Turk. J. Fish.& Aquat. Sci. 21(3), 147-158
http://doi.org/10.4194/1303-2712-v21_3_05

PROOF
RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Combined Effects of *Leucas aspera*, Oxy-Cyclodextrin and Bentonite on the Growth, Serum Biochemistry, and the Expression of Immune-Related Gene in Nile Tilapia (*Oreochromis niloticus*)

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Article History
Received 16 October 2020
Accepted 26 December 2020
First Online 29 December 2020

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Keywords
Fish health
Herb
Immune genes
Inclusion complex
Mineral clay

Abstract

This study investigated the effects of a combination of *Leucas aspera*, Oxy-cyclodextrin and sodium bentonite (LOB) on growth, digestive enzyme activity, innate immune response, haematology, and expression of immune-related genes in Nile tilapia. A total of 240 juvenile fish (20.15±0.05g) were randomly distributed into four dietary groups in triplicate and fed respective diets containing a graded level of LOB at 0 g kg^{-1} (Control), 0.3 g kg^{-1} (T1), 0.6 g kg^{-1} (T2) and 0.9 g kg^{-1} diet (T3) for 60 days. After 60 days, higher growth was observed in fish fed T2 diet ($P < 0.05$). Digestive enzyme activities and innate immune parameters were significantly higher in T3 group. Some of the haematological parameters reported statistically higher counts in T2 group ($P < 0.05$), whereas erythrocyte indices and WBC counts were significantly higher in T3 group. Liver- kidney activities were recorded low in T3 group. Urea and creatinine were higher in control group, whereas T2 group recorded the least value. The highest relative expression of IL-1β, IgM-heavy chain, TGF-β and IFN-γ were recorded in T2 group, but TNF-α was upregulated in T3 group. The results showed that 0.6 - 0.9 g kg^{-1} of LOB is recommended for inclusion in diet.

Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important aquaculture species globally representing about 8.3% (4.5 million tons) of the world's major farmed finfish species produced in 2018 (FAO, 2020). However, studies have shown that the ever-increasing human population will require an additional ~ 48 million tonnes of fish by 2050 to meet food fish demand (FAO 2016; Shamna et al., 2020). This could only be made possible through a high-density intensive farming system which is often accompanied with husbandry-related stress and diseases, high mortality, immunosuppression, and growth retardation (Liu et al., 2019; Yousefi et al., 2019; Fawole et al., 2020). Nonetheless, strategies for improving performance and confer immunocompetence in fish under intensive culture system could be achieved via nutritional manipulation. In this regard, several antibiotic-free nutritional approaches have been sought by many researchers including the use of medicinal plants (Yousefi et al., 2019; Garg et al, 2019; Van Doan et al., 2019; Fawole et al., 2020), vitamin-mineral supplements (Shamna et al., 2020), pro- and pre-biotics (Hai, 2015; Cavalcante et al., 2020), sodium bentonite (Jawahar et al., 2018), etc. Furthermore, previous studies have confirmed that dietary inclusion of functional feed additives either alone or in combination help improve the immune system and induce physiological benefits in fish (Ebru and Cengiz, 2016; Fawole et al., 2016; Dawood et al., 2017; Wang et al., 2018; Xia et al., 2020).

Leucas aspera, an important Indian herbal plant of the family *Lamiaceae* with a wide range of medicinal bioactive compounds; such as triterpenoids, oleanolic acid, α-sitosterol and β-sitosterol, nicotine, ursolic acid, glucoside, novel phenolic compounds (4-(24-hydroxy-1-

oxo-5-n-propyltetracosanyl)-phenol) and diterpenes which are responsible for its medicinal properties (Prajapati et al., 2010; Latha et al., 2013; Antony et al., 2014). Previous findings reveal that the extract of *L. aspera* possesses strong anti-inflammatory, analgesic, antipyretic, antioxidant, antibacterial and fungicidal properties (Saundane et al., 2000; Kripa et al., 2011). Oxycyclodextrin (oxy-CD complex) is a water-soluble derivative of oxyresveratrol (oxy), a polyphenol, mainly 2,4',3,5'-tetrahydroxystilbene, found in the heartwood of *Artocarpus lakoocha*. Oxyresveratrol has been reported to possess some health-promoting impact in human such as prevention of inflammation and atherosclerosis, reduction in the occurrence of cardiovascular disease, free radical scavenging and DNA protective properties (He et al., 2019; Venuti et al., 2014). Although oxy has good health benefits, however, it is light sensitive, susceptible to oxidation, and has low bioavailability (He et al., 2019). Cyclodextrin is a cyclic polysaccharide with hydrophilic outer cavity and forms inclusion complex by improving the physicochemical properties with other guest molecules (eg. oxy) without altering the original structure (Venuti et al., 2014). Thus, Oxy-CD complex is prepared as inclusion complex and exhibits antioxidant, anti-inflammatory and anticancer activity (Venuti et al., 2014).

Bentonite, derived from volcanic ash is another important non-nutritive additive, used as a binding agent and a potent adsorbent of mycotoxins, enzymes, and pathogenic microbes in feeds and animal guts (Eya et al., 2008; Ayoola 2016; Jawahar et al., 2018). Studies have shown that the addition of bentonite in poultry or fish diet enhanced growth performance, feed efficiency and utilization (Pasha et al., 2008; Eya et al., 2008; Khanedar et al., 2012), and improve disease resistance (Jawahar et al., 2018). Also, the dietary addition of *Leucas aspera* was reported to improve growth and feed conversion efficiency, boost the immune system and reduced mortality in Nile tilapia (Kurian et al., 2020). According to Meseguer and Cerezuela (2011), the addition of two or more additives often presents three-dimensional patterns viz., synergism, additivity or potentiation. Despite the positive impact of bentonite on animal performance, little emphasis has been placed on its additive effect on fish. Also, there has been no study reported on the effect of a combination of plants material and mineral clay as a feed additive in finfish. Considering the individual attributes of these additives, the present study was designed to explore the potential synergistic effect of *L. aspera* (a medicinal herb), oxy-CD complex (a water-soluble compound of oxyresveratrol), and bentonite (a natural clay) on growth performance, digestive enzyme response, immune status modulation and inflammatory responses in Nile tilapia. The study postulated that the immunostimulatory action of *Leucas aspera* may be augmented by bentonite and oxy-CD complex.

