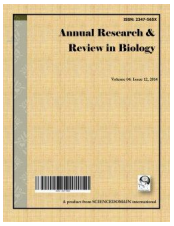

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Growth, Body Composition and Resistance to *Aeromonas hydrophila* Challenge in Juvenile African Catfish (*Clarias gariepinus*) Fed Diets Supplemented with Spirulina (*Arthrospira platensis*)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study evaluates the growth, body composition, and resistance to *Aeromonas hydrophila* challenge in juvenile African catfish (*Clarias gariepinus*) fed diets supplemented with spirulina (*Arthrospira platensis*). Five experimental diets containing different percentages of locally grown dried spirulina (0, 1, 3, 5 and 7%) were fed daily to catfish juveniles at 5% of their body weight. The growth and body composition of the catfish were determined up to 90 days of the study period. At day 91, the catfish were intraperitoneally injected with 10^7 CFU/ml of virulent *A. hydrophila*. Generally, the growth and body composition of the catfish in spirulina inclusion groups showed no

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significant difference with the control group. The survival rate following *A. hydrophila* challenge was significantly low in the control group compared to all of the other treatment groups. After the challenge trial, only the white blood cell count value was significantly higher in all of the groups supplemented with spirulina compared to the control group. We concluded that the locally grown spirulina do not improve growth and body composition, but it increased the catfish resistance towards *A. hydrophila* infection.

Keywords: Clarias gariepinus; Aeromonas hydrophila; Arthrospira platensis; growth performance; body composition; disease resistance.

1. INTRODUCTION

The Clariidae family dominates the freshwater aquaculture production in the Asia Pacific region, representing ~80% of total catfish production. Among the most cultured species are walking catfish (*Clarias batrachus*), bighead catfish (*C. microcephalus*), and African catfish (*C. gariepinus*) [1]. In Malaysia, catfish reported the highest production levels amongst cultured freshwater fish. The total production of this species was approximately 50,534 tons, with an estimated retail value of RM 314 million. Most of the catfish were cultured in freshwater ponds (48,422 tons; 95.8%), followed by cemented tanks (1,242 tons; 2.5%), and canvas tanks (354 tons; 0.7%). It is predicted that the production of catfish will increased in the near future, due to demands and improved farming technology [2].

Spirulina (*Arthrospira* spp.) is a filamentous, blue-green microalgae, photosynthetic and generally regarded as a rich source of essential amino acids, minerals, vitamins, essential fatty acids, and antioxidants [3]. It has been confirmed that the addition of small amounts of microalgae to fish feed can result in pronounced effects on growth, lipid metabolism, body composition, and disease resistance [4]. Moreover, the supplementation of spirulina can provide excellent nutritional value, inducing probiotic effects, and stimulate the immunity of the cultured aquatic animals [5,6]. Spirulina also improved the growth performances of rainbow trout (*Oncorhynchus mykiss*), rohu (*Labeo rohita*), and great sturgeon (*Huso huso*) [7,8,9] and increase disease resistance of great sturgeon and common carp (*Cyprinus carpio*) against bacterial diseases [9,10]. Indeed, spirulina has been reported to increase the nutritional value of rainbow trout, three-spot gourami (*Trichopodus trichopterus*), and African catfish [11,12,13].

Disease outbreaks due to bacterial infection resulted in huge economic losses in aquaculture [14]. In freshwater fish farming, *Aeromonas* spp. infection has been reported as a detrimental bacterial disease in the global catfish industry [15]. The bioactive components in spirulina, which endowed positive health effects and enhance the fish immune system, its utilization as feed supplements could increase the catfish disease resistance towards bacterial infections [16]. The aim of this study are to evaluate the growth, body composition, and resistance to *A. hydrophila* challenge in juvenile African catfish fed with diets supplemented with spirulina (*Arthrospira platensis*).

2. MATERIALS AND METHODS

2.1 Fish and Experimental Unit

A total of 450 healthy African catfish juveniles were purchased from a local fish farm located at Balakong, Selangor, Malaysia. The sizes of the juveniles before the experiment were approximately 2.62 ± 0.04 g in total weight and 7.09 ± 0.05 cm in total length. Prior to the experiment, the fry was acclimatized for two weeks and fed with commercial fish diet (Cargill, Port Klang, Malaysia). Dechlorinated water was used throughout the study period. At the end of the acclimatization period, the fry was randomly placed into the cages at 30 juveniles/cage and in triplicate for each diet treatment.

The rearing facilities included a concrete cemented tank (8.7 m length × 8.0 m width × 0.8 m depth). This allowed 15 cages to fit into the tank and enabling it to hold 1000-L volume of water. This tank also complete with aeration from air pump and drainage system to prevent overflow. The cages were made of 15 mm class D PVC pipe that act as its frame, while black nylon mesh was used to wrap the frame, measuring 8 mm × 300 mm. The dimension of

the cage was 1 m length × 1 m width × 1 m depth.

2.2 Cultivation and Harvesting Process of Spirulina

Spirulina (*A. platensis*) was the species that has been cultivated and used as a supplement for the catfish in this study. The pure stock culture of spirulina was obtained from the Plant Physiology Laboratory, Department of Biology, Faculty of Science, Universiti Putra Malaysia (UPM).

The Kosaric medium was prepared according to Al Mahrouqi et al. [17], as a reference growth medium. A total of 50% of spirulina seeds from stock culture were cultivated in outdoor-covered tanks at the Fish Breeding Center, UPM, by mixing the prepared media with filtered tap water. Aeration was provided at 0.7-L/min with outdoor condition for direct light source.

Light intensity, culture temperature, pH of the culture medium, and the optical density of the cultured spirulina were monitored and recorded daily. As the growth curve reached stationary phase and confirmed by the optical density data, the spirulina was harvested by pumping out the cultured water from the tank onto the algae harvester swing with the filter cloth at a pore size of 37-µm, and were swung until concentrated spirulina was obtained.

The concentrated spirulina was then dried at 60°C (Jeio Tech Co. Inc., Seoul, Korea) for 24 h. In order to obtain the powdery form, the dried spirulina was ground using an electrical blender (Pensonic, Penang, Malaysia) and stored at a dry place before being coopted into the commercial fish feed.

2.3 Experimental Diet Preparation

Five experimental diets were used in the study by manipulating the percentage of spirulina at 0 (control), 1, 3, 5, and 7% in the commercial fish feed. The feeds were then given to the fish throughout the 105 days of the study period, including during the bacteria challenge experiments. The experimental diets included the commercial fish feed (Cargill) and the produced dry powdered locally grown spirulina.

The preparation of the experimental diets began with grinding the commercial fish feed into powder using an electrical blender. The next step involves mixing the commercial fish feed and spirulina powders according to the formulation of the predetermined percentage of spirulina. The fish feed with 0, 1, 3, 5, and 7% of spirulina were mixed thoroughly to achieve a constant dispersion of the spirulina and commercial fish feed. In order to create pellets, 10% of water was added to the total volume of the feed, and the mixture was then compressed, extruded, and cut according to the desired length. The final product was then kept in a dry place prior to its use.

2.4 Fish Growth Performance Determination

Five experimental diets containing different percentages of locally grown dried spirulina (0, 1, 3, 5 and 7%) were fed to catfish fry at 5% of fish body weight for 90 consecutive days. The feed was given at 8.00 AM and 5.00 PM daily, and all of the fishes were monitored to obtain the same volume of feed. At 14 days interval, a total of 45 catfish (15/replicate) were sampled randomly in each group to measure the total weight and length. The parameters on the fish growth performance, such as total biomass increase (TBI), total length (TL), survival rate (SR), specific growth rate (SGR), feeding efficiency (FE), feed conversion rate (FCR), gonadosomatic index (GSI), hepatosomatic index (HSI), and vicerosomatic index (VSI) were also determined [18,19,20,21,22].

