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Effects of dietary supplementation of natural Spirulina on growth performance, hemato-biochemical indices, gut health, and disease resistance to *Aeromonas hydrophila* of Stinging catfish (*Heteropneustes fossilis*) fingerling

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ABSTRACT

Spirulina is a popular edible blue-green alga that has piqued the interest of aqua-feed formulations due to its high concentration of micronutrients, macronutrients, and antioxidants. To investigate the impacts of natural Spirulina as a feed additive on Stinging catfish (*Heteropneustes fossilis*) growth, feed utilization, hematology and serum biochemistry, gut morphology, as well as disease resistance to *Aeromonas hydrophila,* a 60-day feeding experiment was performed. The fish (N = 240), with an initial weight of 4.23 ± 0.50 g, were placed in 12 aquaria tanks (100 L/ tank) and fed twice daily until they were satisfied. Four different experimental diets: 0% (D0, control diet), 1% (D1), 3% (D2), and 5% (D3) natural Spirulina meal were used to feed the fish. This study found that Stinging catfish fed with a 5% Spirulina diet obtained notably higher ($p < 0.05$) specific growth rate, final weight, weight gain, and average daily weight gain than other supplemented regimens. Furthermore, as Spirulina inclusion in diets increased, feed utilization parameters such as feed conversion ratio and protein efficiency ratio improved remarkably ($p < 0.05$). Red blood cell, white blood cell, lymphocytes, neutrophil, monocytes, basophil, hemoglobin, red cell distribution width-standard deviation, and mean corpuscular hemoglobin were all substantially ($p < 0.05$) greater in 5% Spirulina diet-fed fish. The basal diet-fed fish had lower ($p < 0.05$) total protein, globulin, and albumin levels and higher glucose, cholesterol, and triglyceride levels than those fed with other test diets. Compared to other fish groups, light microscopic examination of intestinal tissues revealed that fish fed 3% and 5% Spirulina had well-organized enterocytes, intact epithelial barrier, abundant goblet cells, and lacking luminal cell debris, as well as exhibited no signs of inflammation (edema). Spirulina diets significantly ($p < 0.05$) affected intestinal villi height and width, mucosa width, crypt depth, villi, and lumen area. Furthermore,

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a 5% Spirulina diet-fed fish demonstrated remarkably ($p < 0.05$) greater resistance (68.57%) to *A. hydrophila* during the bacterial challenge trial. Based on the findings, applying natural Spirulina powder at 5% in diets as a feed supplement could benefit *H. fossilis* nutrient utilization, growth performance, health status, as well as disease resistance.

1. Introduction

The stinging catfish (*H. fossilis*) is essential and has great potential in hatcheries and fish farming. Superior growth and survival, an excellent nutritional profile, and customer preference have recently sparked interest in cultivating this species in constrained spaces (Arifa et al., 2022; Nandi et al., 2023). The main restrictions to reproducing this species in aquaculture are a need for more quality fry, a poor production status, and a lack of quality feed and materials (Mahalder et al., 2023; Nandi et al., 2023). Furthermore, disease outbreaks in the catfish aquaculture industry can cause significant production losses (Mamun et al., 2023; Suma et al., 2023). For instance, Stinging catfish are susceptible to bacterial infections, particularly *Aeromonas hydrophila* and such outbreak can result in both external and internal organ damage, leading to significant fish mortality (Monir et al., 2015). In Bangladesh, there is a huge growth in demand for stinging catfish cultivation. However, the wild fry supply is insufficient to meet the needs of commercial aquaculture farms (Ali et al., 2014). Several antibiotics and medicines have been utilized to treat as well as control infectious diseases. Nonetheless, the European Union (EU) and many other countries have prohibited using these harmful substances in aqua-farming systems owing to their negative impacts on fish, the environment, as well as human health (Kari et al., 2022). As a result, there is an increasing need to identify natural bioactive compounds and immunostimulants that modify the fish's immune system without compromising their health (Cao et al., 2020). Using the different types of Cyanobacteria, particularly Spirulina (*Arthrospira platensis*) meal, in fish diets may be a good way to solve these issues.

Microalgae have lately emerged as a natural alternative dietary source of novel bioactive compounds (Yang et al., 2023), and they have garnered significant attention in the aquaculture industry. Spirulina is a microscopic, filamentous, multicellular, and photosynthetic blue-green alga commonly used in animal feed as a safe and functional supplement (Akter et al., 2023; Amer, 2016; Wan et al., 2021). Spirulina's higher quantities of protein, amino acids, fatty acids, vitamins, minerals, pigments, and digestive enzymes are regarded to be beneficial to health (Amer, 2016; Bortolini et al., 2022; Cao et al., 2018; Gogna et al., 2023; Liestianty et al., 2019; Roohani et al., 2019; Wan et al., 2021). Spirulina also exhibits anti-inflammatory and antioxidant characteristics owing to the presence of bioactive components, e.g., chlorophyll, phenol, β-carotene, and phycobiliprotein (Carcea et al., 2015; Chentir et al., 2018; Gogna et al., 2023; L. C. Li et al., 2022; L. Li et al., 2022). Spirulina possesses a smooth body with a delicate cell wall, making it highly digestible (Mathur, 2018). Spirulina meal (SM) in diets increases host defense mechanisms (Amer, 2016; Sattanathan et al., 2020). In addition, Abdulrahman (2014) discovered that the inherent palatability problem in SM might considerably improve fish growth performance while reducing feed waste.

Spirulina is enriched with gamma-linolenic acid, phenolic substances, and phycocyanin which helps aquatic animals resist pathogens and diseases (Mamun et al., 2023; Silva et al., 2021). Numerous studies have been carried out to evaluate the impacts of graded levels of Spirulina inclusion on feed utilization, growth performance, health status, as well as disease resistance of various fish species, including of *Huso huso* (Adel et al., 2016), *Oreochromis niloticus* (Al-Deriny et al., 2020; Amer, 2016; Velasquez et al., 2016), *Botia Dario* (Gogoi et al., 2018), *Clarias gariepinus* (Hamed et al., 2019; Sayed et al., 2021), *Salmo trutta caspius* (Roohani et al., 2019), *Ompok pabda* (Akter et al., 2023), *Astronotus ocellatus* (Mohammadiazarm et al., 2021), *Dicentrarchus labrax* (Güroy et al., 2022), and *Mystus cavasius* (Mamun et al., 2023). However, the influences of SM supplementation on Stinging catfish production are very limited. Meanwhile, determining the optimal levels of supplementation of feed ingredients, particularly Spirulina, could significantly impact large-scale aquaculture production. Hence, the current study sought to examine the influences of Spirulina supplementation at various percentages (0%, 1%, 3%, and 5%) to determine the optimum levels for superior growth and nutrient utilization, hemato-biochemical parameters, gut health, and specific disease resistance in Stinging catfish aquaculture in captivity.

