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Effects of Chinese Herbal Formulas on Gut Microflora to Juvenile Chinese Softshell Turtles *Pelodiscus sinensis* Wiegmann

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ABSTRACT

As a natural "green" feed additive, Chinese herbal medicines (CHMs) are extensively used in aquaculture. Nevertheless, little information is obtainable regarding the performance of CHMs on the composition of gastrointestinal microbiota in healthy juvenile *Pelodiscus sinensis*. Here, we investigated the intestinal microbial community composition of *P. sinensis* fed with artificial feed added with CHM feeding additive. Three hundred juvenile *P. sinensis* (3.92 ± 0.08 g) were randomly divided into 5 groups in quadruplicate. 0.0%, 1.0%, 2.0%, 3.0%, and 4.0% of CHMs were added to the control group (CG), test group 1 (TG1), test group 2 (TG2), test group 3 (TG3), and test group 4 (TG4), respectively. After the aquaculture experiment was completed, the intestinal microbial community of *P. sinensis* was examined by 16S rDNA sequencing method. The results showed that the relative abundance of Proteobacteria and *Firmicutes* were the two most dominant phyla, and there were significant differences in TG1, TG2, TG3, and TG4 compared to the CG. The abundance of phylum Proteobacteria and genus *Citrobacter* increased with the increase of the dose of CHMs ($p < 0.05$), while a clear decrease in the relative abundance of phylum *Firmicutes* and genus *Romboutsia*, genus *unclassified_f_Enterobacteriaceae*, and genus *Clostridium_sensu_stricto_1* was observed in the CHMs groups ($p < 0.05$), which could be considered as the indicators of the medication due to CHMs as a feed additive modulating the gut microbiota. The results of this study indicated that dietary supplementation with 2.0% of CHM feeding additive could significantly improve the intestinal microbiota composition of juvenile *P. sinensis*.

1 | Introduction

The Chinese softshell turtle (*Pelodiscus sinensis* Wiegmann) is an ancient, subaquatic reptile and is a highly valued special aquaculture animal in China [1, 2]. Owing to the rapid development of intensive turtle farming, the *P. sinensis* has undergone viral diseases and parasitic and bacterial attacks [3]. To control disease outbreaks, Chinese herbal medicine (CHM) added into the basal feed could improve immunity, increase the rate of gain, and decrease the diet coefficient in fish under culture. CHM added to the diet is environmentally friendly and cheaper with low side effects to the fish and consumers, which has attracted more and more attention all around the globe [4, 5]. As a natural "green" feed additive, CHM is extensively used in aquaculture [6–10]. *Litopenaeus vannamei* were fed a diet supplemented with 0, 50, 100, 150, and 300 mg/kg of mulberry leaf flavonoids, respectively. The trial lasted for 50 days. The results showed that dietary mulberry leaf flavonoids could increase the intestinal flora diversity of shrimp. The Shannon index was significantly increased and the Simpson index was significantly decreased in the 50 mg/kg group as compared with the 0 mg/kg group ($p < 0.05$). Compared with the no added group, adding mulberry leaf flavonoids into the diet could significantly increase the relative abundances of *Vibrio* (100 and 150 mg/kg) and Shewanella (50, 100, and 150 mg/kg) ($p < 0.05$), whereas significantly decrease the relative abundances of *Lactococcus* (100, 150, and 300 mg/kg) and *Rhodococcus* (50, 100, 150, and 300 mg/kg) ($p < 0.05$) [11]. Yellow catfish (*Pelteobagrus fulvidraco*) were fed a control diet or a diet supplemented with 100 or 400 mg/kg berberine for 60 days. The 400 mg/kg berberine group showed significant improvements in growth performance, intestinal lysozyme activity, compared to the control group ($p < 0.05$). Berberine treatment improved intestinal histological parameters and increased the abundance of probiotic bacteria such as *Bifidobacterium* and *Ruegeria*, while reducing potential pathogenic bacteria such as *Pseudonocardia*, *Vibrio*, *Psychrobacter*, and *Pseudomonas* [12]. Mud crab (*Scylla paramamosain*) were fed a diet supplemented with varying amounts of guava leaves (0, 80, 160, 320, and 640 mg/kg) and fed to mud crabs for 30 days. The results showed that guava leaves, at concentrations ranging from 160 to 320 mg/kg, could promote growth and regulate the intestinal microbiota. *Firmicutes* and *Proteobacteria* were the dominant phyla, with *Proteobacteria* abundance increasing and then decreasing with increasing guava leaf concentration, while *Firmicutes* abundance increased with increasing guava leaves concentration [13].

Gut microflora has grown into a hot issue in life sciences in the context of global systems biology [14]. An interrelationship between CHMs and gut microflora has been revealed by a series of studies [15]. CHMs could exert its holistic effects by way of multiple components that interfered with gut microflora [14]. The intestinal flora of cultivated animals could play an important role in the immunity, nutrition, and disease resistance of aquatic animals [16]. Next-generation sequencing technologies were widely used in microbial ecology, which could furnish information on gut microflora [17, 18]. In fish, the homeostatic equilibrium of gut microflora has played a key role in physiological functions in assimilation, digestion, regulation of intestinal epithelial proliferation, inflammatory response, resistance to infection, and growth [19, 20]. CHMs were used as feed additives, which could enhance the immunity and growth of fish such as *Salmo salar*, *Oncorhynchus masou*, and *Huso dauricus* and influence the composition and quantity of intestinal microflora [6, 18, 21].

It was considered that some CHMs had been proven to regulate intestinal microflora and increase growth and decrease the threat raised by antibiotics to human and animal health [22, 23]. Until now, little information is obtainable regarding the performance of CHMs on the growth performance, serum biochemical parameters, and the composition of gastrointestinal microbiota in healthy juvenile *P. sinensis*. We hypothesized that CHMs' effect on growth performance might modulate gut microbiota and promote growth in healthy juvenile *P. sinensis* due to their bioactive properties. Hence, this study was implemented to investigate the effects of nineteen CHMs feed additives on growth performance and the composition of gastrointestinal microbiota in healthy juvenile *P. sinensis* by 16S rRNA sequencing, which could help to illustrate the molecular mechanism of CHMs and also make a future prospect for the modernization of CHMs.