2.5 Proximate Composition of Experimental Diets and Catfish's Fillets

The proximate composition of spirulina, experimental diets, and catfish fillets (muscle below the dorsal fin) including the moisture, ash, crude protein (Kjeldahl's protein), crude lipid, and crude fiber were determined based on previous study [23]. A total of 15 catfish fillets (5/replicate) were used for each group.

The moisture content calculation was based on the percentage of wet weight and the formula is:

% of wet weight = (a - b) / a

where a = weight of sample used, and b = dry weight of sample.

The percentage of the ash calculation is as follows:

% of ash = [{(weight of ash + weight of crucible) – (weight of crucible)/ weight of sample} × 100]

The calculation of crude protein content is as follows:

$$\% \text{ of nitrogen} = \frac{(I_s - I_b) \times N \times 1.4}{W}$$

% of protein = % nitrogen × conversion factor

where W = weight of sample, I_s = volume of H_2SO_4 to titrate BH_3O_3, I_b = volume of H_2SO_4 to titrate blank, and N = normality of NaOH

The calculation of the fat content in percentage of dry basis sample is as follows:

% of crude lipid in sample = (M – T / W) × 100

where W = weight of sample (g), M = weight aluminum cup with extracted lipid (after drying) (g), and T = weight of empty aluminum cup.

The calculation of the crude fiber content is as follows:

$$\% \text{ crude fiber} = \frac{S - A}{W} \times 100,$$

where W = weight of sample (g), S = weight of crucible + dried ash residue, and A = weight of crucible + ash.

2.6 Preparation of *Aeromonas hydrophila* for Challenge Experiment

The *A. hydrophila* stock was obtained from the Department of Aquaculture, Faculty of Agriculture, UPM. The isolate was confirmed as *A. hydrophila* based on the biochemical tests, PCR and sequencing analysis. The isolate was previously isolated from diseased catfish during *A. hydrophila* outbreak [24].

The isolate was cultured in a tryptic soy agar (TSA) (Merck, Darmstadt, Germany) and incubated for 24 h at 37°C. The bacterial stock was prepared by culturing the 25 colony of *A. hydrophila* in a 100-mL of tryptic soy broth (Merck) and incubated in the orbital incubator shaker (BioMaker, Zhejiang, China) at 0.75 ×*g* for 24 h at 37°C. The bacterial concentrations was then calculated based on standard ten-fold serial dilution and spread plated onto *Aeromonas* medium base agar (Oxoid, Hampshire, United Kingdom), and further incubated for 24 h at 37°C. The bacteria colonies that grew out of it were counted, and its concentration was calculated.

2.7 Challenge of Catfish with *Aeromonas hydrophila*

On day 91, a total of 2-mL bacteria stock containing 10^7 CFU/mL of virulent *A. hydrophila* was intraperitoneally injected into all catfish groups using a 21G (0.80 mm diameter and 38 mm in length) syringe needle (Terumo, NJ, USA). The fish were first anesthetized using MS222 (Sigma-Aldrich, Kuala Lumpur, Malaysia) at a rate of 140 mg/L [25]. Then, they were placed with its abdomen facing upwards and supported by a moistened foam bed to ensure that it remained in an upside-down position. The needle was inserted into the midline of the abdomen, posterior to the pectoral fins. The experiment was observed for the following 14 days. A total of 60 catfish (20/replicate) were challenged for each group.

Throughout the infection period, clinical signs and mortality were recorded daily. The dead catfish were immediately removed from the cages. Bacteria swabs from organs such as skins, livers, kidneys and spleens were cultured in the TSA. Isolation and identification of bacteria were conducted to confirm the catfish mortalities were caused by *A. hydrophila* infection [24].

2.8 Blood Sample Analysis

Before (day 91) and after (day 106) challenging catfish with *A. hydrophila*, three catfish from each cage (n = 9/group) were randomly sampled. The blood from caudal peduncle was collected for hematological profiles. The blood samples were collected using the caudal venous puncture method. The sterile needle attached to a syringe 1 cc/mL (Terumo) was inserted under the skin of ventral midline of the caudle peduncle of the anaesthetized catfish. The collected blood were transferred into the vacutainer tube (EDTA-K_2 4 ml, HmBG, Johor, Malaysia) and sent to Hematology Laboratory, Faculty of Veterinary Medicine, UPM within 12 h for hematological profile analysis. The blood parameters were analyzed for the red blood cell count (RBC), white blood cell count (WBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). The results

were compared between before and after the bacteria challenge experiment.

2.9 Histopathology Analysis

At the same time of blood sample analysis collection, two catfish from each cage (n = 6/group) were randomly sampled and placed into the water containing MS222 at a rate of 300 mg/L to be euthanized [25]. Tissues obtained from spleens, livers, and kidneys of the catfish were fixed in 10% buffered formalin. The tissues were further processed for dehydration and infiltration using tissue-processing machine (Leica TP1020, Leica, Heidelberger, Germany). It was then embedded using paraffin and sectioned (8 µm) using the microtome jung multicut (Leica Jung Multicut 2045 Microtome, Leica). Hematoxylin and eosin staining (H&E) method were applied onto the tissues slides. The photos were recorded and analyzed using Nikon Eclipse 50i Japan and The Nikon NIS-Element D 3.2 Image Analyser (Nikon Instruments Inc., USA).

The histopathological scoring of the catfish's spleens, livers, and kidneys tissue included the aspects of necrosis and congestion. The score and percentage are as follows; 0 = 0% (none), 1 = 1-20% (very mild), 2 = 21-40% (mild), 3 = 41-60% (moderate), 4 = 61-80% (severe), and 5 = 81-100% (very severe). However, the melano macrophage centres (MMC) and white pulp were manually counted. The results were compared before and after the bacteria challenge experiment.

2.10 Data Analysis

The collected data on the nutritional composition of spirulina and experimental diets, growth performances of catfish, hematological profiles of catfish before and after bacteria challenge and survival rates of catfish supplemented with different ratio of spirulina against bacteria challenge were analyzed for the means and standard error of mean (SEM). The determined significant difference (p<0.05) was done using one-way ANOVA with Turkey Post Hoc Multiple Comparisons.

The data of histopathology of catfish before and after bacteria challenge were analyzed by scoring the severity condition of the organ tissues. After the scoring, the data were analyzed for its mean and SE. In order to determine the significant difference (p<0.05) of the data, one-way ANOVA with Turkey Post Hoc Multiple Comparisons was used.

3. RESULTS AND DISCUSSION

3.1 Nutrient Composition of Spirulina and Experiment Diets

The highest proximate composition in the locally grown spirulina was the protein content with the value of 63.07 ± 0.02% and significantly higher (p<0.05) compared to carbohydrate (12.36 ± 0.03%), ash (8.02 ± 0.03%), moisture (6.80 ± 0.01%), crude lipid (5.05 ± 0.03%), and crude fiber contents (4.70 ± 0.04%) (Table 1).

Table 1. The proximate composition (%) of locally grown dried spirulina

Proximate composition	Percentage (%)
Moisture	6.80 ± 0.01^{c}
Ash	8.02 ± 0.03^{d}
Crude protein	63.07 ± 0.02^{f}
Crude lipid	5.05 ± 0.03^{b}
Crude fiber	4.70 ± 0.04^{a}
Carbohydrate	12.36 ± 0.03^{e}

The moisture content showed a decreasing trend as the percent of spirulina increased (Table 2) and there was a significant different (p>0.05) between the control group (0%) with 1%, 3%, and 5% of the spirulina inclusion. Similar trend were seen for the ash content. As the spirulina percent increased, the ash content decreased, and there was a significant different among 0%, 1%, 3% and 7% of spirulina inclusion. However, the crude protein increased as the spirulina increased in the diet, and the inclusion of 7% spirulina significantly (p<0.05) resulted in higher crude protein compared to the other groups. The crude lipid content showed no significant difference (p>0.05) between each group, except that the inclusion of 1% spirulina was significantly lower (p<0.05) compared to the other groups, while the crude fiber showed a significant (p<0.05) decreasing trend as the percent of spirulina increased. The carbohydrate content showed that 0% spirulina inclusion is significantly lower (p<0.05), while each group of spirulina inclusion showed no significant difference (p>0.05) between them.

