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Doctoral thesis

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Norwegian University of Science and Technology
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Mingxu Xie

Trondheim, November 2022

Abstract

Plant proteins are one of the most widely used sources for fish meal (FM) replacement in aquafeed. However, high levels of antinutritional factors do have negative impacts on fish health. Ultra-micro ground mixed plant proteins (uPP) are made up of potato, soybean, pea protein, and wheat gluten followed by heating and ultra-micro grinding (< 200 μm) to reduce the content of antinutritional factors. Probiotics have been used to improve growth, immune responses, and disease resistance of fish. Most probiotics are derived from terrestrial sources rather than fish, however, some probiotic strains used in aquaculture pose challenges. It is urgent to identify potential commensal probiotics isolated from fish intestinal microbiota. Hence, the aim of the current thesis was to evaluate the replacement of FM in the diet by uPP, and identify potential probiotics in the gut microbiota of fish and study their effects on fish health.

The effect of uPP as FM replacement was studied in common carp (*Cyprinus carpio*). They were fed a basal diet in which some FM was replaced by 2.5% or 5% uPP. The results demonstrated that uPP had no effect on growth of common carp. However, dietary 5% uPP did have a negative impact on gut health by upregulating the intestinal expression of inflammation-related genes and reducing *hypoxia inducible factor 1 subunit alpha* expression. Moreover, dietary 5% uPP impaired liver health by increasing serum alanine aminotransferase and aspartate aminotransferase levels and upregulating liver expressions of inflammation-related genes. We also observed a significant alteration of the intestinal microbiota with a reduced relative abundance of *Cetobacterium*, which suggested that *Cetobacterium* might play a beneficial role in fish health. Furthermore, we evaluated the effects of supplementation of *Cetobacterium somerae* on common carp fed 5% uPP. The results indicated that *C. somerae* had the potential to reverse the negative effects of 5% uPP on the gut and liver. Additionally, using fermentation products of *C. somerae* in diets for zebrafish (*Danio rerio*), we noted that *C. somerae* improved liver and gut health and antiviral immunity. These findings suggest that *C. somerae* can be used as a potential probiotic in aquafeed.

In conclusion, the present work demonstrates that a low level of dietary inclusion of uPP (2.5%) can be used to reduce the dependence of FM on common carp, while 5% uPP impairs health. Commensal *C. somerae* can be used as a potential probiotic, reversing the negative effects of the

addition of 5% uPP. This research not only provides a theoretical reference for replacing FM with uPP plant proteins but also encourages the development of probiotics as functional feed additives for fish health.

Keywords: Fish meal, replacement, uPP, *Cetobacterium somerae*, fish health

Abbreviations

Alt	Alanine aminotransferase
ANFs	Antinutritional factors
Ast	Aspartate aminotransferase
cpt1	Carnitine palmitoyl transferase 1
FAO	Food and Agriculture Organization
FM	Fish meal
GI	Gastrointestinal
hif1α	Hypoxia inducible factor 1 subunit alpha
LAB	Lactic acid bacteria
LPS	Lipopolysaccharides
PCoA	Principal coordinates analysis
ppar	Peroxisome proliferator-activated receptor
ppargc1α	Proliferator-activated receptor gamma coactivator 1 alpha
SBM	Soybean meal
SCFAs	Short-chain fatty acids
SPC	Soy protein concentrate
SPI	Soy protein isolate
SVCV	Spring viremia of carp virus
TAG	Triacylglycerols
uPP	Ultra-micro ground mixed plant proteins
ucp2	Uncoupling protein 2

List of papers

This thesis is based on the following original research papers:

1. Xie, M., Xie, Y., Li, Y., Zhou, W., Zhang, Z., Yang, Y., Olsen, R.E., Ran, C., Zhou, Z., 2021. 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*). Aquaculture Reports, 19, 100558. https://doi.org/10.1016/j.aqrep.2020.100558

2. Xie, M., Zhou, W., Xie, Y., Li, Y., Zhang, Z., Yang, Y., Olsen, R.E., Ran, C., Zhou, Z., 2021. Effects of *Cetobacterium somerae* fermentation product on gut and liver health of common carp (*Cyprinus carpio*) fed diet supplemented with ultra-micro ground mixed plant proteins. Aquaculture, 543, 736943. https://doi.org/10.1016/j.aquaculture.2021.736943

3. Xie, M., Xie, Y., Li, Y., Zhou, W., Zhang, Z., Yang, Y., Olsen, R.E., Ringø, E., Ran, C., Zhou, Z., 2022. Stabilized fermentation product of *Cetobacterium somerae* improves gut and liver health and antiviral immunity of zebrafish. Fish & Shellfish Immunology, 120, 56–66. https://doi.org/10.1016/j.fsi.2021.11.017

Author Contributions

Papers 1 & 2: MX planned and carried out the experiment, analyzed the samples and data, and wrote the manuscript; YX and YL performed samples collection and data treatment; WZ participated in collecting samples; ZZ and YY participated in data treatment; REO contributed to the planning and analysis of samples; CR and ZZ designed the experiment and acquired funding and participated in the planning and revising process.

Paper 3: MX planned and carried out the experiment, analyzed the samples and data, and wrote the manuscript; YX participated in carrying out the experiment, analyzing the samples and data, and writing the manuscript; YL and WZ participated in investigating and analyzing data; ZZ and YY contributed to the data analysis; REO and ER provided resources and methodology; CR and ZZ designed the experiment and acquired funding and participated in the planning and revising process.

1. Introduction

The production volumes of fish aquaculture have increased steadily over the past decades and aquaculture has become an important supplier of aquatic products, providing humans with healthy proteins and lipids (Dawood et al., 2019; Schar et al., 2020). Nearly half of all aquatic products currently consumed by humans are provided by aquaculture (Food and Agriculture Organization [FAO], 2020). Fish meal (FM) has been an indispensable high-quality protein source because of its high content of essential amino acids and fatty acids, low carbohydrate content, superior palatability, few antinutritional factors (ANFs), and high digestibility by cultured animals (Miles and Chapman, 2006; Cho and Kim, 2011). The supply of FM, however, has reached its maximum level, and the price tends to fluctuate (Hardy, 2010). The imbalance between supply and demand has prompted intensive research on FM replacements for aquafeed (Jannathulla et al., 2019).

1.1 Plant proteins

Currently, plant proteins are the most important protein sources for human and animal nutrition because of their high content of crude protein and good amino acid profiles. Plant proteins increasingly are used to replace FM for most fish species (Hardy, 2010; Zhou et al., 2018). Soybean (*Glycine max* Linnaeus), barley (*Hordeum vulgare* Linnaeus), canola (*Brassica rapa* Linnaeus), corn (*Zea may* Linnaeus), cottonseed (*Gossypium hirsute* Linnaeus), peas (*Pisum sativum* Linnaeus)/lupins (*Lupinus* sp. Linnaeus), and wheat (*Triticum aestivum* Linnaeus and *T. diccoides*, var. *durum*) are the most often utilized plant ingredients (Gatlin et al., 2007).

1.1.1 Anti-nutritional factors (ANFs) of plant proteins

Although plant proteins contain macronutrients and micronutrients, they are also rich in ANFs, limiting their use (Gatlin et al., 2007; Krogdahl et al., 2015). Generally, ANFs contain two categories: the heat-labile group (e.g., protease or amylase inhibitors, lectins/agglutinin) and the heat-stable group (e.g., saponins, phytosterols, phytic acid, tannins, estrogens, β-conglycinin, non-starch oligosaccharides) (Zhou et al., 2018). Each of these antinutrients has detrimental effects when fed to fish, including reduced growth and nutrient utilization efficiency, intestinal dysfunction, immune alteration, and modulated intestinal microbiota. Table 1 shows the effects of some ANFs in fish.

Table 1. Effects of purified antinutritional factors (ANFs) from plant proteins on fish

ANFs	Fish species	Inclusion rate	Biological effect	Reference
Soybean protease inhibitors	Rainbow trout (*Oncorhynchus mykiss*)	0.37–1.48%	Reduced protein digestibility and increased accumulation of intestinal cysteine, both with a dose-dependent effect	Krogdahl et al., 1994
Soybean trypsin inhibitors	Atlantic salmon (*Salmo salar* L.)	0.105–0.42%	Reduced digestibility of protein and fat, weight gain, and trypsin activity in intestinal content	Olli et al., 1994
Soybean agglutinin	Rainbow trout	6%	Bound *in vivo* to intestinal epithelium; contributed to pathology in soybean-fed salmonids	Buttle et al., 2001
Soy saponins	Turbot (*Psetta maxima*)	0.25–1.5%	Reduced growth, nutrient utilization, increased inflammation, epithelial permeability, enteritis in the distal intestine	Gu et al., 2018
Soy saponins	Zebrafish (*Danio rerio*)	0.33%	Intestinal inflammation	Hedrera et al., 2013
Soy isoflavone	Rice field eel (*Monopterus albus*)	0.25%	Reduced growth; damaged intestinal structure and intestinal barrier; increased intestinal inflammation	Hu et al., 2021
Phytic acid	Grass carp (*Ctenopharyngodon idellus*)	0.8–4%	Suppressed growth and reduce their ability to resist enteritis; decreased fish intestinal antimicrobial ability; aggravated fish intestinal inflammation responses	Zhong et al., 2019
β-conglyc-in in	Turbot	4–8%	Reduced growth and feed utilization, as well as a variety of nonspecific and specific immune responses and intestinal mucosal lesions	Li et al., 2017
β-conglyc-in in	Jian carp (*Cyprinus carpio* var. Jian)	8%	Reduced fish growth; induced inflammation and oxidation, and dysfunction of intestinal digestion and absorption	Zhang et al., 2013
Hydrolys-able annins	Grass carp	1.25%	Impaired protein metabolism; reduced lipids digestion and accumulation; promoted carbohydrate digestion; changed the intestinal environment and bacterial composition	Yao et al., 2019

1.1.2 Main removal method of ANFs

To improve the usability of plant proteins in aquafeed, it is essential to reduce or remove as many ANFs as possible. Several processing technologies and combinations of them have been used to

reduce ANFs in plant proteins (Zhou et al., 2018; Samtiya et al., 2020) (Table 2).

Table 2. Findings of selected investigations in which plant-derived materials were introduced to substitute fishmeal in fish diets

Plant material	Fish species	FM replacement rate	Biological effect	References
Thermal and hydrothermally treated full-fat soybean meal (SBM)	Carp	50%	Body weight gain and body protein retention better than FM group.	Abel et al., 1984
Thermal-treated pea seed meal	African catfish (*Clarias gariepinus*)	33%	Weight gain and protein efficiency ratio higher than fish fed raw pea seed meal.	Davies and Gouveia, 2008
Defatted SBM (roasted and solvent-extracted)	Juvenile cobia (*Rachycentron canadum*)	10–60%	Growth and feed utilization were not affected when the replacement level was up to 40%.	Zhou et al., 2005
Soy protein concentrate (SPC)	Red sea bream (*Pagrus major*)	60–100%	No differences in growth performance up to a level of 70% inclusion.	Biswas et al., 2019
Extruded plant proteins (SBM, SPC, and wheat gluten)	Atlantic cod (*Gadus morhua* L.)	25–100%	The 50% level had higher growth and feed utilization; 100% level had lower hepatosomatic index.	Hansen et al., 2007
Soy protein isolate (SPI)	Juvenile Amur Sturgeon (*Acipenser schrenckii*)	25–100%	Replacement level can be up to 57.64% without inducing negative impacts on growth performance.	Xu et al., 2012
Fermented SBM	Largemouth bass (*Micropterus salmoides*)	15–60%	The 30% replacement level of fermented SBM had no negative impacts on the growth performance, body composition, nutrient utilization, and intestinal health of fish, while the substitution level of FM with SBM was only 15%.	He et al., 2020
Fermented SBM	Atlantic Salmon	30%	Enhancement of health and growth physiology by encouraging the growth of intestinal lactic acid bacteria, improvement of proximal gut health by boosting mucin formation, and an increase of intestinal transcellular water absorption.	Catalán et al., 2018
Enterococcus faecium fermented SBM	Turbot	45%	Increase of antioxidant capacity, suppression of inflammatory responses, and modulation of gut microbiota in turbot.	Li et al., 2020

1.1.2.1 Physical treatment

For some heat-labile ANFs, such as protease inhibitors and lectins, the content can be lowered by heating, such as roasting, boiling, or extruding (Zhou et al., 2018). Trypsin inhibitors in raw

soybeans were reduced from 0.0154 g/kg to 0.0023 g/kg after heating and to 0.0061 g/kg after extrusion (Ari et al., 2012). Heating soybean meal (SBM) for 30 min reduced the trypsin inhibitor activity and increased apparent protein digestibility and body protein content in Nile tilapia (*Oreochromis niloticus*) compared with fish that were fed untreated SBM (Azaza et al., 2009). Cooking can lower the content of lectins in legumes, thus improving their nutritional quality (Maphosa and Jideani, 2017). In addition, micronization, reducing the average diameter, is widely used to lower the levels of ANFs, such as phytate, tannin, and trypsin inhibitors, in beans (Patterson et al., 2017). Pereira and Oliva-Teles (2004) demonstrated that gilthead sea bream (*Sparus aurata* L.) that were fed micronized lupin seed meal grew better than fish that were fed raw lupin.

Thermal inactivation of ANFs is the normal treatment used for terrestrial animals. However, many fish species appear to be particularly sensitive to heat-labile ANFs residues that remain after treatment (Drew et al., 2007; Zhou et al., 2018). Furthermore, thermal treatment also may reduce the protein content and availability of several essential nutrients, including amino acids and vitamins. This will reduce the nutritional value of seed meal (Francis et al., 2001).

1.1.2.2 Chemical treatment

Heat-stable ANFs, such as saponins, phytates, non-starch polysaccharides, and β-conglycinin, are not significantly affected by thermal treatment (Drew et al., 2007). These ANFs can be reduced by extracting the meal, such as SBM, with alcohol or aqueous solutions, generating soy protein concentrate (SPC) and soy protein isolate (SPI), respectively (Francis et al., 2001; Zhou et al., 2018). These products improve the nutritional value of SBM (Drew et al., 2007; Collins et al., 2012). The properties of the two products will vary (Drew et al., 2007). One main difference between the two treatments is that a large part of the lipid soluble saponins are removed with SPC treatments, while they will accumulate with aqueous SPI extraction (Anderson and Wolf, 1995; Lusas and Riaz, 1995). This in part can explain why diets supplemented with SPI reduce the growth in chinook salmon (*Oncorhynchus tshawytscha*), while alcohol-extracted SPC improves weight gain compared with normal heat-treated SBM (Hajen et al., 1993; Bureau et al., 1998).

1.1.2.3 Biological treatment

ANFs in plant proteins also can be reduced by biological treatment, such as biological processing, fermentation, or the addition of enzymes (Diouf et al., 2019; Samtiya et al., 2020). Enzymatic hydrolysis by alcalase and trypsin can reduce the content of β-conglycinin and soybean globulin in SBM (Zhou et al., 2018). Phytase treatment can reduce the negative effects of phytic acid (chelating of phosphorous), including reductions in fish growth and low mineral availability (Spinelli et al., 1983; Gibson et al., 2010). Protease inhibitors, phytic acid, and tannins also can be reduced by fermentation (Coulibaly et al., 2011; Simwaka et al., 2017). Ogodo et al. (2019) demonstrated that the fermentation of corn flour by lactic acid bacteria (LAB) reduced the content of phytase, tannins, and polyphenols. Fermentation of SBM by *Bacillus siamensis* for 24 h reduced the content of glycinin, β-conglycinin, and trypsin inhibitors by 86.0%, 70.3%, and 95.01%, respectively (Zheng et al., 2017). Fermentation also changes the microstructure of the SBM protein, reducing its size and creating a looser network with less β-sheet structure and a more randomly coiled structure (Zheng et al., 2017). The cofermentation of cassava products with *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* has been shown to massively reduce the level of ANFs, improving the nutritional value (Etsuyankpa et al., 2015). Solid-state fermentation by *Bacillus* sp increases the level of soluble polyphenols in wheat bran three times that of raw bran along with the activities of protease, cellulase, and phytase, whereas the content of phytic acid decreases (Tanasković et al., 2021).

1.1.3 Ultra-micro ground mixed plant proteins (uPP)

A novel commercial mixed plant protein uPP (original name: JPC56) that uses a mixture of heating and micronization has just entered the market. It is provided by Joosten (Netherlands) and is composed of several protein sources, including potato protein, pea protein, wheat gluten, and soy protein (Vermeer and Stein, 2019). The mixture is first heated to eliminate trypsin inhibitors and conglycinin and then is micronized by ultra-micro grinding (95% < 200 μm), which is predicted to further reduce many ANFs, including trypsin inhibitors (to 2 mg/g) and β-conglycinin (to 28 mg/g). The manufacturing process increases the surface area for digestive enzymes and improves digestibility in piglets and poultry (Vermeer and Stein, 2019). There is, however, little information about its suitability in fish feeds.

1.2 Probiotics and their application in aquaculture

1.2.1 The definition of probiotics

Lilly and Stillwell used the word “probiotics” to describe “substances secreted by one microorganism which stimulates the growth of another” (Lilly and Stillwell, 1965). The term has since changed slightly and the definition used by the FAO and the World Health Organization is “living microorganisms that are consumed orally and provide demonstrable health advantages to the host” (Hill et al., 2014). Merrifield et al. (2010) defined probiotics in aquaculture as organisms, such as “living, dead, or components of a microbial cell that are added to diets or rearing water and have favorable effects on host health.”

1.2.2 Application of probiotics in aquaculture

The first study of probiotics in aquaculture was conducted by Kozasa (1986), who revealed that dietary *Bacillus toyoi* increased growth of yellowtail (*Seriola quinqueradiata*). Since then, probiotics have been widely used in aquaculture, with the most commonly used genera, including *Bacillus* and LAB (Ringø et al., 2020). In addition, *Aeromonas*, *Alteromonas*, *Arthrobacter*, *Bifidobacterium*, *Clostridium*, *Paenibacillus*, *Phaeobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Rhodosporidium*, *Roseobacter*, *Streptomyces* and *Vibrio*, and microalgae (*Tetraselmis*) and yeast (*Debaryomyces, Phaffia* and *Saccharomyces*) are also used (Van Doan et al., 2021; Ringø et al., 2022).

1.2.2.1 Effects of probiotics on growth performance

During fermentation or growth, bacteria may produce exogenous enzymes or other compounds that promote fish growth (Wu et al., 2012; Hoseinifar et al., 2017; Liu et al., 2017). For example, dietary *Bacillus subtilis* can increase feed efficiency and weight gain of orange-spotted grouper (*Epinephelus coioides*) by providing exogenous enzymes, including protease and lipase (Liu et al., 2012). In rainbow trout (*Oncorhynchus mykiss*), *L. bulgaricus*, *Lactobacillus acidophilus*, and *Citrobacter* improve fish growth by increasing intestinal amylase, trypsin, and alkaline phosphatase activities (Mohammadian et al., 2019). *Enterococcus faecalis* increases not only the activity of digestive enzymes including protease and lipase but also the production of intestinal propionic- and butyric-acid in Javanese carp (*Puntius gonionotus*, Bleeker 1850) (Allameh et al.,

2017). In addition, probiotics can provide vitamin K and B_{12} to the host, with beneficial effects on growth performance (Sugita et al., 1991; Soltani et al., 2019). Yeast was reported to produce polyamines that can enhance intestinal maturation in adult penaeid shrimp (*Penaeus chinensis*) (Wang et al., 2000).

Probiotics can promote nutrient absorption by improving the intestinal morphology and microbiota of fish (e.g., Standen et al., 2015; Soltani et al., 2019). *B. subtilis* Ch9 increases the relative abundance of the intestinal anaerobic bacteria, *Bifidobacterium* and *Lactobacillus*, which benefits growth of grass carp (*Ctenopharyngodon idellus*) (Wu et al., 2012). Dietary *Bacillus amyloliquefaciens* improves intestinal morphology by increasing the heights of the intestinal villi and the numbers of mucus-secreting cells (goblet cells) in Nile tilapia (Reda and Selim, 2015). A multispecies probiotic, including *Lactobacillus reuteri*, *B. subtilis*, *Enterococcus faecium,* and *Pediococcus acidilactici*, increases not only the relative abundance of Firmicutes but also the intestinal populations of intraepithelial leucocytes, absorptive surface area index, and microvilli density, which benefits growth of tilapia (Standen et al., 2015).

Probiotics can improve defense against bacterial and virus infections (e.g., Hoseinifar et al., 2018; Ran et al., 2021). Dietary *Bacillus cereus* can compete with pathogenic *Aeromonas hydrophila* for glucose or iron (Lalloo et al., 2010). Feeding juvenile shrimp (*Litopenaeus vannamei*) with *Bacillus licheniformis* Mat32, *B. subtilis* Mat43, and *B. subtilis* subsp *subtilis* GatB1 decreases infections induced by infectious hypodermal and hematopoietic necrosis virus, which results in runt-deformity syndrome and stunted growth (Sánchez-Ortiz et al., 2016). Moreover, probiotics can reduce the adherence and colonization of pathogenic bacteria, promote gastrointestinal (GI) tract function, and modulate the GI tract microbiota (e.g., Ringø et al., 2020).

In contrast, some probiotic strains have differential effects on the growth of aquatic animals. *Bacillus amyloliquifaciens* does not improve the growth of tilapia (Silva et al., 2015). Similarly, the addition of *Lactobacillus plantarum* in diets to narrow clawed crayfish (*Astacus leptodactylus*) (Valipour et al., 2019), white shrimp (Huynh et al., 2018), and giant freshwater prawn (*Macrobrachium rosenbergii*) (Dash et al., 2015) does not affect growth. A commercial probiotic containing *Bacillus* spp., *Lactobacillus* spp., and *S. cerevisiae* has no impact on growth of juvenile olive flounder (*Paralichthys olivaceus*) (Niu et al., 2019). This lack of impact most likely is due

to changes in the aquatic animals' gut microbiome, life phases, and culture conditions (Soltani et al., 2019).

1.2.2.2 Effects of probiotics on immunity and disease resistance

Several studies have demonstrated that probiotics can improve immunity and disease resistance in a variety of fish species, such as grass carp (Jiang et al., 2019), grouper (Liu et al., 2012), Nile tilapia (Liu et al., 2017), olive flounder (Hasan et al., 2018), humpback grouper (*Cromileptes altivelis*) (Sun et al., 2018), common carp (*Cyprinus carpio*) (Soltani et al., 2017), and yellow croaker (*Larimichthys crocea*) (Ai et al., 2011).

The addition of probiotics enhances the survival rate of fish infected with pathogenic bacteria by increasing nonspecific immune responses, which in turn improves disease resistance (Soltani et al., 2019). For example, dietary *Lactococcus lactis* improves nonspecific immunity and enhances disease resistance by decreasing proinflammatory cytokines and increasing anti-inflammatory cytokines in the serum of common carp infected with *A. hydrophila* (Kong et al., 2020). *B. subtilis* elevates the activity of serum lysozyme and the alternative complement and survival rate of the grouper against *Streptococcus* sp. and an iridovirus (Liu et al., 2012).

Antibacterial compounds produced by probiotics include antibiotics, bacteriocins, lysozymes, H_2O_2, and acidic substances, which function as a barrier to the growth of opportunistic pathogens (Ringø et al., 2020; Van Doan et al., 2021). *Lactobacilli* isolated from Persian sturgeon (*Acipenser schrenckii*) produce bacteriocin-like inhibitory compounds that can inhibit pathogens in aquatic animals (Ghanbari et al., 2013). Antibacterial substances produced by *L. plantarum*, *Pseudomonas aeruginosa*, and *B. subtilis* isolated from the intestine of rohu (*Labeo rohita*) were reported to be antagonistic to *A. hydrophila* (Giri et al., 2011).

In addition, probiotics compete with pathogens for binding sites and nutrition, so as to prevent the adhesion and colonization of pathogens in the intestine in aquatic animals. The addition of *B. subtilis*, *B. licheniformis*, and *E. faecium* enhances the function of gut microbiota by adhering to the intestinal mucosal epithelium, which might protect rainbow trout from infection by potential pathogens (Merrifield et al., 2010). Gram-positive probiotics inhibit bacterial pathogens of fish by competing for nutrients, such as iron (Lemos and Balado, 2020).

Moreover, some probiotics have interference with quorum-sensing genes that can reduce the virulence of pathogens (Van Doan et al., 2021). *B. licheniformis* T-1, which has the quorum-quenching gene *ytnP*, can increase the survival of zebrafish (*Danio rerio*) infected with *A. hydrophila* (Chen et al., 2020). *Bacillus* quorum-sensing inhibitor-1 isolated from Prussian carp (*Carassius auratus gibelio*) has the ability to degrade quorum-sensing molecules protecting fish against *A. hydrophila* infection (Chu et al., 2011).

