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Effects of *Polygonatum cyrtonema* Hua and Semen Cassiae feed additive on intestinal microflora of Wanxi White Goose

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Abstract

The widespread use of antibiotics in poultry breeding has led to drug resistance of pathogenic microorganisms. With long-term dependence on antibiotics, immunity in poultry has decreased, increased susceptibility and seriously affecting the quality of poultry protein products and consumer health. Chinese medicinal herbs come mainly from nature and are natural products, so are called green feed additives. It has become an important substitute for antibiotics in high-quality livestock products. In this study, Wanxi White Geese were used as the research object to explore the effects of Chinese medicinal herb feed additives on intestinal microorganisms. Semen Cassiae and *Polygonatum cyrtonema* Hua increased the number of operational taxonomic units (OTUs) in intestinal microorganisms of geese, and the number of OTUs increased with dosage. Semen Cassiae or *Polygonatum cyrtonema* Hua has great influence on the abundance of intestinal microbes. The relative abundance of *Clostridiales* was higher than the control (feeding only the basal diet) after treated with Semen Cassiae or *Polygonatum cyrtonema* Hua feed additives. Chinese medicinal herb feed additives inhibited *Erysipelotrichales* and the relative abundance of *Erysipelotrichales* in all treated samples was lower than the control group. Alpha diversity analysis also showed that Chinese medicinal herb feed additives promoted the diversity of intestinal microorganisms. The purpose of this study is for future research and practical application of feed additives.

Keywords: Wanxi white geese; Semen Cassiae; *Polygonatum cyrtonema* Hua; intestinal microorganisms

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Introduction

Wanxi White Geese originated in Lu'an, Anhui Province, and are an excellent regional goose species that has been selected breeding, and has been bred for a long time in China. Wanxi White Geese were included in the National Livestock and Poultry Genetic Resources Conservation Field (serial number: C3411002) in 2008 and have been embodied in the National Animal Genetic Resources Protection List in 2014 in China (Ministry of Agriculture and Rural Affairs of the People's Republic of

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China, 2008 and 2014). The irrational use of antibiotics seriously affects the quality of Wanxi White Geese and human safety.

With the rapid development of poultry breeding, feed safety has attracted wide attention globally, especially the irrational use of it for promoting growth additives (Salim *et al*., 2016, Ndlovu *et al*., 2017, Salim *et al*., 2018, Xu *et al*., 2020, Qin *et al*., 2020). However, residual antibiotics in meat can seriously affect human health and safety (Silbergeld *et al*., 2008, Lin *et al*., 2020). Thus, the Chinese government has issued a decree that antibiotics were prohibited as feed additives in 2020 (Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2019). Poultry dependence on antibiotics for a long time has decreased poultry immunity, increase the incidence of disease, and the cost of disease prevention (Grondel *et al*., 2020, Shang *et al*., 2020, Zhang *et al*., 2020).

In recent years, Chinese medicinal herbs as feed additive have been applied in poultry breeding to replace the antibiotics that gradually became an applied research direction. Some feed additives of Chinese medicinal herbs have been found and successfully used in breeding, such as *Astragalus mongholicus*, *Radix Codonopsis*, *Zingiber officinale* (Wu *et al*., 2009, Wang *et al*., 2011, Zhao *et al*., 2011, Ogueji *et al*., 2017). Although Chinese medicinal herb has made great achievements in the poultry industry, the development of these drugs is mostly concentrated in chicken breeding. Hence, it is urgent to develop Chinese medicinal herb feed additives for geese, especially Wanxi White Geese.

Semen Cassiae (*Catsia tora*) is a seed material which can be used as drug and food. Chemically, it is composed of anthraquinones, fatty acids, unsaponifiable matter, naphthopyrone, amino acids (Liu *et al*., 2010, Feng *et al*., 2014, Dong *et al*., 2017, Luo *et al*., 2019). Luo *et al*. (2011) found that the Semen Cassiae relieved fat degeneration and the inflammatory response in rats with alcoholic fatty liver; decreased alanine transaminase, aspartate transaminase, triglyceride, and total cholesterol; decreased TNF-a, IL-6, and IL-8 levels (Meng *et al*., 2019); and regulated the role of intestinal microorganism stabilization in mice with fatty liver (Luo *et al*., 2020). This research indicates that Semen Cassiae has some nutraceutical value in liver and intestinal disease. However, Semen Cassiae as a feed additive as applied to geese has not been reported.