2. Materials and method

2.1. Diet preparation and chemical composition

Table 1 illustrates the feed ingredients as well as the proximate composition of test diets. All the feed ingredients (except the Acilina meal) were purchased from Bandar Bazar, Sylhet, Bangladesh. The Spirulina (*A. platensis*) was bought from Advanced Chemical Industries Health (ACI Limited, Sylhet, Bangladesh). Preparation of the four iso-nitrogenous diets (crude protein 34%) was performed with various levels of Spirulina supplementation at 0% (D0, control diet), 1% (D1), 3% (D2), and 5% (D3), respectively. Shortly, all the finely grounded feed ingredients, i.e., binder, fish meal, rice bran, mustard oil cake, wheat bran, flour, palm oil, and vitamin-mineral premix, were mixed homogenously with Acilina powder for 30 min and then passed through an extruder (LM40 floating fish feed machine, Henan Lima Machinery Manufacture Co. Ltd., Zhengzhou, China) to prepare 2 mm pellets. Then, the extruded pellets were oven-dried for 8 hrs at 60°C, allowed to cool at room temperature, packed in a plastic bag, and kept in a cool and dry place until use. Proximate analysis of basal and Spirulina-incorporated diets was accomplished following the procedure outlined by the Association of Official Analytical Chemists (AOAC, 1990) as shown in

Table 1
Feed ingredients and proximate composition (% dry matter basis) of test diets.

Ingredients (g/100 g)	D0	D1	D2	D3
Fish meal	42	41	39	37
Spirulina powder	0	1	3	5
Mustard oil cake	20	20	20	20
Rice bran	10	10	10	10
Wheat bran	10	10	10	10
Flour	8	8	8	8
Palm oil	4	4	4	4
Vitamin and minerals premix[a]	3	3	3	3
Binder[b]	3	3	3	3
Total	100	100	100	100
Proximate composition (%)				
Moisture	8.47	8.37	8.41	8.39
Crude protein	34.01	34.07	34.5	34.66
Crude lipid	7.49	7.36	7.56	7.43
Crude fiber	5.59	5.87	5.91	6.15
Ash	7.27	7.34	7.53	7.35
NFE[c]	37.17	36.99	36.09	36.02

[a] Vitamin and mineral premix, each kg of premix contained vitamin A (7000000 IU), vitamin D3 (70000 IU), vitamin E (250 mg), cobalt (150 mg), copper (1200 mg), iodine (325 mg), iron (1500 mg), magnesium (6000 mg), potassium (100 mg), sodium (5.9 mg), manganese (1500 mg), sulfur (0.72%), zinc (9600 mg), DL-methionine (1000 mg), calcium (25.5%) and phosphorus (12.75%)

[b] Carboxymethyl Cellulose

[c] NFE, Nitrogen free extract.

Table 1. In short, the moisture content was determined by subjecting the triplicate feed samples to oven-drying at 105°C for 24 h; crude protein was evaluated employing Kjeldahl method, crude lipid was estimated through n-hexane extraction using Soxhlet apparatus, crude fibre was conducted employing the hot extraction unit, and ash content was assessed with the Muffle furnace at 550 °C for 6 h.

2.2. Experimental fish collection and rearing

One thousand Stinging catfish fingerling (average weight: 4.23 ± 0.50 g) were collected from Shohag Motsho Khamar, Bahubal, Hobigonj, and then immediately moved to the wet laboratory of the Department of Fish Health Management, Faculty of Fisheries, Sylhet Agricultural University (SAU). To acclimatize with the new environment, the fingerlings were stocked in an aquarium (73 ×35 ×38 cm) for seven days and fed with a basal diet that had 6% crude lipid and 36% crude protein. After that, 240 fingerlings were hand-counted and randomly distributed into 12 aquaria tanks (capacity: 100 L/ tank) with a stocking of 20 fish/ tank. Every tank was facilitated with a good water supply and aeration system to ensure that dissolved oxygen and other water quality factors remained at optimum levels. Feeding was done two times a day at 9.00 a.m. and 5.00 p.m. for 60 days until apparent satiation. The experiment consisted of 4 biological treatments with three replicates each. Once in two days, wastes from the tank were siphoned; subsequently, the replacement of 20% of the water was performed.

2.3. Recording of water quality parameters

The experimental fish containing each tank water quality variables including temperature (°C), water pressure (mm Hg), dissolved oxygen (ppm), conductivity (Siemens/ meter), total dissolved solid (ppm), salinity (ppt), and pH were monitored at 7-day intervals throughout the 60-day experiment with a multiparameter probe (HI 9828, YSI Incorporation, Yellow Spring, OH, USA). Moreover, ammonia (ppm) tank water levels were investigated using HACH test kit (HI 28049, HACH, USA).

2.4. Calculation of growth performance

After a 60-day nutritional trial, all the experimental fish were captured and subjected to anesthesia with MS_{222} at a dose of 0.1 g/L water. Following that, the final fish weight was individually recorded, and the survival rate of each replica tank was checked. The total feed intake by each tank containing fish over the experiment was recorded daily. To determine the growth parameters of the fish, the following formulae (Kari et al., 2023) were utilized:

i. Weight gain (g) = Mean final weight (g) - Mean initial weight (g)
ii. Weight gain (%) = (Mean final weight - Mean initial weight)/ (Mean initial weight) × 100
iii. Average daily weight gain (g/day) = (Final weight - Initial weight)/ Days of experiment
iv. Specific growth rate (% /day) = [ln final weight - ln initial weight/ Days of experiment] x100
v. Feed conversion ratio = Feed intake/ Final weight gain
vi. Protein efficiency ratio = (Weight gain/ feed consumed) × percentage of protein in feed
vii. Survival rate (%) = [(Number of fish harvested)/ (Number of fish stocked at the start of the experiment)] × 100

2.5. Hematology and biochemical indices

From each tank, three fish were randomly captured and anesthetized with MS_{222} at a dose of 0.1 g/L water after the termination of the 60-day feeding experiment. In order to collect the blood sample, the caudal vein was punctured using a 1 mL syringe rinsed with anticoagulant (10% EDTA) and then immediately placed in EDTA-coated vials. To collect serum samples, blood was obtained in a 1.5 mL centrifuge tube without any anticoagulant. The hematological parameters were enumerated using an automatic hematology analyzer (PE-7010) in the Department of Physiology, Faculty of Veterinary, Animal and Biomedical Sciences, SAU. Moreover, the biochemical parameters were analyzed by a clinical chemistry analyzer (AGD-2020) at the Laboratory of Fish Disease Diagnosis and Pharmacology, Faculty of Fisheries, SAU. Using kits (AGD Biomedicals Pvt. Ltd., Mumbai, India), serum parameters were analyzed.

