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Effects of prebiotic mixtures on growth performance, intestinal microbiota and immune response in juvenile chu's croaker, *Nibea coibor*

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ABSTRACT

Prebiotics has been known to be growth promoter and immunostimulant in aquatic animals. In this study, we investigated the effects of prebiotics on growth performance, intestinal microbiota, short-chain fatty acids (SCFAs) production and immune response of the marine fish, juvenile chu's croaker (*Nibea coibor*). The fish were fed IG (including 0.5% inulin and 0.5% GOS), GS (0.5% GOS and 0.5% D-sorbitol), IGS (0.33% inulin, 0.33% GOS and 0.33% D-sorbitol) or control diets for 8 weeks. The results showed that the growth performance of the fish was promoted by IG and GS, but not by IGS. The intestinal microbiota in NDC (non-digestible carbohydrates, NDC)-supplemented groups was clearly separated from that of the control, and the highest Shannon and Simpson diversity indices were observed in the IGS group. In the intestine of the croaker, Proteobacteria, Firmicutes, and Bacteroidetes were dominant; among them, 24 taxa revealed a significant difference among groups. Most of these bacteria are able to produce SCFAs, which were significantly increased in all NDC-supplemented groups. Moreover, NDCs were found to activate the immune system of the fish by modulating the serum complements, cytokine levels, lysozyme activities and antioxidant capacity. Furthermore, the results of this study revealed correlations among intestinal microbiota, SCFAs production, innate immunity, antioxidant capacity and digestive enzymes in the croaker fed NDCs. Taken together, our results demonstrated that NDC mixtures might promote growth performance, antioxidant capacity and immune responses of the croaker through modulating the composition of intestinal microbiota and the subsequent SCFAs production, which suggest that NDCs were efficient feed additives for marine fish.

1. Introduction

Aquaculture is one of the fastest growing food production industries and plays a significant role in meeting global protein needs for human [1]. The greater public awareness for healthy food has led to the banning of antibiotics used in aquaculture and an increased interest in the functional feed additives. Prebiotic is a promising feed additive, which is defined as “an effective substrate that is selectively utilized by host microorganisms conferring a health benefit” [2]. Since then, several non-digestible carbohydrates (NDCs), including oligosaccharides, plant cell wall polysaccharides, resistant starches and sugar alcohols were considered as potential prebiotics [3]. Previous studies revealed that prebiotics has beneficial effects on growth performance, feed utilization, carcass composition, intestine microbiota, SCFA levels, immunity system, and disease resistance in aquatic animals [4–6]. For example, galacto-oligosaccharides (GOS) has been widely used in various aquatic animals, such as Asian snakehead (*Channa striata*) [7,8], Caspian roach (*Rutilus rutilus*) [9], common carp (*Cyprinus carpio*) [10,11], hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) [12] and red drum (*Sciaenops ocellatus*) [13]. Also, inulin has benefits in Asian seabass (*Lates calcarifer*) [14], common carp (*C. carpio*) [11], Gilthead seabream (*Sparus aurata*) [15], Nile tilapia (*Oreochromis niloticus*) [16–18], rainbow trout (*Oncorhynchus mykiss*) [19] and white shrimp (*Litopenaeus vannamei*) [20,21]. Although D-sorbitol is not widely used in aquaculture, it has been demonstrated to augment the concentrations of acetate, propionate and butyrate in human fecal cultures *in vitro* [22].

The suggested mechanism action of prebiotics involves changes in bacterial communities, with an increased account of the beneficial bacteria which produce inhibitory compounds, compete with pathogens for nutrients or adhesion sites, inhibit virulent gene expression or

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disrupt quorum sensing in aquatic animals [4,23]. Other mechanisms have been proposed to be related to the induction of the end-products of bacterial fermentation, or interactions between prebiotics and pattern recognition receptors of the hosts [4,24,25]. Microbial fermentation of NDCs mainly stimulates the production of SCFAs, mainly acetate, propionate, and butyrate, which play important roles in keeping the microbial homeostasis and host health [26]. In aquatic animals, it has been reported that SCFAs and their salts serve as a source of energy, promote the proliferation of intestinal epithelial cells, exert the antimicrobial activity by lowering intestinal pH, modulate the composition of intestinal microbiota, and enhance the immune response by increasing the levels of immune components and regulating the expression of immune-related genes [4,27–32]. In grass carp, it has been shown that sodium butyrate enhanced the intestinal immune function of fish by elevating lysozyme and acid phosphatase activities, increasing the contents of complement C3, C4 and immunoglobulin M, down-regulating the pro-inflammatory cytokines (such as TNF-α, IL-1β, IL-6, and IL-8), and up-regulating the anti-inflammatory cytokines (such as IL-10, TGF-β1 and TGF-β2). In addition, sodium butyrate increased enteritis resistance of fish challenged with *Aeromonas hydrophila* [33]. However, the mechanisms of action of SCFA in fish immune system are poorly understood and need extensive research.

Chu's croaker, *Nibea coibor*, a member of the family Sciaenidae, is an economically important marine fish widely distributed in Japan, Korea, India and Southeast China [34]. In our previous works, it has been found the relationship between potential SCFA-producing bacteria and diets in the intestine of *Nibea* [35], and the ability of three NDCs (inulin, GOS, and D-sorbitol) to promote butyrate formation in *in vitro* intestinal cultures of *Nibea* (unpublished data). In this study, these NDCs were used to assess their effects on growth performance, intestinal microbiota, SCFA levels, immune response and antioxidant defense in juvenile chu's croaker. The findings of this study provide a better understanding on the beneficial stimulation of selective prebiotics on the chu's croaker gut microbes (including SCFA-producing bacteria), as well as the effects of microbes and SCFAs on the growth performance and immunity of hosts.