Table 2. The proximate composition (%) of the experimental diets

Proximate composition	% of spirulina				
	0	1	3	5	7
Moisture	12.54 ± 0.01^{a}	12.90 ± 0.01^{d}	12.81 ± 0.01^{cd}	12.71 ± 0.01^{bc}	12.60 ± 0.08^{ab}
Ash	6.80 ± 0.23^{c}	6.63 ± 0.15^{c}	5.21 ± 0.05^{b}	4.89 ± 0.08^{ab}	4.44 ± 0.15^{a}
Crude protein	27.90 ± 0.04^{a}	28.39 ± 0.02^{b}	28.45 ± 0.01^{b}	29.21 ± 0.07^{c}	30.24 ± 0.72^{d}
Crude lipid	8.85 ± 0.28^{bc}	7.12 ± 0.03^{a}	8.31 ± 0.15^{b}	8.75 ± 0.16^{bc}	9.34 ± 0.11^{c}
Crude fiber	5.40 ± 0.09^{e}	4.90 ± 0.07^{d}	4.40 ± 0.09^{c}	3.79 ± 0.03^{b}	2.94 ± 0.05^{a}
Carbohydrate	38.50 ± 0.58^{a}	40.25 ± 0.19^{b}	40.82 ± 0.28^{b}	40.47 ± 0.33^{b}	40.45 ± 0.06^{b}

^{a,b,c,d,e} *Different superscripts indicate significant difference ($p<0.05$) of the same row. Values are presented in mean ± SEM. Number of sample is 15 for each group*

The nutrient contents of spirulina were influenced by several factors, such as cultivation's temperature, light, cell concentration, and the growth medium [26,27]. As spirulina is widely acknowledged as a super food, it is well known for its high protein content [28,29,30]. Our study showed the inclusion of spirulina in the commercial fish feed has significantly increased protein and carbohydrate, due to high contents of both nutrients in the locally grown spirulina. Even though the percent of the inclusion of spirulina in the commercial fish feed was small compared to previous studies [7,13,31], the differences of both nutrients between the experimental diet is rather obvious.

3.2 Growth Performance of Catfish

Catfish supplemented with spirulina does not generally improve growth performance. The TBI, TL, and survival rates showed increasing patterns with increased in spirulina percentage in their diet (Table 3). However, no significant difference ($p>0.05$) was reported between each spirulina inclusion group for TBI and TL with the exception of TBI in 0% and 7% spirulina inclusion diet. The SGR was reported the lowest value in the 0% spirulina diet, and was significantly lower ($p<0.05$) compared to the 7% spirulina diet. However, the rest of the groups reported no significant difference ($p>0.05$) in terms of the SGR. There was no significant ($p>0.05$) pattern for FCR, FE, GSI, HSI, and VSI of the catfish when fed with different percentages of spirulina.

The growth performance of the catfish fed with the prepared experimental diets generally showed no difference when compared to the control diet, and between different percentages of spirulina. In this study, the catfish fed with ≤5% of fish body weight restricted consumption of the feed, and might encounter reduced dietary requirement. Moreover, fish also report species-specific interaction in protein digesting ability, especially from plant sources. Nandeesha et al. [7] showed that catla (*Catla catla*) and rohu fed with different levels of spirulina resulted in non-significant difference of the catla growth performances before and after the experiments, contrary to the significant improvement reported in rohu. In addition, when rainbow trout is fed with ≥5% of *Spirulina platensis,* it reported significantly improved growth performance compared to the control sample, 10 weeks after the experimental trial [32]. In another study, replacing the meal with *S. platensis* for three-spot gourami for 16 weeks resulted in final weight, weight gain, total length, fork length, and body height of fish not showing any significant differences between the treatments at 2.5%, 5%, 10%, and 20% of *S. platensis* inclusion [13], which is also the case in this study . However, all of the fishes fed with *S. platensis* meal reported greater gonadosomatic indices, contrary to our results.

3.3 Nutrient Composition of the Catfish's Fillet

Generally, catfish supplemented with spirulina does not improve its nutritional composition (Table 4). There was no significant difference ($p>0.05$) on moisture, ash, and crude fat composition of the catfish when fed with different rations of spirulina. The crude fiber content significantly ($p<0.05$) increased with increasing spirulina percent in their diet, but the 5% and 7% of spirulina inclusion showed no significant difference ($p>0.05$). The crude protein content reported an increasing trend as the percent of spirulina increased, however, there was no significant difference ($p>0.05$) between all the diet groups. The carbohydrate content of the catfish's fillet in 5% and 7% spirulina diet inclusion differ significantly ($p<0.05$) between them, while the rest were not.

Table 3. Growth performances of catfish supplemented with different ratio of spirulina

Growth parameters	% of spirulina				
	0	1	3	5	7
TBI (g)	44.42 ± 2.39[a]	48.15 ± 3.68[ab]	51.90 ± 2.39[ab]	49.01 ± 2.81[ab]	55.32 ± 4.25[b]
TL (cm)	19.00 ± 0.33[a]	19.56 ± 0.40[a]	19.88 ± 0.37[a]	19.92 ± 0.34[a]	20.00 ± 0.43[a]
Survival (%)	82	83	84	85	87
SGR	4.15 ± 0.06[a]	4.20 ± 0.07[ab]	4.38 ± 0.05[ab]	4.25 ± 0.06[ab]	4.36 ± 0.07[b]
FCR	1.66 ± 0.04[a]	1.61 ± 0.96[a]	1.57 ± 0.06[a]	1.75 ± 0.23[a]	1.47 ± 0.21[a]
FE	0.60 ± 0.01[a]	0.63 ± 0.04[a]	0.64 ± 0.03[a]	0.60 ± 0.08[a]	0.71 ± 0.09[a]
GSI	1.59 ± 0.92[a]	2.64 ± 0.79[a]	1.34 ± 0.56[a]	1.05 ± 0.27[a]	3.90 ± 1.98[a]
HSI	1.65 ± 0.15[a]	1.53 ± 0.13[a]	1.53 ± 0.16[a]	2.99 ± 1.15[a]	1.63 ± 0.14[a]
VSI	4.36 ± 0.71[a]	3.30 ± 0.35[a]	3.98 ± 0.35[a]	3.61 ± 1.03[a]	3.54 ± 0.70[a]

[a,b] *Different superscripts indicate significant difference ($p<0.05$) of the same row. TBI: total biomass increase/fish; TL: total length; SGR: specific growth rate; FCR: feed conversion rate; FE: feeding efficiency; GSI: gonadosomatic index; HSI: hepatosomatic index and VSI: viserosomatic index. Values are presented in mean ± SEM. Number of sample is 15 for each group (5/replicate) at 14 days interval of sampling*