1.2.3 Gut microbiota and *Cetobacterium*

The gut microbiota is composed of aerobic, facultatively anaerobic, and obligate anaerobic bacteria that may be influenced by variables such as diet, age, and the ambient environment of aquatic animals (Ringø et al., 2016). Gut microbiota can be divided into autochthonous (when they are able to colonize the host's gut epithelial surface or are associated with the microvilli) and allochthonous (when they are transient, associated with food particles or present in the lumen) (Ringø et al., 2003; Tlaskalová-Hogenová et al., 2004; Ringø et al., 2016). The gut microbiota contributes to nutritional metabolism, enhances intestinal function, and improves intestinal immunity (Wang et al., 2018). Additionally, the gut microbiota can protect the host from being infected with pathogens by competing for nutrients or producing bacteriocins and proteinaceous toxins (e.g., Kamada et al., 2013; Qi et al., 2019; Xiong et al., 2019). The composition of fish gut microbiota differs greatly from that of humans and other terrestrial mammals, with Fusobacteria and Proteobacteria predominating over Firmicute and Bacteroides (Kostic et al., 2013). Host-associated probiotics, which are originally isolated from the rearing water or the GI tract of the host to improve growth and health of the host, display the best benefits in their own original environment (e.g., Lazado et al., 2015; Van Doan et al., 2020). For example, probiotics isolated from the intestine of Atlantic cod (*Gadus morhua* L.) inhibit pathogens at temperatures close to the cod culture temperature in this case 13°C, but the antibacterial activity is reduced at 20°C (Caipang et al., 2010). In recent years, studies increasingly have shown that host-associated probiotics can promote growth, survival rate, immune response, and disease resistance of aquatic animals in aquaculture (Wu et al., 2014; Lazado et al., 2015; Thy et al., 2017; Ringø et al., 2018; Van Doan et al., 2018). Although the mechanisms underlying the difference between host-associated probiotics and probiotics obtained from other sources are unclear, probiotics

derived from aquatic animals remain to have great potential as probiotics for application in aquaculture.

The commensal Fusobacteria makes up the majority of gut microbiota in fish (Larsen et al., 2014; Zhang et al., 2017). Fusobacteria is detected in the core gut microbiota of zebrafish (Roeselers et al., 2011). Meng et al. (2021) demonstrated that the infection with spring viremia of carp virus (SVCV) increased Proteobacteria, while the abundance of Fusobacteria decreased, suggesting that Fusobacteria in mucosal tissues plays a key role in the antiviral response. *Cetobacterium*, which belongs to Fusobacteria, has been detected as the most abundant genus in freshwater fish, including bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), channel catfish (*Ictalurus punctatus*) (Larsen et al., 2014), common carp (Van Kessel et al., 2011), giant Amazonian fish (*Arapaima gigas*) (Ramírez et al., 2018), goldfish (*Carassius auratus*) (Tsuchiya et al., 2008), ayu (*Plecoglossus altivelis*) (Tsuchiya et al., 2008), zebrafish (Wang et al., 2021a), and Nile tilapia (Ray et al., 2017). *C. somerae* (formerly termed Bacteroides type A) is a micro-aerotolerant, rod-shaped Fusobacterium that does not generate spores (Tsuchiya et al., 2008). *Cetobacterium* can produce vitamin B_{12} (Roeselers et al., 2011), butyrate (Bennett and Eley, 1993), acetate, and propionate (Hao et al., 2017), all of which have been shown to benefit fish health. In addition, the abundance of *C. somerae* is high in fish, such as carp and tilapia that does not need a dietary supply of vitamin B_{12}, but not in fish such as the eel and channel catfish that need dietary vitamin B_{12}, implying that *C. somerae* is associated with vitamin B_{12} production in the fish gut (Sugita et al., 1991). Acetate, propionate, and butyrate can enhance intestinal health by maintaining the integrity of the intestinal barrier and by preventing the development of inflammation (Lewis et al., 2010; Brown et al., 2013). Wang et al. (2021b) reported that the presence of *C. somerae* was related to higher acetate levels. The addition of acetate also increased glucose consumption in zebrafish, which indicated a role for acetate in *C. somerae* function.

1.2.4 Dietary supplementation of probiotics in aquafeed containing plant proteins

Probiotics can be used to degrade ANFs in the diet, and thereby, can increase the digestibility of plant proteins (Bairagi et al., 2004; Salehi et al., 2022). The issue is not well studied, but several studies have been promising (Ringø and Song, 2016). Supplementation of 1% *Lactobacillus* sp. in the diet to striped mullet (*Mugil cephalus*) containing plant protein sources has been shown to

improve weight gain and feed utilization compared with a plant protein diet (El-Tawil et al., 2012). Feeding *Bacillus* sp. plus sweet potato (*lpomoea batatas*) to Humpback grouper increases growth and immune parameters following infection with *Vibrio alginolyticus* compared with a control diet (Azhar, 2013). Dietary plant proteins supplemented with probiotics increase the innate immunity compared with a plant protein diet alone in Senegalese sole (*Solea senegalensis*) (Batista et al., 2016). The underlying mechanism of the combined use of probiotics and plant proteins needs additional investigation.

1.3 Common carp and zebrafish

Common carp is one of the oldest farmed fish in the world and its aquaculture dates back to 6000 BC in the Henan Province, China (Nakajima et al., 2019). They can tolerate extreme water quality conditions, including low temperature and dissolved oxygen, which make them suitable for being cultured in a variety of aquatic ecosystems (Zambrano et al., 2006; Weber and Brown, 2009). Owing to the advantages of rapid growth, early maturation, and high nutritional value, common carp is one of the most important freshwater fish cultured in the world (Weber and Brown, 2009; Phelps et al., 2010). According to the FAO, the output of common carp exceeded 4.18 million tons in 2018 (FAO, 2020).

Zebrafish were first used to study molecular pathways of forwarding genetics by George Streisinger in the late 1960s (Grunwald and Eisen, 2002). Initially, zebrafish were used to study embryonic biology, but now they are used in a variety of other fields (Ulloa et al., 2011; Jørgensen, 2020). Zebrafish have many advantages as a fish model, including the small size, transparent embryos, fast growth, short generation time, and the completion of the full genome sequencing (Yoder et al., 2002; López Nadal et al., 2020). With the development of genome editing techniques, such as CRISPR/Cas and Zinc finger nuclease, zebrafish have become an important fish model in basal and mechanistic studies of fish health, nutrient interactions, gut microbiota, and gut immunity (Urnov et al., 2010; Jørgensen, 2020; López Nadal et al., 2020).

In this study, common carp and zebrafish were used as commercial fish and model fish, respectively, to study the effects of adding plant proteins and *C. somerae* in fish diets. Conducting research with the two fish models offers several advantages. For example, common carp can produce enough blood cells to classify diverse immune cell subtypes and then do transcriptomic

analysis (Secombes et al., 1983; Romano et al., 1998; Joerink et al., 2006). Zebrafish may have similar nutritional requirements with those of farmed herbivores such as carp and tilapia (Ulloa et al., 2011). Moreover, because both common carp and zebrafish are members of the Cyprinidae family, combining animal models will produce results that are easy to compare among these species, allowing them to provide the “best of both worlds” of a small model of zebrafish with genetic diversity and common carp, a large-body size fish with well-defined genetically highly inbred lines (Henkel et al., 2012).

2. Aims of the study

The current thesis has the following research questions:

1. What are the effects and levels of ultra-micro ground mixed plant proteins (uPP) in fish diets for FM replacement?

2. Can any potential probiotics in the gut microbiota be used as a biological treatment to counteract the negative effects caused by plant proteins including uPP?

Therefore, the objectives of the research are:

1. To investigate the levels of uPP replacing FM and seek commensal bacteria that may mitigate the negative effects associated with the addition of uPP.

2. To study the effects of the *C. somerae* fermentation product on the health of fish that have been fed diets containing uPP.

3. To evaluate whether the commensal *C. somerae* can be used as a probiotic to improve fish health.

3. Summary of results

3.1 Paper 1: "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*)."

We investigated the impact of replacing FM with 2.5% or 5% uPP on the health of common carp. Results showed that uPP had no effects on growth and survival of the fish (Figure 1 in **Paper 1)**. We observed, however, that 5% uPP impaired gut and liver health by upregulating the expression of inflammation-related genes in elevating serum contents of alanine aminotransferase (Alt) and aspartate aminotransferase (Ast). None of these effects were seen with 2.5% uPP but improved expression of occludin and defensin in the gut (Figures 3, 4, 6, and 7 in **Paper 1)**.

Adding 5% uPP to carp diets reduced the α-diversity index (Table 3 in **Paper 1)** and the relative abundance of Fusobacteria at the phylum level and *Cetobacterium* at the genus level, and increased the relative abundance of Proteobacteria, Actinobacteria, and Verrucomicrobia compared with the control and the 2.5% uPP groups (Tables 4 and 5 in **Paper 1;** Figure 1). Moreover, principal coordinates analysis (PCoA) at the phylum and genus levels indicated that the composition of gut microbiota of fish that were fed the 5% uPP diet was different from the control group and the 2.5% uPP group (Figure 1). We did not observe any differences in gut microbiota between the control group and the 2.5% uPP group. According to these results, we inferred that the commensal bacterium *Cetobacterium* might play an important role in maintaining fish health.

In conclusion, the addition of 2.5% uPP can be used to replace some FM, but increasing the inclusion to 5% uPP has a detrimental effect on fish gut and liver as well as on the intestinal microbiota. The results of intestinal microbiota provided evidence that some commensal bacteria could be adopted as fermentation strains or as dietary supplements in combination with plant proteins to replace FM.

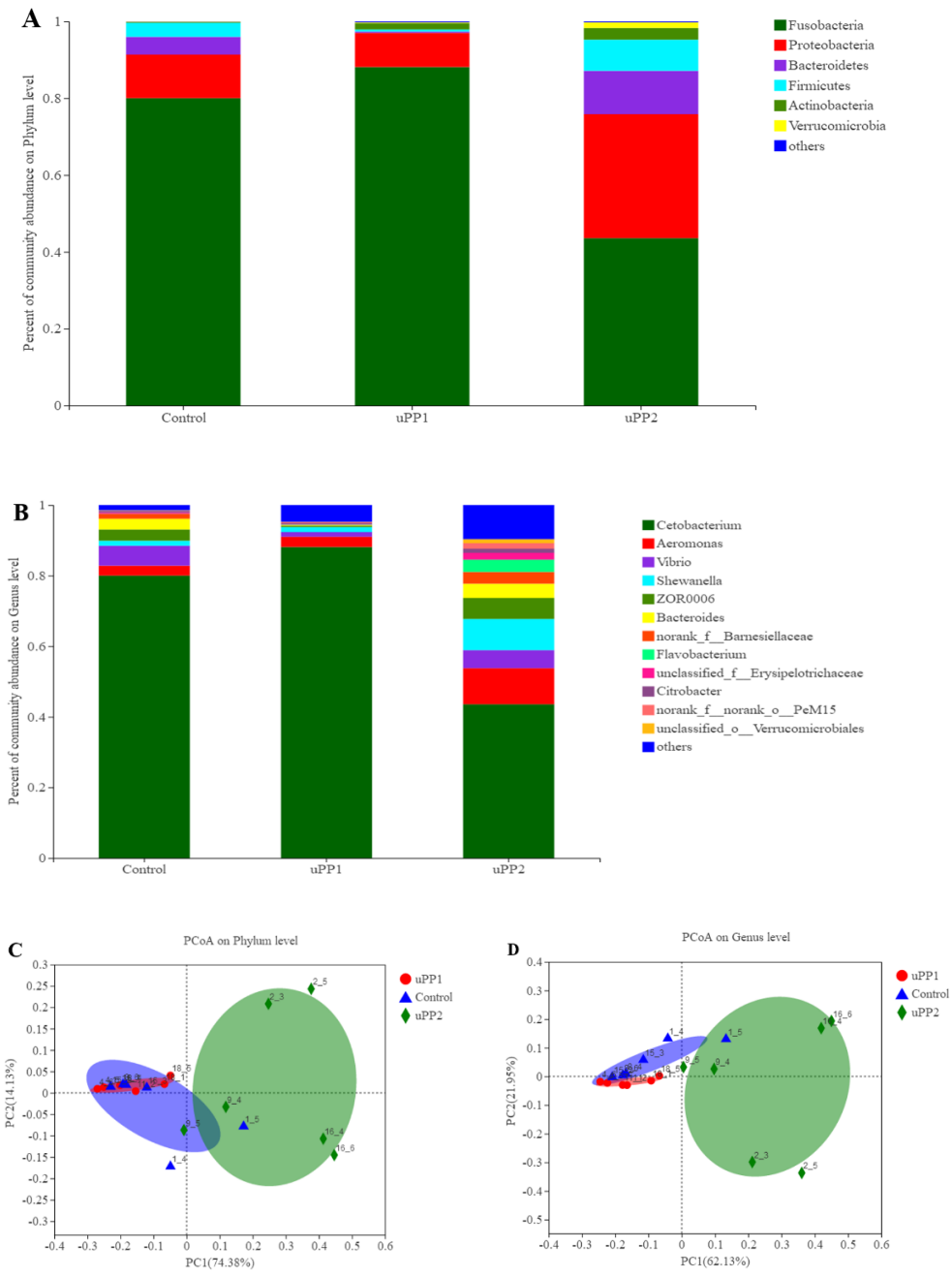

Figure 1. Effects of ultra-micro ground mixed plant proteins (uPP) supplemented diet on the gut microbiota of common carp. (A) Relative abundance (%) at the phylum level of gut bacteria of common carp fed the uPP supplemented diet or the control diet, n = 6. (B) Relative abundance (%)

at the genus level of gut content bacteria of common carp fed the uPP supplemented diet or the control diet, n = 6. (C) PCoA at the phylum level for the bacterial communities of common carp fed with experimental diets (n = 6). (D) PCoA at the genus level for the bacterial communities of common carp fed with experimental diets, n = 6. uPP, ultra-micro ground mixed plant proteins; uPP 1, 2.5% of the uPP inclusion level in basal diet; uPP 2, 5% of the uPP inclusion level in basal diet. PCoA, principal coordinates analysis. This figure is taken from **Paper 1.**

3.2 Paper 2: "Effects of *Cetobacterium somerae* fermentation product on gut and liver health of common carp (*Cyprinus carpio*) fed diet supplemented with ultra-micro ground mixed plant proteins"

In this study, the effect of a fermentation product of *C. somerae* was tested as a possible moderator of the negative effects of uPP. As expected, dietary 5% uPP compromised the intestinal and liver health of carps. The addition of the *C. somerae* fermentation product reversed these effects by reducing the inflammatory score of the intestine and the liver (Figure 2). This was supported by reduced expression of inflammation-related genes (Figures 3 and 9 in **Paper 2)** as well as reduced serum levels of lipopolysaccharides (LPS), LPS-binding protein, Alt and Ast (Figures 5 and 8 in **Paper 2)**. In addition, 5% uPP supplementation induced fatty liver of carps, whereas the *C. somerae* fermentation product reduced the formation of lipid droplets and the expression of lipogenesis genes (*fatty acid synthase*, *peroxisome proliferator-activated receptor* [*ppar*] *gamma* and *ppar beta*) and increased the expression of lipolysis genes (*carnitine palmitoyl transferase 1* and *ppar alpha*) in the liver. This result suggested that the fermentation product from *C. somerae* had the potential to prevent and heal fatty liver in carps.

In summary, our study suggested that dietary uPP can impair gut and liver health and induce fatty livers, whereas *C. somerae* fermentation product can improve gut and liver health of common carp and decrease lipid deposition in the liver. This study contributed to developing feed additives to improve the efficacy of FM replacement with plant proteins.

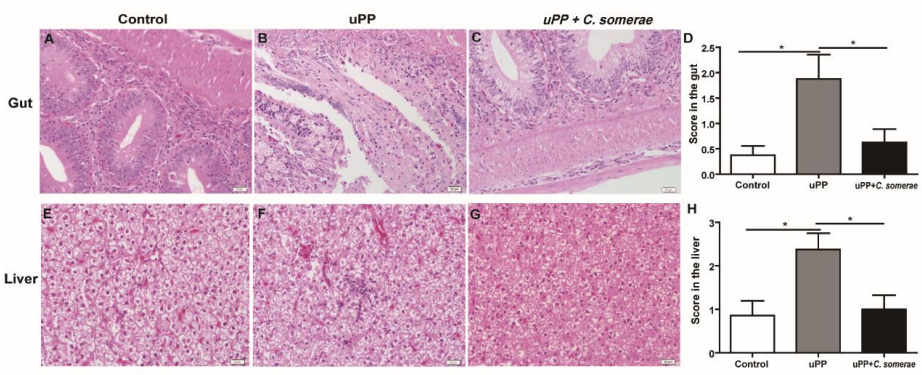

Figure 2. Effects of 5% ultra-micro ground mixed plant proteins (uPP) and 5% uPP supplemented with *C. somerae* fermentation product (uPP + *C. somerae*) on the hematoxylin and eosin-stained sections of the gut (A, B, C, and D) and liver (E, F, G, and H) of common carp. Dietary uPP significantly increased the inflammation score of the gut and liver compared with the control group ($P < 0.05$), whereas the *C. somerae* fermentation product significantly reduced the inflammation score compared with the uPP group ($P < 0.05$). All statistical metrics are detailed in **Paper 2.** uPP, ultra-micro-ground mixed plant proteins. This figure is modified from Figures 2 and 7 in **Paper 2.**

3.3 Paper 3: "Stabilized fermentation product of *Cetobacterium somerae* improves gut and liver health and antiviral immunity of zebrafish"

In the present work, dietary supplementation of *C. somerae* had no effects on zebrafish survival rate, weight gain, or feed conversion rate when compared with those fed a practical basal diet (total crude fat 13.62%) (Figure 1 in **Paper 3)**. The addition of *C. somerae* did reduce intestinal inflammatory score and pro-inflammatory gene (*interleukin1 beta*) expression, and increased the expression of hypoxia inducible factor 1 subunit alpha (Figures 2 and 3 in **Paper 3)**. This result showed that *C. somerae* has the potential to improve gut health of zebrafish. This was further supported by findings of lower intestinal malondialdehyde content, higher total antioxidant capacity, and an upregulation of antioxidative genes, including *glutathione peroxidase 1 alpha* and *Copper/Zinc superoxide dismutase* (Figure 4 in **Paper 3)** compared with the control group. The composition of gut microbiota in *C. somerae* and the control group was different at the phylum level, and *C. somerae* supplementation reduced the relative abundance of Proteobacteria while it increased Firmicutes and Actinobacteria (Figure 5 and Table 3 in **Paper 3)**.

The reduced lipid droplets in fish supplemented with *C. somerae* were further supported by the reduced liver triacylglycerols content and increased expression of lipid oxidation–related genes (*uncoupling protein 2* [*ucp2*], *carnitine palmitoyl transferase 1* [*cpt1*], and *proliferator-activated receptor gamma coactivator 1 alpha* [*ppargc1α*]) (Figure 2). Compared with the control group, the serum Alt level was decreased in the *C. somerae* group (Figure 2). The results indicated that dietary *C. somerae* has beneficial effects on liver health of zebrafish.

Interestingly, zebrafish that were fed the *C. somerae* diet had a higher survival rate after challenge with SVCV (Figure 7 in **Paper 3)**. More genes in the antiviral pathway were upregulated after the SVCV challenge in the spleen of zebrafish relative to the control group (Figure 7 in **Paper 3)**. These results also indicated that the underlying mechanism of *C. somerae* involved type I interferon signaling, which is explained in detail in **Paper 3.**

This study elucidated the finding that *C. somerae* can be used as a potential probiotic strain with beneficial effects on the gut and liver health and antiviral immunity of zebrafish.

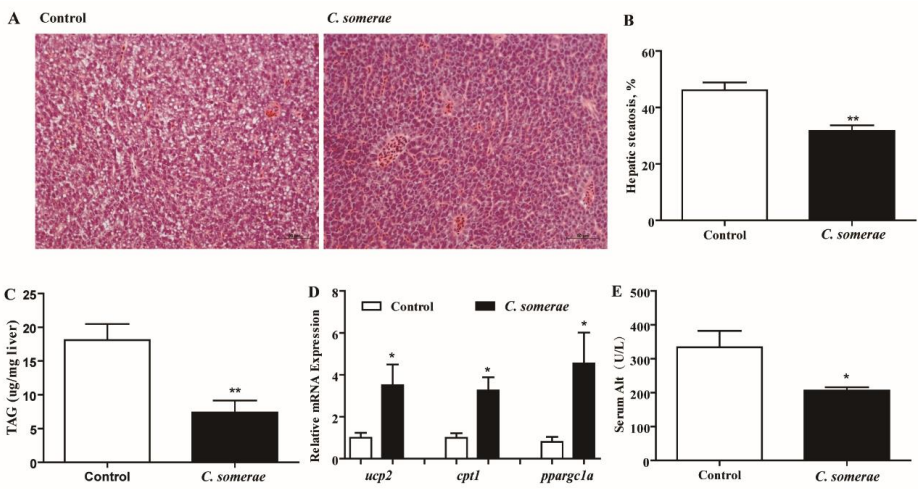

Figure 3. Effects of control and *C. somerae* diets on the lipid fat. (A) Representative liver histology image by hematoxylin and eosin staining. The scale bar is 50 μm. (B) Score of hepatic steatosis (%). (C) TAG in the liver. (D) Expression of lipolysis-related genes. (E) Serum Alt. Data were represented as the means (± SEM) (n = 6). *, $p < 0.05$ and **, $p < 0.01$ comparison with the control group. TAG, triacylglycerols; *ucp2*, *uncoupling protein 2*; *cpt1*, *carnitine palmitoyl*

transferase 1; *ppargc1α*, *proliferator-activated receptor gamma coactivator 1 alpha*; Alt, alanine aminotransferase. The figure is modified from figure 6 in **Paper 3.**

4. Discussion

This thesis evaluated the effects of uPP supplementation on health of common carp and documented the effects on fish performance and health (intestine and liver) (**Papers 1 and 2)**. Based on the effects on intestinal microbiota reported in **Paper 1,** we then explored the potential of some commensal gut bacteria to improve gut and intestinal health when combined with uPP as FM replacement (**Papers 2 and 3)**.

These observations indicated that 2.5% uPP of diet can be used to replace FM to common carp, but increasing the inclusion levels to 5% uPP impaired gut and liver health (**Paper 1)**, which was further confirmed in **Paper 2.** In addition, 5% uPP negatively affected intestinal microbiota by increasing the relative abundance of Proteobacteria and reducing Fusobacteria and *Cetobacterium* abundance, suggesting that *Cetobacterium* might be used as fermentation strains or dietary additives in combination with uPP. Interestingly, adding a fermentation product of *C. somerae* to the diets reversed the negative effects caused by 5% uPP (**Papers 1 and 2)** and improved the gut and liver health indices of common carp **(Paper 2)**. Likewise, adding *C. somerae* to a practical basal diet for zebrafish improved gut and liver health and enhanced the antiviral immunity of zebrafish when compared with the basal diet (**Paper 3)**. The findings of this thesis have provided a reference for FM replacement with plant proteins as well as a deeper understanding of the use of *C. somerae* as a potential probiotic additive in commercial feeds.

4.1 *C. somerae* supplementation can reverse the negative effects of the dietary higher level of uPP on fish

Plant proteins currently are viewed as the best sources to replace FM because they are available in large quantities and at a relatively low cost (Daniel, 2018). ANFs in plant proteins, however, have restricted their use (Chou et al., 2004; Lin and Luo, 2011; Zhang et al., 2018). One intriguing new alternative source is uPP, which is a commercial plant protein product consisting of mixtures of potato protein, pea protein, wheat gluten, and soy protein. Both heating and ultra-micro grinding (95% < 200 μm) will reduce the level of ANFs like trypsin inhibitors to 2 mg/g, and β-conglycinin to 28 mg/g. In **Paper 1,** we found that a low-level addition of uPP at 2.5% can be used to further improve FM replacement, but dietary 5% uPP impaired gut and liver health of common carp. Interestingly, dietary 5% uPP increased Proteobacteria abundance and decreased

the abundance of Fusobacteria compared with the control group, which agreed with findings from previous studies (Guo et al., 2017; Zhang et al., 2019b), suggesting negatively affected fish health and welfare. At the genus level, the addition of 5% uPP lowered the relative abundance of *Cetobacterium*, which has been reported to generate Vitamin B_{12} and short-chain fatty acids (SCFAs), including acetate, propionate, isobutyrate, and butyrate, all of which are favorable to fish health (Tsuchiya et al., 2008; Zhang et al., 2019b). Therefore, the alteration of 5% uPP on the intestinal microbiota may have a detrimental impact on fish health and may be partially responsible for the unfavorable phenotypes observed in the gut and liver. 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 reveals that uPP has a dosage impact on the intestinal microbiota, which is consistent with the overall observations of gut and liver health associated with 2.5% uPP. Studies have shown that many bacteria isolated from fish intestines can further improve the utilization of plant proteins and improve fish health by reducing the ANFs and increasing the available nutrients (Mukherjee et al., 2016; Soltani et al., 2019). Heikkinen et al. (2006) discovered that SBM considerably changed the abundance of intestinal bacterial species in juvenile rainbow trout, implying that it might be feasible to manipulate the intestinal microbiota to help the host respond to dietary changes. Similarly, from the results of the gut microbiota in **Paper 1,** we inferred that *Cetobacterium* could play an important stabilizing role in the intestine.

