High-throughput sequencing–based analysis of the intestinal microbiota of broiler chickens fed with compound small peptides of Chinese medicine

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ABSTRACT The objective of this study was to determine the effects of compound small peptides of Chinese medicine (**CSPCM**) on the intestinal microbiota of broilers. A total of thirty-six 1-day-old Arbor Acres broilers were assigned to 6 dietary treatments that include 250, 500, and 750 g/T of CSPCM in feed, 100 g/T of *Bacillus subtilis* and *Clostridium butyricum* in feed, and 100 g/T of 50,000 IU xylanase in feed. Each treatment had 2 replicates with 2 cages (3 birds per cage). The jejunal digesta samples were collected from chickens at 42 d. Operational taxonomic unit analysis showed that adding CSPCM at a concentration of 750 g/T of feed can increase the number of operational taxonomic unit samples than other groups. Compared with the control group, adding 250 g/T of CSPCM of feed can improve content of *Lactobacillus*, *Cupriavidus*, *Ochrobactrum*, *Candidatus_Arthromitus*, *Acinetobacter*, and *Sphingomonas.* Adding 500 g/T of CSPCM in feed resulted in varying degrees of improvement in *Candidatus_Arthromitus*, *Acinetobacter*, and *Sphingomonas.* Adding 750 g/T of CSPCM in feed can increase the content of *Lactobacillus* and *Candidatus_Arthromitus.* In PICRUSt function prediction analysis, CSPCM acts on the body by creating an environment suitable for the growth of beneficial bacteria. Adding 250 g/T of CSPCM in feed can improve amino acid metabolism, endocrine system function, membrane transport, and cell mobility function. Adding 500 g/T of CSPCM in feed can improve replication and repair and membrane transport function. Adding 750 g/T of CSPCM in feed can increase carbohydrate metabolism, replication and repair, and membrane transport function. Adding *B. subtilis* and *C. butyricum* in feed increased replication and repair and membrane transport function. Adding xylanase in feed increased membrane transport function. In conclusion, this study demonstrated that dietary supplementation of CSPCM to broiler diets increased beneficial flora content, metabolism of carbohydrates, amino acid metabolism, the deposition of proteins, renewal of bacteria, and maintenance of vigorous vitality. Among the 3 additive quantities of 250 g/t, 500 g/t, and 750 g/t of CSPCM in feed, 250 g/t of CSPCM improved parameters that are necessary for improved growth and production.

Key words: broiler, compound small peptide of Chinese medicine, high-throughput sequencing–based analysis, intestinal microflora

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INTRODUCTION

Immediately after birth, the gastrointestinal tract of the animal is colonized by a complex and diverse microbial ecosystem. The bacterial invasion and the gut microbial composition have an enormous impact on the host's health and well-being (Mohd Shaufi et al., 2015; Alexander et al., 2018; Tong et al., 2018). Chickens have proportionally shorter intestines and shorter transit digestion times than mammals, but do not appear to be less efficient at digestion than their mammalian counterparts (Inês and Mingan, 2018). The chicken digestive system is adapted to extract energy from difficult-to-digest food sources (Mcwhorter et al., 2010). This may be explained, in part, by the fact that the chicken gastrointestinal tract is home to a complex microbial community, the chicken gut microbiota, which underpins the link between diet and health (Xu et al., 2016). The digestive tracts of animals are complex microecosystems that include gut microbiota

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that exist in a dynamic symbiosis between the host, microbiota, and the environment (Yuan et al., 2019). It has been estimated that the microbes in our bodies collectively make up to 100 trillion cells, 10-fold the number of human cells, and suggested that they encode 100-fold more unique genes than our own genome (Hu et al., 2017). Gut microbiota are an integral component of their host. Microbiota play a key role in host fitness, including the proliferation of enterocytes, the defense against pathogens, the production of secondary metabolites, and the digestion of complex carbohydrates. This microecosystem can perform numerous metabolic functions that change as the composition of the microbiome changes (Cullen et al., 2017). Gut microbes are important indicators of animal health. In different parts of the intestine, the species distribution of bacterial populations varies greatly owing to different pH values and nutritional status (Sánchez-Moya et al., 2017). Most of the intestinal flora are symbiotic types, mainly anaerobic bacteria, such as *Bifidobacterium*, *Eubacteria*, and digestive cocci, which are present in a constant amount, and have physiological functions such as vitamin synthesis, protein antagonism, and biological antagonism, which can maintain the health of the host (Qiao et al., 2018). The gut microbiota is also an essential stimulus that results in the maturation of the animal's gut immune homeostasis and the intestinal immune response (Jing et al., 2018). The symbiotic flora living in the intestine shows a good interaction with the host's immune system (Qin et al., 2010; Mohd Shaufi et al., 2015). Broilers as an important economic animal, the most important thing is to improve their digestion and absorption of nutrients, at the same time to avoid drug residues. The gut flora of broilers metabolizes the food that enters the gut, digesting it into nutrients that can be absorbed. In this experiment, by improving the addition of compound small peptides of Chinese medicine (**CSPCM**) into the feed, the effect of improving intestinal flora was achieved, thus increasing the deposition of nutrients and finally achieving the goal of improving broiler economic benefits.

