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The effects of fish meal replacement with ultra-micro ground mixed plant proteins (uPP) in practical diet on growth, gut and liver health of common carp (*Cyprinus carpio*)

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ABSTRACT

Plant proteins are widely used for fish meal replacement in aquafeeds, but anti-nutritional factors in plant protein reduce fish growth performance and impair fish health. The present work aimed to study the effects of improving fish meal replacement percentage with ultra-micro ground mixed plant proteins (uPP) on growth, gut and liver health of common carp. Carps were fed with a practical basal diet with partial fish meal replacement by plant proteins or the basal diet supplemented with 2.5 % or 5% uPP for 16-week. Results indicated that uPP addition did not affect growth and survival of common carp at a supplementation level up to 5% ($p > 0.05$). However, 5% uPP up-regulated the intestinal expression of inflammation related genes ($p < 0.05$) and reduced *HIF-1α* expression ($p < 0.05$). Moreover, dietary 5% uPP increased serum ALT ($p = 0.06$) and AST level ($p < 0.05$) and up-regulated liver expressions of inflammation related genes ($p < 0.05$). The Simpson diversity index of gut microbiota was lower in 5% uPP group compared to control ($p < 0.05$). The relative abundance of Fusobacteria and *Cetobacterium* was lower ($p < 0.05$), while Proteobacteria including *Shewanella* and *Citrobacter* was higher in the 5% uPP group compared to control ($p < 0.05$). In contrast, 2.5 % uPP did not increase inflammatory and injury parameters in fish intestine and liver, but rather improved the expression of occludin and defensin in the intestine compared with control ($p < 0.05$). Moreover, no significant differences were found in gut microbiota between 2.5 % uPP group and control. Together, our study suggests that low-level uPP addition can be adopted to further improve fish meal replacement, while dietary 5% uPP impairs gut and liver health of common carp and negatively affects intestinal microbiota.

1. Introduction

Fish meal has been the ideal protein source for aquafeed. Fish meal is well balanced with respect to essential amino acids, fatty acids and minerals, has a low carbohydrate content, and is devoid of anti-nutritional factors with high palatability and digestibility (Gatlin et al., 2007). However, the global production of fish meal cannot meet the rapidly growing demand for aquafeeds and the price of FM is steadily increasing (Hardy, 2010; Gu et al., 2018; Cruz-Suárez et al., 2007).

Alternative protein sources have been studied to replace fish meal in aquafeed. Among them much attention has been focused on plant proteins due to their low price, high protein content and acceptable amino acid composition (Santigosa et al., 2011; Al-Thobaiti et al., 2018). Studies have shown that plant protein such as soybean, barley, canola, corn, cottonseed, peas/lupins, and wheat can replace fish meal and is widely used in aquafeeds (Gatlin et al., 2007; El-Saidy and Gaber, 2002; Brinker and Friedrich, 2012; Ibrahem and Ibrahim, 2014). However, the use of plant proteins is restricted because they contain many anti-nutritional factors (ANFs), e.g. phytic acid, protease inhibitors, lectins, saponins, antivitamins and allergens (Gatlin et al., 2007). The presence of antinutritional factors affects the utilization of protein, minerals or vitamins and results in poor growth and feed utilization in

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Table 1
Feed formulation and chemical composition of diets for common carp (dry matter, g/kg).

Ingredient	g/kg DM		
	Control	2.5 % uPP	5% uPP
Rice bran	190.0	178.0	159.0
Flour	200.0	200.0	200.0
Soybean meal	200.0	200.0	200.0
Rapeseed meal	105.0	116.0	129.0
Fish meal	80.0	55.0	30.0
Poultry by-product meal	50.0	50.0	50.0
DDGS	100.0	100.0	100.0
uPP	0.0	25.0	50.0
Bentonite	10.61	8.31	9.80
Lys-HCl	1.4	1.0	0.7
Methionine	0.49	0.49	0.50
Choline chloride (50 %)	2.0	2.0	2.0
$Ca(H_2PO_4)_2$	20.0	20.0	20.0
Soybean oil	30.0	33.7	38.5
VC phosphate	0.5	0.5	0.5
Fish premix (1%) [a]	10.0	10.0	10.0
Crude protein (%)	32.47	32.88	32.99
Crude fat (%)	9.53	9.64	9.67
Crude ash (%)	16.50	8.38	8.32
Moisture (%)	5.77	6.92	7.25

[a] Provided by Beijing Sino-Norway Joint Aquaculture Technology Co., Ltd. The product meets NRC standard.

several fish species (Zhou et al., 2017; Francis et al., 2001; Rumsey et al., 1994; Refstie et al., 2005; Pham et al., 2010; Wang et al., 2017; Yin et al., 2020). Some antinutritional factors also cause pathological damages in fish tissues and organs (Gu et al., 2018; Knudsen et al., 2008; Wang et al., 2017). In turbot (*Scophthalmus maximus*), addition of soya-saponins (2.5−15 g kg^{-1}) led to inflammatory changes in distal intestine (Bai et al., 2019). The histopathological damages are commonly accompanied by induction of nuclear factor *NF-κB* and the downstream activation of inflammatory cytokines, chemokines, and *TNFα* related genes (Trejo-Escamilla et al., 2016; De Santis et al., 2015). Moreover, many studies have shown that replacing fish meal with plant protein will affect the gut microbes of fish such as rainbow trout (*Oncorhynchus mykiss*) (Heikkinen et al., 2006), Atlantic salmon (*Salmo salar*) (Green et al., 2013), and Atlantic cod (*Gadus morhua L.*) (Ringø et al., 2006). In practical aquafeed, partial replacement of fish meal by plant proteins is common. Further improvement of replacement percentage of fish meal has the benefit of deceasing cost and saving the continuously decreasing resources.

Plant proteins can be processed to reduce the level of anti-nutritional factors before being used in feeds (Zhou et al., 2017). The main removal methods include physical treatment, chemical treatment and biological treatment. In particular, micronization of plant proteins can reduce the antinutritional content (Khattab and Arntfield, 2009). Moreover, micronization increases surface area of the protein particles, which might enable digestive enzymes to work and decrease impact of ANFs. Studies have shown the usage of processed plant proteins in aquafeed, which led to improved performance of aquatic animals (Arntfield et al., 2001; Moniruzzaman et al., 2020). In this study, we used ultra-micro ground mixed plant protein (uPP), which was made by mixing high-quality protein sources including potato protein, pea protein, wheat gluten and soya protein. In addition to the routine procedure of heating and solvent extraction that removes trypsin inhibitor and conglycinin, the mixture of plant proteins was subjected to micronization by ultra-micro grinding (< 200 μm), which was expected to further decrease the negative effect of antinutrients. On the basis of plant protein in a practical diet, uPP was added to further replace fish meal. Common carp (*Cyprinus carpio*) is one of the most important cultivated fish species in the world (Vandeputte, 2003) with four million tons annual production (Nedoluzhko et al., 2020), and can utilize plant proteins (Suprayudi et al., 2015; Yılmaz et al., 2004), thus it is important to conduct such researches on this species. In the present study, we evaluated the effects of further fish meal replacement by uPP in practical diet on growth, gut and liver health of common carp.