2. Materials and methods

2.1. Experimental diets

The control diet containing 45% crude protein and 9% lipid was purchased from Yuequn (Guangdong, China). The NDCs-added diets, including IG (including 0.5% inulin and 0.5% GOS), GS (0.5% GOS and 0.5% D-sorbitol), and IGS (0.33% inulin, 0.33% GOS and 0.33% D-sorbitol), were prepared in the feeding trials. The NDCs were purchased from Yuanye (Shanghai, China). Each of the NDC mixtures used in this study was dissolved in sterile purified water, and then sprayed homogeneously with commercial feed pellets. The control diet was sprayed with equivalent sterile purified water. Feeds were dried at 45 °C for about 48 h and stored at −20 °C.

2.2. Experimental design

All animal handling procedures were reviewed and approved by the ethics committee of the "Regulations for the administration of affairs concerning experimental animals". Healthy juvenile chu's croaker were obtained from a local hatchery in Raoping (Guangdong, China). The fish were acclimatized in sea cages (3.0 × 3.0 × 3.0 m) and fed a control diet for 2 weeks. An 8-week feeding trial was conducted at the experimental station of Shantou University. A total of 240 fish were randomly distributed into 12 cages (1.0 × 2.0 × 1.5 m, 20 fish per cage). Fish of every three cages were fed with experimental diets (either control, IG, GS or IGS diet). Fish in each cage were weighed for initial analysis and weights were determined weekly during the trial. Fish were fed to apparent satiation twice daily (06:00 and 18:00), and the feeding rate was approximately 3% of body weight adjusted accordingly. During the experiment, temperatures ranged from 20 to 30 °C, pH was about 8.8, salinity was about 36‰ and dissolved oxygen was higher than 5.5 mg/L.

2.3. Growth performance

At the end of the experiment (8 weeks), total fish biomass in each cage (20 fish per cage) was weighed. The data of total fish biomass in each treatment (3 replicate cages per treatment, 20 fish per cage) were used to calculate the growth parameters. Weight gain (WG), specific growth rate (SGR)), feed conversion ratio (FCR), and survival rate (SR) were calculated as follows:

Weight gain rate (%) = (final body weight - initial body weight)/initial body weight × 100

Specific growth rate (%/day) = (In final body weight- In initial body weight)/feeding days × 100

Feed conversion ratio (FCR) = total feed consumption/(final body weight - initial body weight)

Survival rate (%) = final number of fish/initial number of fish × 100

2.4. Sample collection

At the end of week 8, eight fish from each cage were randomly selected for sampling (blood, intestine and gut content). Blood samples were drawn, kept at 4 °C for 4 h, and centrifuged at 3500 rpm for 20 min. The serums of the six fish of each treatment were pooled, yielding four replicates per treatment and kept at −80 °C until immune parameters analysis. The intestinal tracts were aseptically removed with sterile dissecting instruments and contents were gently squeezed out and harvested into sterile tubes. After removal of contents, foreguts were sampled and harvested into new sterile tubes. The foreguts (for antioxidant parameters analysis) and gut contents (for intestinal microbiota and SCFAs analyses) of six fish of each treatment were pooled, respectively. All samples (four replicates per treatment) were stored at −80 °C until further analyses.

2.5. Intestinal microbiota analysis

DNA extraction was performed as described previously [36,37] with some modifications. One-hundred mg of content was separately suspended in a 1-mL CTAB lysis buffer and added 2.5 mg/mL lysozyme. The solution was mixed, vortexed and then incubated at 65 °C for 15 min. The lysates were purified twice by extraction with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v), and the residual phenol was removed by extraction with an equal volume of chloroform: isoamyl alcohol (24:1, v/v). After centrifugation at 12,000 rpm for 10 min, nucleic acids were precipitated with isopropyl alcohol at −20 °C, rinsed with 75% cold ethanol and suspended in 100 μL of sterile water. Finally, 1 μL of RNase A solution was added, and the mixture was incubated at 37 °C for 15 min. The extracted DNA was ascertained using 1% agarose gel electrophoresis and measured using NanoDrop ND-2000 (Thermo Scientific, USA), and diluted to 1 ng/μL using sterile water.

The V4 region of 16S rRNA gene was amplified by PCR using universal primers: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [38] with the barcode. Each PCR reaction was carried out in a 30 μL volume containing 10 ng of DNA, 15 μL of Phusion® High-Fidelity PCR Master Mix with GC buffer (New England Biolabs, USA), 0.2 μM forward and reverse primers and 2 μL of distilled water. Thermal cycling conditions were as follows: 1 min of initial denaturation at 98 °C; 30 cycles of 98 °C for 10 s, 50 °C for 30 s and 72 °C for 30 s; and a final elongation step at 72 °C for 5 min. The PCR products were purified by Gel Extraction Kit (Thermo Scientific, USA). Sequencing libraries were generated using Ion Plus Fragment

Table 1
Growth performance of chu's croaker fed the experimental diets.

Parameter	Control	IG	GS	IGS
Initial weight (g)	13.58 ± 0.07^{a}	13.33 ± 0.04^{a}	13.78 ± 0.32^{a}	13.65 ± 0.09^{a}
Final weight (g)	36.61 ± 0.43^{b}	40.56 ± 0.81^{b}	42.42 ± 2.14^{a}	40.00 ± 0.93^{b}
Weight gain (%)	169.6 ± 4.4^{b}	204.3 ± 7.1^{a}	207.4 ± 8.4^{a}	193.1 ± 8.7^{b}
Specific growth rate (% day^{-1})	1.77 ± 0.03^{b}	1.99 ± 0.04^{a}	2.00 ± 0.05^{a}	1.92 ± 0.05^{b}
Feed conversion rate	1.34 ± 0.08^{a}	1.22 ± 0.06^{a}	1.08 ± 0.04^{b}	1.24 ± 0.02^{a}
Survival rate (%)	88.33 ± 1.67^{a}	90 ± 2.89^{a}	90 ± 2.89^{a}	88.33 ± 1.67^{a}

The data of total fish biomass in each treatment (3 replicate cages per treatment, 20 fish per cage) were used to calculate above growth parameters. Control diet: basal diet; IG diet: 0.5% galacto-oligosaccharides and 0.5% inulin diet; GS diet: 0.5% GOS and 0.5% D-sorbitol diet; IGS diet: 0.33% GOS, 0.33% inulin and 0.33% D-sorbitol diet.
Values are presented as mean ± SE.
Values with different superscripts are significantly different between diets ($P < 0.05$).

