Aquaculture 479 (2017) 333–341

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Modulation of growth performance, immunity, and disease resistance in narrow-clawed crayfish, *Astacus leptodactylus leptodactylus* (Eschscholtz, 1823) upon synbiotic feeding

Omid Safari[a,*], Marina Paolucci[b]

[a] Department of Fisheries, Faculty of Natural Resources and Environment, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Department of Sciences and Technologies, University of Sannio, Benevento, Italy

ARTICLE INFO

Keywords:
Crayfish
Enterococcus faecalis
Pediococcus acidilactici
Growth performance
Immunity
Digestive enzymes
Challenge

ABSTRACT

In the present study, a 126-day experiment was carried out under controlled conditions to compare the effects of prebiotic (galactooligosaccharide), probiotics (*Enterococcus faecalis* and *Pediococcus acidilactici*), and synbiotics (galactooligosaccharide + *Enterococcus faecalis* and galactooligosaccharide + *Pediococcus acidilactici*) on the growth performance, indices of nutritional efficiency, *in vivo* apparent digestibility coefficients of nutrients, digestive enzymes, hemolymph indices, and biological responses of juvenile crayfish *Astacus leptodactylus leptodactylus* (6.18 ± 0.31 g) against 48-h *Aeromonas hydrophila* exposure challenge. The maximum specific growth rate (2.32% day^{-1}), voluntary feed intake (2.68% body weight day^{-1}), and survival rate (93.67%) as well as the minimum feed conversion ratio (2.07) were observed in the juvenile crayfish fed with the galactooligosaccharide + *Enterococcus faecalis* diet. The highest means of *in vivo* apparent digestibility coefficients of organic matter (85.33%), crude protein (91.67%), crude fat (82.33%), and gross energy (83.67%) were measured in the crayfish fed with the galactooligosaccharide + *Enterococcus faecalis* diet ($p < 0.05$). Moreover, the highest ratios of presumptive autochthonous lactic acid bacteria to total viable aerobic heterotrophic bacteria were observed in the crayfish fed with the synbiotic diets ($p < 0.05$). It was found that feeding crayfish with the galactooligosaccharide + *Enterococcus faecalis* diet indicated the highest activities of phenoloxidase, superoxide dismutase, lysozyme, and nitric oxide synthase ($p < 0.05$). The mean survival rate of *Aeromonas hydrophila* injected crayfish fed with the galactooligosaccharide + *Enterococcus faecalis* diet (77.67%) was higher than that of the crayfish fed with the control (8.33%) and other diets (58.33–72.33%) ($p < 0.05$). Finally, considering all the tested levels, 7.53 log CFU *E. faecalis* g^{-1} + 10 g kg^{-1} galactooligosaccharide used in the diet was observed to be the optimum diet.

1. Introduction

The freshwater crayfish, *Astacus leptodactylus leptodactylus*, is one of the economically significant farmed crayfish species in the world (Holdich, 1993; Karimpour et al., 2011); however, like other crayfish species, *A. leptodactylus* has suffered increased outbreaks of disease in intensive culture. To combat its pertinent diseases and increase its sustainable production, feed additives such as organic salts, nucleotides, probiotics, paraprobiotics, prebiotics, symbiotics, and phytoproducts can be used (Castex et al., 2014; Dash et al., 2015; Ng and Koh, 2016; Safari et al., 2014a). Some additives act as immunostimulants; while others balance the gastrointestinal microbiota of aquatic species and are critical in their growth and welfare (Hoseinifar et al., 2015a; Llewellyn et al., 2014).

Synbiotics, the combined form of probiotics and prebiotics, are critically considered as potential feed additives as a result of growing interests in and worries about achieving a sustainable aquaculture industry (Cerezuela et al., 2011). Synergistic effects of synbiotics on growth performance, indices of nutritional efficiency, carcass composition, activities of digestive enzymes, hemato-immunological parameters, and stress resistance of cultivable aquatic species have been reported in previous studies (Cerezuela et al., 2011; Ringø et al., 2014; Rodriguez-Estrada et al., 2009).

Pediococcus acidilactici (Hoseinifar et al., 2011; Neissi et al., 2013) and *Enterococcus faecalis* (Allameh et al., 2015; Rodriguez-Estrada et al., 2013, 2009; Safari, 2016), as two types of gram-positive cocci (Vos et al., 2009), have the potential to adhere to and colonize the gastrointestinal tract of aquatic species. The beneficial effects of using *P.*

* Corresponding author at: Department of Fisheries, Faculty of Natural Resources and Environment, Ferdowsi University of Mashhad, P.B. 91773-1363, Mashhad, Iran.
E-mail address: omidsafari@um.ac.ir (O. Safari).

http://dx.doi.org/10.1016/j.aquaculture.2017.05.049
Received 23 September 2016; Received in revised form 29 May 2017; Accepted 31 May 2017
Available online 09 June 2017

acidilactici (Neissi et al., 2013; Villamil et al., 2010) and *E. faecalis* (Allameh et al., 2015; Rodriguez-Estrada et al., 2013; Safari, 2016) in the diet on the growth performance, health status, and hemato-immunological parameters of aquatic species have been described in the literature.

Galacto-oligosaccharide (GOS), a β-galactosidase-catalysed lactose, is a hydrolyzed product of dairy products (Gosling et al., 2010). GOS, a prebiotic in the aquafeed industry, has positive effects on the biological indices of Atlantic salmon (*Salmo salar*) (Grisdale-Helland et al., 2008), common carp (*Cyprinus carpio*) (Hoseinifar et al., 2016), Caspian roach (*Rutilus rutilus*) (Hoseinifar et al., 2013), red drum (*Sciaenops ocellatus*) (Zhou et al., 2010), and freshwater crayfish (*Astacus leptodactylus leptodactylus*) (Safari, 2016). It is essential to consider the chemical properties of prebiotics such as the degree of polymerization (DP), biological origin (fungi or plant), structure (molecular weight), and *in vitro* fermentation conditions to select suitable prebiotics in the aquafeed industry (Ringø et al., 2010; Safari et al., 2014a). In order to maximize the population of beneficial gut microbiota after the cessation of treatment, synbiotics, which are the best combination of a probiotic and special prebiotic substrate, have recently received due attention in the aquafeed industry (Hoseinifar et al., 2015b; Llewellyn et al., 2014). In this regard, the positive *in vitro* effects of galactooligosaccharide (GOS) on some probionts (*P. acidilactici* + *E. faecalis*) and the *in vivo* effects of these synbiotics on immunity and growth indices of juvenile rainbow trout, *Oncorhynchus mykiss* (Hoseinifar et al., 2015a, 2015b), and crayfish *A. leptodactylus leptodactylus* (Safari, 2016) have been discussed in the literature. To the best of researchers' knowledge, little information is available presenting the proper evaluation of the effects of dietary synbiotics in crayfish (Safari, 2016). Hence an evaluation of a range of effective synbiotics would be of significant value in the establishment of a sustainable astaciculture industry.

The highest SGR and the lowest FCR levels were reported in the diet of juvenile *Astacus leptodactylus* containing mannan-oligosaccharide (MOS; 3.0 g kg^{-1}) (Mazlum et al., 2011). The positive effects of using a combination of 2.25 g kg^{-1} MOS and 1.5 g kg^{-1} fructo-oligosaccharide (FOS) in the diet of adult *A. leptodactylus leptodactylus* on the growth indices, nutritional efficiency, and immune responses against air and bacterial exposure challenges were observed in a study conducted by (Safari et al., 2014a). Utilization of FOS (2.0 to 10.0 g kg^{-1}) in the diet of red swamp crayfish, *Procambarus clarkii*, increased the activities of antioxidant enzymes (Dong and Wang, 2013). Therefore, the aim of the present study was to evaluate the *in vivo* effects of two selected synbiotics (galactooligosaccharide + *Enterococcus faecalis* and galactooligosaccharide + *Pediococcus acidilactici*) on the growth performance, nutrient digestibility, immune responses, and stress resistance of juvenile narrow-clawed crayfish, *Astacus leptodactylus leptodactylus*.