Table 2
Primer sequences for *q*PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	GAAGTGTGGTGTGGACATCCGTAA	AGACTCATCGTACTCCTGCTTGCT
NF-κB p65	AACCAAGAACCAGCCGTACAAGC	ACTGTGTATCCTCCGCTCCTGTAG
Hif-1α	GTTCTGCGTACCTGGTGCTCATC	AAAGTGTGGCGGCTGAGAAAGG
TNFα	GCTGTCTGCTTCACGCTCAA	CCTTGGAAGTGACATTTGCTTTT
IL1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA
IL10	GCTGTCACGTCATGAACGAGAT	CCCGCTTGAGATCCTGAAATAT
TGFβ	ACGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA
ZO-1	CCGAAGCTTTGACAGCAAAC	GGTTGATCTTCTCCACTGACTC
Occludin	GACGCCATGGATGAGTACAA	GTGGTTGAGTTTGGCTTTCAG
Defensin	GGGATTCGATTTGGACGTGTGG	GTGGACAACCCTGGTGACTAACA

2. Materials and methods

2.1. Fish husbandry and experimental diets

All experiments and animal care procedures were approved by Feed Research Institute of the Chinese Academy of Agricultural Sciences chaired by the China Council for Animal Care (Assurance No. 2018-AFFRI-CAAS-001). Juvenile carp (average initial weight 2 g) were randomly allocated into eighteen 100-L tanks. The type of culturing system was recirculating system. The fish were weighed at start of the experiment. Each treatment included 6 replicate tanks. uPP was provided by Joosten (Joosten, Netherlands). It was made by mixing different plant protein sources including potato protein, pea protein, wheat gluten and soya protein, followed by heating, solvent extraction, and ultra-micro grinding (< 200 μm). The basal dietary formulation and proximate composition are described in Table 1. uPP was supplemented to the basal diet at 2.5 % and 5%. The chemical composition of feeds was tested according to Mandal et al. (2010) and was given in Table 1. Fish were fed to apparent satiation three times daily at 08:00, 13:00 and 17:00. During the 16-week feeding trial, water temperature was 26 °C; pH was 7.0–7.2; the dissolved oxygen was > 6.0 mg/L; the total ammonia was < 0.01 mg/L.

2.2. Growth measurements and sampling

Fish was fasted for 24 h before measurements and sampling. The weight of fish was measured for each tank. Body length was also measured. Survival, weight gain, feed conversion ratio, daily feeding rate, fatness and hepatosomatic index of fish were calculated as previously described (Wu et al., 2020).

For serum biochemical parameter analysis, four fish were sampled per tank. Blood was collected from fish caudal vein with a sterile 1 ml syringe. The blood samples were put into Eppendorf tubes with no anticoagulant, followed by centrifugation at 4000 *g* for 10 min (4 °C) to collect the serum. Serum samples were pooled by tanks and frozen at −80 °C before analysis.

2.3. Histological analysis

The intestinal and liver samples were collected from 6 fish per treatment to obtain 6 replicates. The samples were washed with sterile PBS and fixed with 4% paraformaldehyde, then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). A light microscope (Leica DMIL-LED, Germany) was used to observe the tissue morphology. Images scoring was evaluated by referring to the criteria described previously (Liu et al., 2016). The scoring scale was from 1 to 10.

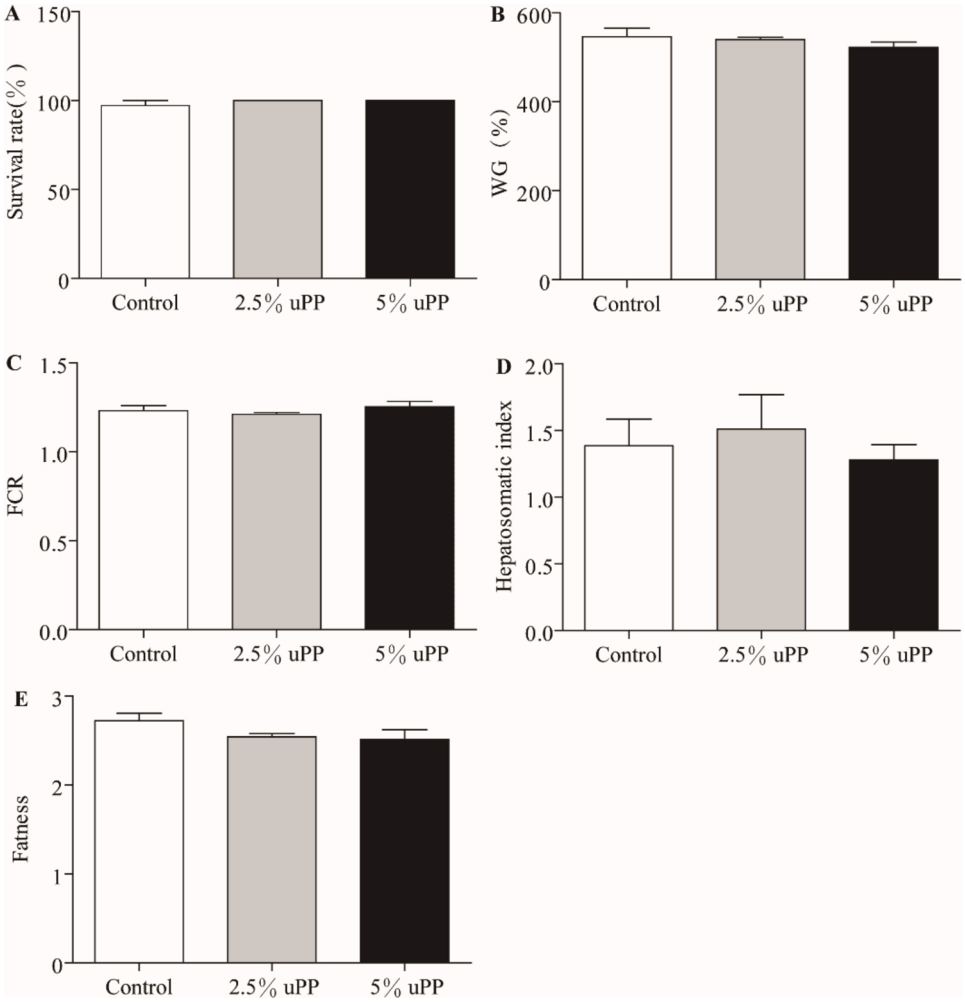

Fig. 1. Effects of uPP supplemented diet on the survival rate, WG, FCR hepatosomatic index and fatness of common carp. (A) survival rate (%), (B) WG (%), (C) FCR, (D) Hepatosomatic index and (E) fatness of common carp fed different diets. Data represent the means (± SEM) of six replicates of each treatment. No significant difference was observed among groups.

