RESEARCH ARTICLE

The effect of Spirulina (*Arthrospira platensis*) feed supplement on proteins, lipids, carbohydrates, and microbiota of juvenile Nile Tilapia (*Oreochromis niloticus*)

Hafidh Al Mahrouqi[1,2*]**, Sergey Dobretsov**[2,3]**, Roberto Tefilo Abdala-Daz**[1]
[1] Malaga University, Ecology Depertment, Faculty of Sciences, 29071 Malaga, Spain
[2] College of Agricultural and Marine Sciences, Sultan Qaboos University, Al Khoud 123, P.O. Box 34, Oman
[3] Centre of Excellence in Marine Biotechnology, Sultan Qaboos University, Al Khoud 123, P.O. Box 50, Oman

Abstract: Tilapia is the second most farmed fish in tropical and sub-tropical countries worldwide. This study investigated the effect of replacing fish meal by *Spirulina platensis* (groups fed with 5%, 10% and 15% Spirulina) on the growth of Nile tilapia *O. niloticus* juveniles. Intestinal microbiota of tilapia has been studied by MiSeq Illumina sequencing. No significant differences ($P > 0.05$) were observed for the protein, carbohydrate and lipids of fish fed with control food and different concentrations of spirulina. However, the Margalef species richness of the intestinal bacteria of tilapia fed with spirulina was significantly higher ($P < 0.05$) compared to the control samples. Evenness was significantly higher ($P < 0.05$) for tilapia fed with food substituted with the highest concentration of spirulina. Also, few potentially probiotic genera, like *Bacillus* and *Actinomycetes* were detected in the gut of the experimented Tilapia. On the other hand, the highest percent of potentially pathogenic genera was recorded for the genera *Pseudomonas* and *Corynebacterium*. In most cases, the highest abundances of potentially pathogenic species were found in control samples. As manipulation of the feed was shown to improve Tilapia microbiota, which could lead to further improvements in aquaculture production.

Keywords: Microbiota, *Spirulina platensis*, Nile tilapia, gut bacteria, MiSeq Illumina

Correspondence to: Hafidh Al Mahrouqi, Malaga University, Ecology Depertment, Faculty of Sciences, 29071 Malaga, Spain; College of Agricultural and Marine Sciences, Sultan Qaboos University, Al Khoud 123, P.O. Box 34, Oman; E-mail: hali@squ.edu.om

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1 Introduction

As the aquaculture industry continues to increase global seafood supply, segments of the industry remain dependent upon capture fisheries for feed ingredients. In 2016, 9% of the total fisheries supply was used to produce fish meal and fish oil (FAO, 2018), the majority of which were used to produce aquafeeds (Auchterlonie, 2016). This has prompted numerous studies evaluating alternative sources of protein and lipid for aquafeeds (Callet et al., 2017; Stevens et al., 2018; Belghit et al., 2019; Twibell et al., 2020).

Microalgae have the potential to replace fish meal and fish oil in aquafeeds (Sirakov et al., 2015; García-Márquez et al., 2020). The nutritional quality of microalgae is high, with a crude protein content of up to 71% and a lipid content of up to 40%, which are comparable with that of terrestrial plant and animal sources (Ravindran et al., 2016). Microalgae are an important source of Omega-3, fatty acids, eicosapentaenoic acid, and docosahexaenoic acid which provide important health benefits and lead to high consumption as dietary supplements (Adarme-Vega et al., 2012).

Tilapia is the second most farmed fish in tropical and sub-tropical countries worldwide and its production is over 90 countries for aquaculture and fisheries (Prabu et al., 2019). There are some biological reasons that make tilapia a good culture species such as: resistance to diseases, high tolerance to high ammonia concentrations and low dissolved oxygen in water (Boyd, 2004). It's a common freshwater tropical aquaculture species, fast growing and matures in 10 months. Tilapias are omnivorous species that can utilize cyanobacterial blue-green algae as a food source (Chow and Woo, 1990).

