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Research Article

Dietary Effects of *Lactobacillus plantarum* Combined with Galactooligosaccharide on Immunological and Biochemical Parameters, Gut Microbiota, Digestive Enzyme Activity, Body Composition, and Stress Resistance in Narrow-Clawed Crayfish, *Pontastacus leptodactylus* (Eschscholtz, 1823)

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The effects of galactooligosaccharide (GOS) and varying levels of *Lactobacillus plantarum* (LP) on some physiological parameters as well as air exposure stress resistance in clawed crayfish (*Pontastacus leptodactylus*) were investigated. During a 97-day trial, 216 crayfish were fed with four experimental diets (in triplicates) including the control diet (without GOS and LP), GLP7 (GOS 2%+LP 10^7 CFU.g^{-1} diet), GLP8 (GOS 2%+*LP* 10^8 CFU.g^{-1} diet), and GLP9 (GOS 2%+LP 10^9 CFU.g^{-1} diet). At the end of the trial, 26% higher amounts of the total hemocyte count (THC) and semigranular cells (SGC) and 27% higher hyaline cells (HC) were observed in GLP7. A significant improvement in lysozyme value was observed in GLP7 and GLP8. Moreover, superoxide dismutase was significantly higher in GLP9, whereas the catalase (CAT) activity did not change in the experimental groups. Unlikely, plasma glucose levels decreased in all the GLP treatments. In terms of intestinal microbiota, autochthonous lactic acid bacteria (LAB) remarkably increased in all the GLP-supplemented groups, while total autochthonous intestinal heterotrophic bacteria counts (TVC) did not change in GLP-supplemented groups. However, 14 days after switching to the basal diet, no reduction was detected in TVC, but LAB levels decreased in GLP7 and GLP9 treatments. Dietary administration of GLP could beneficially modulate digestive enzyme activity, including protease, lipase, and amylase. Furthermore, higher lipid and gross energy were observed in GLP9. However, GLP-supplemented diets could not improve growth performance parameters. After 24 h subjecting to air exposure stress, although no mortality was detected, crayfish fed GLP-supplemented diets were healthier through enhanced THC, SGC, and HC levels in GLP7 and GLP9 and CAT activity in GLP7 and GLP8. These results proved the positive impacts of dietary supplementation with combined GOS and LP, with GLP8 and GLP9 as optimum diets, on immunochemical parameters, intestinal microbiota, digestive enzyme activity, and stress resistance in *P. leptodactylus*.

1. Introduction

The interest in animal welfare is growing along with the expanding aquaculture industry. In addition to ethical issues related to animal welfare, stressed animals are susceptible to microbial and environmental diseases, mainly due to the depression of the immune system. Farmed aquatics are regularly exposed to different stressors associated with the applied techniques. Thus, researchers and aquaculturists are trying to improve the animals' welfare in commercial facilities.

The administration of dietary supplements to improve aquatics' immune system is one of the most effective methods to alleviate the effects of the present stressors during the culture period. In that sense, the diet's prebiotic, probiotic, and synbiotic supplements have been studied in several shellfish, such as *Cherax tenuimanus* ([1]), *Cherax destructor* ([2]), *Pontastacus leptodactylus* [3–5], *Penaeus monodon* [6, 7], *Penaeus japonicus* [8, 9], and *Penaeus vannamei* [10, 11].

Narrow-clawed crayfish (*Pontastacus leptodactylus*), the only native crayfish species in Iran [12], is noticed as a candidate for aquaculture diversity. Its potential aquaculture features, high nutrient value, economic importance, and high consumer demands make this species a suitable choice [13]. Today, the reduction of the wild resources, along with some negative factors that limit the success of introduction to new resources, lead to increased attention and research on crayfish artificial propagation and culture under controlled conditions [12, 14, 15]. In addition to common stressors of the cultural environments, the increasing global demand for live crustaceans [16], freshwater crayfish, is usually faced with major problems during commercial harvest and postharvest procedures [17]. So far, numerous studies have been reported that handling and air exposure during live transportation negatively affect health statute and cause an immunological disturbance [18–21] and eventually poor survival (S. [22]). Therefore, surveying the function of different immune parameters in response to air exposure and finding an appropriate feed additive, which can improve these parameters following stressful conditions, can be desirable.

Nedaei et al. [3] used a GOS-supplemented diet for *P. leptodactylus* and found positive effects on immune responses and resistance of treated animals subjected to air exposure challenge. In another study, the administration of probiotic *Lactobacillus plantarum* to increase the ability of narrow-clawed crayfish to air exposure challenge revealed positive results [5]. The same improvements in immunity and resistance ability of crayfish against air or bacterial exposure challenge obtained in other studies used different supplements such as *L*-carnitine [23], fructo- and mannano-oligosaccharides [1, 21], trehalose [24], and synbiotic (galactooligosaccharide and mannanoligosaccharide with or without *Enterococcus faecalis*) [25]. However, to our best knowledge, there is no available data regarding the effects of the combination of GOS and *L. plantarum* in form of a synbiotic on *P. leptodactylus*. Given the importance of *P. leptodactylus* as valuable species on one side and the importance of resistance against environmental stress as well, the present study is designed to assess the effects of GOS+*L. plantarum* (as synbiotic) on immune parameters, intestinal microbiota, digestive enzyme activity, carcass composition, and resistance against air exposure stress.

2. Material and Methods

2.1. Experimental Diets. The basal diet composition (Table 1) was prepared according to Miandare et al. [26]. All ingredients are well-grounded, mixed thoroughly, pelleted (cold-press), dried at 40°C, and kept at 4°C until feeding. The basal diet, without any GOS or *L. plantarum* supplementation, is considered the control. A combination of 20 g kg^{-1} diet Vivinal-GOS® (Friesland Foods Domo Company, Zwolle, The Netherlands) and three different levels of *L. plantarum* (10^7,10^8, and 10^9 CFU g^{-1} diet) (Zistyar Varna Company, Superzist, Iran) (synbiotic treatment) was applied to prepare the supplemented experimental diets, according to Merrifield et al. [27]. In this way, four treatments named as control (basal diet), GLP7 (20 g kg^{-1} diet GOS+10^7 CFU g^{-1} diet *L. plantarum*), GLP8 (20 g kg^{-1} diet GOS+10^8 CFU g^{-1} diet *L. plantarum*), and GLP9 (20 g kg^{-1} diet GOS+10^9 CFU g^{-1} diet *L. plantarum*) are considered. The experimental diets were renewed every four days to preserve viability of probiotic bacteria [28].