Table 4. Proximate composition of catfish's fillet after 90 days of experiment

Proximate composition	% of spirulina				
	0	1	3	5	7
Moisture	65.53 ± 0.61[a]	65.21 ± 1.07[a]	61.95 ± 0.90[a]	65.28 ± 2.71[a]	63.57 ± 1.43[a]
Ash	6.08 ± 0.23[a]	6.01 ± 0.18[a]	5.44 ± 1.29[a]	6.44 ± 0.20[a]	5.69 ± 0.38[a]
Crude fiber	1.40 ± 0.06[a]	1.62 ± 0.01[b]	1.83 ± 0.04[c]	2.07 ± 0.020[d]	2.13 ± 0.02[d]
Crude protein	20.64 ± 0.99[a]	21.43 ± 0.77[a]	21.43 ± 1.15[a]	22.57 ± 1.55[a]	23.69 ± 2.21[a]
Crude fat	0.45 ± 0.21[a]	0.52 ± 0.21[a]	0.22 ± 0.036[a]	0.43 ± 0.11[a]	0.49 ± 0.11[a]
Carbohydrate	5.90 ± 0.76[ab]	5.22 ± 0.69[ab]	9.13 ± 1.99[b]	2.09 ± 0.73[a]	5.55 ± 1.41[ab]

[a,b,c,d] *Different superscripts indicate significant difference ($p<0.05$) of the same row. Values are presented in mean ± SEM. A total of 15 catfish's fillets (5/replicate) were used for each group*

The nutritional composition of the catfish fillet generally showed insignificant differences of nutrient contents in all of the treatment groups, with the exception of crude fiber and carbohydrate. Similarly, supplementation of *Spirulina* did not change moisture and protein contents in rainbow trout muscle [32], protein, carbohydrate, fat, ash, and fiber in African catfish muscle [11], and moisture and ash in Nile tilapia (*Oreochromis niloticus*) muscle [16]. Moreover, according to Jafari et al. [12], *S. platensis* inclusion at ≥5 weight percent of loading in rainbow trout culture can be beneficial in terms of nutrient compositions of the fish fillet.

3.4 Survival Rate of Catfish against *Aeromonas hydrophila* Challenge

The highest survival rate of catfish against *A. hydrophila* challenge was the catfish supplemented with 7% spirulina (73.33%), which is significantly higher ($p<0.05$) compared to the control group, but not with the other diet groups (Table 5). All of the groups with inclusions of spirulina also showed significantly higher ($p<0.05$) survival rates compared to the control group. The lowest survival rate (36.67%) of catfish against *A. hydrophila* challenge was the catfish supplemented with 0% spirulina, where more than half of the cultured catfish did not survive the bacterial infection.

Table 5. Survival rate (%) of catfish supplemented with different ratio of spirulina following *Aeromonas hydrophila* challenge

Percentage of spirulina	Survival rates (%)
0	36.67 ± 0.00[a]
1	71.67 ± 1.67[b]
3	71.67 ± 1.67[b]
5	70.00 ± 10.00[b]
7	73.33 ± 10.00[b]

[a,b] *Different superscripts of the same column indicate significant difference ($p<0.05$). Values are presented in mean ± SEM. A total of 60 catfish (20/replicate) were challenged in each group*

The challenged fish showed clinical signs and symptoms such as skin ulcerations, bloated stomach, pale gills, and hemorrhagic internal

organs. Bacteria isolation and identification from the death fish organ's revealed the presence of *A. hydrophila*.

In this study, catfish supplemented with spirulina at even 1%, is able to survive up to 71.67% against the *A. hydrophila* challenge, whereas the catfish in the control group reported only 36% survivability. This showed that spirulina helps improve the immune system, lower stress, and normalize the body condition of the catfish [33]. The availability of phytopigments (phycobilins, phycocyanin, allophycocyanin, and xanthophylls) in the spirulina act as antioxidant which helps to protect the cellular component versus the oxidative stress [34] contributes to the survival rate of catfish against the *A. hydrophila* challenge. Vonshak [26] stated that spirulina possessed C-phycocyanin that stimulates the immune capacity. Furthermore, Nakono *et al*. [35] mentioned that the carotenoids content in spirulina improve fishes' health and increase its ability to fight infections by reducing stress levels. Indeed, the enhancement of the chemotaxis and phagocytosis, which is the nonspecific immune responses, can be achieved by feeding the spirulina to the reared fish [16]. Moreover, spirulina stimulate the activity of phagocytosis [36].

3.5 Hematological Profiles of Catfish Before and After Challenged by *Aeromonas hydrophila*

The results of catfish's blood parameters (RBC, Hb, PCV, MCV, MCHC and WBC) before being challenged with *A. hydrophila* did not show any significant difference ($p>0.05$) between different percentages of spirulina inclusion (Table 6). The RBC of the catfish before infection reported the lowest value, at 2.80 ± 0.14 ($\times10^{12}$/L) for catfish supplemented with 3% spirulina, while the highest value was 3.09 ± 0.14 ($\times10^{12}$/L) for catfish supplemented with 0% spirulina, while the Hb of the catfish before the infection reported the lowest value of 125.80 ± 7.32 g/L for catfish supplemented with 3% spirulina and the highest value was 138.00 ± 5.02 g/L for catfish supplemented with 0% spirulina. Moreover, before *A. hydrophila* infection, the PCV of the catfish showed mostly similar value of ~0.30 L/L for all the diet groups and the value of PCV of the catfish supplemented with 7% spirulina reported the highest value of 0.33 ± 0.02 L/L. The MCV of the catfish before the infection reported the lowest value of 102.40 ± 3.44 fL for catfish supplemented with 1% spirulina, and the highest value of 107.80 ± 1.16 fL for catfish supplemented with 7% spirulina, while MCHC reported the lowest value of 415.80 ± 5.81 g/L for catfish supplemented with 7% spirulina, and the highest value of 447.20 ± 15.39 g/L for catfish supplemented with 1% spirulina. Finally, in the case of the WBC, the readings showed that the lowest value was 0.07 ± 0.02 ($\times10^{9}$/L) for catfish supplemented with 5% spirulina, and highest value of 0.24 ± 0.09 ($\times10^{9}$/L) for catfish supplemented with 1% spirulina.

The hematological profile of the catfish blood after being challenged by *A. hydrophila* reported no significant difference ($p>0.05$) on the readings of the RBC and Hb for all of the used experimental diets. However, significant difference ($p<0.05$) between certain groups of catfish supplemented with different ratio of spirulina was observed for PCV, MCV, MCHC, and WBC parameters. The RBC of the catfish after infection reported the lowest value at 2.32 ± 0.10 ($\times10^{12}$/L) for catfish supplemented with 7% spirulina, and the highest value of 2.62 ± 0.10 ($\times10^{12}$/L) for catfish supplemented with 5% spirulina, while in the case of Hb, the lowest value was reported to be 27.60 ± 0.68 (g/L) for catfish supplemented with 7% spirulina, and the highest value was reported to be 8.98 ± 0.23 (g/L) for catfish supplemented with 5% spirulina. The PCV of the catfish after infection reported the lowest value being 26.00 ± 0.71 L/L for catfish supplemented with 7% spirulina, and the highest value was reported to be 32.00 ± 1.14 L/L for catfish supplemented with 3% spirulina. Significant difference ($p<0.05$) value was observed from these two readings, but not with the other diet groups. Moreover, in the case of MCV, the lowest value was reported to be 112.20±3.64 fL for catfish supplemented with 7% spirulina, while the highest value was reported to be 126.80±1.53 fL for catfish supplemented with 1% spirulina. These two groups diet readings were significantly different ($p<0.05$) from each other, but not with the other diet groups. Moreover, in the case of the MCHC, the lowest value was reported to be 27.60 ± 0.68 g/L for catfish supplemented with 3% spirulina while the highest value was reported to be 31.40 ± 0.75 g/L for catfish supplemented with 7% spirulina. The WBC of the catfish after infection showed the lowest value being 4.22 ± 0.50 ($\times10^{9}$/L) for catfish supplemented with 0% spirulina, and the highest value reported to be 82.48 ± 25.63 ($\times10^{9}$/L) for catfish supplemented with 7% spirulina. There was a significant difference ($p<0.05$) between the 0% group diets, with 1%, %, 5% and 7% of the supplemented spirulina groups diets.

