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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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Original

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 / Ashouri, G.; Mahboobi Soofiani, N.; Hoseinifar, S. H.; Jalali, S. A. H.; Morshedi, V.; Valinassab, T.; Bagheri, D.; Van Doan, H.; Torfi Mozanzadeh, M.; Carnevali, O.. - In: AQUACULTURE. - ISSN 0044-8486. - 518:(2020). [10.1016/j.aquaculture.2019.734638]

Availability:

This version is available at: 11566/283168 since: 2024-03-27T14:31:52Z

Publisher:

Published

DOI:10.1016/j.aquaculture.2019.734638

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(Article begins on next page)

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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1 **Abstract**

2 A 42-day study was conducted to determine the effect of incorporating dietary low molecular
3 weight sodium alginate (LMWSA), extracted from the brown algae, *Undaria pinnatifida* and
4 *Macrocystis pyritera*, and *Pediococcus acidilactici* MA 18/5 M (PA), Lallemand Animal Nutrition
5 S.A., Blagnac, France, on growth performance, antioxidant defense activity, intestinal lysozyme
6 gene (LYZ) expression, histo-morphology, microbiota, and digestive enzymes activity of Asian
7 sea bass (*Lates calcarifer*) juveniles. Six experimental diets were formulated including: Diet (1) a
8 basal diet (Control), Diet (2) 5 g LMWSA kg⁻¹ diet, Diet (3) 10 g LMWSA kg⁻¹ diet, Diet (4) 0.9
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
10 (6) 10 g LMWSA kg⁻¹ diet + 0.9 × 10⁷ CFU PA g⁻¹ diet were fed to Asian sea bass, *L. calcarifer*
11 (12.0 ± 0.2 g). The results showed that fish fed PA alone (Diet 4) and the combination of both
12 supplements (Diet 5) had the greatest weight gain. Fish fed Diet 6 and those fed Diet 1 (Control)
13 had the highest and lowest villus height, apparent villus surface and crypt depth, respectively. Fish
14 fed diets administered with PA (Diet 4) or synbiotics (Diets 5 and 6) showed higher total viable
15 and lactic acid bacteria counts than all the other groups. The evaluated digestive enzymes activities
16 including total protease, trypsin, lipase, and α-amylase remarkably increased by administration of
17 LMWSA or its combination with PA. Moreover, liver antioxidant enzymes activities including
18 superoxide dismutase, catalase, and Glutathione S-transferase pronouncedly enhanced following
19 the administration of LMWSA or its combination with PA. Supplementing diet with blends of 10
20 g kg⁻¹ of LMWSA and PA (Diet 6) more pronouncedly enhanced c-type and g-types LYZ
21 expression in comparison with those fed 5 g kg⁻¹ of LMWSA and PA (Diet 5). Based on the results
22 obtained, it can be claimed incorporating diet with LMWSA and PA separately or in symbiotic
23 form had promising results as functional feed additives in juvenile Asian sea bass *L. calcarifer*.

24

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

26

27 **1. Introduction**

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

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

8 It is well proved that supplementing aqua-feeds with different kinds of immunostimulants mainly
9 probiotics, prebiotics, phytobiotics, parabiotics and synbiotics not only can fortify immune
10 competence but also can promote growth performance in cultured aquatic species (Ringø et al.,
11 2014; Abdel-Tawwab, 2016; Ringø and Song, 2016; Hoseinifar et al., 2018b). For example, lactic
12 acid bacteria (LAB) determined as the most promising probiotics in aquaculture due to their
13 abilities to stimulate growth, reproductive performance, gastrointestinal function, immune
14 responses, and enhance disease resistance (Gioacchini et al., 2010, 2012; Giorgini et al., 2010;
15 Ringø et al., 2018). LAB have been considered as profitable bacteria of the fish intestinal
16 microbiome and mostly can be isolated from the various fish species intestinal tract (Dimitroglou
17 et al., 2011; Falcinelli et al., 2017). Among different species of LAB, *Pediococcus acidilactici* (PA)
18 reported to have promising effects on growth performance, digestive enzymes activities,
19 antioxidant and stress resistance (Castex et al., 2009, 2010; Hoseinifar et al., 2017d; Taridashti et
20 al., 2017), gut microbiota and morphology (Ferguson et al., 2010; Merrifield et al., 2010; Abid et
21 al., 2013; Standen et al., 2013), as well as enhance immune gene expression (Abid et al., 2013) in
22 various cultured finfish species.

23 On the other hand, herbal extracts contains bioactive ingredients with antioxidant and
24 antimicrobial activities, which can provoke growth, appetite, immune competence, and also
25 ameliorate signs of stress in cultured aquatic species (Citarasu, 2010; Holdt and Kraan, 2011; Jiao
26 et al., 2011; Abdel-Tawwab, 2016; Abdel-Tawwab et al., 2018; Adeshina et al., 2019). Among
27 medical herbs, seaweeds could be considered as good sources of bioactive and environmentally
28 friendly compounds with antibacterial (Gonzalez del Val et al., 2001), antioxidant potential (Yuan
29 and Walsh, 2006; Chandini et al., 2008), antiinflammatory (Kang et al., 2008), anti-coagulant
30 (Pushpamali et al., 2008), and anti-viral (Sinha et al., 2010) properties. Low molecular weight
31 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 (Ramos, 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 \pm 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

1 Nutrition S.A., Blagnac, France) as described by Merrifield et al. (2011). The selected dosages of
2 the PA and LMWSA were added to the basal diet as previously suggested by Castex et al. (2010)
3 and Van Doan et al. (2016b), respectively to prepare experimental feeds including: Diet (1) a basal
4 diet (Control), Diet (2) 5 g LMWSA kg⁻¹ diet, Diet (3) 10 g LMWSA kg⁻¹ diet, Diet (4)
5 0.9×10^7 CFU PA g⁻¹ diet, Diet (5) 5 g LMWSA kg⁻¹
6 diet + 0.9×10^7 CFU PA g⁻¹ diet, and Diet (6) 10 g LMWSA kg⁻¹
7 diet + 0.9×10^7 CFU PA g⁻¹ diet. Each dietary treatment was tested in
8 triplicate. Feeding was carried out two times a day at 10:30 and 16:30 h
9 up to fish visual satiation during the husbandry trial. A half an hour
10 after feeding, uneaten feed was siphoned then dried (60 °C for 24 h) and
11 weighed for evaluation of feed efficiency.

12 2.2. Fish growth and sampling

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

20 $WG (\%) = ((BWf (g) - BWi (g)) / BWi (g)) \times 100$; $SGR (\% \text{ day}^{-1}) = [(\ln BWf - \ln BWi) / t] \times 100$;

21 $FCR = FI (g) / BWf (g)$;

22 $PER (\%) = (WG (g) / PI (g)) \times 100$;

23

24

Table 1Proximate 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

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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

1 using a digital microscope for evaluating their condition (Firdaus-Nawi et al., 2013). A
2 computerized microscopic image analyzer (Digimizer 4.1.1) was used to determine histo-
3 morphometric factors such as villus height (from top of villus to opening of crypts), width
4 (averages width of one-third and two-third of villus height) as well as crypt depth (from villus base
5 to muscular layer) and intestinal muscular layer thickness (from sub-mucosal layer to serous layer)
6 (Geyra et al., 2001), and apparent villus surface (by multiplying average of width by height in
7 3.14) (Iji et al., 2001) of ten villi from foregut and midgut sections of a fish.

8

9 2.4. Intestinal microbiota analysis

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

23

24 2.5. Digestive enzyme analyses

25 The intestinal tract of fish was homogenized as described by Gisbert et al. (2016) and samples were
26 kept in -80 °C for further analysis. Total alkaline proteases were assayed as described by Walter
27 (1984) using the azo-casein as substrate. Trypsin (EC 3.4.21.4) activity was measured with
28 BAPNA (N- α -benzoyl-dlarginine-p-nitroanilide, 1 mM in 50 mM Tris-HCl, pH 8.2, 20 mM
29 CaCl₂) that according to Erlanger et al. (1961). Bile salt-activated lipase (EC 3.1.1) activity was
30 assayed using p-nitrophenyl myristate in cholate buffer (0.25 mM TrisHCl + 0.25 mM 2-
31 methoxyethanol + 5 mM sodium cholate, pH = 9.0) as described by Iijima et al. (1998). Alpha-

1 amylase (EC 3.2.1.1) activity was measured using 0.3% soluble starch dissolved in Na₂HPO₄
2 buffer (pH = 7.4) as substrate as described by Métais and Bieth (1968). Alkaline phosphatase
3 (ALP) was measured by means of an autoanalyzer (Technicon RA-1000, Technicon Instruments,
4 New York, NY, USA) using commercial clinical investigation kits (Pars Azmoon Kit, Tehran,
5 Iran; www.parsazmun.com). The Bradford's method was used for evaluating soluble protein of
6 crude enzyme extracts using bovine serum albumin as standard (Bradford, 1976).

