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Enrichment of rainbow trout (*Oncorhynchus mykiss*) fingerlings diet with microbial lysozyme: Effects on growth performance, serum and skin mucus immune parameters

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ABSTRACT

A two-month study was conducted to determine the influence of different levels of microbial lysozyme (LZ) contents (0, 0.5, 1.0, and 1.5 g kg^{-1} of diet) on growth performance, serum and skin mucus immune parameters as well as intestinal immune-related genes expression in rainbow trout *Oncorhynchus mykiss* fingerlings (5.5 ± 0.1 g). Growth performance and feed utilization were not affected significantly by dietary LZ. Fish fed LZ-supplemented diets had higher serum total immunoglobulins concentration than the control group. In addition, fish fed 1.5 g LZ kg^{-1} diet had the highest skin mucosal total protein and immunoglobulin contents compared to other experimental groups. Furthermore, skin mucosal lysozyme and alkaline phosphatase activities as well as intestinal tumor necrosis factor-α and interlukine-1β relative genes expression were higher in fish fed 1.0 and 1.5 g LZ kg^{-1} diets than the other groups. Overall, the present results clearly showed that LZ powder can be considered as a potential immunostimulant in *O. mykiss* fingerlings, but in the long term period it may result in negative effects on intestinal health as a consequence of inducing pro-inflammatory cytokines gene expression in the intestine.

1. Introduction

Rainbow trout is among one of the most important cultured fish in several countries. Nowadays, intensive and super-intensive culture systems are common for trout culture. There are increasing concerns about using antibiotics in rainbow trout culture to overcome infectious diseases. Using immunostumulants as bio-friendly agents is a sustainable way for reducing side effects of applying antibiotics in aquaculture [1–6]. Among different kinds of immunostimulant classes (*e.g.* probiotic, prebiotic and symbiotic), lysozyme (muramidase, EC 3.2.1.17) (LZ), which is a natural endogenous antibiotic has been proven to induce specific and non-specific immune responses in teleosts [7–9]. Lysozyme as a mucolytic and antibacterial enzyme is widely distributed in animal body fluids (*e.g.* saliva, mucus, tears, serum) [10]. This enzyme has several physicochemical properties including high resistant to acid digestion and proteases in gastrointestinal tract, heat-resistant, low-molecular-weight, as well as non-toxic; which is makes it as a promising immunostimulant in aquaculture [9,11,12]. In addition, LZ act as natural immunostimulant with antibacterial, antiviral, antimetastatic and anti-inflammatory properties suggesting this enzyme may be a potential antibiotic alternative [10]. A plethora of studies available on positive effects of dietary LZ on growth enhancement [9,13], humoral (*e.g.* alternative complement pathway hemolysis, lysozyme and myeloperoxidase activities, total Ig) and cellular (*e.g.* phagocytosis activity of neutrophils and macrophages, respiratory burst, lymphocyte proliferation and antibody secretion) immune responses [7–9,14], disease resistance [9], oxidative stress [15], intestinal microbiome and intestinal morphology [9,15–18], as well as cytokines gene expression [15,18] in fish, poultry and pig. However, to our best knowledge, no research has been determined immunomodulatory impact of exogenous LZ on health condition in aquaculture fish species. Thus, in our study we want to evaluate the influence of dietary LZ inclusion on performance, immune response and intestinal cytokines gene expression in rainbow trout *Oncorhynchus mykiss* fingerlings.

