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Influence of dietary sodium alginate and Pediococcus acidilactici on liver antioxidant status, intestinal lysozyme gene expression, histomorphology, microbiota, and digestive enzymes activity, in Asian sea bass (Lates calcarifer) juveniles

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1 **Influence of dietary sodium alginate and Pediococcus acidilactici on liver antioxidant status,**
2 **intestinal lysozyme gene expression, histomorphology, microbiota, and digestive enzymes**
3 **activity, in Asian sea bass (Lates calcarifer) juveniles**

4 **Running head:** functional feed additives in diet Asian sea bass

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Abstract

A 42-day study was conducted to determine the effect of incorporating dietary low molecular weight sodium alginate (LMWSA), extracted from the brown algae, Undaria pinnatifida and Macrocystis pyritera, and Pediococcus acidilactici MA 18/5 M (PA), Lallemand Animal Nutrition S.A., Blagnac, France, on growth performance, antioxidant defense activity, intestinal lysozyme gene (LYZ) expression, histo-morphology, microbiota, and digestive enzymes activity of Asian sea bass (Lates calcarifer) juveniles. Six experimental diets were formulated including: Diet (1) a basal diet (Control), Diet (2) 5 g LMWSA kg-1 diet, Diet (3) 10 g LMWSA kg-1 diet, Diet (4) 0.9 × 107 CFU PA g-1 diet, Diet (5) 5 g LMWSA kg-1 diet + 0.9 × 107 CFU PA g-1 diet, and Diet (6) 10 g LMWSA kg-1 diet + 0.9 × 107 CFU PA g-1 diet were fed to Asian sea bass, L. calcarifer (12.0 ± 0.2 g). The results showed that fish fed PA alone (Diet 4) and the combination of both supplements (Diet 5) had the greatest weight gain. Fish fed Diet 6 and those fed Diet 1 (Control) had the highest and lowest villus height, apparent villus surface and crypt depth, respectively. Fish fed diets administered with PA (Diet 4) or synbiotics (Diets 5 and 6) showed higher total viable and lactic acid bacteria counts than all the other groups. The evaluated digestive enzymes activities including total protease, trypsin, lipase, and α-amylase remarkably increased by administration of LMWSA or its combination with PA. Moreover, liver antioxidant enzymes activities including superoxide dismutase, catalase, and Glutathione S-transferase pronouncedly enhanced following the administration of LMWSA or its combination with PA. Supplementing diet with blends of 10 g kg-1 of LMWSA and PA (Diet 6) more pronouncedly enhanced c-type and g-types LYZ expression in comparison with those fed 5 g kg-1 of LMWSA and PA (Diet 5). Based on the results obtained, it can be claimed incorporating diet with LMWSA and PA separately or in symbiotic form had promising results as functional feed additives in juvenile Asian sea bass L. calcarifer.

Keywords: Antioxidant, Asian sea bass, Lysozyme, Prebiotic, Probiotic, Sodium alginate

1. Introduction

The diversification in aquaculture by introducing new aquatic species is one of the main FAO's projects for sustainable aquaculture (FAO, 2018). Asian sea bass (Lates calcarifer) is one of the most promising candidate species for extending marine cage culture programs in tropical and

subtropical regions (Singh, 2000; Mathew, 2009). It has many characteristics that makes it unique candidate for aquaculture such as fast growth rate, high fecundity and easy reproduction in captivity, high resistance to environmental and culture conditions (Mathew, 2009). The production of this species increased from ca. 20,000 tons in 1998 to 90,000 tons in 2017 in the major producing countries (Khang et al., 2018). However, intensification of this fish may result in poor water quality and crowding stress that could have deleterious effects on their welfare, which may lead to the repeated occurrence of infectious diseases (Ringø et al., 2010b; Romero et al., 2012).

It is well proved that supplementing aqua-feeds with different kinds of immunostimulants mainly probiotics, prebiotics, phytobiotics, parabiotics and synbiotics not only can fortify immune competence but also can promote growth performance in cultured aquatic species (Ringø et al., 2014; Abdel-Tawwab, 2016; Ringø and Song, 2016; Hoseinifar et al., 2018b). For example, lactic acid bacteria (LAB) determined as the most promising probiotics in aquaculture due to their abilities to stimulate growth, reproductive performance, gastrointestinal function, immune responses, and enhance disease resistance (Gioacchini et al., 2010, 2012; Giorgini et al., 2010; Ringø et al., 2018). LAB have been considered as profitable bacteria of the fish intestinal microbiome and mostly can be isolated from the various fish species intestinal tract (Dimitroglou et al., 2011; Falcinelli et al., 2017). Among different species of LAB, Pediococus acidilactici (PA) reported to have promising effects on growth performance, digestive enzymes activities, antioxidant and stress resistance (Castex et al., 2009, 2010; Hoseinifar et al., 2017d; Taridashti et al., 2017), gut microbiota and morphology (Ferguson et al., 2010; Merrifield et al., 2010; Abid et al., 2013; Standen et al., 2013), as well as enhance immune gene expression (Abid et al., 2013) in various cultured finfish species.

On the other hand, herbal extracts contains bioactive ingredients with antioxidant and antimicrobial activities, which can provoke growth, appetite, immune competence, and also ameliorate signs of stress in cultured aquatic species (Citarasu, 2010; Holdt and Kraan, 2011; Jiao et al., 2011; Abdel-Tawwab, 2016; Abdel-Tawwab et al., 2018; Adeshina et al., 2019). Among medical herbs, seaweeds could be considered as good sources of bioactive and environmentally friendly compounds with antibacterial (Gonzalez del Val et al., 2001), antioxidant potential (Yuan and Walsh, 2006; Chandini et al., 2008), antiinflammatory (Kang et al., 2008), anti-coagulant (Pushpamali et al., 2008), and anti-viral (Sinha et al., 2010) properties. Low molecular weight sodium alginate (LMWSA) is one of the sodium alginate derivatives that extracted from brown

1 seaweed, emerged as novel prebiotic with better properties than sodium alginate presenting lower
2 molecular weight, higher solubility and fermentation (MacArtain et al., 2007; Van Doan et al.,
3 2014, 2016a). In this sense, previous studies demonstrated positive influences of LMWSA on
4 growth, feed efficiency and immune responses in fish species (Van Doan et al., 2014, 2016a, b;
5 2017).
6 Deeper knowledge regarding the intestinal tissue changes, the variation of microbial flora coupled
7 with digestive enzymes levels are necessary to find out the influence of functional feed additives
8 (i.e. pro-pre and synbiotics) on the physiology of a cultured fish species. Therefore, the antioxidant
9 capacity determined by antioxidant enzymes evaluation as well as the intestinal immune-related
10 gene expression will allow us to elucidate the molecular pathways in which different classes of
11 immunostimulants can improve general health and immune competence of farmed fish. Thus, the
12 current study aimed to assess the effects of LMWSA and PA individually or in synbiotic form on
13 growth, digestive enzyme activities, histological architecture of intestine, antioxidant enzymes as
14 well as intestinal lysozyme gene (LYZ) expression in Asian sea bass L. calcarifer juveniles.

