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Chinese yam peel enhances the immunity of the common carp (*Cyprinus carpio* L.) by improving the gut defence barrier and modulating the intestinal microflora

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ABSTRACT

The Chinese yam peel (CYP) is a by-product of yam processing that is rich in various nutrients and a good source for feed additives. This study investigated the effects of CYP on the intestinal microbiota and gut defence barrier of the common carp (*Cyprinus carpio* L.). Different groups of experimental fish were fed a normal control diet (NC), a low CYP diet (LYP) and a high CYP diet (HYP) for 8 weeks. After the feeding trial, the fish were assessed for intestinal enzyme activity, intestinal histology, immune-related gene expression, intestinal SCFAs and intestinal microbiota. Our results indicated that the intestinal integrity and antioxidant enzyme (CAT and SOD) activity in the common carp were enhanced following CYP supplementation. The mRNA levels of anti-inflammatory (*TGF-β*), tight binding protein (*occludin* and *ZO-1*) and pathway factor genes (*TLR4* and *NF-κB*) were significantly upregulated in the HYP group ($P < 0.05$), which was accompanied by an increase in the level of pro-inflammatory *IL-1β* in the gut ($P < 0.05$). High-throughput sequencing revealed that *Fusobacteria*, *Proteobacteria*, and *Bacteroidetes* bacteria were most abundant in the microbial community in the gut of the common carp. The relative abundances of *Bacteroides*, *Flavobacterium* and *Lactobacillus* were increased, while the abundances of pathogenic microorganisms such as *Enterobacteriaceae, Shewanella, Pseudomonas* and *Vibrio* were reduced after treatment with CYP. Furthermore, the concentrations of acetic acid, propionic acid, butyric acid and total short-chain fatty acids (SCFAs) in the gut were also increased ($P < 0.05$). Finally, our results revealed correlations between gut microbiota, SCFAs, non-specific immunity and antioxidant enzymes in CYP-fed carp. These results suggest that CYP-supplemented feed could improve the immunity of the common carp by modulating the intestinal microflora and enhancing the gut defence barrier and has the potential to be used as an immunostimulating feed additive in aquaculture.

1. Introduction

The common carp (*Cyprinus carpio* L.) is an economically important freshwater fish that is widespread in northern China. However, the aquaculture environment has been deteriorating, leading to frequent fish diseases, which have generated great obstacles to the healthy development of the aquaculture industry [1]. Although chemical drugs exert important effects on preventing different kinds of aquatic diseases [2], long-term or excessive use of such drugs will destroy immune defences, increase the amounts of drug residues and enhance drug resistance against pathogens in fish [3]. As types of food additives, Chinese herbal medicine or plant extracts, such as *Paeoniae* radix [4], *Coptis chinensis* Franch [5]. and triptolide [6], are rich in various nutrients, trace elements and biologically active substances. Experiments in humans and rats have identified their biological effects, including the enhancement of immunity, the promotion of growth, the inhibition of pathogens, and the protection of the liver and gallbladder [7–10]. Additionally, several other herbal medicines have been applied in aquaculture, such as *astragalus* [11] and *radix rehmannia* [12], for intensive fish farming. They can be widely used as new feed additives instead of antibiotics [13]. However, the results of studies on the effects of Chinese yams on aquaculture are not clear.

The Chinese yam (*Dioscorea opposita* Thunb.), which is native to northern China, is currently widely grown in most northern areas of

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China and has been used in Chinese medicine for a long time to improve gastrointestinal function, reduce anorexia, and treat diarrhoea [14]. To date, there have been many studies on the extraction and function of various components of the Chinese yam [15,16]. However, few studies on the use of yam peel generated as processing waste have been reported. In yam processing, the yam peel is usually discarded as waste, which not only pollutes the environment but also wastes resources [17]. A previous study found that yam peel contains various active ingredients, such as diosgenin, polysaccharides, flavonoids and polyphenols [18]. Therefore, how to utilize the peel effectively and promote its secondary development is a key problem for the sustainable development of agriculture and environmental protection.

The gut is the largest and most complex ecosystem in organisms. Intestinal microorganisms and their metabolites are involved in many physiological activities, such as nutrient metabolism and immune regulation [19]. Conversely, intestinal flora disturbances will lead to the dysregulation of the immune response and the destruction of the intestinal barrier, leading to disease [20]. It is worth noting that the use of Chinese herbal medicine could have a beneficial effect on immunity by affecting intestinal flora stability and regulating intestinal bacteria [21]. Previous studies have found that yam can improve the composition of the intestinal flora, increase the amounts of short-chain fatty acids, and protect the intestinal barrier to promote the health of mice [22,23]. Yam polysaccharide is the main active component that can regulate the intestinal flora and restore intestinal homeostasis [24]. However, the effects of the yam peel on gut microbiology are unclear.

In this study, we aimed to investigate the effects of Chinese yam peel on the immunity and intestinal microbes of the common carp. For this purpose, intestinal morphology, immune-related gene expression, the intestinal microbial community structure and SCFA metabolites were studied.

2. Materials and methods

2.1. Experimental animal acclimation and cultivation

All animal experiments conducted in this study were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Henan Normal University. Common carp were purchased from the Yanjin Fishing Ground of Xinxiang City, Henan Province. Chinese yam peel was purchased from Kondarl Feed Co., Ltd. The main active components are listed in additional file 1. The fish were examined thoroughly and acclimatized to the outdoor cement tank (400 cm × 200 cm × 120 cm) with aerated freshwater. After 2 weeks of acclimation, 225 carp (75.19 ± 1.56 g) in good health were selected. They were randomly divided into 3 groups, the normal control group (NC), the low-CYP diet group (1% added, LYP) and the high-CYP diet group (2% added, HYP), including triplicates in each group comprising 75 fish. The experiment lasted for 8 weeks. Feeding was carried out 4 times a day according to a 3% weight, which was adjusted every two weeks, at 8:30, 11:30, 14:30 and 17:30 daily. The water quality parameters during the experiment were checked regularly and maintained as follows: dissolved oxygen concentration of 6 mg/L, amino nitrogen level of 0.01 mg/L, water temperature of 26–28 °C and pH 7.5–7.9.

2.2. Sampling collection

After the feeding experiment, all the fish were fasted for 24 h and anaesthetized with MS-222 (10 mg/L) solution before sampling. Each fish body was cleaned with 70% ethanol. Guts of 6 fish from each trial bucket were sampled for tissue RNA extraction and enzyme activity detection. Another 6 fish were used to obtain samples from the mid-gut, which were used for morphological observation by haematoxylin-eosin staining (HE) and scanning electron microscopy (SEM). The intestinal contents from another 8 fish were carefully removed by gently squeezing the mid gut with sterile forceps in a sterile environment. One part of the sample was subjected to DNA extraction, and the other part was used for the determination of short-chain fatty acids. If the sample could not be extracted immediately, the sample was frozen in liquid nitrogen for at least 4 h and then quickly transferred to a −80 °C refrigerator for subsequent DNA extraction.

