Role of dietary *Schizochytrium* sp. in improving disease resistance of zebrafish through metabolic and microbial analysis

Yanyan Shi

Xiamen University College of Chemistry and Chemical Engineering

Xingyu Cao

Xiamen University College of Chemistry and Chemical Engineering

Zhidong Ye

Xiamen University College of Chemistry and Chemical Engineering

Yiyuan Xu

Xiamen University College of Chemistry and Chemical Engineering

Yiming Wang

Xiamen University College of Chemistry and Chemical Engineering

Zhipeng Li

Jimei University

Wei Hang

Xiamen University College of Chemistry and Chemical Engineering

Ning He (✉ hening@xmu.edu.cn)

Xiamen University <https://orcid.org/0000-0002-2698-877X>

Research

Keywords: Schizochytrium sp., zebrafish, metabolic profiling, intestine microbiota, disease resistance

Posted Date: October 14th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-88323/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Aquaculture on June 1st, 2021. See the published version at <https://doi.org/10.1016/j.aquaculture.2021.736631>.

Abstract

Background

As an essential nutrient for farmed fish, the fish oil from wild fish has been partly replaced by vegetable ingredients to prevent overfishing. *Schizochytrium* sp., a type of marine microalgae, is considered as a promising alternative to fish oil for improving growth and fatty acid profile in fish. However, there lacks a comprehensive understanding of disease resistance on microalgae supplementation of fish. In the present study, to understand the pathogen-resistant mechanisms of diets enriched with microalgae, the effects of dietary *Schizochytrium* sp. on the intestine microbial and metabolic profile of zebrafish were investigated.

Results

The challenge trial with *Edwardsiella piscicida* showed that 120 g/kg *Schizochytrium* sp. supplementation had a significantly higher survival rate of zebrafish. Additionally, higher goblet cell density was observed in zebrafish fed with *Schizochytrium* sp. Metabolomic analysis of humoral fluids indicated that the diet supplemented with *Schizochytrium* sp. boosted the TCA cycle, energy supply, taurine metabolism, and L-serine metabolism, whereas decreased cholesterol metabolism in zebrafish. The microbiome analysis revealed that a 120 g/kg *Schizochytrium* sp. supplemented diet could remarkably increase the abundance of beneficial bacteria (i.e., *Lactobacillus*, *Dorea*, *Butyricicoccus*, and *Pseudoxanthomonas*), and reduce several potential pathogens (i.e., *Flavobacterium*, *Pseudomonas*, *Citrobacter*, and *Mycoplasmas*). Combined omics analysis indicated that some *Dorea* and *Butyricicoccus* species might be candidate probiotics with disease resistance.

Conclusions

Dietary supplement of *Schizochytrium* sp. could improve the survival rate of zebrafish when infected with *Edwardsiella piscicida*. It further revealed that *Schizochytrium* sp. as feed additive had the potential to improve metabolism and the intestine health by dual-omics analysis, and thus enhance disease resistance of zebrafish. Our research provides a novel insight into developing the fundamental understanding of disease resistance in aquatic animals fed with microalgae. Moreover, this experiment shed substantial light on the screening of probiotic candidates with immunomodulatory properties.

Background

Dietary fish oil, mainly produced from wild-caught ocean fish, provides essential nutrients for farmed fish [1]. However, due to the rapid global expansion of the aquaculture industry and the limited availability of wild fish resources, fish oil access is gradually decreasing [2–4]. To satisfy the increasing needs of the aquaculture industry, researchers have focused on finding fish oil alternatives[5]. Compared with land-based crops, microalgae have a nutritional quality that is more similar to fish oil, especially in terms of proteins, lipids, vitamins, and minerals, and, hence, they are considered as a promising alternative to fish

oil and could enhance sustainability in aquaculture [6]. Studies have shown that marine microalgae diets that are rich in long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can improve the growth, nutritional performance, and fatty acid profile of fish [3, 7, 8]. Moreover, Sheikhzadeh *et al.* have found that dietary supplementation with 2.5% *Spirulina platensis* could improve mucosal immune responses and disease resistance in rainbow trout [9].

Schizochytrium sp., a type of marine microalgae, has been preferred as a potential replacement for fish oil because of its ease in large-scale heterotrophic cultivation and high lipid content [10]. Our previous study found that *Schizochytrium* sp. is rich in LC-PUFA, especially DHA (above 40% of total lipids) [11]. To our knowledge, many researchers have focused on improving growth and the ratio of LC-PUFA with dietary containing with *Schizochytrium* sp. both in juvenile and adult fish, such as the Atlantic salmon (*Salmo salar*) [12, 13], Pacific white shrimp (*Litopenaeus vannamei*) [14], channel catfish (*Ictalurus punctatus*) [15], seabream (*Sparus aurata*) [16], and zebrafish (*Danio rerio*) [8]. Nevertheless, few studies have shown that dietary *Schizochytrium* sp. significantly improves the non-specific immunity [17], and modulates the intestinal microbiota of both the Nile tilapia (*Oreochromis niloticus*) [18] and the rainbow trout (*Oncorhynchus mykiss*) [19]. Therefore, there is a need for a more comprehensive and more in-depth understanding of the *Schizochytrium* sp. supplementation's internal effects on host health.

Recently, technologies such as high-throughput sequencing and metabolomics have considered being a rapid expansion in research related to fish immunity [20]. It is well known that the intestinal microbiota plays essential roles in nutrient distribution, regulation of innate immunity, and maintenance of intestinal tissue integrity, which can be modulated by diets [21]. Because of the central role played by metabolism in immunity, metabolomics is rapidly established as a critical analytical tool in immunity studies [22]. However, few studies have been conducted in an integrated manner to evaluate the influence of dietary microalgae on metabolism and microbiota in fish.

