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Changes in intestinal microbiota, immunity and metabolism caused by mixed *Lactiplantibacillus plantarum* and *Bacillus subtilis*-fermented feed in Bamei pigs

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Abstract

Background The Chinese pig breed Bamei faces numerous challenges, such as antibiotic abuse, feed shortages, weaning stress, low immunity and disease resistance after weaning. Probiotic-fermented feed is an ideal profile that can improve the intestinal microbiota, promote the digestion and absorption of nutrients, and improve immunity. However, the combined effect of long-term intake of probiotic-fermented feeds on the intestinal microbiota, intestinal metabolic profiles, and immunity in pigs is not well understood. Here, we investigated the effects of feeding basal feed, *Lactiplantibacillus*-fermented feed, *Bacillus subtilis*-fermented feed, mixed-fermented feed, and antibiotic-added feed for 100 days on the gut microbiota, immunity, and metabolism of Bamei pigs after feeding five different fermented feeds by using 16S rDNA high-throughput sequencing, enzyme-linked immunoassay, and untargeted metabolomics, respectively.

Results 16S rDNA sequencing revealed that after the piglets were fed five different feeds for 50 days, the structure of the intestinal microbiota of the Bamei pigs was significantly altered, and feeding the mixed *Lactiplantibacillus* (L.) *plantarum* and *Bacillus* (B.) *subtilis*-fermented feed not only increased the α-diversity of the intestinal microbiota and the relative abundance of *Lactobacillus*, but also suppressed the growth of the conditional pathogens, *Clostridium* and *Streptococcus*. The Sobs and Shannon indices were significantly lower ($p < 0.05$) on Day 10 in Group A, which was fed feed supplemented with antibiotics. Feeding mixed-fermented feed not only significantly increased the production of anti-inflammatory cytokines, but also significantly decreased the production of several proinflammatory cytokines and inhibited the TLR4/MyD88/NF-κB inflammatory-related signaling pathway ($p < 0.05$), even more so than antibiotics. The results of untargeted metabolomics showed that feeding mixed-fermented feed improved the metabolism of Bamei pigs by increasing the content of narceine and alpha-cephalin; promoting bile secretion; and facilitating the synthesis of phenylalanine, tyrosine, and steroid hormones. ATP-binding cassette (ABC) transporters were significantly enriched in the antibiotic group.

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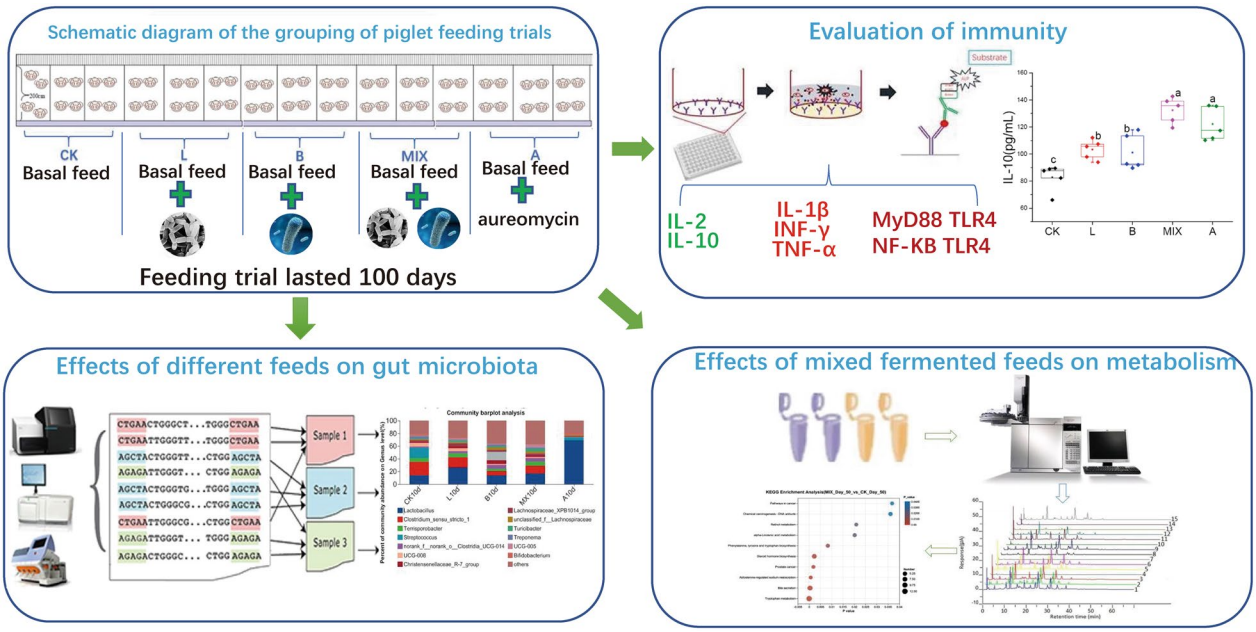
Conclusion The mixed *L. plantarum* QP28-1a and *B. subtilis* QB8a-fermented feed not only improved the intestinal microbiota structure and metabolic profiles and regulated the metabolic pathways of tryptophan, phenylalanine, and steroid hormone biosynthesis, but also improved the immunity of Bamei pigs. This research provides an ideal, healthful, and environmentally sustainable approach for Bamei pig breeding and conservation.

Keywords Bamei pigs, Probiotics, Lactic acid bacteria, *Bacillus*, Fermented feed, Intestinal microbiota, Metabolites, Immunity

Graphical Abstract



Background

Bamei pigs (*Sus scrofa*) are mainly distributed in the western provinces of China, such as Qinghai and Gansu, and are favored by the local people for their outstanding traits, delicious meat, strong environmental adaptability, and tolerance of coarse feeding [1, 2]. However, currently, Bamei pigs are facing many challenges, including a lack of feed, high costs for transportation and storage, high-cold and high-altitude growing environments, excessive use of antibiotics, weaning stress, and the weak resistance of piglets, which can lead to weight loss, diarrhea, and even death [3–6]. In particular, weaning stress causes intestinal disorders, low feed intake, low immunity, diarrhea and other problems, and growth performance significantly decreases, which seriously threatens the healthy growth of piglets [7]. To alleviate weaning stress, antibiotics are often added to piglet nursery feeds to stimulate the establishment of the immune system, improve antimicrobial resistance, and promote the growth of piglets, but the overuse of antibiotics also causes serious problems such as the increased resistance of pathogens, drug residues in meat products, and environmental pollution, which poses a direct threat to human health [8].

Accumulated evidence indicates that probiotics and their metabolites have a positive effect on improving performance and preventing disease in livestock and have the potential to replace antibiotics [9]. It has been reported that probiotics can stabilize the gastrointestinal barrier, regulate the function of intestinal microbial homeostasis, produce antimicrobial substances, promote the activity and absorption of digestive enzymes, regulate the immune system, and inhibit the ability of pathogens to colonize and infect mucous membranes [10]. At the same time, probiotics also have a variety of functions such as antioxidant, anti-inflammatory, anti-allergic and antiviral [9], and contribute to the prevention of a variety of diseases, including diabetes, obesity, cancer, cardiovascular disease and inflammatory bowel disease [11]. Therefore, the application of probiotics in the animal feeding industry has long-term development prospects.

Probiotic-fermented feed not only improves the absorption level of feed nutrients and the degradation of antinutritional factors and toxic and hazardous substances that may exist in the feed, but also promotes animal growth, maintains the microecological balance of the intestinal tract, and improves the immunity of animals [12, 13]. The most widely used probiotics in animal breeding mainly include lactic acid bacteria (LAB), *Bacillus*, and yeast [14]. The main effects of LAB-fermented feed on piglets include **a**. Improving the microecological environment of the digestive tract and maintaining the balance of the gastrointestinal microbiota. After the fermented feed containing LAB enters the gastrointestinal tract, a large number of live bacteria become dominant and grow and colonize rapidly, and the interaction of the flora is conducive to inhibiting the growth of harmful flora and ensuring the microecological balance of the gastrointestinal tract [15]. **b** Providing more nutrients, aromatic odor and good palatability. LAB-fermented feeds can produce organic acids, which cause the fermented feed to have an acidic flavor. Some organic acids can partially remove antinutritional factors in feeds [16], reduce the content of phytic acid and crude fiber, and increase the palatability of feeds [17]. **c** Increasing immunity in weaned piglets and enhancing disease resistance. Lactic acid produced by LAB can reduce the redox potential and pH in the intestinal tract, and some harmful bacteria gradually die due to changes in the internal environment to prevent disease [18]. On the other hand, as a nonspecific immunomodulator, it can promote the formation of the immune system in the gastrointestinal tract, regulate the expression of immune-related cytokines, increase the level of antibodies in animals, and kill invading pathogens, thus enhancing the resistance of animals to various diseases, and decreasing morbidity and mortality rates [19]. Fermented feed supplemented with *B. subtilis* can produce extracellular enzymes such as protease, cellulase, and amylase, which are beneficial for degrading large-molecule nutrients, such as proteins, polysaccharides, and fats in feed that are difficult for animals to absorb; thus, these enzymes increase digestibility, promote the absorption of nutrients in the intestinal tract, and promote the growth of the animals [20].

16S rRNA high-throughput sequencing technology is an efficient method for studying the structural composition of the intestinal microbiota. Bacteria have conserved and variable regions spaced in the 16S rRNA gene, and the taxonomic characteristics of each bacterium and the relationships between bacteria can be obtained after the high-throughput sequencing of the 16S rRNA of bacteria [21]. Metabolomics is a systematic biological method for the qualitative and quantitative detection and analysis of small-molecule metabolites in organisms using mass spectrometry, high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) [22]. Metabolomics is applicable to the statistical analysis of endogenous and exogenous metabolites and metabolic pathways in organisms, and can effectively and rapidly capture subtle differences in metabolite changes in biological organisms, moreover, it has been widely used in the fields of drug discovery and development [23], disease control [24], and toxicity evaluation [25] and has become an important research tool in the agricultural and animal husbandry industries.

Considering previous studies on the positive effects of probiotics on the gut, immunity, and metabolism, we hypothesized that feeding Bamei pigs probiotic-fermented feeds improved the gut microbiota, immunity, and metabolic profile. Despite numerous studies demonstrating the benefits of probiotic-fermented feeds, the combined effects of the long-term feeding of probiotic-fermented feeds on the intestinal microbiota, immunity, and metabolic profiles of weaned Bamei pigs are not clearly understood. In this study, 16S high-throughput sequencing technology, metabolomics and enzyme immunoassay were combined to analyze the effects of feeding fermented feeds supplemented with potential probiotics on the structure of the intestinal microbiota, intestinal health, immunity, metabolic pathways and metabolite variability of newly weaned Bamei pigs, aiming to investigate the influence and mechanisms of mixed *L. plantarum* QP28-1a and *B. subtilis* QB8a fermented feeds on the intestinal microbiota, immunity, metabolism and health of Bamei pigs, as well as to provide part of the theoretical basis for the breeding of Bamei pigs, the replacement of antibiotics with probiotics, and the protection of Bamei pig breeds.