2. Materials and method

2.1. Experimental diets

A basal diet (384.1 g kg^{-1}, crude protein; 128.5 g kg^{-1}, crude fat; 14.93 MJ kg^{-1}, gross energy) as the control diet (Safari et al., 2014a) was developed by WUFFDA (windows-based user-friendly feed formulation, done again; University of Georgia, Georgia, USA) software (Table 1). To prepare the experimental diets, galactooligosaccharide (GOS; Friesland Foods Domo Co., the Netherlands; DP: 5) as a prebiotic as well as *E. faecalis* (Nichi Nichi Pharmaceutical Co., LTD, Japan; 7.53 log CFU g^{-1}) and *P. acidilactici* (Bactocell®, Lallemand Inc., Montreal, QC, Canada;7.53 log CFU g^{-1}) as probiotics were employed. Both of the probiotic strains utilized in the present study were in lyophilized forms. To obtain De Man, Rogosa and Sharpe (MRS) (Merck, UK) broth, the culturing conditions of *E. faecalis* (24 h at 30 °C) and *P. acidilactici* (48 h at 37 °C) were applied. Fresh colonies of the probionts were obtained after re-culturing them on MRS agar (Merck, UK). The number of bacteria was estimated by serial dilutions plated on MRS agar plates in triplicate and were then counted in groups receiving *E. faecalis* and *P. acidilactici* diets after 24 h of incubation at 30 °C and 37 °C, respectively. The control and experimental diets were prepared as follows: (1) control, (2) GOS (10 g kg^{-1}), (3) *E. faecalis* (EnF; 7.53 log CFU g^{-1}), (4) *P. acidilactici* (PeA; 7.53 log CFU g^{-1}), (5) GOS (10 g kg^{-1}) + EnF (7.53 log CFU g^{-1}), and (6) GOS (10 g kg^{-1}) + PeA (7.53 log CFU g^{-1}).

Table 1
Composition (g kg^{-1} dry matter) of the control diet fed with the juvenile crayfish (6.18 ± 0.31 g).

	g kg^{-1} (dry-weight basis)
Ingredient	
Menhaden fish meal[a]	147
Soybean meal[a]	175
Corn gluten[a]	112
Wheat flour[a]	289
Corn starch[b]	49
Fish oil[a]	42
Canola oil[a]	41
Soy lecithin[a]	50
Cholesterol[d]	5
Glucosamine[c]	10
Choline chloride (70%)[d]	15
Vitamin C (stay)[d]	10
Vitamin premix[d,e]	20
Mineral premix[d,e]	15
Carboxymethyl cellulose[c]	19.9
Ytterbium oxide[c]	0.1
Chemical composition	
Dry matter	874.2
Crude protein	384.1
Crude fat	128.5
Crude fiber	28.9
Nitrogen free extract	420.6
Ash	37.9
Gross energy (MJ kg^{-1})	14.93
Crude fat/crude protein	0.33

[a] Behparvar Aquafeed Co, Iran.
[b] Scharloo Chemical Co, Spain.
[c] Sigma, Germany.
[d] Kimia Roshd Co. Iran.
[e] Mineral premix contains (mg kg^{-1}) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; antioxidant (BHT), 100. Vitamin premix contains (mg kg^{-1}) E, 30; K, 3; thiamine, 2; riboflavin, 7; pyridoxine, 3; pantothenic acid, 18; niacin, 40; folacin, 1.5; choline, 600; biotin, 0.7 and cyanocobalamin, 0.02.

2.2. Crayfish sampling

Six hundred healthy juvenile crayfish (6.18 ± 0.31 g) were obtained from Shahid Yaghoobi reservoir (35° 9′ 36″ N 59° 24′ 18″ E, Khorasan Razavi, Iran) and stocked at a density of 25 crayfish per 1000-L tank (2 × 1 × 0.5 m) in a semi-recirculating system with a daily water exchange rate of 35% with four replicates for each experimental diet. Each tank was fitted with 25 plastic tubes (diameter of 4 cm and length of 12 cm), which served as hiding places for the animals. Unconsumed food was collected and weighed 3 h after feeding. Water temperature was maintained at 25.5 °C throughout the feeding trial. DO (6.4 ± 0.19 mg l^{-1}), pH (7.08 ± 0.44), hardness (141 ± 5.7 mg l^{-1} as $CaCO_3$), un-ionized ammonia (< 0.06 mg l^{-1}), and nitrite contents (< 0.6 mg l^{-1}) were evaluated every week. The animals were held on L:D 14:10 cycle. Briefly, each diet was randomly assigned to a tank of crayfish, and the crayfish were fed 4% of their body weight three times a day (8:00, 14:00, and 20:00) for 126 days. Biometry was conducted on the first and last days of the experiment.

2.3. Evaluation of the growth performance and carcass quality

At the end of the feeding trial, each crayfish was individually weighed (± 0.01) on an electronic scale (AND, Japan). All parameters were corrected based on the feed ingested. Growth parameters, survival rate, and nutrient efficiency indices were calculated as follows (Glencross et al., 2007; Safari et al., 2014a):

$$\text{Specific Growth Rate (SGR;\%day}^{-1}) = [(\ln W_f - \ln W_i)/t] \times 100$$

$$\text{Survival Rate (\%)} = (\text{Final Individual Numbers} / \text{Initial Individual Numbers}) \times 100$$

$$\text{Voluntary Feed Intake (VFI;\%body weight day}^{-1}) = [(\text{Feed}_{\text{consumed (DM)}})/(W_{mean} \times t)]$$

$$\text{Feed Conversion Ratio (FCR)} = (\text{Feed}_{\text{consumed}}/W_{gain})$$

$$\text{Protein Efficiency Ratio (PER)} = (W_{gain}/\text{Crude protein}_{\text{consumed}})$$

$$\text{Protein Productive Value (PPV;\%)} = 100 \times [(\text{Protein}_{\text{retained}})/(\text{Protein}_{\text{consumed}})]$$

In the above-mentioned equations, W_i, W_f, W_{mean}, W_{gain}, t, and $\text{Feed}_{\text{consumed}}$ represent initial weight, final weight, mean weight, weight gain (g), time period (day), and consumed feed (g), respectively.

2.4. Calculation of in vivo nutrient apparent digestibility

In vivo ADCs of organic matter (ADC_{OM}), crude protein (ADC_{CP}), crude fat (ADC_{CF}), and gross energy (ADC_{GE}) of the experimental diets were calculated according to the following equation (Safari et al., 2014b):

$$ADC_{test} = 100 \times (1 - (\text{Marker}_{test} \times \text{Nutrient}_{feces}/\text{Marker}_{feces} \times \text{Nutrient}_{test}))$$

In the presented equation, the terms Marker_{test} and Marker_{feces} represent the marker (0.1 g kg^{-1} ytterbium oxide, Yb_2O_3, in the diet) contents of the diet and feces, respectively. Furthermore, Nutrient_{test} and Nutrient_{feces} represent the nutritional parameters of concern (for example, protein or energy) in the diet and feces, respectively.

2.5. Biochemical analyses

2.5.1. Hemolymph indices

On the 126th day, 6 crayfish from each tank (24 crayfish per treatment) were killed 24 h after the last feeding. All assays were done without pooling samples with four replicates. According to the previously described protocol (Safari et al., 2014a), hemolymph was obtained from ventral sinus using a needle (25G), pooled, and stored with application of the following two methods: (1) 1 ml microtube without any anticoagulant agent (see Section 2.5.2) and (2) 1 ml microtube containing 0.4 ml Alsever as an anticoagulant. Briefly, 125 μl of plasma obtained from the later protocol was used to measure the following hemolymph indices: THC measured with hemocytometer cell (Beco, Hamburg, Germany) (Jiang et al., 2004), hyaline cell (HC) count, and semi- and large granular cell (SGC and LGC, respectively) count *via* hemolymph extension at room temperature (25 °C), fixation at methanol for 1 min, staining by the method of May-Grunwald-Giemsa, and then counting with light microscope (Pousti and Adib Moradi, 2004). The total plasma protein was estimated using the biuret method.

2.5.2. The activities of phenoloxidase, superoxide dismutase, lysozyme, and nitric oxide synthase

The remaining anticoagulated hemolymph (250 μl) was centrifuged at 700 × *g* for 20 min at 4 °C to separate the hemocytes from the plasma. In addition, the supernatant fluid was employed for plasma determinations (Safari et al., 2014a; Zhang et al., 2011). All activities of the enzymes were standardized based on the protein concentration. Phenoloxidase (PO) activity was assayed using a spectrophotometer by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) at the final reading of the medium at 490 nm (Hernández-López et al., 1996; Safari et al., 2014a). The activity of superoxide dismutase (SOD) was measured by observing the inhibition of ferricytochrome C reduction at the final reading of the medium at 550 nm (Cooper et al., 2002). The lysozyme (LYZ) activity was determined by observing a decrease in its absorbance in comparison with suspension of *Micrococcus lysodeikticus* without plasma at the final reading of the medium at 530 nm (Ellis, 1990). The activity of nitric oxide synthase (NOS) was measured using the assay kit (Nanjing Jiancheng Bioengineering Institute, China) (Marzinzig et al., 1997).