Poultry products have become indispensable in daily life, and the safety and efficiency of the poultry industry have become more important. In poultry diets, antibiotics have been used for preventing and treating disease. However, indiscriminate use of these substances may lead to the occurrence of undesirable side effects such as drug residues, drug resistance (Roth et al., 2019), and liver (Han et al., 2019) damage in broilers. Each Chinese medicine has its best producing area, but prevention, treatment, and diagnosis of diseases are under the theory of traditional Chinese medicine (**TCM**). If TCM can be taken orally into the gastrointestinal tract, it must depend on the metabolism of intestinal flora to produce effective components that are easy to absorb or to produce new pharmacological components. And the toxicity of TCM can be decompositioned through the metabolism of intestinal flora. Intestinal flora can produce abundant enzymes to metabolize flavonoids and saponins into aglycones to exert their pharmacological activities. Under the theory of TCM, TCM is used for the prevention, treatment, and diagnosis of diseases and has the function of rehabilitation and health care. As food safety issues increasingly attract the attention of consumers to healthy poultry industry and the use of natural drugs, TCM as a feed additive is of great significance to improve disease resistance and maintain intestinal stability in broilers (Wang et al., 2019). Many reports have pointed out that TCM could improve the intestinal flora structure and create a good intestinal environment, such as Chinese parsnip root, *Astragalus membranaceus*, and *Atractylodes macrocephala* Koidz, which can modulate the composition of gut microbiota and upregulate the metabolism of gut microbiota (Feng et al., 2019; Dongsheng et al., 2019). A multitude of studies supports the use of herbal medicines, herbal formulas, and phytochemicals, such as Gegen Qinlian Decoction formula, Qushi Huayu Decotion formula, and quercetin, which could upregulate the beneficial gut microbiota (Meng et al., 2018). Feeding incorporated diets with appropriate *A. membranaceus* regulated the intestinal flora by promoting the proliferation of *Lactobacillus* and inhibiting the growth of coliform bacteria (Guo et al., 2019). Probiotics combined with *Astragalus* polysaccharide administration in feed displayed synergistic modulation effects on intestinal microbiota (Li et al., 2009). China will ban the use of antibiotics as additives from 2020. The search for antibiotic substitutes has become a hot topic. Existing feed additives include TCM, probiotics, enzyme preparation, and so on. The probiotics used in this experiment are *Bacillus subtilis* and *Clostridium butyrate*. Previous studies have shown that adding *B. subtilis* and *C. butyrate* to feed can improve the growth performance of broiler chickens through beneficial effects on intestinal flora and the host (Jacquier et al., 2019). Enzyme preparation used in this experiment was 5,000 IU of xylanase. Xylanase can regulate the activity of intestinal flora and promote the degradation of feed fiber components (Munyaka et al., 2016; Konieczka et al., 2018). The peptide activities have been described, including antimicrobial and antifungal properties, blood pressure–lowering effects, cholesterol-lowering ability, antithrombotic effects, enhancement of mineral absorption, immunomodulatory effects, and localized effects on the gut (Salavati et al., 2019). Researchers discovered that ingesting hydrolyzed soy protein results in faster and more efficient absorption than consumption of protein or amino acid mixtures (Maebuchi et al., 2007). Soy peptides can increase the diversity of intestinal flora in animals (Dimidi et al., 2019) and can recombine lactic acid bacteria, inhibit bacteria, and increase the total number of aerobic bacteria in laying sacs, the jejunum, and the cecum (Kalmendal and Tauson, 2012). Adding 2% soy peptide to the diet can significantly reduce the number of jejunal colon cancer and increase the number of lactobacilli (Kim and Isaacson, 2015). A large number of studies have shown that TCM and peptides could

improve the intestinal flora structure and create a good intestinal environment, but there is limited research in the area of TCM in poultry. High-throughput sequencing analysis was used to investigate the effect of CSPCM on the intestinal flora of broilers, thereby promoting the application of CSPCM in healthy breeding of broilers. Therefore, in this study, CSPCM (containing 64.8% of sbean peptide, 25% of wheat germ powder, 10% of *Astragalus* hydrolysate, and 0.2% of vitamin C) were added to the broiler chicken feed. Traditional Chinese medicine can increase the action site through the formulation to enhance its effect, but there are not many reports investigating the effects of compound peptide of Chinese medicine on the intestinal microorganisms of broilers. Therefore, we studied the effect of CSPCM on intestinal flora to provide evidence for the clinical application of CSPCM.

MATERIALS AND METHODS

Bird Management

A total of 36 Arbor Acres broilers were purchased from Hebei Dawu Agricultural Group Poultry Company Ltd. (Baoding, China). The CSPCM, *B. subtilis*, *C. butyricum*, and xylanase were provided by HeBei TaiFeng Biotechnology Co., Ltd. (Handan, China). In this experiment, thirty-six 1-day-old broilers were randomly assigned to 6 dietary treatments. The 6 groups were A (control group), B (250 g/T of CSPCM in feed), C (500 g/T of CSPCM in feed), D (750 g/T of CSPCM in feed), E (100 g/T of *B. subtilis* and *C. butyricum* in feed), and F (100 g/T of 50,000 IU xylanase in feed). Each treatment had 2 replicates with 2 cages (3 birds per cage) as per dietary treatments. The cages are suspended 3-layer cages with the size of 100 × 120 × 100 cm. This experiment used round feeders and water nipples, and water and feed were available ad libitum. The house was artificially ventilated, and continuous light regimens were provided. All chicks were raised in an environmentally controlled room (34°C to 36°C) during 1 to 14 d; then, the temperature was gradually decreased to 26°C until the end of this experiment. The experiments complied with the Guide to the Care and Use of Experimental Animals (National Research Council, 2011) with respect to experimentation and care of birds under study. This experiment has been approved by American Association for Laboratory Animal Science.

Experiment Procedures and Sampling

Broilers were reared for 42 d, and chicks were inoculated with the Newcastle disease–avian influenza combined vaccine at 7 d and inoculated with the live Newcastle disease vaccine at 21 d. At 42 d, the broilers were euthanized, and jejunal digesta samples were placed in frozen storage tubes and quickly placed in dry ice.

Sample Processing, Laboratory Analyses, and Calculation

DNA Extraction Total microbial genomic DNA samples were extracted using the DNeasy PowerSoil Kit (QIAGEN, Inc., Hilden, Germany), following the manufacturer's instructions, and stored at −20°C before further analysis. The quantity and quality of extracted DNAs were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis, respectively.

16S rRNA Gene Amplicon Sequencing PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 515F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 907R (5′-GGACTACHVGGGTWTCTAAT-3′). Sample-specific 7-bp bar codes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μl of Q5 reaction buffer (5 ×), 5 μl of Q5 High-Fidelity GC buffer (5 ×), 0.25 μl of Q5 High-Fidelity DNA Polymerase (5U/μl), 2 μl (2.5 mM) of dNTPs, 1 μl (10 μM) of each forward and reverse primer, 2 μl of DNA template, and 8.75 μl of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 2 min, followed by 25 cycles consisting of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension for 5 min at 72°C. PCR amplicons were purified using Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA). After the individual quantification step, amplicons were pooled in equal amounts, and paired-end 2 × 300 bp sequencing was performed using the Illumina MiSeq platform t Shanghai Personal Biotechnology Co., Ltd. (MiSeq Reagent Kit v3, Shanghai, China).

