Furthermore, we annotated 21 phyla, 35 classes, 70 orders, 125 families, and 234 genera in pigeon milk. The analysis of alpha diversity indicated that both the Chao1 and Shannon indices of the microbiota in meconium (F0) and pigeon milk (MN) were significantly higher than those observed in FN (Figure 1). This finding suggests a relatively high richness and diversity of bacteria in the first feces and pigeon milk.

3.2 Fecal microbiota dynamics during squab development

The taxonomic composition of species at multiple taxonomic levels within feces is illustrated in Figures 2A,B. In the feces of F0, the microbial phyla with the highest abundance were Firmicutes, Proteobacteria, and Bacteroidota, while at the genus level, the most abundant genera are *Enterococcus*, *Prevotella_9*, and *Acinetobacter*. The Firmicutes phylum constituted the most significant proportion, followed by Actinobacteriota, Proteobacteria, and Bacteroidota in the feces of FN. Correspondingly, the dominant microbial genera included *Lactobacillus*, *Limosilactobacillus*, *Turicibacter*, *Escherichia-Shigella*, *Bifidobacterium*, and *Ligilactobacillus*.

The taxonomic composition analysis at the genus level revealed dynamic alterations in these microbes throughout different developmental stages (F0, F7, F14, and F21), as shown in Figure 2C. *Enterococcus* (20.82%), *Prevotella_9* (12.86%), and *Acinetobacter* (12.70%) were the primary microbiota in F0; however, their relative abundance declined rapidly, becoming extremely low in F7, F14, and F21. Conversely, the prominent probiotics, *Lactobacillus* and *Limosilactobacillus*, were present in minimal quantities in F0, accounting for 0.20 and 0.11%, respectively, but their levels increased rapidly, reaching approximately 70% in F14 and F21. Additionally, the probiotic *Ligilactobacillus* exhibited a similar trend, and these three probiotics maintained a stable presence in the later three stages. Notably, *Bifidobacterium* had a high content only in F7, accounting for 15.76%, while its levels were extremely low in other stages. *Escherichia-Shigella* was predominantly present in F14, accounting for 15.37%, but its abundance was lower in other groups, specifically 3.21% in F0, 0.14% in F7, and 1.14% in F21. *Turicibacter* had minimal presence in F0, but its levels increased gradually, peaking at 11.51% in F21. The analysis of inter-group differences (Supplementary Table S1) revealed significant discrepancies in the microbial composition of F0 compared

¹ <https://www.kegg.jp/>

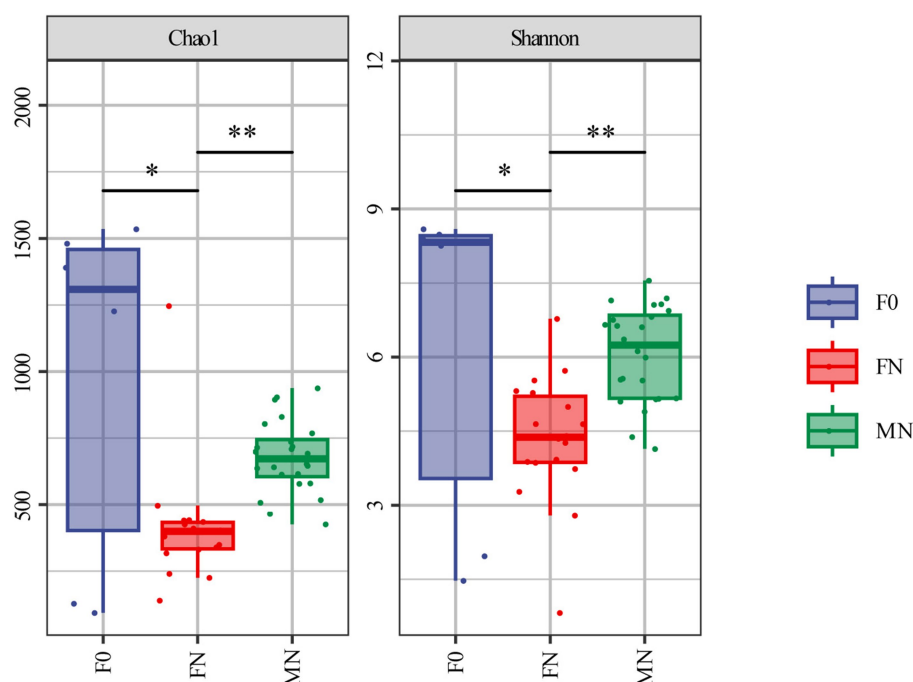


FIGURE 1

Alpha diversity analysis by Chao1 and Shannon index. *The difference is significant ($0.01 \leq p < 0.05$). **The difference is extremely significant ($p < 0.01$). Error bars show standard errors.

to other stages. In contrast, the fecal microbiota compositions from 1 week to 4 weeks of age exhibited similarities, with no notable differences observed. Principal coordinate analysis (PCoA) (Figure 2D) based on Bray–Curtis distances calculated from the adjusted ASV, indicated a marked separation of F0 samples from those of other stages, while samples from the latter clustered together. This suggests a distinct microbial community in meconium compared to subsequent stages, corroborating the findings from the inter-group difference analysis.

Predicted microbial functions (Figure 3) demonstrated that the squab gut microbiota plays a crucial role in the metabolism of various substances, including carbohydrates, amino acids, lipids, cofactors, and vitamins. Additionally, these microbiota are involved in genetic information processing, which encompasses replication and repair, folding, sorting, degradation, and translation. These metabolic pathways are essential for supporting the nutritional requirements and growth of squabs, particularly during early developmental stages. The microbial taxa contributing to these functions could play a significant role in shaping the gut ecosystem and promoting optimal growth.

3.3 The pigeon milk microbial characteristics

The predominant phylum in pigeon milk was Firmicutes, accounting for 81.86% of the relative abundance, followed by Actinobacteria (16.39%), Proteobacteria (1.06%), and Bacteroidetes (0.37%). The dominant microbial genera included *Limosilactobacillus* (36.15%), *Ligilactobacillus* (20.58%), *Lactobacillus* (17.68%), *Bifidobacterium* (13.02%), and *Aeriscardovia* (2.65%) (Figure 4A), all

of which were classified as probiotics, collectively exceeding 90% in total abundance. At the genus level, there was notable variation in microbial composition across different developmental stages (M0, M7, M14, and M21) (Figure 4B). *Limosilactobacillus* and *Ligilactobacillus* emerged as key genera, exhibiting a significant upward trend, particularly in the later stages. Specifically, the abundance of *Limosilactobacillus* increased substantially from approximately 24.55% in the initial stage to 42.54% in M14 and further to 53.34% in M21. Similarly, *Ligilactobacillus* showed a marked increase from 9.24% in M0 to 23.98% in M21. In contrast, *Bifidobacterium* initially rose from 14.02% in M0 to 37.51% in M7, but then experienced a steep decline to 0.14% in M14 and 0.43% in M21. The patterns observed for *Aeriscardovia* and *Lactobacillus* were notably different, with high abundance in the early stage followed by a gradual decrease. Inter-group difference analysis (Supplementary Table S2) indicated significant differences between M0 and M7 compared with the other groups. PCoA visually supported these findings (Supplementary Figure S1).

We predicted the microbial functions in pigeon milk and discovered that the milk microbiota of pigeons shares similar functionalities with the squab gut microbiota, primarily involved in metabolic pathways (Supplementary Figure S2).

3.4 Effects of pigeon milk and meconium microbes on gut microbiota establishment in squabs

To investigate whether pigeon milk and meconium microbiota are transmitted to the gut of squabs, we conducted a comparative analysis

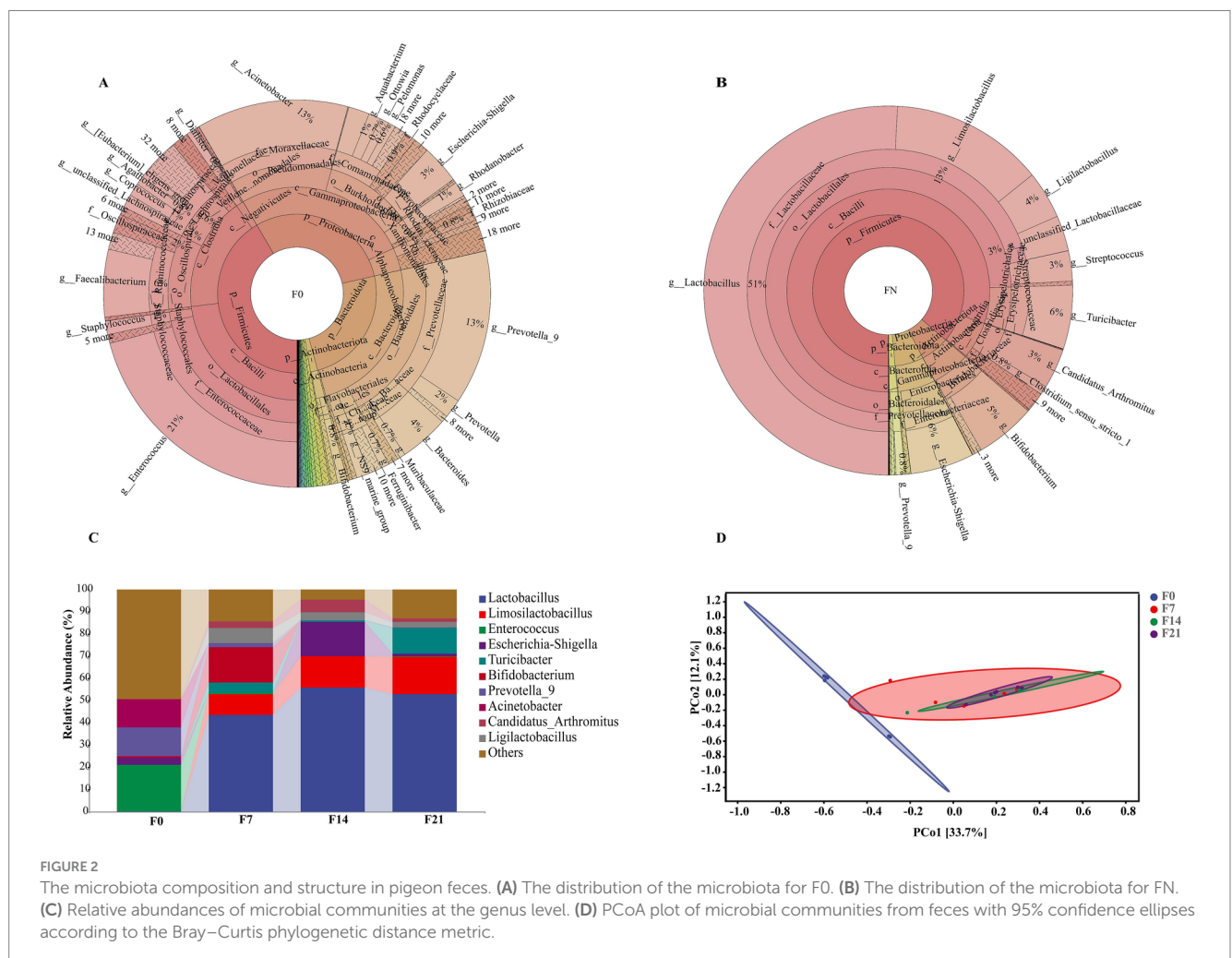
at the genus level of microbiota in pigeon milk and meconium, comparing these with squab feces during the three post-feeding stages (Table 1). The number of shared microbes between meconium and squab feces gradually decreased from 281 to 152 over time. Correlation analysis of the abundance of shared microbes revealed extremely low correlations between meconium and feces at all stages. Similarly, the number and abundance correlation of shared microbes between colostrum and squab feces exhibited a decreasing trend, with the number of shared genera diminishing from 82 to 68 and the abundance correlation declining from 0.922 to 0.883. Notably, the number and abundance correlation of shared microbes between colostrum and feces at all stages was the highest compared to other stages of pigeon milk. Despite the lower number of shared microbes between pigeon milk and feces at various stages compared to meconium, the correlation of abundance for the shared microbes across these stages consistently remained higher than that of meconium.

Venn diagram analysis (Figure 5) investigating the shared microbes between colostrum and squab feces across the three post-feeding stages unveiled a consistent presence of 47 microbial genera within the post-feeding squab gut. Notably, the majority of these genera were also detected in the prenatal gut, indicating their continuous relevance throughout developmental stages. The cumulative abundance of these 47 genera varied across different groups (F0, F7, F14, F21, M0, M7, M14, M21), with percentages

reaching 74.14, 88.42, 93.22, 86.30, 97.93, 97.87, 97.55, and 95.57%, respectively (Supplementary Table S3), emphasizing their status as the predominant microbial communities in squab intestines. Among this select group, *Lactobacillus*, *Limosilactobacillus*, *Bifidobacterium*, *Ligilactobacillus*, and *Aeriscardovia* stood out for their relatively high abundances. These five probiotics are not only primary microorganisms found in colostrum but also dominate the fecal microbiota of post-feeding squabs. Their total contributions across the groups sequentially amounted to 1.125, 76.937, 73.544, 72.425, 90.653, 91.736, 90.672, and 87.204% (Supplementary Table S4), further underscoring their significance in shaping the gut microenvironment of postnatal squabs.

4 Discussion

Numerous studies have demonstrated that the colonization of gut microbiota begins during the embryonic period. Shortly after birth, an even greater diversity of microbiota establishes residence in the intestinal tract, gradually forming a stable and diverse microbial community that plays a crucial role in maintaining host health (Oakley et al., 2014). Consequently, we examined the microbial composition of pigeon feces across multiple growth stages, analyzed their dynamic changes, and predicted their functional roles within the



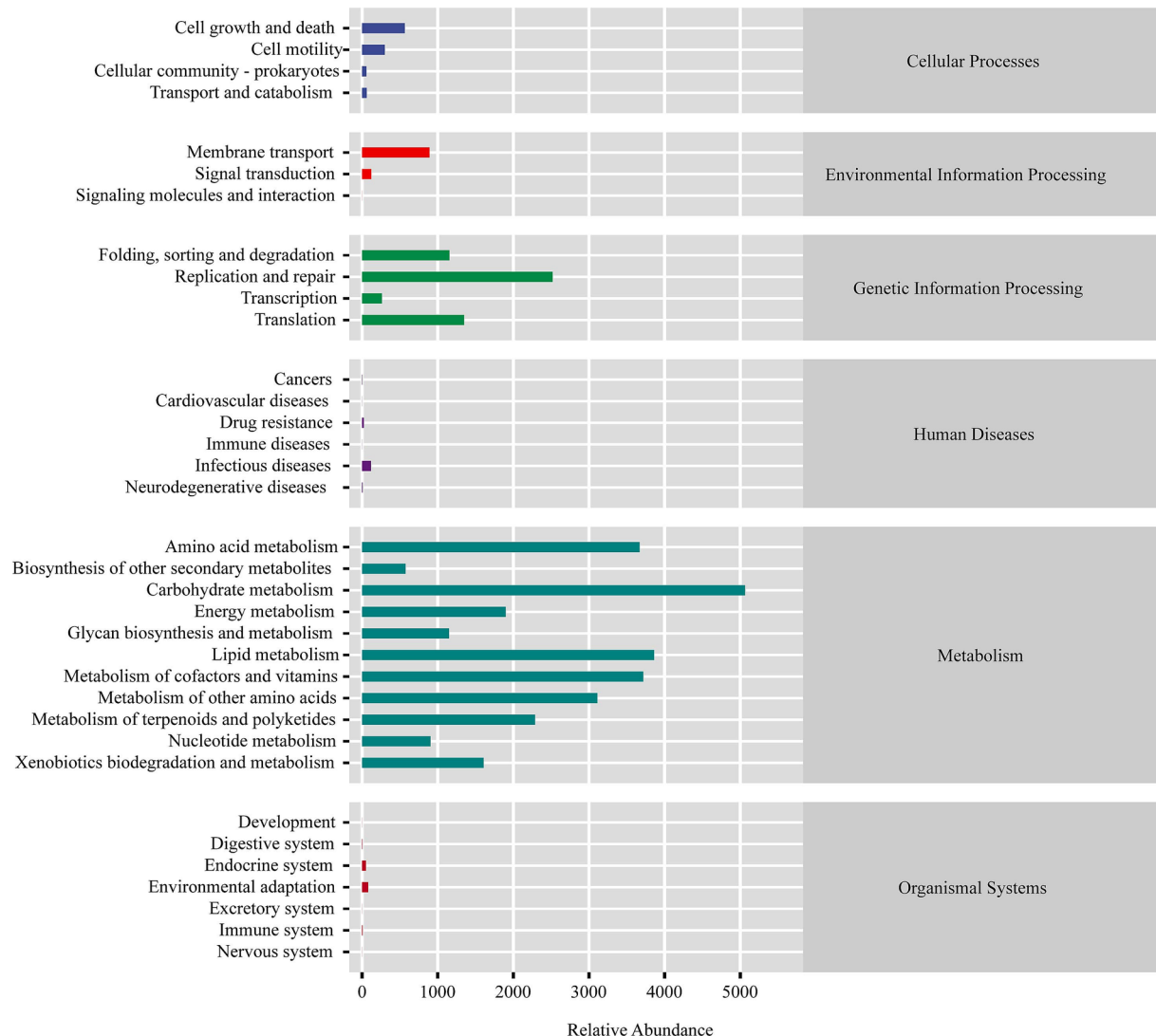


FIGURE 3
KEGG pathway enrichment analysis of feces microbiota from FN.

gut. Diet serves as a critical driver for the establishment of gut microbiota, and pigeon milk, as the sole source of nourishment for newborn pigeons, plays a significant role in shaping their gut microbiota. Therefore, we also analyzed the microbial composition of pigeon milk at different growth stages, aiming to elucidate its role in the establishment of the gut microbiota in young pigeons.

The analysis of taxonomic composition and inter-group differences revealed significant alterations in the fecal microbiota between F0 and subsequent groups, whereas changes from F7 to F21 were relatively minor. This finding suggests that the intestines of White King pigeons are already colonized with a diverse array of microbiota prior to birth, and that these gut microbiota undergo substantial changes during the first week after hatching, followed by a period of relative stability. According to [Dietz's et al. \(2019\)](#) research, prenatal colonization has been documented in rock pigeons, while [Xu et al. \(2022\)](#) found that the microbiota in the ileum of pigeons experiences significant variations in the first week post-hatching. These findings align with our research outcomes.

As pigeons aged, the abundance of Firmicutes in their feces gradually increased, ultimately becoming the dominant bacterial group, accounting for 97.74% at 21 days old. Firmicutes represent the most prevalent bacterial group in birds and is ubiquitous in the intestines of domestic chickens, turkeys, ducks, and other poultry ([Wei et al., 2013](#); [Yang et al., 2020](#)). These Firmicutes species play a crucial role in facilitating digestive processes and nutrient absorption in their hosts by breaking down a diverse array of compounds, including carbohydrates, polysaccharides, sugars, and fatty acids, with the aid of digestive enzymes as catalysts ([Wen et al., 2021](#)). For example, Firmicutes produce short-chain fatty acids as fermentation byproducts, which can be directly absorbed into the host's intestinal wall as an energy source ([den Besten et al., 2013](#)). An abundance of Firmicutes has been associated with weight gain in various animals. In domestic chickens, a positive correlation has been observed between Firmicutes abundance and both weight gain and immune function ([Zhang et al., 2015](#)). In this study, within the phylum Firmicutes, the Lactobacillaceae family exhibited a predominant

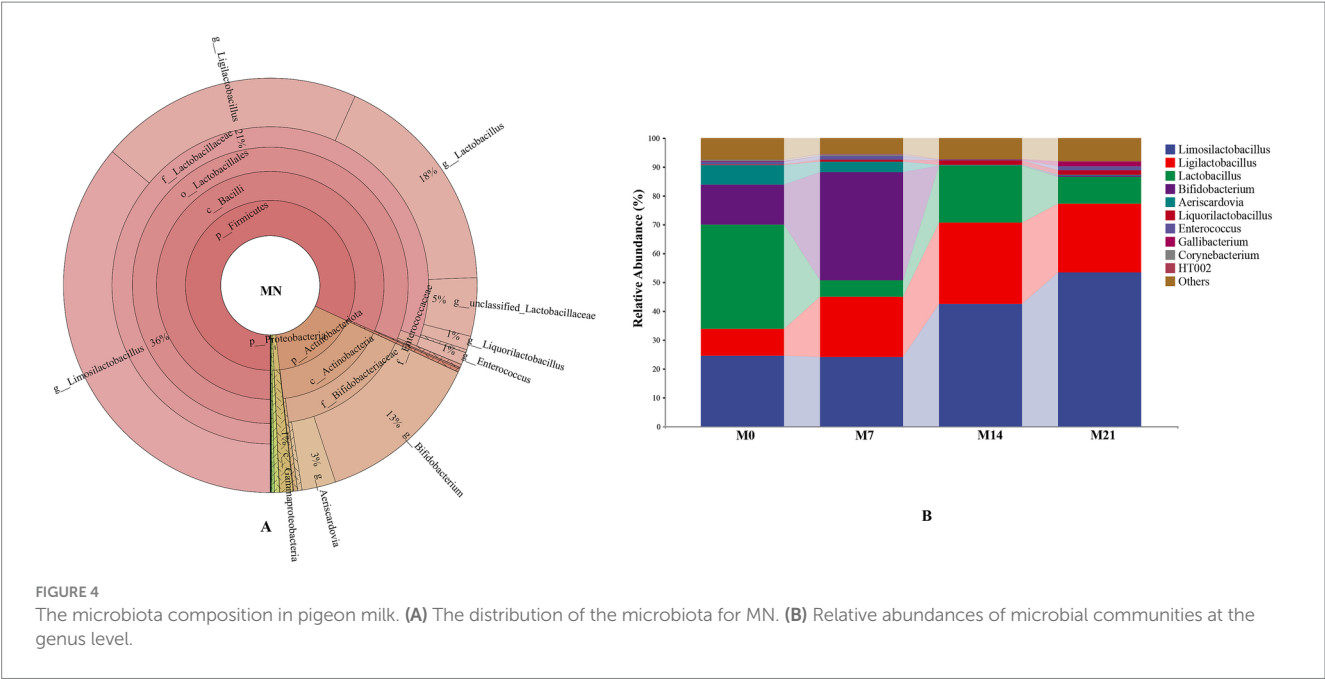


TABLE 1 The shared microbes analysis of meconium, pigeon milk, and squab feces.

	F0			M0			M7			M14		M21
	F7	F14	F21	F7	F14	F21	F7	F14	F21	F14	F21	F21
The number of shared microbes	281	106	152	82	62	68	75	56	54	40	38	51
Correlation coefficient (<i>r</i>)	0.028	−0.010	−0.020	0.922	0.843	0.883	0.477	0.201	0.217	0.517	0.568	0.410
<i>p</i> -value	0.639	0.917	0.808	0.000	0.000	0.000	0.000	0.137	0.115	0.001	0.000	0.003

The *r* was interpreted as follows: $0 < r < 1$ indicates a positive correlation, and $-1 < r < 0$ indicates a negative correlation. The strength of the correlation was classified based on common definitions: $0.00 \leq |r| \leq 0.19$ was considered very weak, $0.20 \leq |r| \leq 0.39$ was considered weak, $0.40 \leq |r| \leq 0.59$ was considered moderate, $0.60 \leq |r| \leq 0.79$ was considered strong, and $0.80 \leq |r| \leq 1.0$ was considered very strong. Statistical significance was assessed using *p*-values: *p*-value < 0.01 indicated a highly significant correlation, $0.01 \leq p\text{-value} < 0.05$ indicated a significant correlation, and *p*-value ≥ 0.05 indicated no significant correlation (Yoshii et al., 2022).

relative abundance, primarily comprising *Lactobacillus* (53.03%), *Limosilactobacillus* (16.80%), and *Ligilactobacillus* (2.42%). Notably, *Limosilactobacillus* and *Ligilactobacillus* are newly classified genera that were separated from *Lactobacillus* in 2020. These genera are common gut probiotics known for maintaining gut microbiota homeostasis, enhancing immune function, promoting nutrient absorption, and alleviating inflammation to improve gut health, with widespread applications in daily life (Jiang et al., 2022; Luo et al., 2023; Rychlik, 2020; van Zyl et al., 2020; Yao et al., 2021). The high concentration of probiotics in the intestines of squab pigeons likely represents an adaptation to the high protein and fat content of pigeon milk. Furthermore, the proliferation of these probiotics may be a key factor contributing to the rapid growth and development of squab pigeons.

Diet is the primary factor shaping the gut microbiota in birds. Pigeon milk, as the exclusive food source for early-stage squabs, directly introduces its microbes into the gut via the digestive tract, significantly contributing to the establishment of the gut microbiota in these young birds. *Lactobacillus* dominated the microbial composition of pigeon colostrum and remained the predominant bacterial group in squab feces, although it was present in extremely low levels in meconium. Within just 7 days, the *Lactobacillus* content surged to 43.26%, with a gradual upward trend thereafter.

Furthermore, the shared dominant bacteria (*Limosilactobacillus*, *Ligilactobacillus*, and *Bifidobacterium*) in both squab feces and pigeon milk exhibited similar trends across the four developmental stages. Specifically, *Limosilactobacillus* showed a steady increase in abundance; *Ligilactobacillus* initially rose and then declined, while *Bifidobacterium* increased consistently within the first 7 days before rapidly dropping to very low levels. Correlation analysis revealed an exceptionally high correlation in the abundance of shared microbes between colostrum and feces from all post-feeding stages. This underscores that pigeon milk not only provides essential nutrients, but also a rich microbiome, particularly abundant in probiotics, which significantly contributes to the establishment of the gut microbiota. Notably, the impact of colostrum was the most pronounced. Upon reaching the gut, dominant bacteria such as *Lactobacillus* from colostrum rapidly colonize and proliferate, inhibiting the growth of preexisting microbiota, thereby becoming the primary dominant flora within 1 week.

Although newborn pigeons were colonized by a diverse array of gut microbiota at birth, the majority of these microbes dissipated over time, leaving only a small fraction behind. The retained microbial populations, however, exhibited an extremely low correlation with the fecal microbiota of nestlings after they began feeding, suggesting that prenatally colonized microbes are transient inhabitants rather than



FIGURE 5
The Venn diagram of the colostrum and feces at post-feeding stages.

true gut colonizers. Furthermore, the retained microbial flora is suppressed by the true colonizers that originate from pigeon milk. The disappearance of prenatal microbes is likely a dynamic process influenced by multiple factors, including environmental interactions, diet, and immune system development. These factors collectively facilitate the colonization of the gut by microbes that are better adapted to the postnatal environment (Donald and Finlay, 2023). Notably, despite their minimal impact on shaping the gut microbiota of postnatal nestlings, prenatally colonized gut microbes harbored multiple potential pathogens, including *Escherichia-Shigella*, *Klebsiella*, *Staphylococcus*, and *Pseudomonas* (Supplementary Table S5). Although these pathogens may be suppressed by dominant probiotics such as lactobacilli and their abundance remain low (van Zyl et al., 2020), they still pose a potential threat to the gut health of nestlings. Consequently, further research is warranted to explore strategies for reducing and controlling the prenatal colonization of pathogenic bacteria.

