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Day Immunity Enrichment of Freshwater Catfish Gulsa (*Mystus cavasius*) by Supplement Dietary Inclusion of Black Cumin Seed (*Nigella sativa*)

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Abstract

The present study was undertaken to evaluate the effects of dietary black cumin Nigella sativa seed on growth performance, feed utilization and disease resistance in freshwater catfish gulsa (*Mystus cavasius*) against the pathogenic bacteria Aeromonus hydrophila. Fish (1.2±0.07 g) were fed with experimental diet, containing control as commercial feed, black cumin seed powder (BCP) 1%, and black cumin seed extract (BCE) 0.5% for 90 days culture period. After 90 days, 10 fish from each treatment group, in triplicate, were challenged with the pathogenic bacteria Aeromonas hydrophila via intraperitoneal injection. The fish were then monitored daily for 7 days to observe clinical signs and record mortality rates. The growth parameters, including weight gain, specific growth rate, protein efficiency ratio, and survival rate, showed a significant increase ($p<0.05$) in fish fed BCE and BCP diets compared to the control group. In fish fed the BCE diet, the feed conversion ratio dropped considerably ($p<0.05$). Fish fed with a BCE-supplemented diet showed enhanced levels of red blood cells, hemoglobin, white blood cells, hematocrit, mean corpuscular hemoglobin, and immunoglobulin M (IgM) in Mystus cavasius compared to those fed with the BCP diet and the control group. The total mortality of fish challenged by A. hydrophila decreased in BCE diet group. These findings indicate that supplementing the diet with black cumin extract has beneficial effects on the growth and immune system of *Mystus cavasius*.

Keywords: *Black Cumin Seed, Freshwater Catfish Gulsa, Mystus cavasius, Immunity*

Introduction

In Bangladesh, aquaculture has grown to be a significant sector. After China and India, Bangladesh is the third-largest producer of inland fish worldwide. Fresh water aquaculture has become an important economic activity in Bangladesh, because it accounts for the majority of Bangladesh's fish production (Islam 2001). In 2023-2024, this sector accounted for 20.27% of the agricultural GDP and 2.81% of the national GDP (BER 2024). 60% of the country's requirement for animal protein comes from fish. *Mystus cavasius*, commonly known as the Gangetic mystus and locally referred to as gulsa tengra, is a species of catfish belonging to the Bagridae family. This freshwater species is regarded as one of the most important Small Indigenous Species (SIS) and is found in both flowing and standing waters throughout Bangladesh. Gulsa is highly popular across Bangladesh, India, Pakistan, Nepal, Myanmar, Indo-china, Sri Lanka, Malaysia, Thailand, and West Africa due to its rich protein content, along with essential micronutrients, vitamins, and minerals (Ross *et al.*, 2003). Because of its quick growth rate, omnivorous eating style, ease of adaptability to artificial diet and high protein content, it is one of the best species for aquaculture (Mamun *et al.*, 2023).

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Effective management, which is linked to a healthy feed supply and a favorable culture environment, is essential to the success of any fish culture. Nonetheless, the careless and unplanned application of fertilizer and feed contributed to fish production failures, and overstocking in intensive culture system made fish increased stress and more vulnerable to infections. Disease has been identified to be a significant contributing factor to the decline in fish productivity in both farmed and wild environments (Aftabuddin *et al.*, 2016).

