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Effects of Biotronic® Top3, a feed additive containing organic acids, cinnamaldehyde and a permeabilizing complex on growth, digestive enzyme activities, immunity, antioxidant system and gene expression of barramundi (*Lates calcarifer*)

Ebrahim Sotoudeh [a,*], Moha Esmaeili [b]

[a] Department of Fisheries, Faculty of Nano and Bio Science and Technology, Persian Gulf University, 75169 Bushehr, Iran
[b] Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, TAS, Australia

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ABSTRACT

The Biotronic® Top3 (OCAP: organic acids, cinnamaldehyde, and permeabilising complex) is used for improving microbial community in monogastric animals. This research was aimed to test the five dosages of OCAP (0%: control, 0.05%: OCAP0.05, 0.10%: OCAP0.1, 0.15%: OCAP0.15%, and 0.20%: OCAP0.2) in barramundi (*Lates calcarifer*) (8.3 ± 0.3 g) for eight weeks. The results showed a linear relation between OCAP level and growth rate, feed conversion ratio, protease, lipase, lysozyme in serum and mucus, alternative complement activity (ACH50) in serum, catalase, glutathione peroxidase, and malondialdehyde levels ($P < 0.05$). Accordingly, the OCAP0.2 treatment had the best performance in the abovementioned parameters. While there was no significant difference in expression of heat shock proteins (HSP70, HSP90) and tumour necrosis factor (TNF-a) among groups, interleukins (IL-1β, IL-8) were significantly lower in the OCAP0.15 and OCAP0.2 group than others. The expression of IGF-1 followed the growth rate trend. Generally, feeding fish with the OCAP0.2 diet improved growth rate, digestive enzymes activities, immune response, antioxidant system, and gene expressions in barramundi. As the linear relation was observed, supplementing more levels of OCAP to reach a plateau is recommended.

1. Introduction

One of the biggest obstacles to developing aquaculture has been the limitations of freshwater (FAO, 2020). Therefore, mariculture and coastal aquaculture have been among the best candidates for providing food for the next century. This kind of aquaculture already accounts for more than 40% of total aquaculture production (82 million tonnes) in 2018 (FAO, 2020). Barramundi (*Lates calcarifer*) is one of the most common fish species farmed around the world. Australia, Singapore, Saudi Arabia, Malaysia, India, Indonesia, Vietnam, Thailand, the United States, Poland, and the United Kingdom are the biggest producers (FAO, 2020). Recently, the complete farming cycle of this fish species in Iran has been localised. It is predicted barramundi to be one of the main marine aquaculture species in the next decades in Iran.

Growth performance and feed efficiency determine the profitability of any aquaculture system. Therefore, any actions that can improve these two parameters, such as dietary manipulations, can be a forward step to achieve sustainability of aquaculture (Asgari et al., 2020; Ghosi Mobaraki et al., 2020; Asadi et al., 2021). Biomin company (Getzersdorf, Austria) recently produced a feed supplement named Biotronic® Top3 (OACP), which contains formic, propionic and lactic acids alongside cinnamaldehyde and the Biomin® Permeabilizing Complex, a proprietary complex. Biomin® Permeabilizing Complex increases membrane permeability to help organic acids and cinnamaldehyde to get into the bacterial cell. It is claimed that it can balances intestinal microbiota, inhibit the growth of gram-negative bacteria like *Vibrio* sp., *Aeromonas* sp., and improve the survival rate of fish. Only three studies investigated the effect of this supplement on farm animals (Stensland et al., 2015; Benţea et al., 2016; Menanteau-Ledouble et al., 2017). Infected rainbow trout (*Oncorhynchus mykiss*), when fed supplemented diets with OACP, had a mortality rate of 30% compared to 75% in control group (Menanteau-Ledouble et al., 2017). When pigs were fed this supplement

* Corresponding author.
E-mail addresses: e.sotoudeh@pgu.ac.ir (E. Sotoudeh), moha.esmaeili@utas.edu.au (M. Esmaeili).

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(1.5% in diet), no improvement in growth rate and feed conversion ratio (FCR) was observed (Stensland et al., 2015). However, in chickens, improved growth and FCR with supplementing OACP were reported (Benţea et al., 2016).

Many studies have reported an improvement in growth, immunity, and antioxidant response by feeding fish with organic acids (see reviews (Hoseinifar et al., 2017; Ng and Koh, 2017)) as well as our studies (Matani Bour et al., 2018; Badzohreh et al., 2020; Sotoudeh et al., 2020; Sangari et al., 2021). Recently aquaculture researchers reported that cinnamaldehyde positively affected growth performance, digestive enzymes activity, antioxidant capability, immune system and intestinal microbiota in channel catfish (*Ictalurus punctatus*) (Mousa et al., 2021), tongue sole (*Cynoglossus semilaevis*) (Wang et al., 2021), Nile tilapia (*Oreochromis niloticus*) (Abd El-Hamid et al., 2021), grass carp (*Ctenopharyngodon idella*) (Zhou et al., 2020), and striped snakehead (*Channa striatus*) (Harikrishnan et al., 2021).

There is no long-term study in fish to investigate the effect of OACP on immunity, antioxidant system and gene expression let alone in marine species. Therefore, this study was designed to examine how OACP can improve growth performance, digestive enzyme, immunity, antioxidant response, and gene expression of barramundi.

2. Material and methods

2.1. Ethics statement

To optimise handling and minimise animal stress, the guidelines, adopted from the Declaration of Helsinki (1975) and the Society for Neuroscience Animal Care and Use guidelines (1998) were approved for implementation by the Medical Ethics Committee, School of Medical Sciences of the Tarbiat Modares University on the 28th day of Farvardin, 1385 (April, 17, 2006) was used.

2.2. Experimental diets

Five isonitrogenous (450 g crude protein/kg feed) and isoenergetic (21 kJ/g) diets were formulated by Lindo software. These protein, lipid and energy levels are sufficient for barramundi (Glencross, 2006). The OACP supplement was provided by Etouk Farda Feed Additives Co (Iran, Tehran). The treatments include control, OCAP0.05, OCAP0.1, OCAP0.15, and OCAP0.2, which 0%, 0.05%, 0.1%, 0.15%, and 0.2% OCAP were added, respectively. The dosage was selected based on previous work (Menanteau-Ledouble et al., 2017) and the company recommendation. The dietary ingredients were first dried and mixed carefully and became homogeneous. After complete mixing, liquid ingredients such as Kilka fish oil, sunflower oil, and lecithin were weighed carefully and added gradually to the mixture. The resulting mixture was compressed by a meat grinder (Electrokar EC-1, Tehran, Iran) to form pellets with a 2 mm diameter. Then, pellets were spread out on a tray and dried in an oven to $\geq$ 90% dry matter at 60 °C for 24–48 h. After dried, the feeds were packed in suitable packages and kept at 4 °C. The chemical compositions of experimental diets are presented in Table 1.