5 Conclusion

This study dynamically monitored the microbial composition of squab feces and pigeon milk using 16S rRNA sequencing technology. Our findings revealed a diverse microbiota present in the meconium of newborn squabs, indicating prenatal colonization in White King pigeons. Minimal variations were observed in the fecal microbial composition of squabs aged 7 to 21 days, with the dominant phyla identified as Firmicutes, Actinobacteriota, and Proteobacteria, in descending order. At the genus level, the predominant microbiota included *Lactobacillus*, *Limosilactobacillus*, and *Turicibacter*. Notably, the major bacteria present in pigeon milk were all probiotics, such as *Limosilactobacillus*,

Ligilactobacillus, *Lactobacillus*, *Bifidobacterium*, and *Aeriscardovia*, collectively accounting for over 90% of the total microbial abundance. An analysis of the abundance correlation among shared microbes demonstrated a remarkably low correlation between meconium and feces from other developmental stages, whereas colostrum exhibited the highest correlation with feces from all post-feeding stages. This demonstrates that colostrum serves as the most crucial driving factor for the establishment of the postnatal gut microbiota in squabs. While the microbiota present in meconium appear to have a limited impact on the later development of gut microbiota, they do harbor multiple pathogenic bacteria that could potentially serve as sources of pathogens in the gut later on. To ensure the intestinal health of squabs, it is crucial to investigate the factors influencing prenatal colonization and to block potential transmission routes of pathogenic bacteria. Additionally, emphasis should be placed on disinfecting breeding eggs and incubators to minimize the introduction of pathogenic microorganisms into the embryo via the eggshell and their subsequent colonization in the gut.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov/PRJNA1146154>.

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee of China West Normal University. The

studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XX: Conceptualization, Funding acquisition, Methodology, Writing – original draft. ZW: Conceptualization, Data curation, Methodology, Writing – original draft. YJ: Project administration, Resources, Writing – original draft. LZ: Methodology, Writing – review & editing. CZ: Conceptualization, Writing – review & editing. LL: Methodology, Resources, Writing – review & editing. HL: Resources, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1481529/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

PCoA plot of microbial communities from milk with 95% confidence ellipses according to the Bray–Curtis phylogenetic distance metric.

SUPPLEMENTARY FIGURE S2

KEGG pathway enrichment analysis of milk microbiota.

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EDITED BY

Franck Carbonero,
Washington State University Health Sciences
Spokane, United States

REVIEWED BY

Tünde Pusztahelyi,
University of Debrecen, Hungary
Xingxiang Chen,
Nanjing Agricultural University, China

*CORRESPONDENCE

Baichuan Deng
✉ dengbaichuan@scau.edu.cn

[†]These authors have contributed equally to this work

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Effect of compound polysaccharide on immunity, antioxidant capacity, gut microbiota, and serum metabolome in kittens

Yixuan Xie^{1†}, Shiyao Jian^{1†}, Limeng Zhang^{2†} and Baichuan Deng^{1*}

¹College of Animal Science, South China Agricultural University, Guangzhou, China, ²Guangzhou Qingke Biotechnology Co., Ltd., Guangzhou, Guangdong, China

Introduction: This study was conducted to investigate the effects of compound polysaccharides (CP), composed of *Astragalus* polysaccharide and *Poria cocos* polysaccharide, on immunity, antioxidant capacity, gut microbiota, and serum metabolome in kittens.

Methods: A total of 14 4-month-old kittens, with an average body weight of 2.39 kg, were used in a 56-day experiment. They were randomly assigned to the control (CON) group ($n = 7$) and CP group ($n = 7$). Blood samples and fresh feces were collected at the end of the experimental period.

Results: The results displayed that supplementation with CP increased the concentrations of serum immunoglobulin A, immunoglobulin G, interleukin 6, and tumor necrosis factor- α ($p < 0.05$). However, there was no difference in the concentrations of serum amyloid A between the two groups ($p > 0.05$). Furthermore, the serum biochemical parameters of all the kittens were within the reference range. The relative abundance of beneficial bacteria (*norank_f__Butyrivibrionaceae* and *Bacteroides plebeius*) was higher in the CP group ($p < 0.05$), while the opportunistic pathogen (*Anaerotruncus*) was lower in the CP group ($p < 0.05$). In addition, serum metabolomic analysis demonstrated that the differential metabolites, including arachidonic acid, dihomogamma-linolenic acid, and glycine, and the relevant metabolic pathway, including glyoxylate and dicarboxylate metabolism, glycine, serine, and threonine metabolism, and biosynthesis of unsaturated fatty acids, were implicated in regulating immune function in the kitten after CP treatment.

Conclusion: CP supplementation can enhance immune function in kittens and increase the relative abundance of beneficial gut microbiota, and does not lead to generalized inflammation. Dietary supplementation with CP may generate nutritional benefits in kittens, and this study offers insight into the development of functional pet food for kittens.

KEYWORDS

Astragalus polysaccharide, immunity, kitten, pet food, *Poria cocos* polysaccharide, microbiota, metabolomics

1 Introduction

Newborn kittens rely on maternal antibodies in the colostrum to guarantee an adequate serum immunoglobulin level, which is essential for proper immune response (Rossi et al., 2021). A previous study has indicated that the maternal antibody titers in cats decrease between 4 and 14 weeks after delivery, and weaning is a challenging stage for neonatal mammals (Lei Liu et al., 2020; Pereira et al., 2023). Kittens are susceptible to diseases due to reduced maternal antibodies, immature immune systems, separation from their mother, and

relocation during the weaning period (Gore et al., 2021). Nutrition plays a vital role in the health of weaning kittens by meeting their nutritional requirements for the immune system and organ development, with long-term effects on overall health (Rossi et al., 2021). The most important impact of nutrition occurs during the development of their immune system (Cunningham-Rundles et al., 2005). The gut microbiota plays an important role in gut health and immune system development (Jia et al., 2011; Mengxia Wang et al., 2013). In addition, the composition of gut microbiota in kittens is modulated by diets and the weaning period process (Hooda et al., 2013; Jia et al., 2011). Therefore, the post-weaning period is when optimal nutrition can help support the immune system.

Plant polysaccharides, serving as functional ingredients, have attracted much attention due to their natural, non-toxic properties and multiple biological effects in recent years (Zhang et al., 2023). *Astragalus* polysaccharide (APS) is an important active component extracted from dried roots of *Astragalus*, known as “Huangqi” in China (Xu et al., 2023). APS is a kind of water-soluble heteropolysaccharide, mainly composed of nine ratios of monosaccharides, namely, glucose, galactose, arabinose, rhamnose, mannose, xylose, fucose, fructose, and ribose (Han et al., 2019; Wang et al., 2023). A large number of *in vivo* and *in vitro* studies have demonstrated the immunomodulatory, antioxidant, and antitumor effects of APS (Huang et al., 2017; Li C. et al., 2022; Li et al., 2009; Zhao et al., 2022). In addition, researchers have confirmed that APS can enhance the abundance and diversity of intestinal microbiota (Yang et al., 2022; Zhao W. et al., 2023). Similarly, *Poria cocos* polysaccharide (PP), derived from *Poria cocos*, comprises ribose, arabinose, xylose, mannose, glucose, and galactose. There are two kinds of polysaccharides in the sclerotium of *Poria cocos*: water-soluble polysaccharides and alkali-soluble polysaccharides. The main active components are water-soluble polysaccharides (Tan et al., 2023; Wang D. et al., 2020; Wang K. et al., 2020). PP exhibits various biological activities, such as antioxidation, immunomodulation, antidepression, and antitumor functions (Khan et al., 2018; Lai et al., 2022; Zhang et al., 2018). However, there is a lack of studies on the effect of APS or PP supplementation on kittens. Thus, this study aimed to investigate the effect of compound polysaccharide (CP) on immunity, antioxidant capacity, gut microbiota, and serum metabolome in kittens. CP was composed of APS and PP, and the experimental diet was supplemented with 0.1% APS and 0.2% PP. We hypothesized that dietary supplementation with CP may enhance the immunity of weaning kittens as maternal antibody titers decrease and endogenous antibody production is activated during this period.

2 Materials and methods

2.1 Polysaccharides

APS and PP were purchased from Xi'an Jinshuo Fruit Industry Co., Ltd. APS was extracted from the roots of *Astragalus*, and PP was extracted from *Poria cocos*. CP was composed of APS and PP, and the experimental diet was supplemented with 0.1% APS and 0.2% PP.

2.2 Animal and housing

A total of 14 healthy weaning shorthair kittens (10 males and 4 females) from 4 litters, with a mean body weight (BW) of 2.39 ± 0.78 kg

and aged 4 months, were included in this study. The kittens were weaned at 3 months of age and then fed the basal diet for 1 month. During the months 3–4, the kittens remained with their litters and in the rooms they were born. At the end of the pre-test period, kittens aged 4 months received the veterinary examination and were relocated from the nursery to another laboratory room (these were considered mildly challenging events). After relocation, the kittens were individually housed at the laboratory in Qingke Biotechnology Co., Ltd. (Guangzhou, China) under controlled temperature and light conditions (12-h light/dark cycle, temperature $23.5 \pm 1^\circ\text{C}$, relative humidity $60 \pm 10\%$) during the test period. Each housing cage (1.1 m $\times$ 0.7 m $\times$ 0.7 m) had separate areas for feeding, defecation, and resting. The kittens were allowed to socialize with each other and play outside the cages during the non-feeding time. Furthermore, the kittens can access various toys and interact with humans during the day. The cages and rooms were cleaned and sanitized daily. All kittens were dewormed and vaccinated 1 month before the experiment. No drugs, such as antibiotics, were administered during the experiment. Kittens had free access to fresh water throughout the study.

2.3 Diet and experiment design

In a completely randomized experimental design, kittens were randomly assigned to the control group (CON, $n = 7$, two females and five males) and CP group (CP, $n = 7$, two females and five males) based on their initial BW, gender, and litter. This experiment lasted for 56 days. The CON kittens were fed a basal diet, while the CP kittens were fed a basal diet supplemented with 0.1% APS and 0.2% PP. Each kitten was housed separately with ad-libitum access to food.

The diet met the nutrient requirements for raising cats, according to the Association of American Feed Control Officials (AAFCO), and the diet was baked food. The food intake was recorded daily, and BW was recorded on days 0 and 56. The experimental diets were prepared by Qingke Biotechnology Co., Ltd. (Guangzhou, China). The ingredients and analyzed chemical compositions of the basal diet are shown in Table 1.

2.4 Serum sample collection and analysis

On day 56, blood samples were collected before feeding. The samples were centrifuged at 3500 rpm at room temperature for 15 min, and the supernatants were collected and stored at -80°C for further analysis. Serum biochemical parameters, including albumin (ALB), globulin (GLO), total protein (TP), albumin/globulin (ALB/GLO), aspartate aminotransferase (AST), alanine transaminase (ALT), amylase (AMY), creatine kinase (CK), creatinine (CRE), urea nitrogen (BUN), urea nitrogen/creatinine (BUN/CRE), glucose (GLU), triglyceride (TG), calcium (Ca), and inorganic phosphorus (IP), were measured by using an automatic biochemical analyzer (SMT-120VP, Chengdu Seamaty Technology Co., Ltd., Chengdu, China). The concentrations of serum immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and serum amyloid A (SAA) were detected by using the commercial feline enzyme-linked immunosorbent assay (ELISA) kits (MEIMIAN, Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China). The levels of serum total antioxidant capacity (T-AOC), glutathione

TABLE 1 Dietary ingredients and analyzed chemical compositions of the basal diet.

Items	CON	CP
Ingredients (as-is basis, %)		
Chicken meal	50.00	50.00
Fish meal	20.00	20.00
Duck meal	12.00	12.00
Chicken liver	5.00	5.00
Krill meal	2.00	2.00
Sweet potato flour	2.00	2.00
Tapioca	2.00	1.70
Salmon oil	1.00	1.00
Whey protein powder	1.00	1.00
Vitamin and mineral	1.00	1.00
Calcium hydrogen phosphate	1.00	1.00
Alfalfa	0.80	0.80
Yolk powder	0.50	0.50
Blueberries	0.50	0.50
Plantain seed	0.50	0.50
Calcium carbonate	0.50	0.50
Glucosamine hydrochloride	0.10	0.10
Yucca powder	0.05	0.05
Chondroitin sulfate	0.05	0.05
<i>Astragalus</i> polysaccharide	–	0.1
<i>Poria cocos</i> polysaccharide	–	0.2
Chemical compositions		
DM, %	94.70	94.70
OM, % DM basis	92.60	92.60
CP, % DM basis	44.35	44.35
EE, % DM basis	20.60	20.60
CF, % DM basis	2.10	2.10

CON, basal diet group; CP, compound polysaccharide diet group; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; CF, crude fiber.

peroxidase (GSH-Px), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5 16S rRNA high-throughput sequencing analysis

On day 56, fresh feces samples were collected and stored at -80°C for further analysis. Total microbial genomic DNA was extracted from feces using E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., USA) following the instructions of the manufacturer. The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with primer pairs 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGA CTACNVGGGTWTCTAAT-3') by T100 Thermal Cycler PCR thermocycler (BIO-RAD, USA). The Polymerase Chain Reaction (PCR) mixture includes 4 μL of $5 \times$ FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL

of forward and reverse primers (5 μM), 0.4 μL of FastPfu polymerase, 0.2 μL of BSA, 10 ng of template DNA, and ddH₂O to a final volume of 20 μL . The PCR amplification cycling was carried out with the initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with single extension at 72°C for 10 min, and ending at 10°C . The PCR product was extracted from 2% agarose gel and purified using the PCR Clean-Up Kit (YuHua, Shanghai, China) following the manufacturer's instructions and quantified using Qubit 4.0 (Thermo Fisher Scientific, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina PE300/PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Raw FASTQ files were de-multiplexed using an in-house Perl script and then quality-filtered by fastp version 0.19.6 and merged by FLASH version 1.2.7. Then, the optimized sequences were clustered into amplicon sequence variants (ASVs) using UPARSE 7.1 with a 97% sequence similarity level. The most abundant sequence from each ASV was selected as a representative sequence. To minimize the effects of sequencing depth on the alpha and beta diversity measures, the number of 16S rRNA gene sequences from each sample was rarefied to 20,000, which still yielded an average Good's coverage of 99.09%. Based on the ASV information, the subsequent analysis, including the alpha diversity and beta diversity, was conducted using QIIME 2 software.

2.6 Serum untargeted metabolomics analysis

The UPLC-Orbitrap-MS/MS analysis, as described in previous research, served as an untargeted metabolomic approach to identify serum metabolic profiles (Xin et al., 2018). Compound Discoverer 2.1 software (Thermo Fisher Scientific, USA) was applied for automated data processing. Metabolites were detected by searching the mzCloud library and mzVault library. Additionally, the potential metabolite markers were filtrated according to the fold change (FC) ($\text{FC} > 1.2$) and t -test ($p < 0.05$). Moreover, these differential metabolites were mapped to the KEGG database for pathway analysis using MetaboAnalyst 5.0.

2.7 Statistical analysis

The experimental data were preprocessed by Microsoft Excel 2019. SPSS 26.0 and GraphPad Prism 8.0 software were applied for statistical analysis and graphical presentation. Independent sample Student's t -test was performed for the comparisons between the two groups. Statistical significance was defined as a p -value of <0.05 . The results were expressed as the mean $\pm$ standard error (mean $\pm$ SE).

3 Results

3.1 Effects of CP supplementation on growth performance and serum biochemistry in kittens

As shown in Table 2, there were no significant differences ($p > 0.05$) in IBW, FBW, WG, and DFI between the two groups.

TABLE 2 Growth performance of kittens during the experiment.

Items	CON	CP	P-value
IBW, kg	2.30 ± 0.29	2.48 ± 0.34	0.701
FBW, kg	3.03 ± 0.26	3.31 ± 0.40	0.570
WG, kg	0.73 ± 0.11	0.83 ± 0.12	0.546
DFI, g/d	68.96 ± 2.68	75.51 ± 5.93	0.334

CON, basal diet group; CP, compound polysaccharide diet group; IBW, initial body weight; FBW, final body weight; WG, weight gain; DFI, daily feed intake.

The effects of dietary CP supplementation on the serum biochemical parameters in kittens are shown in Table 3. The CP supplementation did not affect the serum biochemistry parameters, including ALB, TP, GLO, ALB/GLO, AST, ALT, AMY, CK, CRE, BUN, BUN/CRE, GLU, TG, Ca, and IP ($p > 0.05$). The serum biochemical parameters of all the kittens were within the reference range.

3.2 Effects of CP supplementation on serum antioxidant capacity, inflammatory cytokines, and immunoglobulins in kittens

The effects of dietary CP supplementation on serum antioxidant capacity are presented in Table 4. There were no differences ($p > 0.05$) in the concentrations of serum antioxidant parameters, including T-AOC, GSH-Px, MDA, CAT, and SOD between the two groups. In addition, CP treatment did not affect the serum IgM, IL-8, IL-1 β , and IL-10 ($p > 0.05$, Figures 1C, 2C–E). However, supplementation with CP increased the levels of serum IgA, IgG ($p < 0.01$, Figures 1A,B), IL-6 ($p < 0.01$, Figure 2A), and TNF- α ($p < 0.001$, Figure 2B).

3.3 Effects of CP supplementation on serum inflammatory markers in kittens

The concentration of serum SAA was not affected by dietary CP supplementation ($p > 0.05$, Figure 2F).

3.4 Effects of CP supplementation on serum metabolomics in kittens

Biomarkers in serum can objectively reflect physiological changes. The UPLC-Orbitrap-MS/MS analysis was used to evaluate the serum metabolic profile. In this study, a total of 759 metabolites were detected in the two groups. Furthermore, potential metabolite markers were screened out using the standard of $FC > 1.2$ and $p < 0.05$, resulting in 50 differential metabolites between the two groups. Dietary administration of CP upregulated 33 metabolites and downregulated 17 metabolites. The volcano plot displayed that the primary potential metabolite markers were glucoiberin, 5-aminoimidazole ribonucleotide, arachidonic acid (ARA), dihomo-gamma-linolenic acid (DGLA), cytidine, *N*-acetyltylucine, hydroxychloroquine, bisphenol A, retinal, cetirizine, 2,3-bis-*O*-(geranylgeranyl)glycerol 1-phosphate, thymine, isopropyl alcohol, glycine, and glyceric acid (Figure 3A).

In addition, as shown in Figure 3B, the KEGG enrichment analysis further explored the effects of dietary CP supplementation on serum metabolic pathways. The results demonstrated that the most dominant

TABLE 3 Effect of CP on serum biochemical parameters.

Items	CON	CP	p-value
ALB, g/L	30.31 ± 1.18	30.51 ± 1.29	0.958
TP, g/L	61.50 ± 2.28	61.70 ± 2.37	0.891
GLO, g/L	31.19 ± 1.88	31.17 ± 2.18	0.695
ALB/GLO	0.99 ± 0.06	1.01 ± 0.09	0.269
AST, U/L	22.86 ± 1.78	22.28 ± 1.58	0.779
ALT, U/L	46.14 ± 2.75	49.29 ± 4.83	0.276
AMY, U/L	897.00 ± 74.66	859.14 ± 66.88	0.751
CK, U/L	293.71 ± 97.18	213.00 ± 54.73	0.094
CRE, μ mol/L	83.53 ± 5.13	84.74 ± 3.62	0.169
BUN, mmol/L	7.08 ± 0.35	6.73 ± 0.36	0.927
BUN/CRE	87.07 ± 7.37	79.82 ± 4.08	0.154
GLU, mmol/L	3.51 ± 0.26	3.33 ± 0.24	0.834
TG, mmol/L	0.41 ± 0.03	0.45 ± 0.04	0.181
Ca, mmol/L	2.33 ± 0.03	2.31 ± 0.05	0.051
IP, mmol/L	2.03 ± 0.08	2.29 ± 0.09	0.894

CON, basal diet group; CP, compound polysaccharide diet group; ALB, albumin; GLO, globulin; TP, total protein; ALB/GLO, albumin/globulin; AST, aspartate aminotransferase; ALT, alanine transaminase; AMY, amylase; CK, creatine kinase; CRE, creatinine; BUN, urea nitrogen; BUN/CRE, urea nitrogen/creatinine; GLU, glucose; TG, triglyceride; Ca, calcium; IP, inorganic phosphorus.

TABLE 4 Effects of dietary CP supplementation on serum antioxidant capacity.

Items	CON	CP	p-value
T-AOC, mM	0.78 ± 0.04	0.76 ± 0.05	0.478
GSH-Px, pmol/mL	4.50 ± 0.81	4.32 ± 1.08	0.735
MDA, nmol/mL	1.41 ± 0.25	1.28 ± 0.23	0.332
CAT, U/mL	0.28 ± 0.17	0.48 ± 0.29	0.141
SOD, U/mL	9.86 ± 0.79	10.03 ± 0.89	0.715

CON, basal diet group; CP, compound polysaccharide diet group; T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase.

pathways were purine metabolism, glyoxylate and dicarboxylate metabolism, glycine, serine, and threonine metabolism, biosynthesis of unsaturated fatty acids, pyrimidine metabolism, glycerolipid metabolism, retinol metabolism, pentose phosphate pathway, and glutathione metabolism.

3.5 Effects of CP supplementation on the fecal microbiota in kittens

The effects of dietary CP supplementation on fecal microbiota are illustrated in Figures 4, 5. The Venn diagram displayed 286 shared ASVs between the two groups, with 664 ASVs in the CON group and 556 ASVs in the CP group, respectively (Figure 4A). No differences ($p > 0.05$) in the Shannon diversity index and the Ace index were observed between the CON and the CP groups (Figures 4B,C).

At the phylum level, the dominant bacteria included *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, *Proteobacteria*, and *Fusobacteriota* (Figure 5A). At the family level, the predominant bacteria included

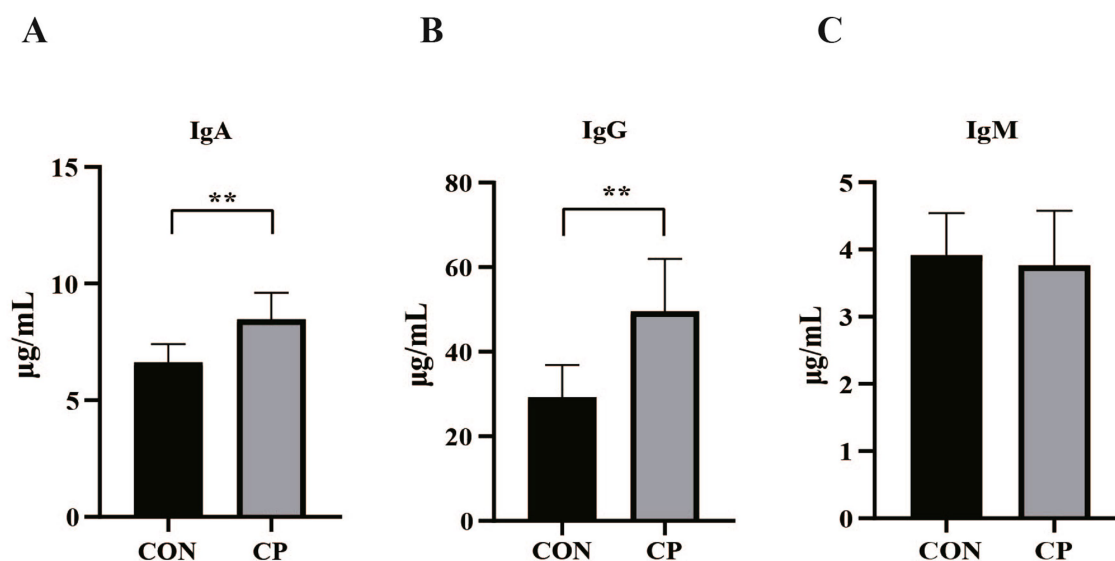


FIGURE 1

Effects of CP supplementation on the concentrations of serum immunoglobulins in kittens. (A) IgA, immunoglobulin A; (B) IgG, immunoglobulin G; (C) IgM, immunoglobulin M. Data are presented as mean $\pm$ SE. The symbol (*) indicates statistically significant differences (** $p < 0.01$).

Peptostreptococcaceae, *Lachnospiraceae*, *Erysipelotrichaceae*, *Coriobacteriaceae*, *Prevotellaceae*, *Streptococcaceae*, *Enterobacteriaceae*, and *Peptococcaceae* (Figure 5B). At the genus level, the dominant bacteria consisted of *Peptoclostridium*, *Blautia*, *Collinsella*, *Streptococcus*, *Holdemanella*, *Prevotella*, *Escherichia-Shigella*, *unclassified_f_Lachnospiraceae*, and *Peptococcus* (Figure 5C). Moreover, the relative abundance of *norank_f_Butyricicoccaceae* and *Bacteroides plebeius* was enriched in the CP group, while the relative abundance of *Anaerotruncus* was enriched in the CON group ($p < 0.05$, Figures 5D–F).

4 Discussion

4.1 Dietary supplementation with CP increased the concentrations of serum IgA, IgG, IL-6, and TNF- α

Macrophages play an essential role in both adaptive and innate immunity as the antigen-presenting cells, secreting pro-inflammatory cytokines, and cleaning foreign antigens (Deng et al., 2023). Previous studies have found that PP can increase the production of pro-inflammatory cytokines IL-6, TNF- α , and IL-12 by inducing M1 macrophage polarization (Hu et al., 2023; Zhao R. et al., 2023). The concentrations of IL-6, TNF- α , and IL-1 β were significantly enhanced after PP treatment, with immune regulation attributed to the activation of the Ca²⁺/PKC/p38/NF- κ B signaling pathway (Pu et al., 2019). In addition, cellular tests indicated that PP can combine with mannose receptors in macrophages, enhancing the levels of TNF- α and mRNA expression in cells (Zhang et al., 2024).