Uses of drug or antibiotic are common practice to get rid of fish diseases but its misuse resulted in spread of disease resistance pathogen, drug residues in food, are now serious health concern particularly buyers are rigid on food safety issues. The inclusion of growth hormones, antibiotics and pesticides in fish and animal feed is legally prohibited in Bangladesh (BP 2010). Traditional Chinese and Indian herbs have long been used to boost the human immune system (Galina *et al*., 2009). Researchers are currently investigating immunostimulants from probiotics and medicinal plants in aquaculture for managing diseases as alternative use of antibiotics. The use of yeast supplements in *M. cavasius* culture shows a promise as a growth stimulant and a substitute for antibiotics in the prevention of disease (Banu *et al*., 2020). Punitha *et al.*, 2008 have found good effects of plant-derived natural products on innate and adaptive immune responses, as well as on the prevention and control of fish diseases. Medicinal herbs contain various bioactive compounds and have been utilized as traditional medicine (Dügenci *et al*., 2003). Research has shown that adding *N. sativa* (black cumin powder) or its good ingredients (thymoquinone) to fish diets can improve growth performance, feed conversion, and flesh quality (El-Hack 2021). Additionally, fish immune systems, blood profile, guards against pathogenic bacteria invasion, and lessens their oxidative stress reactions to heavy metal toxicants is improved by adding of *N. sativa* or its derivatives into their diets. *Nigella sativa* powder and its oil showed antimicrobial actions opposed to different diseases of freshwater fishes caused by pathogens: *Aeromonas hydrophila*, *Flavobacterium columnare*, and *Pseudomonas fluorescens* (Aly *et al*., 2019; Dey *et al*., 2020; Mohammed and Arias 2016). Black cumin seed (*N. sativa*) powder was found a highly effective in promoting growth and enhancing immune function in rohu, rainbow trout, common carp, and tilapia (Latif *et al*., 2021; Bektas *et al*., 2018; Khandoker *et al*., 2016; Wafaa *et al*., 2014 Yilmiz *et al*., 2012). So, far no information is available on a comparison between the extract and powder of *N. sativa* on growth, immunity and disease resistant in *M. cavasius* against pathogenic bacteria. Therefore, the current study investigated the potential efficacy of *N. sativa* on growth performance, blood parameters, and feed utilization of *M. cavasius* and its immune resistance to *Aeromonas hydrophila* infection.

Material and Methods

Black cumin seed extracts

Nigella sativa (BC) was grinded into powder and the extraction was prepared according to Sultana et al. (2009). The black cumin seed powder (BCP) 20 g was extracted by adding 80% methanol in air-sealed bottles which shaken continuously at room temperature for 24 hrs. The black cumin seed extract (BCE) was filtered through 125 mm size filter paper (Whatman No. 1). The extract was concentrated using a rotary evaporator and weighed and kept at -4°C for further analysis.

Experimental diets

Three iso-nitrogenous (35% crude protein) diets were formulated by adding BCP and BCE at 1.0 % (BCP 1.0) and 0.5% (BCE 0.5) with the formulated feed while the diet without addition of those served as control. (Table 1). The ingredients were blended with water until a stiff dough formed. The pelleted feeds were made by using a pelleting machine (die 0.6mm) and dried under the sunlight for 5-6 hrs. The BC enriched feed was kept in a plastic container at 4°C for further use.

Table 1. Ingredients (%) of experimental diets for *Mystus cavasius*

Ingredients	Control	BCP	BCE
Fish meal	40.0	40.0	40.0
Mustard oil cake	10.0	10.0	10.0
Soybean meal	10.0	10.0	10.0
Rice bran	30.0	30.0	30.0
Wheat flour	2.0	2.0	2.0
Wheat bran	6.0	6.0	6.0
Vitamin mineral mix	2.0	2.0	2.0
Black cumin seed	0.0	1.0	0.5

Experimental design

M. cavasius fry were collected from the Sharnalata Agro-Fisheries Ltd., Mymensingh and brought to the Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) in oxygen-filled polythene bags and allowed to acclimate in tanks for two weeks. After that, fish were randomly assigned to each of the nine tanks (50 per tank) and treated with 1% salt solution before being released into the experimental tanks. The commercial floating pellets (Mega Feed Co. Ltd., Bangladesh) containing approximately 37% crude protein, 8% lipid, 16% Ash and 12 % moisture were fed to the fish in control and during acclimatization. Fish were fed twice a day at 8.00 am and 16.00 pm for 90 days. Fish sample was collected and measured weight and length in every two weeks. Based on the weight gain, the feeding rate was adjusted. Fish excreta were removed by siphoning each morning. Every day, 30% of the water in each tank was partially exchanged and was continuously aerated. At the final harvest, fish were collected to measure weight, length for growth performance.

Sample collection

Fish from each tank were anesthetized with MS-222 at the end of the feeding trail and their weights were recorded. The blood sample (2.0 ml) from 15 fish for each replication was collected by using a syringe and stored in tubes containing EDTA (BD Microtainer®, UK), and stored for hematological analysis. A subsample of the collected blood was centrifuged at 5000 rpm for 10 min, while serum sample was used to immunoglobulin M (IgM) analysis.