2.3. Determination of intestinal enzyme activity

The intestinal samples were homogenized with approximately 10 vol (w/v) of 0.1 M PBS (pH 7.4) and centrifuged at 4 °C for 5 min (3000 rpm/min). The supernatants were collected and kept frozen at −80 °C until the enzyme activity assay was performed. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and lysozyme (LZM) were measured using a commercial assay kit (Nanjing Institute of Bioengineering, Nanjing, China) according to the manufacturer's protocol.

2.4. Histochemical observations of the mid-gut of the common carp

Paraffin sections Each gut sample was rinsed with PBS, fixed with 10% formalin solution, dehydrated with an ethanol gradient after 24 h, and embedded in paraffin. The wax block was cut at a thickness of 4 μm using a microtome (Leica, Germany), subjected to haematoxylin/eosin (HE) staining and observed under an optical microscope (ZEISS, Germany). Images were obtained at 100× and 400× magnification.

Scanning electron microscopy The intestinal samples were washed with PBS and then fixed with 2.5% glutaraldehyde solution for 2 h. The samples were dehydrated in 30%, 50%, 70%, 80%, 90%, and 100% alcohol for 15 min. Finally, the samples were soaked in t-butanol for 15 min and frozen at 4 °C. The obtained samples were observed under a scanning electron microscope (JEOL, Japan).

2.5. Short chain fatty acids in the intestinal contents

Fresh intestinal content samples were ground for 2 min with a tissue disrupter (JingXin, China). Then, each sample was thoroughly homogenized and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was aspirated, and H_2SO_4 was added to adjust the pH to 2–3. The samples was then filtered through a 0.45 μm filter, and the content of SCFAs in the sample was analysed by gas chromatography (Agilent Technologies 7890 B, America) with a HP-INNOWAX capillary column (30 m × 0.32 mm × 0.5 μm) (Agilent, America). Ten microliters of standard acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and isovaleric acid standard were added to ultrapure water to yield a total volume of 1 mL, which was diluted to generate 6 different concentration gradients, and a standard curve was prepared by loading and analysing the samples. Nitrogen (purity of 99.99%) was used as a carrier gas with a flow rate of 8 mL/min; a volume of 1 μL of each aliquoted sample was automatically injected into the inlet, which was maintained at a temperature of 240 °C.

2.6. Quantitative real-time PCR (qPCR)

The forward and reverse primers were designed according to the cDNA sequences of the immune-related genes using Primer 6.0, and 18s was used as the internal reference gene. One microgram of total RNA was obtained using a TRIzol reagent RNA kit (TaKaRa) and gDNA was removed by the gDNA Eraser (PrimeScript RT reagent kit with gDNA Eraser, TaKaRa, RR047A) before the first strand cDNA synthesized, and reverse transcription was carried out according to the instructions of the PrimeScript RT Reagent Kit (TaKaRa, Dalian). Real-time PCR was performed using a Roche LightCycle 96® real-time PCR instrument (Roche, Switzerland). The reaction system included 5 μL SYBR® Premix Ex Taq™ (TaKaRa, Dalian), 0.3 μL PCR forward primer (10 μmol/L), 0.3 μL PCR reverse primer (10 μmol/L), 1 μL cDNA template and 3.4 μL

sterile water. The amplification procedure used a two-step method: predenaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Three replicates were performed per sample, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression level of each gene.

2.7. Genomic DNA extraction and illumina high-throughput sequencing of the 16 S rRNA gene

The genomic DNA of the intestinal content samples was extracted using a genomic DNA extraction kit (QIAamp, Germany). The DNA concentration was measured using a NanoDrop 2000, and the DNA integrity was detected by agarose electrophoresis. The resulting qualified total DNA samples were used for the high-throughput sequencing of 16 S rRNA. The V3–V4 region of the 16 S rDNA gene was amplified with the barcoded primers V338F (5′-ACTCCTACGGGAGGCAGCA-3′) and V806R (5′-ATGCAGGGACTACHVGGGTWTCTAAT-3′). The amplicons were extracted from 1% agarose gels, purified by an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. Then, the purified amplicons were paired-end sequenced by an Illumina Miseq PE 300. The obtained PE reads were assembled using FLASH (Version 1.2.11) and analysed using QIIME (V.1.9.1). The operational taxonomic units (OTUs) were clustered by using a 97% similarity cutoff with UPARSE (Version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16 S rRNA gene sequence was analysed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16 S rRNA database according to a confidence threshold of 70%. According to the species annotations, the number of sequences in each sample corresponding to each classification level (Kingdom, Phylum, Class, Order, Family) was labelled. The diversity indexes were analysed by the MOTHUR program (Version 1.33.3, http://www.mothur.org).

2.8. Statistical analysis

The results are represented as the mean ± standard deviation. The data were analysed for significance by one-way ANOVA followed by a Duncan multiple comparison test using SPSS software version 11.0. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Intestinal enzyme activities

The intestinal enzyme activities of the experimental group and the control group are shown in Table 1. Compared with those in the NC group, SOD, CAT and LZM activity in the HYP group were all significantly increased ($P < 0.05$), and CAT and LZM activity displayed the same tendency in the LYP group ($P < 0.05$). However, MDA activity showed no significant difference in the CYP treatment groups compared with the NC group ($P > 0.05$).

Table 1
Intestinal enzyme activity indexes of common carp (*Cyprinus carpio* L.) from different treatment groups.

Index	Group		
	NC	LYP	HYP
SOD (*mM*)	0.79 ± 0.22[a]	0.82 ± 0.19[a]	0.94 ± 0.03[b]
CAT (*U/mgprot*)	3.31 ± 0.39[a]	5.95 ± 0.13[b]	6.17 ± 0.18[b]
MDA (*nmol/ml*)	2.23 ± 0.05	3.38 ± 0.41	3.24 ± 0.52
LZM (*μg/ml*)	7.88 ± 0.46[a]	9.20 ± 0.11[b]	9.45 ± 0.36[b]

Note: Values are mean ± SEM (n = 4) and values with different letters within the same row are significantly different at $P < 0.05$.