2.5.3. Digestive enzyme activities

On the 126th day, the live crayfish (12 individuals per treatment) were transported to the laboratory, ice-anesthetized, and then dissected using a scalpel. The hepatopancreas was removed, rinsed in distilled water, dried with paper towels, homogenized (30 g/70 ml distilled water) using a homogenizer (DI 18 Disperser). The homogenate was then centrifuged at 10,000 × *g* for 25 min at 4 °C, and the supernatant was stored in liquid nitrogen. All hepatopancreas tissues were collected one by one without pooling. The measurement of digestive enzyme activities follows Safari et al. (2014a). Briefly, the amylase activity was measured using a starch as the substrate at the final reading of the medium at 550 nm with a UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec 2000) (Coccia et al., 2011). Lipase activity was measured by α-naphthyl caprylate as substrate at the final reading of the medium at 540 nm (López-López et al., 2003). Alkaline protease activity was determined using azocasein as substrate at the final reading of the medium at 366 nm (Fernández Gimenez et al., 2001). In the present study, specific enzyme activity was defined as enzyme units (U) per milligram of total protein.

2.6. Chemical analysis

The analysis of dry matter by oven drying at 105 °C, crude protein (N × 6.25, Kjeldahl system: Buchi Labortechnik AG, Flawil, Switzerland), crude fat (Soxtec System HT 1043: Foss Tecator, AB), ash (muffle furnace, 550 °C), gross energy (Parr bomb calorimeter model 1266, Parr Instrument Co., Moline, IL), and crude fiber after digestion with H_2SO_4 and NaOH was performed according to the standard methods (AOAC, 2005). The nitrogen-free extract (NFE) was calculated by subtraction of dry matter from crude protein, crude fat, crude fiber, and ash contents. The organic matter was calculated by subtraction of dry matter from ash content. Ytterbium oxide was determined in diets and feces by inductively coupled plasma-atomic absorption spectrophotometery (ICP; GBC Integra XL, Australia).

2.7. Bacteriological analysis

At the onset of the feeding trial, the total viable count (TVC) of aerobic heterotrophic bacteria and presumptive lactic acid bacteria (LAB) levels in hepatopancreas were determined by random sampling of 20 crayfish from the stock. As described in a study conducted by (Safari et al., 2014a), at the end of the experiment, the crayfish (12 individuals per treatment) were transported alive to the laboratory, ice-anesthetized, rinsed with benzalkonium chloride (0.1% for 60 min), and dissected using a scalpel. Subsequently, hepatopancreas was removed and homogenized with sodium chloride (0.9 w/v) using a homogenizer (DI 18 Disperser). The homogenate was then centrifuged at 5000 × *g* for 5 min at 4 °C. One hundred microliters of the prepared samples was spread onto plate count agar (PCA; Merck Co.) and de Man, Rogosa and Sharpe media (MRS; Merck Co.) at four replicates to

Table 2
The mean (± SD[1]) of initial weight (g), final weight (g), specific growth rate (% day^{-1}), voluntary feed intake (% BW day^{-1}), feed conversion ratio, survival rate (%), protein efficiency ratio (PER) and protein productive value (PPV, %) of crayfish fed with the experimental diets after 126 days (n = 4)[2].

	Control	GOS[3]	*E. faecalis* (EnF)	*P. acidilactici* (PeA)	GOS + *E. faecalis* (GOSEnF)	GOS + *P. acidilactici* (GOSPeA)	*p*-Value
Initial weight (g)	6.17 ± 0.01^{a}	6.18 ± 0.01^{a}	6.17 ± 0.02^{a}	6.16 ± 0.03^{a}	6.16 ± 0.01^{a}	6.17 ± 0.03^{a}	0.847
Final weight (g)	37.40 ± 0.50^{a}	44.68 ± 0.64^{b}	58.09 ± 0.92^{d}	48.87 ± 0.61^{c}	114.61 ± 2.99^{f}	102.11 ± 2.44^{e}	0.0001
Specific growth rate (% BW day^{-1})	1.43 ± 0.01^{a}	1.57 ± 0.01^{b}	1.78 ± 0.01^{d}	1.64 ± 0.01^{c}	2.32 ± 0.02^{f}	2.23 ± 0.02^{e}	0.0001
Voluntary feed intake (% BW day^{-1})	1.64 ± 0.01^{a}	1.88 ± 0.01^{b}	2.12 ± 0.02^{c}	2.19 ± 0.01^{d}	2.68 ± 0.01^{f}	2.33 ± 0.05^{e}	0.0001
Feed conversion ratio	3.42 ± 0.02^{f}	2.92 ± 0.02^{e}	2.55 ± 0.01^{c}	2.77 ± 0.01^{d}	2.07 ± 0.20^{a}	2.32 ± 0.02^{b}	0.0001
Survival rate (%)	44.00 ± 1.00^{a}	49.67 ± 0.58^{b}	73.00 ± 1.00^{d}	66.33 ± 1.15^{c}	93.67 ± 0.58^{f}	91.67 ± 0.58^{e}	0.0001
Protein efficiency ratio	1.25 ± 0.02^{a}	1.74 ± 0.01^{b}	2.34 ± 0.02^{d}	1.98 ± 0.02^{c}	3.29 ± 0.02^{f}	3.10 ± 0.01^{e}	0.0001
Protein productive value (%)	41.67 ± 0.58^{a}	49.47 ± 0.41^{b}	56.67 ± 0.95^{d}	52.67 ± 0.75^{c}	63.33 ± 0.87^{f}	60.33 ± 0.78^{e}	0.0001

[1] Standard deviation.
[2] Different superscripts within a row indicate significant differences at $p < 0.05$.
[3] GOS: galactooligosaccharide.

determine TVC and LAB, respectively. The plates were incubated at room temperature (25 °C) for 5 days, and colony-forming units (CFU) g^{-1} were calculated from the plates containing 30–300 colonies (Hoseinifar et al., 2015a).

2.8. Bacterial exposure challenge

The challenge test was initiated on the 127th day of the feeding trial. Twelve crayfish from each diet tank received an injection of 1×10^{8} cells ml^{-1} *Aeromonas hydrophila* (ATCC 49040) through the base of the fifth thoracic leg with 20 ml bacterial stock suspension (Safari et al., 2014a; Sang et al., 2009). The injected crayfish were marked before being transferred back into their original tanks to avoid repeated sampling. Forty eight hours after the injection, the infected crayfish were monitored for survival rate, THC, HC, SGC, and LGC.

2.9. Statistical analysis

All percentage data were transformed using arcsine method. After confirming the homogeneity of variance and normality of the data by Leaven's and Kolmogorov-Smirnov tests (Zar, 2007), respectively, ANOVA was run to compare the results of treatments at four replicates. Duncan's test was used to compare the significant differences between the results of treatments ($p < 0.05$). To perform the analyses, SPSS™ version 19 was used. All results were presented as mean ± SD.

3. Results

3.1. Growth performance, survival rate, and in vivo ADCs of nutrients and gross energy

In comparison with the crayfish fed with the control diet, administration of prebiotic (GOS), probiotics (EnF and PeA), and synbiotics (GOSEnF and GOSPeA) in the diet of juvenile crayfish significantly improved ($p < 0.05$) the growth performance (final weight, SGR, FCR, and VFI), survival rate, and nutritional efficiency indices (PER and PPV) (Table 2). Juvenile crayfish fed with the diet containing GOSEnF revealed the highest survival rate, final weight, SGR, VFI, PER, and PPV, and the lowest FCR (Table 2). The crayfish fed with the diet containing GOSPeA were placed in the second rank after the GOSEnF treatment (Table 2). Final weight, SGR, survival rate, PER, and PPV in the crayfish fed with the diet containing EnF were significantly higher than those of the crayfish fed with the GOS-, PeA, and control diets (Table 2). In this regard, the level of FCR in the crayfish fed with the diet containing EnF was significantly lower than that of the crayfish fed with the above-mentioned diets. The levels of PER and PPV in the crayfish fed with the diet containing PeA were significantly higher than those of the crayfish fed with GOS and control diets (Table 2). Finally, the crayfish fed with the diet containing GOSEnF showed the highest values for *in vivo* ADCs of organic matter, crude protein, crude fat, and gross energy (Table 3).