Growth performance estimation

The following indicators were used to assess the growth performance and feed utilization: weight gain (WG), weight gain% (WG%), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (S). The growth performance, feed efficiency, and biological indices were calculated using the following formulas (Chakraborty *et al*., 2021).

$$\text{WG (g)} = \text{Final average weight (g)} - \text{Initial average weight (g)}$$

$$\text{Percentage (\%) weight gain} = \frac{\text{Average final fish weight} - \text{Average initial fish weight}}{\text{Average initial fish weight}} \times 100$$

$$\text{Specific growth rate (SGR \% per day)} = \frac{\ln W2 - \ln W1}{T2 - T1} \times 100$$

$$\text{FCR} = \frac{\text{Total feed intake (g)}}{\text{Total weight gain of fish (g)}}$$

$$\text{Survivability (\%)} = \frac{\text{Final number of fish survived}}{\text{Number of actual fish stocked}} \times 100$$

$$\text{Condition Factor (\%)} = \frac{\text{Final average weight (g)}}{\text{Final length}^3\text{ (cm)}} \times 100$$

where, W_1 = The initial average body weight (g) at time T_1 (day); W_2 = The final average body weight (g) at time T_2 (day).

Water quality analysis

Water temperature, pH and dissolved oxygen were recorded everyday by using thermometer, pH meter and dissolved oxygen meter (YSI Model 58, USA) respectively. Nitrogen compounds (NH_3-N, and NO_2-N) were analyses bi-weekly by colorimetric method of HACH kit (model DR 6000, HACH Co., USA).

Hematological analysis

Hemoglobin (Hb), white blood cells (WBC), red blood cells (RBC), hematocrit (Hct%), and lymphocytes (LYMPH%) of blood were measured using Blaxhall and Daisley's (1973) techniques. The mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) were calculated using the following formula

$MCV = Hct/RBC \times 10$; $MCHC = Hb/Hct \times 100$;

$MCH = Hb/RBC \times 10$

Immunoglobulin M was measured according to Siwicki and Anderson (1993).

Challenge test for bacterial with aeromonas hydrophila

Ten fish were chosen at random from each tank and challenged with a virulent strain of *A. hydrophila* that was identified and processed at the Institute of Biotechnology and Genetic Engineering, BSMRAU (Mamun *et al.*, 2023). Some fish were sacrificed to analysis hematological parameters. Following cleaning with wet cotton soaked in 70% alcohol, 0.1 ml of *A. hydrophila* was injected intraperitoneally into each experimental fish (Ahmed and Haque 2009). The dead fish were removed immediately. The fish's survival rate was then monitored twice daily for ten days.

Statistical analysis

Throughout the experiment, data were collected, recorded, and stored on a computer spreadsheet. Based on the information gathered in the three samples for every treatment, the mean value and standard deviation were determined. The Kolmogorov-Simirnov test and the Bartlett test were used to check all of the data for homogeneity of variance and normality of distribution. The data was evaluated using one-way analysis of variance (ANOVA) to test for significant differences between the treatments. In order to determine whether the main effects were significant, Tukey's multiple range test was used to compare the mean values ($p < 0.05$) between treatments using SPSS statistics 10.0 software.

Results

Water quality

The growth rate and yield of *M. cavasius* were high as the water quality parameters in all tanks were observed to be retained in acceptable ranges during the culture period. The parameters of water quality except NO_2-N were not significantly different between treatments and control (Table 2). A natural photoperiod (light period ranging from 12 h to 13 h) was present in all tanks.

Table 2. Water quality parameters (Mean ± standard deviation) in *M. cavasius* fed with the experimental diets in culture period

Parameter	Control	BCP 1.0	BCE 0.5
Temperature (°C)	29 ± 0.64^a	29 ± 0.57^a	29 ± 0.63^a
pH	8.13 ± 0.08^a	8.05 ± 0.06^a	8.12 ± 0.04^a
DO (mg/l)	7.34 ± 0.11^a	7.31 ± 0.1^a	7.26 ± 0.16^a
NO_2-N	0.132 ± 0.05^a	0.05 ± 0.01^b	0.12 ± 0.03^a
NH_3-N	0.07 ± 0.19^a	0.04 ± 0.02^a	0.04 ± 0.02^a

Different superscripts mean within the same row differ significantly ($p < 0.05$).

Growth performance, feed efficiency, and biological indices

The body weight development throughout the experiment period is presented in Figure (1). The growth metrics such as final body weight, weight gain, and specific growth rate of *M. cavasius* were higher in fish fed supplemented black cumin seed than that of in control. Moreover, the fish body weight was significantly ($p<0.05$) greater in fish fed supplemented with BCP diet than in fish fed with BCE diet (Table 3).