15 ## 2. Materials and Methods

16 ### 2.1. Fish husbandry and diets

17 Fish were transferred from a private company (Ramoz, Bushehr, Iran) to the laboratory of Aquatic
18 Research, Persian Gulf University, Bushehr, Iran (28°91′N, 50°82′E), where the study was carried
19 out. Juveniles of Asian sea bass (L. calcarifer) with initial weight of 12.0 ± 0.2 g (mean ± SE) were
20 kept in two 1000 L fiberglass tanks (200/fish m3) and adjusted to the husbandry system (for two
21 weeks). Then, they were transferred to experimental system including 18 polyethylene tanks (300-
22 L), which were randomly stocked at a density of 20 fish per tank. Tanks were previously supplied
23 with 200 L of disinfected and filtered sea water and around half of water was daily changed. Water
24 quality parameters were monitored daily by a 340i Multimeter (WTW, Weilheim, Germany) to
25 ensure the fish welfare. The ranges of water salinity, temperature, dissolved oxygen content and
26 pH were 38.9–39.5‰, 21.5–24.5 °C, 4.8–5.9 mg l-1 and 7.8–8.1, respectively and photoperiod
27 was natural (28°91′N, 50°82′E) during the experimental period.
28 For preparing the experimental diets, a basal feed (Beyza, Shiraz, Iran; Table 1) was firstly milled,
29 then supplemented with the selected levels of low molecular weight sodium alginate (LMWSA)
30 and lyophilized Pediococcus acidilactici (PA, MA 18/5 M, Bactocell® PA 10; Lallemand Animal

Nutrition S.A., Blagnac, France) as described by Merrifield et al. (2011). The selected dosages of the PA and LMWSA were added to the basal diet as previously suggested by Castex et al. (2010) and Van Doan et al. (2016b), respectively to prepare experimental feeds including: Diet (1) a basal diet (Control), Diet (2) 5 g LMWSA kg-1 diet, Diet (3) 10 g LMWSA kg-1 diet, Diet (4) 0.9 × 107 CFU PA g-1 diet, Diet (5) 5 g LMWSA kg-1 diet + 0.9 × 107 CFU PA g-1 diet, and Diet (6) 10 g LMWSA kg-1 diet + 0.9 × 107 CFU PA g-1 diet. Each dietary treatment was tested in triplicate. Feeding was carried out two times a day at 10:30 and 16:30 h up to fish visual satiation during the husbandry trial. A half an hour after feeding, uneaten feed was siphoned then dried (60 °C for 24 h) and weighed for evaluation of feed efficiency.

2.2. Fish growth and sampling

A day before sampling, fish were being anaesthetized (2-phenoxyethanol, 0.3 ml l-1) and individually weighed (BWf) at accuracy of 0.1 g. Five fish from each replicate were sacrificed with an overdose of the same anesthetic to measure their hepatosomatic index (HSI), viscerosomatic index (VSI) (Ali et al., 2017). For measuring digestive enzymes activities and oxidative status, five fish per tank were euthanized with overdose the anesthetic, and the alimentary tract and liver were dissected instantly on ice surface, then stored at -80 °C. All morphometric indices were calculated as follows:

WG (%) = ((BWf (g) – BWi (g)) / BWi (g)) × 100; SGR (% day-1) = [(ln BWf – ln BWi)/t] × 100;

FCR = FI (g) / BWf (g);

PER (%) = (WG (g) / PI (g)) × 100;

Table 1

Proximate analysis of Asian sea bass (*Lates calcarifer*) feed[a].

Nutrient	Composition (%)
Moisture	10.0
Crude protein	48.0
Crud lipid	16.0
Crude fibre	2.0
Ash	10.0
Nitrogen-free extract	14.0

[a] Digestible energy is 19.2 MJ kg^{-1}

1
2
3
4 K factor (%) = (BWf (g) / standard length (cm) 3) × 100;
5 HSI (%) = (liver weight (g) / whole body weight (g)) × 100; VSI (%) = (Visceral weight (g) /
6 whole body weight (g)) × 100;
7 Fish survival (%) = 100 × (final amount of fish) / (initial amount of fish)
8
9 Where:
10 WG = Weight gain, BWf = final body weight, BWi = initial body weight, SGR = Specific growth
11 rate, FCR = Feed conversion ratio, FI = Feed intake, PER = Protein efficiency ratio, PI = Protein
12 intake, K factor = Fulton's condition factor, HSI = Hepatosomatic index, VSI = Viscerosomatic
13 index
14
15 2.3. Microscopy
16 After finishing the husbandry trial, three fish per tank was eviscerated and intestine was transferred
17 in 10% buffered formaldehyde (pH: 7.4, 24 h at 25 °C) and then replaced with fresh buffer
18 formaldehyde. Samples were dehydrated by graded series of ethanol, cleared with xylene,
19 embedded in paraffin (Akhundov and Federove, 1994), and cut in serial sections (3–5 μm thick).
20 Hematoxylin and eosin as well as Giemsa-stained sections were then photographed and studied

using a digital microscope for evaluating their condition (Firdaus-Nawi et al., 2013). A computerized microscopic image analyzer (Digimizer 4.1.1) was used to determine histo-morphometric factors such as villus height (from top of villus to opening of crypts), width (averages width of one-third and two-third of villus height) as well as crypt depth (from villus base to muscular layer) and intestinal muscular layer thickness (from sub-mucosal layer to serous layer) (Geyra et al., 2001), and apparent villus surface (by multiplying average of width by height in 3.14) (Iji et al., 2001) of ten villi from foregut and midgut sections of a fish.