Polygonatum cyrtonema Hua., a perennial herb, has some anticancer, anti-oxidation, anti-pathogenic microorganisms, and antivirus roles (Liu *et al*., 2004, Su *et al*., 2007, Liu *et al*., 2009, Ding *et al*., 2010, Zeng *et al*., 2018). Furthermore, *Polygonatum cyrtonema* Hua. is widely used as a dual-purpose drug and food in the treatment and health protection of humans, but its application as a feed additive in goose breeding has not been reported.

To promote the development of the Wanxi White Goose industry and accelerate the alternative antibiotics industry, Semen Cassiae and *Polygonatum cyrtonema* Hua. were used as feed additive alternatives to antibiotics to feed the goslings. The 16S rDNA sequencing was used to investigate the intestinal microorganisms to provide a theoretical basis for the use of Chinese medicinal herbs as alternative antibiotics.

Materials and Methods

The goslings were allowed to have free access to pelleted food and drinking water. The experimental protocol was approved by the Animal Care and Use Committee of West Anhui University. The experimental procedures were carried out in accordance with the guidelines of the Welfare and Ethical of Treatment of Experimental Animals in China (T/CALAS 73-2019). The basal diet was obtained from Anhui Xinhua Animal Husbandry Technology Co. of China and nutritional content is shown in Table 1.

Table 1: Nutritional content of basal diet fed to Wanxi White goslings

Crude protein ≥	Crude fiber ≤	Crude fat ≥	Crude ash ≤	Calcium	Total phosphorus ≥	Sodium chloride	Moisture content
19.0	7.00	2.50	9.00	0.60–1.40	0.55	0.30–0.70	13.0

Note: Dietary composition: corn, high quality puffed soybean meal, fish meal, secondary meal, stone powder, calcium hydrogen phosphate, sodium chloride, lysine, methionine, copper sulphate, zinc sulphate, manganese sulphate, ferrous sulphate, vitamin A, vitamin D, vitamin E, vitamin B, vegetable oil, phytase

The extract was obtained at a raw drug concentration of 1 g/ml by soaking 20 kg of dried Semen Cassiae (Aoxiangtang, China) or *Polygonatum cyrtonema* Hua (Shenghaitang, China) 19 times in distilled water, extraction in a water bath at 100 °C for 2 h, and finally centrifuging (3500 rpm, 15 min) and preparation to 1000 mL. Then, 1L of extract was well mixed with 2 kg of basal diet, dried and set aside as a premix. The premix was added to the basal diet and mixed to obtain the corresponding feeds: J2, J4, J6 were formed by adding 2%, 4%, and 6% Semen Cassiae; H2, H4, H6 were formed by adding 2%, 4%, and 6% *Polygonatum cyrtonema* Hua to the basal diet.

A total of 140 goslings were randomly divided into seven groups: CK (receiving only the basal diet), J2, J4, J6, H2, H4, and H6 group. The experiment lasted for 30 d. At the end of the feeding experiment, three geese were randomly selected from each group and faeces was collected, which was sent for 16S rDNA sequencing.

The CTAB method was used to extract the total DNA of all samples, which were uniformly diluted to 100 ng/µL for polymerase chain reaction (PCR) detection of the target genes. Primers were synthesized according to the variable region of V3–V4 in the hypervariable region of 16S rDNA (F: 5'-CCTAYGGGRBGCASCAG-3', R: 5'-GGACTACNNGGGTATCTAAT-3'), and a sequenced adaptor at was added at the end of the primer. PCR amplification was performed on 50 µl PCR reaction mixtures containing 100 ng of the DNA extract, 1× Taq reaction buffer, 20 pmol of each primer, 200 µM each dNTP, and 1.5 U of Taq DNA polymerase (Sangon Biotech, China). Samples were amplified using PCR with the following protocol: 5 min denaturation at 95 °C, 30 cycles of 1 min at 94 °C, 50 s at 55 °C, and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. Amplicon libraries were then prepared by pooling 10 ng of each PCR product. The PCR product was purified, quantified, and homogenized to form a sequence library. The completed library was tested using a library quality test and certified library was sequenced using an Illumina HiSeq 2500.

The original data were spliced and the spliced sequences were mass-filtered, chimeras were removed, and the high-quality tagged sequences were obtained. The sequences were clustered at a level of similarity of 97%, and 0.005% of all sequences were sequenced as the threshold to filter operational taxonomic units (OTUs). According to the Silva database information, the bacterial community was annotated with RDP Classifier software, and the confidence threshold was 0.8. The alpha and beta diversity of different samples were analyzed using Mothur, version v.1.30 software, and the principal components (PCA) were analysed using R software.