2.6. Histological observation of intestine

At the end of the trial, the fish were euthanized. Then, excision, rinsing in normal saline, and fixation of each fish intestine in 10% buffered formalin for 72 hr was performed. After that, the gut samples (9 fish from each treatment) were dehydrated in graded ethanol, embedded in paraffin, and sectioned at 5–6 μm (Thermo Scientific, Microm HM-325, USA). Using the hematoxylin-eosin solution (H&E), sections of the tissues were stained. To check for any signs or damage, the tissue sections on the glass slides were observed and analyzed using a light microscope (Carl Zeiss GmbH, 415500–0057–000, Germany). Following the method of Bullerwell et al. (2016), villus height, villus area, villus width, crypt depth, lumen area, and intestinal wall thickness were recorded by utilizing an image analysis application software (ImageJ, Wayne Rasband). As many villi as possible were recorded, up to 10 villi per slide and no less than five. If more than ten were able to be recorded, the villi were selected to be as equally spaced around the intestine sample as possible. The exclusion of the slides with fewer than six suitable villi per slide was considered.

2.7. Experimental infection with *Aeromonas hydrophila*

2.7.1. Collection and maintenance of *A. hydrophila*

A. hydrophila was previously isolated from disease-infected catfish, *Clarias batrachus* (Rahman et al., 2021), where its identification was later confirmed by PCR as well as gene sequence (GB Accession number: ON833076.1). The isolate was obtained from the Laboratory of Fish Disease Diagnosis and Pharmacology, Department of Fish Health Management, SAU. Using nutrient agar slants, subcultures were maintained at 4 °C, while tryptone soya (TSB, Himedia, India) was used for bacterial inoculation. An overnight (12 hrs) incubation of the broth was performed in a shaker at 37 °C and harvested at 0.8 optical density (OD) at 600 nm. Using 1.5% TSB with 20% glycerol, the stock culture was preserved at − 20 °C until use.

2.7.2. LD_{50} of *A. hydrophila*

Following Reed and Muench (1938), the mean lethal dose (LD_{50}) was estimated in Stinging catfish. With adequate aeration, the fingerlings were able to maintain in aquarium tanks (10 fish/ aquarium). To eradicate the waste materials as well as to maintain better water quality, 20% of the tank's water was siphoned off every alternate day. The tank water hydrological variables were found to be optimum levels: temperature (27.02 ± 0.09 °C), pH (7.99 ± 0.13), DO (5.12 ± 0.12 ppm), and ammonia (0.13 ± 0.03 ppm). The trial was done in triplicates. An overnight growing of the isolate was performed at 37 °C on the TSB medium, and cell suspensions were prepared in phosphate-buffered saline (PBS). A 0.1 mL *A. hydrophila* inoculation (10^2 to 10^9 CFU/ mL) was injected intra-peritoneally, while 0.1 mL of PBS was injected to control fish group. On a daily basis, the mortality of the experimental fish was recorded for ten days.

2.7.3. Challenge trial

After a 60-day feeding experiment, 15 fish from each treatment group, including the positive control, were injected with 0.1 mL of *A. hydrophila* (LD_{50}–2.3 × 10^7 CFU/mL) and fed with various experimental diets. The fish injected with 0.1 mL PBS served as a negative control. During the bacterial challenge, fish were checked three times a day, i.e., morning (7.30 a.m.), afternoon (3.30 p.m.), and night (10.30 p.m.), to observe any signs of infection. The numbers of bacterial infected fish were recorded every day throughout the 15-day challenge trial and then eliminated to estimate percent survival.

2.8. Statistical analysis

Data were analyzed by utilizing one-way analysis of variance (ANOVA), and the mean values were ranked and compared by using Duncan multiple range tests, employing the software program SPSS version 20.1 for Windows. Statistically significant differences were considered when $p < 0.05$. Data are expressed as mean ± standard error (M ± SE).

3. Results

3.1. Water quality parameters

Table 2 represents the hydro-ecological variables of tank water over a 60-day experiment. In this study, the quality of the water parameters, including temperature, water pressure, DO, conductivity, salinity, TDS, pH, as well as ammonia contents, was not remarkably ($p > 0.05$) impacted by different levels of Spirulina-treated diets.

3.2. Feed utilization and growth parameters

The feed utilization and growth indices of fish fed with graded levels of Spirulina diets are mentioned in Table 3. Remarkable variations ($p < 0.05$) were noted in the fish feed utilization and growth parameters in terms of final weight (FW), weight gain (WG), average daily weight gain (ADWG), protein efficiency ratio (PER), feed conversion ratio (FCR), as well as specific growth rate (SGR) among the treatment groups of Spirulina. *H. fossilis* fed with a D3 diet (5% Spirulina) showed the notably ($p < 0.05$) greatest SGR, FW, WG, and ADWG in comparison with the rest test diets. Additionally, the fish's feed utilization capacity was remarkably ($p < 0.05$) improved with the elevation of Spirulina incorporation levels in diets. However, different diet groups did not influence ($p > 0.05$) the fish survival rate.

3.3. Hematological parameters

Blood hematological parameters of *H. fossilis* fed with Spirulina diets are depicted in Table 4. The majority of the hematological indices were substantially ($p < 0.05$) varied among the treated groups of Spirulina diets, except for eosinophil (Eos) and platelet distribution width (PDW).

Table 2
Hydro-ecological parameters of tank water during the experiment. Data are expressed as mean ± standard error (M ± SE).

Parameters	Diets (% Spirulina)			
	D0	D1	D2	D3
Temperature (°C)	27.22 ± 0.22	27.50 ± 0.29	26.90 ± 0.06	26.90 ± 0.13
Pressure, mm (Hg)	756.30 ± 1.30	753.73 ± 2.20	754.57 ± 1.34	756.03 ± 0.50
DO (ppm)	4.97 ± 0.21	4.92 ± 0.31	5.53 ± 0.09	5.52 ± 0.05
Conductivity (Siemens/ meter)	177.12 ± 18.09	178.88 ± 25.12	194.30 ± 21.49	181.23 ± 21.63
TDS (ppm)	84.83 ± 8.58	88.00 ± 9.25	86.92 ± 11.57	92.50 ± 11.05
Salinity (ppt)	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03
pH	7.60 ± 0.09	7.50 ± 0.09	7.50 ± 0.09	7.50 ± 0.09
NH_3 (ppm)	0.25 + 0.02	0.27 ± 0.04	0.35 ± 0.03	0.15 ± 0.01

DO: Dissolved oxygen, TDS: Total dissolved solid.