Studies have demonstrated the successful use of microalgal biomass as a feed additive or fish meal replacement for a wide range of aquaculture species, generally with positive effects on fish growth and quality, including Nile tilapia *Oreochromis niloticus*, Linnaeus (Mai et al., 2013; Vizcaíno et al., 2014; Pakravan et al., 2017; Sarker et al., 2018; García-Márquez et al., 2020).

Spirulina platensis is a filamentous cyanobacterium with a good nutritional profile due to its high protein content (60-70%), variety of vitamins, minerals, essential fatty acids, and antioxidant pigments, such as carotenoids (Belay et al., 1996, James et al., 2006). Studies conducted using Spirulina as a supplement and as partial substitution in diets have reported enhanced growth performance, meat quality, and immune responses of aquaculture species (Palmegiano et al., 2008; Adel et al., 2016; Cao et al., 2018; Sheikhzadeh et al., 2019; Van et al., 2020).

Recently, technologies such as next-generation sequencing (NGS) have allowed a rapid expansion in research related to fish nutrition. It is known that the microbiota of the digestive tract of aquaculture species plays important roles in the host's physiology, digestion, and energy homeostasis (Tarnecki et al., 2016; Butt and Volkoff, 2019). Microbiota of host species regulates immune response and maintenance of intestinal tissue integrity (Nayak, 2010; Gonçalves and Gallardo-Escárate, 2017; Tarnecki et al., 2017), which can be modulated by different diets and farming conditions (Tapia-Paniagua et al., 2019). Few studies used 454 pyrosequencing and MiSeq Illumina to characterize gut microbial communities associated with larvae, juveniles, juveniles, and adults of cultivated Nile Tilapia (Giatsis et al., 2015; Shi et al., 2020). However, little is known about the influence of dietary *S. platensis* on the intestinal microbiota of cultivated Nile tilapia *O. niloticus*. A previous study analyzed intestinal microbiota after feeding with 3% of Spirulina resulting in no apparent modification when compared with the control diet (Plaza et al., 2019). The effect of a higher percentage ($> 3\%$) of Spirulina on intestinal microbiota of tilapia has not been previously tested. We hypothesise that Spirulina feed additives have influence on the intestinal microbiota of Nile Telibia, but have no significant effect on biochemical composition of the muscle. The objective of the present study was to analyze the influence of the replacement of fish meal by Spirulina on the proteins, lipids, and carbohydrates of Nile tilapia juveniles as well as their intestinal microbiota.

2 Materials and Methods

2.1 Ethical statements

In this study, the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and RD 53/2013) were applied for the manipulation of animals.

2.2 Microalgae

Spirulina platensis strain was obtained from the Ecology and Geology Department of the University of Málaga (code: BEA0007B). Before the experiment, Spirulina was grown using the standard Zarrouk medium.

2.3 Experimental feeds and feeding trial

This study was conducted in the glasshouse in a controlled environment at the Agricultural Experiment Station, College of Agricultural and Marine Sciences, Sultan Qaboos University from May to July 2019. Control diet and diets with 3 different percentages of spirulina were formulated, with each treatment consisting of three replicate tanks. 12 tanks with 15 fish each with a capacity of 100 liter of water and air bubbles running 24/7. The water was changed weekly.

Two diets were used in the experiment. One included the commercial tilapia fish feed (ARASCO, Saudi Arabia Tilapia Feed CP 36). It was used as a control. The modified formulated feed was manufactured using three different percentages of spirulina (5, 10 and 15%). The protein content was adjusted by varying the levels of soybean and spirulina. The protein content was managed to be the same as the imported feed. All ingredients of the formulated feed were grounded and homogenized in a mixer and pelleted using the pelleting press to get a pellet of 2 mm. Finally, pellets were dried at room temperature and stored at -20°C before the experiment.