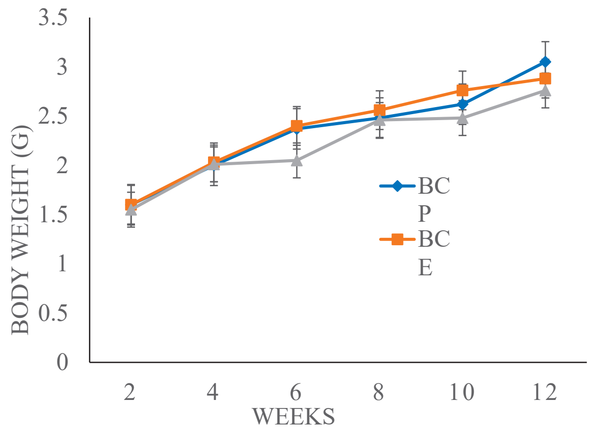

Figure 1. The growth increment of freshwater catfish *M. cavasius* fed with black cumin seed supplemented diet during culture period

Table 3. The growth performance and feed utilization (Mean ± standard deviation) of freshwater catfish *M. cavasius* fed black cumin seed supplemented diet at the end of culture period

Parameter	Control	BCP 1.0	BCE 0.5
Initial Weight (g)	1.27 ± 0.64	1.14± 0.57	1.27 ± 0.63
Final Weight (g)	2.76 ± 0.16[b]	3.05± 0.44[a]	2.88 ± 0.17[b]
Weight gain (g)	1.49 ± 0.15[c]	1.91 ± 0.44[a]	1.61 ± 0.13[b]
Weight gain (%)	117.1 ± 10.5[c]	168.3 ± 5.9[a]	127 ± 13.8[b]
SGR (%/day)	0.86 ± 0.05[b]	1.1 ± 0.16[a]	0.91 ± 0.04[b]
FCR	1.9 ± 0.19[a]	1.65 ± 0.36[b]	1.86± 0.16[a]
Survivability (%)	48 ± 0.06[b]	59 ± 0.03[a]	59 ± 0.07[a]
CF (%)	0.72± 0.06[b]	0.9± 0.01[a]	0.85± 0.13[a]

Different superscripted means within the same row differ significantly (p <0.05). SGR= Specific growth rate; FCR= Feed conversion ratio; CF= Condition factor.

Feed conversion ratio (FCR) means how much feed is utilized by fish efficiently which also called feed utilization. The low production costs and improved fish feed utilization efficiency is expressed by a low FCR score. A statistical study revealed that the FCR of fish fed with the BCP diet was significantly lower than that of the BCE diet and the control ($p < 0.05$). In addition, the condition factor of fish health fed with BCP and BCE supplemented diets was very well compared to fish fed with un-supplemented diet or control. The fish survival rate was found the highest (59%) in the BCP and BCE treatments whereas it was the lowest (48%) in the control after 90 days of rearing (Table 3).

Hematological parameters

The several blood parameters were significantly different between treatments. All parameters except MCV of *M. cavasius* fed with supplemented black cumin seed extract (BCE) diet were significantly higher ($p<0.05$) than the BCP diet and control (Table 4).

Table 4. Effects of black cumin seed supplemented diet on hematological parameters (Mean ± standard deviation) in freshwater catfish *M. cavasius* at the end of culture period.

Blood parameters (%)	Control	BCP 1.0	BCE 0.5
Hb (g/dl)	5.03 ± 0.67[b]	4.5 ± 2.57[c]	5.6 ± 2.1[a]
WBC ($\times10^3$ cells μL^{-1})	38 ± 1.1[b]	13.85 ± 1.6[c]	41.1 ± 0.23[a]
RBC ($\times10^6$ cells μL^{-1})	1.53 ± 0.22[b]	1.45 ± 0.92[b]	1.62 ± 0.64[a]
Hct %	21.87± 2.8[b]	20.7±14[b]	22.8 ± 11[a]
MCV(fL)	143.1±2.7[a]	140.13±11[a]	138.5±11[a]
MCH (pg)	32.83 ± 0.42[b]	31.9 ± 2.1[b]	34.73± 3.8[a]
MCHC (g/dl)	22.97 ± 0.15[b]	22.97± 3.3[b]	25.27± 3.97[a]
IgM (gm/L)	0.14± 0.05[b]	0.16± 0.12[b]	0.24± 0.23[a]