Table 3
Feed utilization and growth indices of *H. fossilis* fed with Spirulina and control diet groups for 60 days. Data are expressed as mean ± standard error (M ± SE).

Parameters		Diets (% Spirulina)		
	D0	D1	D2	D3
IW (g)	4.25 ± 0.14	4.23 ± 0.11	4.27 ± 0.13	4.25 ± 0.13
FW (g)	10.27 ± 0.09^{c}	11.09 ± 0.14^{c}	15.29 ± 1.10^{b}	17.25 ± 0.13^{a}
WG (g)	6.02 ± 0.02^{b}	6.86 ± 0.02^{b}	11.01 ± 0.09^{a}	13.00 ± 0.70^{a}
WG (%)	141.45 ± 2.51^{c}	162.11 ± 3.37^{c}	257.81 ± 3.02^{b}	307.50 ± 4.57^{a}
ADWG (g/ day)	0.10 ± 0.00^{c}	0.10 ± 0.01^{c}	0.14 ± 0.01^{b}	0.22 ± 0.01^{a}
FCR	2.52 ± 0.11^{a}	2.04 ± 0.08^{a}	1.32 ± 0.07^{b}	1.13 ± 0.06^{c}
PER	1.17 ± 0.05^{c}	1.44 ± 0.05^{c}	2.21 ± 0.11^{b}	2.57 ± 0.16^{a}
SGR (% day^{-1})	1.47 ± 0.02^{c}	1.61 ± 0.02^{c}	2.12 ± 0.01^{b}	2.33 ± 0.11^{a}
SR (%)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

IW, Initial weight; FW, Final weight; WG, Weight gain; ADGW, Average daily weight gain, PER, Protein efficiency ratio; FCR, Feed conversion ratio; SGR, Specific growth rate; and SR, Survival rate. Various superscript letters in the same row are remarkably different ($P < 0.05$).

Table 4
Hematological parameters of Stinging catfish fed with various levels of Spirulina incorporated diets. Results are presented as mean ± SE (n = 3).

Parameters			Diets (% Spirulina)	
	D0	D1	D2	D3
WBC (10^9/ L)	109.40 ± 1.45^{c}	114.44 ± 2.28^{c}	123.76 ± 1.36^{b}	142.05 ± 3.48^{a}
Neu (%)	0.67 ± 0.02^{c}	0.70 ± 0.01^{bc}	0.78 ± 0.03^{b}	1.23 ± 0.03^{a}
Lym (%)	82.07 ± 0.58^{c}	83.00 ± 1.53^{bc}	85.60 ± 0.91^{ab}	88.10 ± 0.59^{a}
Mon (%)	9.60 ± 0.30^{c}	9.94 ± 0.06^{c}	11.53 ± 0.43^{b}	12.90 ± 0.55^{a}
Eos (%)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01
Bas (%)	0.03 ± 0.00^{ab}	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}	0.05 ± 0.01^{a}
RBC (10^{12}/ L)	0.73 ± 0.05^{c}	1.54 ± 0.51^{b}	2.07 ± 0.12^{a}	2.04 ± 0.07^{a}
HGB (g/L)	2.57 ± 0.07^{c}	3.69 ± 0.66^{b}	4.69 ± 0.10^{ab}	5.07 ± 0.07^{a}
HCT (%)	0.10 ± 0.02^{c}	0.19 ± 0.02^{b}	0.30 ± 0.01^{a}	0.20 ± 0.01^{b}
MCV (fL)	98.73 ± 1.10^{b}	103.83 ± 3.40^{b}	135.07 ± 0.12^{a}	103.10 ± 5.37^{b}
MCH (pg)	3.10 ± 0.17^{b}	3.02 ± 0.04^{b}	9.17 ± 0.29^{a}	8.76 ± 0.41^{a}
MCHC (g/L)	33.79 ± 1.06^{c}	24.58 ± 3.63^{d}	70.41 ± 0.52^{b}	75.61 ± 0.54^{a}
RDW-CV (fL)	0.33 ± 0.05^{c}	0.55 ± 0.01^{a}	0.43 ± 0.06^{b}	0.55 ± 0.00^{a}
RDW-SD (fL)	65.5 ± 0.50^{d}	166.43 ± 1.69^{c}	210.73 ± 0.46^{b}	213.23 ± 1.37^{a}
PLT (10^9/L)	1338.00 ± 19.55^{a}	1274.67 ± 6.89^{b}	1126.00 ± 2.31^{c}	1251.33 ± 28.05^{b}
MPV (fL)	7.07 ± 1.10^{b}	8.57 ± 0.51^{b}	11.1 ± 1.010^{a}	8.27 ± 0.64^{b}
PDW (%)	16.9 ± 1.01	16.93 ± 10.00	16.83 ± 0.76	16.53 ± 0.30
PCT (mL/L)	11.08 ± 10.00^{c}	31.18 ± 0.75^{a}	13.43 ± 1.50^{b}	1.46 ± 0.50^{d}

WBC: White blood cell; Neu: Neutrophil; Lym: Lymphocytes; Mon: Monocytes; Eos: Eosinophil, Bas: Basophil; RBC: Red blood cell; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW-CV: red cell distribution width-coefficient of variation, RDW-SD: Red cell distribution width-standard deviation; PLT: Platelet; MPV: Mean platelet volume; PDW: Platelet distribution width; PCT: Procalcitonin. Various superscripts in each row imply remarkable differences ($p < 0.05$).

The mean values of white blood cell (WBC), neutrophil (Neu), lymphocytosis (Lym), monocytes (Mon), basophil (Bas), mean corpuscular hemoglobin (MCH), hemoglobin (HGB), and red cell distribution width-standard deviation (RDW-SD) were remarkably ($p < 0.05$) elevated with Spirulina increment in diets. A 5% Spirulina diet-fed fish showed a higher red blood cell (RBC) rather than other treatments, although no obvious difference was noted in the RBC of the 3% Spirulina diet. The hematocrit (HCT), mean corpuscular volume (MCV), red cell distribution width-coefficient of variation (RDW-CV), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV), as well as procalcitonin (PCT), were statistically ($p < 0.05$) differed among the fish groups but did not show any specific trend.

3.4. Serum biochemical indices

Table 5 illustrates the serum biochemistry parameters of Stinging catfish fed with various test diets. A notably ($p < 0.05$) greater glucose (GLU), cholesterol (CHOL), triglyceride (TRIG), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) were found in the control diet-fed fish group compared to other test fish. Conversely, D3 (5% Spirulina inclusion) diet-fed fish showed remarkably ($p < 0.05$) higher total protein (TP), albumin (ALB), as well as globulin (GLOB) contents when compared to other treatment groups. However, the alkaline phosphatase (ALKP) and albumin-globulin ratio (AG-ratio) levels were not influenced ($p > 0.05$) by the various degrees of Spirulina supplementation in diets.