Sequence Analysis

The Quantitative Insights Into Microbial Ecology (**QIIME**, version 1.8.0) pipeline was used to process the sequencing data, as previously described (Caporaso et al., 2010). In brief, raw sequencing reads with exact matches to the bar codes were assigned to respective samples and identified as valid sequences. The low-quality sequences were filtered using the following criteria: sequences that had a length of <150 bp, sequences that had average Phred scores of <20, sequences that contained ambiguous bases, and sequences that contained mononucleotide repeats of >8 bp. All sequences are filtered, denoised, merged, nonchimeric, and nonsingleton to filter out high-quality sequences. Paired-end reads were assembled using FLASH (Magoc and Salzberg, 2011). After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (**OTU**) at 97% sequence identity by UCLUST (Edgar, 2007). A representative sequence was selected from each OTU using

Table 1. Sequencing depth of each sample.

Sample I	A	B	C	D	E	F
Sample 1	28,338	40,096	35,013	23,730	33,546	38,531
Sample 2	41,839	42,960	34,962	35,644	44,139	33,883
Sample 3	45,858	38,764	38,171	32,682	39,732	32,671
Sample 4	44,556	33,722	39,453	32,821	42,042	34,125
Sample 5	28,064	35,542	40,353	33,301	44,696	38,185
Sample 6	24,006	32,517	41,532	41,305	44,639	31,763

default parameters. Operational taxonomic unit taxonomic classification was conducted by BLAST searching the representative sequences set against the Greengenes Database (DeSantis et al., 2006) using the best hit. An OTU table was further generated to record the abundance of each OTU in each sample and the taxonomy of these OTU. Operational taxonomic units containing less than 0.001% of total sequences across all samples were discarded. To minimize the difference of sequencing depth across samples, an averaged, rounded rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under the 90% of the minimum sequencing depth for further analysis.

Bioinformatics and Statistical Analysis

Sequence data analyses were mainly performed using QIIME and R packages (version 3.2.0). Operational taxonomic unit–level alpha diversity indices, such as Chao1 richness estimator, Abundance-based Coverage Estimator (**ACE**) metric, Shannon diversity index, and Simpson index, were calculated using the OTU table in QIIME. Operational taxonomic unit–level ranked abundance curves were generated to compare the richness and evenness of OTU among samples. Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using UniFrac distance metrics. Differences in the UniFrac distances for pairwise comparisons among groups were determined using the Student t test and the Monte Carlo permutation test with 1,000 permutations and visualized through the box-and-whisker plots. Taxon abundances at the genus levels were statistically compared among samples or groups by the methodology of Metastats (White et al., 2009) and visualized as violin plots. Linear discriminant analysis effect size was performed to detect differentially abundant taxa across groups using the default parameters. Partial least squares discriminant analysis was also introduced as a supervised model to reveal the microbiota variation among groups, using the "plsda" function in R package "mix Omics." Microbial functions were predicted by PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states), based on high-quality sequences.

RESULTS

Sequencing Depth

The sequencing depth was 95% of the minimum sample sequence size. In this experiment, the minimum sequence amount was 23,730, and the sequencing depth was 22,544 (Table 1).

Operational Taxonomic Unit Analysis

Operational taxonomic units were analyzed for each sample at 97% identity. We obtain 145, 44, 101, 858, 54, and 82 OTU samples from A, B, C, D, E, and F group, respectively, with 274 shared OTU between the 6 groups (Figure 1A).

Alpha Diversity Analysis

The alpha diversity indices of jejunal ecosystems are shown in Table 2. No differences in the Chao1 index, the ACE index, and the Simpson index were found between the 6 groups.

On the whole, we found that the rank abundance curve was smooth. In the later period, the polyline length of the first sample in D group was relatively long, and the OTU number was relatively large, but the species uniformity was relatively low, and the polyline of other samples was relatively flat, with no significant difference in uniformity and richness. Species accumulation curves tend to be gentle, indicating that the species in the

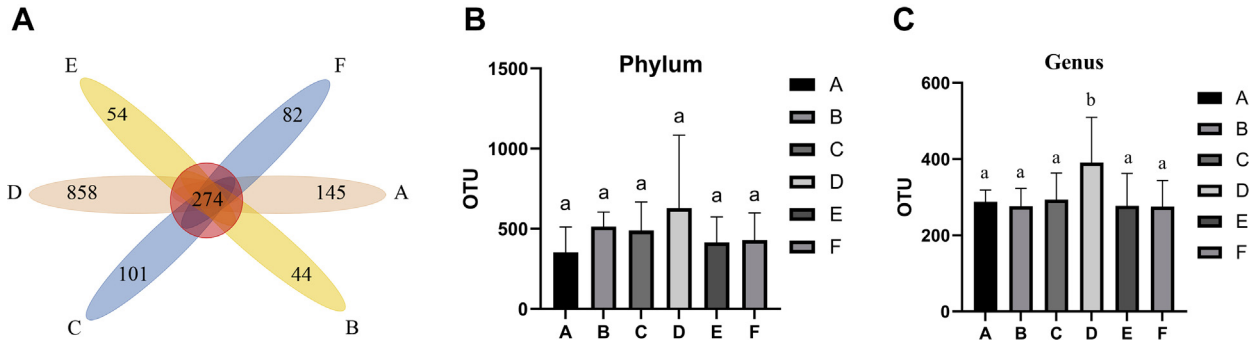

Figure 1. (A) Petals figure: Each petal represents a group, the number in the middle represents the number of OTU shared by all groups, and the number on the petal represents the number of OTU unique to the group. (B) Microbial flora bar plot at the phylum level. (C) Microbial flora bar plot at the genus level: the same lowercase letters mean no significant difference ($P > 0.05$), whereas different lowercase letters mean significant difference ($P < 0.05$). Abbreviation: OTU, operational taxonomic unit.

Table 2. Alpha diversity indices of jejunal ecosystems in broilers.