Materials and Methods

Diet Preparation

The whole plant of *L. aspera* grown at the Kerala University of Fisheries and Ocean Studies (KUFOS) was collected, washed with tap water, dried and ground into fine powder for extraction. Five grams (5g) of plant powder was added into a conical flask containing 50 mL distilled water and boiled for 20 minutes at a temperature of 100˚C. The aqueous extract was filtered using Whatman No.1 filter paper and stored at 4˚C. The

Table 1. Formulation and proximate composition of experimental diets (g kg^{-1})

Ingredients (g kg^{-1})	Control	0.3 g kg^{-1}	0.6 g kg^{-1}	0.9 g kg^{-1}
Fish meal	270	270	270	270
Corn meal	200	199	203	206
Soybean meal	270	270	270	270
Wheat flour	60	60	60	60
Rice bran	150	150	150	150
LOB	0	0.3	0.6	0.9
Cellulose	30	30.7	26.4	23.1
Soybean oil	5	5	5	5
Premix[1]	10	10	10	10
Vitamin C[2]	5	5	5	5
Proximate composition of the experimental diets (g kg^{-1} dry matter basis)				
Dry matter	916.06	916.13	916.20	916.29
Ash	100.10	100.18	100.21	100.23
Crude Fibre	48.33	49.50	50.14	50.25
Crude lipid	68.41	69.23	69.51	70.03
Crude protein	322.09	322.15	322.38	322.44
GE (Cal g^{-1})[3]	4055	4050	4024	4012

Control (0g kg^{-1}), T1 (0.3 g kg^{-1} of LOB), T3 (0.6g kg^{-1} LOB) and T4 (0.9g kg^{-1} of LOB).

[1]Vitamin and trace mineral mix supplemented as follows (IU kg–1 or g kg–1 diet): retinyl acetate 1,085,000 IU; cholecalciferol 217,000 IU; D, L-a-tocopherol acetate 0.5 g; thiamin nitrate 0.5 g; pyridoxine hydrochloride 0.5 g; niacin 3 g; folic 0.05 g; cyanocobalamin 10 g; Ca pantothenate 1 g kg-1; inositol 0.5 g; zinc 1 g; copper 0.25 g; manganese 1.32 g; iodine 0.05 g; sodium 7.85 g.

[2]Vitamin C 98%

[3] GE = Gross energy

extract was lyophilized using ilShin Biobase freezer drier, and the lyophilized powder was stored at -20°C until diet preparation. The experimental diet was prepared by supplementing different levels of *L. aspera* extract, oxy-CD complex and sodium bentonite (LOB) into the basal diet at 0 g kg^{-1} (control), 0.3 g kg^{-1} of LOB (T1), 0.6 g kg^{-1} of LOB (T2), and 0.9 g kg^{-1} (T3) of LOB (in the ratio 1:2:3; Table 1). The diets were mixed evenly, pelletized, and dried in a vacuum freeze dryer for 15h. Thereafter, all diets were ground properly to ensure even mixing and extruded by a 5 mm mesh sieve. The prepared diets were stored at -20°C until the start of the experiment. Fish were fed with the prepared diets for 60 days.

Fish and Experimental Design

Nile tilapia, *Oreochromis niloticus*, used in the present study were transferred from a fish farm in Alleppey district, Kerala, India, to the KUFOS wet laboratory where the fish were acclimatized for two weeks in a 1000 L aerated fibre tank, and fed a control diet. After acclimatization, 240 fish were randomly distributed into twelve 150-L glass tanks at a density of 20 fish per tank (average weight 20.15±0.05g), assigned to four dietary groups in triplicate, and fed their respective diet for 60 day trial period. The fish were fed *ad libitum* twice a day at 9.00 h and 17.00 h. Throughout the experimental period, water quality parameters were monitored daily viz., temperature 28 ± 2°C, pH 7.7 ± 0.24, and dissolved oxygen 5.1 ± 0.31 mg L^{-1}.

Growth Performance

Fish were starved for 24 h before weighing for the determination of growth performance indices. Weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate were calculated as described below:

$$WG = \text{final weight (g)} - \text{initial weight (g)};$$

$$SGR\ (\%) = 100 \times (\ln \text{final weight} - \ln \text{initial weight}) / \text{duration of experiment};$$

$$FCR = \text{feed given (dried weight)} / \text{weight gain (wet weight)};$$

$$\text{Survival rate}\ (\%) = (\text{final fish number} / \text{initial fish number}) \times 100.$$

Digestive Enzyme Assay

For the digestive enzyme analysis, four fish were anaesthetized with clove oil and was euthanized by severing the spinal cord. The intestine was removed and rinsed with cold normal saline solution, homogenized and centrifuged (2500 x *g* for 10 min). The resultant supernatants were collected and kept at -80°C until further analysis. The protein content of the intestinal tissue was quantified following Bradford's method (Bradford, 1976) using bovine serum albumin as the standard. The α-amylase was estimated by the starch-hydrolysis method (Robyt and Whelan, 1968). The lipase activity was estimated by the method of Bier (1955). The total proteolytic activity was estimated based on the Casein-hydrolysis method described by Walter (1984).

