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RESEARCH ARTICLE

Effects of Earthworm Antimicrobial Peptides and Probiotics on Intestinal Flora of Yellow-feathered Broilers

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Abstract: Earthworm antimicrobial peptides combined with probiotics were supplemented to the yellow-feathered broiler diet, and the cecal intestinal flora was subjected to 16S rDNA Qualcomm sequencing. The results showed that the total number of species in S1 (control group) was significantly higher than S2 (earthworm antimicrobial peptides) and S3 (Compound probiotic preparation), S4 (earthworm antibacterial peptide + composite probiotic preparation) (P<0.05), the colony structure of S1 is significantly different from S2, S3, and S4 (P<0.05). At the phylum level of each test group, the dominant bacterial groups were *Firmicutes*, *Bacteroides*, and *Proteobacteria*. Among them, S2, S3, and S4 were significantly higher than S1 in the relative abundance of *Firmicutes* and *Bacteroides* (P<0.05), the relative abundance of *Proteobacteria* was significantly lower than S1 (P<0.05). At the genus level, S2, S3, and S4 were significantly higher than S1 in the relative abundance of *Lactobacillus* (P<0.05), where S4 is higher than S3 (P>0.05). Earthworm antimicrobial peptides combined with compound probiotics can increase the number of beneficial bacteria and reduce the number of harmful bacteria to regulate intestinal flora, indicating that earthworm antimicrobial peptides combined with compound probiotics can be used as new green antibiotics in animal production.

Keywords: *Earthworm antibacterial peptides, Probiotic, 16S rDNA*

Topraksolucanı Antimikrobiyal Peptitleri ve Probiyotiklerinin Etlik Piliçlerin Bağırsak Florası Üzerine Etkileri

Öz: Etlik piliç rasyonlarına, topraksolucanı antimikrobiyal peptitleri ile birlikte probiyotikler ilave edildi ve sekum flora 16S rDNA Qualcomm sekanslamaya tabi tutuldu. Sonuçlar, S1'deki (kontrol grubu) toplam tür sayısının S2 (topraksolucanı antimikrobiyal peptit ilaveli), S3 (bileşik probiyotik preparatı ilaveli) ve S4'ten (topraksolucanı antibakteriyel peptid + kompozit probiyotik preparatı ilaveli) anlamlı derecede yüksek olduğunu (P<0.05) ve S1'in koloni yapısının S2, S3, S4'ten önemli ölçüde farklı olduğunu gösterdi (P<0.05). Her test grubunun filum seviyesinde, baskın bakteri grupları *Firmicutes*, *Bacteroides* ve *Proteobacteria* idi. Bunlar arasında, S2, S3 ve S4 gruplarında *Firmicutes* ve *Bacteroides*'in nispi yoğunlukları S1'den önemli ölçüde yüksekti (P<0.05), *Proteobacteria*'nın nispi yoğunluğu ise S1'den önemli ölçüde düşüktü (P<0.05). Cins düzeyinde, S2, S3 ve S4'ün nispi *Lactobacillus* yoğunluğu S1'den önemli ölçüde yüksekti (P<0.05), ancak S4'ün bu oranı S3'ten daha yüksekti (P>0.05). Farklı probiyotik türlerinin topraksolucanı antimikrobiyal peptitleri ile birlikte kullanımı, yararlı bakteri sayısını arttırabilir ve zararlı bakteri sayısını ise azaltarak bağırsak florasını düzenleyebilir. Bu da hayvansal üretimde, probiyotik karmaları ile topraksolucanı antimikrobiyal peptitlerinin bir arada yeni nesil antibiyotikler olarak kullanılabileceğinin göstergesidir.

Anahtar sözcükler: *Topraksolucanı antibakteriyel peptitleri, Probiyotik, 16S rDNA*

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Introduction

Since the discovery of penicillin in 1929 [1], antibiotics have become a protective shield for human health. However, with the widespread application of antibiotics in clinical practice, problems such as "superbacteria" and drug-resistant genes have emerged, especially the "abuse" of antibiotics in animal husbandry, which makes the problem of antibiotic resistance more and more serious. It poses a serious threat to human health and the development of animal husbandry, so it is urgent to seek alternative products.

Antimicrobial peptides refer to polypeptides with antibacterial activity in insects, which have strong alkaline, broad spectrum antibacterial activity and weak positive characteristics. Subsequently, antimicrobial peptides were also found in mammals such as pigs, cattle, sheep, and amphibians such as *Xenopus laevis*. Although different antimicrobial peptides from different sources have the same antibacterial effect, different antimicrobial peptides have different antibacterial effects, different bactericidal activities and different antibacterial spectrum, they all have research effects. In addition, from the known antibacterial peptides, antibacterial peptides with different protein structures have different antibacterial effects and mechanisms. Since this kind of active peptides have a wide spectrum and high bactericidal activity to bacteria, they are named "antibacterial peptides" [2-4].

Probiotics are active microorganisms beneficial to the host by colonizing the body and changing the composition of a specific part of the host flora. By regulating the immune function of the host mucosa and the system, or by regulating the balance of intestinal flora, promoting nutrient absorption to maintain intestinal health, thus producing single microorganisms or mixed microorganisms with precise composition in favor of health, also known as probiotics or compound microecological preparation [5,6].

