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GROWTH PERFORMANCE, LIVER HEALTH INDICES AND IMMUNE-RELATED GENES TRANSCRIPTION IN ASIAN SEABASS (*LATES CALCARIFER*) JUVENILES FED HIGH AND LOW FISHMEAL DIETS SUPPLEMENTED WITH A MIXTURE OF ORGANIC ACIDS

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Abstract

This research was conducted to determine the effects of a mixture of organic acid (OAs) in low fishmeal diets on Asian seabass (*Lates calcarifer*) juveniles (54.2 g). There were two dietary groups including high fishmeal diet (HFM, 45.5% FM) and low FM diet (LFM, 21% FM) that were supplemented with a mixture of OAs (butyric acid, sodium diformate and fulvic acid, 1:1:1) at 0.5 and 1.0% levels. Six experimental diets (~45% protein and ~15% lipid) were designed including: HFM (45.5% FM), HFM$^{+0.5}$ (HFM diet + 0.5% OAs), HFM$^{+1.0}$ (HFM diet + 1.0% OAs), LFM (21% FM), LFM$^{+0.5}$ (LFM diet + 0.5% OAs), and LFM$^{+1.0}$ (LFM diet + 1.0% OAs). Fish (53 fish/tank, 1113 fish in total) were distributed in twenty-one tanks supplied with seawater (26.5°C, 46.0 ppt). Each treatment had three replicates. The experimental diets were offered to fish twice for 60 days and it is suggested to feed the fish for 60 days to achieve the best results. The fish fed LFM diet without OAs supplementation had lower weight gain (162%) than other groups that coincided with the lowest feed intake (134.1 g). Fish fed LFM$^{+1.0}$ had the highest gut *Lactobacillus* bacteria colonies count. HFM$^{+0.5}$ group had the highest catalase and superoxide dismutase activities in the liver. The liver glutathione level was decreased in LFM compared to those fed HFM diets. LFM$^{+1.0}$ group had the highest amount of liver malondialdehyde value ($P<0.05$). Fish fed HFM$^{+0.5}$ and LFM$^{+1.0}$ diets had the highest values of liver alanine aminotransferase. The largest lipid vesicles were in the liver of the fish fed with HFM$^{+0.5}$, LFM and LFM$^{+0.5}$ diets and the smallest ones were in the fish fed with HFM$^{+1.0}$. Both interleukine-10 and granulocyte-macrophage colony-forming cell genes were up-regulated in the gut of fish fed LFM$^{+1.0}$ and HFM$^{+1.0}$ diets after 30 and 60 days, respectively. Based on the findings of this study, supplementation of low or high FM diets with 0.5% OAs mixture is recommended for *L. calcarifer* juveniles.

Key words: acidifiers, antioxidant enzymes, feed conversion ratio, liver enzymes, liver histoarchitecture

The market of formulated feed production for aquaculture is estimated to reach USD 71.6 billion by 2025 (Markets, 2021) as 70% of the world aquaculture yield depends on aquafeed and its cost accounts for ~40%–70% of the total production expenses (Chakraborty et al., 2019; Kok et al., 2020). Nevertheless, about 70 to 80% of fishmeal (FM) and fish oil (FO) are used in aquafeed industry (FAO, 2020) and this trend cannot be sustainable in long term period due to their inflated price and ecological concerns (*e.g.* fishing pressure on small pelagic fish stocks) (Chakraborty et al., 2019). Therefore, finding alternative ingredients to spare FM and FO in aquafeeds is an integral goal for reduction of the marine-derived feed ingredients in aquaculture production (Kok et al., 2020).

In this sense, various terrestrial vegetal and animal sources were used as ingredients in aquafeeds formulation to increase the sustainability and profit margins of the aquafeed industry (Kok et al., 2020; Naylor et al., 2021). Nevertheless, deficiencies of some nutrients (*e.g.* amino and fatty acids), lower palatability and digestibility and the presence of anti-nutritional factors (ANFs) in alternative feedstuffs may negatively affect growth and welfare of farmed aquatic species (Gatlin et al., 2007; Hua et al., 2019). In this regard, several researchers reported that dietary FM sparing by using vegetal proteins may retard growth, reduce nutrients digestibility, metabolic disorders, and immunosuppression in marine carnivorous fish species (Yaghoubi et al., 2016; Zhang et al., 2016; Abbasi et al., 2020; Hernández et al., 2020). Moreover, various alternative protein sources (APS) can drastically alter gut microbiome which may deteriorate health, immunity and growth of fish (Krogdahl et al., 2003; Hartviksen et al., 2014; Zhang et al., 2018; Zhou et al., 2018; Kononova et al., 2019). Furthermore, FM sparing with plant or animal by-product proteins may induce metabolic disorders and liver disease symptoms such as swollen hepatocytes, steatosis, multifocal necrosis, increased plasma levels of the metabolic enzymes (Hu et al., 2013; Bian et al., 2016; Amer et al., 2019; Zhang et al., 2019; Chaklader et al., 2019, 2020; Ye et al., 2019; Zhou et al., 2020; Hosseini Shekarabi et al., 2021). At the molecular level, FM substitution with various vegetal proteins may up-regulate pro-inflammatory genes or down-regulate the anti-inflammatory genes (*e.g.* interleukin 10) that can adversely affect fish health (Azeredo et al., 2017; Zhang et al., 2021).

There are several remedies to enhance the efficiency and alleviate the symptoms of ANFs present in APS on the health of fish such as supplementing these diets with crystalline amino acids (Nunes et al., 2014), probiotics (Back et al., 2020), protein hydrolysate (Hlordzi et al., 2022), and organic acids (OAs) (Sotoudeh et al., 2020; Sangari et al., 2021; Yarahmadi et al., 2022). Among the above-mentioned additives, OAs have shown promising effects in promoting feed efficiency, growth and welfare of fish fed low-FM aquafeeds (Ng and Koh, 2017). Organic acids are organic compounds that are mainly produced through the microbial fermentation of carbohydrates and have one or more carboxyl groups. These additives by reducing pH have antibacterial effects (Ng and Koh, 2017). In addition, OAs by reducing gut's pH not only can modulate its microbiome by inducing the colonization of beneficial bacteria (e.g. lactic acid bacteria) but also they can control the propagation of infectious opportunistic bacteria (Jaafar et al., 2013). Moreover, OAs by modulating the gut's pH can trigger the secretion of neurotransmitters that consequently affect the digestive enzymes activities and may enhance digestibility of nutrients (Pandey and Satoh, 2008; Ng and Koh, 2017; Khorshidi et al., 2022). Furthermore, OAs can attach to some receptors on immune cells that are present in the gut-associated lymphoid tissue (GPR41, GPR43, and GPR109A) and consequently affect the expression of some immune-related genes which modify fish health competence (Hoseinifar et al., 2017; Ng and Koh, 2017; Tran et al., 2020). Also, OAs by increasing the bioavailability of minerals such as selenium, copper, zinc can affect antioxidative status of fish (Ma et al., 2014; Zhang et al., 2016).

