INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20F–001/2020/24–1–91–100
DOI: 10.17957/IJAB/15.1412
http://www.fspublishers.org

Full Length Article

Effect of Chinese Herbal Medicines Supplementation on the Diversity and Structure of Intestinal Microbiota in Broiler Cecum

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Received 03 January 2020; Accept 07 February 2020; Published 20 April 2020

Abstract

The excessive use of antibiotics disrupts the balance of normal microflora, and stimulates the development of drug-resistant bacteria, which threaten not only the human health but also the environment. This study was conducted to provide some insights into the effect of different Chinese herbal medicines (CHM) supplementation on the diversity and composition of broilers intestinal microbiota by high-throughput sequencing. A total of 420 one-day-old Arbor Arbor broilers were divided into seven groups: fed basal diet (CT), or basal diet supplemented with 0.1% monensin (MON), *Cynanchum atratum* Bunge (CA), *Radices paeoniae alba* (RPA), *Morus alba* L. (MAL), *Astragalus membranaceus* (AM), or *Eucommia ulmoides* Oliver (EUO) for 42 days. The intestinal microbiota of the cecum was analyzed by high-throughput sequencing (16S rRNA genes). Taxonomic components of intestinal microbiota revealed that the cecum microbiota of CHM groups was clearly distinguished from the MON and CT groups. CHM significantly influenced the relative abundances of ceca microbiota genera at phylum and genus level via increasing the abundance of beneficial bacterial and decreasing the colonization of pathogens, especially in CA, EUO, RPA and MAL groups. Functional profiling of bacterial communities revealed that CHM increased the enrichment of several functional categories, such as cellular processes, metabolism, organismal systems and immune system. In conclusion, the present study showed that CHM influenced the diversity and taxonomic composition of broiler ceca microbiota, and the functional categories were clearly disparate. These results improved our comprehension of the influence of CHM on structure and activity of intestinal microbiota in broiler, and contributed to the development of antibiotic alternative feeding strategies. © 2020 Friends Science Publishers

Keywords: Chinese herbal medicines; Broiler; Intestinal microbiota; Cecum; Antibiotic alternative; 16S rRNA sequencing

Introduction

Antibiotics, as growth promoters, were widely used in poultry feed industry, in order to improve the feed utilization efficiency and reduce the rates of mortality for more than 50 years. However, the excessive use of antibiotics disrupts the balance of normal microflora, stimulate the proliferation of drug-resistant bacteria, and increase the accumulation of antibiotic residues in not only animal products but also the environment (Park and Kim 2014). Therefore, there is an urgent need to develop and provide antibiotic-free feed for farm animals.

In response to the above request, a number of potential alternatives, such as probiotics, prebiotics, essential oils, and antimicrobial peptides (Kim *et al.* 2019; Kazemi *et al.* 2019; Wang *et al.* 2019). Tradition Chinese herbal medicines (CHM), usually comprising of multiple medicinal plants, has shown its unique strengths in treating diseases or unbalances via ameliorating human or animal health (Zhang *et al.* 2011; Gong *et al.* 2014). In recent years, CHM have also shown its regulatory role in nutritional regulation, immuno-enhancement and gut health in animals, as feed supplementation (Kong *et al.* 2007; Yin *et al.* 2008; 2009). Numerous studies also have suggested that CHM could serve as a potential effective substitute of the conventional antibiotic related drugs commonly used to suppress stress and enhance immune and anti-microbial activities in commercial animal production (Liu *et al.* 2011). However, the mechanism of CHM is still unclear in regulating the nutritional metabolic processes in animals.

Intestinal microbiota is known to play an important role in maintaining local immunity, enhancing nutrient utilization, and alleviating the influence of exogenous stimulus on the performance and production of animals

To cite this paper: Fu C, Y Zhang, Q Yao, X Wei, T Shi, P Yan, X Liu (2020). Effect of Chinese herbal medicines supplementation on the diversity and structure of intestinal microbiota in broiler cecum. *Intl J Agric Biol* 24:91–100

(Zhang *et al.* 2007; Crhanova *et al.* 2011; Diao *et al.* 2012). Several studies reported that CHM supplementation enhanced the intestinal health via regulating the balance of intestinal microbiota in poultry and swine (Gong *et al.* 2014). The mushroom *Agaricus bisporus* treatment (with 20–30 g/kg diet) increased the counts of Lactobacillus and Bifidobacterium, and improved the intestinal health of broilers (Giannenas *et al.* 2010; Kavyani *et al.* 2012). Crude *Acanthopanax senticosus* extract supplementation reduced the colonization of coliform bacteria in ileum of swine (Yin *et al.* 2008). Besides, some CHM polysaccharides (such as Lentinus edodes and Tremella) are used as feed additives to improve the productivity of broilers and swine via reducing the abundance of *Bacterides* spp. and *Escherichia coli*, and improving the density of *Bifidobacteria* and *Lactobacilli* in cecum microbiota (Guo *et al.* 2004; Gong *et al.* 2014).