7 8 2.6. Quantification of lipid peroxidation and antioxidant enzymes

9 For evaluating the activity of antioxidant stress enzymes, fish livers were quickly dissected and
10 washed in ice-cold phosphate buffer (pH = 7.4). Individual livers were divided in two parts to
11 determine malondialdehyde (MDA) levels and antioxidant enzymes activity. The samples were
12 frozen in liquid N₂ immediately, and stored at -80 °C until homogenate preparation. Individual
13 livers were homogenized in 1: 5 weight: volume (w: v) ice cold 100 mM K-phosphate (KH₂PO₄),
14 pH 7.5, with 1.8% NaCl, and 0.1 mM phenyl-methylsulphonyl fluoride (PMSF) (Regoli et al.,
15 2012). The samples were homogenized on ice by using glass pestle for 30–60s. Then, the
16 homogenates were centrifuged at 12000 g for 15 min at 4 °C. Supernatants were collected without
17 the lipid phase, immediately subdivided into small aliquots (150 µl) and stored at -80 °C until their
18 analysis (Regoli et al., 2012). Superoxide dismutase (SOD, E C 1.15.1.1) activity was measured
19 according to McCord and Fridovich (1969). The activity of catalase (CAT, E C 1.11.1.6) was
20 determined using the method described by Aebi (1984). The spectrophotometric assay for
21 glutathione S-transferase (GST, E C 2.5.1.18) activity was based on the GST-catalysed reaction
22 between glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The GST-
23 catalysed formation of GS-DNB produces a dinitrophenyl thioether that can be detected at 340 nm
24 (Habig and Jacoby, 1981). Glutathione reductase (GR, E C 1.6.4.2) activity was determined using
25 the method described by Meister (1989). Alkaline phosphatase (E 3.1.3.1) was quantified using a
26 commercial kit (Pars Azmon Co., Tehran, Iran) according to the manufacturer protocol. The MDA
27 level was measured according to Ringwood et al. (2003) and expressed as nano-moles of MDA
28 per gram wet weight.

29

30 2.7. RNA extraction and intestinal c-type and g-type lysozyme genes expressions analyses

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

28

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	ATTACACCCACAACCTGACACATAG	58	243
	Reverse	GCAGCGAGCTTCTGACTGATGAT		
g-type	Forward	AGAGTCCAGGGCTGGAAAT	58	102
	Reverse	GTGTGTCCACCTCCACCTG		
<i>EFla</i>	Forward	TGCCACTGTTCCTTTGT	56	99
	Reverse	CGCTCAATTTCCATCCCTT		

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3 2.8. Statistical analyses

4 Analyses were performed using SPSS the Statistical Software System v16.0 (SPSS, Chicago,
5 Illinois, USA). Means and standard error (SE) were calculated for each parameter measured. All
6 data tested for normality and homogeneity of the variance by One-Sample KolmogorovSmirnov
7 Test and Levene Test, respectively. Based on the main subjective of the study, all results presented
8 focusing on the effect of prebiotic and probiotic and their combination were analyzed by two-
9 way ANOVA analyses with LMWSA And PA established as fixed factors. Duncan's test (Duncan,
10 1955) was used for multiple comparisons and both were carried out at significance of $P < 0.05$.

11 3. Results

12 3.1. Growth performance

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

Table 3

Growth performances and feed utilization (mean \pm 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 ^o Pro
BW _f (g)	27.3 \pm 0.7 ^b	28.8 \pm 0.5 ^b	28.1 \pm 0.4 ^b	30.8 \pm 0.6 ^a	30.9 \pm 0.6 ^a	28.7 \pm 0.4 ^b	ns	**	**
WG (%)	130.0 \pm 5.1 ^c	143.7 \pm 2.4 ^b	134.6 \pm 2.9 ^{bc}	160.5 \pm 7.9 ^a	154.6 \pm 5.0 ^a	136.6 \pm 10.8 ^{bc}	ns	**	***
SGR (% day ⁻¹)	2.1 \pm 0.1 ^c	2.3 \pm 0.0 ^b	2.2 \pm 0.0 ^b	2.5 \pm 0.1 ^a	2.4 \pm 0.1 ^{ab}	2.2 \pm 0.1 ^b	ns	*	ns
FCR	1.04 \pm 0.01 ^a	1.02 \pm 0.02 ^{bc}	1.07 \pm 0.03 ^a	1.02 \pm 0.04 ^{bc}	1.00 \pm 0.00 ^c	1.07 \pm 0.04 ^a	*	ns	ns
PER (%)	3.6 \pm 0.1 ^a	3.6 \pm 0.1 ^a	3.5 \pm 0.1 ^a	3.5 \pm 0.2 ^a	3.7 \pm 0.1 ^a	3.5 \pm 0.1 ^a	ns	ns	ns
K factor (%)	1.2 \pm 0.0 ^a	1.2 \pm 0.0 ^a	1.3 \pm 0.0 ^a	1.3 \pm 0.0 ^a	1.3 \pm 0.0 ^a	1.3 \pm 0.1 ^a	ns	ns	ns
HSI (%)	3.3 \pm 0.1 ^a	3.6 \pm 0.3 ^a	3.7 \pm 0.3 ^a	3.7 \pm 0.2 ^a	3.5 \pm 0.3 ^a	3.0 \pm 0.2 ^a	ns	ns	ns
VSI (%)	11.9 \pm 0.3 ^a	11.5 \pm 0.4 ^a	10.8 \pm 0.4 ^a	11.3 \pm 0.2 ^a	11.1 \pm 0.2 ^a	10.2 \pm 0.2 ^a	ns	ns	ns
Fish Survival (%)	100 \pm 0.0 ^a	100 \pm 0.0 ^a	100 \pm 0.0 ^a	100 \pm 0.0 ^a	100 \pm 0.0 ^a	100 \pm 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 \times 10⁷ CFU g⁻¹ PA), Diet 5 (5 g kg⁻¹ LMWSA + 0.9 \times 10⁷ CFU g⁻¹ PA), and Diet 6 (10 g kg⁻¹ LMWSA + 0.9 \times 10⁷ CFU g⁻¹ PA).

Abbreviations: 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).

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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 \pm 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 ^o Pro
Villus height (μ m)	347.8 \pm 15.1 ^c	445.5 \pm 23.8 ^b	454.4 \pm 17.2 ^b	464.4 \pm 13.1 ^b	437.8 \pm 29.2 ^b	526.7 \pm 6.8 ^a	**	**	***
Villus width (μ m)	117.8 \pm 6.0 ^b	116.7 \pm 9.8 ^b	114.4 \pm 12.5 ^b	130.0 \pm 7.7 ^b	122.2 \pm 5.3 ^b	176.6 \pm 25.1 ^a	*	**	***
Apparent villus surface (μ m ²)	1286 \pm 198 ^c	1632 \pm 147 ^b	1632 \pm 326 ^b	1896 \pm 320 ^{ab}	1679 \pm 142 ^b	2920 \pm 235 ^a	**	**	***
Crypt depth (μ m)	274.4 \pm 15.8 ^c	366.7 \pm 28.8 ^b	353.3 \pm 21.6 ^b	384.5 \pm 19.2 ^b	361.1 \pm 43.7 ^b	441.7 \pm 9.2 ^a	*	**	***
Muscular layer thickness (μ m)	45.5 \pm 2.3 ^a	36.7 \pm 2.5 ^b	25.0 \pm 3.2 ^c	36.7 \pm 1.9 ^b	27.5 \pm 1.8 ^c	48.9 \pm 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 \times 10⁷ CFU g⁻¹ PA), Diet 5 (5 g kg⁻¹ LMWSA + 0.9 \times 10⁷ CFU g⁻¹ PA), and Diet 6 (10 g kg⁻¹ LMWSA + 0.9 \times 10⁷ 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).