Therefore, *C. somerae* was anaerobically isolated from the zebrafish intestine, and the fermentation product of *C. somerae* was added to diets containing 5% uPP to evaluate the effects on fish health (**Paper 2)**. As expected, feeding *C. somerae* significantly reduced histological damage and also the expression of inflammation-related genes compared with the uPP group. In addition, we observed that *C. somerae* reduced gut-blood translocation of LPS, LPS-binding protein, and tissue damage indicators Ast and Alt. In addition, the upregulation of several genes showed improved intestinal integrity (e.g., zonula occludens) and reduced liver inflammation compared with the uPP group. We also observed increased expression of hepatic lipolysis genes as well as the reduced formation of lipid droplets. These observations agreed with findings from Pagnini et al. (2010) who showed that a range of probiotic products were able to reduce intestinal inflammation by maintaining epithelial barrier integrity. Similarly, *L. lactis* in rainbow trout

(Ulluwishewa et al., 2011) and *B. subtilis* in grass carp (Sun et al., 2020) can improve the intestinal barrier by regulating the expression of tight junction proteins. In addition, *Cetobacterium* ferments sugar to butyrate, which has been shown to enhance the intestinal epithelial barrier. This may be one reason for the favorable effects of *C. somerae* on gut health reported in **Paper 2.** Moreover, the increased expression of *hypoxia inducible factor 1 subunit alpha* (*hif1α*) in fish that were fed *C. somerae* in **Paper 2** could explain the improvement of gut health, because Hif1α also regulates intestinal tight junctions and the expression of antimicrobial peptide-related genes (Zheng et al., 2015). Several studies have shown that plant proteins tend to cause fatty liver in fish (Messina et al., 2007; Yin et al., 2018; Zhang et al., 2019a). The administration of *B. subtilis* reduces the fatty liver by preventing fatty acid synthesis and increasing fatty acid β-oxidation in grass carp (Zhao et al., 2020). Supplementation with *B. subtilis* BS1 or *L. plantarum* LP1 can reduce the density of lipid droplets and crude lipid in the liver and upregulate the expression of lipolysis genes in Chinese perch (*Siniperca chuatsi*) (Feng et al., 2021). In zebrafish, *Lactobacillus rhamnosus* GG inclusion reduces hepatic lipid accumulation and improves gut health by regulating the inflammatory response and gut permeability parameters (Bruch-Bertani et al., 2020). Furthermore, most studies of the anti-inflammatory mechanism of butyrate are related to the inhibition of *nuclear factor κB*, and *nuclear factor κB* inhibition can further lead to the lower expression of cytokine genes, including *tumor necrosis factor alpha*, *interleukin1 beta*, and other pro-inflammatory cytokines (Meijer et al., 2010).

Based on these findings, we concluded that *C. somerae* supplementation reversed the negative effect of 5% uPP in fish diets and improved gut and liver health. The *C. somerae* fermentation product has the potential to be developed as a novel feed additive to improve the efficacy of FM replacement by plant proteins.

4.2 *C. somerae* as a potential probiotic to improve fish health

Since the European Union banned the use of antibiotics other than coccidiostats or histomonostats in feed additives in 2003, finding alternatives to antibiotics has become the goal of many researchers (Ringø et al., 2020). *Bacillus* and LAB are promising alternatives and are widely used in aquaculture (e.g., Van Doan et al., 2018; Wang et al., 2019; Ringø et al., 2020). Certain

probiotic strains, however, also pose safety concerns but generally are regarded as safe for use, such as *B. cereus* in European sea bass (*Dicentrarchus labrax*) (Aboyadak et al., 2016), *L. rhamnosus* GG in zebrafish (He et al., 2017), and *L. plantarum* JCM1149 in hybrid tilapia (*Oreochromis niloticus*♀ ×*Oreochromis aureus*♂) (Liu et al., 2016). As a result, it is of great value to isolate and identify novel probiotics derived from the intestinal commensal bacteria of fish and apply them to aquaculture.

In **Paper 1,** we found that *Cetobacterium* was the most predominant genus of gut microbiota of common carp, which is consistent with many previous results (Roeselers et al., 2011; Ramírez et al., 2018; Hassenrück et al., 2021). In our lab, Zhang et al. (2019b) demonstrated that SCFAs, including acetate, propionate, isobutyrate, and butyrate, can be produced by culturing *C. somerae in vitro* for 12 h, among which the content of acetate was the highest. Dietary *C. somerae* and its production of acetate have the ability to improve glucose homeostasis (Wang et al., 2021b). Furthermore, introducing *C. somerae* to the rearing water of zebrafish reveals that *C. somerae* can stably colonize the gut of zebrafish and increase the concentrations of acetate, propionate, and butyrate (Wang et al., 2021b). Therefore, we evaluated the effects of *C. somerae* on the health of zebrafish.

As expected, dietary *C. somerae* improved the gut health of fish by reducing the intestinal inflammatory score, the expression of inflammation-related genes, and the level of serum LPS, as well as increasing Hif1α expression (**Papers 2** and **3)**. These beneficial influences could be linked to the effects of SCFAs produced by *C. somerae*, especially butyrate, which can enhance the gut function by improving the intestinal epithelial barrier and mucosal structure, reduce the inflammatory responses, maintaining intestinal homeostasis (Leonel and Alvarez-Leite, 2012; Jesus et al., 2019). SCFAs also can stimulate the intestinal epithelium to consume oxygen and reduce the oxygen level in the intestinal microenvironment (Kelly et al., 2015; Zhang et al., 2019b). Butyrate produced by *Cetobacterium* might be responsible for the decrease in intestinal partial oxygen pressure (Zhang et al., 2019b). In addition, SCFAs lower the intestinal pH and inhibit the growth of pathogens that are sensitive to pH, which is beneficial to intestinal health (Xie et al., 2017). The inclusion of sodium butyrate improves the activity of immunity parameters and reduces the mortality of Nile tilapia challenged with *A. hydrophila* (Abd El-Naby et al., 2019).

Similarly, Mirghaed et al. (2019) discovered that the addition of SCFAs boosted the immune response and improved the survival rate of rainbow trout infected with *Streptococcus iniae*. *Cetobacterium* isolated from freshwater fish was reported to have antibacterial action against the genus *Aeromonas* and human pathogenic bacteria (Larsen et al., 2014), which is consistent with the antiviral results of *C. somerae* reported in **Paper 3.** In addition, dietary sodium butyrate improves fish intestine antioxidant capacity in many ways, including reducing the malondialdehyde level, increasing the activity of intestinal antioxidant enzymes like superoxide dismutases, and increasing the mRNA levels of antioxidant enzymes, such as glutathione peroxidase-1a (Abdel-Latif et al., 2020), which can explain the effects of dietary *C. somerae* on the increase in the intestinal antioxidant capacity in zebrafish (**Paper 3)**. Similar to this result, *B. subtilis* has been reported to increase antioxidant capacity in grass carp (Tang et al., 2019) and tilapia (Liu et al., 2017).

SCFAs also play an important role in regulating lipid metabolism by promoting fatty acid β-oxidation and inhibiting lipid synthesis in mammals (den Besten et al., 2013). Furthermore, SCFAs have been shown to increase the expression of *ppargc1α* and *ucp* in brown adipose tissue, enhancing fatty acid β-oxidation in mice (Gao et al., 2009). In grass carp, dietary sodium butyrate can reduce lipid levels in the hepatopancreas and lipid droplets in the hepatocytes (Zhou et al., 2019). These findings are in accordance with the results of reduced lipid droplet accumulation and lipid metabolism–related genes in the liver reported in **Paper 2** and **Paper 3.** In addition, the observed reduction in serum Alt and Ast reported in **Paper 2** and **Paper 3** could be explained by the effects of *C. somerae* metabolites, such as butyrate, which can improve liver function, thereby leading to lowered levels of serum Ast and Alt.

Merrifield et al. (2010) summarized the selection criteria of probiotics. A probiotic must be indigenous to the fish or the ambient environment, nonpathogenic, not contain antibiotic-resistant genes encoded by plasmids, be able to adhere to or grow well in intestinal mucus, have the ability to be used as a feed additive, colonize on the surface of the intestinal epithelium, be beneficial to growth, antagonize pathogens, produce useful extracellular digestive enzymes or vitamins, and be easy to store and use. However, it is unlikely for a probiotic to fulfill all criteria. Thus, Wang et al. (2019) proposed that a probiotic in aquaculture should meet some basic criteria, including safety,

adaptability to the host intestine, improvement of fish health and welfare, and convenience for storage and administration. Results from this thesis (**Papers 1, 2,** and **3)** showed that *C. somerae* exhibited some properties as a probiotic, such as safety, usage as a feed additive, benefits for liver and gut health of fish, reduction of hepatic steatosis, and resistance to SVCV infection. Therefore, we suggest *C. somerae* can be applied to aquatic feed as a probiotic.

5. Concluding remarks and future perspectives

In summary, this study revealed that dietary supplementation of 2.5% uPP can be adopted to further improve FM replacement, whereas dietary 5% uPP impaired gut and liver health of common carp. Notably, the addition of 5% uPP negatively changed the gut microbiota, significantly reducing the relative abundance of *Cetobacterium*. Our further results showed that the addition of *C. somerae* reversed the negative impact of 5% uPP inclusion on fish health, indicating that it can be used as a feed additive to improve the percentage of FM replacement by plant proteins. Results in zebrafish showed that the supplementation of *C. somerae* improved liver and gut health, as well as antiviral immunity, suggesting that *C. somerae* can be used as a potential probiotic to improve fish health.

Some issues still need to be investigated and addressed. In **Paper 2** and **Paper 3,** *C. somerae* exhibited beneficial effects on fish health, but the effector molecules of *C. somerae* were unclear. Therefore, further studies are required to identify the functional components or metabolites of *C. somerae* responsible for promoting fish health. In **Paper 2,** feeding common carp with *C. somerae* repaired the unfavorable impact of uPP inclusion, but how *C. somerae* interacted with ANFs in plant proteins should be further investigated. Additional research also is needed to determine the effects of dietary *C. somerae* on fish that are fed diets containing other sources and inclusion levels of plant proteins. Moreover, future experiments in stomach fish should be conducted to elucidate the influence of fish species on the application of *C. somerae.*

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Paper 1

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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.

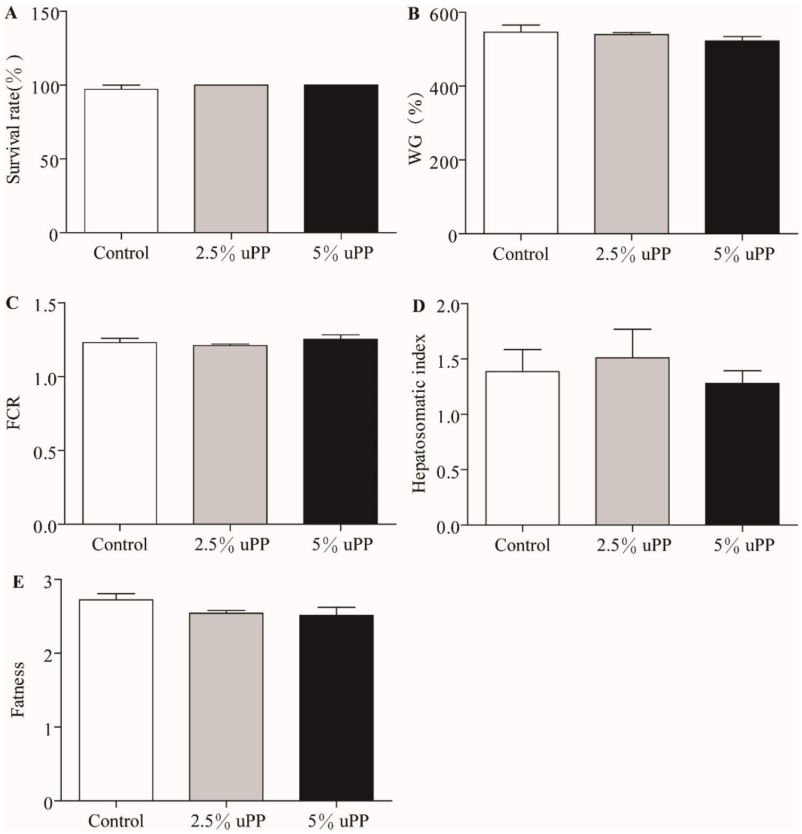

Fig. 1. Effects of uPP supplemented diet on the survival rate, WG, FCR hepatosomatic index and fatness of common carp. (A) survival rate (%), (B) WG (%), (C) FCR, (D) Hepatosomatic index and (E) fatness of common carp fed different diets. Data represent the means (± SEM) of six replicates of each treatment.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

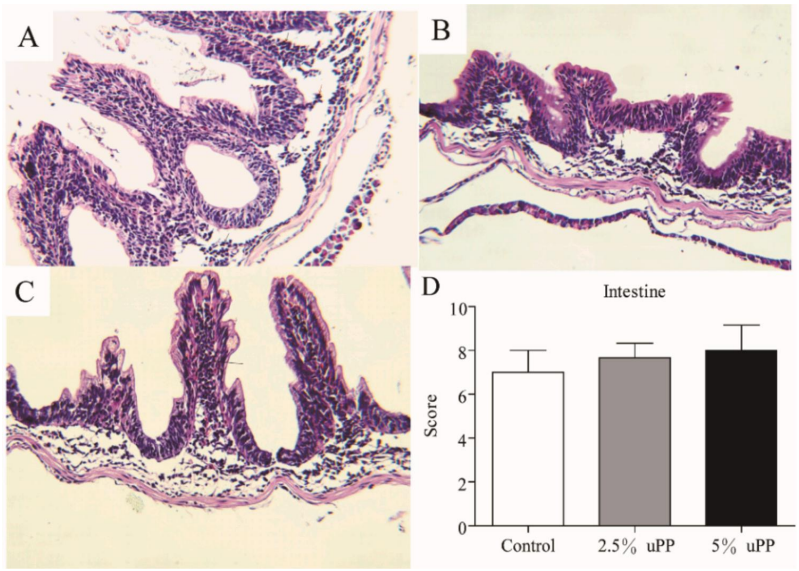

Fig. 2. Representative histomorphological images from HE-stained sections of the distal intestine of common carp fed different diets. (A) Control; (B) 2.5 % uPP; (C) 5% uPP; (D) Histological score of intestine (n = 6). No significant difference in histological score was observed among groups.

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

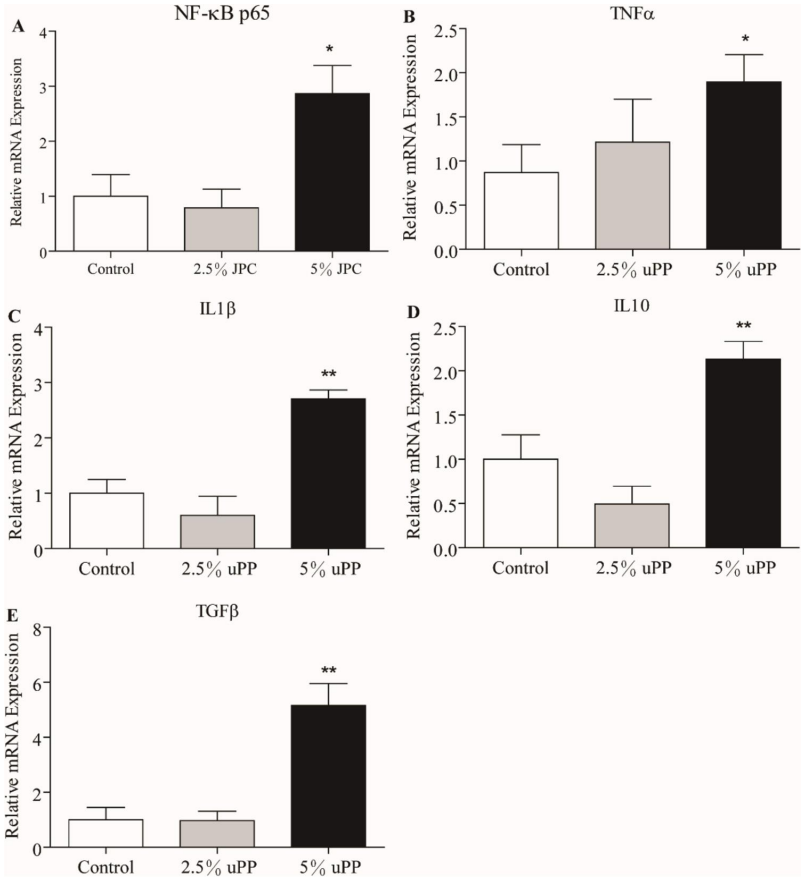

Fig. 3. Relative mRNA expression of inflammation related genes in the intestine of the common carp fed different diets. Data are expressed as mean ± SEM (n = 6). A single and double asterisk denote $p < 0.05$ and $p < 0.01$, respectively.

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

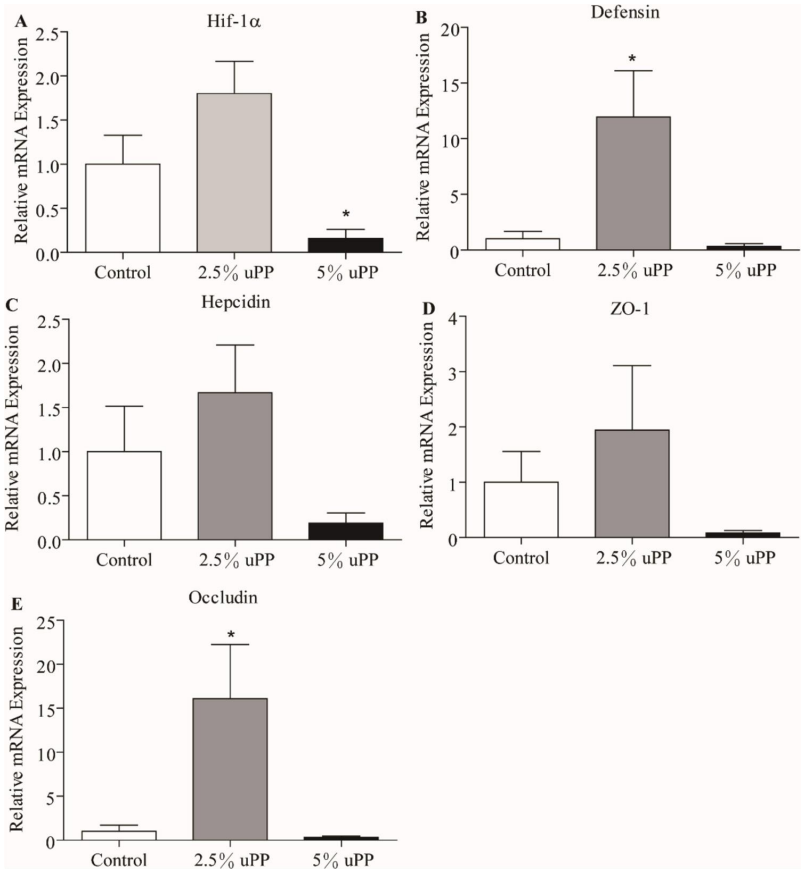

Fig. 4. Relative mRNA expression of genes related to intestinal health in common carp fed different diets. Data are expressed as mean ± SEM (n = 6). Data of Hepcidin, Defensin, and Occludin expression were analyzed by non-parametric Jonckheere-Terpstra test followed by post hoc test. A single asterisk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

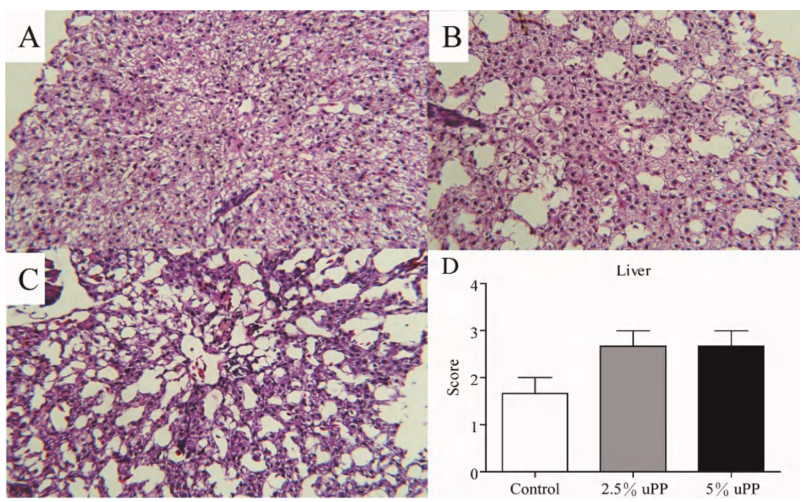

Fig. 5. Representative histomorphological images from HE-stained sections of the liver of common carp fed different diets. (A) Control; (B) 2.5 % uPP; (C) 5% uPP; (D) Histological score of liver (n = 6). No significant difference in histological score was observed among groups.

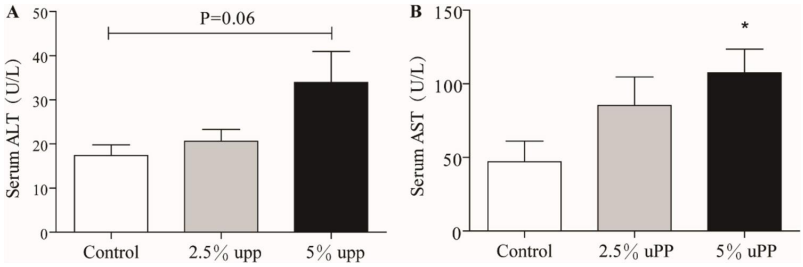

Fig. 6. Serum ALT and AST levels of the common carp fed different diets. Data are expressed as mean ± SEM (n = 6). The ALT data were analyzed by non-parametric Jonckheere-Terpstra test followed by post hoc test. A single asterisk denotes $p < 0.05$.

