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Varieties of immunity activities and gut contents in tilapia with seasonal changes

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ABSTRACT

We performed 16S rDNA sequencing of tilapia fecal samples to analyze changes in tilapia gut contents after cultivation of the fish in the presence of sandwich-like floating beds of Chinese medicinal herbs (5 and 10% planting-areas; 5% *Polygonum cuspidatum*). The interactive effects between water quality and blood and hepatic pro- and anti-inflammatory concentrations were also assessed. Our results showed that the water quality (i.e., NO_3^--N, NO_2^--N, TP removal rates) improved, and the abundance of Chloroflexi and Cyanobacteria increased. The abundance of Bacteroidetes, Verrucomicrobia, Saccharibacteria, and Actinobacteria showed both significant seasonal decreases and increases in the presence of *P. cuspidatum* (increases in August and decreases in July). Fish blood and hepatic IL-10 and IFN-γ levels (together with fish sampled in September) significantly increased in the *P. cuspidatum* group sampled in August, while those of TNF-α (10% sandwich-like, *P. cuspidatum*), IL-1β (*P. cuspidatum*), IL-8 (5% sandwich-like in September, S905S) significantly decreased. Heat shock proteins 60 and 70 levels significantly increased in the *P. cuspidatum* group, and complement C3 and C4 concentrations significantly increased in S905S. This study demonstrated that enhanced immunity through the regulation of pro- and anti-inflammatory proteins was sustained throughout development until harvest, particularly in fish grown with *P. cuspidatum*.

1. Introduction

The study of traditional Chinese medicinal herbs and natural products has made great contributions to the prevention and treatment of illnesses. Four categories of disease (cancer, 20.9%; cardiovascular, 19.2%; oral/gastrointestinal, 9.8%; and inflammatory/immune, 9.0%) account for the majority of the research in this field [1]. Medicinal herbs are often prescribed as multi-herb compounds. The main active compounds in extracts (e.g. emodin [2], cassane diterpenes [3], iridoids [4], rutin, and resveratrol [5–7]) have revealed that the anti-inflammatory activities are exerted through the regulation of cytokine expression. Floating-bed cultivation with medicine-food-homology plants, e.g. *Houttuynia cordata Thunb*, *Polygonum cuspidatum,* and *Mentha haplocalyx Briq*. (*Yuxingcao*, *Huzhang*, and *Bohe* in Chinese, respectively), improved water quality in tilapia ponds [8], an effect similar to the addition of *Ipomoea aquatica Forsk* (a vegetable) [9], and simultaneously enhanced the disease resistance of tilapia against *Streptococcus* infection [10]. Using medicinal herbs instead of vegetables will therefore be more beneficial to and accepted by fish farmers, although the usage of Chinese medicinal herbs in aquaculture is not widespread. *H. cordata Thunb* mainly contains volatile oils, flavonoids, and phenolics [5,11]. *P. cuspidatum* contains phenolics [6,7], stilbene, and anthraquinone (emodin and physcion) [12,13]. *M. haplocalyx Briq*. contains peppermint oil (active against phytopathogenic fungi) [14], menthol, and menthone. The plants release allelochemicals against bacteria pathogenic in fish and can also absorb active compounds that can enhance fish health through changes in the gut; there appears to be crosstalk between fish immune responses and their gut microbiota [15], although this remains poorly understood. To determine the differences arising from different floating-bed cultivation conditions (control; 5% and 10% of a combination of *Houttuynia cordata Thunb*, *Mentha haplocalyx Briq*., and *Ipomoea aquatica Forsk*; or 5% *Polygonum cuspidatum*), we performed 16S rDNA sequencing of the total gut contents from tilapia.

The inflammatory process is controlled by a wide range of mediators, e.g. heat shock proteins (HSPs), complement components, cytokines, etc. [16]. The mononuclear phagocyte system of the liver contains many immunologically active cells, whose activation results in the increased transcription of pro- and anti-inflammatory cytokines. Interleukin 10 (IL-10) is the most important anti-inflammatory cytokine and

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is a potent inhibitor of interferon-γ (IFN-γ). Pro-inflammatory cytokines are used to activate neutrophils, and include tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) [6]; these cytokines can have multiple functions in multiple locations [6]. TNF-α is often involved in different signaling pathways to regulate apoptosis in cells. While IL-1β is released by monocytes and macrophages, IL-6 plays a role in neuronal responses to injuries. IL-8 is a chemokine of the immune system and is produced mainly by macrophages and epithelial cells. Inflammatory symptoms result from the recruitment of cells of the innate and adaptive immune systems and the production of cytokines and chemokines [17]. HSPs not only aid the correct folding of damaged proteins that act as sensors of redox changes in the cell [18], but also play important roles in both innate and adaptive immunity [19]. The most important members of the HSP family are HSP60, HSP70, and HSP90 [20]. Complement C3 and C4 play key roles in the complement system, and their deficiencies or overexpression are associated with many infectious or immune diseases [21].

The gut microbiome not only strengthens the digestive and immune systems in fish but is itself affected by several host-associated factors [22,23]. It is well known that many compounds are potentially bioactive, such as those from medicinal plants and their derivatives, and affect innate and adaptive immunities, as well as growth, in fish [24]. For that reason, there has been considerable interest in the use of medicinal plants in aquaculture in an effort to provide safe and eco-friendly replacements for antibiotics and chemical compounds, as well as to enhance immunity and control fish diseases.

The gut contains many antimicrobial peptides and cytokines that play roles in innate immunity, while the serum contains immunoglobulins and cytokines secreted by T cells that function in adaptive immunity [15,24,25]. Our recent studies have shown that at the time of fish harvest, fish immunity was enhanced when they were raised in floating beds cultivated with Chinese medicinal herbs [10]. This may have resulted in inflammatory responses through increase in proinflammatory protein levels or transcripts after feeding with resveratrol at day 45 [6,7]. However, whether enhanced immunity is present from the developing fry until harvest is unclear. In this study we sequenced 16S rDNA from fecal samples of fish reared in floating beds with different Chinese medicinal herbs, to explore crosstalk between water quality, immunity, and intestinal microbiota.