In the present study, zebrafish was selected as the model organism to evaluate the effect of *Schizochytrium* sp. in the diet on the metabolic profile and intestinal microbiota. A 56-day feeding trial using a diet enriched with *Schizochytrium* sp. was administered daily to zebrafish. Metabolomic profiling of the humoral fluid and the intestinal microbial community structure were examined by gas chromatograph-mass spectrometer (GC-MS) analysis and 16S rDNA gene sequencing technology, respectively. Additionally, zebrafish were challenged with *Edwardsiella piscicida* (*E. piscicida*) to verify the disease resistance of *Schizochytrium* sp. enriched diets. Our results provide a novel insight into developing the fundamental understanding of metabolic responses and disease resistance in zebrafish fed with microalgae.

Materials And Methods

Microalgal strains and culture conditions

Schizochytrium sp. ATCC MYA 1381 was maintained in our laboratory. The fermentation process was consistent with our previous study[11]. In brief, the seed culture (10% v/v) was transferred to a 3 L fermenter and incubated at 28 °C for 10 days. The biomass was harvested by centrifugation (12,000 × *g*, 10 min), and then dried in a vacuum freeze dryer. The resulting cells were stored at -20 °C until further use.

Diet preparation

Three iso-nitrogenous, iso-energetic, and iso-lipidic experimental diets were prepared according to Sarker *et al.*, [23] with different *Schizochytrium* sp. content: 0 g/kg (C), 60 g/kg (S1), and 120 g/kg (S2), respectively. Details of the feed formula are provided in Supplemental Table 1. We used the whole dried cells of *Schizochytrium* sp. instead of fish oil in the S1 and S2 groups. The diet preparation followed the methods by Duan *et al.* [20]. Crude protein, lipid, and ash of the trial diets were measured by the guidelines of the Association of Official Analytical Chemists.

Zebrafish treatment and sample collection

Healthy juvenile zebrafish (body weights of approximately 0.59 g) were purchased from Guangzhou Flower Bird Fish and Bug market (Guangzhou, China). The zebrafish were acclimatized to the trial conditions for 14 days before conducting the feeding trial. The water quality parameters were as follows: a temperature of 25 ± 0.5 °C, pH 7.8, and 6.0 ± 0.5 mg/L of dissolved oxygen. The zebrafish were fed 4% of their body weight daily. Uneaten food and feces were removed from the tanks, and one-third of the culture water was renewed with fresh water once a day.

After acclimation, fish were split into three groups with replicate tanks (48 L) containing 50 fish per tank and fed with the corresponding experimental feed. The culture conditions were identical to those of the acclimation stage. The fish were fed twice a day, namely at 08:00 and 17:00, for 56 days. On day 56, eight fish from each group were sampled for GC-MS analysis. Intestines from three individuals of each group were pooled together as one sample to reduce individual variation. Three replicates were performed for the microbiome analysis.

Challenge experiment

Eighty fish from each group were challenged with *E. piscicida* isolated from diseased Nile tilapia in southeastern China. These fish were infected by an intraperitoneal injection of *E. piscicida* at 6×10^3 CFU/fish. The concentration of bacteria selected was identified through previous experiments that the 96-h median lethal concentration (96 h LC₅₀) of adult zebrafish exposure to *E. piscicida* is 8×10^3 CFU/fish. The symptoms were observed twice daily for 144 h, and the cumulative deaths were collated. As a negative control, 20 fish from each group were challenged with a saline solution and maintained in aquarium water. This experiment involved the same consistent feeding strategy as the other experiments. Survival curves were estimated with Kaplan-Meier's analysis, using SPSS software (SPSS v. 20.0: SPSS, Chicago, IL, USA). The experimental protocols were permitted by the Institutional Animal Care and Use Committee of Xiamen University, Xiamen, China.

Histological analysis

The foreguts from three fish per tank were sampled on the 56th day and fixed for 24 h in 4% paraformaldehyde solution. They were then dehydrated, equilibrated, and embedded in paraffin wax, following standard histological techniques [24]. The tissue was sectioned to a 4 μm thickness with a microtome (Leica, RM2016, Wetzlar, Germany). After being stained with hematoxylin and eosin (HE), the sections were observed and photographed under a light microscope (Leica DM500, Wetzlar, Germany).

Sample preparation for GC-MS

Zebrafish were cut on ice into pieces and weighed. Pieces from each individual were placed in tubes with 1 mL/g sterile saline, then mixed by vortexing, and centrifuged for 10 min at 4 $^{\circ}\text{C}$ to collect humoral fluid. The GC-MS sample preparation was performed as described in Jiang *et al.*, [25] with moderate modifications. Briefly, 200 μL ice-cold methanol was added to 100 μL humoral fluid to quench the metabolites, and then added 3 μL internal standard (100 $\mu\text{g}/\text{mL}$ ribitol) (Sigma, St. Louis, USA). After centrifugation (12,000 $\times g$, 15 min), the transferred supernatant was dried in a vacuum freeze dryer for 24 h. The resulting pellets were applied to further analysis.

GC-MS analysis

Derivatization was performed according to previously described [26]. First, the dried pellet was dissolved in 80 μL of 20 mg/mL methoxyamine hydrochloride (Sigma, St. Louis, USA) containing pyridine and incubated for 1.5 h at 37 $^{\circ}\text{C}$. Subsequently, 80 μL N-methyl-N-trimethyl-silyl-trifluoroacetamide (MSTFA, Sigma, St. Louis, USA) was added and incubated for 0.5 h at 37 $^{\circ}\text{C}$. The samples were then centrifuged at 12,000 $\times g$ for 10 min at 4 $^{\circ}\text{C}$. Chemical analysis of the samples was performed using the Agilent 7890-5975C GC-MS solution system (Agilent, Sacramento, California, USA). The injector temperature was held at 270 $^{\circ}\text{C}$. Using the splitless model, 0.5 μL of the derivatized sample was injected into a Dodecyl Benzene Sulfonate (DBS) column of 30 m in length, 250 μm in inner diameter, and 0.25 μm in thickness. The temperature program of the GC oven is 85 $^{\circ}\text{C}$ for 5 min. The temperature is raised to 285 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}/\text{min}$, and finally to 310 $^{\circ}\text{C}$ at a rate of 20 $^{\circ}\text{C}/\text{min}$. Helium was used as carrier gas with a flow rate of 1 mL/min. The scanning mass range was set at 50–600 m/z , and the ionization energy of the electron impact ionization (EI) was 70 eV.