2.4 Fish sampling

Tilapia juveniles were obtained from (Issa Al Sultani Aquaculture Company, Oman) with an average weight of 30 g from the same hatched group. After 90 days of feeding, (15 fish per tank, n = 15 per experimental group) juveniles were euthanized by an overdose of isoeugenol (clove oil) followed by spine severing according to the requirements of the European Union (2010/63/EU) for animal experiments. Then, specimens were individually weighted and stored at -20°C until use. For the evaluation of intestinal microbiota, whole intestines were removed and kept at -80°C for further analysis.

2.5 Biochemical composition (protein, lipids and carbohydrate) of tilapia

The total nitrogen in tilapia was determined using a C/H/N Elemental Analysis 2400 (PerkinElmer, USA). Briefly, the dry biomass of tilapia was oxidized in the analyser at 600°C and the resulting C, N, H peaks were compared with a known mass value of an acetanilide standard. Obtained N values were presented as a percentage of dry weight.

The amount of total proteins in the dry biomass of spirulina was calculated using the nitrogen conversion factor of 6.25 (Jones, 1931).

The quantification of carbohydrates in tilapia samples was performed using the DuBois method (DuBois et al., 1956). In order to do this, 5 mg of dry sample was hydrolysed in 5 mL of 1 M H_2SO_4 in a water bath at 100°C for 1 hour. Then, the sample was centrifuged at 2,000 g (Sartorius 2-16PK, Sigma, Germany) for 10 minutes. One mL of phenol 5% was added to 1 mL of supernatant and incubated for 40 minutes at room temperature. After that, 5 mL of concentrated H_2SO_4 was added to the mixture. Finally, the absorbance at 485 nm was measured using a spectrophotometer (UV Mini-1240, Shimadzu, USA). Glucose was used as a standard.

Lipids were measured using the gravimetric method (Folch et al., 1957). Briefly, 200 mg of dry sample was homogenized in 5 mL of chloroform:methanol (2:1) with butylhydroxytoluene 0.01%. After that, 2 mL of 0.88% KCl was added to separate chloroform and lipids. The lipid fraction was filtered, dried with N_2 and weighted.

2.6 Evaluation of the intestinal microbiota

2.6.1 DNA extraction

Bacterial samples were collected from the intestinal lumen of tilapia in control and groups fed with 5%, 10%, and 15% spirulina. There were 3 replicates for each treatment. DNA was extracted individually from the lumen intestinal content according to Martínez et al. (1998), with modifications described by Tapia-Paniagua et al. (2014) and Alcalde-Rico et al. (2016). The DNA quality was analysed in a 1.5 % agarose gel electrophoresis in the presence of ethidium bromide. The concentration and quality of DNA was assessed using a nanodrop spectrophotometer (Thermo Fisher, USA).

2.6.2 Illumina MiSeq sequencing

Microbial communities associated with the tilapia lumen were sequenced using Illumina MiSeq next generation sequencing of bacterial V3/V4 hypervariable regions of 16S rRNA genes at the Molecular Research (MR DNA) company (Shallowater, TX, USA). The genes were sequenced using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). A full description of the PCR and sequencing can be found in (Dobretsov and Rittschof, 2020). Sequence data were processed using the MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA) (Dowd et al., 2008). Sequences shorter than 150bp and chimeras were removed. Operational Taxonomic Units (OTUs) were classified using BLASTn against RDPII (http://rdp.cme.msu.edu) and NCBI (http://ncbi.nlm.nih.gov) databases. The raw sequences from the project were deposited in the NCBI database (BioProject SUB13190644).

2.7 Statistical analysis

Statistical analysis (ANOVA) was performed to determine the significant differences in protein, carbohydrate, and lipids of the tilapia muscle using the software STATISTICA (V.7). Values were expressed as mean ± standard deviation (SD) (n = 15) using analysis of variance (Two-way ANOVA) to compare the control with different treatments.

For the microbiota, there were 3 replicates for Illumina MiSeq sequencing and the plots represent mean values. Microbial diversity indices and evenness were calculated using the PRIMER (Plymouth, UK) software. Differences between treatments and the control were analysed using the Dunnet test. A Bray-Curtis similarity matrix of abundances of OTUs was generated with the PRIMER software. Principal component analysis based on the Bray-Curtis similarity matrix was conducted using the PRIMER software.