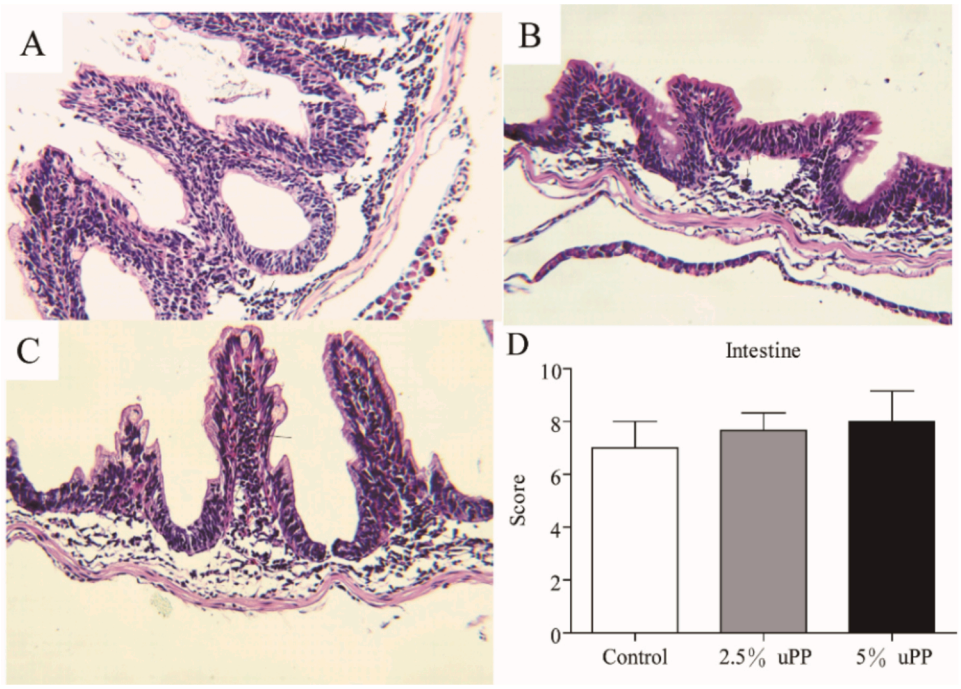

Fig. 2. Representative histomorphological images from HE-stained sections of the distal intestine of common carp fed different diets. (A) Control; (B) 2.5 % uPP; (C) 5% uPP; (D) Histological score of intestine (n = 6). No significant difference in histological score was observed among groups.

2.4. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

Serum AST and ALT levels were measured by AST and ALT assay kits, respectively (Jiancheng Bioengineering Ins., Nanjing) following the manufacturer's instructions.

2.5. 16S rRNA gene sequencing

16S *r*RNA gene sequencing was used to analyze gut microbiota. Gut content samples were collected 6 h after the last feeding. Samples from 3 fish per tank were pooled. DNA extraction, sequencing, and data analysis were conducted as previously described (Wu et al., 2020)

2.6. qRT-PCR analysis

Total RNA was extracted using the TRIzon method. The integrity of RNA was evaluated by 1.5 % agarose gel electropheresis. *c*DNA synthesis was performed with the Fast King gDNA Dispelling RT SuperMix (TIANGEN, KR118). Expression of nuclear factor-κB (*NF-κB*), interleukin 1 beta (*IL-1β*), tumour necrosis factor alpha (*TNF-α*), transforming growth factor beta (*TGF-β*), Interleukin 10 (*IL10*), hypoxia inducible factor 1 subunit alpha (*HIF-1α*), defensin, *ZO-1* and *Occludin* were determined using *q*PCR. Primer sequences are listed in Table 2. qPCR reactions were conducted using SYBR Green Premix Ex Taq™ II (TaKaRa) in iQ5 multicolor real-time PCR detection system (Bio-Rad). The PCR conditions were as follows: 95 °C for 10 min, 45 cycles (95 °C 15 s, 58 °C 30 s and 72 °C 30 s). Data were analyzed by $2^{-\Delta\Delta CT}$ method using β-Actin as reference.

2.7. Statistical analysis

All statistics are from at least three independent experiments, using software GraphPad Prism 8.0 or Microsoft Office Excel 2010. The data was expressed in the form of mean ± SE. Data were analyzed by one way analysis of variance (ANOVA) with post hoc test. Normality of data was tested by one-sample Kolmogorov-Smirnov test. Variance homogeneity of the data was examined with Levene's test. When assumptions were violated, data were analyzed by non-parametric Jonckheere-Terpstra test followed by post hoc test. Differences with p values < 0.05 were considered as significant.

3. Results

3.1. Growth performance and feed utilization of common carp

After 16-week feeding, the survival percentage, weight gain rate, feed conversion, fatness and hepatosomatic index of common carp were tested and the results were presented in Fig.1. Compared to the control group, fish fed diets supplemented 2.5 % uPP and 5% uPP exhibited no significant difference in survival percentage, weight gain rate, feed conversion, fatness and hepatosomatic index ($p > 0.05$, Fig. 1).

3.2. Effects of uPP on intestinal health of common carp

To further characterize the effects of uPP on intestinal health in common carp, we examined the intestinal tract morphology. Although the HE image showed signs of inflammation in the intestine of common carp fed with 2.5 % uPP or 5% uPP diet, the effect was minor and no significant difference in inflammatory score was observed for the two replacement groups compared with control (Fig. 2).