Traditional CHM, such as *Cynanchum atratum* (Baiwei, CA), *Radix Paeoniae Alba* (Baishao, RPA), *Morus alba L.* (Sangbaipi, MAL), *Astragalus membranaceus* (Huangqi, AM), and *Eucommia ulmoides* Oliver (Duzhong, EUO), have been demonstrated to have anti-inflammatory activities, and prevent diarrheal incidence, which is closely related to the balance of intestinal microbiota (de Oliveira *et al.* 2016; Zhou *et al.* 2016; Hu *et al.* 2018; Jo *et al.* 2018; Che *et al.* 2019). Our previous studies found that addition of these above CHMs influenced the growth performance, immune organ index and antioxidant activity, while the regulatory mechanism was unclear (Fu *et al.* 2018). The present study was conducted to provide some insights into the effect of these five different kinds of CHM treatment on diversity and composition in broilers intestinal microbiota by high-throughput sequencing. Meanwhile, in order to elucidate the beneficial effect of CHM treatment in bacterial taxa and their functions in broilers, we also compared the difference of intestinal microbiota community among broilers fed CHM diet, antibiotic diet and basal diet without antibiotic. The results obtained from this study might provide useful information to develop a more efficient CHM formulation, for replacing the antibiotics and develop antibiotic-free animal feed.

Materials and Methods

Diet preparation

The diets were conducted following the nutrient demands of broilers (NRC 1994), with comparable protein and energy ratios to allow for equal protein intake, as shown in Table S1. The feeding study comprised a series of seven diets: a basal diet (CT), and five CHM diets, with 0.1% CHM supplementation, including *C. atratum*, *R. paeoniae alba*, *M. alba*, *A. membranaceus* and *E. ulmoides*, which were well known for the anti-inflammatory activities (as shown in Table S2). In addition, an antibiotic group, with 0.1% monensin supplementation in basal diet (MON) was conducted. All the CHM were purchased from a local Chinese medicine shop, and then were shattered and sifted out before they were added into the diets.

Animal experiments

A total of 420 healthy 1d Arbor Arbor broilers were obtained from a local hatchery. All the birds were kept in stainless steel (three-tiered battery cages with 0.9 m length, 0.6 m width and 0.6 m height) and raised wire netted floors in an open house under the natural conditions, and divided into seven diet groups (6 replicates per group with 10 birds per replicate), with 23L:1D lighting regime for the first three days and then 16L:8D lighting regime till the end. The brooding temperature was at 35ºC (65% relative humidity, RH) for the first two days and then gradually reduced to 30ºC on day 7 and 26ºC on day 21, after which animals were maintained at room temperature.

DNA extraction and 16S rRNA gene sequencing

On 42 d, six chickens per treatment were selected randomly and received an intramuscular administration of 0.2 mL/kg pentobarbital sodium ten minutes before the blood withdrawal. Cecal contents were collected using swabs and immediately frozen at -80^{o}C. To alleviate pain, microbial genomic DNA extraction was performed with TruSeq Nano DNA LT Sample Prep Kit (Illumina, San Diego CA, USA) according to manufacturer's instruction. Agarose gel electrophoresis was conducted to test the integrities of gDNA. The DNA concentrations were detected with a Qubit and DNA quality was assessed with a Nanodrop 1000 (NanoDrop Technologies, Thermo Scientific, USA).

As previously described by Tong *et al.* (2018), specific PCR primers (520F: GCACCTAAYTGGGYDTAAAGNG and 802R: TACNVGGGTATCTAATCC) with barcode for 16S rRNA were synthesized in V4 variable region. The PCR was constituted by the following reagents: 2 μL DNA template, with 11.25 μL ddH_2O, 5 μL reaction buffer (5 ×), 5 μL high GC buffer (5 ×), 0.5 μL dNTP (10 mM), 1 μL Forward primer (10 μM), 1 μL 10 μM Reverse primer, and 0.25 μL DNA Polymerase (Q5). The PCR was performed at 98ºC for 30 s of predenaturation, followed by 30 cycles with 98°C for 15 s, 50ºC for 30s, 72ºC for 30s and finishing at 72°C for 5 min. Products of polymerase chain reaction were detected with agarose gel electrophoresis (2%). A gel recovery kit (Axygen, Union City, CA, USA) was used to retrieve the target fragment. For defining the sample mixing ratio according to the sequencing requirement, qRT-PCR was conducted for the recovery products with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, CA, USA). The TruSeq Nano DNA LT Library Prep Kit (Ilumina, USA) was used to prepare the sequencing libraries. The self-connected fragments of linker was removed by magnetic bead screening using the BECKMAN AMPure XP Beads (Backman, Brea, CA, USA) and the library enrichment product was purified for sequencing. Then, 2% agarose gel

electrophoresis was used to select and purify the library.

Quality of the library was tested by using Agilent High Sensitivity DNA Kit (Bioanalyser, Agilent Technologies, California, USA) on Agilent Bioanalyzer. Then, the V4 region from 16S rRNA gene was sequenced by using the Illumina MiSeq platform and MiSeq Reagent Kit version 3 with 600 cycles and a paired-end read length of 2×300 bp. A Quant-iT PicoGreen dsDNA Assay Kit was using to test the quality of the libraries on a Promega QuantiFluor fluorescence quantification system.