3.2. Intestine histology

The mechanical barrier of the intestinal epithelium was examined by HE staining and SEM. HE staining revealed that the LYP and HYP groups featured greater microvilli density, more complete microvillus structure and increased goblet cell numbers (Fig. 1A; Fig. 1B). The histological scores of the heights of intestinal villi in the HYP group showed a remarkable increase compared to those in the NC group ($P < 0.05$) but not in the LYP group (Fig. 1D). However, the thickness of the muscle layer in the two trial groups displayed no significant difference compared to that in the NC group ($P < 0.05$) (Fig. 1E). SEM photos (Fig. 1C) revealed that the intestinal mucosal folds in the midgut of the LYP and HYP groups showed an increase in wavy protrusions compared to those in the NC group, and the number intestinal microvilli were increased compared to those in the NC group and were arranged in clusters.

3.3. Determination of intestinal SCFAs

The contents of six SCFAs in the intestinal contents were determined by GC analysis and calculated via calibration curves. Our results showed that the contents of total SCFAs (Fig. 2A) and acetic acid (Fig. 2B) in the LYP and HYP groups were both significantly higher than those in the control group ($P < 0.05$). Additionally, dietary supplementation with high concentrations of Chinese yam peel remarkably increased the contents of propionic acid (Fig. 2C) and butyric acid (Fig. 2D) in comparison with those found in the NC group ($P < 0.05$) but not in the LYP group ($P > 0.05$). However, the contents of isobutyric acid, isovaleric acid and valeric acid showed no differences among all the groups ($P > 0.05$, data not shown).

3.4. Expression levels of genes involved in immunity

The expression profiles of immune-related genes and tight junction protein genes in the midgut of the common carp were examined. The mRNA levels of the tight junction protein genes *occludin* and *ZO-1* showed a significant increase ($P < 0.05$) following the addition of the Chinese yam peel (Fig. 3A; Fig. 3B). As shown in Fig. 3D, the mRNA levels of the pro-inflammatory immune factor *IL-1β* were upregulated in the HYP group ($P < 0.05$) but not in the LYP group (Fig. 3E). The anti-inflammatory factors *TGF-β* (Fig. 3F) and *IL-10* (Fig. 3C) also showed a remarkable tendency towards increase compared with the control group, although there was no statistically significant difference in *IL-10* expression ($P > 0.05$). In addition, Fig. 3G and H show the mRNA levels of the pathway factor genes *TLR4* and *NF-κB*. *TLR4* expression in the LYP ($P < 0.01$) and HYP group ($P < 0.05$) was significantly higher than that in the control group. Changes in *NF-κB* mRNA levels were only observed in the HYP group ($P < 0.05$).

3.5. Structural modulation of the intestinal microbiota during CYP treatment

To study the effect of different concentrations of Chinese yam peel (CYP) on the intestinal microbiota of the common carp, high-throughput sequencing of the bacterial 16 S rRNA V3–V4 region was used to investigate the structural changes. After the low-quality sequences were removed, a total of 1,723,685 sequences with an average length of 434.99 bp were obtained from the samples. In total, 500 OTUs were collected from the samples with a similarity level of 97%. As shown in Fig. 4A, the composition of the intestinal microbiota at the phylum level showed significant changes. Each sample at the phylum level was mainly composed of *Fusobacteria, Proteobacteria,* and *Bacteroidetes*, *Fusobacteria* was found to be the most abundant bacterium in all groups, representing 56.24% (NC), 80.57% (LYP), and 92.68% (HYP) of

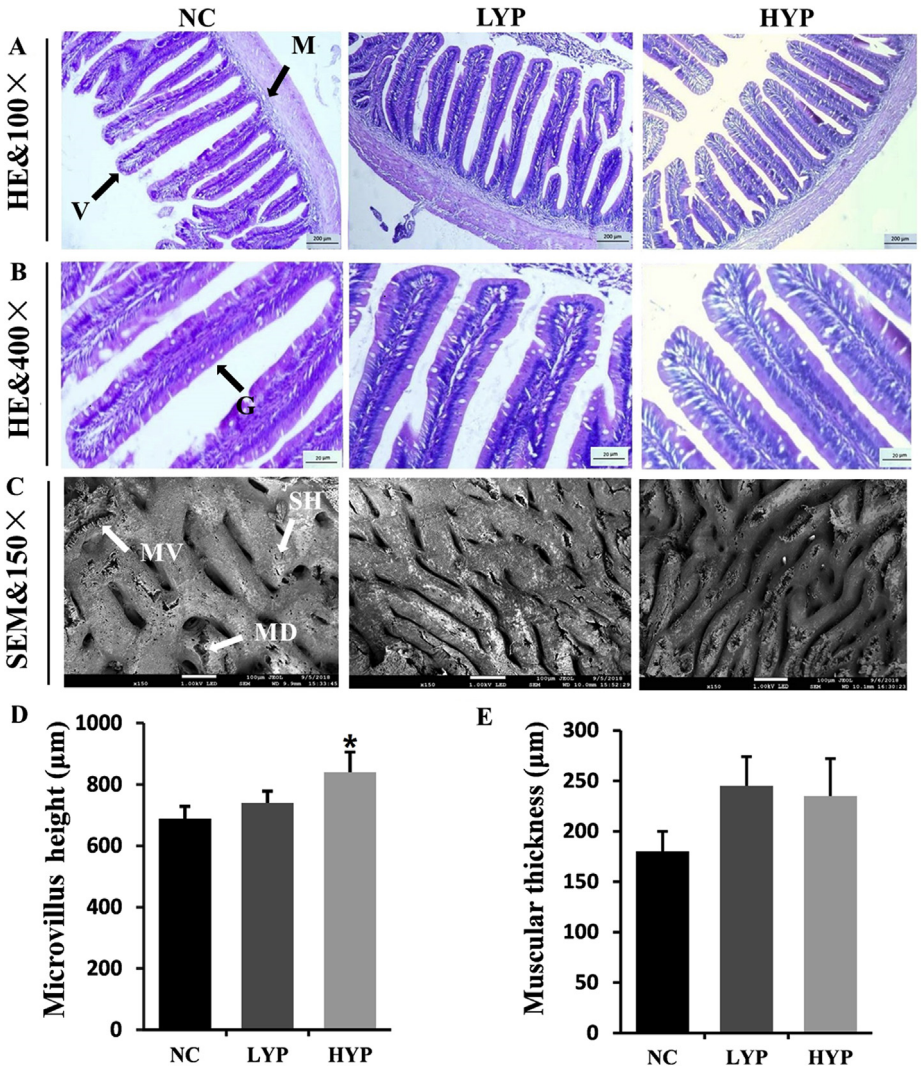

Fig. 1. Histological sections from the mid-intestine of common carp (*Cyprinus carpio* L.) from different treatment groups. (A) HE staining 100× (B) HE staining 400× (C) SEM observation of the epithelial tissue 150× (D) Microvillus height (μm) (E) Muscular thickness (μm). "*":indicates $P < 0.05$. ("MD"-Mechanical damage; "MV"-Microvilli; "SH"-Secretory hole; "V"-Villous; "M"-Muscular, "G"-Goblet cell).

the total, respectively. Compared to the control group, adding Chinese yam peel to the diet significantly increased the relative abundances of *Fusobacteria* and *Bacteroidetes* (Fig. 4B; Fig. 4D). However, *Proteobacteria* exhibited an opposite trend with increasing doses of Chinese yam peel (Fig. 4C). Furthermore, as shown in Fig. 4E, the ratio of *Firmicutes/Bacteroidetes* was remarkably decreased in the HYP group ($P < 0.05$).