Materials and methods

Strains and fermentation

L. plantarum QP28-1a and *B. subtilis* QB8a for fermented feeds were supplied by the Ion Beam Bioengineering Laboratory of Zhengzhou University. The strains were isolated and screened from the feces of Bamei pigs on the Tibetan Plateau. *L. plantarum* QP28-1a has broad-spectrum bacteriostatic activity, high acid-producing capacity and good tolerance [26], and *B. subtilis* QB8a possesses broad-spectrum bacteriostatic activity and high cellulase activity.

Basal feed: The basal feeds were prepared with reference to the "Pig Feed Standard NY/T 65-2004" promulgated by the Chinese Ministry of Agriculture and the method of Wu et al. [2]. The materials and nutritional levels are shown in Table 1.

Table 1 Ingredient composition and nutrient of the basal feed

Material	(w/w %)	Nutrition level[2]	
Premix[1]	1	Metabolizable energy (kcal/kg)	3085
Lysine	0.16	Methionine (%)	0.22
Dicalcium phosphate	0.32	Available phosphorus (%)	0.23
Salt	0.52	Total phosphorus (%)	0.47
Rape stalk	0.83	Ca (%)	0.60
Stone powder	1.15	Lysine (%)	0.75
Soybean oil	2.58	Crude protein (%)	14.00
Rapeseed meal	4.10		
Alfalfa silage	10.50		
Wheat bran	14.51		
Soybean meal	11.15		
Corn	53.15		

[1] Premix provided per kilogram of diet: VA 16000 IU, VD 33000 IU, VE 35 mg, VK 33 mg, VB 12.5 mg, VB 26 mg, VB 63 mg, VB 120.25 mg, Nicotinic acid 25 mg, pantothenic acid 15 mg, biotin 0.15 mg, Cu 150 mg, Fe 80 mg, Zn 80 mg, Mn 10 mg, Se 0.2 mg

[2] Nutrient levels were measured by near-infrared reflectance spectroscopy

Table 2 Trial grouping and addition of bacteria or antibiotics

Group	Feed formula	Concentration of additive
CK	Basal feed	–
L	Basal feed + *L. plantarum* QP28-1a	1×10^6 CFU/g
B	Basal feed + *B. subtilis* QB8a	1×10^6 CFU/g
MIX	Basal feed + *L. plantarum* QP28-1a + *B. subtilis* QB8a	1×10^6 CFU/g (1:1)
A	Basal feed + Aureomycin (antibiotic)	50 mg/kg

Antibiotic feed: Fifty milligrams of Aureomycin was added to each kg of basal feed to obtain antibiotic feed, as shown in Table 2.

Fermented feed: Fifty kilograms of water was added to every 100 kg of basal feed, and different bacterial solutions were added according to the grouping in Table 2, mixed well, packed into 100-L plastic buckets, and then sealed. The Bamei pigs were fed after fermentation for 7 days at room temperature (15–25 °C). Three tons of each kind of feed were prepared. The total number of days of the fermentation cycle of the feeds was 107 days, and the Bamei pigs were fed for 100 days and then slaughtered.

Animals and experimental design

The feeding trial was carried out with 60 crossbred 30-day-old Bamei pigs (Duroc × Long White × Bamei) with similar genetic backgrounds and an initial weight of approximately 10 kg. All 60 Bamei pigs were randomly assigned to five treatment groups ($n = 12$) according to Table 2. The control check (CK) group was fed basal feed; Group L was fed *L. plantarum* QP28-1a-fermented feed; Group B was fed *B. subtilis* QB8a-fermented feed; Group MIX was fed a mixed-fermented feed (the ratio of strains added was 1:1), and Group A was fed antibiotic feed. The piglets were numbered and vaccinated after deworming, and the pens were cleaned and disinfected regularly.

The whole feeding trial lasted for 100 days, and the Bamei pigs were given free access to water during the trial. The pigs were fed four times a day at 8:00, 11:00, 15:00, and 19:00, and the feed intake and feed residue were recorded for each treatment group, using the small amount of feed remaining as a criterion for feed intake. The feces of all groups were collected on the 10th and 50th days of the feeding trial, and nine tubes of fresh fecal samples were collected from each treatment group for high-throughput sequencing analysis and microbiology and metabolomics analysis. After 100 days of feeding, the pigs were slaughtered and nine tubes of spleen samples were collected from each treatment group for the detection of immune-related cytokine production. All the samples were stored in an ultralow temperature refrigerator at – 80 °C for later use.

Extraction of fecal genomic DNA and sequencing of 16S rDNA

To investigate the effect of feeding five different feeds on the intestinal microbiota of Bamei pigs, the high-throughput sequencing of fecal samples after 10 and 50 days of feeding was performed, with three fecal samples randomly selected from each treatment group. Total genomic DNA was extracted from the fecal samples of Bamei pigs using a DNA Kit D3350-02 (Omega Biotek, Norcross, GA, USA). After checking the quality of the extracted DNA using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), the 16S rDNA V3–V4 variable region of the fecal samples was amplified according to the method of Wang et al. [27], using primers 338F (5′-ACTCCTACGGGAGG CAGCAG-3′) and 806R (5′-GGACTACHVGGGTWT CTAAT-3′). PCR products were purified, quantified and then analyzed by sequencing using Illumina's MiSeq PE300 platform (Shanghai Majorbio Biopharm Technology Co. Ltd., China).

Sequence analysis of high-throughput sequencing of fecal samples

The sequencing data were analyzed using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 16 October 2023), and then the FLASH software FLASH (http://ccb.jhu.edu/software/FLASH/, accessed on 25 October 2023) was used to assemble the clean reads into contigs. Low-quality sequences were removed using QIIME software (version

1.9.1, the Knight and Caporaso laboratories, University of Colorado, USA). Sequences were clustered into operational taxonomic units with 97% similarity using Uparse (version 7.0.1001; https://drive5.com/uparse/, accessed on 16 October 2023) and operational taxonomic units (OTUs) were obtained [28]. The Silva Database (http://www.arb-silva.de/, accessed on 21 October 2023) was used to annotate taxonomic information [29]. The α-diversity index and β-diversity were calculated and analyzed using Mothur software (http://www.mothur.org, accessed on 16 October 2023) and QIIME software (version 2.15.3). Linear discriminant analysis (LEfSe) was performed using Python software (version 2.7, https://www.python.org/, accessed on 26 October 2023) to identify species that differed significantly between treatments.

Evaluation of immunity in Bamei pigs

To assess the effects of five different diets on the immunity of Bamei pigs, nine immune-related cytokines or receptor proteins, namely, interleukin 2 (IL-2), interleukin 10 (IL-10), interleukin 1β (IL-1β), interferon γ (INF-γ), tumor necrosis factor α (TNF-α), Toll-like receptor 2 (TLR2), myeloid differentiation factor 88 (MyD88), Toll-like receptor 4 (TLR4), and nuclear factor kappa B (NF-KB), were selected as indicators of immunity. All 60 pigs were slaughtered after 100 days of feeding different diets, and the spleens of nine individual pigs from each treatment group were randomly sampled, for a total of 45 spleen samples. The spleen samples were cut into small pieces and stored in sterile tubes frozen in liquid nitrogen. Five spleen samples were randomly selected from each treatment group ($n=5$/group) and assayed for the production of immune-related cytokines and receptor proteins using ELISA kits (Beijing Dogesce Biotechnology Co., Ltd., Beijing, China) according to the methods of Liu et al. [30].

Preparation of samples for nontargeted metabolomics analysis

To compare the effects of feeding different diets on fecal metabolites in Bamei pigs, a total of 20 samples ($n=4$/group) from Day 50 were analyzed using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS, UHPLC-Q Exactive HF-X, Thermo Fisher, MA, USA). Fifty milligrams of Bamei pig fecal sample was accurately weighed in a 2-mL sterile centrifuge tube. One 6-mm-diameter grinding bead was added, and then 400 μL of extraction solution (methanol:water = 4:1) was added for the extraction of metabolites from the fecal samples, which contained 0.02 mg/mL L-2-chlorophenylalanine as an internal standard. The fecal sample solution was placed in a frozen tissue grinder for 6 min (− 10 °C, 50 Hz) and then placed at low temperature for ultrasonic extraction (5 °C, 40 kHz). The sample was then centrifuged at − 20 °C for 30 min to precipitate the proteins. The sample was then centrifuged in a high-speed centrifuge for 15 min (4 °C, 13,000 g), and the supernatant was pipetted into the injection vials for analysis using a UPLC–MS/MS platform.

UHPLC–MS/MS analysis

Chromatographic conditions: The sample injection volume was 3 μL, and a 100 mm × 2.1 mm i.d., 1.8 μm HSS T3 column was used. Mobile phase A was 95% water + 5% acetonitrile (containing 0.1% formic acid), and mobile phase B was 47.5% acetonitrile + 47.5% isopropanol + 5% water (containing 0.1% formic acid). The column temperature was set at 40 °C, and the flow rate was 0.40 mL/min.

Mass spectrometry conditions: Positive and negative ion scanning modes were used, with the mass scanning range set to 70–1050 m/z. The auxiliary gas flow rate was set to 13 psi. The sheath gas flow rate was set to 50 psi, and the auxiliary gas heating temperature was set to 425 °C. The positive-mode ion spray voltage was set to 3500 V, and the negative-mode ion spray voltage was set to − 3500 V. The normalized collision energies were set to 20–40–60 V, and the cyclic collision energy was set. The resolution of the primary mass spectrometer was set to 60,000; the resolution of the secondary mass spectrometer was set to 7500, and the data were collected in DDA mode.

Metabolomics data analysis

The UPLC–MS/MS data were imported into the metabolomics software Progenesis QI (Waters Corporation, Milford, USA) for baseline filtering, peak identification, integration, retention time correction, peak alignment, etc., and then a data matrix was obtained, which consisted of the retention time (RT), mass-to-charge ratio (m/z), and peak intensity. These metabolites were subsequently annotated using HMDB (http://www.hmdb.ca/, accessed on 25 November 2023), the KEGG database (http://www.genome.jp/kegg/, accessed on 3 November 2023), Metlin (https://metlin.scripps.edu/, accessed on 11 November 2023), and the self-constructed database of Shanghai Meiji Biological Co. The matched metabolome data matrix was uploaded to the Meiji cloud platform (cloud.majorbio.com, accessed on 22 November 2023) for metabolomics analysis. The criteria for screening differentially abundant metabolites included the following: 1. metabolites with a VIP ≥ 1 in the OPLS-DA model were considered significant; 2. FC ≥ 2, i.e., metabolites with a onefold or greater difference between the control and experimental groups were considered significant; and 3. p-value < 0.05. Metabolite cluster analysis, Spearman

correlation analysis of differentially abundant metabolites with environmental factors were performed using R 4.0.1 software.

Statistics and analysis

IBM SPSS 22.0 (Chicago, IL, USA) was used to calculate and analyze the data. Significant differences between treatment groups were analyzed by one-way ANOVA using Tukey's post hoc multiple comparisons method, with $p < 0.05$ and $p < 0.01$ indicating significant and highly significant differences, respectively, whereas $p > 0.05$ indicated that no significant differences existed. The experiments for each sample were carried out using three to five repeats, and the calculated and measured values are expressed as the mean ± standard deviation.