Furthermore, research has proven the immunomodulatory effect of APS by enhancing the activities of macrophages, natural killer cells, T lymphocytes, and B lymphocytes, leading to the secretion of cytokines (Li C. et al., 2022; Zhao R. et al., 2023). Dietary supplementation with APS has been shown to increase the serum

TNF- α , IL-1 β , and IgA in weaned piglets by activating the TLR4-mediated MyD88-dependent signaling pathway (Wang K. et al., 2020). Treatment with APS could resist the infection of the pathogen *Brucella* and alleviate the symptoms of mice with brucellosis by activating the macrophages and upregulating the production of TNF- α , IL-12, and IFN- γ (Shi et al., 2019). Furthermore, supplementation with APS effectively increased serum concentrations of IL-6, TNF- α , and IFN- γ in weaning rex rabbits (Sun et al., 2016).

The above results demonstrate that APS and PP display immunostimulatory functions by enhancing the secretions of pro-inflammatory cytokines and immunoglobulins. Similar results were observed in our study, where CP supplementation increased the concentrations of serum IL-6, TNF- α , IgA, and IgG in kittens.

Previous studies have indicated that IL-6 and TNF- α were pro-inflammatory cytokines, which mediate in many inflammatory diseases and contribute to developing autoimmune disorders and cancer (Waldner and Neurath, 2014). However, a number of studies have also demonstrated that IL-6 is a multifunctional cytokine that can induce B cells into mature plasma cells, thus improving antibody production (Urashima et al., 1996). Moreover, IL-6 is involved in the proliferation and differentiation of T cells, and mice lacking IL-6 often show deficiencies in T cell effector function and memory recall (Kopf et al., 1994; Twohig et al., 2019; Urashima et al., 1996). Furthermore, IL-6 plays an essential role in protecting mice from *Escherichia coli* infection by efficiently inducing neutrophils in the bloodstream (DALRYMPLE et al., 1996).

TNF- α is indispensable for adaptive immune responses as it is necessary for developing lymphoid organs and can activate lymphocytes, thus enhancing the secretion of antibodies and cytokines to eliminate infection (Li et al., 2001). For example, TNF- α is essential for mice to eliminate hepatocytes infected by hepatitis B virus in the immune response to hepatitis B virus. TNF- α also plays an important role in regulating cytokines derived from astrocytes, which are involved in responding to microbial infections (Phulwani et al., 2008). In brief, IL-6 and TNF- α , crucial immunomodulators, play an

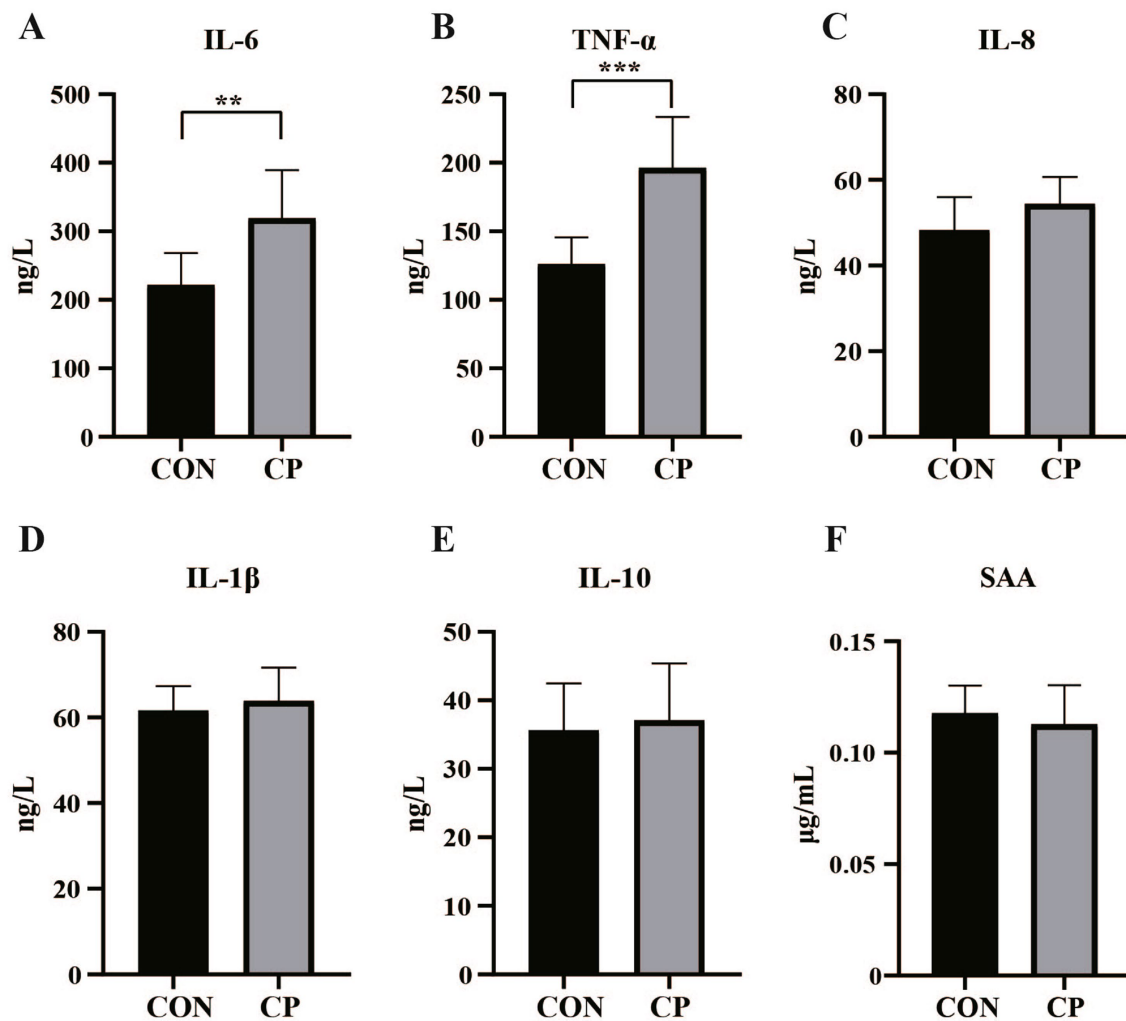


FIGURE 2

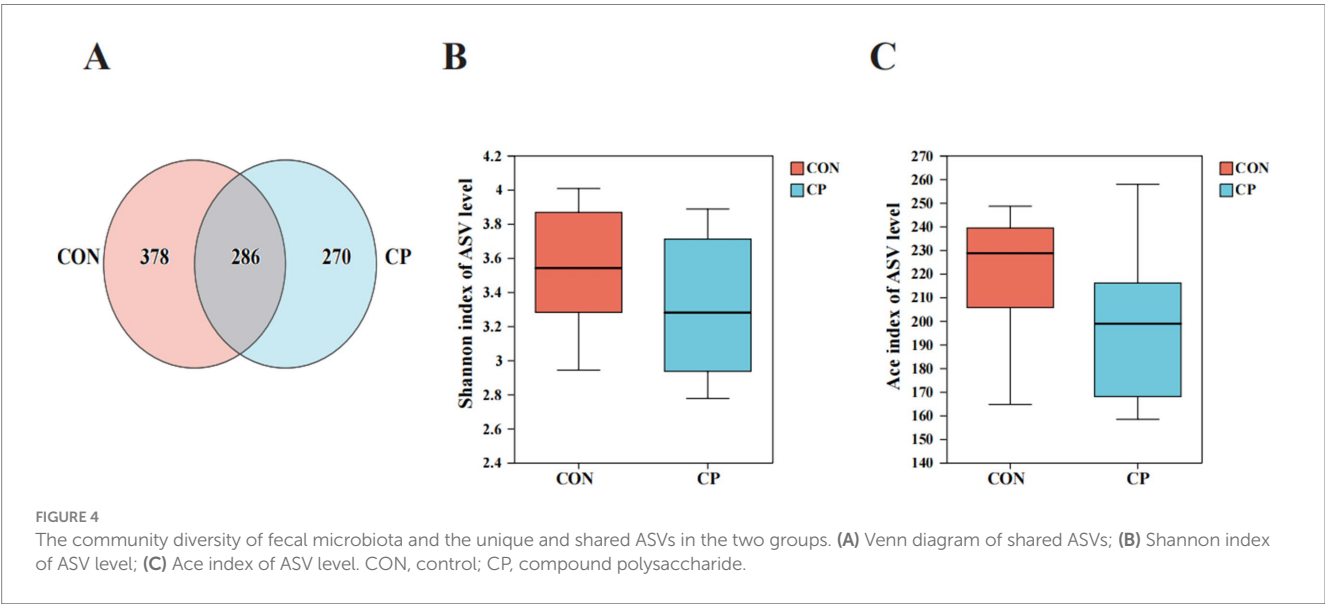
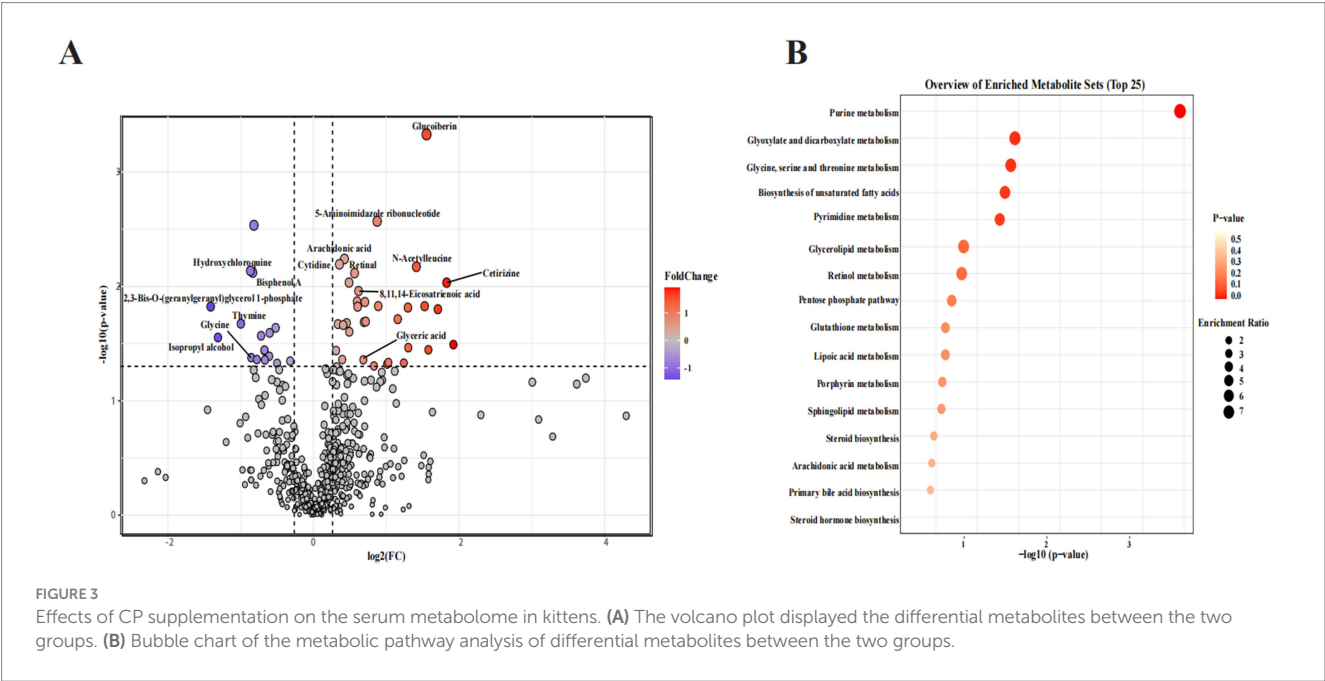
Effects of CP supplementation on the concentrations of serum inflammatory cytokines and markers of inflammation in kittens. (A) IL-6, interleukin 6; (B) TNF- α , tumor necrosis factor- α ; (C) IL-8, interleukin 8; (D) IL-1 β , interleukin 1 β ; (E) IL-10, interleukin 10; (F) SAA, serum amyloid A. Data are presented as mean $\pm$ SE. The symbol (*) indicates statistically significant differences (** p < 0.01 and *** p < 0.001).

important role in resisting foreign pathogens and injuries by mediating the activation of lymphocytes and dendritic cells and inducing the production of antibodies.

Generally, the systemic inflammation response can improve immunity and resistance to infection, benefiting the host. However, an excessive pro-inflammatory response harms the whole health (Bistrrian, 2004). A moderate increase in IL-6 and TNF- α can enhance the overall immunity of kittens. To confirm that the immune enhancement of CP supplementation did not lead to generalized inflammation, we measured the concentration of serum SAA. SAA, one of the serum acute phase proteins (APPs), serves as an inflammatory marker for cats. In addition, SAA is a major positive APP, which is more sensitive than white blood cell counts for early detection of inflammation (Rossi, 2023; Tuna and Ulutas, 2022). Under the stimulation of inflammatory cytokines, such as IL-6 and TNF- α , SAA is synthesized by hepatocytes in the liver and released into the circulating blood, resulting in a systemic inflammatory response (Ishioka and Hayakawa, 2019). The concentration of serum SAA increases during acute or chronic

inflammation (Waugh et al., 2022). In this study, no difference in the concentration of serum SAA was found after CP treatment. Moreover, the concentrations of serum SAA in both groups fell within the normal reference range, as reported in previous research (Waugh et al., 2022). Thus, it is clear that the immune enhancement effect of CP treatment did not cause generalized inflammation.

However, there are many studies demonstrating that APS and PP display an anti-inflammatory effect through a decrease in the production and secretion of inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α (He et al., 2022; Ming et al., 2022; Ng et al., 2024; Wu et al., 2022), which are contrary to our results. It is worth noting that the anti-inflammatory effects of APS and PP are displayed in the inflammation model or hosts experiencing inflammation. Conversely, the pro-inflammatory effect can be observed in the immunosuppression model or hosts under immunosuppressive conditions. Therefore, the effects of supplementation with CP depend on the immune status of the host. In this study, kittens were more susceptible to diseases because of the reduced maternal antibodies,



immature immune system, and challenging events in their early life; thus, dietary supplementation of CP enhanced the levels of serum IL-6, TNF- α , IgA, and IgG, which contributed to improving their overall immunity of the kittens.

4.2 The untargeted serum metabolomics analysis reveals the metabolic mechanism of CP supplementation in kittens

The untargeted serum metabolomics analysis was applied to uncover the potential mechanism of CP supplementation in regulating inflammatory responses in kittens. In this study, serum metabolomic analysis revealed that the metabolic markers significantly altered by CP supplementation were ARA, DGLA,

glycine, and glyceric acid. Moreover, further pathway analysis revealed the metabolic pathways regulated by the above differential metabolites, which play a role in the immunomodulatory effects of kittens.

Previous studies indicated that glycine plays an important part in regulating inflammatory cytokines by inhibiting the activities of macrophages, monocytes, and T lymphocytes. Furthermore, the level of serum glycine is negatively correlated with the level of TNF- α (Gu et al., 2012; Li et al., 2001; Yao et al., 2015). In our study, pathway analysis indicated that glycine and glyceric acid were the enriched metabolites in the glycine, serine, and threonine metabolism and glyoxylate and dicarboxylate metabolism pathways. In addition, supplementation with CP decreased the level of glycine and increased the concentration of TNF- α in serum, which is consistent with the results in the above studies.

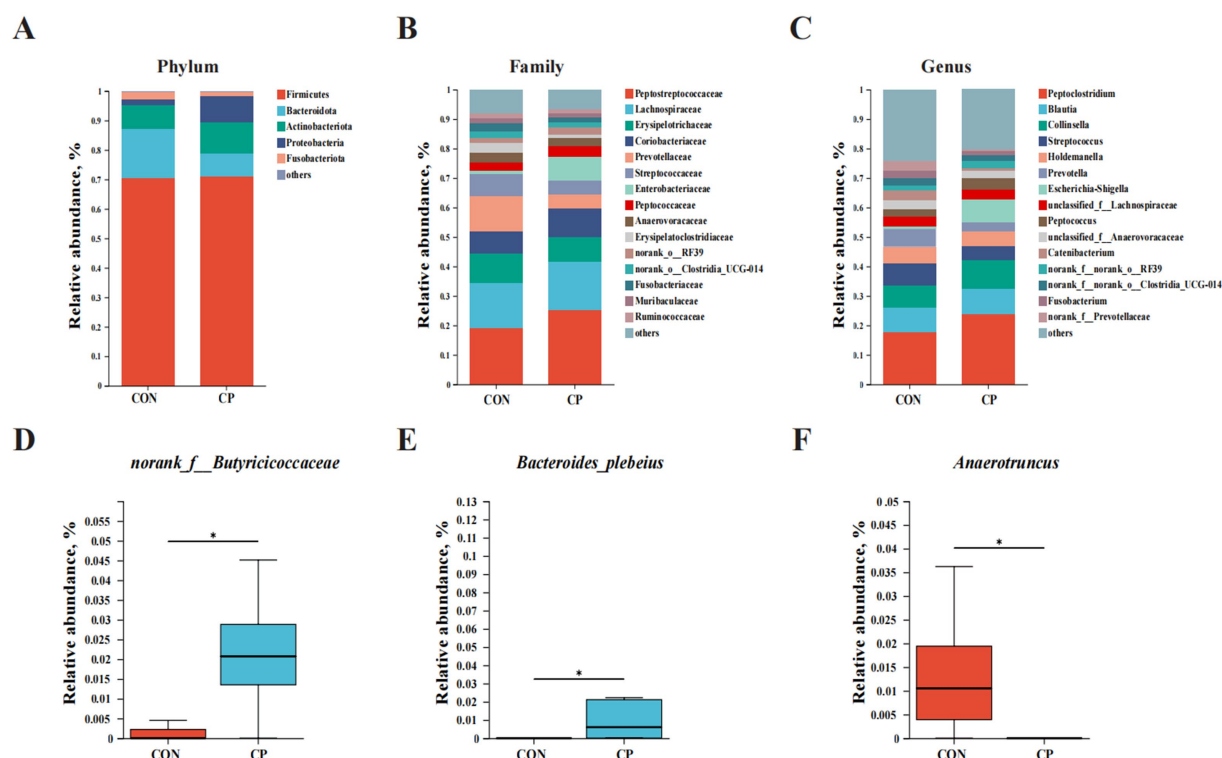


FIGURE 5

Effects of CP supplementation on intestinal microbiota composition in kittens. Barplot analysis of microbial composition at (A) phylum, (B) family, and (C) genus levels. (D–F) The relative abundance of differential bacteria. CON, control; CP, compound polysaccharide. The symbol (*) indicates statistically significant differences (* $p < 0.05$).

DGLA and ARA are both omega-6 polyunsaturated fatty acids, and the balance between DGLA and ARA is important in regulating inflammatory processes in the body (Mustonen and Nieminen, 2023). Previous research has demonstrated that DGLA, serving as a precursor for the synthesis of ARA, can be metabolized via delta-5 desaturase reaction to ARA in cats (Trevizan et al., 2012). ARA plays an essential role in inflammatory processes by stimulating the production of pro-inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β (Monmai et al., 2020). Previous studies have proved that ARA is the main precursor of eicosanoids, such as prostaglandins, leukotrienes, thromboxanes, lipoxins, hepxilins, hydroxyeicosatetraenoic acids, and epoxyeicosatrienoic acids, which are mediators of inflammation (Calder, 2008; Ming Luo, 2006; Panigrahy et al., 2010). Specifically, ARA is present in the membranes of most mammalian cells and can be released from the cell membrane through mobilization by various phospholipase enzymes; thus, free ARA can participate in the synthesis of eicosanoids (Ming Luo, 2006; Calder, 2008; Panigrahy et al., 2010). For example, ARA can be converted to hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids by cytochrome P450 enzymes (Panigrahy et al., 2010). These ARA-derived eicosanoids were found to modulate the production of inflammatory cytokines (Esser-von Bieren, 2017). For example, the concentrations of TNF- α and IL-6 in cell-free supernatants increased after the monocytes were exposed to leukotriene B4 (Rola-Pleszczynski and Stankova, 1992). The above research indicated that the biosynthesis of unsaturated fatty acids and related metabolites plays a crucial role in immune regulation. Our results revealed that CP supplementation upregulated the concentrations of ARA and DGLA in

serum, with consequent effects on the biosynthesis of unsaturated fatty acids. In our study, supplementation with CP increased the concentrations of serum DGLA, ARA, TNF- α , and IL-6 and decreased the level of glycine, suggesting that DGLA, ARA, and glycine may serve as mediators for CP in regulating immune function in kittens.

4.3 Dietary supplementation with CP did not generate a phylogenetic change in the fecal bacterial composition of kittens

The results showed that the predominant phyla were *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, *Proteobacteria*, and *Fusobacteriota*, which were consistent with fecal microbial composition in healthy cats as described in previous research (Bierlein et al., 2021; Garcia-Mazcorro et al., 2011). Furthermore, there were no differences in the relative abundance of these predominant phyla between the two groups, suggesting that the supplementation of CP did not alter the predominant phyla in kittens. However, the supplementation with CP increased the relative abundance of *norank_f__Butyricococcaceae* and *Bacteroides plebeius* ($p < 0.05$) while decreasing the abundance of *Anaerotruncus* ($p < 0.05$).

The *Butyricococcaceae* family is a group of essential butyrate-producing bacteria. Previous studies have indicated the beneficial effects of *Butyricococcaceae* on improving the intestinal health of the host (Ameer et al., 2023; Guo et al., 2022; Yu et al., 2023). The *Bacteroides plebeius* served as probiotics, and it was the producer of

propionate in the canine intestine (Kaelle et al., 2023; Sun et al., 2023). Moreover, studies in cats have indicated that *Bacteroides plebeius* are significantly abundant in healthy cats compared to those with chronic enteropathy (Li X. et al., 2022; Marsilio et al., 2019). Previous research has indicated that *Bacteroides plebeius* can enhance the barrier function of the intestinal mucosa by modulating the gut microbiome, reducing protein consumption in rats with chronic kidney disease, and increasing the abundance of probiotics while minimizing damage to the intestinal mucosal barrier (Pei et al., 2022).

Anaerotruncus is an opportunistic pathogen that has been linked to inflammatory bowel disease as it can induce the production of pro-inflammatory cytokines and cause immune disorder (Liu et al., 2021). A previous study demonstrated that Fuzi polysaccharide treatment significantly decreased the relative abundance of *Anaerotruncus*, and the mRNA expression of IL-6 was negatively correlated with the relative abundance of *Anaerotruncus* in the immunosuppressive mouse model, which is consistent with our findings (Tu et al., 2023). In a word, administration with CP caused several alterations in the relative abundance of certain bacteria groups, leading to some beneficial effects in kittens. However, CP treatment did not generate a phylogenetic change in the fecal bacterial composition in kittens.

To the best of our knowledge, this is the first time to evaluate the effect of compound polysaccharides on immunity, antioxidant capacity, gut microbiota, and serum metabolome in kittens. However, it is important to note that there are some limitations to this study. A major limitation of our study is that while the supplementation of CP did generate some advantageous effects on kittens, we cannot determine the individual contributions of each polysaccharide in CP to the observed results. In addition, the time-dependent effects of CP administration were not assessed during the study. Clearly, more research is needed to investigate the respective effects of APS and PP to evaluate their potential beneficial properties in kittens. Moreover, further studies should consider increasing the sampling frequency to evaluate the effect of time and the interaction between diet and time of CP treatment in kittens.

5 Conclusion

The serum biochemistry parameters were within the normal range for kittens, and all were under normal physiological conditions. Supplementation with CP increased the concentrations of serum immunoglobulins and inflammatory cytokines, but it did not affect the level of serum SAA. These results indicate that dietary supplementation with CP has the potential to support the immunity of kittens and increase the relative abundance of beneficial gut microbiota. It is a favorable immunonutritional supplement in pet food.

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Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI, accession PRJNA1226968.

Ethics statement

The animal study was approved by the Experimental Animal Ethics Committee of South China Agricultural University (protocol code 2021a030). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YX: Conceptualization, Investigation, Methodology, Resources, Validation, Writing – original draft. SJ: Writing – review & editing, Conceptualization, Formal analysis, Methodology, Visualization. LZ: Data curation, Investigation, Resources, Software, Supervision, Writing – review & editing. BD: Supervision, Funding acquisition, Project administration, Writing – review & editing.

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Conflict of interest

LZ was employed by Guangzhou Qingke Biotechnology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Spase Stojanov,
Institut "Jožef Stefan," Slovenia
Cristina Casto Rebollo,
Universitat Politècnica de València, Spain

*CORRESPONDENCE

Seongbeom Cho
✉ chose@snu.ac.kr

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Dynamics of the canine gut microbiota of a military dog birth cohort

Jae-Uk An¹, Seung-Hyun Mun¹, Woo-Hyun Kim^{1,2},
Je Kyung Seong^{1,2}, Kyoungwan Park³ and Seongbeom Cho^{1,2,4*}

¹College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul, Republic of Korea, ²Korea Mouse Phenotyping Center (KMPC), Seoul National University, Seoul, Republic of Korea, ³Military Working Dogs Training Center, Gangwon, Republic of Korea, ⁴Comparative Medicine Disease Research Center (CDRC), Seoul National University, Seoul, Republic of Korea

Introduction: We systematically tracked early life stages in a military dog birth cohort to investigate canine gut microbiota dynamics related to environmental exposure during growth. This study utilized 16s rRNA amplicon sequencing-based analysis with molecular epidemiology of *Enterococcus faecalis* within a controlled environment at a military dog training center.

Methods: We examined shifts in gut microbiota diversity and taxonomic composition across four growth stages (lactation, weaning, starter, puppy) in three littermate groups. Additionally, *E. faecalis* dynamics was analyzed to confirm strain sharing among littermate groups.

Results: Gut microbiota changed rapidly during early growth, stabilizing at the puppy stage. This is supported by increased similarity in taxonomic composition among littermate groups, as they experienced an increased shared external environment and consumed the identical diets. *E. faecalis* strain sharing among littermate groups increased as dogs aged. Nine *E. faecalis* cluster types were identified; three specific types (type I, II, and IX) dominated in each littermate group during lactation. With greater exposure to the shared external environment, cluster type I gradually assumed dominance across all groups. Despite the dynamic shifts in microbiota, we found five genera within the core microbiota, *Bacteroides*, *Peptoclostridium*, *Fusobacterium*, *Lactobacillus*, and *Blautia*.

Discussion: This study is the first to explore the dynamic nature of early-life canine gut microbiota, illustrating its transition to stability and its resilience to environmental perturbations within the controlled training environment of a military dog birth cohort.

KEYWORDS

military dogs, birth cohort, gut microbiota, growth, controlled environment, 16s rRNA amplicon sequencing, *Enterococcus faecalis*, Canine parvovirus-2

1 Introduction

Gut microbiota includes all the microorganisms in the gastrointestinal (GI) tract, including bacteria, viruses, fungi, archaea, and protozoa (Honneffer et al., 2014). It forms a complex symbiotic relationship with the host, facilitating nutrient digestion and absorption and/or preventing the entry of exogenous pathogens (Hooda et al., 2012).