In animal breeding, intestinal health plays an important role in the prevention and control of pathogenic microorganisms and the digestion and utilization of feed [7,8].Digestive system not only is a site for digestion and absorption of dietary nutrients, but also provides protection against pathogens and toxins and has a large microbiome and immune cells [9]. The microbiota in the gastrointestinal tract is associated with a broad range of functions within the host, including the fermentation of complex macronutrients, nutrient and vitamin production, cellulose fermentation, protection from pathogens, maintenance of the balance of the immune system, and physiological metabolism in distal organs or tissues [10-13]. When the body is affected by some abnormal factors, intestinal barrier damage, bacteria, and other pathogenic agents can enter, engraftment in the intestinal tract through the blood circulation to achieve internal organs organ enteritis causes a series of reactions and systemic infection, cause severe infection situation, bring irreparable economic benefits to farms. Intestinal tract is not only the main place for nutrient digestion and absorption, but also has a very important defense function. Intestinal environmental imbalance will lead to a series of intestinal diseases. Increased intestinal bacteria and toxins with the probability of intestinal infection, inflammation and other problems hinder the digestion and absorption of nutrients, resulting in reduced performance and even death of animals. In order to maintain animal health and improve the quality of products, digestion, absorption and synthesis of nutrients in poultry are often promoted by improving the dietary ratio. Antimicrobial peptides (AMP) are a family of peptides that exhibit a range of antimicrobial activities. Studies have found that through isolation of key growth nutrients, penetration of bacterial membranes, and other related mechanisms have been identified as key regulators of interactions between symbiotic microorganisms and host tissues [14]. Both antimicrobial peptides and probiotics could regulate the intestinal flora of animals. AMPs exhibit a broad spectrum of antimicrobial activity and inhibit microbial cells by interacting with their membranes or by other mechanisms, such as inhibition of cell-wall synthesis or suppression of nucleic acid or protein synthesis [15]. In prior analyses, we have found that cathelicidin-WA can enhance the barrier function of the intestinal epithelium, protecting hosts from enterohemorrhagic *E. coli* O157:H7 infection [16]. The results suggest that probiotics-feeding may enhance the immunodefense system mediated by AvBDs but not by cytokine, against infection by Gram-negative bacteria [17]. Further, the data showed that SGAMP could effectively enhance the contents of IEL, mast cells, and goblet cells in the intestine [16]. All the above studies showed that dietary antimicrobial peptides or probiotics can improve the changes of intestinal microbial environment of animals, Nowadays, fly maggot antimicrobial peptides have been widely used in poultry disease treatment and aquatic research, but they have not been found to be used as feed additives in broiler humoral immunity and intestinal health [18].

Therefore, this experiment intends to add earthworm antimicrobial peptides and probiotics to the yellow-feathered broiler's diet. Through 16S rDNA high-throughput sequencing of the contents of the cecum of chickens, the results of the sequencing are used to analyze the effects of earthworm antimicrobial peptides combined with probiotics on the cecum of yellow-feathered broilers. To explore whether the combined effect of the two is better than the effect of a single addition, and to provide a theoretical basis for the application of earthworm antimicrobial peptides in the poultry breeding industry, the current study was performed [19,20].

Material and Methods

Ethical Statement

The study was approved by the Animal Experimentation Ethics Committee of the School of Animal Science and Technology, Shihezi University. All chickens were kept experimentally and euthanized in strict accordance with the guidelines of the committee. During the test, all efforts were made to minimize the suffering of the animals.

Animal Feeding Experiment and Management

Two hundred and forty healthy 1-day-old yellow-feathered broilers with similar body weight were purchased from a local hatchery and randomly divided into 4 treatment groups with 6 replicates in each treatment and 10 broilers in each replicate. The treatments were basal diet group (S1 group), basal diet +100 mg/kg earthworm antimicrobial peptide group (S2 group), basal diet +200 mg/kg compound probiotics group (S3 group), basal diet +100 mg/kg earthworm antimicrobial peptide +200 mg/kg compound probiotics group (S4 group). Corn-soybean meal diet was used in the experiment, and the basal diet was prepared according to the NRC (1994) broiler nutritional standard, and its composition and nutritional level were listed in *Table 1*. The composition and nutrient level of the basal diet are shown in *Table 1*. Throughout the entire study, the indoor temperature for chickens was monitored at constant temperature. It was 32~35°C on the first day, then gradually decreased and remained at 22°C for the last two weeks. According to the commercial conditions, the implementation of the artificial lighting scheme of 23 h of all-day lighting. The chickens had free access to food and water. Other immunization and disinfection measures were performed in strict accordance with the farm procedures, and the test period was 64 days. The activity unit was 100 mg/kg, and the compound probiotic preparations (1000 mg/kg *yeast*, 200 mg/kg *Lactobacillus*, 500 mg/kg *Bacillus subtilis*) were purchased from Shaanxi Longzhou Biological Co., Ltd. (China).