GC-MS data processing and statistical analysis

The original GC-MS data were conducted as previous reports [27]. Each peak was identified by alignment with the mass spectra from the NIST 2.2 (National Institute of Standards and Technology, USA) library. The data of the identified metabolites were normalized and imported into SIMCA software (version 14.1; Umetrics, Umeå, Sweden) for principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Differential metabolites were identified with variable importance in the projection (VIP) > 1.0 obtained from OPLS-DA and p -values < 0.05 obtained from two-tailed Student's t -tests. The pathway enrichment analysis of differential metabolites was conducted with MetaboAnalyst 4.0 [28].

Intestinal microbiome analysis

Intestinal microbial DNA was extracted and purified by HiPure Stool DNA Kit (Magen, Guangzhou, China), followed by the manufacturer's protocols. For microbial analysis, the primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT) were used to amplify the 16S rDNA V3-V4 region. The amplicons were purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA).

Purified products were pooled in equimolar amounts and paired-end sequenced (PE250) on an Illumina MiSeq platform. UPARSE (version 9.2.64) was used to truncate the clustering of operational taxa units (OTUs) with 97% similarity, and the UCHIME algorithm was used to identify and delete abnormal gene sequences. The representative sequence was classified into organisms for each OTU according to the SILVA database (version 132), using the RDP classifier (version 2.2) with 90% confidence. The Chao and Shannon indices were determined using QIIME (version 1.9.1), following previous research protocols[29]. Non-metric multidimensional scaling (NMDS) was performed based on the unweighted UniFrac distances. The most differentially abundant taxon between the groups was identified by the linear discriminant analysis (LDA) of effect size (LEfSe) method.

Correlation analysis between bacterial taxonomy and differential metabolites

Spearman's correlation analysis assessed the relationship between differential metabolites and bacterial taxonomy (at the genus level) with the psych package in R. *p* values was adjusted with a false discovery rate (FDR). Any adjusted *p*-value of less than 0.05 was statistically significant.

Results

Effect of *Schizochytrium* sp. supplementation on fish response to *E. piscicida* infection

To investigate whether *Schizochytrium* sp. affected the host's susceptibility to bacterial infection, zebrafish were infected with *E. piscicida* (6×10^3 CFU/fish). After being infected with *E. piscicida*, the cumulative survival rates of zebrafish were 87.5% and 70% when fed with 120 g/kg and 60 g/kg *Schizochytrium* sp., respectively. These results are higher than the survival rate of fish without *Schizochytrium* sp. supplementation (62.5%; Fig. 1). No deaths were recorded in fish from the negative control group that was injected with saline solution. These results indicate that *Schizochytrium* sp. diets could protect zebrafish from *E. piscicida* infection.

Intestinal histological structure in zebrafish fed with *Schizochytrium* sp. diets

The intestinal morphology of zebrafish fed with *Schizochytrium* sp. diets is presented in Fig. 2. Our results demonstrated that intestinal morphology was changed by the supplement of *Schizochytrium* sp.

The density of goblet cells increased in zebrafish fed with *Schizochytrium* sp. as a diet supplement when compared with the control group.

Effects of *Schizochytrium* sp. supplementation on metabolite profiles in zebrafish

There was a clear separation between the three groups (the zebrafish fed two different doses of the supplement and one control without *Schizochytrium* sp.) in the PCA score plot (Fig. 3A). Further models of OPLS-DA between zebrafish fed with or without *Schizochytrium* sp. were conducted (Fig. 3B-D). Three permutation tests indicated that all OPLS-DA models contained high discrimination and predictive capability (Supplemental Figure S1).

We used the criteria of $VIP > 1$ and $p < 0.05$ for multivariate and univariate statistical analyses, respectively, to identify 17 differential metabolites between the zebrafish fed with 0 and 120 g/kg *Schizochytrium* sp. Similarly, 12 differential metabolites between the zebrafish fed with 0 and 60 g/kg *Schizochytrium* sp. Moreover, six differential metabolites existed between the zebrafish fed with 60 and 120 g/kg *Schizochytrium* sp. All the differentially abundant metabolites in any two groups are visually displayed in a heatmap plot (Fig. 3E). A cross-comparison Venn diagram illustrated the variation among the three collections of differential metabolites to further characterize the metabolites involved in immune resistance. All the differential metabolites formed 7 clusters (Supplemental Fig. 2). Detailed descriptions of these metabolites are included in Supplemental Table 2.

The pathway analysis revealed eight primary disturbed pathways of humoral fluid in zebrafish, including (1) taurine and hypotaurine metabolism; (2) starch and sucrose metabolism; (3) glycine, serine, and threonine metabolism; (4) primary bile acid biosynthesis; (5) pentose phosphate pathway; (6) aminoacyl-tRNA biosynthesis; (7) glyoxylate and dicarboxylate metabolism; and (8) galactose metabolism (Fig. 4).

Effects of *Schizochytrium* sp. supplementation on the intestinal microbiota of zebrafish

The nine most dominant phyla were Proteobacteria, Fusobacteria, Actinobacteria, Planctomycetes, Firmicutes, Bacteroidetes, Tenericutes, Verrucomicrobia, and Chloroflexi (Fig. 5A). Bacteroidetes were less abundant in zebrafish fed with 120 g/kg *Schizochytrium* sp. supplemented diet than the control group (Fig. 5B). The NMDS showed that both the *Schizochytrium* sp. supplemented diets were separated from the control group with nm1s1 (Fig. 5C). The intestinal microbial compositions of zebrafish fed with *Schizochytrium* sp. were completely different from that fed without *Schizochytrium* sp. However, the Chao index and Shannon index indicated no statistically significant difference among the three groups (Supplemental Fig. 3).