2 | Materials and Methods

2.1 | Feed Preparation and Feeding Experiment

Nineteen CHMs were bought from Bozhou Qingyi Chinese Medicinal Materials Technology Co., Ltd. (Bozhou, China). Each CHM sample was crushed into a powder screening either 100 mesh for mixture as feeding additive. The experimental design and feeding procedure of juvenile *P. sinensis* were quoted as described in the previous paper [2]. The experimental diets were retained for forty-nine days, and the final body weight of *P. sinensis* was gauged using a digital scale. Prior to sacrifice, all experimental *P. sinensis* were euthanized by decapitation after being anesthetized with 250 mg/L of ethyl 3-amino benzoate methane sulfonate (MS-222) (Sigma, St. Louis, MO, USA) [24]. This method was employed to minimize pain and distress to the animals. The blood of *P. sinensis* was drawn by decapitation. The turtles were euthanized by decapitation. The intestine samples of *P. sinensis* were accumulated and conserved at −80°C until DNA extraction.

2.2 | Statistical Analyses

The experimental data were externalized by mean ± S.D and analyzed by factorial analysis of variance (ANOVA) (single-factor), which evaluated the effect of CHMs additives on the growth performances of *P. sinensis*. The significance of differences between the experimental groups was contrasted by Duncan's multiple range tests by SPSS 22.0, and $p < 0.05$ was considered significant.

2.3 | DNA Extraction and PCR Amplification

Thirty-seven samples were obtained for microbial sequencing. The sample numbers of CG, TG1, TG2, TG3, and TG4 were as follows: 7, 6, 8, 8, and 8. The microbial DNA of thirty-seven samples was extracted by commercial magnetic bead DNA isolation kit (Hangzhou, China). The 16S V3-V4 region was amplified using common primer pair for the Illumina platform as previously described [24]. The PCR protocol was performed as follows: an initial denaturation at 95°C for 4 min, followed by 32 cycles of denaturing at 95°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 50 s, and single extension at 72°C for 8 min. The PCR mixtures contain 5 × *TransStart* FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, *TransStart* FastPfu DNA Polymerase 0.4 μL, BAS 0.2 μL template DNA 10 ng, and finally ddH$_2$O up to 20 μL. The PCR products were purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Purified amplicons were pooled in equimolar proportions and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.4 | Processing of Sequencing Data

The raw data were quality-filtered using QIIME (version 1.17) with the three criteria described by Sun et al. [25]. Operational taxonomic units (OTUs) with a 97% similarity cutoff [26] were gathered using UPARSE pipeline (version 7.1), and chimeric sequences were distinguished and cleared up. The representative sequences based on the 16S rRNA database were resolved by RDP Classifier [27] using a confidence threshold of 0.7. Alpha diversity analysis consisted of PD whole tree, Shannon index, Chao1, and observed species. Jackknifed beta diversity comprised both weighted and unweighted UniFrac distances computed with 10 times of subsampling, and these distances were visualized by PCoA [28]. The microbial taxa differentially represented between groups at the genus level were distinguished using LEfSe [29]. Differences between populations were analyzed by a one-way ANOVA ($p < 0.05$) [30]. The PICRUSt software package and the sequenced reference were performed to analyze the metagenomic function [28, 31, 32].

3 | Results

3.1 | Sequencing Coverage

To compare the intestinal microbes of experimental groups and the CG group, the 16S V3-V4 region was performed to examine the microbial communities of 37 intestine samples by the Illumina MiSeq sequencing platform. In total, 30,294 valid sequence mean numbers and 3314 OTUs were examined from the experimental groups and CG group. The average number of OTUs was 490.62 per group (Table 1). The diversity and richness indexes relative to each gut sample at a genetic distance of 3% are shown in Table S1. The obtained OTUs were allocated 38 phyla, 94 classes, 260 orders, 469 families, 995 genera, and 1829 species with taxonomic names. Most of the OTUs were detected in each sample. In total, 711 species were shared as core microbiota among the experimental groups and CG group (Figure S1).

3.2 | Effects of Dietary CHMs on the Composition of Intestinal Microflora

The barplot figure showed the distribution of bacterial phyla and genus in the five group samples (Figure 1). *Proteobacteria* was the most abundant phylum in TG1, TG2, TG3, and TG4 groups, accounting for 41.48%, 47.42%, 67.47%, and 75.05%, respectively (Figure 1(a)). The abundance of phylum *Proteobacteria* increases with the increase of the dose of CHMs for juvenile turtles. The second most dominant phylum in TG1, TG2, TG3, and TG4 groups was *Firmicutes*, which was responsible for 27.69%, 32.63%, 9.01%, and 11.58%, respectively. *Firmicutes* was the most abundant phylum in the CG group (42.58%), whereas the next most dominant phylum was *Proteobacteria* (37.25%). *Bacteroidetes* was the third predominant phylum in five groups. At the genus level, 995 different bacterial genera were distributed among five groups (Figure 1(b) and Table S1). The abundance of genus *Citrobacter* increases with the increase of CHMs concentration. Meanwhile, the relative abundance of genu *Romboutsia*, genu *unclassified_f_Enterobacteriaceae*, and genu *Clostridium_sensu_stricto_1* showed a downward trend in the five groups. At the phylum level, the relative abundance of *Firmicutes* ($p = 0.04402$) and *Proteobacteria* ($p = 0.04976$) were significant differences among experimental groups and the CG group (Figure 2(a)), in the meantime, at the genus level, the relative abundance of *Citrobacter* ($p = 0.03063$) presented significant differences in the five groups, and the relative abundance of *unclassified_f_Enterobacteriaceae* ($p = 0.00932$) only showed significant differences in the CG, TG2, TG3, and TG4 groups (Figure 2(b)).