Different superscripted means within the same row differ significantly ($p < 0.05$). Hb=hemoglobin; RBC= red blood cell; WBC=white blood cell; Hct= hematocrit; MCH= mean corpuscular; MCHC= mean corpuscular hemoglobin

Challenge test with aeromonas hydrophila

Though, there was not observed any mortality of fish until 7th day after receiving an intraperitoneal injection of *A. hydrophila,* the total number of

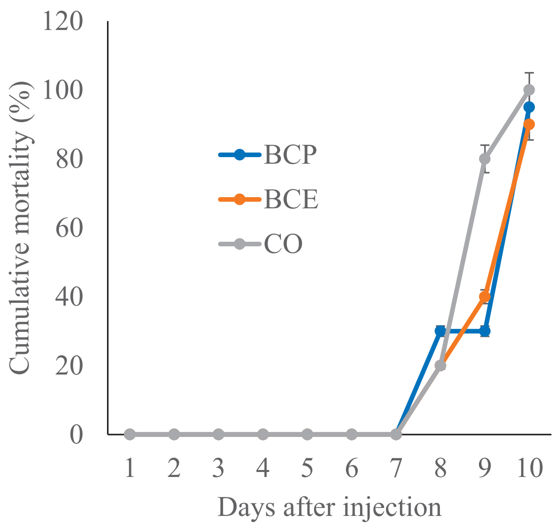

Figure 2. The cumulative fish mortality (%) of *M. cavasius* fed with black cumin seed supplemented diet challenged by *A. hydrophila* for 10 days

death fish was significantly (p <0.05) increased on 8th day of the injection (Figure 2). Additionally, the cumulative mortality of *M cavasius* fed with BCE supplemented diet for 90 days was the lowest among the treatments.

Discussion

A medicinal plant has potential activities to be used as growth promoter (Yilmaz *et al*., 2012), appetizer (Zaki *et al*., 2012), immunomodulator (Abdal-Tawwab *et al*., 2018a; Latif *et al*., 2020), anti-stress and antioxidants (Abdal-Tawwab *et al*., 2018b) in aquaculture practices. Ahmad *et al*. (2013) observed that *N. sativa* seed and oils have been used as traditional treatment for digestive tract, immune system support, kidney and liver functions, as well as general well-being. *N. sativa* seed meal improved the immunity in Tilapia *Oreochromis mossambicus* (Yilmaz *et al.,* 2012), natural growth performance and antioxidant in Rohu *Labeo rohita* (Latif *et al*., 2020). Kandoker *et al*. (2016) also found that *N. sativa* extract was enhanced the immunity in Common carp *Cyprinus carpio.* The enriched immunity in the climbing perch *Anabas testudineus* was investigated by Khatun *et al*. (2015). Moreover, there are few studies on the growth performance and immunity of different fish species fed with black cumin seed powder and extract separately. The comparison between *N. sativa* seed powder and its extract effects on growth performance, feed utilization, blood parameters, immunity, and resistance of disease in freshwater catfish Gulsa *M. cavasius* was investigated in the present study.

The growth performance was significantly high and decreased feed conversion ratio of fish fed *N. sativa* seed powder in the present study that might impute essential vitamins, minerals, amino acids, increased feed intake, and nutrient digestibility (Al-Jassir 1992). In addition, the bioactive compounds (thymoquinone, thymol, dithymoquinone, nigellicine and nigellidine) to fish diets might have improved the growth rate, feed conversion ratio, and flesh quality (El-Hack 2021; Ahmed *et al*., 2013). Wafaa *et al*. (2014), who noticed Nile tilapia (*Oreochromis niloticus*) increased significantly (p<0.05) the growth performance and reduced the FCR in fish fed with *N. sativa* extract diet than other diets. Bektas *et al*. (2018) have studied black cumin seed powder as feed supplement for growth promoter and enriched immunity in Rainbow trout *(Oncorhynchus mykiss*). The growth rate of *O. mykiss* improved in fish fed with *N. sativa* oil supplemented diet which was similar to the current results (Oz *et al*., 2018; Diab *et al*., 2008). Moreover, the growth index enhanced in Common carp (*Cyprinus carpio*) fingerlings fed with 1% (Al-Dubakel *et al*., 2012) and Rohu (*labeo rohita)* fed with 2.5% (Latif *et al*., 2021) *N. sativa* seed meal supplemented diets. In contrary, the dietary supplementing of black cumin seed in Tilapia *(O. mossambicus)* diet was not significantly effect on the growth performance but increased survival rate of fish (Yilmaz *et al*., 2012). All above aforementioned reports were achieved the same results with the present study. The cumulative effects of minerals (iron, copper, phosphorus, and zinc), vitamins (folic acid, riboflavin, thiamin, pyridoxine, and vitamin E), saponins, and a different types of alkaloids, which are present in the black cumin seed (Al-Jassir *et al*., 1992), may be enhanced the growth performance of *M. cavasius*.