Table 1. *Dietary nutrient levels at different stages in each experimental group*

Items	Content, %
Ingredients	
Corn	56.32
Soybean meal	34.80
Soybean oil	4.00
limestone	1.00
mountain flour	1.50
$CaHPO_4$	1.80
NaCL	0.32
L - lysine	0.10
DL - lysine	0.16
Total	100
Nutrient levels	
ME/(MJ/Kg)	12.88
CP	21.80
Ca	0.90
AP	0.44
Lys	1.14
Met	0.5

Composite premix: Cu: 8 mg, Fe: 100.0 mg, Mn: 120.0 mg, I: 0.7 mg, Se: 0.35 mg, Zn: 100 mg per kg; Multivitamins per kg of diet provide: Vit. A: 12.000 IU, Vit. D_3: 3.000 IU, Vit. E: 7.5 IU, Vit. K: 21.5 mg, Vit. B_1: 0.6 mg, Vit. B_2: 4.8 mg, Vit. B_6: 1.8 mg, Vit. B_{12}: 9 μg, Niacin 10.5 mg, D-pantothenic acid: 7.5 mg, Folic acid: 0.15 mg

The Sample Collection

On day 64, a total of 12 yellow-feathered broilers (male) were randomly selected for each replicate in each experimental group. After slaughter, the caecal contents of chickens were removed by opening the abdominal cavity and stored at -80°C.

Sample Testing

16S rDNA sequencing was commissioned by Shanghai Zhongke New Life Science Co., LTD. The samples were sent to Shanghai Zhongkexin Life Biotechnology Co., LTD for detection. The sequencing process was as follows: Firstly, the DNA of the samples was extracted by fecal genome DNA extraction kit, and the V3-V4 variable region was amplified and sequenced by Illumina Miseq sequencing platform after the detection was qualified by 1% agarose gel electrophoresis. There is a certain proportion of Dirty Data in the Raw Data obtained by sequencing. In order to make the results of information analysis more accurate and reliable, the original Data should be spliced, filtered and de-chimeric to obtain valid Data. OTUs (Operational Taxonomic Units) clustering and species classification analysis were then performed based on available data.

Results

In this experiment, 1265441 original Tags sequences were obtained, and the average value of each sample in Q20 and Q30 was in 98% and 94%, and the quality of the sequencing data was good, which could be used for further analysis of species abundance and diversity.

After sequencing results of Illumina MiSeq platform were obtained, all sequences were clustered with classifiable operating units according to 97% similarity. OTUs statistics were performed on the samples, as shown in *Fig. 1.* S1 obtained 1.904 OTUs numbers, 982 of which were unique; S2 obtained 1285 OTU numbers. S3 obtained 1202 OTU numbers, only 215; S4 got 1388 OTUs, 313 unique. Group S1 shared 702 OTUs with S2, S3 and S4. SI and S2 have 822 OTU numbers, S1 and S3 have 789 OTU numbers, S1 and S4 have 842 OTU numbers.

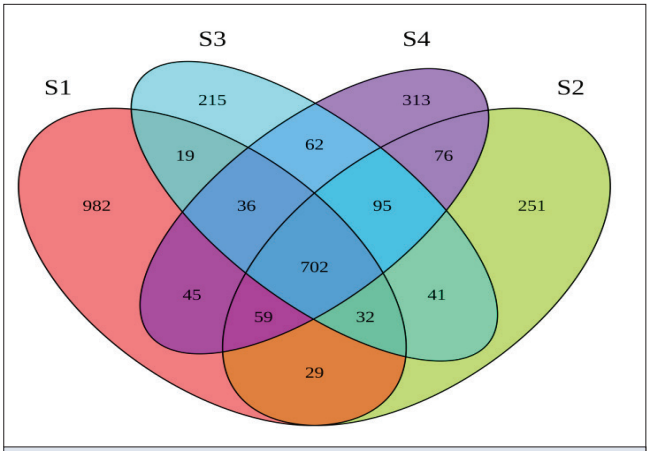

Fig 1. Venn plot of cecal intestinal flora of yellow-feathered broilers in different treatments

As shown in *Table 2*, there was no significant difference in Shannon and Simpson species richness among experimental groups (P>0.05). ACE and Chao 1 reflected the total number of species. The total number of species in S1 was significantly higher than S2, S3 (P<0.05), S4 (P>0.05), S2 was higher than S3 (P>0.05). Good coverage reaction sequencing depth, all test groups were 1, satisfying the sequencing depth.

As shown in *Fig. 2*, the Rarefaction Curve indicates that the predicted species richness of the tested samples is high. When the Curve tends to be flat, it indicates that the amount of sequencing data is reasonable. When Shannon index ranged from 3 to 5, the curve tended to be flat, indicating that the species discovered from the randomly selected sequencing number of each sample tended to be saturated, that is, nearly all OTUs were detected. The data are reliable and comprehensive.

Principal Component Analysis (PCA) is a method to simplify data Analysis and dimensionality reduction of multidimensional data, so as to extract the most important elements and structures in the data. Therefore, samples with high similarity in community structure tend to cluster together, while those with large difference in community structure tend to disperse. As shown in *Fig. 3*, S2, S3 and S4 are aggregated together with high similarity in colony structure, indicating that there is little difference in diversity of intestinal flora among S2, S3 and S4, and S2, S3 and S4 have high dispersion degree with S1. Based on Unweighted UnifracBeta distance, it can be obtained from *Fig. 4* the bacterial colony structure diversity of S1 was significantly different from S2, S3 and S4 (P<0.05).