Asian seabass (*Lates calcarifer*) has promising characteristics for aquaculture such as high fecundity, high growth rate, optimal feed conversion ratio and high tolerance to various culture conditions (Mozanzadeh et al., 2021). Its global production reached 108,000 tons in 2019 (Tveteras et al., 2019). Several studies confirmed the positive effects of OAs such as sodium diformate (SDF, Reyshari et al., 2019), butyric acid (BA, Alamifar et al., 2020), propionic acid (PA, Salehi et al., 2023) on growth and health of this species. However, the possible synergistic effects of OAs mixture on performance of this species need to be evaluated too. In fact, the efficacy of a mixture of OAs could have a synergistic effects on the gut health compared to any single OAs (Sangari et al., 2021; Sotoudeh et al., 2020). Thus, this research was carried out to examine the effects of a mixture of OAs in low-FM diets on growth and health status of *L. calcarifer*.

Material and methods

Experimental feeds

Six isonitrogenous (~45%) feeds were formulated by the inclusion of 45.5% or 21.0% of FM to produce two groups of diets including high FM (HFM) and low FM (LFM) diets, respectively. Then two levels (0.5 and 1.0%) of a mixture of OAs (BA, SDF, and fulvic acid (FA), ~1:1:1 ratio) were added to the diets to produce the experimental feeds (Table 1). Butyric acid (Merck, Germany, 99%, liquid), SDF (conjugated salt of formic acid, HCOOH••HCOO-Na, contained 195 g/kg sodium + 390 g/kg formic acid + 381 g/kg formate + 34 g/kg silicate and water; Formi® NaDF, Addcon Nordic AS, Norway) and FA (FulvoFeed, HuminTech. Germany, 17% fulvic acid + 1% humic acid) were equally mixed together then added to the experimental diets. Six experimental diets were designed including: HFM (45.5% FM), HFM$^{+0.5}$ (HFM diet + 0.5% OAs), HFM$^{+1.0}$ (HFM diet + 1.0% OAs), LFM (21% FM), LFM$^{+0.5}$ (LFM diet + 0.5% OAs), and LFM$^{+1.0}$ (LFM diet + 1.0% OAs). HFM$^{+0.5}$ and LFM$^{+0.5}$ diets were supplemented with the mixture of 1.6, 1.6 and 1.8 g kg^{-1} of BA, SDF and FA, meanwhile HFM$^{+1.0}$ and LFM$^{+1.0}$ diets were supplemented with the blend of 3.2, 3.2 and 3.6 g kg^{-1} of BA, SDF and FA. First all dry ingredients were weighed and blended with 15 min, then oils and lecithin were added and mixed for 10 min. The OAs mixture was dissolved in distilled water and poured on the mixture and mixed for 10 minutes to form a dough. A meat grinder was used to pellet the dough with desired size of 4 mm. The standard AOAC methods (2005) were applied to evaluate biochemical composition of the feeds.

Feeding trial

The research trial was conducted in a marine fish hatchery center, Bandar Imam Khomeini, Khuzestan, Iran. About 1200 *L. calcarifer* juveniles were purchased (Ramooz, Bushehr, Iran) and transferred then stocked into a 15 m^3 circular concrete tank and were acclimated with new culture condition for two weeks. During the two-week acclimation period they were fed with the control diet twice a day. After acclimation period, 1113 fish (54.2±0.2 g, mean ± standard deviation) were stocked into twenty one 2000-L rectangular concrete tanks in a flow-through system (2 L min^{-1}). Each treatment had three replicates. In each tank 53 fish were stocked and each dietary treatment was replicated in triplicate. The experimental diets were offered to fish two times daily (09:00 and 17:00). The unfed pellets were syphoned and weighted after being dry in an oven (60ºC, 24h). The mean values of temperature, salinity, pH and dissolved oxygen of seawater were 26.5±1.5°C, 46.0±0.2 ppt, 7.9±0.3 and 6.2±0.5 mg/L, respectively with 12 h light 12 h dark photoperiod condition.

Table 1. Formulation (g/kg) and proximate composition (%) of experimental diets

Ingredients[a]	Experimental diets					
	HFM	$\text{HFM}^{+0.5}$	$\text{HFM}^{+1.0}$	LFM	$\text{LFM}^{+0.5}$	$\text{LFM}^{+1.0}$
Fishmeal[b]	455	455	455	210	210	210
Soybean meal[c]	80	80	80	125	125	125
Corn gluten[c]	80	80	80	120	120	120
Wheat gluten[c]	80	80	80	120	120	120
Poultry meal[d]	145	145	145	300	300	300
Wheat middling	42	42	42	–	–	–
Beef gelatin	10	10	10	10	10	10
DL-methionine	1	1	1	2.5	2.5	2.5
L-lysine	2	2	2	5	5	5
Fish oil[b]	20	20	20	20	20	20
Soybean oil[c]	20	20	20	20	20	20
Soy lecithin[e]	20	20	20	20	20	20
Butyric acid[f]	–	1.6	3.2	–	1.6	3.2
Sodium diformate[g]	–	1.6	3.2	–	1.6	3.2
Fulvic acid[h]	–	1.8	3.6	–	1.8	3.6
Cellulose	10	5	–	10	5	–
Vitamin premix[i]	10	10	10	10	10	10
Mineral premix [j]	10	10	10	10	10	10
L-ascorbic acid (50%)[k]	5	5	5	5	5	5
Dicalcium phosphate	10	10	10	12.5	12.5	12.5
Proximate composition (%)						
Moisture	9.0	9.0	8.0	10	8.0	8.0
Crude protein	45.7	46.2	46.1	44.9	45.8	44.8
Crude lipid	15.1	16.5	16.1	14.9	15.3	15.8
Ash	9.8	9.7	9.3	9.5	9.4	9.9

[a] Composition of ingredients as % dry-weight basis [fishmeal (60.5% crude protein, 18.0% crude lipid); soybean meal (41% crude protein, 4.2% crude lipid); corn gluten (71.4% crude protein, 4.1% crude lipid); wheat gluten (53.3% crude protein, 2.8% crude lipid); poultry meal (51.2% crude protein, 15.5% crude lipid), gelatin (85% crude protein, crude lipid, 4.2); wheat middling (12% crude protein, 3.0% crude lipid)].

[b] Parskilka Mazandaran, Iran (Clupeonella sp.).

[C] Product of Kesht Va Sanat Shomal Vegetable Oil Factories Complex (Neca, Iran).

[d] Nazdaneh Sepahan, Isfahan, Iran.

[e] Behpak industrial company, Behshahr, Mazandaran, Iran.

[f] Merck, Germany.