Furthermore, we investigated the effects of uPP inclusions on the expression of genes related to intestinal health. Dietary 5% uPP significantly up-regulated the intestinal expressions of inflammation related genes including *NF-κB p65*, *TNF-α*, *IL-1β*, *IL10* and *TGF-β* ($p < 0.05$, Fig. 3). Moreover, fish fed the 5% uPP diet had significantly lowered expression of *HIF-1α* compared to the control ($p < 0.05$). Hepcidin and ZO-1 tended to be reduced in the 5% uPP group as well but these were not statistically significant. In contrast, 2.5 % uPP did not induce the intestinal expression of inflammatory genes, and the relative expression of defensin and occludin was significantly up-regulated in 2.5 % uPP group, compared to the control ($p < 0.05$; Fig. 4). Together, our data indicated that dietary 5% uPP can negatively affect intestinal health in common carp, while 2.5 % uPP diet showed no apparent negative effect

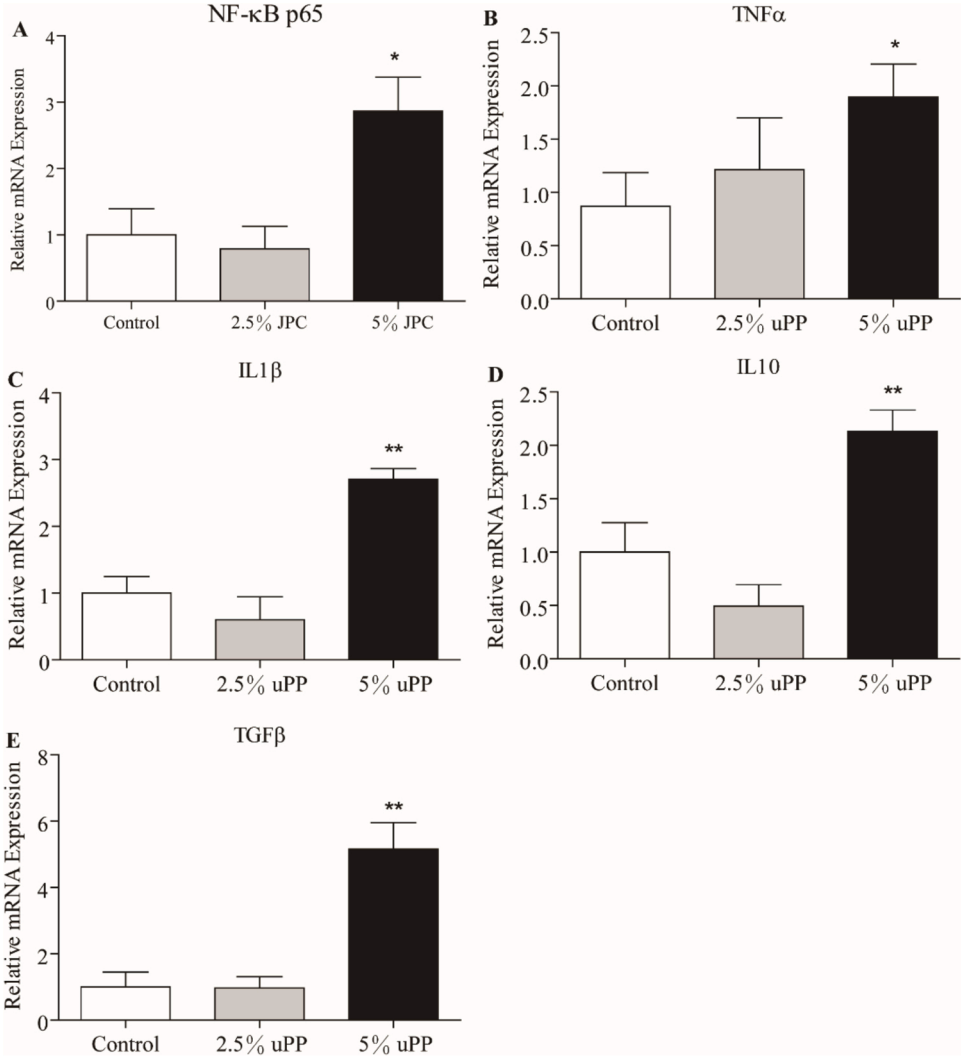

Fig. 3. Relative mRNA expression of inflammation related genes in the intestine of the common carp fed different diets. Data are expressed as mean ± SEM (n = 6). A single and double asterisk denote $p < 0.05$ and $p < 0.01$, respectively.

on the intestine, but rather improved gut health in terms of the expression of tight junction proteins and antimicrobial peptides.

3.3. Effects of uPP on liver health in common carp

As for intestine, there were no significant effect of diet on liver injury score at the end of trial (Fig. 5). However, compared to control group, fish fed the 5% uPP diet had significantly higher serum AST level ($p < 0.05$), and the serum ALT level showed a up-regulated tendency ($p = 0.06$), suggesting compromised liver health (Fig. 6). Moreover, 5% uPP increased the mRNA expression of genes related to inflammation in liver, including *TNF-α*, *IL-1β*, *IL10* and *TGF-β* ($p < 0.05$, Fig. 7). In contrast, the 2.5 % uPP diet showed no significantly negative effect on the liver health.

3.4. Effects of uPP on gut microbiota of common carp

The α-diversity indexes of the intestinal microbiota of fish fed different diets were presented in Table 3. The Simpson diversity index was significantly lower in 5% uPP diet group compared with control ($p < 0.05$). No significant difference was observed between the control group and the group of 2.5 % uPP ($p > 0.05$).

At phylum level (Table 4, Fig. 8A), Fusobacteria was the most abundant phylum with the relative abundances being 80.40 %, 88.09 % and 43.71 % in the control, 2.5 % uPP and 5% uPP groups, respectively. The relative abundance of Fusobacteria was significantly lower in 5% uPP group compared with the other groups ($p < 0.05$). In contrast, the relative abundance of Proteobacteria was significantly higher in the 5% uPP group compared with control and 2.5 % uPP group ($p < 0.05$). Furthermore, the relative abundance of Actinobacteria and Verrucomicrobia was significantly higher in the 5% uPP group compared with control ($p < 0.05$). The abundance of Bacreroidetes was not significantly different among groups.

At the genus level (Table 5, Fig. 8B), *Cetobacterium* was the most abundant genus, with the relative abundance in the 5% uPP group being significantly lower than the other groups ($p < 0.05$). The relative abundance of *Shewanella* was on the other hand significantly higher in the 5% uPP group compared with other groups ($p < 0.05$) as was *Citrobacter* ($p < 0.05$).

The results of PCoA on phylum and genus level indicated that the gut bacterial composition of fish fed the 5% uPP diet was different from the control group and the 2.5 % uPP group (Fig. 8C). In addition, no significant difference in gut microbiota was observed between control group and the 2.5 % uPP group (Fig. 8C).