Bioinformatics and statistical analysis

Firstly, preliminary screening was conducted to test the quality of the original high-throughput sequencing data via QIIME software. Sequence should be ≥ 150 bp in length, and sequences with ambiguous base N were deleted. The sequence, that passed preliminary screening, was identified and assigned into the corresponding sample according to the quality screening and Barcode information. The obtained sequences were classified via operational taxonomic unit (OTU), with the representative sequence one used for further analysis. The diversity level of each sample was evaluated on the basis of the density structure of OTU in different samples, and sparse curves were drawn to indicate the depth of sequencing. The unique components of each sample were analyzed at different level, and the statistical difference between different groups was tested. Based on the species composition and distribution in each sample, the interaction correlation network was constructed. The microbial metabolic function in each sample was predicted according to the high-throughput sequencing results.

Results

Sequence quality assessment

As the sequencing process produced many questionable sequences, a further estimation was acquired to improve the reliance of the effective sequences. The valid sequences of each group ranged from 38320 to 52531, and the high-quality sequences of each group ranged from 34349 to 48230.

OTU classification analysis

The reliable sequences were merged. The OTU were partitioned at 97% sequence similarity. The representative sequence of the OTU was identified via selecting the most abundant sequence in each OTU. In the ceca contents, over 564 and 269 OTUs were got at phylum and genus level respectively. In addition, there were 1–6 OTUs that unclassified from all samples.

The indexes of Simpson, Chao1, ACE and Shannon were used to show the alpha diversity of the intestinal microbiota community. The comparison of results showed no significant difference among all seven groups (Table S3, P>0.05). In Fig. 1, it is evident that the rarefaction curve of all groups extended to the right completion of the x-axis, indicating that for reflecting the diversity of microorganisms in all groups, the depth of the present sequencing was sufficient.

Influence of CHM supplementation on the taxonomic components of intestinal microbiota

After the microbial OTU representative sequences were taxonomically classified, the differences of intestinal microbiota composition at several taxonomic levels were analyzed. The relative abundances of five different taxonomic levels were shown in Fig. 2 (phylum and genus) and Fig. 3 (class, order and family). All microbiota sequences of seven groups were divided into 9 phyla (Fig. 2A). The preponderant bacteria at phylum level were *Bacteroidetes* (45.87–72.26%), *Firmicutes* (24.91–35.03%), *Verrucomicrobia* (0.08–8.03%), and *Proteobacteria* (0.75–

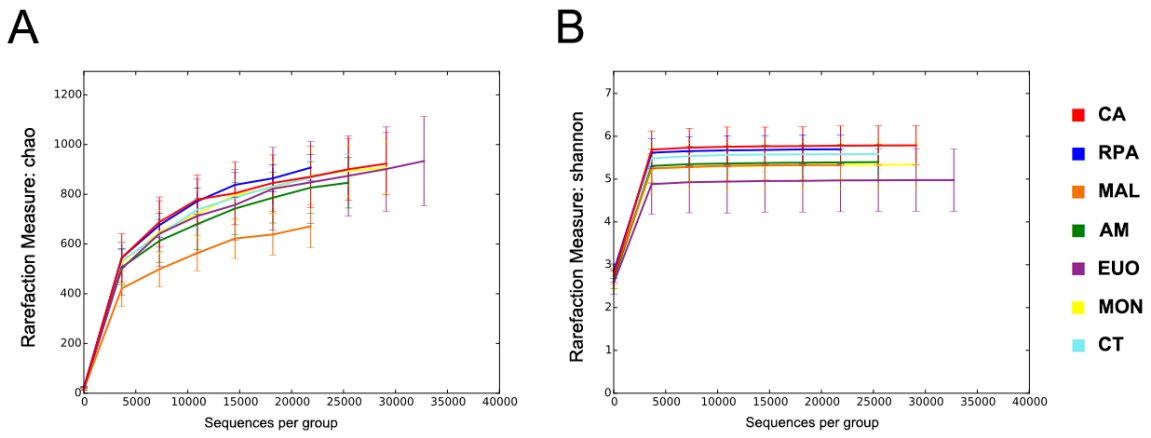

Fig. 1: Microbial rarefaction curves based on Chao index (A) and Shannon (B) index. Each group was distinguished by different colors of lines