Library Kit 48 rxns (Thermo Scientific, USA), and then assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific, USA). Finally, the libraries were sequenced on an IonS5^{TM}XL platform (Thermo Fisher Scientific, USA) and 400 bp single-end reads were generated.

2.6. Short-chain fatty acid analysis

The gut contents were suspended in ten volumes (w/v) of 2-ethylbutyric acid (internal standard) solution (pH was adjusted to 2–3 by adding H_2SO_4). Samples were mixed thoroughly (for 2 min) and centrifuged (13,000 rpm, 10 min, 4 °C). The supernatants were filtered (using a 0.2-μm nylon syringe filter) into 1.5 mL headspace vials for the SCFA analysis by GC6890N gas chromatography. Gas chromatography with a polar HP-INNOWAX capillary column (30 m × 0.25 mm x 0.25 μm) and an FID detector (Agilent Technologies, USA) was used. A 1-μL injection volume of sample was automatically injected into an inlet, which was kept at 230 °C with 10:1 split ratio. Nitrogen was used as the carrier gas at a flow rate of 1.0 mL/min. The detector temperature was 250 °C, and the injector temperature was 230 °C. The flow rates of nitrogen, hydrogen, and air as auxiliary gases were 25, 40, 450 mL/min, respectively. The initial oven temperature was 50 °C, which was maintained for 6 min and then raised to 230 °C at 15 °C/min and held at the temperature for 5 min.

2.7. Immune parameter and digestive enzyme analyses

The serum activity of lysozyme (LZM) was assessed using the detection kits (Jiancheng, Nanjing, China). The contents of complements (C3 and C4), immunoglobulin M (IgM), interleukin (IL-1β, −6, and −10), tumor necrosis factor α (TNF-α), and transforming growth factor β (TGF-β) were detected using ELISA Kits for fish (Yanhui, Shanghai, China).

The foreguts were homogenized in ten volumes (w/v) of cold physiological saline and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatants were collected and stored at −80 °C until the further analysis. Total superoxide dismutase (T-SOD), catalase (CAT), malondialdehyde (MDA), total antioxidant capacity (T- AOC), and activities of trypsin, lipase, and amylase were measured using the diagnostic reagent kits (Jiancheng, Nanjing, China) according to the manufacturer's instructions.

2.8. Bioinformatics

Single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Quality filtering and chimera checking of the raw reads was performed using Cutadapt (Version 1.9.1) [39] and UCHIME algorithm [40], respectively. Sequences were assigned to operational taxonomic units (OTUs) based on a similarity cutoff of 97% using Uparse software (Version 7.0.1001) [41]. The representative sequences from each OTU were taxonomically classified using the SILVA reference database with a confidence threshold of 80%. The alpha-diversity indices (Shannon and Simpson indices) were generated using QIIME (Version 1.7.0) [42]. Heatmap was plotted using Microsoft office excel 2007. Non-metric multi-dimensional scaling (NMDS) was used to visualize the weighted unifrac distances among samples performing in R software using the VEGAN package [43].

2.9. Statistical analyses

Quantitative data were expressed as the mean ± SE. Data comparisons between treatment groups were done running a One-Way ANOVA with Tukey's test in GraphPad Prism (Version 6,0). The *P*-value of less than 0.05 was considered to be statistically significant. LEfSe [44] was used to identify discriminatory bacterial groups among all experimental groups. When the alpha value of Kruskal-Wallis and Wilcoxon test was less than 0.05, and the logarithmic LDA score reached 3.5, the relative abundances of bacterial taxa were considered significant by LEfSe analysis. Correlation analysis of microbial genera, SCFA levels, innate immunity, antioxidant capacity, and digestive enzyme activities was subjected to Pearson correlation analysis using GraphPad Prism (Version 6.0). The arcsine-transformation of bacterial relative abundance was analyzed using Microsoft Office Excel 2007.

2.10. Data submission

The 16S rRNA gene sequences obtained in this study have been submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP182367.

3. Results

3.1. Survival rate and growth performance

The growth performance of fish during an 8-week feeding trial is shown in Table 1. The results revealed that the WG and SGR in the IG group were significantly higher than those in the control group ($P < 0.05$). Compared to the control group, the final weight, WG, and SGR were significantly higher, as well as FCR was significantly lower than those in the GS group ($P < 0.05$). No significant difference was observed in the survival rate and growth performance between IGS group and the control ($P > 0.05$).

3.2. Intestinal microbiota

A total of 1,186,143 quality-filtered V4 region reads were obtained from 12 samples, and the numbers of OTUs ranged from 666 to 1622 for each sample. An NMDS plot of these data showed that all three NDC-supplemented groups (IG, GS and IGS groups) were clearly separated from the control group (Fig. 1). The IGS group showed a higher