4. Discussion

In this study, we investigated the effects of further fish meal

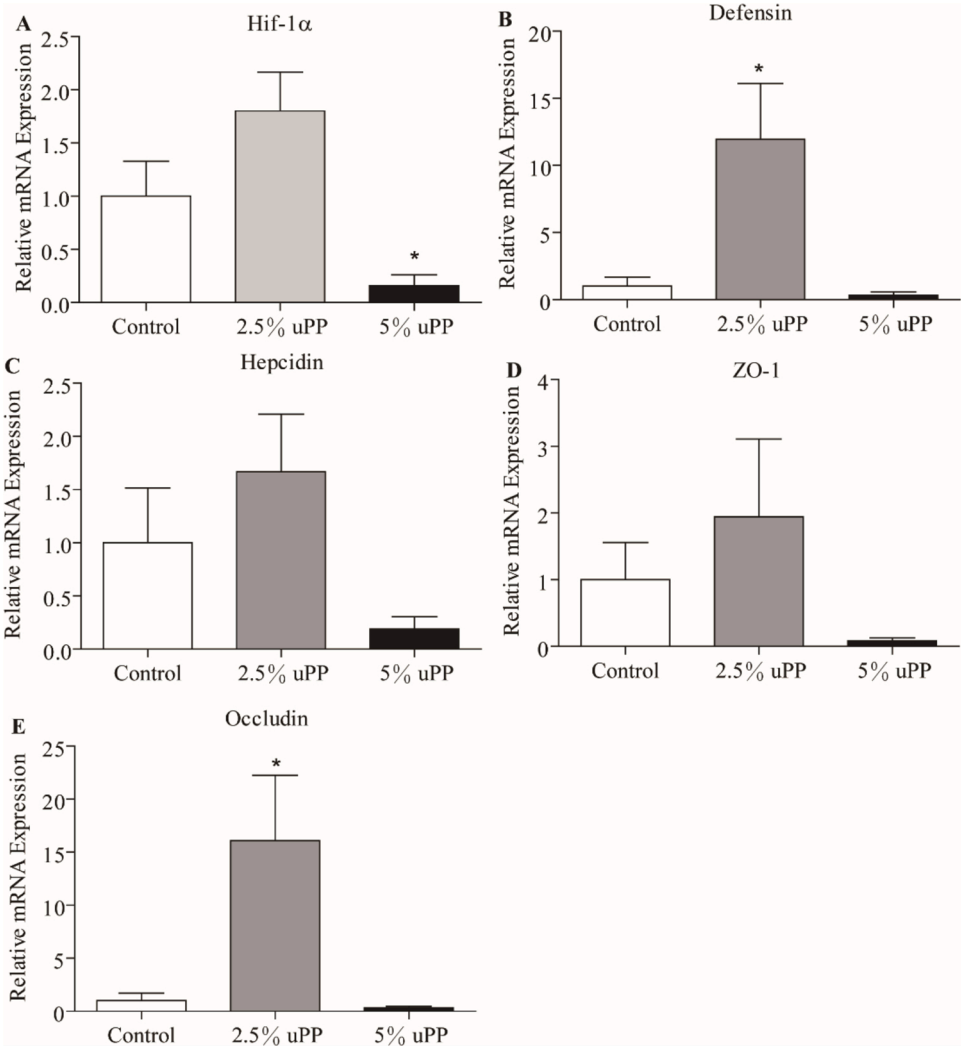

Fig. 4. Relative mRNA expression of genes related to intestinal health in common carp fed different diets. Data are expressed as mean ± SEM (n = 6). Data of Hepcidin, Defensin, and Occludin expression were analyzed by non-parametric Jonckheere-Terpstra test followed by post hoc test. A single asterisk denotes $p < 0.05$.

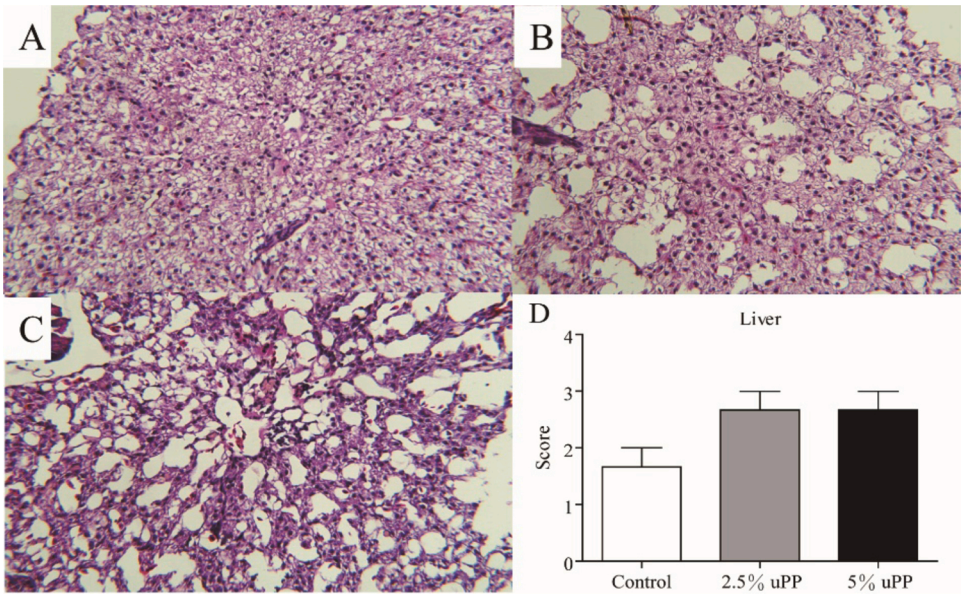

Fig. 5. Representative histomorphological images from HE-stained sections of the liver of common carp fed different diets. (A) Control; (B) 2.5 % uPP; (C) 5% uPP; (D) Histological score of liver (n = 6). No significant difference in histological score was observed among groups.

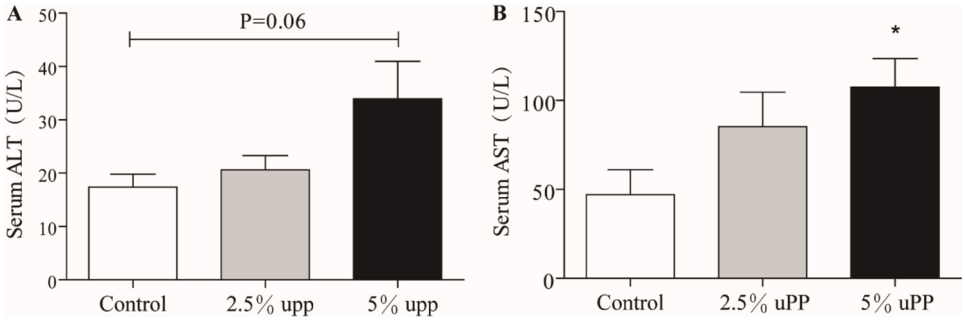

Fig. 6. Serum ALT and AST levels of the common carp fed different diets. Data are expressed as mean ± SEM (n = 6). The ALT data were analyzed by non-parametric Jonckheere-Terpstra test followed by post hoc test. A single asterisk denotes $p < 0.05$.

