COIX SEED, CHINESE YAM, AND SEMEN EURYALES COMPLEXITY MODULATING AND STABILITY ENHANCING EFFECT ON GUT BACTERIAL COOCCURRENCE PATTERNS IN MICE

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Abstract. Chinese medicine plays a crucial role in maintaining the equilibrium of the intestinal microbiome, and this microbiome, in turn, plays a pivotal role in mediating the pharmacological effects of Chinese medicine on the host. Despite the significance of this interplay, there is a paucity of studies investigating the impact of orally administered Traditional Chinese Medicine (TCM) on the co-occurrence patterns of intestinal gut bacteria. In the present study, we incorporated Coix seed, Chinese yam, Semen euryales, and their combination into the diet of mice. Subsequently, we assessed the effects of these TCM on the diversity, community composition, and co-occurrence networks of gut bacteria by oral administration. Our findings underscored the substantial influence of the three TCM varieties and their mixture on the diversity and community composition of gut bacteria. Notably, all TCM utilized in our study exhibited the ability to enrich beneficial gut bacteria and inhibit pathogenic bacteria within the intestinal microbiome. Furthermore, oral administration of Coix seed, Chinese yam, Semen euryales, or their combinations resulted in increased thymus and spleen indices. Examination of network topological features revealed marked dissimilarities between the control group and the TCM oral administration groups. The co-occurrence pattern of bacteria in the TCM oral administration groups exhibited less complexity but greater stability compared to that in the control groups. These observations suggest that the bacterial community in TCM oral administration groups is more resilient to disturbances than in the control groups.

Keywords: *traditional Chinese medicine, gut microbial community, network analysis, network complexity, natural connectivity*

Introduction

Traditional Chinese medicine (TCM), as one of the most significant components of complementary and alternative medicines, have been used in and around China for thousands of years (Feng et al., 2019). Recently, in western countries such as the US, the UK and Germany, TCM is also increasingly being used to treat diseases (Scheid et al., 2015; Liu and Salmon, 2010; Rashrash et al., 2017). Traditional Chinese Medicine is the outcome of the combined action of multiple bioactive compounds, usually showing relatively fewer adverse effects within the appropriate dosage range for taking and demonstrating excellent therapeutic effects, and involving a large variety of medical practices (Li et al., 2021). It is predictable that TCM will play an increasingly important role in fighting diseases in the future (Feng et al., 2019).

In recent years, gut microbiota has been emerging as a new frontier in understanding the advancement and development of human health. A huge number of microbes are living in the gastrointestinal tract of healthy people (Rashrash et al.,

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2017). The composition and function of the gut microbiota are intimately associated with human health and play a crucial role in sustaining physiological homeostasis (Zhang et al., 2019). Alterations in the gut microbiota may disrupt the metabolism of nutrients and lead to diseases, such as antibiotic-associated diarrhea (Schwabe and Jobin, 2013). Previous studies have proved that TCM is conductive to maintain the homeostasis of the intestinal microbiome (Chang et al., 2015; Zhou et al., 2016). When Oral TCM enter the intestinal tract, they must come into contact with microorganisms in the body (Shi et al., 2014; Gong et al., 2020b). The composition of the intestinal microbiota must inevitably be regulated by the components of TCM (Akyildiz et al., 2010). Chinese yam has been shown to increase the probiotics *Bifidobacterium* and *Lactobacillus* and decrease the potential pathogens *Enterococcus* and *Clostridium perfringens* (Zhang et al., 2019). Coix seed and Lotus seed also have been proved that they could decrease pH in the succus gastricus, and then improve the structure of gut microflora (Li et al., 2021). In line with this trend, an increasing number of studies are demonstrating that the plant molecules which are employed in Traditional Chinese Medicine interact with intestinal bacteria subsequent to oral administration (Feng et al., 2019). As reviewed by Feng et al. (2019), TCM can regulate the composition and metabolism of the intestinal microbiota, which can transform the TCM compounds. However, microbes do not exist as isolated individuals, but co-occur with multipe species through the exchange of information, energy and matter, and forming complex interaction networks for its community assembly and function (Shi et al., 2016; Hallam and McCutcheon, 2015; Ma et al., 2016). The co-occurrence patterns among microbiota have been well demonstrated in various habitat types, such as the human gut (Sung et al., 2017), grassland (Wang et al., 2018) and agricultural ecosystem (Fan et al., 2018). However, little information is available on the topological changes in the co-occurrence interactions of TCM on intestinal bacteria.