3.5. Histopathology of mid-gut tissues

Histological alterations in the mid-gut of *H. fossilis* fed with Spirulina diets are shown in Figs. 1 and 2. Microscopic analysis revealed that the intestinal wall of Stinging catfish consists of four layers from the lumen, i.e., mucosa, submucosa, muscular (muscular internal and external), and serosa (Figs. 1B and 2B). Spirulina-fed fish displayed an intact epithelial barrier, lacked luminal cell debris, and well-organized enterocytes, as well as exhibited no signs of inflammation (edema). The intestinal mucosa consists of a simple epithelium with abundant goblet cells as well as a lamina propria with clear lacteal. However, numerous signs of damage were noted in mid-gut samples from fish fed with 1% spirulina incorporated and a control diet. Light microscopy revealed the morphological alterations and several signs of intestinal damage, which included high enterocyte vacuolization and absence of distinct lamina propria, lacteal, mucosal, and sub-mucosal tissues clearly disrupted compared to 3% and 5% Spirulina supplemental diets (Fig. 1).

Table 5
Blood biochemical indices of *H. fossilis* fed with Spirulina supplemented and control diets. Data expressed as mean ± SE (n = 3).

Parameters	Diets (% Spirulina)			
	D0	D1	D2	D3
GLU (mg/dL)	57.00 ± 3.46^{a}	39.67 ± 2.33^{b}	44.00 ± 2.31^{b}	26.67 ± 2.91^{c}
TP (g/dL)	3.40 ± 1.16^{b}	4.85 ± 0.89^{ab}	5.61 ± 0.96^{ab}	7.97 ± 1.27^{a}
ALB (g/dL)	2.12 ± 0.1^{b}	2.24 ± 0.04^{b}	2.72 ± 0.09^{a}	2.90 ± 0.05^{a}
GLOB (g/dL)	2.07 ± 0.03^{b}	2.22 ± 0.06^{ab}	3.03 ± 0.48^{ab}	3.84 ± 0.66^{a}
CHOL (mg/dL)	182.67 ± 3.75^{a}	164.00 ± 4.58^{b}	164.00 ± 4.51^{b}	127.00 ± 3.46^{c}
TRIG (mg/L)	42.67 ± 4.48^{a}	41.00 ± 2.08^{a}	32.67 ± 3.17^{b}	26.67 ± 2.60^{b}
SGPT (u/L)	31.33 ± 3.28^{a}	27.33 ± 2.03^{a}	13.33 ± 1.20^{b}	12.00 ± 1.73^{b}
SGOT (u/L)	28.67 ± 2.96^{a}	26.33 ± 1.45^{a}	14.00 ± 1.73^{b}	12.33 ± 1.76^{b}
ALKP (u/L)	18.65 ± 2.02	17.29 ± 1.89	14.11 ± 1.73	13.33 ± 1.81
AG ratio	1.03 ± 0.04	1.01 ± 0.01	0.95 ± 0.16	0.80 ± 0.12

GLU: Glucose, TP: Total protein, ALB: Albumin, GLOB: Globulin, CHOL: Cholesterol, TRIG: Triglyceride, SGPT: Serum glutamic pyruvic transaminase; SGOT: Serum glutamic oxaloacetic transaminase, ALKP: Alkaline phosphatase, AG ratio: Albumin-globulin ratio. Various superscripts in each row imply remarkable differences ($p < 0.05$).

3.6. Measurements of intestinal micro-morphology

The histology of the Stinging catfish intestine is shown in Table 6. The histological measurements, including villi width and height, mucosa width (intestinal wall thickness), crypt depth, and villi area of the midgut, were substantially ($p < 0.05$) influenced by various levels of Spirulina and their highest levels were noted in 5% diet. Conversely, a remarkably higher ($p < 0.05$) lumen area was observed in 0% and 1% Spirulina-fed fish.

3.7. LD_{50} of *A. hydrophila*

The mean lethal dose (LD_{50}) was estimated in *H. fossilis* to be 2.3×10^7 CFU/ mL.

3.7.1. Resistance to bacterial infection

Kaplan Meyer's investigation demonstrated noteworthy variations in percent survival when the *H. fossilis* fed with various levels of Spirulina incorporated diets (Fig. 3). The survival of Stinging catfish upon challenged with *A. hydrophila* was recorded in D3 (68.57 ± 2.57%), D2 (51.28 ± 1.39%), D1 (25.00 ± 1.11%), D0 (16.67 ± 1.31%). However, compared with the control diet, substantially greater post-challenge survival was noted in D3 and D2 diet groups ($p = 0.004$ and 0.030, respectively).

4. Discussion

In the aquaculture feed innovation sector, SM has gained quick popularity as a protein supplement and additive material (Altmann and Rosenau, 2022; C. Li et al., 2022; L. Li et al., 2022; Masten Rutar et al., 2022; Ramírez-Carmona et al., 2022). Additionally, the identification of suitable levels of Spirulina supplementation meal in fish diets is required to encourage sustainable production, economic growth, and health benefits. Thus, the current study observed the feed utilization indices, growth performance, blood parameters, health of the gut, as well as disease resistance trial in Stinging catfish to identify the optimum levels of Spirulina supplementation during aqua-feed formulation.

The study of water hydro-ecological factors is highly significant in all aquatic species to keep all the conditions of the rearing system at the highest level (Siddique et al., 2022). Water quality is a critical component that controls various features that affect broodfish reproductive activities, growth, maturity, ovulation, breeding, embryonic development, egg hatching and juvenile growth (Pankhurst and Munday, 2011). From this present observation, it was depicted that the tank water hydro-ecological variables were not remarkably impacted by different levels of Spirulina diets. The possible outcomes might be due to the use of water from the same source. Throughout the experiment, the quality of the water parameters was within the optimum levels for *H. fossilis* aquaculture (B Santhosh and N.P, 2017; Bhatnagar and Singh, 2010; Ekubo and Abowei, 2011). It has been emphasized that an ideal water quality range would minimize stress and increase fish survival rate (Nandi et al., 2023).