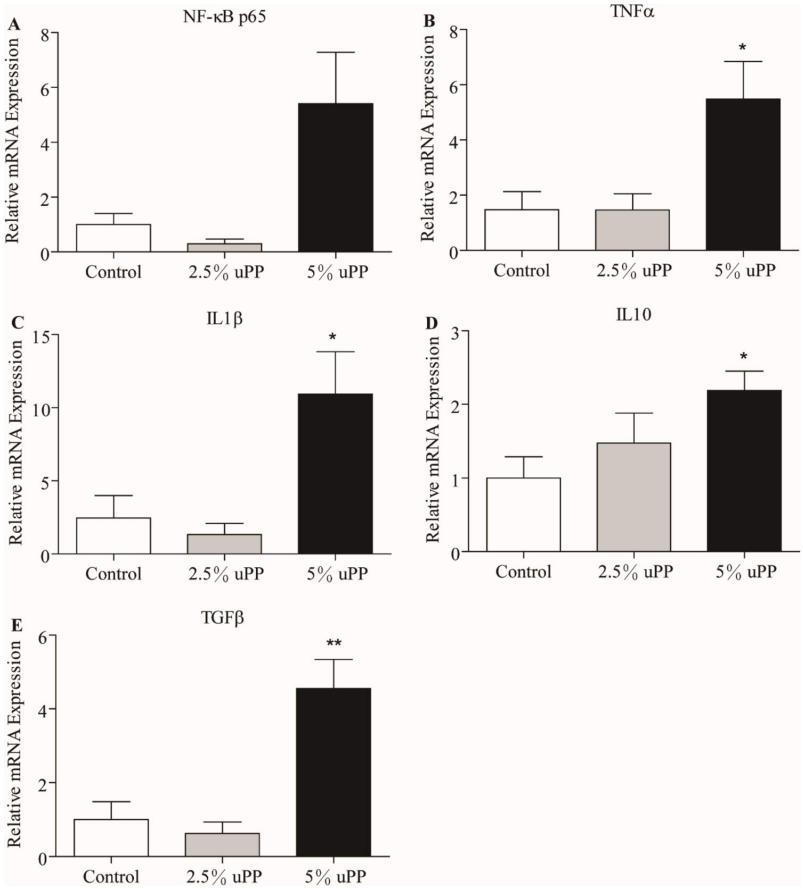

Fig. 7. Relative mRNA expression of inflammation related genes in the liver of the common carp fed different diets. Data are expressed as mean ± SEM (n = 6). Data of NF-κB p65 expression were analyzed by non-parametric Jonckheere-Terpstra test followed by post hoc test. A single and double asterisk denote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

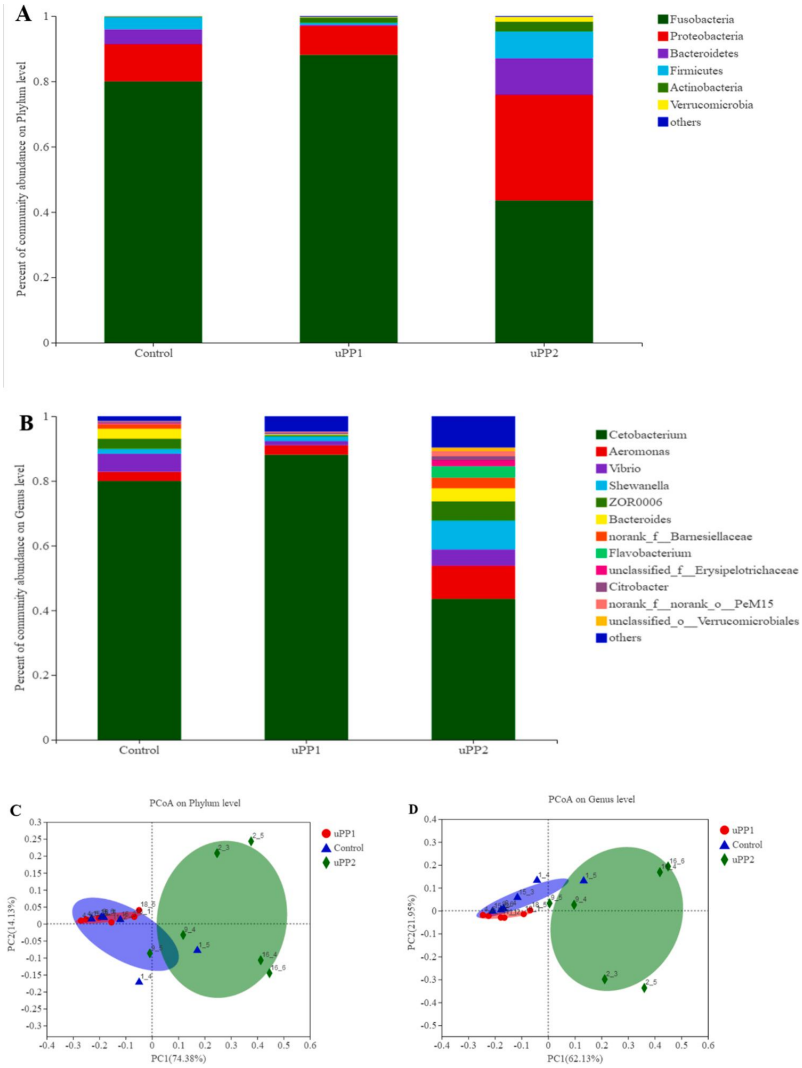

Fig. 8. The intestinal microbiota of common carp fed with control or uPP supplemented diets. Relative abundance at phylum (A) and genus (B) level of the gut microbiota; (C) Principal coordinates analysis (PCoA) of the intestinal microbiota on phylum level; (D) Principal coordinates analysis (PCoA) of 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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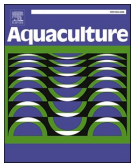

Effects of *Cetobacterium somerae* fermentation product on gut and liver health of common carp (*Cyprinus carpio*) fed diet supplemented with ultra-micro ground mixed plant proteins

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ABSTRACT

Improving the replacement percentage of fish meal by plant proteins is of value for the aquaculture industry. *Cetobacterium* is the dominant commensal bacterium in many fish species. However, the function and potential beneficial effects of *Cetobacterium* is unknown. In this study, carps were fed with a practical basal diet with partial fish meal replacement by plant proteins (Control), basal diet with improved fish meal replacement percentage by adding ultra-micro ground mixed plant proteins (uPP), or the uPP diet supplemented with the fermentation product of a commensal bacterial strain *Cetobacterium somerae* XMX-1 (uPP + XMX-1). The effects of *C. somerae* XMX-1 fermentation product on gut and liver health of carp fed uPP supplemented diet were evaluated. After 4-week feeding, growth performance, feed utilization, biometric parameters, inflammatory score, expression of inflammation-related and lipid metabolism related genes were evaluated. The levels of LBP, LPS, AST and ALT in serum were assessed. 16S rRNA gene pyrosequencing was used to evaluate intestinal microbiota. Results showed that dietary uPP and uPP + XMX-1 had no effect on survival rate and growth ($p > 0.05$). However, dietary uPP compromised gut and liver health of fish. The expressions of inflammation related genes in gut and liver and the levels of LBP, LPS, AST and ALT in serum were significantly increased in uPP group versus control ($p < 0.05$). Interestingly, uPP + XMX-1 significantly decreased the expressions of inflammation related genes in gut and liver, as well as the levels of LBP, LPS, AST and ALT in serum compared to the uPP group ($p < 0.05$). In addition, uPP + XMX-1 significantly reduced the formation of lipid droplets in liver and significantly up-regulated the expression of lipogenesis genes and down-regulated the expression of lipolysis genes ($p < 0.05$) in comparison to the uPP group. Taken together, our study suggests that dietary uPP can impair gut and liver health and induce fatty liver, whereas *C. somerae* XMX-1 fermentation product can improve gut and liver health of common carp and decrease lipid deposition in liver, suggesting that XMX-1 fermentation product can be used as feed additive to improve the efficacy of fish meal replacement by plant proteins.

1. Introduction

In recent years, aquaculture has gradually become the main way to improve the global supply of aquatic products. The intensive aquaculture depends on the development of aquatic feed industry (Tacon and Metian, 2008). Fish meal has always been an indispensable high-quality protein source in aquatic feed with the characteristics of high essential amino acid and fatty acid content, low carbohydrate content, good palatability, low anti-nutritional factors, and good digestion and absorption by farmed animals (Burel et al., 2000). However, on the one hand, the global supply of fishmeal has been declining; on the other hand, with the rapid development of aquaculture, the demand for fish meal is increasing rapidly, which leads to the price of fish meal soaring (Brinker and Friedrich, 2012). Therefore, finding alternative protein sources that that can partially or completely replace fish meal has become a very urgent task (Gómez-Requeni et al., 2004).

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Significant progress has been made in plant proteins replacement of fish meal in fish feed, which reduces the cost and the discharge of nitrogen, phosphorus and other nutrients, and slows down the pressure on the environment and resources (Torstensen et al., 2008; Hardy, 2010). Despite these advances, most plant proteins contain different types of antinutritional factors (ANFs), which interfere with the normal growth and nutrition metabolism of fish, and even cause deaths when improperly consumed (Gilani et al., 2012; Król et al., 2016). Dietary β-conglycinin induced inflammation and oxidation in juvenile Jian carp (*Cyprinus carpio* var. Jian), leading to intestinal digestion and absorption dysfunction, and ultimately affected growth (Zhang et al., 2013). In juvenile turbot (*Scophthalmus maximus*), dietary β-conglycinin and glycinin decreased digestive enzymes activities, negatively changed intestinal histology and caused intestinal immune dysfunction (Gu et al., 2016). Adding soya-saponins to the diet for 8 weeks caused enteritis and damaged the intestinal barrier function in juvenile turbot (Gu et al., 2018). At present, there are many ways to remove antinutritional factors of plant proteins, mainly including physical, chemical and biological treatment (Muzquiz and Wood, 2007).

Common carp (*Cyprinus carpio*) is an important freshwater fish species, and the scale of aquaculture is expanding year by year (Monier, 2020). In previous study, we improved fish meal replacement in practical diet by using ultra-micro ground mixed plant protein (uPP), in which the antinutritional factors are removed by physical methods (Xie et al., 2021). We found that dietary 5% uPP damaged intestinal and liver health of common carp and negatively affected intestinal microbiota. 16S rRNA gene sequencing of gut microbiota showed that 5% uPP significantly reduced the abundance of *Cetobacterium* compared with the control group. Therefore, we infer that supplementation of *Cetobacterium* in combination with uPP might counteract the negative effects associated with uPP addition. In the present study, we added the fermentation product of *Cetobacterium somerae* in combination with 5% uPP in practical diet of carp. The effects of *Cetobacterium* fermentation product on gut and liver health of carp fed uPP supplemented diet was evaluated. To our knowledge, this is the first study about the beneficial function of the fish commensal bacterium *Cetobacterium somerae*. This study provides a basis for the development of sustainable dietary additive derived from the commensal bacteria of fish.

2. Materials and methods

2.1. Bacteria and culture conditions

Cetobacterium somerae XMX-1 was originally isolated from zebrafish intestine in our laboratory and it was preserved in China General Microbiological Culture Collection Center with its preservation number CGMCC no.18908. XMX-1 was anaerobically cultured on GAM Agar medium (Haibo, China) at 28 °C for 24 h. Colonies of XMX-1 were selected and anaerobically cultured in GAM Broth medium (Haibo, China) at 28 °C for 18 h to reach the concentration of 10^8 CFU/mL. Then 100 mL of XMX-1 culture was added into 100 g of rice husk powder to reach the final concentration of 10^8 CFU/g. The mixture was dried at room temperature to remove moisture and was add to the diet.

2.2. Fish husbandry and feeding

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). The experimental carp (initial body weight 54.36 ± 0.28 g) were purchased from a farm (Hebei, China) and transported to the International Agricultural High-tech Industry Park (Hebei, China), Chinese Academy of Agricultural Sciences. The purchased carp were domesticated in circulating water for 15 days before the test.

Carps with the same size were randomly divided into 3 groups, with 8 90-L tanks in each group at a density of 5 fish per tank. In the control group, carps were fed with basal diet, while carps in the experimental groups were fed with basal diet supplemented with 5% uPP (Joosten, Netherlands), or basal diet supplemented with 5% uPP and 2.5 g/kg fermentation product of XMX-1. Feed formulation and proximate analysis shown in Table 1.

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

Ingredient	g/kg DM		
	Control	uPP	uPP + XMX-1
Rice bran	190.0	159.0	159.0
Flour	200.0	200.0	200.0
Soybean meal	200.0	200.0	200.0
Rapeseed meal	105.0	127.5	127.5
Fish meal	80.0	30.0	30.0
Poultry by-product meal	50.0	50.0	50.0
DDGS	100.0	100.0	100.0
uPP	0.0	50.0	50.0
XMX-1	0.0	0.0	2.5
Bentonite	10.0	10.0	7.5
Lys-HCl	2.0	2.0	2.0
Methionine	0.5	0.5	0.5
Choline chloride (50%)	2.0	2.0	2.0
$Ca(H_2PO_4)_2$	20.0	20.0	20.0
Soybean oil	30.0	38.5	38.5
VC phosphate	0.5	0.5	0.5
Fish premix (1%) [a]	10.0	10.0	10.0
Crude protein (%)	32.64	32.75	32.37
Crude fat (%)	10.35	10.39	10.56
Crude ash (%)	7.5	6.96	7.04
Moisture (%)	6.16	5.38	5.6

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

Fish were fed to apparent satiation three times daily at 8:00, 13:00 and 17:00, respectively. Food intake and death were counted every day. During the 4 weeks experiment within a recirculating system, the water inflow was 120 L/h, the water temperature was controlled at 26.5 °C, the dissolved oxygen was >6.0 mg/L, the pH was 7.0–7.2, and the ammonia nitrogen content was <0.05 mg/L.

2.3. Growth measurements and sampling

At the end of the experiment, the weight, liver and intestine of fish were weighted and body length was measured after 24 h fasting. The weight gain rate (WG, %), feed coefficient (FCR), survival rate (%), fatness, hepatosomatic index and intestinal somatic index were calculated according to the method (Bai et al., 2017). The serum, liver, intestine and intestinal contents samples of carp were collected after 24 h fasting. The intestinal contents samples of carp were collected after 6 h fasting. The samples for histological analysis were immediately placed in 4% paraformaldehyde, and the remaining samples were immediately stored in liquid nitrogen for subsequent analysis.

2.4. Hematoxylin and eosin analysis and Oil Red O staining

Eight fish from each treatment group with 8 replicates were taken for gut and liver histological analysis. The samples were embedded in paraffin, sectioned, stained with hematoxylin eosin (H&E), and observed by microscope (Leica DMIL-LED, Germany). The morphological changes of liver and intestine were scored as 0, 1, 2 or 3 (Liu et al., 2016). In addition, 8 fish from each treatment group were randomly sampled to obtain 8 replicates for liver Oil Red O staining. The processing and details for obtaining images were determined according to Zhang et al. (Zhang et al., 2019b).

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
TNFα	GCTGTCTGCTTCACGCTCAA	CCTTGGAAGTGACATTTGCTTTT
IL-1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA
IL10	GCTGTCACGTCATGAACGAGAT	CCCGCTTGAGATCCTGAAATAT
TGFβ	ACGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA
HIF1α	GTTCTGCGTACCTGGTGCTCATC	AAAGTGTGGCGGCTGAGAAAGG
ZO-1	CCGAAGCTTTGACAGCAAAC	GGTTGATCTTCTCCACTGACTC
Hepcidin	ACATGCGTCTGCTTCCTCC	CTGGTTCTCCTGTGGTGCTT
CPT1	CAGATGGAAAGTGTTGCTAATGAC	TGTGTAGAAGTTGCTGTTGACCA
PPARα	TGAACAAAGCCAAAGCACGC	TGGAGAGTGTCCATGTCGTG
FAS	GACAGGCCGCTATTGCTATT	TGCCGTAAGCTGAGGAAATC
PPARβ	GAGGCCATATTTGCGATGCTC	TCTCTCGTCACAAAGCCCTTC
PPARγ	GTCAAGTCCGAGATGCACC	GGATGACCTGAGCATTGAAGC

2.5. Serum biochemical parameter analysis

The blood samples were collected from 8 fish in each treatment group with 8 replicates by the way of taking blood form caudal vein, followed by centrifugation at 4000 rpm for 10 min. The supernatant was collected as serum, and was used to analyze serum biochemical parameter. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity was evaluated by kits (Jiancheng Bioengineering Ins., China). The level of serum LPS and LPS binding protein (LBP) were detected by ToxinSensor™ Chromogenic LAL Endotoxin (Genscript, China) and LBP (Jiangsu Meimian industrial Co., Ltd., China) assay kits, respectively.

2.6. Quantitative real-time PCR analysis

Eight fish from each treatment group were randomly sampled to obtain 8 replicates for gut and liver cytokine gene expression analysis. TRIzol method was used to extract total RNA from liver and intestinal samples. 1 μg RNA was taken from each sample and transformed into cDNA. SYBR Green PremixEx Taq™ II (TaKaRa) was used for RT-*q*PCR using cDNA as template. The detailed methods were described in the literature (Wu et al., 2020; Xie et al., 2021). The primers were listed in Table 2 and synthesized by Shenggong in Shanghai. *β-actin* was used as the reference gene, and data was analyzed according to $2^{-\Delta\Delta CT}$ method.

2.7. 16S ribosomal RNA gene sequencing

Gut microbiota of carp in the experiment was analyzed using 16S rRNA gene sequencing. 6 h after the last feeding, the intestinal contents samples of carp were collected from 12 fish in each treatment group to get 6 replicates. DNA extraction, sequencing, and data analysis was conducted according to the method previously described (Zhang et al., 2019b).

2.8. Statistical analysis

Data was expressed in the form of mean ± S.E.M. The difference among groups was analyzed by one-way ANOVA with post hoc test. Variance homogeneity of data was examined by Levene's test. If variance homogeneity could be met, the data was analyzed by Duncan's test. If not, data was examined by Tamhane's T2 test. All analyses were conducted by Graphpad prism 5.0. Difference was considered as significant when p values < 0.05.

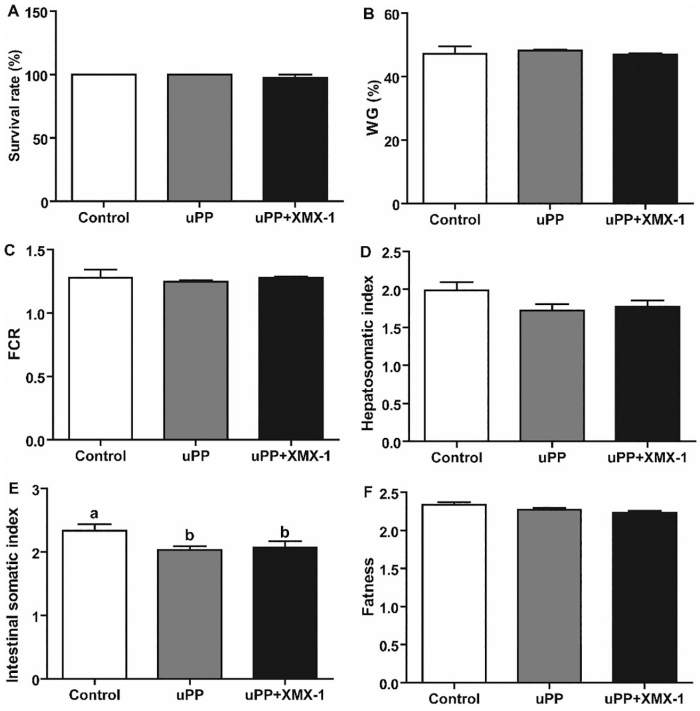

Fig. 1. Effects of uPP and uPP + XMX-1 diet on the survival rate, WG (%), FCR, hepatosomatic index, intestinal somatic index and fatness of common carp. (A) survival rate (%), (B) WG (%), (C) FCR, (D) Hepatosomatic index, (E) Intestinal somatic index and (F) Fatness of common carp fed different diets. Data represent the means (±SEM) of 8 replicates of each treatment. Labeled means without a common letter differ, $p < 0.05$.

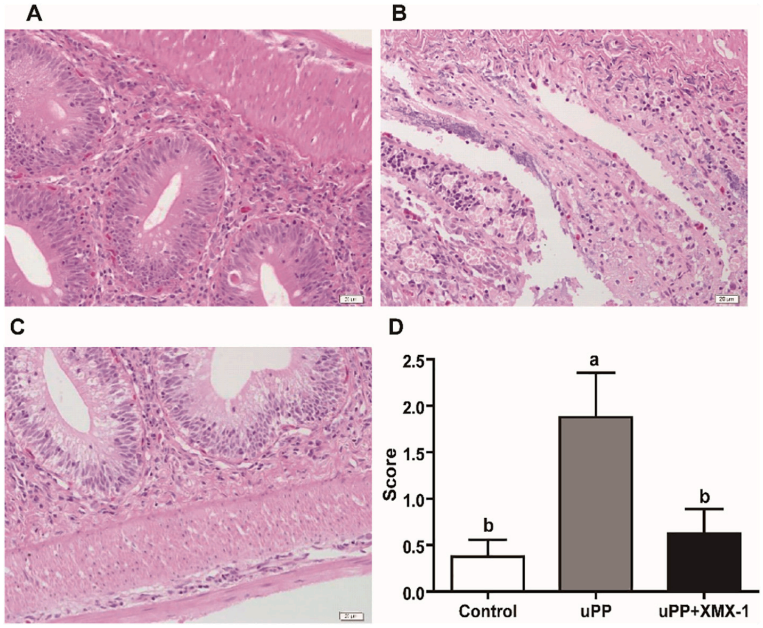

Fig. 2. Effects of uPP and uPP + XMX-1 diet on the HE-stained sections of the gut of common carp. (A) Control, (B) uPP and (C) uPP + XMX-1. Data represent the means (±SEM) ($n = 8$). Labeled means without a common letter differ, $p < 0.05$.

3. Results

3.1. Growth performance, feed utilization and biometric parameters

After a 4-week feeding, as shown in the Fig. 1, no significant differences were observed among the three groups in survival rate, WG (%), FCR, fatness, hepatosomatic index ($p > 0.05$). However, fish fed the uPP diet or uPP + XMX-1 diet significantly decreased intestinal somatic index compared to fish fed the control diet ($p < 0.05$). In addition, compared with the control group, hepatosomatic index in the uPP group had an increased trend ($p = 0.06$).

3.2. Effects of uPP and uPP + XMX-1 diet on the intestinal health in common carp

As can be seen from Fig. 2, there were signs of inflammation in the intestine of common carp fed with uPP diet. Moreover, the inflammatory score had a significant increase in the uPP group compared to the control group ($p < 0.05$). However, fish fed uPP + XMX-1 diet significantly decreased inflammatory score compared to fish fed the uPP diet ($p < 0.05$).

In addition, the expression of genes related to intestinal health was detected. The expressions of inflammation related genes including *NF-κB p65*, *TNFα*, *IL-1β*, *IL10* and *TGFβ* were significantly increased in the uPP diet group ($p < 0.05$, Fig. 3). However, fish fed the uPP + XMX-1 diet significantly decreased inflammation related genes compared to fish fed the uPP diet ($p < 0.05$). Moreover, compared to the control group, dietary uPP significantly down-regulated the expression of *HIF1α* and Hepcidin ($p < 0.05$, Fig. 4), while the expression of *HIF1α*, Hepcidin and *ZO-1* was reversed in the uPP + XMX-1 group compared with uPP group ($p < 0.05$). Then we detected the levels of serum LBP and LPS in common carp. Results showed that dietary uPP significantly increased the level of LBP and LPS in serum compared to the control group, suggesting gut damage ($p < 0.05$, Fig. 5). However, the levels of serum LBP and LPS were reversed in the uPP + XMX-1 group ($p < 0.05$).

3.3. Effects of uPP and uPP + XMX-1 diet on gut microbiota of common carp

As can be seen from Fig. 6A and Table 3, at phylum level, Proteobacteria was the most abundant phylum with the relative abundances being 63.49%, 74.68% and 63.76% in the control, uPP and uPP + XMX-1 groups, respectively, followed by Firmicutes, Actinobacteriota, Bacteroidota and Fusobacteriota. Among the three groups, the uPP group had the highest abundance of Proteobacteria and the lowest abundance of Firmicutes and Fusobacterita, though there were no significant differences.

At the genus level (Fig. 6B; Table 4), *Shewanella* was the most abundant genus with the relative abundances being 24.69%, 46.15% and 6.26% in the control, uPP and uPP + XMX-1 group, respectively. Moreover, the relative abundance of *Shewanella* was significantly decreased in the uPP + XMX-1 group compared to uPP group ($p < 0.05$).

In addition, there was no significant differences between groups for PCoA analysis, suggesting that the uPP diet and uPP + XMX-1 diet had no marked effects on the autochthonous microbiota ($p > 0.05$, Fig. 6C, D).

3.4. Effects of uPP and uPP + XMX-1 diet on the liver health in common carp

Further, we investigated the effects of uPP and XMX-1 fermentation product on the liver health of common carp. As can be seen from Fig. 7, dietary uPP significantly increased the inflammatory score in the liver of common carp compared to the control group ($p < 0.05$). In contrast, fish fed uPP + XMX-1 diet significantly decreased the inflammatory score compared with uPP ($p < 0.05$).

Then we detected the levels of serum ALT and AST in common carp. Results showed that dietary uPP significantly increased the levels of ALT and AST in serum compared to control, indicating liver injury ($p < 0.05$, Fig. 8). However, the levels of serum ALT and AST were reversed in the uPP + XMX-1 group ($p < 0.05$). Furthermore, we detected the expression

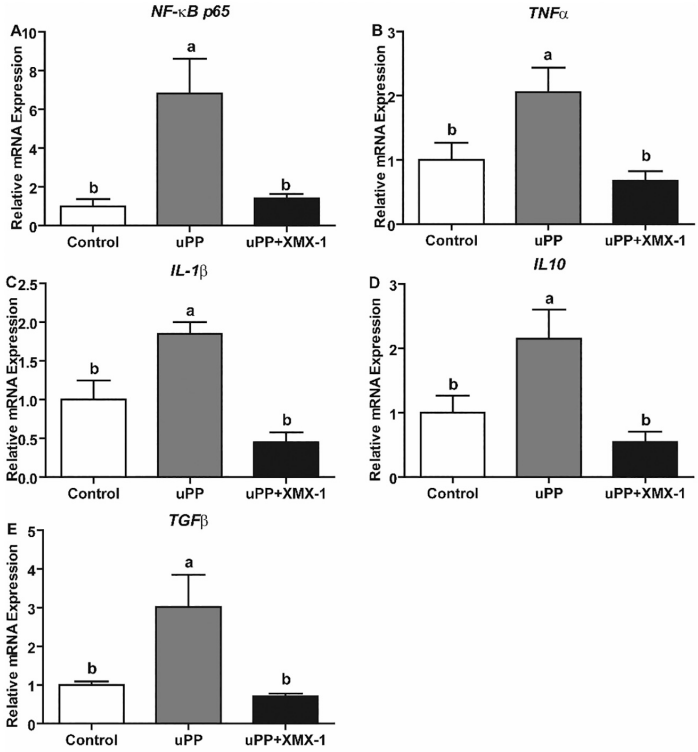

Fig. 3. Effects of uPP and uPP + XMX-1 diet on the expression of inflammation related genes in gut. (A) *NF-κB p65*, (B) *TNFα*, (C) *IL-1β*, (D) *IL10* and (E) *TGFβ*. Data represent the means (±SEM) ($n = 8$). Labeled means without a common letter differ, $p < 0.05$.