Results

The weight of goslings with Semen Cassiae and *Polygonatum cyrtonema* Hua feed additives was substantially higher than the CK group. The weight of goslings treated with H2 and H4 was higher than the CK group at 10, 20, and 30 d. Over time, the growth advantage of the H2 and H4 groups became more obvious (Figure 1). The weight of goslings treated with J4 was substantially higher than the CK at 10, 20, and 30 d, and the weight of goslings treated with J2 and J6 was substantially higher than the CK group at 20 d and 30 d (Figure 2). All the above indicate that Semen Cassiae and *Polygonatum cyrtonema* Hua feed additives can substantially improve the growth of Wanxi White Geese.

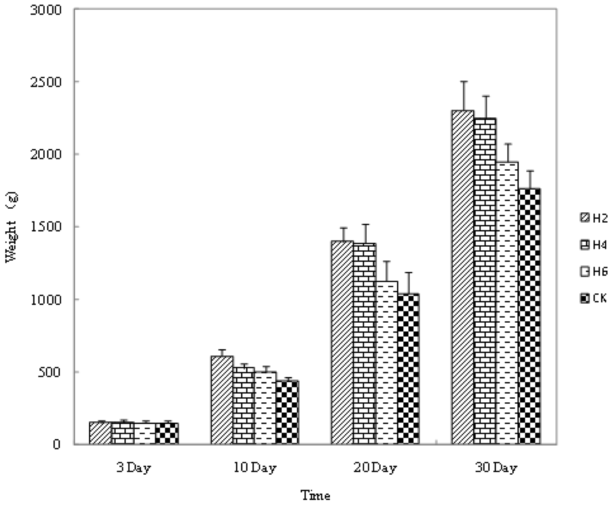

Figure 1. Weight changes of in Wanxi White geese fed *Polygonatum cyrtonema* Hua. feed additive

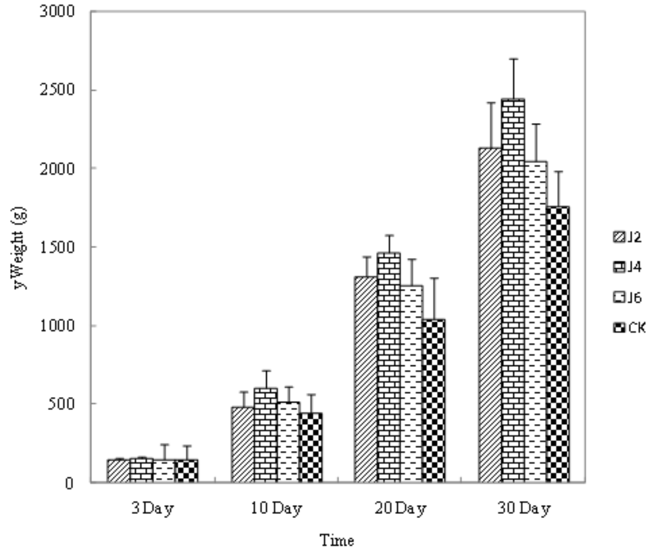

Figure 2. Weight changes of in Wanxi White geese fed Semen Cassiae feed additive

After the quality control of the raw data, a total of 1,308,527 clean sequences and 1156155 filtered sequences were obtained. The content of GC was ~55%, and the Q20 (%) and Q30 (%) of all test samples were above 93% and 80%, respectively (Table 2). This indicates that the sequencing quality was high enough to meet the requirements of subsequent test analysis.