To investigate the potential variations of gut microbiota interactions in response to oral TCM, we performed high-throughput sequencing (16S rRNA) and network-based analysis at 25 samples under Oral administration of three kinds of traditional Chinese medicine, including of Coix seed, Chinese yam, Semen euryales. Coix seeds and lotus seeds are the mature kernels and seeds of Coix lacryma-jobi L. var. ma-yuen Stapf (Roman.) (Zhu, 2017) and Nelumbo nucifera Gaertn (Zhang et al., 2015), respectively, which are rich in a variety of nutrients and medicinal active ingredients and have numerous physiological and pharmacological effects, involving strengthening the spleen, inhibiting dampness, oxidative stress and inflammation, and regulating endocrine and immune functions (Wu et al., 2013; Zhang et al., 2014; Li et al., 2021). The tubers of Chinese yam are rich in flavonoids, proteins and polysaccharides (Shujun et al., 2006), which have various pharmacological effects, such as immune regulation, antioxidant, anti-inflammatory and improvement of gastrointestinal function. Yam tubers (Dioscorea opposita Thunb) contain flavonoids, proteins and polysaccharides (Shujun et al., 2006) with various pharmacological effects, such as immunoregulatory, antioxidant, anti-inflammatory and improvement of gastrointestinal function (Luo et al., 2016; Yang et al., 2015). We aimed to address the following questions: (i) Are the intestinal microbial composition, diversity and community structure different under oral administration different kinds of traditional Chinese medicine? (ii) Does oral administration of TCM affect the complex and stability of intestinal microorganisms?

Materials and methods

Animals and experimental design

Thirty-five weaned normal male mice, with weights of 34.92 ± 2.95 g, were bought from Changchun Yi Si Experimental Animal Co., LTD. After the experimental mice were purchased, they were first fed with ordinary feed for 1 week in the same cage to ensure that the mice adapted to the feeding environment and reached similar basal levels of intestinal flora. Then, the mice were randomly divided into 5 groups, one group was used as the normal control group; The mice in the normal diet group were given normal mouse chow (maintenance chow were purchased from Daqing Traditional Chinese Medicine Hospital), while the mice in the TCM diet group (Coix seed, Chinese yam, Semen euryales and their mixture) were given a custom-made mixed chow, which was prepared by mixing TCM with the above normal mouse chow flour at 1:3 and then pressed into shaped with the above diet for 3 weeks, and fresh stool samples were collected from each of the mice. The animal experiment program shall be formulated and implemented in accordance with the relevant regulations of the Animal Experiment Ethics Committee of Beihua University.

Calculation of immune organs indices

At the end of the experiments, we weighed the mice and sacrificed them using a cervical dislocation method. Thymus and spleen of the experimental mice were collected, washed and weighed. Then the thymus index and the spleen index were calculated according to the following formulas:

$$\text{Thymus or spleen index} = \text{thymus or spleen weight (mg)}/\text{body weight (g)}$$

which as described by Xu et al., 2015). Feces were aseptically collected in sterile centrifuge tubes and stored at −80°C.

DNA extraction and polymerase chain reaction

DNA was extracted from collected feces using TIANamp Stool DNA Kit (TIANGEN Biotech, Beijing, China). The V3-V4 region of bacterial 16S were subject to PCR using a primer pair (338F, 5′-ACT CCT ACG GGA GGC AGC A-3′; and 806R, 5′-GGA CTA CHV GGG TWT CTA AT-3′). After quantification, amplicons from different group mice feces were pooled in equimolar concentrations. Sequencing was performed using Illumina Miseq sequencing according to the standard protocol of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Details on DNA extraction, quality control, and raw data processing refer to Wang et al. (2015).

Network analysis

In this study, co-occurrence network analysis was used to explore the interactions between soil bacterial species at different precipitation gradient. Bacterial communities were analyzed for co-occurrence network at the OTU level. The Spearman correlation method was chosen for the assessment of correlations of bacterial species. The values of Spearman’s correlation coefficient $r > |0.8|$ with significance $P < 0.01$ were screened for the construction of the co-occurrence network. The correlation matrix analysis was performed using the ‘Hmisc’ package of R software. The sub-network properties were analysis with the ‘igraph’ R package (Wang et al., 2023). Network visualizations were performed with Gephi version 0.9.2. Natural connectivity was used to provide a sensitive discernment of the robustness of the network structure. The stability of the network was assessed by removing nodes from the static network to evaluate the rate of robustness degradation, and we assessed network robustness by natural connectivity (Peng and Wu, 2016).