3.2. Hemolymph indices and the activities of phenoloxidase (PO), superoxide dismutase (SOD), lysozyme (LYZ), and nitric oxide synthase (NOS)

Feeding the juvenile crayfish with the diets containing GOS, EnF, PeA, GOSEnF, and GOSPeA, in comparison with the control diet, considerably enhanced the THC, HC, SGC, and LGC (Table 4). Juvenile crayfish fed with the GOSEnF indicated the highest values for hemolymph indices and the activities of PO, SOD, LYZ, and NOS. Furthermore, hemolymph indices and the activities of PO, LYZ, and NOS in the crayfish fed with EnF diet were substantially higher than those of the crayfish fed with the GOS, PeA, and control diets (Table 4). After bacterial exposure challenge, the highest values of hemolymph indices were observed in the crayfish fed with the GOSEnF (Table 5). The crayfish fed with the GOSEnF diet demonstrated the highest values for survival rate and hemolymph indices (Table 5). As depicted in Fig. 1, during 48-h post-challenge, the survival rate in the injected crayfish fed with the GOSEnF diet was higher than that of the crayfish fed with other diets.

3.3. Digestive enzyme activities

As illustrated in Fig. 2, digestive enzyme activities including alkaline protease, lipase, and amylase in the juvenile crayfish fed with the prebiotic, probiotic, and synbiotics diets were significantly higher than those of the crayfish fed with the control diet. The highest activities of alkaline protease, lipase, and amylase appeared in the crayfish fed with the GOSEnF diet (Fig. 2a, b, c).

3.4. Microbiological analysis

The ratio of presumptive autochthonous lactic acid bacteria count to total viable aerobic heterotrophic bacteria count extracted from the hepatopancreas in the juvenile crayfish fed with the GOSEnF and GOSPeA diets demonstrated the highest values (Fig. 3). The EnF diet yielded significantly higher values than the PeA, GOS, and control diets (Fig. 3).

Table 3
The mean (± SD[1]) of *in vivo* ADCs of organic matter (ADC_{OM}, %), crude protein (ADC_{CP}, %), crude fat (ADC_{CF}, %) and gross energy (ADC_{GE}, %) of crayfish fed with the experimental diets after 126 days (n = 4)[2].

	Control	GOS[3]	*E. faecalis* (EnF)	*P. acidilactici* (PeA)	GOS + *E. faecalis* (GOSEnF)	GOS + *P. acidilactici* (GOSPeA)	*p*-Value
In vivo ADC_{OM}	61.33 ± 0.58^a	69.33 ± 0.87^b	78.67 ± 0.72^d	73.33 ± 0.91^c	85.33 ± 0.60^f	81.67 ± 0.52^e	0.0001
In vivo ADC_{CP}	75.67 ± 0.52^a	78.67 ± 0.95^b	84.33 ± 0.65^d	80.67 ± 0.67^c	91.67 ± 0.81^f	87.33 ± 0.75^e	0.0001
In vivo ADC_{CF}	54.67 ± 1.85^a	69.67 ± 1.56^b	75.33 ± 1.95^d	72.33 ± 1.95^c	82.33 ± 1.21^f	79.67 ± 1.85^e	0.0001
In vivo ADC_{GE}	61.33 ± 1.17^a	65.67 ± 1.78^b	74.67 ± 1.29^d	70.33 ± 1.48^c	83.67 ± 0.98^f	79.67 ± 1.18^e	0.0001

[1] Standard deviation.
[2] Different superscripts within a row indicate significant differences at $p < 0.05$.
[3] GOS: galactooligosaccharide.

Table 4
The mean (± SD[1]) of total hemocyte count (THC, $\times 10^5$ cells ml^{-1}), hyaline count (HC, $\times 10^5$ cells ml^{-1}), semi-granular count (SGC, $\times 10^5$ cells ml^{-1}), large-granular count (LGC, $\times 10^5$ cells ml^{-1}), phenoloxidase activity (PO, U min^{-1}), superoxide dismutase activity (SOD, U min^{-1}), lysozyme activity (LYZ, U min^{-1}) and nitric oxide synthase activity (NOS, U min^{-1}) of crayfish fed with experimental diets after 126 days (n = 4)[2].

	Control	GOS[3]	*E. faecalis* (EnF)	*P. acidilactici* (PeA)	GOS + *E. faecalis* (GOSEnF)	GOS + *P. acidilactici* (GOSPeA)	*p*-Value
THC ($\times 10^5$ cells ml^{-1})	81.67 ± 1.25^a	96.33 ± 1.78^b	105.67 ± 1.41^d	99.33 ± 1.75^c	114.33 ± 2.51^f	111.67 ± 2.01^e	0.0001
HC ($\times 10^5$ cells ml^{-1})	73.33 ± 0.85^a	86.67 ± 0.95^b	99.33 ± 0.57^d	93.67 ± 0.71^c	109.67 ± 1.12^f	104.67 ± 0.98^e	0.0001
SGC ($\times 10^5$ cells ml^{-1})	21.67 ± 2.12^a	32.67 ± 3.25^b	42.67 ± 2.14^d	37.91 ± 2.01^c	48.81 ± 4.15^f	45.75 ± 2.87^e	0.0001
LGC ($\times 10^5$ cells ml^{-1})	17.33 ± 1.16^a	32.33 ± 1.85^b	47.33 ± 1.47^d	41.33 ± 1.45^c	52.67 ± 1.12^e	49.33 ± 1.23^d	0.0001
Phenoloxidase activity (U min^{-1})	2.20 ± 1.12^a	3.47 ± 1.47^b	5.27 ± 1.25^d	4.80 ± 1.85^c	6.77 ± 1.56^f	6.07 ± 1.75^e	0.0001
Superoxide dismutase activity (U min^{-1})	2.17 ± 0.06^a	4.88 ± 0.52^b	5.86 ± 0.01^b	5.12 ± 0.15^b	6.69 ± 0.12^d	6.20 ± 0.13^c	0.0001
Lysozyme activity (U min^{-1})	4.14 ± 0.25^a	5.34 ± 0.81^b	6.88 ± 0.35^d	6.13 ± 1.12^c	8.87 ± 0.86^f	8.22 ± 0.97^e	0.0001
Nitric oxide synthase activity (U min^{-1})	1.95 ± 0.85^a	2.47 ± 0.97^b	3.64 ± 0.51^d	3.12 ± 0.90^c	5.11 ± 0.72^f	4.93 ± 0.95^e	0.0001

[1] Standard deviation.
[2] Different superscripts within a row indicate significant differences at $p < 0.05$.
[3] GOS: galactooligosaccharide.

Table 5
The mean (± SD[1]) of total hemocyte count (THC, $\times 10^5$ cells ml^{-1}), hyaline count (HC, $\times 10^5$ cells ml^{-1}), semi-granular count (SGC, $\times 10^5$ cells ml^{-1}), large-granular count (LGC, $\times 10^5$ cells ml^{-1}), and survival rate (%) of crayfish exposed to bacterial exposure challenge (n = 4)[2].

	Control	GOS[3]	*E. faecalis* (EnF)	*P. acidilactici* (PeA)	GOS + *E. faecalis* (GOSEnF)	GOS + *P. acidilactici* (GOSPeA)	*p*-Value
THC ($\times 10^5$ cells ml^{-1})	81.75 ± 0.57^a	96.48 ± 0.58^b	105.91 ± 0.57^d	99.54 ± 0.58^c	114.66 ± 0.57^f	111.95 ± 0.58^e	0.0001
HC ($\times 10^5$ cells ml^{-1})	78.64 ± 0.96^a	98.44 ± 2.14^b	123.08 ± 1.12^d	112.00 ± 0.15^c	144.66 ± 0.71^f	133.53 ± 1.16^e	0.0001
SGC ($\times 10^5$ cells ml^{-1})	23.31 ± 0.45^a	36.09 ± 0.77^b	53.03 ± 0.96^d	45.18 ± 0.82^c	64.38 ± 1.04^f	58.42 ± 0.48^e	0.0001
LGC ($\times 10^5$ cells ml^{-1})	18.59 ± 0.78^a	36.70 ± 0.58^b	58.65 ± 0.88^d	49.30 ± 0.61^c	69.47 ± 1.06^f	62.94 ± 1.02^e	0.0001
Survival rate (%)	8.33 ± 0.08^a	58.33 ± 2.25^b	68.33 ± 1.12^d	62.33 ± 3.14^c	77.67 ± 3.11^f	72.33 ± 2.13^e	0.0001

[1] Standard deviation.
[2] Different superscripts within a row indicate significant differences at $p < 0.05$.
[3] GOS: galactooligosaccharide.