Table 6. Hematological profiles of catfish before and after challenged by *Aeromonas hydrophila*

Blood parameter	0% spirulina		1% spirulina		3% spirulina		5% spirulina		7% spirulina	
	Before	After	Before	After	Before	After	Before	After	Before	After
RBC ($\times10^{12}$/L)	3.09±0.14	2.46±0.16^{a}	2.90±0.10	2.44±0.08^{a}	2.80±0.14	2.54±0.12^{a}	2.98±0.07	2.62±0.10^{a}	3.04±0.20	2.32±0.10^{a}
Hb (g/L)	138.00±5.02	8.46±0.42^{a}	132.60±5.03	8.94±0.30^{a}	125.80±7.32	8.74±0.27	133.40±4.31	8.98±0.23^{a}	136.20±8.92	8.10±0.25^{a}
PCV (L/L)	0.30±0.02	30.60±1.72^{ab}	0.30±0.017	31.20±0.97^{ab}	0.30±0.02	32.00±1.14^{b}	0.31±0.01	30.60±1.63^{ab}	0.33±0.02	26.00±0.71^{a}
MCV (fL)	103.20±5.77	125.20±2.94^{ab}	102.40±3.44	126.80±1.53^{b}	106.20±1.80	125.20±2.96^{ab}	103.00±3.16	117.80±3.88^{ab}	107.80±1.16	112.20±3.64^{a}
MCHC (g/L)	438.20±22.11	27.80±0.66^{a}	447.20±15.39	28.80±0.20^{ab}	423.60±7.63	27.60±0.68^{a}	434.00±8.24	29.40±0.93^{ab}	415.80±5.81	31.40±0.75^{b}
WBC ($\times10^{9}$/L)	0.09±0.04	4.22±0.50^{a}	0.24±0.09	48.88±10.37^{b}	0.10±0.06	44.22±13.51^{b}	0.07±0.02	63.46±10.85^{b}	0.16±0.06	82.48±25.63^{b}

^{a,b} Different superscripts indicate significant difference ($p<0.05$) of the same row after infection by A. hydrophila only. However, there was no significant difference ($p<0.05$) between all blood parameters of the same row before A. hydrophila infection. Values are presented in mean ± SEM. A total of 9 catfish (3/replicate) were used for each group before and after the challenged by Aeromonas hydrophila

Table 7. Histopathological scoring of the catfish's organs tissue before and after challenged by *Aeromonas hydrophila*

Organ	Histopathological changes	Before *A. hydrophila* challenge					After *A. hydrophila* challenge				
		0%	1%	3%	5%	7%	0%	1%	3%	5%	7%
Kidney	Congestion	0.20±0.20	0.00±0.00	0.20±0.20	0.40±0.24	0.00±0.00	2.00±0.00	2.00±0.00	1.60±0.24	2.00±0.32	1.60±0.24
	Necrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.80±0.20	1.40±0.24	1.60±0.24	1.80±0.37	1.80±0.20
Spleen	MMC	2.20±0.58	1.40±0.51	1.80±0.66	1.40±0.51	2.20±0.73	2.60±0.92^{a}	11.20±4.79^{c}	6.20±2.71^{b}	10.40±4.99^{c}	10.40±1.25^{c}
	Congestion	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.60±0.24	1.20±0.20	1.20±0.20	1.80±0.20	1.40±0.25
	Necrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.40±0.25	1.00±0.00	1.40±0.25	1.40±0.24	1.20±0.20
	White pulp	0.40±0.24	0.80±0.20	0.40±0.24	0.40±0.24	0.60±0.24	2.20±0.37	2.00±0.32	1.80±0.37	1.60±0.24	2.20±0.45
Liver	Congestion	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.60±0.24	1.40±0.24	1.40±0.24	1.60±0.24	1.20±0.20
	Necrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.80±0.37	1.20±0.20	1.80±0.37	1.80±0.20	1.80±0.37

^{a,b} Different superscripts indicate significant difference ($p<0.05$) of the same row after A. hydrophila challenge group only. However, there was no significant difference ($p>0.05$) between all spirulina diets in term of histopathological changes before A. hydrophila challenge. Values are presented in mean ± SEM. A total of 6 catfish (2/replicate) were used for each group before and after challenged by Aeromonas hydrophila

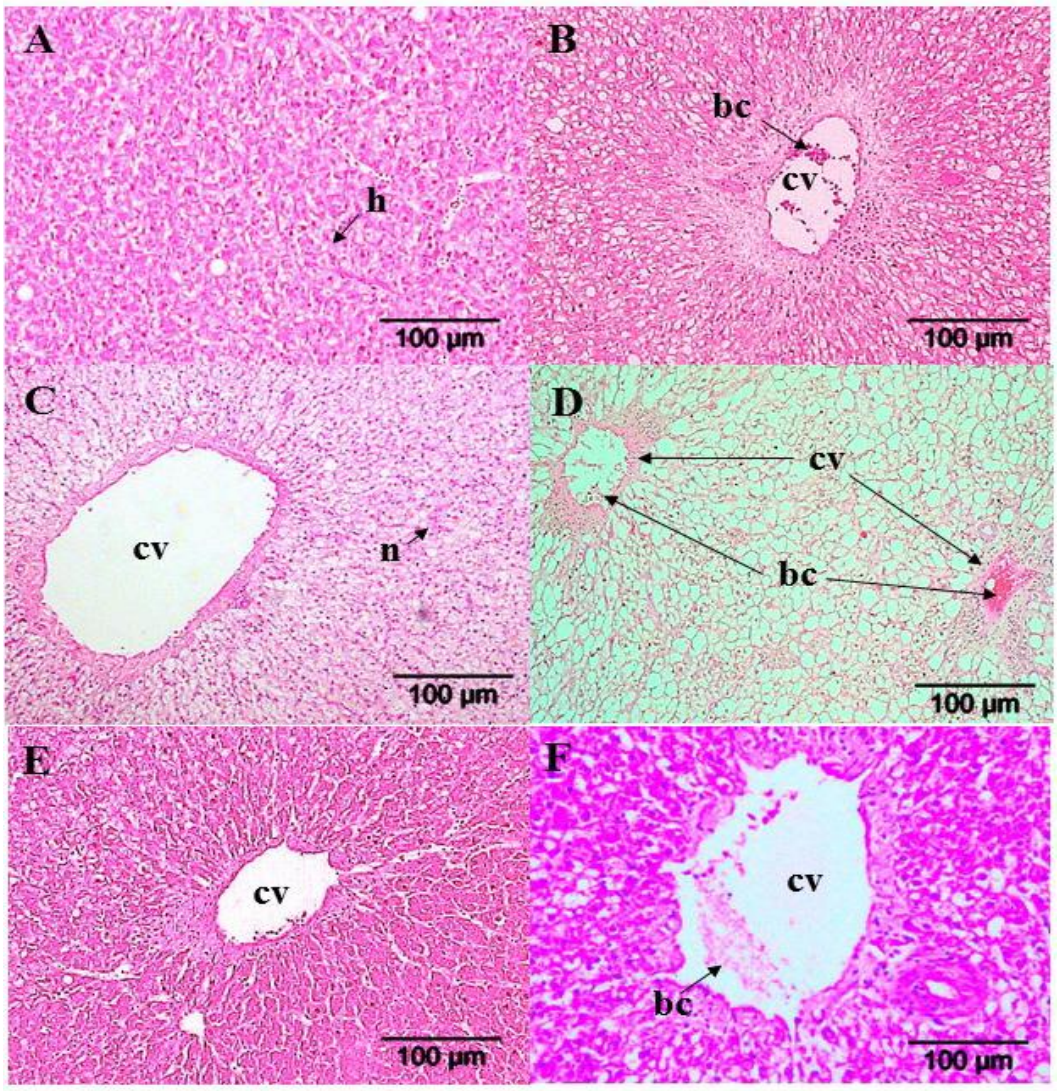

Fig. 1. Catfish liver stained with H&E under 100× magnification. A) Hepatic tissue in normal condition before *A. hydrophila* infection showed hepatocytes (h) and without any hemorrhage; B) Catfish liver supplemented with 0% spirulina after the infection showed central vein (cv) with blood congestion (bc); C) Catfish liver supplemented with 1% spirulina after the infection showed central vein (cv), generalized necrosis (n); D) Catfish liver supplemented with 3% spirulina after the infection showed central vein (cv) with blood congestion (bc); E) Catfish liver supplemented with 5% spirulina after the infection showed central vein (cv) with what mild blood congestion; F) Catfish liver supplemented with 7% spirulina after the infection showed central vein (cv) with blood congestion (bc)