Results

Bacterial community composition

Our experiments resulted in a total of 1,486,041 (ranging from 34,322 to 51,309 reads per sample) high-quality sequences of bacterial communities with an average length of 424 bp. Based on 97% similarity, a total of 392 OTUs were detected, belonging to 50 families, 95 genera, and 154 species; The four most predominant genus of bacterial community were Lactobacillus (58.2%), Muribaculaceae (10.6%), Staphylococcus (6.3%), Saccharimonas (4.62%), and Rikenellaceae RC9 gut group (3.4%). The relative abundance of Lactobacillus significantly increased from control to all the TCM feeding groups (*Fig. 1*), while Staphylococcus, Rikenellaceae RC9 gut group and Aerococcus showed an opposite pattern. Among them, the variation of Lactobacillus is the largest, followed by Staphylococcus and Aerococcus (*Fig. 1*).

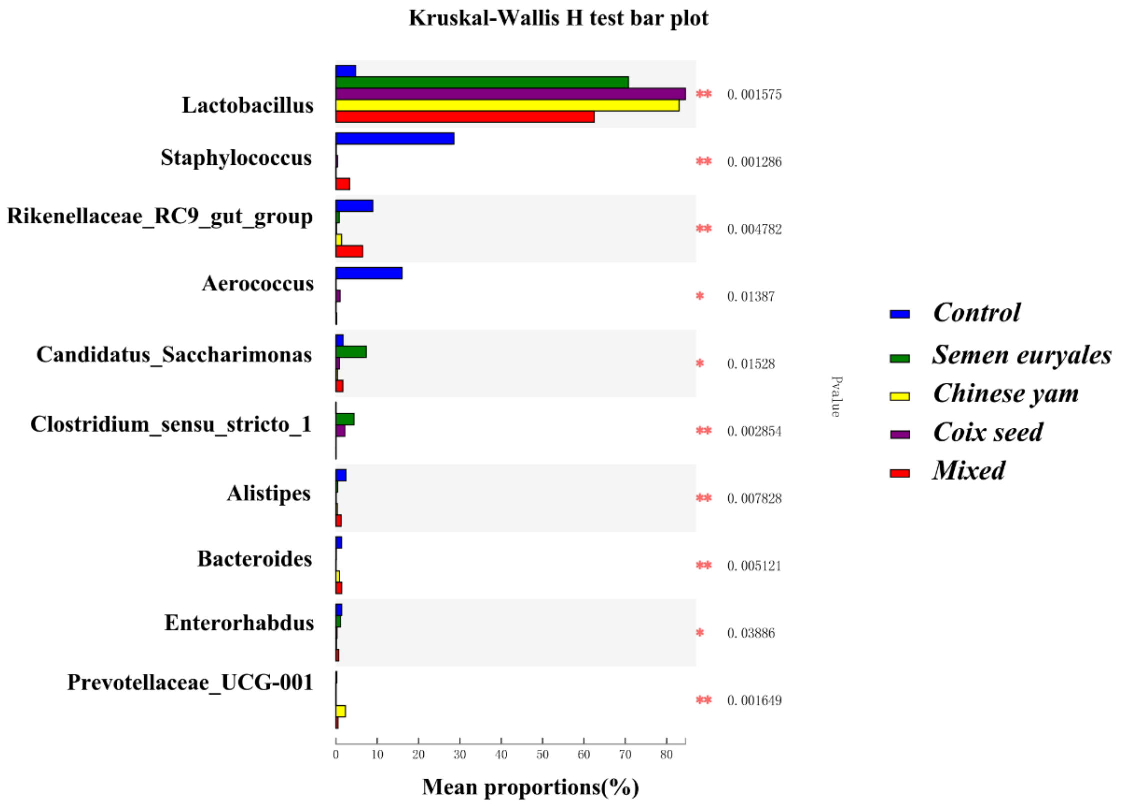

Figure 1. *Wilcoxon rank-sum test revealed the bacterial populations with significant differences in the relative abundance between control to the TCM feeding groups.* $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Gut bacterial diversity and community structure

Alpha diversity, including Shannon index, Sobs index, Ace index and Chao index of bacteria in mice gut under different kinds of traditional Chinese medicine feeding group are significantly different. Bacterial alpha diversity in control group was significantly higher in control group than Chinese yam, Semen euryales and mix group.