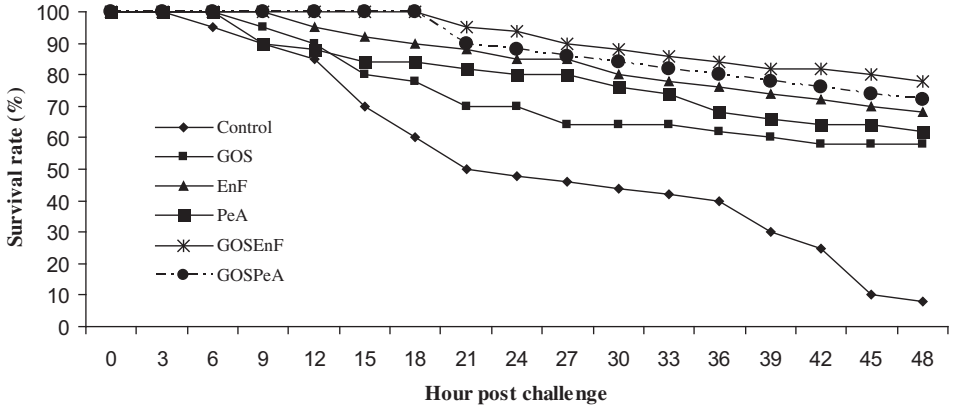
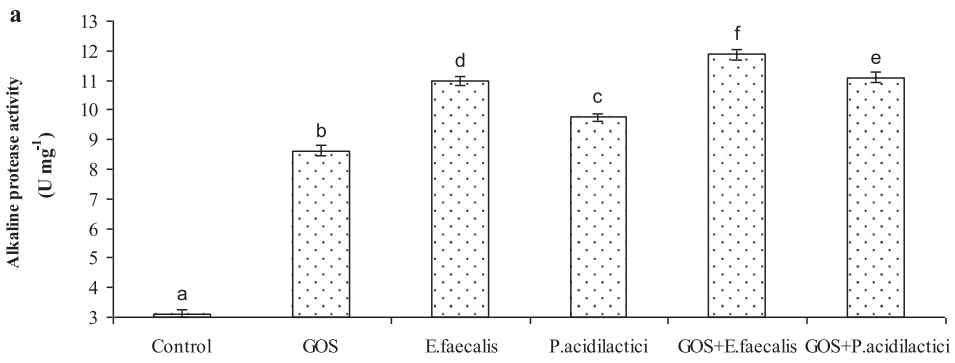
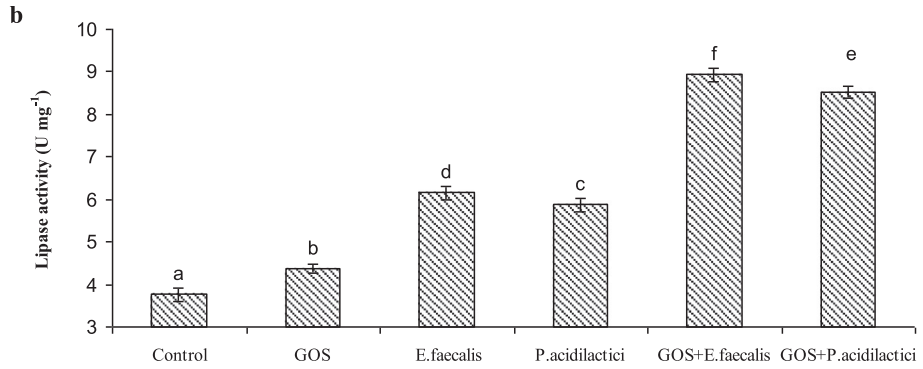


Fig. 1. Survival rate (%) of crayfish injected with *Aeromonas hydrophila* during 48 h post-challenge and fed with experimental diets (GOS: galactooligosaccharide; EnF: *Enterococcus faecalis*; PeA: *Pediococcus acidilactici*; GOSEnF: GOS + *Enterococcus faecalis*; GOSPeA: GOS + *Pediococcus acidilactici*) with four replicates.





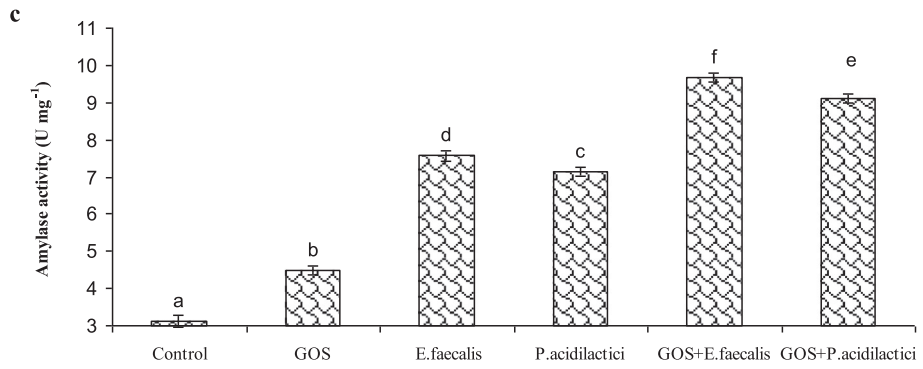


Fig. 2. The mean (± SD) activities (U mg^{-1}) of (a) alkaline protease, (b) lipase, and (c) amylase in the hepatopancreas of crayfish fed with the experimental diets after 126 days with four replicates (GOS: galactooligosaccharide). Different letters indicate significant differences ($p < 0.05$).

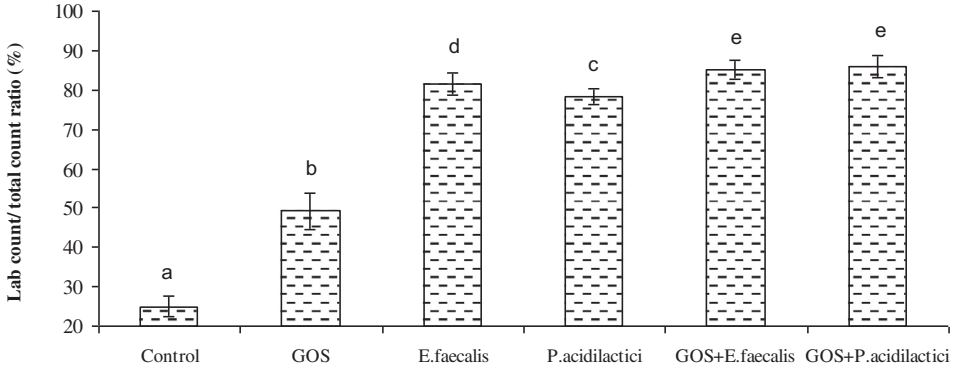


Fig. 3. The mean (± SD) ratio (%) of presumptive autochthonous lactic acid bacteria (lab) count (CFU g^{-1}) to total viable heterotrophic aerobic bacteria count (CFU g^{-1}) of hepatopancreas extracted from crayfish fed with experimental diets after 126 days with four replicates (GOS: galactooligosaccharide). Different letters indicate significant differences ($p < 0.05$).

4. Discussion

4.1. Growth performance, nutrient efficiency indices, in vivo ADCs of nutrients, bacterial flora in hepatopancreas, and digestive enzymes of hepatopancreas

Short-chain fatty acids (SCFA; acetic, propionic, and butyrate acids) produced during fermentation process in gastrointestinal tract have confirmed the positive effects on the growth performance (weight gain, SGR, and FCR), immune responses, and survival rate (Hoseinifar et al., 2015a; Maslowski and Mackay, 2011). Butyrate acts as a main energy source for colonic epithelial cells and plays a key role in epithelium maintenance (Maslowski and Mackay, 2011). Moreover, it can increase the *in vivo* ADCs of organic matter, crude protein, crude fat, and gross energy (Safari, 2016; Safari et al., 2014a; Ye et al., 2011) and finally improve the feed efficiency (Buentello et al., 2010). Furthermore, *via* stimulation of the immune responses, butyrate leads stress-resistance of the host (Maslowski and Mackay, 2011). As a factor increasing disease resistance, butyrate could down-regulate the expression of invasion genes in *Salmonella* sp. (Van Immerseel et al., 2006). The results of a study conducted by (Safari, 2016) showed that butyrate was found to be the main SCFA during fermentation of *E. faecalis* and *P. acidilactici* in GOS. The changes in the ratios of LAB to TVC in hepatopancreas, which is extracted from the crayfish fed with the experimental diets, may be beneficial for the crayfish. This benefit could be possibly obtained by increasing the nonspecific immune responses and concentrations and/or production of volatile fatty acids in general and butyrate in particular as the by-products of the fermentation process in GIT (Hoseinifar et al., 2015a; Safari, 2016). The mentioned point is in line with the findings reported by Hoseinifar et al. (2015a). The mentioned study has reported that butyrate is the main SCFA obtained from fermentation of *P. acidilactici* in the different prebiotics. However, this issue merits further investigations.