[g] Conjugated salt of formic acid, HCOOH··HCOO-Na, contained 195 g/kg sodium + 390 g/kg formic acid + 381 g/kg formate + 34 g/kg silicate and water; Formi® NaDF, Addcon Nordic AS.

[h] FulvoFeed, HuminTech, Germany.

[i] Vitamin premix (IU/kg of premix): ascorbic acid, 350000; retinol, 1000000000; cholecalciferol, 500000000; tocopherols, 500000; vitamin K_3, 960000; thiamine, 980000; riboflavin, 800000; pyridoxine, 990,000; folic acid, 950000; cobalamin, 10000; biotin, 20000; niacin, 995000; pantothenic acid, 980,000.

[j] Mineral premix mg/kg of premix: magnesium, 6,400; copper, 2000; ferrous, 11,000; zinc, 7,000; selenium, 100; iodine, 300; cobalt, 50; natrium, 5,000. ATA Company, Tabriz, Iran.

[k] Rooyan Darou, Semnan, Iran.

Sampling

Sampling of fish was done before the beginning of the trial (day 0, three fish per tank), at days 30 (six fish per tank) and 60 (six fish per tank). Fish were fasted a day before each sampling. The biometry of each fish was done individually. Six fish of each tank were anesthetized (2-phenoxyethanol, 300 mg/L) and were bled from the caudal vein. Blood was allowed to clot, centrifuged (5000 *g*, 4°C, 10 min), then sera were maintained in a –80°C freezer. The liver of the same fish was dissected then kept in a –80°C freezer. In addition, the gut of the same fish was dissected and kept in –80°C freezer for further evaluation of immune-related genes expression. For liver histological evaluation, three fish of each tank were sacrificed, eviscerated and liver was dissected and transferred in buffer formalin (10%, pH = 7.0) for a day, then were rinsed with alcohol and kept in 70% alcohol.

Gut microbiome

After 30 days and at the end of the husbandry trial, three fish from each tank were sacrificed with overdose of 2-phenoxsyethanol (1000 mg/L), their skin disinfected (70% ethanol), eviscerated and their gut totally dissected and washed with serum saline (0.85% NaCl). Guts were properly homogenized and diluted 10 to 7 times for determining total viable heterotrophic aerobic bacteria (TVC) and LAB colonies, respectively (Hoseinifar et al., 2011). One hundred µL of diluted samples was spread on plate count agar (Merck) and deMan, Rogosa and Sharpe agar (Merck) media for evaluating TVC and LAB, respectively then incubated at 25°C for 5 days (Mahious et al., 2006), and count of colonies (CFU)/g was determined (Rawling et al., 2009).

Antioxidant and liver enzymes

The standard methods were used to evaluate catalase (CAT, Aebi et al., 1984), superoxide dismutase (SOD, McCord and Fridovich, 1969), glutathione content (GSH, Beutler et al., 1963), malondialdehyde (MDA, Buege and Aust, 1978) and soluble protein level (Bradford, 1976) in the sera. Commercial diagnostic kits (Pars Azmoon Co., Tehran, Iran) were used to analyze liver and serum alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Liver histology

The fixed liver dehydrated in a graded series of ethanol, cleared with xylene, embedded in paraffin, and cut in serial sections (5 µm thickness) was cut with a conventional microtome (LeicaRM 2125 RT) and stained with hematoxylin and eosin. The sections were studied using an optical microscope (BH2; Olympus, Norfolk, USA) and histomorphometric parameters including the position of hepatocyte nuclei, hypertrophy and the size of the lipid vesicle in the liver on ten sections per fish were analyzed by Quick Photo Micro 2.3 software.

Gene transcription

To determine the transcription levels of interleukin-10 (IL-10) and granulocyte-macrophage colony-forming cells (GMCFC) in the gut of fish, total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instruction and kept at –80°C. The level and purity of extracted RNA were determined by a spectrophotometer (NanoDrop Technologies, Wilmington, USA) and agarose gel (1%). cDNA synthesis was carried out by employing an iScript cDNA Synthesis Kit (Bio-Rad CA, USA) and polymerase chain reaction (PCR) was performed in duplicate for each sample with the SYBRgreen method in an iQ5 iCycler thermal cycler (Bio-Rad). The sequences of specific primers used are presented in Table 2. The reactions were conducted according to Abbaszadeh et al. (2019). β-actin was used as reference gene in each sample. The iQ5 optical system software (ver. 2.0) was used to analyze the obtained data.

Statistics

Data were represented as means ± standard deviation (n = 3 replicates). Levene's and Shapiro–Wilk's tests were used to evaluate homogeneity of variance and normality of the data. A two-way ANOVA analysis (SPSS 23.0, Chicago, IL, USA) was used to evaluate the effects of dietary FM content and the OAs mixture levels and their interactions. To compare means a one-way ANOVA analysis and Tukey test were used. In all cases, $P<0.05$ was considered as significant.

Results

Growth

Fish survival was between 96.6 and 100% (Table 3). $HFM^{+0.5}$ and LFM groups had the highest and lowest growth performance, respectively ($P<0.05$). Feed intake (FI) in the fish fed HFM diets was higher than in those fed LFM diets and the inclusion of 1.0% OAs mixture significantly enhanced FI. The highest and lowest values of FCR were in the fish fed HFM and $LFM^{+0.5}$ diets, respectively. After 60-day feeding trial, feeding with LFM diet without the OAs supplementation markedly reduced specific growth rate (SGR), nevertheless, supplementing 0.5% OAs mixture enhanced growth rate in fish fed $LFM^{+0.5}$ (Table 3, $P<0.05$). Furthermore, LFM diet reduced FI compared to the fish fed $HFM^{+0.5}$, $HFM^{+1.0}$ and $LFM^{+1.0}$ diets. LFM and $HFM^{+0.5}$ had the highest and lowest FCR values, respectively. Growth and FCR were adversely affected by LFM level; meanwhile OAs mixture inclusion in diet elevated growth and FI ($P<0.05$).

Table 2. Primer sequences and amplification efficiencies

Gene name	Sequences of primers	Accession number	Length	Efficiency
IL-10	Forward: CCAATGTGCAACAACCAGTG Reverse: TTCGACGGTCTGATCTAGCA	XM_018686737.1	149	97%
GMCFC	Forward: ACCCTCTGCCCCAGTTCTTC Reverse: TCTGAGCCAGTGTGGTTGC	XM_035655954.1	115	97%
β-actin	Forward: CACAGCTAACGGATTCACTCTG Reverse: TTCCATGGCTGAACTTTGGG	XM_018667666.1	134	97%

Abbreviations: IL-10: interleukin 10; GMCFC: granulocyte-macrophage colony-forming cell.