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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

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

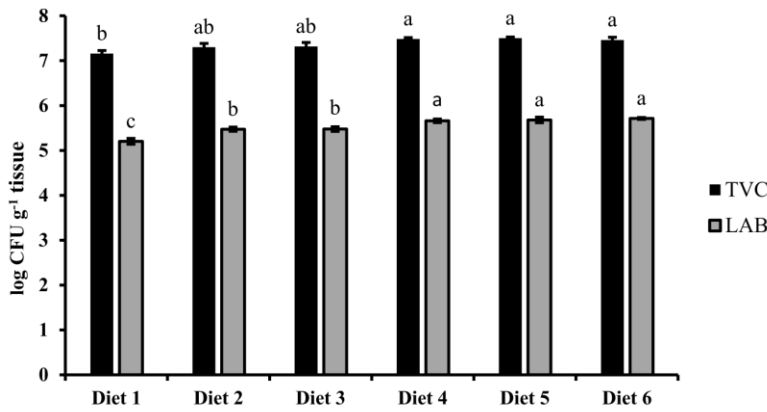


Fig. 1. Total viable (black columns) and lactic acid (gray columns) bacteria counts from the posterior intestinal region (mean \pm S.E.) of the Asian sea bass (*Lates calcarifer*) fed different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA): Diet 1 (control),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

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 5 3.4. Digestive enzymes activities

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

Table 5
 Specific digestive enzymes activity ($\text{U/mg protein} \cdot \text{min}^{-1}$) 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 ($\text{mU mg protein}^{-1}$)	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 ($0.9 \times 10^7 \text{ CFU g}^{-1}$ PA), Diet 5 (5 g kg^{-1} LMWSA + $0.9 \times 10^7 \text{ CFU g}^{-1}$ PA), and Diet 6 (10 g kg^{-1} LMWSA + $0.9 \times 10^7 \text{ CFU g}^{-1}$ 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).

9
 10 Specifically, the total protease activity was higher in fish fed diets enriched with synbiotics (Diets
 11 5 and 6) than the other experimental groups ($P < 0.05$). Fish fed PA incorporated diet (Diet 4) or
 12 combined with LMWSA (Diets 5 and 6) had higher trypsin activity than other experimental groups
 13 ($P < 0.05$). Administration of $10 \text{ g LMWSA kg}^{-1}$ diet solely or combined with PA resulted in
 14 higher lipase activity in the intestine of the fish belong to these groups ($P < 0.05$). The activity of
 15 α -amylase was significantly increased in fish fed immunostimulants supplemented diets (Diet 5)
 16 compared to the control one ($P < 0.05$).

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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 \pm 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 ^a Pro ^o
SOD ($\mu\text{mol mg protein}^{-1}$)	9.0 \pm 0.9 ^b	10.8 \pm 0.2 ^{ab}	11.1 \pm 0.4 ^{ab}	13.2 \pm 0.4 ^a	13.2 \pm 0.5 ^a	13.4 \pm 0.8 ^a	ns	**	*
CAT ($\mu\text{mol min}^{-1}$ mg protein ⁻¹)	43.3 \pm 0.8 ^c	48.0 \pm 1.2 ^b	48.1 \pm 1.4 ^b	48.6 \pm 0.9 ^b	57.8 \pm 2.5 ^a	60.5 \pm 1.6 ^a	***	***	***
GST (nmol min ⁻¹ mg protein ⁻¹)	25.3 \pm 1.1 ^c	34.9 \pm 1.9 ^b	36.5 \pm 1.0 ^b	34.2 \pm 1.6 ^b	57.3 \pm 1.4 ^a	58.1 \pm 1.7 ^a	***	***	***
GR (nmol min ⁻¹ mg protein ⁻¹)	6.2 \pm 0.8 ^a	6.5 \pm 0.9 ^a	5.3 \pm 0.7 ^a	4.6 \pm 0.4 ^a	5.8 \pm 0.7 ^a	5.1 \pm 0.5 ^a	ns	ns	ns
ALP (U min ⁻¹ mg protein ⁻¹)	2.8 \pm 0.2 ^a	2.4 \pm 0.1 ^a	2.7 \pm 0.2 ^a	2.8 \pm 0.1 ^a	2.5 \pm 0.1 ^a	2.7 \pm 0.1 ^a	ns	ns	ns
MDA (nmol g ⁻¹ wet weight)	287.3 \pm 12.1 ^a	321.5 \pm 14.7 ^a	292.6 \pm 15.2 ^a	319.3 \pm 17.7 ^a	305.5 \pm 14.6 ^a	296.1 \pm 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 \times 10⁷ CFU g⁻¹ PA), Diet 5 (5 g kg⁻¹ LMWSA + 0.9 \times 10⁷ CFU g⁻¹ PA), and Diet 6 (10 g kg⁻¹ LMWSA + 0.9 \times 10⁷ 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).

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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 ($P < 0.05$).

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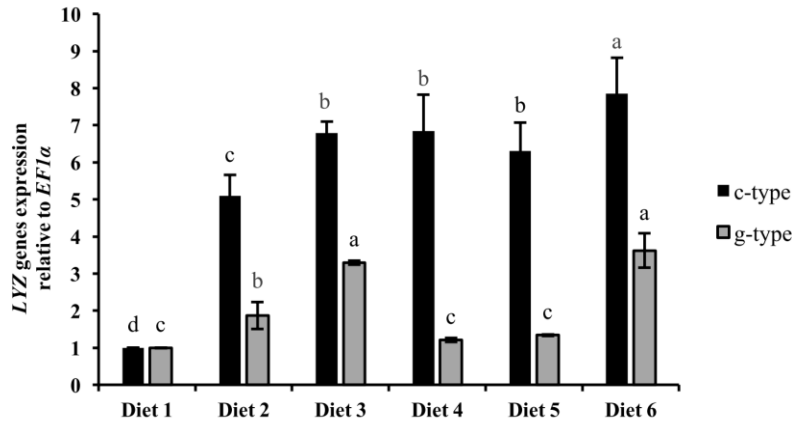


Fig. 2. Relative expression profiles of c-type (black columns) and g-type (gray columns) lysozyme genes (*LYZ*) in intestine of Asian sea bass (*Lates calcarifer*) fed with different levels of dietary low molecular sodium alginate (LMWSA) and *Pediococcus acidilactici* (PA): Diet 1 (control), Diet 2 (5 g kg⁻¹ LMWSA), Diet 3 (10 g kg⁻¹ LMWSA), Diet 4 (0.9 × 10⁷ CFU g⁻¹ PA), Diet 5 (5 g kg⁻¹ LMWSA + 0.9 × 10⁷ CFU g⁻¹ PA), and Diet 6 (10 g kg⁻¹ LMWSA + 0.9 × 10⁷ 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).

1

2 4. Discussion

3 The present study showed that fish fed diets supplemented exclusively with PA (Diet 4) or in
 4 combination with 5 g LMWSA kg⁻¹ diet (Diet 5) had better growth rate than other experimental
 5 groups probably as a consequence of stimulation of the vitamins synthesis and the increasing of
 6 enzymatic activity that may improve feed digestibility (Ringø et al., 2010a; Azimirad et al., 2016;
 7 Dawood et al., 2016; Hoseinifar et al., 2019a; Modanloo et al., 2017). In line with findings of the
 8 present study, it has been exhibited that administration of dietary PA in green terror, *Aequidens*
 9 *rivulatus* (Neissi et al., 2013), black swordtail, *Xiphophorus helleri* (Hoseinifar et al., 2015c), and
 10 oriental bream, *Abramis brama orientalis* fry (Asadi Khomami et al., 2016) or in combination with
 11 *Saccharomyces cerevisiae* in Pollack, *Pollachius pollachius* larvae (Gatesoupe, 2002) or
 12 galactooligosaccharide in rainbow trout, *Oncorhynchus mykiss* (Hoseinifar et al., 2015b, 2017d),
 13 common carp, *Cyprinus carpio* (Modanloo et al., 2017), or fructooligosaccharide in angelfish,
 14 *Pterophyllum scalare* (Azimirad et al., 2016) remarkably promoted growth performance. However,
 15 the obtained findings evidenced that supplementation of LMWSA alone did not promote WG of
 16 Asian sea bass (*L. calcarifer*) as confirming previous results obtained with grouper (*Epinephelus*
 17 *fuscoguttatus*) fed with sodium alginate and κ- carrageenan (Cheng et al., 2008).
 18 Interestingly, the present study revealed growth increase in fish fed with 5 g kg⁻¹ LMWSA + PA
 19 (Diet 5) compared with fish fed Diets 2 and 3, suggests a synergistic effects between PA and
 20 LMWSA. The improvement of growth might be due to fermentation of LMWSA by PA leading
 21 to a better utilization of this prebiotic. In agreement with these