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2. Materials and methods

2.1. Experimental setup

This study was carried out in the laboratory in Gorgan University of Agricultural and Natural Resources, Gorgan, Iran. One hundred and twenty *O. mykiss* fingerlings purchased from a private farm and were transferred to the laboratory then adapted to experimental condition for two weeks. Following adaptation to experimental condition, fish (initial body weight (BW_i) = 5.5 ± 0.1 g) were randomly distributed among twelve fiberglass 200-L tanks (n = 10 fish per tank) supplied with filtered fresh water and continuous aeration, and 50% of water was exchanged daily. The mean values of water temperature, dissolved oxygen and pH were 16.2 ± 0.9 °C, 6.1 ± 0.2 mg L^{-1} and 7.6 ± 0.2, respectively. A commercial feed (Coppens Co., Netherland; 48% crude protein, 22% crude fat, 8.8% ash, 0.9% fiber; 20.7 MJ kg^{-1} digestible energy) was supplemented with the (Microbial lysozyme 10%, fermented lysozyme use as feed additives, Zhejiang Aegis Biotech Co., Ltd) graded levels of LZ powder according to the recommended dose reported in the previous studies [9,13] including: 0 (control), 0.5, 1.0 and 1.5 g LZ kg^{-1} diet, respectively. In this regard, the basal diet was ground in order to produce a fine powder and then supplemented with the above-mentioned LZ dosages, then thoroughly mixed and water was added to produce dough according to Ref. [19]. The dough was pelleted using a meat grinder to obtain pellets with size of 2 mm, then pellets were dried in a convection oven at 25 °C for 24 h and stored at −20 °C until used.

Fish were manually-fed three times (0800, 1200 and 1600) at rate of 3% of BW_i daily for two months. Feeding ratio was corrected based on biometry which was done every 10 days. Utmost care was considered to avoid feed loose.

3. Immunological parameters

3.1. Sample collection

At the end of feeding trial, fish were fasted for a day and weight (BW_f) were measured, individually to assess growth performance and feed utilization parameters. Nine fish of each experimental treatment were randomly bled from the caudal vein with syringe after anaesthetizing by clove powder (500 mg L^{-1}). Then after, blood was allowed to clot (at 4 °C for 4 h), centrifuged and stored at −80 °C until use. For evaluating skin-related lymphoid immune system, mucus was collected according to Ross et al. [20] method. In this regard, at the end of feeding trial fish (9 fish per treatment) was anaesthetized with the same anesthetic and individually was transferred to a polyethylene bag containing 10 mL of 50 mM NaCl (Sigma, Steinheim, Germany) and gently rubbed for approximately 1 min to mucus collection. Thereafter, obtained mucus was prepared and stored at −80 °C until use according to Khodadadianzou et al. [21].

3.2. Immunological parameters

The levels of LZ in mucus was determined using a turbidimetric assay according to Ellis [22] by measuring the lytic activity of *O. mykiss* serum against lyophilized *Micrococcus luteus* (Sigma, St Louis, MO, USA). Serum and skin mucosal total immunoglobulin (Ig) was measured using the method described by Siwicki et al. [23]. Total protein concentration was determined by using a commercial kit (Zist Shimi kits, Iran) according to the manufacture instruction. Alkaline phosphatase activity of the mucus was measured using a commercial kit (Pars Azmon Co, Tehran, Iran) according to the manufacturer protocol.

3.3. RNA extraction and relative mRNA expression of intestinal cytokines

After the feeding period, 3 fish from each replicate (n = 9 fish per treatment) were randomly euthanized with overdose anesthetic, and immediately eviscerated on ice surface, then intestine was dissected for intestinal cytokines gene expression assays. The intestine was frozen immediately in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA of samples was isolated using BIOZOL RNA extraction kit (Bioflux-Bioer, China) according to the manufacturer's instructions. For prohibiting contamination of samples with genomic DNA, total RNA was treated with DNase I (Fermentas, Lithuania). Afterwards, the quantity and concentration of RNA were measured using nanodrop spectrophotometer (Nanodrop technology, Wilmington, DE, USA) at 260 and 280 nm (A260:A280 > 1.8 were selected for further experiments) following electrophoresis on a 1.5% agarose gel. The reverse transcription was used to synthesize the first-strand cDNA using transcription Kit (Fermentas, Lithuania) [19]. The conserved regions of *Cyprinus carpio* Gene Bank were used for designing the primers sequences by means of by Oligo7 software (Table 1). Quantitative real-time PCR assays were performed in triplicate to study the effects of dietary lysozyme inclusion on the expression of intestinal cytokines (TNF-α, IL-1β) in *O. mykiss* as described by Miandare et al. [19]. For normalizing the expression of the cytokine genes, β-actin was used as the housekeeping gene. Data was analyzed using the iQ5 optical system software (Bio-Rad) and DDCt method.