3.3 | Community Structures

The structure of the gut microbiota of *P. sinensis* was analyzed by PCoA in the experimental groups and CG group. These results disclosed there were significant differences in gut microbial community structure among the experimental groups and CG group. The first two principal component scores explained 40.25% and 26.90% of the total variations (Figure 3), indicating that CHMs feeding additive contributed to changes in the constituents of the intestinal microflora of *P. sinensis*. Bacterial communities from the turtles in CG were separated from those from the turtles in TG1, TG2, TG2, and TG4 groups, which were also confirmed by the NMDS analysis (Figure 3(b)). As is known to all, stress value < 0.2 could be described by a two-dimensional point graph of NMDS, which has some explanatory significance; stress value < 0.1 could be regarded as a good sort; stress value < 0.05 shows good typicalness [33]. Therefore, the observed stress value was 0.108 on the genus level of the intestinal microflora of *P. sinensis*, which suggested the NMDS ordination plot had some explanatory significance among the experimental groups and CG group.

To study the differences in gut microbiota among the experimental groups and the CG group, taxon abundance was used by LEfSe difference analysis. A total of 39 microbial biomarkers in gut microbiota among five groups were identified ($p < 0.05$, LDA > 3) (Figure 4). *Corynebacterium*_1 (TG2) and unclassified_f__*Enterobacteriaceae* (CG) of microbial biomarkers exhibited an extremely distinct difference ($p < 0.01$) (Table S2). The gut microbiota of *P. sinensis* in the TG1 group share most bacterial class, order, family, and genera compared to those in CG, TG2, TG3, and TG4 groups, i.e., 25 genera in the TG1 group. The Proteobacteria, *Actinobacteria*, *Firmicutes*, and *Chloroflexi* phyla were more abundant in TG1 group. Compared to those of the TG1, TG2, and TG3 groups, the relative abundances of phylum *Firmicutes* and genus unclassified_f__*Enterobacteriaceae* were higher in the CG group. The *Bacteroidetes* phylum was only found in the CG group. In contrast, the abundances of phylum Proteobacteria, class *Gammaproteobacteria*, were highest in CG, TG1, TG3, and TG4 groups. The *Actinobacteria* phylum was only found in TG1 and TG2 groups. Likewise, the phylum Proteobacteria, class *Gammaproteobacteria*, was the most abundant and was found in TG4 only, compared to TG1, TG2, TG3, and CG groups.

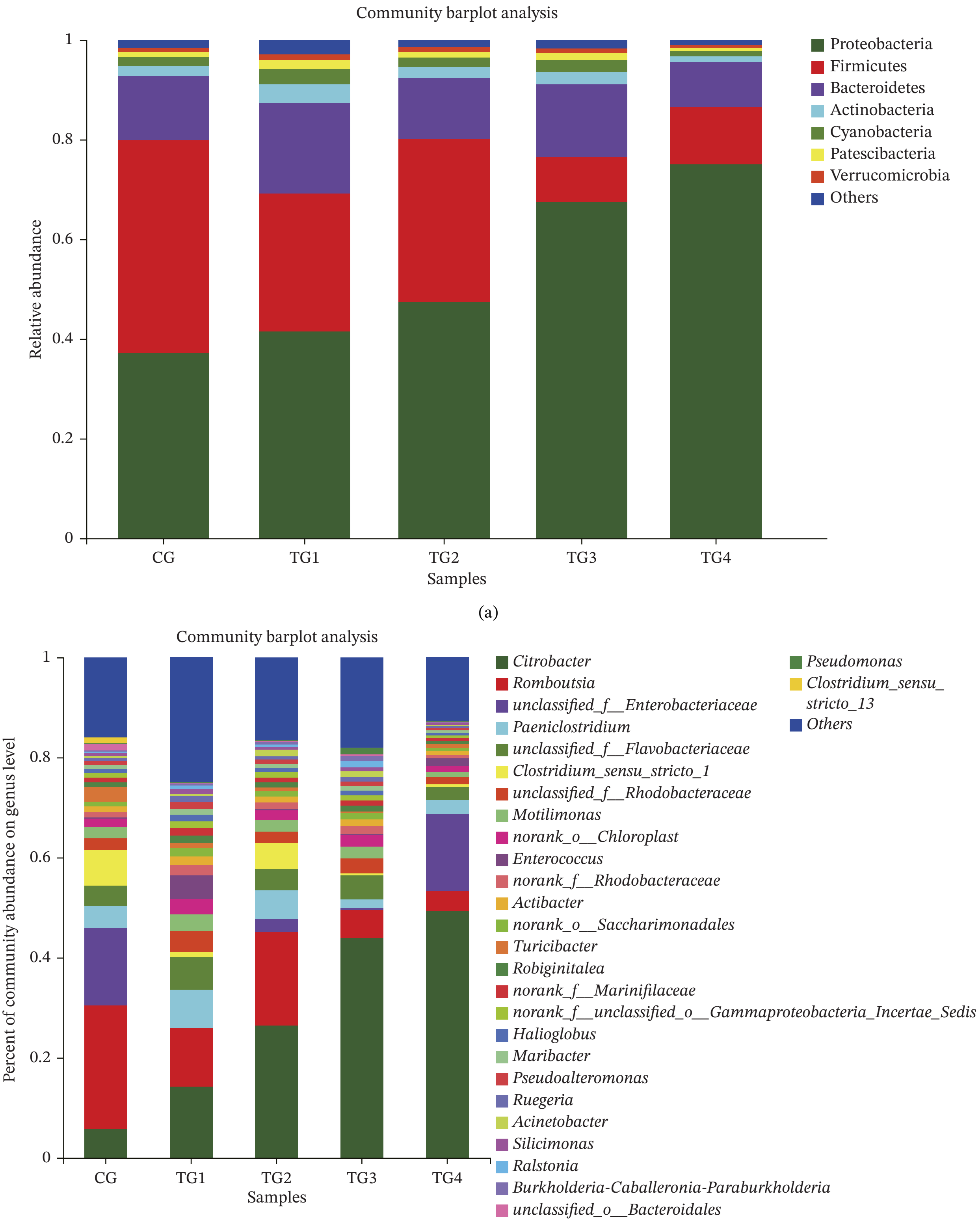
3.4 | Functional Profiles of *P. sinensis* Microbiomes Predicted by PICRUSt

The predictive functional profiling of the intestinal microflora of the experimental groups and CG group was predicted by PICRUSt analysis (Figure 5). In this study, three hundred and seventy-eighty pathways were significantly illustrated in KEGG level 3 (Table S2), which were categorized into 5 functional groups belonging to the organismal systems, cellular processes, human diseases, genetic information processing, metabolism, and environmental information processing. "Metabolism" was the most prevailing classification, of which "Carbohydrate metabolism", "Global and overview maps" and "amino acid metabolism" were the top three abundant pathways, followed by "human diseases" and "organismal systems". Environmental information processing was the fewest category. Notably, ABC transporters, biosynthesis of amino acids, carbon metabolism and two-component system were the most abundant pathways in TG1, TG2, TG3, and TG4 groups, while those pathways were fewer abundant in the CG group (Figure 5). In the top 30 enriched KOs, there were some KOs with significant differences

TABLE 1 | Number of sequence number, valid sequence number, base number, valid mean length, valid min length, valid max length, OTU counts, and total OTUs in CG, TG1, TG2, TG3, TG3, and TG4 groups by high-throughput sequencing.