2.2. Crayfish and Culture System. Healthy crayfish were caught from Aras Dam Lake (western Azerbaijan Province, Iran) and transferred to the laboratory of Aquatic Live Food and Nutrition Station (Ghazian, Bandar Anzali, Iran). All individuals were acclimated to the experimental indoor condition in 1000 l fiberglass tanks for two weeks and fed with the basal diet prior to the feeding trial. At the start of the experiment, two hundred and sixteen crayfish with an initial weight of 27.52 ± 0.45 g were randomly stocked into twelve 300 l cylindrical fiberglass tanks at a density of 18 crayfish per treatment with three replicates. All the experimental treatments were fed with the corresponding diet two times a day (8:00 and 15:00) at the rate of 1.5% of the crayfish body weight for 97 days. All treatments were equipped with static aerated freshwater and 90% of water exchanged on a one-day interval basis. During the experimental period, water temperature, dissolved oxygen, and pH maintained at 23-25°C, 7.20-8.05 mg l^{-1}, and 6.70-7.20, respectively. Moreover, to prevent cannibalistic behavior within the molting period, 18 numbers of PVC pipes supplied for each tank.

2.3. Experimental Design and Sample Collection. At the end of the 97-day feeding trial, all crayfish from each tank are counted and weighed to calculate the growth performance indices [29] and molting frequency [15] using the following formula:

$$\text{Weight gain (WG)} = 100 \times \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{initial weight (g)}}, \quad (1)$$

Table 1: Composition and proximate analysis of the basal diet.

Ingredients used in basal diet	(%)
Fish meal	37.6
Wheat flour	15
Soybean meal	16.1
Corn meal	5
Starch meal	5
Crayfish meal	2
Gelatin	2
Fish oil	7.8
Cholesterol	0.5
Cellulose	3
Choline chloride	0.5
Methionine	1
Lysine	1
Vitamin C	0.5
Vitamin premix[a]	1
Mineral premix[a]	2
Proximate composition of basal diet	*(%)*
Dry matter	92.96
Crude protein*	44
Crude lipid*	16
Ash*	3.6
Fiber*	1.68
Moisture	7.53
NFE[b]	36.68
GE (kcal 100 g^{-1})[c]	471

[a]Vitamin premix and mineral premix according to Miandare et al. [26]. [b]Nitrogen – free extracts (NFE) = dry matter – (crude protein + crude lipid + ash + fiber). [c]Gross energy (kcal 100 g^{-1}). *% dry weight basis.

$$\text{Protein efficiency ratio (PER)} = \frac{\text{weight gain}}{\text{crude protein consumed}}, \quad (2)$$

$$\text{Lipid efficiency ratio (LER)} = \frac{\text{weight gain}}{\text{crude lipid consumed}}, \quad (3)$$

$$\text{Specific growth rate (SGR)} = 100\ \frac{\ln\ \text{final weight} - \ln\ \text{initial weight}}{\text{Time}}, \quad (4)$$

$$\text{Feed conversation ratio (FCR)} = \frac{\text{feed intake (g)}}{\text{weight gain (g)}}, \quad (5)$$

$$\text{Condition factor (CF)} = \frac{\text{Body weight (g)} \times 100}{\text{total length}^3\,\text{(cm)}}, \quad (6)$$

$$\text{Survival rate (SR)} = \left(\frac{\text{final number}}{\text{initial number}}\right) \times 100, \quad (7)$$

$$\text{Molting frequency (MF)} = \left(\frac{\text{molting crayfish}}{\text{total number of crayfish}}\right) \times 100. \quad (8)$$

At the end of the 97-day feeding trial, nine crayfish were randomly sampled from each treatment. All the samples rinsed thoroughly with normal saline before hemolymph withdrawal and dissecting the hepatopancreas to evaluate digestive enzyme activity. Moreover, three individuals were randomly sampled from each treatment and kept frozen at -80°C for analyzing proximate body composition based on the method described by AOAC [30]. The intestinal microbiota assay was conducted by the whole intestine of nine 48 h starved samples from each treatment. At the end of the 97-day feeding trial and 14 days after switching from the experimental diets to the basal diet, this last process was carried out once again. The remaining animals were regarded for assessment of air-exposure stress.

2.4. Hemolymph Biochemical and Immunological Index Assay

2.4.1. Hemolymph Sampling. About 1 ml hemolymph was extracted from the base of the fifth thoracic leg of each crayfish, using a 2 ml syringe equipped with a 23-gauge needle. Each collected sample was instantly transferred into a 2 ml microtube containing 1 ml precooled anticoagulant solution (18.7 $g\,l^{-1}$ glucose, 8 $g\,l^{-1}$ sodium citrate, 0.5 $g\,l^{-1}$ citric acid, and 4.2 $g\,l^{-1}$ NaCl) [31]. A part of the anticoagulant-hemolymph mixture (200 μl) was used to measure the hemocyte count, and the rest of the mixture was centrifuged for 10 min at 700 × g at 4°C. Separated plasma was stored at -20°C to measure other immune indices.

2.4.2. Total and Differential Hemocyte Counts (THC and DHC). THC and DHC were evaluated according to the method described by Fotedar et al. [32] and Nedaei et al. [3]. The three basic types of hemocytes (SGC, LGC, and HC) were recognized as stated by Hose et al. [33].

2.4.3. Total Plasma Proteins (TPP) and Glucose. TPP was examined following the Bradford procedure [34] using bovine serum albumin as standard (Sritunyalucksana et al.). Plasma glucose content was analyzed spectrophotometrically, using a commercially available kit (Pars Azmoun Co., Iran).