2.4. Intestinal microbiota analysis

Before sampling, fish were starved for a day then sample preparation for intestinal microbiota evaluation was carried out as described by Hoseinifar et al. (2011). Three fish per tank were randomly sampled for determining total viable bacteria (TVC) and LAB colonies. The fish were killed by an overdose of the anesthetic (2-phenoxyethanol, 1 ml l-1) and before exenterating of the intestinal tract, the skin was disinfected with 70% ethanol. Intestines were processed individually for each fish. The entire intestinal tract was dissected and adherent adipose tissues carefully separated, washed thoroughly with sterile saline (0.85% NaCl) and homogenized (IKA, Ultra-turrax®, USA). After diluting homogenate to 10-7, 1000 µl of the homogenate were cultivated into plate count agar (PCA, Merck, Germany) using pour-plate technique and 100 µl of the homogenate were cultivated onto deMan, Rogosa and Sharpe agar media (MRS, Merck, Germany) using spread-plate technique for evaluating TVC and LAB, respectively. Plates were incubated at room temperature (25 °C) for five days (Mahious et al., 2006) and number of colonies (CFU) g-1 were determined (Rawling et al., 2009).

2.5. Digestive enzyme analyses

The intestinal tract of fish was homogenized as descried by Gisbert at al. (2016) and samples were kept in -80 °C for further analysis. Total alkaline proteases were assayed as described by Walter (1984) using the azo-casein as substrate. Trypsin (EC 3.4.21.4) activity was measured with BAPNA (N-α-benzoyl-dlarginine-p-nitroanilide, 1 mM in 50 mM Tris–HCl, pH 8.2, 20 mM CaCl2) that according to Erlanger et al. (1961). Bile salt-activated lipase (EC 3.1.1) activity was assayed using p-nitrophenyl myristate in cholate buffer (0.25 mM TrisHCl + 0.25 mM 2-methoxyethanol + 5 mM sodium cholate, pH = 9.0) as described by Iijima et al. (1998). Alpha-

amylase (EC 3.2.1.1) activity was measured using 0.3% soluble starch dissolved in Na_2HPO_4 buffer (pH = 7.4) as substrate as described by Métais and Bieth (1968). Alkaline phosphatase (ALP) was measured by means of an autoanalyzer (Technicon RA-1000, Technicon Instruments, New York, NY, USA) using commercial clinical investigation kits (Pars Azmoon Kit, Tehran, Iran; www.parsazmun.com). The Bradford's method was used for evaluating soluble protein of crude enzyme extracts using bovine serum albumin as standard (Bradford, 1976).

2.6. Quantification of lipid peroxidation and antioxidant enzymes

For evaluating the activity of antioxidant stress enzymes, fish livers were quickly dissected and washed in ice-cold phosphate buffer (pH = 7.4). Individual livers were divided in two parts to determine malondialdehyde (MDA) levels and antioxidant enzymes activity. The samples were frozen in liquid N_2 immediately, and stored at -80 °C until homogenate preparation. Individual livers were homogenized in 1: 5 weight: volume (w: v) ice cold 100 mM K-phosphate (KH_2PO_4), pH 7.5, with 1.8% NaCl, and 0.1 mM phenyl-methylsulphonyl fluoride (PMSF) (Regoli et al., 2012). The samples were homogenized on ice by using glass pestle for 30–60s. Then, the homogenates were centrifuged at 12000 g for 15 min at 4 °C. Supernatants were collected without the lipid phase, immediately subdivided into small aliquots (150 μl) and stored at -80 °C until their analysis (Regoli et al., 2012). Superoxide dismutase (SOD, E C 1.15.1.1) activity was measured according to McCord and Fridovich (1969). The activity of catalase (CAT, E C 1.11.1.6) was determined using the method described by Aebi (1984). The spectrophotometric assay for glutathione S-transferase (GST, E C 2.5.1.18) activity was based on the GST-catalysed reaction between glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The GST-catalysed formation of GS-DNB produces a dinitrophenyl thioether that can be detected at 340 nm (Habig and Jacoby, 1981). Glutathione reductase (GR, E C 1.6.4.2) activity was determined using the method described by Meister (1989). Alkaline phosphatase (E 3.1.3.1) was quantified using a commercial kit (Pars Azmon Co., Tehran, Iran) according to the manufacturer protocol. The MDA level was measured according to Ringwood et al. (2003) and expressed as nano-moles of MDA per gram wet weight.

2.7. RNA extraction and intestinal c-type and g-type lysozyme genes expressions analyses

After finishing the husbandry period, five fish of each tank were randomly euthanized with overdose of the anesthetic, and immediately hindgut part of intestine was dissected on ice and was transferred in liquid nitrogen then stored at -80 °C until RNA extraction (Rasmussen, 2001). Total RNA was extracted using Qiagen RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instruction and eluted in DNase/RNase free water (Qiagen, 2012), then the extracted RNA was treated with DNase I (Thermo Scientific Fisher, USA) for avoiding pollution with genomic DNA. A Nanodrop spectrophotometer (Pico200, Picodrop Co., UK) was used for quantifying total RNA extraction at 260 and 280 nm (A260: A280 ~ 1.8–2 were selected for further experiments) followed by electrophoresis on 1% agarose gel (Sambrook et al., 1989).
First-strand cDNA was synthesized using 1 µg of DNase I-treated RNA and oligo dT primer and random hexamer primers according to the supplier's instruction (RevertAid cDNA synthesis kit, Thermo Scientific, USA). The cDNA was subsequently used in quantitative real-time PCR (qPCR) for evaluating intestinal chicken-type (c-type) and goose-type (g-type) LYZ genes as described by Hoseinifar et al. (2017e). The elongation factor 1-alpha (EF1α) gene was selected as the internal control (Fu et al., 2013).
Specific primers for amplification the partial sequences of c-type and g-type LYZ genes, as well as EF1α reference gene, were designed using Oligo 7.56 (Molecular Biology Insights, Inc), according to the nucleotide sequences available in GeneBank (Table 2). qPCR amplifications were conducted with an ABI StepOne Real-Time PCR System (Applied Biosystems Foster, CA, USA), in a total volume of 20 µl containing 1 µl of template cDNA, 1 µl of each forward and reverse genespecific primers (10 Um), 0.2 µl of 50 × ROX reference dye (Genet Bio, South Korea), 10 µl of 2 × SYBR Green qPCR Master Mix (Prime QMaster Mix, Genet Bio, South Korea) and 6.8 µl H2O. The thermal cycling conditions were as follow: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 94 °C for 15 s, primer annealing at the optimized temperatures (Table 2) for 30 s and elongation at 72 °C for 30 s. The specificity and quality of amplifications were checkout using melting-curve analysis. qPCR assays were performed in triplicates and relative expression of each lysozyme gene was quantified using 2-ΔΔCt method (Pfaffl et al., 2002).