Fish Blood Collection, Immunological and Haematological Analysis

Upon the completion of the 60-day trial, four fish were randomly selected from each replicate tank and anaesthetized with clove oil (5 ml L^{-1}). Blood samples were collected via the caudal vein using 1 ml hypodermic syringe, placed in an Eppendorf tube, and allowed to clot in a slanted position for 1 h at room temperature and stored in 4 C for 4 h. The samples were centrifuged at 1500 x *g*, for 5 min at 4°C, yellow-straw serum sample collected and stored at -20°C until assay. Fresh blood samples were collected from the caudal vein using a 1 mL heparin-coated syringe and were stored at 4°C for haematological analysis.

Lysozyme and Peroxidase Activity

The serum lysozyme activity was assayed following the method described by Parry et al. (1965) and described in our earlier study (Kurian et al., 2020). The peroxidase activity was evaluated by the methods of Quade and Roth (1997) and Cordero et al. (2016).

Respiratory Burst Activity and Antiprotease Assay

Respiratory burst activity was carried out as described by Secombes (1990) for measurement of the production of oxygen radicals from phagocytes. The serum antiprotease assay was assayed as described by Zuo and Woo (1997).

Haematological Analysis

Blood samples that were mixed with heparin were used for haematological analysis. Red blood cells count (RBCs) and white blood cells count (WBCs) were determined using a haemocytometer according to Sarder et al. (2001). Microhematocrit method was followed for determining haematocrit as described by Brown (1988). Level of haemoglobin was estimated by Sahli's method (Blaxhall and Daisley, 1973).

Liver and Kidney Function Test

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities were determined following the method of Reitman and Frankel (1957). Acid and alkaline phosphatase activity was estimated in line with Michell et al. (1970) and Estiarte et al. (2008). The serum creatinine level was

measured colourimetrically according to the method of Henry (1974) and the serum urea level was measured according to the method of Patton and Crouch (1977).

RNA Extraction, cDNA Synthesis and Real Time-PCR

For gene expression studies, the liver of the selected fish was carefully collected and transfer to RNA later. Total RNA was extracted using TRIzol (Sigma). Purity and quality of RNA were detected using Nanodrop (Thermo Fisher Scientific) as the 260:280 ratios were between 1.8-2.0. Afterwards, complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (iScript cDNA synthesis kit, Bio-Rad) according to the manufacturer's protocol. Specific primers for gene expression were designed using primer premier 5 software to amplify the selected genes with β-actin as a housekeeping gene (Table 2). The RT-PCR analysis was carried out using CFX96 Touch Real-time PCR detection system (Bio-Rad) and Sso Advanced Universal SYBR green supermix (Bio-Rad) following the manufacturer's instructions and as per the method described by Mahfouz (2015).

Statistical Analysis

The normality of the data was checked and confirmed by the Kolmogorov- Smirnov test. Then the data were analysed by one-way ANOVA using SAS Computer Program for least significant differences among the treatments. Where differences occur, the Duncan's Multiple Range Test was used to separate the mean. The mean values were considered significantly different when $P< 0.05$.

Results

Growth Performance

After 60 days of feeding, the growth indices in terms of final weight (FW), weight gain (WG) and specific growth rate (SGR) was found to be significantly higher in Nile tilapia fed 0.6 g/kg LOB-based diet than the control and other supplemented groups ($P < 0.05$). However, the feed conversion ratio (FCR) showed an opposite trend with the highest value record in the control fed fish compared to the lowest value seen in 0.6 g/kg LOB group ($p < 0.05$). Supplementation of LOB did not affect the survival of the fish (Table 3).

Digestive Enzymes Activity

Fish fed with 0.9 g/kg LOB diet recorded a significantly ($P < 0.05$) higher amylase, lipase, and protease enzyme activities compared with other supplemented groups and the control (Table 4).

Serum Immune Responses

The dietary supplementation of LOB had a significant impact on the innate immune response of Nile tilapia. The serum lysozyme, peroxidase, respiratory burst and antiprotease activities were found to increase with increasing level of LOB compared to the lowest

Table 2. Primers used for real - time PCR

Gene	Sequence (5' – 3')	Product size (bp)	Reference
IFN-γ	F: TGACCACATCGTTCAGAGCA R: GGCGACCTTTAGCCTTTGT	128	Yilmaz et al [83]
TNF-α	F: GAGGTCGGCGTGCCAAGA R: TGGTTTCCGTCCACAGCGT	119	Chen et al [84]
IgM-heavy chain	F: AGGAGACAGGACTGGAATGCACAA R: GGAGGCAGTATAGGTATCATCCTC	171	Pang et al [87]
TGF- β	F: TGCGGCACCCAATCACACAAC R: GTTAGCATAGTAACCCGTTGGC	105	Harms et al [88]
IL-1 β	F: AAGATGAATTGTGGAGCTGTGTT R: AAAAGCATCGACAGTATGTGAAAT	175	Zhi et al [85]
β-actin	F: ACAGGATGCAGAAGGAGATCACAG R: GTACTCCTGCTTGCTGATCCACAT	155	Zhi et al [85]

Table 3. Growth performance and feed utilization of *O. niloticus* fed on diets containing different levels of LOB (*L. aspera*, oxy-cyclodextrin and bentonite).