Sample ID	Sequence number	Valid sequence number	Base number	Valid mean length	Valid min length	Valid max length	OTU counts	Total OTUs
CG2	47,987	30,294	19,962,667	416.0016	265	440	815	3314
CG3	47,518	30,294	19,903,354	418.8593	229	452	752	3314
CG4	51,602	30,294	21,174,812	410.3487	213	440	611	3314
CG5	43,530	30,294	18,403,614	422.78	312	477	119	3314
CG6	38,385	30,294	16,415,103	427.6437	290	430	238	3314
CG7	39,909	30,294	16,222,757	406.4937	292	433	250	3314
CG8	46,100	30,294	18,898,774	409.9517	278	441	647	3314
TG1_1	46,719	30,294	19,574,690	418.9878	291	452	728	3314
TG1_2	48,783	30,294	20,248,620	415.0753	202	437	718	3314
TG1_3	47,806	30,294	20,015,929	418.6907	340	503	783	3314
TG1_4	38,409	30,294	16,182,579	421.3226	262	497	564	3314
TG1_5	47,615	30,294	20,239,464	425.0649	255	441	536	3314
TG1_6	44,854	30,294	19,066,509	425.0793	201	441	928	3314
TG1_7	52,611	30,294	21,233,951	403.6029	259	460	74	3314
TG1_8	47,918	30,294	19,961,140	416.5687	203	441	680	3314
TG2_1	33,466	30,294	14,199,740	424.3035	402	440	620	3314
TG2_2	31,013	30,294	13,289,033	428.4988	256	430	85	3314
TG2_3	46,714	30,294	19,773,962	423.2984	227	452	165	3314
TG2_4	42,938	30,294	18,016,171	419.5857	290	447	171	3314
TG2_5	40,916	30,294	16,716,531	408.5573	398	437	714	3314
TG2_6	43,844	30,294	17,802,164	406.0342	384	431	803	3314
TG3_1	45,825	30,294	19,021,506	415.0901	396	431	174	3314
TG3_2	40,777	30,294	17,488,556	428.8829	271	431	78	3314
TG3_3	41,883	30,294	17,835,240	425.8348	337	430	832	3314
TG3_4	45,040	30,294	18,896,685	419.5534	262	452	444	3314
TG3_5	40,934	30,294	17,486,402	427.1853	242	452	546	3314
TG3_6	45,142	30,294	19,255,178	426.5469	229	449	779	3314
TG3_7	48,198	30,294	20,185,253	418.7986	262	514	754	3314
TG3_8	44,530	30,294	18,671,179	419.2944	257	441	803	3314
TG4_1	40,847	30,294	17,522,470	428.9781	237	431	21	3314
TG4_2	44,169	30,294	18,754,569	424.6093	205	447	474	3314
TG4_3	40,351	30,294	17,305,596	428.8765	341	431	70	3314
TG4_4	41,837	30,294	17,791,361	425.2542	262	437	643	3314
TG4_5	46,319	30,294	19,554,055	422.1606	229	447	694	3314
TG4_6	50,963	30,294	21,139,237	414.7958	225	440	692	3314
TG4_7	42,420	30,294	18,135,946	427.5329	265	435	105	3314
TG4_8	45,830	30,294	19,610,153	427.889	277	435	43	3314
Average	44,154.11	30,294	18,539,322.97	420.22	274.22	446.62	490.62	3314

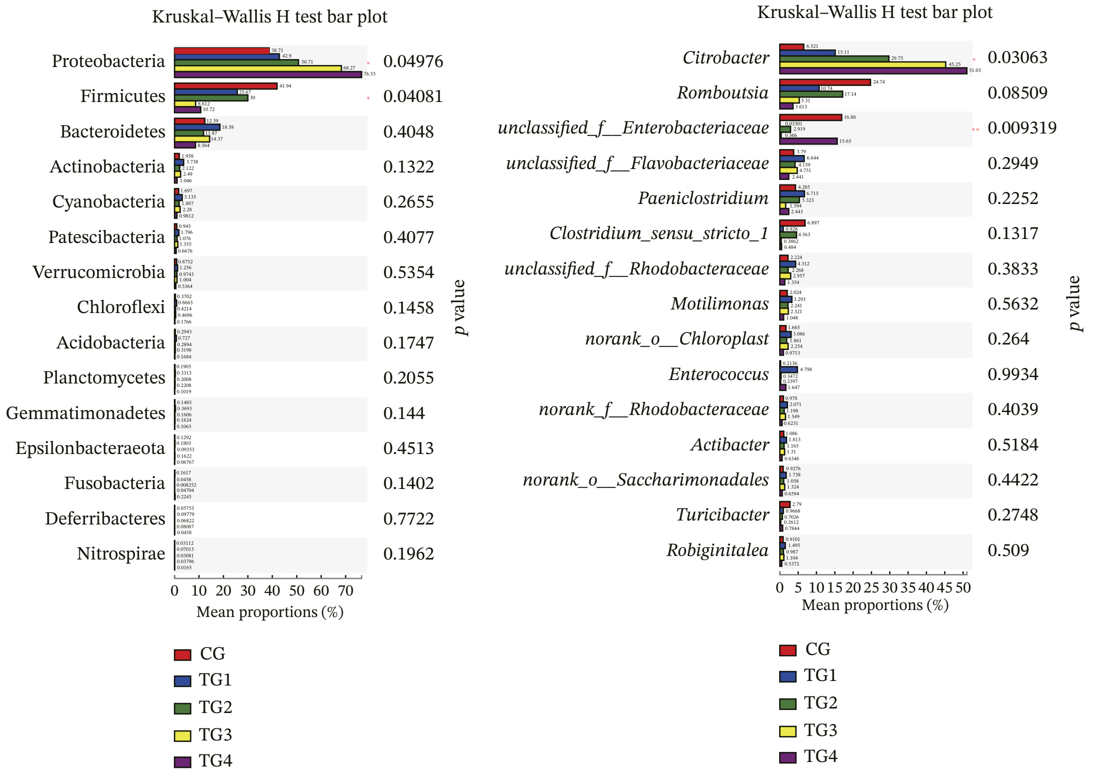
in the TG1, TG2, TG3, and TG4 groups compared with those of the CG group by comparing means across multiple groups analysis: ANOVA, namely, ko00230 (purine metabolism), ko02020 (two-component system), ko03010 (ribosome), ko02024 (quorum sensing), ko00240 (pyrimidine metabolism), and ko00640 (propanoate metabolism). ko00270 (cysteine and methionine metabolism), ko00250 (aspartate, alanine, and glutamate metabolism), ko00650 (butanoate metabolism), ko00970 (aminoacyl-tRNA biosynthesis), ko00680 (methane metabolism), ko03440 (homologous recombination), and ko00920 (sulfur metabolism) (Figure 6). Among them, the mean proportions of ko02020 (two-component system) were higher.