As shown in *Table 3* and *Fig. 5*, *Bacteroidetes*, *Proteobacteria*, *Deiron-bacilli*, *Tautrophs*, *Epsilonbacteraeota*, *Verrucobacteriaceae*, *Actinomycetes*, *Cyanobacteria* and *Firmicutes* were the top 10 dominant flora in relative abundance. *Firmicutes*, *Bacteroidetes* and *Proteobacteria*

Table 2. *Effects of earthworm antimicrobial peptides and probiotics on cecal intestinal microflora α diversity of yellow-feathered broilers*

Parameter	S1	S2	S3	S4
Shannon	6.55±0.18	6.18±0.13	6.28±0.22	6.36±0.87
Simpson	0.96±0.005	0.95±0.003	0.95±0.01	0.95±0.04
ACE index	1140.68±117.1^{a}	941.91±67.04^{b}	883.29±38.11^{b}	1006.55±44.17^{ab}
Chao1 index	1146.84±110.34^{a}	902.86±56.75^{b}	849.35±39.48^{b}	961.67±34.12^{ab}
Good coverage	1	1	1	1

^{a,b,c} Means within a row followed by the different superscripts differ significantly (P<0.05)

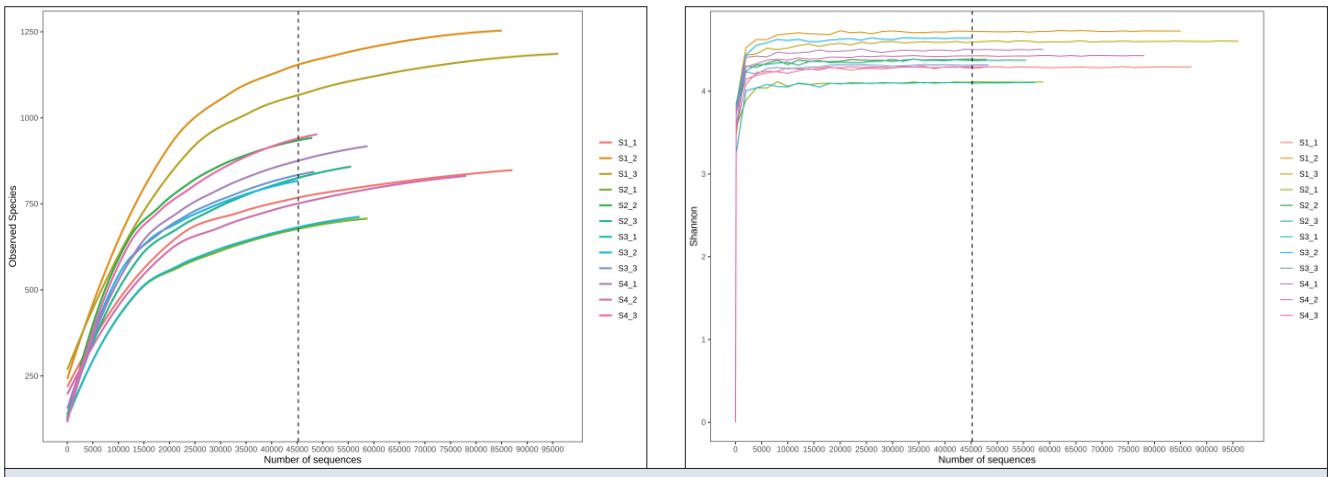

Fig 2. Sample Rarefaction Curve (A) Shannon Curve (B) (The abscissa are all randomly selected sequence numbers)

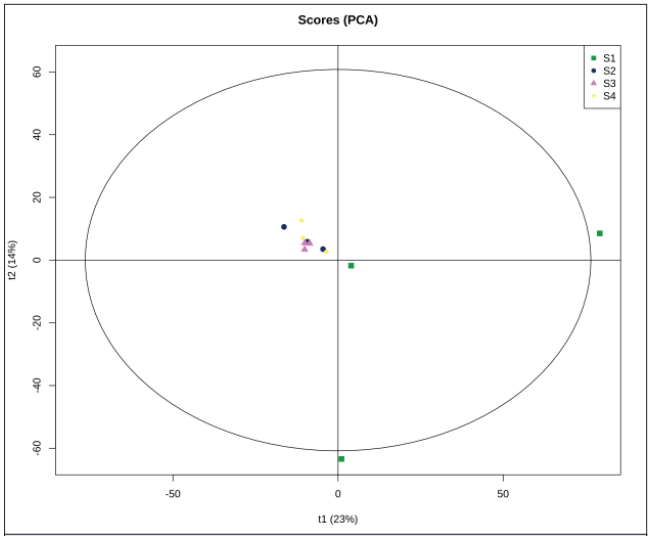

Fig 3. PCA of cecal intestinal flora of yellow-feathered broilers treated with different treatments

Table 3. Relative abundance of species in cecal intestinal microflora level of yellow-feathered broilers with different treatments

Parameter	S1	S2	S3	S4
Thick wall door	0.49	0.53	0.53	0.57
Bacteroidetes	0.21[a]	0.40[b]	0.34[b]	0.34[b]
Deformation of the fungus door	0.11[a]	0.03[b]	0.02[b]	0.03[b]
Deferrobacterium phylum	0.11[a]	0.00[b]	0.01[b]	0.01[b]
Put the door	0.01	0.02	0.03	0.01
Epsilonbacteraeota	0.03	0.00	0.00	0.01
Warts micro bacteria	0.01	0.01	0.03	0.01
Actinobacillus	0.01	0.00	0.01	0.00
Cyanophyta	0.01	0.00	0.00	0.00
Soft wall door	0.00	0.00	0.00	0.00
Other	0.01	0.00	0.00	0.00

[a,b,c] Means within a row followed by the different superscripts differ significantly (P<0.05)