To further understand the changes in the microbial community, a heat map of the relative abundances of the top 45 most abundant bacteria at the genus level is shown in Fig. 5A. The relative abundances of *Enterobacteriaceae*, *Shewanella, Pseudomonas* and *Vibrio* were decreased in the CYP-supplemented group compared to those in the

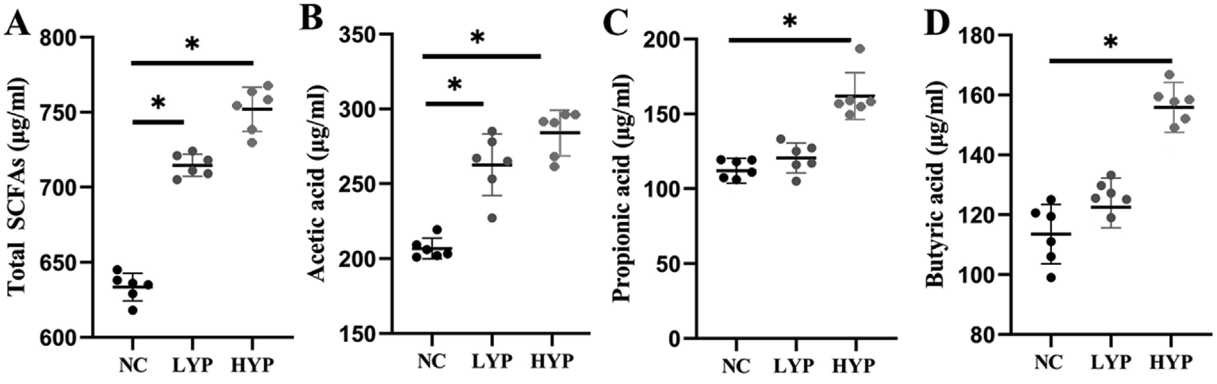

Fig. 2. Effect of Chinese yam peel on (A) total SCFAs, (B) acetic acid, (C) propionic acid and (D) butyric acid. "*"indicates $P < 0.05$ compared with the NC group (n = 6).

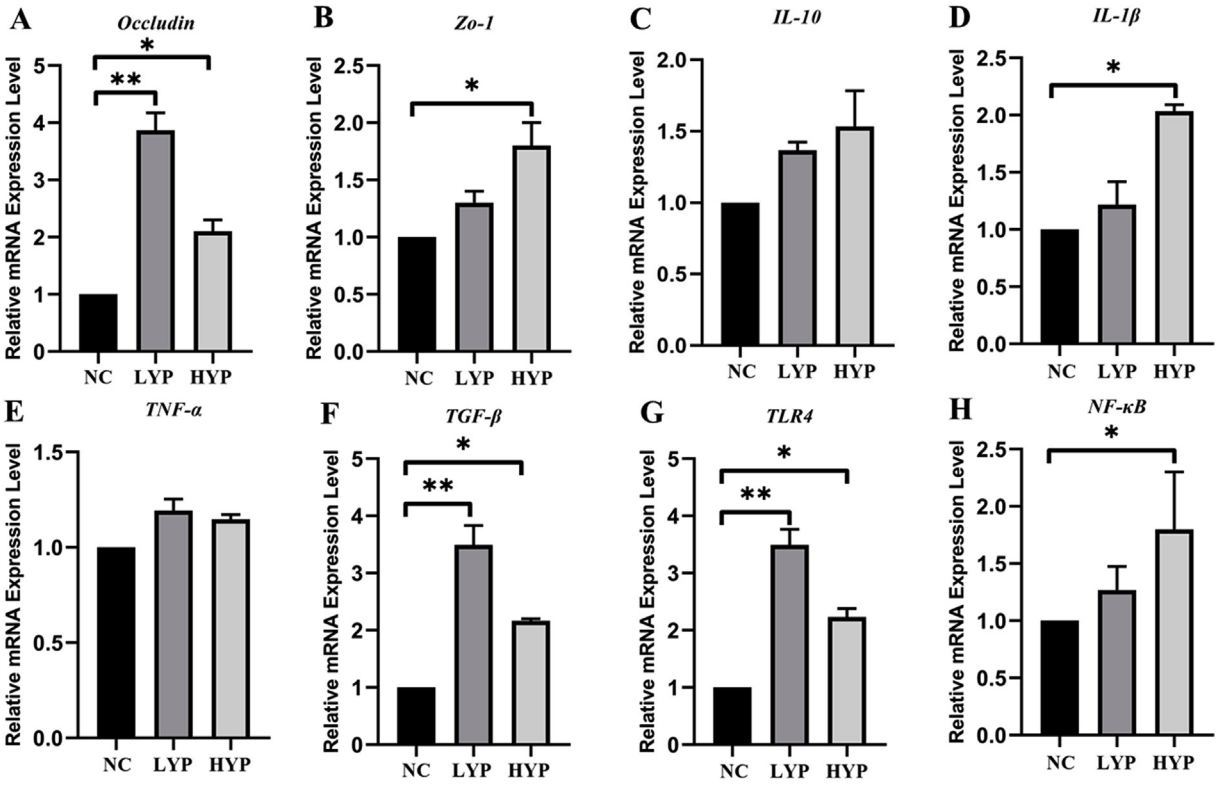

Fig. 3. The mRNA levels of genes involved in immune responses and tight binding proteins in the mid-gut of the common carp following the addition of CYP. Values are given as the mean ± SD (n = 3). The mRNA expression level values were normalized to those of β-actin and are expressed as a ratio with the control. "*"indicates $P < 0.05$; "**" indicates $P < 0.01$ compared with the NC group.

control group. *Plesiomonas, Desulfovibrio*, *Lactobacillus* and *Verrucomicrobiaceae* were more abundant in the LYP group than that in the NC group. Moreover, the *Propionivibrio, Roseomonas, Rhodobacter, Gemmobacter, Desulfovibrionaceae, Pasteurellaceae* and *Flavobacterium* abundances were increased in the HYP group compared with those in the control.

