

Effects of kudzu vine (*Pueraria lobata*) on the intestinal microflora and volatile fatty acids in meat rabbits

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Abstract

Adding flavonoid-rich kudzu vine (*Pueraria lobata*) meal to animal feed can improve intestinal microflora. Here, we investigated the effects of kudzu vine meal on the growth performance, caecal microbial diversity, caecal microbial number, intestinal pH, and volatile fatty acids (VFAs) of meat rabbits. Two hundred weaned Ira rabbits were randomly divided into four groups. Meat rabbits in the control group (group A) were fed a basal diet without kudzu vine meal; those in the three test groups were fed experimental diets comprising 15% (group B), 25% (group C), and 35% (group D) kudzu vine meal. The daily feed intake and mortality of meat rabbits decreased significantly after adding kudzu vine meal to their diet. The feed-to-gain ratio and mortality rate in group D were 3.5 and 7.5%, respectively, significantly lower than those in the control group. The richness and evenness of the bacterial diversity decreased significantly after kudzu vine consumption. Kudzu vine meal also altered the composition of the dominant gut bacterial groups. The intestinal total VFAs were the highest in group B, followed by group D, and significantly higher in both than those in the control group. Hence, adding kudzu vine meal significantly decreased the mortality rates of meat rabbits, decreased the feed-to-gain ratio, and inhibited *Escherichia coli* and *Salmonella* growth in the intestinal flora, while altering the intestinal composition of the dominant gut bacterial groups and VFA content. Thus, group D conditions are recommended to achieve optimal results in meat rabbits.

Introduction

Kudzu vine (*Pueraria lobata*) is a high-quality legume forage, distributed over approximately 400,000 hm² in China, primarily along the Yangtze River and to the south of it, yielding 30 billion kg annually. However, the plant is primarily cultivated for its roots, while the vine remains unutilised. Testing of wild kudzu vine indicated that these plant parts contain 20.89–29.2% crude protein (CP), 26.55–34.19% crude fibre (CF), 19.4–37.21% acid detergent fibre (ADF), 29.6–47.21% neutral detergent fibre (NDF), 1.56–3.44% calcium (Ca), and 0.24–0.48% total phosphorus (TP) (Zhou et al. 2007; Gulizia and Downs 2020; Li et al. 2021; Downs et al. 2022). The CP, Ca, and TP contents of the vine powder are similar to those of alfalfa powder; however, the crude fat (EE), NDF, ADF, and crude ash (Ash) contents are considerably higher in the former. Previous studies have investigated the use of kudzu vine for feeding goats, meat rabbits, broiler chickens, deer, and cattle, with improvements reported in the production performance and immune function of Boza goats (Li et al. 2021). However, replacing other forage in the diet of rabbits with kudzu vine powder does not significantly alter body length, slaughter traits, or feed consumption (Du et al. 2015). Meanwhile, the flavonoid extracts from kudzu vine improve broiler chicken quality and body fat deposition, thus enhancing immunity (Lv and Ou-Yang 2017). Kudzu vine can also be used in grazing, cut-and-carry feeding systems, or mixed with grass to produce good quality silage (Glass and Al-Hamdani 2016). Young bulls grazing on kudzu pastures exhibit a daily weight gain of 568–735 g. Moreover, kudzu vine is rich in isoflavones, such as puerarin (Li et al. 2014); flavonoids affect metabolic regulation and improve animal intestinal health (Zhao et al. 2022). Glass and Al-Hamdani (2016) evaluated the nutritional value of kudzu. They reported that 'kudzu can be considered a good source of

animal feed, especially the leaf, the content of which exceeds most criteria recommended by the National Research Council.'

In summary, compared to alfalfa powder, kudzu vine is more widely available, abundant, easier to preserve and use, and of higher quality. However, studies in China or internationally have not investigated the effects of kudzu vine supplementation on the intestinal tract and metabolism of rabbits. To explore kudzu vine as a potentially valuable resource and to provide novel ideas and methods for rationally developing and utilising new resources, we added different kudzu vine proportions to pelleted feed.

We investigated the effects of kudzu on the growth performance, caecal microbial diversity, caecal microbial numbers, intestinal pH, and volatile fatty acids (VFAs) of meat rabbits. The primary aim of this study was to investigate the effects of kudzu vine meal supplementation on the intestinal tracts of rabbits and to determine the most suitable proportion of dietary kudzu vine to achieve optimal results.

Materials and Methods

This experiment was approved by the Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agricultural Sciences (No. 20220308002).

Collection and diet preparation of Kudzu

In September 2022, fresh kudzu was collected at the junction of Taining County, Fujian Province and Guangchang County, Jiangxi Province, dried, and crushed for use. The sampling area is 800–1,000 m above sea level, and the geographical coordinates are 116°20'–117°24' east longitude and 26°51'–27°47' north latitude, belonging to the subtropical monsoon climate. Dry matter nutrients of Kudzu (refer to 2.4.1 for the determination method): crude protein 17.9%, crude fibre 22.96%, calcium 0.75%, total phosphorus 0.43%.

Animals, diets, and experimental design

To investigate the effects of kudzu vine meal on meat rabbits, 200 healthy and weaned Ira rabbits with similar body weights $[(0.88 \pm 0.13) \text{ kg}]$ at 35 ± 2 days old (provided by Fujian Province in China) were randomly divided into four groups using a single-factor randomised block design. Meat rabbits were fed pellets made from raw materials containing different proportions of kudzu vine meal. The four groups (A, B, C, and D) were fed diets with 0%, 15%, 25%, and 35% kudzu vine meal, respectively. Pellets were made using a pellet machine (Yangzi 210 model-7.5 kw, Yangzi Seiko Technology Co., Ltd.), and the nutritional levels were calculated and tested. Table 1 shows the composition and nutrient levels of the diets, with the detection methods presented in Section 2.1. Rabbits were grouped, dewormed, and vaccinated during the seven-day pre-test period, followed by a main test 42-day period.

Table 1
Composition and nutrient levels of diet (air-dry basis) (%)

Ingredient	Group A	Group B	Group C	Group D
Corn	29.0	24.0	14.0	14.0
Rice bran	2.0	2.0	2.0	2.0
Wheat Middling	5.0	5.0	5.0	4.0
Soybean meal	11.0	8.0	4.0	1.0
Kudzu vine	0	15.0	25.0	35.0
Barley malt Sprouts	6.0	4.0	2.0	2.0
Rice bran meal	6.0	9.0	24.0	25.0
Unified	22.0	13.0	4.0	2.0
Beet slag	14.0	15.0	15.0	9.0
NaCl	0.5	0.5	0.5	0.5
CaHPO ₄	0.5	0.5	0.5	0.5
Premix ¹⁾	4.0	4.0	4.0	4.0
Total	100.0	100.0	100.0	100.0
Nutrient level	Group A	Group B	Group C	Group D
Dry matter	88.45	90.74	91.36	90.54
DE/(MJ/KG) ²⁾	10.03	10.16	10.18	10.12
CP	14.33	14.33	14.55	14.23
CF	12.00	12.84	12.75	12.77
EE	3.69	3.06	3.79	3.19
NDF	29.66	36.17	34.31	33.3
ADF	22.38	28.43	26.09	23.21
Hemicellulose	7.28	7.74	8.22	10.09
Pectin (Galacturonic acid)	16.58	28.48	31.76	38.76
NFC	38.26	33.06	38.71	35.17
Ca	0.97	1.04	1.3	1.3
TP	0.59	0.53	0.83	0.94

Ingredient	Group A	Group B	Group C	Group D
Ash	10.06	10.38	6.64	11.11

¹⁾ Premix provided the following per kg of diet: VA 120000 IU, VD₃ 20000 IU, VE350 IU, Cu (as copper sulphate) 440 mg, Zn (as zinc sulphate) 1035 mg, VK₃ 30 mg, VB₁ 30 mg, VB₂ 200 mg, Fe (as ferrous sulphate) 1,000 mg, Mn (as manganese sulphate) 477 mg, NaCl 5–6%.