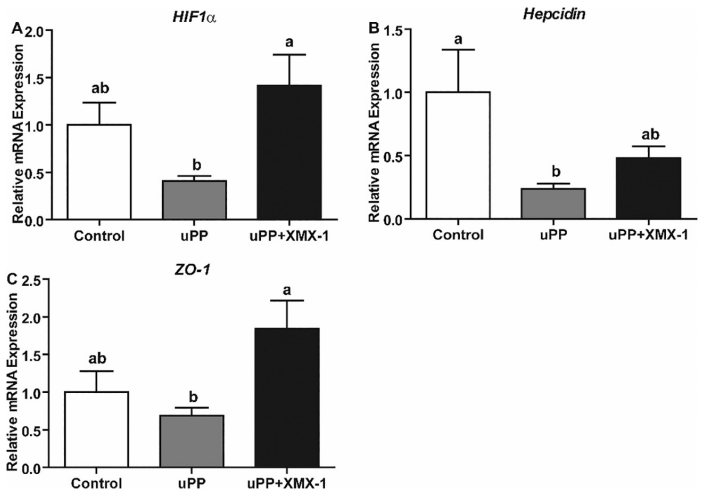

Fig. 4. Effects of uPP and uPP + XMX-1 diet on the expression of gut health related genes (A) *HIF1α*, (B) *Hepcidin* and (C) *ZO-1*. Data represent the means (±SEM) (n = 8). Labeled means without a common letter differ, $p < 0.05$.

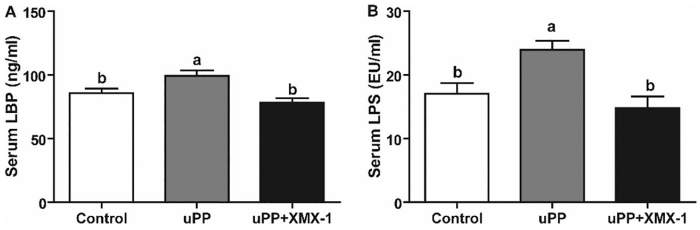

Fig. 5. Effects of uPP and uPP + XMX-1 diet on the level of LBP and LPS in serum (A) LBP and (B) LPS. Data represent the means (±SEM) (n = 8). Labeled means without a common letter differ, $p < 0.05$.

of inflammation related genes in liver. Results showed that dietary uPP significantly increased the expressions of *NF-κB p65*, *TNFα, IL-1β* and *TGFβ*, whereas dietary uPP + XMX-1 significantly decreased their expressions compared with uPP ($p < 0.05$, Fig. 9).

3.5. Effects of uPP and uPP + XMX-1 diet on hepaticlipid depositionof common carp

After a 4-week feeding, the liver of zebrafish was assessed by oil red staining. uPP significantly increased the formation of lipid droplets in liver compared with control, while the formation of lipid droplets was significantly decreased in the uPP + XMX-1 group versus uPP group (Fig. 10). Furthermore, we detected the expression of genes related to lipid metabolism. Compared to the control group, uPP addition significantly up-regulated the expression of lipogenesis genes including *FAS*, *PPARγ* and *PPARβ*, and down-regulated the expression of lipolysis genes including *CPT1* and *PPARα* ($p < 0.05$, Fig. 11). However, dietary uPP + XMX-1 significantly down-regulated the expression of lipogenesis genes including *FAS*, *PPARγ* and *PPARβ*, while up-regulated the expression of lipolysis genes including *CPT1* and *PPARα* compared to the uPP group ($p < 0.05$, Fig. 11).

4. Discussion

Cetobacterium have been found in the intestines of many freshwater and marine fish, which is dominant in the intestinal microbiota (Merrifield et al., 2013; Hassenrück et al., 2020). In hybrid sturgeon (*Acipenser baerii* × *Acipenser schrenckii*), the formulated feed group caused dysbiosis of intestinal microbiota compared with the bloodworm group, with a concomitant decrease in the abundance of *Cetobacterium* (Hao et al., 2020). Notably, the metabolites of *Cetobacterium* are rich in vitamin B12, which is inferred to promote the health of fish (Sugita et al., 1991; Bhute et al., 2020). In the present work, common carp was fed with uPP and uPP + XMX-1 diet for 4 weeks. Growth performance, liver and intestinal health of fish were observed. There were no significant differences among the three groups in survival rate, WG (%), FCR, fatness, hepatosomatic index. However, the uPP group decreased intestinal somatic index and hepatosomatic index, and compromised liver and intestinal health of common carp. In contrast, uPP + XMX-1 supplementation showed positive effects on the liver and intestinal health of common carp compared to the uPP group.

Plant proteins are one of the ideal protein sources to replace fish meal because of their wide range of sources, low price and high nutritional value (Gatlin III et al., 2007). Studies on rainbow trout (*Oncorhynchus mykiss*) and European perch (*Dicentrarchus labrax*) showed that the highest proportion of single replacement of fish meal with soybean meal and other plant proteins could reach 30% ~ 40%, without affecting the growth of fish (Kaushik et al., 2004; Dias et al., 2005). In the present work, uPP was subjected to ultra-micro grinding to further decrease the negative effect of antinutrients. Similarly, our results indicated that dietary uPP had no effect on the growth of common carp.

The gut of fish is not only responsible for obtaining nutrients and energy from the feed, but also actively participates in the immune response, metabolism and a variety of stress reactions in the body, which plays an important role in the growth and health of fish (Grosell et al., 2010). Many studies have reported that plant proteins replacement in the diet can compromise gut health in different fish species, including Atlantic salmon (*Salmo salar L.*) (Hartviksen et al., 2014), juvenile turbot (Gu et al., 2018), juvenile hybrid grouper (♀*Epinephelus fuscoguttatus*×♂*E. lanceolatu*) (Yin et al., 2020) and common carp (Xie et al., 2021). In the present work, dietary uPP negatively affected the structure of intestine of common carp and increased the expression of inflammation related genes. Previous studies have shown that the secretion of inflammatory cytokines will lead to the destruction of the integrity of intestinal mucosal epithelium and the increase of intestinal permeability (Johnson et al., 2006; Zhang et al., 2010; Zhang et al., 2018).

Besides, some studies show that acute stress can damage the intestinal epithelial barrier of Atlantic salmon, and aggravate the penetration of intestinal bacteria across the intestinal epithelial barrier (Olsen et al., 2002). Furthermore, the damage of intestinal epithelial barrier can make the intestinal LPS pass through the intestinal epithelial barrier, resulting in a significant increase in blood LPS (Zhang et al., 2019b). In agreement with these studies, we observed that dietary uPP significantly increased the serum LBP and LPS level compared to the control group. LPS can cause systemic inflammatory response syndrome, and LBP plays an important role in regulating the pathogenicity of LPS. LPS may activate multiple signal pathways and transcription factors in cells such as monocyte macrophages, inducing the expression of inflammatory cell genes, and then leading to a series of pathological processes (Krasity et al., 2015). The levels of serum LBP and LPS were reversed in the uPP + XMX-1 group. Many researches have proved that probiotics including *Lactococcus lactis* in rainbow trout and *Bacillus subtilis* in grass carp (*Ctenopharyngodon idella*) can improve barrier by regulating the expression of tight junction proteins (Ulluwishewa et al., 2011; Sun et al., 2020). It has been reported that butyrate, which is one of metabolites of *Cetobacterium* (Bennett and Eley, 1993), can enhance the intestinal epithelial barrier (Canfora et al., 2015), which might contribute to the positive effect of XMX-1 fermentation product on gut health of carp. The metabolites responsible for the beneficial effects of *Cetobacterium someare* on intestinal health and the underlying mechanisms derserve further investigation.

Dietary uPP down-regulated the expression of *Hepcidin*. It has been proved that hepcidins are related to iron metabolism and antimicrobial property in mammals (Nicolas et al., 2002; Walker et al., 2004). In fish, studies showed the role of antibacterial or antifungal effects of hepcidins (Lauth et al., 2005; Wang et al., 2009). In contrast, XMX-1 fermentation product increased the expression of *Hepcidin*. Intriguingly, a previous study showed that *Cetobacterium* exhibited the ability of inhibiting the growth of other bacterial strains (Larsen et al., 2014), which might be associated with its effect on the expression of antimicrobial peptides of host. Furthermore, the expression of *HIF1α* is related to intestinal health, which can also regulate intestinal tight junction and the expression of antimicrobial peptide related genes (Zheng et al., 2015). In this regard,

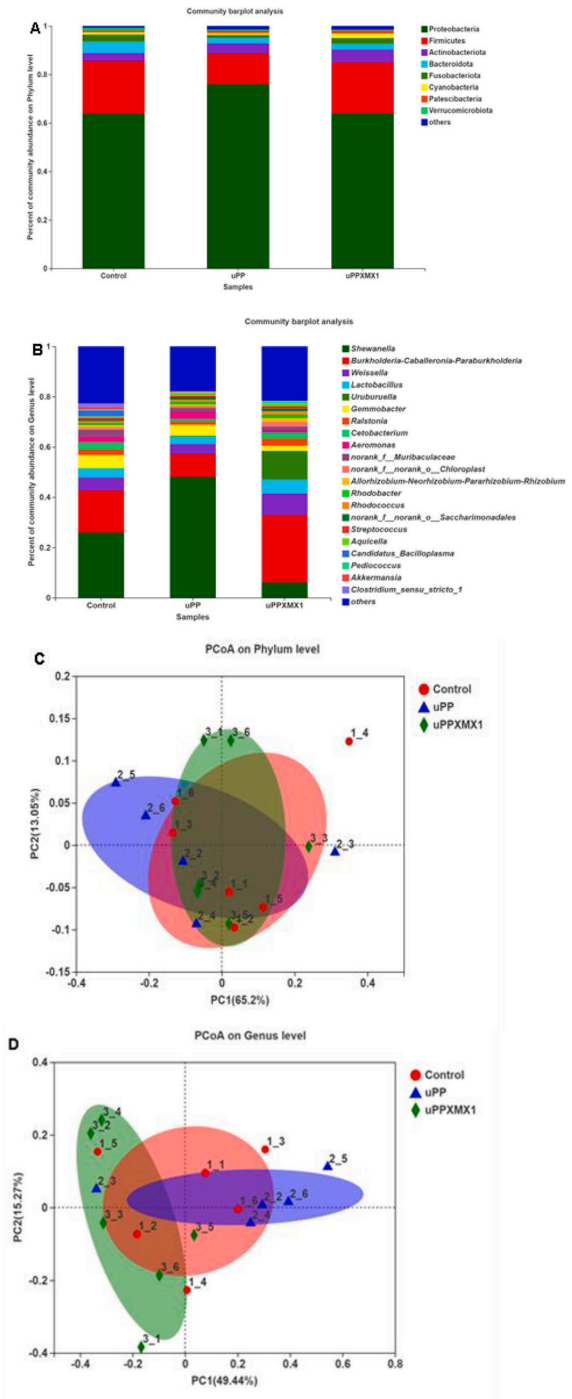

Fig. 6. Effects of uPP and uPP + XMX-1 diet on the intestinal microbiota of common carp. Relative abundance at phylum (A) and genus (B) level of the gut microbiota; Principal coordinates analysis (PCoA) of the intestinal microbiota on phylum (C) and genus (D) level of the gut microbiota ($n = 6$).

Table 3
The relative abundance of main phyla in the intestinal microbiota of common carp fed with different diets.

Bacteria Phylum	Control	uPP	uPP + XMX-1
Proteobacteria	63.49 ± 7.23	74.68 ± 9.64	63.76 ± 4.12
Firmicutes	21.93 ± 4.95	13.59 ± 6.98	21.33 ± 4.02
Actinobacteria	3.45 ± 0.69	4.32 ± 1.10	5.50 ± 0.64
Bacteroidetes	4.74 ± 1.21	2.45 ± 1.45	2.37 ± 0.77
Fusobacteria	2.73 ± 1.80	1.10 ± 0.61	2.00 ± 1.12
Cyanobacteria	0.99 ± 0.42	0.83 ± 0.39	1.84 ± 0.40
Patescibacteria	0.76 ± 0.18	0.99 ± 0.27	1.28 ± 0.31
Verrucomicrobiota	1.21 ± 0.64	0.26 ± 0.04	0.52 ± 0.22
Planctomycetota	0.19 ± 0.06	0.68 ± 0.44	0.60 ± 0.26
Chloroflexi	0.11 ± 0.02	0.23 ± 0.10	0.33 ± 0.14

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

Bacteria Genus	Control	uPP	uPP + XMX-1
Shewanella	24.69 ± 10.06^{ab}	46.15 ± 13.11^{a}	6.26 ± 4.06^{b}
Burkholderia-Caballeronia-Paraburkholderia	17.21 ± 4.64	9.77 ± 4.30	28.37 ± 8.15
Weissella	5.28 ± 0.83	4.15 ± 1.93	8.59 ± 1.92
Uruburuella	0.02 ± 0.01	0.22 ± 0.18	9.25 ± 7.89
Lactobacillus	3.59 ± 0.66	3.26 ± 1.71	5.75 ± 1.44
Gemmobacter	5.66 ± 2.80	4.28 ± 1.35	2.04 ± 0.48
Ralstonia	2.30 ± 0.57	1.30 ± 0.74	3.12 ± 1.08
Cetobacterium	2.71 ± 1.79	1.08 ± 0.61	1.20 ± 1.12
Aeromonas	2.16 ± 1.55	2.97 ± 1.84	0.82 ± 0.46
Muribaculaceae	2.70 ± 0.91	1.60 ± 1.04	1.74 ± 0.53

Labeled means without a common letter differ, $p < 0.05$.

the positive effect of XMX-1 fermentation product on *HIF1α* expression might contribute to its effect on intestinal health.

Studies in fish have demonstrated fish meal replacement by plant proteins can affect liver health (Xie et al., 2021). In the present work, uPP significantly increased the expressions of *NF-κB p65*, *TNFα*, *IL-1β* and *TGFβ* in liver. However, the effects of uPP were reversed by dietary uPP + XMX-1 supplementation. *NF-κB* is an important transcription factor (Li and Verma, 2002). Most studies of anti-inflammatory mechanism of butyrate are related to the inhibition of *NF-κB*, and the inhibition of *NF-κB* can further lead to decreased expression of cytokine genes, including *TNFα*, *IL-1β* and other pro-inflammatory cytokines (Meijer et al., 2010; Sossai, 2012). Therefore, the anti-inflammatory effect of XMX-1 fermentation product might be associated with butyrate production. AST and ALT are mainly distributed in hepatocytes, and their activity in serum is very low. When hepatocytes are injured, ALT and AST are released from hepatocytes, and their activity in serum increases, which is consistent with the degree of liver damage (Nyblom et al., 2004; Zhang et al., 2019b). Interestingly, the levels of serum ALT and AST were reversed in the uPP + XMX-1 group compared with uPP fed fish, indicating that the liver injury induced by dietary uPP was relieved in the uPP + XMX-1 group.

Studies on Japanese seabass (*Lateolabrax japonicas*) (Zhang et al. 2019c), sea bass (*Dicentrarchus labrax*) (Messina et al., 2010) and hybrid grouper (Yin et al., 2018) have shown that fish meal replacement by plant proteins can induce fatty liver. In the present study, we also found that dietary uPP significantly improved the formation of lipid droplets in liver and up-regulated the expression of lipogenesis genes, while down-regulated the expression of lipolysis genes. Consistent with our results, previous study showed that Japanese seabass fed a diet with plant proteins induced fatty liver symptom (Zhang et al., 2019a). Excessive accumulation of lipid in the liver will form fatty liver, which damages the liver function of fish, and reduces growth performance, feed utilization efficiency, immunity and anti-stress ability of fish (Qin et al., 2020). Interestingly, the formation of lipid droplets and the expression of lipogenesis genes were significantly down-regulated, whereas the

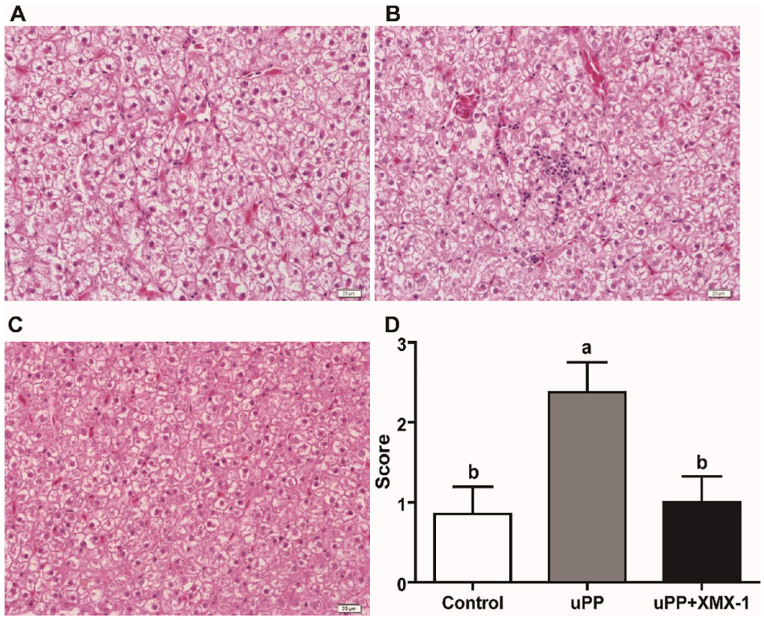

Fig. 7. Effects of uPP and uPP + XMX-1 diet on the HE-stained sections of the liver of common carp. (A) Control, (B) uPP and (C) uPP + XMX-1. Data represent the means (±SEM) (n = 8). Labeled means without a common letter differ, $p < 0.05$.

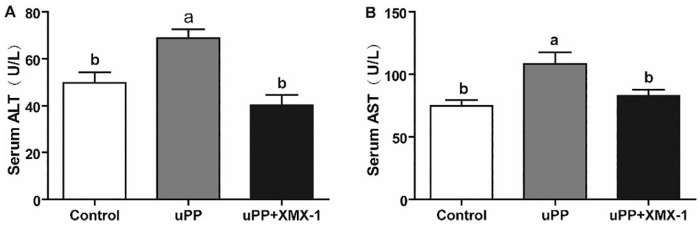

Fig. 8. Effects of uPP and uPP + XMX-1 diet on the level of AST and ALT in serum (A) AST and (B) ALT. Data represent the means (±SEM) (n = 8). Labeled means without a common letter differ, $p < 0.05$.

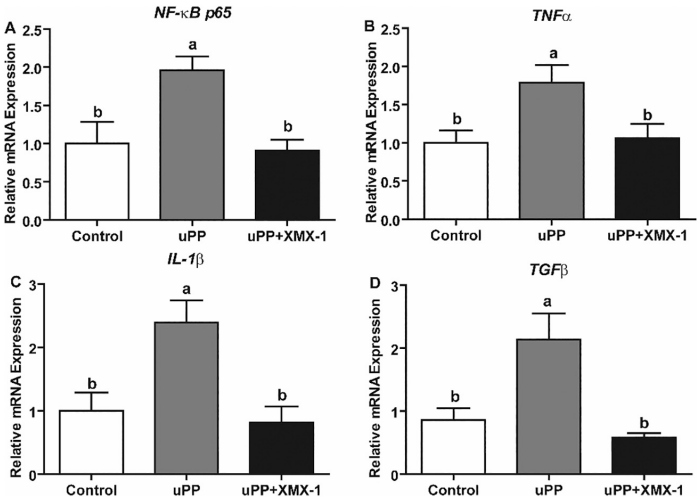

Fig. 9. Effects of uPP and uPP + XMX-1 diet on the expression of inflammation related genes in liver. (A) *NF-κB p65*, (B) *TNFα*, (C) *IL-1β* and (D) *TGFβ*. Data represent the means (±SEM) (n = 8). Labeled means without a common letter differ, $p < 0.05$.

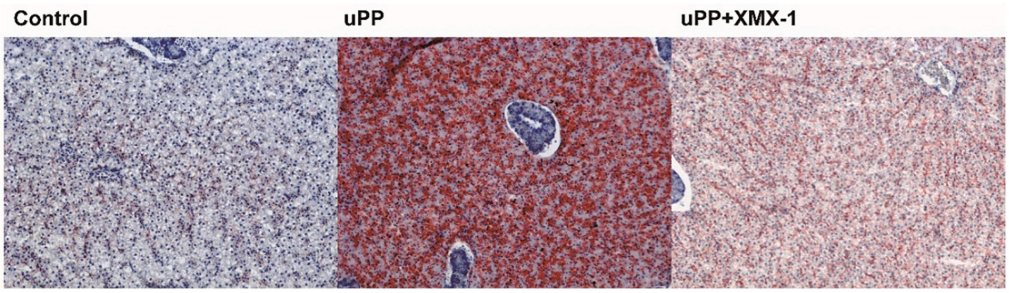

Fig. 10. Oil Red O staining of liver in common carp fed with Control, uPP or uPP + XMX-1 diet. (A) Control, (B) uPP and (C) uPP + XMX-1. Data represent the means (±SEM) (n = 8). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of lipolysis genes was significantly up-regulated in the uPP + XMX-1 group, indicating the beneficial effect of *C. somerae* fermentation product in reducing hepatic lipid deposition.

5. Conclusions

In conclusion, our study showed that dietary uPP exhibited no effects on the growth performance of common carp but compromised gut and liver health. *C. somerae* XMX-1 fermentation product protected gut and liver health of fish by improving the structure of gut and liver and decreasing the expression of inflammation related genes compared to the uPP group. In addition, compared with uPP group, *C. somerae* product decreased the levels of LPS, LBP, AST and ALT in serum, and reduced lipid deposition by decreasing the formation of lipid droplets and significantly up-regulating the expression of lipogenesis genes and down-regulating the expression of lipolysis genes in liver. Therefore, *Cetobacterium somerae* XMX-1 fermentation product has the potential to be developed as novel feed additive to improve fish health.

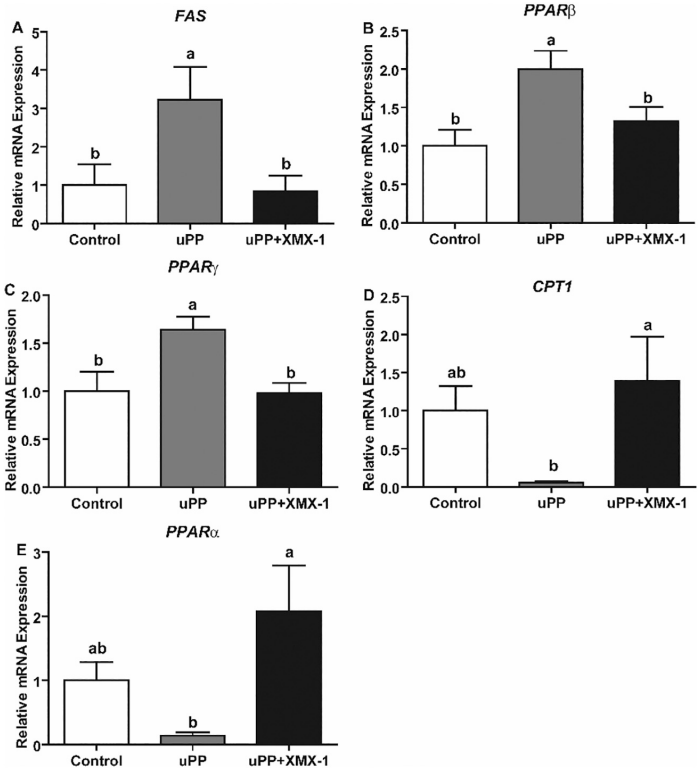

Fig. 11. Effects of uPP and uPP + XMX-1 diet on the expression of lipid metabolism related genes in liver. (A) *FAS*, (B) *PPARβ*, (C) *PPARγ*, (D) *CPT1* and (E) *PPARα*. Data represent the means (±SEM) (n = 8). Labeled means without a common letter differ, $p < 0.05$.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Paper 3

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Stabilized fermentation product of *Cetobacterium somerae* improves gut and liver health and antiviral immunity of zebrafish

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ABSTRACT

Probiotics are widely used in aquafeeds and exhibited beneficial effects on fish by improving host health and resisting pathogens. However, probiotics applied to aquaculture are mainly from terrestrial sources instead of the host animal. The purpose of the work was to evaluate the effects of stabilized fermentation product of commensal *Cetobacterium somerae* XMX-1 on gut, liver health and antiviral immunity of zebrafish. A total of 240 zebrafish were assigned to the control (fed a basal diet) and XMX-1 group (fed a basal diet with 10 g XMX-1/kg diet). After four weeks feeding, growth performance, feed utilization, hepatic steatosis score, TAG, lipid metabolism related genes and serum ALT were evaluated. Furthermore, serum LPS, the expression of Hif-1α, intestinal inflammation score, antioxidant capability and gut microbiota were tested. The survival rate and the expression of antiviral genes were analyzed after challenge by spring viremia of carp virus (SVCV). Results showed that dietary XMX-1 did not affect growth of zebrafish. However, dietary XMX-1 significantly decreased the level of serum LPS, intestinal inflammation score and intestinal MDA, as well as increased T-AOC and the expression of Hif-1α in zebrafish intestine ($p < 0.05$). Furthermore, XMX-1 supplementation decreased the relative abundance of Proteobacteria and increased Firmicutes and Actinobacteria. Additionally, XMX-1 supplementation significantly decreased hepatic steatosis score, hepatic TAG, serum ALT and increased the expression of lipolysis genes versus control ($p < 0.05$). Zebrafish fed XMX-1 diet exhibited higher survival rate after SVCV challenge. Consistently, dietary XMX-1 fermentation product increased the expression of *IFNφ2* and *IFNφ3* after 2 days of SVCV challenge and the expression of *IFNφ1*, *IFNφ2* and *MxC* after 4 days of SVCV challenge in the spleen in zebrafish versus control ($p < 0.05$). In conclusion, our results indicate that dietary XMX-1 can improve liver and gut health, while enhancing antiviral immunity of zebrafish.