Table 1
Primers sequences and amplification efficiencies.

Gene name	Sequences of primers	Accession no	Efficiency
β-actin	Forward: AGACATCAGGGTGTCATGGTTGGT Reverse: CTCAAACATGATCTGTGTCAT	M24113.1	97%
IL1β	Forward: ACCAGCTGGATTTGTCAGAAG Reverse: ACATACTGAATTGAACTTTG	AB010701.1	98%
TNF-α	Forward: GGTGATGGTGTCGAGGAGGAA Reverse: TGGAAAGACACCTGGCTGTA	AJ311800.1	97%

3.4. Statistical analyses

Data were analyzed using SPSS version 19 (Chicago, IL, USA). One-way analysis of variance was performed at a significance level of 0.05 following the confirmation of normality (Shapiro–Wilk's test) and homogeneity (Levene's test) of variance. Duncan's multiple-range procedure was used for multiple comparisons.

4. Results

The results the present study showed that growth performance and feed utilization were not significantly affected by dietary LZ in *O. mykiss* ($P > 0.05$, Table 2).

Results showed that fish fed 0.5, 1.0, and 1.5 g LZ kg^{-1} diets had higher serum total immunoglobulins concentration than the control group ($P < 0.05$, Fig. 1). The highest total serum Ig were noticed in rainbow trout fed 1.0 and 1.5 g LZ kg^{-1} diets. However, no significant difference in total serum Ig was found between 1.0 and 1.5 g LZ kg^{-1} diets ($P > 0.05$).

Regarding mucosal immune response, fish in 1.5 g LZ kg^{-1} diet had higher total protein (Fig. 2a) and Ig (Fig. 2b) in the mucus than the control and other supplemented groups ($P < 0.05$). Similarly, fish fed 1.0 and 1.5 g LZ kg^{-1} diets had higher mucosal lysozyme (Fig. 3a) and alkaline phosphatase (Fig. 3b) activities than the control and 0.5 g LZ kg^{-1} group ($P < 0.05$).

For intestinal cytokines gene expression, fish in 1.0 and 1.5 g LZ kg^{-1} groups showed significant higher relative TNF-α (Fig. 4a) and IL1β (Fig. 4b) gene expression compared to the control and 0.5 g LZ kg^{-1} group ($P < 0.05$).

Table 2
Growth performance of rainbow trout fed with different dietary lysozyme levels (g kg^{-1}) for 8 weeks experimental period (means ± SD).

Index	Control	0.5 g kg^{-1}	1 g kg^{-1}	1.5 g kg^{-1}
BW_i (g)[a]	5.44 ± 0.08	5.42 ± 0.05	5.50 ± 0.04	5.48 ± 0.07
BW_f (g)[b]	27.62 ± 1.7	29.19 ± 0.3	29.58 ± 0.4	30.07 ± 0.75
WG (%)[c]	407.6 ± 32.2	438.6 ± 2.1	437.1 ± 8.7	441.4 ± 12.6
SGR (% day)[d]	2.70 ± 0.10	2.80 ± 0.06	2.80 ± 0.02	2.83 ± 0.03
FCR[f]	1.02 ± 0.08	0.95 ± 0.01	0.94 ± 0.01	0.93 ± 0.02
Survival (%)[e]	100	100	100	100

[a] Initial body weight.
[b] Final body weight.
[c] Weight gain (%) = $(BW_f - BW_i)/BW_i$.
[d] Specific growth rate (%) = $((\ln BW_f - \ln BW_i)/t) \times 100$, where t is 60 days.
[e] Survival (%) = (number of fish in each group remaining at the end of each phase/initial number of fish) × 100.
[f] Feed conversion ratio = feed intake (g)/weight gain (g).