2. Materials and methods

2.1. Animals and sample collection

Fertilized eggs of *O. niloticus* were obtained from the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences (Yixing, China). The fish fry were cultivated in a pond (20 m × 30 m), and acclimatized for two weeks prior to the experiment. The male genetically-improved farmed tilapia (GIFT) juveniles (4.7–5.5 cm, 15.4–24.3 g, $n = 39600$) were assigned to four treatment groups and each treatment was performed in triplicate (twelve ponds in total; $n = 3300$ per pond, 1333 m^2, 1.5 m deep). The fish were fed once a day (the recommended 4% of body weight). The feed was purchased from Jiangsu Zhe Ya Food Co. Ltd, China. The four treatment groups were as follows: One group in the nonfloating bed control group (designated as S700C, S800C, and S900C for July, August, and September sampling, respectively); groups in sandwich-like floating beds with 5% or 10% planting-areas consisting of equal parts *H. cordata Thunb*, *M. haplocalyx Briq.*, and *I. aquatica Forsk* (S705S, S805S, and S905S; and S710S, S810S, and S910S, respectively); and the final group in beds with 5% *Polygonum cuspidatum* (S705P, S805P, and S905P). The total yield of each pond, the biological parameters (total length and weight) of each fish, and the feed coefficient were measured. All pond water and the gut contents of fish in the four treatment groups were collected on the 18th of July, August and September 2017.

At each sampling point, livers were sampled for protein activity, and the gut contents from three fish were pooled and processed for high-throughput sequencing analysis ($n = 3$ pooled samples) [7]. The foregut collections were done as previously described [26,27].

2.2. Water quality determination

The pond water quality was assessed from July to September as follows: Mixed samples, consisting of central and surrounding water were collected at a depth of 10–15 cm and stored in a sterile glass bottle (500 mL). The electrical conductivity (EC), oxidation-reduction potential (ORP), pH, and dissolved oxygen (DO), were measured in situ with a YSI EXO2 Multiparameter Sonde (USA). The total nitrogen (TN), ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), total phosphorus (TP), phosphate (PO_4^{3-}-P), total organic carbon (TOC), COD_{Mn}, and algal chlorophyll (Chl.a) were analyzed as described [28].

2.3. DNA extraction, PCR amplification of 16S rDNA, amplicon sequencing, and sequence data processing

Total bacterial DNA extraction [7] and PCR amplification [29] were performed as previously described. All PCR products were quantified using Quant-iT™ dsDNA HS Reagent and pooled. High-throughput sequencing analysis of bacterial rRNA genes was performed on the purified, pooled sample using the PacBio Sequel platform at Biomarker Technologies Corporation (Beijing, China). The splicing and filtering of the raw data (PE Reads), and cleaning of chimera were performed using Lima v1.7.0 and UCHIME v4.2 (Effective Tags), respectively. Finally, the average length, GC content, Q20 [quality value > 20/total number of bases], Q30 [quality value > 30/total number of bases], and effective ratio were calculated.

Taxonomy classifications [7] and statistical analyses [30] were performed as previously described. We aimed to identify the "core microbiome" of each group and the "core intestinal fecal microbiome" of all intestinal fecal samples. The bacterial community indices applied here included Chao1, Ace, Shannon, and Simpson. We used the unweighted UniFrac distance metric to estimate α/β diversity (Mothur v.1.30 and QIIME), and clustering was performed using principal component analysis (PCA) of UniFrac distance matrices.

2.4. Changes in bacterial communities

We applied a previously-used relative abundance threshold (0.5%) [31] to focus our analysis on PCR-reproducible operational taxonomic units (OTUs). We used significant increases or decreases in the bacterial community from the databank constructed by a set of comparisons (e.g. control group S700C vs. case group S705S, S700C vs. S800C) to calculate the variation. Differences could range from the phylum to the species level.

2.5. Determination of pro-/anti-inflammatory cytokines

For the biochemical analyses, livers from 3 individuals per group at each sampling point were collected and washed thoroughly with ice-cold physiological saline (0.86% NaCl), and their surfaces dried with absorbent paper. Whole liver samples were homogenized on ice with cold 0.86% saline (1:9, w/v), and then centrifuged at 2500 rpm at 4 °C for 10 min. Blood from 3 individuals per group at each sampling point were centrifuged at 2500 rpm at 4 °C for 10 min before being stored overnight at 4 °C.

Using mammalian antibodies against fish blood and tissue cytokines [32], the supernatants were analyzed for blood and hepatic IL-10 (CAS number H009), IFN-γ (CAS number H025), TNFα (CAS number H052), IL-1β (CAS number H002), IL-8 (CAS number H008), HSP60 (CAS number H264-1), HSP70 (CAS number H264-2), HSP90 (CAS number

H264-3), complement C3 (CAS number H186-1), and complement C4 (CAS number H186-2) using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China; the protocol is attached as supplementary material) referred to in our previous studies [5,6,17]. The cytokine levels were based on their respective binding with antibody. Proteins were quantified spectrophotometrically with a PowerWave XS2 (at an accurate nm determination point) (BioTek instruments Inc, Vermont, USA) and a standard curve using known concentrations of the target antigen.

2.6. Data analysis

The degree of decline in water quality indexes was determined by dividing the decrease in water quality (value in treatment groups - controls) by the original value (control). The relationship between the selected taxonomy group (abundant phyla, genera, classes, orders, or families), the observed OTUs or the bacterial community index (Chao1), and the apparent nutrient digestibility was calculated using SPSS 13.0 software. For all parameters, data were compared using a one-way analysis of variance (ANOVA) at the end of each bioassay. A comparison of means was performed using Fisher's least significant difference test and the Duncan multiple range test with a significance level of $P < 0.05$, markedly significance level of $P < 0.01$.

3. Results

3.1. Water quality measurements

EC, ORP, pH, and DO ranged from 0.175 to 0.346 mS/cm, 67–84 mV, 7.4–9.5, 5.9–8.2 mg/L, respectively. The decline in water quality for TOC, $COD_{Mn,}$ Chl.a, TN, NH_4^+-N, NO_3^--N, NO_2^--N, TP, and PO_4^{3-}-P were 10.24–65.83%, 1.30–46.35%, 18.86–60.09%, 4.57–53.21%, 6.94–48.79%, 17.48–72.50%, 41.18–95.45%, 30.15–78.16%, and 14.73–100.00%, respectively (Table 1). The total yields of the control, 5% and 10% sandwich-like, and 5% *Polygonum cuspidatum* groups were 20,578, 23,415, 23,478 and 22,451 kg/hm^2, respectively; the total lengths were 257 ± 13, 263 ± 11, 267 ± 10, and 271 ± 12 cm, respectively; total weights were 767 ± 25, 814 ± 34, 848 ± 36, and 795 ± 17 g, respectively; and the feed coefficients for those groups were 1.2397, 1.1977, 1.2016, and 1.3101, respectively.