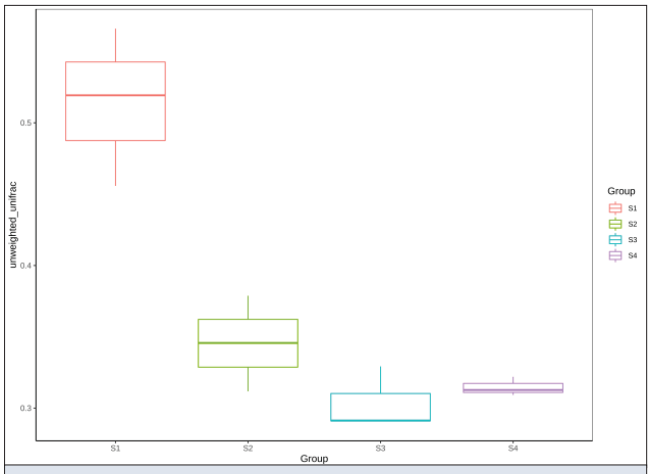

Fig 4. Weihted Box figure of cecal intestinal flora of yellow-feathered broilers treated with different treatments

were the top three dominant phyla in relative abundance of each experimental group. The relative abundance of *Firmicutes* was S4 > S3 > S2 > S1, and there was no significant difference among all groups (P>0.05). The relative abundance of S1 was significantly lower than S2, S3 and S4 (P<0.05). In relative abundance of *Proteobacteria*, S1 was significantly higher than S2, S3 and S4 (P<0.05), but there was no significant difference among S2, S3 and S4 (P>0.05). The relative abundance of S1 was significantly higher than S2, S3 and S4 (P<0.05). There was no significant difference in the relative abundance of *Microbacteria*, *Actinobacter*, *Cyanobacteria*, *Firmicutes*, *Epsilonbacteraeota* and *Actinobacter* among experimental groups (P>0.05).

As *Table 4* and *Fig. 4* shows, at the genus level, the top 10 dominant flora in relative abundance were *Bacteroidetes*, *Faecalis*, *Alistipes*, *Koala bacillus*, *Mucispirillum*, *Ruminococcus*

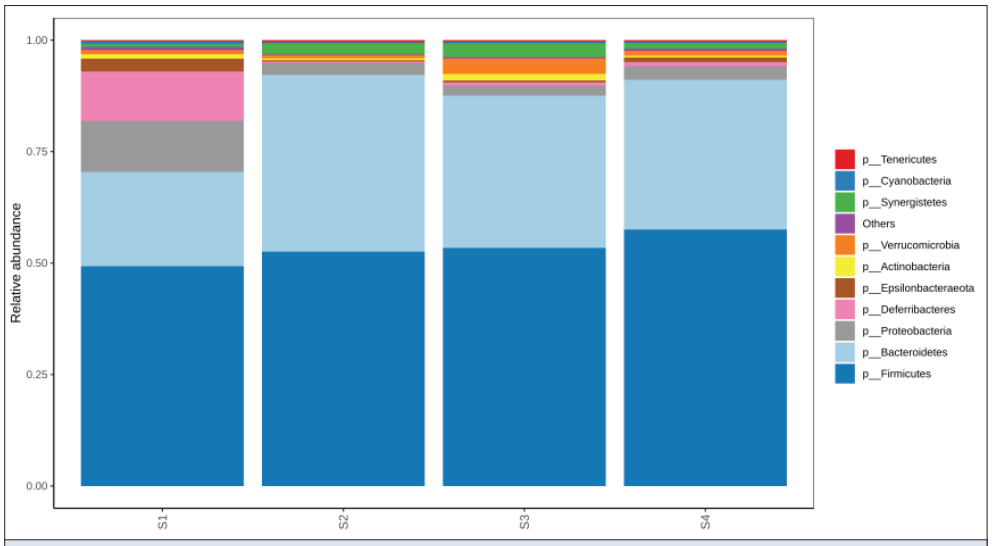

Fig 5. Histogram of relative abundance of species in cecal intestinal flora level of yellow-feathered broilers with different treatments

Table 4. *Species relative abundance of cecal intestinal flora of yellow-feathered broilers under different treatments*

Microorganism	S1	S2	S3	S4
Bacteroides	0.11^{c}	0.30^{a}	0.23^{b}	0.23^{b}
Fecal coli	0.10	0.06	0.05	0.09
Alistipes	0.04^{b}	0.08^{a}	0.09^{a}	0.07^{a}
Koala bacillus	0.02^{b}	0.07^{a}	0.06^{a}	0.04^{ab}
Mucispirillum	0.11^{a}	0.00^{b}	0.01^{b}	0.01^{b}
Ruminococcaceae UCG-014	0.04	0.05	0.04	0.05
Ruminococcus torques group	0.04	0.03	0.03	0.04
Unclassified genus of bacteria	0.02	0.03	0.05	0.02
Lactobacillus	0.01^{b}	0.04^{a}	0.02^{b}	0.03^{b}
Rumen fungi NK4A214	0.01	0.02	0.02	0.02
Other	0.51	0.34	0.41	0.40

^{a,b,c} Means within a row followed by the different superscripts differ significantly (P<0.05)