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

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

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

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

16 that could be regarded as complementary for the appropriate synthesis and secretion of the
17 digestive enzymes (Ringø and Gatesoupe, 1998; Bairagi et al., 2002; Hoseinifar et al., 2017c).
18 Moreover, the presence of probiotics can stimulate the synthesis of endogenous digestive enzymes
19 (Mohapatra et al., 2012). As reviewed by Hoseinifar et al. (2017c) the effects of these functional
20 feed additives on digestive enzyme activities are species specific and depends on different
21 parameters such as the composition of autochthonous intestinal microbiome, dose, type and
22 duration of administration, experimental condition as well as fish life stage. In the present study,
23 protease activity in fish fed immunostimulants-supplemented diets was higher than the control and
24 synbiotics groups had highest protease activity. Likewise, Ye et al. (2011) reported that
25 supplementing diet with FOS (2.5 g kg⁻¹ diet) and MOS (2.5 g kg⁻¹ diet) as well as *Bacillus clausii*
26 (1×10^7 CFU g⁻¹) in Japanese flounder (*Paralichthys olivaceus*) increased protease activity
27 compared with the control group. Moreover, Zhang et al. (2015) described an improvement in
28 protease activity in triangular bream (*M. terminalis*) fed diets supplemented with FOS (3 g kg⁻¹
29 diet) and 1×10^7 CFU g⁻¹ of *B. licheniformis*. Therefore, synergistic effects of preand probiotics
30 on gut microflora and digestive enzymes activities may exert better effects than individual
31 application of each of them (Hoseinifar et al., 2017d). Similar results were noticed in case of

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

10 Previous studies proved that most of the probiotics stimulates the synthesis of antioxidant enzyme
11 such as SOD and glutathione for efficient removal of free radicals for maintaining a balanced
12 oxidative status (Li et al., 2012; Bartoskova et al., 2013; Hoseinifar et al., 2019b; Van Doan et al.,
13 2019). Different studies reported that seaweeds extracts such as alginate increased the phagocytic
14 and the respiratory burst activities that induced up-regulation of the antioxidant enzymes genes
15 that act as defense mechanism against immune-related damages especially reactive oxygen species
16 (Chiu et al., 2008; Yeh et al., 2008; Harikrishnan et al., 2011). Furthermore, previous studies
17 revealed the positive effects of prebiotics, herbal extracts, and short chain fatty acids on up-
18 regulation of antioxidant enzymes gene expression in different fish species that may promote
19 translation and/or post-translational processes of these antioxidant enzymes (Esteban et al., 2014;
20 Hoseinifar et al., 2017b; Safari et al., 2017b). In the current study, individual or combined
21 administration of diets with LMWSA and PA significantly improved SOD, CAT, and GST
22 activities, especially in fish fed with synbiotics reflecting improvement in the general health status
23 of fish. In line with these findings, antioxidant enzymes activities improved by diet supplemented
24 with PA in Pacific blue shrimp, *Litopenaeus stylirostris* (Castex et al., 2009), with yeast in
25 European sea bass, *Dicentrarchus labrax* (Tovar-Ramirez et al., 2010), with FOS + *B.*
26 *licheniformis* in *M. terminalis* (Zhang et al., 2013), with FOS in turbot, *Scophthalmus maximus*
27 (Guerreiro et al., 2014), *Lactobacillus sakei* + *Navicula* sp. In Pacific red snapper, *Lutjanus peru*
28 (Reyes-Becerril et al., 2014), *B. subtilis* + MOS in Mrigal carp, *Cirrhinus mrigala* (Kumar et al.,
29 2018) and with PA + galactooligosaccharide in rainbow trout, *O. mykiss*
30 (Hoseinifar et al., 2016a).

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

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

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

3 Declaration of competing interest

4 There is no conflict of interest to declare.

5 Acknowledgement

6 This project was funded by the Isfahan University of Technology. Special thanks to Prof. Sabihe
7 Soleimani-Zad, Dr. Takavar Mohamadian and Dr. Rezvan Mohammadi-Nezhad for their help
8 with various stage of this project. We would like to acknowledge the efforts of the personnel of
9 Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Iran.

10

11 **References**

12 Abdel-Tawwab, M., 2016. Feed Supplementation to Freshwater Fish: Experimental Approaches.
13 LAP LAMBERT Academic, Berlin, Germany.

14 Abdel-Tawwab, M., Adeshina, I., Jenyo-Oni, A., Ajani, E.K., Emikp, B.O., 2018. Growth,
15 physiological, antioxidants, and immune response of African catfish, *Clarias gariepinus* (B.), to
16 dietary clove basil, *Ocimum gratissimum*, leaf extract and its susceptibility to *Listeria*
17 *monocytogenes* infection. *Fish Shellfish Immunol.* 78, 346–354.

18 Abid, A., Davies, S., Waines, J.P., Emery, M., Castex, M., Gioacchini, G., Carnevali, O.,
19 Bickerdike, R., Romero, J., Merrifield, D.L., 2013. Dietary synbiotic application modulates
20 Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity. *Fish*
21 *Shellfish Immunol.* 35, 1948–1956.

22 Abou-El-Atta, M.E., Abdel-Tawwab, M., Abdel-Razek, N., Abdelhakim, T.M.N., 2019. Effects
23 of dietary probiotic *Lactobacillus plantarum* and whey protein concentrate on the productive
24 parameters, immunity response, and susceptibility of Nile tilapia, *Oreochromis niloticus* (L.), to
25 *Aeromonas sobria* infection. *Aquacult. Nutr.* <https://doi.org/10.1111/anu.12957>.

26 Adeshina, I., Jenyo-Oni, A., Emikpe, B.O., Ajani, E.K., Abdel-Tawwab, M., 2019. Stimulatory
27 effect of dietary clove, *Eugenia caryophyllata*, bud extract on growth performance, nutrient
28 utilization, antioxidant capacity, and tolerance of African catfish, *Clarias gariepinus* (B.), to
29 *Aeromonas hydrophila* infection. *J. World Aquac. Soc.* 50, 390–405.

30 Aebi, H., 1984. Catalase in vitro. *Methods Enzymol.* 105, 121–126.

1 Akhter, N., WU, B., Memon, A.M., Mohsin, M., 2015. Probiotics and prebiotics associated with
2 aquaculture: a review. *Fish Shellfish Immunol.* 45, 733–741.

3 Akhundov, M.M., Fedorov, K.E., 1994. Effects of exogenous estradiol on the formation of ovaries
4 in juvenile sterlet *Acipenser ruthenus*. *J. Ichthyol.* 35, 109–120.

5 Akter, M.N., Sutriana, A., Talpur, A.D., Hashim, R., 2016. Dietary supplementation with mannan
6 oligosaccharide influences growth, digestive enzymes, gut morphology, and microbiota in juvenile
7 striped catfish, *Pangasianodon hypophthalmus*. *Aquacult. Int.* 24, 127–144.

8 Ali, S.S.R., Ambasankar, K., Praveena, P.E., Nandakumar, S., Syamadaya, J., 2017. Effect of
9 dietary fructooligosaccharide supplementation on growth, body composition, hematological and
10 immunological parameters of Asian seabass (*Lates calcarifer*). *Aquacult. Int.* 25, 837–848.

11 Asadi Khomami, S., Mooraki, N., Valipour, A., Kakoolaki, S., 2016. The effects of dietary
12 probiotic *Pediococcus acidilactici* on the growth performance and survival rate of oriental bream
13 fry (*Abramis brama orientalis*). *Iran. J. Aquat. Anim. Health* 2, 55–66.

14 Azimirad, M., Meshkini, S., Ahmadifard, N., Hoseinifar, S.H., 2016. The effects of feeding with
15 synbiotic (*Pediococcus acidilactici* and fructooligosaccharide) enriched adult *Artemia* on skin
16 mucus immune responses, stress resistance, intestinal microbiota and performance of angelfish
17 (*Pterophyllum scalare*). *Fish Shellfish Immunol.* 54, 516–522.