Table 2. Statistics of sequencing data

Sample	Clean Reads (M)	Clean Bases (G)	Clean Q20 (G)	Clean Q30 (G)	Average Length (bp)
CK-1	0.052(49.8%)	0.024(75.5%)	0.023(97.9%)	0.022(92.8%)	456
CK-2	0.052(49.7%)	0.024(75.8%)	0.023(97.5%)	0.022(91.7%)	458.5
CK-3	0.055(49.7%)	0.025(75.0%)	0.024(97.5%)	0.023(92.0%)	454
J2-1	0.073(47.4%)	0.033(70.2%)	0.031(93.9%)	0.026(80.9%)	446
J2-2	0.069(49.8%)	0.031(74.4%)	0.030(97.5%)	0.028(91.6%)	449.9
J2-3	0.044(49.8%)	0.020(74.3%)	0.019(97.5%)	0.018(91.4%)	449.5
J4-1	0.047(49.8%)	0.021(74.8%)	0.020(97.3%)	0.019(91.5%)	452.5
J4-2	0.066(49.8%)	0.030(74.1%)	0.029(97.5%)	0.027(91.9%)	448.1
J4-3	0.077(49.7%)	0.035(75.5%)	0.034(97.4%)	0.032(91.9%)	457.4
J6-1	0.057(49.7%)	0.026(75.0%)	0.025(97.5%)	0.024(91.9%)	454.2
J6-2	0.075(49.8%)	0.034(75.3%)	0.033(97.7%)	0.031(92.5%)	455.6
J6-3	0.077(49.8%)	0.035(74.6%)	0.034(97.8%)	0.032(92.8%)	450.6
H2-1	0.052(49.7%)	0.024(75.1%)	0.023(97.6%)	0.022(91.8%)	455.1
H2-2	0.078(49.8%)	0.035(75.0%)	0.035(97.5%)	0.033(92.1%)	453.6
H2-3	0.077(49.8%)	0.035(74.6%)	0.034(97.2%)	0.032(91.0%)	451.3
H4-1	0.048(49.8%)	0.022(74.5%)	0.021(97.4%)	0.020(91.3%)	450.5
H4-2	0.069(49.8%)	0.031(74.9%)	0.031(97.5%)	0.029(91.7%)	453.1
H4-3	0.057(49.8%)	0.026(74.6%)	0.025(97.8%)	0.024(92.3%)	451.3
H6-1	0.062(49.7%)	0.028(74.9%)	0.028(97.5%)	0.026(91.4%)	453.4
H6-2	0.074(49.7%)	0.033(74.4%)	0.033(97.4%)	0.030(91.3%)	450.4
H6-3	0.048(49.8%)	0.022(74.6%)	0.021(97.6%)	0.020(91.8%)	451.1

J2, J4, J6 were formed by adding 2%, 4%, and 6% Semen Cassiae; H2, H4, H6 were formed by adding 2%, 4%, and 6% *Polygonatum cyrtonema* Hua to the basal diet; CK, control

Analysis of all OTUs showed that the Semen Cassiae and *Polygonatum cyrtonema* Hua feed additive increased the number of intestinal microorganisms in geese (Figure 3). In the *Polygonatum cyrtonema* Hua feed additive, the number of intestinal microorganisms increased with additive concentration, and the number of microorganisms was higher than the CK group. In addition, the number of microorganisms in the Semen Cassiae feed additive treatments was also higher than the CK group. In J6, the number of microorganisms was slightly higher than the J2, J4, and CK groups. This indicates that Chinese herbal feed additives can affect the number of intestinal microbes in geese.

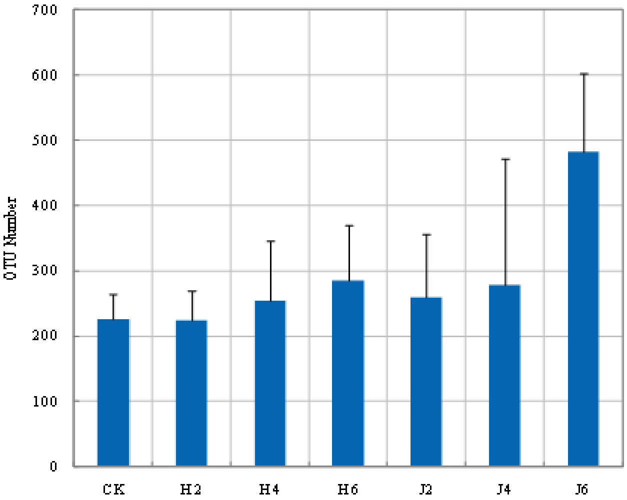

Figure 3. The operational taxonomic units (OTU) number of different treatments
J2, J4, J6 were formed by adding 2%, 4%, and 6% Semen Cassiae; H2, H4, H6 were formed by adding 2%, 4%, and 6% *Polygonatum cyrtonema* Hua to the basal diet; CK, control

Venn diagrams indicated that Chinese herbal feed additives also had an impact on the composition of intestinal microorganisms in geese (Figure 4A). In the *Polygonatum cyrtonema* Hua feed treatments, 106 kinds common OTUs were been counted, with 15, 27, 46, and 59 species in the CK, H2, H4 and H6 groups, respectively. The number of specific microorganisms increased with the concentration of *Polygonatum cyrtonema* Hua. The addition of *Polygonatum cyrtonema* Hua. can change the composition of intestinal microorganisms in geese. Similarly, Semen Cassiae additives can also change the composition of intestinal microorganisms in geese, but the effect was different from the *Polygonatum cyrtonema* Hua. Only 75 kinds common OTUs were counted in all groups, and there were differences in the OTUs specific to different concentrations of Semen Cassiae feed additives (Figure 4B). Of these, OTUs of J2 and J4 were 45 and 17, respectively, whereas the specific OTUs of J6 increased to 342, indicating that the higher concentration of Semen Cassiae had a greater impact on the composition of intestinal microorganisms in geese.