UCG-014 and *Ruminococcus* torques Group, unclassified bacteria, *Lactobacillus,* rumen bacteria family NK4A214. The dominant flora in S1 were *Bacteroides*, *Faecalis*, and *Mucispirillum*; in S2 and S3 were *Bacteroides*, *Faecalis*, and *Phascolarctobacterium*; in S4, *Bacteroides*, *Faecalis*, and *Alistipes*. In the relative abundance of *Bacteroides*, S1 was significantly lower than S2, S3 and S4 (P<0.05), and S2 was the highest with no significant difference from S3 and S4 (P>0.05). In relative abundance of *Faecalis*, S1 was higher than S4, S2 and S3 (P>0.05); In the relative abundance of *Alistipes*, S2, S3 and S4 were significantly higher than S1 (P<0.05), and S3 had the highest relative abundance with no significant difference with S2 and S4 (P>0.05). In the relative abundance of *Koala bacillus*, S2 and S3 were significantly higher than S1 (P<0.05), and S4 was higher than S1 (P>0.05). In relative abundance of *Mucispirillum*, S1 was significantly lower than S2, S3 and S4 (P<0.05). In the relative abundance of *Lactobacillus*, S2, S3 and S4 were significantly higher than S1 (P<0.05), and S2 was the highest. There was no significant difference in the relative abundance of *UCG-014*, *Ruminococcus torques group* and NK4A214 of *Ruminococcus* among all groups (P>0.05).

LefSe (LDA Effect Size) analysis can be used to find with significant differences in abundance between groups through comparative analysis between and within groups. The differences are expressed by LDA Score, and the larger THE LDA Score value is, the greater the impact of species is. A total of 47 species, including 6 S2, 8 S3 and 33 S4, showed significant differences in abundance between groups. As shown in *Fig. 4*, *Fig. 5*, compared with other groups, S2 has significant differences in species including *Bacteria*, *Negativicutesc*, *Selenomonadales*, *Rikenellaceae* and *Alistipes*. The LDA Score of Bacteria was greater than 4, followed by the LDA Score of *Negativicutesc*, *Selenomonadales* and *Rikenellaceae* was greater than 3.5. S3 Compared with other groups, the species with significant differences are *ultradClostridiabacterium*, *Bacteroidales*, *Bacteroidetes*, *Bacteroidaceaec*, *Bacteroidia*, *Bacteroides*, *Phascolarctobacterium*, *Acidaminococcaceae*, *Itured-Firmicutesbacterium*, the largest affect is the *ulturedClostridiabacterium* and order, phylum, family, class, and genus of Bacteroides, LDA Score were greater than 3.5; Compared with other groups, the species of S4 group were *Betaproteobacteriales*, *Rhizobiales*, *Burkholderiaceae*, *Rhizobiaceae,* *Ochrobactrum*, Other, *Magnetospirillaceae*, *Chitinophagaceae*, Other, *Chitinophagales*, *Asticcacaulis*, *Caulobacter*, Other, *Rhodobacteraceae*, Other, *Acidobacteria*, *Rhodobacterales*, *Diplorickettsiles*, *Planctomycetes*, *Oxyphotobacteria*, *Chloroplast*, *Ralstonia*, *Pararhizobium_Rhizobium*, *Unclassified*, *Planococcaceae*, *Blastocatellia - Subgroup4*, *Altererythrobacter*, *Moraxellaceae*,

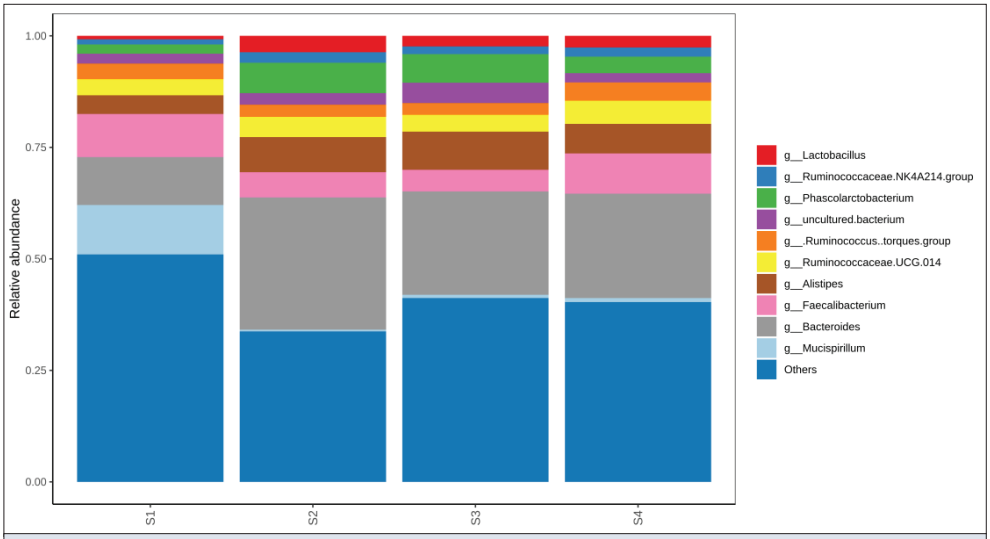

Fig 6. Column chart of species relative abundance in cecal intestinal flora of yellow-feathered broilers treated with different treatments