2.4.4. Phenoloxidase (PO), Lysozyme (LYZ), Superoxide Dismutase (SOD), and Catalase (CAT) Activities. PO activity was investigated spectrophotometrically by measuring the formation of dopachrome from L-dihydroxy phenylalanine (LDOPA, Sigma) following Hernández-López et al. [35]. LYZ activity was determined based on turbidimetric assay following the method described by Ellis [36]. SOD activity was measured calorimetrically using a commercially available kit (ZellBio GmbH, Germany). The enzyme activity unit is considered the amount of the sample that will catalyze decomposition of 1 μ mole of O_2^- to H_2O_2 and O_2 in one minute [5]. CAT activity was measured calorimetrically using a commercially available kit (ZellBio GmbH, Germany). The enzyme activity unit was considered the amount of the sample that will catalyze decomposition of 1 μ mole of H_2O_2 to H_2O and O_2 in one minute [5].

2.5. Intestinal Microbiota Assay. Surveying the intestinal microbiota of crayfish fed the experimental diets was carried

out as described by Hoseinifar et al. [37] and [5]. Sampling was done at two separate times; first, at the end of the 97-day feeding period and second, 14 days after switching from the experimental diets to the basal diet. Briefly, the samples from each sampling time were rinsed with distilled water, disinfected with 0.1% benzalkonium chloride and subsequently rinsed again with sterilized distilled water to eliminate surface bacteria. Then, the whole intestine of each individual (i.e., they were not pooled) was separated, rinsed with sterile saline (0.85% NaCl), and homogenized (Potter-Elvehjem Tissue Homogenizer, USA) to isolate the autochthonous intestinal microbial communities. To determine the total intestinal heterotrophic (aerobic) bacteria (TIHB) and lactic acid bacteria (LAB), hundred microliters of the obtained homogenate were spread on plate count agar (PCA) (Merck, Germany) and de Man, Rogosa, and Sharpe (MRS) agar media (Merck, Germany), respectively. Plates were incubated at room temperature (25°C) for 5 days [38], and colony forming units (CFU g^{-1}) were calculated from statistically viable plates (i.e., plates containing 30–300 colonies) [39]. All the processes are done in triplicates.

2.6. Digestive Enzyme Activity Assessment. The hepatopancreas crude extract of the crayfish was used to measure digestive enzyme activity at the end of the feeding trial. Samples were prepared based on the method described by Coccia et al. [40]. Measurement of amylase activity according to Rick and Stegbauer [41], total protease activity based on Anson [42], and lipase activity according to López-López et al. [43] was done. Alkaline phosphatase (ALP) activity was measured by a commercial assay kit (Pars Azmoun Co., Iran) based on Nedaei et al. [3].

2.7. Air Exposure Stress Assay. After a 97-day feeding period, twelve polystyrene boxes were applied to induce experimental air exposure stress, each treatment with 3 replicates. For this purpose, soaked filter papers were stretched on the bottom of each box to maintain proportional humidity, and 3 crayfish from each treatment were transferred in these boxes and exposed to atmospheric air (23–25°C) [3, 19]. After 24 h exposure to atmospheric air, the survival rate of crayfish calculated and the hemolymph was withdrawn from each experimental animal separately to evaluate immunological and biochemical parameters including THC, LGC, SGC, HC, PO, LYZ, SOD, CAT, TPP, and glucose.

2.8. Statistical Analysis. Significant differences between four experimental groups were verified by one-way ANOVA followed by Duncan's multiple range test after checking the normality and homogeneity of variance. Student's *t*-test was used to compare the immune indices between two different time points. Broken-line regression analysis (quadratic model) was used to determine the optimum diet based on the measured parameters. Differences considered statistically significant at $P < 0.05$. Data are expressed as means ± SE. SPSS 21.0 package (SPSS Inc., Chicago, IL, USA) was applied to perform statistical analyses.

3. Results

3.1. Growth Performance and Survival Rate. At the end of the culture period, no significant differences were observed in terms of growth performance and survival rate between crayfish fed GLP-supplemented diets and the controls ($P > 0.05$) (Table 2). No mortality was recorded in crayfish fed GLP-supplemented diet or the control groups after a 24 h air exposure challenge.

3.2. Hemolymph Indices. After a 97-day feeding trial, the number of different hemocytes changed in different treatments (Table 3). The crayfish fed GLP7 showed significantly higher THC, SGC, and HC than the controls diet ($P < 0.05$) and higher SGC than the negative control ($P < 0.05$). However, the number of LGC remained the same in all groups ($P > 0.05$). After the air exposure stress, the number of THC, SGC, LGC, and HC remained unchanged only in the GLP7 ($P > 0.05$). In the control, all the indices except LGC increased significantly after the challenge ($P < 0.05$). The same was observed in the negative control, except in HC ($P < 0.05$). In contrast, in the GLP7 and GLP8, all the indices remained unchanged, but the LGC significantly increased in GLP8 ($P < 0.05$). In GLP9, all the indices increased after the air exposure challenge ($P < 0.05$). For hemolymph indices, GLP7 and GLP8 were considered as the optimum diet by the broken-line regression analysis (Table 3).

3.3. Plasma Immunological and Biochemical Parameters. The plasma levels of the PO and CAT remained unchanged in all the treatments at the end of the feeding trial (Table 4). However, the plasma levels of the LYZ increased in crayfish fed GLP7 and GLP8, with the former treatment demonstrating the highest value. The plasma level of the SOD only increased significantly ($P < 0.05$) in the GLP9 treatment. The levels of TPP in plasma increased significantly in all the experimental treatments, with the highest level in GLP7, whereas the plasma levels of GLC decreased significantly in all the experiments, with the lowest value in GLP9. After the air exposure stress, the crayfish fed GLP7 demonstrated the same levels of the PO, LYZ, SOD, and TPP. However, the level of the CAT increased significantly in this treatment, whereas the level of GLC decreased significantly ($P < 0.05$). In the GLP8 group, plasma levels of the PO, SOD, and TPP remained unchanged after the air exposure stress. Both the LYZ and CAT increased significantly, and the plasma level of the GLC decreased significantly ($P < 0.05$). Considering the GLP9 group, only the PO and CAT remained unchanged after the air exposure stress. The plasma levels of the LYZ and TPP significantly increased, whereas the values of the SOD and GLC significantly decreased (Table 4). For plasma immunological and biochemical parameters, GLP8 and GLP9 were chosen as the optimum diet by the broken-line regression analysis (Table 4).