Group	Chao1	ACE	Simpson	Shannon
A	570.11 ± 156.13[a]	582.96 ± 157.89[a]	0.88 ± 0.13[a]	5.66 ± 1.37[a]
B	548.66 ± 84.53[a]	564.92 ± 82.07[a]	0.89 ± 0.03[a]	5.16 ± 0.57[a]
C	549.37 ± 216.68[a]	565.83 ± 277.74[a]	0.86 ± 0.11[a]	4.89 ± 1.18[a]
D	710.73 ± 535.09[a]	745.54 ± 573.51[a]	0.87 ± 0.09[a]	5.36 ± 1.4[a]
E	452.08 ± 175.53[a]	471.73 ± 188.43[a]	0.89 ± 0.07[a]	4.94 ± 1.11[a]
F	460.21 ± 175.06[a]	475.94 ± 183.88[a]	0.83 ± 0.11[a]	4.48 ± 0.11[a]

Each mean represents values from 6 birds

The data in this experiment are mean ± SD, and significant differences among groups are set at a value of $P < 0.05$ and $P < 0.01$. The same lowercase letter indicates no difference.

Abbreviation: ACE, Abundance-based Coverage Estimator.

specimen will not increase significantly with the increase in the sample size (Figure 2B).

Analysis of the Intestinal Flora Structure

The 6 major species in each group were *Lactobacillus*, *Cupriavidus*, *Ochrobactrum*, *Candidatus_Arthromitus*, *Acinetobacter*, and *Sphingomonas*. At the phylum level, most of them belong to *Proteobacteria* and *Firmicutes*, accounting for about 84.1% of the total sequence. In B group, the abundance of *Firmicutes* was significantly lower than that in D group (24.27% vs. 75.28%, respectively, $P < 0.05$), and the abundance of *Proteobacteria* (64.07% vs. 19.32%, respectively, $P < 0.05$) was significantly higher than that in D group. In A group, the abundance of Bacteroidetes was significantly higher than that in E (9.43% vs. 2.33%, respectively, $P < 0.05$) and F (9.431% vs. 2.44%, respectively, $P < 0.05$) groups. At the genus level, *Lactobacillus* is the main bacterial genus in A, B, C, D, E, and F groups; *Cupriavidus* is the main bacterial genus in B group (Figure 3).

Linear Discriminant Analysis Effect Size Analysis

As per the linear discriminant analysis distribution histogram, there were 30 taxa with statistical difference. Among the 30 taxa, 16 taxonomic units with the highest average abundance corresponding to A group are Bacteroidales (4.95%), Bacteroidia (4.95%), Bacteroidetes (4.98%), *S24_7* (4.78%), Deltaproteobacteria (3.999%), Desulfovibrionaceae (3.99%), Desulfovibrionales (3.99%), *Coprococcus* (3.67%), *Dehalobacterium* (3.04%), *Limnohabitans* (3.07%), Veillonellaceae (3.64%), *TM7* (2.86%), *TM7_3* (2.86%), Erysipelotrichales (3.4%), Erysipelotrichia (3.4%), Erysipelotrichaceae (3.4%), *Acidovorax* (2.5%), Bacteroidaceae (4.31%), *Bacteroides* (4.31%), Porphyromonadaceae (4.2%), *Parabacteroides* (4.2%), *Methylibium* (2.57%) were the taxonomic elements with the highest average richness corresponding to B group (3.11%), *Bifidobacterium* (2.97%), Bifidobacteriales (2.97%), Bifidobacteriaceae (2.97%), Bdellovibrionaceae (3.11%). *Clostridium* (3.56%) is the highest average abundance taxon of C group. The highest average abundance taxon corresponding to D group is *Oscillospira* (4.06%) and *Streptococcus* (3.66%). The taxon with the highest average abundance corresponding to E group was Bdellovibrionales (3.24%).

As per the evolutionary branch graph, the important microbial groups of A group are Bacteroidetes radiating outward to Bacteroidia, Bacteroidales, and *S247* families. Erysipelotrichia radiates outward to Erysipelotrichales and finally to Erysipelotrichaceae. Deltaproteobacteria radiate outward to Desulfovibrionales and finally to Desulfovibrionaceae (Figure 4A).

The important microbial groups of B group are Bacteroidaceae to *Bacteroides*. Porphyromonadaceae extends outward to *Paraberoides*. Bifidobacteriales

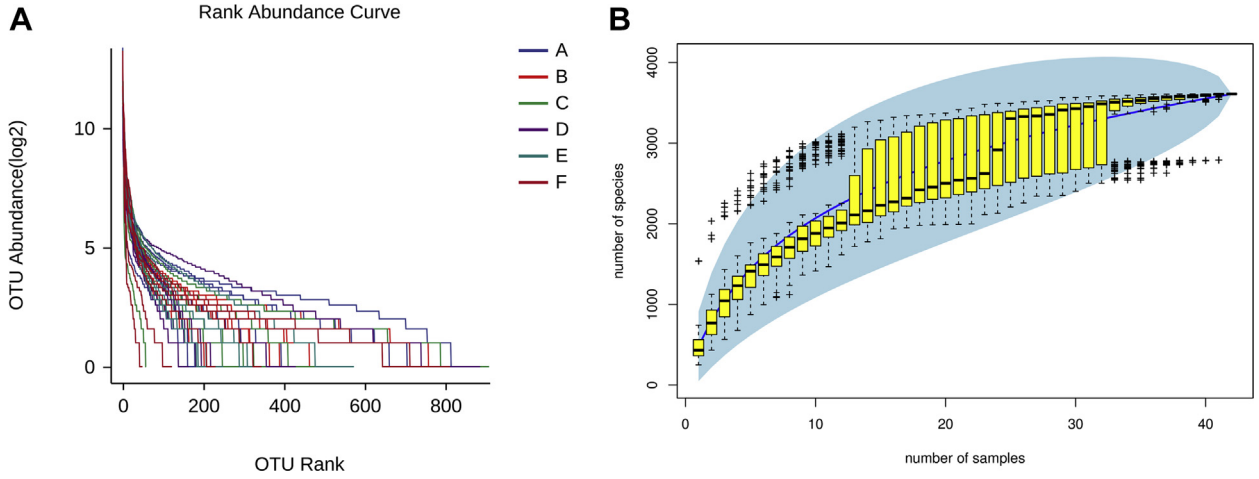

Figure 2. (A) Rank abundance and (B) species accumulation curve of jejunal microorganisms. Abbreviation: OTU, operational taxonomic unit.