Based on the results of LEfSe, *Butyrivibrio*, *Dorea*, *Lactobacillus*, *Pseudoxanthomonas*, *Chthoniobacter*, *Rubellimicrobium*, *Alsobacter*, *Mesorhizobium*, *Neochlamydia*, *Methyloceanibacter*, *Rhodopirellula*, *Bradymonas*, *Candidatus Protochlamydia*, *Roseomonas*, *Ensifer*, *Pseudoxanthomonas*, *Actinomycetospira*, *Schlesneria*, *Candidatus Paracaedibacter*, *Paludisphaera*, and *Mycobacterium* were the dominant genera in zebrafish fed with 120 g/kg *Schizochytrium* sp. *Pseudomonas*, *Flavobacterium*,

Chitinilyticum, *Citrobacter*, *Mycoplasma*, *Fluviicola*, *Coriobacteriaceae* UCG-002, *Sediminibacterium*, *RB41*, *Novosphingobium*, *Zoogloea*, *Delftia*, *Acidovorax*, *Stella*, *Alistipes*, *Shewanella*, and *Crenobacter* were the dominant genera in the control group which without *Schizochytrium* sp. supplemented.

Correlations between differential metabolites and bacterial genera

Spearman's correlation analysis was performed to investigate the relationship between the differential metabolites and the 37 bacterial genera that were statistically changed in the group fed with 120 g/kg *Schizochytrium* sp. when compared to the group fed without *Schizochytrium* sp. The genus *Sediminibacterium* was negatively correlated with metabolites such as taurine, L-serine, citric acid, creatinine, D-glucose, D-galactose, phosphorylethanolamine, and myo-inositol (Fig. 6). *Flavobacterium* and *Shewanella* were negatively correlated with taurine and pantothenic acid, while the former was also negatively associated with L-serine and citric acid. In contrast, *Butyricicoccus*, *Dorea*, *Bradymonas*, and *Actinomycetospora*, were positively correlated with five metabolites: taurine, creatinine, D-glucose, D-galactose, and pantothenic acid. In addition, *Dorea*, *Bradymonas*, and *Actinomycetospora* also showed a positive correlation with L-serine and citric acid.

Discussion

In recent decades, different microalgae species have been tested for use in aquaculture applications [8, 9]. Microalgae contain many bioactive components, such as omega-3 fatty acids, β -glucans, and flavonoids. These nutrients may modulate fish physiology, thereby promoting the general well-being and better overall health of fish [30]. In line with previous reports, the dietary consumption of the microalga *Schizochytrium* sp. dramatically improved the survival ability of zebrafish after the fish were challenged with *E. piscicida*.

Furthermore, the density of goblet cells was increased in the treatment groups, which is similar to the observations in golden pompano (*Trachinotus ovatus*) fed with a *Schizochytrium* sp. diet [17]. Goblet cells can synthesize and secrete mucin glycoproteins, which can cover the surface of the gastrointestinal tract epithelium, protecting the intestine from infection [31]. The elevated goblet cell density observed in our study suggests that dietary supplementation with *Schizochytrium* sp. improves intestinal homeostasis and assists the zebrafish in resisting the pathogen.

Treating fish with varying doses of *Schizochytrium* sp. led to different metabolism responses and microbial composition. Both diets with *Schizochytrium* sp. markedly increased phosphorylethanolamine concentrations, creatinine, myo-inositol, glucose, galactose, and decreased benzoic acid. Moreover, the 120 g/kg *Schizochytrium* sp. diet dramatically reduced the abundance of Bacteroidetes when compared to the control diet. These findings indicated a conspicuous influence of *Schizochytrium* sp. on the metabolic profiles and intestinal microbial community in zebrafish.

Diets with *Schizochytrium* sp. alter the colonic metabolite profiles of zebrafish

PCA and OPLS-DA analyses revealed a clear separation of metabolites in the humoral fluid of fish fed different diets, suggesting differences in the metabolic profiles. The carbohydrates, such as D-glucose, D-galactose, maltose, and myo-inositol, were found to increase in zebrafish fed with 120 g/kg *Schizochytrium* sp. These carbohydrates are mainly involved in starch and sucrose metabolism, galactose metabolism, and the pentose phosphate pathway. The pentose phosphate pathway and galactose metabolism are related to energy metabolism. The starch and sucrose metabolism can influence the tricarboxylic acid cycle (TCA cycle) through aerobic glycolysis [32]. A previous study also demonstrated that boosting the TCA cycle and energy supply enhanced the survival rate of zebrafish infected with *Vibrio alginolyticus* [33]. These elevated concentrations of carbohydrates indicate that diets with *Schizochytrium* sp. could increase the disease resistance of zebrafish by enhancing the TCA cycle and energy supply. Notably, citrate, the most important intermediate of the TCA cycle, was also increased in the zebrafish fed with 120 g/kg *Schizochytrium* sp. compared with zebrafish fed the control diet. Higher citrate levels also indicate an intensification of the TCA cycle and an enhanced energy supply.

The diet with 120 g/kg *Schizochytrium* sp. also increased the concentration of taurine. Taurine has many essential biological functions, including the stabilization of cell membranes and antioxidation [34]. Increased taurine has been reported to protect organisms against oxidant effects [35]. Leukocytes contain high concentrations of taurine, which can increase respiratory bursts and reduce tissue damage [36]. Therefore, the increase in taurine levels observed in this study suggests that a *Schizochytrium* sp. diet might enhance the innate immunity against bacterial infections.