3 Results

3.1 Protein, Carbohydrate and Lipids of tilapia muscle

There were no significant differences ($P > 0.05$) observed for the protein, carbohydrate, and lipids of fish fed with control food and different concentrations of spirulina (Figure 1, 2 and 3). The highest protein content (30.83 ± 0.18%) was found in fish fed with 5% spirulina and the lowest one (29.26 ± 0.73%) was observed in tilapia fed with 10% spirulina (Figure 1). Similarly, the highest carbohydrate content (43.41 ± 0.46%) was observed in fish fed with 5% spirulina (Figure 2). However, the lowest carbohydrate content (41.22 ± 0.73%) was in the control group. The highest percent of lipids was found in the control group (5.50 ± 0.65%) and the lowest one (4.40 ± 0.21%) was observed in fish fed with 15% of spirulina (Figure 3). There were no significant differences between treatments ($P > 0.05$).

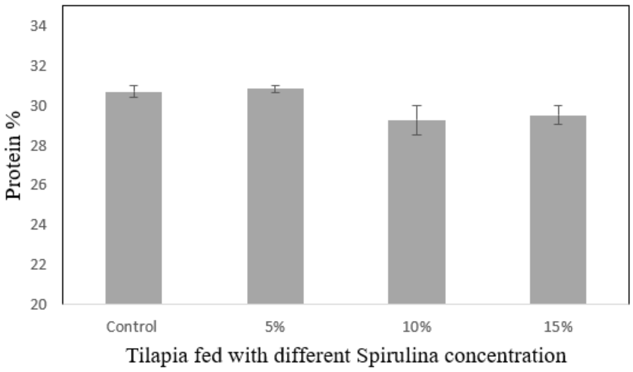

Figure 1. Protein content (%) of different groups of fishes fed different Spirulina concentration control, 5, 10 and 15%). Values are presented as mean ± standard error (n = 3).

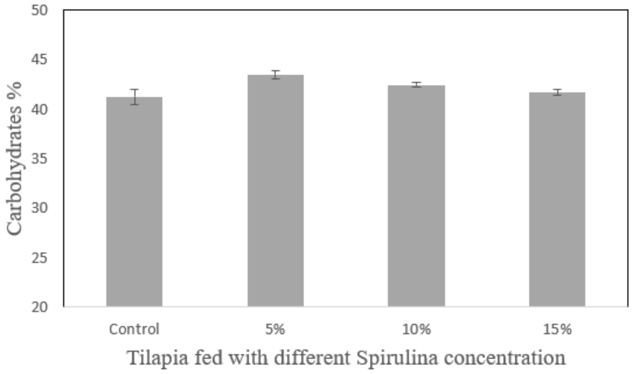

Figure 2. Carbohydrate content (%) of different group of fishes fed different spirulina concentrations 5, 10 and 15%. Control group was fed with a standard food. Values are presented as mean ± standard error (n = 3).

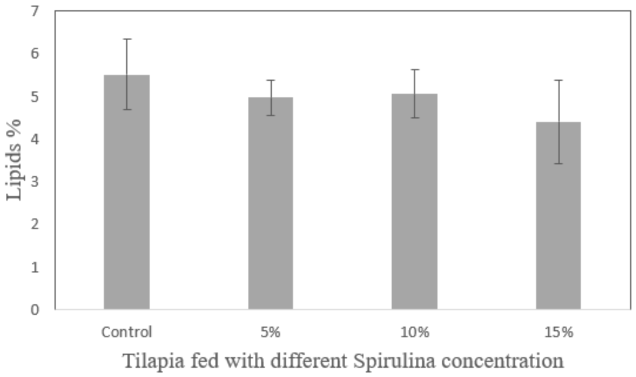

Figure 3. Lipids content (%) of different group of fishes fed different spirulina concentration control, 5, 10 and 15%. Control group was fed with a standard food. Values are presented as mean ± standard error (n = 3).