Based on the Bray-Curtis distance, non-metric multidimensional scaling (NMDS) was used to visualize the pattern of bacterial community structure (*Fig. 2*). NMDS analysis showed that the gut bacterial community structure of mice was significantly different under different TCM feeding conditions. Consistent with the NMDS ordination, PERMANOVA results showed that there were highly significant differences in bacterial communities between the different treatment groups. ($R^2 = 0.56$, $P = 0.001$).

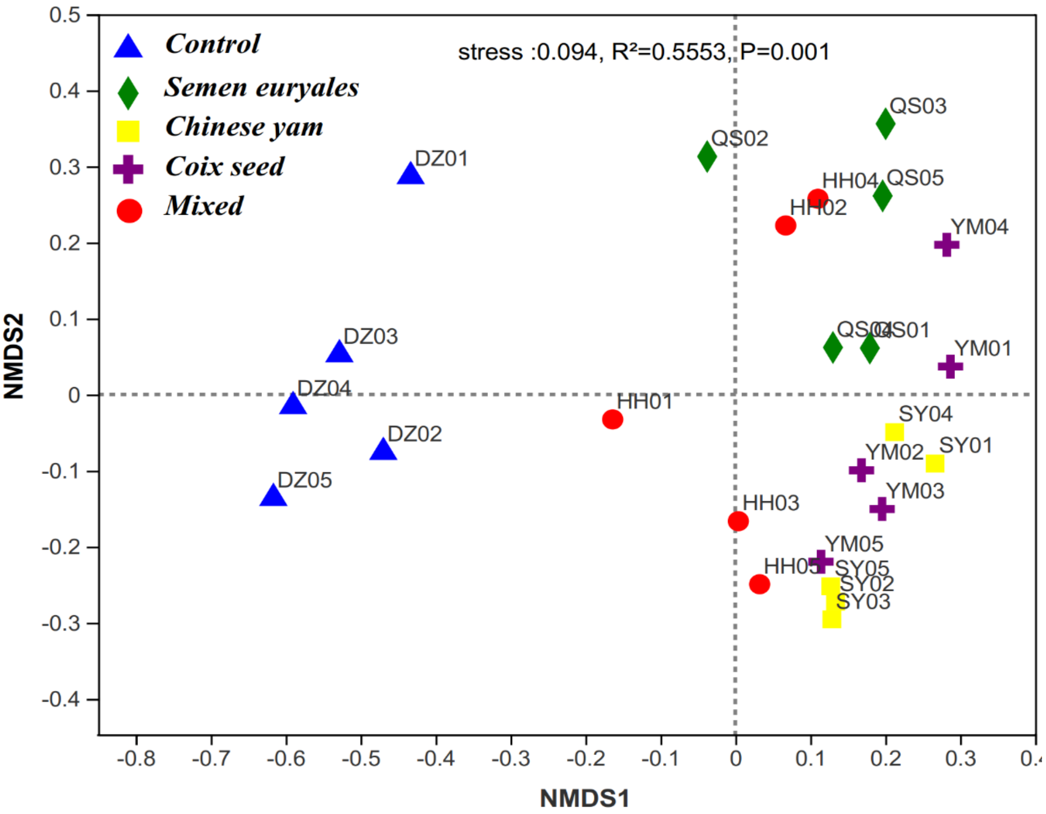

Figure 2. *Non-metric multidimensional scaling (NMDS) ordination plot of gut bacterial communities of control and the TCM feeding groups. PERMANOVA analysis showed significant differences in bacterial communities between the control group and the TCM feeding groups*

Key characteristics of gut bacterial networks

To determine the influence of TCM on the co-occurrence patterns of bacteria in the mice gut, five networks (each feeding treatment) were constructed (*Fig. 3*). Compared to the corresponding random network, the average path length and clustering coefficient of the five networks obtained in this experiment are significantly larger, indicating that the network obtained in this study has the characteristics of a “small world” (*Table 1*). The value of modularity is higher than that of the random network and greater than 0.4, indicating that the constructed networks had modular structures

(*Table 1*). For each sequenced sample, we generate sub-networks from the networks of different TCM treatments by saving the taxa presented in each treatment following the functional subgraph() in the graph package. A number of network-level topological features were calculated for sub-networks. Multiple network topology metrics consistently showed that microbial co-occurrence pattern in control group differed profoundly from the Coix seed, Chinese yam, Semen euryales feeding group (*Fig. 4, Table 1*). Control group formed larger networks with more nodes than the Coix seed, Chinese yam, and Semen euryales feeding group networks; meanwhile the control networks contained less connections (edges) between nodes. These results demonstrate that the interaction pattern of microorganisms in control and mixed group were more compact and aggregated than in Coix seed, Chinese yam, and Semen euryales feeding group. These results indicate that network complexity, as indicated by node number, edge number and average degree, was similar between control and mix, but significantly higher than Coix seed, Chinese yam and Semen euryales feeding group.