Additionally, zebrafish fed with 120 g/kg *Schizochytrium* sp. significantly increased their concentrations of amino acid relatives (such as L-serine) compared with zebrafish fed without *Schizochytrium* sp. supplements. It has been demonstrated that L-serine could modulate the metabolome of tilapias to improve their innate immunity and eliminate pathogens, thereby improving the survival rate in tilapias infected with *Streptococcus iniae* [37]. Thus, increasing L-serine concentration in zebrafish via a diet with 120 g/kg *Schizochytrium* sp. might have a beneficial effect on pathogen resistance.

A previous study reported that an n-3 highly unsaturated fatty acid supplementation could reduce the cholesterol content in *Ctenopharyngodon idella* [38]. Similarly, in our study, zebrafish fed with the DHA-rich marine microalga *Schizochytrium* sp. supplemented diets contained less cholesterol than zebrafish fed a control diet. The cholesterol content may affect immune cell function, whereas reducing cholesterol in T cells may enhance CD8⁺ T cell immune activity [39]. In our study, the DHA-rich marine microalga *Schizochytrium* sp. might have improved the immune capacity of zebrafish by reducing their cholesterol content.

Diets containing *Schizochytrium* sp. alter the intestinal microbiota structure in zebrafish

Schizochytrium sp. added to the diet resulted in differences in the bacterial community structure in zebrafish intestines. In concordance with the previous reports on zebrafish [40], the phyla Proteobacteria,

Fusobacteria, Actinobacteria, Planctomycetes, and Firmicutes were dominant regardless of diet. Additionally, a significant decrease in Bacteroidetes was detected in the zebrafish fed with 120 g/kg *Schizochytrium* sp. *Schizochytrium* sp. is rich in omega-3 LC-PUFAs, especially DHA, and a recent study recorded a significant increase in the fatty acid content in zebrafish fed *Schizochytrium* sp. diets [8]. Therefore, the reduced abundance of Bacteroidetes in zebrafish fed with 120 g/kg *Schizochytrium* sp. supplemented diet implies that the abundance of Bacteroidetes in zebrafish is related to the dietary fat levels.

Some beneficial bacterial genera, including *Lactobacillus*, *Dorea*, *Butyricicoccus*, and *Pseudoxanthomonas*, were enriched in the zebrafish fed with 120 g/kg *Schizochytrium* sp. supplemented diet. *Lactobacillus* could be used as an immunostimulant and growth promotor. For instance, it enhanced the immune response and disease resistance in tilapia [41]. Furthermore, a higher abundance of *Dorea* was associated with greater resistance in human feces to pathogenic bacterial infections [42], while *Butyricicoccus* was shown to decrease lesion sizes and inflammation in a rat colitis model [43]. Finally, *Pseudoxanthomonas* could influence the host immune response through a microbiome signature in tumors [44].

Interestingly, the relative abundances of some harmful bacterial genera (including *Flavobacterium*, *Shewanella*, *Pseudomonas*, *Citrobacter*, and *Mycoplasmas*) were depleted after the zebrafish were fed with 120 g/kg *Schizochytrium* sp. The presence of *Flavobacterium* may explain the changes in the immunometabolism in fish and affects the nutrient metabolism, immune response, and related signaling pathways in fish. Additionally, several species are known to cause diseases in freshwater fish [45]. *Shewanella*, *Pseudomonas*, and *Citrobacter* are pathogens or opportunistic pathogens of fish [46–48]. Most *Mycoplasma* species are specific commensals or parasites of eukaryotes. For instance, *Mycoplasma mobile* is a parasitic bacterium that binds to the gills of freshwater fish and causes necrosis [49]. This study demonstrates that dietary *Schizochytrium* sp. could optimize the intestinal microbial composition in zebrafish, enhance their immunity, and decrease the host's pathogen invasion.

To understand the composition and function of microbial communities, we undertook a Spearman's correlation analysis of the associations between metabolite features with microorganisms [50]. Our results indicated a close association between the circulating metabolites and the altered microbiome. It was observed that two beneficial bacterial genera in the Firmicutes phylum (namely *Dorea* and *Butyricicoccus*) correlated with some functional metabolites (i.e., taurine, D-glucose, and D-galactose). The genus *Dorea* is associated with glucose metabolism and plays a role in the activity of the immune system [51]. Several species of the genus *Butyricicoccus* are butyrate producers, and butyrate has several beneficial properties that are essential for maintaining intestinal homeostasis and resistance to specific enteric pathogens [52]. A previous study has also shown that some species of *Butyricicoccus* are associated with mucosa, and their presence is reduced in patients with ulcerative colitis. As mentioned previously, taurine is involved in stabilizing cell membranes and immune capacity of the body. Therefore, we speculate that the observed improvement in the immune capacity of zebrafish was related to the

changes in the abundance of *Dorea* and *Butyricicoccus*. Therefore, the potential probiotic species with two genera are worth investigating further.

Conclusions

The present study showed that *Schizochytrium* sp. supplementation enhanced the disease resistance in zebrafish. The mechanism involved in the improved immunology were explained via metabolomics and microbiome analysis. The altered metabolic pathways mainly involved the TCA cycle as well as the energy, taurine, L-serine, and cholesterol metabolisms. The abundance of beneficial bacteria, such as *Lactobacillus*, *Dorea*, *Butyricicoccus*, and *Pseudoxanthomonas* increased, whereas harmful pathogens (*Flavobacterium*, *Pseudomonas*, *Citrobacter*, and *Mycoplasma*) decreased in zebrafish fed with 120 g/kg *Schizochytrium* sp. Spearman's correlation analysis showed that some species of the genera *Dorea* and *Butyricicoccus* might be probiotics. Furthermore, the density of goblet cells in the intestine increased significantly in the zebrafish fed with *Schizochytrium* sp. when compared to the control group. Therefore, we conclude that *Schizochytrium* sp. supplementation has a positive effect on the immunity of zebrafish by improving their metabolic process and intestinal health.