Table 2
Primers sequences used for analysis of intestinal lysozyme gene expression and their amplification characteristics.

Primer name		Sequence	Annealing Temperature (°C)	Product Length (bp)
c-type	Forward	ATTACACCCCACAACCTGACACATAG	58	243
	Reverse	GCAGCGAGCTTCTGACTGATGAT		
g-type	Forward	AGAGTCCAGGGCTGGAAAT	58	102
	Reverse	GTGTGTCCACCTCCACCTG		
EF1α	Forward	TGCCACTGTTGCCTTTGT	56	99
	Reverse	CGCTCAATTTTCCATCCCTT		

2.8. Statistical analyses

Analyses were performed using SPSS the Statistical Software System v16.0 (SPSS, Chicago, Illinois, USA). Means and standard error (SE) were calculated for each parameter measured. All data tested for normality and homogeneity of the variance by One-Sample KolmogorovSmirnov Test and Levene Test, respectively. Based on the main subjective of the study, all results presented focusing on the effect of prebiotic and probiotic and their combination were analyzed by two-wayANOVA analyses with LMWSA And PA established as fixed factors. Duncan's test (Duncan, 1955) was used for multiple comparisons and both were carried out at significance of $P < 0.05$.

3. Results

3.1. Growth performance

The results exhibited supplementing diet with PA singularly (Diet 4) or in combination with 5 g LMWSA kg-1 diet (Diet 5) promoted weight gain and specific growth rate compared with the other treatments ($P < 0.05$, Table 3). However, feed utilization parameters including feed conversion ratio was improved in fish fed Diet 5, but protein efficiency ratio as well as somatic indices were not affected by different diets ($P > 0.05$, Table 3).

Table 3
Growth performances and feed utilization (mean ± S.E.) of the Asian sea bass (*Lates calcarifer*) fed different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA).

Growth performance	Experimental diets						Two-way ANOVA		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Prebiotic	Probiotic	Pre*Pro
BW_f (g)	27.3 ± 0.7^b	28.8 ± 0.5^b	28.1 ± 0.4^b	30.8 ± 0.6^a	30.9 ± 0.6^a	28.7 ± 0.4^b	ns	**	**
WG (%)	130.0 ± 5.1^c	143.7 ± 2.4^b	134.6 ± 2.9^{bc}	160.5 ± 7.9^a	154.6 ± 5.0^a	136.6 ± 10.8^{bc}	ns	**	***
SGR (% day^{-1})	2.1 ± 0.1^c	2.3 ± 0.0^b	2.2 ± 0.0^b	2.5 ± 0.1^a	2.4 ± 0.1^{ab}	2.2 ± 0.1^b	ns	*	ns
FCR	1.04 ± 0.01^a	1.02 ± 0.02^{bc}	1.07 ± 0.03^a	1.02 ± 0.04^{bc}	1.00 ± 0.00^c	1.07 ± 0.04^a	*	ns	ns
PER (%)	3.6 ± 0.1^a	3.6 ± 0.1^a	3.5 ± 0.1^a	3.5 ± 0.2^a	3.7 ± 0.1^a	3.5 ± 0.1^a	ns	ns	ns
K factor (%)	1.2 ± 0.0^a	1.2 ± 0.0^a	1.3 ± 0.0^a	1.3 ± 0.0^a	1.3 ± 0.0^a	1.3 ± 0.1^a	ns	ns	ns
HSI (%)	3.3 ± 0.1^a	3.6 ± 0.3^a	3.7 ± 0.3^a	3.7 ± 0.2^a	3.5 ± 0.3^a	3.0 ± 0.2^a	ns	ns	ns
VSI (%)	11.9 ± 0.3^a	11.5 ± 0.4^a	10.8 ± 0.4^a	11.3 ± 0.2^a	11.1 ± 0.2^a	10.2 ± 0.2^a	ns	ns	ns
Fish Survival (%)	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a	ns	ns	ns

Diet 1 (control), Diet 2 (5 $g\,kg^{-1}$ LMWSA), Diet 3 (10 $g\,kg^{-1}$ LMWSA), Diet 4 (0.9×10^7 CFU g^{-1} PA), Diet 5 (5 $g\,kg^{-1}$ LMWSA + 0.9×10^7 CFU g^{-1} PA), and Diet 6 (10 $g\,kg^{-1}$ LMWSA + 0.9×10^7 CFU g^{-1} PA).
Abreviations: WG = Weight gain, BW_f = final body weight, BW_i = initial body weight, SGR = Specific growth rate, FCR = Feed conversion ratio, FI = Feed intake, PER = Protein efficiency ratio, PI = Protein intake, K factor = Fulton's condition factor, HSI = Hepatosomatic index, VSI = Viscerosomatic index.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns: not significant.
Different superscript letters in each row represent significant differences among groups (Duncan's test, $P < 0.05$).

3.2. Intestinal histomorphology

Fish fed diet supplemented with singular PA or in combination with 10 g LMWSA kg-1 diet (Diet 6) and those fed basal diet (Diet 1) had highest and lowest villus height, apparent villus surface and crypt depth, respectively (Table 4, P < 0.05). The villus width value was greatest in fish fed diet incorporated with PA and 10 g LMWSA kg-1 diet (Diet 6). The muscular layer thickness values in fish fed Diets 1 and 6 were higher than fish fed Diets 3 and 5 (P < 0.05).

Table 4
Intestine morphological parameters (mean ± S.E; n = 9) of the Asian sea bass (*Lates calcarifer*) fed different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA).