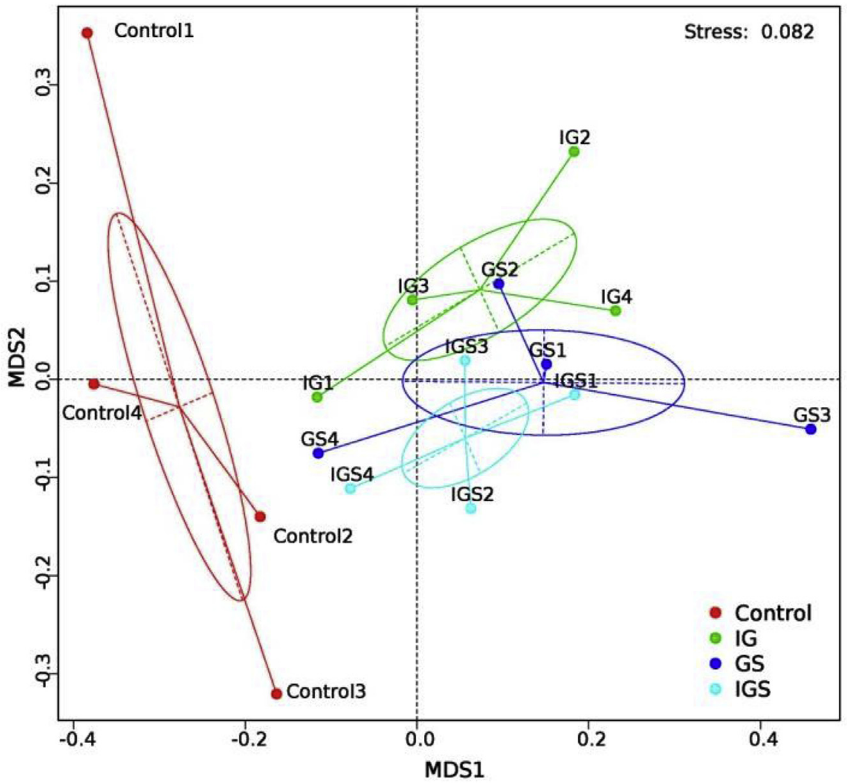

Fig. 1. Nonmetric multidimensional scaling (NMDS) plot of the intestinal microbiota of chu's croaker fed four different diets containing different non-digestible carbohydrates (NDCs). NMDS plot was based on weighted unifrac distances of the intestinal microbiota of fish fed four different diets (IG, GS, IGS, and control diet, respectively). Each point represents a sample and the colors represent the different treatments. Control diet: basal diet; IG diet: 0.5% galacto-oligosaccharides and 0.5% inulin diet; GS diet: 0.5% GOS and 0.5% D-sorbitol diet; IGS diet: 0.33% GOS, 0.33% inulin and 0.33% D-sorbitol diet. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Alpha diversity indices based on the intestinal microbiota of different treatments.

	Control	IG	GS	IGS
Shannon	6.96 ± 0.25^{ab}	7.31 ± 0.32^{b}	7.61 ± 0.42^{ab}	8.22 ± 0.20^{a}
Simpson	0.97 ± 0.003^{b}	0.98 ± 0.007^{b}	0.98 ± 0.006^{b}	0.99 ± 0.002^{a}

Control diet: basal diet; IG diet: 0.5% galacto-oligosaccharides and 0.5% inulin diet; GS diet: 0.5% GOS and 0.5% D-sorbitol diet; IGS diet: 0.33% GOS, 0.33% inulin and 0.33% D-sorbitol diet.
Values are presented as mean ± SE (four replicates per treatment).
Values with different superscripts are significantly different between diets ($P < 0.05$).

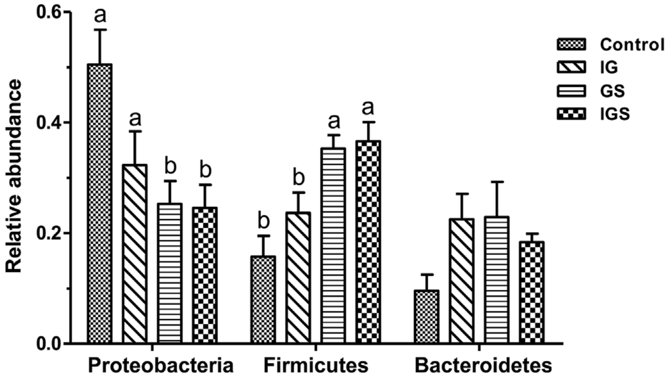

Fig. 2. The predominant phyla in the intestine of chu's croaker fed four different diets containing different non-digestible carbohydrates (NDCs). Control diet: basal diet; IG diet: 0.5% galacto-oligosaccharides and 0.5% inulin diet; GS diet: 0.5% GOS and 0.5% D-sorbitol diet; IGS diet: 0.33% GOS, 0.33% inulin and 0.33% D-sorbitol diet. Different superscripts suggest significantly different between diets ($P < 0.05$).

Shannon diversity (compared with the IG group) and the highest Simpson diversity (among four groups), whereas the IG and GS groups exhibited no differences compared with the control group (Table 2).

At the phylum level, three dominant phyla were identified in all groups, including Proteobacteria, Firmicutes and Bacteroidetes. Proteobacteria was increased, while Firmicutes was decreased in GS and IGS groups compared to either control or IG group (Fig. 2). At the genus level, the most abundant genera were identified as *Bacteroides* (control: 4.27%; IG: 10.39%; GS: 4.28%; IGS: 4.76%), *Acinetobacter* (control: 9.47%; IG: 7.19%; GS: 3.28%; IGS: 6.06%), *Lawaonia* (control: 7.51%), *Bifidobacterium* (IG: 7.45%), *Staphylococcus* (control: 5.73%) and *Prevotella 7* (GS: 5.16%).

A total of 24 taxa showed significantly different among the groups (Fig. 3). In GS group, a decrease in the relative abundance of Proteobacteria and an increase in the relative abundance of *Clostridium sensu strict* 1, Clostridiaceae 1, Clostridiales and Clostridia (phylum Firmicutes) were found. In IG group, a significant increase occurred in the relative abundance of *Bifidobacterium*, Bifidobacteriaceae, Bifidobacteriales (phylum Actinobacteria), *Parabacteroides* (phylum Bacteroidetes) and *Enterococcus durans* (phylum Firmicutes). Thirteen discriminant taxa in IGS group were classified as Firmicutes (i.e. Lachnospiraceae, *Ruminococcus flavefaciens* and *Blautia*), Verrucomicrobia (*Akkermansia muciniphila* and Verrucomicrobiaceae), Bacteroidetes (*Bacteroides nordii* and *Prevotella* 1), and Actinobacteria (*Paenarthrobacter*).

3.3. SCFA levels

The concentrations of SCFAs in the NDCs-supplemented groups were higher than that in the control (Fig. 4). The levels of butyrate and total SCFAs in the IG group were significantly higher than those in the control group ($P < 0.05$). Compared to the control, propionate, butyrate, and total SCFAs production were significantly higher in the GS group ($P < 0.05$). Levels of acetate, propionate, butyrate and total SCFAs in IGS group were also higher than that in the control group ($P < 0.05$).