Abbreviations

LC-PUFA: Long-chain polyunsaturated fatty acids; EPA:Eicosapentaenoic acid; DHA:Docosahexaenoic acid; *E. piscicida*:*Edwardsiella piscicida*; GC-MS:Gas chromatograph-mass spectrometer; LC₅₀:Median lethal concentration; HE:Hematoxylin and eosin; MSTFA:N-methyl-N-trimethyl-silyl-trifluoroacetamide; DBS:Dodecyl Benzene Sulfonate; EI:Electron impact ionization; PCA:Principal component analysis; OPLS-DA:Orthogonal partial least-squares discriminant analysis; VIP:Variable importance in the projection; OTUs:Operational taxa units; NMDS:Non-metric multidimensional scaling; LEfSe:The linear discriminant analysis effect size; TCA cycle:tricarboxylic acid cycle.

Declarations

Ethics approval

The experimental protocols were permitted by the Institutional Animal Care and Use Committee of Xiamen University, Xiamen, China.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was financially supported by the National Natural Science Foundation of China (31871779, 21676221).

Authors' contributions

YS conducted the animal work and most of the laboratory work and wrote the manuscript. XC and YZ helped to conduct the animal trial and part of the

laboratory work and helped to revise the manuscript. YX and YW helped to revise the manuscript. NY oversaw the development of the study and reviewed the last version of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We gratefully acknowledge the bioinformatic analysis of Gene Denovo Biotechnology Co., Ltd (Guangzhou, China).

References

1. Ye Y, Barange M, Beveridge M, Garibaldi L, Gutierrez N, Anganuzzi A, Taconet M. FAO's statistic data and sustainability of fisheries and aquaculture: Comments on Pauly and Zeller (2017). *Mar Pol.* 2017;81:401-5.
2. Sarker P, Gamble M, Kelson S, Kapuscinski A. Nile tilapia (*Oreochromis niloticus*) show high digestibility of lipid and fatty acids from marine *Schizochytrium* sp. and of protein and essential amino acids from freshwater *Spirulina* sp. feed ingredients. *Aquac Nutr.* 2016;22:109–19.
3. Gedi M, Magee K, Darwish R, Eakpetch P, Young I, Gray D. Impact of the partial replacement of fish meal with a chloroplast rich fraction on the growth and selected nutrient profile of zebrafish (*Danio rerio*). *Food Funct.* 2019;10:733–45.
4. Pontecorvo G, Schrank W. The expansion, limit and decline of the global marine fish catch. *Mar Pol.* 2012;36:1178–81.
5. Tacon A, Metian M. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture.* 2008;285:146–58.
6. Badvipour S, Eustance E, Sommerfeld M. Process evaluation of energy requirements for feed production using dairy wastewater for algal cultivation: Theoretical approach. *Algal Res.* 2016;19:207–14.
7. Gbadamosi O, Lupatsch I. Effects of dietary Nannochloropsis salina on the nutritional performance and fatty acid profile of Nile tilapia, *Oreochromis niloticus*. *Algal Res.* 2018;33:48–54.
8. Byreddy AR, Yoganantharjah P, Gupta A, Gibert Y, Puri M. Suitability of Novel Algal Biomass as Fish Feed: Accumulation and Distribution of Omega-3 Long-Chain Polyunsaturated Fatty Acid in

- Zebrafish. Appl Biochem Biotechnol. 2018;188:112–23.
9. Sheikhzadeh N, Mousavi S, Hamidian G, Firouzmandi M, Khani Oushani A, Mardani K. Role of dietary *Spirulina platensis* in improving mucosal immune responses and disease resistance of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 2019;510:1–8.
 10. Khan MI, Shin JH, Kim JD. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microb Cell Fact. 2018;17:36.
 11. Li Z, Meng T, Ling X, Li J, Zheng C, Shi Y, Chen Z, Li Z, Li Q, Lu Y, He N. Overexpression of malonyl-CoA: ACP transacylase in *Schizochytrium* sp. to improve polyunsaturated fatty acid production. J Agr Food Chem. 2018;66:5382–91.
 12. Miller MR, Nichols PD, Carter CG. Replacement of fish oil with thraustochytrid *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. Comp Biochem Phys A. 2007;148:382–92.
 13. Sprague M, Walton J, Campbell PJ, Strachan F, Dick JR, Bell JG. Replacement of fish oil with a DHA-rich algal meal derived from *Schizochytrium* sp. on the fatty acid and persistent organic pollutant levels in diets and flesh of Atlantic salmon (*Salmo salar*, L.) post-smolts. Food Chem. 2015;185:413–21.
 14. Wang Y, Li M, Filer K, Xue Y, Ai Q, Mai K. Replacement of fish oil with a DHA-rich *Schizochytrium* meal on growth performance, activities of digestive enzyme and fatty acid profile of Pacific white shrimp (*Litopenaeus vannamei*) larvae. Aquac Nutr. 2017;23:1113–20.
 15. Li MH, Robinson EH, Tucker CS, Manning BB, Khoo L. Effects of dried algae *Schizochytrium* sp., a rich source of docosahexaenoic acid, on growth, fatty acid composition, and sensory quality of channel catfish *Ictalurus punctatus*. Aquaculture. 2009;292:232–6.
 16. Ganuza E, Benítez-Santana T, Atalah E, Vega-Orellana O, Ganga R, Izquierdo MS. Crypthecodinium cohnii and *Schizochytrium* sp. as potential substitutes to fisheries-derived oils from seabream (*Sparus aurata*) microdiets. Aquaculture. 2008;277:109–16.
 17. Xie J, Fang H, Liao S, Guo T, Yin P, Liu Y, Tian L, Niu J. Study on *Schizochytrium* sp. improving the growth performance and non-specific immunity of golden pompano (*Trachinotus ovatus*) while not affecting the antioxidant capacity. Fish Shellfish Immunol. 2019;95:617–23.
 18. Souza FP, Lima ECS, Urrea-Rojas AM, Suphoronski SA, Facimoto CT, Bezerra Junior JDS, Oliveira TES, Pereira UP, Santis GWD, Oliveira CAL, Lopera-Barrero NM. Effects of dietary supplementation with a microalga (*Schizochytrium* sp.) on the hemato-immunological, and intestinal histological parameters and gut microbiota of Nile tilapia in net cages. PLoS One. 2020;15:e0226977.
 19. Lyons PP, Turnbull JF, Dawson KA, Crumlish M. Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquac Res. 2017;48:2438–52.
 20. Duan Y, Wang Y, Liu Q, Dong H, Li H, Xiong D, Zhang J. Changes in the intestine microbial, digestion and immunity of *Litopenaeus vannamei* in response to dietary resistant starch. Sci Rep. 2019;9:6464.