3.2. OTU collection and biogeography effects on α/β diversity

Optimization-circular-consensus sequences (CCS) ranged from 2900 to 3227, and OTUs ranged from 196 to 318 (Table S1). Thirty-eight OTUs were gathered based on species abundance-clustering images at the phylum level (Fig. 1). Fusobacteria (44.04%), Firmicutes (18.45%), and Planctomycetes (17.40%) were the three main phyla for the different groups (Fig. 1), while uncultured Cetobacterium (44.01%), unclassified (27.92%), and uncultured Planctomycetaceae (11.90%) were the three main bacterial species. Except for the three main phyla, the abundance of Bacteroridetes, Tenericutes, Cyanobacteria, and Chloroflexi varied significantly between groups (Fig. S1). When looking at all the samples, the top 10 phyla were similar between groups (Fig. S2). We found no significant differences in the Ace, Chao1, Simpson, and Shannon diversity indices of the bacterial communities between the treatment groups and the control group (Table S1). Indeed, different plant species and sampling times accounted for the largest variation in β diversity measured by both weighted and unweighted UniFrac matrices (50.97% along PC1, Fig. S3, $P < 0.05$). While 11.07% of the variation was explained by PC2, which accounted for water quality, the intestinal absorptivity rates of chemicals from plants in the sampled individuals was similar to that reported in a previous study [7] (Fig. S3).

3.3. Variation in specific OTU abundances

When we compared groups using the unweighted UniFrac distance, we found a highly significant increase in the S710S group compared to S705P group in July (Fig. 2). When compared to S800C and S805P, the S810S group was significantly decreased, and S805S was significantly increased compared to the S810S group in August. In September, S905P was significantly increased compared to the S905S group. When we looked at floating beds cultivated with *P. cuspidatum*, the values in September were markedly higher than those in July ($P \leq 0.01$), while the value in August was significantly higher than those in July ($P \leq 0.05$). The values in August were significantly lower than those in September. For the 5% planting-area beds, the values in July were significantly lower than in August, whereas for the 10% planting-area beds, the values in July were significantly higher than in September and August.

We found significant changes in the phylum Actinobacteria when comparing S705S: S805P, S805P: S800C, S810S: S910S, S900C: S905S, and S905S: S910S. Phylum Tenericutes was initially present in the S810S group; phylum BRC1 was initially present in the S905P, S805S, S905S, and S910S groups; phylum TM6 [Dependentiae] was initially present in the S805S, S805P, and S905S groups; and phylum Microgenomates began to appear in the S905P, S805S, and S905S groups. However, compared to floating-bed cultivation groups, phylum Chloroflexi was absent from the S700C, S800C, and S900C groups, while phylum Cyanobacteria was absent from the S800C and S900C groups. Phylum Firmicutes in the S705S group (0.275 ± 0.093) significantly increased compared to the S700C group (0.0481 ± 0.0179, p = 0.0324). When we compared S705P to S805P, phylum Bacteroidetes (0.0086 ± 0.0013 vs. 0.0002 ± 0.0002, p = 0.0000), Verrucomicrobia (0.1150 ± 0.0161 vs. 0.0304 ± 0.0092, p = 0.0085), and Saccharibacteria (0.0058 ± 0.0017 vs. 0.0015 ± 0.0007, p = 0.0423) significantly decreased, and Actinobacteria (0.0049 ± 0.0011 vs. 0.0551 ± 0.0147, p = 0.0257) significantly increased. Phylum Actinobacteria in S900C (0.0002 ± 0.0002) significantly increased compared to S905P (0.0163 ± 0.0085, p = 0.0298).

When compared to the S700C group, phylum Firmicutes

Table 1
The water removal rate of sandwich-like and *P. cuspidatum* pond from July to October (%, $n = 3$).

Month	Group	TOC	COD_{Mn}	Chl.a	TN	NH_4^+-N	NO_3^--N	NO_2^--N	TP	PO_4^{3-}-P
July	S705S	42.34	21.06	57.28	17.29	35.85	45.21	60.00	64.80	60.00
	S710S	65.83	46.32	60.09	53.12	6.94	49.36	95.45	78.16	100.00
	S705P	55.10	46.35	60.09	53.21	13.43	72.50	70.00	79.41	60.00
August	S805S	12.95	1.30	37.91	18.51	33.12	−27.92	81.18	30.15	14.73
	S810S	10.24	3.88	−13.24	4.59	−8.79	28.17	−40.76	34.39	48.64
	S805P	−4.21	3.86	18.86	4.57	23.11	27.89	41.18	34.04	50.00
September	S905S	12.34	18.89	38.54	9.65	37.86	17.48	43.52	41.67	66.67
	S910S	25.89	24.20	21.81	17.64	48.79	34.95	62.18	61.11	60.00
	S905P	27.63	8.96	50.68	35.94	35.92	17.48	40.00	−8.33	60.00