(a)

(b)

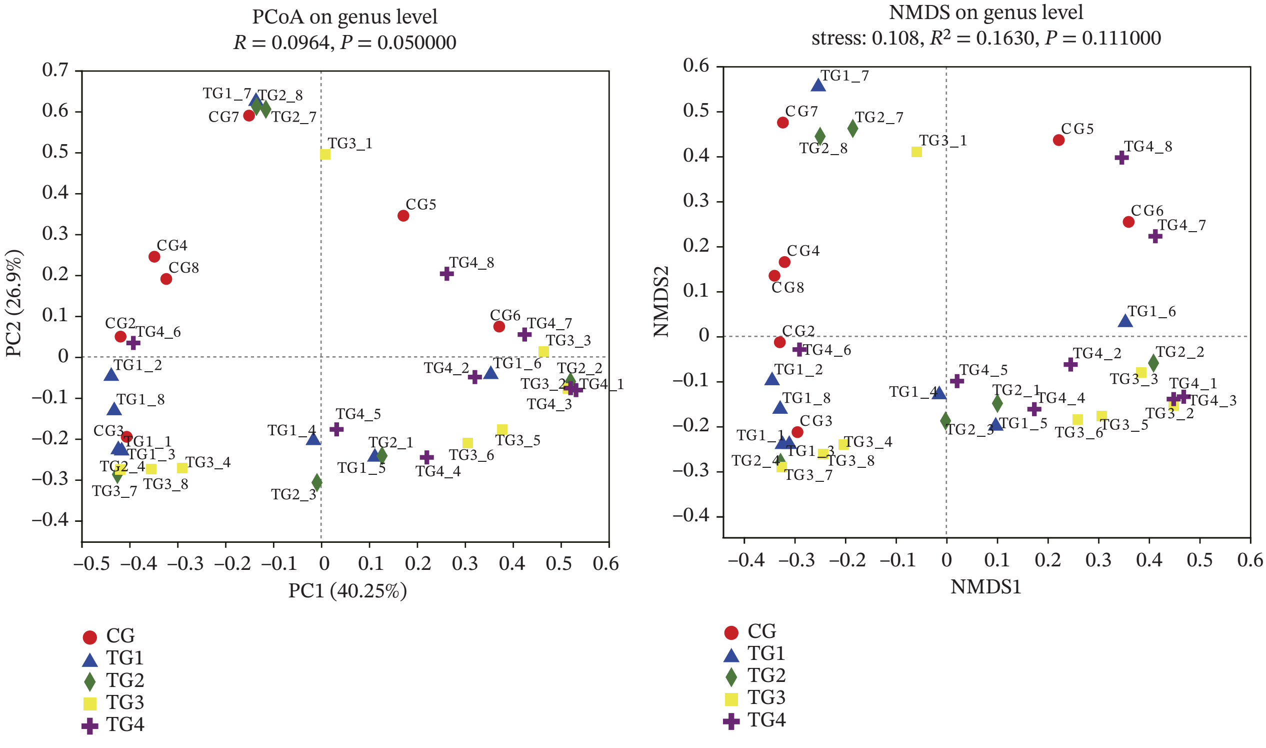
FIGURE 1 | Gut bacterial community at the phylum (a) and genus (b) levels. (a, b) Relative abundance of bacterial groups in the guts of 37 *P. sinensis*. Less than 1% abundance of the phyla or genus was merged into others.



(a)

(b)

FIGURE 2 | Statistical comparison of the relative abundance (a, b) of gut microbiota among CG, TG1, TG2, TG3, and TG4 groups. (a) Comparison of dominant phyla in the five groups. (b) Comparison of dominant genera in the five groups. $p < 0.05$ was considered statistically significant.



(a)

(b)

FIGURE 3 | Statistical comparison of the relative abundance (a, b) of gut microbiota among CG, TG1, TG2, TG3, and TG4 groups. (a) Comparison of dominant phyla in the five groups. (b) Comparison of dominant genera in the five groups. $p <$ was considered statistically significant.