Intestinal morphology	Experimental diets						Two-way ANOVA		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Prebiotic	Probiotic	Pre*Pro
Villus height (μm)	347.8 ± 15.1^c	445.5 ± 23.8^b	454.4 ± 17.2^b	464.4 ± 13.1^b	437.8 ± 29.2^b	526.7 ± 6.8^a	**	**	***
Villus width (μm)	117.8 ± 6.0b	116.7 ± 9.8b	114.4 ± 12.5b	130.0 ± 7.7b	122.2 ± 5.3b	176.6 ± 25.1a	*	**	***
Apparent villus surface ($μm^2$)	1286 ± 198^c	1632 ± 147^b	1632 ± 326^b	1896 ± 320^{ab}	1679 ± 142^b	2920 ± 235^a	**	**	***
Crypt depth (μm)	274.4 ± 15.8^c	366.7 ± 28.8^b	353.3 ± 21.6^b	384.5 ± 19.2^b	361.1 ± 43.7^b	441.7 ± 9.2^a	*	**	***
Muscular layer thickness (μm)	45.5 ± 2.3^a	36.7 ± 2.5^b	25.0 ± 3.2^c	36.7 ± 1.9^b	27.5 ± 1.8^c	48.9 ± 3.1^a	ns	ns	***

Diet 1 (control), Diet 2 (5 $g\,kg^{-1}$ LMWSA), Diet 3 (10 $g\,kg^{-1}$ LMWSA), Diet 4 (0.9×10^7 CFU g^{-1} PA), Diet 5 (5 $g\,kg^{-1}$ LMWSA + 0.9×10^7 CFU g^{-1} PA), and Diet 6 (10 $g\,kg^{-1}$ LMWSA + 0.9×10^7 CFU g^{-1} PA).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns: not significant.
Different superscript letters in each row represent significant differences among groups (Duncan's test, $P < 0.05$).

3.3. Intestinal microbiota

Total viable and lactic acid bacteria counts from the posterior
intestinal region of Asian sea bass (L. calcarifer) increased following supplementing diets with LMWSA solely or in combination with PA compared to fish fed a basal diet (Diet 1) (Fig. 1). Two-way ANOVA of TVC and LAB data indicated a significant effects of interaction between

LMWSA and PA ($P < 0.0001$). In this sense, fish fed diets administered with PA (Diet 4) or synbiotics (Diets 5 and 6) had relatively higher TVC and LAB counts than other groups ($P < 0.05$).

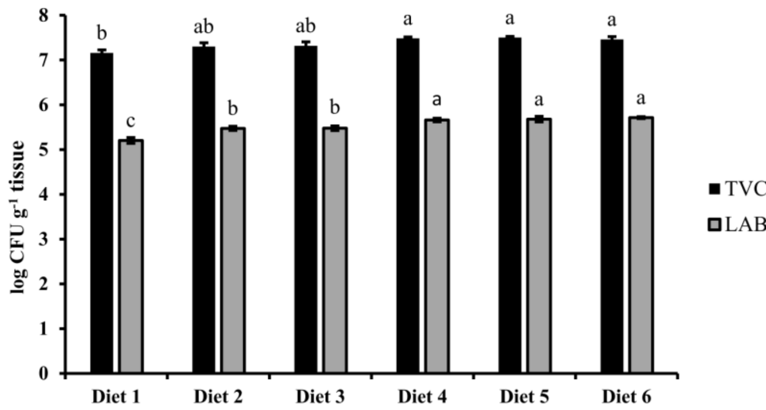

Fig. 1. Total viable (black columns) and lactic acid (gray columns) bacteria counts from the posterior intestinal region (mean ± S.E.) of the Asian sea bass (*Lates calcarifer*) fed different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA): Diet 1 (control), Diet 2 ($5\,g\,kg^{-1}$ LMWSA), Diet 3 ($10\,g\,kg^{-1}$ LMWSA), Diet 4 ($0.9 \times 10^7\,CFU\,g^{-1}$ PA), Diet 5 ($5\,g\,kg^{-1}$ LMWSA + $0.9 \times 10^7\,CFU\,g^{-1}$ PA), and Diet 6 ($10\,g\,kg^{-1}$ LMWSA + $0.9 \times 10^7\,CFU\,g^{-1}$ PA). Different superscript letters in each column represent significant differences among groups (Duncan's test, $P < 0.05$) n = 6 (pooled from 12 fish).

3.4. Digestive enzymes activities

The obtained results illustrated the enhancement of specific activity of all evaluated digestive enzymes including total protease, trypsin, lipase and α-amylase activity by singular administration of LMWSA or in combination with PA compared to the control group (Table 5).

Table 5
Specific digestive enzymes activity (U/mg protein.min^{-1}) in gut of Asian sea bass (*Lates calcarifer*) fed different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA).

Digestive enzyme activity	Experimental diets						Two-way ANOVA		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Prebiotic	Probiotic	Pre*Pro
Total protease (U mg protein^{-1})	12.5 ± 1.4^{c}	22.6 ± 2.3^{b}	24.9 ± 3.0^{b}	28.8 ± 3.1^{b}	37.5 ± 2.6^{a}	40.2 ± 2.4^{a}	***	***	***
Trypsin (U mg protein^{-1})	0.27 ± 0.01^{c}	5.5 ± 0.01^{bc}	0.29 ± 0.01^{b}	0.31 ± 0.00^{a}	0.32 ± 0.01^{a}	0.32 ± 0.00^{a}	ns	***	**
Lipase (U mg protein^{-1})	11.1 ± 0.3^{c}	12.0 ± 0.9^{c}	17.3 ± 0.8^{ab}	14.6 ± 0.7^{b}	15.3 ± 1.0^{b}	18.2 ± 0.5^{a}	***	**	**
α-Amylase (mU mg protein^{-1})	16.7 ± 3.3^{b}	36.7 ± 8.8^{ab}	40.0 ± 5.8^{ab}	40.0 ± 9.7^{ab}	63.3 ± 3.3^{a}	40 ± 9.1^{ab}	ns	*	*

Diet 1 (control), Diet 2 ($5\,g\,kg^{-1}$ LMWSA), Diet 3 ($10\,g\,kg^{-1}$ LMWSA), Diet 4 ($0.9 \times 10^7\,CFU\,g^{-1}$ PA), Diet 5 ($5\,g\,kg^{-1}$ LMWSA + $0.9 \times 10^7\,CFU\,g^{-1}$ PA), and Diet 6 ($10\,g\,kg^{-1}$ LMWSA + $0.9 \times 10^7\,CFU\,g^{-1}$ PA).
*$P < 0.05$; **$P < 0.\ 01$; ***$P < 0.001$; ns: not significant.
Different superscript letters in each row represent significant differences among groups (Duncan's test, $P < 0.05$). n = 6 (pooled from 12 fish).