²⁾ DE, hemicellulose, and NFC were calculated values, while others (CP, CF, EE, NDF, ADF, Ca, TP, and Ash) were measured values. Hemicellulose = NDF – ADF; NFC = 100 – CP – EE – NDF – Ash.

Feeding and management

All meat rabbits were housed in the same intelligent rabbitry at the Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agricultural Sciences. Meat rabbits were raised in single cages in overlapping 3-storey blocks, with all meat rabbits in each group maintained under the same management and hygiene conditions. Before the experiment, the barn was thoroughly cleaned, including floor cleaning and disinfection, and meat rabbit cages were disinfected. Food was provided twice daily, and the meat rabbits were allowed free access to food and water. The health of the meat rabbits was regularly monitored, and detailed records were maintained throughout the study period.

Sample collection and laboratory analyses

Conventional nutrient composition testing

The nutrient composition was assessed using the following methods: CP (Kjeldahl method), coarse fibre (CF; washing method), EE (Soxhlet extraction), NDF, ADF (paradigm washing method), galacturonic acid (carbazole sulphate colourimetric method), Ca (flame spectrophotometer method), TP (molybdenum antimony anti-colourimetric method), crude ash, and Ash (weighing method).

Determination of growth performance indicators

Initial weights were recorded after the beginning of the formal test period and final weights at the end of the test period. Additionally, feed intake and leftovers were accurately weighed and recorded daily for each group of rabbits. Measurements were used to calculate the average daily feed intake, average daily gain, and feed-to-gain ratio (F/G).

Intestinal pH and caecal fermentation parameters

At the end of the feeding study, eight rabbits of similar weight were selected from each group (N = 32 rabbits), fasted for 12 h with unrestricted access to water, and euthanised by carotid artery bleeding.

Measurement of pH: The pH values of the contents from the caecum and three different locations of the stomach (anterior, middle, and posterior) were measured using a PHB-4 portable pH meter, and the average value was recorded.

Determination of ammoniacal nitrogen ($\text{NH}_3\text{-N}$): The contents of the caecum were placed in a centrifuge tube, and $\text{NH}_3\text{-N}$ was measured using the indophenol blue colourimetric method.

Determination of VFA content: The caecum contents (0.09 g) were accurately weighed into a 2-mL centrifuge tube, and 1 mL of 50% acetonitrile (ACN) was added before the sample was ground and centrifuged for 10 min (4°C, 13,000 rpm). After extracting 1 mL of supernatant, 1.8 mL of 50% ACN containing 0.01 g of sample was added to the solution and centrifuged for 30 s; 20 µL of supernatant was then aspirated. Subsequently, 10 µL of 200 mM₃-NPH·HCl, 10 µL of 200 mM EDC·HCl, 80 µL of 50% ACN, 50 µL of 7% pyridine, and 1 µL of isotope internal standard solution were added with vortex mixing for 1 min. The sample was centrifuged at 13,000 rpm and 4°C for 1 min and then centrifuged at 40°C for 30 min. Twenty microlitres of the derivatised reaction solution were then added to 280 µL of 50% ACN (containing 0.1% formic acid), vortexed for 30 s, and centrifuged (13,000 rpm, 10 min, 4°C) before the supernatant was aspirated into a sample bottle. The acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and hexanoic acid contents were then determined using the LC-30 high-performance liquid chromatography system and SCIEX QTRAP5500 (Sciex, Singapore) mass spectrometer LC-30 (Shimadzu, Singapore). The Kinetex C18 (2.6 µm 100 × 3.00 mm; Phenomenex, Los Angeles, CA, USA) chromatographic column was utilised under the following separation conditions: column temperature: 40°C, flow rate: 0.7 mL/min, injection volume: 2 µL, and sample chamber temperature: 4°C with water (A) and acetonitrile (B) mobile phases. The known characteristic ions were collected using the sMRM mode and integrated using Sciex OS-Q software. The content was calculated using a standard curve.

Determination of bacterial community counts in the caecum

Standard curve preparation: Total bacteria, *Lactobacillus*, *Escherichia coli*, *Bifidobacterium*, and *Salmonella typhi* bacteria were quantitatively detected in the collected caecum contents. First, according to the corresponding primers (Table 2), a pre-experiment was performed to confirm that the solubility curve was normal and that the product was single, indicating that the required primers were available. The product from the pre-experiment was recovered for TA cloning, and the clone with the highest number of concatenated bases was selected as standard. The length of the entire plasmid was recorded before the plasmid was extracted. Samples were prepared with graduated dilution, and qPCR was performed on the prepared samples, with three replicates performed for each sample. The samples were removed from the machine and used to calculate the R² values, amplification efficiencies, and standard curves.

Table 2
Primer sequences of bacteria

Bacteria names	Primer sequences (5' to 3')	Product length/bp	Reference
Total bacteria	F: ACTCCTACGGGAGGCAGCAG R: GGACTACHVGGGTWTCTAAT	140	Maeda et al. (2003)
<i>Lactobacillus</i>	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	341	Zhao et al. (2019)
<i>Escherichia coli</i>	F: CATGCCGCGTGTATGAAGAA R: CGGGTAACGTCAATGAGCAAA	149	Penders et al. (2005)
<i>Bifidobacterium</i>	F: GATTCTGGCTCAGGATGAACGC R: CTGATAGGACGCGACCCCAT	243	Echarri (2011)
Salmonella typhi	F: AAGACGTATTCTCCATCTCCGG R: ACGGCCGCCTTCATCTTTGGACAG	119	Muhmood (2021)

qPCR on-board detection: The DNA samples to be sent for testing were diluted 10 times, used as qPCR templates, and amplified using the Qingke 2 × T5 Fast qPCR Mix (SYBR Green I). The amplification system components were 2 × T5 Fast qPCR Mix (SYBR Green I), 10 µM Primer F, 10 µM Primer R, template (gDNA), and ddH₂O. Amplification was performed via pre-denaturation at 95°C for 2 min, 95°C for 15 s, 56°C for 20 s, 72°C for 20 s, followed by 40 cycles of 95°C for 5 s, 65°C for 20 s, and 95°C for 15 s; melting curve analysis was then conducted. The Ct values were obtained from the qPCR detection, and the absolute amount of each bacterium species was calculated according to the Ct value.

Detection method for microbial diversity in the caecum

Sequencing process: The collected caecal contents were sent to Nanjing Aowaisen Technology Co., Ltd. for testing. The DNA was extracted, and the genomic DNA was detected using 1% agar gel electrophoresis. PCR amplification was then performed, and specific barcoded primers were synthesised according to the V3–V4 region. The PCR products were sorted by size into amplified target bands using 1% agarose gel electrophoresis and purified using the Agencourt AMPure XP (Pasadena, CA, USA) nucleic acid purification kit.

A Miseq library was constructed, and 'Y'-shaped adapters were connected to magnetic beads to screen adapter dimers for removal. PCR amplification was used to enrich the library templates, denaturation with sodium hydroxide generated single-stranded DNA fragments, and Miseq on-machine sequencing was performed. The end of the DNA fragment complementary to the primer base was fixed onto the chip, while the other end randomly complementing another nearby primer was also fixed to form a

'bridge.' PCR amplification was then used to generate DNA clusters and the DNA amplicons were rendered single-stranded by adding modified DNA polymerase and dNTP with four fluorescent labels. Only one nucleotide was synthesised in each cycle. The surface of the reaction plate was scanned with a laser to read the nucleotide types aggregated in the first round of each template sequence. The 'fluorescent' and 'termination' groups were chemically cleaved, with the 3'-end viscosity restored, and the aggregation continued for the second nucleotide. The results of the fluorescent signals collected in each round were counted to determine the sequence of the template DNA fragment.