1. Introduction

The fish intestinal microbiota is a dynamically changing flora composed of aerobic bacteria, facultative anaerobes and absolute anaerobes [1]. The host provides a nutrient-rich environment for the microbiota that modulates host behaviors, including feeding behavior, digestion and absorption processes and immune response [2,3]. The balance of microbiota is a key factor in maintaining the overall health of fish [4]. Studies have shown that the number of microorganisms associated with the fish digestive tract samples is approximately 10^7-10^8 bacteria per gram weight intestinal tissue [5]. Fusobacteria is a dominant phylum in fish gut microbiota [6,7], in which *Cetobacterium* is an important commensal bacterium which accounts for more than 70% of the gut microbial community in many fish species, such as common carp (*Cyprinus carpio*) [8], bass (*Dicentrarchus labrax*) [9], Nile tilapia (*Oreochromis niloticus*) [10].

Studies in fish have shown that probiotics are beneficial effects on host health by resisting pathogens and improving feed digestibility

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through digestive enzymes, as well as producing fatty acids, bacteriocins, vitamin B12 [11–14]. For example, dietary *Bacillus subtilis* and *B. cereus* NY5 could enhance gut health of juvenile Nile tilapia by improving intestinal immune system, morphology and the composition of gut microbiota [15]. In addition, Ran et al. found that yeast supplementation improved gut health by increasing gut microvilli length and trypsin activity and decreasing the expression of hsp70, as well as reducing the activity of intestinal alkaline phosphatase in Nile tilapia [16]. Furthermore, probiotics play an important role in treating different kinds of chronic liver damage. Dietary VSL#3 probiotics significantly improved hepatic steatosis induced by high fat diet in mice [17]. Additionally, *Lactobacillus rhamnosus* PL60 supplementation exhibited the effects of anti-obesity and the reduction of liver steatosis in obese mice [18]. However, probiotics used in aquaculture, mostly from terrestrial animals, instead of the environment where aquatic animals live or the host animal [19]. Recently, more and more attention has been paid to probiotics isolated from the host's intestines in aquaculture and their application to the host to improve the health and welfare. For example, a study in rainbow trout (*Oncorhynchus mykiss*, Walbaum) showed that LAB, such as *Lactococcus* and *L. lactis* isolated rainbow trout and rearing environment, inhibited the growth of fish pathogens [20]. Dietary *L. lactis* ssp. *lactis* CLFP 100, *Leuconostoc mesenteroides* CLFP 196 and *L. sakei* CLFP 202 isolated from gut microbiota of salmonids increased complement and lysozyme activities in brown trout (*Salmo trutta*) [21]. Additionally, *B. subtilis* AB1 which was obtained from fish gut supplementation enhanced immune system and inhibited the infection of *Aeromonas* sp. in rainbow trout [22]. Dietary *B. clausii* DE5 and *B. pumilus* SE5 isolated from the gut of juvenile grouper *Epinephelus coioides* (*Malabar grouper*) improved growth and the development of gastrointestinal tract of fish by increasing intestinal digestive enzymes activities and lysozyme and SOD activities [23].

Virus diseases can affect fish health and result in economic loss in farmed animal industry [24]. Furthermore, there are no effective treatments or preventive measures for these viral diseases [25]. Spring viremia of carp virus (SVCV) belongs to the family Rhabdoviridae, and its genome is composed of negative-sense single-stranded RNA. SVCV can cause spring viremia of carp in Europe, America, and Asia, and has resulted in huge economic losses due to its high mortality rate especially in common carp [26]. The World Organization for Animal Health has listed SVCV as a disease that must be declared, and it was also included in the list of animal diseases issued in 2008 in China [27,28].

Zebrafish (*Danio rerio*) is an ideal model for studying vertebrate biology with many advantages such as transparent embryos, high reproductive rate and genetic similarity to humans [29]. Many studies have shown that adding probiotics to diet for zebrafish has positive effects on fish health [30–32]. In the present work, stabilized fermentation product of *C. somerae* was added to diet for zebrafish. We evaluated the effects of *C. somerae* fermentation product on growth, liver, gut health of zebrafish, as well as antiviral immunity. Our results demonstrated that *C. somerae* fermentation product exhibited positive effects on liver and gut health of zebrafish. Furthermore, *C. somerae* increased the ability of zebrafish to resist SVCV infection. So far, this is the first report about the effect of *C. somerae* on anti-viral immunity of fish to our knowledge. Our results suggested *C. somerae* as a potential probiotic could be applied in aquaculture.

2. Materials and methods

2.1. Bacteria culture

The protocols of cultivating bacteria were conducted according to the previous method with minor modifications [33]. We anaerobically isolated *Cetobacterium somerae* XMX-1 from zebrafish's intestines and got its preservation number CGMCC no.18908 in the China General Microbiological Culture Collection Center. The strain XMX-1 was named after the abbreviation of Mingxu Xie, who isolated the strain from the intestine of zebrafish reared in our facility. The colonies of XMX-1 were inoculated to GAM Broth medium (Haibo, China) and was anaerobically cultured in an anaerobic incubator (Electrotek, England) for 24 h to attain primary seed fermentation broth of XMX-1. The secondary seed fermentation broth was anaerobically cultured in GAM Broth medium at 5% inoculation amount for 24 h to obtain the fermentation product of XMX-1. Then 3 ml sterilized calcium carbonate (1 M) was added into 100 ml of the XMX-1 fermentation product with the concentration of 10^8 CFU/ml. The addition of calcium carbonate increased the pH after XMX-1 fermentation and make fermentation product more stable. 100 ml of the fermentation product was mixed with 100 g of rice husk powder. Finally, the mixture was dried at room temperature and added to the diet.

Table 1
Ingredients and proximate composition of diets for zebrafish (dry matter, g/kg diet).

Ingredient (g/kg diet)	Control	XMX-1
Flour	200	200
Soybean meal	196	196
Fish meal	450	450
Choline chloride	2	2
Monocalcium phosphate	20	20
Soybean oil	100	100
VC phosphate	1	1
Bentonite	27	17
Vitamin premix[a]	2	2
Mineral premix[b]	2	2
XMX-1	0	10
Total	1000	1000
Moisture (%)	6.28	6.66
Crude ash (%)	11.89	11.09
Crude protein (%)	42.68	42.51
Crude fat (%)	13.62	13.95

[a] Containing the following (g/kg vitamin premix): thiamine, 0.438; riboflavin, 0.632; pyridoxine·HCl, 0.908; D-pantothenic acid, 1.724; nicotinic acid, 4.583; biotin, 0.211; folic acid, 0.549; vitamin B-12, 0.001; inositol, 21.053; menadione sodium bisulfite, 0.889; retinyl acetate, 0.677; cholecalciferol, 0.116; *dl*-α-tocopherol-acetate, 12.632.

[b] Containing the following (g/kg mineral premix): $CoCl_2{\cdot}6H_2O$, 0.074; $CuSO_4{\cdot}5H_2O$, 2.5; $FeSO_4{\cdot}7H_2O$, 73.2; NaCl, 40.0; $MgSO_4{\cdot}7H_2O$, 284.0; $MnSO_4{\cdot}H_2O$, 6.50; KI, 0.68; Na_2SeO_3, 0.10; $ZnSO_4{\cdot}7H_2O$, 131.93; Cellulose, 501.09.

2.2. Animals feeding and sample collection

This work was conducted in Institute of Feed Research, Chinese Academy of Agricultural Sciences, Beijing, China. All animal experiments and care procedures were allowed by the Feed Research Institute of the Chinese Academy of Agricultural Sciences chaired by the China Council for Animal Care (Assurance No. 2018-AF-FRI-CAAS-001). A total of 240 one-month adult zebrafish, with an average initial weight of 0.28 ± 0.003 g, were randomly assigned to 2 treatments: the control and XMX-1 groups. And there are 120 zebrafish in each group. During 4-week feeding, zebrafish in the control group were fed a basal diet and in the XMX-1 group were fed a basal diet supplement with 10 g XMX-1/kg diet, which was the best additive dosage after preliminary research results. The protocols of breeding and housing were performed as previously described [34]. The formulation and proximate composition of diets were listed in Table 1. Zebrafish in each treatment were housed in 6 replicate tanks with 20 zebrafish in each tank. Fish were fed twice (08:30 and 16:30) a day, with 6% of the total body weight.

After 4-week feeding, fish in each tank were weighed after 24 h fasting. The survival rate, feed conversion ratio and weight gain of zebrafish were calculated as previously described [35]. Protocols for collecting liver, intestine, spleen, serum and intestinal content samples were conducted according to the previous description [35].

Table 2
Primer sequences for *q*RT-PCR analysis.

Gene	Nucleotide sequence of primers (5′ to 3′)	Size, bp	GenBank accession No.
RPS11	F: ACAGAAATGCCCCTTCACTG R: GCCTCTTCTCAAAACGGTTG	146	NM_213377.1
UCP2	F: TGCCACCGTGAAGTTTATTG R: CCTCGATATTTCACCGGACC	261	XM_009305562.3
CPT1	F: GCATTGACCTTCAGCTCAGC R: CTGCCAACACCAGCACGAAC	150	XM_005166476.4
PGC1a	F: CCCCTTTGCCCTGACCTGCCTGAG R: GAAGGACAGCTCTGATCACTGGCATTGG	296	XM_017357140.2
IL-1β	F: GGCTGTGTGTTTGGGAATCT R: TGATAAACCAACCGGGACA	218	NM_212844.2
GPX1a	F: GAGGCACAACAGTCAGGGATT R: CTTCATTCTTGCAGTTCTCCTGGT	126	NM_001007281.2
Cu/Zn SOD	F: GTCGTCTGGCTTGTGGAGTG R: TGTCAGCGGGCTAGTGCTT	113	Y12236
SVCV_G	F: TGCTGTGTTGCTTGCACTTATYT R: TCAAACKAARGACCGCATTTCG	150	DQ097384
IFNφ1	F：GAGCACATGAACTCGGTGAA R：TGCGTATCTTGCCACACATT	105	NM_207640.1
IFNφ2	F: CCTCTTTGCCAACGACAGTT R: CGGTTCCTTGAGCTCTCATC	125	NM_001111082.1
IFNφ3	F: TTCTGCTTTGTGCAGGTTTG R: GGTATAGAAACGCGGTCGTC	137	NM_001111083.1
MxC	F: GAGGCTTCACTTGGCAACTC R: TTGTTCCAATAAGGCCAAGC	172	NM_001007284.2

2.3. Histology staining

The liver and intestine were fixed in formalin and then mounted in paraffin blocks. Experimental methods were performed as previously described [36]. Images were obtained by microscopy (Leica DMIL-LED, Germany). Score, villus height and muscle layer height of gut, hepatic steatosis were measured as previously described [37,38].

2.4. Detection of TAG content

The liver samples were homogenized in PBS, and the TAG was obtained according to the method previously described [36]. Free glucose reagent (Sigma-Aldrich, Shanghai) and triglyceride reagent (Sigma-Aldrich, Shanghai) were used to quantify TAG.

2.5. Detection of serum LPS and ALT

Zebrafish blood was collected by tail cutting, and then centrifuged at 1467×*g* for 10 min to obtain the supernatant, namely serum. Serum LPS and ALT were determined using the Serum ToxinSensor™ Chromogenic LAL Endotoxin assay kit (Genscript, China) and ALT assay kits (Jiancheng Bioengineering Ins., Nanjing), according to the manufacturer's instructions, respectively.

2.6. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from liver, intestine and spleen using TRIzol (Invitrogen). For each sample, 1 μg RNA was transformed into cDNA. Experimental methods about RT-*q*PCR reaction were performed according to the previous description [36]. All primers used in the experiment were listed in Table 2. Ribosomal protein S11 gene (*rps11*) was used as the internal control, and all data were statistically analyzed by $2^{-\Delta\Delta CT}$ method.

2.7. Detection of intestinal total antioxidant capacity and malondialdehyde content

To detect the intestinal levels of total antioxidant capacity (T-AOC) and malondialdehyde (MDA) content, intestines of zebrafish were weighed and homogenized with PBS at a concentration of 10%. And then centrifuged at 10000×*g* for 10 min at 4 °C. The supernatant was taken to analyze T-AOC and MDA using the T-AOC assay kit (Cominbio, Suzhou, China) and the lipid peroxidation MDA assay kit (Beyotime Biotechnology, Shanghai, China), respectively, based on manufacturers' instructions.

2.8. Western blot analysis

Zebrafish liver was homogenized in ice-cold HBSS buffer mixed with phosphatase inhibitors. The quantification of protein was measured by

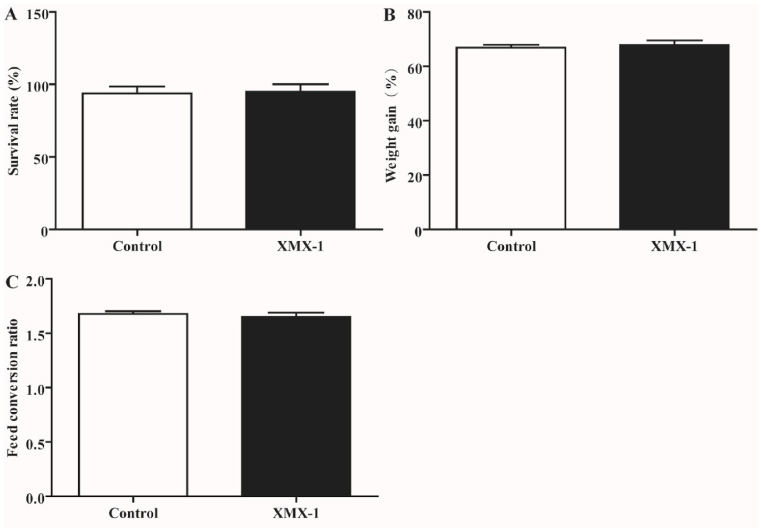

Fig. 1. Effects of XMX-1 diet on the survival rate, weight gain (%) and feed conversion ratio. (A) Survival rate (%), (B) weight gain (%), (C) feed conversion ratio. Data were represented as the means (±SEM) (n = 6).

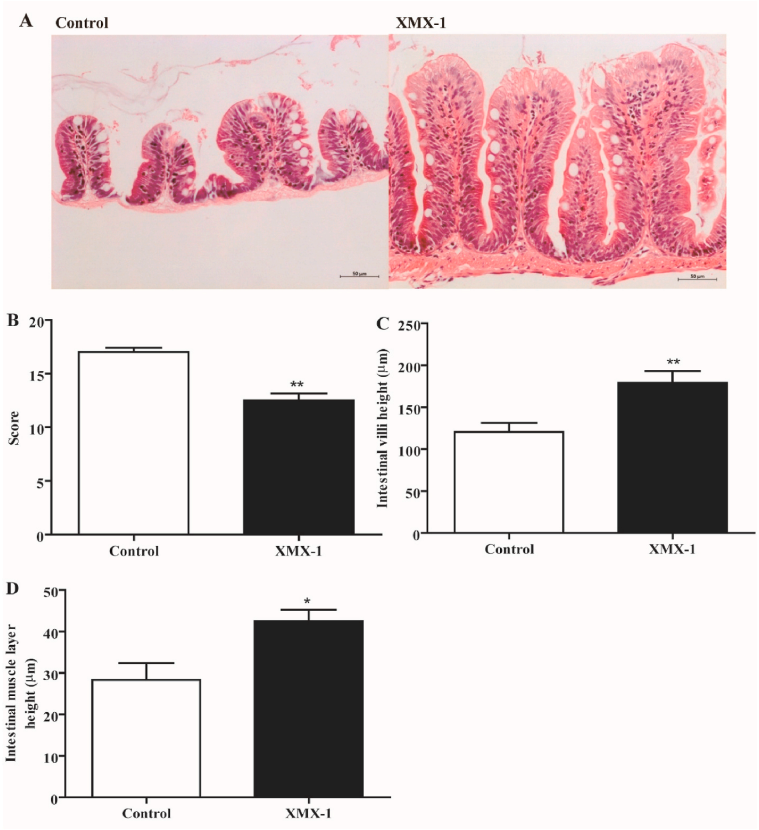

Fig. 2. Effects of dietary control and XMX-1 on intestinal morphology of zebrafish (A) Representative intestinal histology image by H&E staining. The scale bar is 50 μm. (B) Histological score of gut, (C) Score of intestinal villi height, (D) Score of intestinal muscle layer. Data were represented as the means (±SEM) (n = 6). *, $p < 0.05$ and **, $p < 0.01$ comparison to the control group.

BCA protein assay kit (Beyotime). Experimental methods about immunoblot analysis were performed as previously described [36]. Antibodies against GAPDH (Sigma, SAB2708126, 1:2000) and Hif-1α (Bioworld, BS3514, 1:1000) were used in the experiment.

2.9. 16S ribosomal RNA gene sequencing

Gut content of zebrafish was collected from each treatment group 6 h after the last feeding. Experimental methods including DNA extraction, sequencing, and data analysis were conducted based on the presentation [36]. Microbiota sequencing data in this study are available from the National Center for Biotechnology Information (NCBI) under accession number PRJNA742829.

2.10. Virus challenge

The protocols of virus challenge were performed according to the previous description [35]. During the last week of feeding, fish from every group were adapted to the temperature of 22 °C. Then SVCV was added to reach 10^6 TCID 50/ml by bath immersion. During SVCV challenge, fish were not fed. Record the fish death and calculate the survival rate for 14 days. On the 2nd and 4th day of the challenge, MS222 was used to anesthetize the zebrafish, then the spleen was dissected to extract RNA, and the expression of zebrafish antiviral genes was tested.

2.11. Statistical analyses

All data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc. CA, USA). All data were represented as mean ± SEM. Differences between treatments were evaluated by independent samples *t*-test. The test of Log-rank (Mantel-Cox) was applied to show survival rate after SVCV challenge. Difference was regarded as significant when *p* values < 0.05.

3. Results

3.1. Growth performance and feed utilization of zebrafish

After 4-week feeding, results of survival rate, feed conversion ratio and weight gain were presented in Fig. 1. Compared to the control group, survival rate, feed conversion ratio and weight gain showed no differences in the XMX-1 group ($p > 0.05$). However, weight gain was 1% higher in the XMX-1 group of fish versus control. Interestingly, feed conversion ratio was 0.02% lower in zebrafish fed the diet supplemented with XMX-1 than in those fed the control diet.

3.2. Effects of XMX-1 diet on the gut health of zebrafish

Results showed that XMX-1 diet significantly reduced intestinal inflammatory score of zebrafish ($p < 0.01$) (Fig. 2A and B). Additionally,

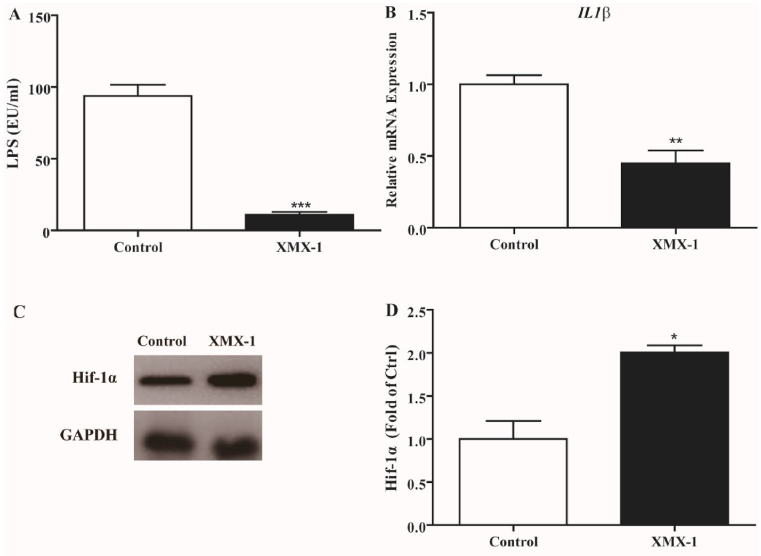

Fig. 3. Effects of XMX-1 diet on the level of serum LPS, the expression of *IL-1β* and western blot analysis of Hif-1α in intestine (A) LPS, (B) *IL-1β*, (C, D) Western blot analysis of Hif-1α. Data were represented as the means (±SEM) (n = 6). *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.0001$ comparison to the control group.

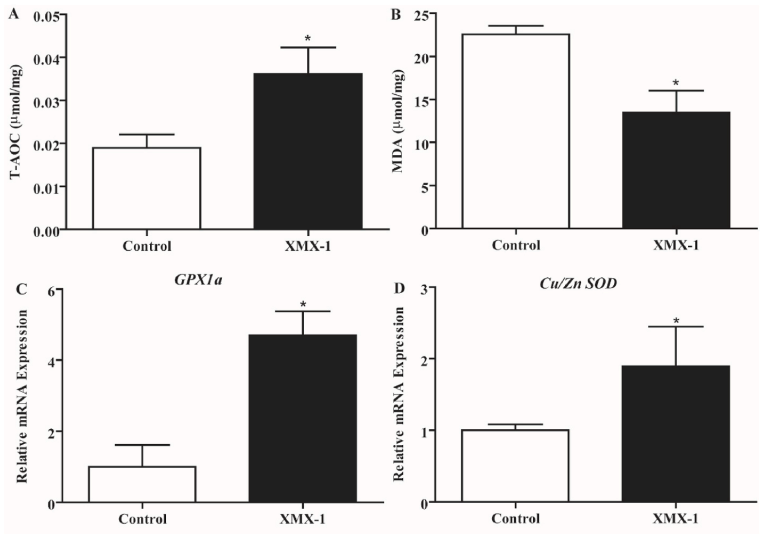

Fig. 4. Effects of XMX-1 diet on the level of T-AOC, MDA and the expression of *GPX1α* and *Cu/Zn SOD* in intestine (A) T-AOC, (B) MDA, (C) *GPX1α*, (D) *Cu/Zn SOD*. Data were represented as the means (±SEM) (n = 6). *, $p < 0.05$ comparison to the control group.

dietary XMX-1 significantly increased the inflammatory villi height ($p < 0.01$) (Fig. 2C) and muscle layer height ($p < 0.05$) (Fig. 2D) of zebrafish. Further, we also found that the LPS in serum was significantly lower in zebrafish fed diet supplemented with XMX-1than in fish fed the control diet ($p < 0.0001$) (Fig. 3A). The expression of pro-inflammatory interleukin-1β (*IL-1β*) was significantly decreased in the XMX-1 group compared with that in the control group ($p < 0.01$) (Fig. 3B). Dietary XMX-1 significantly increased the expression of HIF1α in gut ($p < 0.05$) (Fig. 3C and D). Then, we detected the effects of dietary XMX-1 on the intestinal antioxidant capacity of zebrafish. Results showed that T-AOC in gut was significantly higher in zebrafish fed diet supplemented with XMX-1 than in fish fed the control diet ($p < 0.05$) (Fig. 4A). Additionally, dietary XMX-1 significantly down-regulated the level of MDA in gut compared with the control group ($p < 0.05$) (Fig. 4B). Furthermore, dietary XMX-1 significantly up-regulated the expression of intestinal *GPX1α* and *Cu/Zn SOD* ($p < 0.05$) (Fig. 4C and D).