The Shannon index showed that both low and high concentrations of CYP remarkably increased the diversity of the gut flora (Fig. 5B) ($P < 0.05$). Additionally, principal coordinate analysis (PCoA) (Fig. 5C) revealed that the structure of the intestinal microbiota differed between the CYP-treated group and the control group. These results indicated that there was a significant structural difference in terms of beta diversity between the CYP supplemental group and the control group.

3.6. Correlation analysis

Correlation analysis identified multiple significant associations between altered microbial communities and SCFAs, antioxidant enzyme activity, and organism immune-related genes. As shown in Fig. 6, there were strong correlations between *Cetobacterium*, *Aeromonas*, *Bacteroides*, and *Lactobacillus* in the intestine with various parameters. Tight junction proteins, antioxidant enzymes and SCFAs are positively correlated with *Cetobacterium* and *Lactobacillus* but negatively correlated with *Aeromonas* and *Bacteroides*. Specifically, *Cetobacterium* was strongly positively correlated with *ZO-1*, *IL-1β*, *NF-κB*, total SCFAs, acetic acid, and LZM, CAT and MDA activity, whereas *Aeromonas* and *Bacteroides* were negatively correlated with these factors. *Lactobacillus* was positively correlated with total SCFAs, acetic acid, *ZO-1*, and LZM and CAT activity.

4. Discussion

The intestine is the main organ involved in the digestion and absorption of nutrients, and it is also the largest immune organ in the body. It has a strong barrier against harmful microorganisms and various toxins [25]. The immune system, as a line of defence against pathogenic microorganisms, is critical to maintaining the health of the body [26]. In this study, the changes in intestinal morphology, SCFAs, immune related gene expression and microbes were investigated to determine the effects of the additive Chinese yam peel on common carp flora and immunity.

The antioxidant system is the most important immune defence system in vertebrates. The activities of enzymes in this system usually indirectly indicate the ability of the host to remove reactive oxygen species (ROS). Catalase (CAT) and superoxide dismutase (SOD) are antioxidant enzymes that are commonly used as functional parameters to evaluate immune potential [27]. In this study, the activities of CAT and SOD in the gut were all increased ($P < 0.05$), indicating that the intestinal antioxidant capacity, including the neutralization of superoxide anions and surplus free radicals, was enhanced. This result is in agreement with the results of studies of the use of *Porphyra yezoensis* polysaccharides (PPs) in grass carp [28] and *Paeoniae radix* polysaccharides in *Litopenaeus vannamei* [29]. Lysozyme (LZM) is a protease with antibacterial activity that has an inhibitory effect on gram-negative bacteria by degrading polysaccharides in the cytoderm. Related research found that the activity or expression of LZM was increased when common carp feed was supplemented with polysaccharides [30], when *Oreochromis niloticus* were fed *Dendrophthoe falcata* [31] or when *Labeo rohita* were injected with β-glucan [32]. In the present study, LZM activity was also significantly increased when carp were supplemented with CYP. The possible reason for this is that the active components in yam peel changed the intestinal flora, a proportion of which promoted the secretion of lysozyme in the intestinal tract. These results suggested

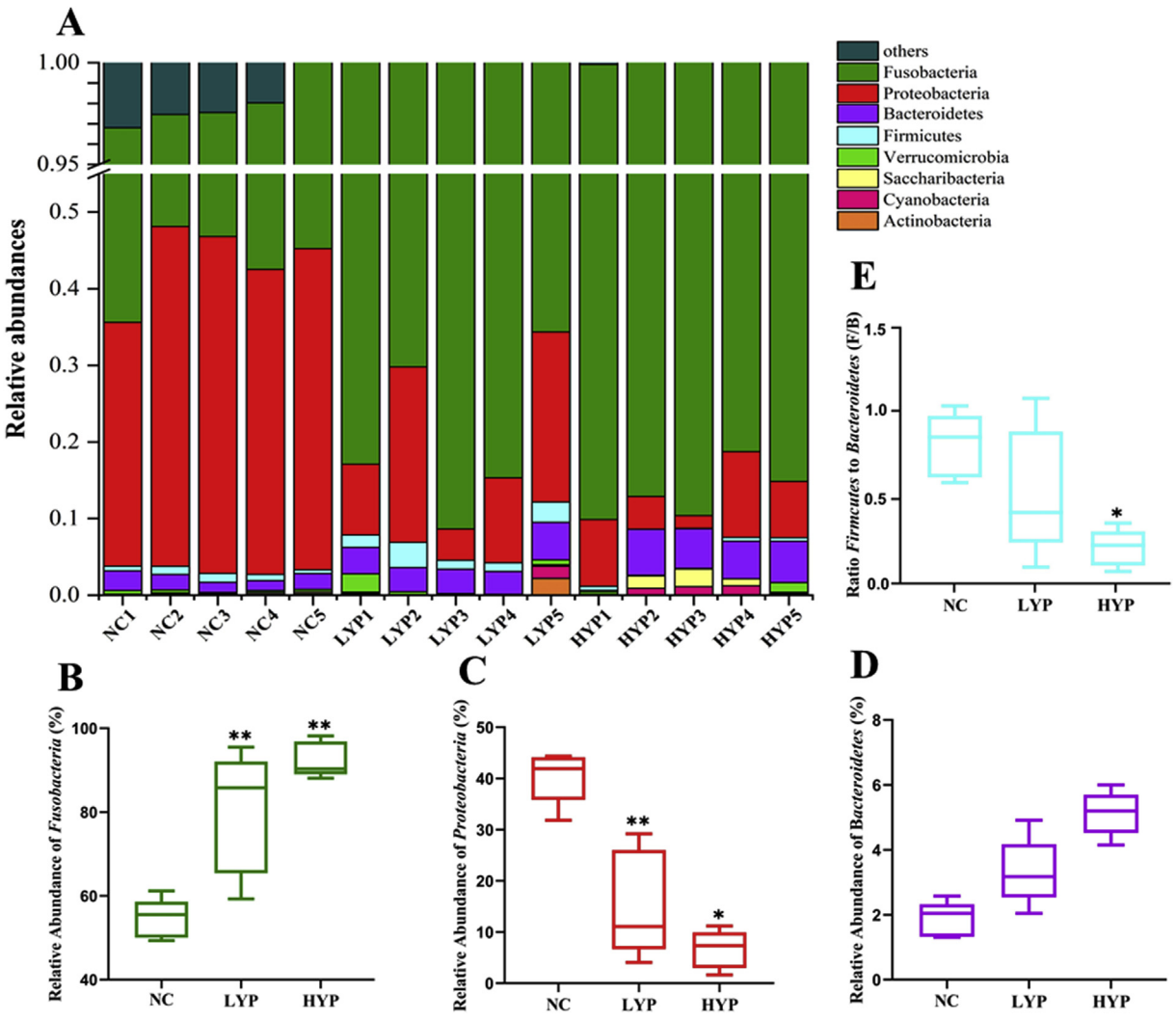

Fig. 4. The bacterial composition of the different communities at the phylum level. (A) Relative abundances of major phyla in faecal microbiomes from fish in the CYP-treated and control groups. (B) Relative abundance of *Fusobacteria* in each group. (C) Relative abundance of *Proteobacteria* in each group. (D) Relative abundance of *Bacteroidetes* in each group. (E) Ratio of *Firmicutes/Bacteroidetes*. "*"indicates $P < 0.05$; "**" indicates $P < 0.01$ compared with the NC group.