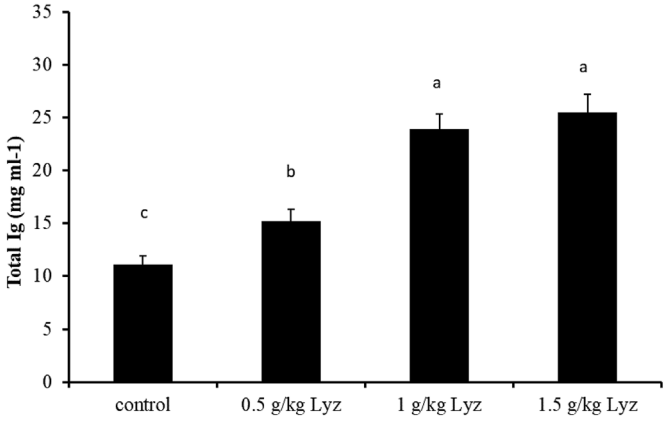

Fig. 1. Serum total immunoglobulin levels of the rainbow trout fingerlings fed different levels of dietary microbial lysozyme. Bars assigned with different superscripts are significantly different ($P < 0.05$); Values are presented as the mean ± S.E.

5. Discussion

The results of present study showed that not only innate immune responses improved in fish fed LZ supplemented diets, but also intestinal cytokines genes expression up-regulated by dietary LZ. It has been postulated that dietary LZ can induce non-specific immune responses through noticeable regulation of intestinal cytokines and antioxidant genes expression as well as modulating intestinal microbiome (*e.g.* increasing *Lactobacillus* counts) [15]. In addition, it is assumed that immuno-stimulating process of LZ associated with the LZ-digested products of bacterial envelop structure that promote the innate immune reaction [24,25], antibodies and cytokines secretion [15] as well as can activate nuclear factor κB(NF-κB) pathway, which induce the expression of pro-inflammatory cytokine genes [15,26].

Immunoglobulins are heterodimeric glycoproteins that play a vital role in recognizing natural antigens and found in the skin, gill and gut mucus, bile as well as systemically found in the plasma of fish [27]. In our study, higher serum total Ig content was noticed in fish fed 1.0 and 1.5 g LZ kg^{-1} diets that was coincided with the over-expression of intestinal inflammatory cytokines (TNF-α and IL1β), suggesting immunomodulatory effects of LZ on specific immune responses. In accordance with this result, supplementing diet with immunostimulants such as date palm fruit extract (DPFE) [28], white bottom mushroom powder (*Agaricus bisporus*) (WBMP) [21] and a symbiotic (galactooligosaccharide and *P. acidilactici*) Modanloo et al. [29] increased serum total Ig in common carp (*Cyprinus carpio*).