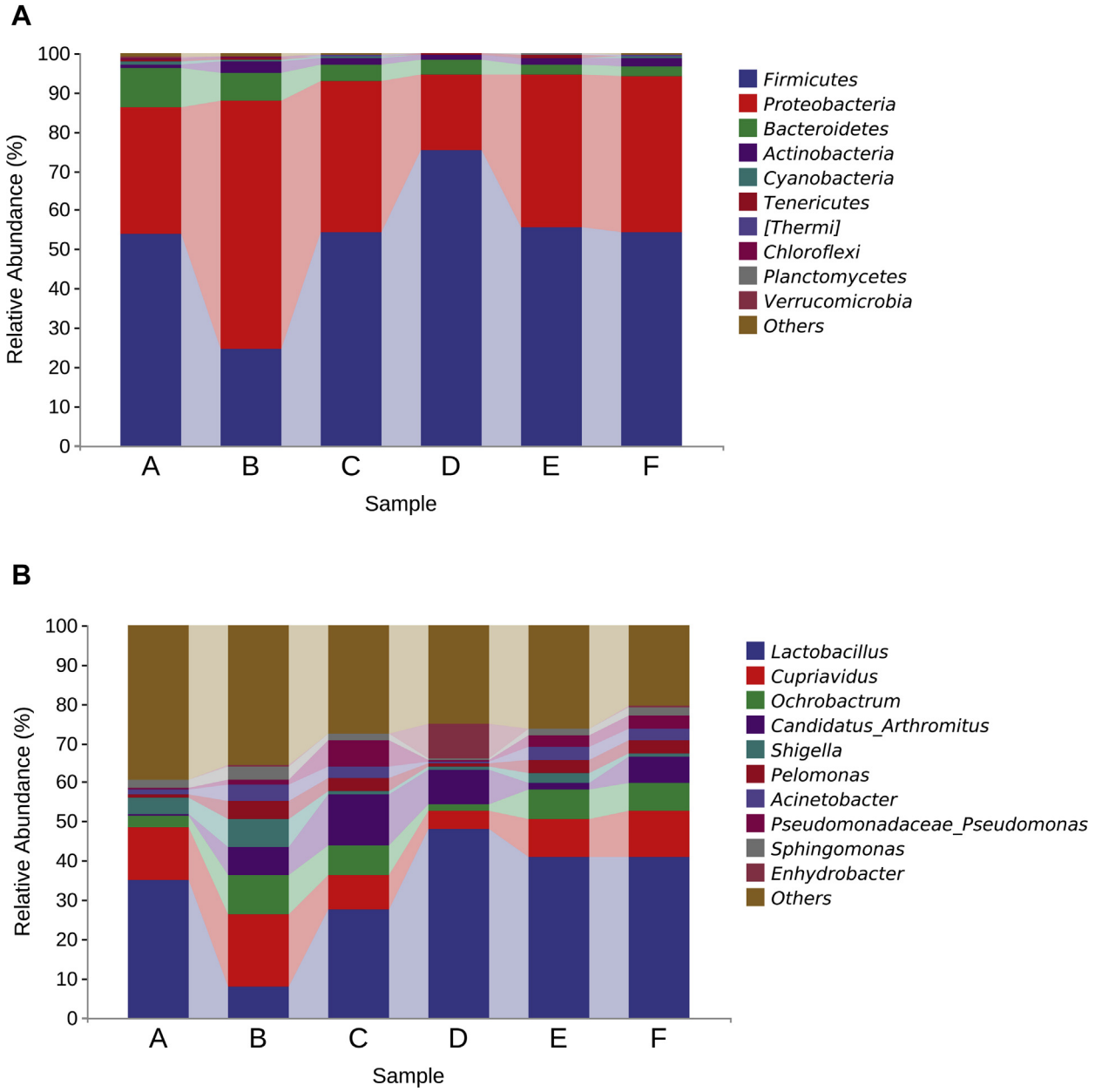

Figure 3. (A) The relative abundances of the major bacteria at the phylum level. (B) The relative abundances of the major bacteria at the genus level. (The horizontal coordinate is arranged according to the sample name, each bar graph represents a sample, and colors are used to distinguish each taxon. The vertical coordinate represents the relative abundance of each taxon. The longer the column, the higher the relative abundance of the taxon in the corresponding samples.)

extends outward to Bifidobacteriaceae and finally to *Bifidobacterium*. Bdellovibrionaceae extended to *Bdellovibrio*. The important microbial groups in D group are *Oscillospira* and *Streptococcus*. *Clostridium* plays an important role in C group. The important microbial group in E group is Bdellovibrionales (Figure 4B).

Beta Diversity Analysis

The core flora of A group was significantly different from that of the other 5 groups (Figure 5). The core flora of A group was significantly different from that of the other 5 groups, with the most significant difference between B group and A group as well as E group and F group. Weighted UniFrac distance and unweighted UniFrac distance of B group vs. B group are the shortest. Weighted UniFrac distance and unweighted UniFrac distance of D group vs. A group are longer than the distance of others groups vs. A group (Figure 6).

PICRUSt Function Prediction Analysis

It can be seen that low concentration of and 500 g/t of CSPSM in feed, enzymes, and probiotics have no effect on carbohydrate metabolism function, whereas 750 g/t of CSPSM in feed can improve carbohydrate-metabolizing function. In the "Amino Acid Metabolism" item, only amino acid–metabolizing function in B group is higher than that in A group. In the "Endocrine System" item, only endocrine system function in B group