21. Eichmiller JJ, Hamilton MJ, Staley C, Sadowsky MJ, Sorensen PW. Environment shapes the fecal microbiome of invasive carp species. *Microbiome*. 2016;4:44.
22. Everts B. Metabolomics in Immunology Research. *Methods Mol Biol*. 2018;1730:29–42.
23. Sarker PK, Kapuscinski AR, Lanois AJ, Livesey ED, Bernhard KP, Coley ML. Towards sustainable aquafeeds: complete substitution of fish oil with marine microalga *Schizochytrium* sp. improves growth and fatty acid deposition in juvenile Nile Tilapia (*Oreochromis niloticus*). *PLoS One*. 2016;11:e0156684.
24. Qiao R, Sheng C, Lu Y, Zhang Y, Ren H, Lemos B. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Sci Total Environ*. 2019;662:246–53.
25. Jiang M, Gong Q, Lai S, Cheng Z, Chen Z, Zheng J, Peng B. Phenylalanine enhances innate immune response to clear ceftazidime-resistant *Vibrio alginolyticus* in *Danio rerio*. *Fish Shellfish Immunol*. 2019;84:912–9.
26. Zeng ZH, Du CC, Liu SR, Li H, Peng XX, Peng B. Glucose enhances tilapia against *Edwardsiella tarda* infection through metabolome reprogramming. *Fish Shellfish Immunol*. 2017;61:34–43.
27. Li Z, Ling X, Zhou H, Meng T, Zeng J, Hang W, Shi Y, He N. Screening chemical modulators of benzoic acid derivatives to improve lipid accumulation in *Schizochytrium limacinum* SR21 with metabolomics analysis. *Biotechnol Biofuels*. 2019;12:209.
28. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, Wishart DS, Xia J. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res*. 2018;46::W486-W94.
29. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*. 2009;75:7537–41.
30. Bravo-Tello K, Ehrenfeld N, Solis CJ, Ulloa PE, Hedrera M, Pizarro-Guajardo M, Paredes-Sabja D, Feijoo CG. Effect of microalgae on intestinal inflammation triggered by soybean meal and bacterial infection in zebrafish. *PLoS One*. 2017;12:e0187696.
31. Gaskins HR. Microbial modulation of innate defense: Goblet cells and the intestinal mucus layer. *Am J Clin Nutr*. 2001;73::1131S-41S.
32. Huang D, Zou Y, Abbas A, Dai B. Nuclear magnetic resonance-based metabolomic investigation reveals metabolic perturbations in PM2.5-treated A549 cells. *Environ Sci Pollut Res Int*. 2018;25:31656–65.
33. Yang MJ, Cheng ZX, Jiang M, Zeng ZH, Peng B, Peng XX, Li H. Boosted TCA cycle enhances survival of zebrafish to *Vibrio alginolyticus* infection. *Virulence*. 2018;9:634–44.
34. Lu J, Feng J, Cai S, Chen Z. Metabolomic responses of *Haliotis diversicolor* to organotin compounds. *Chemosphere*. 2017;168:860–9.
35. Lu J, Shi YY, Cai SH, Feng JH. Metabolic responses of *Haliotis diversicolor* to *Vibrio parahaemolyticus* infection. *Fish Shellfish Immunol*. 2017;60:265–74.

36. Lin Y, Shiao S. Dietary lipid requirement of grouper, *Epinephelus malabaricus*, and effects on immune responses. *Aquaculture*. 2003;225:243–50.
37. Du CC, Yang MJ, Li MY, Yang J, Peng B, Li H, Peng XX. Metabolic mechanism for L-Leucine-induced metabolome to eliminate *Streptococcus iniae*. *J Proteome Res*. 2017;16:1880–9.
38. Montenegro LF, Descalzo AM, Cunzolo SA, Perez CD. Modification of the content of n-3 highly unsaturated fatty acid, chemical composition, and lipid nutritional indices in the meat of grass carp (*Ctenopharyngodon idella*) fed alfalfa (*Medicago sativa*) pellets. *J Anim Sci*. 2020;98::skaa084.
39. Ma X, Bi E, Lu Y, Su P, Huang C, Liu L, Wang Q, Yang M, Kalady MF, Qian J. Cholesterol induces CD8⁺ T cell exhaustion in the tumor microenvironment. *Cell Metab*. 2019;30:143 – 56. e5.
40. Navarro-Barron E, Hernandez C, Llera-Herrera R, Garcia-Gasca A, Gomez-Gil B. Overfeeding a High-Fat Diet Promotes Sex-Specific Alterations on the Gut Microbiota of the Zebrafish (*Danio rerio*). *Zebrafish*. 2019;16:268–79.
41. Van Nguyen N, Onoda S, Van Khanh T, Hai PD, Trung NT, Hoang L, Koshio S. Evaluation of dietary heat-killed *Lactobacillus plantarum* strain L-137 supplementation on growth performance, immunity and stress resistance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*. 2019;498:371–9.
42. Kampmann C, Dicksved J, Engstrand L, Rautelin H. Composition of human faecal microbiota in resistance to *Campylobacter* infection. *Clin Microbiol Infect*. 2016;22:61–8.
43. Eeckhaut V, Machiels K, Perrier C, Romero C, Maes S, Flahou B, Steppe M, Haesebrouck F, Sas B, Ducatelle R, et al. *Butyricicoccus pullicaecorum* in inflammatory bowel disease. *Gut*. 2013;62:1745–52.
44. Riquelme E, Zhang Y, Zhang L, Montiel M, Zoltan M, Dong W, Quesada P, Sahin I, Chandra V, San Lucas A. Tumor microbiome diversity and composition influence pancreatic cancer outcomes. *Cell*. 2019;178:795–806.
45. Bernardet J-F, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P. Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatidis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Evol Microbiol*. 1996;46:128 – 48.
46. Kačániová M, Klūga A, Kántor A, Medo J, Žiarovská J, Puchalski C, Terentjeva M. Comparison of MALDI-TOF MS Biotyper and 16S rDNA sequencing for the identification of *Pseudomonas* species isolated from fish. *Microb Pathog*. 2019;132:313–8.
47. Paździor E. *Shewanella putrefaciens* - A new opportunistic pathogen of freshwater fish. *J Vet Res*. 2016;60:429–34.
48. Sood N, Pradhan PK, Ravindra, Verma DK, Yadav MK, Mishra RK, Kumar U, Swaminathan TR, Sood NK. Large-scale mortality in cultured tilapia *Oreochromis niloticus* due to infection with *Shewanella putrefaciens* in India. *J World Aquacult Soc*. 2020;51:563–70.
49. Austin B, Austin DA. Bacterial fish pathogens: Disease of farmed and wild fish. In: editors. Cham: Springer International Publishing; 2016. pp. 603–42.