3.3. Effects of XMX-1 diet on gut microbiota of zebrafish

It could be seen that the relative abundance of Proteobacteria was

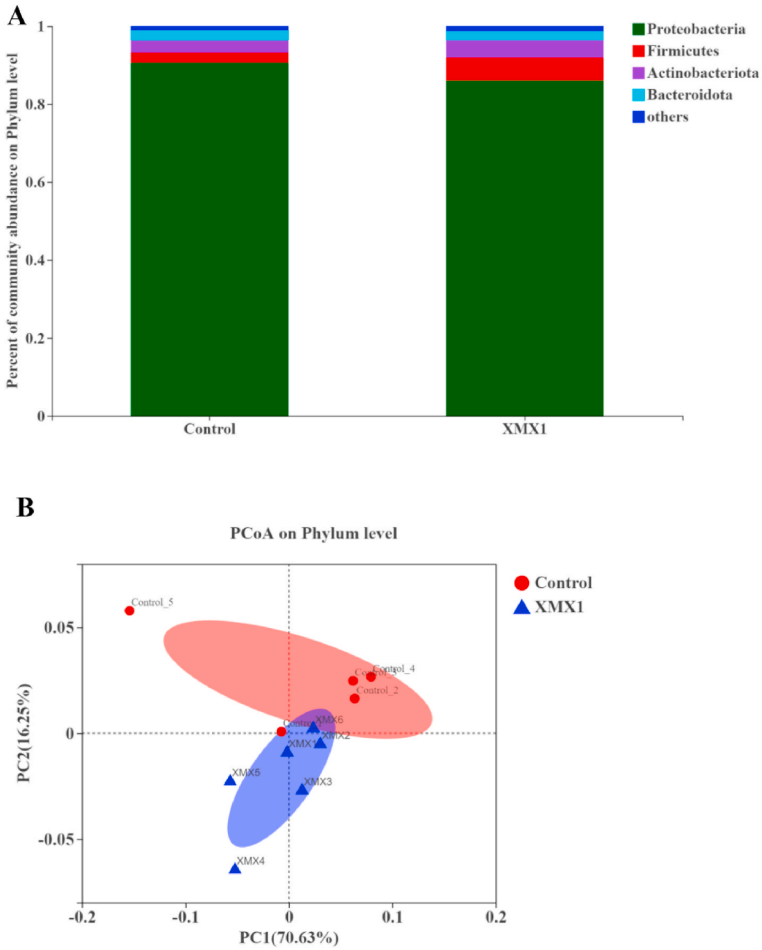

Fig. 5. Effects of dietary XMX-1 fermentation product on the gut microbiota of zebrafish. Staked bar chart (A) of relative abundance of bacterial phylum of intestinal microbiota; Principal coordinates analysis (PCoA) (B) of the gut microbiota at phylum level (n = 6). XMX1 represents the group of XMX-1.

Table 3
The relative abundance of main phyla in the intestinal microbiota of zebrafish fed with different diets.

Bacteria Phylum	Control	XMX-1
Proteobacteria	90.28 ± 3.40	86.30 ± 2.32
Firmicutes	2.66 ± 0.82	5.71 ± 1.61
Actinobacteria	3.19 ± 0.23[a]	4.48 ± 0.39[b]
Bacteroidetes	2.74 ± 2.39	2.19 ± 0.99
Cyanobacteria	0.27 ± 0.17	0.20 ± 0.09
Planctomycetota	0.17 ± 0.06	0.20 ± 0.10
Fusobacteria	0.23 ± 0.11	0.06 ± 0.03
Others	0.45 ± 0.24	0.90 ± 0.20

lower and Firmicutes was higher in the XMX-1 group compared with the other groups at phylum level (Fig. 5A and Table 3). In addition, dietary XMX-1 fermentation product significantly increased the relative abundance of Actinobacteria compared with control group ($p < 0.05$) (Fig. 5A and Table 3). The results of PCoA showed that the composition of gut microbiota in XMX-1 and control group was different at phylum level (Fig. 5B).

3.4. Effects of XMX-1 diet on the liver health of zebrafish

As can be seen from Fig. 6A, H&E staining showed that dietary XMX-1 reduced lipid droplets accumulation in the liver. Additionally, XMX-1 diet significantly decreased the score of hepatic steatosis compared with the control group ($p < 0.01$) (Fig. 6B). Furthermore, the TAG content in the liver was significantly lower in zebrafish fed diet supplemented with XMX-1than in fish fed the control diet ($p < 0.01$) (Fig. 6C). Meanwhile, we found that dietary XMX-1 significantly increased the expression of genes related to lipid oxidation including uncoupling protein 2 (*UCP2*), carnitine palmitoyl transferase 1 (*CPT1*) and proliferator-activated receptor γ coactivator 1α (*PGC-1α*) ($p < 0.05$) (Fig. 6D). Additionally, XMX-1 supplementation significantly decreased the serum ALT compared with the control group ($p < 0.05$) (Fig. 6E).

3.5. Dietary XMX-1 protected zebrafish against SVCV infection

After 4-week feeding, the effects of XMX-1 on zebrafish challenged by SVCV were presented in Fig. 7. Results showed that the survival rate exhibited an increasing tendency in zebrafish fed diet supplemented with XMX-1 than in fish fed the control diet ($p = 0.06$) (Fig. 7A). Then

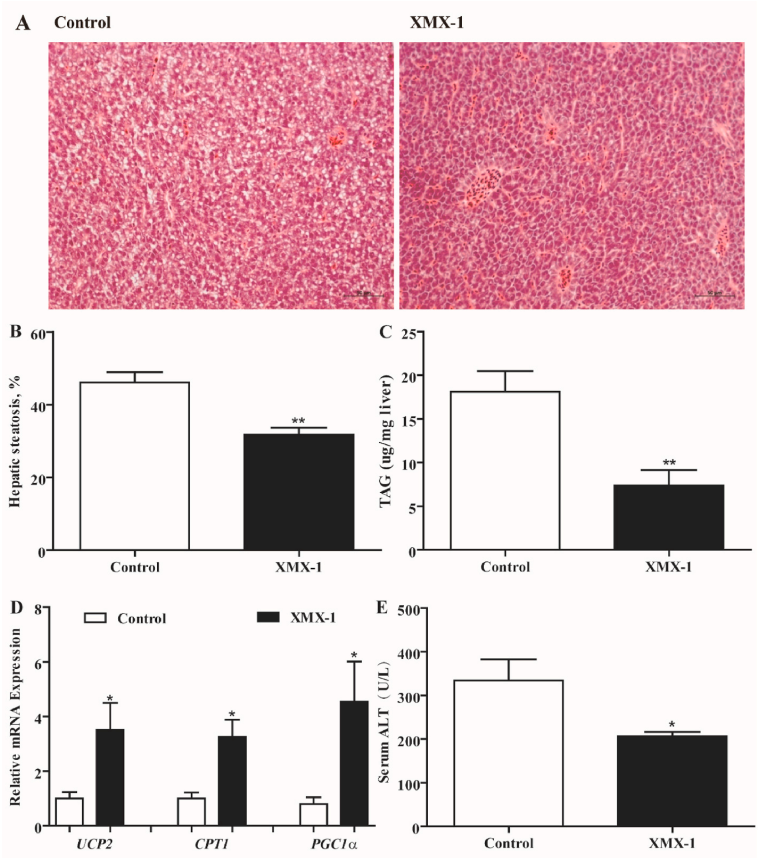

Fig. 6. Effects of control and XMX-1 diet on the lipid fat. (A) Representative liver histology image by H&E staining. The scale bar is 50 μm. (B) Score of hepatic steatosis (%), (C) TAG, (D) The expression of lipolysis related genes, (E) Serum ALT. Data were represented as the means (±SEM) (n = 6). *, $p < 0.05$ and **, $p < 0.01$ comparison to the control group.

we investigated the expression of antiviral genes of type 1 IFNs and MXs in the spleen two and four days after challenge. In agreement with the survival data, our results indicated that dietary XMX-1 significantly inhibited the replication of SVCV in fish compared to the control group ($p < 0.05$) (Fig. 7B), and increased the expression of antiviral genes including interferon $\varphi 2$ (*IFNφ2*) ($p < 0.01$) (Fig. 7C) and interferon $\varphi 3$ (*IFNφ3*) ($p < 0.05$) (Fig. 7D) two days after challenge. Similarly, on the 4th day after challenge, the replication of SVCV exhibited a decreasing tendency in fish fed diet supplemented with XMX-1 compared to the control group four days after challenge ($p = 0.06$) (Fig. 7E). Furthermore, dietary XMX-1 significantly increased the expression of antiviral genes including interferon $\varphi 1$ (*IFNφ1*), *IFNφ2* and myxovirus resistance C (*MxC*) compared to the control group ($p < 0.05$) (Fig. 7F, G, H). These results indicated that dietary XMX-1 enhanced antiviral IFN signaling after SVCV challenge and the anti-viral mechanism of XMX-1 involves type I IFN signaling.

4. Discussion

Metabolites of *Cetobacterium* which is dominant in fish intestines, such as short-chain fatty acids (SCFAs) including acetate, propionate and butyrate and vitamin B12 can enhance fish health [33,34,39]. In the present study, zebrafish was fed with control and XMX-1 diet for 4 weeks. Growth, liver and gut health of zebrafish, as well as antiviral immunity were evaluated. There are no significant differences in growth. However, weight gain was higher in the XMX-1 group of fish versus control and feed conversion ratio was lower in zebrafish fed the diet supplemented with XMX-1 than in those fed the control diet. In addition, dietary XMX-1 exhibited positive effects on liver and gut health of zebrafish compared with the control group. Furthermore, XMX-1 supplementation increased the ability of zebrafish to resist SVCV infection.

The increase of LPS in blood indicates that the intestinal epithelium barrier is damaged, which leads to the intestinal LPS entering the blood through the intestinal epithelial barrier [36,40]. *IL-1β* is a typical pro-inflammatory factor, which plays a very important role in immune response [41]. However, *IL-1β* often causes chronic diseases in the gut, such as chronic enteritis, colitis when overexpressed [42]. Consistent with these studies, we found that dietary XMX-1 fermentation product significantly reduced the level of serum LPS compared with the control group. In addition, the expression of *IL-1β* was significantly decreased in XMX-1 group compared with the control group, which indicating that dietary XMX-1 fermentation product inhibited the level of inflammation in zebrafish intestine. Hypoxia inducible factor (HIF) is considered to be an important regulator of intestinal homeostasis and a therapeutic target for colitis [43–45]. Results of western blot showed that the protein expression of HIF-1α was significantly increased in the XMX-1 group compared with the control group. Recent studies showed that HIF1 α

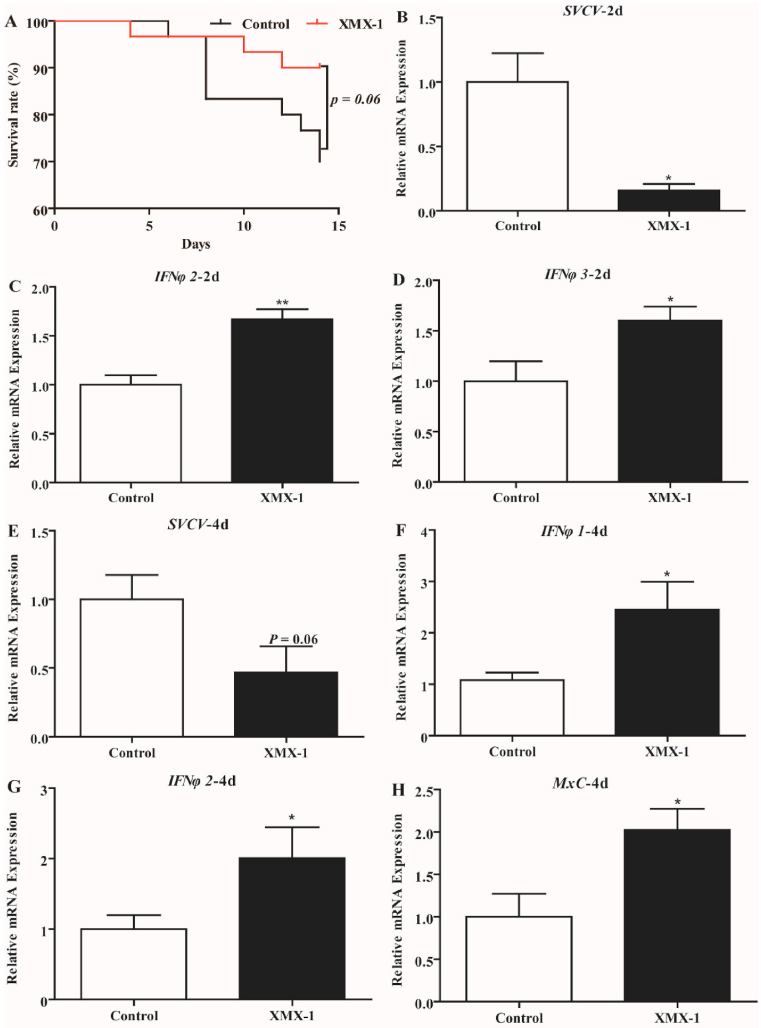

Fig. 7. Effects of dietary XMX-1 on survival rate (A) of zebrafish post SVCV infection and the expression of (B) SVCV-2d, (C) *IFNφ2*-2d, (D) *IFNφ3*-2d, (E) SVCV-4d, (F) *IFNφ2*-4d, (G) *IFNφ3*-4d, (H) *MxC*-4d in the spleen of zebrafish fed XMX-1 and control diets two and four days after SVCV challenge. For the survival rate, Log-rank (Mantel-Cox) test was used to analyze. Data were represented as the means (±SEM) (n = 6). *, $p < 0.05$ and **, $p < 0.01$ comparison to the control group.

could improve gut health by increasing the expression of gut health related genes including intestinal tight junction and antimicrobial peptides [45,46]. Furthermore, it was reported that HIF1 α could alleviate intestinal microbial imbalance and regulate intestinal microbiota [36].

Studies have shown that the balance of oxidation and reduction of the body will be broken after long-term intake of high-fat diet, further causing oxidative stress, and causing damage to tissues, cells, DNA, protein and other biological macromolecules [47]. Numerous studies have found that beneficial additives could increase the antioxidant enzyme activity and total antioxidant capacity of aquatic animals, and reduce the level of MDA [48,49]. The increase of MDA content indicates that the lipid peroxidation of the whole body is enhanced, indicating cell damage induced by oxidative stress [50]. In the present study, intestinal MDA in XMX-1 group was significantly lower than it in the control group. Similarly, the study in grass carp (*Ctenopharyngodon idella*) showed the level of MDA was decreased in fish fed with *B. subtilis* [51]. T-AOC is one of the important indexes to reflect the total antioxidant capacity of the body [52]. In the current work, the value of T-AOC was increased in zebrafish intestine by XMX-1 fermentation product supplementation, as well as antioxidant enzymes activities such as gpx1a and CU/Zn SOD. Similar to this finding, Liu et al., found that dietary *B. subtilis* could significantly increase T-AOC of tilapia [53]. These results indicated that dietary XMX-1 fermentation product could enhance antioxidant activities in zebrafish by reducing the level of MDA and increasing the level of T-AOC. Song et al. proved that phenylpropanoid compound S5 can improve the antioxidant enzyme activity of EPC cells, inhibit the oxidative damage caused by SVCV, prevent the death EPC cell, and reduce the proliferation of SVCV [54]. Liu et al. reported that coumarin D5 significantly increased the activity of antioxidant enzymes in zebrafish, maintained the oxidation-reduction homeostasis of zebrafish, reduced the oxidative damage caused by SVCV infection in zebrafish, and improved the survival rate of zebrafish infected with SVCV

[55].

Recent studies showed that SCFAs can improve intestinal health by keeping the integrity of intestinal barrier, producing mucus, preventing inflammation and reducing the risk of colorectal cancer [56,57]. For example, butyrate improved gut health by activating AMP-Activated Protein Kinase [58]. SCFAs can decrease the expression of pro-inflammation related genes through the activation of FFAR2 and FFAR3 receptors in asthma inflammation [59]. Additionally, SCFAs have great potential in the treatment of inflammatory diseases by inhibiting oxidative stress [60]. For example, Huang et al. showed that butyrate inhibited oxidative stress induced by type 2 diabetes and relieved renal injury [61]. Therefore, we speculated that SCFAs from *Cetobacterium* played an important role in improving gut health of zebrafish in the study, which needs more research.

SCFAs which were kind of metabolites of *Cetobacterium* can be used as substrates for lipid synthesis and regulate lipid metabolism [62,63]. SCFAs can increase the expression of lipolysis related genes such as *PGC-1α* and *UCP* in brown adipose tissue of mice [64]. It was reported that acetate promoted liver fatty acid metabolism as a precursor for palmitate and stearate synthesis [65]. Additionally, butyrate can promote the oxidation of fatty acids to resist obesity and insulin resistance in mice [66]. den Besten et al. showed that dietary propionate increased the expression of *UCP2* and the ratio of AMP: ATP in mitochondria and activated the rate of β-oxidation and reduced fat production [67]. Furthermore, the increase of ALT in serum indicates liver damage [36]. Consistent with these studies, our results showed that dietary XMX-1 fermentation product improved liver health, in which SCFAs as metabolites of *Cetobacterium* may play significant role and needs further research.

Type I IFNs is an important part of antiviral immune response of fish, which can directly inhibit virus replication and also induce the expression of corresponding antiviral proteins to reduce the virus [68,69]. Studies have proven that Mx proteins are important components when animals are in the antiviral state induced by interferons [70]. In addition, spleen is the target organ of SVCV attack, so we take the spleen samples to detect the expression of antiviral related genes in zebrafish after SVCV challenge [71,72]. Our results showed that dietary XMX-1 fermentation product increased the survival rate of zebrafish challenged by SVCV, as well as the expression of *IFNφ2* and *IFNφ3* after 2 days of SVCV challenge and the expression of *IFNφ1*, *IFNφ2* and *MxC* after 4 days of SVCV challenge. In line with our data, dietary *Saccharomyces cerevisiae* improved the survival rate of juvenile *Litopenaeus vannamei* after WSSV injection [73]. Harikrishnan et al. proved that the survival rate of olive flounder (*Paralichthys olivaceus*) fed with *Lactobacillus* and *Sporolac* was improved after lymphocystis disease virus challenge [74]. Similarly, a study in gibel carp (*C. gibelio*), Li et al. found that *Clostridium butyricum* could protect against the infection of *Carassius auratus* herpesvirus and the expression of antiviral related genes including MX was also increased [1].

Studies have proven that gut microbiota plays an important role in regulating the host's antiviral immunity response [75]. For example, Ichinohe et al. showed that antibody titer against influenza virus was significantly reduced after antibiotic treatment in mice, indicating that the gut microbiota exhibited a key role against virus infection [76]. In addition, using a zebrafish model, Galindo-Villegas et al. proved that conventional zebrafish exhibited higher capability to resist SVCV infection than germ-free fish, which indicates gut microbiota possesses an important role in antiviral immune [77]. In mammals, numerous studies have proven that commensal bacteria had the ability to resist viral infection [75]. Further research by Varyukhina et al. showed that probiotics *Bacteroides polymorpha* and *L. casei* interfere with the attachment of rotavirus, thereby blocking rotavirus infections *in vitro* [78]. Probiotics *Bacillus* have shown beneficial effects to improve the survival against white spot syndrome virus challenge by enhancing the immunity of shrimp [79,80]. Similarly, feeding shrimp with diet containing probiotics improves immunity and reduces the infections caused by infectious hypodermal and hematopoietic necrosis virus [81]. Interestingly, it has been demonstrated that probiotics exhibited its contribution to antiviral immunity through producing specific substances [75, 82].

5. Conclusion

In conclusion, the present work showed that the supplementation of XMX-1 stabilized fermentation product did not affect the survival rate, feed conversion ratio and weight gain of zebrafish. Our results demonstrated that dietary XMX-1 improved gut and liver health of zebrafish, and altered the composition of gut microbiota. Furthermore, XMX-1 fermentation product supplementation protected zebrafish from SVCV infection. These results indicate that XMX-1 fermentation product benefits the health of zebrafish and enhanced antiviral immunity, which suggests its potential usage as dietary supplement for aquaculture.

CRediT authorship contribution statement

Mingxu Xie: Formal analysis, Data curation, Writing – original draft. **Yadong Xie:** Formal analysis, Data curation, Writing – original draft. **Yu Li:** Methodology, Investigation. **Wei Zhou:** Investigation, interpretation of data. **Zhen Zhang:** Formal analysis, Resources. **Yalin Yang:** Formal analysis, Resources. **Rolf Erik Olsen:** Resources, Methodology. **Einar Ringø:** Formal analysis, Methodology. **Chao Ran:** Data curation, Project administration, Funding acquisition. **Zhigang Zhou:** Project administration, Supervision, Funding acquisition.

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Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos Zoology	Breeding events of birds in relation to spring temperature and environmental phenology
1978	Egil Sakshaug	Dr. philos Botany	The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton
1980	Arnfinn Langeland	Dr. philos Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation
1984	Eivin Røskaft	Dr. philos Zoology	Sociobiological studies of the rook *Corvus frugilegus*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exposed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos Zoology	Biochemical genetic studies in fish
1985	John Solem	Dr. philos Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains
1985	Randi E. Reinertsen	Dr. philos Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds
1986	Bernt-Erik Sæther	Dr. philos Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach
1986	Torleif Holthe	Dr. philos Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna
1987	Helene Lampe	Dr. scient Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires
1987	Olav Hogstad	Dr. philos Zoology	Winter survival strategies of the Willow tit *Parus montanus*
1987	Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway

1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987	Bjørn Åge Tømmerås	Dr. scient Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction
1988	Hans Christian Pedersen	Dr. philos Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care
1988	Tor G. Heggberget	Dr. philos Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure
1988	Marianne V. Nielsen	Dr. scient Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*)
1988	Ole Kristian Berg	Dr. scient Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.)
1989	John W. Jensen	Dr. philos Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth
1989	Helga J. Vivås	Dr. scient Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*
1989	Reidar Andersen	Dr. scient Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture
1990	Bengt Finstad	Dr. scient Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season
1990	Hege Johannesen	Dr. scient Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams
1990	Tor Jørgen Almaas	Dr. scient Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues
1990	Magne Husby	Dr. scient Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*
1991	Tor Kvam	Dr. scient Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway
1991	Jan Henning L'Abêe Lund	Dr. philos Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular
1991	Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug	Dr. scient Zoology	Reflectometric studies of photomechanical adaptation in superposition eyes of arthropods
1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway

1991	Odd Terje Sandlund	Dr. philos Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism
1991	Nina Jonsson	Dr. philos Zoology	Aspects of migration and spawning in salmonids
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks
1992	Arne Vollan Aarset	Dr. philos Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels and some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Botany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Botany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses

1994	Morten Bakken	Dr. scient Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*
1994	Arne Moksnes	Dr. philos Zoology	Host adaptations towards brood parasitism by the Cockoo
1994	Solveig Bakken	Dr. scient Botany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1994	Torbjørn Forseth	Dr. scient Zoology	Bioenergetics in ecological and life history studies of fishes.
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions
1995	Hanne Christensen	Dr. scient Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*
1995	Svein Håkon Lorentsen	Dr. scient Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition
1995	Chris Jørgen Jensen	Dr. scient Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport
1995	Vidar Moen	Dr. scient Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations
1995	Hans Haavardsholm Blom	Dr. philos Botany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae
1996	Ola Ugedal	Dr. scient Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines
1996	Christina M. S. Pereira	Dr. scient Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996	Jan Fredrik Børseth	Dr. scient Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics
1996	Gunnar Henriksen	Dr. scient Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region
1997	Gunvor Øie	Dr. scient Botany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spruce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters
1997	Ole Reitan	Dr. scient Zoology	Responses of birds to habitat disturbance due to damming
1997	Jon Arne Grøttum	Dr. scient Zoology	Physiological effects of reduced water quality on fish in aquaculture

1997	Per Gustav Thingstad	Dr. scient Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997	Torgeir Nygård	Dr. scient Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as
1997	Signe Nybø	Dr. scient Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway
1997	Atle Wibe	Dr. scient Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry
1997	Rolv Lundheim	Dr. scient Zoology	Adaptive and incidental biological ice nucleators
1997	Arild Magne Landa	Dr. scient Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation
1997	Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*
1997	Jarle Tufto	Dr. scient Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997	Trygve Hesthagen	Dr. philos Zoology	Population responses of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997	Trygve Sigholt	Dr. philos Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997	Jan Østnes	Dr. scient Zoology	Cold sensation in adult and neonate birds
1998	Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins
1998	Thor Harald Ringsby	Dr. scient Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998	Erling Johan Solberg	Dr. scient Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998	Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity
1998	Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro
1998	Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservation biological approach
1998	Bente Gunnveig Berg	Dr. scient Zoology	Encoding of pheromone information in two related moth species
1999	Kristian Overskaug	Dr. scient Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999	Hans Kristen Stenøien	Dr. scient Botany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway