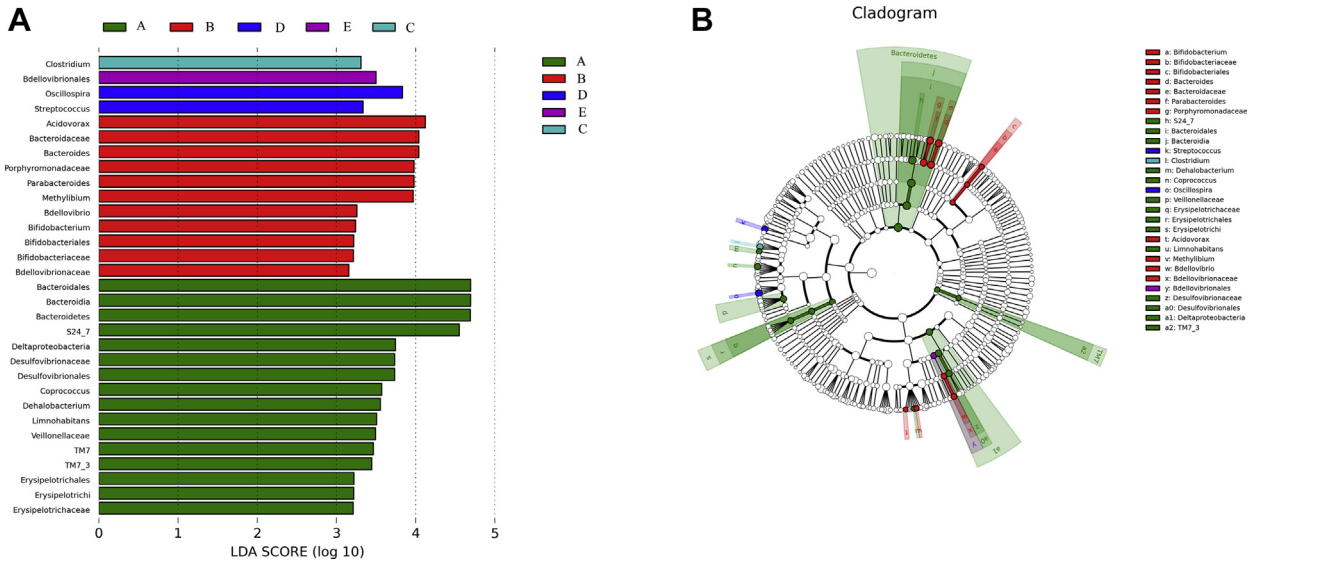

Figure 4. (A) Distribution histogram and evolutionary branch graph based on LDA value: The histogram of the LDA score shows the biomarkers with statistics difference between groups. The influencing degree of species was expressed by the length of the bar in the histogram. (B) Cladogram: the circle radiated inside out demonstrated the classification—from the phylum to genus. Each small circle at different classification represents a taxon, and the diameter of the circle is proportional to the relative abundance. The species not with significant differences are colored by yellow, and biomarkers are colored by different groups. Red and green dots represent the core bacterial populations in the respective group. Abbreviation: LDA, linear discriminant analysis.

was higher than that in A group. Regarding this item, C, D, and E groups differed from A group. The cell mobility function of B group is slightly higher than that of A group (Figure 7).

DISCUSSION

Bioactive peptides are protein-derived components, which when used by animals provide beneficial impacts on their health (Salavati et al., 2019). Studies have shown that dietary differences affect the abundance of bacteria in the gut of broiler chickens (La-Ongkhum et al., 2011; Mohammed et al., 2019). Microbial diversity is a new health marker, and it is important to maintain the stability and performance of the ecosystem (Ji et al., 2017). The decrease of intestinal flora diversity may directly affect the physiological function of intestinal microbes. Traditional Chinese medicine is an important substitute for antibiotics. The effects of CSPCM on intestinal flora of broiler chickens are discussed as follows.

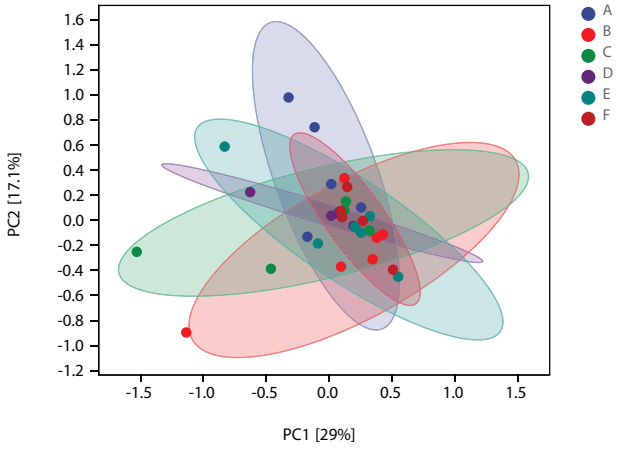

Figure 5. PLS-DA discriminant analysis diagram. Each point represents a sample. The points of the same color belong to the same group. The points of the same group are marked by ellipses. If the samples belonging to the same group are closer to each other and the points of different groups are farther apart, it indicates that the classification model is more effective. Abbreviation: PLS-DA, partial least squares discriminant analysis.

The rank abundance curve was smooth, which indicates high evenness among samples (Torok et al., 2011). The species accumulation curves tend to be smooth, and there was no difference between the groups, indicating that the species in the specimen will not increase significantly with the increase in the sample size. This showed that the sample size was sufficient and data analysis could be carried out (Geng et al., 2018). This is the basis for the analysis of other results.

Alpha diversity analysis is to analyze the species diversity of independent samples. The Chao1 estimator and the ACE estimator of D group are higher than those in other groups. The higher the value, the richer the species, indicating that 750 g/t of CSPSM in feed can improve the species evenness, but the sample within the group varies greatly. From rank abundance distribution curves, we find that the curves of the first sample are more smooth than other samples' curve in D group. The Simpson index of B group is the highest; the higher the value, the higher the species diversity of samples, which indicates that 250 g/t of CSPSM in feed can improve the dominant OTU and evenness of intestinal flora. In this experiment, 97% similar sequences were classified as an OTU, which can represent species richness to a certain extent. The Shannon diversity index of A group is the highest; the higher the value, the higher the species diversity of samples, which indicates that this group has