Results

Effects of different feeds on gut microbial diversity of Bamei pigs

The high-throughput gene sequencing of 16Sr RNA in fecal bacterial DNA on Days 10 and 50 was performed to compare the effects of different feeds on the structure of the intestinal microbiota of the Bamei pigs. The number of reads per sample in the raw data was not less than 3.86×10^5, and after quality control, the sequences were clustered into 1565 OTUs with 97% similarity. The α-diversity of the bacterial community was assessed using the Sobs index and Shannon index. After the feed fermentation test lasted for 10 days, the Sobs index and Shannon index of Group A with added antibiotics were significantly lower ($p < 0.05$) than those of the other groups (Fig. 1A, B), and the diversity indices of Groups L, B, and MIX were slightly greater than those of Group CK. By the 50th day (Fig. 1C, D), there was no significant difference in the Sobs index between the groups, while the Shannon index was significantly lower ($p < 0.05$) in the Group CK than in the other groups.

Principal component analysis (PCA) was used to visualize differences in gut bacterial community structure and diversity between groups (Fig. 1E, F). The results showed that after feeding five different feeds, the representative points were significantly separated between groups and clustered within groups, indicating that feeding different diets significantly altered the composition of the bacterial microbiota in the feces of the Bamei pigs. The results of the hierarchical clustering analysis (Fig. 1A) of the bacterial community structure of all fecal samples ($n = 30$) also showed the samples from the same treatment group clustered together, with the different treatment groups being farther apart. Overall, feeding fermented feeds supplemented with potential probiotic strains increased the diversity of the gut microbiota in Bamei pigs, whereas feeding feeds supplemented with antibiotics significantly decreased the diversity of the gut microbiota.

Comparison of the gut microbial composition in Bamei pigs

To investigate the microorganisms contained in the different treatment groups as well as their relative abundance, community bar graphs were used to analyze the composition of the gut microbiota at the genus level on Days 10 and 50, as shown in Fig. 2B and C. The relative abundance of *Lactobacilli* was greater in Group L (27.1%), Group B (15.1%), Group MIX (17.1%), and Group A (59.3%) than in the Group CK (13.2%). The relative abundance of *Clostridium* in Groups L, B, and MIX was significantly lower than that in Group CK. *Streptococcus* disappeared in Groups L, B, and MIX treated with the potential probiotics. *Pediococcus* (14.1%) dominated only in Group B. On Day 50, the relative abundance of *Lactobacilli* was maintained at 9.6%, 9.5%, 9.5%, and 11.3% in Groups L, B, MIX, and A, respectively, which was significantly greater than that of the Group CK (1.3%). The relative abundance of *Streptococcus* was much greater in Groups CK and A. The relative abundance of *Clostridium* was greater in Group B than in Group B on Day 10.

Linear discriminant analysis effect size (LEfSe) analysis was employed to assess the relative abundance of the fecal microbiota in the five groups. LEfSe analysis on Day 10 revealed 32 significant taxa between the groups (LDA > 3) (Fig. 2D). Group CK was enriched with Clostridiaceae and Streptococcaceae families, *Clostridium*_sensu_stricto_1_group and *Streptococcus* genera; Group L was enriched with Clostridiaceae family; Group B was enriched with *Pediococcus* genus and Christensenellaceae, Eggerthellaceae and Anaerovoracaceae families; Group MIX was enriched with Erysipelotrichaceae and Oscillospiraceae families, *Terrisporobacter*, *Turicibacter* and *Romboutsia* genera; Group A was enriched with Lactobacillaceae and Coriobacteriaceae families, *Collinsella*, *Prevotella* and *Catenibacterium* genera. LEfSe analysis on Day 50 revealed 26 significant taxa between the groups (LDA > 3) (Fig. 2E). Group CK was enriched with Streptococcaceae family and *Streptococcus* genus; Group L was enriched with *Prevotella* genus; Group B was enriched with Clostridiaceae and Atopobiaceae families, *Clostridium*_sensu_stricto_1, *Pediococcus* and *Cellulosilyticum* genera; Group MIX was enriched with Erysipelotrichaceae family, *Turicibacter* and *Romboutsia* genera; Group A was enriched with Oscillospiraceae, and Bifidobacteriaceae families, *Bifidobacterium*, *Prevotellaceae*_NK3B31_group, *Blautia* and *Oscillospira* genera.

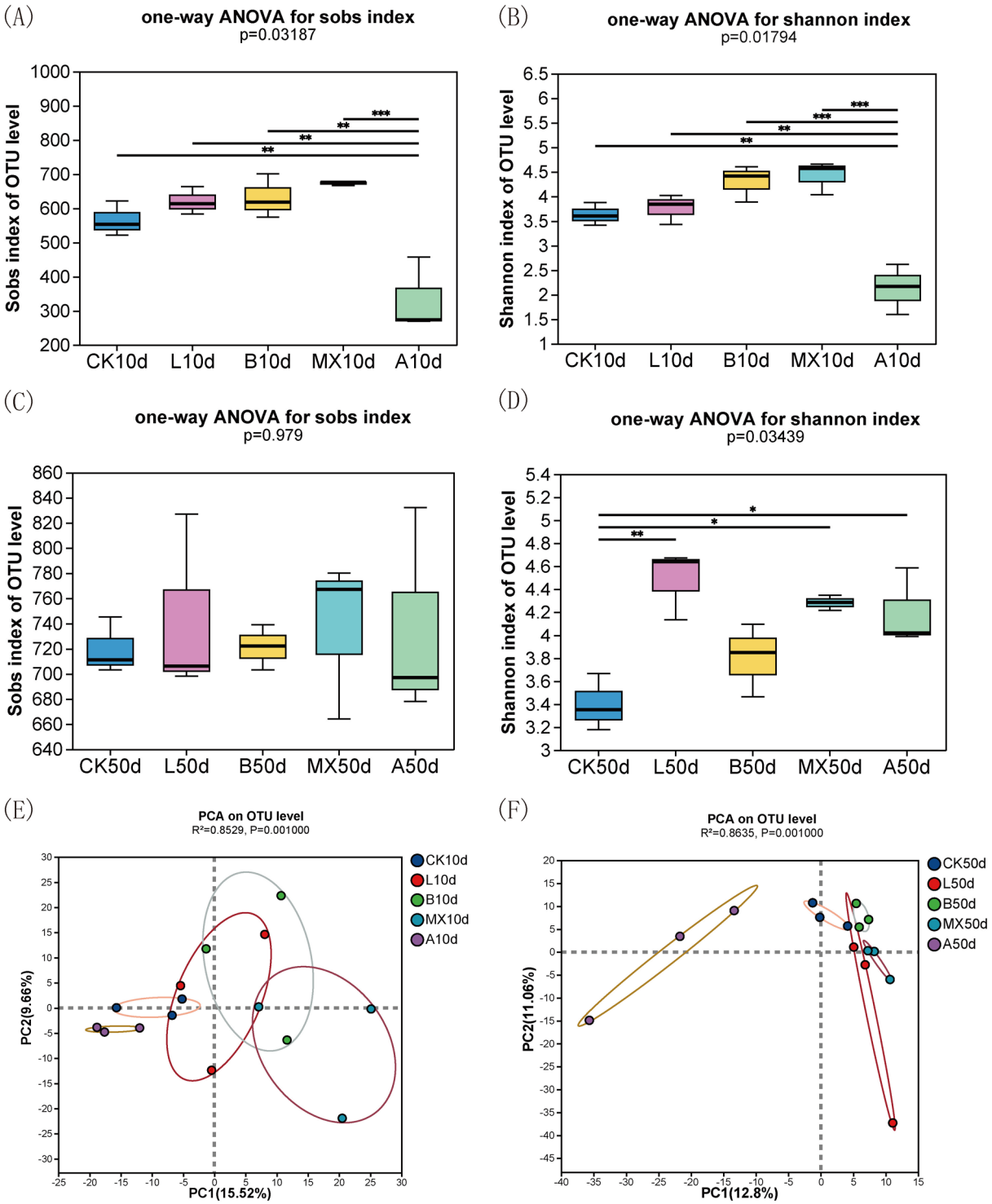


Fig. 1 Effect of feeding five different diets on α-diversity of the intestinal microbiota of Bamei pigs and PCA analysis. **A** Sobs index of OUT level on day 10; **B** Shannon index of OUT level on day 10; **C** Sobs index of OUT level on day 50; **D** Shannon index of OUT level on day 50; **E** PCA analysis of bacterial community on OUT level at day 10; **F** PCA analysis of bacterial community on OUT level at day 50