	Experimental groups			
Variables	Control	0.3 g kg^{-1}	0.6 g kg^{-1}	0.9 g kg^{-1}
Final weight (g $fish^{-1}$)	92.56 ± 0.35^{c}	93.34 ± 0.24^{c}	110.10 ± 0.38^{a}	100.73 ± 0.45^{b}
Weight gain (g $fish^{-1}$)	71.88 ± 0.30^{c}	72.75 ± 0.21^{c}	89.67 ± 0.37^{a}	80.23 ± 0.45^{b}
Specific growth rate (% day^{-1})	2.49 ± 0.004^{c}	2.51 ± 0.005^{c}	2.80 ± 0.019^{a}	2.65 ± 0.008^{b}
Feed conversion ratio	0.86 ± 0.003^{a}	0.85 ± 0.002^{a}	0.72 ± 0.002^{c}	0.79 ± 0.003^{b}
Survival rate (%)	100%	100%	100%	100%

Mean (± SE) value in the same row with different superscript differ significantly ($p<0.05$).

value recorded in the control group (Figure 1-4; P<0.05). The highest response was noticed in fish fed 0.9g kg^{-1} of the LOB.

Haematological Parameters

The supplementation of the concoction of LOB significantly (P<0.05) altered the haematological parameters of Nile tilapia (Table 5). The inclusion of 0.6 g/kg LOB resulted in statistically higher counts of red blood cells, haematocrit, and haemoglobin concentration compared to other dietary groups (P < 0.05). However, other erythrocyte indices (MCV, MCH, MCHC) and WBC counts were significantly higher in fish fed 0.9 g/kg diet (P < 0.05).

Liver and Kidney Function

After 60-days of feeding, the activities of AST and ACP were decreasing with the increasing levels of the LOB. The lowest value of AST and ACP was recorded in fish fed 0.9g/kg LOB-based diet (P<0.05). Also, ALT and ALP activities were significantly lower in fish fed 0.9 g/kg diet compared to other groups, however, no statistical differences were observed among the fish fed 0.3g/kg, 0.6 g/kg and the control (P>0.05). The activity of urea and creatinine were found to be higher in fish fed the control diet whereas 0.6 g/kg LOB-fed fish recorded the least value (P<0.05; Table 6).

Expression of Immune-Related Genes

The effects of LOB supplementation on immune-related gene expression in Nile tilapia is shown in Figure 5 (a)-(e). Significantly (P < 0.05) higher expression level of interleukin-1 beta (IL-1β), immunoglobulin M (IgM), transforming growth factor-beta (TGF-β) and interferon-gamma (IFN-γ) were recorded in fish fed 0.6 g/kg LOB-based diet compared to the control group. Furthermore, the relative expression of tumour necrosis factor-alpha (TNF-α) was upregulated in fish fed 0.9 g/kg diet than other dietary groups (P < 0.05).

Discussion

Aquaculture is one of the fastest-growing food-producing sectors in the world. Among the farmed fishes, tilapias are the second most important finfish cultured after carp (Waite et al., 2015). However, considering the limitation of land and water resources, increased intensification of aquaculture operation is one of the viable alternatives to meet up with global seafood demand. This strategy often results in an increased incidence of diseases and immune suppression; hence, it is necessary to maintain the health of farmed fishes to meet the global demand for chemical/antibiotic-free seafood (Bulfon et al., 2015). Previous studies have reported that the inclusion of medicinal plants in the fish diet has a favourable impact on growth, immunity and

Table 4. Digestive enzyme activities of *O. niloticus* fed on diets containing different levels of LOB (*L. aspera*, oxy-cyclodextrin and bentonite).

	Experimental groups			
Parameters	Control	0.3 g kg^{-1}	0.6 g kg^{-1}	0.9 g kg^{-1}
Amylase (U mg^{-1})	2.19±0.77^{c}	5.32±0.44^{b}	6.18±0.53^{b}	9.02±0.61^{a}
Lipase (U mg^{-1})	1.48±0.02^{c}	2.0±0.04^{b}	2.2±0.05^{b}	5.11±0.09^{a}
Protease (U mg^{-1})	7.66±0.15^{c}	9.63±0.14^{b}	10.27±0.04^{b}	14.51±0.06^{a}

Mean (± SE) value in the same row with different superscript differ significantly (p<0.05).

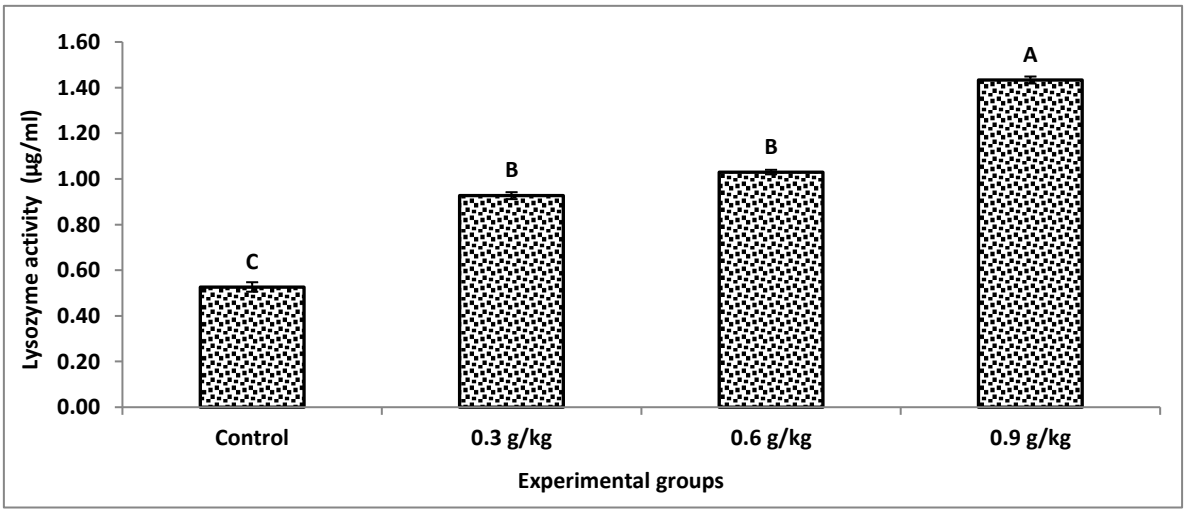

Figure 1. Serum lysozyme activity of *O. niloticus* fed experiment diets containing different concentration of LOB (mean ± SE, n=4). Bars assigned with different superscripts are significantly different (P<0.05)