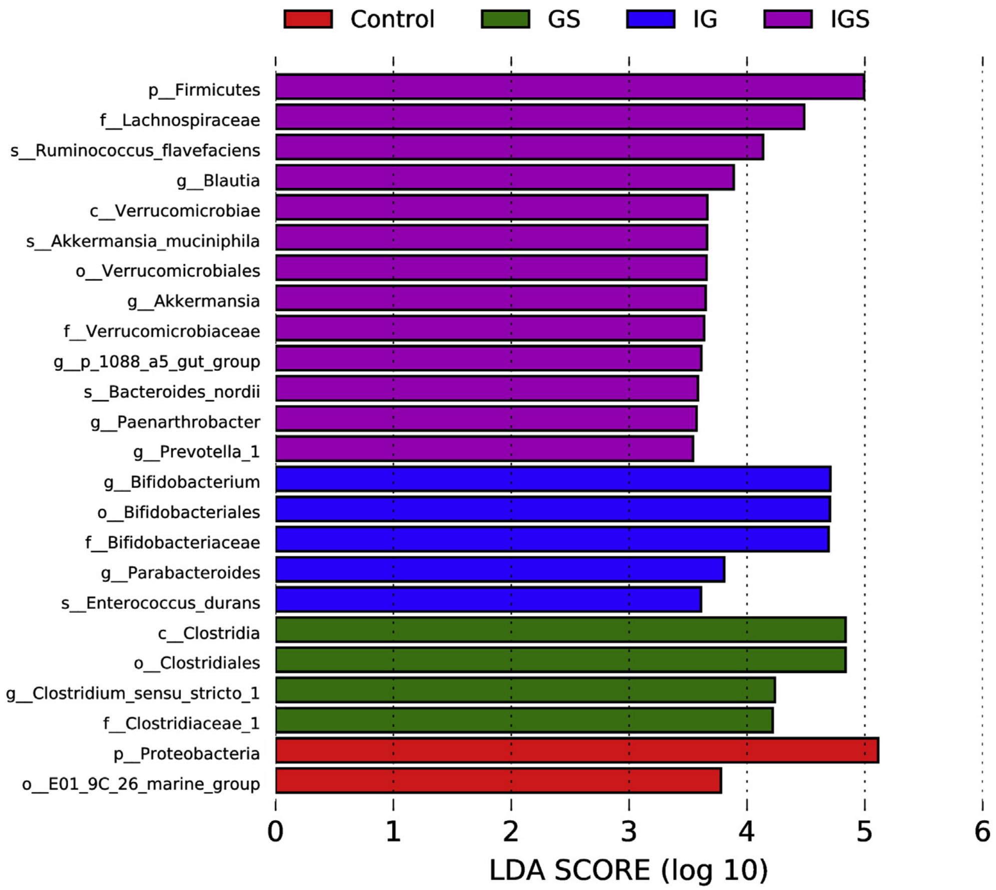

Fig. 3. Linear discriminant analysis (LDA) of the intestinal microbiota of chu's croaker fed four different diets containing different non-digestible carbohydrates (NDCs). The alpha value of Kruskal-Wallis and Wilcoxon test was less than 0.05 and the logarithmic LDA score reached 3.5, the relative abundances of bacterial taxa were considered significant by LEfSe analysis among four treatment groups. Control diet: basal diet; IG diet: 0.5% galacto-oligosaccharides and 0.5% inulin diet; GS diet: 0.5% GOS and 0.5% D-sorbitol diet; IGS diet: 0.33% GOS, 0.33% inulin and 0.33% D-sorbitol diet. The significantly different taxa include phylum (p), class (c), order (o), family (f), genus (g) and species (s).

3.4. Innate immunity, antioxidant capacity and digestive enzymes

The contents of C3, C4, IgM, IL-1β, IL-10 and TGF-β were significantly increased in all NDC-supplemented groups ($P < 0.05$) (Fig. 5). IL-6 and TNF-α were significantly increased in both IG and IGS groups ($P < 0.05$). Compared to the control samples, significantly increased activities of LZM, amylase and lipase (in IG group), LZM and lipase (in GS group), as well as a significantly increased content of T-AOC content and activity of amylase (in IGS group) were observed ($P < 0.05$). On the contrary, a significant decrease in intestinal MDA contents was found in all NDC-supplemented groups ($P < 0.05$). For CAT, T-SOD and trypsin activities, no significant differences were found when comparing NDC-supplemented groups and control group ($P > 0.05$).

3.5. Correlation analysis

To further elucidate the beneficial effect of the altered microbial community on SCFA, innate immunity, antioxidant capacity and digestive enzymes, a Pearson correlation analysis of the interaction matrix were performed in present study. The microbial taxa (at the genus level) were restricted to the discriminatory representatives (from LDA analysis) and potential SCFA-producers. As shown in Fig. 6, there was a significant positive correlation between the relative abundance of microbiota and SCFA contents in the intestine. It was found that 4, 9, 9 and 9 genera were significantly positively correlated with the levels of acetate, propionate, butyrate and total SCFAs, respectively. Among them, *Akkermansia* and *Paenarthrobacter* were significantly positively correlated with acetate and propionate, while *Anaerostipes*, *Blautia*,