Table 1. *Bacterial network topological features at control and the TCM feeding groups*

	CK	**Semen euryales**	**Chinese yam**	**Coix seed**	**Mixed**
Links	1190	1308	1164	947	1140
Positive	82.7%	92.89%	86.34%	77.93%	82.19%
Negative	17.2%	7.11%	13.66%	22.07%	17.81%
Nodes	332	276	284	286	317
Density	0.022	0.034	0.029	0.023	0.023
Average degree	7.16	9.478	8.197	6.622	7.192
Modularity	1.139	0.792	1.001	1.199	1.172
Average path length	8.968	2.522	2.776	4.376	5.818
Clustering coefficient	0.799	0.86	0.826	0.814	0.819

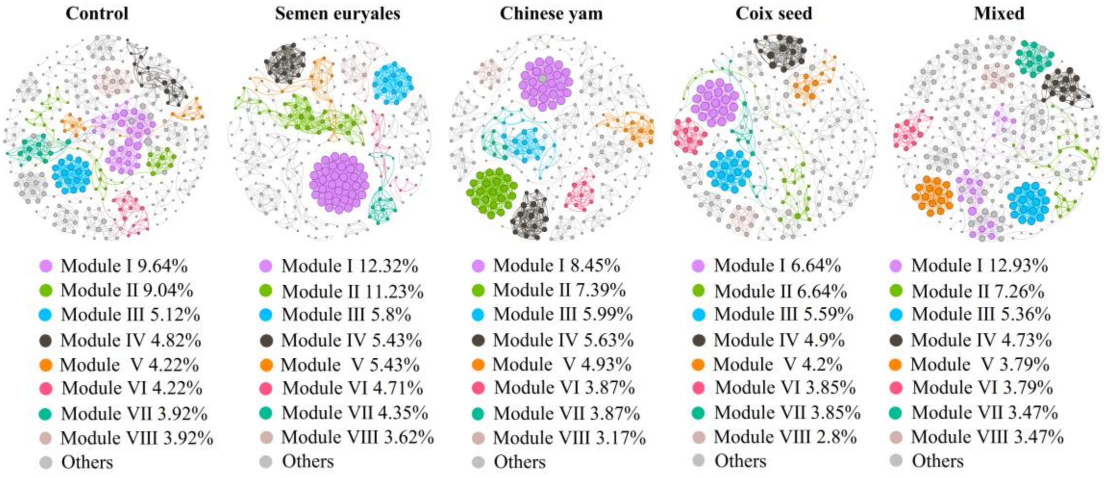

Figure 3. *The co-occurrence patterns among OTUs revealed by network analysis of gut bacterial communities of control and the TCM feeding groups. The nodes were colored according to different types of modularity classes, respectively. A connection stands for a strong (Spearman's r > 0.6 or r < –0.6) and significant (P-value < 0.01) correlation*