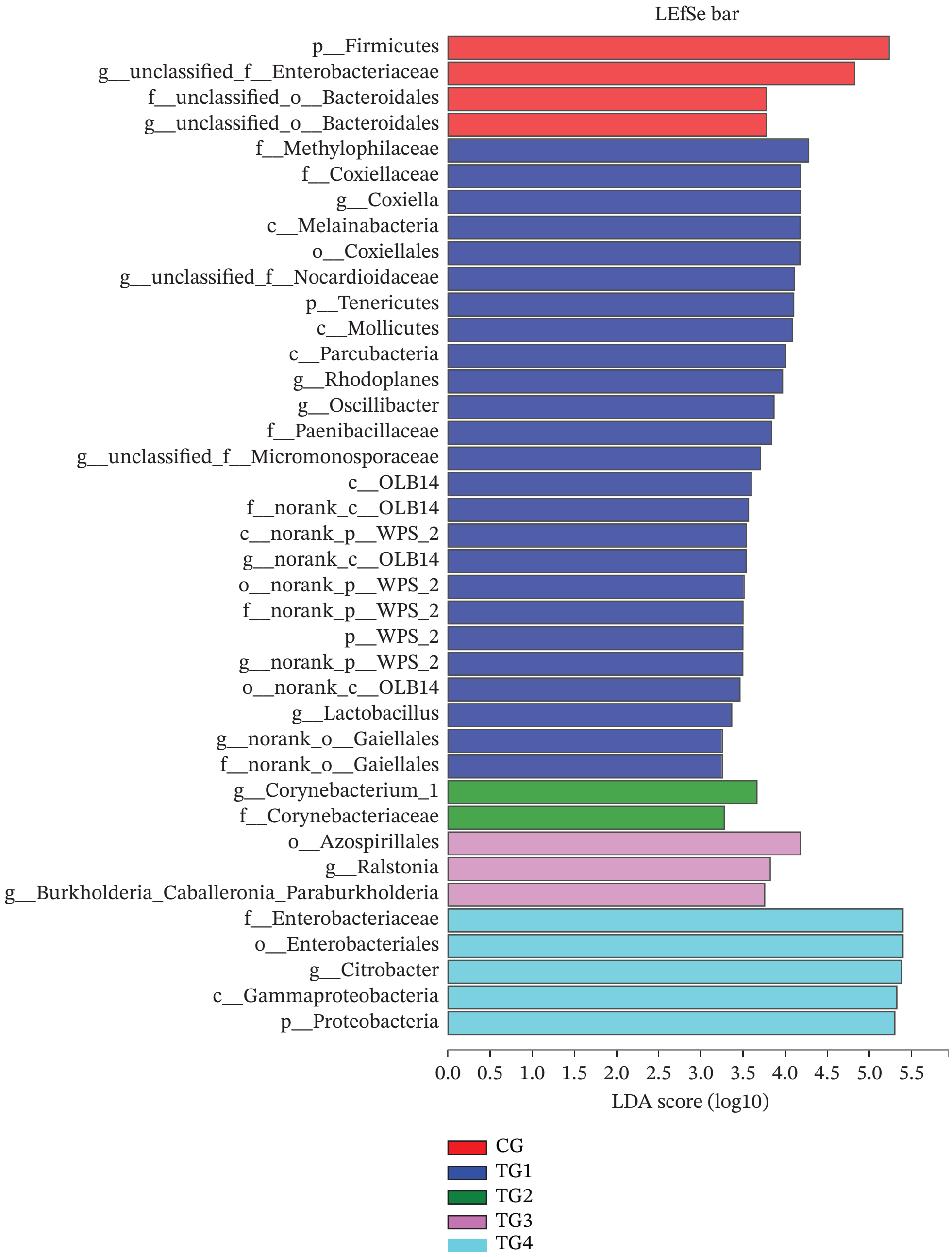


FIGURE 4 | Analysis of differences in the microbiota among CG, TG1, TG2, TG3, and TG4 groups using LEfSe software (linear discriminant analysis [LDA] coupled with effect size measurements). Only taxa meeting an LDA significant threshold of 3 are shown ($p < 0.05$). For taxa, which were defined as unclassified.

4 | Discussion

The complicated composition of the gut microbiota formed a host's health and metabolism, which played a critical role in the biotransformational processes of organic matter [34]. Gut microbiota was affected by many factors, for example, diet, geographical locations, hygiene, host genotype, and medication [35]. CHMs can adjust the composition of the gut microbiota and safeguard the function of the intestinal mucosal barrier to restore gut microbiome homeostasis. Meanwhile, human intestinal bacteria metabolized or biotransformed effective compositions of CHMs, whose metabolites can be taken in more easily and can even decrease or increase toxicity and then display significantly different biological effects [36, 37]. Discovering a core microbiome has been a main goal for many researchers who are keen on understanding intestinal flora [38]. Roeselers et al. first investigated that a core intestinal microbiota was present in the zebrafish [39]. In the present study, 711 OTUs were shared as core microbiota among the CG, TG1, TG2, TG3, and TG4 groups. Eichmiller et al. reported that the core intestinal flora of invasive carp species was compared between wild fish and farmed fish, and only five shared OTUs were recognized [40]. While others regarded nutrition or trophic level as the main modifier for the composition of the gut microbiota [41], Eichmiller et al. consider the environment as the primary driver for population structure assembly [40].

It is said that the *Firmicutes*, Fusobacteria, and Proteobacteria phylum, were the most abundance in the intestinal microbiota of