2.3. Fish and husbandry trial

The husbandry trial was carried out at the laboratory of the Aquatic Research (Persian Gulf University, Bushehr, Iran). Fish were purchased from a private local farm (Ramoz, Boushehr province, Iran) and transferred to the laboratory. Upon fish arrival, they were acclimated to the experimental condition for 14 days and fed with a commercial diet (21 Beyza, Shiraz, Iran; particle size: 2 mm, 48% crude protein, 18% crude fat, 10.7% ash, 10% moisture and 2.2% fibre). Two hundred and seventy fish (8.3 ± 0.3 g) were stocked into 15 tanks (300 L capacity, 18 fish per tank, triplicate). Tanks were filled with filtered and disinfected (chlorine, 10 ppm) sea water (42.0 ± 0.6 ppt), and about twenty percent of water was exchanged daily. The average temperature and pH were 28.0 ± 0.5 °C and 7.5 ± 0.5, ammonia-nitrogen 0.09 ppm, and the photoperiod were 12 L: 12D (Light: Dark). During 8 weeks of the feeding trial, fish were fed with the diets two times a day (10:00 and 16:00 hr) at apparent satiation. Each dietary treatment was carried out with three replicates.

Table 1
Ingredients and proximate composition of the experimental diets included different levels of OACP.

Ingredients	Experimental diets				
	Control	OACP0.05	OACP0.1	OACP0.15	OACP0.2
Fish meal	30	30	30	30	30
Soybean meal	30.2	30.2	30.2	30.2	30.2
Wheat meal	13.28	13.28	13.28	13.28	13.28
Corn gluten	7	7	7	7	7
Wheat gluten	5	5	5	5	5
Fish oil	6	6	6	6	6
Sunflower oil	2	2	2	2	2
Soy lecithin	1	1	1	1	1
Mineral mix[1]	1	1	1	1	1
Vitamin mix[1]	1	1	1	1	1
L-lysine	0.5	0.5	0.5	0.5	0.5
DL-methionine	0.5	0.5	0.5	0.5	0.5
Dicalcium phosphate	1	1	1	1	1
Yeast	1	1	1	1	1
Antioxidant	0.02	0.02	0.02	0.02	0.02
OACP[2]	0	0.05	0.1	0.15	0.2
Cellulose	0.5	0.45	0.4	0.35	0.3
Proximate analysis (%)[4]					
Protein	45.8	45.6	45.2	45.09	45.5
Lipid	15.1	14.9	15.1	15.3	15.2
Ash	11.3	11.2	10.8	11.2	10.9
Moisture	8.5	8.7	8.2	8.5	8.4
Gross energy (kJ g^{-1})[3]	21.2	21.4	21.8	21.61	21.2

[a] Composition of ingredients as % dry-weight basis: fish meal (70% crude protein, 11.8% crude lipid); soybean meal (45.0% crude protein, 5% crude lipid); corn gluten (70% crude protein, 3.8% crude lipid); wheat gluten (58% crude protein, 2.8% crude lipid).
[3] Vitamin and Mineral premix U kg^{-1} of premix: vitamin A, 5,000,000 IU; vitamin D3, 500,000 IU; vitamin E, 3000 mg; vitamin K3, 1500 mg; vitamin B1, 6000 mg; vitamin B2, 24,000 mg; vitamin B5, 52,000 mg; vitamin B6, 18,000 mg; vitamin B12, 60,000 mg; Folic acid, 3000 mg; nicotinamide 180,000 mg; antioxidant, 500 mg, copper, 3000 mg; zinc, 15,000 mg; manganese, 20,000 mg; Iron, 10,000 mg; potassium iodate, 300 mg, career up to1 kg, Damloran pharmaceutical company, Broujerd, Iran.
[2]OACP: a mixture of an organic acid blend, cinnamaldehyde and permeabilizing complex (Biomin Iran, Tehran, Iran).
[3] Calculated on gross energy values of 23.6 kJ g^{-1} proteins, 39.5 kJ g^{-1} fat and 17.2 kJ g^{-1} carbohydrates (NRC, 2011).
[4] The proximate analysis of diets was done according to standard AOAC method (AOAC, 2000).

2.4. Growth performance

At the end of the feeding trial, all fish were fasted for 24 h and were then anesthetised with the clove oil stock solution (50–70 ppm) (Esmaeili et al., 2017a). The survival rate and growth indices, including final weight, specific growth rate (SGR), FCR, daily feed intake (DFI), and condition factor (CF), were determined using standard methods and relationships in all fish. The abovementioned parameters were calculated as follows:

SGR (%BW/day): specific growth rate (% body weight/day) = ((ln final weight – ln Initial weight) / days) × 100)

Weight gain (%) = ((final body weight – initial body weight)/ initial body weight) × 100

FCR: feed conversion ratio = feed intake (g) / weight gain (g)

DFI (%BW/day): Daily feed intake= total feed fed (g)/ average body weight (g) /days

Condition factor = (body weight (g)/total length^3 (cm)) ×100

Survival rate (%) = number of fish in each group remaining on day 56/initial number of fish) × 100

2.5. Digestive enzyme activities

Amylase, lipase, and protease measurements were done in the collected digestive tracts from each fish (three fish per replicate) after homogenising samples on ice by an electric homogeniser. The Bradford method (Bradford, 1976) was used to measure the total protein content of the supernatant using bovine serum albumin (BSA) as a standard. For measuring protease activities using tyrosine as a standard (Hidalgo et al., 1999), enzyme reaction mixtures consisted of 1% (w/v) casein in water (0.25 ml), buffer (0.25 ml), and enzyme sample (0.1 ml) were incubated for 1 h at 37 °C. Trichloroacetic acid 8% (w/v) stopped the reaction and absorbance were measured at 280 nm (Hidalgo et al., 1999). Lipase activity was assayed by using 0.53 mM pnitrophenyl myristate dissolved in 0.25 mM Tris – HCl, 0.25 mM 2 – methoxy ethanol and 5 mM sodium cholate buffer (pH= 9.0). Briefly, 5 μL of enzyme extract in 0.5 ml of the substrate was incubated for 15 min at 30 °C. The reaction was stopped by the addition of 0.7 ml of acetone:n-heptane (5:2), the extract was centrifuged for 3 min at 6080 g and 4 °C, and absorbance was recorded at 405 nm. In the blank, acetone: n-heptane was added to the substrate before the addition of enzyme extract (Iijima et al., 1998; Ravardshiri et al., 2021). Specific activity (U) was expressed as:

Lipase activity = (A (sample (280 nm)) × value × 1000)/(15 × 16500 × mg protein)

Dissolved starch in 100 ml buffer containing 20 mM sodium phosphate and six mM NaCl (pH = 6.9) was used to measure amylase (Bernfeld, 1955; Worthington, 1991). Briefly, 250 μL of enzyme extract was incubated for 3–4 min at 25 °C. This was followed by the addition of 0.5 ml dinitrosalicylic acid (DNS) and incubation for 5 min at 100 °C and the addition 5 ml water. Absorbance was recorded at 540 nm, and the quantity of maltose released was determined from a standard curve prepared from maltose solution (Ravardshiri et al., 2021). One unit was calculated as the quantity of enzyme that released one μmol of maltose in 1 min. Specific activity (U) was expressed as:

Amylase activity= (Maltose released (μmol))/(3 × mg protein)

2.6. Blood collection and serum and skin mucus sample preparation

Seven fish from each tank were sampled for immune response and antioxidant activities. Four fish samples were pooled to provide two sub-samples and three fish samples were pooled to provide one sub-sample. The stock solution of clove oil (50 mg/L) anesthetised fish for preventing stress (Esmaeili et al., 2017b), and blood samples were collected quickly by venipuncture of the caudal vein using a sterile 5-ml syringe. In the next step, blood was kept in the fridge for 2 h for blood clotting, and then serum was collected after centrifuging in 3000*g* at 4 °C (Esmaeili et al., 2017b). A small piece of sterilised cotton was used to collect skin mucus at the same area of each fish on the body surface. Then, the collected samples were immediately suspended in 1 ml of phosphate buffer saline (PBS, pH = 7.2) and centrifuged (2000*g*, 10 min, 4 °C) (Esmaeili et al., 2019).

2.7. Antioxidant activity and Immune response

The antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and malondialdehyde (MDA) in serum were determined using an analysis ELISA kits (ZellBio, GmbH, Germany) according to the protocol. Serum lysozyme was determined by the gram-positive bacteria sensitive to the lysozyme enzyme via using bacteria (*Micrococcus lysodeikticus*) (Clerton et al., 2001; Tukmechi et al., 2007). The amount of the sample resulted in a decrease in absorbance of 0.001/min, considered a unit of lysozyme activity. For the determination of alternative complement activity (ACH50), the hemolysis of rabbit RBCs (RaABC) was performed (Amar et al., 2000), which was described in our previous works (Ramezanzadeh et al., 2020a; Zeilab Sendijani et al., 2020).

2.8. Evaluation of relative expression of genes

For measuring expression of IGF-1, HSP70, and HSP90 in liver; IL-1β, IL-8, and TNF-α, in the kidney (Table 2), the frozen tissue samples were homogenised in liquid nitrogen. Four fish samples were pooled to provide two sub-samples and three fish samples were pooled to provide one sub-sample. The RNA extraction was done by using a RiboExTM reagent (GeneAll®, Seoul, South Korea). The 2-step RT-PCR kit (BIOFACTTM, Daejeon, South Korea) was used for cDNA synthesising from 2 μg of RNA. For measuring these six genes, real-time PCR was performed in a total volume of 20 μL including RealQ plus 2x Master Mix Green (Ampliqon), forward and reverse primers, cDNA and nuclease-free water, using a EcoTM Real-Time PCR system (Illumina®). Table 2 showed the primer sequencing and GenBank accession numbers for all genes. Real-time PCR was performed at 95 °C for 15 min, followed by 40 cycles at 95 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Logarithmic serial dilution (five serial dilutions) with three replicates was used to obtain the efficiencies of PCR. After PCR amplification, melt-curve analysis was conducted to confirm that there was only one amplified product. The data for the relative abundance of mRNA transcript was obtained by conducting three independent biological replicates, which were performed in duplicate to calculate the threshold cycle (Ct) value. The comparative Ct ($2^{-\Delta\Delta CT}$) method was used (Livak and Schmittgen, 2001).

2.9. Statistical analysis

This experiment was conducted in a completely randomised design with five treatments and three replications. Shapiro–Wilk and Levene's tests were used to test for normality and homogeneity of variance, respectively. All data were analysed by one-way analysis of variance

Table 2
Target genes and primer sequences of barramundi.

Gene[a]	Sequences of primers	Accession number
IL-1β	F: CCTGTCGCATTTCAGTACGG R: ATTTCCACCGGCTTGTTGTC	XM_018669006
IL-8	F: TCTGACTGTTCCTGAGGCTATC R: GACGTCCAATGGGCTTTCT	XM_018705196
TNF-α	F: AGAGCATCAAACCCAGCTCA R: CTCAATGCGTGACGAAGATCAAAA	XM_018695468
hsp-70	F: CTGGAGTCCTACGCTTTCAA R: CTTGCTGATGATGGGGTTAC	HQ646109
hsp-90	F: ACGATGATGAGCAGTATGCC R: CAAACAGGGTGATGGGGTA	XM_018661637
IGF-1	F: ACGCTGCAGTTTGTATGTGG R: CCTTAGTCTTGGGAGGTGCA	XM_018697285
Beta actin	F: AACCAAACGCCCAACAACT R: ATAACTGAAGCCATGCCAATG	XM_018667666

[a] IL-1β, interleukin-1β; IL-8, interleukin-8; TNF-α, tumor necrosis factor-like; hsp-70, heat shock cognate 70 kDa protein; hsp-90, heat shock cognate 90 kDa protein; IGF-1, insulin-like growth factor 1.

(ANOVA) using SPSS (version 22.0 for Windows). Duncan's multiple range tests were used to assess differences among six treatments in growth performance, digestive enzymes, immune response, antioxidant activities and gene expression. For providing a comprehensive analysis, we applied orthogonal polynomial contrasts to determine if the OACP level had linear and/or quadratic relations with measured parameters (Zaretabar et al., 2021). Pearson's correlation analysis among parameters was conducted using SPSS (version 22.0 for Windows).