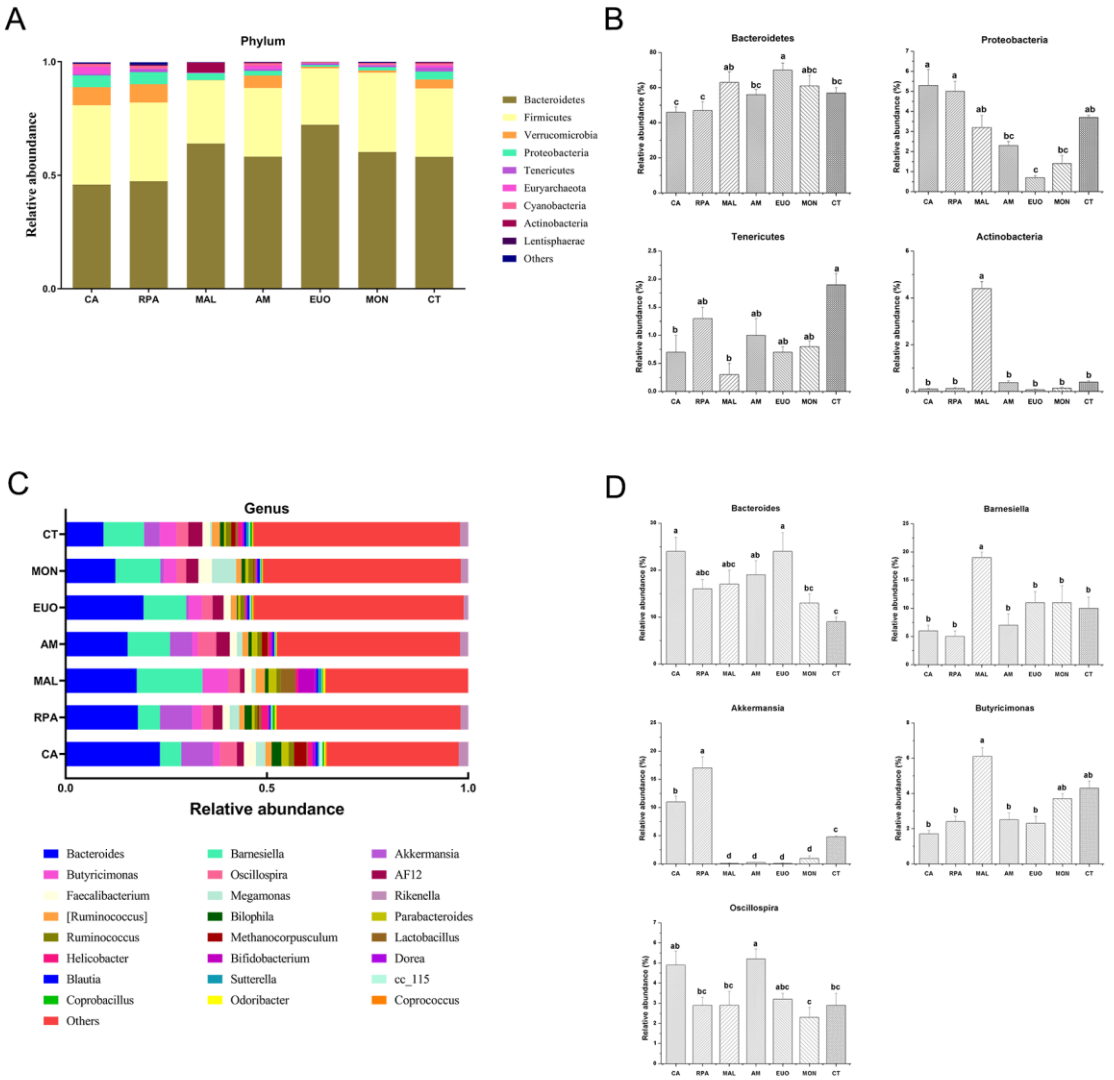

Fig. 2: Alternation in intestinal microbial taxonomic composition with different Chinese herbal medicines exposure. (A) Graph represents the OTUs at different taxonomical levels: phylum. (B) The change in the relative abundance of phyla *Bacteroidetes*, *Proteobacteria*, *Tenericutes* and *Actinobacteria*. (C) Graph represents the OTUs at different taxonomical levels: genus. (D) The change in the relative abundance of genus *Bacteroides*, *Barnesiella*, *Akkermansia*, *Butyricinas* and *Oscillospira*. Metastats analysis was applied to identify the significantly differentially abundant genera among groups. Different letters above the bars denotes significantly differentially abundant genera among groups (P<0.05)

5.35%). Meanwhile, EUO supplementation notably improved the relative abundance of *Bacteroidetes* (P=0.0063), but reduced the relative density of *Proteobacteria* (Fig. 2B, P=0.0013) compared with control. Besides, CA and MAL addition reduced the relative density of *Tenericutes* (P=0.0842) compared with control, while MAL improved the relative density of *Actinobacteria* (P<0.0001) compared to the other groups.

The inspection of taxonomic structures at level of genus showed that the most ample taxa (top 25) while the rest of the lower frequent taxa was identified as 'others' (Fig. 2C). The results showed that 14 dominant genera (*Bacteroides*, *Barnesiella*, *Akkermansia*, *Butyricimonas*, *Oscillospira*, *AF12*, *Faecalibacterium*, *Megamonas*, *Rikenella*, [*Ruminococcus*], *Bilophila*, *Parabacteroides*, and *Ruminococcus*), which constituted more than 1% of the complete sequences on average, were present in the microbial communities. However, at genus level the most significant changes included *Bacteroides*, *Barnesiella*, *Butyricimonas*, *Akkermansia* and *Oscillospira.* A considerable increase of *Bacteroides* in CA and EUO groups was found compared with control and other groups (Fig. 2D, P=0.0069). A prominent increase in *Barnesiella* (P=0.0015) and *Butyricimonas* (P=0.0484) was found in