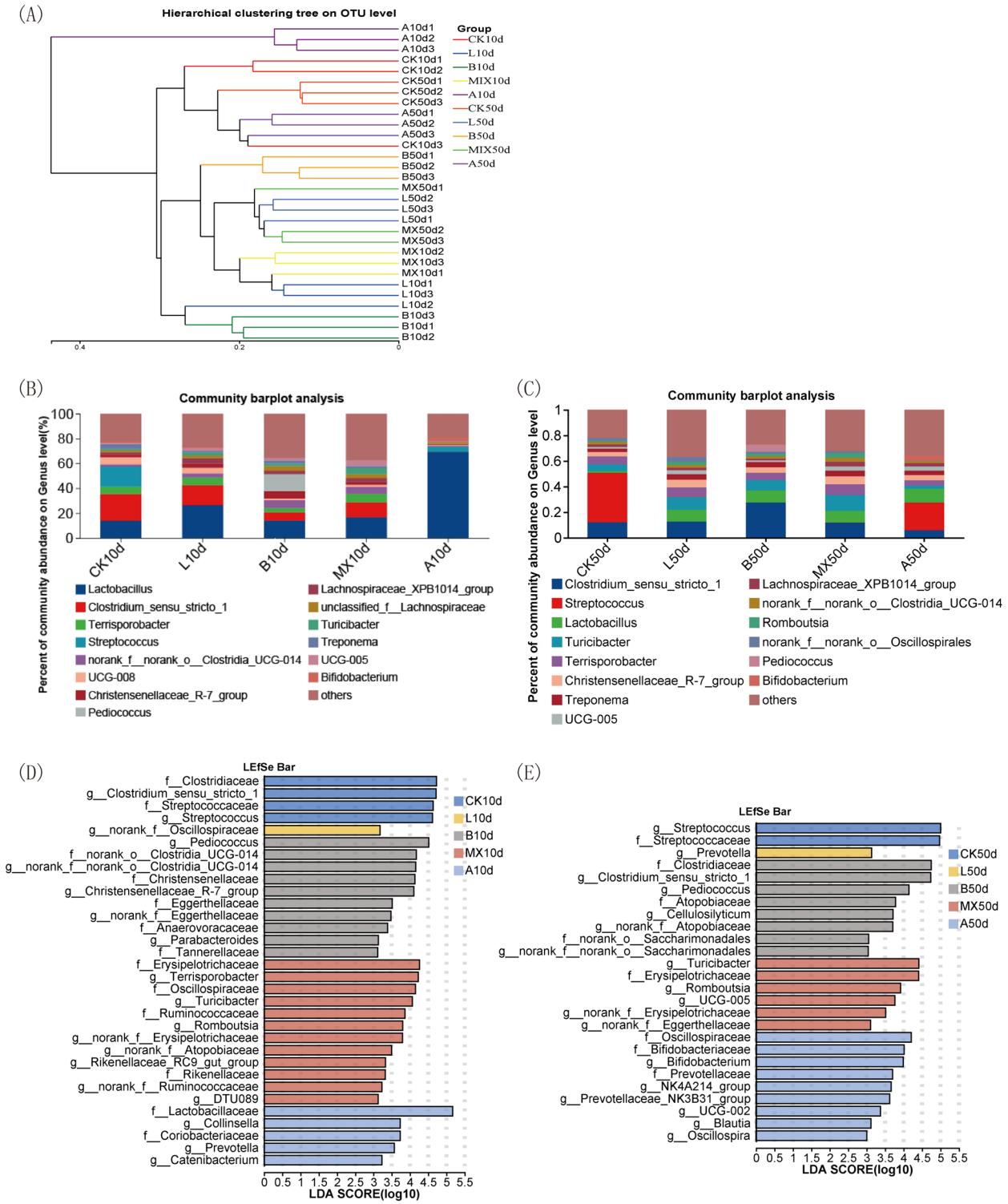


Fig. 2 Analysis of the composition of the intestinal bacterial community of the Bamei pigs after feeding 10 and 50 days. **A** Hierarchical clustering analysis of the bacterial composition of all feces samples; **B** bacterial taxonomic distributions and relative abundances on genus level at day 10; **C** bacterial taxonomic distributions and relative abundances on genus level at day 50; **D** comparison of bacterial variations using LEfSe analysis at day 10; **E** comparison of bacterial variations using LEfSe analysis at day 50

Effect of fermented feed on the immunity of Bamei pigs

The ELISA results showed that using the five different feeds for 100 days significantly affected the production of immune-related cytokines in the spleens of Bamei pigs, as shown in Fig. 3. The production of IL-2 and IL-10 was significantly greater ($p < 0.05$) in Groups L, B, and MIX, which were fed fermented feeds, and in Group A, which was fed antibiotic feed, than in Group CK, which was fed basal feed. IL-1β, INF-γ, TNF-α, TLR2, TLR4, MyD88, and NF-κB were all lower in Groups L, B, MIX, and A than in Group CK, and it is particularly noteworthy that IL-1β, INF-γ, TLR2, TLR4, MyD88, and NF-κB were significantly lower in Group MIX than in Group CK ($p < 0.05$).

Comparison of the fecal metabolic profiles of Bamei pigs

The metabolic profiles of the fecal samples from each treatment group on Day 50 were analyzed using the UPLC–MS/MS platform. The results of total metabolome ion count and metabolite identification showed (Table 3) that a total of 6599 mass spectrometry peaks were detected and that 1663 metabolites were identified in positive ion mode, while 8560 mass spectrometry peaks were detected and 1113 metabolites were identified in

Table 3 Statistics of total ion number and identification of metabolites in fecal samples of Bamei pigs after feeding 50 days

Ion mode	All peaks	Identified metabolites	Metabolites in library	Metabolites in KEGG
pos	6599	1663	1514	866
neg	8560	1115	1076	519

(1) Ion mode: the ion mode of the substance detected by the mass spectrometer; (2) all peaks is the number of mass spectral peaks extracted by the software; (3) identified metabolites: the number of metabolites identified after comparing the mass spectral data with databases; (4) metabolites in library: the number of metabolites annotated to public databases such as HMDB and Lipidmaps; (5) metabolites in KEGG is the number of metabolites annotated to the KEGG database

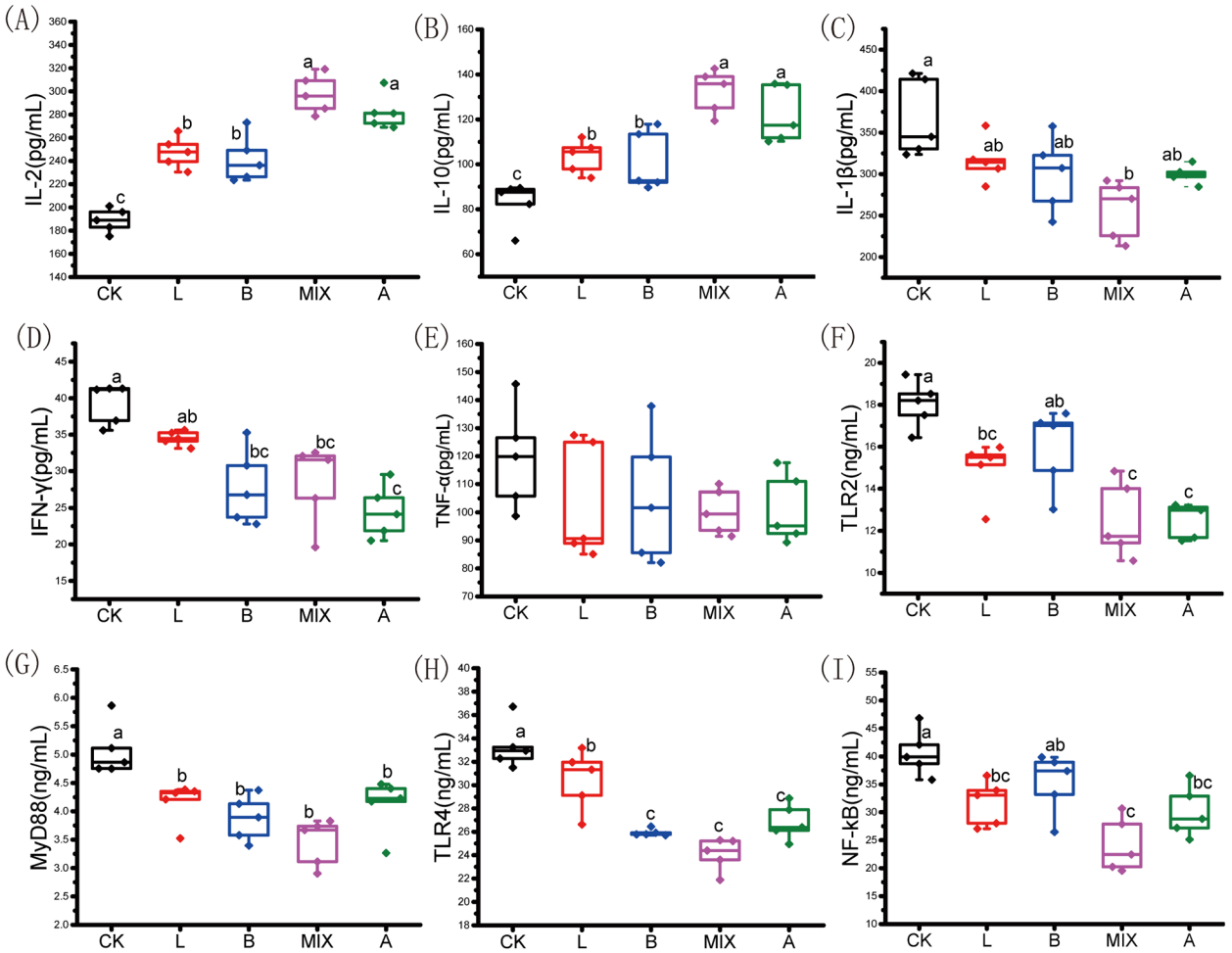


Fig. 3 Effect of feeding different feeds for 100 days on immunity of the Bamei pigs. Production of the immune-related cytokine **A** IL-2, pg/mL; **B** IL-10, pg/mL; **C** IL-1β, pg/mL; **D** INF-γ, pg/mL; **E** TNF-α, pg/mL; **F** TLR2, ng/mL; **G** MyD88, ng/mL; **H** TLR4, ng/mL; **I** NF-KB, ng/mL

negative ion mode. Based on the metabolic profiles of fecal samples from different fecal treatment groups, principal component analysis (PCA) was performed to evaluate the similarity of samples within groups and the difference in samples between groups. As shown in Fig. 4A and B, the samples within the different treatment groups were close to each other, whereas there was a significant difference between the treatment groups, indicating that feeding different feeds can significantly affect the metabolic profiles of Bamei pigs. The QC samples were centered and clustered together, indicating that the UPLC–MS/MS system used for the metabolome assay was stable and that the data were reliable. Venn diagrams revealed 1304 cationic metabolites and 961 anionic metabolites in the cecum of the six treatment groups. Among them, the CK group on the 0th day had the most unique metabolites.

Variable importance in projection (VIP) analysis and clustered heatmaps were jointly used to visualize the differentially abundant metabolites in Groups L, B, MIX, and A compared to Group CK. After 50 days of feeding, the levels of the metabolites miroprofen, dukunolide D, 3-hydroxykynurenamine, alizarin complexone, and narceine were significantly greater ($p < 0.05$) in the feces of Group L than in those of Group CK (Fig. 5A); the levels of neolinustatin, alpha-cephalin, 1,6-anhydro-N-acetyl-beta-muramate, alizarin complexone, aciclovir, narceine, aflatoxin B2, and avenanthramide 1p were significantly greater ($p < 0.05$) in Group B (Fig. 5B); the levels of 3-methyl-3-butenyl apiosyl-(1–6)-glucoside,