18 Bairagi, A., Ghosh, K.S., Sen, S.K., Ray, A.K., 2002. Enzyme producing bacterial flora isolated
19 from fish digestive tracts. *Aquacult. Int.* 10, 109–121.

20 Bartoskova, M., Dobsikova, R., Stancova, V., Zivna, D., Blahova, J., Marsalek, P., Zelníckova,
21 L., Bartos, M., Di Tocco, F.C., Faggio, C., 2013. Evaluation of ibuprofen toxicity for zebrafish
22 (*Danio rerio*) targeting on selected biomarkers of oxidative stress. *Neuroendocrinol. Lett.* 34, 102–
23 108.

24 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities
25 of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

26 Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2018. MicroRNAs and their role on fish
27 oxidative stress during xenobiotic environmental exposures. *Ecotoxicol. Environ. Saf.* 148, 995–
28 1000.

29 Castex, M., Lemaire, P., Wabete, N., Chim, L., 2009. Effect of dietary probiotic *Pediococcus*
30 *acidilactici* on antioxidant defences and oxidative stress status of shrimp *Litopenaeus stylirostris*.
31 *Aquaculture* 294, 306–313.

1 Castex, M., Lemaire, P., Wabete, N., Chim, L., 2010. Effect of probiotic *Pediococcus acidilactici*
2 on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio*
3 *nigripulchritudo* challenge. *Fish Shellfish Immunol.* 28, 622–631.

4 Chandini, S.K., Ganesan, P., Bhaskar, N., 2008. In vitro antioxidant activities of three selected
5 brown seaweeds of India. *Food Chem.* 107, 707–713.

6 Cheng, A.C., Chen, Y.Y., Chen, J.C., 2008. Dietary administration of sodium alginate and κ -
7 carrageenan enhances the innate immune response of brown-marbled grouper *Epinephelus*
8 *fuscoguttatus* and its resistance against *Vibrio alginolyticus*. *Vet. Immunol. Immunopathol.* 121,
9 206–215.

10 Chiu, S.T., Tsai, R.T., Hsu, J.P., Liu, C.H., Cheng, W., 2008. Dietary sodium alginate
11 administration to enhance the non-specific immune responses, and disease resistance of the
12 juvenile grouper *Epinephelus fuscoguttatus*. *Aquaculture* 277, 66–72.

13 Citarasu, T., 2010. Herbal biomedicines: a new opportunity for aquaculture industry. *Aquacult.*
14 *Int.* 18, 403–414.

15 Daniels, C.L., Merrifield, D.L., Boothroyd, D.P., Davies, S.J., Factor, J.R., Arnold, K.E., 2010.
16 Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus*
17 *gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture* 304,
18 49–57.

19 Dawood, M.A.O., Koshio, S., Ishikawa, M., Yokoyama, S., 2016. Effects of dietary inactivated
20 *Pediococcus pentosaceus* on growth performance, feed utilization and blood characteristics of red
21 sea bream, *Pagrus major* juvenile. *Aquacult. Nutr.* 22, 923–932.

22 Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C.L., Güroy,
23 D., Davies, S.J., 2011. Microbial manipulations to improve fish health and production: a
24 Mediterranean perspective. *Fish Shellfish Immunol.* 30, 1–16.

25 Dimitroglou, A., Merrifield, D.L., Mote, R., Davies, S.J., Spring, P., Sweetman, J., Bradley, G.,
26 2009. Dietary mannan oligosaccharide supplementation modulates intestinal microbiology
27 ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J.*
28 *Anim. Sci.* 87, 3226–3234.

29 Duncan, D.B., 1955. Multiple range and multiple F tests. *Biometrics* 11 (1), 1–42. [https://](https://doi.org/10.2307/3001478)
30 doi.org/10.2307/3001478.

1 Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new
2 chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95, 271–278.

3 Esteban, M.Á., Cordero, H., Martinez-Tome, M., Jimenez-Monreal, A., Bakhrouf, A., Mahdhi, A.,
4 2014. Effect of dietary supplementation of probiotics and palm fruits extracts on the antioxidant
5 enzyme gene expression in the mucosae of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish*
6 *Immunol.* 39, 532–540.

7 Falcinelli, S., Picchiatti, S., Rodiles, A., Cossignani, L., Merrifield, D.L., Taddei, A., Maradonna,
8 F., Olivotto, I., Gioacchini, G., Carnevali, O., 2015. *Lactobacillus rhamnosus* lowers lipid content
9 by changing gut microbiota and host transcription of genes involved in lipid metabolism in
10 zebrafish. *Sci. Rep.* 5, 1–11.

11 Falcinelli, S., Rodiles, A., Hatef, A., Picchiatti, S., Cossignani, L., Merrifield, D.L., Unniappan,
12 S., Carnevali, O., 2017. Dietary lipid content reorganizes gut microbiota and probiotic *L.*
13 *rhamnosus* attenuates obesity and enhances catabolic hormonal milieu in zebrafish. *Sci. Rep.* 7, 1–
14 15.

15 Falcinelli, S., Rodiles, A., Unniappan, S., Picchiatti, S., Gioacchini, G., Merrifield, D.L.,
16 Carnevali, O., 2016. Probiotic treatment reduces appetite and glucose level in the zebrafish model.
17 *Sci. Rep.* 6, 1–13.

18 FAO, 2018. *The State of World Fisheries and Aquaculture - Meeting the Sustainable.*
19 *Development Goals.*, Rome, pp. 227. <http://www.fao.org/3/i9540en/i9540en.pdf>.

20 Ferguson, R., Merrifield, D.L., Harper, G.M., Rawling, M.D., Mustafa, S., Picchiatti, S., Balcázar,
21 J.L., Davies, S.J., 2010. The effect of *Pediococcus acidilactici* on the gut microbiota and immune
22 status of on-growing red tilapia (*Oreochromis niloticus*). *J. Appl. Microbiol.* 109, 851–862.

23 Firdaus-Nawi, M., Yusoff, S.M., Yusof, H., Abdullah, S.Z., ZamriSaad, M., 2013. Efficacy of
24 feed-based adjuvant vaccine against *Streptococcus agalactiae* in *Oreochromis* spp. in Malaysia.
25 *Aquacult. Res.* 45, 87–96.

26 Fu, G.H., Bai, Z.Y., Xia, J.H., Liu, F., Liu, P., Yue, G.H., 2013. Analysis of two lysozyme genes
27 and antimicrobial functions of their recombinant proteins in Asian sea bass. *PLoS One* 8, 1–12.

28 Gatesoupe, F.J., 2002. Probiotic and formaldehyde treatments of *Artemia nauplii* as food larval
29 Pollack, *Pollachius pollachius*. *Aquaculture* 212, 347–360.

30 Geraylou, Z., Souffreau, C., Rurangwa, E., Maes, G.E., Spanier, K.I., Courtin, C.M., Delcour, J.A.,
31 Buyse, J., Ollevier, F., 2013. Prebiotic effects of arabinoxylan oligosaccharides (AXOS) on

1 juvenile Siberian sturgeon (*Acipenser baerii*) with emphasis on the modulation of the gut
2 microbiota using 454 pyrosequencing. *Fed. Eur. Microbiol. Soc.* 86, 357–371.

3 Geyra, A., Uni, Z., Sklan, D., 2001. Enterocyte dynamics and mucosal development in the
4 posthatch chick. *Poult. Sci.* 80, 776–782.

5 Gioacchini, G., Giorgini, E., Merrifield, D.L., Hardiman, G., Borini, A., Vaccari, L., Carnevali,
6 O., 2012. Probiotics can induce follicle maturational competence: the *Danio rerio* case. *Biol.*
7 *Reprod.* 86, 1–11.

8 Gioacchini, G., Maradonna, F., Lombardo, F., Bizzaro, D., Olivotto, I., Carnevali, O., 2010.
9 Increase of fecundity by probiotic administration in zebrafish (*Danio rerio*). *Reproduction* 140,
10 953–959.

11 Giorgini, E., Conti, C., Ferraris, P., Sabbatini, S., Tosi, G., Rubini, C., Vaccari, L., Gioacchini, G.,
12 Carnevali, O., 2010. Effects of *Lactobacillus rhamnosus* on zebrafish oocyte maturation: an FTIR
13 imaging and biochemical analysis. *Anal. Bioanal. Chem.* 398, 3063–3072.

14 Gisbert, E., Mozanzadeh, M.T., Kotzamanis, Y., Estévez, A., 2016. Weaning wild flathead grey
15 mullet (*Mugil cephalus*) fry with diets with different levels of fish meal substitution. *Aquaculture*
16 462, 92–100.