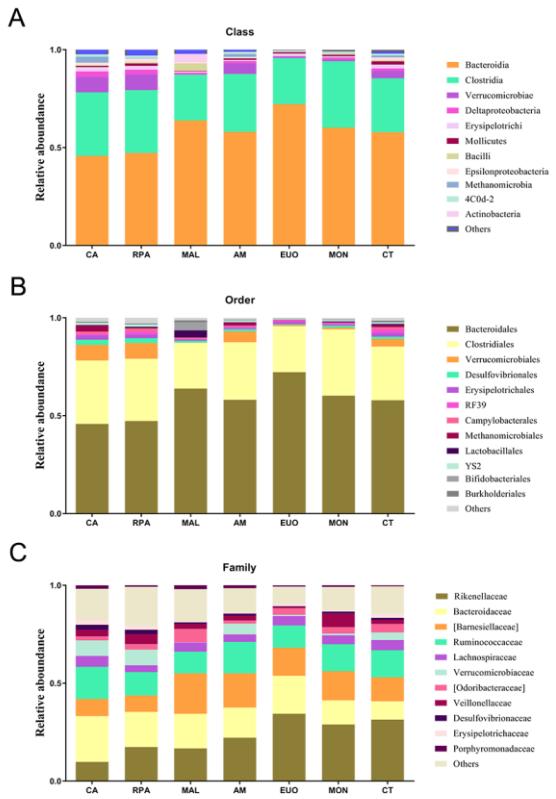

Fig. 3: Graph represents the OTUs at different taxonomia levels: class (A); order (B); family (C)

MAL group, compared with those in other groups. Compared to control, the proportion of *Akkermansia* was higher in RPA and CA groups, and MAL, AM, EUO, and MON groups decreased the relative abundance of *Akkermansia* (*P<0.0001*). Compared with antibiotic and control groups, AM group significantly increased the level of *Oscillospira*, which was decreased in RPA and MAL groups (*P = 0.0372*).

To further discriminate the difference induced by CHM supplementation compared with basal diet and antibiotic group, LEfSe was also performed to found out significant abundance of bacterial taxa at genus level (Fig. 4). Data showed that compared with MON and CT groups, 4 genera and 2 genera were respectively increased and decreased in response to CA supplementation (Fig. 4A; *P*<0.05); 7 genera and 3 genera were induced and reduced in RPA group (Fig. 4B; *P*<0.05). MAL supplementation increased the enrichment of 15 genera in intestinal microbial, but decreased the abundance of 7 genera (Fig. 4C; *P*<0.05). However, 6 genera and 8 genera showed significant decrease in AM and EUO group, respectively (Fig. 4D and E; *P*<0.05). Data further showed that three of the five CHM (CA, RPA, and MAL) supplementation increased the relative abundance of *B_42*, and *Luteimonas* (*P*<0.05). All of five CHM supplementation decreased the relative abundance of *Sutterella*, and *Ralstonia* (*P*<0.05).

Comparative analysis of intestinal microbiota among CHM, antibiotic and control groups

In order to clearly achieve the difference of CHM groups in community structures, partial least squares discriminant analysis model (PLS-DA), as well as ANOSIM analysis and Adonis analysis were used with 999 permutations. It illustrated that the bacterial communities among all seven groups were clearly distinguished (R=0.5070, P=0.001 by ANOSIM; R^2=0.3150, P=0.001 by Adonis), especially for CA, RPA, and MAL groups (Fig. 5A). Combined with previous results, we further analyzed the difference of bacterial communities for CHM group with MON group and control group. Data showed that CA treatment resulted in significant difference in community structures of bacteria (R=0.7341, P=0.004 by ANOSIM; R^2=0.2081, P=0.005 by Adonis) compared with MON and control groups, similar to that of RPA group (R=0.4860, P=0.001 by ANOSIM; R^2=0.1912, P=0.001 by Adonis) and MAL group (R=0.5481, P=0.001 by ANOSIM; R^2=0.2540, P=0.001 by Adonis) group.

Impact of CHM supplementation on the predicted functions of intestinal microbiota

To predict the functional profiling of bacterial communities, the PICRUSt was used based on the closest reference genomes (Langille *et al.* 2013). And then the predicted genes were transformed to KEGG functional categories. Although there were some restrictions in predicting process, the inspected functional categories may also define some insights about the microbiome-regulated pathways influenced by CHM supplementation. We found that, compared with other groups, CA, RPA, and MAL groups respectively increased 3 different kinds of functional categories enrichment in intestinal bacterial communities (Fig. 6A, *P*<0.05). While compared with other six groups, EUO supplementation significantly induced 9 functional categories enrichment in intestinal bacterial communities (*P* < 0.05). In order to further analysis the influence of CHM on intestinal bacterial communities, we calculated the difference of predicted microbial functions in CA, RPA, MAL and EUO groups with MON and CT group, respectively. Results revealed that CA supplementation induced 4 functional categories enrichment in intestinal bacterial communities, including signal transduction, cardiovascular diseases, xenobiotics biodegradation and metabolism, and cellular processes and signaling, which was similar to that of RPA (Fig. 6B–C; *P* < 0.05). But CA and RPA exposure also reduced the abundance of 6 functional categories, including cell growth and death, metabolic diseases, nucleotide metabolism, replication and repair, translation and immune system diseases (*P* < 0.05). MAL supplementation increased the enrichment of intestinal microbiota functioned in enzyme families, and decreased 1 functional categories (genetic information processing)