Table 3. Growth performance of *L. calcarifer* juveniles fed different experimental diets. Data are presented as the mean ± SD of three replicates

Diets	Parameters					
	IBW (g)	FBW (g)	SGR (% IBW day^{-1})[1]	FI (g fish^{-1})[2]	FCR[3]	Survival (%)[4]
			30 days			
HFM	54.5 ± 0.1	128.1±0.4 e	2.9±0.0 e	102.0±1.4 a	1.4±0.1 c	100.0±0.0
HFM$^{+0.5}$	54.4 ± 0.1	141.5±0.5 a	3.2±0.0 a	102.3±3.5 a	1.2±0.1 ab	99.0±1.1
HFM$^{+1.0}$	54.3 ± 0.1	139.3±0.3 b	3.1±0.0 b	108.4±1.2 a	1.3±0.0 bc	98.0±0.0
LFM	54.2 ± 0.1	123.7±0.2 f	2.8±0.0 f	83.1±4.3 b	1.2±0.0 ab	100.0±0.0
LFM$^{+0.5}$	54.7 ± 0.1	131.4±0.4 d	2.9±0.0 d	85.6±5.1 b	1.1±0.0 a	100.0±0.0
LFM$^{+1.0}$	54.3 ± 0.1	137.3±0.3 c	3.1± 0.0 c	101.4±3.8 a	1.2±0.0 ab	99.0±1.1
			Two-way ANOVA			
Replacement level		0.001	0.001	0.001	0.001	1.000
Acidifier level		0.001	0.001	0.001	0.001	1.000
Interaction		0.001	0.001	0.001	0.093	1.000
			60 days			
HFM		179.8±6.4 a	2.0±0.1 a	148.6±13.2 ab	1.3±0.2 ab	100.0±0.0
HFM$^{+0.5}$		188.0±1.7 a	2.1±0.0 a	167.3±3.9 a	1.2±0.0 a	97.7±1.3
HFM$^{+1.0}$		179.6±9.1 a	2.0±0.1 a	152.9±4.9 a	1.3±0.1 ab	96.6±0.7
LFM		142.1±6.8 b	1.6±0.1 b	134.1±22.6 b	1.5±0.1 b	98.9±0.9
LFM$^{+0.5}$		163.5±1.3 ab	1.8±0.0 ab	143.1±13.8 ab	1.4±0.1 ab	100.0±0.0
LFM$^{+1.0}$		179.9±0.6 a	2.0±0.0 a	168.1±13.3 a	1.3±0.1 ab	98.9±0.0
			Two-way ANOVA			
Replacement level		0.01	0.002	0.050	0.037	0.720
Acidifier level		0.041	0.022	0.002	0.474	0.944
Interaction		0.046	0.038	0.203	0.082	0.931

Values within a column with a common letters are not significantly different from the other dietary groups ($P>0.05$). The significance of the two main effects (fishmeal replacement level and acidifier level) and their interaction were analyzed using two-way ANOVA.

Abbreviations: IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR: specific growth rate; K, Fulton's condition factor; FI, feed intake; FCR, feed conversion ratio.

[1] Specific growth rate (SGR, %/day) = 100 × [(ln FBW – ln IBW)/number of feeding days].

[2] Feed intake (FI, g fish^{-1}) = total feed intake per tank (g) / number of fish.

[3] Feed conversion ratio (FCR) = total feed intake (g) / weight gain (g).

[4] Survival (%) = 100 × (final number of fish / initial number of fish).

Table 4. Total viable and lactic acid bacterial counts from intestinal region (mean ± SD, n = 3) of *Lates calcarifer* juveniles fed the experimental diets

Parameters	Experimental diets						Two-way ANOVA		
	HFM	HFM$^{+0.5}$	HFM$^{+1.0}$	LFM	LFM$^{+0.5}$	LFM$^{+1.0}$	Replacement level	Acidifier level	Interaction
					30 days				
TVC ($\times 10^5$ CFU/g)	0.63±0.0 b	1.02±0.05 a	1.0±0.0 a	0.96±0.04 a	0.25±0.02 c	1.0±0.07 a	0.001	0.001	0.001
LAB ($\times 10^2$ CFU/g)	0.04±0.0 b	0.08±0.0 b	1.05±0.08 a	0.11±0.0 b	0.04±0.0 b	1.26±0.08 a	0.045	0.001	0.035
					60 days				
TVC ($\times 10^5$ CFU/g)	1.0±0.1 c	2.03±0.1 a	0.9±0.1 c	1.12±0.1 c	1.66±0.3 b	1.13±0.2 c	0.940	0.001	0.001
LAB ($\times 10^2$ CFU/g)	0.34±0.07 b	0.26±0.05 b	0.3±0.1 b	0.15±0.05 c	0.34±0.1 b	0.49±0.1 a	0.202	0.001	0.001

Gut microbiome

After 30-day husbandry period, HFM and LFM$^{+0.5}$ groups had lower TVC count compared to the other groups (Table 4). HFM$^{+0.5}$ and LFM$^{+0.5}$ groups had the highest TVC level after 60 days. After 30-day feeding trial, HFM$^{+1.0}$ and LFM$^{+1.0}$ groups had higher LAB colonies than the other ones. Meanwhile, after 60-day feeding period, LFM$^{+1.0}$ and LFM groups had the highest and lowest LAB counts, respectively.

Liver antioxidants responses

After 30-day feeding trial, the inclusion of 0.5% OAs mixture in diets enhanced liver CAT activity ($P<0.05$, Table 5). HFM$^{+0.5}$ group had the lowest liver SOD activity. HFM and LFM groups without the OAs mixture supplementation had lower liver GSH levels than other treatments. The highest MDA level was in the liver of the fish fed HFM$^{+0.5}$, and HFM and LFM groups had the lowest values.

HFM$^{+0.5}$ had the highest liver CAT activity, and the lowest values were in the groups fed HFM, HFM$^{+1.0}$ and LFM diets after 60 days. The highest liver SOD activities were in HFM$^{+1.0}$ group and LFM groups had the lowest value. HFM$^{+0.5}$ and LFM$^{+1.0}$ treatments had higher liver GSH levels than other ones. LFM$^{+1.0}$ had the highest amount of liver MDA, but HFM and HFM$^{+1.0}$ had the lowest values. The interaction of dietary FM level and the OAs level markedly affected the amounts of SOD activity and MDA level in the liver of fish.

Liver enzymes

The levels of liver ALP and ALT activities were not influenced by the experimental diets after 30 days (P>0.05, Table 6). The inclusion of 0.5% OAs mixture in HFM and LFM diets significantly enhanced AST activity in the liver of fish. LFM$^{+1.0}$ and HFM$^{+1.0}$ had the highest and lowest serum ALP levels, respectively. LFM and HFM$^{+0.5}$ treatments had the highest and lowest ALT values, respectively (P<0.05). HFM$^{+0.5}$ and HFM$^{+1.0}$ groups had lower serum AST values than the other groups.