17 Gonzalez del Val, A., Platas, G., Basilio, A., Cabello, A., Gorrochategui, J., Suay, I., Pelaez, F.,
18 2001. Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria
19 (Canary Islands, Spain). *Int. Microbiol.* 4, 35–40.

20 Guerreiro, I., Pérez-Jiménez, A., Costas, B., Oliva-Teles, A., 2014. Effect of temperature and short
21 chain fructooligosaccharides supplementation on the hepatic oxidative status and immune
22 response of turbot (*Scophthalmus maximus*). *Fish Shellfish Immunol.* 40, 570–576.

23 Habig, W.H., Jacoby, W.B., 1981. Assays for differentiation of glutathione S-transferases.
24 *Methods Enzymol.* 77, 398–405.

25 Harikrishnan, R., Kim, M.C., Kim, J.S., Han, Y.J., Jang, I.S., Balasundaram, C., Heo, M.S., 2011.
26 Immunomodulatory effect of sodium alginate enriched diet in kelp grouper *Epinephelus bruneus*
27 against *Streptococcus iniae*. *Fish Shellfish Immunol.* 30, 543–549.

28 Heidarieh, M., Mirvaghefi, A.R., Akbari, M., Farahmand, H., Sheikhzadeh, N., Shahbazfar, A.A.,
29 Behgar, M., 2012. Effect of dietary Ergosan on growth performance, digestive enzymes, intestinal
30 histology, hematological parameters and body composition of rainbow trout (*Oncorhynchus*
31 *mykiss*). *Fish Physiol. Biochem.* 38, 1169–1174.

1 Holdt, S.L., Kraan, S., 2011. Bioactive compounds in seaweed: functional food applications and
2 legislation. *J. Appl. Psychol.* 23, 543–597.

3 Hoseinifar, S.H., Mirvaghefi, A., Mojazi Amiri, B., Rostami, H.K., Merrifield, D.L., 2011. The
4 effects of oligofructose on growth performance, survival and autochthonous intestinal microbiota
5 of beluga (*Huso huso*) juveniles. *Aquacult. Nutr.* 17, 498–504.

6 Hoseinifar, S.H., Sharifian, M., Vesaghi, M.J., Khalli, M., Esteban, M.Á., 2014. The effect s of
7 dietary xylooligosaccharide on mucosal parameters, intestinal microbiota and morphology and
8 growth performance of Caspian white fish (*Rutilus frisii kutum*) fry. *Fish Shellfish Immunol.* 39,
9 231–236.

10 Hoseinifar, S.H., Eshaghzadeh, H., Vahabzadeh, H., Peykaran Mana, N., 2015a. Modulation of
11 growth performances, survival, digestive enzyme activities and intestinal microbiota in common
12 carp (*Cyprinus carpio*) larvae using short chain fructooligosaccharide. *Aquacult. Res.* 47, 3246–
13 3253.

14 Hoseinifar, S.H., Mirvaghefi, A., Amoozegar, M.A., Sharifian, M., Esteban, M.Á., 2015b.
15 Modulation of innate immune response, mucosal parameters and disease resistance in rainbow
16 trout (*Oncorhynchus mykiss*) upon synbiotic feeding. *Fish Shellfish Immunol.* 45, 27–32.

17 Hoseinifar, S.H., Roosta, Z., Hajimoradloo, A., Vakili, F., 2015c. The effects of *Lactobacillus*
18 *acidophilus* feed supplement on skin mucosal immune parameters, intestinal microbiota, and
19 stress resistance and growth performance of black swordtail (*Xiphophorus helleri*). *Fish Shellfish*
20 *Immunol.* 42, 533–538.

21 Hoseinifar, S.H., Hoseini, S.M., Bagheri, D., 2016a. Effects of galactooligosaccharide and
22 *Pediococcus acidilactici* on antioxidant defence and disease resistance of rainbow trout,
23 *Oncorhynchus mykiss*. *Ann. Anim. Sci.* 17, 217–227.

24 Hoseinifar, S.H., Zoheiri, F., Dadar, M., Rufchaei, R., Ringø, E., 2016b. Dietary
25 galactooligosaccharide elicits positive effects on non-specific immune parameters and growth
26 performance in Caspian white fish (*Rutilus frisii kutum*) fry. *Fish Shellfish Immunol.* 56, 467–
27 472.

28 Hoseinifar, S.H., Ahmadi, A., Khalili, M., Raeisi, M., Van Doan, H., Caipang, C.M., 2017a. The
29 study of antioxidant enzymes and immune-related genes expression in common carp (*Cyprinus*
30 *carpio*) fingerlings fed different prebiotics. *Aquacult. Res.* 48, 5447–5454.

1 Hoseinifar, S.H., Dadar, M., Khalili, M., Cerezuela, R., Esteban, M.Á., 2017b. Effect of dietary
2 supplementation of palm fruit extracts on the transcriptomes of growth, antioxidant enzyme and
3 immunerelated genes in common carp (*Cyprinus carpio*) fingerlings. *Aquacult. Res.* 48, 3684–
4 3692.

5 Hoseinifar, S.H., Dadar, M., Ringø, E., 2017c. Modulation of nutrient digestibility and digestive
6 enzyme activities in aquatic animals: the functional feed additives scenario. *Aquacult. Res.* 48,
7 3987–4000.

8 Hoseinifar, S.H., Mirvaghefi, A., Amoozegar, M.A., Merrifield, D., Ringø, E., 2017d. In vitro
9 selection of a synbiotic and in vivo evaluation on intestinal microbiota, performance and
10 physiological response of rainbow trout (*Oncorhynchus mykiss*) fingerlings. *Aquacult. Nutr.* 23,
11 111–118.

12 Hoseinifar, S.H., Safari, R., Dadar, M., 2017e. Dietary sodium propionate affects mucosal immune
13 parameters, growth and appetite related genes expression: Insights from zebrafish model. *Gen.*
14 *Comp. Endocrinol.* 243, 78–83.

15 Hoseinifar, S.H., Sun, Y.-Z., Caipang, C.M., 2017f. Short chain fatty acids as feed supplements
16 for sustainable aquaculture: an updated view. *Aquacult. Res.* 48, 1380–1391.

17 Hoseinifar, S.H., Khodadadian Zou, H., Van Doan, H., Kolangi Miandare, H., Hoseini, S.M.,
18 2018a. Evaluation of some intestinal cytokines genes expression and serum innate immune
19 parameters in common carp (*Cyprinus carpio*) fed dietary loquat (*Eriobotrya japonica*) leaf extract.
20 *Aquacult. Res.* 49, 120–127.

21 Hoseinifar, S.H., Sun, Y., Wang, A., Zhou, Z., 2018b. Probiotics as means of diseases control in
22 aquaculture, A Review of current knowledge and future perspectives. *Front. Microbiol.* 9, 2429.

23 Hoseinifar, S.H., Hosseini, M., Paknejad, H., Safari, R., Jafari, A., Yousefi, M., Van Doan, H.,
24 Mozanzadeh, M.T., 2019a. Enhanced mucosal immune responses, immune related genes and
25 growth performance in common carp (*Cyprinus carpio*) juveniles fed dietary *Pediococcus*
26 *acidilactici* MA18/5M and raffinose. *Dev. Comp. Immunol.* 94, 59–65.

27 Hoseinifar, S.H., Khodadadian Zou, H., Paknejad, H., Hajimoradloo, A., Van Doan, H.,
28 2019b. Effects of dietary white-button mushroom powder on mucosal immunity, antioxidant
29 defence, and growth of common carp (*Cyprinus carpio*). *Aquaculture* 501, 448–454.

1 Huynh, T.G., Shiu, Y.L., Nguyen, T.P., Truong, Q.P., Chen, J.C., Liu, C.H., 2017. Current
2 applications, selection, and possible mechanisms of actions of synbiotics in improving the growth
3 and health status in aquaculture: a review. *Fish Shellfish Immunol.* 64, 367–382.

4 Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile salt activated lipase
5 from the hepatopancreas of red seabream, *Pagrus major*. *Fish Physiol. Biochem.* 18, 59–69.

6 Iji, P.A., Saki, A., Tivey, D.R., 2001. Body and intestinal growth of broiler chicks on a commercial
7 starter diet. 1. Intestinal weight and mucosal development. *Br. Poult. Sci.* 42, 505–513.