The current findings revealed that the dietary administration of SM at approximately 5% in Stinging catfish diets had remarkable impacts on the growth parameters in terms of SGR, FW, WG, and ADWG. Spirulina, a rich source of essential nutrients, might have contributed to enhanced growth performance. Additionally, the 5% Spirulina-fed fish showed better FCR and PER in comparison with other diet groups. FCR plays an important part in determining profit and loss and estimating feed requirements in aquaculture. The current data demonstrated a declining trend in FCR for the experimental diets as SM increased. Compared to prior research that used lower amounts of SM supplementation for

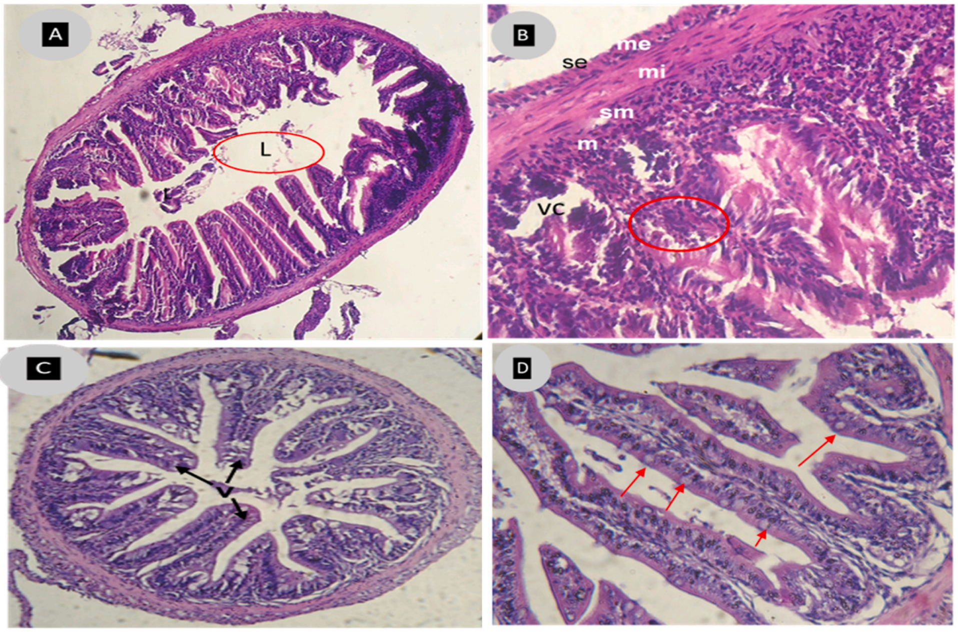

Fig. 1. Histological analysis of midgut of *H. fossilis* fed a control diet (**A: 10X**). Transverse section showing short and stout villi (v) large lumen space (L) and thin wall (mi and me) (**B: 40X C: 20X**). Histo-epithelium section clearly displayed the enterocyte vacuolization (vc) and disruption of enterocytes (circle) (**C: 10X and D: 40X**). Fish fed with the Spirulina diet at 1% showed long villi and small lumen space, as well as an increasing number of goblet cells.

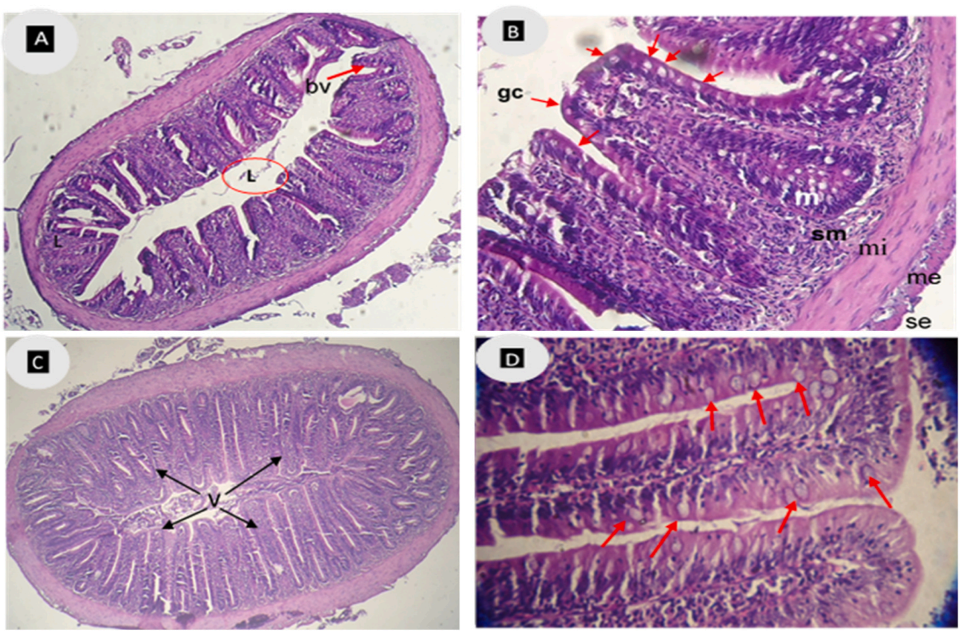

Fig. 2. Histological analysis of midgut of *H. fossilis* at 3% (**A: 10X; B: 40X**) and 5% (**C: 10X; D: 40X**) Spirulina-fed fish. Transverse section showing elongated villi (v) sub-branch of villi (bv) with reduced lumen area (L) thick intestinal wall with four layers of intestine *viz.,* i) mucosa (m) ii) submucosa (sm) iii) muscularis [muscularis interna (mi) and muscularis externa (me)] iv) serosa (se). (**B & D: 40X)** Higher magnification showing intact architecture villi (v) and increased goblet cells (red arrows).

African catfish, a higher supplementation significantly affected FCR value (De Chavez and Bolivar, 2018; Promya and Chitmanat, 2011; Raji et al., 2019; Rosenau et al., 2021). However, according to Becker (2007) the feed ingredients, rearing condition, and age of the catfish differed between tests, and SM's carbohydrate, protein, and fat content can vary. A great source of protein, vitamins, and mineral elements in SM might be the reason to boost the feed efficiency and growth in fish. The overall outcomes might be linked to improved nutrient utilization and digestibility of feed due to enhanced palatability, resulting from the inclusion of SM. Moreover, an outstanding result in the D3 diet indicated that the feed formulated with 5% Spirulina was suited better in the fish bodies than other inclusion levels of this test ingredient. The latest reports by Liu et al. (2019) and C. Li et al. (2022); L. Li et al. (2022) documented that the SM serves as a functional additive that lessens the detrimental impacts on fish growth and productivity. Parallel findings were noted in many previous studies (Belal et al., 2012; Hassaan et al., 2021; Ibrahem et al., 2013; Kabir et al., 2015; Kabir et al., 2019; Kim et al., 2013; Sayed and Fawzy, 2014; Simanjuntak et al., 2018; Sirakov et al., 2012). A study by Adel et al. (2016) documented that juvenile great sturgeon (*Huso huso*) fed with a 10 g/100 g *Spirulina platensis* supplemented diet exhibited remarkably greater growth performance than those fed with other test diets. A significant positive outcome was also found when Tilapia (*O. niloticus*) was fed with Spirulina at levels up to 10 g/kg in diets (Mabrouk et al., 2022). Indeed, spirulina has been found as an essential element of tilapia natural diets (Velasquez et al., 2016). Likewise, when Pabda (*O. pabda*) fed with 2% fish protein

Table 6
Intestinal morphology from fish fed with Spirulina incorporated and control diets (n = 3). Data are expressed as mean ± standard error (M ± SE).