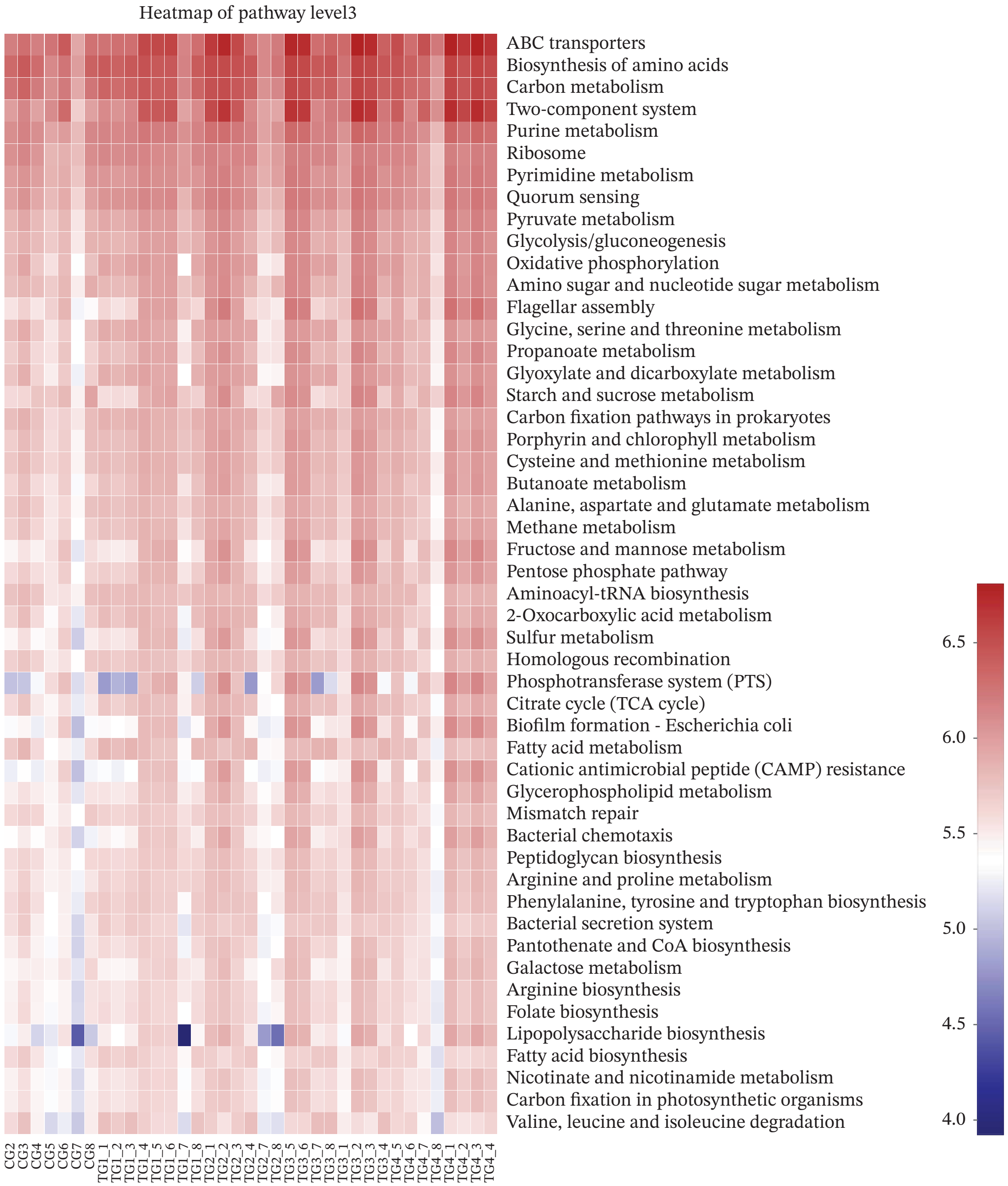


FIGURE 5 | Heatmap of significantly different functional profiles inferred by PICRUSt2 red colors represent higher abundance and blue colors lower abundance. To represent relative changes across the samples, the relative abundance was normalized to a *Z*-score. The left-side color bars indicate the KEGG pathway categories. All pathways within the functional profiles for metabolism, genetic information processing, environmental information processing, and cellular processes were significantly different among CG, TG1, TG2, TG3, and TG4 groups.

most fish species studied to date including freshwater species [40–42] and marine [43], and they can comprised up to 90% of the communities. However, *Firmicutes* were identified as the primary phylum in the allochthonous microbiome of *Oreochromis niloticus* [44], and Bacteroidetes, Actinobacteria, and Proteobacteria were confirmed as the most frequent phyla in tilapia [45]. In the current study, Proteobacteria, *Firmicutes*, and Bacteroidetes were the most ubiquitous and ascendant phyla in TG1, TG2, TG3, TG4 groups and CG group. The relative abundance of Proteobacteria ($p = 0.04976$) and *Firmicutes* ($p = 0.04402$) were the two most dominant phyla, with significant differences in experimental groups compared with the CG group (Figure 1(a)). The abundance of phylum Proteobacteria increases with the increase of the dose of CHMs for juvenile turtles, while a clear decrease in the relative abundance of *Firmicutes* was observed in the CHMs groups. These results obviously suggested the higher abundance of phylum Proteobacteria among the turtles of the CHMs group, which might result from the selection pressure within the gut due to long-term TCM feeding, as demonstrated by several studies [46]. Previous investigations implied that the immune system carried out a critical role in the establishment of sustainable host-microbe relationships, contributing thus to the