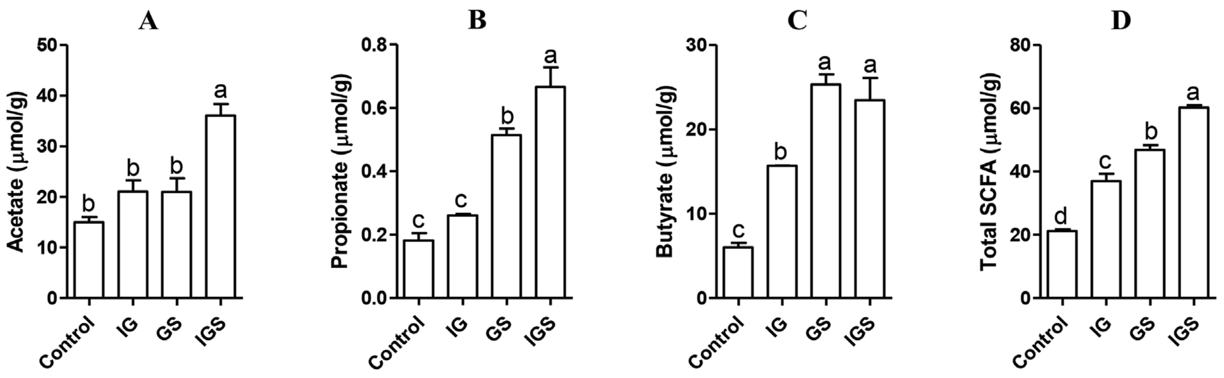

Fig. 4. Intestinal short-chain fatty acids (SCFAs) contents of chu's croaker fed four different diets containing different non-digestible carbohydrates (NDCs). (A) acetate, (B) propionate, (C) butyrate and (D) total SCFA in intestinal contents of the croaker. Control diet: basal diet; IG diet: 0.5% galacto-oligosaccharides and 0.5% inulin diet; GS diet: 0.5% GOS and 0.5% D-sorbitol diet; IGS diet: 0.33% GOS, 0.33% inulin and 0.33% D-sorbitol diet.

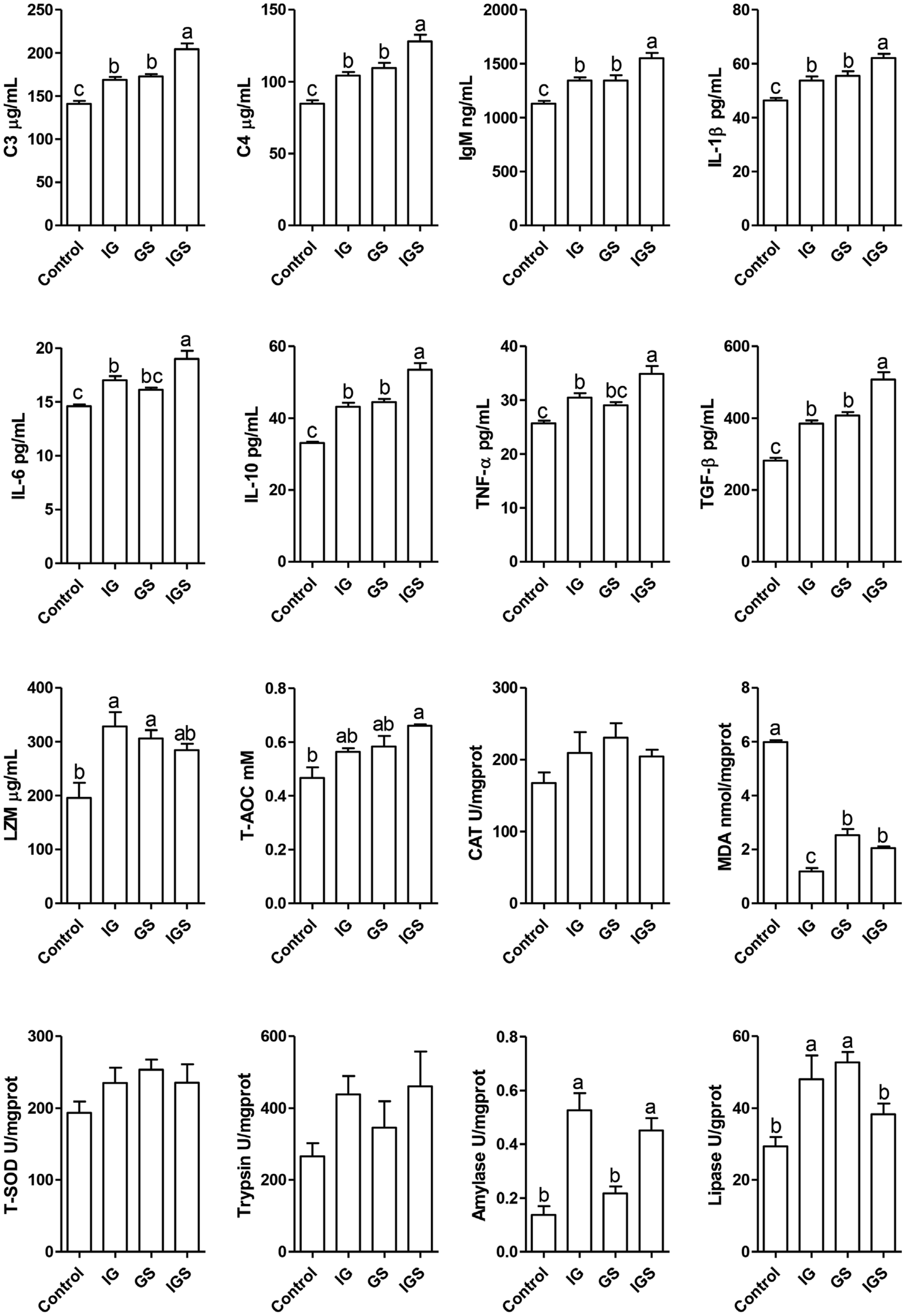

Fig. 5. Non-specific immunity parameters and digestive enzymes in the intestine of chu's croaker fed four different diets containing different non-digestible carbohydrates (NDCs). Control diet: basal diet; IG diet: 0.5% galacto-oligosaccharides and 0.5% inulin diet; GS diet: 0.5% GOS and 0.5% D-sorbitol diet; IGS diet: 0.33% GOS, 0.33% inulin and 0.33% D-sorbitol diet. Different letters indicate the significant differences between diets ($P < 0.05$).