Table 5. Liver antioxidant enzymes (U mg protein^{-1}), glutathione content (mmol g^{-1} mg protein) and lipid peroxidation (nmol g^{-1} tissue) in *L. calcarifer* fed different experimental diets

Diets	Parameters			
	Catalase	Superoxide dismutase	Glutathione	MDA
		Beginning (Day 0)		
HFM	11.8±2.3	3.0±0.9	1.0±0.2	406.9±26.1
HFM$^{+0.5}$	12.4±3.1	2.9±0.8	1.0±0.2	398.0±28.0
HFM$^{+1.0}$	11.7±1.0	2.9±0.3	1.2±0.4	407.5±32.6
LFM	11.1±1.7	2.9±0.3	1.1±0.2	395.6±43.0
LFM$^{+0.5}$	13.0±1.8	3.0±0.5	1.2±0.2	402.2±66.5
LFM$^{+1.0}$	12.2±1.7	3.0±0.7	1.2±0.2	412.8±21.6
		30 days		
HFM	10.8±3.2 b	2.8±0.6 a	1.0±0.2 b	293.5±45.0 d
HFM$^{+0.5}$	13.4±1.7 a	1.4±0.3 b	1.4±0.3 a	754.8±70.0 a
HFM$^{+1.0}$	12.2±1.9 a	2.8±0.6 a	1.4±0.4 a	587.6±231.5 b
LFM	10.5±1.8 b	2.1±0.6 a	0.9±0.2 b	293.5±87.0 d
LFM$^{+0.5}$	11.6±3.1 ab	2.9±0.4 a	1.3±0.1 a	491.0±132.2 c
LFM$^{+1.0}$	10.2±2.4 b	2.5±0.3 a	1.3±0.3 a	661.6±145.8 b
Two-way ANOVA				
Replacement level	0.214	0.183	0.023	0.024
Acidifier level	0.005	0.055	0.001	0.001
Interaction	0.287	0.001	0.548	0.014
		60 days		
HFM	5.0±1.4 d	1.7±0.6 b	0.6±0.1 b	402.8±96.9 c
HFM$^{+0.5}$	18.8±3.7 a	2.0±0.1 a	0.9±0.2 a	545.8±89.5 ab
HFM$^{+1.0}$	5.8±2.0 d	0.8±0.2 d	0.7±0.2 b	410.0±67.3 c
LFM	7.1±0.7 d	1.3±0.2 c	0.5±0.2 b	441.4±76.7 b
LFM$^{+0.5}$	9.1±1.5 c	1.6±0.6 b	0.6±0.1 b	476.3±125.2 b
LFM$^{+1.0}$	12.0±3.2 b	1.2±0.2 c	0.9±0.2 a	629.0±143.0 a
		Two-way ANOVA		
Replacement level	0.550	0.241	0.004	0.061
Acidifier level	0.001	0.001	0.179	0.035
Interaction	0.001	0.025	0.014	0.004

Data are presented as the mean ± SD of three replicates. Values within a row with a common letters is not significantly different from the other dietary groups (P>0.05).

Values within a column with a common letters are not significantly different from the other dietary groups (P>0.05). The significance of the two main effects (fishmeal replacement level and acidifier level) and their interaction were analyzed using two-way ANOVA.

Table 6. Liver (U mg protein^{-1}) and serum (U L^{-1}) enzymes in *L. calcarifer* fed different experimental diets

Diets	Parameters					
	Liver (U mg protein^{-1})			Serum (U L^{-1})		
	ALP	ALT	AST	ALP	ALT	AST
Beginning (Day 0)						
HFM	3.9±1.3	0.3±0.1	1.5±0.5	46.8±4.0	2.1±0.6	9.6±3.3
HFM$^{+0.5}$	4.2±1.3	0.3±0.1	2.1±0.9	46.5±2.5	1.9±0.6	12.9±1.7
HFM$^{+1.0}$	4.1±0.8	0.3±0.1	2.3±0.7	45.7±3.4	2.0±0.6	10.0±3.2
LFM	4.1±0.9	0.3±0.1	2.8±0.7	42.2±2.5	2.1±0.5	10.2±3.7
LFM$^{+0.5}$	4.1±0.8	0.2±0.1	2.6±0.8	44.5±2.5	1.9±0.6	12.4±3.5
LFM$^{+1.0}$	3.8±0.8	0.3±0.1	1.7±0.7	45.9±3.3	2.3±0.3	9.6±3.6
30 days						
HFM	3.4±0.8	0.10±0.0	0.10±0.0 c	26.1±5.6 ab	2.0±0.7 ab	11.4±2.3 a
HFM$^{+0.5}$	2.7±0.8	0.14±0.0	2.30±1.2 a	24.3±2.4 b	0.8±0.3 c	5.1±1.5 c
HFM$^{+1.0}$	2.9±0.7	0.06±0.0	1.10±0.7 b	21.2±2.9 c	1.1±0.3 b	5.4±1.6 c
LFM	2.0±0.5	0.10±0.0	0.10±0.0 c	24.0±4.5 b	2.8±0.6 a	10.7±2.9 a
LFM$^{+0.5}$	2.0±0.8	0.16±0.1	2.00±0.6 a	26.3±2.2 ab	1.7±0.8 b	10.7±1.1 a
LFM$^{+1.0}$	2.5±1.1	0.15±0.0	0.70±0.4 b	32.2±5.0 a	2.3±0.6 ab	8.2±1.4 b
Two-way ANOVA						
Replacement level	0.551	0.058	0.718	0.001	0.337	0.002
Acidifier level	0.131	0.101	0.001	0.001	0.044	0.001
Interaction	0.669	0.124	0.876	0.094	0.005	0.001
60 days						
HFM	7.2±1.4 b	0.08±0.0 b	1.00±0.4 a	9.3±0.7 ab	3.6±1.2 b	7.2±2.8 d
HFM$^{+0.5}$	9.3±1.9 a	0.15±0.0 a	0.05±0.0 b	7.6±1.6 ab	4.1±1.6 b	23.9±10.8 b
HFM$^{+1.0}$	1.4±0.5 d	0.10±0.0 b	0.22±0.1 b	7.9±1.9 ab	5.0±1.9 ab	19.6±7.9 b
LFM	1.8±0.5 d	0.04±0.0 c	0.10±0.0 b	6.1±2.2 b	20.2±5.9 a	43.8±2.5 a
LFM$^{+0.5}$	1.5±0.7 d	0.13±0.0 b	0.06±0.0 b	6.6±1.6 b	7.4±3.1 ab	14.9±7.2 c
LFM$^{+1.0}$	2.3±0.9 c	0.18±0.0 a	0.08±0.1 b	10.1±2.6 a	7.4±3.3 ab	14.6±7.2 c
Two-way ANOVA						
Replacement level	0.257	0.001	0.002	0.001	0.596	0.001
Acidifier level	0.199	0.001	0.001	0.001	0.001	0.001
Interaction	0.006	0.001	0.051	0.001	0.003	0.001

Data are presented as the mean ± SD of three replicates. Values within a row with a common letters are not significantly different from the other dietary groups (P>0.05).