8 Jiao, G., Yu, G., Zhang, J., Ewart, H.S., 2011. Chemical structures and bioactivities of sulfated
9 polysaccharides from marine algae. *Mar. Drugs* 9, 196–223.

10 Kang, J.Y., Khan, M.N., Park, N.H., Cho, J.Y., Lee, M.C., Fujii, H., Hong, Y.K., 2008.
11 Antipyretic, analgesic, and anti-inflammatory activities of the seaweed *Sargassum fulvellum* and
12 *Sargassum thunbergii* in mice. *J. Ethnopharmacol.* 116, 187–190.

13 Khang, P.V., Phuong, T.H., Dat, N.K., Knibb, W., Nguyen, N.H., 2018. An 8-year breeding
14 program for Asian sea bass *Lates calcarifer*: genetic evaluation, experiences, and challenges. *Front.*
15 *Genet.* 9, 1–12.

16 Kumar, P., Jain, K.K., Sardar, P., 2018. Effects of dietary synbiotic on innate immunity,
17 antioxidant activity and disease resistance of *Cirrhinus mrigala* juveniles. *Fish Shellfish Immunol.*
18 80, 124–132.

19 Li, W.F., Huang, Q., Li, Y.L., Rajput, I.R., Huang, Y., Hu, C.H., 2012. Induction of synbiotic
20 strain *Enterococcus faecium* EF1 on the production of cytokines, superoxide anion and
21 prostaglandin E2 in a macrophage cell line. *Pak. Vet. J.* 32, 530–534.

22 MacArtain, P., Gill, C.I., Brooks, M., Campbell, R., Rowland, I.R., 2007. Nutritional value of
23 edible seaweeds. *Nutr. Rev.* 65, 535–543.

24 Mahious, A.S., Gatesoupe, F.J., Hervi, M., Metailler, R., Ollevier, F., 2006. Effect of dietary inulin
25 and oligosaccharides as prebiotics for weaning turbot (*Psetta maxima*). *Aquacult. Int.* 14, 219–
26 229.

27 Mathew, G., 2009. Taxonomy, identification and biology of Sea bass (*Lates calcarifer*). In: Imelda,
28 J., Edwin, J.V., Susmitha, V. (Eds.), *Course Manual: National Training on Cage Culture of Sea*
29 *Bass*. CMFRI & NFDB, Kochi, pp. 38–43.

30 McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymatic function for
31 erythrocyte (hemocypren). *J. Biol. Chem.* 244, 6049–6055.

1 Meister, A., 1989. On the biochemistry of glutathione. In: Taniruchi, N., Higashi, T., Sakamoto,
2 S., Meister, A. (Eds.), *Glutathione Centennial: Molecular Perspective and Clinical Implications*.
3 Academic Press, San Diego, CA, pp. 3–22.

4 Merrifield, D.L., Balcazar, J., Daniels, C., Zhou, Z., Carnevali, O., Sun, Y., Hoseinifar, S.H.,
5 Ringø, E., 2014. Indigenous lactic acid bacteria in fish and crustaceans. In: Ringø, E., Merrifield,
6 D.L. (Eds.), *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. Wiley-Blackwell
7 Scientific Publication, London, United Kingdom, pp. 128–168.

8 Merrifield, D.L., Bradley, G., Harper, G., Baker, R., Munn, C., Davies, S., 2011. Assessment of
9 the effects of vegetative and lyophilized *Pediococcus acidilactici* on growth, feed utilization,
10 intestinal colonization and health parameters of rainbow trout (*Oncorhynchus mykiss*, Walbaum).
11 *Aquacult. Nutr.* 17, 73–79.

12 Merrifield, D.L., Harper, G.M., Dimitroglou, A., Ringø, E., Davies, S.J., 2010. Possible influence
13 of probiotic adhesion to intestinal mucosa on the activity and morphology of rainbow trout
14 (*Oncorhynchus mykiss*) enterocytes. *Aquacult. Res.* 41, 1268–1272.

15 Métais, P., Bieth, J., 1968. Détermination de l' α -amylase. *Ann. Biol. Clin.* 26, 133–142.

16 Modanloo, M., Soltanian, S., Akhlaghi, M., Hoseinifar, S.H., 2017. The effects of single or
17 combined administration of galactooligosaccharide and *Pediococcus acidilactici* on cutaneous
18 mucus immune parameters, humoral immune responses and immune related genes expression in
19 common carp (*Cyprinus carpio*) fingerlings. *Fish Shellfish Immunol.* 70, 391–397.

20 Mohapatra, S., Chakraborty, T., Prusty, A.K., Das, P., Paniprasad, K., Mohanta, K.N., 2012. Use
21 different microbial probiotic in the diet of rohu, *Labeo rohita* fingerlings: effect on growth, nutrient
22 digestibility and retention, digestive enzyme activities and intestinal microflora. *Aquacult. Nutr.*
23 18, 1–11.

24 Neissi, A., Rafiee, G., Nematollahi, M., Safari, O., 2013. The effect of *Pediococcus acidilactici*
25 bacteria used as probiotic supplement on the growth and non-specific immune responses of green
26 terror, *Aequidens rivulatus*. *Fish Shellfish Immunol.* 35, 1976–1980.

27 Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for
28 group-wise comparison and statistical analysis of relative expression results in real-time PCR.
29 *Nucleic Acids Res.* 30, 1–10.

1 Pushpamali, W.A., Nikapitiya, C., Zoysa, M.D., Whang, I., Kim, S.J., Lee, J., 2008. Isolation and
2 purification of an anticoagulant from fermented red seaweed *Lomentaria catenata*. *Carbohydr.*
3 *Polym.* 73, 274–279.

4 Qiagen, Co, 2012. RNeasy® mini handbook. Available online at: [http://mace.ihes.fr/
5 data/protocol/1/RNeasy%20Plus%20Mini.pdf](http://mace.ihes.fr/data/protocol/1/RNeasy%20Plus%20Mini.pdf) 48.

6 Rasmussen, R., 2001. Quantification on the light cycler. In: Meuer, S., Witter, C., Nakagawa, K.
7 (Eds.), *Rapid Cycler Real-Time PCR, Methods and Applications*. Springer Press, Heidelberg, pp.
8 21–34.

9 Rawling, M.D., Merrifield, D.L., Davies, S.J., 2009. Preliminary assessment of dietary
10 supplementation of Sangrovit on red tilapia (*Oreochromis niloticus*) growth performance and
11 health. *Aquaculture* 294, 118–122.

12 Regoli, F., Bocchetti, R., Filho, D.W., 2012. Spectrophotometric assays of antioxidants. In: Abele,
13 D., Vázquez-Medina, J.P., Zenteno-Savín, T. (Eds.), *Oxidative Stress in Aquatic Ecosystems*.
14 Wiley-Blackwell, A John Wiley & Sons, Ltd., Publication, Chichester, UK, pp. 367–380.

15 Reyes-Becerril, M., Angulo, C., Estrada, N., Murillo, Y., Ascencio-Valle, F., 2014. Dietary
16 administration of microalgae alone or supplemented with *Lactobacillus sakei* affects immune
17 response and intestinal morphology of Pacific red snapper (*Lutjanus peru*). *Fish Shellfish*
18 *Immunol.* 40, 208–216.

19 G. Ashouri, et al. *Aquaculture* 518 (2020) 734638
20 9

21 Ringø, E., Dimitroglou, A., Hoseinifar, S.H., Davies, S.J., 2014. Prebiotics in finfish: an update.
22 In: Merrifield, D., Ringø, E. (Eds.), *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*.
23 Wiley-Blackwell Publishing, Oxford, UK, pp. 360–400.

24 Ringø, E., Gatesoupe, F.J., 1998. Lactic acid bacteria in fish: a review. *Aquaculture* 160, 177–203.

25 Ringø, E., Hoseinifar, S.H., Ghosh, K., Van Doan, H., Beck, B.R., Song, S.K., 2018. Lactic acid
26 bacteria in finfish—an update. *Front. Microbiol.* 9, 1–37.

27 Ringø, E., Løvmo, L., Kristiansen, M., Bakken, Y., Salinas, I., Myklebust, R., Olsen, R.E.,
28 Mayhew, T.M., 2010a. Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a
29 review. *Aquacult. Res.* 41, 451–467.