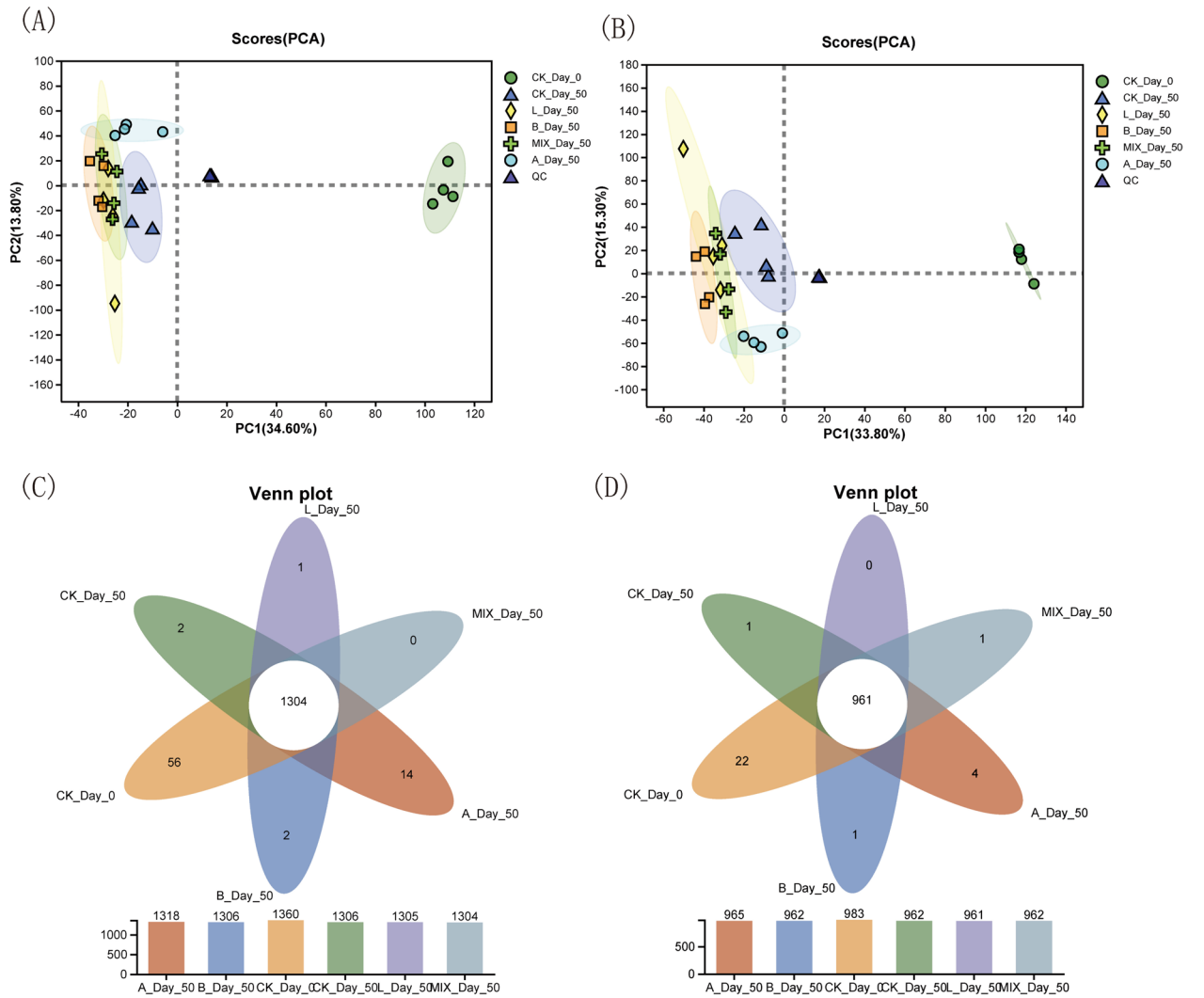


Fig. 4 Comparison of metabolites in the feces of Bamei pigs after 50 days of feeding different diets. **A** PCA analysis of cationic metabolites; **B** PCA analysis of anionic metabolites; **C** Venn diagram of cationic metabolites; **D** Venn diagram of anionic metabolites

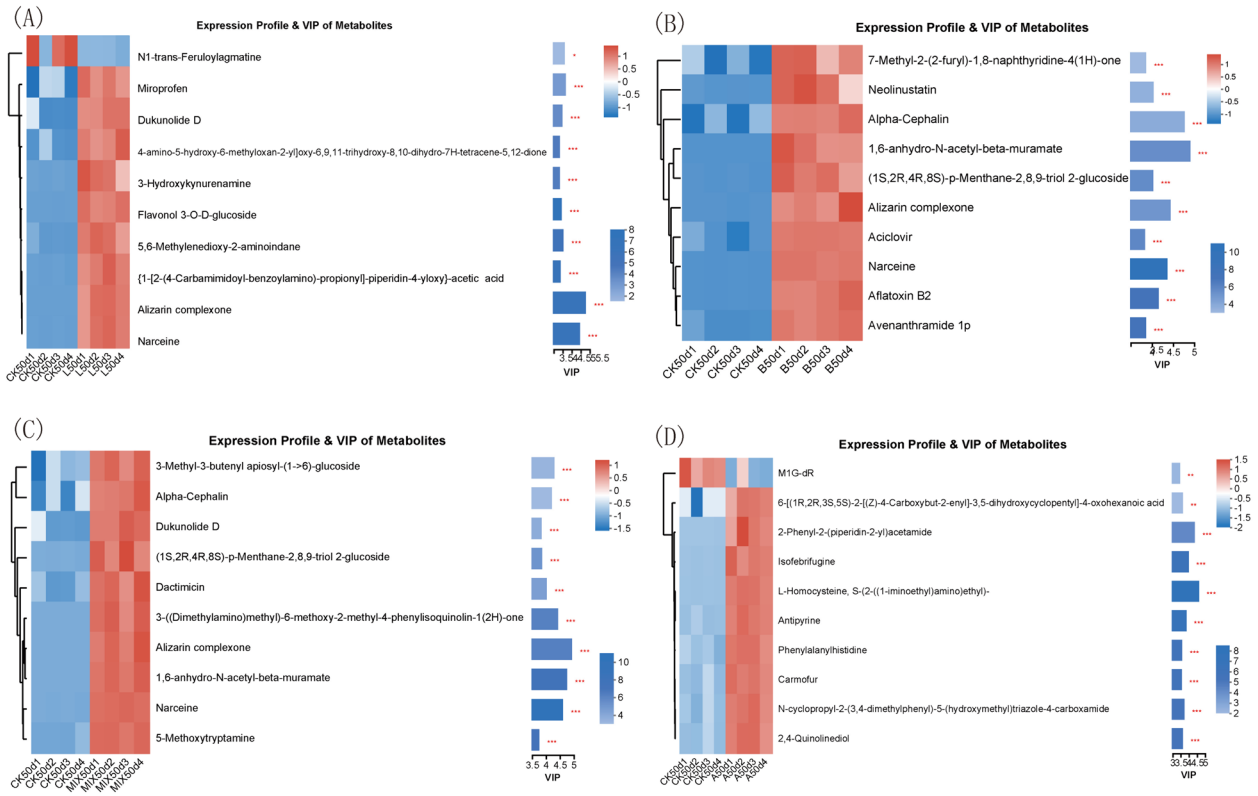


Fig. 5 **A** Expression profile and VIP of metabolites between the L and CK groups; **B** expression profile and VIP of metabolites between the B and CK groups; **C** expression profile and VIP of metabolites between the MIX and CK groups; **D** expression profile and VIP of metabolites between the A and CK groups. Differences were considered statistically significant at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ level

alpha-cephalin, dukunolide D, alizarin complexone, 1,6-anhydro-N-acetyl-beta-muramate, and narceine were significantly greater ($p < 0.05$) in Group MIX (Fig. 5C); and the levels of 2-phenyl-2-(piperidin-2-yl) acetamide, isofebrifugine, antipyrine, phenylalanylhistidine, and carmofur were significantly greater ($p < 0.05$) in the group (Fig. 5D).

Correlation analysis of differentially abundant metabolites with differential gut microbiota and immune-related cytokines

Correlation analyses of differential fecal metabolites with differential fecal gut microbiota or immune-related cytokines were performed to determine the relationships between differentially abundant metabolites and differential microbiota or immunity. The results of Pearson correlation analysis of differential fecal metabolites with immune-related cytokines (Day 50) showed (Fig. 6A) that the immune factor IL-2 was significantly positively correlated with the differentially abundant metabolites nona-4,7-dienedioylcarnitine ($r = 0.54$; $p < 0.01$), and 9-F1-phytoprostane ($r = 0.57$; $p < 0.05$) and significantly negatively correlated with 6-hydroxyhexanoic acid ($r = -0.46$; $p < 0.01$) and (±)-(Z)-2-(5-tetradecenyl) cyclobutanone ($r = -0.46$; $p < 0.01$). The immune factor IL-10 showed a significant positive correlation with cyclophosphamide ($r = 0.44$; $p < 0.05$) and 9-F1-phytoprostane ($r = 0.49$; $p < 0.05$) and a significant negative correlation with 6-hydroxyhexanoic acid ($r = -0.57$; $p < 0.01$), xanthine ($r = -0.49$; $p < 0.01$). IFN-γ was significantly positively correlated with 6-hydroxyhexanoic acid ($r = 0.51$; $p < 0.05$), oxypurinol ($r = 0.56$; $p < 0.01$), and xanthine ($r = 0.58$; $p < 0.01$). MyD88 was significantly positively correlated with 6-hydroxyhexanoic acid ($r = 0.50$; $p < 0.05$). IL-1β was significantly positively correlated with 6-hydroxyhexanoic acid ($r = 0.69$; $p < 0.01$), xanthine ($r = 0.49$; $p < 0.05$). TLR4 was significantly positively correlated with 6-hydroxyhexanoic acid ($r = 0.54$; $p < 0.05$) and (±)-(Z)-2-(5-tetradecenyl) cyclobutanone ($r = 0.46$; $p < 0.05$) and was significantly negatively correlated with cyclophosphamide ($r = -0.45$; $p < 0.05$) and 9-F1-phytoprostane ($r = -0.60$; $p < 0.01$). TLR2 was significantly positively correlated with xanthine ($r = 0.48$; $p < 0.05$) and with (±)-(Z)-2-(5-tetradecenyl) cyclobutanone ($r = 0.65$; $p < 0.01$) and was significantly negatively correlated with cyclophosphamide ($r = -0.46$; $p < 0.05$).

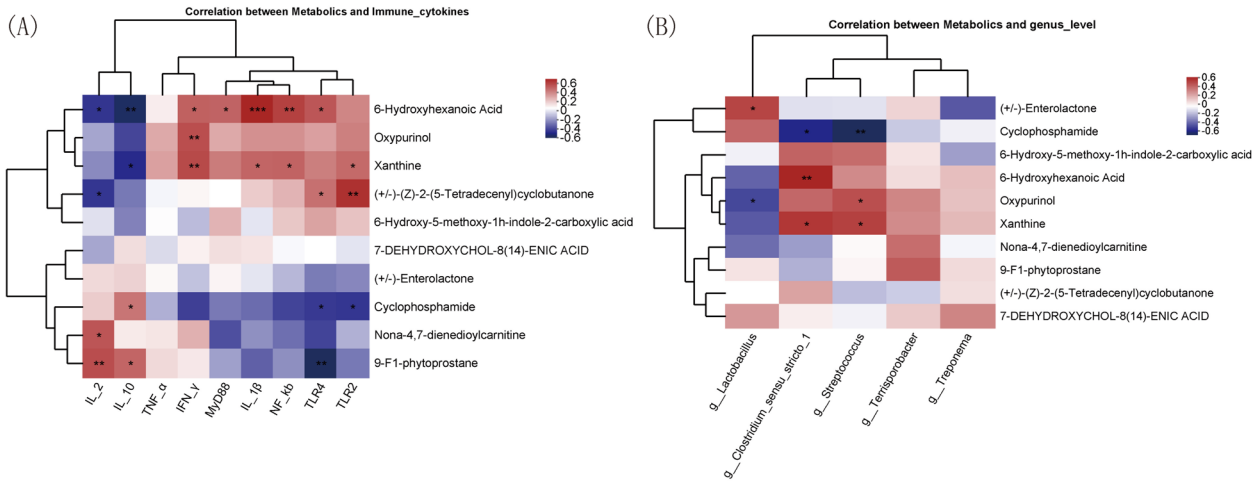


Fig. 6 **A** Pearson correlation analysis between differential metabolites and immune-related cytokines; **B** Pearson correlation analysis between differential metabolites and differential gut microbiota on genus level

The results of a Pearson correlation analysis of differentially abundant metabolites with gut differential microbiota in feces showed (Fig. 6B) that g_*Lactobacillus* was significantly positively correlated with (±)-enterolactone (r = 0.49; $p < 0.05$) and significantly negatively correlated with oxypurinol (r = -0.47; $p < 0.05$). g_ *Clostridium*_ sensu_stricto_1 showed a significant positive correlation with 6-hydroxyhexanoic acid (r = 0.60; $p < 0.01$) a xanthine (r = 0.40; $p < 0.05$) and a significant negative correlation with cyclophosphamide (r = -0.56; $p < 0.05$). The relative abundance of g_*Streptococcus* was significantly positively correlated with that of oxypurinol (r = 0.46; $p < 0.05$) and xanthine (r = 0.51; $p < 0.05$) significantly negatively correlated with that of cyclophosphamide.