Specifically, the total protease activity was higher in fish fed diets enriched with synbiotics (Diets 5 and 6) than the other experimental groups ($P < 0.05$). Fish fed PA incorporated diet (Diet 4) or combined with LMWSA (Diets 5 and 6) had higher trypsin activity than other experimental groups ($P < 0.05$). Administration of 10 g LMWSA kg-1 diet solely or combined with PA resulted in higher lipase activity in the intestine of the fish belong to these groups ($P < 0.05$). The activity of α-amylase was significantly increased in fish fed immunostimulants supplemented diets (Diet 5) compared to the control one ($P < 0.05$).

3.5. Antioxidant enzymes activity

In the present study, activities of liver antioxidant enzymes such as SOD, CAT and GST increased significantly ($P < 0.05$) following treatment with singular or mixture administration of LMWSA with PA compared to the control group (Table 6); however, other antioxidant indices including GR, Alkaline phosphatase (ALP) and MDA (Table 6) were not influenced by different dietary treatments ($P > 0.05$). Liver SOD activity enhanced remarkably in fish fed diets supplemented with PA (Diet 4) or in combination with LMWSA (Diets 5 and 6) in comparison with other experimental groups ($P < 0.05$). Fish fed diets supplemented with synbiotics had significantly higher CAT and GST activities (Table 6) than fish fed diets solely containing immunostimulant or respect to the control group.

Table 6
Specific antioxidant activities as well as malondialdehyde concentration (mean ± S.E.) in the liver of Asian sea bass (*Lates calcarifer*) fed different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA).

Antioxidant activity	Experimental diets						Two-way ANOVA		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Prebiotic	Probiotic	Pre*Pro
SOD (μmol mg protein^{-1})	9.0 ± 0.9^{b}	10.8 ± 0.2^{ab}	11.1 ± 0.4^{ab}	13.2 ± 0.4^{a}	13.2 ± 0.5^{a}	13.4 ± 0.8^{a}	ns	**	*
CAT (μmol min^{-1} mg protein^{-1})	43.3 ± 0.8^{c}	48.0 ± 1.2^{b}	48.1 ± 1.4^{b}	48.6 ± 0.9^{b}	57.8 ± 2.5^{a}	60.5 ± 1.6^{a}	***	***	***
GST (nmol min^{-1} mg protein^{-1})	25.3 ± 1.1^{c}	34.9 ± 1.9^{b}	36.5 ± 1.0^{b}	34.2 ± 1.6^{b}	57.3 ± 1.4^{a}	58.1 ± 1.7^{a}	***	***	***
GR (nmol min^{-1} mg protein^{-1})	6.2 ± 0.8^{a}	6.5 ± 0.9^{a}	5.3 ± 0.7^{a}	4.6 ± 0.4^{a}	5.8 ± 0.7^{a}	5.1 ± 0.5^{a}	ns	ns	ns
ALP (U min^{-1} mg protein^{-1})	2.8 ± 0.2^{a}	2.4 ± 0.1^{a}	2.7 ± 0.2^{a}	2.8 ± 0.1^{a}	2.5 ± 0.1^{a}	2.7 ± 0.1^{a}	ns	ns	ns
MDA (nmol g^{-1} wet weight)	287.3 ± 12.1^{a}	321.5 ± 14.7^{a}	292.6 ± 15.2^{a}	319.3 ± 17.7^{a}	305.5 ± 14.6^{a}	296.1 ± 13.8^{a}	ns	ns	ns

Diet 1 (control), Diet 2 (5 g kg^{-1} LMWSA), Diet 3 (10 g kg^{-1} LMWSA), Diet 4 (0.9×10^{7} CFU g^{-1} PA), Diet 5 (5 g kg^{-1} LMWSA + 0.9×10^{7} CFU g^{-1} PA), and Diet 6 (10 g kg^{-1} LMWSA + 0.9×10^{7} CFU g^{-1} PA).
*P < 0.05; **P < 0. 01; ***P < 0.001; ns: not significant.
Different superscript letters in each row represent significant differences among groups (Duncan's test, $P < 0.05$) n = 6 (pooled from 12 fish).

3.6. Intestinal lysozyme genes expression

The results of the intestinal lysozyme gene expression revealed both c-type and g-types LYZ genes expression significantly influenced by dietary immunostimulants (Fig. 2). The results showed that both c-type and g-type types LYZ genes expression gradually up-regulated with increasing LMWSA levels in the diets ($P < 0.01$). Moreover, fish fed diet supplemented with PA had higher c-type LYZ gene expression than the control group, but g-type LYZ was not influenced by the singular administration of PA alone. Two-way ANOVA of c-type and g-type types LYZ genes expression data indicated a significant effects of interaction between LMWSA and PA ($P < 0.0001$). Regarding synbiotics, supplementing diets with blends of 10 g LMWSA kg-1 diet and PA more pronouncedly enhanced both c-type and g-types LYZ genes expression in comparison with those fed the diet 5 ($P < 0.05$).

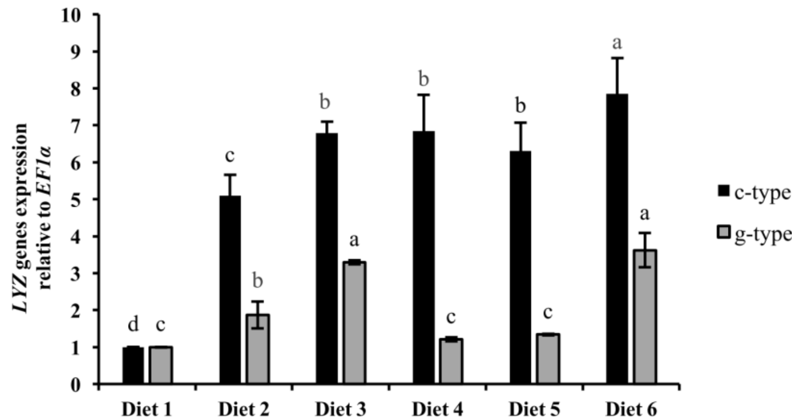

Fig. 2. Relative expression profiles of c-type (black columns) and g-type (gray columns) lysozyme genes (*LYZ*) in intestine of Asian sea bass (*Lates calcarifer*) fed with different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA): Diet 1 (control), Diet 2 (5 g kg^{-1} LMWSA), Diet 3 (10 g kg^{-1} LMWSA), Diet 4 (0.9 × 10^{7} CFU g^{-1} PA), Diet 5 (5 g kg^{-1} LMWSA + 0.9 × 10^{7} CFU g^{-1} PA), and Diet 6 (10 g kg^{-1} LMWSA + 0.9 × 10^{7} CFU g^{-1} PA). Different superscript letters in each column represent significant differences among groups (Duncan's test, $P < 0.05$) n = 6 (pooled from 12 fish).