Analysis of sequencing information: After the sequencing run, the raw data were stored in the Fastq format. Quality control was performed using Trimmomatic and Pear. Flash and Pear were used to merge the sequences at both ends according to the PE overlap relationship. Chimeric sequences were removed from the Fasta files using the UCHIME method based on the Silva database, and the self-alignment (*de novo*) method was used for unknown databases, and the removal of short sequences that did not adhere to the requirements. Valid data statistics were performed, and the final quality sequence distribution statistics obtained after data pre-processing were subsequently clustered using the UPARSE method for operational taxonomic unit (OTU) analysis. All sequences were clustered according to 97% similarity and UCLUST was used to plot a Venn diagram of the OTU distribution that was common and unique to all samples.

Species annotation and taxonomic analysis: The RDP Classifier algorithm was used to perform a comparative analysis of the OTU representative sequences and annotate the species information for communities at each phylogenetic level (kingdom, phylum, class, order, family, genus, and species) so that the species classification information corresponding to each OTU could be obtained.

Statistical analysis

Data were presented as mean $\pm$ standard deviation. Statistical analysis was performed using SPSS 19.0 software. One-way ANOVA (least significant difference [LSD]) and Duncan multiple comparison method were used to test for significance, with $P < 0.05$ indicating a significant difference and $P < 0.01$ indicating a highly significant difference.

Results

Effects of different kudzu vine meal proportions on the growth performance of meat rabbits

Adding different proportions of kudzu vine meal significantly affected the mortality rate, average daily gain, average daily feed intake, and F/G of the rabbits (Table 3). The mortality rate of group A was significantly lower than group D ($P < 0.05$). The average daily feed intake was also significantly lower for groups B, C, and D than for group A ($P < 0.05$); the lowest F/G of 3.50 observed in group D was significantly lower than those obtained for groups A, B, and C ($P < 0.05$).

Table 3
Effects of different kudzu vine meal proportions on the growth performance of meat rabbits

Item	Group A	Group B	Group C	Group D
Initial weight (kg)	0.89 ± 0.13	0.87 ± 0.11	0.89 ± 0.097	0.87 ± 0.084
Final weight (kg)	2.49 ± 0.25	2.39 ± 0.27	2.54 ± 0.14	2.38 ± 0.12
Mortality rate (%)	20.00 ± 13.91 ^a	15.00 ± 5.77 ^{ab}	10.00 ± 8.16 ^b	7.50 ± 5.00 ^b
Average daily gain ADG (g)	32.70 ± 1.80	30.90 ± 20.00	32.70 ± 3.80	32.80 ± 2.40
Average daily feed intake ADFI (g)	155.80 ± 12.20 ^a	130.40 ± 21.80 ^b	138.70 ± 5.50 ^b	115.40 ± 17.10 ^b
Feed-to-Gain ratio(F/G)	4.66 ± 0.36 ^a	4.12 ± 0.34 ^a	4.14 ± 0.43 ^a	3.50 ± 0.31 ^b

In the same row, values with different lowercase letter superscripts indicate highly significant differences ($P < 0.01$), values with different lowercase letter superscripts indicate significant differences ($P < 0.05$), while values with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Intestinal pH and caecal fermentation parameters of meat rabbits

Significant differences were observed for stomach pH, total VFAs, acetic acid, and propionic acid in each group (Table 4). Specifically, the gastric pH showed an increasing trend as the proportion of dietary kudzu increased, with a significantly higher value in group D than in group A ($P < 0.05$). The $\text{NH}_3\text{-N}$ content was the highest in group A and gradually decreased as the proportion of kudzu increased, with no significant difference observed between groups C and D ($P > 0.05$) and significantly lower amounts observed in groups D and C than that in group A ($P < 0.01$ and $P < 0.05$ respectively). Total VFAs, acetic acid, and propionic acid contents were essentially the same in all groups, with significantly higher contents observed only in group B than in group C ($P < 0.01$). No significant differences were observed between groups A, B, and D ($P > 0.05$), and significant differences were observed only between groups B and D ($P < 0.05$).

Table 4
Effects of different kudzu proportions on intestinal pH and caecal fermentation parameters of meat rabbits

Item	Group A	Group B	Group C	Group D
pH of stomach	1.49 ± 0.18 ^b	1.62 ± 0.27 ^{ab}	1.59 ± 0.32 ^{ab}	1.79 ± 0.16 ^a
pH of caecum	6.61 ± 0.27	6.97 ± 0.16	6.75 ± 0.35	6.95 ± 0.17
NH ₃ -N (µg/g)	201.30 ^{Aa}	167.77 ^{ABab}	129.97 ^{Bb}	152.26 ^{ABb}
Total VFAs (mg/g)	1.19 ± 0.05 ^{ABab}	1.41 ± 0.41 ^{Aa}	0.98 ± 0.17 ^{Bb}	1.18 ± 0.09 ^{ABab}
Acetic acid (mg/g)	0.73 ± 0.07 ^{ABab}	0.88 ± 0.30 ^{Aa}	0.57 ± 0.09 ^{Bb}	0.71 ± 0.07 ^{ABab}
Propionic acid (mg/g)	0.17 ± 0.02 ^{ABab}	0.20 ± 0.04 ^{Aa}	0.13 ± 0.02 ^{Bc}	0.15 ± 0.02 ^{ABbc}
Butanonic acid (mg/g)	0.13 ± 0.03	0.17 ± 0.07	0.13 ± 0.04	0.16 ± 0.03

In the same row, values with different uppercase letter superscripts indicate highly significant differences ($P < 0.01$), values with different lowercase letter superscripts indicate significant differences ($P < 0.05$), while values with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Effects of different kudzu vine meal proportions on caecal bacteria community counts of meat rabbits

Different proportions of kudzu vine meal had different effects on the number of bacteria in the rabbit caeca. Table 5 shows that the number of total bacteria, *E. coli*, and *S. typhi* exhibited a decreasing trend as the proportion of kudzu increased. The total number of bacteria was significantly lower in group D than in groups A, B, and C ($P < 0.01$), while the numbers of *E. coli* and *Lactobacillus* were the lowest in group D. However, this difference was not significant ($P > 0.05$). The contents of *Lactobacillus* showed an increasing trend, with the highest content in group D. However, the increase was not significant ($P > 0.05$). Moreover, the abundance of *Bifidobacterium* was the highest in group A, but this difference was not significant ($P > 0.05$).

Table 5
Effects of different kudzu vine meal proportions on caecal bacteria community counts of meat rabbits I g (copy number/ μ L)

Item	Group A	Group B	Group C	Group D	P value
Total bacteria	10.02 $\pm$ 0.12 ^{Aa}	9.82 $\pm$ 0.18 ^{Aa}	9.76 $\pm$ 0.27 ^{Aa}	9.34 $\pm$ 0.26 ^{Bb}	0.0012
<i>Bifidobacterium</i>	6.86 $\pm$ 0.12	6.70 $\pm$ 0.13	6.80 $\pm$ 0.22	6.52 $\pm$ 0.51	0.3028
<i>Lactobacillus</i>	3.91 $\pm$ 0.15	4.04 $\pm$ 0.16	4.08 $\pm$ 0.34	4.15 $\pm$ 0.42	0.6203
<i>Escherichia coli</i>	3.74 $\pm$ 0.11	3.89 $\pm$ 0.25	3.73 $\pm$ 0.19	3.69 $\pm$ 0.19	0.3811
<i>Salmonella typhi</i>	5.88 $\pm$ 0.25	5.71 $\pm$ 0.18	5.72 $\pm$ 0.31	5.54 $\pm$ 0.40	0.3911

In the same row, values with different uppercase letter superscripts indicate highly significant differences ($P < 0.01$), values with different lowercase letter superscripts indicate significant differences ($P < 0.05$), while values with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Effects of different kudzu proportions on the caecal bacteria microbial species of meat rabbits

Venn diagram feature distribution

The number of OTUs that were common or unique in each group was calculated based on the eigenvalue OTU abundance table and interpreted using a Venn diagram. An OTU present in only one subgroup was considered unique to that subgroup. Each circle in Fig. 1 represents one sample/group, and the number of circles and overlapping circles represents the number of OTUs shared between different groups. A total of 1064 OTUs were common to all groups, while 640, 45, 80, and 62 unique OTUs were obtained for groups A, B, C, and D, respectively, indicating significant differences in the composition of caecal microbiota following treatment. At the 97% sequence identification threshold, group A OTUs were classified into 31 phyla, 80 classes, 149 orders, 207 families, and 341 genera; group B included 15 phyla, 23 classes, 41 orders, 64 families, and 149 genera; group C comprised 21 phyla, 47 classes, 89 orders, 124 families, and 237 genera; and group D involved 20 phyla, 38 classes, 72 orders, 104 families, and 194 genera.