Thought, the growth rate, feed utilization, survival rate of fish fed with the cumin seed powder supplemented diet showed significantly (p<0.05) higher and lower FCR than that in fish fed with *N. sativa* extract supplemented diet and un-supplemented diet, but the immunity and disease resistant were significantly (p<0.05) greater in fish fed with *N. sativa* extract diet than other diets in the present study. The hematological parameters except MCV of *M. cavasius* rose in fish fed with *N. sativa* extract diet among the treatments. Whatever the black cumin seed extract in *M. cavasius* diet must be included to improve the growth performance and broad immune parameters. A defense mechanism of fish body in opposition to pathogen is an important innate immune system which contains of granulocytes, monocytes, macrophages, humoral elements such as immunoglobulins (IgM) and complement system. A strong immune system is an important way to prevent the fish diseases. The immune parameters index explained in a detailed for understanding a health condition of *M. cavasius.* The blood parameters of fish such as RBC, WBC, Hb, Hct, MCH, MCHC, MCV, platelet and IgM were measured in this study. White blood cell from the immune parameters of fish has a censorious significance in the cellular defense mechanism and resistance to infectious diseases (Whyte, 2007). All blood parameters except MCV of *M. cavasius* fed with black cumin extract increased significantly (p<0.05) compared to other treatments which was the same with the findings of Dorucu et al. (2009) and Altinterim and Dorucu (2009). The immunoglobulins value of fish blood was the greatest in the BCE treatment among others. Dorucu *et al*. (2009) investigated the immunomodulators in diet may affect the production of circulating IgM in fish. A clear up-regulation of immunoglobulin M was an evident in fish fed with black cumin supplemented diet compared to the control (Mahboub *et al*., 2022). Celik Atunoglu *et al.* (2017) reported that the immunity of Rainbow trout (*O. mykiss*) enhanced in the methanolic extract of black cumin seed. The immunological status of *M. cavasius* enriched upon dietary exposure to black cumin seed extract (Dey *et al*., 2022) which might have stimulated the beneficial bacteria and stimulated a better feed utilization to all bioactive compounds in black cumin seed.

In challenge test, the maximum cumulative mortality of *M. cavasius* was observed in the control and the lowest in fish fed with black cumin seed extract diet in the present study. Khandoker *et al*. (2016) and Mohamad and Abasali (2010) were found the lowest mortality in *C. carpio* fed 4% black seed oil supplemented diet. Yilmaz *et al*. (2013) investigated that the survival rate increased in fish fed with black cumin supplemented diet. Black cumin seed contains a variety of bioactive compounds which might have activated and protected the intestinal microbiota and thus, prevent opposed to the invading pathogen (Dey *et al*., 2020; Mahboub *et al*., 2022).

Conclusions

The growth performance and feed utilization of fish fed with *N. sativa* seed powder supplemented diet improved compared to fish fed with *N. sativa* extract and control diets. Thought, the growth performance and feed utilization of *M. cavasius* were not significantly different in fish fed with black cumin seed extract and control, the immunity and disease resistant enriched in fish fed with black cumin seed extract supplemented diet. *N. sativa* seed extract has an important immunostimulant compounds which may enhance the innate immune system and disease resistance of *M. cavasius* fed with BCE diet in the present investigation. The result recommended that black cumin extract can be used as supplement at the rate of 0.5% with *M. cavasius* diet which improved the growth and immunity of fish.

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Authorship Contribution Statement

Hasan Sharif implemented the experiment, and collected the data; M. R. Banu wrote the draft; M. M Rahman, M. R. Banu, and M. Das supervised the research and revised the manuscript. The manuscript was also critically assessed by all authors for its intellectual quality, and they all ultimately approved its publication.

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.

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