3.2 Diversity of microbial communities in tilapia gut

The species richness (Margalef) of the intestinal bacteria of tilapia fed with spirulina was significantly higher ($P < 0.05$) compared to the control samples (Table 1). Evenness was significantly higher ($P < 0.05$) for tilapia fed with food substituted with the highest concentration of spirulina. The Shannon diversity was not significantly different ($P > 0.05$) between all treatments. On the other hand, the Simpson diversity was significantly higher ($P < 0.05$) in tilapia fed with 15% spirulina (Table 1).

Table 1. Mean diversity estimates (± standard deviation) for microbial communities associated with the intestinal lumen of tilapia fed with standard food (control) and food replaced by 5%, 10% and 15% of Spirulina. Significantly different values compare to the control according to Dunnet-test are highlighted

Sample	Species richness	Evenness J'	Shannon diversity H'	Simpson diversity
Control	3.068 ± 0.03	0.454 ± 0.22	1.22 ± 0.56	0.543 ± 0.26
5%	3.165 ± 0.03	0.458 ± 0.33	1.23 ± 0.18	0.549 ± 0.13
10%	3.220 ± 0.03	0.466 ± 0.31	1.28 ± 0.85	0.502 ± 0.27
15%	3.114 ± 0.03	0.663 ± 0.17	1.20 ± 0.88	0.733 ± 0.08

3.3 Composition of microbial communities in tilapia gut

In total, 1,415,042 OTUs from the tilapia's lumen intestinal samples were obtained. These sequences correspond to 15 phyla, 33 classes, 108 families and 161 genera of bacteria. Bacteria belonging to the phylum Proteobacteria and the phylum Fusobacteria are dominated and comprised of more than 60% of all sequences. The class Fusobacteriia dominated in all samples (Figure 4). Classes Actinobacteria, Gamma- and Alpha-Proteobacteria were less abundant.

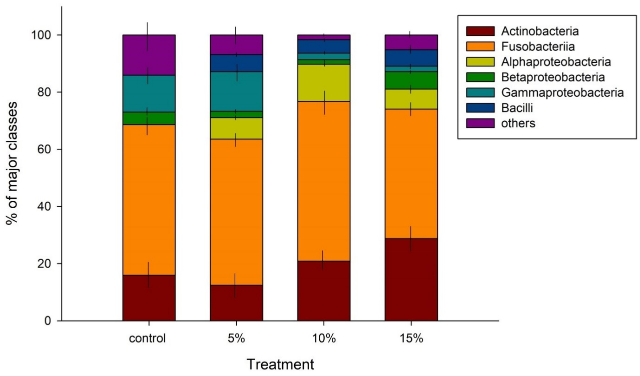

Figure 4. Major bacterial classes in the intestinal lumen of tilapia fed with standard food (control) and food replaced by 5%, 10% and 15% of spirulina. The data are the mean of 3 replicates ± standard error (SE).

The top abundant genus detected in tilapia intestinal samples was *Cetobacterium*, which relative density was more than 45% (Figure 5). Other less abundant genera were *Pseudomonas*, *Actinomyces*, and *Corynebacterium*. The genus *Hyphomicrobium* was absent in the control group but was abundant in the fish fed with spirulina (abundance 3-10%). Similarly, *Achromobacter*, *Xanthobacter* and *Geobacillus* were found only in tilapia fed with spirulina. In opposite, *Plesiomonas* was present only in the control group (abundance 1.7%) but absent in the fish fed with spirulina. The differences in microbial communities are further supported by the principal component analysis (PCA, Figure 6). The dissimilarity between the samples was mainly explained by the first principal component (PC1- variation 41.5%) and the second component (PC2 variation 27.1%). PCA illustrates that fish microbial communities in the control group were different from the intestinal communities of tilapia fed with spirulina, except one fish sample fed with 15% spirulina (Figure 6).