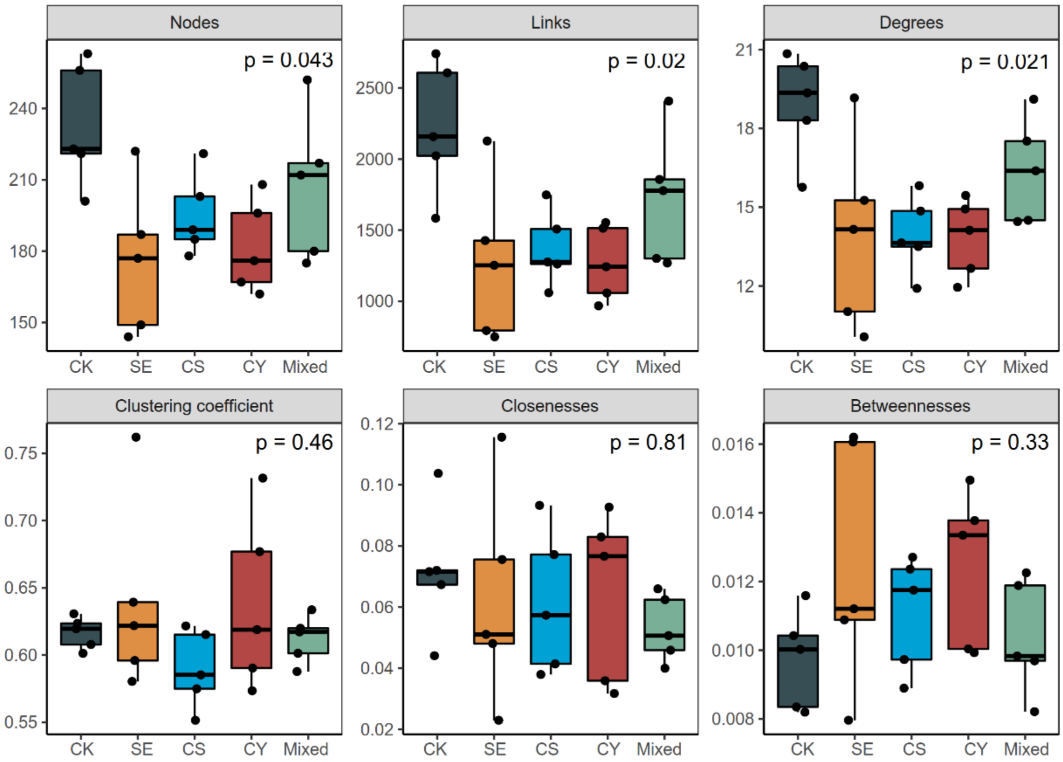

Figure 4. *Comparison of node-level topological feature of gut bacterial community co-occurrence patterns among control and the TCM feeding groups. CK: control, SE: Semen euryales, CS: Coix seed, CY: Chinese yam, Mixed: both of Semen euryales, Coix seed and Chinese yam*

Finally, we conducted a natural connectivity analysis to test the robustness of the bacteria under different feeding goups. The results show (*Fig. 5*) that, Oral administration of Coix seed, Chinese yam, Semen euryales or their mixtures enhanced the robustness of bacterial networks to node attacking when compare with control group.

These results indicating that oral administration of TCM could strengthens the bacterial stability of gut microbiome. Afterwards, natural connectivity was recalculated with nodes removed.

Effects of TMC on thymus and spleen indices and body weight gain

To investigate the immune regulatory effects of Coix seed, Chinese yam, Semen euryales and mixed of them, both of these TCM were orally administered to mice for 30 consecutive days. Compared to the control group, feeding with Chinese yam, Semen euryales and mixed of them did not significantly altered spleen index in mice, but have higher values in them except the Semen euryales groups (*Table 2*). In contrast, the thymus indices were significantly higher in all the TCM feeding groups than that in the control group.

When observing the effect of traditional Chinese medicines feeding on body weight gain, it is noted that the Coix seed group has a significantly higher weight gain when compared to the Control group. Specifically, the weight gain in the Coix seed group is noticeably greater. Furthermore, the Chinese yam group shows a remarkable increase in weight gain that is significantly higher than both the Coix seed group and the Semen euryales group. This indicates that Chinese yam has a more potent impact on weight gain compared to these other two types of traditional Chinese medicines. However, among the other groups, namely the mixed group and potentially other combinations, no significant differences are detected in terms of weight gain. These findings provide valuable insights into the differential effects of these specific traditional Chinese medicines on the body weight gain of mice, highlighting the distinct roles and magnitudes of impact of Coix seed and Chinese yam in particular.