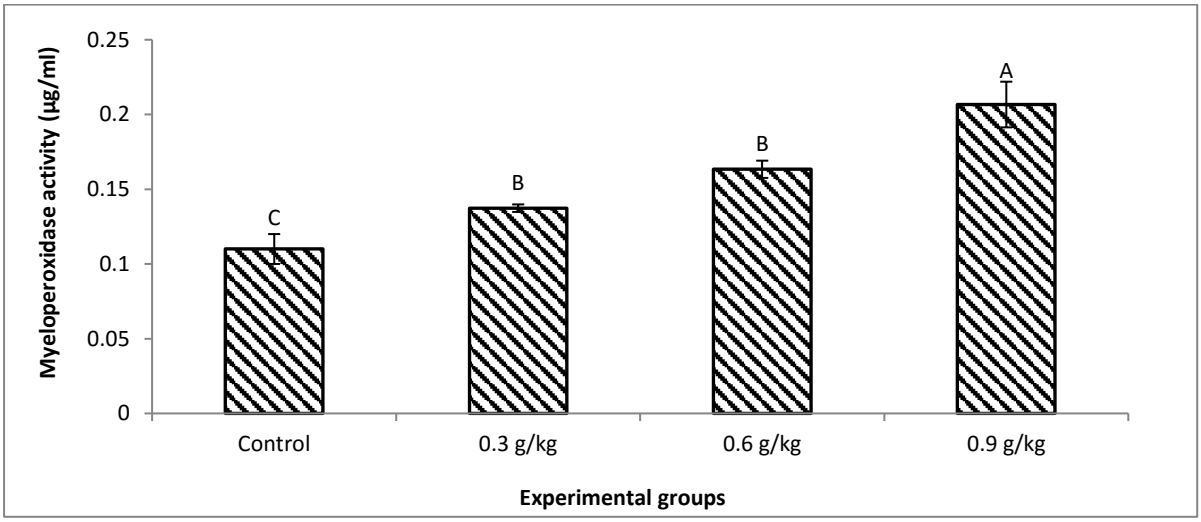

Figure 2. Serum peroxidase activity of *O. niloticus* fed experiment diets containing different concentration of LOB (mean ± SE, n=4). Bars assigned with different superscripts are significantly different (P<0.05).

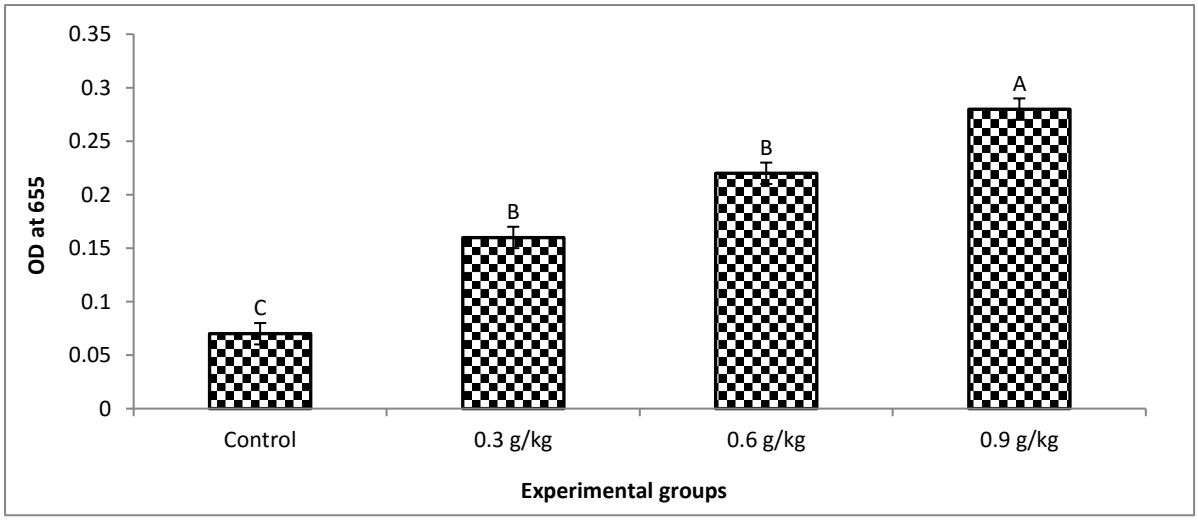

Figure 3. Serum respiratory burst activity of *O. niloticus* fed experiment diets containing different concentration of LOB (mean ± SE, n=4). Bars assigned with different superscripts are significantly different (P<0.05).