3.4. Intestinal Microbiota. At the end of the feeding trial, no autochthonous LAB was isolated from the intestinal microbiota of the control group, but significantly higher amounts were detected in all the GLP-supplemented groups as well as

Table 2: Growth performance and survival rate of narrow-clawed crayfish fed with different experimental diets after 97 days.

Parameter	Experimental diets				
	Control	$Control_{(ng)}$	GLP7	GLP8	GLP9
Initial weight (g)	27.05 ± 0.78^{a}	28.37 ± 0.55^{a}	27.50 ± 0.83^{a}	26.47 ± 0.73^{a}	25.74 ± 0.84^{a}
Final weight (g)	40.19 ± 1.94^{a}	39.33 ± 3.92^{a}	39.62 ± 2.45^{a}	37.07 ± 1.56^{a}	39.62 ± 1.84^{a}
WG (%)	44.17 ± 6.96^{a}	41.07 ± 14.08^{a}	42.10 ± 8.79^{a}	32.96 ± 5.60^{a}	42.11 ± 6.60^{a}
PER	1.00 ± 1.16^{a}	0.93 ± 0.32^{a}	0.96 ± 0.20^{a}	0.75 ± 0.13^{a}	0.96 ± 0.15^{a}
LER	2.76 ± 0.43^{a}	2.57 ± 0.88^{a}	2.63 ± 0.55^{a}	2.06 ± 0.35^{a}	2.63 ± 0.41^{a}
SGR (% day^{-1})	0.37 ± 0.05^{a}	0.34 ± 0.10^{a}	0.36 ± 0.06^{a}	0.29 ± 0.04^{a}	0.36 ± 0.05^{a}
FCR	3.46 ± 0.41^{a}	3.73 ± 0.64^{a}	3.66 ± 0.52^{a}	3.96 ± 0.31^{a}	3.60 ± 0.32^{a}
CF	3.38 ± 0.14^{a}	3.14 ± 0.20^{a}	3.09 ± 0.20^{a}	3.29 ± 0.07^{a}	3.37 ± 0.11^{a}
Survival (%)	77.78 ± 0.00^{a}	75.93 ± 1.85^{a}	81.48 ± 4.90^{a}	77.78 ± 3.21^{a}	72.22 ± 3.21^{a}
MF (%)	59.26 ± 4.90^{a}	72.22 ± 8.48^{a}	66.67 ± 8.49^{a}	61.11 ± 3.21^{a}	74.07 ± 6.68^{a}

Data in the same row with different superscripts are significantly different ($P < 0.05$). Values are presented as mean ± SE. WG: weight gain; PER: protein efficiency ratio; LER: lipid efficiency ratio; SGR: specific growth rate; FCR: feed conversion rate; CF: condition factor; MF: molting frequency; Control: basal diet; $Control_{(ng)}$: basal diet+2% GOS; GLP7: GOS+*L. plantarum* (2% + 10^7 CFU g^{-1} diet); GLP8: GOS+*L. plantarum* (2% + 10^8 CFU g^{-1} diet); GLP9: GOS+*L. plantarum* (2% + 10^9 CFU g^{-1} diet).

Table 3: Differential hemocyte counts of narrow-clawed crayfish fed with different experimental diets after 97 days feeding trials (1^{st} sampling time; 1^{st} ST) and 24 h post air exposure challenge (2^{nd} sampling; 2^{nd} ST).

Parameter		Experimental diets					
		Control	$Control_{(ng)}$	GLP7	GLP8	GLP9	OD
THC (10^6 cells ml^{-1})	1^{st} ST	11.47 ± 0.34^{aA}	13.05 ± 0.03^{abA}	14.40 ± 0.26^{bA}	12.82 ± 0.56^{abA}	12.20 ± 0.38^{aA}	GLP7, GLP8
	2^{nd} ST	13.60 ± 0.10^{aB}	14.53 ± 0.35^{abB}	15.20 ± 0.32^{bA}	14.30 ± 0.06^{abA}	14.70 ± 0.26^{abB}	GLP9
LGC (10^6 cells ml^{-1})	1^{st} ST	1.60 ± 0.12^{aA}	1.75 ± 0.03^{aA}	1.90 ± 0.06^{aA}	1.93 ± 0.02^{aA}	1.70 ± 0.06^{aA}	GLP7, GLP8
	2^{nd} ST	2.17 ± 0.18^{aA}	2.00 ± 0.06^{aB}	2.20 ± 0.10^{aA}	2.40 ± 0.06^{aB}	2.17 ± 0.09^{aB}	GLP8
SGC (10^6 cells ml^{-1})	1^{st} ST	4.07 ± 0.09^{aA}	4.55 ± 0.03^{bA}	5.13 ± 0.12^{cA}	4.53 ± 0.23^{bA}	4.37 ± 0.09^{abA}	GLP7, GLP8
	2^{nd} ST	4.77 ± 0.09^{aB}	5.17 ± 0.15^{abB}	5.33 ± 0.12^{bA}	5.00 ± 0.06^{bA}	5.15 ± 0.10^{bB}	GLP7, GLP9
HC (10^6 cells ml^{-1})	1^{st} ST	5.80 ± 0.17^{aA}	6.75 ± 0.03^{abA}	7.37 ± 0.13^{bA}	6.35 ± 0.32^{abA}	6.13 ± 0.23^{aA}	GLP7, GLP8
	2^{nd} ST	6.67 ± 0.13^{aB}	7.37 ± 0.26^{abA}	7.67 ± 0.12^{bA}	6.90 ± 0.06^{abA}	7.38 ± 0.25^{abB}	GLP7

THC: total hemocyte count; LGC: large-granular cells; SGC: semigranular count; HC: hyaline count. Data in the same column with different superscript small letters are significantly different among experimental treatments within the same sampling time ($P < 0.05$). Superscript capital letters indicate significant difference ($P < 0.05$) between different sampling times for each treatment. Values are presented as mean ± SE ($n = 3$). Control: basal diet; $Control_{(ng)}$: basal diet+2% GOS; GLP7: GOS+*L. plantarum* (2% + 10^7 CFU g^{-1} diet); GLP8: GOS+*L. plantarum* (2% + 10^8 CFU g^{-1} diet); GLP9: GOS+*L. plantarum* (2% + 10^9 CFU g^{-1} diet); OD: optimum diet.

the negative control (Table 5). At this time, the amounts of autochthonous LAB bacteria were significantly higher in GLP8 than that of the GLP9. However, considering the TVC levels at the same time, there was no significant difference between GLP-supplemented groups with the control. Fourteen days after switching from GLP-supplemented diets to the basal diet, no autochthonous LAB detected in the control again. The amount of these bacteria was significantly higher in the GLP-supplemented groups, with no significant difference between them. In this time, no significant difference was found in the levels of TVC between all the experimental groups ($P > 0.05$). However, the LAB levels decreased significantly ($P < 0.05$) in GLP7 and GLP9 after switching to the basal diet, whereas the number of TVC bacteria remained unchanged ($P > 0.05$).