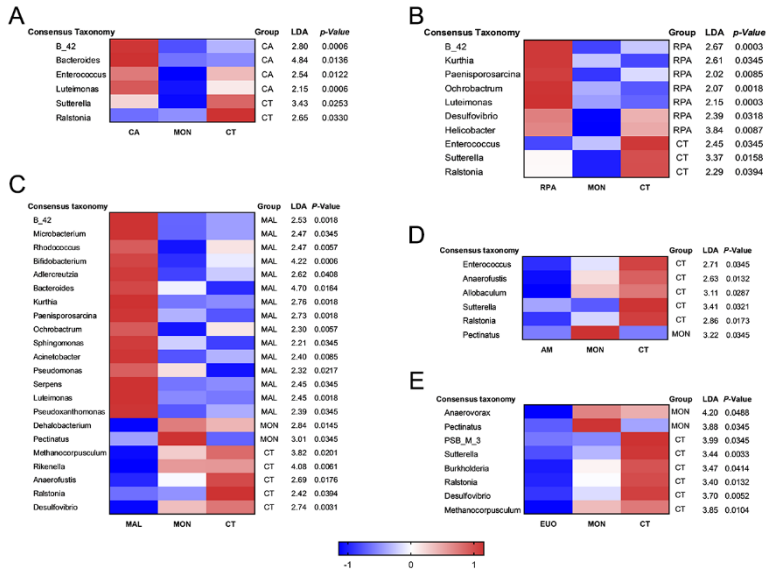

Fig. 4: Heat map of relative abundance of bacterial genera detected in the samples. The heat map values were Z-score normalized from the abundance of bacterial genera. Differential abundance was statistically tested using the linear discriminant analysis effect size (LEfSe) method. Bacterial genera that were significantly more abundance in one group than the other groups were marked on the right side of heat map, as well as the log LDA score, and p-value determined by LEfSe test

enrichment (Fig. 6D; $P < 0.05$). EUO group induced the enrichment of 5 functional categories (including nervous system, amino acid metabolism, digestive system, immune system, and endocrine system), but decreased the relative abundance of microbiota functioned in signal transduction (Fig. 6E; $P < 0.05$). While MON supplementation increased the enrichment of microbiota functioned in cell growth and death, metabolic diseases, nucleotide metabolism, and replication and repair ($P < 0.05$).

Discussion

Antibiotics were widely used in animals, to maintain health and elevate productivity, which resulted antibiotic resistance and threatened public health (Huang *et al.* 2018). Therefore, it is urgent to develop safe substitutes to antibiotics in animal feed. So far, numerous natural growth promoters have been exploited as substitutes to antibiotics in livestock production, including CHM (Gong *et al.* 2014). Intestinal microorganism, involved in interactional system, play a vital role in regulating the host metabolism (Tong *et al.* 2018). Antibiotics were reported to reduce the growth of pathogens or the production of growth-depressing metabolites from gut microbiota (Crabbé *et al.* 2019). Besides, some CHM also showed its regulatory role in modulating intestinal microbiota (Guo *et al.* 2004; Che *et al.* 2019). However, it is still unclear about the influence of CHM on intestinal microbiota community in broilers. In the current study, 16S rRNA sequencing was conducted to figure out the structure and difference of intestinal microbiota in the cecum of broilers fed five different CHMs. Meanwhile, the bacterial community of broilers fed with or without antibiotic was also analyzed to elucidate the influence of CHM on intestinal microbiota when replacing antibiotic. And the current study might contribute to the development of antibiotic alternative feeding strategies for maintaining health and improving performance of animals.

The OTU analysis showed the diversity of ceca microbiota in all groups. The alpha diversity indices (Chao1, ACE, Simpson, and Shannon) also indicated that there was no significant difference in the species' richness and evenness among the seven groups investigated. The evaluation of rarefaction curves showed that two curves both tended to reach a plateau. The sequenced data obtained from each group was suggested competent to cover the majority of biodiversity within the samples (Mancabelli *et al.* 2016).

Furthermore, taxonomic classification in the ceca of broilers revealed that, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, and *Proteobacteria* were the predominant bacterial phyla, according to the other findings (Sergeant *et al.* 2014; Huang *et al.* 2018; Choi *et al.* 2018). Previous studies showed that *Bacteroidetes* phylum played a role in maintaining a healthy gut via altering the morphology and function of gut as well as the immune system (Kim and Milner 2007; Mazmanian *et al.* 2008; Thomas *et al.* 2011). The phyla *Bacteroidetes*, *Tenericutes*, *Firmicutes*, and *Actinobacteria* were reported to be probiotics, and participated in the regulation of host health via translating the feeds into microbial fermentation end production, and mediating the usage of nitrogenous substances, the bioconversion of bile acids, as well as preventing pathogen