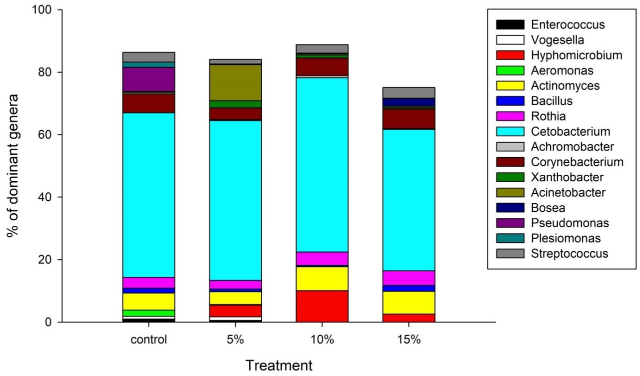

Figure 5. Major bacterial genera in the intestinal lumen of tilapia fed with standard food (control) and food replaced by 5%, 10% and 15% of spirulina. The data are the mean of 3 replicates. The sum of dominant genera is lower than 100% due to the presence of unidentified species.

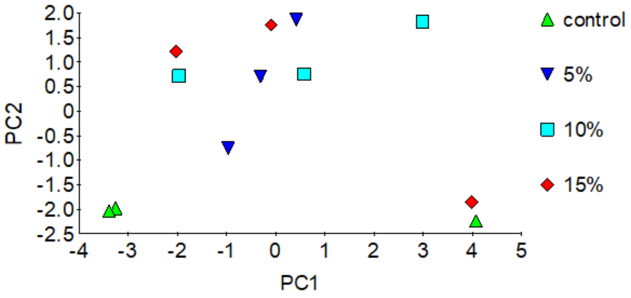

Figure 6. Principal component analysis of major bacterial genera in the intestinal lumen of tilapia fed with standard food (control) and food replaced by 5%, 10% and 15% of spirulina.

3.4 Potentially probiotic and pathogenic genera in tilapia gut

In the tilapia's lumen intestinal we were able to detect few potentially probiotic genera, like *Bacillus* and *Actinomycetes* (Table 2). The highest abundance was observed for the genera *Actinomycetes*. The percent of this genus increased in tilapia fed with spirulina. Additionally, potentially pathogenic genera were recorded (Table 2). These include *Enterococcus*, *Vogesella*, *Pseudomonas*, and *Streprococcus* among others. The highest percent of pathogenic genera was recorded for the genera *Pseudomonas* and *Corynebacterium*. In most of cases, the highest abundances of potentially pathogenic genera were found in control samples (Table 2). Fish fed with spirulina contained either less or no potentially pathogenic genera.

Table 2. Percent of potentially probiotic and pathogenic genera in the intestinal lumen of tilapia fed with standard food (control) and food replaced by 5%, 10% and 15% of spirulina

Genera	Control	5%	10%	15%
Potentially probiotic genera				
Actinomycetes	5.46	4.12	7.67	7.29
Bacillus	1.50	0.83	0.47	1.90
Potentially pathogenic genera				
Enterococcus	0.93	0.54	0	0
Vogesella	0.92	1.12	0	0
Aeromonas	1.98	0.23	0	0
Corynebacterium	6.03	3.81	5.63	6.28
Pseudomonas	7.75	0.19	0.25	0.13
Pleisomonas	1.74	0	0	0
Streptococcus	3.09	1.46	2.68	3.4

4 Discussion

In this study, tilapia were fed diets with different Spirulina concentrations and a control diet without Spirulina. The use of Spirulina in tilapia feed resulted in a decrease in the cost of feed, improved the feed conversion ratio and the growth performance, as well as increased the survival rate and immunity of fish (El-Sheekh et al., 2014). In our study, Spirulina clearly shows the same level of proteins, lipids, and carbohydrates in tilapia muscle in comparison to the control. Different findings have been reported by Siringi et al. (2021). The crude protein, lipid, and ash content were significantly different in Nile tilapia fed with 8 g of Spirulina per 1 kg of the diet. The differences could be explained by the differences in experimental conditions and concentrations of Spirulina in both studies.