4. Discussion

The present study showed that fish fed diets supplemented exclusively with PA (Diet 4) or in combination with 5 g LMWSA kg-1 diet (Diet 5) had better growth rate than other experimental groups probably as a consequence of stimulation of the vitamins synthesis and the increasing of enzymatic activity that may improve feed digestibility (Ringø et al., 2010a; Azimirad et al., 2016; Dawood et al., 2016; Hoseinifar et al., 2019a; Modanloo et al., 2017). In line with findings of the present study, it has been exhibited that administration of dietary PA in green terror, Aequidens rivulatus (Neissi et al., 2013), black swordtail, Xiphophorus helleri (Hoseinifar et al., 2015c), and oriental bream, Abramis brama orientalis fry (Asadi Khomami et al., 2016) or in combination with Saccharomyces cerevisiae in Pollack, Pollachius pollachius larvae (Gatesoupe, 2002) or galactooligosaccharide in rainbow trout, Oncorhynchus mykiss (Hoseinifar et al., 2015b, 2017d), common carp, Cyprinus carpio (Modanloo et al., 2017), or fructooligosaccharide in angelfish, Pterophyllum scalare (Azimirad et al., 2016) remarkably promoted growth performance. However, the obtained findings evidenced that supplementation of LMWSA alone did not promote WG of Asian sea bass (L. calcarifer) as confirming previous results obtained with grouper (Epinephelus fuscoguttatus) fed with sodium alginate and κ- carrageenan (Cheng et al., 2008).

Interestingly, the present study revealed growth increase in fish fed with 5 g kg-1 LMWSA + PA (Diet 5) compared with fish fed Diets 2 and 3, suggests a synergistic effects between PA and LMWSA. The improvement of growth might be due to fermentation of LMWSA by PA leading to a better utilization of this prebiotic. In agreement with these

results, LMWSA in synbiotic form with Lactobacillus plantarum or kefir increased growth in Nile tilapia, O. niloticus (Van Doan et al., 2016a, 2017). Abou-El-Atta et al. (2019) recommended the use of probiotic L. plantarum with whey protein concentrate in diets to improve the growth, antioxidant, and immunity responses Nile tilapia. The growth enhancement were observed in fish fed Diets 4 and 5 might be also associated with significant improvements in microvilli height and density, elevation of digestive enzyme activities as well as metabolic changes as a consequence of intestinal microbiome communities variation toward beneficial bacterial communities (Dimitroglou et al., 2009; Akhter et al., 2015; Falcinelli et al., 2015, 2016; Huynh et al., 2017). It has been proved that different factors such as fish species, experimental condition, dose and duration of immunostimulants administration may affect the results (Hoseinifar et al., 2016b; BurgosAceves et al., 2018).

In the current study, different aspects of intestinal histoarchitecture such as villus height, width, and apparent villus surface as well as crypt depth improved in fish fed with Diet 6 (PA + 10 g LMWSA kg-1 diet) indicating pronounced positive effects of synbiotic compared to the singular administration of pre- or probiotic. As mentioned above, the enhancement of the microvilli height in fish fed Diet 6 appears positively related to higher nutrient uptake by increasing absorption surface that associated with improvement in integrity of brush borders and digestibility of nutrients (Hoseinifar et al., 2017f). Synbiotics and prebiotics can interact directly with enterocytes by producing short chain fatty acids during prebiotic fermentation. They may also trig different immune and antioxidant related gene expression that may promote the integrity of the brush boarders (Hoseinifar et al., 2017f). Meanwhile, a pronounced increase in antioxidant enzymes activity and redox status in the intestine in fish fed synbiotics has been described and it was assumed that the brush boarder integrity from reactive oxygen species and free radicals could be preserved (Safari et al., 2018). In addition, the alternation of the intestinal morphology also coincident with modifications in the intestinal microbiota especially a significant increase in LAB in fish fed diets administered with PA and synbiotics. Similar to our results, administration of diets with synbiotics such as Bacillus spp. + mannan-oligosaccharide (MOS) in European lobster, Homarus gammarus L, (Daniels et al., 2010), and Bacillus licheniformis + fructooligosaccharide (FOS) in triangular bream, Megalobrama terminalis (Zhang et al., 2015) enhanced intestinal microvilli length.

It has been suggested that administration of pre-, pro-, or synbiotics can elevate the establishment of LAB in the intestine microbiome (Merrifield et al., 2014). The obtained findings exhibited increase of LAB levels following dietary administration of LMWSA suggest possible utilization of LMWSA as energy source. Similarly, it has been reported TVC and LAB levels significantly increased in Siberian sturgeon (Acipenser baerii) juvenile and Caspian white fish (Rutilus frisii kutum) fry fed diets incorporated with arabinoxylan-oligosaccharide (2%) (Geraylou et al., 2013) and xylooligosaccharide (2 and 3%) (Hoseinifar et al., 2014), respectively. The results of the present study also illustrated that the administration mixture of LMWSA and PA increased intestinal ecosystem capacity for establishing LAB colonies compared with the diet solely supplemented with LMWSA. The obtained results clearly showed that feeding on combined LMWSA and PA resulted in significant alteration of intestinal microbiota, and the increase in LAB level.