To the best of researchers' knowledge, the present study is the first one addressing the effects of synbiotics on the diet of crayfish. However, this complementary study was line with the previous studies addressing *Penaeus japonicus* fed with Bacillus (*B. licheniformis* and *B. subtilis*) + isomaltooligosaccharide (Zhang et al., 2011), *Litopenaeus vannamei* fed with *Bacillus* sp. + oligosaccharides (Munaeni et al., 2014), *L. vannamei* fed with *B. subtilis* + *P. acidilactici* and β-glucan (Wongsasak et al., 2015), *L. vannamei* fed with *Vibrio alginolyticus* + oligosaccharide (Nurhayati et al., 2015), ovate pompano fed with *B. subtilis* + fructooligosaccharide (Zhang et al., 2014), hybrid surubim fed with inulin + *Weissella cibaria* (Mourino et al., 2012), and rainbow trout fed with *E. faecalis* + MOS (Rodriguez-Estrada et al., 2009) and *E. faecalis* + FOS (Mehrabi et al., 2012). Considering the origin prebiotics, type of probiont species, *in vivo* hydrolysis conditions (pH, substrate availability, digesta viscosity, redox potential, *etc.*), habitat conditions, and characteristics of testing the animals (dominant feeding regime, gastrointestinal evacuation rate, diet formulation, initial weight, nutritional history, and feeding period) may contribute to describing the discrepancy in the obtained findings. The results of this trial revealed the superior biological effects of EnF, in comparison with PeA, on the similar substrate, *i.e.* GOS. A possible explanation for this finding may be attributed to production of exocrine metabolites and bioactive products including vitamins and antibiotics or any unknown by-products obtained during the fermentation process. However, microbial interactions, nutritional history, genetic (Konstantinov et al., 2004), and substrate and immune system interactions (Safari et al., 2014a) can be considered as the potential parameters.

The data obtained in this study indicated the synergistic effects of synbiotics used in the experimental diets on nutrient retention. One of the most effective ways to decrease the environmental impacts of aquaculture is to increase nutrient retention efficiency in general and PPV in particular. It has been reported that SCFA can modulate lipid synthesis and potentially can improve the metabolic pathways related to nutrient retention (Marcil et al., 2002). A probable explanation for the mentioned finding may be attributed to the structure-function relationship of the synbiotics in order to modulate the beneficial microbiota of gastrointestinal tract (GIT) in the aquatic species (Buentello et al., 2010; Safari et al., 2014a; Ye et al., 2011).

In vivo ADCs increment could possibly up-regulate the activities of specific digestive enzymes (Buentello et al., 2010; Ringø et al., 1995). In the present study, the activities of digestive enzymes, namely alkaline protease, lipase, and amylase, in the crayfish fed with synbiotic diets were found to be higher than those of the crayfish fed with the control diet. Feeding Japanese flounder, namely *Paralichthys olivaceus*, with the diet containing *B. clausii* + FOS + MOS increased the activities of protease and amylase digestive enzymes (Ye et al., 2011). The positive effects of synbiotics on growth performance may be associated with improved nutrient digestibility, which could be the result of boosting the digestive enzymes, as a result of which the host is allowed to degrade more nutrients (Ringø et al., 1995; Ye et al., 2011). Facilitating the digestion and absorption process in the crayfish with application of any technique can be promising in astaciculture to formulate more economically practical diets.

4.2. Hemolymph indices and air and bacterial exposure challenges

THC and different hemocyte counts (HC, SGC, and LGC) play key roles in maintaining the health of crustacean species (Jussila et al., 1997) and participate in the storage and release of the prophenoloxidase system and cytotoxicity (Johansson et al., 2000). Dietary manipulations with application of feed additives such as probiotics, prebiotics, and synbiotics can increase the animal resistance through pathogen inhibition pathways in GIT, a number of which are competition for territory in GIT, reduction in pH, and release of natural antibiotics from beneficial microbial populations (Li et al., 2007; Manning and Gibson, 2004). Feeding black tiger shrimp, *Penaeus monodon*, with the diet containing a probiont bacterium increased the activity of the innate immune system (Rengpipat et al., 2000). β-1,3-Glucan increased phagocytic activity of hemocyte and production of superoxide anion and lowered the clotting time in the involved black tiger shrimp (Chang et al., 2000). Moreover, it promoted the immune system against white spot syndrome virus (Chang et al., 2003). Feeding western king prawn, *Penaeus latisulcatus*, with test diets containing *Pseudomonas synxantha* and *P. aeruginosa* probiotics, in comparison with the control diet, decreased the proportion of HC and increased the proportion of SGC and THC (Hai et al., 2009). In this trial, incorporation of synbiotics into diets may stimulate and enhance the proliferation rate of juvenile crayfish hemocytes to compensate the loss of hemocytes due to *Aeromonas hydrophila* injection. Biological exposure challenge of blue crab, *i.e. Callinectes sapidus* (Johnson, 1976), and lobster, *Homarus americanus* (Stewart et al., 1967), to the harmful bacteria decreased THC content. A decreasing trend was reported in THCs of Chinese shrimp, *Fenneropenaeus chinensis*, and marron, *Cherax tenuimanus*, after being injected with *Vibrio anguillarum* and *V. mimicus*, respectively (Sang et al., 2009; Thaithongnum et al., 2006).

The survival rate of juvenile crayfish fed with the synbiotic diets after injection was higher than that of the crayfish fed with the control diet. Decapods species, namely shrimp, prawn, and crayfish, only rely on innate immune responses against microbial invasion (Zhang et al., 2011). In addition, defense activities could begin *via* hemolymph migration to the injection site and hemocytolysis (Sang et al., 2009). Injection of a lipopolysaccharide to kuruma prawns led to three-fold increase in the hemocyte proliferation rate (Sequeira et al., 1996). In this regard, abiotic and biotic factors such as life cycle, food intake, disease outbreaks, pollutants, and environmental stress can affect the quantity and quality of circulating hemocyte count (Sang et al., 2009). Finally, with the aim of offering sustainable astaciculture and higher survival rate of *Aeromonas hydrophila*-injected crayfish, incorporation of the GOSEnF- (77.67%) and GOSPeA- (72.33%) into diets approved the

positive effects of synbiotics. However, further studies are required to determine the best synbiotics, dose level, feed formulation, and manufacturing conditions.

5. Conclusion

Feeding juvenile crayfish with the diet containing 7.53 log CFU *E. faecalis* g^{-1} + 10 g kg^{-1} galactooligosaccharide demonstrated the highest values for survival rate (93.67%), final weight (114.61 g), SGR (2.32% day^{-1}), PER (3.29), and PPV (63.33%) and the lowest FCR (2.07). Furthermore, in comparison with the control diet, inclusion of this synbiotic in the crayfish diet significantly improved hemolymph indices including THC, HC, SGC, and LGC and stimulated immune responses against *Aeromonas hydrophila* injection.

Acknowledgment

This research was supported by the Research & Technology Deputy of Ferdowsi University of Mashhad under Project No. 39595.

References

Allameh, S.K., Ringø, E., Yusoff, F.M., Daud, H.M., Ideris, A., 2015. Dietary supplement of *Enterococcous faecalis* on digestive enzyme activities, short-chain fatty acid production, immune system response and disease resistance of Javaneses carp (*Puntinus gonionotus*, Bleeker 1850). Aquac. Nutr. http://dx.doi.org/10.1111/anu.12397.

AOAC, 2005. Official Methods of Analysis, 18th ed. AOAC International, Maryland, USA.

Buentello, J.A., Neill, W.H., Gatlin, D.M., 2010. Effects of dietary prebiotics on the growth, feed efficiency and non-specific immunity of juvenile red drum *Sciaenops ocellatus* fed soybean-based diets. Aquac. Res. 41, 411–418.

Castex, M., Daniels, C., Chim, L., 2014. Probiotic applications in crustaceans. In: Merrifield, D., Ringø, E. (Eds.), Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics. John Wiley & Sons, Ltd, Chichester, UK, pp. 290–319.

Cerezuela, R., Meseguer, J., Esteban, M.A., 2011. Current knowledge in synbiotic use for fish aquaculture: a review. J. Aquaculture Res. Dev. 1, 1–7.

Chang, C.-F., Chen, H.-Y., Su, M.-S., Liao, I.-C., 2000. Immunomodulation by dietary β-1, 3-glucan in the brooders of the black tiger shrimp *Penaeus monodon*. Fish Shellfish Immunol. 10, 505–514.

Chang, C.-F., Su, M.-S., Chen, H.-Y., Liao, I.-C., 2003. Dietary β-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. Fish Shellfish Immunol. 15, 297–310.

Coccia, E., Varricchio, E., Paolucci, M., Coccia, E., Varricchio, E., Paolucci, M., 2011. Digestive enzymes in the crayfish *Cherax albidus*: polymorphism and partial characterization. Int. J. Zool. 2011, 1–9.