Alpha diversity

In the alpha diversity index, the Chao1 index reflects the richness of the community, the Shannon, Simpson, and PD_whole_tree indices reflect the diversity of the community, and the Good's coverage reflects the sequencing depth. No significant difference was observed among the groups under Good's coverage ($P > 0.05$), indicating that the sequencing depth of each group was similar. However, the OTU number, Chao, and Shannon indices were significantly lower in groups B, C, and D than in group A ($P <$

0.05). While no significant difference was observed between groups A and D for PD_whole_tree ($P > 0.05$), the results were significantly lower than those for groups B and C ($P < 0.05$). No significant difference was observed among the groups using the Simpson method. These results indicate that the richness and diversity of bacterial communities were higher in group A than in the other groups (Table 6).

Table 6
Alpha diversity index

Item	Group A	Group B	Group C	Group D	<i>P</i> -value
OTU number	1152.40 ± 221.47 ^a	897.20 ± 87.03 ^b	923.60 ± 46.58 ^b	911.00 ± 94.00 ^b	0.0201
Shannon index	7.07 ± 0.37 ^a	6.30 ± 0.23 ^b	6.36 ± 0.23 ^b	6.50 ± 0.06 ^b	0.0037
Simpson index	0.95 ± 0.03	0.94 ± 0.02	0.96 ± 0.01	0.95 ± 0.01	0.7575
PD_whole_tree	93.68 ± 18.14 ^a	72.95 ± 8.29 ^b	76.81 ± 7.86 ^b	81.18 ± 10.20 ^{ab}	0.0687
Chao1 index	1506.61 ± 230.78 ^a	1202.38 ± 85.55 ^b	1250.69 ± 158.91 ^b	1201.84 ± 116.12 ^b	0.0211
Good's coverage	0.99	0.99	0.99	0.99	

In the same row, values with different lowercase letter superscripts indicate highly significant differences ($P < 0.01$), values with different lowercase letter superscripts indicate significant differences ($P < 0.05$), while values with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Relative abundance(at phylum level)

A total of 32 phylum-level bacterial microorganisms were isolated in the rabbit caeca.

Verrucomicrobiota, Bacteroidota, and Firmicutes were the dominant phyla (Fig. 2), with relative abundances of 94.99%, 95.22%, 96.2%, and 96.6% in groups A, B, C, and D, respectively. The relative abundance of Desulfobacterota was between 1–5%. A total of ten phyla were detected in group A, five in group B, nine in group C, and nine in group D. Compared with group A, fewer Acidobacteriota, Planctomycetota, Gemmatimonadota, Spirochaetota, and Campilobacterota were detected in group B, and fewer Spirochaetota in groups C and D (Table 7). Significant differences were observed only for Desulfobacterota, Patescibacteria, and Euryarchaeota. Desulfobacterota was the lowest in group A, with significantly lower abundance than in group B ($P < 0.05$). However, the difference in abundance between group A and groups C and D was not significant ($P > 0.05$). Patescibacteria was significantly higher in group A than in groups B ($P < 0.01$) and D ($P < 0.05$). The Euryarchaeota content was significantly higher in group D than in all other groups ($P < 0.05$ for each). The content of Proteobacteria was the highest in group A, however, there was no statistically significant difference.

Table 7
Relative abundance of the caecal microflora of meat rabbits at the phylum level (%)

Item	Group A	Group B	Group C	Group D	P-value
Verrucomicrobiota	17.63 ± 12.31	24.93 ± 5.36	20.80 ± 7.29	16.34 ± 7.42	0.4114
Firmicutes	53.01 ± 2.04	48.03 ± 7.76	50.08 ± 8.44	57.05 ± 14.43	0.4667
Bacteroidota	24.35 ± 10.58	22.26 ± 9.32	25.32 ± 5.47	23.21 ± 9.10	0.9508
Desulfobacterota	1.34 ± 0.95 ^b	3.18 ± 1.38 ^a	2.05 ± 0.80 ^{ab}	1.82 ± 1.23 ^{ab}	0.1009
Proteobacteria	0.88 ± 1.14	0.65 ± 0.67	0.64 ± 0.21	0.56 ± 0.22	0.8956
Patescibacteria	0.59 ± 0.41 ^{Aa}	0.30 ± 0.30 ^{ABab}	0.07 ± 0.06 ^{Bb}	0.08 ± 0.11 ^{ABb}	0.0210
Cyanobacteria	0.70 ± 1.46	0.08 ± 0.12	0.06 ± 0.05	0.07 ± 0.05	0.4537
Acidobacteriota	0.24 ± 0.45	0	0.04 ± 0.05	0.02 ± 0.04	0.3397
Planctomycetota	0.01 ± 0.02	0	0.01 ± 0.01	0.01 ± 0.01	0.708
Actinobacteriota	0.55 ± 0.41	0.46 ± 0.23	0.57 ± 0.20	0.41 ± 0.21	0.7839
Gemmatimonadota	0.14 ± 0.30	0	0.06 ± 0.07	0.04 ± 0.08	0.5566
Spirochaetota	0.16 ± 0.21 ^a	0 ^b	0 ^b	0 ^b	0.321
Euryarchaeota	0.06 ± 0.06 ^b	0.08 ± 0.06 ^{ab}	0.13 ± 0.08 ^{ab}	0.23 ± 0.18 ^a	0.103
Campilobacterota	0.01 ± 0.01	0	0.01 ± 0.01	0.04 ± 0.07	0.5131

In the same row, values with different uppercase letter superscripts indicate highly significant differences ($P < 0.01$), values with different lowercase letter superscripts indicate significant differences ($P < 0.05$), while values with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Relative abundance(at genus level)

At the genus level (Fig. 3), *Akkermansia*, *Muribaculaceae*, the NK4A214_group, uncultured, and *Clostridia*_UCG-014 constituted the dominant bacterial groups, with a relative abundance > 5%. In addition, some groups, such as the *Lachnospiraceae*_NK4A136_group, *Bacteroides*, *Ruminococcus*, UCG-010, *Fusicatenibacter*, the *Clostridia*_vadinBB60_group, *Desulfovibrio*, and dgA-11_gut_group, showed a relative abundance of 1–5%. No significant differences were observed among the four groups at the genus level except for *Muribaculaceae*, which showed significant differences between group B and groups A, C, and D ($P > 0.05$), and RF39, which was significantly more abundant in group D compared to group A ($P < 0.05$). Of the bacterial communities with a relative abundance < 1%, *Monoglobus* was significantly higher in group D than in the other groups ($P < 0.01$; Table 8).