Effects of different feeds on intestinal metabolic pathways in Bamei pigs

The results of metabolic pathway enrichment analysis based on the KEGG database are shown in Fig. 7. The main intestinal metabolic pathways significantly enriched ($p < 0.05$) in Group L compared with Group CK were tyrosine and tryptophan biosynthesis, bile secretion, tyrosine metabolism, tryptophan metabolism. The significantly enriched metabolic pathways ($p < 0.05$) in Group B were arginine biosynthesis, lysine degradation, steroid hormone biosynthesis, bile secretion, and tryptophan metabolism. The significantly enriched metabolic pathways ($p < 0.05$) in Group MIX were phenylalanine, tyrosine and tryptophan biosynthesis, tyrosine metabolism, chemical carcinogenesis-DNA adducts, steroid hormone biosynthesis, bile secretion, and tryptophan metabolism. The main intestinal metabolic pathways significantly enriched in Group A compared to Group CK included ($p < 0.05$) central carbon metabolism in cancer, histidine metabolism, phenylalanine metabolism, ABC transporters, and tryptophan metabolism.

Considering the outstanding effect of mixed *L. plantarum* QP28-1a and *B. subtilis* QB8a-fermented feeds on improving the intestinal microbiota and enhancing immunity, a more detailed metabolic pathway topology analysis based on the KEGG database was implemented. Three metabolic pathways with high importance and significance were screened, as shown in Fig. 8A, including tryptophan metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, and steroid hormone biosynthesis. The number of differentially abundant metabolites enriched in these three metabolic pathways was 8, 5, and 8, respectively, as shown in Fig. 8B–D. In the metabolic pathway tryptophan metabolism, which was significantly enriched in Group MIX compared with the CK group (Fig. 8B), the differentially abundant metabolites 5-methoxy-indoleacetate, 5-hydroxyindole-acetylglycine, 5-methoxy-trytamine, 3-indoleglycol-aldehyde, 4-(2-amino-3-hydroxyphenyl)-2,4-dioxobutanoate, and 3-hydroxy-kynurenamine were upregulated, and the differentially abundant metabolites serotonin, 6-hydroxymelatonin, and N-formyl-anthranilate were downregulated. In the phenylalanine, tyrosine and tryptophan biosynthesis metabolic pathway (Fig. 8C), the differentially abundant metabolites 3-dehydroshikimate, shikimate, shikimate 3-phosohate, and L-arogenate were upregulated and the differentially abundant metabolite phenylpyruvate was downregulated. In the steroid hormone biosynthesis pathway (Fig. 8D), the differentially abundant metabolites 18-hydroxy-corticosterone, tetrahydro-corticosterone, cortisol, urocortisone, and cortolone were upregulated and the differentially abundant metabolite aldosterone was downregulated.

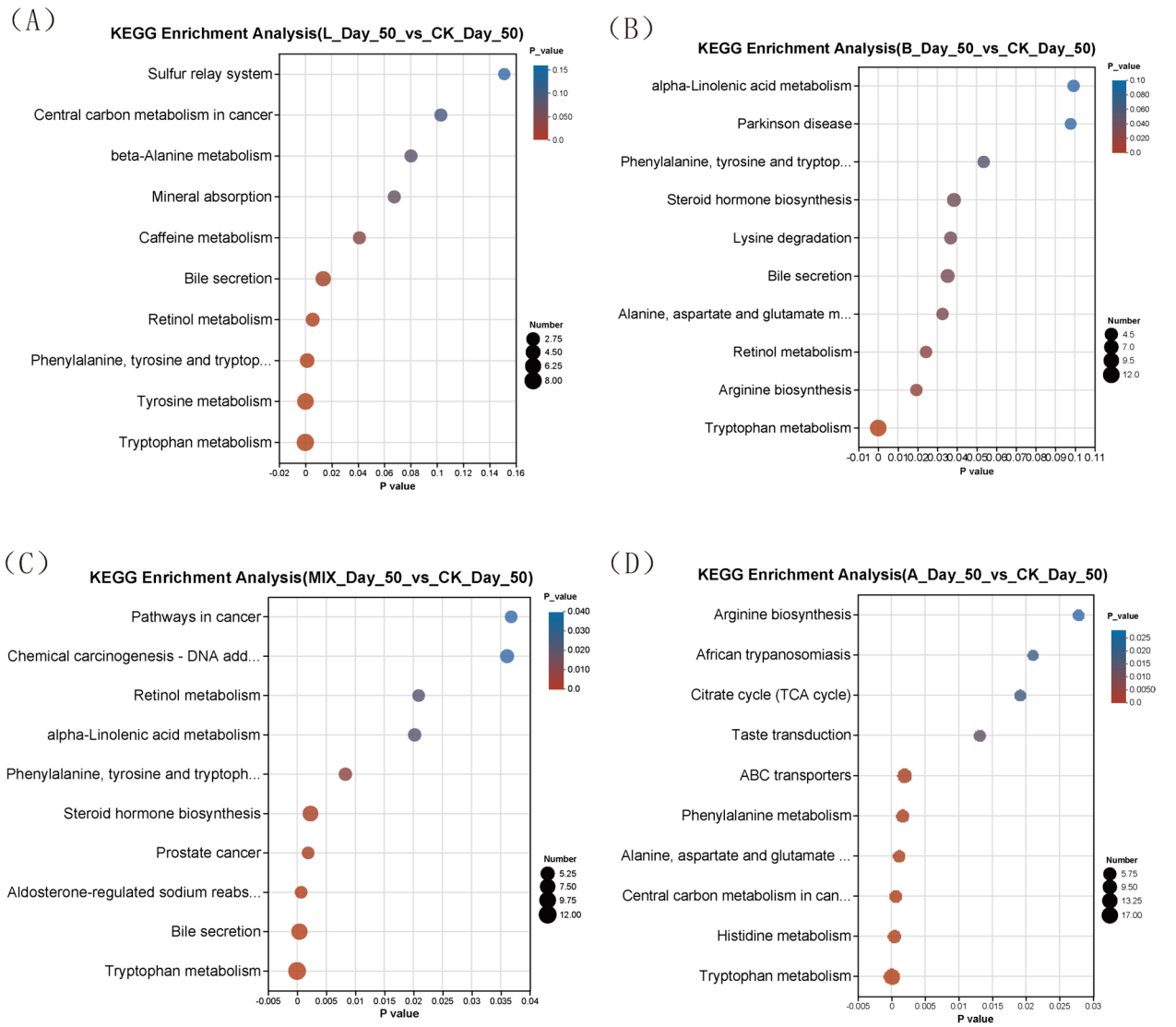


Fig. 7 The top metabolic pathway enrichment analyses based on KEGG database between the CK group and other treatment groups on day 50. **A** Group L VS group CK; **B** group B VS group CK; **C** group MIX VS group CK; **D** group A VS group CK

Discussion

Bamei pig breeding and conservation are facing severe challenges from various factors such as high feed costs, high weaning stress, low immunity after weaning, poor ecological environment, and African swine fever outbreaks. Antibiotics are the main drugs used for preventing and treating diseases. However, the misuse of antibiotics can lead to bacterial resistance and drug residues, which are extremely harmful to human and animal health and to environmental safety. In addition, the long-term use of antibiotics in pigs can further cause a decline in immunity and increase the risk of disease transmission. Therefore, choosing suitable antibiotic alternatives to maintain the symbiotic microenvironment between gut microorganisms and hosts is the key to solving the current problems of sustainable development in the Bamei pig farming industry. The advantages of probiotic-fermented feeds include the inability of antibiotics to maintain intestinal health, enhance immunity, and improve the metabolic profile of pigs.

Fermented feed improves the gut microbiota

Gut health requires a balance of symbiotic bacteria, probiotics and pathogens, and probiotics are vital for regulating the gut microbiota [31]. Feeding fermented feed to Bamei pigs changed the structure of the intestinal microbiota, and both the Sobs index and Shannon index increased, improving the diversity of the

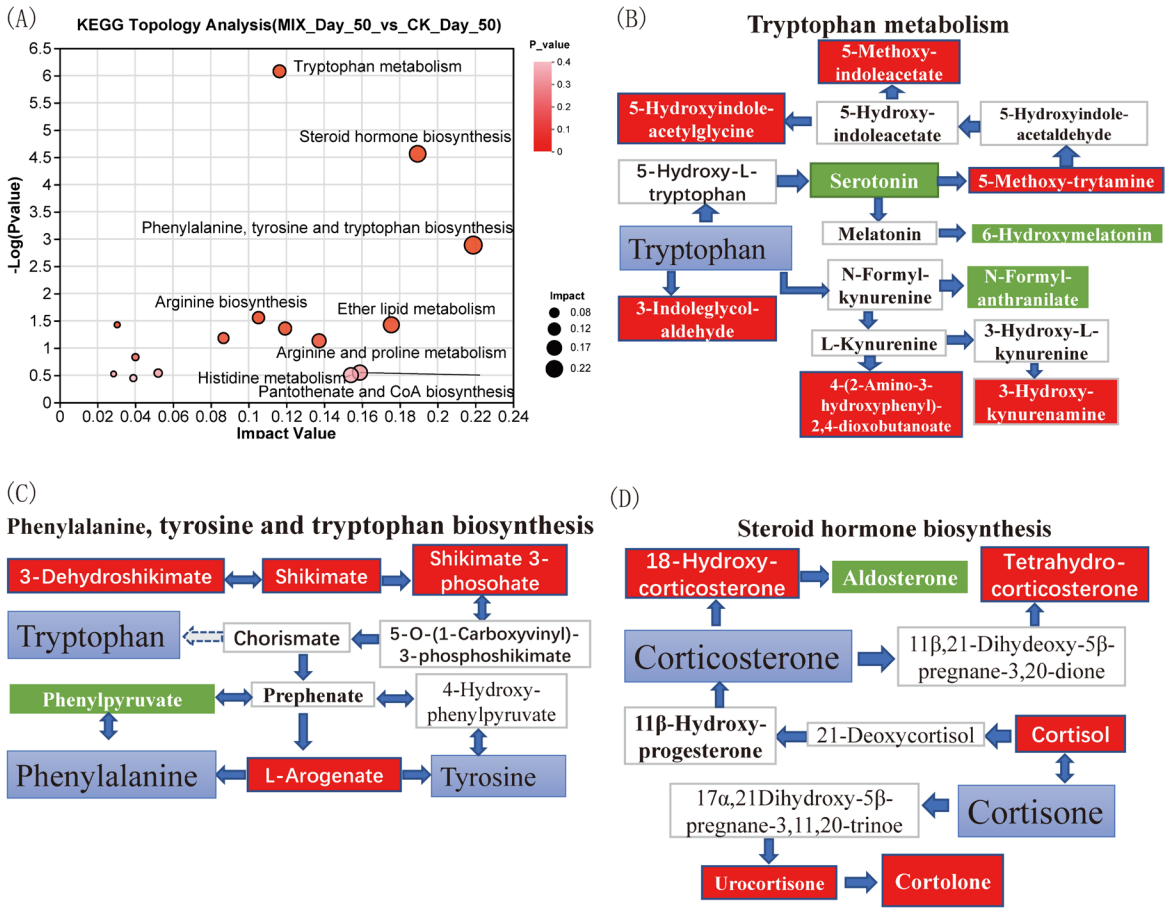


Fig. 8 Metabolic pathway enrichment topology analysis of differential metabolites based on KEGG database after feeding mixed-fermented feeds for 50 days in group MIX. **A** KEGG topology analysis; **B** pathway enrichment network diagram for the metabolic pathway tryptophan metabolism; **C** pathway enrichment network diagram for the metabolic pathway phenylalanine, tyrosine and tryptophan biosynthesis; **D** pathway enrichment network diagram for the metabolic pathway steroid hormone biosynthesis. Note: Each bubble in **A** represents a KEGG Pathway; the horizontal axis represents the importance of metabolites in the pathway; the vertical axis represents the enriched significance of metabolites involved in the pathway -log10 (*p*-value); the size of the bubbles represents the Impact Value. In **B**–**D**, the colored metabolites are the differential metabolites that were enriched, red represents the upregulated metabolites and green represents the downregulated metabolites