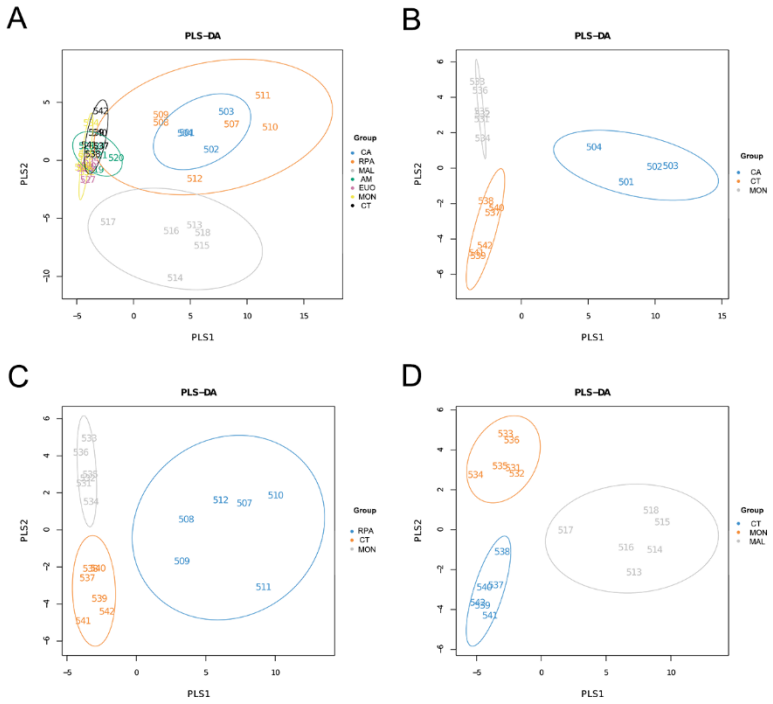

Fig. 5: Influence of different CHM supplementation on bacterial community structure. Partial least squares discriminant analysis (PLS-DA) of all samples (A) based on UniFrac distances calculated from OTU abundance matrix. PLS-DA for CA (A), RPA (B) or MAL (D) group with antibiotic and control groups

colonization (Tan *et al.* 2009; Latha and Dhanasekaran 2013; Sergeant *et al.* 2014; Corrigan *et al.* 2015; Latha *et al.* 2016). While the abundance of the phylum *Proteobacteria*, including several pathogens such as *Sutterella* and *Ralstonia* genus, was related with pro-inflammatory response and metabolic disorder, such as glucose homeostasis and diabetic phenotype (Qin *et al.* 2012; Oakley and Kogut 2016). The current study found a higher population of phylum *Bacteroidetes* (including the genera *Bacteroides*, *Barnesiella*, and *Butyricimonas*) was enriched in CHM groups (such as the CA, EUO and MAL groups) compared to the control group, and the relative abundance of *Actinobacteria* was more in MAL group than the other groups. Furthermore, EUO, CA and MAL treatment reduced the enrichment of the phyla *Proteobacteria* and *Tenericutes* respectively, compared to control. These results suggested that CHM supplementation improved the density of beneficial bacteria and reduced the enrichment of pathogens at the phylum level.

Further analysis also revealed that the genera *Akkermansia* (Phylum: *Verrucomicrobia*) and *Oscillospira* (Phylum: *Firmicutes*) were highly abundant in CA, RPA and AM groups, which were found to have anti-inflammatory and anti-diabetic activity (Hansen *et al.* 2013; Shin *et al.* 2014; Konikoff and Gophna 2016). Besides, the abundance of *Butyricimonas* and *Barnesiella* genus were higher in MAL group than the others, suggested as core components of the poultry microbiota by other researchers (Oakley *et al.* 2014; Oakley and Kogut 2016; Wei *et al.* 2013; 2016), and received much attention for their potential probiotic capabilities.

Consistently, the comparison of CHM groups with MON and CT groups using LEfSe methods also indicated that CHM supplementation regulated the diversity and composition of intestinal microbiota in broiler cecum, and increased the favorable bacteria genus, especially in MAL group. Interestingly, the phylum *Actinobacteria*, including genera *Microbacterium*, *Rhodococcus*, *Bifidobacterium*, and *Adlercreutzia*, was highly enriched in MAL groups. The phylum *Actinobacteria* is reported to produce many secondary metabolites, which are potent antibiotics, although it makes up a small proportion in host intestine (Jensen *et al.* 2007; Ramesh and Mathivanan 2009). Previous studies also showed that the supplementation of prebiotics on various terrestrial decreased the colonization of potentially pathogenic bacteria via increasing the amount of health-protecting bacteria e.g., *Bifidobacterium* and *Adlercreutzia* (De Maesschalck *et al.* 2015; Johnson *et al.* 2015; Chen *et al.* 2016). Therefore, these results indicated that MAL might be more beneficial for potential favorable bacteria. Data also showed that MAL increased the abundance of several pathogenic bacteria, such as *Ophingomonas*, *Acinetobacter*, *Pseudomonas*, which belongs to phylum *Proteobacteria*. These genera were reported to be associated with human disease, and the spread and accumulation of antibiotic resistance genes