Parameters	Diets (% Spirulina)			
	D0	D1	D2	D3
Villus length (mm)	0.30 ± 0.00[c]	0.35 ± 0.01[bc]	0.38 ± 0.00[b]	0.49 ± 0.01[a]
Villus width (mm)	0.08 ± 0.00[b]	0.09 ± 0.00[b]	0.14 ± 0.01[a]	0.14 ± 0.01[a]
Crypt depth (mm)	0.03 ± 0.00[c]	0.03 ± 0.00[c]	0.04 ± 0.00[b]	0.05 ± 0.00[a]
Intestinal wall (mm)	0.09 ± 0.00[b]	0.09 ± 0.00[b]	0.14 ± 0.00[a]	0.16 ± 0.00[a]
Villus area (mm^2)	0.03 ± 0.00[c]	0.03 ± 0.00[c]	0.05 ± 0.00[b]	0.09 ± 0.00[a]
Lumen area (mm^2)	0.81 ± 0.01[a]	0.77 ± 0.00[a]	0.60 ± 0.01[b]	0.13 ± 0.01[c]

Various superscripts in each row imply remarkable differences ($p < 0.05$).

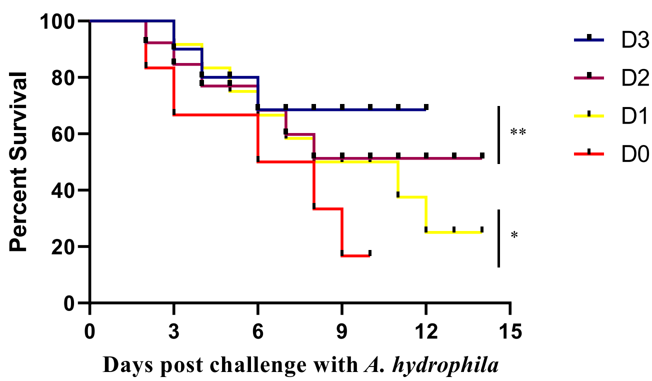

Fig. 3. The Kaplan Meyer's survival analysis of Stinging catfish after intraperitoneal challenge with *A. hydrophila* for ten days. The percent survival curves exhibiting the results of the bacterial challenge trial, where n = 15 for each treatment group. Asterisks * and * * show statistically remarkable variations among treatment groups and infected control at $p < 0.05$ and $p < 0.01$, respectively.

hydrolysate (FPH) incorporated diets resulted in superior feed utilization as well as growth performance (Suma et al., 2023).

According to Altmann and Rosenau (2022), the use of spirulina has already been tested for various fish species, albeit high substitution levels typically result in decreased growth performance and increase FCR in carnivorous fish. While the majority of studies give positive results for fed fish with spirulina, several studies indicated that SM has no effects on fish, for example, Pabda (*Ompok pabda*) (Akter et al., 2023), Thinlip mullet (*Liza ramada*) (Abdel-Latif et al., 2022), Guppy (*Poecilia reticulata*) (Dernekbaşı, 2010), Tilapia (*O. mossambicus*) (Al-Deriny et al., 2020; Hussein et al., 2013),Tilapia (*Oreochromis niloticus*) (Shalata et al., 2021), Hybrid Red Tilapia (*O. niloticus* x *mossambicus*) (Ungsethaphand et al., 2010), Three-spot gourami (*Trichopodus trichopterus*) (Khanzadeh et al., 2016), Kenyi cichlids (*Maylandia lombardoi*) (Karadal et al., 2017), Coral trout (*Plectropomus leopardus*) (Yu et al., 2018), and Gold fish (*Carassius auratus*) (Yousefi et al., 2022). The effect of spirulina on fish development performance appears to be species-specific, and more fish species studies are needed to evaluate the possible usage of spirulina in fish diets. Furthermore, the presence of anti-nutritional factors in plant protein-based and various feed processing methods may negatively impact aquatic species' growth performance during the feed trial (Abdul Kari et al., 2021; Kari et al., 2022; Spínola et al., 2022).

Hematological analysis is necessary to evaluate the fish's physiological condition and health status. Previous reports stated that the fish's hematological parameters are greatly influenced by nutrition and feeding (Sorta Basar Ida Simanjuntak and Eko, 2018), gender (Acharya and Mohanty, 2014), and diseases (Simanjuntak et al., 2011). According to this study's findings, fish-fed diets containing 5% Spirulina had a substantially greater WBC, Neu, Lym, Mon, and Bas. These outcomes indicated that 5% of Spirulina-fed fish had an improved defense mechanism in comparison with other treatment groups. Spirulina's nutrients and bioactive compounds such as antioxidants and immune-modulators might enhance fish immunity, bolstering WBC, NEU, LYM, MON, and BAS counts for improved defense. Similar observations were also found in *Oncorhynchus mykiss* (Yeganeh et al., 2015), *O. niloticus* (Mahmoud et al., 2018), *Plectropomus leopardus* (Yu et al., 2018), *D. labrax* (El-Bab, 2022). Stinging catfish fed with fermented water spinach meal at approximately 50 g/100 g showed corresponding results (Nandi et al., 2023). Phycocyanin extract from Spirulina improves the blood hematological parameters of fish (Hassaan et al., 2021). Conversely, the control diet-fed fish had lower HGB and RBC content as compared to other treatment groups, which corroborated with other studies (Raji et al., 2018; Suma et al., 2023; Yeganeh et al., 2015). WBC and RBC are essential biomarkers for evaluating the health state of fish (Kari et al., 2022; Suma et al., 2023). In the current investigation, the values of MCH and HCT were remarkably different among the treated groups of SM. El-Bab (2022) recorded identical results when *D. labrax* was fed with various levels of Spirulina and/or chitosan nanoparticles-based diets. Furthermore, the variations of MCV, MCHC, RDW-SD, RDW-CV, MPV, and PCT in this study indicate variations in fish physiological condition and health status when fed with graded levels of Spirulina incorporated diets. In this study, ALB and GLOB levels were highest with 5% SM supplementary meals. A higher concentration of ALB and GLOB is linked to a greater innate immune response and is regarded as a solid indicator of immune system activation (Siwicki et al., 1994; Wiegertjes et al., 1996). In summary, the differing hematological parameters among the groups treated with SM indicate changes in fish physiology and health.