However, this symbiotic relationship can be altered and reconstructed by numerous factors, including dietary or living environmental changes, diseases, antibiotic use, genetics, and aging (Cho and Blaser, 2012; O'Toole and Jeffery, 2015; Million et al., 2017). Among those factors, strong perturbations, including infectious disease and antimicrobial application, can cause dysbiosis, a disrupted symbiotic state (Sommer et al., 2017).

In clinical studies, environmental factors during early life stages play a critical role in gut microbiota establishment and development (Kelsen and Wu, 2012). A well-established gut microbiota at the early life stages may assist the host in developing resilience to microbial dysbiosis and diseases (Lozupone et al., 2012; Parkin et al., 2021). Gut microbiota management can be a powerful tool for preventing and treating various diseases (Markandey et al., 2021; Waller et al., 2021). The dysbiosis patterns in humans and dogs are relatively similar (Coelho et al., 2018). Understanding the development of healthy canine gut microbiota in early life stages can provide a valuable cornerstone for health care and disease treatment in adult dogs.

Microbiota is greatly affected by external factors; thus, controlling for confounding factors is important when studying microbiota changes (Karahana, 2024). Domestic dogs interact with various external factors in indoor and outdoor environments (Pocar et al., 2023). Therefore, excluding confounding factors is difficult. The health state of military dogs used for special purposes is crucial, and they live systematically under strict veterinarian management. They are born and raised in a controlled training environment (Military Dog Training Center), and variations in individual dietary habits and living conditions are minimal. The living environment characteristics of these military dogs make them suitable models for studying canine gut microbiota.

Enterococci, particularly *E. faecalis* and *E. faecium*, are crucial components of the gut microbiota in humans and animals and have been suggested to be early gut colonizers (Fisher and Phillips, 2009; Gilmore et al., 2013). Colonization with *E. faecalis* may help maintain gut microbiota homeostasis by reducing the risk of infectious diseases and stimulating immune responses in early life stages (Wang et al., 2008). In many instances, sequencing-based metagenomic analysis, such as 16S rRNA analysis or shotgun metagenomics, cannot accurately differentiate between closely related microbial strains owing to technical limitations, such as sequencing errors (Yan et al., 2020). In contrast, culture-based molecular methods allow distinguishing between different species with similar phenotypic characteristics and between strains belonging to the same species (Martin-Platero et al., 2008). In addition to 16S rRNA metagenomic analysis, analyzing genetic relationships using culture-based methods can be an important key factor in understanding the evolution and dynamics of microbial communities at the strain level (Leventhal et al., 2018; Lou et al., 2021; Goyal et al., 2022; Wolff et al., 2023).

This study aimed to track the dynamics of the gut microbiota of dogs at early life stages (lactation/weaning/starter/puppy) by using a birth cohort group in a military dog training center. We analyzed the gut microbiota of military dogs that were born and raised in a training center without contact with external environments. The α - and β -diversities, taxonomic composition, and predictive functional profile were analyzed by growth stage based on 16S rRNA amplicon sequencing-based analysis to determine gut microbiota changes. Additionally, the strain-level

dynamics of *E. faecalis*, a representative fecal indicator bacterium, were analyzed using rep-PCR to confirm the sharing, proliferation, and introduction of bacterial strains among littermates.

2 Materials and methods

2.1 Fecal sample collection from military dogs

Fecal samples were collected from 27 dogs born and raised at the Military Working Dog Training Center located in Gangwon-do, Republic of Korea, between May 2016 and March 2017. Since the puppies were delivered from three different mothers, they were classified into three groups (A, B, and C), each comprising puppies from the same mother. There were 7, 10, and 10 dogs in groups A, B, and C, respectively. The breed of the dogs in group A was German Shepherd, and that of groups B and C was Belgian Malinois. To collect the fecal samples, a clean and independent space was provided for each dog in the morning after feeding. If a fresh fecal sample could not be collected at any time point for a particular individual, all samples from that individual across the seven time points were excluded from the analysis. Consequently, the final numbers of animals included in the analysis were six, eight, and six animals in groups A, B, and C, respectively. The fecal samples were collected in sterilized containers immediately after excretion and placed into Styrofoam packages containing a sufficient amount of ice packs to maintain ice-cold conditions ($\sim 4^{\circ}\text{C}$) during transportation to the laboratory.

Sampling was performed at seven points between 4 and 28 weeks of age, and the sampling points were grouped into four growth stages according to growth and dietary changes (Figure 1): lactation (4 weeks of age), weaning (6 weeks of age), starter (8 and 11 weeks of age), and puppy (14, 21, and 28 weeks of age). The feed differed depending on the growth stage: mother's breast milk only during the lactation period; mother's breast milk with a starter commercial diet (Royal Canin, Aimargues, France) during the weaning period; starter and puppy commercial diets only (Royal Canin) during the starter and puppy stages, respectively. The diet and its nutrients composition provided to military dogs in our study are explained in order to provide complete information to the reader. The nutritional composition of the commercial diets was crude protein (min) 28.0%, crude fat (min) 20.0%, crude fiber (max) 3.4%, and moisture (max) 10.0% for the starter commercial diet; and crude protein (min) 32.0%, crude fat (min) 12.0%, crude fiber (max) 3.4%, and moisture (max) 10.0% for the puppy commercial diet. The feed and/or its ingredients was not changed/manipulated for this study, compared to the age-specific feed originally provided at Military working dogs Training center.

According to the growth stages, military dogs were provided with different living spaces. During the lactation and weaning stages, the siblings lived together, sharing the living space with their mother (Figure 1). During the starter stage, sibling dogs were housed separately from their mother in individual living spaces, with three to four dogs per space. Although their sleeping and feeding areas were separated, these spaces were connected to a shared fenced backyard playground, allowing all siblings to interact with one another. However, this backyard was not

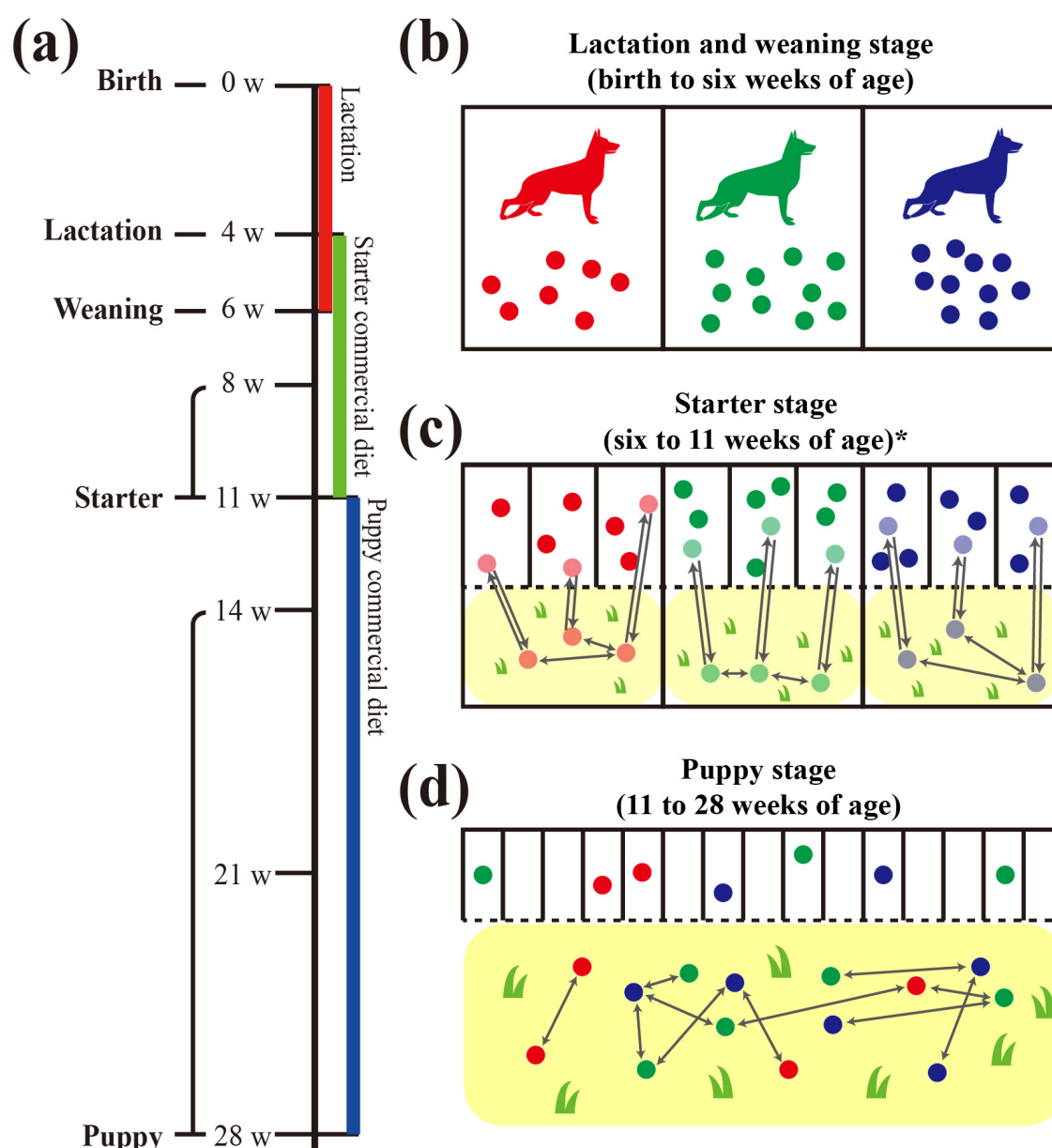


FIGURE 1

Graphical diagram of dog growth and living environment in the military dog training center. Schematic diagram for changes in the living environment from birth to 28 weeks of age of three littermate groups. **(a)** Sampling was performed at 4, 6, 8, 11, 14, 21, and 28 weeks of age, and the dietary stages were divided into lactation (4 weeks of age), weaning (6 weeks of age), starter (8 and 11 weeks of age), and puppy (14, 21, and 28 weeks of age). Colored bars below the timeline indicate the provided diet: lactation diet in red, starter commercial diet in green, and puppy commercial diet in blue. **(b)** Separate living spaces (indoors, cement, metal fence) were provided for each littermate group with the mother dog from birth to the weaning stage (6 weeks of age). **(c)** Three to four littermates shared one indoor living space (cement, metal fence), and separate backyards (outdoors, soil and grass) were also provided for each littermate group. **(d)** An individual living space and shared outdoor training field were provided for each puppy. In the training field, contact occurred freely between puppies, regardless of littermate group.

shared with other sibling groups. From the puppy stage, each individual dog lived independently in a separate space, using the same building regardless of the sibling group. Furthermore, they started to perform outdoor field training from this stage (14 weeks of age) to develop qualities suitable to military dogs. This age was a period of adaptation to field training, which actively began from the age of 21 weeks.

Among groups A, B, and C, only the puppies in group B exhibited diarrhea suspected to be infectious bowel

disease around 13 weeks of age, while those in groups A and C were healthy and without any disease symptoms. At 13 weeks and 6 days of age, Canine parvovirus-2 (CPV-2) infection was diagnosed in group B using the “Antigen Rapid CPV Ag Test Kit” (BIONOTE Inc., Gyeonggi-do, Korea). Accordingly, group B received antibiotics (enrofloxacin, cefazolin, amoxicillin/clavulanic acid, and metronidazole) and intravenous fluid therapy for approximately two weeks until the symptoms vanished.

2.2 Study design

Figure 1 shows an overview of the sampling timeline. To analyze healthy canine gut microbiota according to age, we included fecal samples from 4, 6, 8, 11, 14, 21, and 28 weeks-old dogs in groups A and C, and 4, 6, 8, and 11 weeks-old dogs in group B. The CPV-2 infection at 13 weeks in group B along with antibiotic treatment for 2 weeks could be a confounding factor; hence, we excluded the samples older than 11 weeks of age (14, 21, and 28 weeks of age) in group B.

To analyze the recovering potential of gut microbiota against CPV-2 infection during the early life stages of military dogs, we compared the gut microbiota of eight dogs in group B (CPV-infected at 13 weeks) with that of six dogs in group C (healthy, without symptoms; control group, CON) from 8 to 28 weeks of age. For clarity of the analysis, we designated group B as “group CPV” and group C as “group CON.” Further, group CPV was subdivided into CPV-high and -low based on the infection dose of CPV-2; the five dogs with a higher cycle threshold (Ct) value of the VP2 gene of CPV-2 relative to the bacterial 16S rRNA in the gut microbiota were assigned to the “CPV-high” (mean $\Delta Ct = 1.670$; 95% CI, 1.409–1.931) and the three dogs with a lower Ct value relative to 16S rRNA in the gut microbiota were assigned to the “CPV-low” group (mean $\Delta Ct = 5.504$; 95% CI, –6.245, –4.763; [Supplementary Figure 1](#)). Four of the five dogs in the group CPV-high died between weeks 11 and 14, and the fifth dog died between weeks 21 and 28. All three dogs in group CPV-low survived after 28 weeks of age.

2.3 Isolation and rep-PCR analysis of *Enterococcus faecalis* for culture-dependent genetic diversity analysis

Repetitive sequence-based PCR (rep-PCR) was performed to investigate whether the genetic diversity among *E. faecalis* belonging to the gut microbiota of each individual is similar to the taxonomic composition of the gut microbiota between individuals.

To isolate *Enterococcus* sp., 1 g of fecal sample was homogenized in 9 mL of EnterococcoselTM broth (Becton Dickinson and Company, United States) and incubated at 37° for 18 h. One loop of enriched broth was streaked on an EnterococcoselTM agar plate (Becton Dickinson and Company, United States) and incubated at 37°C for 18–20 h, and six suspected colonies were picked for each plate. Genomic DNA was extracted from each colony using the InstageneTM matrix (Bio-Rad, United States), and *E. faecalis* was identified using the *Enterococcus* genus-specific primer set and *E. faecalis* species-specific primer set as previously reported ([Kariyama et al., 2000](#)).

In total, 81 *E. faecalis* strains were isolated, with each strain obtained from the fecal sample of a unique individual dog within the healthy group. The rep-PCR was performed using BOXA1R primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') and PCR mixture as follows, 12.5 μ L of 2X Lamp *taq* master mix (Biofact, Korea), 2 μ L of primer (25 pmol/ μ L), 5 μ L of DNA template, and distilled water to attain a final volume of 25 μ L. PCR reactions were performed using the conditions previously described ([Koeuth et al., 1995](#)). The extracted DNA quality was assessed using a Nanodrop

spectrophotometer (Thermo Fisher Scientific, United States), and samples having a 260/230 absorbance ratio value above 1.8 were used for further analysis. PCR products were electrophoresed on a 1.0% agarose gel containing DNA staining solution and imaged using Gel-doc (Bio-Rad, United States). Band patterns from rep-PCR were analyzed using BioNumerics version 6.6 (Applied Maths, Belgium). Dendrograms were generated based on the Dice similarity coefficient with the unweighted pair group method with arithmetic mean using parameters as follows: 3% of optimization and 1.5% of band matching tolerance. Effective number of species (ENS) of Shannon (e^H) and Simpson (1/D) ([Simpson, 1949](#)) diversity indices were used to calculate the genetic diversity of *E. faecalis* isolated at each week of age based on 80% band pattern similarity criteria. The 80% band pattern similarity criteria were applied based on previously established thresholds for rep-PCR studies ([Versalovic et al., 1991](#); [Koeuth et al., 1995](#)).

2.4 Metagenomic DNA extraction and bacterial 16S rRNA amplicon sequencing

Immediately after being transported to the laboratory, the fecal samples were homogenized with a 10-fold dilution in 0.85% NaCl solution. A 250 μ L aliquot of each diluted sample was used for metagenomic DNA extraction using the FastDNATM SPIN Kit for Soil (MP Biomedicals, Irvine, CA, United States) and FastPrep[®]-24 Classic Instrument (MP Biomedicals). The DNA concentration and purity were assessed using PicoGreen (Invitrogen, Carlsbad, CA, United States) and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The V3–V4 region of the 16S rRNA gene was amplified using bacterial primers (Forward: 341F, 5'-CCT ACG GGN GGC WGC AG-3'; Reverse: 805R, 5'-GAC TAC HVG GGT ATC TAA TCC-3') as described by [Klindworth et al. \(2013\)](#). A limited-cycle PCR was subsequently performed to add multiplexing indices and Illumina-compatible sequencing adapters for library preparation. The 16S rRNA gene amplicons were pooled and normalized using PicoGreen, and the library size was validated using the LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, Waltham, MA, United States). Sequencing was conducted using the MiSeqTM platform (Illumina, San Diego, CA, United States) with a paired-end read length of 2 $\times$ 300 bp, provided by Macrogen (Seoul, Korea).

Raw sequence data were subjected to quality control to ensure clean and reliable reads for downstream analysis. Adapter sequences were removed using Scythe (v0.994) and low-quality bases were trimmed using Sickel. Reads shorter than 38 bp after adapter trimming were discarded to produce high-quality and clean data for further analysis.

2.5 Taxonomic assignment using 16S rRNA amplicon sequencing

Bioinformatics analysis was performed using QIIME2 (2023.9) ([Bolyen et al., 2019](#)). The raw sequence reads were processed using the Divisive Amplicon Denoising Algorithm 2 (DADA2)

plugin (Callahan et al., 2016) within QIIME2, ensuring high-quality and reliable data for downstream analysis. In the denoising process, primer sequences were automatically removed from the raw reads, and low-quality bases were filtered based on a Phred quality score threshold ($\geq Q20$). Reads with ambiguous bases or consistently low-quality regions were discarded, while the remaining reads were truncated at the position where the average quality dropped below the threshold to minimize sequencing errors. Additionally, identical reads were dereplicated to reduce redundancy and computational load, and chimeric sequences were identified and removed *de novo* to eliminate amplification artifacts. Low-abundance amplicon sequence variants (ASVs) ($< 0.25\%$ relative abundance per sample) were removed to exclude spurious ASVs. The alignment of ASVs was accomplished using MAFFT (Katoh et al., 2002) and phylogenetic tree was analyzed with the FastTree2 plugin (Price et al., 2010). Taxonomic assignment was performed using the SILVA database (release 138) (Quast et al., 2013). After taxonomic assignment, archaeal, eukaryotic, mitochondria, and chloroplasts ASVs were filtered out to collect bacterial ASVs.

After taxa and abundance filtering, 7,031,749 sequence reads were obtained, ranging from a minimum of 19,936 to a maximum of 120,662 (1st quartile: 43,681.25, Median: 51,883.5, 3rd quartile: 64,507.25) from the 130 samples (42, 46, and 42 samples from each group). Taxonomic annotation identified a total of 9 phyla, 13 classes, 31 orders, 52 families, and 115 genera. During taxonomic assignment, sequences that could not be confidently classified at the genus or species levels were handled based on their taxonomic resolution at higher ranks. Among the seven unassigned genera observed in the dataset, two genera (*g__uncultured*; *s__uncultured_bacterium* and *g__uncultured*; *s__uncultured_Allobaculum*) within the family Erysipelotrichaceae were collapsed into a single genus, *uncultured_Erysipelotrichaceae*, due to their shared family-level classification. In contrast, the remaining five unassigned genera were associated with unique families; thus, they were retained as distinct taxa to preserve the taxonomic diversity within the dataset.

2.6 Taxonomic diversity analysis based on 16s rRNA amplicon sequencing: α - and β -diversity analyses

To further address differences in sequencing depth across samples, rarefaction was performed at a depth of 19,936 reads, which corresponds to the lowest sequencing depth among all samples. Using these normalized and rarefied datasets, four α -diversity indicators were calculated, namely Shannon diversity (ENS), Richness (observed features), Pielou's evenness, and Faith's phylogenetic diversity. In addition, to identify the difference in taxonomic composition between growth stages and the variance within stages, a β -diversity analysis based on the generalized UniFrac distance (gUniFrac, $\alpha = 0.5$) was performed (Chen et al., 2012). β -diversity allows the comparison and verification of compositional differences in gut microbiota between individuals, and non-metric multidimensional scaling (NMDS) indirectly provides a value for compositional differences within groups. Large difference in the taxonomic composition of the gut microbiota

between individuals within the same group is reflected in a wide dispersion of their coordinates on the NMDS plot. To quantify within-group dispersion, we calculated the mean and standard deviation of all pairwise gUniFrac distances within groups, and between-group significance was assessed using a *t*-test. All α - and β -diversity indices were calculated using the QIIME2 and vegan packages in R and visualized on the NMDS plot using the "Vegan" and "ggplot2" packages in R (Oksanen et al., 2007; Bolyen et al., 2019).

2.7 Predictive functional profiling based on taxon

To determine the predicted metabolic pathways corresponding to individual microbiota, a phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2) was performed based on the MetaCyc database (Krieger et al., 2004; Douglas et al., 2020). To assess significant differences in predicted functional pathway abundances between growth stages (Lactation-Weaning vs. Starter-Puppy stages), the Welch's *t*-test adjusted with the Benjamini-Hochberg false discovery rate (FDR) method was used (differences in mean proportion > 0.1 , *q*-value < 0.05).

2.8 Relative quantification of CPV-2

To quantify the infectious dose of CPV-2 in the metagenomic DNA of the eight dogs in groups CPV-low and CPV-high, we conducted quantitative real-time PCR (qRT-PCR) using the VP2 gene of CPV-2 as the gene of interest and the bacterial 16s rRNA as the endogenous control (Kumar and Nandi, 2010; Bacchetti De Gregoris et al., 2011). The VP2 gene was detected from the metagenomic DNA of the eight dogs in groups CPV-low and CPV-high at 14 weeks of age but not detected at 8, 11, 21, and 28 weeks of age. The VP2 gene was not detected from the metagenomic DNA of the six dogs in group CON at all ages.

Serial 2-fold dilutions of metagenomic DNA at concentrations of 1×2^0 ng/ μ L to 1×2^{-4} ng/ μ L were prepared in TE buffer to generate a standard curve of 16s rRNA and CPV-2. The assay showed linearity over the dilution range with R^2 values of 0.984 and 0.990 and reaction efficiencies of 103.3% and 108.6%, respectively. The qRT-PCR was performed using the PowerSYBR® Green PCR Master Mix (Applied Biosystems, Waltham, MA, United States) and the QuantStudio™ three Real-Time PCR System thermal cycler operated by QuantStudio™ Design and Analysis desktop software v1.5.0 (Thermo Fisher Scientific, Waltham, MA, United States). Each qRT-PCR was performed in a total volume of 50 μ L, containing 1 μ L template DNA, 1 pmol of each primer, 25 μ L 2X qPCR master mix, and 23 μ L nuclease-free water. The qRT-PCR conditions consisted of initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by melting curve analysis. Amplification and denaturation data were acquired for further analysis. All experiments were performed in triplicate. The relative quantity of CPV-2 was calculated using the value of ΔCt as follows: $\Delta Ct = Ct(\text{Gene of interest : CPV-2}) - Ct(\text{Endogenous control : 16s rRNA})$.

2.9 Statistical analyses

To investigate the association between alpha-diversity of gut microbiota and canine growth stages, we utilized linear mixed-effect model in R version 4.4.0. The dependent variable in our model was four alpha diversity indices. The independent variable was a categorical variable “weeks of age” and “growth stage,” respectively. To control for potential confounding factors, we used the littermate group and sex as fixed effects, and the individual puppy as a random effect to account for repeated measures within the same puppy. For post-hoc pairwise multiple comparison, the *p*-value was corrected by Bonferroni method.

To analyze the difference in taxonomic composition across growth stages, pairwise permutational multivariate ANOVA (PERMANOVA) with 999 random permutations using *adonis* function was performed. We performed Beta Dispersion analysis using the *betadis* function in R to validate the homogeneity of multivariate dispersion across groups. The results indicated significant differences in dispersion (ANOVA, $p < 0.001$), suggesting caution in interpreting PERMANOVA results. The littermate group and sex were included in the formula of *adonis* as covariates. For *post hoc* pairwise multiple comparison, the *p*-value was corrected using the Bonferroni method.

To robustly investigate the significant differences in the relative taxa abundance among growth group, we used two statistical tools—generalized linear models implemented in multivariate association with linear models (MaAsLin2) and analysis of composition of microbiomes with bias correction 2 (ANCOM-BC2). Zero imputation was not performed because both differential abundance analysis tools have built-in mechanisms to handle zero values without additional preprocessing. The dependent variable in our model was relative abundance of family-level taxa for MaAsLin2 and absolute abundance of family-level taxa for ANCOM-BC2. The independent variable was a categorical variable growth stage, with the lactation stage as a reference. To control for potential confounding factors, we used the stage and sex as fixed effects, and the littermate group as a random effect to account for repeated measures. For MaAsLin2, we used LOG transformation, minimum abundance of 0.001%, individual prevalence of 10%. For ANCOM-BC2, we used minimum abundance of 100 read counts, individual prevalence of 10%, and structure zero “TRUE” option. In addition, log-fold changes were obtained from a log-linear model and *p*-values were calculated using a two-sided Z-test for ANCOM-BC2 analysis. The *q*-value was calculated using the Benjamini-Hochberg method for multiple testing correction in both the MaAsLin2 and ANCOM-BC2 analyses.

We used the Kruskal-Wallis test for alpha diversity of microbial taxa between CPV-high, CPV-low, and CON group across age of weeks 8, 11, 14, 21, and 28. To investigate the significant differences in the relative taxa abundance associated with CPV-infection, we used generalized linear models implemented in MaAsLin2, adjusting for covariate sex. The dependent variable in our model was relative abundance of genus-level taxa. The independent variable was three CPV groups including CPV-high, CPV-low and CON groups. For MaAsLin2, we used LOG transformation, minimum abundance of 0.001%, individual prevalence of 10%. The *q*-value was calculated using the Benjamini-Hochberg method for multiple testing correction.

3 Results

3.1 Growth stage-associated changes in gut microbiota α -diversity in healthy military dogs

For all four α -diversity indices, the α -diversity was lowest during lactation and increased with growth to weaning, starter, and puppy (Figure 2a). The α -diversity showed significant differences across the four growth stages in the richness and Faith's PD indices (linear mixed-effect regression model, $p < 0.05$). For Shannon (ENS) index, no differences were observed between lactation and weaning. For Pielou's evenness, no differences were observed between lactation and weaning and between starter and puppy. According to the ages of weeks, the upward trend of α -diversity plateaued at approximately 14 weeks of age, with a similar α -diversity level maintained thereafter.