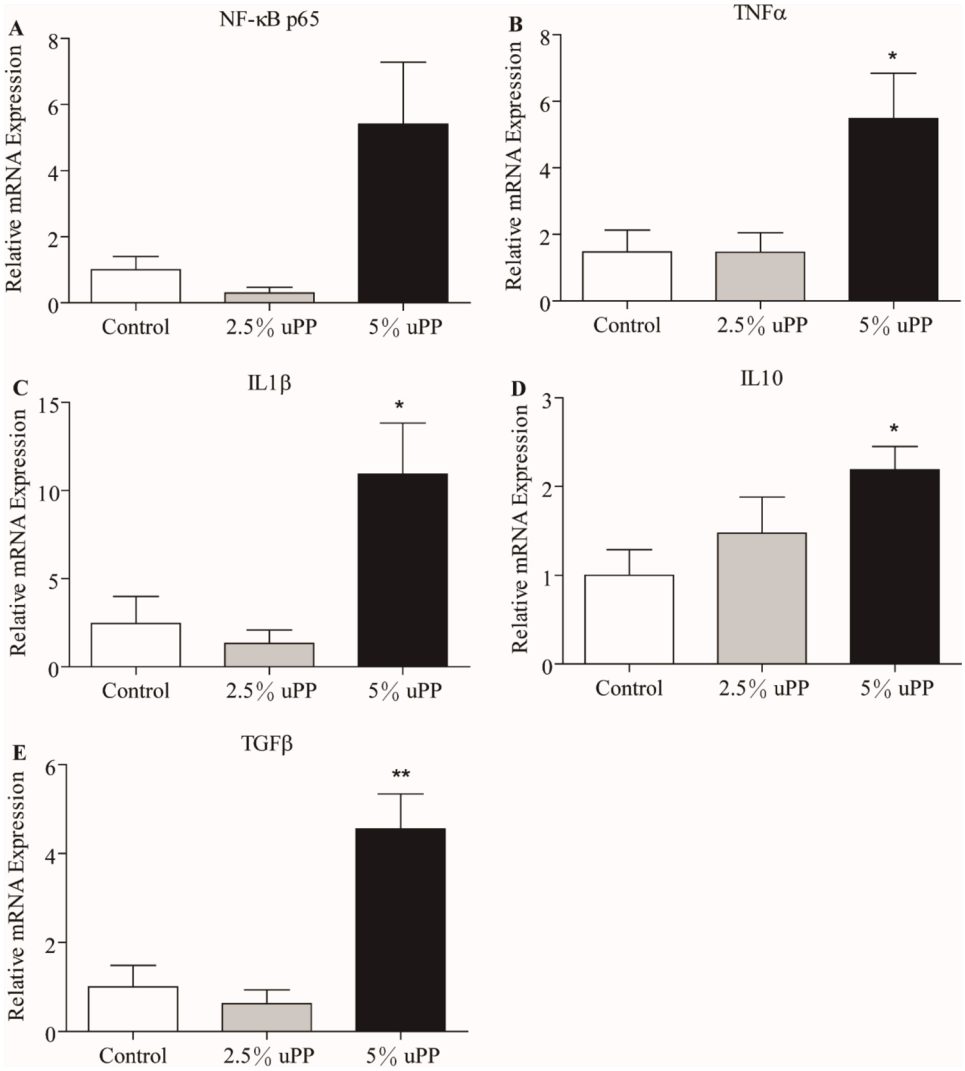

Fig. 7. Relative mRNA expression of inflammation related genes in the liver of the common carp fed different diets. Data are expressed as mean ± SEM (n = 6). Data of NF-κB p65 expression were analyzed by non-parametric Jonckheere-Terpstra test followed by post hoc test. A single and double asterisk denote $p < 0.05$ and $p < 0.01$, respectively.

replacement by uPP in practical diet on growth performance and gut/liver health of common carp. After a 16-week feeding trial, SR, WG, FCR, fatness and hepatosomatic index of common carp were not significantly different among the three groups. However, dietary 5% uPP can compromise gut and liver health of common carp.

In the present work, there were no significant differences in SR, WG, FCR, fatness and hepatosomatic index of common carp fed 2.5 % uPP or 5 % uPP diets versus control. While some studies reported that plant proteins replacement of fish meal did not impact growth performance in fish (Erdal et al., 2006; Ye et al., 2019; El-Saidy and Gaber, 2002), most results showed that replacing fish meal with plant protein significantly compromised the survival, weight gain, SGR, feed intake, and feed conversion ratio (Ma et al., 2019; Lin et al., 2010). The different results among studies may be attributed to replacement percentage, vegetable

Table 3
Simpson and ACE index of the intestine microbiota of carp fed with different diets.

Parameters	Control	2.5 % uPP	5% uPP
Simpson	0.66984 ± 0.23[a]	0.78 ± 0.15[a]	0.26501 ± 0.16[b]
ACE	152.72 ± 73.92[a]	223.07 ± 105.73[a]	219.57 ± 70.02[a]

Values represent the means (± SEM) of six replicates. Means without a common letter were significantly different ($p < 0.05$).

Table 4
The relative abundance of main phyla in the intestinal microbiota of common carp fed with different diets.

Bacteria Phylum	Control	2.5 % uPP (uPP1)	5% uPP (uPP2)
Fusobacteria	80.40 ± 6.59[a]	88.09 ± 7.41[a]	43.71 ± 3.65[b]
Proteobacteria	11.33 ± 3.07[a]	8.86 ± 7.51[a]	33.06 ± 2.72[b]
Firmicutes	3.53 ± 1.81[ab]	7.94 ± 2.01[a]	0.55 ± 0.32[b]
Actinobacteria	0.21 ± 0.07[a]	2.98 ± 0.72[ab]	1.68 ± 0.70[b]
Bacteroidetes	4.40 ± 2.30	10.66 ± 5.54	0.35 ± 0.18
Verrucomicrobia	0.06 ± 0.04[a]	1.40 ± 0.68[ab]	0.22 ± 0.10[b]

Values represent the means (± SEM) of six replicates. Means without a common letter were significantly different ($p < 0.05$).

protein sources, pre-treatment methods of the vegetable proteins, as well as fish species and feeding regime. In particular, the level of anti-nutritional factors in vegetable proteins may play an important role in terms of the outcome of FM replacement. In our study, micronization of plant proteins can reduce the negative effects of anti-nutritional factors in uPP, which may contribute to the positive results of further FM replacement.