that the addition of CYP to the feed could increase the antioxidant capability and inhibit the proliferation of intestinal pathogenic bacteria in common carp.

The intestinal tract is the most important organ involved in fish digestion, nutritional absorption and intestinal immunity, especially in fish without a stomach. The integrity of the morphology could reflect the health state and the absorptive function of the intestinal tract to a certain extent. In this study, microvilli damage (MD) was remarkably alleviated after the addition of CYP, suggesting improved structural integrity of the intestine. Additionally, wrinkles in the intestinal mucosal projections distribute functional cells so they can absorb nutrients, so the increase in the microvilli and mucosal folds in this research indicated the increase of the absorbent surface area and the food contact area in the intestinal digestion. The epithelial cells of the mucosa are composed of a single layer of columnar epithelial cells, which are scattered among many goblet cells. The goblet cells secrete digestive enzymes and mucus, thereby protecting the epithelial cells and lubricating the food to make it easy to pass. In the present study, the number of goblets was also increased following the addition of CYP to the feed, suggesting an increase in digestion and absorptive capacity.

Tight junction protein is an important component of the intestinal mucosal mechanical barrier [33]. *Occludin* and occluded small band protein (*ZO-1*) can form a stable connection with each other and are important for maintaining the integrity of the intestinal mucosal barrier [34]. Previous studies have reported the enhancement of the mRNA expression of *ZO-1* and *occludin* through the administration of Astragalus [35] and *Bletilla striata* polysaccharide [36]. Moreover, some studies have shown that phenolic compounds affect the ability of these proteins to tightly bond and enhance barrier integrity [37]. In this work, the addition of yam peel increased the mRNA expression levels of *occludin* and *ZO-1* in the intestine, indicating that yam peel promotes the protective effect of the intestinal barrier. This is consistent with the examination of the intestinal morphological structure.

Cytokine genes are important mediators of the immune system and anti-infective defence mechanisms secreted by macrophages or monocytes. During the process of inflammation, interleukin 10 (*IL-10*) and transforming growth factor-β (*TGF-β*), as important anti-inflammatory cytokines in fish, not only inhibit the release of excessive cytokines but also control the inflammatory process [38]. The transcription of *IL-10* and *TGF-β* mRNA in this research was increased after adding CYP, which was consistent with the results in the common carp following the administration of palm fruit extract [39]. Pro-inflammatory cytokines, such as *TNF-α* and *IL-1β*, are biomarkers of immune-regulatory factors expressed early in fish infection and play an important role in the regulation of inflammation. The expression of *IL-1β* and *TNF-α* are generally considered to be predictors of the presence of inflammatory