3.2 Growth stage-associated changes in gut microbiota β -diversity in healthy military dogs

We found high dispersion of individuals within the same age group on the NMDS plot during the lactation-weaning period (4–6 weeks), which decreased as dogs grew (Figure 2b). At 8–11 weeks, during the starter period, the dispersion degree continuously decreased in the NMDS plot. We quantitatively measured the change in gut microbiota composition using the gUniFrac distance (Figure 2c). The dispersion of individuals was significantly smaller at 14 weeks of age compared to other ages (0.24, $p < 0.05$, *t*-test) (puppy stage). From the lactation to the weaning stage (4–6 weeks), the average gUniFrac distance was 0.56. These were significantly higher than the average values of 0.24–0.37 (minimum to maximum), found after the starter stage (8–28 weeks) (*t*-test, $p < 0.05$). Additionally, gUniFrac pairwise distance of 4–6, 6–8 weeks of ages were significantly higher compared to that of 8–11, 11–14, 14–21, and 21–28 weeks of ages. In the microbial community difference analysis, no significant change was identified from four periods; 4–6, 8–11, 11–14, 21–28 weeks of ages (PERMANOVA, $p > 0.05$) (Supplementary Table 1). Except for four periods, significant differences were identified across the ages of weeks. Although PERMANOVA results revealed significant differences between groups, Beta Dispersion analysis showed heterogeneity in within-group dispersion, which may influence the interpretation of PERMANOVA results.

3.3 Changes in the gut microbiota taxonomic composition during the growth of healthy military dogs: phylum- and family-level analyses

The five major phyla with high average relative abundance in canine microbiota, from the lactation to the puppy stage, were Bacillota (avg. 49.89%), Bacteroidota (avg. 29.38%), Fusobacteriota (avg. 11.88%), Pseudomonadota (avg. 6.27%), and Actinomycetota

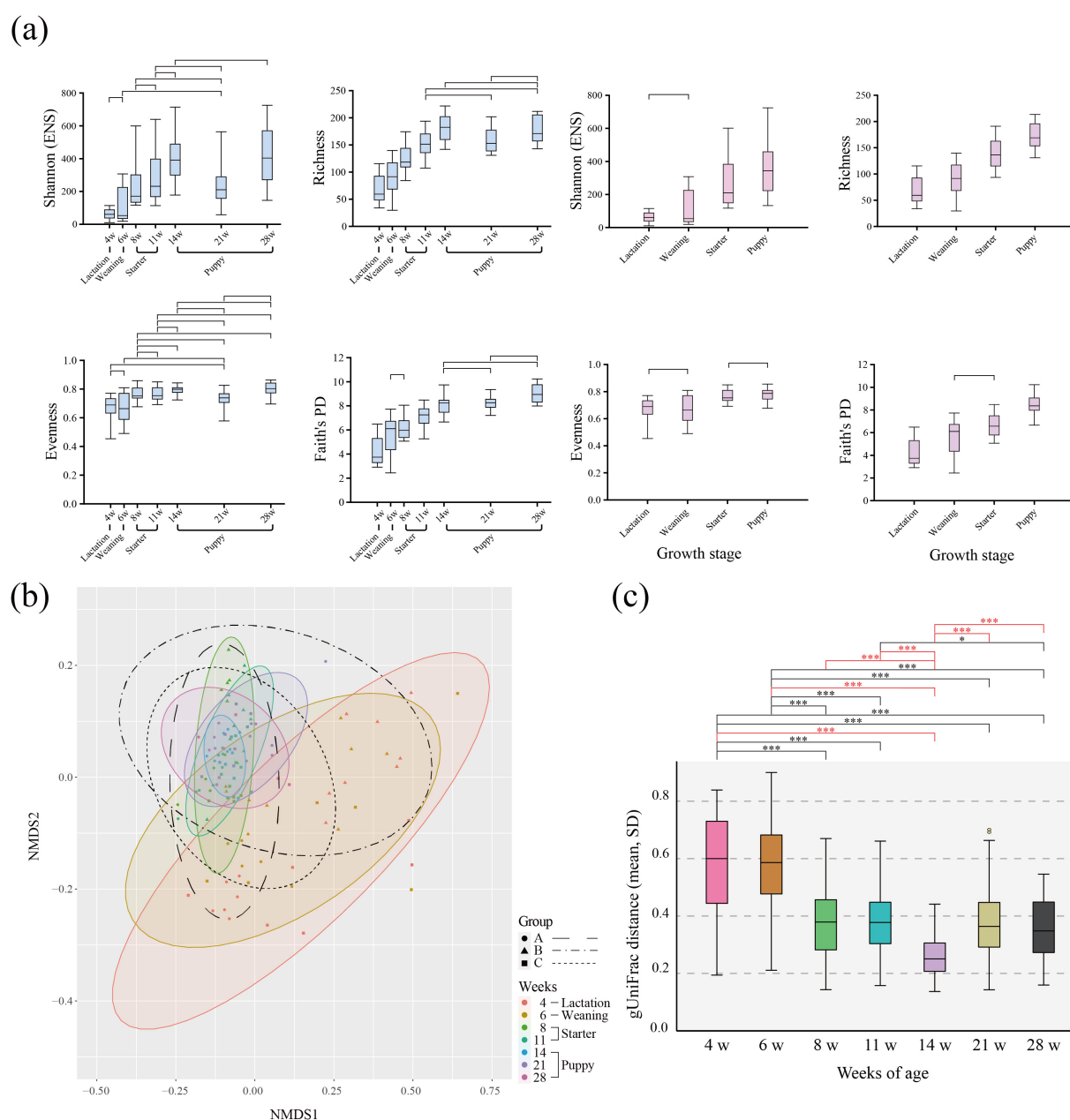
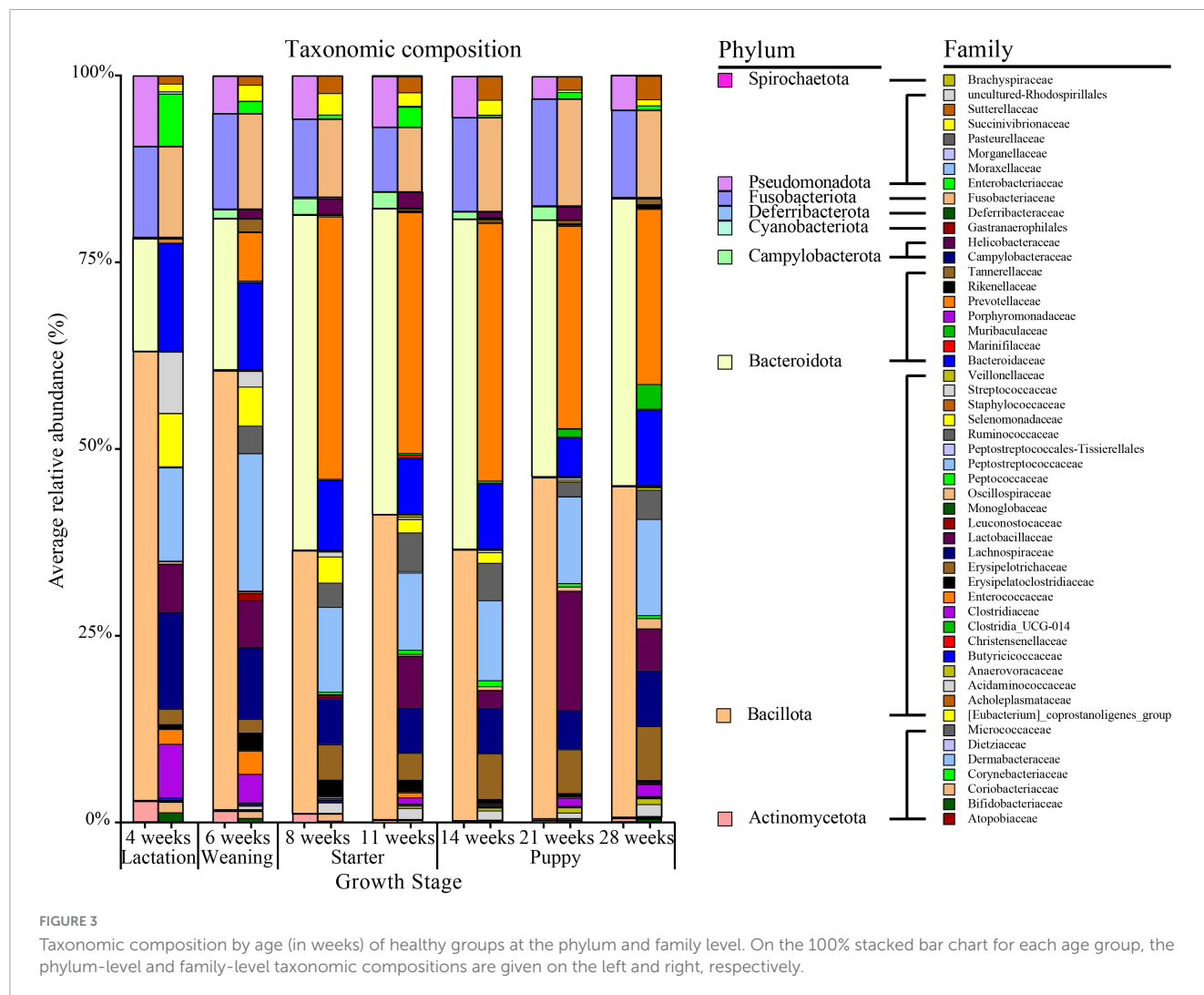


FIGURE 2

α - and β -diversity of gut microbiota according to the age (in weeks) of healthy 116 individual military dogs. **(a)** Box-and-whiskers plots represent the inter-quartile range and minimum to maximum values of α -diversity [Shannon (ENS), Richness, Pielou's evenness, Faith's PD] for age and dietary stages. To verify statistical significance, the linear mixed effect model adjusted by Bonferroni correction was performed. Only non-significant (adjusted p -value ≥ 0.05) pair of weeks of ages were marked on box plot. **(b)** Non-metric multidimensional scaling (NMDS) plot using the generalized UniFrac distance matrix based on relative abundance. Color-shaded ellipses indicate 95% confidence intervals for the multivariate t -distribution around the centroids of the groupings, with the number of weeks as a factor. An ellipse with a black border indicates 95% confidence intervals for each group; each group is denoted by a different shape. Group A: circle, dashed line, Group B: triangle, dash-single dotted line, Group C: square, dotted line. **(c)** Generalized UniFrac (gUniFrac) pairwise distance to the growth stage of each individual. Significance was calculated using the non-parametric Kruskal-Wallis test, adjusted by Bonferroni correction. p -value < 0.05 , $** < 0.01$, $*** < 0.001$.

(avg. 1.38%) (Figure 3 and Supplementary Figure 2). The average relative abundance of Bacillota was 60.23% during the lactation stage (4 weeks) but decreased to 42.23% at the puppy stage (14–28 weeks). The Bacteroidota accounted for 15.12% and 20.35% of the taxa during the lactation (4 weeks) and weaning (6 weeks) stages, respectively, and nearly doubled to 40% after the starter stage (11 weeks), which was sustained until the

puppy stage (28 weeks, 40%). The Bacillota/Bacteroidota (B/B) ratio was 3.98 and 2.90 in the lactation and weaning stages, respectively, decreasing to 0.89 and 1.08 in the starter and puppy stages, respectively. The Actinomycetota showed average relative abundances of 2.80%, 1.55%, and 1.19% at 4, 6, and 8 weeks of age, respectively, decreasing to less than 0.5% on average at the starter stage (11 weeks). Two phyla, Fusobacteriota and Pseudomonadota,



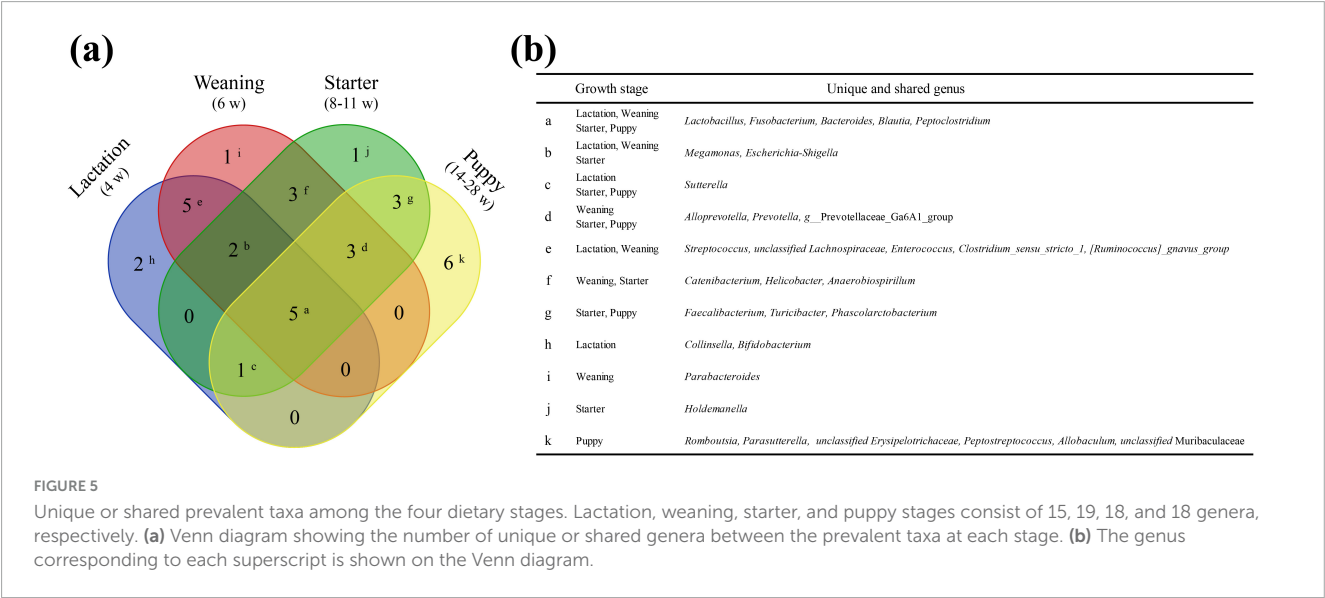
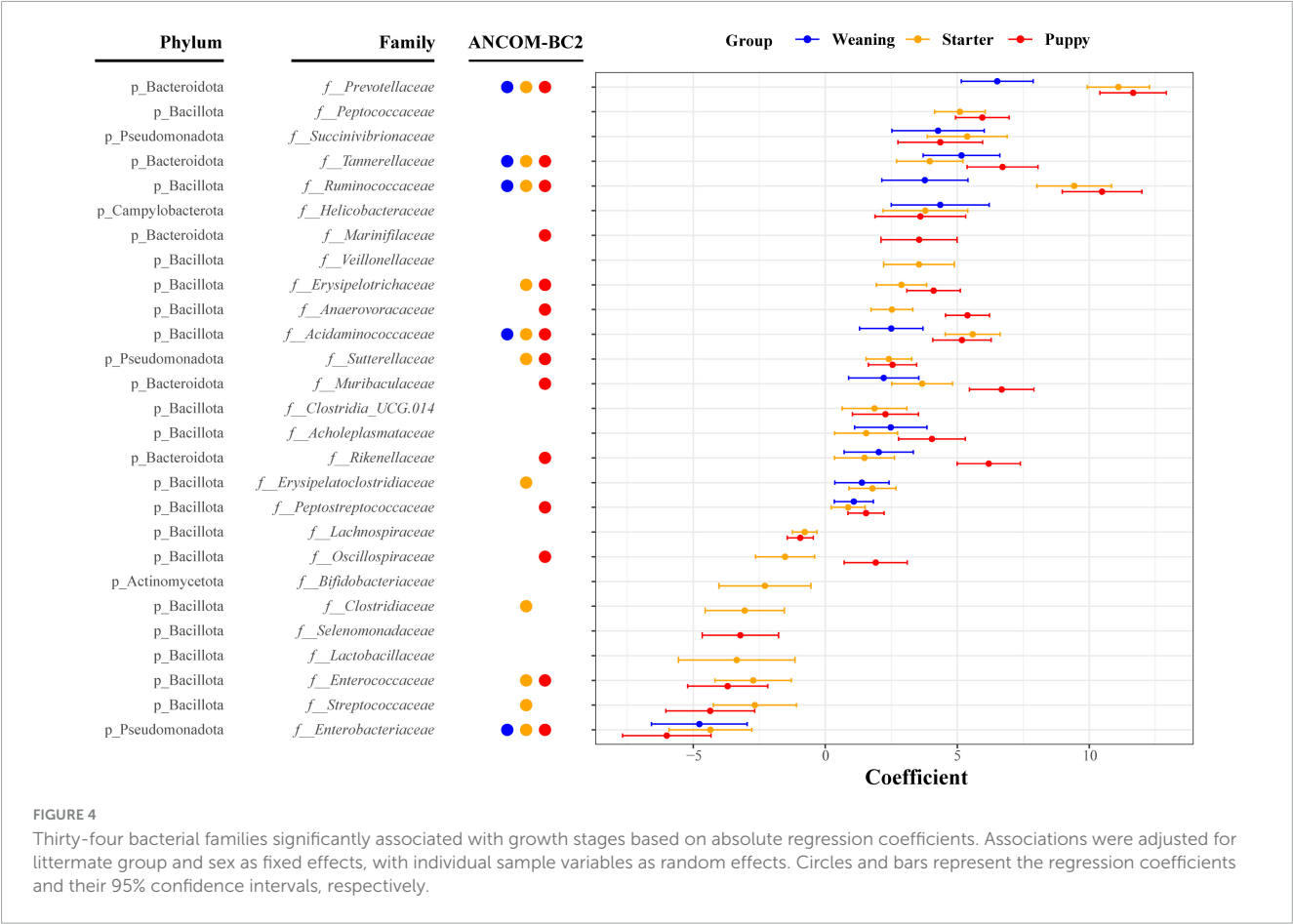
maintained a constant relative abundance regardless of the growth stage.

In the differential relative abundance analysis with the reference of lactation stage, we identified 27 families which are significantly associated with the growth stages (Linear regression model, $q < 0.05$) (Figure 4). Overall, weaning, starter, and puppy stages were associated with a similar set of families, with regression coefficients showing an increasing pattern as dogs grew from weaning to puppy stages. Amongst the largest positive coefficients, the coefficient of *Prevotellaceae* was 6.5 for weaning, 11.1 for starter, and 11.7 for puppy stage. The coefficient of *Ruminococcaceae* was 3.8 for weaning stage, 9.4 for starter, and 10.5 for puppy. The coefficient of *Muribaculaceae* was 2.2 for weaning, 3.7 for starter, and 6.7 for puppy stage. Amongst the largest negative coefficients, the coefficient for *Enterobacteriaceae* was -4.8 for weaning, -4.3 for starter, and -6.0 for puppy stage. The coefficient for *Enterococcaceae* was -2.3 for starter and -3.7 for puppy stage. Among 27 differently abundant families identified by MaAsLin2 analysis, the significances of 17 families remained robust in the ANCOM-BC2 analysis. Specifically, the absolute abundances of five families—*Prevotellaceae*, *Tannerellaceae*, *Ruminococcaceae*, *Acidaminococcaceae*, and *Enterobacteriaceae*—were significantly

different in the weaning, starter, and puppy stages compared to the lactation stage. In contrast, the absolute abundances of 12 families, including *Enterococcaceae*, *Sutterellaceae*, and *Peptostreptococcaceae*, were significantly different in the starter and puppy stages compared to the lactation stage.

3.4 Changes in the gut microbiota taxonomic composition during the growth of healthy military dogs: prevalent taxa at the genus level

We annotated 114 genera from 116 healthy individuals, of which 32 were selected as prevalent taxa genera based on the relevant criteria (individual prevalence $> 50\%$, average relative abundance $> 1\%$). The five core genera identified from all four stages were *Fusobacterium* (avg. 11.57%), *Peptoclostridium* (avg. 11.01%), *Bacteroides* (avg. 10.03%), *Lactobacillus* (avg. 5.91%), and *Blautia* (avg. 4.37%) (Figure 5). For each growth stage (lactation, weaning, starter, and puppy stages), 15 (91.29%), 19 (86.98%), 18 (89.86%), and 18 (86.28%) prevalent taxa genera were identified.



3.5 Changes in lactic acid bacteria (LAB) relative abundance and diversity during growth

To analyze the changes in LAB composition in the canine gut microbiota, we evaluated the relative abundance of 13 LAB genera, namely *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*,

Weissella, *Leuconostoc*, *Vagococcus*, *Tetragonococcus*, *Pediococcus*, *Oenosoccus*, *Carnobacterium*, *Aerococcus*, and *Bifidobacterium*, at the different growth stages (Supplementary Figure 3). Among the 13 genera, seven genera (*Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Weissella*, *Leuconostoc*, and *Bifidobacterium*) were identified in the gut microbiota of military dogs. The LAB relative abundance in the gut microbiota was the highest in the

lactation stage (17.99%) with high abundance of *Streptococcus* and *Bifidobacterium*, during which the dogs were breastfed. As dogs grew, the LAB relative abundance decreased to an average value of 4.81% at the starter stage and 8.57% at the puppy stage (14–28 weeks). There was a sharp decline in the relative abundance of LAB to 1.69% at 8 weeks (initiation of starter stage) and to 2.97% at 14 weeks (initiation of puppy stage). Both decline valleys were partially recovered after stage adaptation. At the puppy stage, *Leuconostoc* were not identified. Notably, a higher relative abundance of the genera *Streptococcus*, *Enterococcus*, and *Bifidobacterium* was identified in the lactation (11.15%) and weaning (5.83%) stages than in the starter (0.95%) and puppy (0.55%) stages.

3.6 *Enterococcus faecalis* strain-level dynamics

Next, we conducted rep-PCR-based molecular epidemiologic analysis for 81 *E. faecalis* isolates from lactation- ($n = 15$), weaning- ($n = 16$), starter- ($n = 28$), and puppy- ($n = 22$) stage dogs. In total, 26 rep-PCR band patterns were identified and grouped into nine cluster types (Cluster I–IX, criteria: 80% band pattern similarity). In the lactation stage, five rep-PCR cluster types (Cluster I, II, IV, VII, and IX) were identified, which were not shared between groups A, B, and C (Supplementary Figure 4). Meanwhile, in the weaning/starter stage, 5–6 cluster types (Cluster I, II, VI, VII, IX and Cluster I, II, III, VII, VIII, IX) were identified, with Cluster I as the predominant type in all three groups. At the puppy stage, three cluster types (Cluster I, III, V) were identified, with Cluster I being the predominant type in both cases among the three groups.

By comparing the diversity of *E. faecalis* rep-PCR patterns for the four growth stages, we confirmed that the strain diversity [Shannon (e^H) and Simpson ($1/D$)] of *E. faecalis* decreased with growth (Supplementary Figure 4). The *E. faecalis* strain diversity was the highest at the lactation stage (4 weeks of age, $e^H = 4.3038$, $1/D = 4.7733$) and lowest at the puppy stage (14–28 weeks of age, $e^H = 1.7796$, $1/D = 1.4808$). As the military dogs grew, the diversity changes of *E. faecalis* strains based on the rep-PCR showed decreasing pattern with the gUniFrac distances within group (Supplementary Figure 4).

3.7 Different relatively dominant functions of microbiota during growth: predictive functional profiling based on taxonomic composition

The relatively dominant functions in two groups, lactation-weaning stages and starter-puppy stages, were predicted using PICRUSt2 (Figure 6). Twenty-five pathways with significant differences ($q < 0.05$) were identified between the two groups. Ten of these pathways, including sugar degradation (lactose and galactose), proteinogenic amino acid (L-methionine, L-alanine) biosynthesis, and cell wall (peptidoglycan) biosynthesis, were significantly higher in the lactation-weaning group than in the starter-puppy group. The other 15 pathways, including fatty acid biosynthesis, lipopolysaccharide biosynthesis, and enzyme cofactor

(vitamin) biosynthesis, were predicted to be relatively higher in the starter-puppy group than in the lactation-weaning group.

3.8 Gut microbiota at early life stages against CPV-2 infection: α - and β -diversity analysis

The gut microbiota of healthy dogs (group CON) was compared with that of dogs accidentally infected with CPV-2 at around 13 weeks of age (group CPV-low and CPV-high). At 8 and 11 weeks of age, before CPV-2 infection, α -diversity indices showed only slight differences between groups (Figure 7a). At 14 weeks of age, one week after CPV-2 infection, the α -diversity index of group CPV-high noticeably decreased compared to that of group CON, whereas that of group CPV-low was similar to that of group CON. At 21 weeks of age, 8 weeks after CPV-2 infection, the diversity of group CPV-low regarding Shannon, Faith's PD and richness α -diversity indices decreased sharply, while that of group CON was similar to the values at 14 weeks of age, i.e., without decrease (Figure 7a). At 28 weeks of age, 15 weeks after CPV-2 infection, the α -diversity indices of individuals in group CPV-low were partially restored to a level comparable to that of group CON.

In the NMDS plot showing the β -diversity index, there were no notable differences in the distribution of individuals between the CPV-high, CPV-low, and CON groups at weeks 8 and 11 (Figures 7b, c). However, at week 14, 1 week after CPV-2 infection, a higher gUniFrac distance was observed in dogs in group CPV-high compared to that of groups CON and CPV-low. The gUniFrac distance of the paired growth ages were used to quantify the changes in β -diversity due to CPV-2 infection (Figure 7c). Except for the 11–14 w average pairwise distance (0.69 ± 0.15 , 95% CI) of the group CPV-high, all pairwise distances were 0.31–0.42.

3.9 Recovering the potential of gut microbiota at early life stages against CPV-2 infection: taxonomic composition analysis

At 8 and 11 weeks of age, the taxonomic composition at the phylum level was similar in all the individuals from the CPV-low, CPV-high, and CON groups (Figure 8). However, at 14 weeks, after CPV-2 infection, the taxonomic composition of CPV-high was notably different from that of group CON. The taxonomic composition of CPV-low also changed compared to that of group CON but to a lesser extent than between group CPV-high and group CON.