After 60 days of feeding trial, the highest ALP activity in the liver was in fish fed HFM and the lowest values were in the fish fed with LFM, LFM$^{+0.5}$ and LFM$^{+1.0}$ diets (Table 6, P<0.05). The lowest level of ALT activity was in the fish fed with LFM$^{+0.5}$ diet and the greatest values were in the fish fed with HFM$^{+1.0}$ and LFM$^{+1.0}$ diets. The AST activity in the liver of fish fed with HFM$^{+0.5}$ was higher than the other groups.

The highest amounts of ALP in serum were in the fish fed with LFM$^{+1.0}$ diet and the lowest amounts were in the fish fed with LFM and LFM$^{+0.5}$ diets after 30 days. The greatest serum ALT value was in the fish fed with LFM diet, and the lowest values were in the fish fed with HFM and HFM$^{+0.5}$. LFM and HFM groups had the highest and lowest serum AST levels, respectively.

Liver histoarchitecture

The evaluation of the liver histoarchitecture revealed that the nuclei position in hepatocytes and hypertrophy index was not influenced by FM replacement level and the OAs content (Table 7, P>0.05). Liver histology of fish fed the experimental diets exhibited increased lipid deposition in hepatocytes and normal cells had clear hexagonal shape with obvious nuclei. However, the largest lipid vesicles size was in the fish fed with LFM$^{+0.5}$ diet at day 30, and the smallest ones were in the fish fed with HFM, HFM$^{+1.0}$ and LFM diets. At the end of the trial, the largest lipid vesicles size were in the liver of the fish fed with HFM$^{+0.5}$, LFM and LFM$^{+0.5}$ diets and the smallest ones were in fish fed HFM$^{+1.0}$ diet (Figure 1).

Table 7. Histological evaluation of the liver of *L. calcarifer* juveniles fed the experimental diets

	Parameters					
	30 day			60 day		
Diets	Nuclei position	Hypertrophy	Lipid vesicle size	Nuclei position	Hypertrophy	Lipid vesicle size
HFM	1.5±0.6	1.5±0.5	3.3±1.1 c	1.0±0.2	1.5±0.6	3.1±0.9 b
HFM$^{+0.5}$	1.5±0.6	2.0±0.6	4.2±1.2 b	1.8±0.5	1.8±0.6	4.8±1.2 a
HFM$^{+1.0}$	1.0±0.2	1.0±0.2	2.3±0.9 c	1.0±0.2	1.0±0.1	2.3±1.1 c
LFM	1.3±0.6	1.8±0.5	2.8±1.2 c	1.3±0.5	1.8±1.0	4.0±1.2 a
LFM$^{+0.5}$	2.3±1.0	2.0±0.8	4.7±1.6 a	2.0±0.7	2.5±0.6	4.5±1.8 a
LFM$^{+1.0}$	1.2±0.5	1.3±0.6	3.8±1.0 b	1.0±0.2	1.5±0.6	3.4±0.9 b
Two-way ANOVA						
Replacement level	0.312	0.174	0.080	0.425	0.057	0.013
Acidifier level	0.058	0.121	0.001	0.119	0.113	0.001
Interaction	0.263	0.615	0.001	0.848	0.712	0.118

Data are presented as the mean ± SD of three replicates.

Table 8. Two-way analysis results of dietary FM replacement and acidifier levels on interleukin-10 (IL-10) and granulocyte-macrophage colony-forming cells (GMCFC) genes transcription in the gut of *L. calcarifer* juveniles

	Sampling period			
	30 day		60 day	
	IL-10	GMCFC	IL-10	GMCFC
Two-way ANOVA				
Replacement level	0.008	0.001	0.001	0.248
Acidifier level	0.001	0.001	0.004	0.001
Interaction	0.006	0.001	0.011	0.001

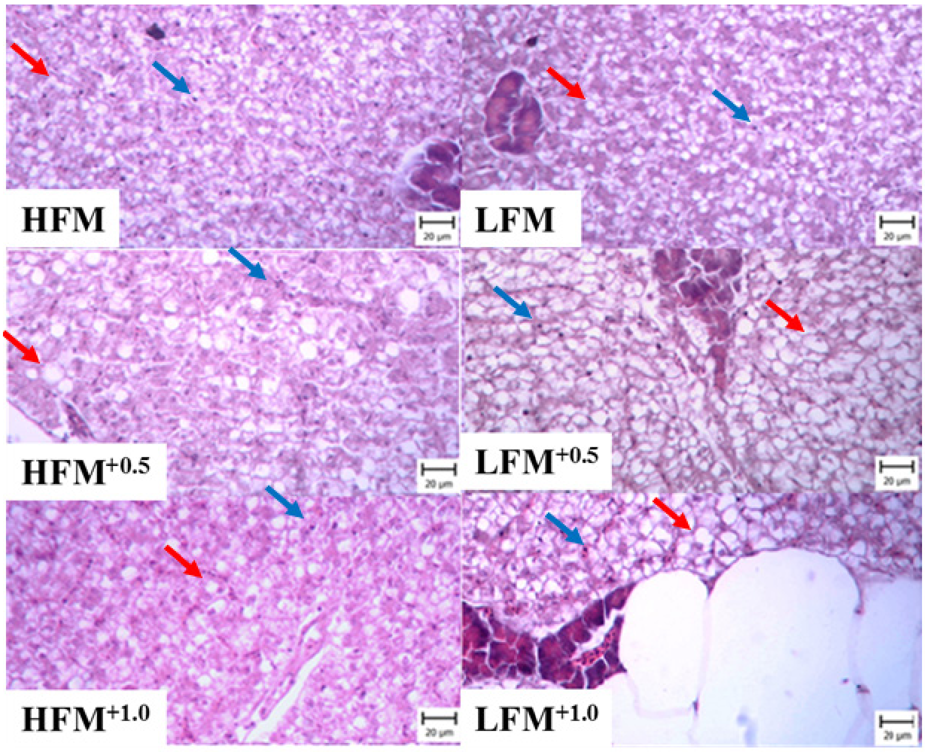

Figure 1. Histomorphology features of the liver in *L. calcarifer* juveniles fed the experimental diets at day 60. Blue arrows show the nucleus position and the red arrows show lipid vesicle