Cooper, R.U., Clough, L.M., Farwell, M.A., West, T.L., 2002. Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J. Exp. Mar. Biol. Ecol. 279, 1–20.

Dash, G., Raman, R.P., Pani Prasad, K., Makesh, M., Pradeep, M.A.A., Sen, S., 2015. Evaluation of paraprobiotic applicability of *Lactobacillus plantarum* in improving the immune response and disease protection in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). Fish Shellfish Immunol. 43, 167–174.

Dong, C., Wang, J., 2013. Immunostimulatory effects of dietary fructooligosaccharides on red swamp crayfish, *Procambarus clarkii* (Girard). Aquac. Res. 44, 1416–1424.

Ellis, A.E., 1990. Lysozyme assays. In: Stolen, J.S., Flecher, T.C., Anderson, D.P., Robertsen, B.S., Van Muiswinkel, W.B. (Eds.), Techniques in Fish Immunology, pp. 101–103.

Fernández Gimenez, A., García-Carreño, F., Navarrete del Toro, M., Fenucci, J., 2001. Digestive proteinases of red shrimp *Pleoticus muelleri* (Decapoda, Penaeoidea): partial characterization and relationship with molting. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 130, 331–338.

Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquac. Nutr. 13, 17–34.

Gosling, A., Stevens, G.W., Barber, A.R., Kentish, S.E., Gras, S.L., 2010. Recent advances refining galactooligosaccharide production from lactose. Food Chem. 121, 307–318.

Grisdale-Helland, B., Helland, S.J., Gatlin, D.M., 2008. The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or galactooligosaccharide on the growth and feed utilization of Atlantic salmon (*Salmo salar*). Aquaculture 283, 163–167.

Hai, N. Van, Buller, N., Fotedar, R., 2009. Effects of probiotics (*Pseudomonas synxantha* and *Pseudomonas aeruginosa*) on the growth, survival and immune parameters of juvenile western king prawns (*Penaeus latisulcatus* kishinouye, 1896). Aquac. Res. 40, 590–602.

Hernández-López, J., Gollas-Galván, T., Vargas-Albores, F., 1996. Activation of the prophenoloxidase system of the brown shrimp *Penaeus californiensis* Holmes. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 113, 61–66.

Holdich, D.M., 1993. A review of astaciculture: freshwater crayfish farming. Aquat. Living Resour. 6, 307–317.

Hoseinifar, S.H., Mirvaghefi, A., Merrifield, D.L., Amiri, B.M., Yelghi, S., Bastami, K.D., 2011. The study of some haematological and serum biochemical parameters of juvenile beluga (*Huso huso*) fed oligofructose. Fish Physiol. Biochem. 37, 91–96.

Hoseinifar, S.H., Khalili, M., Khoshbavar Rostami, H., Esteban, M.Á., 2013. Dietary galactooligosaccharide affects intestinal microbiota, stress resistance, and performance of Caspian roach (*Rutilus rutilus*) fry. Fish Shellfish Immunol. 35, 1416–1420.

Hoseinifar, S.H., Mirvaghefi, A., Amoozegar, M.A., Merrifield, D.L., Ringø, E., 2015a. *In vitro* selection of a synbiotic and in vivo evaluation on intestinal microbiota, performance and physiological response of rainbow trout (*Oncorhynchus mykiss*) fingerlings. Aquac. Nutr. http://dx.doi.org/10.1111/anu.12373.

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

Hoseinifar, S.H., Ahmadi, A., Raeisi, M., Hoseini, S.M., Khalili, M., Behnampour, N., 2016. Comparative study on immunomodulatory and growth enhancing effects of three prebiotics (galactooligosaccharide, fructooligosaccharide and inulin) in common carp (*Cyprinus carpio*). Aquac. Res. http://dx.doi.org/10.1111/are.13156.

Jiang, G., Yu, R., Zhou, M., 2004. Modulatory effects of ammonia-N on the immune system of *Penaeus japonicus* to virulence of white spot syndrome virus. Aquaculture 241, 61–75.

Johansson, M.W., Keyser, P., Sritunyalucksana, K., Söderhäll, K., 2000. Crustacean haemocytes and haematopoiesis. Aquaculture 191, 45–52.

Johnson, P.T., 1976. Bacterial infection in the blue crab, *Callinectes sapidus*: course of infection and histopathology. J. Invertebr. Pathol. 28, 25–36.

Jussila, J., Jago, J., Tsvetnenko, E., Dunstan, B., Evans, L.H., 1997. Total and differential haemocyte counts in western rock lobsters (*Panulirus cygnus* George) under post-harvest stress. Mar. Freshw. Res. 48, 863.

Karimpour, M., Harlioğlu, M.M., Aksu, Ö., 2011. Status of freshwater crayfish (*Astacus leptodactylus*) in Iran. Knowl. Manag. Aquat. Ecosyst. http://dx.doi.org/10.1051/kmae/2011032.

Konstantinov, S.R., Awati, A., Smidt, H., Williams, B.A., Akkermans, A.D.L., De Vos, W.M., 2004. Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the guts of weaning piglets. Appl. Environ. Microbiol. 70, 3821–3830.

Li, P., Burr, G.S., Gatlin, D.M., Hume, M.E., Patnaik, S., Castille, F.L., Lawrence, A.L., 2007. Dietary supplementation of short-chain fructooligosaccharides influences gastrointestinal microbiota composition and immunity characteristics of Pacific white shrimp, *Litopenaeus vannamei*, cultured in a recirculating system. J. Nutr. 137, 2763–2768.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. http://dx.doi.org/10.3389/fmicb.2014.00207.

López-López, S., Nolasco, H., Vega-Villasante, F., 2003. Characterization of digestive gland esterase–lipase activity of juvenile redclaw crayfish *Cherax quadricarinatus*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 135, 337–347.

Manning, T.S., Gibson, G.R., 2004. Prebiotics. Best Pract. Res. Clin. Gastroenterol. 18, 287–298.

Marcil, V., Delvin, E., Seidman, E., Poitras, L., Zoltowska, M., Garofalo, C., Levy, E., Agellon, L., Zhang, P., Jiang, X., Mendelsohn, L., Tall, A., Basson, M., Emenaker, N., Hong, F., Behall, K., Black, D., Borenfreund, E., Schmid, E., Bendich, A., Franke, W., Bush, R., Milligan, L., Chou, J., Takahashi, S., Cioe, L., McNab, A., Hubbell, H., Davidson, N., Magun, A., Haan, J. De, Gevers, W., Parker, M., Field, F., Mathur, S., Fishman, P., Simmons, J., Brady, R., Freese, E., Peris, P.G., Alvarez, M.C., Griffin, M., Price, G., Bazzell, K., Cox, R., Ghosh, N., Guo, Z., Yuan, C., Wei-Lavery, T., Fang, Y., Garvin, R., Nishida, H., Nishida, T., Hormia, M., Linder, E., Lehto, V., Jenkins, D., Wolever, T., Jenkins, A., Brighenti, F., Vuksan, V., Rao, A., Cunnane, S., Ocana, A., Corey, P., Vezina, C., Kaptein, A., de Wit, E., Princen, H., Kaptein, A., Roodenburg, L., Princen, H., Kruh, J., Levrat, M., Favier, M., Moundras, C., Rémésy, C., Demigné, C., Morand, C., Levy, E., Garofalo, C., Rouleau, T., Gavino, V., Bendayan, M., Levy, E., Mehran, M., Seidman, E., Levy, E., Ménard, D., Levy, E., Sinnett, D., Thibault, L., Nguyen, T., Delvin, E., Ménard, D., Levy, E., Thibault, L., Delvin, E., Ménard, D., Levy, E., Thibault, L., Ménard, D., Loirdighi, N., Ménard, D., Delvin, E., Levy, E., Loirdighi, N., Ménard, D., Levy, E., Mehran, M., Levy, E., Gurbindo, C., Bendayan, M., Seidman, E., Mehran, M., Seidman, E., Marchand, R., Gurbindo, C., Levy, E., Mortensen, P., Clausen, M., Moundras, C., Behr, S., Demigne, C., Mazur, A., Remesy, C., Orly, J., Schramm, M., Perrine, S., Rudolph, A., Faller, D., Phan, C., Tso, P., Prasad, K., Sinha, P., Roediger, W., Roediger, W., Ruemmele, F., Ruemmele, C., Levy, E., Seidman, E., Salter, A., Wiggins, D., Sessions, V., Gibbons, G., Skretting, G., Gjernes, E., Prydz, H., Spalinger, J., Seidman, E., Lepage, G., Ménard, D., Gavino, V., Levy, E., Sperker, B., Mark, M., Budzinski, R., Stan, S., Delvin, E., Seidman, E., Rouleau, T., Bendayan, M.S., Yotov, W., Levy, E., Taghibiglou, C., Rudy, D., Iderstine, S. Van, Aiton, A., Cavallo, D., Cheung, R., Adeli, K., Toscani, A., Soprano, D., Soprano, K., Tso, P., Gollamudi, S., Wetterau, J., Lin, M., Jamil, H., Yang, L., Kuksis, A., Myher, J., Younes, H., Levrat, M., Demigne, C., Remesy, C., 2002. Modulation of lipid synthesis, apolipoprotein biogenesis, and lipoprotein assembly by butyrate. Am. J. Physiol. Gastrointest. Liver Physiol. 283, G340–G346.