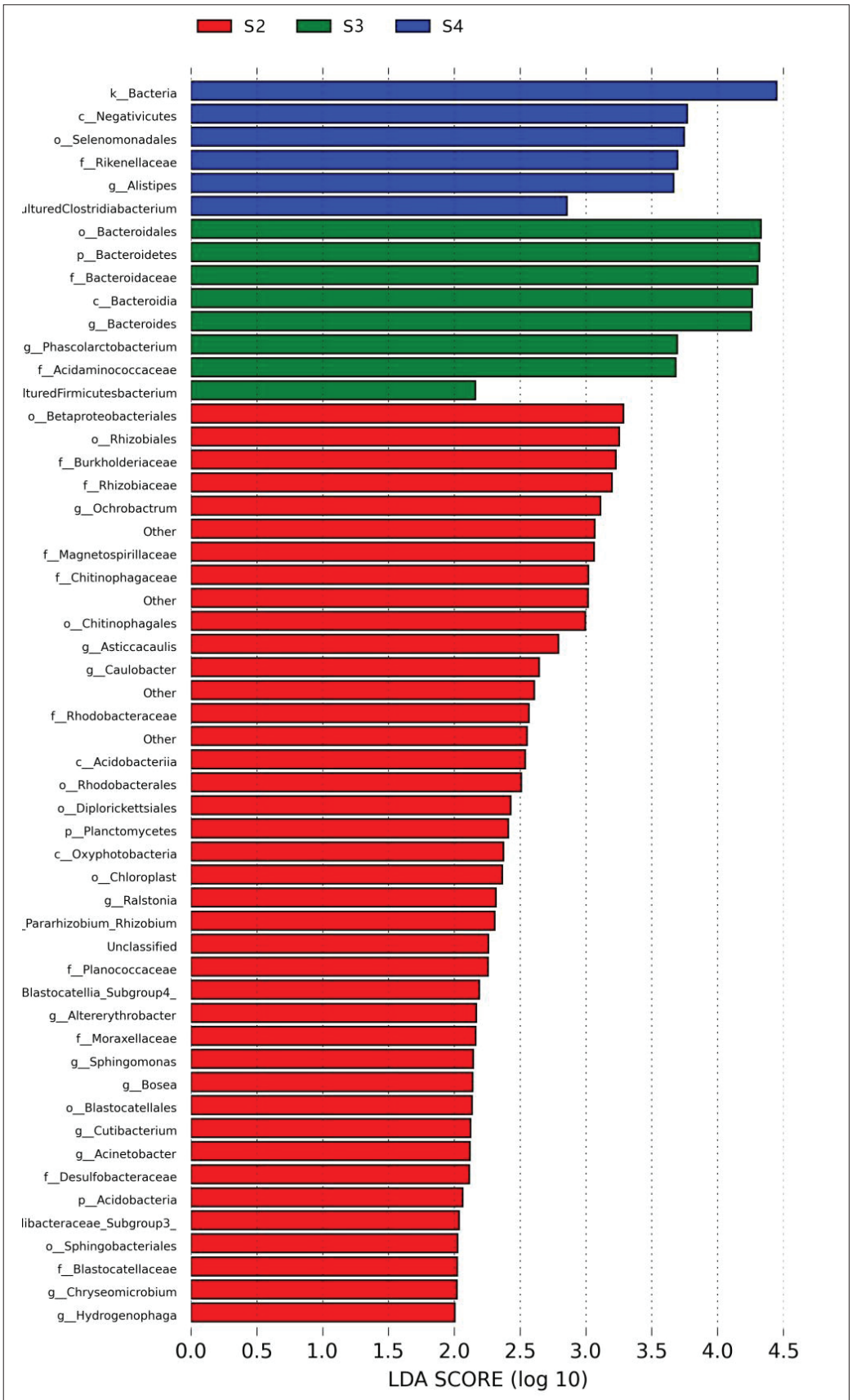

Fig 7. Column chart of cecal intestinal flora LAD of yellow-feathered broilers treated with different treatments. The length of the histogram represents the size of the impact of significantly different species, and different colors represent different samples

Sphingomonas, *Bosea*, *Blastocatelales*, *Cutibacterium*, *Acinetobacter*, *Desulfobacteraceae*, *Acidobacteria*, *Ibacteraceae _Subgroup3*, *Sphingobacteriales*, *Blastocatellaceae*, *Chryseomicrobium*, and *Hydrogenophaga*, Among them, *Betaproteobacteriales*, *Rhizobiales* and *Burkholderiaceae* had the greatest influence with LDA Score greater than 3, *Rhizobiaceae*, *Ochrobactrum,* Other, *Magnetospirillaceae*, *Chitinophagaceae*, Other, *Chitinophagales*. Species with no significant difference between S1 and other groups (*Fig. 6*, *Fig. 7*).