3. Results

3.1. Growth performance and survival rate

According to Table 3, in fish fed OACP higher than 0.1% in the diet, final weight and FCR were improved and declined, respectively compared with control ($P < 0.05$). The final weight for OACP0.1, OACP0.15, and OACP0.2 was 33.76, 34.35, and 33.43 respectively, significantly higher than the others ($P < 0.05$). There was no significant difference in DFI, CF, and survival rate values among treatments.

According to Fig. 1, IGF-1 has a linear relation with the OACP level in diets which was compatible with growth data. Further, OACP0.1, OACP0.15, and OACP0.2 treatments had a significantly higher value of IGF-1 than others ($P < 0.05$).

3.2. Digestive enzymes

The results indicated that protease activity (U/mg protein) and lipase (U/mg protein) had a linear relation with the level of OACP, and the barramundi fed dietary OACP0.2 displayed the highest value of protease (5.85) and lipase (1.12) ($P < 0.05$). Fish fed dietary OACP0.15 and OACP0.2 had significantly higher values of protease and lipase as compared with those fed other groups. There was no significant difference in amylase activity across treatments (Table 4).

3.3. Antioxidant activities

In the present research, OACP0.15 and OACP0.2 groups had the best performance regarding CAT, GPX, and MDA ($P < 0.05$) (Fig. 2). These treatments had high values of GPX (59.3, 58.32), and CAT (34.96, 33.9) than the others. Further, the MDA value for OACP0.15 (1.36) and OACP0.2 (1.57) treatment were significantly lower than other groups ($P < 0.05$). The SOD did not show any change among treatments. There was a linear relationship between these parameters and the OACP levels in the diet, suggesting that adding more levels should be tried.

3.4. Immune response

The result of the present study indicated that ACH50 in serum and lysozyme activity in serum and mucus had a linear relation with the OACP levels in diets. In this way, OACP0.2 had the best results (124.9., 51.6, and 71.13 respectively) as compared with control regarding immune system response (Fig. 3). According to Fig. 4, there was no significant difference in HSP70, HSP90, and TNFa across treatments, showing that the fish was not under stress or immune response was not activated. However, IL-1b and IL-8 had a decreased linear relation with the OACP levels, and OACP0.15 and OACP0.2 treatments had the lowest levels compared to the control group.

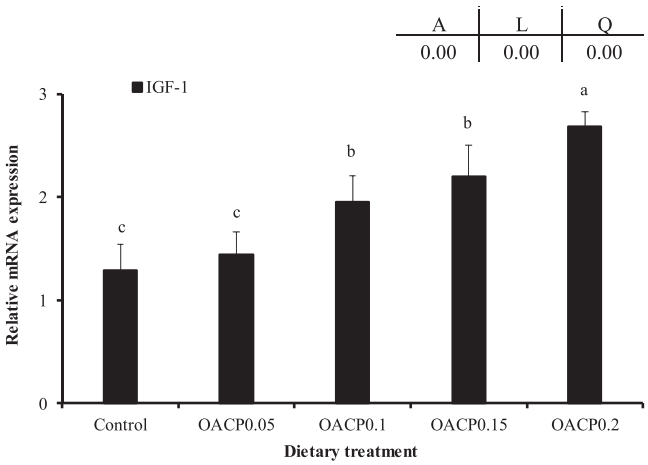

Fig. 1. Relative mRNA expression of Insulin-like growth factor 1 (IGF-1) in liver of barramundi fed experimental diets containing different levels of Biotronic® Top3 (OCAP: organic acids, cinnamaldehyde, and permeabilising complex). Letters a, b, and c indicate significant differences in treatment, according to Duncan's multiple range tests ($P < 0.05$). Letters A, L, and Q at the top of the plot show the P-value of ANOVA, linear relation and quadratic relations, respectively.

3.5. Correlation between measured parameters

In the present study, a positive correlation between digestive enzyme activities (protease and lipase) with growth performance, lysozyme in serum, CAT, GPX, and IGF-1 were observed ($P < 0.01$). Also, we found a strong negative correlation between these enzymes with FCR, MDA, IL1b, and IL8 ($P < 0.01$) (Table 5). The OACP level had a strong positive correlation with SGR, protease, lipase, lysozyme, ACH50, catalase, GPX, and IGF-1 ($P < 0.01$).

4. Discussion

4.1. Growth performance and survival rate

Although several studies investigated the effect of organic acids or cinnamaldehyde (the most abundant compounds in cinnamon) on animals, the exact mechanism has not been discovered yet. The result of the

Table 3
Growth performance and feed utilization of barramundi fed diets contained different level of OACP for 8 weeks.

Parameter	Dietary treatments					P value		
	Control	OACP0.05	OACP0.1	OACP0.15	OACP0.2	ANOVA	Linear	Quadratic
Initial weight (g)	8.4 ± 0.47	8.2 ± 0.46	8.1 ± 0.51	8.3 ± 0.53	8.5 ± 0.55	–	–	–
Final weight (g)	28.57 ± 1.35^{b}	30.05 ± 1.67^{b}	33.76 ± 1.34^{a}	34.35 ± 1.02^{a}	33.43 ± 0.92^{a}	0.00	0.00	0.00
Weight gain (%)	358.1 ± 25.5^{b}	421.3 ± 33.2^{a}	407.9 ± 8.5^{a}	398.2 ± 10.7^{a}	416.2 ± 14.8^{a}	0.01	0.08	0.07
SGR (%BW/day)	2.22 ± 0.08^{b}	2.32 ± 0.09^{a}	2.52 ± 0.07^{a}	2.55 ± 0.06^{a}	2.51 ± 0.05^{a}	0.00	0.00	0.00
FCR	1.28 ± 0.08^{a}	1.20 ± 0.09^{ab}	1.1 ± 0.05^{bc}	1.0 ± 0.04^{c}	1.03 ± 0.04^{c}	0.03	0.00	0.00
DFI (%BW/day)	0.59 ± 0.03	0.53 ± 0.04	0.54 ± 0.01	0.56 ± 0.03	0.53 ± 0.02	0.06	0.07	0.10
Condition factor	1.26 ± 0.26	1.43 ± 0.16	1.36 ± 0.28	1.47 ± 0.34	1.43 ± 0.38	0.92	0.42	0.67
Survival rate (%)	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	–	–	–

Values are represented means ± SDM of triplicate tanks; means without letter labels are not significantly different. According to Duncan's multiple range tests, the letters a and b indicate significant differences in the treatments ($P < 0.05$).
SGR: specific growth rate, FCR: feed conversion ratio, DFI: Daily feed intake

Table 4
Digestive enzyme activities in digestive tracts of barramundi fed diets contained different level of OACP for 8 weeks.