Table 8
Relative abundance of the caecal microflora of meat rabbits at the genus level (%)

Item	Group A	Group B	Group C	Group D	P-value
Akkermansia	17.57 ± 12.39	24.93 ± 5.36	20.8 ± 7.29	16.34 ± 7.42	0.4115
Muribaculaceae	17.20 ± 8.61 ^a	8.41 ± 2.18 ^b	11.26 ± 4.47 ^{ab}	8.75 ± 7.89 ^{ab}	0.1471
NK4A214_group	11.24 ± 4.09	10.49 ± 3.04	10.73 ± 5.81	14.18 ± 6.94	0.6608
uncultured	11.82 ± 2.05	12.09 ± 2.82	8.79 ± 2.23	8.6 ± 3.27	0.0903
Clostridia_UCG-014	6.83 ± 2.02	5.88 ± 2.45	5.10 ± 0.89	6.52 ± 3.67	0.6991
Lachnospiraceae_NK4A136_group	4.33 ± 1.35	2.53 ± 0.57	3.99 ± 3.26	3.65 ± 2.49	0.5990
Christensenellaceae_R-7_group	2.86 ± 0.39	2.55 ± 0.94	2.58 ± 0.76	3.13 ± 1.46	0.7561
Bacteroides	2.92 ± 1.56	6.18 ± 5.12	4.70 ± 2.34	4.56 ± 2.61	0.4779
RF39	2.44 ± 1.03 ^b	2.33 ± 0.69 ^b	3.18 ± 0.70 ^{ab}	4.16 ± 2.08 ^a	0.1223
Ruminococcus	1.88 ± 1.14	1.46 ± 0.83	1.11 ± 0.39	0.99 ± 0.13	0.2579
UCG-010	1.64 ± 0.7	1.33 ± 0.6	2.04 ± 0.67	2.31 ± 1.14	0.2659
Fusicatenibacter	1.58 ± 0.21	1.50 ± 0.91	1.08 ± 0.19	1.60 ± 0.75	0.5011
Clostridia_vadinBB60_group	1.52 ± 1.14	1.29 ± 0.68	2.17 ± 1.17	1.60 ± 1.01	0.5797
Desulfovibrio	1.25 ± 0.89 ^b	3.14 ± 1.38 ^a	2.02 ± 0.8 ^{ab}	1.76 ± 1.19 ^{ab}	0.0827
dgA-11_gut_group	1.00 ± 0.93	3.22 ± 4.50	3.53 ± 2.92	3.88 ± 2.63	0.4506
Alistipes	0.96 ± 0.55	1.77 ± 1.19	1.26 ± 0.36	0.91 ± 0.63	0.2819
Bacteroidetes_BD2-2	0.69 ± 0.24	0.62 ± 0.37	0.59 ± 0.22	0.54 ± 0.15	0.8321
Monoglobus	0.67 ±	0.73 ±	0.74 ±	1.15 ±	0.0045

Item	Group A	Group B	Group C	Group D	P value
	0.15 ^{Bb}	0.19 ^{Bb}	0.19 ^{Bb}	0.24 ^{Aa}	

In the same row, values with different uppercase letter superscripts indicate highly significant differences ($P < 0.01$), values with different lowercase letter superscripts indicate significant differences ($P < 0.05$), while values with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Relative abundance of different bacteria

The Kruskal–Wallis test is a non-parametric test that can determine differences from the means of three or more independent data sets. Differentially abundant species at the OTU level can be obtained from multiple datasets with biological repetitions using Kruskal–Wallis, with $P < 0.05$ indicating significantly different species.

Table 9
Relative abundance of different bacteria at the genus level using the Kruskal–Wallis detection method (%)

Item	Group A	Group B	Group C	Group D
g__Candidatus_Saccharimonas	0.54	0.30	0.05	0.06
g__Lachnospiraceae_UCG-006	0.07	0.12	0.12	0.17
g__Monoglobus	0.67	0.73	0.74	1.15
g__Negativibacillus	0.19	0.17	0.06	0.02
g__Lachnoclostridium	0.07	0.03	0.02	0.03
g__Oxalobacter	0.03	0.05	0.08	0.16
g__Lachnospiraceae_AC2044_group	0.04	0	0	0
g__Gordonibacter	0.02	0.05	0.11	0.05
g__Ruminococcus_torques_group	0	0.03	0.03	0
g__Anaerotruncus	0	0.02	0.03	0.01
g__Catenibacillus	0.02	0.01	0.01	0.03
g__Methanobrevibacter	0.03	0.07	0.11	0.15
g__Parvibacter	0	0.02	0.02	0.02
g__Lachnospira	0	0.00659	0.00659	0.00659

The differentially abundant species (at the genus level) are listed in Table 9. Those with relative abundances $> 0.02\%$ included the six major species: *Candidatus_Saccharimonas*,

*Lachnospiraceae*_UCG-006, *Negativibacillus*, *Monoglobus*, *Lachnoclostridium*, and *Oxalobacter* (Fig. 3). Ten differentially abundant species were observed in group A, of which four were significantly higher than in the other groups: *Lachnospiraceae*_AC2044_group (Firmicutes), *Negativibacillus* (Firmicutes), *Lachnoclostridium* (Firmicutes), and *Candidatus_Saccharimonas* (Patescibacteria Patellaria). Twelve differentially abundant species were obtained in group B, of which two were significantly more abundant than in the other groups: *Ruminococcus_torques_group* (Firmicutes) and *Parvibacter* (Actinobacteriota). Twelve differentially abundant species were obtained in group C, two of which were significantly more abundant than in other groups: *Anaerotruncus* (Firmicutes) and the *Ruminococcus_torques_group* (Firmicutes). Out of the 11 differentially abundant species obtained from group D, five were significantly more abundant than in other groups: *Lachnospiraceae*_UCG-006 (Firmicutes), *Monoglobus* (Firmicutes), *Catenibacillus* (Firmicutes), *Oxalobacter* (Proteobacteria), and *Methanobrevibacter* (Euryarchaeota) (Fig. 4).

Correlation analysis between caecum VFA and different bacterial species of meat rabbits

Butanoic acid was positively correlated with all bacterial species, excluding *Lachnoclostridium*, *Negativibacillus*, the *Lachnospiraceae*_AC2044_group, and *Catenibacillus*. It had the strongest correlation with *Parvibacter* and the *Lachnospiraceae*_AC2044_group ($r > 0.8$). Valeric, propionic, acetic, isovaleric, and isobutyric acids were positively correlated with *Lachnoclostridium*, *Negativibacillus*, *Ruminococcus_torques_group*, and *Parvibacter*, however, these correlations were not significant ($P < 0.05$). The strongest correlation was observed in *Lachnoclostridium* (valeric acid: $r = 0.4$; other acids $r = 0.8$; Fig. 5).

Discussion

Effect of kudzu on meat rabbit growth performance

Dietary fibre is an important component of rabbit feed, accounting for 40–50% of the diet. The importance of fibre lies in its effect on the speed at which chyme passes through the digestive tract, its impact on the function of the mucosa, and its role as a microbial substrate. The digestion, absorption and utilization of nutrients in rabbits is 7–19% before entering the caecum, and the utilization rate of NDF level is 5–43% (de Blas and Wiseman 2010). The CF levels of diet is too high or too low will adversely impact meat rabbit growth. This study investigated the effect of feed on the growth performance of rabbits by maintaining consistent CF levels. The results indicated that the F/G of the rabbits decreased significantly as the proportion of kudzu increased, which may be due to the abundant total flavonoids in kudzu vine (the total flavonoid content in kudzu vine is 2.73%, while that in kudzu root is 4.81%) (Kong and Jiang 2009). Flavonoids enhance appetite and digestive juice secretion, increasing digestion and food absorption and reducing the amount of energy required from food. This, in turn, increases the average daily gain and reduces the average daily feed intake and F/G. The study is similar

to this conclusion: Feeding Boer goats with kudzu grass powder instead of alfalfa powder significantly increased the average daily weight gain. When 75% of the grass powder was replaced by kudzu vine, this led to an optimal average daily gain and a 15.49% decrease in the F/G, possibly because the high puerarin content in kudzu promotes animal growth (Li et al. 2021). Zhang and Gu (1998) added 60% kudzu leaf powder to the diet of growing rabbits and found that their daily weight gain could reach 20.37 g. The flavonoids in kudzu root also significantly improved the growth performance of broiler chickens (Zhang et al. 2017).