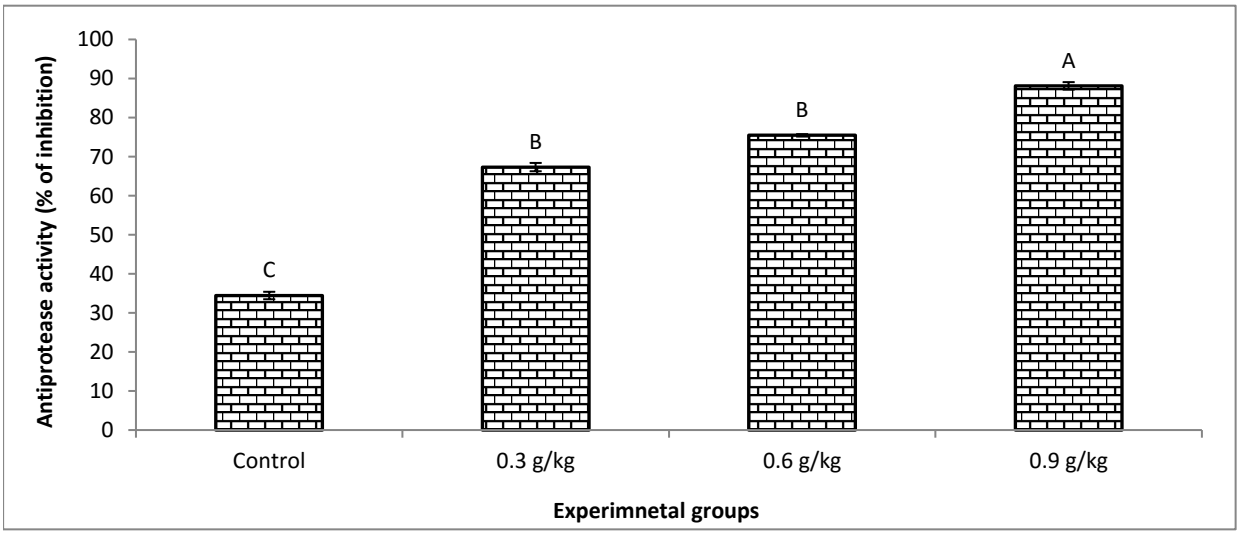

Figure 4. Serum antiprotease activity of *O. niloticus* fed experiment diets containing different concentration of LOB (mean ± SE, n=4). Bars assigned with different superscripts are significantly different (P<0.05).

Table 4. Digestive enzyme activities of *O. niloticus* fed on diets containing different levels of LOB (*L. aspera*, oxy-cyclodextrin and bentonite).

	Experimental groups			
Parameters	Control	0.3 g kg^{-1}	0.6 g kg^{-1}	0.9 g kg^{-1}
Amylase (U mg^{-1})	2.19±0.77^{c}	5.32±0.44^{b}	6.18±0.53^{b}	9.02±0.61^{a}
Lipase (U mg^{-1})	1.48±0.02^{c}	2.0±0.04^{b}	2.2±0.05^{b}	5.11±0.09^{a}
Protease (U mg^{-1})	7.66±0.15^{c}	9.63±0.14^{b}	10.27±0.04^{b}	14.51±0.06^{a}

Mean (± SE) value in the same row with different superscript differ significantly (P<0.05).

Table 5. Haematological parameters of *O. niloticus* fed on diets containing different levels of LOB (*L. aspera*, oxy-cyclodextrin and bentonite).

	Experimental groups			
Parameters	Control	0.3 g kg^{-1}	0.6 g kg^{-1}	0.9 g kg^{-1}
RBC (10^{7} mL^{-1})	1.07±0.01^{b}	1.35±0.02^{b}	1.74±0.01^{a}	1.36±0.01^{b}
WBC (10^{5} mL^{-1})	1.45±0.01^{d}	1.67±0.01^{c}	1.92±0.01^{b}	2.18±0.02^{a}
Hematocrit (%)	16.43±0.07^{d}	20.5±0.10^{c}	30.14±0.02^{a}	25.56±0.05^{b}
Hemoglobin (g %)	1.31±0.01^{c}	1.65±0.01^{b}	1.92±0.01^{a}	1.67±0.01^{b}
MCV (μm^{3})	219.6±0.56^{c}	222.97±0.30^{b}	224.93±.18^{b}	228.5±0.30^{a}
MCH (pg)	36.08±0.17^{c}	37.48±0.08^{c}	40.36±0.21^{b}	44.38±0.79^{a}
MCHC (%)	20.39±0.25^{c}	24.5±0.17^{b}	25.5±0.07^{b}	29.3±0.19^{a}

Mean (± SE) value in the same row with different superscript differ significantly (P<0.05).

Table 6. Hepatic and renal markers in *O. niloticus* fed on diets containing different levels of LOB (*L. aspera*, oxy-cyclodextrin and bentonite).

	Experimental groups			
Parameters	Control	0.3 g kg^{-1}	0.6 g kg^{-1}	0.9 g kg^{-1}
AST (U/L)	68.71±0.19^{a}	55.68±0.33^{b}	48.43±0.21^{b}	30.49±0.31^{c}
ALT (U/L)	88.77±0.35^{a}	84.28±0.19^{a}	81.82±0.31^{a}	62.74±0.34^{b}
ALP (U/L)	14.23±0.07^{a}	11.23±0.12^{a}	8.63±0.25^{a}	5.17±0.61^{b}
ACP (U/L)	41.36±0.94^{a}	31.00±0.70^{b}	24.3±0.78^{b}	10.8±0.20^{c}
Creatinine (mg/dL)	1.03±0.03^{a}	0.89±0.00^{b}	0.54±0.01^{c}	0.74±0.01^{b}
Urea (mg/ dL)	22.19±0.04^{a}	19.13±0.06^{b}	15.36±0.05^{c}	18.13±0.05^{b}

Mean (± SE) value in the same row with different superscript differ significantly (P<0.05).