Marzinzig, M., Nussler, A.K., Stadler, J., Marzinzig, E., Barthlen, W., Nussler, N.C., Beger, H.G., Morris, S.M., Brückner, U.B., 1997. Improved methods to measure end products of nitric oxide in biological fluids: nitrite, nitrate, and S-nitrosothiols. Nitric Oxide 1, 177–189.

Maslowski, K.M., Mackay, C.R., 2011. Diet, gut microbiota and immune responses. Nat. Immunol. 12, 5–9.

Mazlum, Y., Yılmaz, E., Genç, M.A., Guner, O., 2011. A preliminary study on the use of mannan oligosaccharides (MOS) in freshwater crayfish, *Astacus leptodactylus* Eschscholtz, 1823 juvenile diets. Aquac. Int. 19, 111–119.

Mehrabi, Z., Firouzbakhsh, F., Jafarpour, A., 2012. Effects of dietary supplementation of

synbiotic on growth performance, serum biochemical parameters and carcass composition in rainbow trout (*Oncorhynchus mykiss*) fingerlings. J. Anim. Physiol. Anim. Nutr. (Berl.) 96, 474–481.
Mourino, J.L.P., Do Nascimento Vieira, F., Jatoba, A.B., Da Silva, B.C., Jesus, G.F.A., Seifert, W.Q., Martins, M.L., 2012. Effect of dietary supplementation of inulin and *W. cibaria* on haemato-immunological parameters of hybrid surubim (*Pseudoplatystoma* sp.). Aquac. Nutr. 18, 73–80.
Munaeni, W., Yuhana, M., Widanarni, W., 2014. Effect of micro-encapsulated synbiotic at different frequencies for luminous vibriosis control in white shrimp (*Litopenaeus vannamei*). Microbiol. Indones. 8, 73–80.
Neissi, A., Rafiee, G., Nematollahi, M., Safari, O., 2013. The effect of *Pediococcus acidilactici* bacteria used as probiotic supplement on the growth and non-specific immune responses of green terror, *Aequidens rivulatus*. Fish Shellfish Immunol. 35, 1976–1980.
Ng, W.-K., Koh, C.-B., 2016. The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. Rev. Aquac. http://dx.doi.org/10.1111/raq.12141.
Nurhayati, D., W, Yuhana, M., 2015. Dietary synbiotic influence on the growth performances and immune responses to co-infection with infectious myonecrosis virus and *Vibrio harveyi* in *Litopenaeus vannamei*. J. Fish. Aquat. Sci. 10, 13–23.
Pousti, I., Adib Moradi, M., 2004. Comparative Histology and Histotechnique, 4th ed. University of Tehran Publishing (315 pp).
Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasaveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191, 271–288.
Ringø, E., Strøm, E., Tabachek, J.-A., 1995. Intestinal microflora of salmonids: a review. Aquac. Res. 26, 773–789.
Ringø, E., Olsen, R.E.E., Gifstad, T.Ø., Dalmo, R.A.A., Amlund, H., Hemre, G.-I.I., Bakke, A.M.M., 2010. Prebiotics in aquaculture: a review. Aquac. Nutr. 16, 117–136.
Ringø, E., Dimitroglou, A., Hoseinifar, S.H., Davies, S.J., 2014. Prebiotics in finfish: an update. In: Aquaculture Nutrition. John Wiley & Sons, Ltd, In, pp. 360–400.
Rodriguez-Estrada, U., Satoh, S., Haga, Y., Fushimi, H., Sweetman, J., 2009. Effects of single and combined supplementation of *Enterococcus faecalis*, mannan oligosaccharide and polyhydroxybutyrate acid on growth performance and immune response of rainbow trout *Oncorhynchus mykiss*. Aquac. Sci. 57, 609–617.
Rodriguez-Estrada, U., Satoh, S., Haga, Y., Fushimi, H., Sweetman, J., 2013. Effects of inactivated *Enterococcus faecalis* and mannan oligosaccharide and their combination on growth, immunity, and disease protection in rainbow trout. N. Am. J. Aquac. 75, 416–428.
Safari, O., 2016. In Vitro Selection of Synbiotic and *In Vivo* Evaluation of Growth Performance and Biological Indices of Narrow Clawed Crayfish (*Astacus leptodactylus leptodactylus*). FUM Publishing, Mashhad, Khorasan Razavi Province, Iran, pp. 65.
Safari, O., Shahsavani, D., Paolucci, M., Atash, M.M.S., 2014a. Single or combined effects of fructo- and mannan oligosaccharide supplements on the growth performance, nutrient digestibility, immune responses and stress resistance of juvenile narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 182. Aquaculture 432, 192–203.
Safari, O., Shahsavani, D., Paolucci, M., Mehraban Sang Atash, M., 2014b. Screening of selected feedstuffs by sub-adult narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 1823. Aquaculture 420–421, 211–218.
Sang, H.M., Ky, L.T., Fotedar, R., 2009. Dietary supplementation of mannan oligosaccharide improves the immune responses and survival of marron, *Cherax tenuimanus* (Smith, 1912) when challenged with different stressors. Fish Shellfish Immunol. 27, 341–348.
Sequeira, T., Tavares, D., Arala-Chaves, M., 1996. Evidence for circulating hemocyte proliferation in the shrimp *Penaeus japonicus*. Dev. Comp. Immunol. 20, 97–104.
Stewart, J.E., Cornick, J.W., Dingle, J.R., 1967. An electronic method for counting lobster (*Homarus americanus* Milne Edwards) hemocytes and the influence of diet on hemocyte number and hemolymph proteins. Can. J. Zool. 45, 291–304.
Thaithongnum, S., Ratanama, P., Weeradechapol, K., Sukhoom, A., Vuddhakul, V., 2006. Detection of *Vibrio harvey*i in shrimp postlarvae and hatchery tank water by the most probable number technique with PCR. Aquaculture 261, 1–9.
Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F., Ducatelle, R., 2006. The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. Avian Pathol. 35, 182–188.
Villamil, L., Figueras, A., Planas, M., Novoa, B., 2010. *Pediococcus acidilactici* in the culture of turbot (*Psetta maxima*) larvae: administration pathways. Aquaculture 307, 83–88.
Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W., 2009. Systematic Bacteriology. Springer New York, New York, NY (1450 pp).
Wongsasak, U., Chaijamrus, S., Kumkhong, S., Boonanuntanasarn, S., 2015. Effects of dietary supplementation with β-glucan and synbiotics on immune gene expression and immune parameters under ammonia stress in Pacific white shrimp. Aquaculture 436, 179–187.
Ye, J.-D.D., Wang, K., Li, F.-D.D., Sun, Y.-Z.Z., 2011. Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder *Paralichthys olivaceus*. Aquac. Nutr. 17, e902–e911.
Zar, J.H., 2007. Biostatistical analysis. In: Journal of Chemical Information and Modeling, 5th ed. Prentice-Hall, Inc, Upper Saddle River, NJ, USA (960 pp).
Zhang, Q., Tan, B., Mai, K., Zhang, W., Ma, H., Ai, Q., Wang, X., Liufu, Z., 2011. Dietary administration of *Bacillus* (*B. licheniformis* and *B. subtilis*) and isomaltooligosaccharide influences the intestinal microflora, immunological parameters and resistance against *Vibrio alginolyticus* in shrimp, *Penaeus japonicus* (Decapoda: Penaeidae). Aquac. Res. 42, 943–952.
Zhang, Q., Yu, H., Tong, T., Tong, W., Dong, L., Xu, M., Wang, Z., 2014. Dietary supplementation of *Bacillus subtilis* and fructooligosaccharide enhance the growth, non-specific immunity of juvenile ovate pompano, *Trachinotus ovatus* and its disease resistance against *Vibrio vulnificus*. Fish Shellfish Immunol. 38, 7–14.
Zhou, Q.-C., Buentello, J.A., Gatlin, D.M., 2010. Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum (*Sciaenops ocellatus*). Aquaculture 309, 253–257.