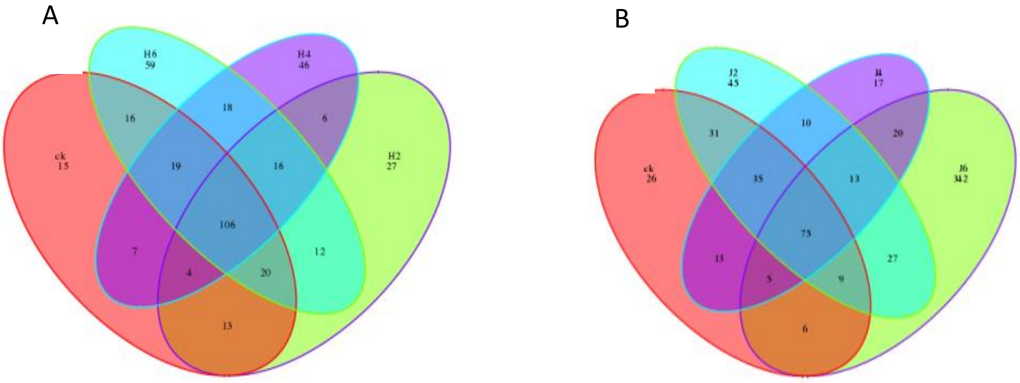

Figure 4. Operational taxonomic unit (OTU) Venn diagrams of different treatments: (A) denotes the OTU Venn diagram of *Polygonatum cyrtonema Hua*, (B) denote the OTU Venn diagram of Semen Cassiae. Ck, control

The OTU sequences were compared to the microbial reference database to obtain the corresponding species classification information. The community composition of each sample was calculated, and the species abundance tables at different classification levels were generated by using QIIME software. The advantages of different treatments were similar; Clostridiales, Erysipelotrichales, Bacteroidales, Bacillales, Enterobacteriales, Pseudomonadales, Rhizobiales total relative abundance was above 97% (Figure 5).