The relative abundance of four of the major phyla in canine gut microbiota, namely Bacillota, Bacteroidota, Campylobacterota, and Pseudomonadota, was significantly changed after CPV-2 infection. The relative abundance of Campylobacterota and Pseudomonadota notably increased in the CPV-2-infected groups (avg. 0.3%–23.10%, 2.96%–22.75%), whereas that of Bacillota and Bacteroidota decreased (avg. 47.38%–20.56%, 46.98%–21.65%). According to the genus-level analysis, there was a notable inconsistency in taxonomic composition among littermates compared to that in

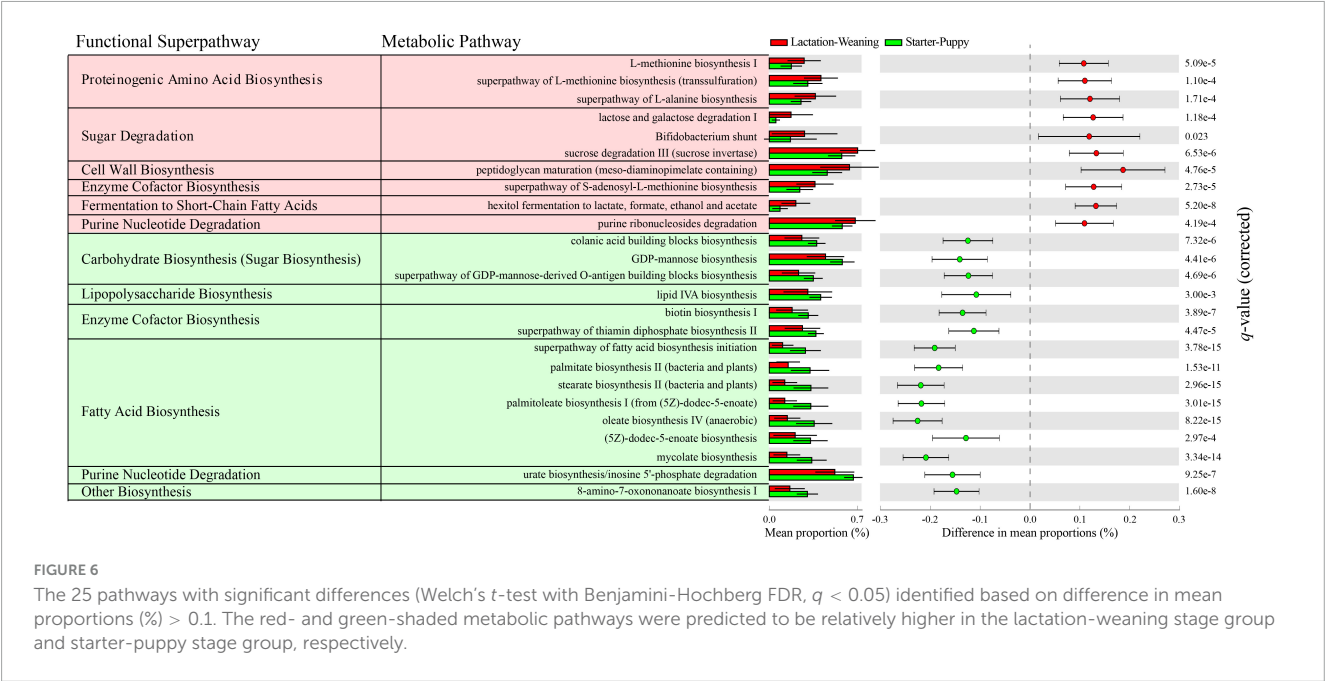


FIGURE 6 The 25 pathways with significant differences (Welch's *t*-test with Benjamini-Hochberg FDR, $q < 0.05$) identified based on difference in mean proportions (%) > 0.1. The red- and green-shaded metabolic pathways were predicted to be relatively higher in the lactation-weaning stage group and starter-puppy stage group, respectively.

the control group at 14 weeks of age (Supplementary Figure 5). Dysbiosis was particularly pronounced in the CPV-high group at 14 weeks old.

In the differential relative abundance analysis with the reference of CON group, we identified seven genus which are significantly associated with the CPV-high group (Linear regression model, $p < 0.05$, Table 1). No significantly differential genus was identified between CPV-low and CON groups. The relative abundance of genus *Helicobacter* was significantly higher in CPV-high group compared to CON group (regression coefficient: 8.59, linear regression model, $p < 0.001$). In contrast, the relative abundance of genus *Faecalibacterium* was significantly lower in CPV-high group than CON group (regression coefficient: -6.01, linear regression model, $p < 0.001$). The relative abundance of genus *Turicibacter* was significantly lower in CPV-high group than CON group (regression coefficient: -6.40, linear regression model, $p < 0.001$). At 21 and 28 weeks of age, the taxonomic composition of the group CPV-low was comparable to that of group CON.

4 Discussion

The gut microbiota is associated with the health status of the host, and microbial dysbiosis could be an important risk factor for various diseases, including metabolic and immune disorders (Carding et al., 2015; Li et al., 2017; Dogra et al., 2020). A well-established gut microbiota at the early life stages may play an important role in the host's ability to resist disease (Kelsen and Wu, 2012). This study tracked the canine gut microbiota dynamics at early life stages against several factors, including growth, diet, external environmental exposure, and CPV-2 infection, in a birth cohort of military dogs born and raised in a controlled training environment. As the gut microbiota can be highly influenced by external factors and controlling confounding factors is essential for its thorough research (Reese and Dunn, 2018), the birth cohort

study design may provide a suitable model for studying canine gut microbiota at early life stages. To our knowledge, this is the first study to analyze canine gut microbiota by 16s rRNA amplicon sequencing-based analysis with molecular epidemiology of *E. faecalis* during the early life of military dogs in a training environment.

Understanding individual taxonomic diversity and inter-individual variation could be the cornerstones for determining the stabilization status of the gut microbiota (Schmitz and Suchodolski, 2016). Here, the gut microbiota taxonomic diversity, i.e., α -diversity, increased during the lactation to starter stages and stagnated at around 14 weeks of age (puppy stage). The gut microbiota of mammals, including humans, increases in α -diversity from birth (Derrien et al., 2019). α -diversity increases plateau at certain time points during growth, and these stagnated points are used as a criterion for gut microbiome stabilization (Yassour et al., 2016). The plateaued state of individual α -diversity at the puppy stage (14 weeks) identified here implies that the individual taxonomic diversity in the gut microbiota of military dogs could be stabilized at this stage.

In the analysis of gut microbiota diversity between individuals, the highest β -diversity dispersion was observed in military dogs at the lactation and weaning stages (4–6 weeks of age). The gut microbiota of human infants is established primarily through the transmission of the mother's gut, skin, fecal, vaginal, and breast milk microbiota (Palmer et al., 2007; Cong et al., 2016). As the naive intestinal environment of infants cannot provide adequate buffering capacity for external microorganisms, the gut microbiota received from their mothers may settle randomly and certain microorganisms may become predominant (Zakosek Pipan et al., 2020). This allows for a high diversity of microbial compositions between individuals even under the same conditions, which could be a potential cause for the high β -diversity at the lactation and weaning stages of military dogs.

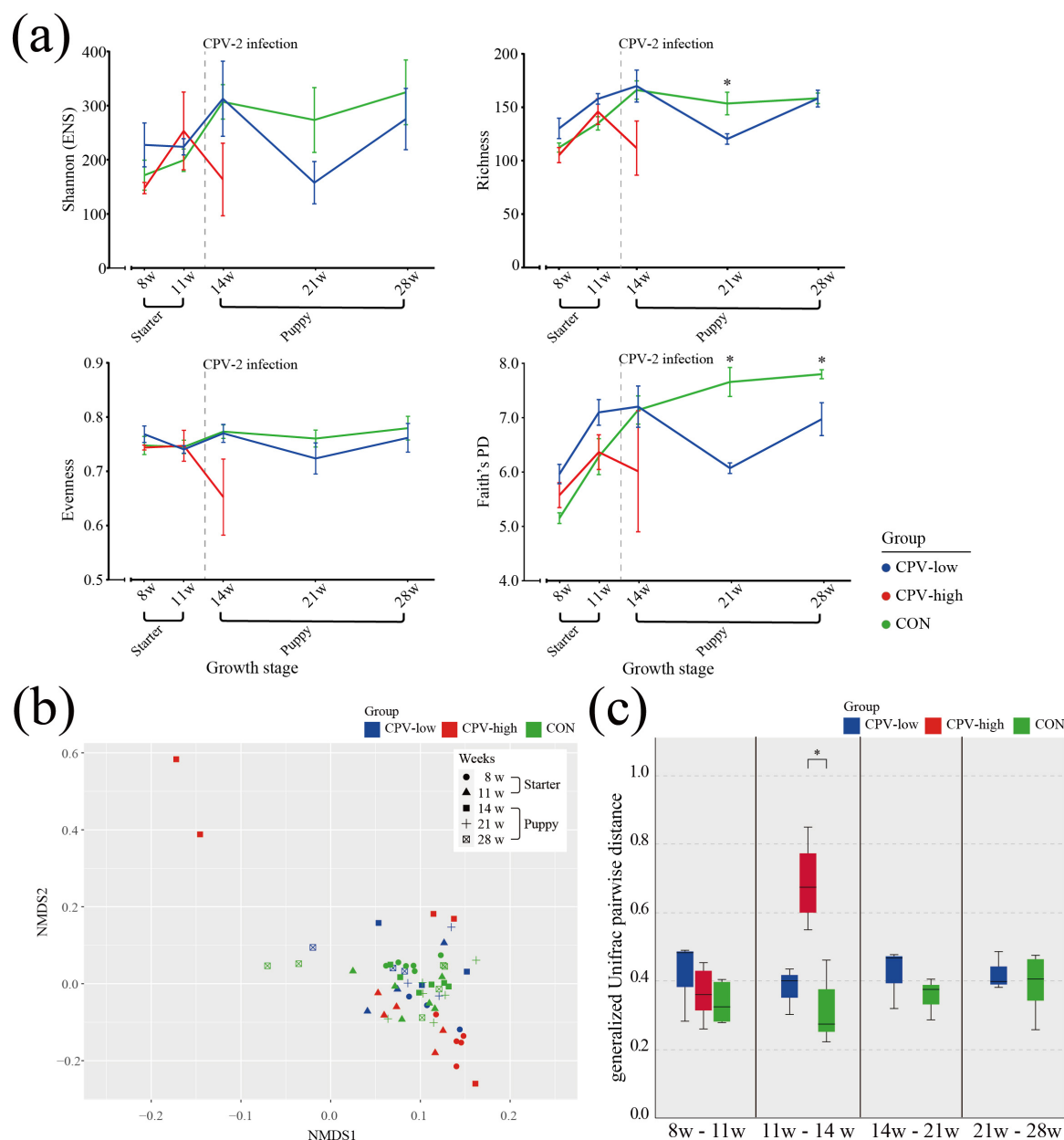


FIGURE 7

α - and β -diversity in the CPV-2 infection and healthy control dogs. **(a)** α -diversity indices, including Shannon (ENS), Richness, Pielou's evenness, and Faith's PD index, from 8 to 28 weeks of age. Each color represents a group; Blue: Canine parvovirus (CPV)-low, Red: CPV-high, Green: CON. Error bars show the standard error of the mean (SEM). Asterisk (*) indicates significance of differences between the control group (CON) and group CPV-low. Significance was calculated using non-parametric Kruskal-Wallis test. **(b)** Non-metric multidimensional scaling (NMS2) plot of CPV-low, CPV-high, and CON groups at 8 and 11 weeks of age before CPV-2 infection, and at 14, 21, and 28 weeks after infection. The plot was obtained based on the generalized UniFrac distance matrix. Color refers to the group (Blue: CPV-low, Red: CPV-high, Green: CON) and the shape of symbols refers to weeks of age (Circle: 8, Triangle: 11, Square: 14, Cross: 21, Squared cross mark: 28). **(c)** Pairwise distance between 11 and 14 weeks of age on the NMS2 plots based on the generalized UniFrac distance matrix of the paired samples of CPV-low, CPV-high, and CON.

During the starter stage (8–11 weeks of age), β -diversity was decreased compared to that at the lactation and weaning stages. We previously reported that a commercial diet may lower the β -diversity of canine gut microbiota (Kim et al., 2017). However, increased exposure to the external environment increases gut microbiota β -diversity (Tasnim et al., 2017). Here, meanwhile, the β -diversity of starter dogs with increased exposure to the external environment, including through play in the backyard, was lower than that of lactating and weaning dogs living exclusively

indoors. Therefore, our results suggest that dietary changes may play a more significant role than housing environmental changes in expanding the gut microbiota diversity. Similarly, Carmody et al. (2015) has shown that major dietary modifications induce more pronounced shifts in gut microbiota composition in mice compared to housing environmental changes. In contrast, husbandry or environmental differences, such as variations in cage bedding or standard chow formulations, tend to produce only subtle or transient microbiota changes (Ericsson and Franklin,

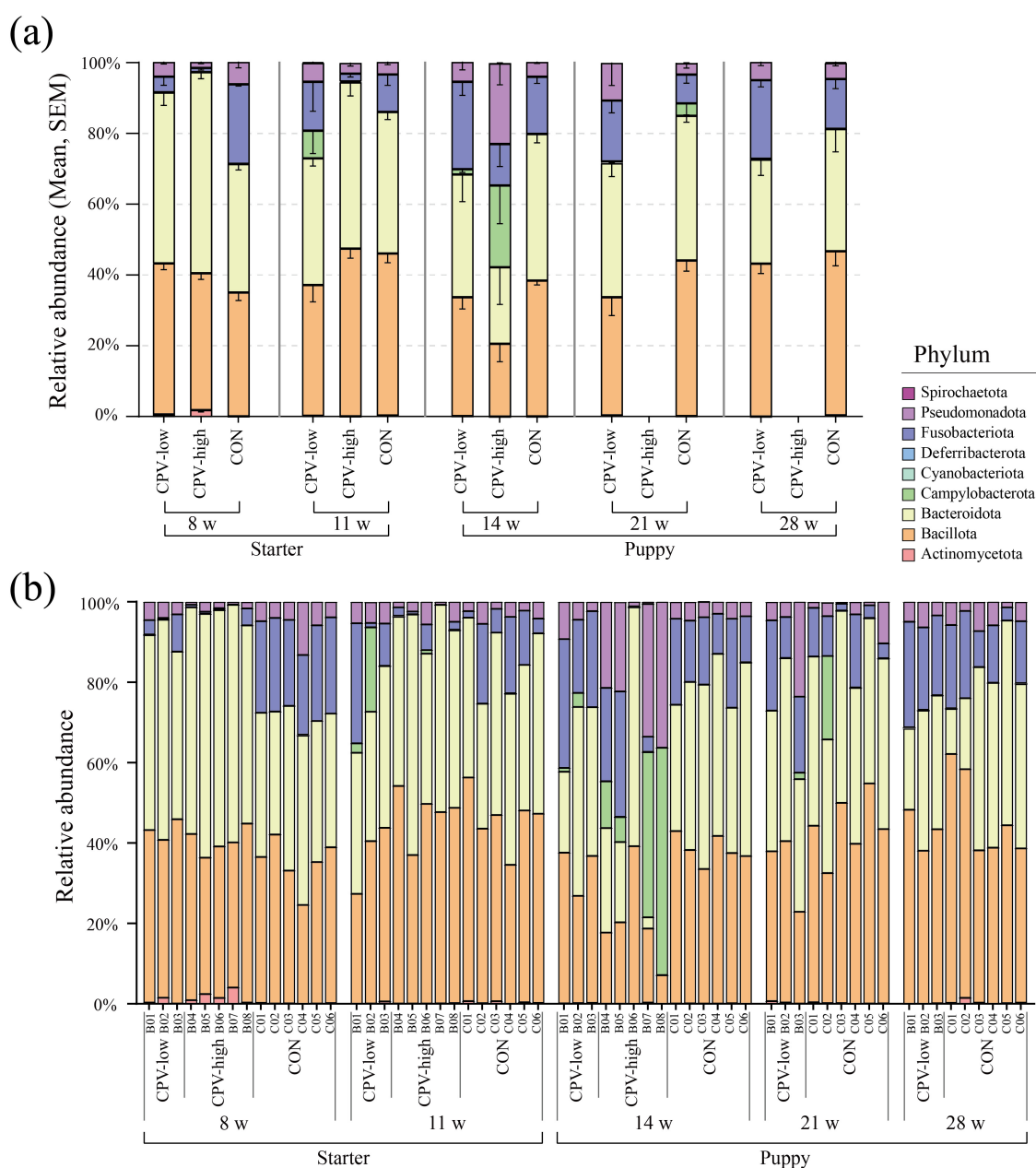


FIGURE 8

Taxonomic composition at the phylum level in Canine parvovirus-2 (CPV-2) infected and control (CON) groups. **(a)** Average relative abundance of phylum in samples used for CPV-2 infection group analysis. **(b)** Taxonomic composition for each individual sample. Each color in the bar chart represents a mean relative abundance of phylum, and the error bar represents the SEM. Only the CPV-low and CON groups were described at 21 and 28 weeks of age because all puppies in group CPV-high died before 21 weeks of age.

2021). Taken together, dietary exert a dominant influence on gut microbiota diversity.

The β -diversity dispersion was the lowest at 14 weeks of age (puppy stage). Although the diet changed from a starter commercial diet to a puppy commercial diet between 11 and 14 weeks of age, dispersion of β -diversity decreased during this period rather than increasing. This suggests that, despite the change in diet, the overall composition of the main nutrients in the two diets remained largely similar, which may explain the observed decrease in dispersion of β -diversity at 14 weeks. Collectively, the increase in α -diversity, which plateaued at 14 weeks of age

(the puppy stage), and the low β -diversity dispersion pattern at week 14 suggest that gut microbiota matures at this age and it is characterized by the homogeneity of the taxonomic composition among individuals of the same age group. Hence, the gut microbiota may have stabilized at 14 weeks, i.e., at the early life stages of military dogs. Moreover, the slight increase that followed during the puppy stage (14–28 weeks) was possibly due to increased exposure to several environmental factors with the initiation of outdoor training activities, including reconnaissance, tracking, and obstacle detection.

TABLE 1 Differentially abundant bacterial genus in canine parvovirus-2 high-dose infection group (CPV-high) and control group (CON).

Bacterial genus	Abundance (%) ^a		MaAsLin2 ^b		
	CPV-high (n = 5)	CON (n = 6)	Coefficient (SE)	P-value	q-value
<i>g_Helicobacter</i>	22.66	0.03	8.59 (1.93)	0.001**	0.04*
<i>g_Peptococcus</i>	0.20	0.96	−2.36 (0.51)	< 0.001***	0.04*
<i>g_Holdemana</i>	0.21	1.66	−3.06 (0.65)	< 0.001***	0.04*
<i>g_Tyzerella</i>	0.02	0.21	−4.33 (0.80)	< 0.001***	0.03*
<i>g_Erysipelatoclostridium</i>	0.11	0.45	−5.04 (1.11)	< 0.001***	0.04*
<i>g_Faecalibacterium</i>	0.46	7.23	−6.01 (1.16)	< 0.001***	0.03*
<i>g_Turicibacter</i>	0.06	2.07	−6.4 (1.05)	< 0.001***	0.03*

^aRelative abundance of bacterial genus in CPV-high and CON groups. ^bP-values from the generalized linear model using MaAsLin on pairwise testing between two groups. q-values were calculated using the Benjamini-Hochberg method for multiple testing correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. SE, standard error.

The *E. faecalis* genetic diversity changes obtained through the culture-based method, interestingly, showed a similar β -diversity change pattern according to the growth stages (positive correlation, Spearman's correlation test, $P < 0.05$). Most single bacterial species constituting the gut microflora consist of a single dominant strain accounting for more than 80% of the population (Truong et al., 2017). Additionally, the strain-level diversity is strongly influenced by the host's geographic distribution (Yan et al., 2020). Previous reports and our results suggest that the gut microbiota shares taxonomic composition between individuals and the dominant strains that occur during stabilization throughout the early life stages of dogs. Introduction and elimination of new strains or strain-level dominance investigation are extremely difficult using 16s rRNA-based taxonomic analysis. Therefore, genome-based studies supported by culture-based studies may provide a better understanding of microbiota changes.

Rep-PCR clusters of *E. faecalis* were not shared between dogs during the lactation stage. However, as dogs grew, rep-PCR clusters of *E. faecalis* were shared between dogs, which implied decreased individual gut microbiota variation. As military dogs grew, the diversity of *E. faecalis* strains decreased, and the shared strain composition between individuals increased. *E. faecalis* is a species found in all environments, including soil, water, animals, and plants (Byappanahalli et al., 2012). Therefore, the *E. faecalis* present in military dogs can spread between the groups through various environments, including the shared playground soil and training tools. Additionally, some clusters were temporarily detected only at specific growth stages and in the littermate group (e.g., cluster IV at the lactation stage in group A). The *E. faecalis* strain belonging to this cluster may have been introduced from the external environment and is a transient member of the gut microbiota.

In a comparative analysis of relative and absolute taxa abundance across different growth stages using MaAsLin2 and ANCOM-BC2, it was found that as the military dogs grew, there was a significant increase in the abundance of Bacteroidota, particularly *Prevotellaceae*. *Prevotellaceae* is a member of the short-chain fatty acid (SCFA) producers known for maintaining intestinal health through its anti-inflammatory effects (Bedarf et al., 2017). Additionally, the absolute abundance of *Ruminococcaceae*, another SCFA producer, significantly increased. SCFAs contribute to intestinal health by exerting anti-inflammatory effects, promoting mucus production, and maintaining intestinal barrier integrity

(Silva et al., 2020). Thus, increased SCFA production indicates enhanced health and stability of the gut microbiota. Conversely, there was a significant decrease in the absolute abundance of *Enterobacteriaceae* with, a family closely associated with dysbiosis, which reduces gut microbiota diversity and stability (Baldelli et al., 2021). Although *Enterobacteriaceae* are linked to dysbiosis, they colonize the intestines early, particularly in newborns, by depleting oxygen in the intestine during the early period of high oxygen concentration (Arrieta et al., 2014). This aids in the colonization of strictly anaerobic bacteria. Therefore, the presence of high proportions of Pseudomonadota, particularly *Enterobacteriaceae*, during early growth can be assumed to be a step in developing and stabilizing a healthy gut microbiota.

The gut microbiota has a fluid structure that is constantly changing through the interactions between constituent microorganisms, some of which change in a correlated relationship (Stubbendieck et al., 2016). Understanding specific genus changes and their correlations in the gut microbiota environment facilitates the understanding of the gut microbiota function, ecology, and physiology. Lactose-degrading LAB such as *Streptococcus*, *Enterococcus*, *Lactobacillus* and *Bifidobacterium*, was higher in the lactation and weaning stages than in the starter and puppy stages. *Lactobacillus* are a representative LAB known as a probiotic that benefits humans and other animals (Douillard and De Vos, 2019). *Lactobacillus* may be an alternative to antibiotics and help the host acquire nutrients and stimulate the immune system against harmful microorganisms (Vieco-Saiz et al., 2019). Lactose, the main component of breast milk, is an essential nutrient for infant growth; however, its excessive accumulation in the intestine can cause osmotic diarrhea and growth retardation (Heyman, 2000). The high LAB richness and their evenness during the lactation and weaning stages appears to aid growth by breaking down and absorbing the lactose delivered from the mother's breast milk. However, LAB evenness and relative abundance decrease as dogs grow into the starter and puppy stages. Interestingly, sharp decline valleys in LAB abundance were observed with dietary changes at 8 weeks (introducing phase of commercial starter feeding only) and 14 weeks (introducing phase of commercial puppy feeding), with partial recovery after stage adaptation.

As LAB may have a role in preventing the colonization of pathogenic and/or opportunistic bacteria in gut microbiota (Vieco-Saiz et al., 2019), the sharp declines in LAB abundance and genetic diversity at dietary transition observed here suggest a

potential risk for gut microbial dysbiosis such as diarrhea. The administration of LAB helps ameliorate gut dysbiosis (Heyman, 2000). Perturbation of the gut microbiota is one of the major causes of growth retardation (Robertson et al., 2019; Vieco-Saiz et al., 2019; Ronan et al., 2021). Our results suggest that the period of growth transition in early life stages may serve as an appropriate time point to provide supplemental therapy (e.g., probiotics) for LAB perturbation control.

Most of the predictive metabolic pathways identified as being relatively high in the lactation-weaning stage group were related to peptidoglycan biosynthesis, proteinogenic amino acid biosynthesis, and sugar degradation. Peptidoglycan and proteinogenic amino acids are essential factors for the multiplication and growth of microorganisms, and sugar degradation could provide resources for microorganisms (Egan et al., 2017; Kaznadzey et al., 2017). The increasing gut microbiota diversity and abundance in canine early life stages is accompanied by microbiota proliferation, with increasing demand for essential elements, including peptidoglycan and proteins. A lack of biosynthesis of peptidoglycan, an essential component of the bacterial cell wall, can negatively affect gut microbiota maturation by weakening the bacterial cell wall and causing bacterial cell lysis (Lovering et al., 2012). The biosynthesis of proteinogenic amino acids, including L-methionine and L-alanine, provides an essential source for synthesizing proteins necessary for bacterial growth (Aliashkevich et al., 2018). In the predictive functional analysis of starter-puppy stages, the major predicted functions were the metabolism of nucleotides, unsaturated fatty acids, and vitamins. Unsaturated fatty acids, such as oleic acid, have an antibacterial action (Zheng et al., 2005), and lipid IV_a lipopolysaccharide evades the host immune system (Steimle et al., 2016). Synthesis of vitamin B, including biotin and thiamine, which are the main pathways at these stages, plays an important effect in gut microbiota composition by providing micronutrients (Yoshii et al., 2019). Our results suggest that the gut microbiota functions were mainly those contributing to increase microorganism diversity and richness and gut microbiota maturation during the lactation and weaning stages. Functions for maintaining the gut microbiota structure predominated in the starter-puppy stages, in which the gut microbiota was already stabilized. The function of maintaining the stability of gut-microbiota may also help with its resilience against microbial perturbation and dysbiosis, and further enhance host immunity through a healthy gut microbiota.

Canine parvovirus-2 infection, the most common cause of viral enteritis in dogs, occurs frequently in puppies younger than 6 months of age (Macintire and Smith-Carr, 1997; Silverstein and Hopper, 2009). The pathogenesis of CPV-2 infection mainly includes the destruction of the crypt epithelial cells of the intestine (Hall, 2012). The destruction of intestinal structures such as crypts and villi, where gut microbiota mainly colonizes, leads to significant changes in the gut microbiota composition. Here, we analyzed the recovering potential of gut microbiota by comparing the gut microbiota of a healthy group (group CON) with that of dogs accidentally infected with CPV-2 at 14 weeks of age (group CPV-low and group CPV-high). In detail, the CPV group was subdivided based on their CPV-2 infectious dose compared with 16S rRNA (Supplementary Figure 1). Additionally, the CPV-high group was excluded from the analysis at 21 and 28 weeks of age due to the death of dogs.