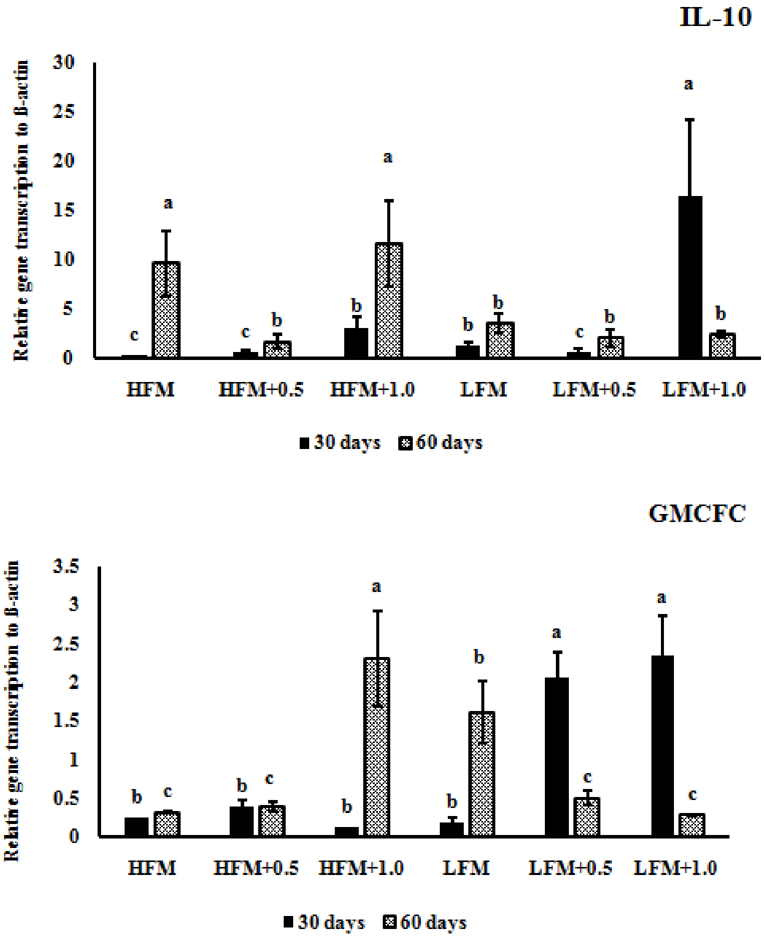

Figure 2. Immune-related genes including interleukin-10 (IL-10) and granulocyte-macrophage colony-forming cells (GMCFC) in the gut of *L. calcarifer* juveniles fed the experimental diets at day 60. Bars with a common superscript letter are not significantly different from the other dietary groups (P>0.05). Data are presented as the mean ± SD of three replicates

Gut immune-related genes

After 30-day feeding period, the highest level of IL-10 relative gene transcription in the gut was in the fish fed with LFM$^{+1.0}$ diet (Figure 2). In addition, LFM$^{+0.5}$ and LFM$^{+1.0}$ groups had higher GMCFC relative transcription level than other groups after 30-day feeding trial (P<0.05). After 60 days, levels of IL-10 gene transcription in the gut of fish fed with the HFM and HFM$^{+1.0}$ diets were higher than other groups. The highest level of GMCFC relative transcription was in the gut of fish fed with HFM$^{+1.0}$ diet (P<0.05). The interaction of dietary FM and the OAs mixture levels affected gut immune-related genes.

Discussion

In this study, LFM diet without the OAs mixture supplementation retarded growth that mostly related to suppressed FI in fish and may be due to lower palatability. But, supplementing diets with the OAs mixture markedly elevated FI and improved FCR that could be contributed to higher growth performance. Organic acids, especially short chain fatty acids (e.g. BA and formate) can act as feed attractant because of their low molecular weights. Similarly, it has been reported that SDF (0.5%) (Reyshari et al., 2019) or PA (1%, Salehi et al., 2023) in *L. calcarifer* significantly enhanced FI and growth performance. In our research, the improvement of FCR values in fish fed diets containing the OAs mixture may indicate the increment of nutrients digestibility. In this sense, OA as a source of energy for rehabilitation of the enterocytes epithelium can promote gut's health (da Silva et al., 2013). Similarly, SDF (Reyshari et al., 2019) and BA (Alamifar et al., 2020) markedly improved feed efficiency in *L. calcarifer.*

Fish gut microbiome has vital role in digestion, health and immunocompetence of fish and investigating the gut microbiome can provide a comprehensive understanding of the dietary effects on the fish health condition (Talwar et al., 2018). It has been proved that dietary administration of OAs can provoke the proliferation of LAB that have health promoting effects on fish (Luckstadt, 2008; Hoseinifar et al., 2017). Dietary OAs by decreasing the gut's pH can fortify the colonization of LAB and strengthen the host's immunocompetence through gut-associated lymphoid tissue (Ng and Koh, 2017). Previous study in *L. calcarifer* fry showed that dietary FM replacement with a mixture of APS remarkably changed hindgut's bacterial communities and 50% dietary FM replacement increased the abundance profile of *Psychrobacter* bacteria in the gut (Gupta et al., 2020). In our study, the OAs mixture relatively increased TVC in the fish gut. Furthermore, supplementing diets with the OAs mixture, especially in fish fed LFM$^{+1.0}$ induced LAB propagation in the gut after 60 days. Similarly, dietary SDF (0.5%) supplementation increased LAB count in the gut of *L. calcarifer* juveniles (Reyshari et al., 2020). Other studies also reported that the population of LAB in the fish gut were increased with OA such as potassium diformate in Nile tilapia (*Oreochromis niloticus*, Elala and Ragaa, 2015), FA in loach (*Paramisgurnus dabryanus,* Gao et al., 2017) and citric acid in turbot (*Scophthalamus maximus* L., Dai et al., 2018). It should be mentioned that in the current research the culture media-dependent methods were used for understanding the gut microbiome, and it is complicated to explain whether the increase or decrease in TVC has a positive or negative influence on the gut's health or which bacterial groups were increased or decreased. Using state-of-the-art techniques such as next generation sequencing (NGS) (Pereira et al., 2017) and denaturing gradient gel electrophoresis (DGGE) (Pedrotti et al., 2015), can provide valuable information regarding the effects of dietary OAs on the fish gut.

In the current research, LFM diet and 1.0% OAs mixture suppressed CAT and SOD activities and decreased GSH level that consequently led to higher MDA levels in the liver that may indicate oxidative stress response in fish. In contrast, it has been reported that citric acid supplementation of a soybean meal-rich diet increased total SOD activity but decreased MDA level in the gut of large yellow croaker (*Larimichthys crocea*) (Zhang et al., 2016). Moreover, BA (0.25–0.5%) enhanced serum SOD activity in *L. calcarifer* (Alamifar et al., 2020). Also, low or medium levels of fulvic acid up-regulated SOD-2 gene expression in zebra fish (*Danio rerio*) (Lieke et al., 2021 a, b).