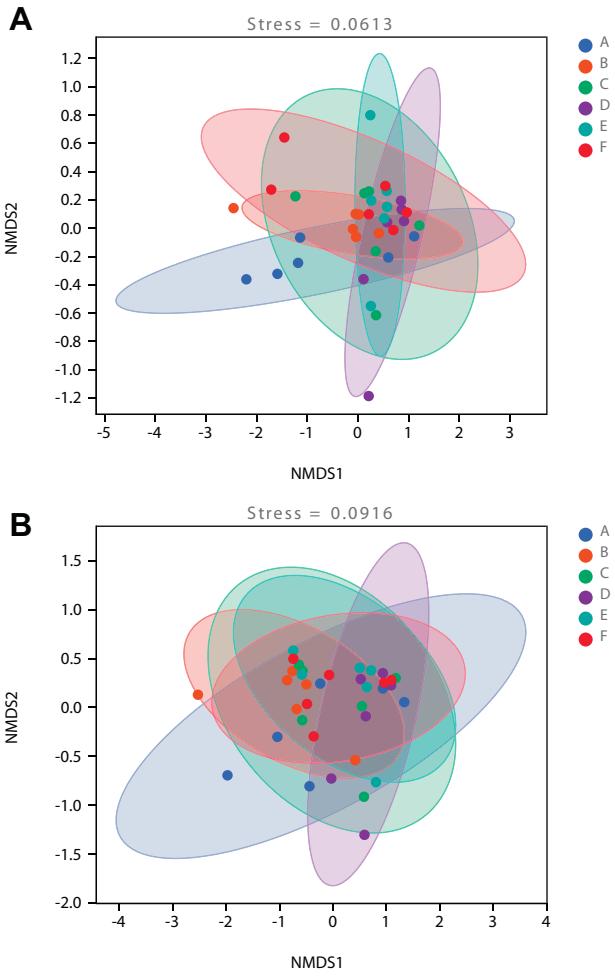

Figure 6. (A) Multiple comparison box plot of unweighted UniFrac distance. (B) Multiple comparison boxplot of weighted UniFrac distance. (The horizontal coordinate corresponds to the statistical comparison between and within each group; the vertical coordinate indicates the corresponding distance value; the border of the box graph represents the interquartile range [IQR]; the horizontal line represents the median value; the upper and lower whisker represents 1.5 times the IQR range outside the upper and lower quartile, respectively; the symbol "+" represents the potential outliers beyond the range.)

the highest diversity and number of rare OTU possibly because other groups can reduce the number of rare OTU.

The results showed that the content of 6 main jejunal bacteria in B group was higher than that in A group. Compared with A group, C group had varying degrees of improvement in *Candidatus_Arthromitus, Acinetobacter*, and *Sphingomonas*. D group showed an effect on the increase of the content of *Lactobacillus* and *Candidatus_Arthromitus*, which can promote digestion, improve carbohydrate metabolism, and promote membrane transportation to achieve the effect of promoting growth (Li et al., 2018; Richards et al., 2019). *Lactobacillus* and *Candidatus_Arthromitus* could produce butyrate, which is the main source of energy for enterocytes and known to be an immune modulator (Jacquier et al., 2019).

The abundance of 11 species of bacteria in C group was higher than that in A, E, and F groups (Figure 3). The 11 species in C group that were different from A group were coincident with the species in B group that were different from A group. The abundance of *Oscillospira* in C group is higher than that in A group but lower than that in E and F groups. The abundance of *Streptococcus* in C group is higher than in A group, but lower than in F group. The abundance of *Streptococcus* in D group was higher than that in A, E, and F groups, indicating that 750 g/t of CSPSM in feed was not conducive to the growth of beneficial bacteria and increased the incidence of broiler diseases. The results are consistent with the study by Brus et al., 2018, which found negative effects at high doses. Combined with the results of the rank abundance curve and OTU simplification and classification, the OTU number data of D group are relatively unstable. Among them, the first sample in D group differs greatly from other data in the group. Adding 750 g/t of CSPSM in feed is not conducive to maintaining the stability of intestinal flora and the growth of beneficial intestinal bacteria. The results showed that B and C groups could increase the abundance of some beneficial bacteria, and the effect of B group was better than that of E and F groups. The probiotics can improve gut-beneficial microorganisms by inhibiting the growth of pathogens (Brus et al., 2018). Enzyme preparation promotes the absorption of nutrients by changing the enzyme activity in the intestine (Guo et al., 2014). Different from enzymes and probiotics, TCM does not act directly on bacteria, but on chicks, creating an environment conducive to the growth of gut-beneficial bacteria.

Linear discriminant analysis effect size was used to identify biomarkers with statistically significant differences between groups. The results showed that *Bacteroides*, *Parabacteroides*, *Bifidobacterium*, and *Bdellovibrio* play an important role in improving intestinal diseases caused by immune system disorders, inhibiting growth of intestinal pathogenic bacteria, scavenging free radicals, improving the host's antioxidant enzyme activity, and reducing the content of MDA(malondialdehyde) in serum and the liver (Borda-Molina et al., 2019), thereby alleviating oxidative damage, delaying aging, and improving intestinal flora disorders caused by the disease.

The main purpose of beta diversity analysis is to investigate the similarity of the community structure among different samples, to naturally decompose the community data structure, and to observe the differences among samples by sorting the samples (Fang et al., 2016). The unweighted UniFrac distance focuses on describing the sample difference caused by the distinct difference of community members, whereas the weighted UniFrac distance focuses on describing the sample difference caused by the change of abundance gradient of community members. As per the analysis of nonmetric multidimensional scaling, partial least squares discriminant analysis, and UniFrac distance. the difference between groups and within groups showed that there were significant differences in the flora of A group and the other 5 groups. It indicated that the CSPSM,