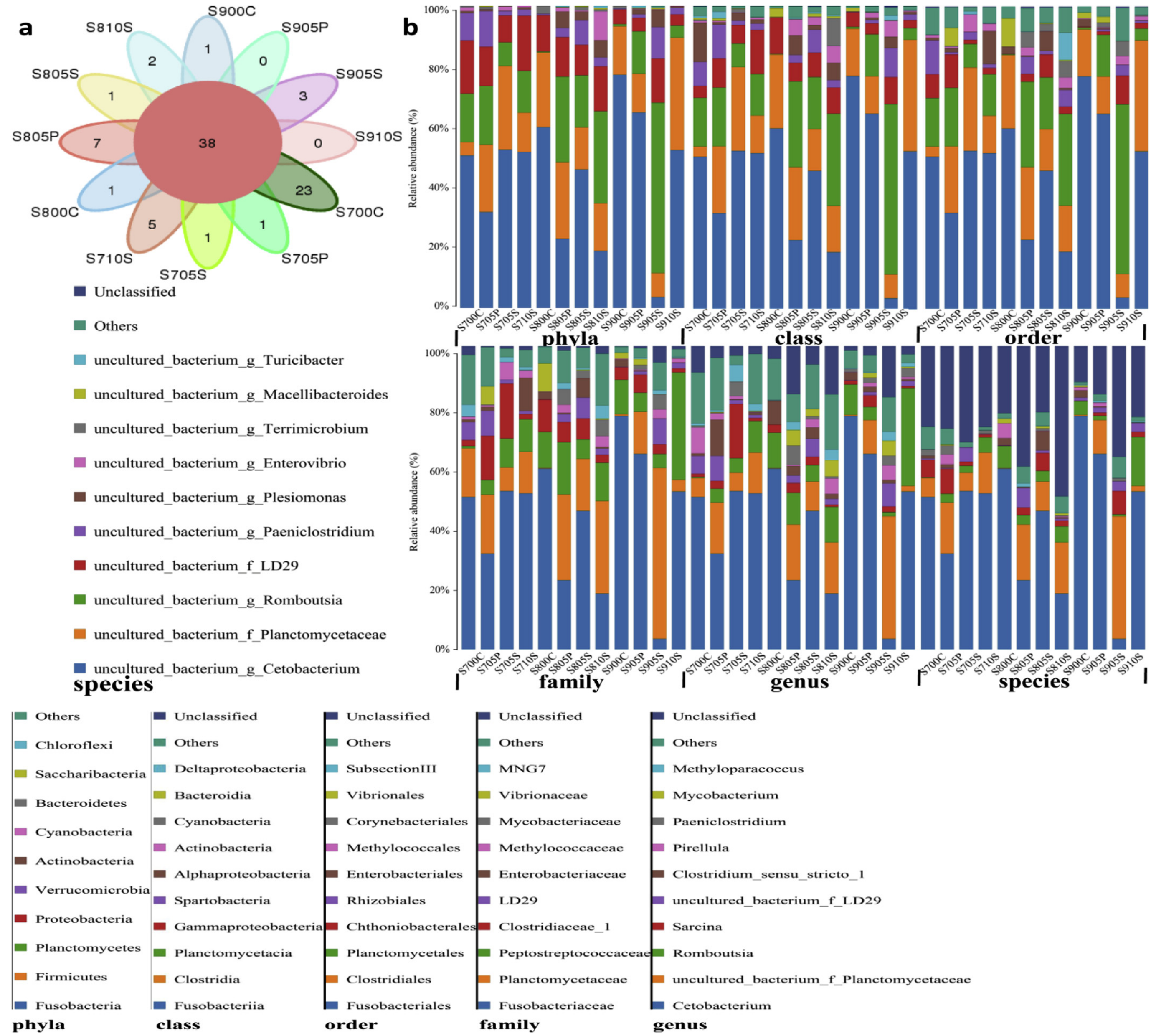

Fig. 1. Species-abundance clustering image for different taxonomic categories ($n = 3$). The relative abundances of the different taxonomic categories in samples as revealed by 16S rRNA gene ribotyping. For each sample type, only taxa with an RA > 0.5% in at least one sample were included in the analysis. Fish were divided into four treatment groups as follows: the control group cultivated without floating beds (termed S700C, S800C and S900C, sampled in July, August, and September, respectively); the groups cultivated with 5% or 10% sandwich-like floating beds planted with a combination of equal parts *H. cordata Thunb*, *M. haplocalyx Briq.*, and *I. aquatica Forsk* (S705S, S805S, and S905S and S710S, S810S, and S910S, respectively); and the group cultivated with 5% *Polygonum cuspidatum* (S705P, S805P, and S905P). (a) The flower figure for sequenced OTUs, (b) the taxonomic categories, ordered phyla, class, order, family, genus, and species.

significantly increased in the S705S group (Table S2, Fig. S4). The orders Corynebacteriales, Legionellales, Rhodobacterales, Chthoniobacterales, Syntrophobacterales, and Verrucomicrobiales significantly decreased, and Clostridiales significantly increased. In the S710S groups, the genus uncultured bacterium *Romboutsia* significantly increased, while others significantly decreased (Table S2). With respect to *P. cuspidatum*, *Clostridium sensu stricto 1*, genus *Romboutsia*, etc. significantly increased, and *Rhodopirellula*, *Candidatus Anammoximicrobium*, etc. significantly decreased. When we compared different groups in July, the genera *Clostridium sensu stricto 1* (Fig. 3), *Comamonas*, etc. in the S705P group was significantly higher than those in the S705S group, while the genera *Cetobacterium* and uncultured bacterium *0319-6G20* were significantly lower. Family uncultured candidate division WS5 bacterium, Prevotellaceae in the S705S group was significantly higher than in the S710S group, while the family Xanthomonadaceae was significantly lower. With respect to genera, uncultured bacterium *Paeniclostridium* in the S705S group was significantly higher than those in the S710S group (4.78% vs. 0.21%, $p = 0.0364$).

With increasing time, the orders Legionellales, Planctomycetales, etc. significantly decreased in the S900C group when compared to the S700C group, while the order Clostridiales significantly increased. The order PeM15, subsection III, family Prevotellaceae and Family I significantly decreased in the S705S group compared to those in the S805S group, while the class Verrucomicrobiae, the order Verrucomicrobiales, and the family Verrucomicrobiaceae significantly increased.

The phyla Bacteroidetes, Verrucomicrobia, and Saccharibacteria; orders Bacteroidales, Oligoflexales, etc.; and families

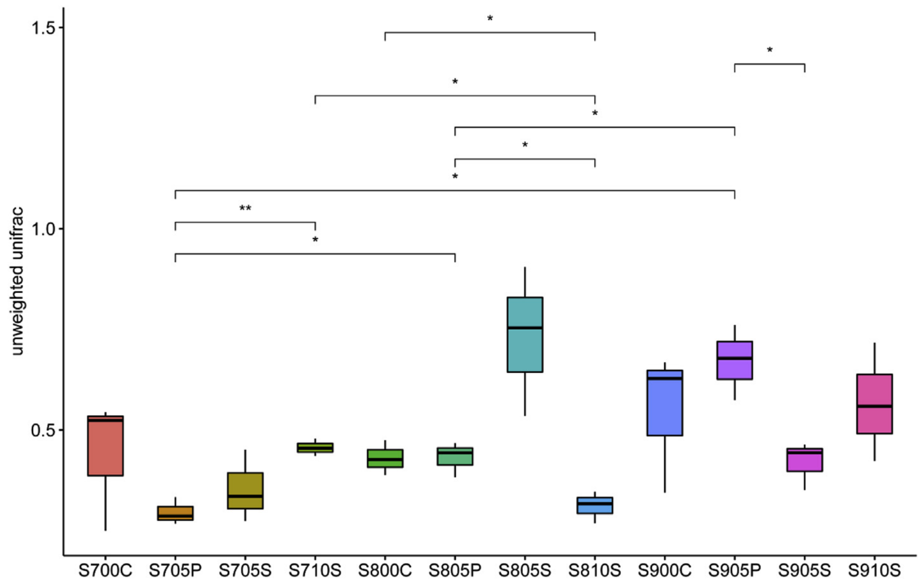

Fig. 2. The box map for the intergroup β-diversity statistical analysis among the treatment groups from July to September by unweighted UniFrac analysis ($n = 3$). Groupings are along the abscissa and the UniFrac distance is along the ordinate (*$P \le 0.05$, **$P \le 0.01$).