The fish skin mucus acts as a frontier defensive barrier to many

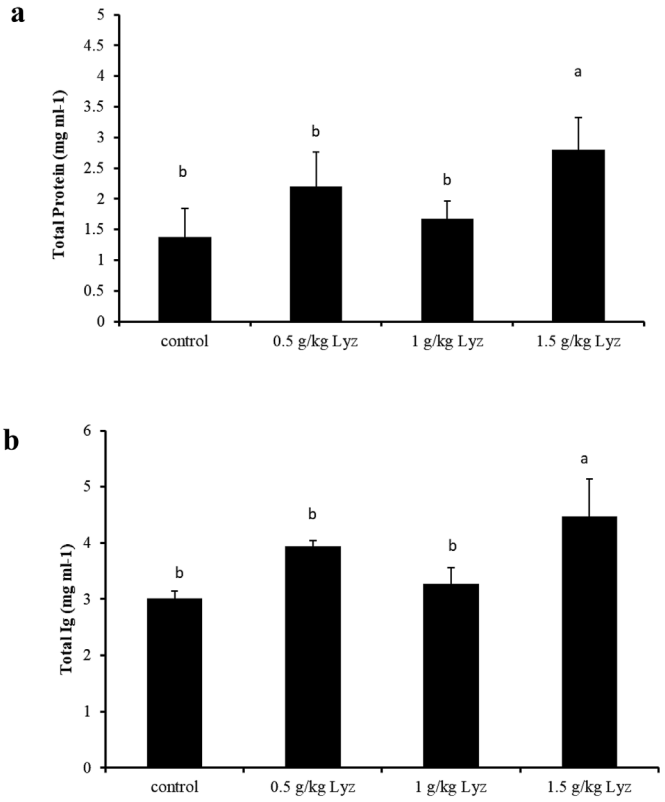

Fig. 2. Skin mucus total protein (a) and total Ig (b) contents in *O. mykiss* fingerlings fed different levels of dietary microbial lysozyme. Bars assigned with different superscripts are significantly different ($P < 0.05$); Values are presented as the mean ± S.E.

infectious pathogens through skin-associated lymphoid tissue including adaptive (*e.g.* B and T lymphocytes, immunoglobulins) and innate (*e.g.* enzymes, antimicrobial peptides and glycoproteins, melanin and natural killer cells) immunity Esteban [30]. Our results indicated noticeable increase in mucosal total protein and Ig concentrations in *O. mykiss* fed diets supplemented with 1.5 g LZ kg^{-1}, which can be associated with development of fish immune competence. In this sense, dietary supplementation of immunostimulants such as fermented *Saccharomyces cerevisiae* in rainbow trout [31], xylactooligosaccharide in Caspian white fish (*Rutilus frisii kutum*) [32], *Lactobacillus acidophilus* in black swordtail (*Xiphophorus helleri*) [33] and herbal plants [loquat (*Eriobotrya japonica*) Hoseinifar et al. [34] and medlar (*Mespilus germanica*) Hoseinifar et al. [35] or symbiotic (galactooligosaccharide (GOS) and *P. acidilactici*) in common carp [29] increased skin mucus total protein or Ig levels.

Mucosal enzymatic activities have protective role against infectious organisms by destroying pathogens cell envelop structure [36], prohibiting their colonization and invasion as well as activating the synthesis of other innate immune components present in mucus (*e.g.* complements and antibodies) [37]. Our results revealed significant increase in mucosal LZ and ALP activities in *O. mykiss* fed 1.0 and 1.5 g LZ kg^{-1} diet in comparison with the other groups, indicating improved non-specific bactericidal activity in these groups. In agreement with this result supplementing diet with vitamin C in Caspian roach (*Rutilus rutilus caspicus*) [38], probiotic (*L. acidophilus*) in black swordtail [33], fermented baker's yeast [31] or Myrtle *(Myrtus communis* L.*)* [39] in rainbow trout fingerlings and date palm fruit extracts in common carp [28] increased skin mucosal ALP activity. Several other studies also reported the positive effects of immunostimulants including GOS [19], symbiotic [40], and medicinal plants [*e.g. Myrtus communis* [41], DPFE [28], WBMP [21], *Cordyceps militaris* [42] and medlar Hoseinifar et al. [35] on skin mucosal LZ activities in different fish species.