Parameters	Dietary treatments							
	Control	OACP0.05	OACP0.1	OACP0.15	OACP0.2	ANOVA	Linear	Quadratic
Protease (u/mg protein)	4.30 ± 0.28^{c}	5.54 ± 0.25^{ab}	4.93 ± 0.15^{bc}	5.80 ± 0.65^{a}	5.85 ± 0.25^{a}	0.00	0.00	0.00
Amylase (u/mg protein)	9.90 ± 2.00	10.77 ± 2.51	9.71 ± 2.41	11.20 ± 1.95	9.55 ± 2.30	0.86	0.95	0.87
Lipase (u/mg protein)	0.69 ± 0.06^{b}	0.59 ± 0.13^{b}	0.69 ± 0.19^{b}	1.25 ± 0.09^{a}	1.12 ± 0.13^{a}	0.00	0.00	0.01

Values are represented means ± SDM of triplicate tanks; means without letter labels are not significantly different. According to Duncan's multiple range tests, the letters a and b indicate significant differences in the treatments ($P < 0.05$).

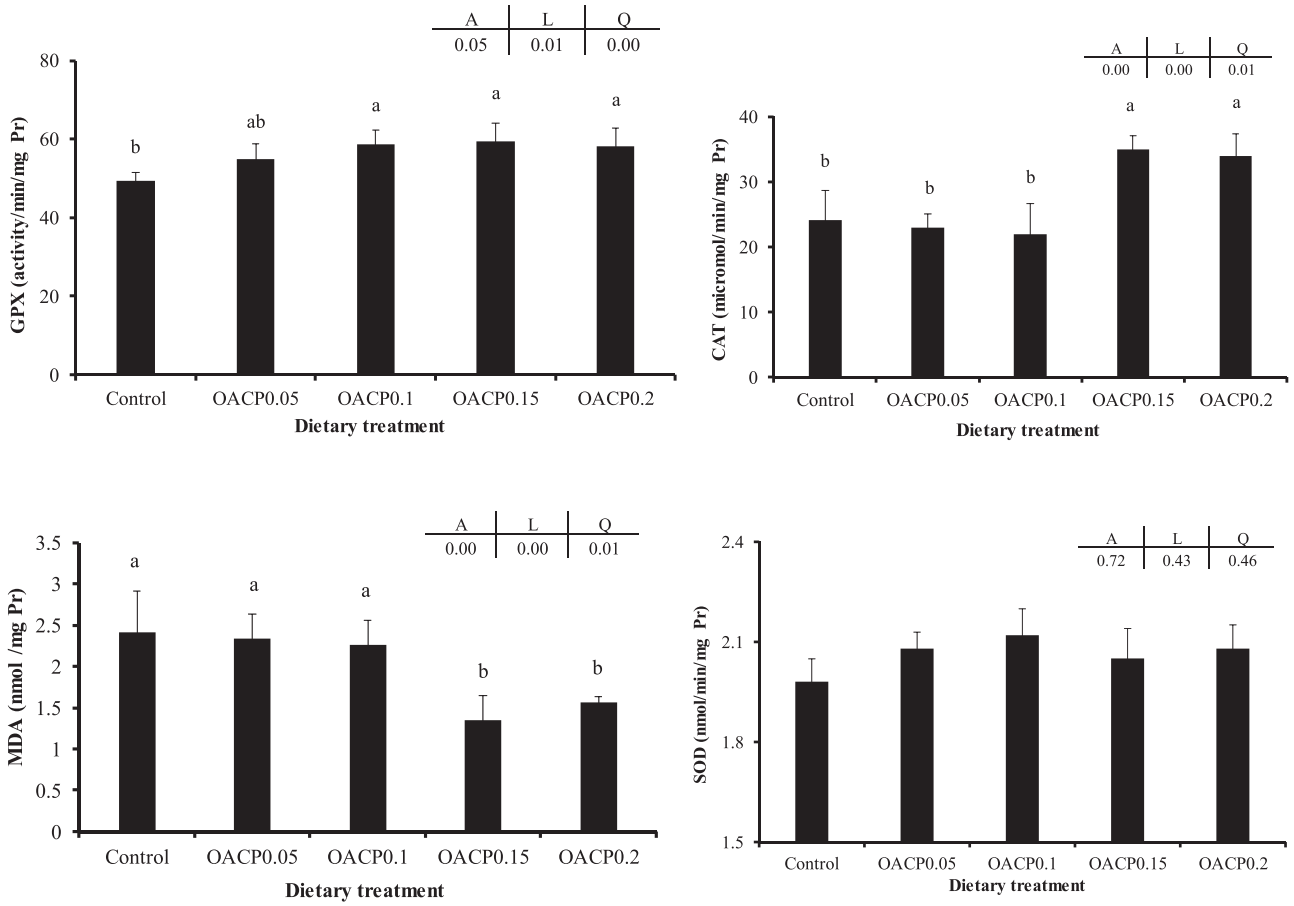

Fig. 2. Antioxidant activities (CAT, SOD, GPX, and MDA) in serum of barramundi fed experimental diets containing different levels of Biotronic® Top3 (OCAP: organic acids, cinnamaldehyde, and permeabilising complex). Letters a, b, and c indicate significant differences in treatment, according to Duncan's multiple range tests ($P < 0.05$). Letters A, L, and Q at the top of the plot show the P-value of ANOVA, linear relation, and quadratic relations, respectively.