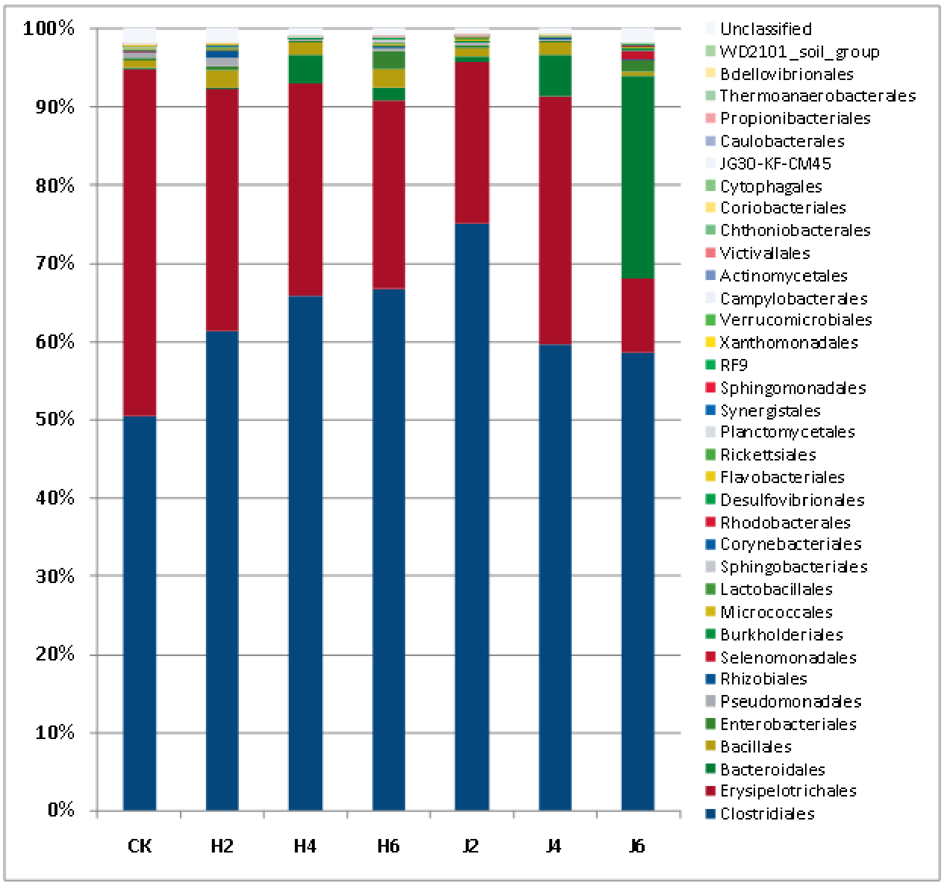

Figure 5. Relative abundance of intestinal microorganisms in Wanxi White goslings
J2, J4, J6 were formed by adding 2%, 4%, and 6% Semen Cassiae; H2, H4, H6 were formed by adding 2%, 4%, and 6% *Polygonatum cyrtonema* Hua to the basal diet; CK, control

Further analysis showed that Chinese herbal additives had a greater impact on the abundance of intestinal microorganisms. After treatment with Chinese herbal feed additives, the relative abundance of Clostridiales was higher than that of the CK group, but different types of Chinese herbal feed additives changed in different ways. In the *Polygonatum cyrtonema* Hua. Treatments, the relative abundance of Clostridiales increased with an increase in the concentration of the additives, showing a positive correlation. Semen Cassiae addition produced the opposite effect. The relative abundance of Clostridiales reached its maximum at low doses and decreased with an increase of Semen Cassiae, indicating that low doses of Semen Cassiae additives can promote the growth of Clostridiales more effectively. It also can be concluded that Chinese herbal feed additives can inhibit the relative abundance of Erysipelotrichales. The relative abundance of Erysipelotrichales in all treatment samples was lower than CK group. The relative abundance of Erysipelotrichales decreased with an increasing dosage of *Polygonatum cyrtonema* Hua, indicating that *Polygonatum cyrtonema* Hua could effectively inhibit the growth of Erysipelotrichales.

According to the annotation information, each classification unit was mapped to the NCBI Taxonomy by MEGAN. According to the analysis, all microorganisms (taxa with an average relative abundance >1%) could be divided into four categories (Figure 6). The first category included

Bacteroidales, mainly composed of Bacteroides and Alistipes; Chinese herbal additives had a relatively large impact on the abundance of Bacteroides.The second component was Cyanobacteria. The third component was Firmicutes, which was the main microbial community. Components could be further divided into four subcategories: Bacillales, Clostridiales, Phascolarctobacterium, and Turicibacter. The fourth part was mainly composed of Gammaproteobacteria, including Pseudomonas and Salmonella. The results show that the relative abundance of microorganisms in different treatments varied, which indicated that Chinese herbal feed additives had an influence on the composition of intestinal microorganisms in geese.