The haematological profile of the catfish before *A. hydrophila* challenge was similar between treatments, indicating that the catfish was in normal condition [37]. However, after the challenge, the haematological profiles differed drastically. These signify that the blood composition encountered major changes when dealing with the infection due to exogenous factor, which include the stress and disease [38]. The reduction of RBC and Hb values can be an indication of poor oxygen transportation capacity of the fish. Amrevuawho et al. [38] stated that the oxygen carrying capacity of the fish was impaired by the bacteria showing poor oxygen transportation capacity. This explains the low value of RBC and Hb after the catfish challenge

with *A. hydrophila*. For the WBC, the value was significantly increased after the infection. As the body experienced pathogen invasion, the body front line defense system, which is the WBC, is activated to fight the infection by the pathogen [39]. Moreover, after the bacteria challenge, the catfish supplemented with spirulina reported higher WBC compared to catfish in the control groups, which explains the high survival rate of catfish against the *A. hydrophila* infection. Ravi et al. [40] pointed out that spirulina have a major impact on the immune system due to it increasing the phagocytic activity of macrophages and stimulating the NK cells, which explains the high value of WBC in catfish supplemented with spirulina.

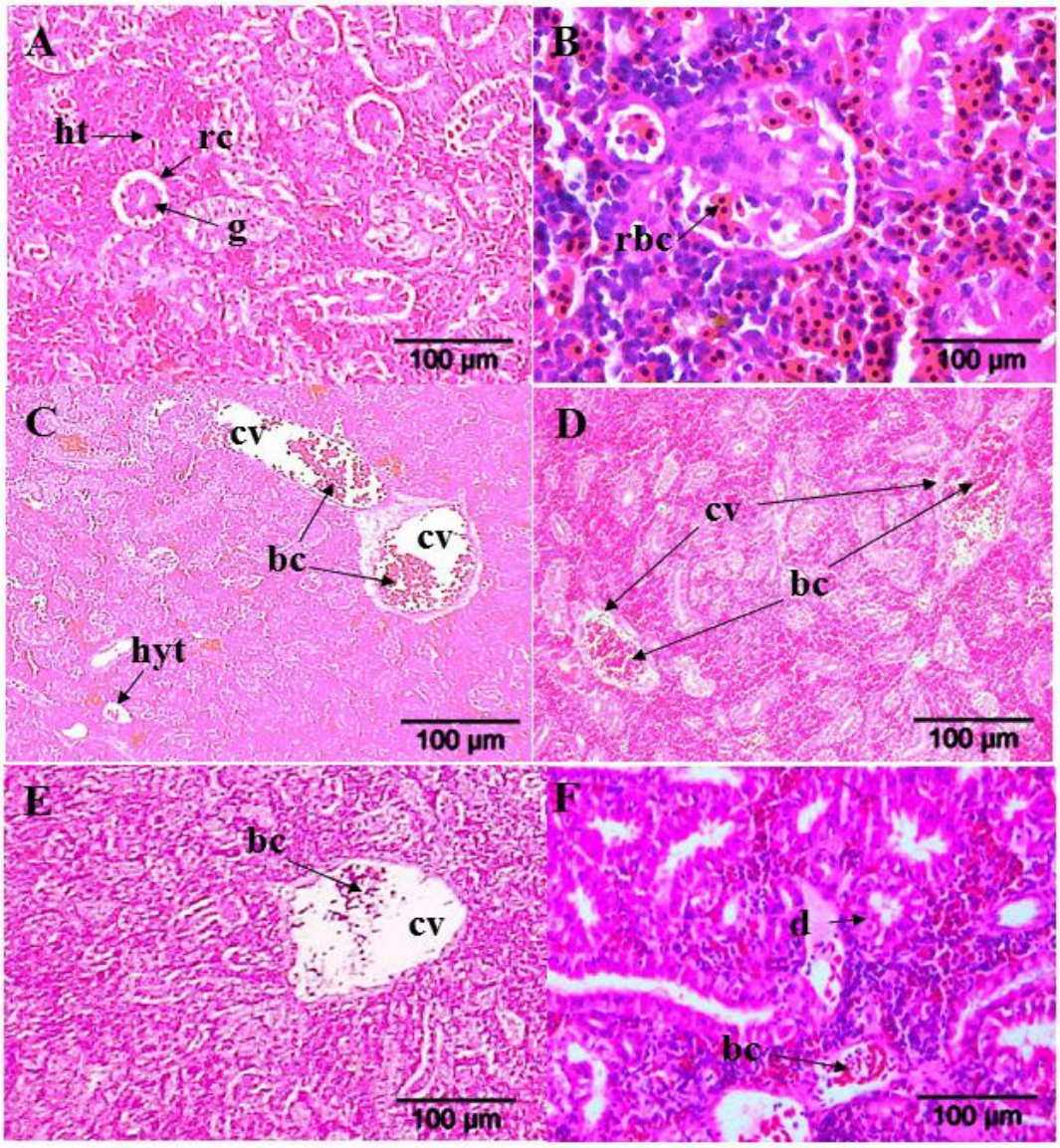

Fig. 2. Catfish kidney stained with H&E. A) Kidney of the catfish before the infection showed well-defined Bowman's capsule and glomerulus (g) of renal corpuscle (rc) and haemopoietic tissue (ht); B and C) Catfish kidney with 0% spirulina diet after the infection showed erythrocyte in glomerular capillaries (e), hypertrophy of glomerulus (hyt), central vein (cv) with blood congestion (bc); D and E) Catfish kidney supplemented with 1% and 3% spirulina showed central vein (cv) with blood congestion (bc); F) Catfish kidney supplemented with 7% spirulina showed degeneration (d) and blood congestion (bc). A, C and E under 40× magnification; B under 400× magnification; D and E under 100× magnification