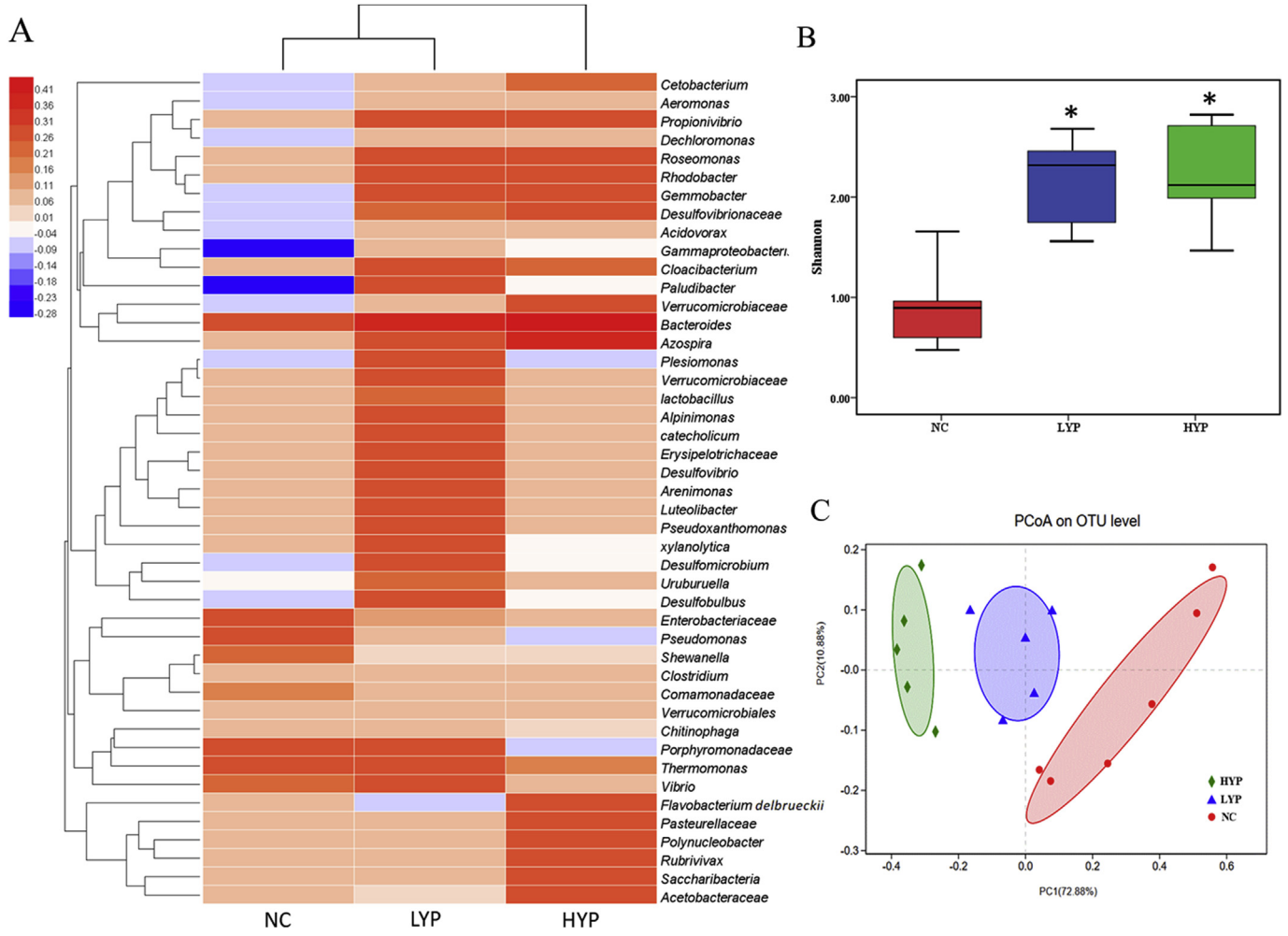

Fig. 5. Changes in the intestinal microbiota at the genus level in the control, LYP and HYP groups. (A) The heatmaps of the specimens show the relative abundances of the main identified bacteria at the genus taxonomic level. Red indicates a higher relative abundance, whereas blue indicates a lower relative abundance. (B) Shannon index of the diversity of the intestinal microbiota after CYP supplementation. (C) Principal coordinate analysis (PCoA) of the unweighted UniFrac scores of the microbial communities. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

responses [40]. Some studies have demonstrated that polysaccharides, as effective immunostimulants, can induce the expression of *TNF-α* and *IL-1β* mRNA and actively regulate the immune system, thereby improving the immune response and disease resistance of fish. The up-regulation of *IL-1β* and *TNF-α* gene expression was observed in carp fed *Rehmannia glutinosa* [41] or *Spirulina platensis* [42]. Additionally, in rainbow trout, evidence indicated that probiotics could effectively stimulate the production of *IL-1β*, *TNF-1* and *TNF-2* [43]. In the present study, we found that *IL-1β* and *TNF-α* were upregulated in the intestine of the carp fed yam peel compared with the control group. These effects might be due to the active components in the peel of the Chinese yam, such as polysaccharides, flavonoids and allantoins. Identical conclusions were reached in studies about the application of *angelica* polysaccharide [44], *astragalus* polysaccharide [45] and *ficus carica* polysaccharide [46]. Additionally, changes in the intestinal microbiota might induce changes in the probiotics in the gut, eliciting a non-specific response and enhancing the superoxide-generating ability of peritoneal macrophages.

TLR4 is an important pattern recognition receptor associated with immunity that binds to ligands, induces *NF-κB* signalling, activates macrophages, and promotes the expression of inflammatory factors [47]. A growing body of evidence suggests that polysaccharides, as potent immunostimulants, are directly involved in the activation of macrophages [48], which cause an immune response by binding to receptors on target cells, ultimately leading to the production of antibacterial molecules [49]. This may be explained by the fact that natural polysaccharides can stimulate macrophages and promote the production of inflammatory factors through the *TLR4/NF-κB* signalling pathway, thereby stimulating an immune response. In mammals, Li et al. [50] have reported that Chinese yam non-starch polysaccharides activate RAW 264.7 macrophages via the *TLR4/NF-κB* signalling pathway and promote NO production and *IL-6* and *TNF-α* mRNA expression. Zhang et al. [51] found that rhubarb polysaccharides could activate macrophage surface *TLR4* complexes and transduce extracellular signals into the cell, leading to *NF-κB* p65 expression. The results of this study demonstrate that Chinese yam polysaccharides can activate macrophages and cause immune responses in common carp.

Short-chain fatty acids (SCFAs) are fermented products of enteric microbial digestive dietary components that improve the intestinal flora structure and intestinal barrier integrity, participate in immune regulation, and effectively prevent intestinal inflammation [52]. At the same time, they can inhibit the proliferation of pathogenic microorganisms, as the pH of the intestinal lumen is reduced by SCFAs [53]. Therefore, the number and relative abundances of SCFAs are considered biomarkers of health status. The results of this study showed that CYP regulates the content of short-chain fatty acids in the intestine of the common carp, especially acetic acid, propionic acid, butyric acid and the total acid content ($P < 0.05$). The main source of SCFA is dietary