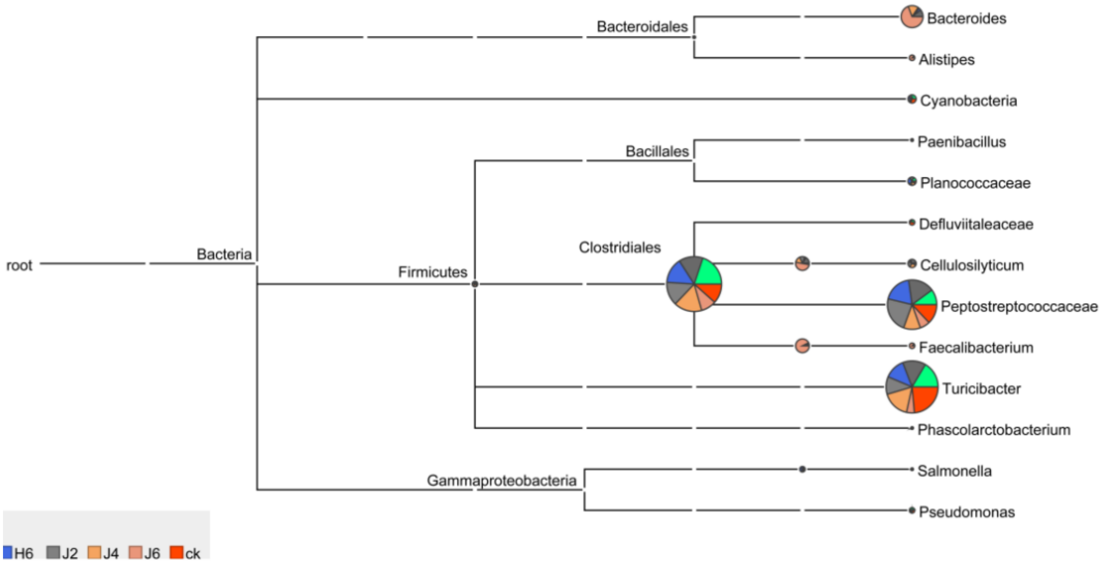

Figure 6 Megan tree diagram: The pie chart at the node represents the proportional contribution of the dietary treatments fed to Wanxi White goslings. J2, J4, J6 were formed by adding 2%, 4%, and 6% Semen Cassiae; H2, H4, H6 were formed by adding 2%, 4%, and 6% *Polygonatum cyrtonema* Hua to the basal diet; CK, control

Rarefaction curves and the Shannon Index showed that the sequenced data were large enough to reflect the vast majority of microbial information in the samples. Alpha diversity can reflect species diversity within the sample. Mothur software was used to evaluate the alpha diversity index of the samples and the number of sequences contained in the sample was standardized. Analysis of all treatments showed that Chinese medicine additives had an influence on the diversity of intestinal microbes. Chinese herbal feed additives substantially increased the species diversity of intestinal microorganisms in geese. Both Chao1 and ACE increased with an increase in dosage of Chinese herbal feed additives and were higher than those of the CK group (except that H2 was slightly lower than the CK group).

Similarly, the Shannon index of the treatment group was higher than the CK group, which also showed an upward trend with Chinese medicine additives. The Simpson index can also reflect the diversity of the microbial communities. The smaller the Simpson index is, the higher the species diversity is. The Simpson indices of *Polygonatum cyrtonema* Hua and Semen Cassiae were lower than the CK group, indicating that the feed additive promoted the diversity of intestinal microbes.

Table 3. Alpha diversity indices of different Chinese herbal additives fed to Wanxi White goslings

GROUPS	*Chao1*	*ACE*	*Shannon*	*Simpson*
CK	313.2553	336.4943	1.9356	0.2621
H2	302.2656	331.5062	2.1525	0.2122
H4	343.0979	352.0529	2.3415	0.1766
H6	373.6764	383.1254	2.3072	0.1876
J2	348.4834	362.0165	2.1779	0.2205
J4	372.8557	402.7670	2.4556	0.1700
J6	526.6805	535.2283	3.7155	0.0709

J2, J4, J6 were formed by adding 2%, 4%, and 6% Semen Cassiae; H2, H4, H6 were formed by adding 2%, 4%, and 6% *Polygonatum cyrtonema* Hua to the basal diet; CK, control

Beta diversity was analyzed using QIIME software to compare the differences in species diversity among different samples. The principal component analysis of all samples showed that the cumulative contribution rate of variance of the first two principal components was 88.24%, which could explain the difference of bacterial community structure in most samples (Figure 7). There were marked differences in the composition of intestinal bacterial communities between the different treatments. All samples could be divided into three parts: the first part mainly included CK, H2, H4, and J4, indicating that low doses of Chinese herbal additives had little effect on the composition of the intestinal microbial community; the second part mainly included J2, H6, indicating that different doses of additives had different effects on the composition of the intestinal microbial community. The third part mainly included the J6 treatment, indicating that high dosage of Semen Cassiae additive has a substantial impact on the composition of the intestinal microbial communities.

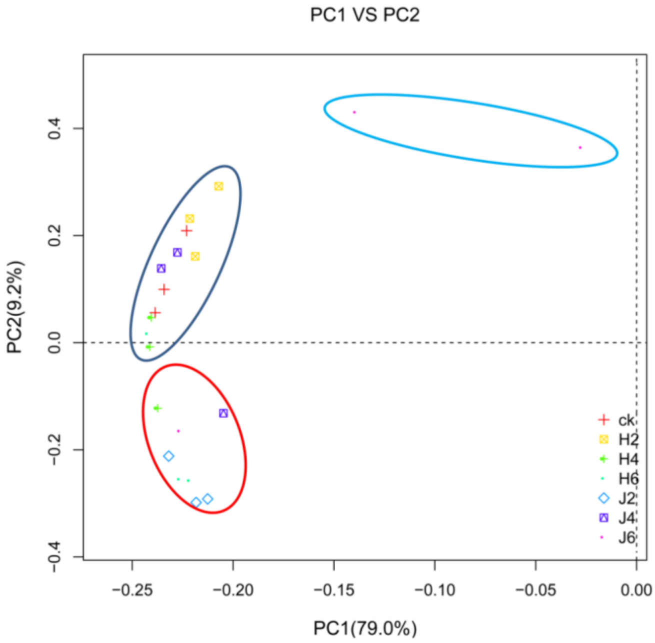

Figure 7. Principal component analysus results of Chinese herbal additives fed to to Wanxi White goslings. J2, J4, J6 were formed by adding 2%, 4%, and 6% Semen Cassiae; H2, H4, H6 were formed by adding 2%, 4%, and 6% *Polygonatum cyrtonema* Hua to the basal diet; CK, control