Considering the fibre type of the diets, with the NDF content negatively and linearly correlated with the mean retention time of the digesta in the ileum-rectum segment (i-rMRT) (de Blas and Wiseman 2010). This indicates that a low dietary NDF content leads to long retention times for digesta in the intestinal tract, which can lead to digestive diseases. In addition, the feeding method of free feeding was adopted in this study. These lead to this resulted highest mortality rate (20%) in group A. Meanwhile, as the proportion of dietary kudzu increased, the NDF content increased, resulting in a significant reduction in the mortality rates as diets with high NDF levels can effectively regulate the pH of the digestive tract and promote gastrointestinal development.

Effect of kudzu on meat rabbit intestinal microflora

Intestinal flora and organisms are mutually symbiotic and interdependent and jointly maintain the dynamic balance of the whole body. In contrast, organisms can provide energy and a nutritional environment for the growth and reproduction of intestinal flora, which can also regulate and protect animal health. Studies have proved that changes in diet can cause changes in intestinal microbial composition within 24 h (David et al., 2014). In this study, the effects of Kudzu on the intestinal flora of weaned Ila rabbits were studied by adding different proportions of kudzu. It was found that the four major phyla in the rabbit gut microbiome were Firmicutes, Bacteroidota, and Verrucomicrobiota, and their relative abundance is greater than 17%. With the increasing amount of kudzu, the dominant phyla did not change; however, relative abundance values varied, and there was no significant difference in the trend (Table 7). A previous study also found that Firmicutes and Bacteroidota were the two most abundant phyla in mammalian faeces (Liu et al., 2022a, 2022b). These results suggest that adding kudzu vine did not alter the dominant phyla in the rabbit gut microbiome but affected the abundance of these phyla. Firmicutes had the highest relative abundance in group D, playing an important role in supplying energy to the host and promoting the development of intestinal epithelial cells (Marcobal et al., 2011). Therefore, it can be explained that when the proportion of kudzu vine is 35%, the change in dietary fibre content changes the relative abundance of Firmicutes, and the increase of this bacterial community is conducive to the development of intestinal epithelial cells and accelerates food digestion and nutrient absorption of domestic rabbits.

There were also some probiotics in the differential bacterial community, and their relative abundance was lower in group A but increased significantly after kudzu vine was added. *Lachnospiraceae*, a potentially beneficial bacteria in the gut, belong to Firmicutes. It is involved in a variety of carbohydrate

metabolism, such as fermentation, leading to the production of acetic acid and butyric acid to provide energy (Langille et al., 2014, 2021). The production may be closely related to the increase in dietary fibre content in the diet. This study is similar to that of Saladrigas-García et al. (2021), who studied the changes of the intestinal microflora in pigs after weaning. They found that the feeding diets with higher dietary fibre increased the abundance and VFAs of *Lachnospira* in the intestines of pigs. Therefore, it can be understood that the diet of the experimental group increased the content of Kudu, and Kudu contains rich soluble fibre, which can promote the production of specific microbial flora and VFAs in caecum microorganisms by regulating the quantity and type of cecum substrates (Wang, 2012; Wang et al., 2018), showing the increase in the relative abundance of *Lachnospira* in groups B, C, and D.

Effect of kudzu on meat rabbit bacterial counts

Lactobacillus and *Bifidobacterium* are beneficial and normal intestinal bacteria (Kang 1985) that can prevent excessive fermentation due to invasive bacteria and opportunistic pathogens. Bacterial metabolites such as acetic and lactic acids can create an acidic environment, thus eradicating the acid-intolerant *E. coli* (Cong et al. 2022). In the animal gut, *Lactobacillus* (*Lactobacillus* spp.) can utilise carbohydrate sugars to produce acidic metabolites, lower the intestinal pH, improve the intestinal microbial environment, and regulate the host immune system (Kim et al. 2016; Tankou et al. 2018). The results of this study showed that adding 25% dietary kudzu resulted in lower levels of intestinal opportunistic pathogens, such as *E. coli* and *Salmonella*, compared to those in group A, along with a tendency for increased levels of *Lactobacillus*. This is because the flavonoids contained in kudzu have a certain moderating effect on the intestinal microorganisms of animals, improving the composition of the gut microbiota, increasing the levels of the beneficial bacteria *Lactobacillus* and *Bifidobacterium*, and inhibiting the growth of harmful bacteria such as *E. coli* (Wang et al. 2018).

Effect of kudzu on meat rabbit VFAs

The caecum is one of the most important digestive organs of rabbits. The caecum's internal environment affects the growth and reproduction of microorganisms and thus affects the health of rabbits (Cheng, 2003). VFA is an important index for evaluating intestinal health in rabbits. $\text{NH}_3\text{-N}$ is a major protein breakdown product and an important substrate for cell protein synthesis by microorganisms of caecum (Kilian, 1998). In this experiment, the $\text{NH}_3\text{-N}$ content of caecum in group B was higher than in groups C and D, which may be due to the highest NDF level in the group B diet. NDF was positively correlated with $\text{NH}_3\text{-N}$ concentration, which was consistent with the results of Peng (2008), who studied the effects of different NDF levels on the performance and caecum fermentation of meat rabbits. This was also confirmed by Morisse et al. (1985), who proposed that the increase in the NDF level in diets resulted in an increase in $\text{NH}_3\text{-N}$ concentration in the caecum. In this study, the NDF level in group A was the lowest; however, the $\text{NH}_3\text{-N}$ concentration in group A was the highest, which may be because the increase of dietary nitrogen content in group A increased the decomposition products in caecum and thus increased the $\text{NH}_3\text{-N}$ content, as reported by Tao (2004). Dietary fibre significantly affects the fermentation of

cecum in test rabbits. With the increase in dietary NDF level, the concentration of $\text{NH}_3\text{-N}$ in the cecum of test rabbits decreased.

The type and content of VFAs are influenced by the amount and type of dietary fibre in the gut and are particularly affected by changes in the composition of the gut microbiota, which in turn affect VFA production (Zhong et al., 2022). Acetic acid, propionic acid, and total VFAs in the caecum are the highest in group B. This may be related to the higher NDF content in the diet of group B. García et al. (2002) showed that the VFA concentration in the caecum increased alongside uronic acid and NDF content. Gidenne (2001) also reported that the increase of soluble fibre in rabbit diets increased the concentration of total VFAs.

Conclusions

When a proportion of 35% dietary kudzu was added to the diet, the F/G, mortality rate decreased significantly, but the richness and evenness of the bacterial community also decreased significantly. However, pathogenic bacteria such as *Escherichia coli* and *Salmonella* in the gut were reduced, and *Lactobacillus* increased. The composition of the dominant bacterial groups in the gut changed, There was no change in the composition of the dominant flora, but there was some change in relative abundance, Firmicutes had the highest relative abundance in group D and the relative abundance of Lachnospira increase after adding kudzu. the $\text{NH}_3\text{-N}$ content of caecum in group B was higher than that in groups C and D. Acetic acid, Propionic acid and Total VFAs in the caecum are highest in group B.

Declarations

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Competing Interests

The authors have no relevant or non-financial interests to disclose.

Author contributions

Gao Chengfang: Methodology, Investigation, Formal analysis, Writing-original draft, Writing-review & editing. Sang Lei: Methodology, Investigation. Sun Shikun: Investigation. Chen Dongjin: Investigation. Wang Jinxiang: Methodology. Xie Xiping: Conceptualization, Project administration, Supervision.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics statement

All procedures involving animals followed the guidelines for the care and use of experimental animals approved by the State Council of the People's Republic of China.

Data Availability

Upon reasonable request, the datasets of this study can be available from the corresponding author.