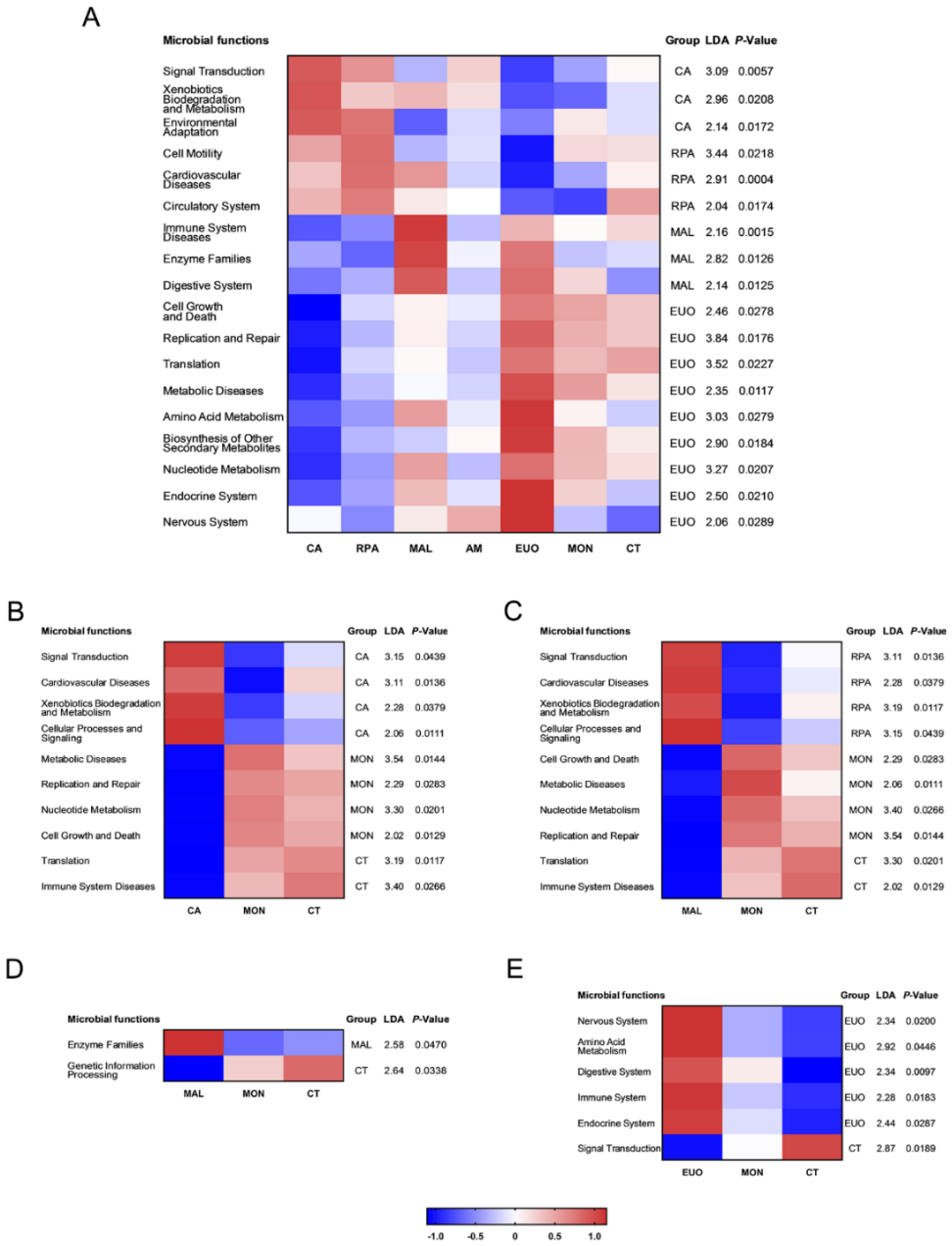

Fig. 6: Predicted microbial functions enriched in intestinal microbial of broilers from different groups. The heat map values were Z-score normalized from PICRUSt count values. Functional categories were taken from the KEGG pathway hierarchy level 2. Linear discriminant analysis effect size (LEfSe) was performed to evaluate the significance of predicted microbial functions among different groups. (A) The difference of predicted microbial functions for all groups. The impact of CA (A), RPA (C), MAL (D), EUO (E) on the predicted functions of intestinal microbiota, compared with MON and control groups were were calculated and showed respectively. Microbial functions that were significantly more abundance in one group than the other groups were marked on the right side of heat map, as well as the log LDA score, and p-value determined by LEfSe test

(Duan *et al.* 2017; El Beaino *et al.* 2018). As CA, EUO, and AM played positive role in decreasing the colonization of phylum *Proteobacteria*, it is suggested that a combination of several CHMs rather than a single one might be more beneficial in maintaining the gut health.

The functional maturation of the microbiome was analyzed using PICRUSt (Langille *et al.* 2013; Buffie *et al.* 2015), and then converted to KEGG functional categories (Choi *et al.* 2018). Identification of genes showed that they were related to cellular processes, environmental information processing, genetic information processing, diseases, metabolism, as well as organismal systems.

Interestingly, the predicted functions enriched in the cecum of CA, RPA, and MAL groups were associated with cellular processes, environmental information processing, and metabolism. However, metabolism and organismal systems, such as nervous system, amino acid metabolism, digestive system, and endocrine system were enriched in the cecum of broilers in EUO group. Our previous study also indicated that EUO supplementation increased the immune organ index and average feed intake in the last two weeks (Fu *et al.* 2018). This may be due to the pharmacological activities of EUO, such as blood pressure reduction, immune regulation, and anti-aging effects (Lee and Weinblatt 2001). In addition, the CHM supplementation (especially for CA and RPA) also decreased the immune system diseases of broilers, which indicated that CHM supplementation regulated the homeostasis of immune system and maintained the balance of immune system, which was consist with Guo *et al.* (2016).

Conclusion

The CHM supplementation in diet regulated the composition of the microbiota communities in broiler cecum, and the combination of several CHM might be more beneficial for the maintenance of gut health and the performance of broilers. Our study might contribute to the development of antibiotic alternative feeding strategies. However, further detail about the regulatory mechanism of intestinal microbiota in influencing the performance of broilers remains to be revealed.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (31902176), the Natural Science Foundation of Shandong Province (ZR2019BC005), the Construction of Subjects and Teams of Institute of Poultry Science (CXGC2018E11), the Shandong Provincial Key Laboratory of Special Construction Project (SDKL201810) and Agricultural scientific and technological innovation project of Shandong Academy of Agricultural Sciences (CXGC2016A04).

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