Comamonadaceae, and Porphyromonadaceae, etc. significantly decreased in the S805P group compared to those in the S705P group, while the phylum Actinobacteria, orders Rhizobiales and Solirubrobacterales, etc., and families Mycobacteriaceae, and Methylococcaceae (Fig. 4), etc. significantly increased.

The families Oligoflexaceae and Porphyromonadaceae, etc.; genera *Macellibacteroides* and *Cellulosilyticum*, etc.; and species uncultured bacterium *Oligoflexaceae*, etc. significantly decreased in the S905P group compared to those in the S705P group. The family Xanthomonadaceae, *Coelastrella_sp._M60*, and Pseudomonadaceae significantly decreased, while the families Verrucomicrobiaceae and Peptostreptococcaceae significantly increased in the S910S group compared to those in the S710S group. The classes Fusobacteriia, uncultured bacterium *Epulopiscium*, uncultured bacterium *Cetobacterium*, and uncultured bacterium Porphyromonadaceae significantly decreased, while the classes Acidimicrobiia, Actinobacteria, and Planctomycetacia, etc., and uncultured bacterium Planctomycetaceae, etc. significantly increased in the S810S group compared to those in the S800C group.

The classes Thermoleophilia and Alphaproteobacteria significantly decreased in the S805S group compared to those in the S805P group. The families FukuN57 and Methylococcaceae, etc. significantly decreased in the S905P group compared to those in the S805P group. The phylum Actinobacteria significantly increased in the S905P group compared to those in the S900C group.

The genera *Epulopiscium*, *Clostridium sensu stricto 1*, *Cellulosilyticum*, and uncultured bacterium *Epulopiscium* significantly decreased in the S900S group compared to those in the S800C group. The genus *Methyloparacoccus* significantly increased in the S905S group compared to those in the S805S group.

The species uncultured bacterium *Epulopiscium*, etc. significantly decreased, while uncultured bacterium TM146 significantly increased in the S805P group compared to those in the S800C group.

3.4. Fish protein concentration assays

The concentrations of the anti-inflammatory protein, IL-10, significantly increased in fish from the groups S705P (blood), S805P (blood and liver), S705S (blood), S805S, and S810S (liver) (Fig. 5). Blood IFN-γ significantly increased in fish from groups S705S, S905S, S805P, S905P, S810S, and S910S, while hepatic IFN-γ significantly increased in fish from groups S805S, S905S, S805P, S905P, and S910S.

From July to September, the blood concentration of the inflammatory protein, TNF-α, significantly decreased in fish grown in the 10% planting-area beds and in the 5% *P. cuspidatum* groups. Hepatic TNF-α significantly decreased in those groups from August to September. Blood IL-1β significantly decreased in the same sampled groups, except for S905S and S710S, while hepatic IL-1β decreased in the 5% *P. cuspidatum* groups from July to August. Blood IL-8 significantly decreased (except in group S805S) and hepatic IL-8 significantly decreased in the S710S, S805S, S805P, and S905S groups.

In July, the blood concentrations of HSP60, HSP70, and HSP90 significantly increased in fish from the 10% planting-area beds and the 5% *P. cuspidatum* group (except for HSP90), while HSP90 significantly increased in the S705S group. In August, HSP60 and HSP90 significantly increased in fish from the 5% and 10% planting-area beds, while HSP70 and HSP90 significantly increased in the 5% *P. cuspidatum* group. In September, HSP60 significantly increased in the 5% *P. cuspidatum* group, HSP70 significantly increased in the 5% planting-area bed group, and the 5% *P. cuspidatum* group, while HSP90 significantly decreased in all treatment groups.

In July however, hepatic HSP proteins significantly increased in fish from the 5% planting-area bed group, while HSP60 significantly decreased and HSP90 significantly increased in fish from the 10% planting-area bed group. Hepatic HSP60 and HSP70 significantly increased in fish from the 5% *P. cuspidatum* group. In August, hepatic HSP proteins significantly increased, except for HSP70 in fish from the 5% *P. cuspidatum* group. In September, HSP proteins significantly increased in fish from the 5% *P. cuspidatum* group, while HSP60 significantly decreased and HSP70 significantly increased in fish from the 5% and 10% planting-area beds. HSP60 significantly increased in fish from the 5% planting-area bed group and HSP90 significantly decreased in the 5% *P. cuspidatum* group.

Blood complement C3 and C4 concentrations significantly decreased in fish from groups S705S and S710S (C3 only) in July and C4 concentration significantly decreased in August. Complement C3 and C4 concentrations significantly increased in groups S905S and S805S and complement C3 significantly increased in groups S805P and S905P. Hepatic C3 and C4 concentrations significantly increased in July and September (C3 only), while C4 concentrations significantly decreased in fish from the 5% *P. cuspidatum* group in August and September.