3.5. Digestive Enzyme Activity. Analyzing the digestive enzyme activity over the experimental period revealed an increasing trend in the GLP-supplemented groups (Table 6). The results showed that protease and lipase activity enhanced in all GLP-supplemented groups with the highest value in GLP9 ($P < 0.05$). Amylase activity increased significantly in all GLP-supplemented groups, regardless of inclusion levels ($P < 0.05$). ALP activity increased significantly in groups fed GLP7 and GLP8 diets ($P < 0.05$). GLP9 was considered the optimum diet based on the results of the broken-line regression analysis of the activities of the digestive enzymes (Table 6).

3.6. Body Proximate Composition. At the end of the experiment, the ash and protein levels in all treatments fed GLP-supplemented diets were remained unchanged ($P > 0.05$). The GLP9 group demonstrated the lowest level of moisture and the highest level of lipid. Also, in this experimental group, the highest level of gross energy (kJ g^{-1}) was measured, too ($P < 0.05$) (Table 7). Based on the results of the

Table 4: Some innate immune parameters of narrow-clawed crayfish fed with different experimental diets after 97 days of feeding trials (1st sampling time; 1st ST) and 24 h post air exposure challenge (2nd sampling; 2nd ST).

Parameter		Experimental diets					
		Control	$Control_{(ng)}$	GLP7	GLP8	GLP9	OD
PO ($U\,ml^{-1}$)	1st ST	1.43 ± 0.12^{aA}	1.30 ± 0.06^{aA}	1.27 ± 0.12^{aA}	1.57 ± 0.03^{aA}	1.27 ± 0.03^{aA}	GLP8
	2nd ST	1.67 ± 0.03^{aA}	1.83 ± 0.26^{aA}	1.53 ± 0.09^{aA}	1.77 ± 0.09^{aA}	1.20 ± 0.06^{aA}	GLP8
LYZ ($U\,ml^{-1}$)	1st ST	5.03 ± 0.80^{aA}	7.70 ± 0.21^{bA}	9.63 ± 0.19^{cA}	7.27 ± 0.39^{bA}	4.03 ± 0.24^{aA}	GLP7, GLP8
	2nd ST	13.33 ± 1.20^{aB}	11.50 ± 0.76^{aB}	10.80 ± 1.61^{aA}	12.33 ± 1.20^{aB}	9.75 ± 0.61^{aB}	GLP9
SOD ($U\,ml^{-1}$)	1st ST	14.40 ± 0.92^{aA}	14.40 ± 0.35^{aA}	18.00 ± 0.92^{abA}	17.80 ± 0.20^{abA}	21.20 ± 1.40^{bB}	GLP9
	2nd ST	24.80 ± 0.72^{cB}	20.00 ± 0.53^{bB}	19.60 ± 1.11^{bA}	19.20 ± 1.25^{bA}	15.30 ± 1.56^{aA}	GLP9
CAT ($U\,ml^{-1}$)	1st ST	4.77 ± 0.64^{aA}	5.47 ± 1.51^{aA}	7.20 ± 0.66^{aA}	4.27 ± 0.41^{aA}	12.00 ± 2.65^{aA}	GLP9
	2nd ST	4.57 ± 0.58^{aA}	5.67 ± 1.09^{abA}	9.83 ± 0.62^{abB}	11.20 ± 1.96^{bB}	6.60 ± 0.29^{abA}	GLP8
TPP ($g\,dl^{-1}$)	1st ST	2.53 ± 0.23^{aA}	3.13 ± 0.18^{abA}	5.47 ± 0.19^{dA}	3.90 ± 0.21^{cA}	3.50 ± 0.23^{bcA}	GLP7, GLP8
	2nd ST	5.57 ± 0.12^{cB}	5.00 ± 0.25^{abB}	5.23 ± 0.15^{bcA}	4.47 ± 0.15^{aA}	4.57 ± 0.15^{aB}	GLP8
GLC ($mg\,dl^{-1}$)	1st ST	71.33 ± 2.03^{cB}	48.33 ± 4.49^{bB}	48.00 ± 4.36^{bB}	50.00 ± 2.65^{bB}	33.67 ± 2.33^{aB}	GLP9
	2nd ST	27.33 ± 4.33^{bcA}	15.67 ± 0.88^{aA}	14.67 ± 2.03^{aA}	31.00 ± 2.31^{cA}	20.00 ± 0.58^{abA}	GLP7

Data in the same column with different superscript small letters are significantly different among experimental treatments within the same sampling time ($P < 0.05$). Superscript capital letters indicate significant difference ($P < 0.05$) between different sampling times for each treatment. Values are presented as mean ± SE ($n = 3$). ND: not detected. Control: basal diet; $Control_{(ng)}$: basal diet+2% GOS; GLP7: GOS+*L. plantarum* ($2\% + 10^7$ CFU g^{-1} diet); GLP8: GOS +*L. plantarum* ($2\% + 10^8$ CFU g^{-1} diet); GLP9: GOS+*L. plantarum* ($2\% + 10^9$ CFU g^{-1} diet); OD: optimum diet.

Table 5: Total viable heterotrophic aerobic bacteria (TVC) and autochthonous lactic acid bacteria (LAB) levels (Log CFU g^{-1}) of narrow-clawed crayfish fed with different experimental diets after 97-day feeding trials and 14 days after switching to basal diets.