Our results suggested that CPV-2 infection may serve as a potential risk factor for gut microbiota dysbiosis, altering the α - and β -diversity and the taxonomic composition at the early phase of infection. Further, the degree of gut microbiota dysbiosis was correlated with the CPV-2 infection dose. Group CPV-high showed significant differences in α - and β -diversity and the taxonomic composition of gut microbiota when compared to group CON. Group CPV-low showed no significant differences in α - and β -diversity relative to group CON but there was a marked change in taxonomic composition, although to a lesser extent than that in group CPV-high. Compared to group CON, an increase in the relative abundance of Pseudomonadota was observed in the composition of gut microbiota in group CPV-high and CPV-low. Pseudomonadota include representative pathogenic genera such as *Escherichia*, *Salmonella*, and *Vibrio* (Mukhopadhyay et al., 2012). *Helicobacter*, are normal indigenous gut microbiota microbes (Pickard et al., 2017). In contrast, *Faecalibacterium*, which was significantly elevated in the CON group, helps reduce intestinal inflammation and produces butyrate, contributing to maintaining a healthy gut microbiota through its probiotic activity (Leylabadlo et al., 2020). Additionally, *Turicibacter* is known to produce SCFAs that play a crucial role in maintaining the health of the intestinal mucosal cells (Yang et al., 2020). This suggests that in the CPV-high group, where the proportions of *Faecalibacterium* and *Turicibacter* are lower, the levels of SCFAs that help sustain gut health might also be reduced.

As no noticeable difference was found in the CPV-low group compared to the CON group immediately after CPV-2 infection, it is interesting that the α -diversity of group CPV-low was notably lower than that of the CON group 8 weeks after CPV-2 infection. Antibiotic use is an important risk factor for dysbiosis, and maximal dysbiosis occurs 2–3 days after antibiotic administration (Kelly et al., 2021). Here, antimicrobial treatment was provided to eight dogs in groups CPV-low and CPV-high immediately after CPV-2 infection detection (at 13 weeks and 6 days of age), and continued for approximately 2 weeks. Therefore, the diversity decreases 8 weeks after CPV-2 infection may be due to perturbation by the antimicrobial treatment applied. Antibiotic application for diarrhea treatment in ruminant delays diversity development and disrupts the stability of early-life gut microbiota (Ma et al., 2020). At 28 weeks of age, approximately 15 weeks after antibiotic administration, the gut microbiota of group CPV-low was comparable to that of group CON regarding α - and β -diversity and taxonomic composition. Intestinal infection and antibiotic application are representative examples of pulsed-perturbations of gut microbiota causing short-term changes, but a well-established and stabilized microbiota has resiliency and can return to its original state (Sommer et al., 2017). Collectively, our results suggest that the disrupted gut microbiota of CPV-low group, due to CPV-2 infection followed by antibiotic treatment, could be recovered to a relatively normal state (resilience) at week 28.

One limitation of this study was the relatively small sample size, which may limit the statistical power of the analyses. Although a linear mixed model was applied to explore the effect of age, the small sample size could reduce the robustness and generalizability

of the results. To improve the statistical validity and reliability of future studies, a larger sample size is required for detecting significant effects and interactions. Another limitation was the lack of paternal lineage information, which could contribute to genetic variability. However, given that the primary focus of this study was the microbiota composition and its temporal changes, we believe that this limitation had minimal impact on the validity of our findings. Future studies could benefit from incorporating detailed genetic background data to explore potential interactions between host genetics and microbiota. Despite these limitations, this study provides a valuable foundation for understanding the growth and dietary changes in gut microbiota in dogs. By employing robust analytical methods and controlling for confounding factors such as diet and environment, our findings offer significant insights into the developmental dynamics of the canine microbiota. These insights can serve as a scientific basis for future studies aiming to comprehensively investigate microbiota-host interactions.

5 Conclusion

This study revealed that canine gut microbiota undergoes rapid changes in microbial composition throughout the early life stages even in the same litter. As dogs grew older, we observed an increase in the microbial ecology diversity of the gut microbiota. Stabilization was achieved at approximately 14 weeks of age, coinciding with the puppy stage and an increase in exposure to the external environment due to systemic training. This stabilization was further corroborated by changes in *E. faecalis* genetic diversity. With heightened exposure to the external environment shared among littermate groups, we observed the propagation of *E. faecalis* strains and the introduction of new strains. Additionally, the shared core microbiome found within the same litter and in different litters emphasizes the influence of shared environments in fostering similar microbiota compositions and strain sharing among military dogs.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/bioproject/>, PRJNA800584.

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Author contributions

J-UA: Conceptualization, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. S-HM: Methodology, Software, Writing – original draft. W-HK: Writing – review and editing. JS: Supervision, Writing – review and editing. KP: Resources, Writing – review and editing. SC: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review and editing.

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EDITED BY

Baichuan Deng,
South China Agricultural University, China

REVIEWED BY

Rangsun Charoensook,
Naresuan University, Thailand
Cunxi Nie,
Shihezi University, China

*CORRESPONDENCE

Shimeng Huang
✉ shimengh@cau.edu.cn

[†]These authors share first authorship

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Comparative analysis of composition and spatial variations in the foregut microbiota of male and female donkeys

Yanwei Wang^{1,2,3†}, Xiaotong Li^{1,2,4†}, Zuowei Li^{1,2,5},
Qiaoqiao Han^{1,2,5}, Tong Hu^{1,2}, Qiyue Zhang^{1,2,4}, Honglei Qu^{1,2},
Haihua Zhang⁵, Yangyan Qu^{2,6}, Donghui Shi⁴, Qiugang Ma^{1,2} and
Shimeng Huang^{1,2*}

¹National Key Laboratory of Livestock and Poultry Nutrition and Feeding, College of Animal Science and Technology, China Agricultural University, Beijing, China, ²Laboratory of Feed grain Safety and Healthy Poultry Farming, Beijing Jingwa Agricultural Science and Technology Innovation Center, Beijing, China, ³College of Life Sciences, Shanxi University, Taiyuan, China, ⁴College of Animal Science and Veterinary Medicine, Jinzhou Medical University, Jinzhou, Liaoning, China, ⁵Hebei Key Laboratory of Specialty Animal Germplasm Resources Exploration and Innovation, College of Animal Science and Technology, Hebei Normal University of Science and Technology, Qinhuangdao, China, ⁶Beijing Sunlon Technology Bio-breeding Innovation Co. Ltd, Beijing, China

Donkeys, as significant herbivorous mammals, also serve as valuable companion animals. Research on gut microbiota has underscored the essential role of microorganisms in maintaining gut health, supporting nutrient metabolism, and regulating immune function. As the gut microbiota is also shaped by factors such as sex, age, diet, environment and genetics, many studies have on the complexity and diversity of hindgut microbial communities, while few studies have focused on the foregut microbiota of donkeys. To address this gap, we conducted high-throughput sequencing of the highly variable V3-V4 region of the 16S rRNA gene from the donkey small intestine (duodenum, jejunum, and ileum) to characterize and compare microbiota composition and abundance between male and female donkeys. A total of 12 healthy and uniformly conditioned Dezhou donkeys (six males and six females, aged 2–3 years, 250 ± 10 kg in weight) were included in the study. The results showed that albumin (ALB), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) levels were significantly higher ($p < 0.05$) in the female group compared to the male group. Additionally, α -diversity indices (Ace, Chao, Simpson, and Sobs) were significantly different ($p < 0.05$) between the groups. The PCoA results indicated significant differences ($p < 0.05$) between male and female donkeys across all intestinal locations ($R^2 = 0.2372$, $p < 0.001$). Similarly, the microbial composition of the jejunum ($R^2 = 0.1875$, $p = 0.019$) and ileum ($R^2 = 0.1776$, $p = 0.007$) showed significant differences between male and female donkeys. Additionally, Firmicutes, Fusobacteriota, Proteobacteria, and Actinobacteriota were the dominant phyla across all gut regions. In male and female donkeys, key genera included *Lactobacillus*, *Streptococcus*, *Sarcina*, and *Escherichia-Shigella*. Linear discriminant analysis effect size (LEfSe) analysis revealed gender-specific enrichment, with *Clostridium_sensu_stricto_1*, *Acinetobacter*, and *NK4A214_group* dominant in female duodenum and jejunum, while *Streptococcus* and *Erysipelotrichaceae_UCG-002* were enriched in males. Similarly, female ileum had enriched *Amnipylo*, *Terrisporobacter*, and *Luteimonas*, whereas males showed higher levels of *Sarcina* and *Streptococcus*. *Blautia* and *Mogibacterium* were enriched in female duodenum and jejunum, while *Fusobacterium*, *Actinobacillus*, and *Moraxella* were more abundant in male ileum. These findings characterize the gut microbiota of healthy donkeys and provide novel insights into the differences between male and female donkeys, offering previously unknown information about donkey gut microbiota.

KEYWORDS

gut microbiota, spatial variations, foregut, donkey, gender

1 Introduction

Many countries around the world have a long history of donkey farming, especially China, where the number of donkeys raised is substantial (Seyiti and Kelimu, 2021). Donkeys contribute significantly to livelihoods and agriculture (Ravichandran et al., 2023). The Dezhou donkey, one of the top five excellent donkey breeds in China, is primarily found in Dezhou City and the surrounding areas of Shandong Province. In 2011, the Dezhou donkey was included in the national list of livestock and poultry genetic resources for protection, and it is recognized as an excellent local breed in Shandong Province, China (Wang et al., 2023). The Dezhou donkey is a medium-sized, well-proportioned breed with strong limbs, highly valued for both its service and meat production (Wang et al., 2023). It is known for its tolerance to roughage, strong adaptability, and good disease resistance. Donkeys play a significant role in various sectors, and their meat is known for its delicious, delicate flavor, being rich in protein, amino acids, and other nutrients (Seyiti and Kelimu, 2021; Wang et al., 2023). In addition, the composition of donkey milk is more similar to that of human milk, including lactose, lipids and proteins, etc. Due to its chemical and nutritional characteristics, it has some potential value in the medical and food fields (Bertino et al., 2022). In recent years, donkey farming in China has attracted increasing attention, with extensive research conducted on donkey growth, reproduction, gut health, and meat quality (Man et al., 2023), highlighting the need for further studies on their nutritional systems to promote sustainable development of the industry.

The intestine plays a key role in digestion and nutrient absorption, with its structure and function closely linked to animal growth and health (Ma et al., 2022; Zhang et al., 2024). This is particularly important for herbivores, which rely on gut bacteria to ferment plant fibers into volatile fatty acids, their main energy source (Husso et al., 2020; Dalile et al., 2019). Research shows that gut microbes significantly influence biometabolic phenotypes, impacting nutrient absorption, tissue development, and immune function (DuPont and DuPont, 2011; González Olmo et al., 2021). Gut microbes are also associated with various diseases; for instance, in horses with colitis, Bacteroidetes were predominant (40%), while healthy horses were dominated by Firmicutes (68%) (Costa et al., 2012). Beyond digestion, the gut microbiota is increasingly recognized for its role in nutrient utilization, digestive tract development, and immunity (Schluter et al., 2020). A recent study showed that donkeys from different regions had different functional differences due to differences in gut microflora. For example, Dezhou donkeys exhibited strong glucose conversion ability and Shigatse donkeys exhibited strong glucose metabolism and utilization ability, which makes them better adapt to the environment (Guo et al., 2023; Liu et al., 2020). Additionally, gender differences have been identified as important factors that interfere with the gut microbiota, and this has been confirmed in humans and mice (Ding and Schloss, 2014; Elderman et al., 2018). As the gut microbiota is shaped by factors such as sex, age, diet, environment and genetics, many studies have shown that fermentation of the hindgut makes its microbial community more complex and diverse, and few studies have focused on the donkey gut microbiota, particularly the foregut. More studies are needed to

explore the gut and fecal microbiota of healthy donkeys, further research is necessary to reveal the microbial colonization patterns in the foregut of male and female Dezhou donkeys.

In this study, the foregut of 12 adult healthy Dezhou donkeys, including six male and six female donkeys, was examined to further explore the characteristics and functional prediction of the contents of the duodenum, jejunum and ileum, and to provide a scientific basis for further revealing the influence of sex on the digestive mechanism of the donkey gastrointestinal tract.

2 Materials and methods

2.1 Animal ethics statement

In this study, the utilization of animals in this study adhered to rigorous ethical standards and was formally approved by the Animal Care and Use Committee of China Agricultural University (Approval no.: AW81704202-1-1).

2.2 Experimental design and data collection

A total of 12 healthy and uniformly conditioned Dezhou donkeys (age: 2–3 years; body weight: 250 ± 10 kg) were selected for this study, comprising six males and six females. All donkeys were raised under the same breeding conditions at Shandong Dong'e Ejiao Co., Ltd., with a standard farm-provided ration offered ad libitum, along with free access to water. The donkeys were housed in single semi-open pens and fed twice daily at 07:00 and 19:00. During this period, none of the donkeys received probiotics or antibiotics for at least 3 months.

All Blood samples were collected in tubes (5 mL) from a jugular vein before feeding. Serum samples were obtained after centrifugation at $3,000 \times g$ for 10 min at 4°C and then snap frozen in liquid nitrogen, awaiting subsequent analysis. The donkeys were slaughtered following a 12-h fast, after which the carcasses were carefully dissected and the intestinal organs removed. Samples were promptly collected from different intestinal locations (duodenum, jejunum, and ileum) of each donkey. All contents were collected, handled, and stored aseptically to prevent contamination. Samples were placed in 5 mL centrifuge tubes, immediately preserved in liquid nitrogen, and then transported to -80°C storage for long-term preservation until DNA extraction.

2.3 Serum biochemical indices

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), total cholesterol (TC), total triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using a chemistry analyzer (Commercial Kit, Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's recommended procedures.

2.4 DNA sequencing and processing

Microbial genomic DNA was extracted from 12 samples using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The extracted DNA served as a template to amplify the V3-V4 hypervariable region of the bacterial 16S rRNA gene, using universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplified products were detected by agarose gel electrophoresis (2% agarose), recovered using the AxyPrep DNA Gel Recovery Kit (Axygen Biosciences, Union City, CA, USA), and quantified with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) to pool in equimolar amounts. Amplicon libraries were sequenced on the Illumina HiSeq 2,500 platform (Illumina, San Diego, CA, USA) for paired-end reads of 250 bp. The amplicons were purified from agarose gels using the AxyPrep DNA Gel Extraction Kit (Corning, Glendale, USA), pooled in equimolar amounts, and sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) following standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Illumina sequencing data were filtered, denoised, concatenated, and de-embedded using QIIME2 to obtain high-quality sequencing data, with each sample region yielding $\geq 50,000$ effective sequences for subsequent bioinformatics analysis. Tags were clustered into ASVs using DADA2. ASV taxonomic assignments were conducted by the RDP classifier (version 2.2) and annotated in the Silva1 database. Alpha diversity indices, including ACE, Chao, Shannon, and Sobs, were calculated using QIIME2 and the R package vegan (v2.5.6). A Venn diagram showing the number of shared and unique ASVs among the different intestinal segments was constructed using the VennDiagram package in R (v3.1.1). Principal Coordinate Analysis (PCoA) and permutational multivariate analysis of variance (Adonis) were carried out using R software (version 3.2.1).¹ Genus-level microbial differences between different intestinal segments were analyzed using vegan v3.5.1, with comparisons made using the Wilcoxon rank sum test or Kruskal-Wallis rank sum test and pairwise comparisons to identify specific variations.

2.5 Statistical analysis

All statistical analyses were conducted using GraphPad Prism (version 9.0; GraphPad Software, La Jolla, CA, USA). Differences were evaluated by groupwise comparisons using Student's *t*-test. Data are shown as means $\pm$ standard errors of the mean (SEMs). $p < 0.05$ were considered statistically significant.

3 Results

3.1 Differences in serum protein metabolism and lipid metabolism indices between adult female and male donkeys

As shown in Figure 1, serum biochemical indices of female and male donkeys were estimated using ALT, AST, ALP, TP, ALB, TC, TG,

HDL-C, and LDL-C levels. The levels of ALB (Figure 1E), TC (Figure 1F), and HDL-C (Figure 1H) in the female donkey group were significantly higher ($p < 0.05$) than those in the male donkey group. No significant differences ($p > 0.05$) were observed between male and female groups for the other indices (ALT, AST, ALP, TP, TG, and LDL-C).

3.2 Analysis of alpha diversity of duodenal, jejunal, and ileal microbiota in adult female and male donkeys

The alpha diversity of gut microbiota in male and female donkeys was assessed using the Ace, Chao, Shannon, Simpson, and Sobs indices. As shown in Figure 2A, the rarefaction curve levels off as the number of sequences increases, indicating that nearly all bacterial species present were captured across samples. The sequences obtained were then analyzed for diversity. Results showed that the Ace (Figure 2B), Chao (Figure 2C), and Sobs (Figure 2F) indices in the M-Jejunum group were significantly lower ($p < 0.05$) than in the F-Jejunum group, while there were no significant differences in Shannon indices (Figure 2D) between groups. This indicates that there is a higher microorganisms community richness in the jejunum of the female donkey. Additionally, the Simpson index in the F-Duodenum and F-Ileum groups was significantly lower ($p < 0.05$) than in the M-Jejunum group (Figure 2E). This shows that there is a higher microorganisms community diversity of male donkey.

3.3 Analysis of gut microbial community of duodenal, jejunal, and ileal microbiota in adult female and male donkeys

Principal Coordinate Analysis (PCoA) of β -diversity, assessed by Bray-Curtis dissimilarity, confirmed spatial differences in microbial function across different gut locations. As shown in Figure 3A, the gut microbiota in the F-Duodenum, F-Jejunum, F-Ileum, M-Duodenum, M-Jejunum, and M-Ileum groups were clustered separately ($R^2 = 0.2372$, $p = 0.001$). The PCoA results showed no significant differences in the microbial structure of the small intestine between adult male (Figure 3B; $R^2 = 0.1694$, $p = 0.170$) and female donkeys (Figure 3C; $R^2 = 0.1108$, $p = 0.513$); however, there was a potential differentiation in the microbial structure of the duodenum between sexes (Figure 3D; $R^2 = 0.1422$, $p = 0.074$), while significant differences were observed in the microbial composition of the jejunum (Figure 3E; $R^2 = 0.1875$, $p = 0.019$) and ileum (Figure 3F; $R^2 = 0.1776$, $p = 0.007$) between male and female donkeys. To illustrate the distribution of common and unique ASVs across samples, we used Venn diagrams to represent the bacterial community ASVs. Clustering the valid labels from all samples, the Venn diagrams showed that the six groups shared a community containing 132 ASVs (Figure 4A), while unique ASVs were identified in M-Duodenum (1236), F-Duodenum (1264), M-Jejunum (855), F-Jejunum (1789), M-Ileum (2032), and F-Ileum (1862). Based on microbial analysis of the duodenum, jejunum, and ileum, the bacterial community shared among female donkeys contained 479 ASVs, whereas the male group shared 246 ASVs (Figures 4B,C). Comparing gut segments between adult male and female donkeys, the F-Duodenum and M-Duodenum

¹ <http://www.r-project.org/>

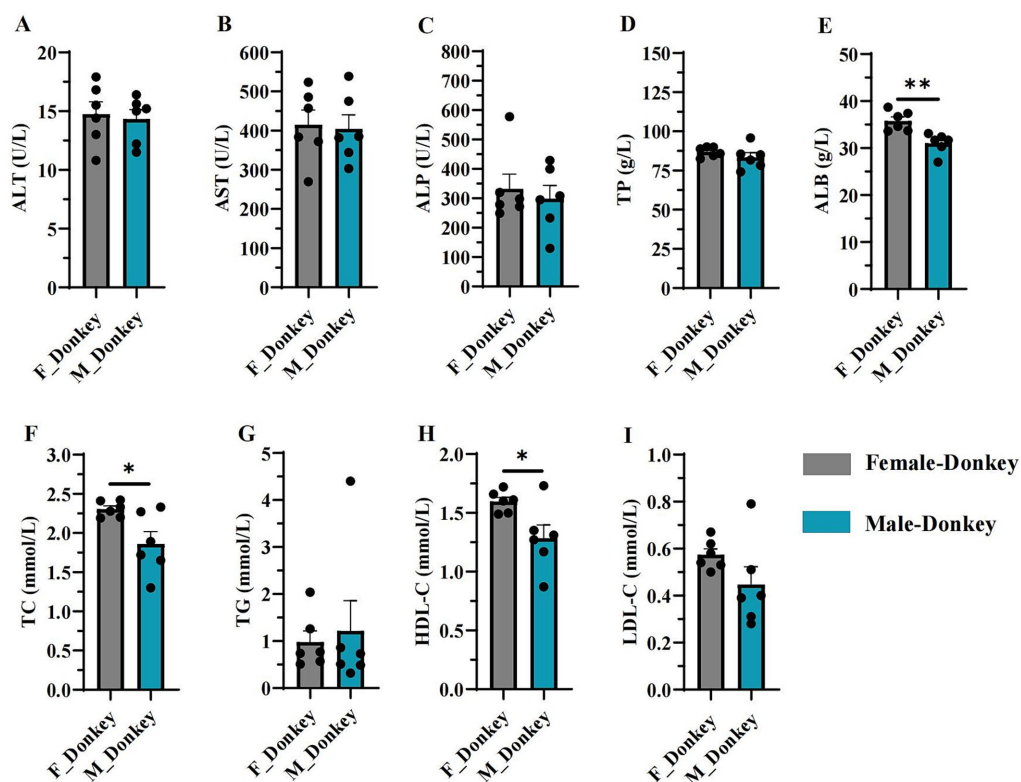


FIGURE 1

Serum biochemical indices in female and male donkeys. (A) ALT level; (B) AST level; (C) Alkaline phosphatase level; (D) *Treponema pallidum* level; (E) Albumin level; (F) Total cholesterol level; (G) Triglyceride level; (H) HDL-C level; (I) LDL-C level. *p < 0.05, **p < 0.01.

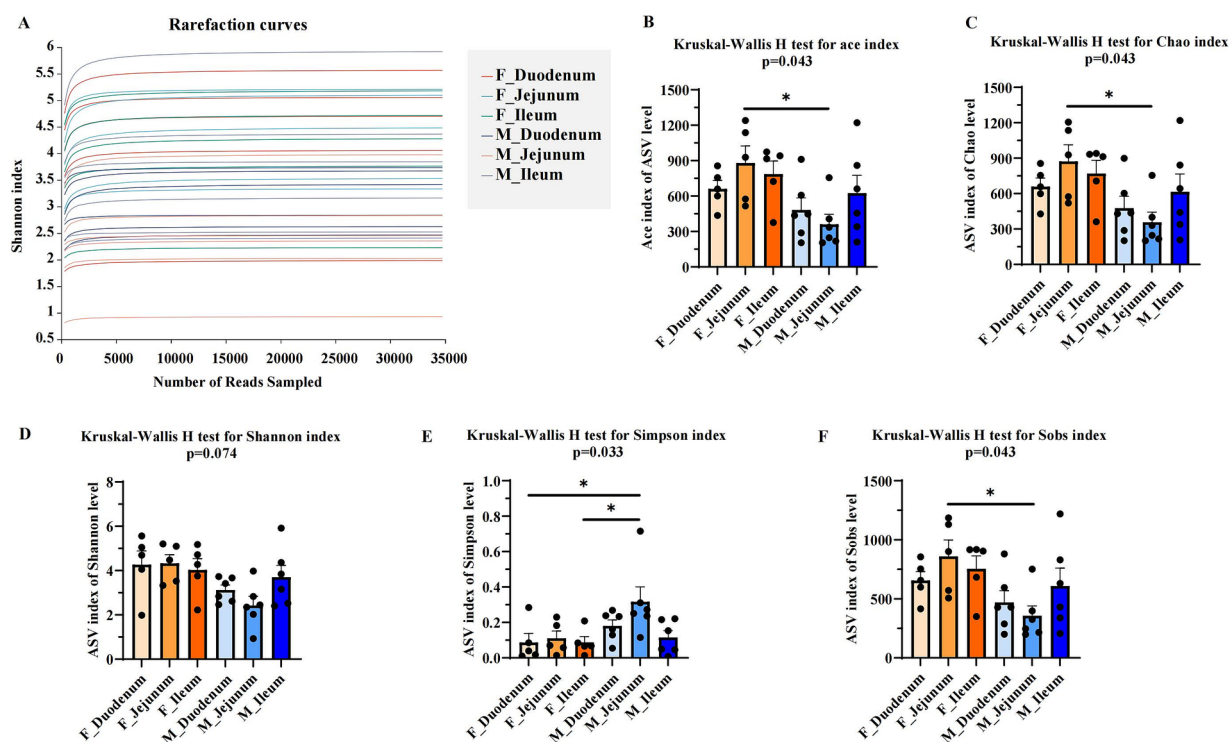


FIGURE 2

Comparison of microbial diversity indices between two groups. Alpha diversity analyses comparing the microbiota of the two groups based on various microbial diversity indices. (A) Rarefaction curves; (B) Kruskal-Wallis H test for the Ace index; (C) Kruskal-Wallis H test for the Chao index; (D) Kruskal-Wallis H test for the Shannon index; (E) Kruskal-Wallis H test for the Simpson index; (F) Kruskal-Wallis H test for the Sobs index. *p < 0.05.

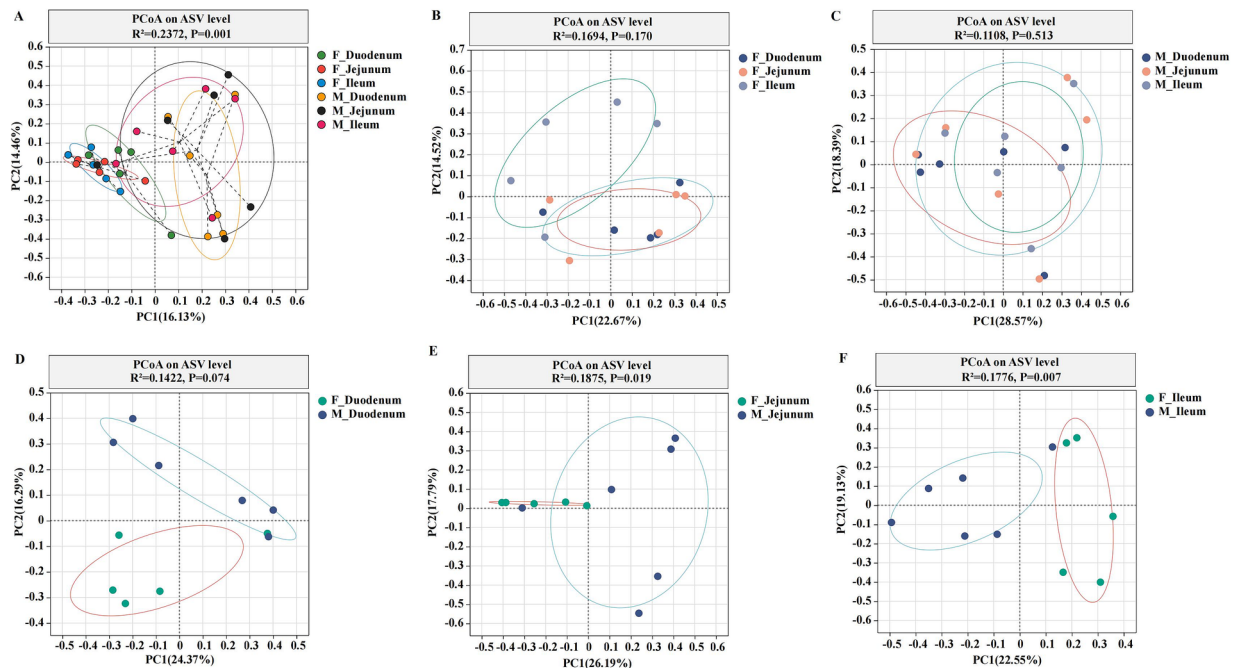


FIGURE 3

Principal Coordinate Analysis (PCoA) plots showing the gut microbial community structure. (A) PCoA of six gut microorganisms; (B) PCoA of gut microorganisms in female donkeys; (C) PCoA of gut microorganisms in male donkeys; (D) PCoA of the duodenum in female and male donkeys (F-Duodenum and M-Duodenum); (E) PCoA of the jejunum in female and male donkeys (F-Jejunum and M-Jejunum); (F) PCoA of the ileum in female and male donkeys (F-Ileum and M-Ileum). PCoA was performed at the ASV level, with each point representing a sample, and the two clusters indicating a significant difference in community structure between the two groups.

groups shared 580 ASVs; the F-Jejunum and M-Jejunum groups shared 513 ASVs; and the F-Ileum and M-Ileum groups shared 585 ASVs (Figures 4D–F).