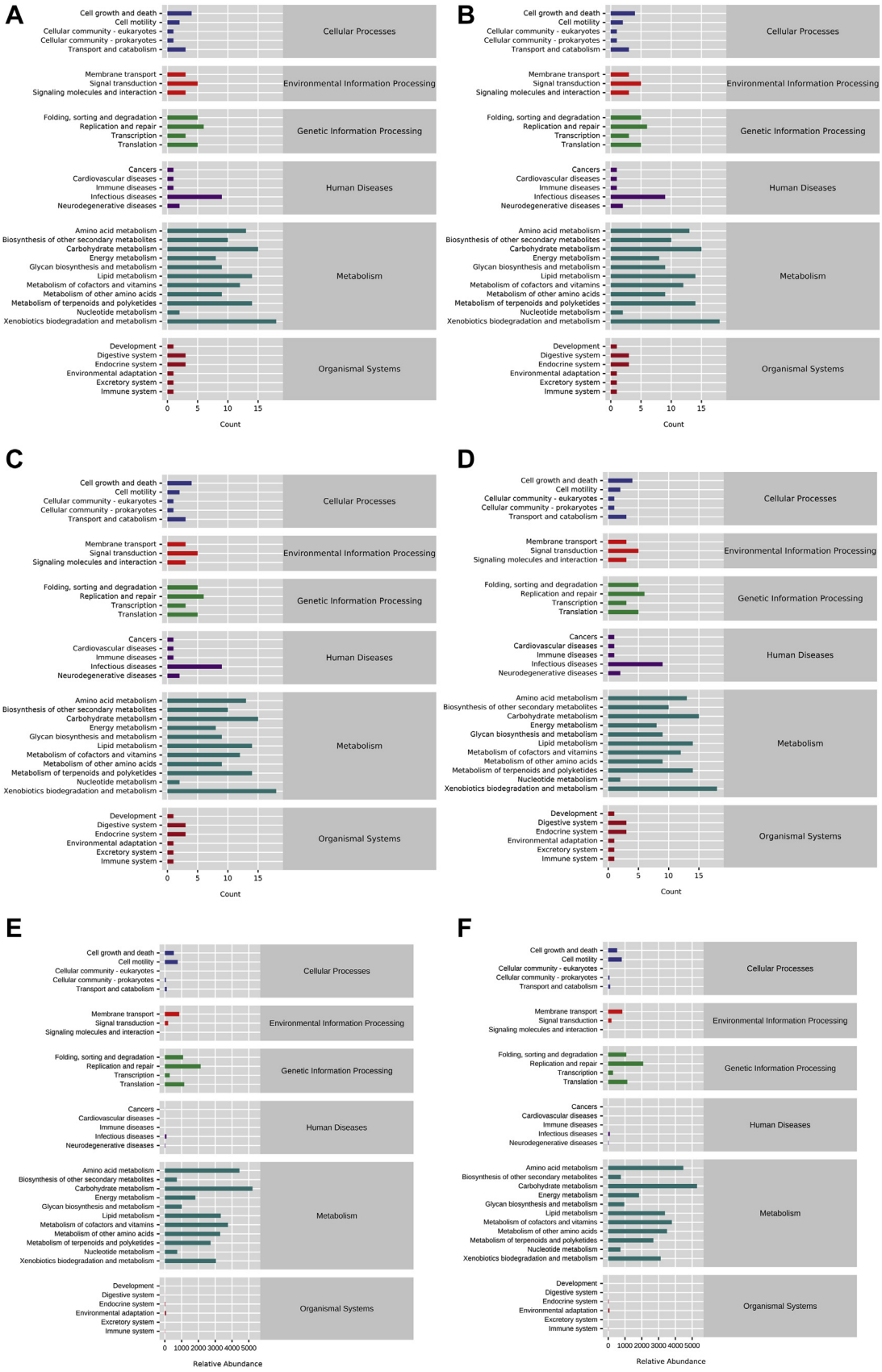

Figure 7. PICRUSt predicted the KEGG (Kyoto Encyclopedia of Genes and Genomes) second-level distribution. Abbreviation: PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states. A, B, C, D, E, and F respectively represent the statistical results of KEGG pathway analysis of Group A, B, C, D, E, and F.

xylanase, and probiotics could change the species and abundance of main intestinal flora. The composition of intestinal flora was stable. The grouping effect was obvious, and the progenitor of intestinal flora could be effectively stabilized. Among them, the sample flora of B group was the most stable. For similarity, clustering is more obvious.

PICRUSt function prediction analysis shows that 250 g/t of CSPSM in feed can improve intestinal amino acid metabolism, endocrine system function, membrane transport function, and cell mobility function. On the one hand, it can improve the digestion of chicken protein to improve protein deposition, and on the other hand, it can improve the vitality of intestinal bacteria. The renewal rate of intestinal microorganisms was very fast, and the bacteria were always replicating and repairing (Zhao et al., 2019). Membrane transport is an important function for intestinal microorganisms, which may be related to massive absorption of substances by bacteria and the massive efflux of substances (Zhao et al., 2018). Intestinal microorganisms help the animal body digest and absorb the substances that are not easy to digest and produce the corresponding nutrients for use. Both processes involve membrane transport. Cell movement is an important function for intestinal microorganisms, which may be related to the continuous migration of intestinal microorganisms to obtain sufficient "food" (Xu et al., 2016). Adding 500 g/t of CSPSM in feed and probiotics can improve "replication and repair" and "membrane transport function" of bacteria, can improve renewal of bacteria, and can maintain more vigorous vitality. Adding 750 g/t of CSPSM in feed can improve carbohydrate metabolism, replication and repair of intestinal bacteria, the function of membrane transport, and the content of pathogenic bacteria, which can not only improve the growth performance of broilers but also improve the probability of disease in broilers. Xylanase improved membrane transport function of bacteria in the gut, allowing the bacteria to help chickens digest indigestible substances. In general, dietary differences will change the composition of intestinal flora in the jejunum of broilers. Adding 750 g/T of CSPCM in feed can not only promote the growth of broilers but also increasethe probability that broilers will catch an infectious disease. This can be explained by the theory of TCM: "excessive and inferior are both diseases." Although there is no harmful residue of TCM, excessive dosage may affect the balance of the body, and the imbalance of the body will increase the risk of disease. Compound small peptides of Chinese medicine can promote bacterial metabolism and increase membrane transport, amino acid metabolism, carbohydrate metabolism, and bacterial regeneration and repair by acting on the intestinal environment of broilers.

The effects of 500 g/T of CSPCM and probiotics of feed on the intestinal flora function of broiler chicken are relatively similar. From PICRUSt function prediction analysis, both of them can improve the renewal of bacteria and aid in maintenance of vigorous vitality. Peptides are not only easier to be absorbed in the gut but also easier to be used by the gut flora, which makes the gut more vital. The flora of broilers that feed on 500g/T and 250g/T are relatively similar.

The effects of 500 g/t of CSPSM in feed and probiotics on the intestinal flora function of broilers were similar, and both could improve the renewal of bacteria and maintain vigorous vitality. The influence of 500 g/t of CSPSM in feed on bacterial flora was similar to that of 250 g/t of CSPSM in feed. Adding 250 g/t of CSPSM in feed can improve the dominant OTU, can improve evenness of intestinal flora, and can improve the growth performance of broilers. The improvement of growth performance of broilers is different from that observed after adding 750 g/t of CSPSM in feed. It can improve the metabolism of carbohydrates, the metabolism of amino acids, and the deposition of proteins.

CONCLUSION

In conclusion, this study demonstrated that dietary supplementation of CSPCM to broilers diets increased beneficial flora content, increased metabolism of carbohydrates, increased amino acid metabolism, increased deposition of proteins, increased renewal of bacteria, and aided in maintenance of vigorous vitality. Among the 3 additive quantities of 250 g/t, 500 g/t, and 750 g/t of CSPCM in feed, 250 g/t of CSPCM improved parameters that are necessary for improved growth and production.

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DISCLOSURES

The authors declare no conflicts of interest.

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