present study indicated that fish growth and FCR have a linear relation with OACP levels in the diets; therefore, more levels should be tested. In the only study on OACP (0.8 g/kg diet) in fish, adding this supplement did not affect the growth rate after 175 days (Menanteau-Ledouble et al., 2017). This output is unlike our study showing that the effectiveness of this supplement can be varied with species, fish size and dosage. In our study even supplementing 0.5 g/kg OACP to diet improved growth performance. More research is required to determine the optimum dosage of this supplement in aquatic species. The OACP contains three different compounds that result in these positive effects. Firstly, it has organic acids (formic, propionic and lactic acids), which can stimulate growth. The most consumed organic acids in animal feeds are propionic acid, followed by fumaric, formic and lactic acids (Ng and Koh, 2017). One of the most popular features of organic acids is antimicrobial activity via lowering the cytoplasmic pH once they entered the cell membranes of microbes, change of membrane fluidity, and inhibiting the synthesis of nutrients (Ng and Koh, 2017). Antimicrobial activity of organic acids against *Streptococcus agalactiae* in Nile tilapia (Libanori et al., 2021) and against *Yersinia ruckeri* in rainbow trout (Villumsen et al., 2020) was observed. Another mechanism is the improvement of the gastrointestinal tract bacterial communities (Ng and Koh, 2017) as observed in aquatic animals (Koh et al., 2016; Pourmozaffar et al., 2019; Busti et al., 2020). Organic acids can also increase nutrient digestibility and availability by lowering gastric and intestinal pH, which can enhance the solubilisation of minerals and act as chelating agents. Many fish studies (Lin and Cheng, 2017; Yao et al., 2019; Javid et al., 2021), as well as our previous study (Matani Bour et al., 2018), reported this effect. Stimulating digestive enzyme activities, improving immunity and antioxidant system are other reasons that can eventually improve growth and feed efficiency by feeding fish with organic acids. Cinnamaldehyde also can improve the growth of fish through antibacterial activity (Abdelhamed et al., 2019), innate immunity (Abd El-Hamid et al., 2021; Harikrishnan et al., 2021), and digestive activity and antioxidant capability (Abd El-Hamid et al., 2021; Wang et al., 2021).

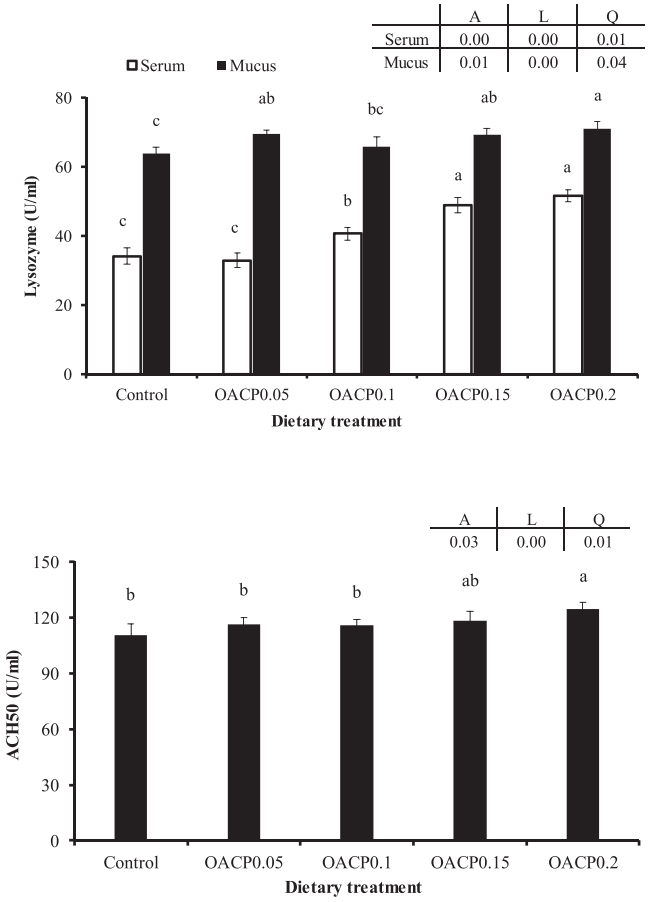

Fig. 3. Immune response parameters (serum and mucus lysozyme activity and serum ACH50 activity) of barramundi fed experimental diets containing different levels of Biotronic® Top3 (OCAP: organic acids, cinnamaldehyde, and permeabilising complex). Letters a, b, and c indicate significant differences in treatment, according to Duncan's multiple range tests ($P < 0.05$). Letters A, L, and Q at the top of the plot show the *P*-value of ANOVA, linear relation and quadratic relations, respectively.

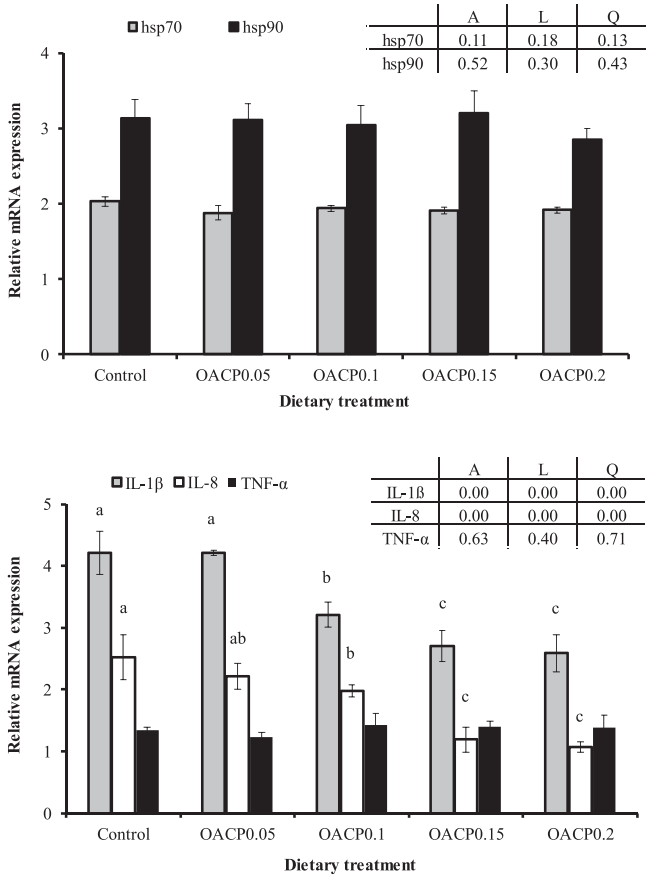

Fig. 4. Relative mRNA expression of heat shock proteins (HSP70 and HSP90) in liver; and interleukins (IL-1β and IL-8) and tumour necrosis factor (TNF-a) in kidney of barramundi fed experimental diets containing different levels of Biotronic® Top3 (OCAP: organic acids, cinnamaldehyde, and permeabilising complex). Letters a, b, and c indicate significant differences in treatment, according to Duncan's multiple range tests ($P < 0.05$). Letters A, L, and Q at the top of the plot show the P-value of ANOVA, linear relation, and quadratic relations, respectively.

Recently, the use of this supplement and other purified phytoadditives attracted the attention of researchers. Although we used cinnamaldehyde with other compounds in OCAP, still can cautiously claim that cinnamaldehyde increased growth by improving immune response, antioxidant activity and digestive enzymes as will be discussed later.