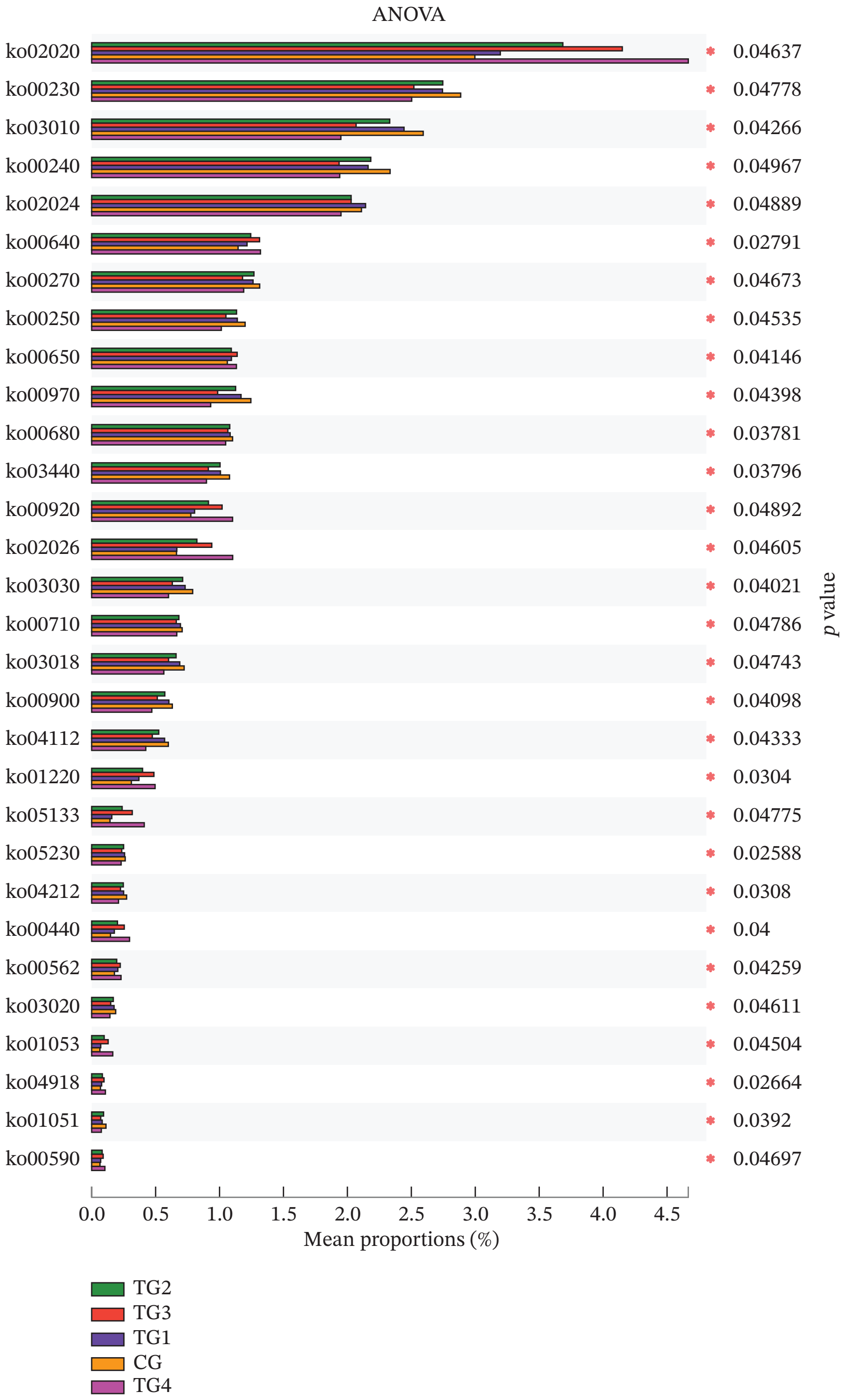


FIGURE 6 | Extended bar plot identifying significant differences between mean proportions of the top 30 KEGG pathways in CG, TG1, TG2, TG3, and TG4 groups. Corrected p values are shown at right.

remarkable diversity of ecosystems [46, 47]. The common carp (*Cyprinus carpio*) was fed the feed added to 1% CHMs. The quantity of some intestinal bacteria and composition of intestinal microbiota in *Cyprinus carpio* were modified by five CHMs [48]. The primary phyla, Proteobacteria, Actinobacteria, and *Verrucomicrobia* of *Carassius auratus* gibelio in the CHMs groups emerged distinctly more abundant compared with those of the control group (MW, $p < 0.01$) [48]. The *Cetobacterium* genus was considered as a common member of the microbiome in *Hypophthalmichthys nobilis*, *Carassius cuvieri*, and *Ctenopharyngodon idellus* [37, 49, 50], which may be recognized as a core genus among carps. At the genus level, the abundance of genus *Citrobacter* in the turtles increased with the increase of CHMs concentration (Figure 1(b)), while an obvious reduction in the relative abundance of genus *Romboutsia*, genus *unclassified_f_Enterobacteriaceae*, and genus *Clostridium_sensu_stricto_1* were observed in the five groups in this study. The relative abundance of *Citrobacter* ($p = 0.03063$) and *unclassified_f_Enterobacteriaceae* ($p = 0.00932$) at the genus level presented a significant difference in the experimental groups (Figure 2(b)), which suggested the increased abundance of genus *Citrobacter* observed after CHMs feeding corresponded mainly to the decrease in the most abundant genus, *unclassified_f_Enterobacteriaceae*. CHMs treatment upgraded intestinal histology and increased the abundance of probiotic bacteria such as *Romboutsia*, while reducing potential pathogenic bacteria such as *Clostridium_sensu_stricto_1*, *unclassified_f_Enterobacteriaceae*. The high abundance of genus *Citrobacter* and the decreasing trend of the abundant genus, *unclassified_f_Enterobacteriaceae* could be considered as the indicators of the medication due to CHMs as feed additive regulating the intestinal microbiota and reducing the incidence of potential pathogens. Caffeic acid regains the relative abundance of the intestinal flora and restrains the increase in the ratio of Firmicutes to Bacteroidetes [51]. The relative abundance of *Lactobacillus* and *Bifidobacterium* in rat intestines was significantly increased by nanopharmaceutical astragalus feed additives. At the same time, the counts of *Escherichia coli* and *Enterococcus* reduced normal levels [52]. The intestinal microbiota could catabolize macromolecular substances in CHMs into alkaloids, bioactive polyphenols, and other active monomers, and CHMs might adjust the structure of the intestinal microbiota [14, 15].

5 | Conclusions

In summary, the addition of 2.0% Chinese herbal mixtures to the diet could significantly improve the intestinal microecological environment of juvenile *P. sinensis*. Future studies would be required to understand the effects of CHMs feed additives on the gut microbial gene catalog found by metagenomic sequencing.

Author Contributions

Dandan Ju, Huimin Xu, and Mingsong Xiao designed the study. Dandan Ju, Huimin Xu, Bing Gong, and Mingsong Xiao performed the experiments. Mingsong Xiao, Dandan Ju, Huimin Xu, and Bing Gong collected and analyzed the data. Mingsong Xiao edited the figures. Mingsong Xiao contributed to the discussion and reviewed/edited the manuscript.

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Disclosure

All authors have read and agreed to the published version of the manuscript.

Ethics Statement

Investigations and protocols were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with the Huainan Normal University Institute of Animal Care and Use Committee. The institutional review board approved this procedure. Our study had been submitted to and approved by the Academic Ethics Committee of Huainan Normal University. All sample collection was undertaken in accordance with relevant Academic Ethics Committee of Huainan Normal University guidelines and regulations.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data used to support the findings of the study are available from the corresponding author upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. *(Supporting Information)*

Figure S1 Scalar–Venn representation of the microbiota among CG, TG1, TG2, TG3, and TG4 groups.

Table S1. Richness and diversity indexes relative to each gut sample.

Table S2. Result of PICRUSt analysis.