While absorbing nutrients, the intestinal tract also acts as a barrier, preventing antigens and pathogens from entering mucosal tissues to cause diseases (Ulluwishewa et al., 2011). The 5% uPP supplementation group showed significantly increased expression of inflammatory cytokines, indicating an elevated intestinal inflammatory status. Similarly, enteritis induced by soybean meal inclusion in the diet has been extensively reported in different fish species, including Atlantic salmon (Urán et al., 2009; Krogdahl et al., 2015), chinook salmon (*Oncorhynchus tshawytscha*) (Booman et al., 2018), turbot (Bai et al., 2017; Gu et al., 2016) and common carp (Urán et al., 2008). In contrast, 2.5 % uPP did not induce intestinal inflammation. Moreover, the relative expression of defensin and occludin was significantly up-regulated in the 2.5 % uPP group compared with control, suggesting an improvement of intestinal health. The discrepancy of results between 2.5 % and 5% uPP supplementation may be attributable to dose response, and the underlying mechanism of the positive effect of 2.5 % uPP supplementation deserves further investigation. Notably, 5% uPP decreased the expression of *HIF1α*. HIF1α is a transcription factor that may regulate the expression of genes related to intestinal tight junction and antimicrobial peptides (Zheng et al., 2015). Moreover, recent study has shown that HIF1α can regulate the intestinal microbiota (Zhang et al., 2019). In this study, the decreased *HIF1α* expression in 5% uPP group is independent of hypoxia as it was mRNA expression result. The potential correlation between *HIF1α* expression and intestinal health in 5% uPP group awaits further study.

FM replacement by plant protein source has also been reported to affect liver health. Studies on pompano (*Trachinotus carolinus*) (Novriadi et al., 2018), Japanese seabass (*lateolabrax japonicus*) (Li et al., 2014), black carp (*Mylopharyngodon piceus*) (Hu et al., 2014), Japanese flounder (*Paralichthys olivaceus*) (Chen et al., 2011) and Atlantic salmon (De Santis et al., 2015) have shown that high levels of plant proteins containing antinutrients negatively affected liver depending on the species and plant protein dose. AST is a transaminase normally found in high concentrations in hepatocyte nucleus and mitochondria. When hepatocytes are damaged, AST leaks into circulation and can be detected in blood (Li et al., 2004; Racicot et al., 1975). Thus, the increase of serum AST is a good indicator of liver damage (Kumar et al., 2010; Zhang et al., 2019). Increased AST level in the blood was reported in Japanese seabass fed diets with SBM inclusion (Li et al., 2014). In the present study, we observed that serum AST level was significantly higher in common carp fed the 5% uPP diet. TNFα and IL1β are typical pro-inflammatory cytokines (Tilg and Moschen, 2010). TGFβ and IL10 are both anti-inflammatory cytokines (Hart et al., 2017; Gaddi et al., 2012), and up-regulation of TGFβ and IL10 is normally accompanied with down-regulated inflammation. However, simultaneous change in the expression of pro- and anti-inflammatory cytokines has also been reported (Falco et al., 2012; Ran et al., 2015), which can reflect a general up- or down-regulation of the inflammatory response. In the present work, the relative expression of *NF-κB*, *TNFα*, *IL1β*, *TGFβ* and *IL-10* were significantly up-regulated in the 5% uPP group compared with control and the 2.5 % uPP group, suggesting a general inflammatory response in the liver. Combined with the results of liver HE staining and gene expression in liver, our results showed that 5% uPP can cause liver inflammation and damage. In contrast, 2.5 % uPP appeared safe for the liver of carp.

Fish intestine harbors a large variety of microbes. The intestinal microbiota plays important roles in the nutrition, metabolism, and immunity of host (Zhou et al., 2017; Corr et al., 2007; Shimada et al., 2013). Studies have shown that plant protein sources can change the composition of the intestinal microbiota as shown in Atlantic salmon (Bakke-McKellep et al., 2007), rainbow trout (Desai et al., 2012), and Asian seabass (*Lates calcarifer*) (Apper et al., 2016). In the present study, the reduction of α-diversity index suggested a negative impact of 5% uPP on the intestinal microbiota. Previous study has shown that Proteobacteria, Firmicutes and Fusobacteria are the predominant phyla in the intestine of carp, accounting for 76.7 % of the total bacteria (Eichmiller et al., 2016). Consistently we observed that Fusobacteria and Proteobacteria were the predominant phyla in carp. Compared with the control group, the abundance of Proteobacteria was significantly increased in 5% uPP group, while the abundance of Fusobacteria was decreased. Our previous studies suggested that higher abundance of Fusobacteria and lower abundance of Proteobacteria was associated with improved fish health and performance and vice versa (Zhang et al., 2019; Guo et al., 2017). Therefore, the alteration of 5% uPP on the intestinal microbiota may lead to negative effects on fish health, and might be at least partially responsible for the negative phenotypes observed in gut and liver. At the genus level, the main Proteobacteria to increase following feeding the fish 5% uPP included *Shewanella* and *Citrobacter* while the main Fusobacteria to be reduced was *Cetobacterium*. *Shewanella* was opportunistic pathogen that could impair the intestinal immune mechanisms in fish (Xiong et al., 2015). Similar increase in Proteobacteria including *Shewanella* was found in northern snakehead fed soybean meal (Miao et al., 2018). Increases in *Citrobacter* may also challenge fish welfare as it is known to be an opportunistic pathogen causing disease in aquatic animals including zebrafish (*Danio rerio*) (Lü et al., 2012), common carp (Karunasagar and Pai, 2010) and rainbow trout (Jeremi et al., 2003). The obligate anaerobic bacterium *Cetobacterium* is one of the main dominant genus of the intestinal microbiota of freshwater fish (Kim et al., 2007; van Kessel et al., 2011; Rawls et al., 2006). *Cetobacterium* can produce vitamin B12 that is beneficial to the host (Tsuchiya et al., 2008), and our previous work has implicated the association of Cetobacterium abundance with fish health and welfare (Zhang et al., 2019; Guo et al., 2017). Deceased abundance of *Cetobacterium* by 5% uPP suggests negative effect on the intestinal microbiome. In contrast, there was no significant difference in the relative abundance of Fusobacteria and Proteobacteria between 2.5 % uPP and the control group, as well as the abundance of *Cetobacterium*. This suggests a dose effect of influence of uPP on the microbiota, and is consistent with the overall results of gut and liver health associated with 2.5 % uPP.