The outer membrane of gram-negative bacteria provides the cell with an effective permeability barrier against external noxious agents, including antibiotics and other bacterial killers (Vaara, 1992). The Permeabilizing ComplexTM blend weakens and permeates the outer membrane of gram-negative bacteria. This tool boosts the antimicrobial activity of both organic acids and cinnamaldehyde against pathogens. The survival rate was 100% for all treatments, and it can be indirectly stated that the tanks were in good condition and free of loaded bacteria. We could not measure antimicrobial activity and did not challenge fish with bacteria. It seems that this complex could be not relative, at least theoretically, to our experiment. The present research is preliminary work, and future investigations focusing on these points are suggested.

Barramundi was fed at ad libitum to monitor any negative effect of this supplement on feed intake. Similarly, other limited studies (Stensland et al., 2015) did not report the adverse impact of OACP on feed intake. This study is the preliminary work, and more studies are required to illustrate the potential positive or negative impact of OACP on fish intake and feed intake regulatory system.

Insulin-like growth factor 1 (IGF-1) stimulates the growth of all cell types in vertebrates and signals to cells that sufficient nutrients are available for growth and inhibit cell apoptosis (Laron, 2001). Cinnamaldehyde increased the expression of IGF-1 (Usta et al., 2002) and modulated the insulin and IGF signalling pathways such as mTOR, Cyclic-AMP signalling and autophagy (Beejmohun et al., 2014). Similarly, when pigs fed organic acid, growth rates along with IGF-1 were increased (Long et al., 2018; Xu et al., 2018) as well as in chickens (Salehizadeh et al., 2019). All these pathways are directly related to growth, and our data is compatible with these outputs.

4.2. *Digestive enzymes*

A positive relationship between final weight and digestive enzyme activities has been reported when fish was fed different supplements including herbs (Esmaeili et al., 2017a; Chowdhury et al., 2021), butyric acid (Aalamifar et al., 2020), probiotics (Castro et al., 2013; Allameh et al., 2017), fermentation products of kitchen waste (Ao et al., 2021), and zinc (Tan et al., 2011; Zhou et al., 2021). Conversely, many researchers did not report this relation when fish fed barberry root (*Berberis vulgaris*) (Ramezanzadeh et al., 2020b). In the current research, OACP stimulated the digestive enzyme activities and eventually increased growth performance. These outputs show that protein and lipid digestion were improved, which makes sense as this fish species is carnivorous. In the current study, while temperature, photoperiod, protein and lipid contents of diets, salinity, and stocking density, were similar among treatments, supplementing OCAP to diets considerably

Table 5
Correlation between investigated factors in of barramundi fed diets contained different level of OACP for 8 weeks.

	OACP level	SGR	Weight gain	FCR	Protease	Lipase	Lysozyme	ACH50	Catalase	GPX	MDA	IGF-1	IL1ab	IL8
OACP level	**1.00**	0.79**	0.47	-0.83**	0.84**	0.75**	0.93**	0.73**	0.70**	0.64**	-0.74**	0.95**	-0.90**	-0.93**
SGR	0.79**	**1.00**	0.55*	-0.96**	0.80**	0.61*	0.70**	0.36	0.51	0.63*	-0.65**	0.73**	-0.75**	-0.69**
Weight gain	0.47	0.55*	**1.00**	-0.57*	0.45	0.02	0.25	0.46	0.11	0.42	-0.20	0.38	-0.18	-0.32
FCR	-0.83**	-0.96**	-0.57*	**1.00**	-0.80**	-0.71**	-0.74**	-0.41	-0.64**	-0.57*	0.74**	-0.75**	0.76**	0.75**
Protease	0.84**	0.80**	0.45	-0.80**	**1.00**	0.64**	0.71**	0.51	0.57*	0.65**	-0.63*	0.81**	-0.74**	-0.78**
Lipase	0.75**	0.61*	0.02	-0.71**	0.64**	**1.00**	0.83**	0.31	0.88**	0.38	-0.88**	0.70**	-0.78**	-0.80**
Lysozyme #	0.93**	0.70**	0.25	-0.74**	0.71**	0.83**	**1.00**	0.65**	0.78**	0.51*	-0.75**	0.89**	-0.92**	-0.88**
ACH50	0.73**	0.36	0.46	-0.41	0.51	0.31	0.65**	**1.00**	0.43	0.42	-0.33	0.72**	-0.59*	-0.71**
Catalase	0.70**	0.51	0.11	-0.64**	0.57*	0.88**	0.78**	0.43	**1.00**	0.31	-0.67**	0.67**	-0.69**	-0.75**
GPX	0.64**	0.63*	0.42	-0.57*	0.65**	0.38	0.51*	0.42	0.31	**1.00**	-0.44	0.59*	-0.56*	-0.66**
MDA	-0.74**	-0.65**	-0.20	0.74**	-0.63*	-0.88**	-0.75**	-0.33	-0.67**	-0.44	**1.00**	-0.65**	0.67**	0.79**
IGF-1	0.95**	0.73**	0.38	-0.75**	0.81**	0.70**	0.89**	0.72**	0.67**	0.59*	-0.65**	**1.00**	-0.92**	-0.93**
IL1b	-0.90**	-0.75**	-0.18	0.76**	-0.74**	-0.78**	-0.92**	-0.59*	-0.69**	-0.56*	0.67**	-0.92**	**1.00**	0.89**
IL8	-0.93**	-0.69**	-0.32	0.75**	-0.78**	-0.80**	-0.88**	-0.71**	-0.75**	-0.66**	0.79**	-0.93**	0.89**	**1.00**

Lysozyme in serum. SGR: specific growth rate; FCR: feed conversion ratio; ACH50: alternative complement activity; GPX: glutathione peroxidase; MDA: malondialdehyde; IGF-1: insulin-like growth factor 1; IL1b: interleukin 1 beta; IL8: interleukin 8.
*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

influenced digestive enzymes. The improved digestion and absorption capacity by increasing the activities of intestinal and digestive enzymes in grass carp and tongue sole (Wang et al., 2021) were reported. Further, an increase in digestive enzyme activities when fish was fed organic acid was observed (Castillo et al., 2014; Aalamifar et al., 2020; Sotoudeh et al., 2020; Javid et al., 2021). The results of our study are somehow in line with those studies. The possible improvement of digestive enzyme activities can be due to the antioxidant properties of OCAP (Suryanti et al., 2018). Results of antioxidant activities in the current study are a further line of evidence as CAT and GPX were higher, and MDA was lower in OCAP0.15 and OACP0.2 treatments. Improved antioxidant defence modified the structure and function of the digestive organ (Matés et al., 1999).