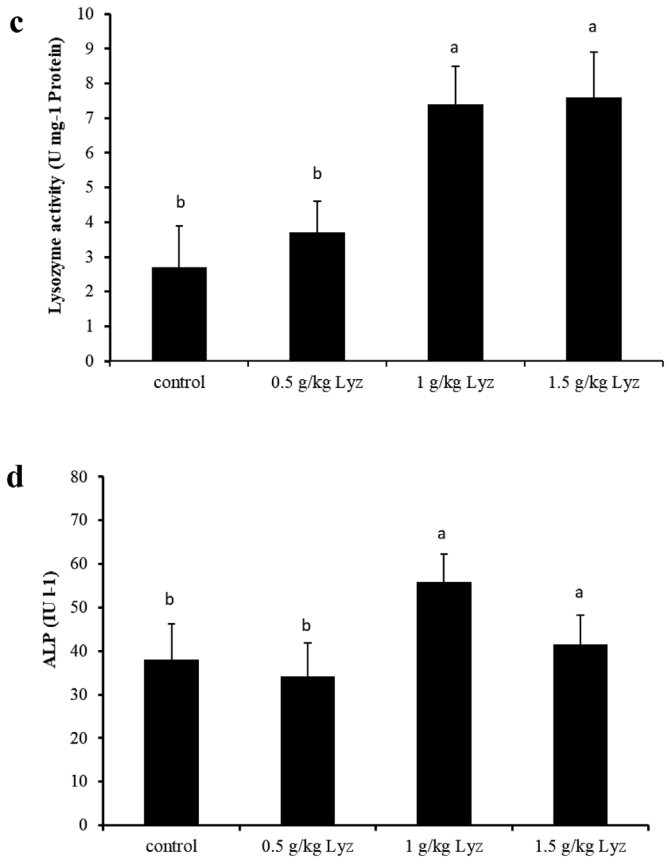

Fig. 3. Skin mucus lysozyme (c) and alkaline phosphatase (d) activities in *O. mykiss* fingerlings fed different levels of dietary microbial lysozyme. Bars assigned with different superscripts are significantly different ($P < 0.05$); Values are presented as the mean ± S.E.

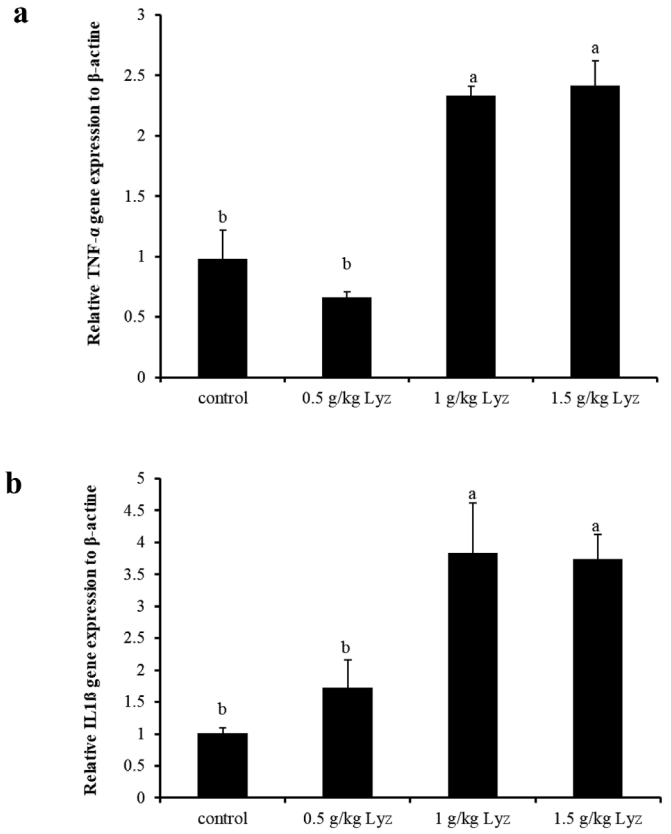

Fig. 4. The effects of different levels of dietary microbial lysozyme on the relative expression of TNF-α (a) and IL1β (b) genes in *O. mykiss* fingerlings intestine. Bars assigned with different superscripts are significantly different ($P < 0.05$); Values are presented as the mean ± S.E.