In conclusion, our results showed that further fishmeal replacement with uPP in a practical diet did not affect the growth performance and

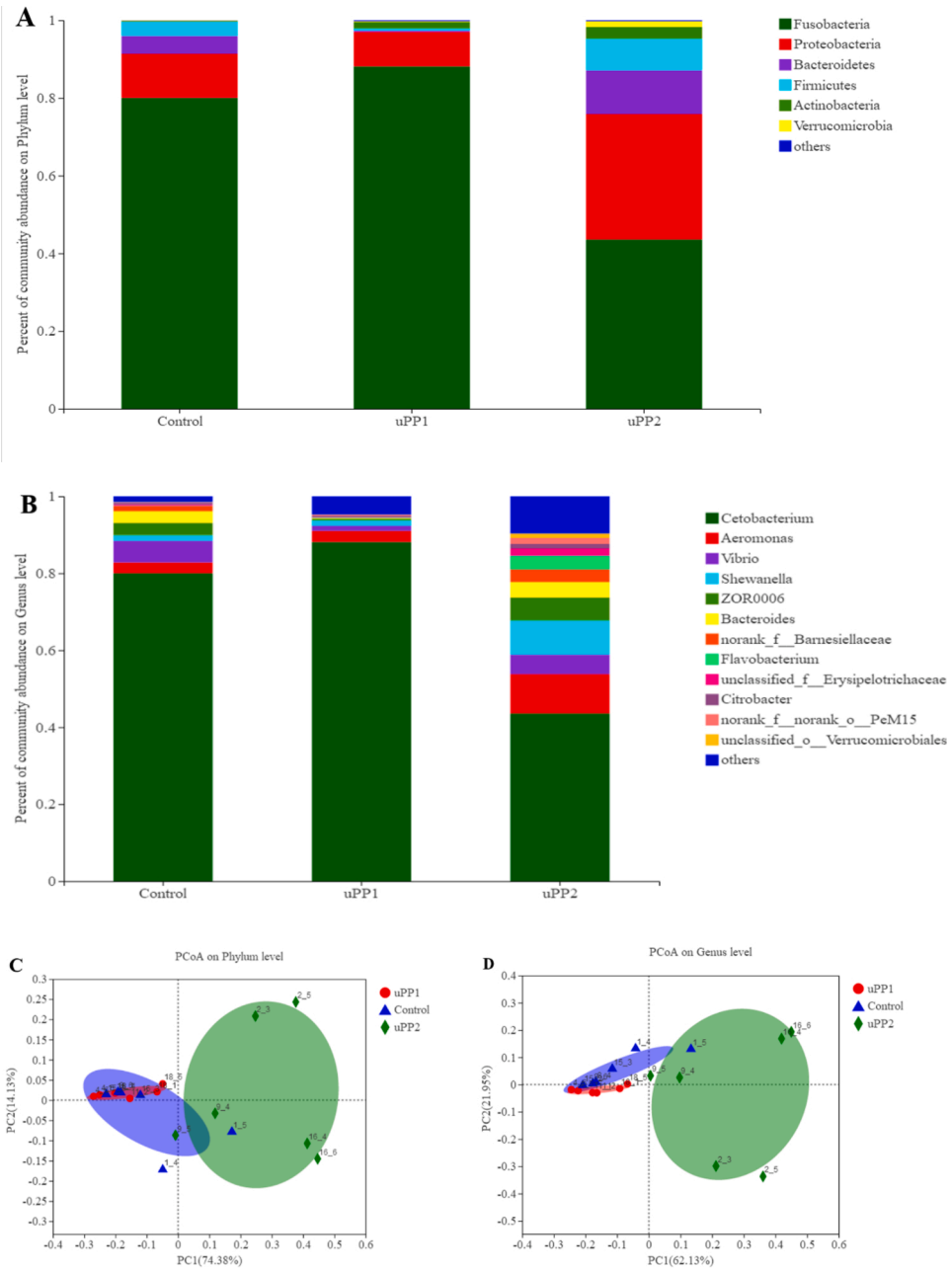

Fig. 8. The intestinal microbiota of common carp fed with control or uPP supplemented diets. Relative abundance at phylum (A) and genus (B) level of the gut microbiota; (C) Principal coordinates analysis (PCoA) of the intestinal microbiota on phylum level; (D) Principal coordinates analysis (PCoA) of the intestinal microbiota on genus level. (n = 6) uPP 1, 2.5 % uPP group; uPP 2, 5% uPP group.

Table 5
The relative abundance of genuses in the intestinal microbiota of common carp fed with different diets.

Bacteria Genus	Control	2.5 % uPP (uPP1)	5% uPP (uPP2)
Cetobacterium	80.38 ± 6.60[a]	88.08 ± 3.66[a]	43.69 ± 7.41[b]
Aeromonas	2.56 ± 1.01	2.88 ± 0.81	10.67 ± 4.71
Vibrio	5.74 ± 1.89	1.29 ± 1.01	4.92 ± 1.53
Shewanella	1.40 ± 0.20[a]	1.38 ± 0.40[a]	9.21 ± 3.26[b]
ZOR0006	1.83 ± 1.10[ab]	0.37 ± 0.27[a]	5.49 ± 1.89[b]
Bacteroides	2.92 ± 1.44	0.12 ± 0.08	3.84 ± 1.77
norank_f_*Barnesiellaceae*	1.32 ± 0.77	0.04 ± 0.03	3.17 ± 1.95
Flavobacterium	0.00 ± 0.00	0.01 ± 0.01	1.86 ± 1.17
unclassified_f_*Erysipelotrichaceae*	0.35 ± 0.23[ab]	0.06 ± 0.04[a]	1.84 ± 0.88[b]
Citrobacter	0.44 ± 0.18[a]	0.36 ± 0.15[a]	1.10 ± 0.30[b]
norank_f_norank_o_PeM15	0.04 ± 0.02[a]	0.26 ± 0.24[a]	1.43 ± 0.57[b]
unclassified_o_*Verrucomicrobiales*	0.06 ± 0.04	0.09 ± 0.03	1.05 ± 0.56

Values represent the means (± SEM) of six replicates. Means without a common letter were significantly different ($p < 0.05$).

survival rate of common carp at a supplementation level up to 5%. However, dietary 5% uPP impaired gut and liver health of common carp while uPP supplementation at 2.5 % showed no significant negative effects. Consistent with the intestinal phenotypes, dietary 5% uPP negatively changed the intestinal microbiota, while no significant difference was found between 2.5 % uPP and control. Together, our results indicated that low-level uPP can be added to practical diet to further reduce fish meal content, while 5% uPP addition may compromise fish health. Further treatment such as biological fermentation may be an effective method to improve the replacement percentage of uPP that can maintain fish health and welfare in parallel of growth performance. The intestinal microbiota results provide a clue that some commensal bacteria can be used as fermentation strains or as dietary additives in combination with fish meal replacement by plant proteins.

Declaration of Competing Interest

The authors report no declarations of interest.

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