Coprococcus and *Ruminococcus* were significantly positively associated with propionate and butyrate. *Eubacterium* was the only genus that showed a significantly positive correlation with acetate, propionate, butyrate and total SCFAs. Additionally, the relative abundance of microbiota and SCFA contents were significantly associated with immune parameters, antioxidant capacity and digestive enzymes. In particular, the results showed a significantly positive correlation between the immune parameters (such as C3, C4, IgM, IL-1β, IL-6, IL-10, TNF-α,

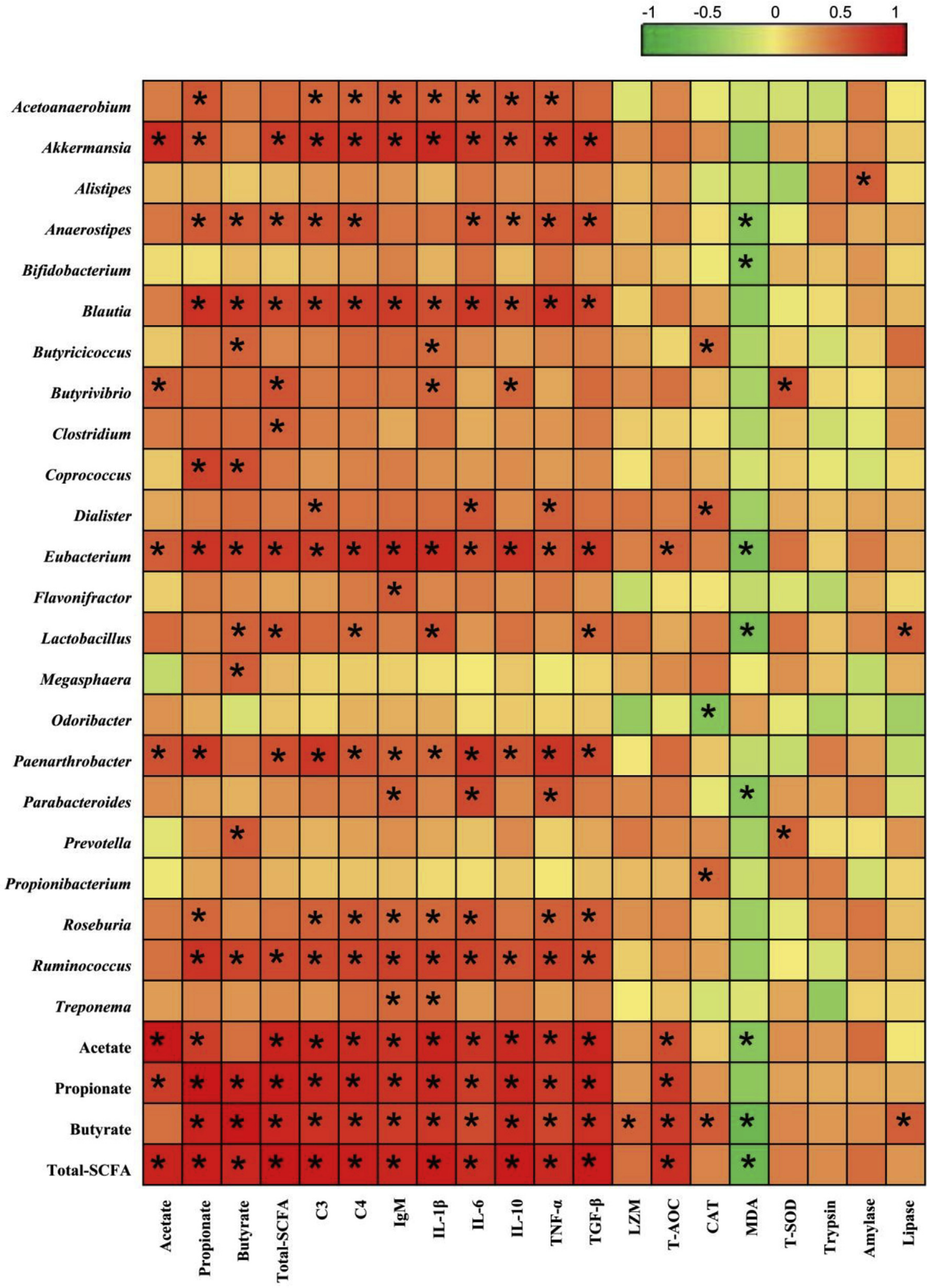

Fig. 6. Correlation analysis of representative microbial genera, SCFA, non-specific immunity parameters and digestive enzymes in the intestine of chu's croaker. The color is according to the Pearson correlation coefficient distribution. Red represents positive correlation, green represents negative correlation, and yellow represents weak correlation. Asterisks represent that the correlation was significant (*$P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

TGF-β) and either bacterial genera (*Akkermansia*, *Blautia*, *Eubacterium*, *Paenarthrobacter*, *Ruminococcus*) or concentrations of SCFAs (acetate, propionate, butyrate and total SCFAs). However, MDA was significantly negatively associated with bacterial genera (i.e. *Anaerostipes*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Parabacteroides*), and production of acetate, butyrate and total SCFAs as well. For digestive enzymes, *Alistipes, Lactobacillus* and butyrate were significantly positively correlated with amylase and lipase, respectively.

4. Discussions

Being the potential substitute of antibiotic, prebiotics has been commonly studied in aquatic animals. In this study, three dietary inulin, GOS, and D-sorbitol mixtures were selected to assess their effects on growth performance, intestinal microbiota, short-chain fatty acids, immune response and antioxidant defense of juvenile chu's croaker. The results of this study would help explore how the prebiotics selectively stimulate the growth of beneficial microbiota (including SCFA-producing bacteria), as well as how this microbiota and its fermentative products (SCFAs) affect host growth, immune response and antioxidant activity. These findings provided a better understanding of the molecular mechanisms of host-microbiota interaction in marine fish, which may be important in healthy and safe aquaculture production.

We found that both IG and GS groups improved the growth performance (i.e. WG, SGR) of chu's croaker, while no significant difference was observed in survival rate and growth performance between IGS and the control groups. The inconsistent results are likely due to differences in types or doses of prebiotics used [4]. In giant freshwater prawn *Macrobrachium rosenbergii*, it has been showed that 0.4% fructooligosaccharide (FOS) increased weight gain and specific growth rate for length and weight of the prawns. However, 0.1% FOS and 2% FOS had no effect on prawns' growth [45]. Another study has reported that GOS improved the growth performance of common carp (*Cyprinus carpio*), including final weight, weight gain, SGR and FCR. However, no significant difference was observed in FOS and inulin group [46]. On the other hand, several studies have reported that prebiotics is able to improve growth performance of fish species [9,16] and are mainly related to an enhancement in nutrient availability through changes in activities of digestive enzymes or intestinal morphology [4]. Our results revealed that the improved growth performance in chu's croaker might be related to the increased activities of lipase in both IG and GS groups. Moreover, it was found that *Alistipes* and *Lactobacillus* were significantly positively correlated with amylase and lipase, respectively. Similarly, several *Lactobacillus* species have been reported to show lipase activity [47]. However, it is difficult to confirm the contribution of gut microbiota exogenous enzymes to the overall digestive enzymatic activity in fish [4], and the related studies need to be investigated in the future.