Tumor necrosis factor-α and IL-1β are cytokines involved in the induction of inflammatory responses and regulation of immune cells [27]. Our study demonstrated up-regulation of intestinal cytokines genes expression in *O. mykiss* fingerlings fed 1.0 and 1.5 g LZ kg^{-1} diets, suggesting dietary LZ trigger an inflammatory response in the intestine of the fish. In addition, the over-expression of immune related gene expression in above-mentioned groups could be associated with the modulation of intestinal microbiome [43]. In agreement with our findings, Abdel-Latif et al. [15] showed the mRNA expressions of pro-inflammatory cytokines such as of interferon-gamma and interleukin-18 were up-regulated in response to inclusion of 90 mg LZ kg^{-1} in broiler chickens. In addition, several studies proved that administration of different classes of immunostimulants including probiotics (*Lactobacillus* sp.) [44], prebiotics (fermentable fiber [45] or GOS [19]), symbiotic (GOS and *P. acidilactici*, Modanloo et al. [29] and herbs such as WBMP (Miandare et al., 2016), Myrtle Safari et al. [41], loquat Hoseinifar et al. [34] up-regulated the gene expression of TNF-α or IL-1β in different fish species. In contrast, Lee et al. [11] reported that injection of hen LZ at a dose of 150 mg kg^{-1} of body weight attenuates inflammation by down-regulation of colon gene expression of pro-inflammatory cytokines (*e.g.* TNF-α, IL-6, IFN-γ, IL-8, and IL-17) and up-regulation of intestinal mucin (MUC1) and anti-inflammatory cytokines (*e.g.* IL-4 and Transforming growth factor-β) in a porcine subjected to dextran sodium sulfate injection. In this sense, it has been reported that pro-inflammatory cytokines such as TNF-α and IL-1β may induce intestinal epithelial barrier dysfunction by increasing epithelial tight junction and permeability [46,47]. Thus, long term feeding of *O. mykiss* fingerlings with 1.0 and 1.5 g LZ kg^{-1} diet may have negative effects on intestinal health as a consequence of inducing pro-inflammatory cytokines responses.

Many previous researches have been proved growth-promoting effect of LZ in poultry, pig as well as fish species [9,13,15]. In addition, studies in terrestrial animals showed that dietary LZ improved intestinal health due to reducing the levels of microbial challenges, which could save extra energy for growth purposes [15,48]. However, our findings indicated supplementing diet with LZ did not improve growth performance and feed utilization in *O. mykiss*. In this case, inclusion of a diet with 100 mg LZ kg^{-1} did not improve growth performance in broiler chickens under clean conditions [49]. The authors of this study speculated that, when broilers were not under an environmental stress, dietary LZ may not improve growth performance, since hygiene condition could not pose a potential challenge to restrict growth or affect the health. Thus, none-stressful condition in the current study might be not trigged dietary LZ to induce growth performance in *O. mykiss* fingerlings as also reported in broiler chickens [49]. On the other hand, biotic (*e.g.* fish species, sex, age, size, health condition, degree of stress) and abiotic (*e.g.* water temperature, pH, toxicants) may change LZ activities in fish and result in contradictory findings among studies [10].

Overall, the present results clearly showed that LZ powder has remarkable effects on humoral and mucosal immune defenses and can therefore be considered as a potential immunostimulant in *O. mykiss* fingerlings diet. However, this dietary supplement did not improved growth performance and in the long term period it may result in negative effects on intestinal health as a consequence of inducing pro-inflammatory cytokines gene expression in the intestine. It is needed to evaluate the protective role of LZ against infectious diseases under experimental challenge as well as investigate the effects of dietary LZ on intestinal anti-inflammatory cytokines in further researches for increasing our knowledge regarding the mode of action and optimum

inclusion level.

Conflicts of interest

There is no conflict of interest to declare.

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