30 Ringø, E., Olsen, R., Gifstad, T., Dalmo, R., Amlund, H., Hemre, G.I., Bakke, A.M., 2010b.
31 Prebiotics in aquaculture: a review. *Aquacult. Nutr.* 16, 117–136.

- 1 Ringø, E., Song, S.K., 2016. Application of dietary supplements (synbiotics and probiotics in
2 combination with plant products and β -glucans) in aquaculture. *Aquacult. Nutr.* 22, 4–24.
- 3 Ringwood, A.H., Hoguet, J., Keppler, C.J., Gielazyn, M.L., Ward, B.P., Rourk, A.R., 2003.
4 Cellular Biomarkers (Lysosomal Destabilisation, Gluthathione and Lipid Peroxidation) in Three
5 Common Estuarine Species: A Methods Handbook. Marine Resources Research Institute, South
6 Carolina Department of Natural Resources, Charleston, USA, pp. 49.
- 7 Romero, J., Feijoó, C.G., Navarrete, P., 2012. Antibiotics in aquaculture-use, abuse and
8 alternatives. In: Carvalho, E.D., David, G.S., Silva, R.J. (Eds.), *Health and Environment in*
9 *Aquaculture*. Tech. Open Science, Rijeka, Croatia, pp. 159–198.
- 10 Safari, R., Hoseinifar, S.H., Nejadmoghadam, S., Jafari, A., 2016. Transcriptomic study of mucosal
11 immune, antioxidant and growth related genes and non-specific immune response of common carp
12 (*Cyprinus carpio*) fed dietary *Ferula* (*Ferula assafoetida*). *Fish Shellfish Immunol.* 55, 242–248.
- 13 Safari, R., Hoseinifar, S.H., Nejadmoghadam, S., Khalili, M., 2017a. Non-specific immune
14 parameters, immune, antioxidant and growth-related genes expression of common carp (*Cyprinus*
15 *carpio* L.) fed sodium propionate. *Aquacult. Res.* 48, 4470–4478.
- 16 Safari, R., Hoseinifar, S.H., Van Doan, H., Dadar, M., 2017b. The effects of dietary Myrtle
17 (*Myrtus communis*) on skin mucus immune parameters and mRNA levels of growth, antioxidant
18 and immune related genes in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* 66, 264–269.
- 19 Saffari, S., Keyvanshokoh, S., Zakeri, M., Johari, S.A., Pasha-Zanoosi, H., 2018. Effects of
20 dietary organic, inorganic, and nanoparticulate selenium sources on growth, hematoimmological,
21 and serum biochemical parameters of common carp (*Cyprinus carpio*). *Fish Physiol. Biochem.* 44,
22 1087–1097.
- 23 Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Gel electrophoresis of DNA. In: Sambrook, J.,
24 Fritsch, E.F., Maniatis, T. (Eds.), *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor
25 Laboratory Press, Cold Spring Harbor, New York, pp. 1564.
- 26 Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defense molecule of fish innate immune
27 system. *Aquacult. Res.* 39, 223–239.
- 28 Singh, R.K., 2000. Growth, survival and production of *Lates calcarifer* in a seasonal rainfed coastal
29 pond of the Konkan region. *Aquaculture* 8, 55–60.

1 Sinha, S., Astani, A., Ghosh, T., Schnitzler, P., Ray, B., 2010. Polysaccharides from *Sargassum*
2 *tenerrimum*: structural features, chemical modification and anti-viral activity. *Phytochemistry* 71,
3 235–242.

4 Soleimani, N., Hoseinifar, S.H., Merrifield, D.L., Barati, M., Abadi, Z.H., 2012. Dietary
5 supplementation of fructooligosaccharide (FOS) improves the innate immune response, stress
6 resistance, digestive enzyme activities and growth performance of Caspian roach (*Rutilus rutilus*)
7 fry. *Fish Shellfish Immunol.* 32, 316–321.

8 Standen, B.T., Rawling, M.D., Davies, S.J., Castex, M., Foey, A., Gioacchini, G., Carnevali, O.,
9 Merrifield, D.L., 2013. Probiotic *Pediococcus acidilactici* modulates both localised intestinal- and
10 peripheral- immunity in tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol.* 35, 1097–1104.

11 Taridashti, F., Delafkar, K., Zare, A., Takami, G.A., 2017. Effects of probiotic *Pediococcus*
12 *acidilactici* on growth performance, survival rate, and stress resistance of Persian sturgeon
13 (*Acipenser persicus*). *J. Appl. Aquac.* 29, 220–232.

14 Tovar-Ramirez, D., Mazurais, D., Gatesoupe, J.F., Quazuguel, P., Cahu, C.L., ZamboninoInfante,
15 J.L., 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene
16 expression of sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* 300, 142–147.

17 Van Doan, H., Doolgindachbaporn, S., Suksri, A., 2014. Effects of low molecular weight agar and
18 *Lactobacillus plantarum* on growth performance, immunity, and disease resistance of basa fish
19 (*Pangasius bocourti*, Sauvage 1880). *Fish Shellfish Immunol.* 41, 340–345.

20 Van Doan, H.V., Hoseinifar, S.H., Esteban, M.Á., Dadar, M., Thu, T.T.N., 2019. In: Atta ur, R.
21 (Ed.), Chapter 2 - Mushrooms, Seaweed, and Their Derivatives as Functional Feed Additives for
22 Aquaculture: an Updated View. Elsevier, pp. 41–90.

23 Van Doan, H., Hoseinifar, S.H., Tapingkae, W., Khamtavee, P., 2017. The effects of dietary kefir
24 and low molecular weight sodium alginate on serum immune parameters, resistance against
25 *Streptococcus agalactiae* and growth performance in Nile tilapia (*Oreochromis niloticus*). *Fish*
26 *Shellfish Immunol.* 62, 139–146.

27 Van Doan, H., Hoseinifar, S.H., Tapingkae, W., Tongsir, S., Khamtavee, P., 2016a. Combined
28 administration of low molecular weight sodium alginate boosted immunomodulatory, disease
29 resistance and growth enhancing effects of *Lactobacillus plantarum* in Nile tilapia (*Oreochromis*
30 *niloticus*). *Fish Shellfish Immunol.* 58, 678–685.

- 1 Van Doan, H., Tapingkae, W., Moonmanee, T., Seepai, A., 2016b. Effects of low molecular weight
2 sodium alginate on growth performance, immunity, and disease resistance of tilapia, *Oreochromis*
3 *niloticus*. *Fish Shellfish Immunol.* 55, 186–194.
- 4 Walter, H.E., 1984. Proteinases: methods with hemoglobin, casein and azocoll as substrates. In:
5 Bergmeyer, H.J. (Ed.), *Methods of Enzymatic Analysis Vol. V.* Verlag Chemie, Weinham, pp.
6 270–277.
- 7 Ye, J.D., Wang, K., Li, F.D., Sun, Y.Z., 2011. Single or combined effects of fructo- and mannan
8 oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body
9 composition, digestive enzyme activity, innate immune response and lipid metabolism of the
10 Japanese flounder *Paralichthys olivaceus*. *Aquacult. Nutr.* 17, 902–911.
- 11 Yeh, S.P., Chang, C.A., Chang, C.Y., Liu, C.H., Cheng, W., 2008. Dietary sodium alginate
12 administration affects fingerling growth and resistance to *Streptococcus* sp. and iridovirus, and
13 juvenile non-specific immune responses of the orange-spotted grouper, *Epinephelus coioides*. *Fish*
14 *Shellfish Immunol.* 25, 19–27.
- 15 Yuan, Y.V., Walsh, N.A., 2006. Antioxidant and antiproliferative activities of extracts from a
16 variety of edible seaweeds. *Food Chem. Toxicol.* 44, 1144–1150.
- 17 Zhang, C.N., Li, W.N., Jiang, G.Z., Lu, K.L., Wang, L.N., Liu, W.B., 2013. Combined effects of
18 dietary fructooligosaccharide and *Bacillus licheniformis* on innate immunity, antioxidant
19 capability and disease resistance of triangular bream (*Megalobrama terminalis*). *Fish Shellfish*
20 *Immunol.* 35, 1380–1386.
- 21 Zhang, C.N., Li, X.F., Xu, W.N., Zhang, D.D., Lu, K.L., Wang, L.N., Tian, H.Y., Liu, W.B., 2015.
22 Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on growth
23 performance, body composition, intestinal enzymes activities and gut histology of triangular bream
24 (*Megalobrama terminalis*). *Aquacult. Nutr.* 21, 755–766.