Experimental diets	LAB		TVC	
	After 97-day feeding trial	14 days after switching to basal diet	After 97-day feeding trial	14 days after switching to basal diet
Control	ND[a]	ND[a]	6.78 ± 0.14^{aA}	7.27 ± 0.23^{abA}
$Control_{(ng)}$	4.52 ± 0.34^{bB}	2.72 ± 0.09^{bA}	8.13 ± 0.08^{aA}	10.27 ± 0.00^{cB}
GLP7	4.40 ± 0.21^{bB}	3.57 ± 0.04^{cdA}	8.06 ± 0.61^{aA}	7.54 ± 0.03^{abA}
GLP8	4.52 ± 0.27^{bA}	3.77 ± 0.05^{dA}	7.44 ± 0.07^{aA}	8.48 ± 1.03^{bA}
GLP9	3.90 ± 0.09^{bB}	2.91 ± 0.32^{bcA}	6.82 ± 0.28^{aA}	6.76 ± 0.40^{aA}

Data in the same column with different superscript small letters are significantly different among experimental treatments within the same sampling time ($P < 0.05$). Superscript capital letters indicate significant difference ($P < 0.05$) between different sampling times for each treatment. Values are presented as mean ± SE ($n = 3$). ND: not detected. Control: basal diet; $Control_{(ng)}$: basal diet+2% GOS; GLP7: GOS+*L. plantarum* ($2\% + 10^7$ CFU g^{-1} diet); GLP8" GOS+*L. plantarum* ($2\% + 10^8$ CFU g^{-1} diet); GLP9: GOS+*L. plantarum* ($2\% + 10^9$ CFU g^{-1} diet).

Table 6: Protease, lipase, amylase, and ALP (alkaline phosphatase) ($U\,mg^{-1}$ protein) of narrow-clawed crayfish fed with different experimental diets after 97 days.

Parameter	Experimental diets					
	Control	$Control_{(ng)}$	GLP7	GLP8	GLP9	OD
Protease	1.45 ± 0.01^{a}	1.51 ± 0.04^{a}	1.63 ± 0.01^{b}	1.74 ± 0.03^{c}	1.84 ± 0.03^{d}	GLP9
Lipase	0.12 ± 0.00^{a}	0.30 ± 0.01^{cd}	0.21 ± 0.01^{b}	0.25 ± 0.02^{bc}	0.32 ± 0.01^{d}	GLP9
Amylase	4.37 ± 0.12^{a}	4.77 ± 0.09^{ab}	5.37 ± 0.12^{bc}	5.83 ± 0.28^{cd}	6.13 ± 0.35^{d}	GLP9
ALP	2.37 ± 0.09^{a}	2.57 ± 0.42^{ab}	3.33 ± 0.12^{c}	3.13 ± 0.12^{bc}	2.73 ± 0.12^{abc}	GLP8

Data in the same row with different superscripts are significantly different ($P < 0.05$). Values are presented as mean ± SE. Control: basal diet; $Control_{(ng)}$: basal diet +2% GOS; GLP7: GOS+*L. plantarum* ($2\% + 10^7$ CFU g^{-1} diet); GLP8: GOS+*L. plantarum* ($2\% + 10^8$ CFU g^{-1} diet); GLP9: GOS+*L. plantarum* ($2\% + 10^9$ CFU g^{-1} diet); OD: optimum diet.

Table 7: Body composition (% dry weight) of narrow-clawed crayfish fed with different experimental diets after 97 days.

Parameter	Experimental diets					
	Control	$Control_{(ng)}$	GLP7	GLP8	GLP9	OD
Moisture*	75.15 ± 0.19^{b}	76.49 ± 0.31^{c}	75.10 ± 0.32^{b}	75.20 ± 0.17^{b}	72.85 ± 0.31^{a}	GLP7
Protein	37.91 ± 0.18^{a}	38.43 ± 0.81^{a}	40.81 ± 0.31^{ab}	40.35 ± 0.23^{ab}	42.70 ± 1.98^{b}	GLP9
Lipid	1.05 ± 0.30^{a}	1.31 ± 0.27^{a}	1.45 ± 0.14^{a}	1.47 ± 0.56^{a}	3.02 ± 0.31^{b}	GLP9
Ash	33.28 ± 0.19^{a}	33.22 ± 0.41^{a}	32.03 ± 1.28^{a}	31.84 ± 1.09^{a}	33.37 ± 1.60^{a}	GLP9
Gross energy (kJ g^{-1})**	9.40 ± 0.77^{a}	9.63 ± 0.30^{a}	10.25 ± 0.67^{a}	10.14 ± 0.17^{a}	11.32 ± 0.59^{b}	GLP9

*Moisture is calculated based on % wet weight. **Gross energy was calculated on the basis of 23.7 kJ g^{-1} of protein and 39.5 kJ g^{-1} of lipid. Data in the same row with different superscripts are significantly different ($P < 0.05$). Values are presented as mean ± SE. Control: basal diet; $Control_{(ng)}$: basal diet +2% GOS; GLP7: GOS+*L. plantarum* (2% + 10^{7} CFU g^{-1} diet); GLP8: GOS+*L. plantarum* (2% + 10^{8} CFU g^{-1} diet); GLP9: GOS+*L. plantarum* (2% + 10^{9} CFU g^{-1} diet); OD: optimum diet.

broken-line regression analysis of the body proximate composition, GLP9 was determined as the optimum diet (Table 7).

4. Discussion

In this work, dietary inclusion of combined GOS and *L. plantarum* at the optimum level (GLP8 and GLP9) exhibited positive effects as immunostimulatory potential through upregulating the crayfish hemolymph indices (THC, LGC, SGC, and HC; GLP8), plasma innate immunity (LYZ, SOD, and CAT; GLP9), and biochemistry (TPP and glucose; GLP8 and GLP9). However, growth performance parameters were unaffected.

Improved growth performance through synbiotic administrated diets has been already reported in many fish and shellfish species [4, 25, 44–47]. In the present study, despite the positive results obtained in term of digestive enzyme activity, dietary *L. plantarum* and GOS could not prompt growth performance and survival rate. Similarly, growth performance parameters were unaffected in MOS fed *P. leptodactylus* [15]. In comparison to the present results, Huynh et al. [48, 49] reported a significant impact of GOS and *L. plantarum* in a synbiotic form on growth performance parameter. It is not clear, but this contradiction may be due to the difference in species, dosage, and experimental set up.