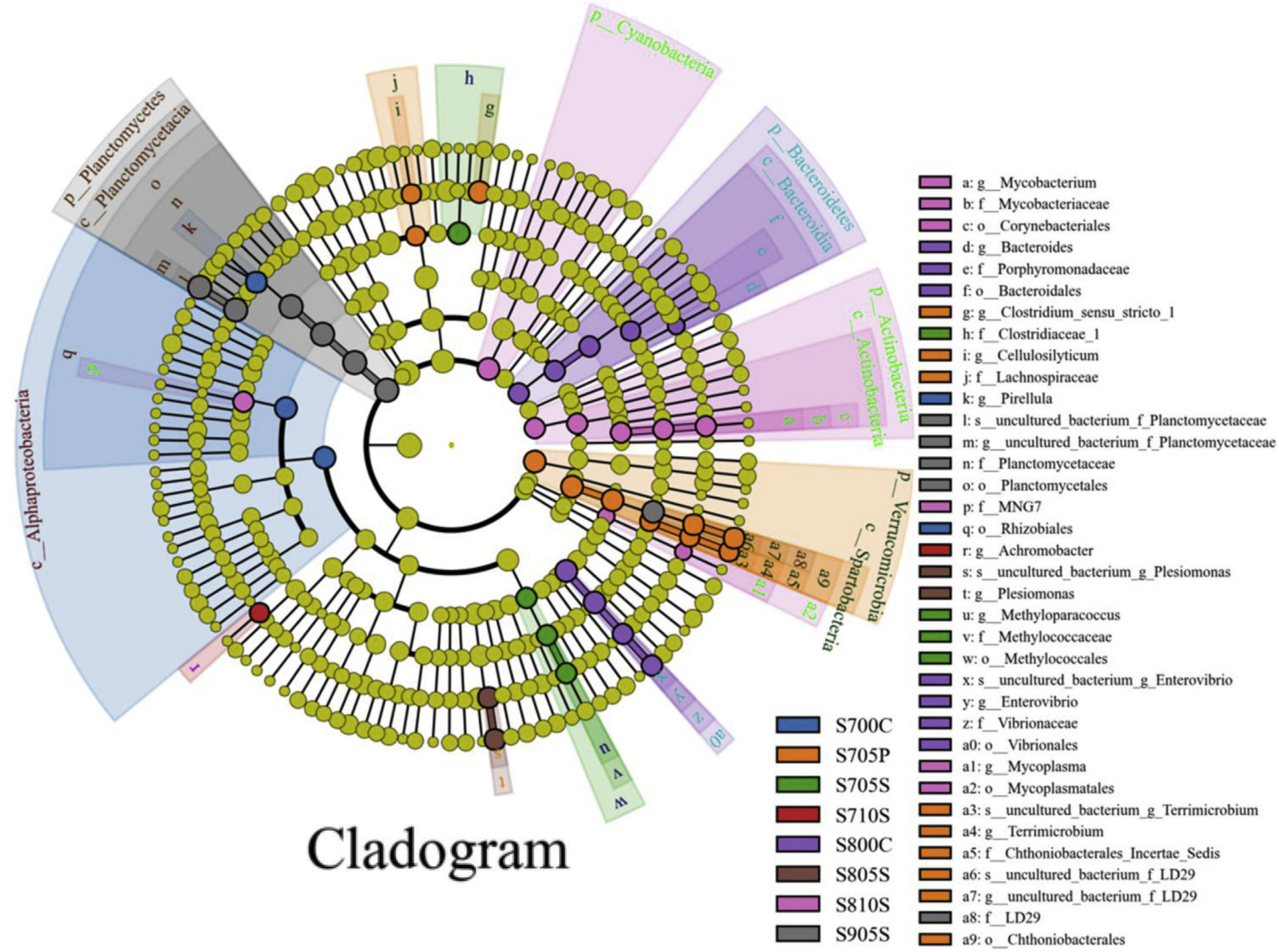

Fig. 3. LEFSe analysis of the evolutionary branching graph for cladogram biomarkers among different treatment groups from July to September. The circles radiating from the inside to the outside of the evolutionary branching map represent the classification levels from phyla to species; each locus on a given circle represents a classification at that level, and the diameter of the locus is proportional to its relative abundance; yellow indicates species without significant differences, while species with significant differences are colored according to the highest abundance for the grouping. Different colors represent different groupings, and nodes with different colors represent microorganisms that play an important role in the grouping. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Blastopirellula (together with *Rhodopirellula*, evolved from *Pirellula*) and Anammoxbacteria belong to the phylum Planctomycetes [33]. Previous studies have shown that Planctomycetes was more abundant in floating-bed-cultivation pond groups than in control groups [7]. In this study, uncultured bacteria *Rhodopirellula/Blastopirellula/Pirellula* were significantly more abundant in the S710S/S810S and S705P/S905P groups.

Cetobacterium is favored when putative probiotics are added to grass carp intestines [34,35]. These studies showed that the abundance of tilapia probiotics (such as *Lactococcus*, *Cetobacterium*, and *Rhodobacter*) was affected less by streptococcal infection than in the controls [34]. In the present study, we found that the abundance of *Cetobacterium* was significantly higher in S705P vs. S705S and lower in S800C vs. S810S and S800C vs. S805P. This suggests that cultivation of floating beds with Chinese medicinal herbs can improve fish health through intestinal changes [34,35] or anti-inflammatory activity [7].

Macellibacteroides and *Anammoximicrobium* present in sulfur- and iron-rich wastewater sediment may promote the biological adaptation to such complex environments [36]. *Macellibacteroides* abundance significantly increased in the *P. cuspidatum* cultivation group in July (S700C vs. S705P↑, S705P↑vs. S705S, S705P↑vs. S905P, S800C↑vs. S805P), while that of *Candidatus Anammoximicrobium* was higher in the S705P group than in the S700C group. When the *Macrophyte acorus calamus* is used to perform pyrene and benzo [a]pyrene degradation, the abundance of aerobic bacteria (*Vogesella*, *Pseudomonas*, *Flavobacterium*, and *Rhizobium*) increases, while anaerobic bacteria (*Longilinea*, *Bellilinea*, *Desulfobacca*, and *Anaeromyxobacter*) accumulate in the groups with sediment microbial fuel cells [37]. *Pseudomonas* significantly increased (S705P↑ vs. S705S) and *Desulfobacca* decreased (S700C↑vs. S705P), which demonstrated that cultivation with *P. cuspidatum* favored aerobic over anaerobic bacteria. Planting with a 10% combination of *Houttuynia cordata Thunb*, *Mentha haplocalyx Briq*. and *Ipomoea aquatica Forsk* may result in the increased oxygen consumption supported by our filed research.