4.3. Antioxidant activities

Investigating antioxidant enzymes and their components such SOD, CAT, GPX and MDA in fish can be reliable biomarkers in terms of growth and health. In the last decades, many researchers have measured these parameters in fish nutritional studies (More than 1900 records, based on the Scopus database). Uncontrolled oxidative activities cause radical damages due to superoxide and H_2O_2, which antioxidant enzymes protect cells from these damages (Matés et al., 1999). In the present research, OACP0.15 and OACP0.2 groups had the best performance regarding CAT, GPX, and MDA. There is no study regarding the impact of OACP on antioxidant activities in animals to compare with the present results. However, cinnamaldehyde is a well-known strong antioxidant (Suryanti et al., 2018) and improves CAT, GPX, and declines MDA. Enhanced antioxidant enzymes when Prussian carp (*Carassius auratus gibelio*) fed malic acid and citric acid (Zhang et al., 2020), rainbow trout fed trans-cinnamic acid (Yılmaz et al., 2019), Nile tilapia fed caffeic acid (Yilmaz, 2019) and potassium diformate (Hassaan et al., 2021) were reported. The treatments with higher growth in our study had a better antioxidant status. It is possible that OACP prevents fish from oxidative stress in our research and eventually resulted in improved growth. Growth, digestive enzyme, and antioxidant relations and identifying cause and effect in their interaction are suggested to be investigated.

4.4. Immune response

Lysozyme is a crucial component of the fish innate immune system that lyses bacterial cell walls and destroys pathogens (Saurabh and Sahoo, 2008). ACH50 also is the second most important and common measured parameter for monitoring the immune system of fish (Zeilab Sendijani et al., 2020). The result of the present study indicated that ACH50 in serum and lysozyme activity in serum and mucus were improved by OACP. Organic acids are well-known in the improvement of the health and immune system of fish. For example, a blend of microencapsulated organic acids (citric and sorbic acid) (Busti et al., 2020), sodium propionate (Hoseinifar et al., 2016), apple cider vinegar (Ahmadniaye Motlagh et al., 2020; Motlagh et al., 2020), sodium butyrate (Mirghaed et al., 2019), and butyric acid (Zarei et al., 2021) improved immune system of fish. While the exact mechanism of action is not clear, it seems that organic acids with modification and improvement of microbial communities in the intestine boost the immune system. Further, the immunostimulatory effect of cinnamon and its components such as cinnamaldehyde well investigated in Nile tilapia (Abdel-Tawwab et al., 2018; Amer et al., 2018) and European Sea Bass (*Dicentrarchus labrax*) (Habiba et al., 2021). Probably cinnamaldehyde protects the mucus layers as the increased gene expression of mucin 2 (MUC2), which is the most important gene to protect the intestinal epithelial cells (Ali et al., 2021). Altogether, OACP0.2 was the best treatment in terms of the immune system.

For a better understanding of the mechanism of action of OACP, the expression of five immune-related genes were investigated. They were heat shock proteins (HSP70 and HSP90), interleukins (IL-1b and IL-8), and tumour necrosis factor-a (TNFa). Heat shock proteins have key roles in proper protein folding of many immune receptors, like pro-inflammatory signalling pathways, toll-like receptor signalling pathways and integrins and these proteins can stimulate immune receptors (Binder, 2014). The TNF is realised by white blood cells when an infection occurs; they release to alert other immune system cells as part of an inflammatory response (Wajant et al., 2003). In the present study, there was no significant difference in heat shock proteins and TNFa across treatments indicating that the fish were not under stress or immune response was not activated. Feeding chicken with organic acids, similar to our data, decreased the expression of IL-1b (Pham et al., 2020) and showed that organic acid has anti-inflammatory functions. In our previous studies, organic acid declined the expression of IL-8 in the gut and liver and mitigated plant protein-induced inflammatory response (Sotoudeh et al., 2020). However, unlike our data, when Nile tilapia fed dietary organic acids, IL-1b TNF-α were overprocessed in the liver and kidney along with increased growth and lysosome (Reda et al., 2016). It seems that any species respond differently at the gene level. Cinnamon

and cinnamaldehyde are well-known anti-inflammatory agents and have lowering effects on TNF-α, interleukins and HSPs (Hong et al., 2012; Zareie et al., 2020; Zhu et al., 2020). These effects were observed for interleukins confirming their anti-inflammatory function. More research on other species is required to test whether these changes are global or species-specific responses.

4.5. Correlation between measured parameters

In the present study, positive correlations between digestive enzyme activities with growth performance, immune response and antioxidant activities were observed. These results supported our hypothesis that digestive enzymes would strongly correlate with growth and other investigated parameters. Similarly, some studies observed a positive correlation between growth rate and digestive enzyme activities (Esmaeili et al., 2017a; Magouz et al., 2020; Zemheri-Navruz et al., 2020). Conversely, no direct relationship between growth rate and digestive enzyme activities was reported as well (Ramezanzadeh et al., 2020b; Zaretabar et al., 2021). We do not know that improved growth elevated digestive enzyme activities or vice versa. A direct positive correlation between digestive enzymes with the immune system and antioxidant response can support the latter. The growth rate was higher in treatments with the improved immune system and antioxidant response.

Strong negative correlations between lysozyme, ACH50, CAT, and GPX with inflammatory response genes (IL1b and IL8) show that these parameters are reliable and accurate indicators of fish health. The OACP level had a strong positive correlation with SGR, protease, lipase, lysozyme, ACH50, catalase, GPX, and IGF-1. These results highlight the positive role of OCAP in our study, which is in line with linear relations. The possible mechanism can be improved the bacterial community and eventually result in all these positive outputs. More research (especially microbiome studies) is required to test how these parameters are changed with OCAP and growth performance in fish.

5. Conclusion

Conclusively, regarding growth performance, digestive enzyme activities, antioxidant response, immune system and inflammatory-related genes, the OCAP0.2 group is introduced as the best treatment. Having a higher activity of lipase and protease, lysozyme, ACH50, CAT, GPX, IGF-1 expression and lower level of MDA and interleukins expressions were reasons for improved growth in this treatment. As there was a linear relationship between these parameters and OCAP level, supplementing more levels of OCAP to diets is recommended to reach the optimum level. Most of the investigated parameters were reported for the first time in fish, and thus, more research is required.

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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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Conflict of interest

There is no conflict of interest to report.

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