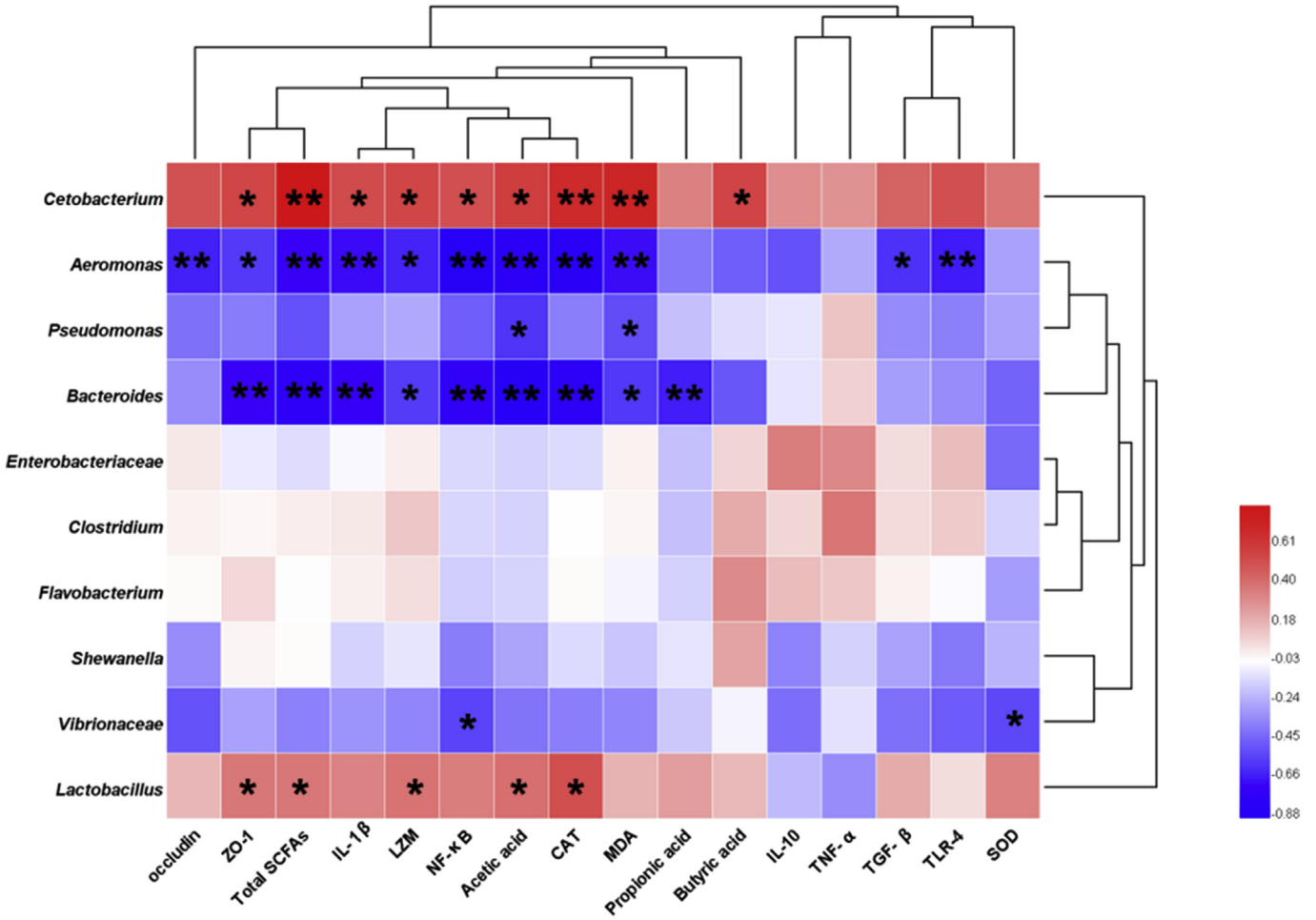

Fig. 6. Correlations between the representative microbial genera, SCFAs, immune-related genes and antioxidant enzymes in the gut. Heatmap constructed according to Spearman correlation coefficients. Red represents a positive correlation and blue represents a negative correlation. "*" represents $P < 0.05$; "**" represents $P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

carbohydrates, especially polysaccharides [54]. There is increasing evidence that polysaccharides have the potential to promote the proliferation of SCFA-producing bacteria, such as *Bacteroides* and *Clostridium*, which produce acetic acid, propionic acid and butyric acid [55]. *Dioscorea opposita* Thunb., a dietary supplement that increases phytochemicals, is a good source of carbon and energy for intestinal microbes and is also rich in Chinese yam polysaccharides. It has been reported that Chinese yam polysaccharides increase the contents of acetate, propionate and butyrate in the rat hindgut and increase the diversity of bacterial communities [22]. In an antibiotic-associated diarrhoea mouse model, orally administered Chinese yam could increase the content of SCFAs [23]. For other herbal extracts, polysaccharides extracted from *Rhizopus nigricans* or *Flammulina velutipes* could also increase the concentration of short-chain fatty acids in the faeces of mice [56,57].

The results of the intestinal flora analysis showed that the microbial community of the common carp mainly consisted of *Fusobacteriales*, *Proteobacteria* and *Bacteroides*. Among the carp, we found that more than 50% of the taxa represented *Fusobacteriales*, which was similar to the findings of van Kessel et al. [58]. Almost all of the *Fusobacteriales* strains currently detected in fish gut samples belong to *Cetobacterium*, which is a common and widely distributed species in the intestinal tracts of freshwater fish. *Cetobacterium* has been reported to produce vitamin B12 [59,60]. Identical conclusions were reached in studies in which the addition of chitosan-silver nanocomposites (CAgNCs) increased the abundance of *Cetobacterium* in zebrafish [61]. *Proteobacteria* are important components of the gut flora of most species, including fish [62]. Most of the *Proteobacteria* found in the intestines of carp belongs to *Aeromonas*. The bacteria of the genus *Aeromonas*, which are widely distributed in nature, exist not only in freshwater rivers and coastal areas but also in tap water and are a conditional pathogen causing human-fish-animal complications [63]. Our research revealed that supplementation with yam peel could reduce the number of *Aeromonas*. This result is consistent with previous studies showing that chitosan can reduce the abundance of *Aeromonas* in the intestine of gibel carp [64]. In addition to *Aeromonas* in the *Proteobacteria,* the abundances of *Enterobacter, Vibrio, Monasisus* and *Acinetobacter* were also found to decrease, which was similar to the results of previous studies [58]. Although these disease-related bacteria are not necessarily pathogenic, the presence of these bacteria suggests that the fish gut may be a potentially pathogenic environment. Therefore, we believe that yam peel may have a positive impact on reducing the number of pathogenic intestinal microbiota and enhancing the ability of hosts to defend against disease. *Firmicutes* and *Bacteroides* have been found to encode a wide range of carbohydrate-related enzymes and to play an important role in the utilization of non-digestible polysaccharides [65]. In this experiment, the decrease in the ratio of *Firmicutes/Bacteroides* may have been related to intestinal health and body weight changes [66]. Specifically, yam peel can reduce the host's absorption of excess energy in food by regulating the relative abundances of *mycobacteria* and *Bacteroides* to prevent the excessive intake of energy to maintain a normal body weight.

Upon analysis of the composition of the intestinal microbial at the genus level, we found that the abundances of *Lactobacillus* and *Flavobacterium* were increased. *Lactobacillus* is a common intestinal probiotic in aquaculture that regulates immune-related cells and activates the body's immune responses against pathogens. Furthermore, *Lactobacillus* can adhere to and colonize the intestinal mucosa, leading to competition at mucosal attachment sites and thereby preventing the colonization of pathogenic bacteria [67]. Zhang et al. showed that *Lactobacillus* could effectively improve the disease resistance, antioxidant capacity and growth performance of *Cyprinus carpio* Huanghe var [68]. Regarding *Flavobacterium*, it has been reported that the addition of *Flavobacterium* can promote immune responses and disease resistance [69]. On the other hand, the abundances of a large number of pathogenic bacteria, such as *Enterobacteriaceae, Shewanella, Pseudomonas* and *Vibrio*, were lower in the treated groups than that in the control group. The inhibition of pathogenic bacteria by *Lactobacillus* probiotics has been widely verified in other fish species [70]. It has been suggested that the beneficial immune effects of CYP may be achieved by increasing the number of probiotics and inhibiting the proliferation of harmful bacteria. However, which specific bacteria show a significant correlation with the immune enhancement effect remains to be further studied.

In our study, cytokine levels, intestinal antioxidant enzymes, and SCFA were significantly associated with the gut microbiota. Although most of the underlying mechanisms are unclear, these findings may be important for future in-depth research. Additionally, this analysis revealed that immunopotentiators interact with the metabolism in the body via the intestinal microbiota to enhance immunity and maintain host health.

Overall, these data suggest that the addition of CYP improves the homeostasis of the common carp gut flora, inhibits the proliferation of pathogens, and promotes the secretion of cytokines and short-chain fatty acids. It provides a theoretical basis for the application of CYP as an immunopotentiators in aquatic products.

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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.2019.10.066.

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