GLU, CHOL, and TRIG were substantially greater in the control diet than in the Spirulina-treated diets in this research. When fish fed with various degrees of SM, it showed analogous outcomes (Mabrouk et al., 2022; Raji et al., 2018). However, Yeganeh et al. (2015) found no significant variations in CHOL and TRIG contents in *O. mykiss*. Blood glucose is an essential indicator, and its highest levels reflect environmental stress on fish (Abdul Kari et al., 2021). Moreover, the TP, ALB, and GLOB values significantly differed among the treatment groups and their highest values were detected in 5% SM, consistent with the earlier research on *O. gourami* (Simanjuntak et al., 2018), *D. labrax* (El-Bab, 2022), *H. fossilis* (Nandi et al., 2023), *O. niloticus* (Refaey et al., 2023), and *O. pabda* (Suma et al., 2023). According to Nya and Austin (2009), ALB and GLOB are correlated with health status and serve as plasma carriers. The mean of SGPT and SGOT was also found alterations in this study. Identical results were noted when fish were fed with various levels of fermented soy pulp (Abdul Kari et al., 2021) as well as fermented water spinach meal-treated diets (Nandi et al., 2023). Overall, results revealed that SM has strong immunomodulatory effects on fish that significantly boosts the health status and immune mechanism.

The intestinal histopathological investigation is one of the vital indicators of fish nutritional status (Caballero et al., 2004; Siddik et al., 2022). Based on the present outcomes, the fish fed with 3% and 5% Spirulina diets exhibited an intact epithelial barrier, lacked luminal cell debris, organized enterocytes, as well as no signs of inflammation (edema), indicating that the inclusion of Spirulina influences intestinal mucosal immunity. A robust mucosal immune system might aid in better nutrient absorption, maintain intestinal barrier integrity, and promotes overall health and disease resistance of fish. In addition, Spirulina-fed fish gut had abundant goblet cells, well-organized villi structure, and lamina propria, with clear lacteal in comparison with the control group. An improved villi structure and reduced lumen in Spirulina-treated fish gut indicates better feed utilization capacity of feed nutrients by fish. These findings also revealed that the Spirulina diet had a good indication to improve intestinal health. Moreover, the elevated quantity of goblet cells is connected to their function in safeguarding the intestinal barriers against harmful microbes by secreting antimicrobial agents and glycoproteins (Knoop and Newberry, 2018). Ren et al. (2022) reported that diets supplemented with SM significantly promote the thickness of the intestinal muscular layer and villi structure in Yellow River carp. Sixty days of feeding trial on Nile tilapia with SM revealed a noteworthy increment of goblet cell numbers (Al-Deriny et al., 2020). Another study by Abu-Elala et al. (2016) stated that various levels of Garlic and Spirulina powder inclusion substantially improved Nile tilapia gut health. Nevertheless, several signs of damage were found in fish mid-gut fed with 0% and 1% Spirulina supplemental diets. Similar histological disorders were also noted in other fish (Abdul Kari et al., 2021; C. Li et al., 2022; L. Li et al., 2022; Nandi et al., 2023; Suma et al., 2023).

In this research, dietary incorporation of various levels of SM had remarkable impacts on villus height and width, crypt depth, mucosa width, villus, and lumen area of the mid intestine. Nutrients enrichment in SM could boost cell regeneration, mucosal health, and gut defense, enhancing intestinal structures and functions. For instance, increased villus height and width provide a larger surface area for nutrient absorption. A deeper crypt depth often corresponds to increased cell turnover and nutrient transport. On the other hand, Spirulina, being a great source of bioactive compounds, has been reported to express various beneficial properties, including antioxidant, anti-inflammatory, and immunomodulatory effects (Calella et al., 2022; Han et al., 2021). These properties could influence the gut's cellular environment. Correspondingly, dietary Spirulina increases villi length and mucosal length in Nile tilapia, as previously documented by Al-Deriny et al. (2020). The study lead by Van Vo et al. (2020) experimented on juvenile barramundi and noticed significant alterations in the height of intestinal fold, microvilli height, and muscular wall thickness, achieving the highest values at 10% enzyme-treated Spirulina treatment. Modulation of the goblet cell density, villi height, intraepithelial lymphocytes, as well as absorption surface area was observed in *O. mykiss* fed with a 5% Spirulina-supplemented diet (Sheikhzadeh et al., 2019). Spirulina may positively influence nutrient intake and protein digestibility to improve all the measurements in fish gut.

The challenge study against *A. hydrophila* revealed that the percentage survival of *H. fossilis* was substantially improved with the elevation of dietary Spirulina inclusion in test diets, and their highest value was noted in D3 (5%) diet. Hence, the control diet-fed fish experienced considerably lower survival when compared to other treatments. The study's outcomes depicted that adding SM to the diet strengthens the defense mechanism, which helps fish become more resistant to certain diseases and pathogens. African Catfish challenged with *A. hydrophila* exhibited remarkably greater survival rates when fed with different levels of Spirulina diets (Nasir et al., 2018). Siddik et al. (2018) documented that Juvenile barramundi fed with 10% FPH showed the highest percent survival after being challenged with *Streptococcus iniae*. The present results were also identical with other species of fish, such as *O. niloticus* (Abdel-Latif and Khalil, 2014; Abdel-Tawwab and Ahmad, 2009; Elabd et al., 2020), *O. mykiss* (Sheikhzadeh et al., 2019), *D. labrax* (Güroy et al., 2022), *Mystus cavasius* (Mamun et al., 2023), and *O. pabda* (Suma et al., 2023). However, to support all the finding, it is must to study on the molecular mechanism to confirmed all the results and calculating the cost involving when produced the feed by spirulina. However, further research on the molecular pathways involving immune-related gene expression of numerous targeted genes, particularly Transforming Growth Factor beta 1, Nuclear Factor kappa-B gene, heat shock protein 90, and Lysozyme G, is required to validate all the findings.

5. Conclusion

In conclusion, the dietary incorporation of natural Spirulina powder in *H. fossilis* diets could significantly enhance feed utilization, growth performance, hemato-biochemical indices, intestinal morphology, and disease resistance against *A. hydrophila.* Based on the present findings, diets supplemented with 5% Spirulina could be used in the aqua-feed business sector for superior growth, health status, as well as disease resistance of Stinging catfish and possibly for other catfish production in captivity.

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CRediT authorship contribution statement

Writing – original draft, Formal analysis, Writing – review, MR, SKN, MAK; Project administration, Conceptualization, Writing – review and editing, Funding, MAAM; Writing – review and editing, MMMA, NKM, AR, LSW, ABT, MMR, SSR; Writing – review and editing, funding, WGB, GTI, ZAK, and MAK. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

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Institutional review board statement

The experiments were approved by Animal Ethics Committee of Sylhet Agricultural University, and performed according to the Animal Ethics Procedures and Guidelines of the People's Republic of Bangladesh.

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