1999	Ingvar Stenberg	Dr. scient Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis
1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gadus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families:
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host specificity as a parameter in estimates of arthropod species richness
1999	Sonja Andersen	Dr. scient Zoology	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial control of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species

2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forest systems
2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient Zoology	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr. philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequences of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr. scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr. scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr. scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr. scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr. scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo salar* L.) parr and smolt
2004	Torkild Bakken	Dr. scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)

2004	Ingar Pareliussen	Dr. scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr. scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr. scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr. scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)
2004	Lene Østby	Dr. scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr. scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr. scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr. scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr. scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr. scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr. scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	PhD Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr. scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr. scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyroid hormone and vitamin A concentrations
2005	Christian Westad	Dr. scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	PhD Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	PhD Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	PhD Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	PhD Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	PhD Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr. scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation

2006	Kari Mette Murvoll	PhD Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds, Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr. scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	PhD Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr. philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr. scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	PhD Biology	*Acesta oophaga* and *Acesta excavata* – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	PhD Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	PhD Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	PhD Biology	Wildlife conservation and local land use conflicts in Western Serengeti Corridor, Tanzania
2006	Anna Maria Billing	PhD Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	PhD Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	PhD Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	PhD Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	PhD Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	PhD Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	PhD Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	PhD Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007	Stig Ulland	PhD Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	PhD Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007	Roelof Frans May	PhD Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	PhD Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007	Julius William Nyahongo	PhD Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania

2007	Shombe Ntaraluka Hassan	PhD Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	PhD Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	PhD Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	PhD Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	PhD Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	PhD Biology	The ecology and behaviour of the Masai Ostrich (*Struthio camelus massaicus*) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	PhD Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, *Taeniopygia guttata*
2008	Sølvi Wehn	PhD Biology	Biodiversity dynamics in semi-natural mountain landscapes - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	PhD Biology	The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations
2008	Katarina Mariann Jørgensen	Dr. scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	PhD Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	PhD Biology	*Arabidopsis thaliana* Responses to Aphid Infestation
2008	Jussi Evertsen	PhD Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	PhD Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	PhD Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	PhD Biology	Cost of rapid growth in salmonid fishes
2008	Line Johansen	PhD Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	PhD Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease
2009	Pål Kvello	PhD Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	PhD Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	PhD Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches

2009	Zsolt Volent	PhD Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	PhD Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	PhD Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	PhD Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments - Focus on formulated diets
2010	Eli Kvingedal	PhD Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	PhD Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	PhD Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. The Case of Serengeti Ecosystem, Tanzania
2010	Anton Tinchov Antonov	PhD Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	PhD Biology	Population Ecology of *Eriophorum latifolium*, a Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	PhD Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	PhD Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	PhD Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters
2010	Hans-Richard Brattbakk	PhD Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	PhD Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	PhD Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	PhD Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS: The science of space experiment integration and adaptation to simulated microgravity
2011	Aline Magdalena Lee	PhD Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics
2011	Christopher Gravningen Sørmo	PhD Biology	Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in Arabidopsis thaliana
2011	Grethe Robertsen	PhD Biology	Relative performance of salmonid phenotypes across environments and competitive intensities
2011	Line-Kristin Larsen	PhD Biology	Life-history trait dynamics in experimental populations of guppy (*Poecilia reticulata*): the role of breeding regime and captive environment
2011	Maxim A. K. Teichert	PhD Biology	Regulation in Atlantic salmon (*Salmo salar*): The interaction between habitat and density
2011	Torunn Beate Hancke	PhD Biology	Use of Pulse Amplitude Modulated (PAM) Fluorescence and Bio-optics for Assessing Microalgal Photosynthesis and Physiology

2011	Sajeda Begum	PhD Biology	Brood Parasitism in Asian Cuckoos: Different Aspects of Interactions between Cuckoos and their Hosts in Bangladesh
2011	Kari J. K. Attramadal	PhD Biology	Water treatment as an approach to increase microbial control in the culture of cold water marine larvae
2011	Camilla Kalvatn Egset	PhD Biology	The Evolvability of Static Allometry: A Case Study
2011	AHM Raihan Sarker	PhD Biology	Conflict over the conservation of the Asian elephant (*Elephas maximus*) in Bangladesh
2011	Gro Dehli Villanger	PhD Biology	Effects of complex organohalogen contaminant mixtures on thyroid hormone homeostasis in selected arctic marine mammals
2011	Kari Bjørneraas	PhD Biology	Spatiotemporal variation in resource utilisation by a large herbivore, the moose
2011	John Odden	PhD Biology	The ecology of a conflict: Eurasian lynx depredation on domestic sheep
2011	Simen Pedersen	PhD Biology	Effects of native and introduced cervids on small mammals and birds
2011	Mohsen Falahati-Anbaran	PhD Biology	Evolutionary consequences of seed banks and seed dispersal in *Arabidopsis*
2012	Jakob Hønborg Hansen	PhD Biology	Shift work in the offshore vessel fleet: circadian rhythms and cognitive performance
2012	Elin Noreen	PhD Biology	Consequences of diet quality and age on life-history traits in a small passerine bird
2012	Irja Ida Ratikainen	PhD Biology	Foraging in a variable world: adaptations to stochasticity
2012	Aleksander Handå	PhD Biology	Cultivation of mussels (*Mytilus edulis):* Feed requirements, storage and integration with salmon (*Salmo salar*) farming
2012	Morten Kraabøl	PhD Biology	Reproductive and migratory challenges inflicted on migrant brown trout (*Salmo trutta* L.) in a heavily modified river
2012	Jisca Huisman	PhD Biology	Gene flow and natural selection in Atlantic salmon
2012	Maria Bergvik	PhD Biology	Lipid and astaxanthin contents and biochemical post-harvest stability in *Calanus finmarchicus*
2012	Bjarte Bye Løfaldli	PhD Biology	Functional and morphological characterization of central olfactory neurons in the model insect *Heliothis virescens*.
2012	Karen Marie Hammer	PhD Biology	Acid-base regulation and metabolite responses in shallow- and deep-living marine invertebrates during environmental hypercapnia
2012	Øystein Nordrum Wiggen	PhD Biology	Optimal performance in the cold
2012	Robert Dominikus Fyumagwa	Dr. Philos Biology	Anthropogenic and natural influence on disease prevalence at the human –livestock-wildlife interface in the Serengeti ecosystem, Tanzania
2012	Jenny Bytingsvik	PhD Biology	Organohalogenated contaminants (OHCs) in polar bear mother-cub pairs from Svalbard, Norway. Maternal transfer, exposure assessment and thyroid hormone disruptive effects in polar bear cubs
2012	Christer Moe Rolandsen	PhD Biology	The ecological significance of space use and movement patterns of moose in a variable environment
2012	Erlend Kjeldsberg Hovland	PhD Biology	Bio-optics and Ecology in *Emiliania huxleyi* Blooms: Field and Remote Sensing Studies in Norwegian Waters

2012	Lise Cats Myhre	PhD Biology	Effects of the social and physical environment on mating behaviour in a marine fish
2012	Tonje Aronsen	PhD Biology	Demographic, environmental and evolutionary aspects of sexual selection
2012	Bin Liu	PhD Biology	Molecular genetic investigation of cell separation and cell death regulation in *Arabidopsis thaliana*
2013	Jørgen Rosvold	PhD Biology	Ungulates in a dynamic and increasingly human dominated landscape – A millennia-scale perspective
2013	Pankaj Barah	PhD Biology	Integrated Systems Approaches to Study Plant Stress Responses
2013	Marit Linnerud	PhD Biology	Patterns in spatial and temporal variation in population abundances of vertebrates
2013	Xinxin Wang	PhD Biology	Integrated multi-trophic aquaculture driven by nutrient wastes released from Atlantic salmon (*Salmo salar*) farming
2013	Ingrid Ertshus Mathisen	PhD Biology	Structure, dynamics, and regeneration capacity at the sub-arctic forest-tundra ecotone of northern Norway and Kola Peninsula, NW Russia
2013	Anders Foldvik	PhD Biology	Spatial distributions and productivity in salmonid populations
2013	Anna Marie Holand	PhD Biology	Statistical methods for estimating intra- and inter-population variation in genetic diversity
2013	Anna Solvang Båtnes	PhD Biology	Light in the dark – the role of irradiance in the high Arctic marine ecosystem during polar night
2013	Sebastian Wacker	PhD Biology	The dynamics of sexual selection: effects of OSR, density and resource competition in a fish
2013	Cecilie Miljeteig	PhD Biology	Phototaxis in *Calanus finmarchicus* – light sensitivity and the influence of energy reserves and oil exposure
2013	Ane Kjersti Vie	PhD Biology	Molecular and functional characterisation of the IDA family of signalling peptides in *Arabidopsis thaliana*
2013	Marianne Nymark	PhD Biology	Light responses in the marine diatom *Phaeodactylum tricornutum*
2014	Jannik Schultner	PhD Biology	Resource Allocation under Stress - Mechanisms and Strategies in a Long-Lived Bird
2014	Craig Ryan Jackson	PhD Biology	Factors influencing African wild dog *(Lycaon pictus*) habitat selection and ranging behaviour: conservation and management implications
2014	Aravind Venkatesan	PhD Biology	Application of Semantic Web Technology to establish knowledge management and discovery in the Life Sciences
2014	Kristin Collier Valle	PhD Biology	Photoacclimation mechanisms and light responses in marine micro- and macroalgae
2014	Michael Puffer	PhD Biology	Effects of rapidly fluctuating water levels on juvenile Atlantic salmon (*Salmo salar* L.)
2014	Gundula S. Bartzke	PhD Biology	Effects of power lines on moose *(Alces alces)* habitat selection, movements and feeding activity
2014	Eirin Marie Bjørkvoll	PhD Biology	Life-history variation and stochastic population dynamics in vertebrates
2014	Håkon Holand	PhD Biology	The parasite *Syngamus trachea* in a metapopulation of house sparrows
2014	Randi Magnus Sommerfelt	PhD Biology	Molecular mechanisms of inflammation – a central role for cytosolic phospholiphase A2
2014	Espen Lie Dahl	PhD Biology	Population demographics in white-tailed eagle at an on-shore wind farm area in coastal Norway

2014	Anders Øverby	PhD Biology	Functional analysis of the action of plant isothiocyanates: cellular mechanisms and in vivo role in plants, and anticancer activity
2014	Kamal Prasad Acharya	PhD Biology	Invasive species: Genetics, characteristics and trait variation along a latitudinal gradient.
2014	Ida Beathe Øverjordet	PhD Biology	Element accumulation and oxidative stress variables in Arctic pelagic food chains: *Calanus*, little auks (*Alle alle*) and black-legged kittiwakes (*Rissa tridactyla*)
2014	Kristin Møller Gabrielsen	PhD Biology	Target tissue toxicity of the thyroid hormone system in two species of arctic mammals carrying high loads of organohalogen contaminants
2015	Gine Roll Skjervø	Dr. philos Biology	Testing behavioral ecology models with historical individual-based human demographic data from Norway
2015	Nils Erik Gustaf Forsberg	PhD Biology	Spatial and Temporal Genetic Structure in Landrace Cereals
2015	Leila Alipanah	PhD Biology	Integrated analyses of nitrogen and phosphorus deprivation in the diatoms *Phaeodactylum tricornutum* and *Seminavis robusta*
2015	Javad Najafi	PhD Biology	Molecular investigation of signaling components in sugar sensing and defense in *Arabidopsis thaliana*
2015	Bjørnar Sporsheim	PhD Biology	Quantitative confocal laser scanning microscopy: optimization of in vivo and in vitro analysis of intracellular transport
2015	Magni Olsen Kyrkjeeide	PhD Biology	Genetic variation and structure in peatmosses (*Sphagnum*)
2015	Keshuai Li	PhD Biology	Phospholipids in Atlantic cod (*Gadus morhua* L.) larvae rearing: Incorporation of DHA in live feed and larval phospholipids and the metabolic capabilities of larvae for the de novo synthesis
2015	Ingvild Fladvad Størdal	PhD Biology	The role of the copepod *Calanus finmarchicus* in affecting the fate of marine oil spills
2016	Thomas Kvalnes	PhD Biology	Evolution by natural selection in age-structured populations in fluctuating environments
2016	Øystein Leiknes	PhD Biology	The effect of nutrition on important life-history traits in the marine copepod *Calanus finmarchicus*
2016	Johan Henrik Hårdensson Berntsen	PhD Biology	Individual variation in survival: The effect of incubation temperature on the rate of physiological ageing in a small passerine bird
2016	Marianne Opsahl Olufsen	PhD Biology	Multiple environmental stressors: Biological interactions between parameters of climate change and perfluorinated alkyl substances in fish
2016	Rebekka Varne	PhD Biology	Tracing the fate of escaped cod (*Gadus morhua* L.) in a Norwegian fjord system
2016	Anette Antonsen Fenstad	PhD Biology	Pollutant Levels, Antioxidants and Potential Genotoxic Effects in Incubating Female Common Eiders (*Somateria mollissima*)
2016	Wilfred Njama Marealle	PhD Biology	Ecology, Behaviour and Conservation Status of Masai Giraffe (*Giraffa camelopardalis tippelskirchi*) in Tanzania
2016	Ingunn Nilssen	PhD Biology	Integrated Enviromental Mapping and Monitoring: A Methodological approach for end users.
2017	Konika Chawla	PhD Biology	Discovering, analysing and taking care of knowledge.
2017	Øystein Hjorthol Opedal	PhD Biology	The Evolution of Herkogamy: Pollinator Reliability, Natural Selection, and Trait Evolvability.

2017	Ane Marlene Myhre	PhD Biology	Effective size of density dependent populations in fluctuating environments
2017	Emmanuel Hosiana Masenga	PhD Biology	Behavioural Ecology of Free-ranging and Reintroduced African Wild Dog (*Lycaon pictus*) Packs in the Serengeti Ecosystem, Tanzania
2017	Xiaolong Lin	PhD Biology	Systematics and evolutionary history of *Tanytarsus* van der Wulp, 1874 (Diptera: Chironomidae)
2017	Emmanuel Clamsen Mmassy	PhD Biology	Ecology and Conservation Challenges of the Kori bustard in the Serengeti National Park
2017	Richard Daniel Lyamuya	PhD Biology	Depredation of Livestock by Wild Carnivores in the Eastern Serengeti Ecosystem, Tanzania
2017	Katrin Hoydal	PhD Biology	Levels and endocrine disruptive effects of legacy POPs and their metabolites in long-finned pilot whales of the Faroe Islands
2017	Berit Glomstad	PhD Biology	Adsorption of phenanthrene to carbon nanotubes and its influence on phenanthrene bioavailability/toxicity in aquatic organism
2017	Øystein Nordeide Kielland	PhD Biology	Sources of variation in metabolism of an aquatic ectotherm
2017	Narjes Yousefi	PhD Biology	Genetic divergence and speciation in northern peatmosses (*Sphagnum*)
2018	Signe Christensen-Dalgaard	PhD Biology	Drivers of seabird spatial ecology - implications for development of offshore wind-power in Norway
2018	Janos Urbancsok	PhD Biology	Endogenous biological effects induced by externally supplemented glucosinolate hydrolysis products (GHPs) on *Arabidopsis thaliana*
2018	Alice Mühlroth	PhD Biology	The influence of phosphate depletion on lipid metabolism of microalgae
2018	Franco Peniel Mbise	PhD Biology	Human-Carnivore Coexistence and Conflict in the Eastern Serengeti, Tanzania
2018	Stine Svalheim Markussen	PhD Biology	Causes and consequences of intersexual life history variation in a harvested herbivore population
2018	Mia Vedel Sørensen	PhD Biology	Carbon budget consequences of deciduous shrub expansion in alpine tundra ecosystems
2018	Hanna Maria Kauko	PhD Biology	Light response and acclimation of microalgae in a changing Arctic
2018	Erlend I. F. Fossen	PhD Biology	Trait evolvability: effects of thermal plasticity and genetic correlations among traits
2019	Peter Sjolte Ranke	PhD Biology	Demographic and genetic and consequences of dispersal in house sparrows
2019	Mathilde Le Moullec	PhD Biology	Spatiotemporal variation in abundance of key tundra species: from local heterogeneity to large-scale synchrony
2019	Endre Grüner Ofstad	PhD Biology	Causes and consequences of variation in resource use and social structure in ungulates
2019	Yang Jin	PhD Biology	Development of lipid metabolism in early life stage of Atlantic salmon (*Salmo salar*)
2019	Elena Albertsen	PhD Biology	Evolution of floral traits: from ecological contex to functional integration
2019	Mominul Islam Nahid	PhD Biology	Interaction between two Asian cuckoos and their hosts in Bangladesh
2019	Knut Jørgen Egelie	PhD Biology	Management of intellectual property in university-industry collaborations – public access to and control of knowledge
2019	Thomas Ray Haaland	PhD Biology	Adaptive responses to enviromental stochasticity on different evolutionary time-scales

2019	Kwaslema Malle Hariohay	PhD Biology	Human wildlife interactions in the Ruaha-Rungwa Ecosystem, Central Tanzania
2019	Mari Engvig Løseth	PhD Biology	Exposure and effects of emerging and legacy organic pollutants in white-tailed eagle (*Haliaeetis albicilla*) nestlings
2019	Joseph Mbyati Mukeka	PhD Biology	Human-Wildlife Conflicts and Compensation for Losses in Kenya: Dynamics, Characteristics and Correlates
2019	Helene Løvstrand Svarva	PhD Biology	Dendroclimatology in southern Norway: tree rings, demography and climate
2019	Nathalie Briels	PhD Biology	Exposure and effects of legacy and emerging organic pollutants in developing birds – Laboratory and field studies
2019	Anders L.Kolstad	PhD Biology	Moose browsing effects on boreal production forests – implications for ecosystems and human society
2019	Bart Peeters	PhD Biology	Population dynamics under climate change ad harvesting: Results from the high Arctic Svalbard reindeer
2019	Emma-Liina Marjakangas	PhD Biology	Understanding species interactions in the tropics: dynamics within and between trophic levels
2019	Alex Kojo Datsomor	PhD Biology	The molecular basis of long chain polyunsaturated fatty acid (LC-PUFA) biosynthesis in Atlantic salmon (*Salmo salar L*): In vivo functions, functional redundancy and transcriptional regulation of LC-PUFA biosynthetic enzymes
2020	Ingun Næve	PhD Biology	Development of non-invasive methods using ultrasound technology in monitoring of Atlantic salmon (*Salmo salar*) production and reproduction
2020	Rachael Morgan	PhD Biology	Physiological plasticity and evolution of thermal performance in zebrafish
2020	Mahsa Jalili	PhD Biology	Effects of different dietary ingredients on the immune responses and antioxidant status in Atlantic salmon (*Salmo salar L.):* possible nutriomics approaches
2020	Haiqing Wang	PhD Biology	Utilization of the polychaete *Hediste diversicolor* (O.F. Millier, 1776) in recycling waste nutrients from land-based fish farms for value adding applications'
2020	Louis Hunninck	PhD Biology	Physiological and behavioral adaptations of impala to anthropogenic disturbances in the Serengeti ecosystems
2020	Kate Layton-Matthews	PhD Biology	Demographic consequences of rapid climate change and density dependence in migratory Arctic geese
2020	Amit Kumar Sharma	PhD Biology	Genome editing of marine algae: Technology development and use of the CRISPR/Cas9 system for studies of light harvesting complexes and regulation of phosphate homeostasis
2020	Lars Rød-Eriksen	PhD Biology	Drivers of change in meso-carnivore distributions in a northern ecosystem
2020	Lone Sunniva Jevne	PhD Biology	Development and dispersal of salmon lice (*Lepeophtheirus salmonis Krøyer*, 1837) in commercial salmon farming localities
2020	Sindre Håvarstein Eldøy	PhD Biology	The influence of physiology, life history and environmental conditions on the marine migration patters of sea trout
2020	Vasundra Touré	PhD Biology	Improving the FAIRness of causal interactions in systems biology: data curation and standardisation to support systems modelling applications

2020	Silje Forbord	PhD Biology	Cultivation of *Saccharina latissima* (Phaeophyceae) in temperate marine waters; nitrogen uptake kinetics, growth characteristics and chemical composition
2020	Jørn Olav Løkken	PhD Biology	Change in vegetation composition and growth in the forest-tundra ecotone – effects of climate warming and herbivory
2020	Kristin Odden Nystuen	PhD Biology	Drivers of plant recruitment in alpine vegetation
2021	Sam Perrin	PhD Biology	Freshwater Fish Community Responses to Climate Change and Invasive Species
2021	Lara Veylit	PhD Biology	Causes and consequences of body growth variation in hunted wild boar populations
2021	Semona Issa	PhD Biology	Combined effects of environmental variation and pollution on zooplankton life history and population dynamics
2021	Monica Shilereyo	PhD Biology	Small Mammal Population Ecology and Ectoparasite Load: Assessing Impacts of Land Use and Rainfall Seasonality in the Serengeti Ecosystem, Tanzania
2021	Vanessa Bieker	PhD Biology	Using historical herbarium specimens to elucidate the evolutionary genomics of plant invasion
2021	Håkon Austad Langberg	PhD Biology	Fate and transport of forever chemicals in the aquatic environment: Partitioning and biotransformation of mixtures of Per- and Polyfluoroalkyl Substances (PFAS) from different point sources and resulting concentrations in biota
2021	Julie Renberg	PhD Biology	Muscular and metabolic load and manual function when working in the cold
2021	Olena Meleshko	PhD Biology	Gene flow and genome evolution on peatmosses (S*phagnum*)
2021	Essa Ahsan Khan	PhD Biology	Systems toxicology approach for evaluating the effects of contaminants on fish ovarian development and reproductive endocrine physiology:A combination of field-, in vivo and ex vivo studies using Atlantic cod (*Gadus morhua*)
2021	Tanja Kofod Petersen	PhD Biology	Biodiversity dynamics in urban areas under changing land-uses
2021	Katariina Vuorinen	PhD Biology	When do ungulates override the climate? Defining the interplay of two key drivers of northern vegetation dynamics
2021	Archana Golla	PhD Biology	Impact of early life stress on behaviour and dorsal raphe serotonergic activity in zebrafish (*Danio rerio*)
2021	Aksel Alstad Mogstad	PhD Biology	Underwater Hyperspectral Imaging as a Tool for Benthic Habitat Mapping

2021	Randi Grønnstad	PhD Biology	Per- and polyfluoroalkyl substances (PFAS) in ski products: Environmental contamination, bioaccumulation and effects in rodents
2021	Gaspard Philis	PhD Biology	Life cycle assessment of sea lice treatments in Norwegian net pens with emphasis on the environmental tradeoffs of salmon aquaculture production systems
2021	Christoffer Høyvik Hilde	PhD Biology	Demographic buffering of vital rates in age-structured populations"
2021	Halldis Ringvold	Dr.Philos	Studies on Echinodermata from the NE Atlantic Ocean - Spatial distribution and abundance of Asteroidea, including taxonomic and molecular studies on Crossaster and Henricia genera- Value-chain results, including test fishery, biology, market and nutritional analysis, on Parastichopus tremulus (Holothuroidea) from the Norwegian coast
2021	Elise Skottene	PhD Biology	Lipid metabolism and diapause timing in Calanus copepods. The impact of predation risk, food availability and oil exposure
2021	Michael Le Pepke	PhD Biology	The ecological and evolutionary role of telomere length in house sparrows
2022	Niklas Erik Johansson	Dr. Philos	On the taxonomy of Northern European Darwin wasps (Hymenoptera: Ichneumonidae).
2022	Jonatan Fredricson Marquez	PhD Biology	Understanding spatial and interspecific processes affecting population dynamics in a marine ecosystem.
2022	Anne Mehlhoop	PhD Biology	Evaluating mitigation measures to reduce negative impacts of infrastructure construction on vegetation and wildlife.
2022	Malene Østreng Nygård	PhD Biology	Integrative biosystematics and conservation genomics – holistic studies of two red-listed plants in Norway
2022	Martin René Ellegaard	PhD Biology	Human Population Genomics in Northern Europe in the Past 2000 years
2022	Gaute Kjærstad	PhD Biology	The eradication of invasive species using rotenone and its impact on freshwater macroinvertebrates
2022	Stefan Vriend	PhD Biology	On the roles of density dependence and environmental fluctuations in driving eco-evolutionary dynamics of hole-nesting passerines
2022	Zaw Min Thant	PhD Biology	Anthropogenic and Environmental factors driving the Human-Elephant Conflict in Myanmar
2022	Prashanna Guragain	PhD Biology	Population analysis and structure and RNA interference to understand salmon lice biology and a review of the principles of controlling infestation in aquaculture facilities.
2022	Ronja Wedegärtner	PhD Biology	Highways up the mountains? Trails as facilitators for redistribution of plant species in mountain areas

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