Administration of functional dietary supplements such as pro-, pre-, and synbiotics is considered as promising feed additives also to enhance digestibility of nutrients by establishing a normal intestinal microflora

that could be regarded as complementary for the appropriate synthesis and secretion of the digestive enzymes (Ringø and Gatesoupe, 1998; Bairagi et al., 2002; Hoseinifar et al., 2017c). Moreover, the presence of probiotics can stimulate the synthesis of endogenous digestive enzymes (Mohapatra et al., 2012). As reviewed by Hoseinifar et al. (2017c) the effects of these functional feed additives on digestive enzyme activities are species specific and depends on different parameters such as the composition of autochthonous intestinal microbiome, dose, type and duration of administration, experimental condition as well as fish life stage. In the present study, protease activity in fish fed immunostimulants-supplemented diets was higher than the control and synbiotics groups had highest protease activity. Likewise, Ye et al. (2011) reported that supplementing diet with FOS (2.5 g kg-1 diet) and MOS (2.5 g kg-1 diet) as well as Bacillus claussi (1 × 107 CFU g-1) in Japanese flounder (Paralichthys olivaceus) increased protease activity compared with the control group. Moreover, Zhang et al. (2015) described an improvement in protease activity in triangular bream (M. terminalis) fed diets supplemented with FOS (3 g kg-1 diet) and 1 × 107 CFU g-1 of B. licheniformis. Therefore, synergistic effects of preand probiotics on gut microflora and digestive enzymes activities may exert better effects than individual application of each of them (Hoseinifar et al., 2017d). Similar results were noticed in case of

trypsin activity. In addition, higher lipase activity was found in specimens of Diet 3 and Diet 6. In this context, previous studies based on the dietary administration of Ergosan (brown sea weeds including Laminaria digitata + Ascophyllum nodosum, 5 g kg-1 diet) in rainbow trout, O. mykiss (Heidarieh et al., 2012), short-chain FOS (1%) in common carp (C. carpio) larvae (Hoseinifar et al., 2015a), FOS (2% and 3%) in Caspian roach (Rutilus rutilus) fry (Soleimani et al., 2012) or MOS (0.6%) in juvenile striped catfish (Pangasianodon hypophthalmus) (Akter et al., 2016) increased lipase activity. In addition, in this study supplementing diet with LMWSA and PA individually or in combination together improved α-amylase activity in Asian sea bass (L. calcarifer).

Previous studies proved that most of the probiotics stimulates the synthesis of antioxidant enzyme such as SOD and glutathione for efficient removal of free radicals for maintaining a balanced oxidative status (Li et al., 2012; Bartoskova et al., 2013; Hoseinifar et al., 2019b; Van Doan et al., 2019). Different studies reported that seaweeds extracts such as alginate increased the phagocytic and the respiratory burst activities that induced up-regulation of the antioxidant enzymes genes that act as defense mechanism against immune-related damages especially reactive oxygen species (Chiu et al., 2008; Yeh et al., 2008; Harikrishnan et al., 2011). Furthermore, previous studies revealed the positive effects of prebiotics, herbal extracts, and short chain fatty acids on up-regulation of antioxidant enzymes gene expression in different fish species that may promote translation and/or post-translational processes of these antioxidant enzymes (Esteban et al., 2014; Hoseinifar et al., 2017b; Safari et al., 2017b). In the current study, individual or combined administration of diets with LMWSA and PA significantly improved SOD, CAT, and GST activities, especially in fish fed with synbiotics reflecting improvement in the general health status of fish. In line with these findings, antioxidant enzymes activities improved by diet supplemented with PA in Pacific blue shrimp, Litopenaeus stylirostris (Castex et al., 2009), with yeast in European sea bass, Dicentrarchus labrax (Tovar-Ramirez et al., 2010), with FOS + B. licheniformis in M. terminalis (Zhang et al., 2013), with FOS in turbot, Scophthalmus maximus (Guerreiro et al., 2014), Lactobacillus sakei + Navicula sp. In Pacific red snapper, Lutjanus peru (Reyes-Becerril et al., 2014), B. subtilis + MOS in Mrigal carp, Cirrhinus mrigala (Kumar et al., 2018) and with PA + galactooligosaccharide in rainbow trout, O. mykiss
(Hoseinifar et al., 2016a).

Considering lysozyme (muramidase, E C 3. 2. 1. 17), it is a natural antibiotic, which has direct lytic activity against Gram-positive bacteria cell walls and indirect bactericidal activity against Gram-negative bacteria acts through stimulating complement system and phagocytes (e.g. polymorphonuclear leucocytes and macrophages) by opsoninic effect (Saurabh and Sahoo, 2008). Lysozymes are categorized into different types including the c-type and g-type that have been identified in some fish species (Fu et al., 2013). In this context, Fu et al. (2013) demonstrated that these two LYZ are important in the defense against pathogenic bacterial such as Vibrio harveyi and Photobacterium damselae. In the present study, intestinal g-type LYZ gene expression increased with increasing LMWSA in diet, meanwhile, both intestinal c-type and g-types LYZ genes expression remarkably increased in fish fed diet supplemented with 10 g LMWSA kg-1 diet + PA (Diet 6) in comparison with other groups. The elevated intestinal c-type and g-types LYZ genes expression indicated immunomodulatory effects of LMWSA and PA that may aid immune competence in fish. In agreement with our findings, it has been demonstrated that supplementing diet with plant-derived products such as Ferula, Ferula assafoetida, (Safari et al., 2016), and loquat, Eriobotrya japonica, (Hoseinifar et al., 2018a), or sodium propionate (Safari et al., 2017a) up-regulates LYZ gene expression in common carp (C. carpio) and it was associated with increasing serum lysozyme activity. Furthermore, it has been revealed that dietary raffinose individually or in synbiotic form with PA increased skin LYZ gene expression in common carp C. carpio (Hoseinifar et al., 2019a). However, Hoseinifar et al. (2017a) reported no significant difference in the head kidney and intestinal LYZ gene expression in common carp C. carpio fingerlings fed dietary FOS, galactooligosaccharide (GOS) or inulin. Moreover, Modanloo et al. (2017) demonstrated that intestinal LYZ gene expression was not affected by single or combined administration of dietary GOS and PA in common carp C. carpio. These discrepancies among different studies in either LYZ genes transcriptional level and enzymatic activity in fish may be the consequence of the different type and dose of prebiotics used (Hoseinifar et al., 2017a, 2018a).

In conclusion, the present study suggests administration of diet with PA solely or in combination with 5 g LMWSA kg-1 diet to enhance growth performance of Asian sea bass (L. calcarifer) juveniles. In addition, administration of diets with these functional feeds especially the combination of PA with 10 g LMWSA kg-1 diet remarkably improved intestinal morphology, intestinal microbial flora, digestive enzymes activities as well as liver antioxidant enzymes as

compared to other experimental groups. Furthermore, transcriptomic study revealed upregulation of intestinal LYZ genes expression in fish fed different immunostimulants.

Declaration of competing interest

There is no conflict of interest to declare.

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