In addition, all NDC-supplemented groups were able to alter the community structure of the intestinal microbiota in chu's croaker *in vivo*, as group IG, GS and IGS were clearly separated of the control group in NMDS plot. The highest Shannon and Simpson diversity indices and microbial diversity could be observed in IGS group. Similarly, mannan oligosaccharides (MOS) increased the species richness and diversity in the intestine of gilthead sea bream (*Sparus aurata*) [48]. The phyla Proteobacteria, Firmicutes and Bacteroidetes were predominant in the intestine of chu's croaker, which was similar to previous studies in zebrafish (*Danio rerio*) and *Nibea diacanthus* [35,49,50]. Proteobacteria is generally associated with dysbiosis or an unstable gut microbial community [51], while Firmicutes and Bacteroidetes are responsible for the metabolism of dietary fiber and other undigested food remnants [52]. In this study, GS and IGS significantly decreased the relative abundance of Proteobacteria and increased the relative abundance of Firmicutes in the intestine of chu's croaker. Moreover, IG enriched *Bifidobacterium*, *Parabacteroides* and *Enterococcus durans,* and GS enriched *Clostridium sensu strict 1*, while IGS significantly increased the relative abundance of *Ruminococcus flavefaciens*, *Blautia*, *Akkermansia muciniphila*, *Bacteroides nordii*, *Prevotella 1* and *Paenarthrobacter*. The prebiotic action on bacterial gut communities works mainly through its fermentation by specific beneficial bacteria, which possess the necessary enzymes to hydrolyse prebiotics and thus are favored relatively to other, eventually less beneficial, bacteria [4]. In particular, most of these bacteria are SCFA-producers, which play an important role in keeping the microbial homeostasis and host health [3,26,56–58]. As previously reported, the non-digestible GOS, D-sorbitol and inulin produced augmented acetate, propionate and butyrate in *in vitro* human fecal cultures [22,59,60]. Similarly, the contents of intestinal SCFAs were increased in all three NDC-supplemented groups, with the greatest changes observed in IGS group. These results suggested that three dietary inulin, GOS, and D-sorbitol mixtures could selectively promote the growth of specific SCFA-producing bacteria, and subsequently increase the SCFA productions in the intestine of chu's croaker. Similar results were also found in Siberian sturgeon (*Acipenser baerii*) fed arabinoxylan-oligosaccharides (AXOS-32-0.30). AXOS-32-0.30 could stimulate the growth of lactic acid bacteria and *Clostridium* sp. with increased concentrations of acetate, butyrate and total SCFAs observed in the hindgut of Siberian sturgeon [61].

Moreover, NDCs activated the immune system by modulating the serum complement and cytokine levels, lysozyme activity and intestinal antioxidant capacity in our study. The contents of serum C3, C4, IgM, IL-1β, IL-10, TGF-β were significantly increased in all NDC-supplemented groups, while IL-6 and TNF-α were significantly increased in IG and IGS groups. Besides, IG and GS groups significantly increased the LZM activities, and IGS significantly increased T-AOC content; whereas a decreased intestinal MDA contents appeared in all NDC-supplemented groups. Similarly, the increased activities of serum IgM levels, LZM, SOD and complements have been reported previously in several fishes fed different prebiotics at different supplementation levels [5,14,15]. Generally, most studies have suggested the beneficial effects of prebiotics in terms of improving the immune system, by increasing the expression of anti-inflammatory cytokines, while reducing the expressions of pro-inflammatory cytokines [6,62]. On the contrary, herein the levels of proinflammatory cytokines (i.e. IL-1β, IL-6, and TNF-α) were significantly increased upon the stimulation of NDCs. This could be explained by the differences in kinds and dosage of prebiotics, sampled tissues, intestinal section or culture conditions among studies. For example, it has been reported that GOS is able to induce the expression of TNF-α in the intestine but not in head kidney, while inulin increased the expression of such gene in both intestine and head kidney of common carp (*Cyprinus carpio*) [11]. In the case of rainbow trout (*Oncorhynchus mykiss*) after challenge with *Aeromonas salmonicida*, the different gut regions revealed the differences in the immune response, with an up-regulation of IL-1β, IL-8, TNF-α and interferon INFγ in the foregut, and down-regulation of TGF-β in the hindgut [63].

Dietary prebiotics are generally reported as immunostimulants in fish, and it is reported that prebiotics activate fish innate immune system in two ways: by directly stimulating the innate immune system, or by enhancing the growth of commensal microbiota [4,24,64]. In our study, the serum complement and cytokine levels, intestinal antioxidant parameters and lipase activity were significantly correlated with dietary prebiotics, SCFA production and intestinal microbiota. Although most of these underlying mechanisms remain unclear, the findings could have important implications for future studies regarding an explanation of the mechanisms that prebiotics are fermented by intestinal microbiota and enhance the production of SCFAs and their role in affecting the host health.

In conclusion, the present study investigated the interaction between the host (the chu's croaker) and the intestinal microbiota under stimulation of three NDC mixtures. We found that different NDC mixtures exhibited different roles in modulating intestinal microbiota, affecting the SCFA productions, innate immunity, antioxidant capacity, digestive enzyme activities and growth performance of chu's croaker to varying degrees. The study findings suggest a potential substitute of three NDC mixtures for antibiotics, thereby contributing to the sustainability of aquaculture and the safety of aquatic products, as well as our understanding of the molecular mechanisms of host-gut microbiota interaction in marine fish species.

Conflicts of interest

There is no conflict of interest to be declared.

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