Immune cell activation increased the transcription of pro- and anti-inflammatory cytokines. When we added rutin [5] and resveratrol [6] to tilapia feed, the levels of pro- and anti-inflammatory cytokines significantly increased and decreased, respectively. In this study, the levels of both blood and hepatic IL-10 (S805P), IFN-γ (S805P, S905S, S905P, and S910S) significantly increased in fish cultivated in beds planted

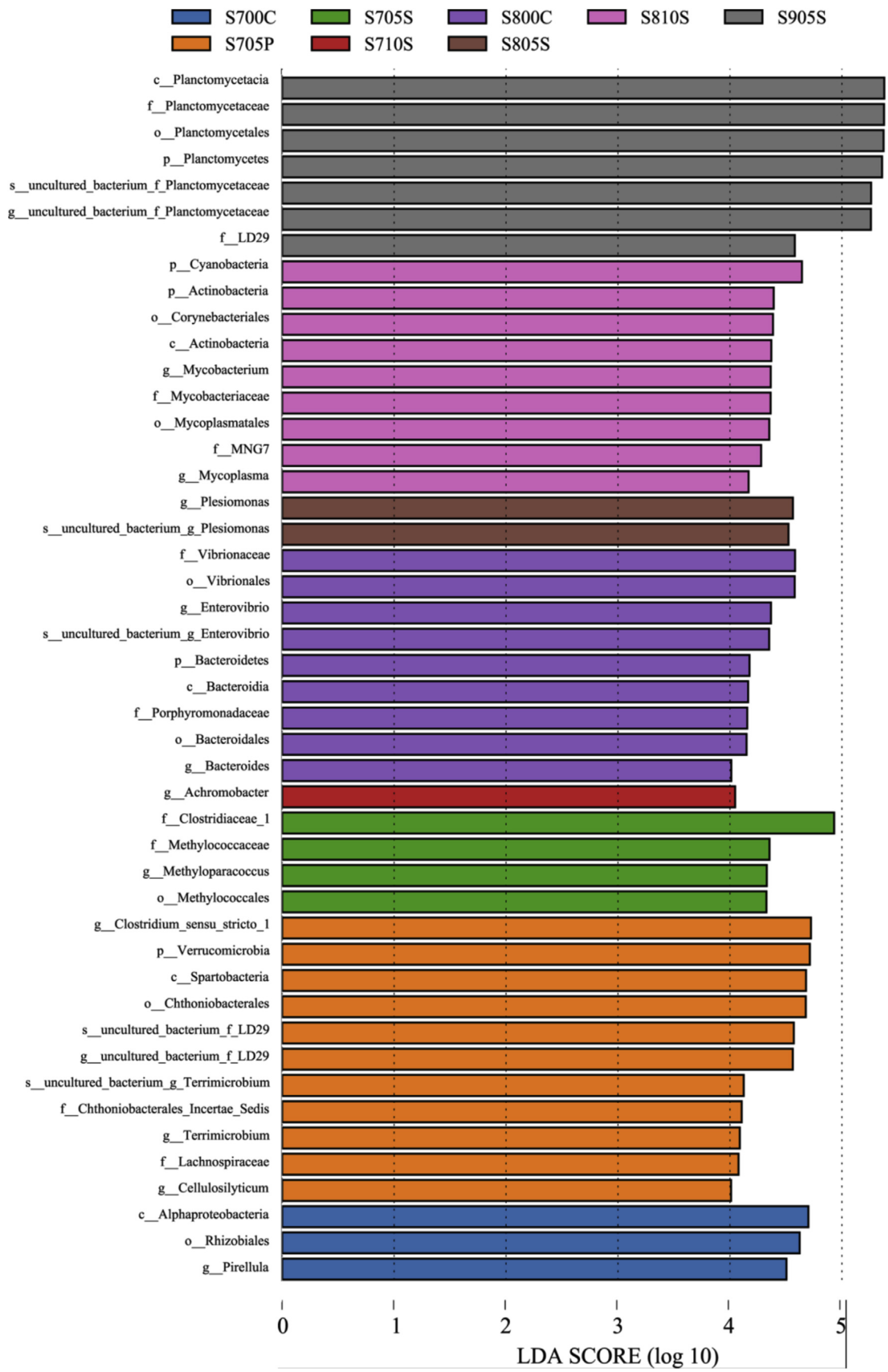

Fig. 4. The significant histogram among different treatment groups from July to September based on the LDA value (LDA score > 4.0). The length of the histogram represents the influence of different species based on LDA Score, and different colors represent the species of different groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