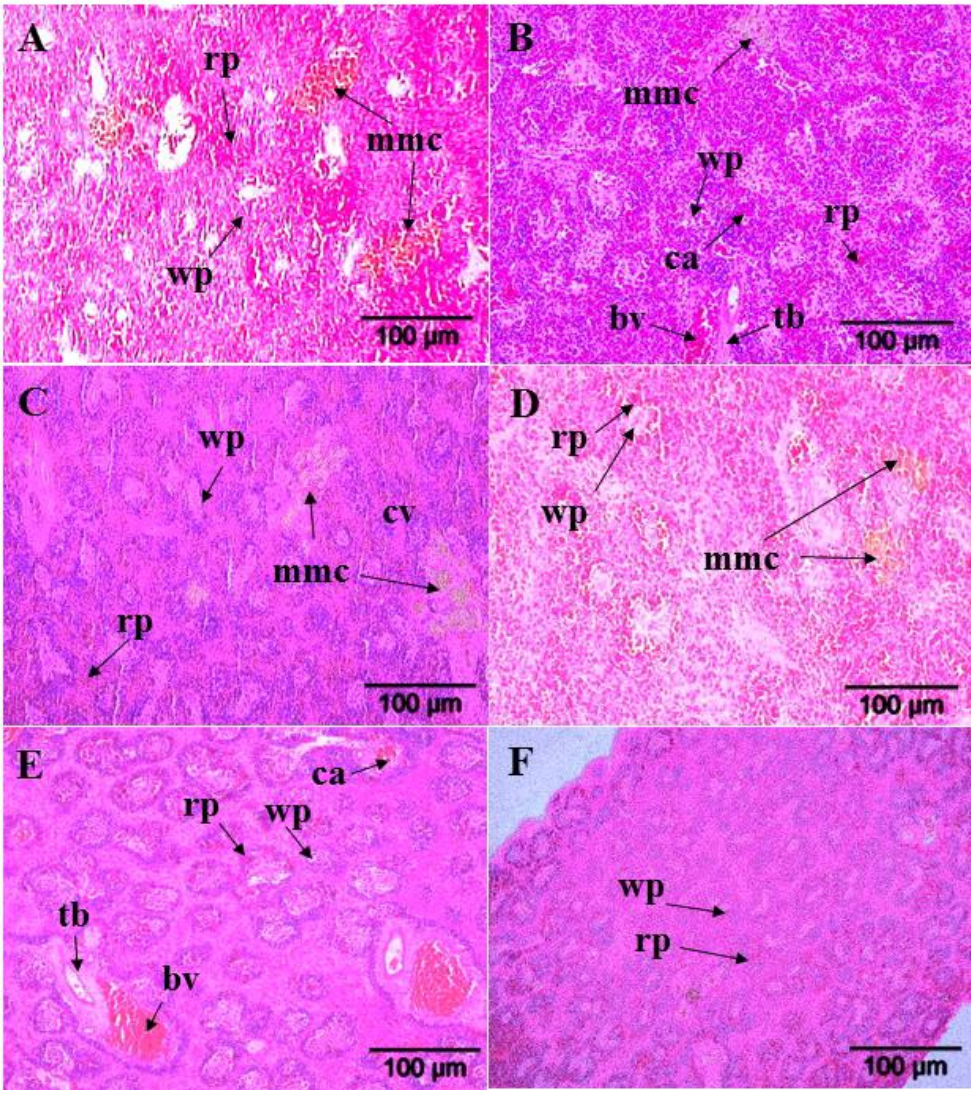

Fig. 3. Catfish spleen stained with H&E. A) Spleen of the catfish before the infection showed white pulp (wp), red pulp (rp) and melanomacrophage centre (mmc); B) Catfish spleen with 0% spirulina diet after the infection showed activation of white pulp (wp), red pulp (rp), melanomacrophage centre (mmc), trabeculae (tb) with blood vessel (bv) within, central artery (ca); C and D) Catfish kidney supplemented with 1% and 3% spirulina showed activation of white pulp (wp), red pulp (rp) and melanomacrophage centre (mmc); E) Catfish kidney supplemented with 5% spirulina showed activation of white pulp (wp), red pulp (rp), trabeculae (tb) with blood vessel (bv) within, central artery (ca); F) Catfish kidney supplemented with 7% spirulina showed activation of white pulp (wp) and red pulp (rp). A, C and F under 40× magnification; B, D and E under 100× magnification

3.6 Histopathological Changes of Catfish Organs Before and After Challenged by *Aeromonas hydrophila*

Generally, there are none to mild histopathological changes of the catfish's organs tissue prior to the *A. hydrophila* challenge. The numbers of MMC present in the spleens were counted, and there was no significant difference (p>0.05) among all of the diet groups. The white pulp in the spleen also scored as none to very mild prior the *A. hydrophila* challenge (Table 7).

However, after the *A. hydrophila* challenge, the congestion and necrosis in the kidneys scored mild for all catfish diets, without any significant difference (p>0.05) between them. The numbers of MMC present in the spleens were significantly higher (p<0.05) in the diet of 1% spirulina compared to 0% inclusion of spirulina, but not in the case of 5 and 7% of spirulina inclusions. The congestion and necrosis in the spleens scored from very mild, to mild in the diet groups, without significant difference (p>0.05) between them. The activation of the white pulps in the spleen after the *A. hydrophila* challenge scored as mild for all diet groups, without any significant difference (p>0.05). The congestion and necrosis of the livers of the catfish in the case of all different diet groups scored from very mild to mild without any significant difference (p>0.05).The histopathological changes and general descriptions of the affected organs are presented in Figs. 1, 2, 3.

Before the challenge period, the hepatic tissue of all catfish was in normal condition. As the catfish were challenged with *A. hydrophila*, there were changes in the histopathology of the catfish. As the liver is an organ that acts as an accumulation and detoxification medium [41], changes in the histology of the liver can be an indicator of invasion of pathogen or the disease infection. Laith and Najiah [42] reported that toxin produced and extracellular products (hemolysin, protease and elastase) of *A. hydrophila* caused necrosis in the liver. In this study, the livers of the infected catfish showed generalized necrosis and blood congestion in the central vein, similar to a previous report [42]. Moreover, Suprapto et al. [43] stated that the structural integrity of the kidney will be loss due to the bacterial toxin attacking the kidney. This is similar to what was observed in the infected catfish, including the congestion in the central vein, hypertrophy of glomerulus and the presence of erythrocyte in glomerular capillaries. Aqius and Roberts [44] reported the presence or the development of MMC in the spleen being associated with chronic inflammatory lesions elsewhere in the body. As *A. hydrophila* is known for causing septicemic condition, the number of MMC increase in the spleens of infected catfishes. MMC also play an important role in focal depositories for resistant intracellular bacteria from which chronic infections could develop [44]. This explains the frequent presence and increase in number of MMC in the infected catfish spleens, which contribute to better adaptation against infections [40].

4. CONCLUSION

In conclusion, catfish supplemented with 1% to 7% of locally grown spirulina did not significantly improve fish growth performances and body composition. However, the application of the spirulina in catfish diet even at 1% would protect catfish against *A. hydrophila* infection.

ETHICAL APPROVAL

The fish were handled and sacrificed according to methods approved by Institutional Animal Care and Use Committee, Universiti Putra Malaysia.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Adan RIY. Catfish culture in Southeast Asia. SEAFDEC Asian Aquac. 2000;22:16-17.
2. Department of Fisheries (DOF). Annual Fisheries Statistic. Department of Fisheries, Ministry of Agriculture and Agro-Based Industry, Malaysia; 2013.
3. Jaime-Ceballos BJ, Hernández-Llamas A, Garcia-Galano T, Villarreal H. Substitution of *Chaetoceros muelleri* by *Spirulina platensis* meal in diets for *Litopenaeus schmitti* larvae. Aquaculture. 2006;260: 215-220.
4. Mustafa MG, Takeda T, Umino T, Wakamatsu S, Nakagawa H. Effects of *Ascophyllum* and *Spirulina* meal as feed additives on growth performance and feed utilization of red sea bream, *Pagrus major*. J Fac Appl Biol Sci Hiroshima Uni.1994;33: 125-132.
5. Muller-Feuga A. The role of microalgae in aquaculture: situation and trends. J Appl Phycol. 2000;12:527-534.
6. Natrah FMI, Yusoff FM, Shariff M, Abas F, Mariana NS. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. J Appl Phycol. 2007;19:711-718.