Based on ASV, phylum, and genus levels, we used heatmaps to illustrate the core species composition across different treatment groups. At the ASV level (Figure 5A), the top 30 species with the highest abundance in the gut-associated microbiota (ASV2565, ASV82, ASV720, ASV97, ASV453, ASV120, ASV19, ASV52, ASV435, ASV442, ASV1106, ASV1536, ASV55, ASV54, ASV42, ASV45, ASV2914, ASV1, ASV2, ASV46, ASV13, ASV2293, ASV2294, ASV6, ASV4, ASV35, ASV10, ASV487, ASV11, and ASV14) were identified among these treatment groups. At the phylum level (Figure 5B), Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, unclassified_k_norank_d_Bacteria, and Fusobacteriota dominated the microbial community in all donkeys. At the genus level (Figure 5C), *unclassified_g_Lactobacillus*, *uncultured_bacterium_g_Sarcina*, *bacterium_RA2114*, *unclassified_g_Streptococcus*, and *unclassified_g_Moraxella* were enriched across these treatment groups.

3.4 Analysis of differential microbiota in duodenal, jejunal, and ileal microbiota in adult female and male donkeys

Next, LEfSe was used to identify bacterial groups that were significantly different between the two management models. As shown in Figure 6A, *Clostridium_sensu_stricto_1*, *Acinetobacter*, *Mogibacterium*, *NK4A214_group*, *Akkermansia*, *Shuttleworthia*,

Cetobacterium, *Acidibacter*, *Butyrivibrio*, *Monoglobus*, and *Clostridium_sensu_stricto_6* were significantly enriched in the F_Duodenum group, while *Streptococcus* and *Erysipelotrichaceae_UCG-002* were significantly enriched in the M_Duodenum group. In Figure 6B, *Clostridium_sensu_stricto_1*, *NK4A214_group*, *Acinetobacter*, *Christensenellaceae_R-7_group*, *Terrisporobacter*, *Ruminococcus*, *Lachnospiraceae_XPB1014_group*, *Akkermansia*, *Mogibacterium*, *Lachnospiraceae_ND3007_group*, *Butyrivibrio*, *Turicibacter*, *Lachnospiraceae_AC2044_group* and *Lachnospira* were significantly enriched in the F_Jejunum group, while *Megasphaera* was significantly enriched in the M_Jejunum group. In Figure 6C, *Amnipyia*, *Terrisporobacter*, *Acidovorax*, *Phoenicibacter*, *Luteimonas*, *Fastidiosipila*, *Dorea*, *Arcobacter*, and *Lachnospiraceae_NK4A136_group* were significantly enriched in the F_Ileum group, whereas *Sarcina*, *Streptococcus*, and *Erysipelotrichaceae_UCG-002* were significantly enriched in the M_Ileum group.

Next, we analyzed differentially abundant microbes across the duodenum, jejunum, and ileum within the same sex. As shown in Figure 7A, *Blautia*, *Mogibacterium*, *Dorea*, *Eubacterium_hallii_group*, *Solobacterium*, *Weissella*, and *Cetobacterium* were significantly enriched in the F_Duodenum group; *Sarcina*, *NK4A214_group*, *Enterococcus*, *UCG-005*, *Ruminococcus*, *Saccharofermentans*, *Pseudomonas*, *Lachnospiraceae_NK4A136_group*, and *Peredibacter* were significantly enriched in the F_Jejunum group; and *Fusobacterium*, *Mannheimia*, *Flavobacterium*, *Conchiformibius*, *Atopostipes*, *Vogesella*, *Guggenheimella*, *Fastidiosipila*, and *Paenaltcaligenes* were enriched in the F_Ileum group. As shown in Figure 7B, *Bifidobacterium*, *Blautia*, *Collinsella*, *Subdoligranulum*, *Eubacterium_hallii_group*, *Dorea*, *Pseudomonas*,

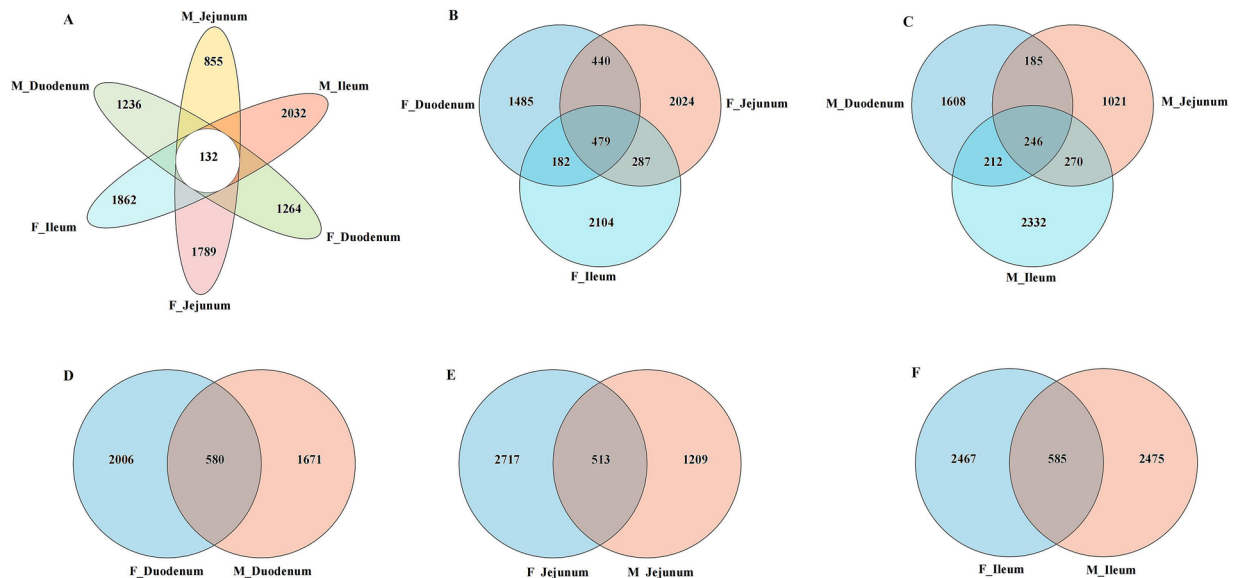


FIGURE 4

Venn diagrams showing the distribution of gut microbiota ASVs between female and male donkeys. (A) Venn analysis of ASVs from six gut microorganisms; (B) Venn analysis of ASVs from female donkeys; (C) Venn analysis of ASVs from male donkeys; (D) Venn analysis of ASVs from the female and male duodenum (F-Duodenum and M-Duodenum); (E) Venn analysis of ASVs from the female and male jejunum (F-Jejunum and M-Jejunum); (F) Venn analysis of ASVs from the female and male ileum (F-Ileum and M-Ileum).

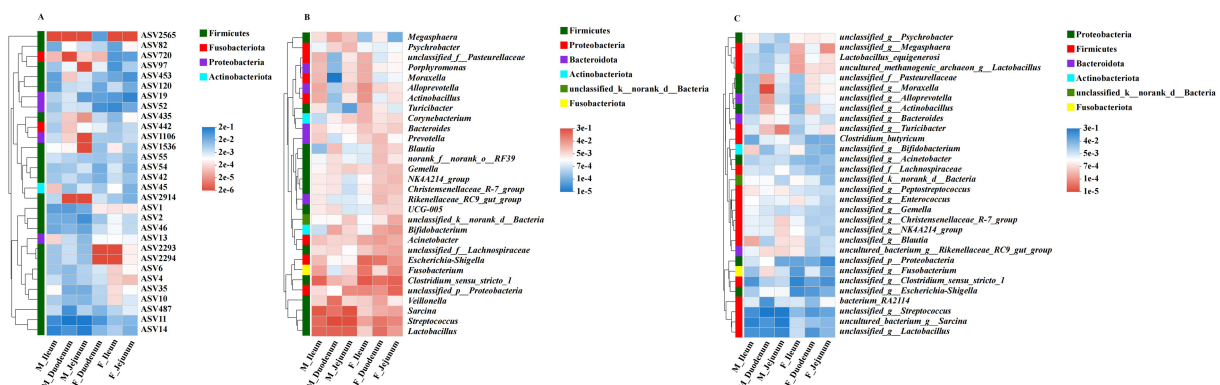


FIGURE 5

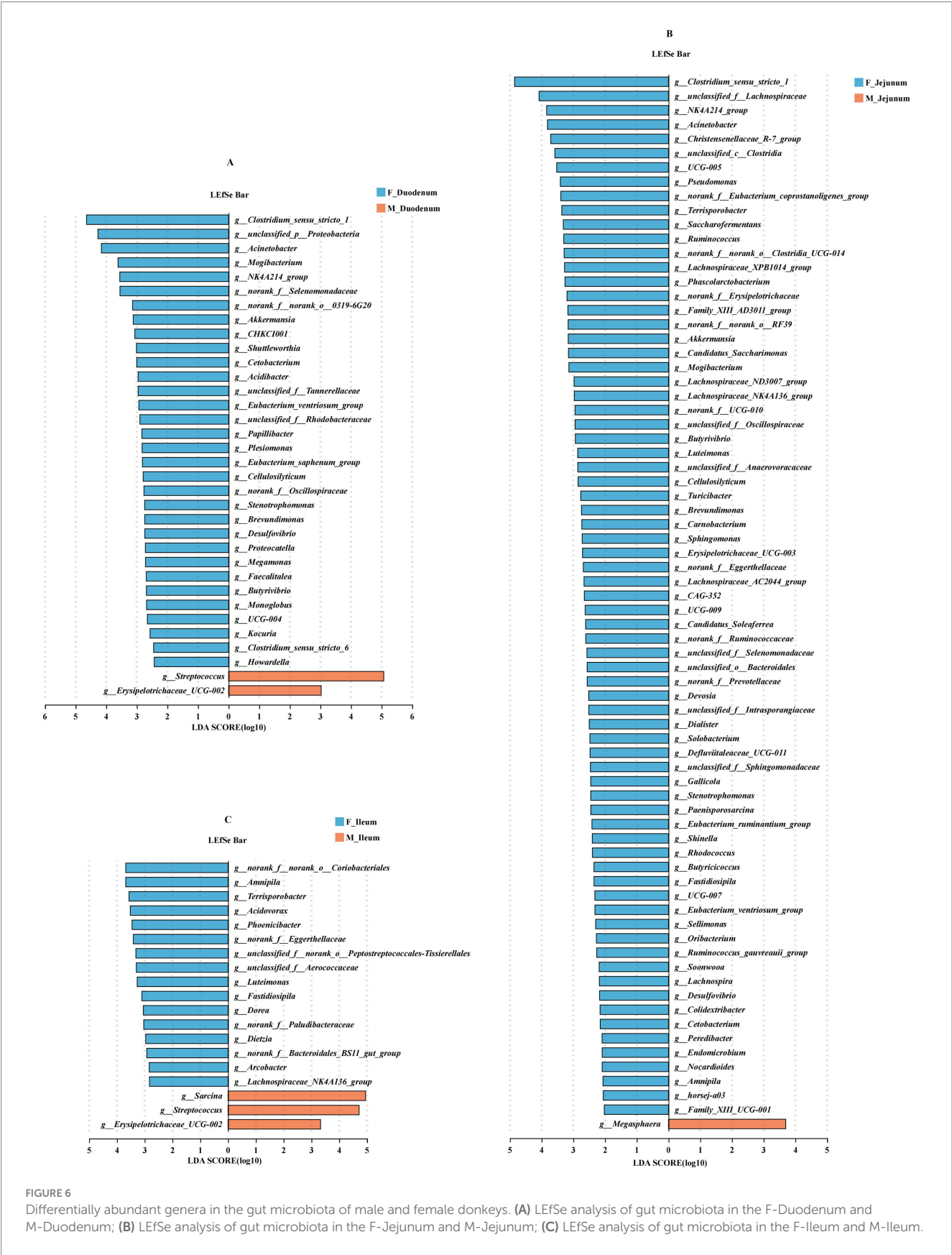
The distribution of important bacterial functions in different groups. (A) Correlation of the dominant affected ASVs in the digesta-associated microbiota across different intestinal locations; (B) Correlation of different intestinal locations with associated microbiota at the genus level; (C) Correlation of different intestinal locations with associated microbiota genera across six phyla.

Erysipelotrichaceae_UCG-003, *Ruminococcus_torques_group*, *Providencia*, *Peptococcus*, *Paenisporosarcina*, and *Pelomonas* were significantly enriched in the M_Duodenum group; *Corynebacterium* was significantly enriched in the M_Jejunum group; and *Fusobacterium*, *Alloprevotella*, *Actinobacillus*, *Moraxella*, *Porphyromonas*, *Bergeyella*, *Mageibacillus*, *Atopostipes*, *Filobacterium*, and *Clostridium_sensu_stricto_6* were enriched in the M_Ileum group.

4 Discussion

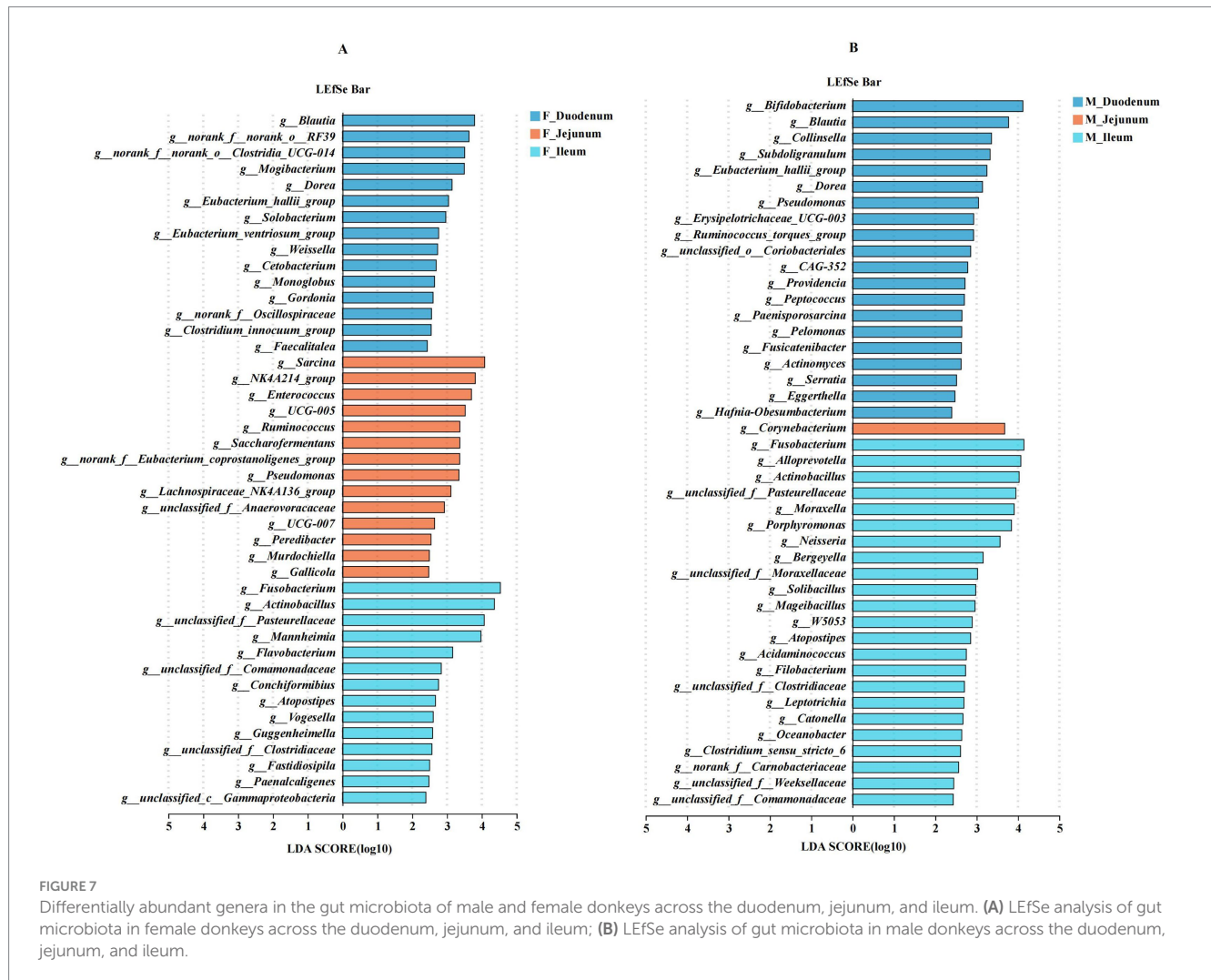
The donkey gut is essential for digestion, with large and complex gut microbes playing a crucial role in breaking down various

indigestible polysaccharides (Ma et al., 2022; Zhang et al., 2024; Glatter et al., 2019; Bäckhed et al., 2005). A previous study has shown that herbivores, especially ruminants, host a diverse and specialized microbiota (Bayané and Guiot, 2011). The mammalian gut microbiota is dynamic and complex, plays a fundamental role in responding and adapting to the host environment, which supports host health and normal reproduction (Ley et al., 2008; Li et al., 2022), and supporting immune function and health maintenance (Bertino et al., 2022; Zhao et al., 2013; Maslowski and Mackay, 2011). The normal gut microbiota contributes to host nutrient metabolism, drug and xenobiotic processing, intestinal barrier integrity, immune modulation, and pathogen resistance (Li et al., 2022; Jandhyala et al., 2015). Our findings characterize the gut microbiota of healthy donkeys and reveal



differences between male and female donkeys, providing novel insights into donkey gut microbiota.

The mammalian gut microbiota plays a vital role in host metabolism and adaptation (Li et al., 2018). In this study,



we examined biochemical indicators in male and female donkey groups and found that ALB, TC, and HDL-C levels were significantly higher in the female group. ALB, a key protein in blood, contributes to vascular endothelial stability, acid-base balance, and the repair of inflammatory damage (Bihari et al., 2020). Additionally, TC transformation is strongly associated with different microbiomes (Bubeck et al., 2023). In previous research on the toxicokinetics of toxins in Dezhou donkeys, including the effects of ochratoxin (OTA) on male donkeys and zearalenone (ZEN) on female donkeys, both of which demonstrated a high absorption rate and slow elimination in Dezhou donkeys (Kang et al., 2023; Qu et al., 2024). Previous research has shown that host genetic influence patterns differ between adult males and females, indicating that gender impacts host metabolism and gut microbiota composition (Zhao et al., 2013; Mueller et al., 2006), which aligns with the findings of our study.

Animal gut microbiota is influenced by both the living environment and sex (de Jonge et al., 2022). Variations in diversity reflect the stability and spatial distribution of the gut microbial environment. In the α -diversity results (Ace, Chao, Simpson, Shannon, and Sobs indices), the Ace, Chao, and Sobs indices were significantly lower in the M-Jejunum group than in the F-Jejunum group, indicating differences in jejunal microbial communities

between male and female donkeys. Differences in gut microbial composition and the host immune system by sex have been reported (Fushuku and Fukuda, 2008), which aligns with our findings. A previous study showed that PCoA at the ASV level revealed distinct spatial variability of gut microbiota across different gut compartments (Donaldson et al., 2016). In our study, the microbial communities within each intestinal location clustered significantly within both male and female groups, with significant differences observed between male and female donkeys in the jejunum and ileum groups, while microbial composition was more consistent between sexes in the duodenum. Previous research also found that microbial richness and diversity are generally higher in the hindgut than in the foregut (Liu et al., 2019), there were significant differences in terms of dominant bacteria among cecum, ventral colon, and dorsal colon, especially between the cecum and dorsal colon sites (Li et al., 2022), suggesting that potential shifts in bacterial communities warrant further investigation. At the ASVs level, our results indicate that the duodenum, jejunum, and ileum have a high number of unique ASVs, reflecting a rich diversity of microbial species. Similarly, previous research on horses revealed that foregut microbiota varies significantly between sections and individuals (Su et al., 2020). Furthermore, Firmicutes emerged as the most prevalent phylum across all intestinal regions, with its

high abundance linked to fiber degradation in herbivores (Liu et al., 2019). In the male donkey group, a unique ASV for Fusobacteriota was identified in the duodenum and one for Proteobacteria in the jejunum. Prior findings suggest that gut microbiota composition varies between sexes, influencing immune properties (Fushuku and Fukuda, 2008). At the phylum level, Firmicutes and Proteobacteria dominated the microbial community in all donkeys, with other identified phyla including Bacteroidota, Actinobacteriota, unclassified_k_norank_d_Bacteria, and Fusobacteriota. Most Firmicutes produce butyric acid that is not absorbed by the intestine, but it provides energy and promotes the development of intestinal epithelial cells (Guo et al., 2023). Interestingly, Steelman et al. (2012) found that bacterial communities in equine gut samples were dominated by Firmicutes and Verrucomicrobia, followed by Bacteroidetes, Proteobacteria, and Spirochaetes, which aligns with our findings. In male donkeys, *Sarcina*, *Streptococcus*, and *Lactobacillus* showed higher relative abundance; these genera within Firmicutes are known to influence gut health differently between sexes. Both *Lactobacillus* and *Streptococcus* have evidence of potential beneficial functions in relation to lipid metabolism (Liu et al., 2019; Ma et al., 2022), illustrating that they play an important role in maintaining intestinal health. Firmicutes is also known to promote short-chain fatty acid (SCFA) production, which supports fat accumulation (Ley et al., 2005). The presence of *Sarcina* often correlates with health complications (Makovska et al., 2023), however, a previous study showed the increased relative abundance of *Sarcina* significantly affected the immune response in rats (Vega-Magaña et al., 2020), our study shows that it seems to appear to be a common member of the gut microbiota. *Streptococcus* has been linked to infectious diseases, triggered by arginine and inhibited by carbon metabolites (Gruening et al., 2006). In contrast, *Lactobacillus* includes acid-tolerant mutualistic bacteria that selectively inhibit pathogens and produce free bile acids to support fat metabolism (Corzo and Gilliland, 1999). The interaction between *Sarcina* and *Lactobacillus* may enhance metabolism in male donkey intestines.

At the genus level, our study found that *unclassified_g_Moraxella* was enriched in the M-Duodenum group. Previous research has shown that stomach bacterial communities are often dominated by Firmicutes, Proteobacteria, and Bacteroidetes, with *Lactobacillus* spp., *Streptococcus* spp., and *Moraxella* spp. as prominent genera (Perkins et al., 2012). These findings indicate differences in microbiota between male and female donkey groups, particularly within Firmicutes. Additionally, *Bifidobacterium* was a dominant genus in the M-Duodenum group, and *Clostridium*, *Lactobacillus* and *Enterococcus* have been noted as primary mucosa-associated genera in the small intestine (Swidsinski et al., 2005). *Clostridium* species in the gut play a vital role in the production of vitamins and short-chain fatty acids, the maintenance of gut homeostasis and the shaping of the mucosal immune system (Marathe et al., 2014). Therefore, there is evidence to speculate that male donkeys have better digestive capacity due to these microbial floras. LEfSe analysis revealed *g_Fusobacterium* as the dominant genus in the F-Ileum group, consistent with findings from fecal studies in diverse horse groups where *Fusobacterium* spp. was prevalent (Costa et al., 2012). This led us to hypothesize that microbial specificity in donkeys may closely relate to gut location and gender. In the M-Ileum group, *g_Sarcina*

and *g_Streptococcus* were predominant, with *Sarcina* spp. known as opportunistic pathogens found in diverse mammalian hosts (Makovska et al., 2023). Notably, *g_Streptococcus* was more abundant in the M-Duodenum than in the F-Duodenum group, differing from prior studies where *Lactobacillus* was dominant in the foregut and *Streptococcus* in the hindgut (Liu et al., 2019). In addition, *g_Clostridium_sensu_stricto_1* dominated in the F-Jejunum group, while *g_Megasphaera* was predominant in the M-Jejunum group. *Clostridium* represents a highly diverse genus with both beneficial and pathogenic species (DuPont and DuPont, 2011). A study shows that a potentially pivotal lipid-lowering role of *Megasphaera* in the gut microbiota (Gao et al., 2022). Further research is needed to explore the link between regional colonization differences in the Dezhou donkey gut and metabolic functions, informing future feeding management and promoting intestinal health in donkeys.

5 Conclusion

This study provides novel insights into the spatial distribution of foregut microbial communities in healthy male and female Dezhou donkeys. Gender was identified as a significant factor influencing gut microbial composition, with notable differences in predicted microbiota functions. Additionally, the microbial composition exhibited distinct distribution patterns across the duodenum, jejunum, and ileum within each gender. These findings enhance our understanding of donkey gut microbiology and clarify the role of the gut in maintaining overall health.

Data availability statement

The raw sequencing data is publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov/>, accession number: PRJNA1257860.

Ethics statement

The animal studies were approved by the Animal Care and Use Committee of China Agricultural University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YW: Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft. XL: Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft. ZL: Data curation, Methodology, Validation, Writing – review & editing. QH: Data curation, Methodology, Validation, Writing – review & editing. TH: Data curation, Methodology, Validation, Writing – review & editing. QZ: Formal analysis, Methodology, Validation, Writing – review & editing. HQ: Validation,

Writing – review & editing. HZ: Investigation, Validation, Writing – review & editing. YQ: Investigation, Validation, Writing – review & editing. DS: Writing – review & editing. QM: Writing – review & editing. SH: Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

YQ was employed by the Beijing Sunlon Technology Bio-Breeding Innovation Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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