Gut microbiotas play important roles in breaking down and absorption of nutrients in fish, and many studies have found that diet is the main factor affecting the metabolism and composition of gut microbiota. In our study, the microbial communities associated with the intestinal lumen of tilapia were investigated. Phylum Proteobacteria and Fusobacteria were the two major microbial dominated groups found in the tilapia intestines. A similar finding was observed by Fan et al. (2017). In another study, the gut microbiota of tilapia sampled from Lakes Awassa and Chamo in Ethiopia were characterised by Illumina MiSeq next-generation sequencing (Bereded et al., 2020). The investigators found dominance of Proteobacteria which suggests that these bacteria are naturally commonly associated with tilapia.

In our study, the genus *Cetobacterium* was the most abundant one in all samples and its density was not significantly affected by the feed. A similar result was observed by Ramírez et al. (2018) who studied the microbiome of giant Amazonian fish (*Arapaima gigas*) in Ecuador. Ray at al. (2017) showed that 90% of the OTUs detected in the fish gut belonged to the genus *Cetobacterium*. This suggests that this genus is widely distributed in the intestinal lumen of freshwater fishes. It encourages carbohydrate metabolism, produces vitamin B12, and indicates healthy intestinal microbiota (Wu et al., 2021).

The diversity of microbes and species richness in tilapia fed with Spirulina was higher compared to the control group. Rosenau et al. (2021), studied microbial communities associated with African catfish during the replacement of fishmeal by Spirulina algae. Similarly, they found that the addition of Spirulina increased bacterial richness. In another study, gilthead sea bream juveniles were fed with a supplement of *Nannochloropsis gaditana* algae (Jorge et al., 2019). The investigators found that microbial richness increased with the dietary supplementation of microalgae. These results suggest that the addition of microalgae to the fish diets could enrich gut microbiota and possibly improve fish health. However, the species and diversity of the gut microbiota is different between different fish species and under different environmental conditions (Yang et al., 2021).

Our study showed that there were significant differences in microbial communities of tilapia fed with Spirulina and controlled ones. The genera *Hyphomicrobium* was found only in Spirulina-fed tilapia. *Hyphomicrobium* is a common gram-negative Alphaproteobacteria commonly associated with fishes (Chen et al., 2018; Mondal et al., 2022). On the opposite, *Plesiomonas* sp. was associated with the control group not fed with Spirulina. This genus belongs to the family Enterobacteriaceae. *Plesiomonas* is commonly associated with fish diseases leading to mass mortality (Behera et al., 2018). It was further supported by our observations that the relative abundances of pathogens were high in the control group but decreased or were absent in tilapia fed with Spirulina.

The relative densities of potentially probiotic strains, like *Bacillus* and *Actynomycetes* were high in tilapia fed with Spirulina. Probiotics are commonly used in aquaculture to improve fish health and prevent diseases. *Bacillus* is among of the most common probiotic strains due to its low toxicity, production of antibiotic substances, and ability to form spores (Kuebutornye et al., 2019). Euanorasetr et al. (2020), identified the presence of *Actinomycetes* in the gut of Nile tilapia and indicated the possibility of using it as a probiotic because of its antibacterial activity. Thus, our results demonstrate the potential benefits of incorporation of Spirulina in aquafeed. It could improve fish gut microbiota by increasing the abundance of probiotic strains, which reduce densities of pathogenic bacteria and improve fish health.

5 Conclusions

1. Microbial communities were different in the fish fed with spirulina and non-fed with spirulina;
2. Diversity of microbial communities in the fish fed with spirulina was higher;
3. The abundance of potentially pathogenic species decreased or they were absent in the fish fed with spirulina;
4. The percent of potentially beneficial strains *Actinomycetes* increased in fish fed with spirulina.

Conflict of Interest

The authors declare no conflict of interest.

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