gut microbiota, which was beneficial for inhibiting the growth and colonization of pathogens in the intestinal tract, and helping to maintain intestinal balance [32]. Tajima et al. [33] also reported that after feeding fermented feed with *Lactobacilli*, the Chao and Shannon indices of the intestinal microbiota of weaned piglets significantly increased. Notably, feeding potentially probiotic-fermented feeds avoided the damage caused by antibiotics to gut diversity. Fouhy et al. [34] reported that the use of excessive antibiotics resulted in reduced gut microbial diversity and impaired gut health in neonates. *Lactobacillus*-fermented feeds produce many organic acids, such as lactic acid, during fermentation, which decreases the pH of the feed, thus effectively inhibiting the growth of some pathogens. In addition, the number of beneficial bacteria such as *Lactobacilli* in the intestinal tract increases, and the number of harmful bacteria decreases after the animals consume the feed [15]. After entering the gastrointestinal tract of animals, *B. subtilis* consumes a large amount of free oxygen and competitively inhibits with other bacteria, reducing the number of aerobic pathogens, such as *E. coli* and *Clostridium* [35]. Van Winsen et al. [36] reported that feeding *L. plantarum*-fermented feed resulted in a decrease in the number of harmful bacteria such as *E. coli* and *Salmonella* while the number of probiotic *Lactobacillus* significantly increased in pig feces. In this study, the relative abundance of *Lactobacillus* increased in Groups L, B, and MIX, which were fed diets supplemented with potential probiotics, and in Group A, which was fed feed supplemented with antibiotics, while the growth of *Clostridium* and *Streptococcus* was effectively inhibited. *Streptococcus* spp., such as Group B *Streptococcus* spp., are conditional

pathogens often found in the genitourinary and intestinal tracts [37], and feeding fermented feeds reduces the risk of infection. LEFSe analysis further revealed bacterial differences between the treatment groups. In conclusion, feeding potential probiotic-fermented feeds increased the diversity of the intestinal microbiota, increased the abundance of beneficial bacteria, and decreased the abundance of harmful bacteria, thus improving the intestinal microbiota of Bamei pigs; in particular, especially the mixed fermentation feed had the most significant effect.

Fermented feed enhances immunity in Bamei pigs

Cytokines are a class of biologically active small-molecule proteins that can regulate cell growth, differentiation, and the immune response by binding to corresponding receptors [38]. Previous studies have shown that probiotic-fermented feeds can not only regulate the intestinal flora, but also enhance host immunity by modulating cytokines [39, 40]. Probiotic-fermented feeds affect immunity mainly through three pathways: anti-inflammatory factors, proinflammatory factors and immunity-related signaling pathways. When exogenous pathogens invade, probiotics can promote the expression of the anti-inflammatory cytokines IL-2 and IL-10 by inducing the differentiation of Th2 cells, thus diminishing harm due to the inflammatory response [41]. The proinflammatory cytokines TNF-α, IFN-γ, and IL-1β can exacerbate mucosal inflammatory responses when stimulated by invasive signals [42]. Sanchez-Muñoz et al. [43] reported that feeding animals diets supplemented with *Lactobacillus* significantly reduced the expression of the proinflammatory cytokines TNF-α and IL-1β and reduced intestinal inflammation. Immunity-related cytokine signaling pathways, particularly the TLR4/MyD88/NF-KB signaling pathway, may also affect host immunity [44]. Previous studies have reported that weaning activates intestinal inflammatory signaling pathways such as the MAPK and NF-kB pathways, triggering inflammation [45]. Jang et al. [46] reported that feeding mice diets supplemented with *Lactobacilli* G-101 inhibited the TLR4/MyD88/NF-KB signaling pathway, thereby alleviating colitis. In this study, feeding mixed *L. plantarum* QP28-1a and *B. subtilis* QB8a-fermented feeds not only significantly increased the production of the anti-inflammatory cytokines IL-2 and IL-10 ($p < 0.05$), but also significantly decreased the production of the proinflammatory cytokines IL-1β and TNF-α, as well as the inflammatory signaling pathway-related cytokines MyD88, TLR4, and NF-KB ($p < 0.05$), thus improving the immunity of freshly weaned Bamei pigs, which was even better than that of antibiotics.

Fermented feeds affect metabolic profiles and pathways

Intestinal metabolism is a complex process, and metabolomics results revealed significant effects on the fecal metabolites and metabolic pathways of Bamei pigs after they were fed different diets. Notably, narceine, an opium alkaloid with antidiabetic effects [47], was significantly enriched in Groups L, B, and MIX compared to Group CK (Fig. 5). The alpha-cephalin enriched in Groups B and MIX had antioxidant effects. Bile acids, the main component of bile, are synthesized by the liver and released into the gastrointestinal tract to aid in the absorption of nutrients, dietary fats, steroids, vitamins and drugs [48]. In this study, the metabolic pathway that promotes bile secretion was enriched in Groups L, B, and MIX. ABC transporter proteins are one of the largest families of transporter proteins discovered to date and are involved in a wide range of biological functions and metabolic processes, transporting proteins, sugars, and lipids across membranes by releasing ATP [49]. Numerous medical studies have revealed that pathogenic bacteria are resistant to drugs, antibiotics, and fungicides through the overexpression of ABC transporter proteins [50, 51]. The significant enrichment of ABC transporter proteins in Group A compared to group CK after feeding for 50 days suggested that the consumption of antibiotic supplemented feed by Bamei pigs resulted in the enrichment of the metabolic pathway of ABC transporter proteins, which led to the body's resistance to antibiotics and harmed the health of the pigs. The results of metabolic pathway topology analyses showed a significant enrichment of amino acid metabolism, especially tryptophan metabolism, in Group MIX, which was highly correlated with nastiness in livestock meat and malodor in feces [52]. The undigested tryptophan in the feed produced indoleacetic acid by various pathways, and indoleacetic acid produced a fecal odor by indoleacetic acid decarboxylase, which caused malodor [53]. The improved metabolic efficiency of tryptophan in Group MIX suggested that mixed-fermented feeds may be able to reduce pork stink and fecal contamination in the environment. Phenylalanine and tryptophan, as essential amino acids for humans, are not only involved in the composition of tissue proteins, but also important precursors of host and microbial metabolism and play important roles in growth and development as well as metabolism [54–56]. Steroid hormones are important for growth, fertility control, and immunomodulation [57–59]. In summary, feeding mixed-fermented feeds increased the content of narceine and alpha-cephalin in the intestinal tract, promoted bile secretion; and favored the synthesis of phenylalanine, tyrosine, and steroid hormones, thus improving the metabolism of Bamei pigs.

Conclusion

Using combined methods of 16S rDNA high-throughput sequencing, ELISA, and metabolomics, this study revealed that feeding five different feeds significantly altered the intestinal microbiota, immunity, and intestinal metabolic profiles of Bamei pigs. Feeding mixed *L. plantarum* QP28-1a and *B. subtilis* QB8a-fermented feeds not only increased the α-diversity of the gut microbiota and the relative abundance of *Lactobacillus*, but also inhibited the growth of the conditional pathogens *Clostridium* and *Streptococcus*. Notably, mixed-fermented feed also increased the production of the anti-inflammatory cytokines IL-2 and IL-10 and decreased the production of the proinflammatory cytokines IL-1β and TNF-α, as well as the inflammatory signaling pathway-related cytokines MyD88/TLR4/NF-KB, thus improving the immunity of freshly weaned Bamei pigs. In addition, feeding mixed-fermented feeds improved the metabolism of Bamei pigs by increasing the content of narceine and alpha-cephalin; promoting bile secretion; and facilitating the synthesis of phenylalanine, tyrosine, and steroid hormones. In conclusion, mixed *L. plantarum* QP28-1a and *B. subtilis* QB8a-fermented feeds improved the gut microbiota, immunity, and metabolism of Bamei pigs and proved to be a promising, healthy and environmentally sustainable solution for Bamei pig breeding and conservation.

Abbreviations

LAB	Lactic acid bacteria
OUT	Operational taxonomic units
LEfSe	Linear discriminant analysis effect size
IL-2	Interleukin 2
IL-10	Interleukin 10
IL-1β	Interleukin 1β
INF-γ	Interferon γ
TNF-α	Tumor necrosis factor α
TLR2	Toll-like receptor 2
MyD88	Myeloid differentiation factor 88
TLR4	Toll-like receptor 4
NF-κB	Nuclear factor-kappa B
PCR	Polymerase chain reaction
PCA	Principal component analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes

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Author contributions

LW: conceptualization, methodology, investigation, data curation, writing original draft; JC: conceptualization, resources, methodology, supervision, writing original draft, data curation; JZ: conceptualization, methodology, resources, formal analysis; FX: methodology, resources, data curation; XL: methodology, investigation, resources; HP: methodology, data curation; MZ and YD: conceptualization, investigation, resources; YC: conceptualization, methodology, resources; GW: conceptualization, methodology, resources, writing review and editing; ZT: conceptualization, funding acquisition, methodology, resources, writing review and editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The use of animals and the experimental protocol was approved by the Life Sciences Ethics Review Board of Zhengzhou University with certificate number ZZUIRB2021-111.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Zhang JX, Yang BC, Wen XC, Sun GQ. Genetic variation and relationships in the mitochondrial DNA D-loop region of Qinghai indigenous and commercial pig breeds. Cell Mol Biol Lett. 2018;23.
2. Wu GF, Tang XJ, Fan C, Wang L, Shen WJ, Ren SE, et al. Gastrointestinal tract and dietary fiber driven alterations of gut microbiota and metabolites in durco x bamei crossbred pigs. Front Nutr. 2022;8.
3. Garrett WS, Gordon JI, Glimcher LH. Homeostasis and inflammation in the intestine. Cell. 2010;140:859–70.
4. Pluske JR, Turpin DL, Kim JC. Gastrointestinal tract (gut) health in the young pig. Anim Nutr. 2018;4:187–96.
5. Barba-Vidal E, Martin-Orue SM, Castillejos L. Review: are we using probiotics correctly in post-weaning piglets? Animal. 2018;12:2489–98.
6. Konowalchuk JD, Rieger AM, Kiemele MD, Ayres DC, Barreda DR. Modulation of weanling pig cellular immunity in response to diet supplementation with 25-hydroxyvitamin D. Vet Immunol Immunop. 2013;155:57–66.
7. Shen YB, Piao XS, Kim SW, Wang L, Liu P, Yoon I, et al. Effects of yeast culture supplementation on growth performance, intestinal health, and immune response of nursery pigs. J Anim Sci. 2009;87:2614–24.
8. Gong J, Yin F, Hou Y, Yin Y. Review: Chinese herbs as alternatives to antibiotics in feed for swine and poultry production: potential and challenges in application. Can J Anim Sci. 2014;94:223–41.
9. Alagawany M, Abd El-Hack ME, Farag MR, Sachan S, Karthik K, Dhama K. The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. Environ Sci Pollut R. 2018;25:10611–8.
10. Elisashvili V, Kachlishvili E, Chikindas ML. Recent advances in the physiology of spore formation for *Bacillus* probiotic production. Probiotics Antimicrobial Proteins. 2019;11:731–47.
11. Azad MA, Sarker M, Li TJ, Yin J. Probiotic species in the modulation of gut microbiota: an overview. Biomed Res Int. 2018;2018.
12. Canibe N, Jensen BB. Fermented and nonfermented liquid feed to growing pigs: effect on aspects of gastrointestinal ecology and growth performance. J Anim Sci. 2003;81:2019–31.
13. Hojberg O, Canibe N, Knudsen B, Jensen BB. Potential rates of fermentation in digesta from the gastrointestinal tract of pigs: effect of feeding fermented liquid feed. Appl Environ Microb. 2003;69:408–18.

14. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastro Hepat. 2017;14:491–502.
15. Humer E, Wetscherek W, Schwarz C, Schedle K. Effects of maize conservation techniques on the apparent total tract nutrient and mineral digestibility and microbial metabolites in the faeces of growing pigs. Anim Feed Sci Tech. 2014;197:176–84.
16. Li SL, Jin ZY, Hu DJ, Yang WW, Yan YY, Nie XB, et al. Effect of solid-state fermentation with *Lactobacillus casei* on the nutritional value, isoflavones, phenolic acids and antioxidant activity of whole soybean flour. Lwt-Food Sci Technol. 2020;125: 109264.
17. Koo B, Kim JW, Nyachoti CM. Nutrient and energy digestibility, and microbial metabolites in weaned pigs fed diets containing Lactobacillus-fermented wheat. Anim Feed Sci Tech. 2018;241:27–37.
18. van Winsen RL, Lipman LJA, Biesterveld S, Urlings BAP, Snijders JMA, van Knapen F. Mechanism of Salmonella reduction in fermented pig feed. J Sci Food Agr. 2001;81:342–6.
19. Chuang L, Wu KG, Pai C, Hsieh PS, Tsai JJ, Yen JH, et al. Heat-killed cells of *Lactobacilli skew* the immune response toward T helper 1 polarization in mouse splenocytes and dendritic cell-treated T cells. J Agr Food Chem. 2007;55:11080–6.
20. Dai CH, Ma HL, He RH, Huang LR, Zhu SY, Ding QZ, et al. Improvement of nutritional value and bioactivity of soybean meal by solid-state fermentation with Bacillus subtilis. Lwt-Food Sci Technol. 2017;86:1–7.
21. Clarridge JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev. 2004;17:840.
22. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 1999;29:1181–9.
23. Everett JR. Pharmacometabonomics in humans: a new tool for personalized medicine. Pharmacogenomics. 2015;16:737–54.
24. Cocci P, Mosconi G, Palermo FA. Changes in expression of microRNA potentially targeting key regulators of lipid metabolism in primary gilthead sea bream hepatocytes exposed to phthalates or flame retardants. Aquat Toxicol. 2019;209:81–90.
25. Potratz S, Tarnow P, Jungnickel H, Baumann S, von Bergen M, Tralau T, et al. Combination of metabolomics with cellular assays reveals new biomarkers and mechanistic insights on xenoestrogenic exposures in MCF-7 cells. Chem Res Toxicol. 2017;30:883–92.
26. Chen J, Pang HL, Wang L, Ma CM, Wu GF, Liu Y, et al. Bacteriocin-producing lactic acid bacteria strains with antimicrobial activity screened from Bamei pig feces. Foods. 2022;11:709.
27. Wang X, Zhang M, Wang WD, Lv HX, Zhang H, Liu Y, et al. The in vitro effects of the probiotic strain, *Lactobacillus casei* ZX633 on gut microbiota composition in infants with diarrhea. Front Cell Infect Mi. 2020;10.
28. Cotta MA, Whitehead TR, Collins MD, Lawson PA. *Atopostipes suicloacale* gen. nov., sp. nov., isolated from an underground swine manure storage pit. Anaerobe. 2004;10:191–5.
29. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6.
30. Liu Y, Lv H, Xu L, Zhang K, Mei Y, Chen J, et al. The effect of dietary lactic acid bacteria on intestinal microbiota and immune responses of crucian carp (Carassius auratus) under water temperature decrease. Front Microbiol. 2022;13: 847167.
31. Feng WW, Ao H, Peng C. Gut microbiota, short-chain fatty acids, and herbal medicines. Front Pharmacol. 2018;9.
32. Kale-Pradhan PB, Jassal HK, Wilhelm SM. Role of lactobacillus in the prevention of antibiotic-associated diarrhea: a meta-analysis. Pharmacotherapy. 2010;30:119–26.
33. Tajima K, Ohmori H, Aminov RI, Kobashi Y, Kawashima T. Fermented liquid feed enhances bacterial diversity in piglet intestine. Anaerobe. 2010;16:6–11.
34. Fouhy F, Guinane CM, Hussey S, Wall R, Ryan CA, Dempsey EM, et al. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob Agents Ch. 2012;56:5811–20.
35. O'Flaherty S, Klaenhammer TR. The role and potential of probiotic bacteria in the gut, and the communication between gut microflora and gut/host. Int Dairy J. 2010;20:262–8.
36. van Winsen RL, Keuzenkamp D, Urlings BAP, Lipman LJA, Snijders JAM, Verheijden JHM, et al. Effect of fermented feed on shedding of Enterobacteriaceae by fattening pigs. Vet Microbiol. 2002;87:267–76.
37. Madrid L, Seale AC, Kohli-Lynch M, Edmond KM, Lawn JE, Heath PT, et al. Infant group B *Streptococcal* disease incidence and serotypes worldwide: systematic review and meta-analyses. Clin Infect Dis. 2017;65:S160–72.
38. Donkor ON, Ravikumar M, Proudfoot O, Day SL, Apostolopoulos V, Paukovics G, et al. Cytokine profile and induction of T helper type 17 and regulatory T cells by human peripheral mononuclear cells after microbial exposure. Clin Exp Immunol. 2012;167:282–95.
39. Markowiak P, Slizewska K. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut Pathog. 2018;10.
40. Lin CH, Chen CC, Chiang HL, Liou JM, Chang CM, Lu TP, et al. Altered gut microbiota and inflammatory cytokine responses in patients with Parkinson's disease. J Neuroinflamm. 2019;16.
41. Lopez P, Gonzalez-Rodriguez I, Gueimonde M, Margolles A, Suarez A. Immune response to *Bifidobacterium bifidum* strains support Treg/Th17 plasticity. PLoS ONE. 2011;6: e24776.
42. Papadakis KA, Targan SR. Tumor necrosis factor: Biology and therapeutic inhibitors. Gastroenterology. 2000;119:1148–57.
43. Sanchez-Munoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. World J Gastroentero. 2008;14:4280–8.
44. Ciesielska A, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. Cell Mol Life Sci. 2021;78:1233–61.
45. Hu CH, Xiao K, Luan ZS, Song J. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J Anim Sci. 2013;91:1094–101.
46. Jang SE, Hyam SR, Han MJ, Kim SY, Lee BG, Kim DH. *Lactobacillus brevis* G-101 ameliorates colitis in mice by inhibiting NF-kappa B, MAPK and AKT pathways and by polarizing M1 macrophages to M2-like macrophages. J Appl Microbiol. 2013;115:888–96.
47. López P, Pereboom-de Fauw DPKHP, Mulder PPJ, Spanjer M, de Stoppelaar J, Mol HGJ, et al. Straightforward analytical method to determine opium alkaloids in poppy seeds and bakery products. Food Chem. 2018;242:443–50.
48. Chiang JYL, Ferrell JM. Bile acid biology, pathophysiology, and therapeutics. Clin Liver Dis. 2020;15:91–4.
49. Lane TS, Rempe CS, Davitt J, Staton ME, Peng YH, Soltis DE, et al. Diversity of ABC transporter genes across the plant kingdom and their potential utility in biotechnology. BMC Biotechnol. 2016;16.
50. Sipos G, Kuchler K. Fungal ATP-binding cassette (ABC) transporters in drug resistance & detoxification. Curr Drug Targets. 2006;7:471–81.
51. Jenness MK, Carraro N, Pritchard CA, Murphy AS. The arabidopsis ATP-BINDING CASSETTE transporter ABCB21 regulates auxin levels in cotyledons, the root pericycle, and leaves. Front Plant Sci. 2019;10:806.
52. Meinert L, Lund B, Bejerholm C, Aaslyng MD. Distribution of skatole and androstenone in the pig carcass correlated to sensory characteristics. Meat Sci. 2017;127:51–6.
53. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. Nat Commun. 2018;9.
54. Liu YL, Hou YL, Wang GJ, Zheng X, Hao HP. Gut microbial metabolites of aromatic amino acids as signals in host-microbe interplay. Trends Endocrin Met. 2020;31:818–34.
55. Gao J, Xu K, Liu HN, Liu G, Bai MM, Peng C, et al. Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. Front Cell Infect Mi. 2018;8.
56. Metges CC. Contribution of microbial amino acids to amino acid homeostasis of the host. J Nutr. 2000;130:1857s-s1864.
57. Srivastava V, Dearth RK, Hiney JK, Ramirez LM, Bratton GR, Dees WL. The effects of low-level Pb on steroidogenic acute regulatory protein (StAR) in the prepubertal rat ovary. Toxicol Sci. 2004;77:35–40.

58. Mandiki SNM, Babiak I, Bopopi JM, Leprieur F, Kestemont P. Effects of sex steroids and their inhibitors on endocrine parameters and gender growth differences in Eurasian perch juveniles. Steroids. 2005;70:85–94.
59. Li MH, Sun LN, Wang DS. Roles of estrogens in fish sexual plasticity and sex differentiation. Gen Comp Endocr. 2019;277:9–16.

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