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Figures

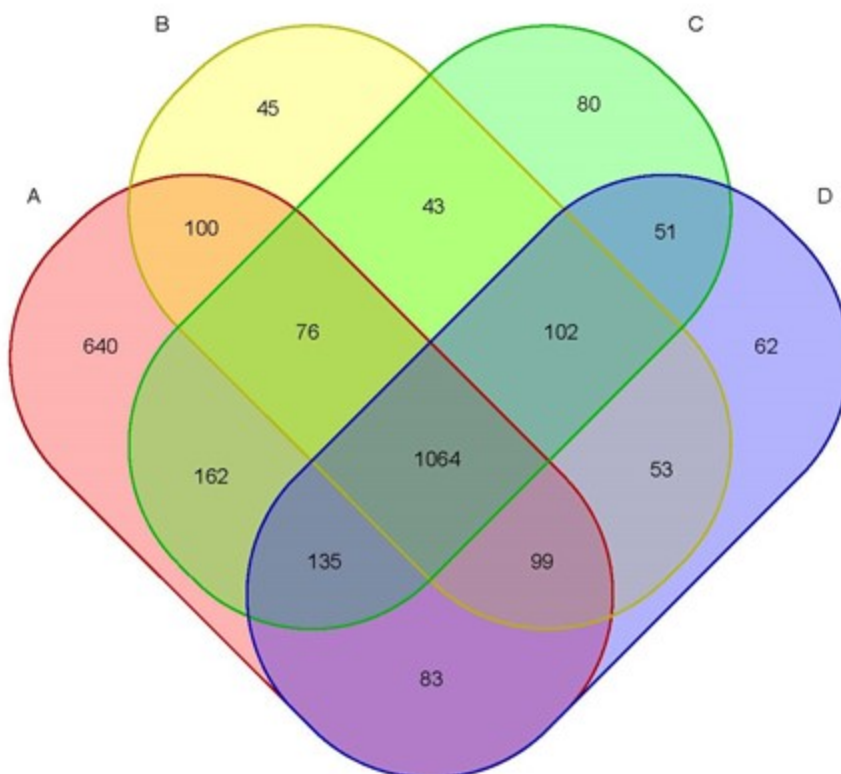


Figure 1

Venn diagram feature distribution

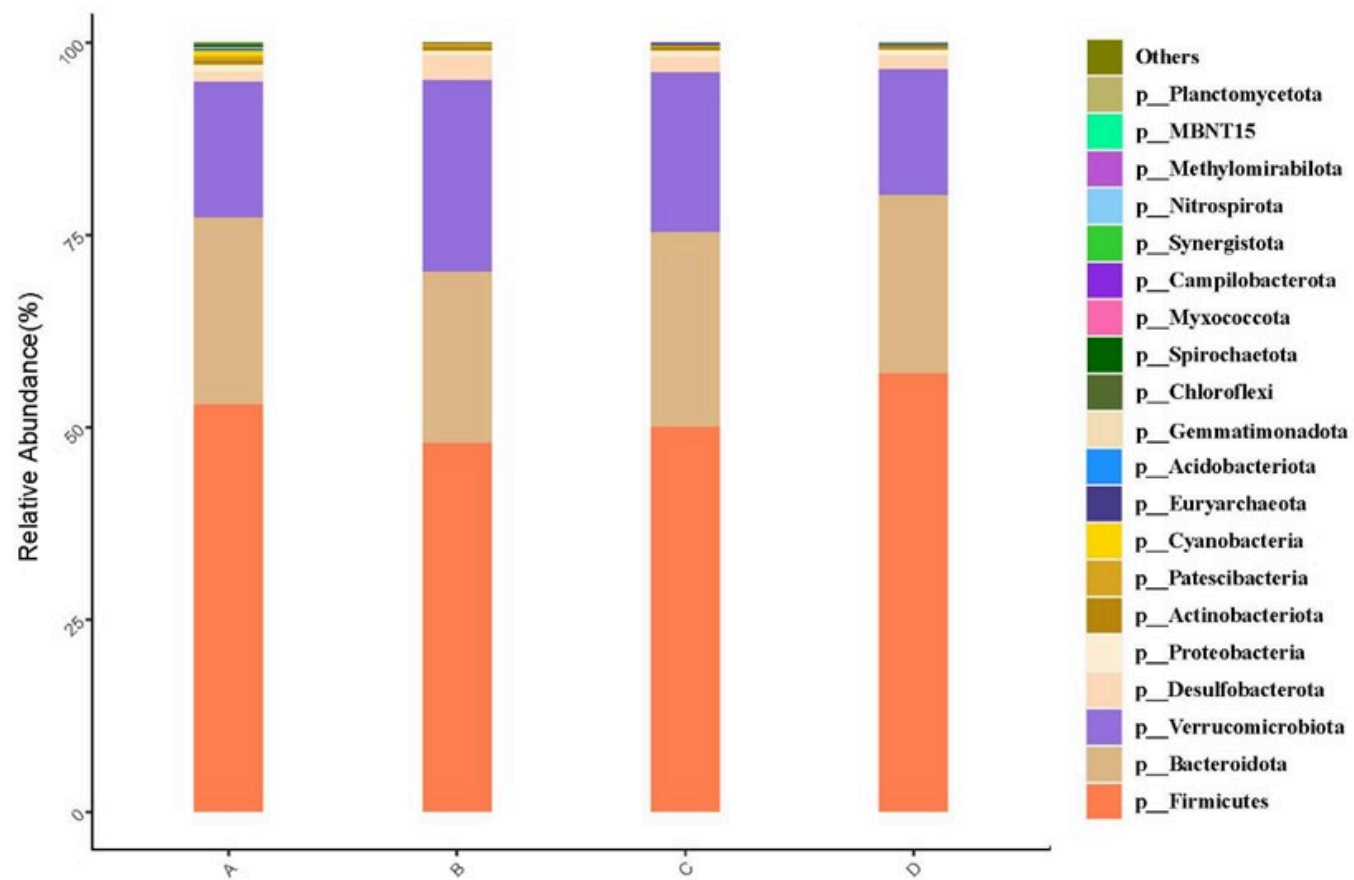


Figure 2

Relative abundance of caecal microflora of experimental rabbits at phylum level(%)