Discussion and Conclusions

Chinese medicinal herbs originate mainly in nature and are natural products, so they are called green feed additives (Sun *et al*., 2005; Liu *et al*., 2011). They have become important substitutes for antibiotics, precipitating research and development into high-quality livestock and poultry products. Chinese medicinal herbs have a low toxicity and low drug resistance, and it is not easy to retain residues in livestock and poultry products, as compared with antibiotics and other synthetic drugs. Therefore, Chinese medicinal herbs as a green feed additive are conducive to proportional application in livestock and poultry products and play a certain role in animal health care.

The intestinal tract of animals hosts a large number of microorganisms, which constitute the intestinal microecological system in the intestinal environment (Zu *et al*., 2016). Intestinal microorganisms participate in a series of life activities, such as regulating the host's nutrient digestion and absorption, immune response and biological antagonism (Zu *et al*., 2016; Pickard *et al*., 2017; Hao *et al*., 2020). The intestinal microorganisms are closely related to the occurrence and development of a variety of diseases (Yamashiro *et al*., 2017; Sittipo *et al*., 2018; Tang *et al*., 2019). Therefore, maintaining the balance of the intestinal microecosystem is particularly important for the physical health of the animals.

A large number of studies have shown that Chinese medicine additives play an important role in regulating the composition of intestinal microflora in animals (Liu *et al*., 2011; Zu *et al*., 2016). Chang *et al*. (2015) found that *Ganoderma lucidum* extract could effectively improve the health status of mice on high-fat diets and their intestinal microbial community was greatly improved. Guo *et al*. (2015) found that the extracts of red ginseng and Semen Coicis could promote the growth of probiotics, such as *Lactobacillus* and *Bifidobacterium*, and inhibit the growth of pathogenic bacteria such as *Escherichia coli*, *Staphylococcus,* and *Salmonella*. It was also found that the extracts of red ginseng and coix seed could improve intestinal flora imbalance and enteritis symptoms, and the effect of red ginseng extract was better. Yan *et al*. (2017) found that oral administration of total saponins and polysaccharides from *Polygonatum kingianum* improved the intestinal microecology by decreasing the abundance of Bacteroidetes and Proteobacteria and increasing that of Firmicutes.

The current study found that Semen Cassiae and *Polygonatum cyrtonema* Hua feed additives also affected the growth of Wanxi white geese and changed the composition of the intestinal microbial community. Semen Cassiae and *Polygonatum cyrtonema* Hua could increase the number of outs in intestinal microorganisms of geese, and the number of OTUS increased with an increase in dosage. Chinese medicinal herb additives had a strong influence on the abundance of intestinal microbes. The relative abundance of Clostridiales was higher than the CK group after treatment with Semen Cassiae or *Polygonatum cyrtonema* Hua feed additives, but the response to different Chinese medicinal herb additives was different. Chinese medicinal herb feed additives could inhibit Erysipelotrichales, and the relative abundance of Erysipelotrichales in all treated samples was lower than the CK group. Alpha diversity analysis showed that Chinese medicinal herb feed additives could promote the diversity of intestinal microorganisms.

However, the specific regulatory mechanism is not yet clear and needs further study in subsequent experiments. In the follow-up study, it is necessary to make a systematic and in-depth study on the metabolism of Chinese medicinal herbs by using the technologies of Chinese medicinal herb, genomics, intestinal macrogenomics, metabonomics and bioinformatics, which is expected to open up a new prospect for the modernization of feed additives.

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Authors' Contributions

Conceptualization, R.H.Z; methodology, R.H.Z. and G.P.X.; validation, C.B.S.; formal analysis, P.J.; investigation, G.P.X.; resources, R.H.Z. and P.J.; data curation, R.H.Z. and G.P.X.; writing−original draft preparation, R.H.Z.; writing−review and editing, R.H.Z.; supervision, C.B.S.; funding acquisition, P.J., All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Declaration

The authors declare that they have no conflicts of interest relative to the content of this paper.

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