Hemolymph biochemical and immune indices as commonly used parameters have already well demonstrated the health status of crustaceans through nutritional manipulation ([2–4, 23, 50]; Huynh Minh [1, 5]). Enhanced immune parameters following immunostimulant-enriched diet feeding could supply greater defense reactions and resistance in crustaceans during stressful situations and diseases. Interestingly, in agreement with a previous study, applying a dietary supplemented with a combination of GOS-*Enterococcus faecalis* and GOS-*Pediococcus acidilactici* enhanced immune parameters such as THC, DHC, LYZ, and SOD activities in *P. leptodactylus* [51]. The significantly increased THC and LGCs were found in *L. vannamei* when fed with synbiotic (cacao pod husk (CPH) pectin and *L. plantarum*) supplemented diet for 7–28 days [52]. In another study, a synbiotic (byproduct of king oyster mushroom and *L. plantarum* 7-40) containing diet improved LYZ activity of *L. vannamei* at the end of the feeding trial [53]. Moreover, feeding by *Bacillus cereus* enhanced THC, LYZ, and TPP values in *Penaeus monodon* [54]. In a study conducted on *Penaeus japonicus*, dietary administration of a combination of isomaltooligosaccharide, *Bacillus licheniformis*, and *Bacillus subtilis* could boost THC, LYZ, and SOD activities ([9]). In addition, dietary supplementations consisting of a combination of *β*-glucan-*B. subtilis* and *β*-glucan-*P. acidilactici* significantly increased SOD and PO activities in *L. vannamei* [11]. In another study conducted on *Apostichopus japonicus*, probiotic mixture of *B. subtilis* and *B. cereus* enhanced LYZ and CAT activities [55]. However, in comparison, neither individual nor a combined dietary of GOS and *L. plantarum* in *L. vannamei* stimulate THC, LYZ, and SOD activities [48, 49]. The conflicting results across studies may be related to species-specific impacts of synbiotics and the effects of various factors such as prebiotic origin, probiotic strain, administration dose, culture period, and the applied experimental species as well as the culture condition which all may affect the results [46, 56, 57]. In the present study, the reduction of plasma glucose concentration after feeding with GLP-supplemented diets may reflect an ideal physiological status in crayfish. Accordingly, several investigators have already pointed to the role of prebiotic, probiotic, and/or their combination to alleviate stress via reducing of cortisol and glucose values [57, 58].

Air exposure stress is considered one of the most practical stressors applied at the end of the nutritional experiments to evaluate the health status of crustaceans [1, 3, 23, 59]. In this regard, the hemolymph immunological indices such as THC and DHC ([19, 60, 61]), PO [20, 62], LYZ [5, 21], SOD, and CAT [63–65], as well as biochemical parameters, e.g., glucose and TPP [17, 18, 66], have already been applied as sensitive biomarkers of air exposure stress effects in shellfish. Similar to previous studies [3, 5], during air exposure stress, no sign of mortality was detected in all GLP-supplemented and control groups. Consistent with the obtained results, studies conducted on marron (*Cherax cainii*) fed pre- or probiotic revealed that 24 h subjecting to air [59] as well as 36 h ([1]) did not alter the survival rate. However, GLP-supplemented crayfish in the present study demonstrated better performance in response to stress due to enhance THC, SGC, HC, and CAT activities compared to the control. Similar results obtained in the previous study on *P. leptodactylus* following *L. plantarum* administration

[5]. Additionally in a study conducted on marron, different MOS inclusion levels could significantly enhance THC and LGC proportion compared to the unsupplemented group after air exposure ([1]). Moreover, higher health status in marron fed *Bacillus mycoides* represented by higher THC levels following air exposure stress [59].

The results demonstrated that all GLP inclusion levels were able to promote PO activity, neither pre- nor poststress. The obtained result is in agreement with those on *P. leptodactylus* [3], *P. monodon* [67], *L. vannamei* [53, 68, 69], *P. japonicus* (Q. [9]), and *Macrobrachium rosenbergii* [70] with no improvement of PO activity but in contrast with the study conducted in *P. leptodactylus* [51] and *L. vannamei* [48, 49, 52] following feeding with different pre- and/or probiotic. These discrepancies proposed that the profitable impacts of immunostimulants may be case-dependent and profoundly influenced by experimental condition.

No significant changes in the number of LGC after air exposure stress may be explained by the changes in the proportion of the differentiated hemocyte ([60]) as well as the time-consuming procedure of hemocyte differentiation [61]. Besides, there is an overlap between LGC and SGC functions, which in the absence of the former, the latter can partly compensate its duties [71]. Moreover, no changes in LYZ activity in the GLP-supplemented groups' postexposure to air may be related to the number of LGC that remained unchanged. These hemocytes considered the main place for LYZ synthesis [72, 73]. Similar to these results, β-glucan and a combination of β-glucan-*B. subtilis* or β-glucan-*P. acidilactici* did not alter LYZ activity in *L. vannamei* postammonia challenge [11]. However, single and combined administration of fructooligosaccharide and mannanoligosaccharide enhanced LYZ level in *P. leptodactylus* after exposure to air [21].

During the innate immunity in crustaceans, activated hemocytes generate a wide range of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [74]. However, there are regulatory mechanisms such as SOD and CAT, two principal antioxidant enzymes [75, 76], which sustain the oxidative stress balance [3]. SOD activity was just enhanced in the control group after air exposure stress, while the GLP-supplemented groups demonstrated lower SOD activity in comparison to the control. These results suggested that crayfish fed GOS and *L. plantarum* may not be subjected to extra oxidative stress, and surplus synthesis of SOD enzyme over the usual condition following air exposure may be unwarranted [5]. Furthermore, increased H_2O_2 level as a byproduct of SOD activity may have repressive effect on SOD enzyme [77] and provide status for CAT secretion [78]. Crayfish in GLP7 and GLP8 demonstrated significant increase in CAT activity after exposure to air. Besides, air exposure stress led to the reduction of the glucose level in all treatments compared to the before exposure time. This may be related to the physiology of the organism to keep down metabolism to expend less energy during stressful condition. In line with these results, previous studies demonstrated a time-dependent and transitory increase in glucose concentration in different crustaceans following exposure to air [19, 79, 80]. Therefore, decrease in the glucose level in the present study may be a physiological reaction to prolonged exposure to air.