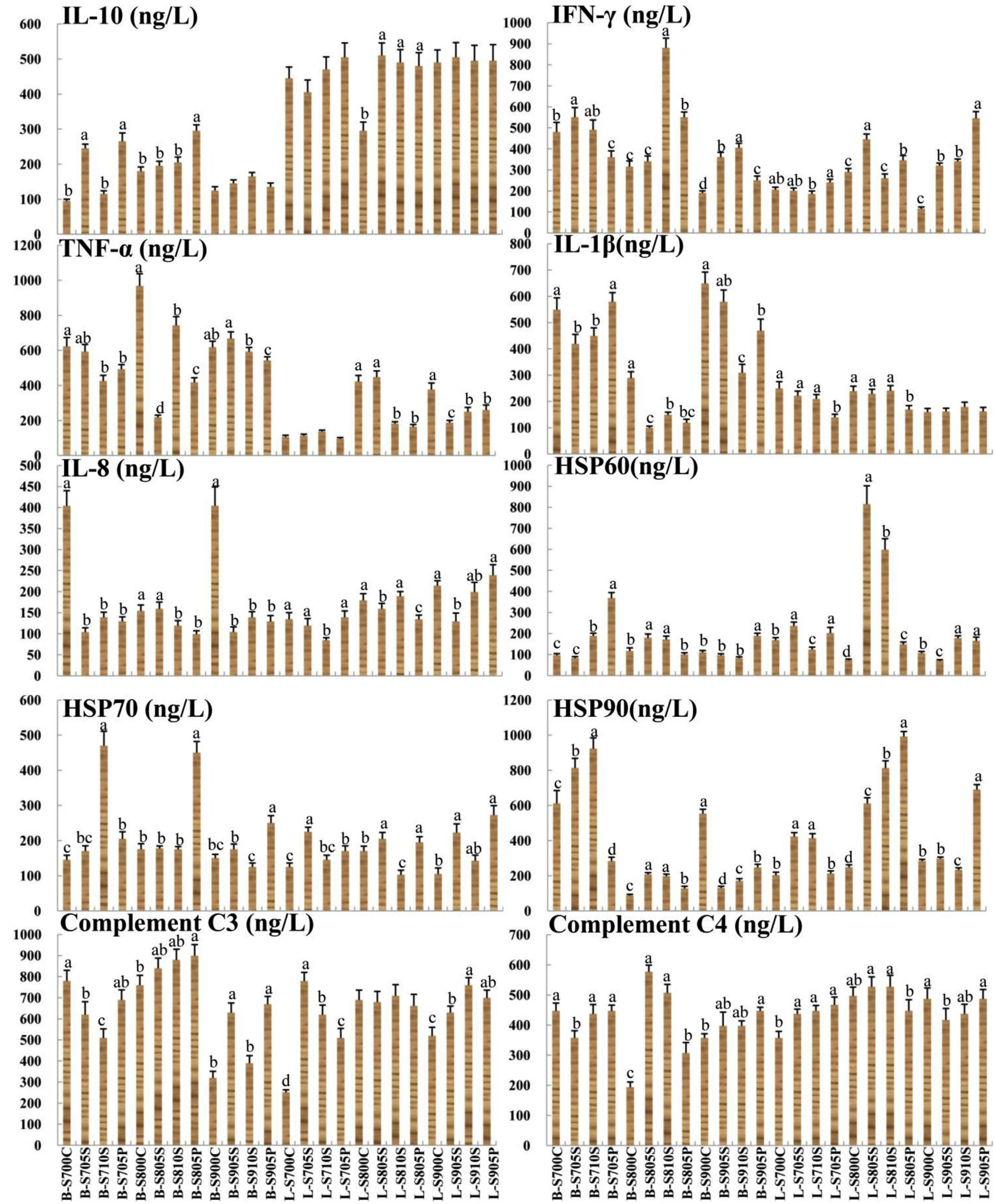

Fig. 5. Fish protein concentrations assessment for different floating bed cultivation groups (n = 3).

with Chinese herbs, while TNF-α (10% planting-area and *P. cuspidatum*), IL-1β (*P. cuspidatum*), and IL-8 (S905S) significantly decreased. Lipid mediators are important endogenous regulators of inflammation and apoptosis [38], and have been associated with the cytokine network of the immune system [39]. Resveratrol is mainly accumulated in the roots of *P. cuspidatum* and GIFT often bite these roots when *P. cuspidatum* is used as a floating bed thereby disturbing the innate immunity of the fish [40]. Resveratrol's effect on the immune system may be associated with tumor necrosis factor-alpha (TNF-α) [41]. High carbohydrate levels increase the transcription levels of *hsp60* and *hsp70* [42]. *IFN-γ*, *TNF-α*, *IL-1β*, and *hsp70* transcription levels increase in tilapia fed with the antimicrobial peptide, Natucin P, which suggests that it may enhance their antioxidant capacity and innate immunity. Natucin P might therefore be a potential alternative to antibiotics when used as a feed additive [43]. Intestinal *TNF-α* and *hsp70* are upregulated in tilapia fed microbial phytase (1000 U/kg) [44]. Fructooligosaccharide (0.4%) increased *hsp70* and *hsp90* levels in blunt snout bream (*Megalobrama amblycephala*) under high-ammonia stress [45]. The different responses to rutin and resveratrol (*hsp90a*) may be due to the time window between transcription and translation [46]. All of these observations suggest that long-term resveratrol administration or ingestion of *P. cuspidatum* roots may improve the immune system through transcriptional regulation of pro- and anti-inflammatory cytokines [5,6]. Our previous studies demonstrated that medicinal plant extracts (rutin and resveratrol) can be absorbed by the intestine [5,6], resulting in inflammatory responses and an advantageous shift (increased beneficial and decreased harmful bacteria) in gut microbiota [7,47]. It has been shown that the gut microbiome may affect immunomodulation [48], disease resistance [49], and adaptive immunity in fish [50], and this has been directly supported by our recent work [7]. The present study showed that floating-bed cultivation with medicinal plants increased the total yield of tilapia 1.12-fold. Furthermore, fish farmers earned additional income through selling the plants to townspeople or to plant extract companies.

The concentrations of complements C3 and C4 significantly increased after 4 weeks in fish fed diets of 3% stinging nettle (*Urtica dioica*), while the immune responses of fish fed 2 and 3% diets improved after 8 weeks [51]. Complements C3 and C4 have been shown to significantly increase ammonium acetate, whereas exogenous taurine can mitigate ammonia toxicity in fish [52]. Complements C3 and C4 significantly increased in the S905S group but decreased in floating beds cultivated with 5% *P. cuspidatum*, which suggests that harmful substances from Chinese medicinal herbs may result in responses leading to decreased complement C3 and C4 levels [53]. Complement C3 and C4 levels decreased in fish fed threonine-deficient diets (0.58% threonine) or diets high in threonine (2.58% threonine) [17]. It has been suggested that orange-peel-derived pectin as a feed additive promotes growth in *O. niloticus* [54]. Whether extracts from *P. cuspidatum* could be a beneficial feed additive has not been determined.

Researchers prefer to use Chinese medicinal herbs such as *Thymus daenensis* (antibacterial and antioxidant with very low toxicity used against *Staphylococcus aureus*) [55], *Ruta graveolens* (*Enterococcus fecalis*) [56], Thai piperaceae plants (*Toxoplasma gondii*) [57], *Rubus parvifolius* L. (antibacterial activity, volatile oil) [58], *Allium sativum* L. Fam. Liliaceae (garlic) [59], *P. cuspidatum* [40], etc., instead of antibiotics against pathogenic bacteria. However, feed additives with active compounds from medicinal herbs can also be used to combat gastrointestinal dysfunction (flavonoid glycosides of *Polygonum capitatum*) [60] and to confer renoprotective effects (*Mentha piperita*) [61]. Our previous findings demonstrated that the proportion of beneficial and harmful microbial taxa increased and decreased respectively with increasing concentrations of medicinal plant extracts [7]. Floating cultivation with Chinese medicinal herbs resulted in probiotic-like effects [47], and it highlighted the potential importance of bacterial interactions in influencing the stability of health-associated gut microbial communities.

The total production of tilapia in China has reached 1.6 billion tons, and heavily-fed tilapia grow faster between the months of July and September. Because of environmental pollution from feed, an effective strategy to reduce infection and disease is dietary supplementation with immunopotentiators. The final goal of the current study was to determine whether the reported immune enhancement [5–7] is sustained from development to harvest. Water and food quality are very important factors affecting fish health. It is well documented that the immune response of fish is seasonal [62] and that innate immunological factors are strongly affected as reflected by significant changes in serum complement concentrations [63]. It is also suggested that seasonal changes can affect humoral immune responses [64]. The present study showed that floating bed cultivation with Chinese medicinal herbs not only enhanced water quality indexes as we previously reported [65], but also has a sustained effect from fish development to harvest.

Author contributions

Y.Z., X.W.B., J.Z.C. conceived and designed the experiments; Y.Z. analyzed the data; L.P.Q., W.W., G.D.H. contributed reagents/materials/analysis tools; Y.Z. contributed to figures preparation; Y.Z. prepared and wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.042.

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