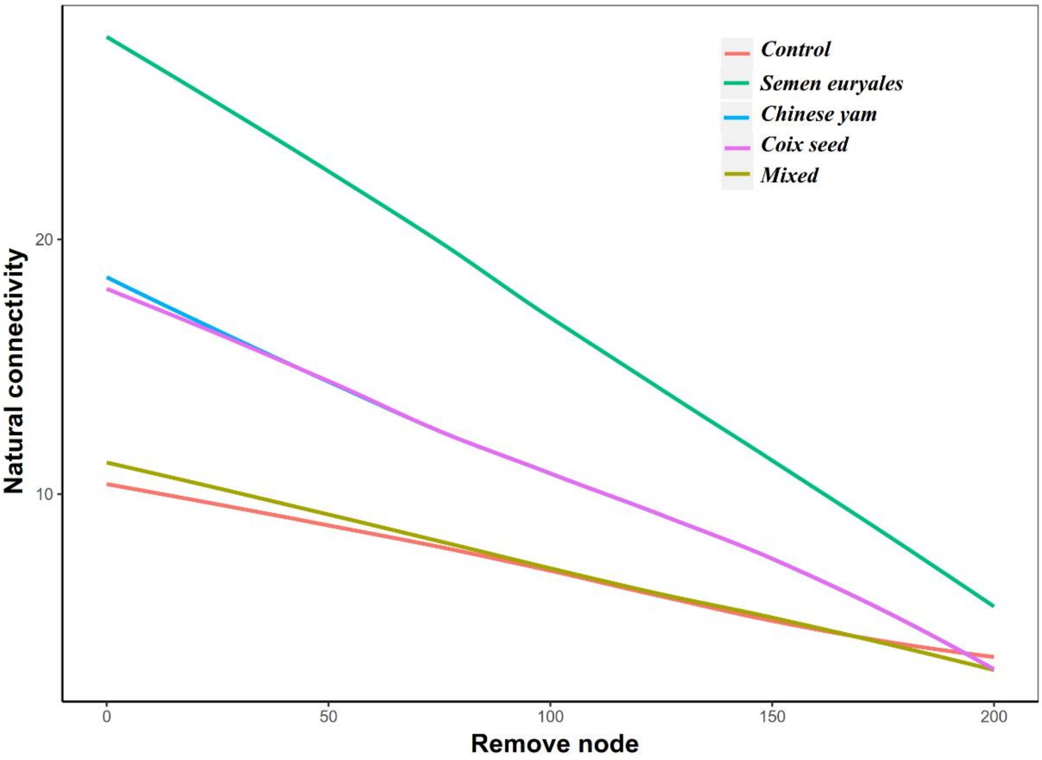

Figure 5. *The natural connectivity of bacterial network of gut bacterial community co-occurrence patterns among control and the TCM feeding groups*

Table 2. *Effect of the traditional Chinese medicines feeding, Semen euryales, Coix seed, Chinese yam and mixed of them, on the indices of immune organs in mice and the terminal body weight and body weight gain of the animals. Data with different lowercase letters indicate significant levels at $P < 0.05$*

	Control	**Coix seed**	**Semen euryales**	**Chinese yam**	**Mixed**
Spleen index	0.023 ± 0.005	0.025 ± 0.008	0.018 ± 0.008	0.028 ± 0.01	0.027 ± 0.007
Thymus index	0.007 ± 0.002a	0.014 ± 0.006bc	0.012 ± 0.007ab	0.011 ± 0.007ac	0.019 ± 0.003b
Terminal weight	38.44 ± 2.48a	37.46 ± 1.81ab	35.04 ± 1.75b	35.98 ± 3.25 ab	35.52 ± 3.16 ab
Weight gain	2.48 ± 0.249ac	1.32 ± 0.683b	1.38 ± 0.944bc	4.46 ± 3.798a	1.86 ± 0.723ab

Discussion

With the current rapidly growing research in TCM and gut microbiome, the researches involving these two areas are significantly accelerated throughout the world. TCM and the intestinal microbiome are intimately associated with human health (Zhang et al., 2020a). Medicine and food homology (MFH) has been practiced for thousands of

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years in China (Yang et al., 2020). MFH can be scientifically and practically used as both food and medicine (Gong et al., 2020a). Therefore, it is essential to explore the relationship between MFH and the gut microbiome for healthcare.

Owing to one of the characteristics of TCM being taken orally, drugs inevitably interact with the intestinal flora. Previous studies have demonstrated that TCM helps maintain the internal homeostasis of the intestinal flora (Chang et al., 2015; Zhou et al., 2016). Taking TCM has also been shown to inhibit the growth of pathogenic microorganisms in the gut, especially harmful bacteria (Wu et al., 2014; Chandrakar and Gupta, 2017). In the present study, we found that oral administration of Coix seed, Chinese yam, Semen euryales or their mixtures can significantly affect the composition of the intestinal microbiome (F*ig. 1*). Feeding TCM groups greatly increased the relative abundance of *Lactobacillus*, while *Staphylococcus Rikenellaceae RC9 gut group* and *Aerococcus* showed an opposite pattern. Lactobacilli are considered to be an essential component of the gut microbiota and have a number of benefits (Turroni et al., 2014). They are referred to as lactic acid producing bacteria and have been shown to be protective at the intestinal barrier by competing with pathogens (Yang et al., 2019). They also induce immune regulation and metabolize carbohydrates into lactic acid, lowering the pH of the gut environment and restraining the development of acid-sensitive pathogenic bacteria (Sengupta et al., 2013; Belenguer et al., 2007). *Staphylococcus* and *Aerococcus* are common intestinal pathogens, which are recognized to be positively correlated with intestinal inflammation (Rasmussen, 2016; Zhang et al., 2020b). Our results indicate that oral administration of Coix seed, Chinese yam, Semen euryales or their mixtures enriched beneficial gut bacteria and inhibited bacterial pathogens in intestinal microbiome. These protective effects may be more or less related to the tissue repairing effect of allantoin and the gut bacteria regulating effect of polysaccharides in Chinese yam (Zhang et al., 2019). While lotus seeds and Coix seeds are rich in probiotic elements such as amino acids, polysaccharides and starch (Xi et al., 2019; Xu et al., 2018). These substances significantly promote the proliferation of Lactobacillus in the colon, jejunum and stomach (Li et al., 2021).