The precise characteristic of the prebiotic is expanding the colonization of host lucrative microflora (lactobacilli and bifidobacteria) [81] as well as probiotic bacteria when applied in the form of synbiotic ([82, 83]). Of interest, production of short-chain fatty acids (SCFA) and other byproducts, e.g., amino acids, polyamins, and vitamins, after fermentation of oligosaccharides by the probiotic and other beneficial bacteria is considered the principal parameters that reflect synbiotic immunostimulatory effects on fish and shellfish species ([83]). Moreover, immunomodulatory activity of GOS and *L. plantarum* may be directly mediated upon recognition and adhesion of the ultraconserved components presented in their cell walls by pattern recognition receptors (PRRs) in cell-free hemolymph. The extracellular signals activated because of this process can be able to stimulate different pathways of cellular and humoral immune reactions [74, 81].

The major purpose of dietary prebiotic and probiotic application in crustacean's aquaculture is elevation of beneficial LAB bacteria, which are not dominant parts of crustacean intestinal microbiota [84, 85]. Interestingly, the results from 97 days of feeding in the present study well exhibited that combination of GOS and *L. plantarum* through diet could beneficially modulate *P. leptodactylus* intestinal microbiota via growth of LAB bacteria. Interestingly, previous study conducted on *L. vannamei* revealed significant increase in intestinal LAB bacteria following GOS and *L. plantarum* supplementation [48, 49]. Moreover, *L. vannamei* received the GOS and *L. plantarum* containing diet presented modified intestinal colonization of *L. plantarum* and decreased domination of *Vibrio harveyi* and *Photobacterium damselae* ([86]). Additionally, a superior increase of the LAB bacteria genus *Pediococcus* was observed in red drum fed with the prebiotic (Grobiotic®-A) enriched diet [87]. In a study conducted on *P. leptodactylus*, single and combined administration of GOS and *E. faecalis* and GOS and *P. acidilactici* enhanced the ratio of LAB bacteria to TVC [51]. In vitro examination previously well exhibited that GOS has been generally utilized by *L. plantarum* as a carbon source [48, 49, 88]. During this catabolic process, GOS is translocated by an extracellular galactoside-pentose-hexuronide (GPH) type LacS permease. Thereafter, intracellular catabolism is carried out along with two cytoplasmic β-galactosidases: LacA hydrolyze GOS into glucose, which further metabolized via the glycolytic pathway, and LacLM hydrolyze GOS into galactose, which eventually metabolized through the Leloir pathways [89]. Therefore, GOS metabolism through diverse pathways can induce growth and colonization of *L. plantarum* and other beneficial specific LAB bacteria. Moreover, analysis of the autochthonous intestinal microbiota revealed that TVC just increased in the GLP7 group. Combined administration of IMO with *B. licheniformis* and/or *B. subtilis* increased TVC in *P. japonisus* ([9]). In contrast, combination of *Bacillus* OJ and IMO in *L. vannamei* significantly decreased TVC [68, 69]. Moreover, 14 days after switching from GLP-supplemented diet to the basal

diet, no reduction was determined in LAB count in GLP8 treatment. Therefore, it could be assumed that GOS and *L. plantarum* combination may provide more sustainable microbial balance through long-lasting colonization and survival of LAB bacteria compared to those fed with individual one [3, 5].

Evaluation of amylase, lipase, and protease levels in the alimentary tract has been proposed as useful indicators for animal's digestive efficiency ([50, 90, 91]). Accordingly, the ability of pre- or/and probiotic to modulate digestive enzyme activity in aquatic animals has been proven previously [92]. In the present study, higher amounts of digestive enzyme activity demonstrated in *P. leptodactylus* through feeding by *L. plantarum* and GOS may be achieved through enhanced population of beneficial microbial community such as LAB, which has already demonstrated wide spectrum of enzymatic potential [93–95]. The increased exogenous enzymes levels produced by bacterial flora might induce biosynthesis and secretion of indigenous digestive enzyme by the host ([96]). In line with the obtained results, improved digestive enzyme activity through single or combined administration of GOS and *L. plantarum* has been reported previously [3, 5, 48, 49]. In another study, the amylase and lipase activities significantly improved in Sobaity (*Sparidentex hasta*) fingerlings after feeding a supplemented diet with 10^6 CFU g^{-1} L. *plantarum* [97]. The higher exogenous and endogenous digestive enzyme activity due to the pre- or/and probiotic administration may ameliorate chemical digestion of protein, lipid, and carbohydrate and consequently result in improved feed efficiency ratio and growth performance ([98]). Accordingly, the highest amounts of protease and lipase activity in crayfish fed with GLP9 diet could be a probable reason for improved body lipid level and gross energy in this group. Interestingly, the utilization of *L. plantarum* in *M. rosenbergii* significantly enhanced body protein [99]. Additionally, in a study conducted on *L. vannamei*, combined application of medicinal herbs and probiotic *Bacillus* sp. through feed significantly enhanced body lipid level [100]. The enhanced body lipid in the crayfish fed with GLP9-supplemented diet suggested that utilized food through synbiotic supplementation could be more efficiently convert into the energy deposition, which is one of the most important features of aquaculture practice [101]. Additionally, higher body lipid accumulation may be achieved by increasing fatty acid absorption efficiency through pre- or/and probiotic utilization in aquatic animals [102].

Overall, the present study clearly revealed that combination of GOS and *L. plantarum* could beneficially affect the well-being of *P. leptodactylus* through improving of immune status including THC, DHC, LYZ, SOD, CAT, TPP, and glucose, intestinal microbiota, digestive enzyme activity, and carcass composition. Additionally, after exposure to air, GOS and *L. plantarum*-fed crayfish were healthier than those fed with the basal diet, by better performance of immune indices like THC, DHC, and CAT. Together, these outputs suggested a combination of 20 g kg^{-1} diet and GOS $+10^9$ CFU g^{-1} diet *L. plantarum* as a suitable immunostimulant for crayfish aquaculture practice.

Data Availability

The data that support the findings of this study are available upon reasonable request to the corresponding author.

Ethical Approval

All experiments were performed following the protocol approved by the committee of ethics of the faculty of sciences of the University of Tehran (357; 8 November 2000).

Conflicts of Interest

The authors have no conflict of interest to declare for the publication of the work herein.

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