There is an increasing interest in using OAs for alleviating the adverse effects of low FM content diets on the health status in marine carnivorous fish species (Benedito-Palos et al., 2016; Estensoro et al., 2016; Zhang et al., 2016; Lin and Cheng, 2017; Chen et al., 2018; Sotoudeh et al., 2020; Sangari et al., 2021; Salehi et al., 2023). A study by Gupta et al. (2020) revealed that plant protein based diet induced cellular degeneration and necrotic foci in the liver of *L. calcarifer* fry but it did not affect serum liver enzymes (AST and lactate dehydrogenase). In the current study, LFM diet enhanced serum ALT and AST. In addition, fish fed LFM$^{+1.0}$ diet had higher levels of liver ALT and serum ALP suggesting 1% OAs mixture may have been excessive to *L. calcarifer*. Also, the inclusion of sodium citrate at 4% enhanced plasma ALT in red hybrid tilapia (*Oreochromis* sp.) and it was associated with extensive hemorrhaging and necrosis in the liver (Romano et al., 2016). Furthermore, supplementing diet with formate at 2% level markedly elevated plasma ALT that coincided with inflammatory responses and hydropic vacuolation in the liver of red hybrid tilapia (Ebrahimi et al., 2017). Additionally, the inclusion of sodium acetate up to 0.2% significantly reduced plasma AST and ALT, but higher levels (0.4%) enhanced their values in golden pompano (*Trachinotus ovatus*) juveniles, suggesting high levels of OAs may induce liver malfunction (Xun et al., 2022).

Several studies reported that FM sparing with terrestrial animal proteins in Japanese seabass (*Lateolabrax japonicus*) (Zhang et al., 2019) and hybrid grouper (Ye et al., 2019; Zhou et al., 2020), or plant protein sources in turbot (Bian et al., 2016), Japanese seabass (Hu et al., 2013) and *L. calcarifer* (Ilham et al., 2018) induced apoptosis, steatosis, fatty liver and liver damages. In the current study, lipid vesicle size in the liver decreased with 1% OA mixture suggesting hypolipidemic effects of OAs. Hypolipidemic impacts of OAs were also reported in rainbow trout (Yilmaz et al., 2018), common carp (Krome et al., 2018), golden pompano (Xun et al., 2022) and American eel (*Anguilla rostrate,* Zhang et al., 2022) fed diets supplemented with humic acid, SDF, potassium diformate and compound OAs, respectively. In this sense, it has been confirmed that OAs by reducing 3-hydroxy-3-methylglutaryl coenzyme A level in the liver can exert their hypolipidemic effects (El-Shenway and Ali, 2016).

The application of nutrigenomics in aquaculture can enhance our knowledge regarding the influences, mode of action and interactive effects of dietary supplements on physiological responses of fish at molecular level (Martin and Król, 2017). Interleukin-10 has remarkable inhibitory impact on various kinds of immune cells by suppressing the expression of pro-inflammatory cytokines, reactive oxygen radicals and deactivating antigen presenting cells (Grayfer et al., 2011; Huo et al., 2019). Moreover, a protective impact on the gut epithelial integrity was described for IL-10 that reduce the permeability of the gut epithelium through raising transepithelial electrical resistance (Al-Sadi et al., 2009). Granulocyte-macrophage colony-stimulating factor stimulates the proliferation and differentiation of a wide range of leucocytes by binding to heteromeric cell-surface receptors on leucocytes and endothelial cells (Hodgkinson et al., 2015). Fishmeal sparing with a mixture of alternative protein sources at 50% simultaneously induced up-regulation of IL-10 and also some pro-inflammatory genes such as IL1-ß, IL-8, IL-17F and TNF-α in the hindgut of *L. calcarifer*, but total replacement did not significantly affect these genes. In the current research, HFM$^{+1.0}$ and LFM$^{+1.0}$ diets up-regulated the transcription of both IL-10 and GMCFC genes after 30 and 60 days of the feeding trial, respectively, suggesting dietary FM content and OAs level had immunomodulatory effects on the expression of immune-related genes. The up-regulation of IL-10 suggesting the protective effects of the OAs mixture on the gut epithelial integrity may be in response to the upregulation of GMCFC gene. Also, dietary sodium butyrate supplementation at 0.25% up-regulated anti-inflammatory cytokines genes (e.g., IL-10 and TGF-β1) and down-regulated pro-inflammatory genes (e.g., IL-8 and TNF-α) in rainbow trout (Mirghaed et al., 2019). Studies in European seabass also demonstrated the up-regulation of IL-10 in the gut after feeding with diets containing butyrate (Terova et al., 2016) or a blend of OAs mixture and herbal extract (Busti et al., 2020). In contrast, supplementing a plant protein rich diet with 0.2% sodium butyrate enhanced the transcription level of TNF-α (a pro-inflammatory cytokine), but did not have effect on IL-10 in European seabass (Rimoldi et al., 2018). It should be mentioned that the cytokine IL-10 can be seen in a negative light when it is up-regulated unnecessarily. The concept of "immune priming" requires that some proinflammatory states be maintained in the absence of infection/disease, and increases of IL-10 can impair this function. For example, it has been reported that IL-10 can weaken the immune defense against *Mycobacterium marinum* infection in zebrafish by restricting interferon gamma-1 response (Harjula et al., 2018). On the other hand, IL-10 deficiency or blockade of its receptor elevated protection against mycobacteria in mice, but the lack of this anti-inflammatory cytokine resulted in harmful lung inflammation and the progression of a mycobacterial disease in the mouse model (Higgins et al., 2009).

Conclusion

In conclusion, the findings of the present research showed that LFM diet could adversely affect growth rate due to lower FI, but supplementing diet with 1% OAs mixture enhanced FI and consequently growth in *L. calcarifer* juveniles. Furthermore, the highest level of LAB count was in fish fed LFM$^{+1.0}$ diet suggesting improvement of gut microbiome. In addition, LFM diet without the OAs mixture supplementation enhanced serum ALT and AST. Furthermore, lipid vesicle size in the liver decreased by inclusion of 1% OAs mixture indicating hypolipidemic effects of OAs. Finally, supplementing diets with 1% of OAs blends up-regulated the transcription of both IL-10 and GMCFC genes suggesting the protective effect of the mixture of OAs on the gut epithelial integrity as well as its immunostimulating impact in the gut of *L. calcarifer*. It is suggested to feed the fish for 60 days to achieve the best results.

Ethical approval

All experiments and samplings were done based on the ethical recommendations in the guide for the care, protection, and use of laboratory animals approved by the Institutional Animal Care and Use Committee of the Iranian Fisheries Science Research Institute.

Competing interests

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.

Authors' contributions

HM: project administration and methodology. TM: conceptualization, methodology, project administration, and supervision. MM: project administration and methodology, and supervision, AR: project administration and methodology, MT: project administration and methodology, MTM: conceptualization, investigation, validation, interpretation of data, and writing original draft.

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Availability of data and materials

Data will be available on reasonable request.

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