50. Meng Z, Liu L, Jia M, Li R, Yan S, Tian S, Sun W, Zhou Z, Zhu W. Impacts of penconazole and its enantiomers exposure on gut microbiota and metabolic profiles in mice. *J Agr Food Chem.* 2019;67:8303–11.
51. Fan Y, Zhang J. Dietary modulation of intestinal microbiota: Future opportunities in experimental autoimmune encephalomyelitis and multiple sclerosis. *Front Microbiol.* 2019;10:740.
52. Trachsel J, Humphrey S, Allen HK. *Butyricicoccus porcorum* sp. nov., a butyrate-producing bacterium from swine intestinal tract. *Int J Syst Evol Microbiol.* 2018;68:1737–42.
53. Devriese S, Eeckhaut V, Geirnaert A, Van den Bossche L, Hindryckx P, Van de Wiele T, Van Immerseel F, Ducatelle R, De Vos M, Laukens D. Reduced mucosa-associated *Butyricicoccus* activity in patients with ulcerative colitis correlates with aberrant claudin-1 expression. *J Crohns Colitis.* 2017;11:229–36.

Supplemental Information

Additional file 1

Supplemental Table 1 Formulation (g/100 g diet) and nutrient composition (%) of control and experimental diets. **Supplemental Fig. 1** Permutation test for the OPLS-DA model. (A) C vs. S1; (B) C vs. S2; (C) S1 vs. S2; R², the explained variance of the model; Q², the predictive ability of the model. C, S1, and S2 represent zebrafish fed with 0, 60, and 120 g/kg *Schizochytrium* sp., respectively. **Supplemental Fig. 2** Venn diagrams showing the number of altered metabolites shared between C and S1 fish (red), C and S2 fish (blue), and S1 and S2 fish (yellow). C, S1, and S2 represent zebrafish fed with 0, 60, and 120 g/kg *Schizochytrium* sp., respectively. **Supplemental Table 2** Differential metabolites among groups. **Supplemental Fig. 3** Alpha diversity of the intestine microbiota in three groups. (A) The Shannon index of OUT level. (B) The Chao index of OUT level.

Figures

Figure 1

Kaplan-Meier survival curves for zebrafish fed with or without *Schizochytrium* sp. diets for 56 days and then challenged with *E. piscicida*. * indicates $p < 0.05$. C, S1, and S2 represent zebrafish fed with 0, 60, and 120 g/kg *Schizochytrium* sp., respectively.

Figure 2

Intestine morphology of zebrafish fed with and without supplementation of Schizochytrium sp. for 56 days. (A) Histological structure of intestine tissues. (B) Changes in the density of goblet cells per group. Black arrowheads indicate goblet cells. C, S1, and S2 represent zebrafish fed with 0, 60, and 120g/kg Schizochytrium sp., respectively. The different lowercase letters indicate a significant difference ($p < 0.05$) between different groups.

Figure 3

Analysis of humoral fluid metabolites from the C, S1, and S2 based on GC-MS. All data were collected from eight fish per group. (A) PCA plots among the C, S1, and S2. OPLS-DA score plots obtained from (B) C vs. S1; (C) C vs. S2; (D) S1 vs. S2. (E) Heatmap and clustering of log concentrations of characteristic metabolites in dietary supplements of *Schizochytrium* sp. C, S1, and S2 represent zebrafish fed with 0, 60, and 120g/kg *Schizochytrium* sp., respectively.

Figure 4

Summary of the pathway analysis. All the matched pathways are arranged based on the $-\log(p)$ values from the pathway impact values from the pathway topology analysis on the X-axis and the pathway enrichment analysis on the Y-axis.

Figure 5

Comparison of intestinal microbiota compositions between groups. (A) Relative abundances of bacterial phyla among the C, S1, and S2 zebrafish, respectively. (B) Changes in the relative abundance of the phyla Bacteroidetes per group. (C) NMDS analysis of bacterial communities based on the unweighted UniFrac distances. (D) LefSe analysis of taxonomic abundance in the intestine. C, S1, and S2 represent zebrafish fed with 0, 60, and 120g/kg Schizochytrium sp., respectively.

Figure 6

Correlation analysis between genera and metabolite concentrations affected by the feed type. Each row in the graph represents a metabolite, each column a genus, and each lattice a Spearman's correlation coefficient between a genus and a metabolite. Positive correlations are indicated in red, and the negative correlations are in blue (* $p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)