disease resistance against pathogenic organism (Fawole et al., 2016; Rashidian et al., 2018; Van Doan et al., 2019; Garg et al., 2019; Hoseinifar et al., 2020). In the present study, dietary LOB supplementation significantly reduces the FCR and improved the FBW, WG, and SGR of Nile tilapia. The better growth observed in 0.6g/kg fed group could be attributed to improved utilization of nutrients resulting from the synergistic effect of the bioactive compounds present in LOB (*L. aspera*, Oxy-cyclodextrin and Bentonite) and their influence on the digestive enzyme function. Furthermore, the higher somatic growth in LOB groups may be associated with increased activities of digestive enzymes (amylase, lipase and protease) required for nutrient hydrolysis. The previous study has reported that increased production of digestive enzymes without hindrances could result in improvement in digestibility and nutrient availability for muscle growth and development (Chesson, 1987; Wang et al., 2018). It is believed, that the absorptive properties of bentonite might have helped in delaying the passage of pre-digested food in the intestine thereby given more time for the digestive enzyme to hydrolyse the nutrients, and also the bioactive substance present in *L. aspera* and Oxy-cyclodextrin to exert their beneficial effect. Herbs have been described as a potent stimulator of pancreatic enzymes involved in nutrients digestion and absorption in livestock animal and fish (Frankic et al., 2009; Citarasu, 2010). Similar to the present study, Hu et al. (2012) reported that the dietary combination of montmorillonite with zinc significantly enhanced the growth performance and improve the integrity of intestinal mucosal and the activity of digestive enzymes in pig. Growth performance and nutrient utilization in rainbow trout and stinging catfish were also improved after feeding a diet supplemented with natural clay (Eya et al., 2008; Jawahara et al., 2018). Wang et al. (2018) reported that dietary Chinese herbal medicines mixture significantly improved the digestive enzyme (erepsin, pepsin, gastric lipase and amylase) activities in Japanese seabass.

Innate immunity is the cardinal defence mechanism in fish and some of the most frequently studied innate immune response parameters in herbal supplemented feeds in teleost include lysozyme (LYZ), respiratory burst (RB), myeloperoxidase (MP),

immunoglobulins (IM), complement system (CS), and antiprotease (AP) (Mahmoud et al., 2017; Yousefi et al., 2019). In this study, the serum LYZ, RB, MP and AP activities were found to be significantly ($P<0.05$) increased with increasing level of LOB and the highest response was observed in fish fed 0.9g kg^{-1}. Enhancement of serum antiprotease, a humoral immune parameter, helps in the defence against bacterial infections by stimulating the production of antimicrobial peptides, complement and immunoglobulins. Also, induction of lysozyme, respiratory burst and myeloperoxidase are an indication of immunological response as reported by previous studies when fish were fed herbal extract alone or in combination with other compounds (Fawole et al., 2016; Baba et al., 2016; Reyes-Cerpa et al., 2018; Yousefi et al., 2019; Kurian et al., 2020). Thus, the higher response of these innate parameters indicates that the dietary LOB supplementation modulated the innate immunity in Nile tilapia, and this could be associated with the polyphenols, alkaloids and flavonoids in *L. aspera* and oxyresveratrol. Van Doan et al. (2019) reported that the dietary inclusion of Assam tea extracts significantly enhanced the serum peroxidase, lysozyme and respiratory burst activities in Nile tilapia. Also, sodium bentonite improved the respiratory burst,