The thymus and the spleen are two important immune organs for the proliferation and growth of immune cells in the body. The thymus is a fundamental organ for immune system development and regulation, and its role is widely recognized. Studies have shown that alterations in the thymus can have significant impacts on immune function (Gulla et al., 2023). Regarding the spleen, it is a key immune organ that participates actively in immune responses and is known to be affected by various factors (Perrotta and Carnevale, 2021). Therefore, the immune organ index is considered to be the main indicator of immune functions (Sun et al., 2016). Previous studies have revealed that various TCM could significantly increase thymus and spleen indices of mice (RenJie, 2008; Bai et al., 2020). In the present study, although not all changes were statistically significant, increases in thymus and spleen indices thymus and spleen indices occurred for all oral administration of Coix seed, Chinese yam, Semen euryales or their mixtures, except spleen indices in Semen euryales feeding group. Previous studies have shown that Chinese yam is rich in proteins, flavonoids and polysaccharides (Shujun et al., 2006), which have various pharmacological functions, such as immunomodulation, anti-inflammation, antioxidation and improvement of gastrointestinal function (Luo et al., 2016; Yang et al., 2015). Coix seeds and Lotus seeds have also been proved that they are rich in starch, proteins, phytosterols, polysaccharides, polyphenols, and other biologically active ingredients that regulate endocrine and immune functions.

Microorganisms have been proved that they were coexist in complex arrays, and in which relations among species are vital for its community assembly and ecosystem function (Shi et al., 2016; Hallam and McCutcheon, 2015). Our results suggest the feeding of TCM, including of Coix seed, Chinese yam, Semen euryales or their mixtures, significance impacts on the complexity of gut microbial networks. The gut microbiome in control group were more compact and aggregated with more nodes, links and degrees than in Coix seed, Chinese yam, Semen euryales and mix feeding groups. Moreover, average path length of networks was larger in the control groups than in the Coix seed, Chinese yam, Semen euryales and mix feeding groups. Networks with small path length are regarded as small-world networks, associated with rapid ecosystem responses to perturbations (Zhang et al., 2018). Therefore, the gut microbial community in TCM feeding groups may be more sensitive to disturbance compared with the microbial community in control groups.

Oral administration of TCM could influence not only bacterial community structure but also the stability of bacterial co-occurrence relationships. In the present study, robustness tests based on natural connectivity also showed that feeding with Coix seed, Chinese yam, Semen euryales or their mixtures enhanced the robustness of bacterial networks to node or edge attacking. The results indicate that the network structure of the gut microbiome in the TCM feeding groups are more stable than in the control groups (*Fig. 5*), which to some extent reflects the less dynamic structure (Costa et al., 2006) and greater ecological stability (Thébault and Fontaine, 2010) of the TCM feeding groups. Increased stability of microbial communities under oral administration was likely due to their higher adaptability to intestinal environmental conditions by increase beneficial gut bacteria. Under oral administration TCM conditions, the dominant species of gut bacterial communities would be more abundant and could outcompete less dominant species, which could enhance the robustness of the co-occurrence relationships among microbes. These suggests that bacterial community in oral administration TCM groups are more resistant to disturbances than in control groups.

In conclusion, our results demonstrated that three kinds of TCM and their mixture significantly influence the diversity and community composition of gut bacteria. All the TCM used in our study have the ability to enriched beneficial gut bacteria and inhibited bacterial pathogens in intestinal microbiome. Oral administration of Coix seed, Chinese yam, Semen euryales or their mixtures increased the thymus and spleen indices. The network topological features of bacterial communities showed dramatic dissimilarity between control group and TMC oral administration groups. The bacterial co-occurrence pattern in TMC oral administration groups was less complex but more stable than that in control groups. These suggests that bacterial community in oral administration TCM groups are more resistant to disturbances than in control groups. In the future study, we would like to investigate the relationships between the co-occurrence pattern with human health.

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Conflict of interest. There are no conflicts of interest to declare.

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