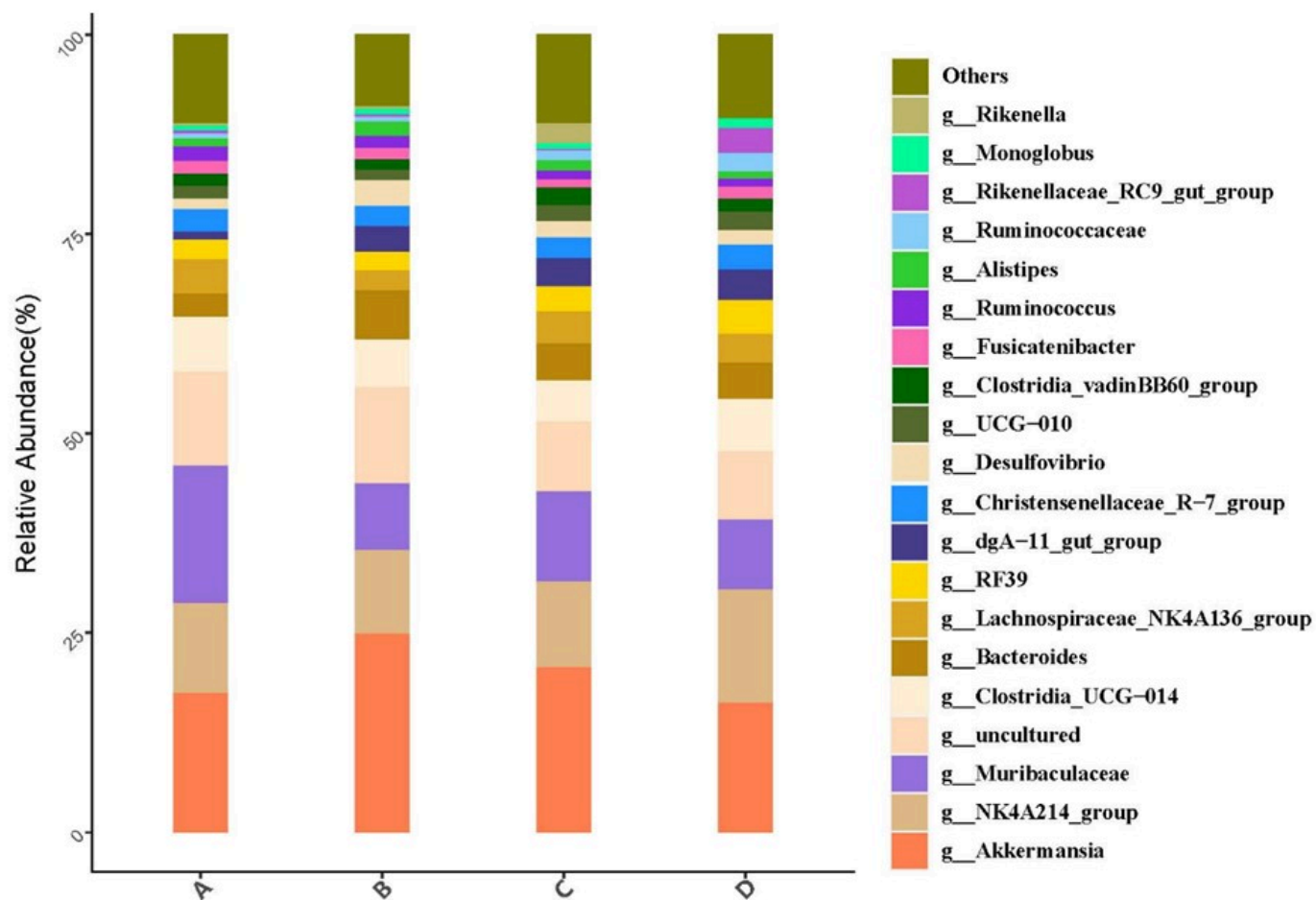


Figure 3

Relative abundance of caecal microflora of experimental rabbits at genus level(%)

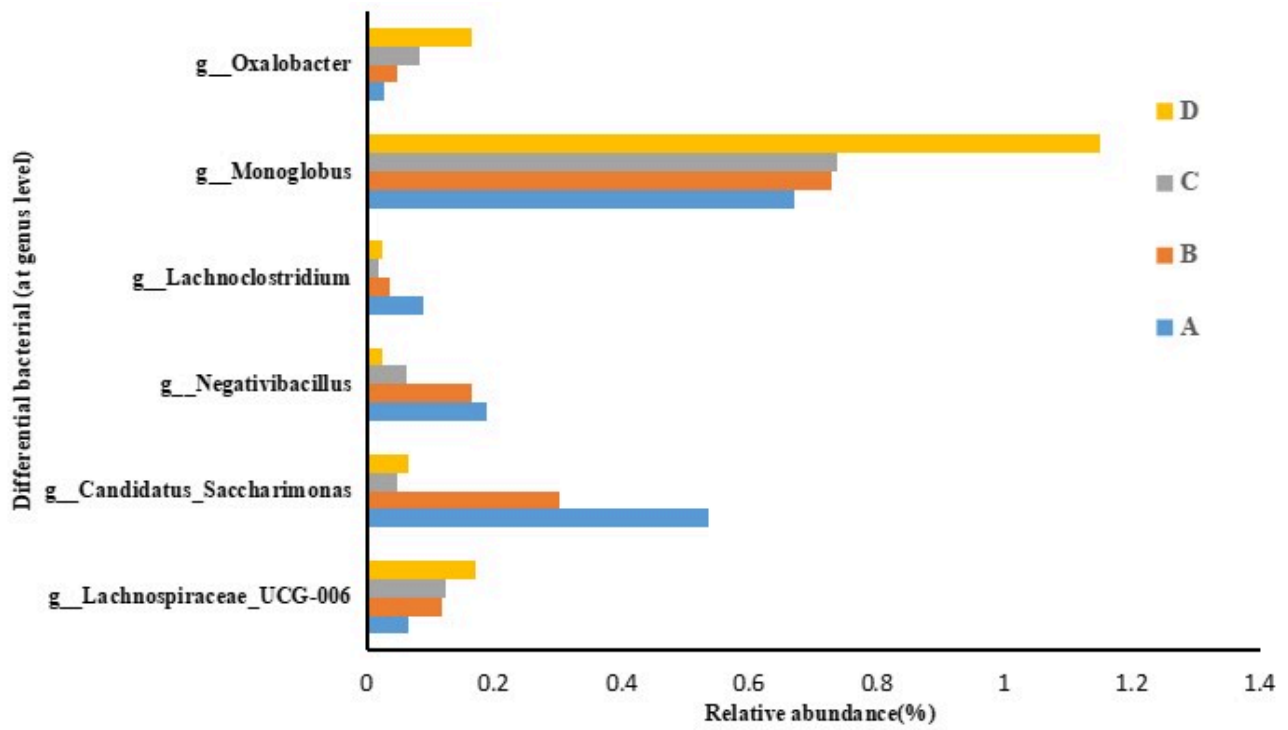


Figure 4

Relative abundance of different bacteria at genus level in Kruskal-Wallis detection method (%)

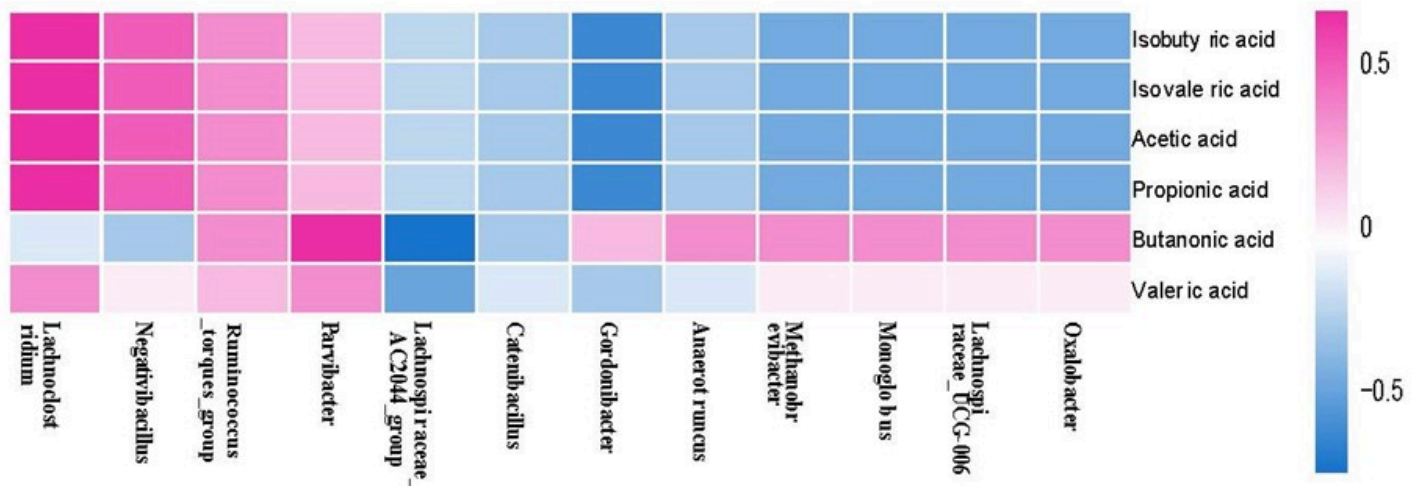


Figure 5

Correlation analysis of volatile fatty acids(VFA) in caecum with species diversity