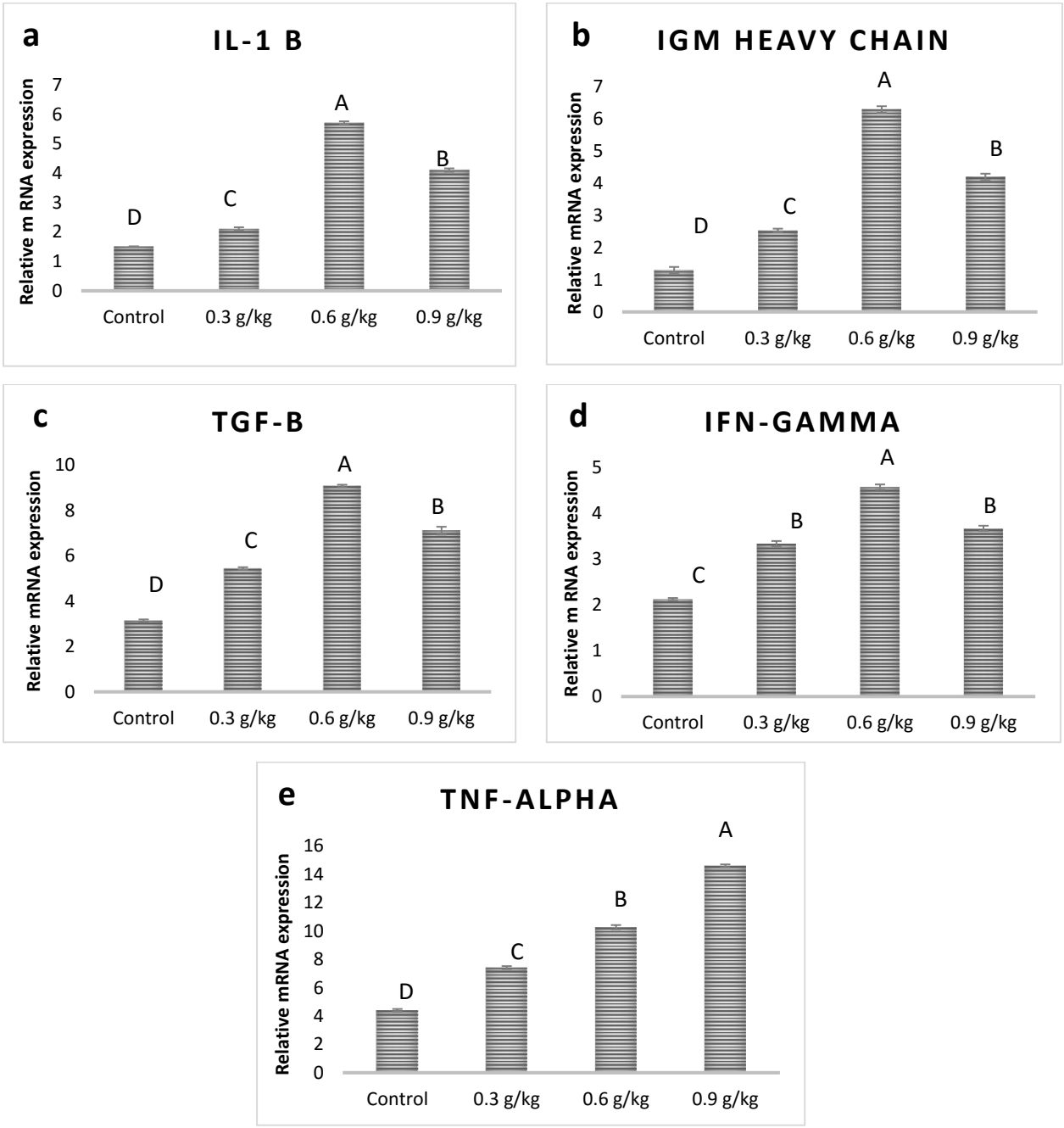

Figure 5. a-e: Relative immune gene expression in *O. niloticus* fed experiment diets containing different concentration of LOB (mean ± SE, n=4). Bars assigned with different superscripts are significantly different ($P<0.05$).

alternative complement activity, phagocytic and lysozyme activities in stinging catfish infected with *Aeromonas hydrophila* (Jawahar et al., 2018).

Haematological indices are one of the widely used indicators for the evaluation of overall health, nutritional and physiological status of fish in response to immunostimulant feed (Fawole et al., 2017; Mbokane and Moyo, 2018). The erythrocyte indices were found to be high in fish fed diet 0.6 g/kg, whereas the WBC count was the highest in fish fed 0.9 g/kg diet. The positive effect of LOB on the haematological parameters suggests that the presence of vitamin C in *L. aspera* increases the absorption of iron from the intestine, and dietary LOB would not cause anaemic condition in the fed fish as indicated by higher erythrocytic index (MCV, MCH and MCHC). The enhanced WBCs which is in line with lysozyme activity, further affirm the potency of LOB in elevating non-specific immunity in Nile tilapia, similar to the previous report in rohu (Fawole et al., 2016), rainbow trout (Taheri Mirghaed et al., 2018), Nile tilapia (Elumalai et al., 2019), and common carp (Yousefi et al., 2019). Elevation of serum AST and ALT levels is commonly used as an indicator of damage to the hepatic tissue and well-being in fish (Hassaan et al., 2019; Adeoye et al., 2020; Moustafa et al., 2020). Results of the present study revealed decreased activity of liver enzymes (AST, ALT and ALP) in fish fed LOB diets, especially 0.9 g/kg fed fish, compared to the control. This indicates that LOB has the potential to protect the hepatocyte integrity as reported in Nile tilapia fed *Silybum marianum* (Hassan et al., 2019). Resveratrol and bentonite have been reported to possess the hepatoprotective property and help to restore normal liver function in stress-induced liver damage in Nile tilapia and pig fed aflatoxin-contaminated diet, respectively (Schell et al., 1993; Jia et al., 2019). Similarly, two biochemical indices in determining renal function are creatinine and urea and increased levels of these indicate a reduction in kidney function (Del Zotti et al., 2008). The results of the kidney function obtained in this study suggest that fish fed different concentrations of LOB has a better functioning kidney than the control fed fish.

Immunostimulants have been found to increase pro-inflammatory responses in fish (Safari et al., 2017; Moustafa et al., 2020). Pro-inflammatory cytokines like TNFα and IL-1β induce inflammatory responses. Moustafa et al. (2020) stated that the transcription of pro-inflammatory cytokines at a moderate level is advantageous to increase fish resistance against infection pathogen and maintain immunological balance. In the present study, significant upregulation of immune genes (TNFα, IL-1β, IgM heavy chain, TGF-β and IFN-γ) were recorded in LOB fed groups. TNFα in fish gets expressed at an early stage of infection and has overlapping functions with IL-1β, promotes phagocytosis and bactericidal properties. Thus, the higher expression of TNFα recorded in all the groups fed with LOB signals activation of phagocytic cells and its readiness to engulf any foreign agents quickly than when in an unstimulated state, and this assertion corresponds with the result of respiratory burst activity earlier reported in this study. In a similar vein, higher relative expression of immune-related (TNFα and IL-1β) gene was noticed in Nile tilapia fed fenugreek supplemented diet (Moustafa et al., 2020). Panprommin et al. (2016) reported that the holy basil extract significantly stimulated the relative expression levels of pro-inflammatory cytokines (IL-1β and IL-8) in Nile tilapia. Furthermore, the upregulated expression of IgM heavy chain, IFN-γ and TGF-β after feeding LOB is an indication of innate immune system improvement and increased ability of the fish to resist pathogen attack. This enhancement effect could be related to the polyphenols and other bioactive elements that form the LOB amalgam. Our result herein is in accord with Panprommin et al. (2016) and Hassaan et al. (2019) who reported significantly higher expression of TGF-β and IgM-2 in Nile tilapia fed holy basil and *Silybum marianum* seed, respectively. Also, Prabu et al. (2016) reported a significantly upregulated expression of IFN-γ2a in healthy and *Aeromonas hydrophila*-challenged *Labeo rohita* fed with fucoidan-rich seaweed extract.

In conclusion, the present study reveals that the combination of *L. aspera*, oxy-cyclodextrin and bentonite synergistically enhanced the growth and digestive enzyme functions, protected the hepatic tissue and stimulated the innate immunity in Nile tilapia. This additive can be utilized as a potential feed supplement without causing any adverse effect on fish health and well-being. Thus, based on the measured parameters, 0.6 - 0.9 g/kg of the LOB is recommended for inclusion to improve good and health status of Nile tilapia.

Acknowledgement

Amitha Kurian is thankful for the KUFOS PhD fellowship provided by the School of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies (KUFOS), Kochi, Kerala, India for completing this study.

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