DISCUSSION

AMP has good potential as a suitable alternative to conventional antibiotics used in the pig and poultry industry [21]. AMP has been reported to benefit growth performance, reduce incidence of diarrhea and increase the rate of weaned pigs [22]. Daneshmand et al.[23] reportedly showed that AMPs can protect broiler chickens from challenging *E. coli* in vivo. The digestive tract microecosystem is an important component of livestock and poultry body weight and has an effect on the metabolism of macromolecules. AMPs has been reported to have beneficial effects on growth performance, intestinal microflora and morphology, immune function and nutrient digestibility of chickens [24,25]. The chicken gastrointestinal (GI) tract is home to a complex microbial community that underlines the links between diet and health. The GI tract is rich in microbial biodiversity, playing home to ≥500 phylotypes or 1 million bacterial genes, which equates to 40-50 times the number in the chicken genome [26]. *In vitro* culture is traditionally used in the study of intestinal microorganisms, but most of them are difficult to be screened and isolated by traditional methods. Therefore, molecular biology is an important direction to explore the composition of intestinal microbiota in the future. At present, intestinal microbes are mostly studied from microbial 16S rRNA sequence. Studies on the cloning and sequencing of 16S rRNA sequence show that more and more intestinal bacteria that cannot be isolated and cultured *in vitro* have been found. The study explored the bacterial community present in water, sediment and intestine samples from an aquaculture site using high-throughput sequencing [27]. This finding indicated that sediment and water are major sources of intestinal microbes. Changes in the qualitative and quantitative composition of the caecal microbiota were less pronounced than in the crop [28]. Daneshmand et al.[23]. used 16S rRNA gene mapping technology to study the diversity of intestinal microbes and found that the diversity and distribution of intestinal microbes were relatively stable. The diversity of intestinal microorganisms of laying hens was investigated by PCR-DOGE test. It was found that the cecum was the most suitable organ for the study of microorganisms in the digestive tract of laying hens. Dietary AMP has been reported to improve intestinal tissue structure and promote growth [29-31].

The results of this experiment showed that the total number of species in S1 was significantly higher than S2, S3 and S4 and the colony structure of S1 was significantly different from S2, S3 and S4. At the phylum level of each experimental group, the dominant flora were *Firmicutes*, *Bacteroides* and *Proteobacteria*. The relative abundance of S2, S3 and S4 in *Firmicutes* and *Bacteroides* was significantly higher than S1, and the relative abundance of Proteobacteria was significantly lower than S1. *Proteobacteria* was the largest phylum in bacteria, including many pathogenic bacteria. Such as *Escherichia coli*, *Salmonella*, *Vibrio Cholera*, *Helicobacter pylori* and other harmful bacteria. On the genus level, S2, S3, and S4 in the *Phascolarctobacterium*, significantly higher than in the relative abundance of the genus lactobacillus S1. the *Phascolarctobacterium* with *Clostridium* difficile bacteria for succinic acid salt to inhibit the growth of clostridium difficile bacteria, lactic acid bacillus genus flora is beneficial to the body, there are few pathogenic, and can improve the body resistance, formany spoilage organisms, and pathogenic bacteria have inhibition. Chicken intestinal microflora plays an important role in immune regulation and disease control. Intestinal microorganisms can be divided into intestinal symbiotic bacteria, conditioned pathogenic bacteria and enterohost pathogenic bacteria [32]. Intestinal opportunistic pathogens, such as facultative anaerobe *Escherichia coli*, are present in low concentrations, but when intestinal homeostasis is disrupted, for example when the body is infected with a virus, *Escherichia coli* proliferates rapidly and leads to intestinal disturbances [33]. According to the report, diacetyl by gram-negative bacteria binding protein reaction of arginine, which interfere with the use of arginine, inhibit the growth of gram negative bacteria , in Newcastle disease virus can be isolated from the dead broilers was added in the diet of earthworm antibacterial peptide and composite probiotic preparations can reduce the relative abundance of harmful bacteria, increase the relative abundance of the beneficial bacteria, thereby regulate the intestinal flora and improve immunity.

LefSe analysis of cecal intestinal flora of yellow-feathered broilers showed that there were 47 species with significant differences in abundance between groups, including 6 species in S2, 8 species in S3 and 33 species in S4. The phylum actinomycetes is a group of prokaryotes and Gram-positive bacteria, once thought to be a cross between bacteria and molds because of their morphology, are prokaryotes without nuclei. Antimicrobial peptides have antibacterial activity against most gram-positive bacteria, gram-negative bacteria, mycoplasma and some

viruses, but have no toxicity to fungi and prokaryotes, so earthworm antimicrobial peptides have no effect on actinomycetes, but compound probiotics can reduce their relative abundance and complement antibacterial peptides. It provides a theoretical basis for earthworm antimicrobial peptides and compound probiotics to maintain the balance of intestinal flora and improve the immune performance of poultry.

After adding earthworm antibacterial peptides and compound probiotics to the diets of yellow-feather broilers, 16S rDNA sequencing analysis of cecum intestinal flora showed that the total number of species in S1 was significantly higher than that in S2, S3 and S4, and the colony structure of S1 was significantly different from S2, S3 and S4 ($P<0.05$). At the phylum level, the dominant bacteria groups of the experimental groups were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, among which the relative abundance of S2, S3 and S4 in *Firmicutes* and *Bacteroidetes* was significantly higher than that of S1 ($P<0.05$), and the relative abundance of *Proteobacteria* was significantly lower than that of S1 ($P<0.05$). The relative abundance of S2, S3 and S4 of *Phascolarctobacterium* and *Lactobacillus* were significantly higher than that of S1 ($P<0.05$). Earthworm antimicrobial peptides and compound probiotics could increase the relative abundance of beneficial bacteria, reduce the relative abundance of harmful bacteria, and regulate the cecal intestinal flora of broilers. Combined use had complementary effects.

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Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethical Statement

The study was approved by the Animal Experimentation Ethics Committee of the School of Animal Science and Technology, Shihezi University.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Author Contributions

HS and JW conceived and supervised this study. JY completed the main experimental content. ZW and SG collected and analyzed data. LD and XZ wrote the first draft of manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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