7. Nandeesha MC, Gangadhara B, Manissery JK, Venkataraman LV. Growth performance of two Indian major carps, catla (*Catla catla*) and rohu (*Labeo rohita*) fed diets containing different levels of *Spirulina platensis*. Bioresour Technol. 2001;80:117-120.
8. Teimouri M, Amirkolaie AK, Yeganeh S. The effects of *Spirulina platensis* meal as a feed supplement on growth performance and pigmentation of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 2013;396-399:14-19.
9. Adel M, Yeganeh S, Dadar M, Sakai M, Dawood MAO. Effects of dietary *Spirulina platensis* on growth performance, humoral and mucosal immune responses and disease resistance in juvenile great sturgeon (*Huso huso* Linnaeus, 1754). Fish Shellfish Immunol. 2016;56:436-444.
10. Khalil SR, Reda RM, Awad A. Efficacy of *Spirulina platensis* diet supplements on disease resistance and immune-related gene expression in *Cyprinus carpio* L. exposed to herbicide atrazine. Fish Shellfish Immunol. 2017;67:119-128.
11. Promya J, Chitmanant C. The effects of *Spirulina platensis* and *Cladophora* algae on the growth performance, meat quality and immunity stimulating capacity of the African sharptooth catfish (*Clarias gariepinus*). IJAB. 2011;13:77-82.
12. Jafari SMA, Rabbani M, Emtyazjoo M, Piryaei F. Effect of dietary *Spirulina platensis* on fatty acid composition of rainbow trout (*Oncorhynchus mykiss*). Aquac Int. 2014;22:1307-1315.
13. Khanzadeh M, Fereidouni AE, Berenjestanaki SS. Effects of partial replacement of fish meal with *Spirulina platensis* meal in practical diets on growth, survival, body composition, and reproductive performance of three-spot gourami (*Trichopodus trichopterus*) (Pallas, 1770). Aquac Int. 2016;24:69-84.
14. Pridgeon JW, Klesius PH. Major bacterial diseases in aquaculture and their vaccine development. CAB Reviews. 2012;27:48.
15. Food and Agriculture Organization of the United Nations (FAO). Fisheries and Aquaculture Department; 2017.
16. Abdel-Tawwab M, Ahmad MH. Live spirulina (*Arthrospira platensis*) as a growth and immunity promoter for Nile tilapia, *Oreochromis niloticus* (L.), challenged with pathogenic *Aeromonas hydrophila*. Aquacult Res. 2009;40:1037-1046.
17. Al Mahrouqi H, Naqqiuddin MA, Achankunju J, Omar H, Ismail A. Different salinity effects on the mass cultivation of spirulina (*Arthrospira platensis*) under sheltered outdoor conditions in Oman and Malaysia. J Algal Biomass Utln. 2015;6:1-14.
18. Howaida RG, Hanlon TH, Mahmoud HH, Eletreby SG. Maturation, fecundity, seasonality and reproduction of two commercially valuable cuttlefish, *Sephia pharaonis* and *S. dollfusi* in Suez Canal. J Fish Res. 1998;36:99-115.
19. Handeland SO, Imsland AK, Stefansson SO. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. Aquaculture. 2008;283:36-42.
20. Gislason H, Daan N, Rice JC, Pope JG. Size, growth, temperature and the natural mortality of marine fish. Fish Fish. 2010;11: 149-158.
21. Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliott M, Farrell AP, Nichols PD. Feeding aquaculture in an era of finite resources. Proc Natl Acad Sci USA. 2009;106:15103-15110.
22. Ighwela KA, Ahmad A, Abol-Munafi AB. The selection of viserosomatic and hepatosomatic indices for the measurement and analysis *Oreochromis niloticus*condition fed with varying dietary maltose levels. Inter J Fauna Biol Stud. 2014;1:18-20.
23. AOAC. Official method of analysis of the association of official analytical chemists. No. 934.06, AOAC, Arlington; 1990.
24. Saleema M. Molecular characterization of *Aeromonas hydrophila* and development of recombinant cells vaccine expressing outer membrane proteins against its in African catfish (*Clarias gariepinus* Burchell). MSc, Universiti Putra Malaysia, Selangor, Malaysia; 2015.
25. Popovic TN, Strunjak-Perovic I, Coz-Rakovac R, Barisic J, Jadan M, Persin Berakovic A, Klobucar SR. Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. J Appl Ichthyol. 2012;28: 553-564.
26. Vonshak A. *Spirulina platensis* (*Arthospira*): Physiology, Cell Biology and Biotechnology. Bristol, USA: Taylor & Francis; 1997.

27. de Oliveira MACL, Monteiro MPC, Robbs PG, Leite SGF. Growth and chemical composition of *Spirulina maxima* and *Spirulina platensis* biomass at different temperatures. Aquac Int. 1999;7:261-275.
28. Khan Z, Bhadouria P, Bisen PS. Nutritional and therapeutic potential of Spirulina. Curr Pharm Biotechnol. 2005;6:373-379.
29. Kulshreshtha A, Jarouliya U, Bhadauriya P, Prasad GBKS, Bisen PS. Spirulina in health care management. Curr Pharm Biotechnol. 2008;9:400-405.
30. Sharma V, Dunkwal V. Development of spirulina based biscuits: A potential method of value addition. Stud Ethno-Med. 2012;6:31-34.
31. Palmegiano GB, Agradi E, Forneris G, Gail F, Gasco L, Rigamonti E, Sicuro B, Zoccarato I. Spirulina as a nutrient source in diets for growing sturgeon (*Acipenser baeri*). Aquacult Res. 2005;36:188-195.
32. Teimouri M, Yeganeh S, Amirkolaie AK. The effects of *Spirulina platensis* meal on proximate composition, fatty acid profile and lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*) muscle. Aquac Nutr. 2016;22:559-566.
33. Kostromina VP, Derkach OV, Symonenkova NV, Riechkina OO, Otroshchenko AO. Evaluation of the efficacy of a plant adaptogen (*Spirulina*) in the pathognic therapy of primary tuberculosis in children. Lik Sprava. 2002;5-6:102-105.
34. Mohan IK, Khan M, Shobha JC, Naidu MU, Prayag A, Kuppusamy P. Protection against cisplatin-induced nephrotoxicity by *Spirulina* in rats. Cancer Chemother Pharmacol. 2006;58:802-808.
35. Nakono T, Yamaguchi T, Sato M, Iwama GK. Biological effects of carotenoids in fish. In: Proceedings of the JSPS International Seminar on Effective Utilization of Marine Food Resource. Kasetsart University, Bangkok. 2003;26-41.
36. Duncan PL, Klesius PH. Effects of feeding *Spirulina* on specific and non-specific immune responses of channel catfish. J Aquat Anim Health. 1996;8:308-313.
37. Sayed AH, Fawzy AM. Effect of dietary supplementation of *Spirulina platensis* on the growth and haematology of the catfish *Clarias gariepinus*. JAB. 2014;5:625-635.
38. Amrevuawho MO, Akinyemi AA, Ezeri GNO, Bankole OM, Takeet OVA. Pathological study of *Clarias gariepinus* (Burchell, 1822) sub-adult artificially infected with *Pseudomonas aeruginosa*. Brazilian J Aquacult Sci Technol. 2014;18:65-70.
39. Castro R, Tafalla C. Overview of fish immunity. In: Mucosal Health in Aquaculture. 1st ed. New York, NY, USA: Academic Press; 2015.
40. Ravi M, De SL, Azharuddin S, Paul SFD. The beneficial effects of spirulina focusing on its immunomodulatory and antioxidant properties. Nutr Diet Suppl. 2010;2:73-83.
41. Brusle J, Anadon GG. The structure and function of fish liver. In: Fish Morphology. Vermont, USA: Science Publishers; 1996.
42. Laith AR, Najiah M. *Aeromonas hydrophila*: Antimicrobial susceptibility and histopathology of isolates from diseased catfish, *Clarias gariepinus* (Burchell). J Aquac Res Dev. 2013;5:1-7.
43. Suprapto H, Sumartiwi L, Prawesthirini S, Handiyatno D, Azmijah A. The isolation of *Aeromonas hydrophila* and *Escherichia coli* from Lou Ham *Cichlasoma synspilum* and studies of their histopathology changes. J Biol Res. 2005;10:139-141.
44. Aqius C, Roberts RJ. Melano-macrophage centres and their role in fish pathology. J Fish Dis. 2003;26:499-509.

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