Effects of synbiotics on immunity and disease resistance of narrow-clawed crayfish, *Astacus leptodactylus leptodactylus* (Eschscholtz, 1823)

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**A R T I C L E  I N F O**

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**A B S T R A C T**

The aim of this study was to evaluate the effects of prebiotics (mannanoligosaccharide and xyloooligosaccharide), probiotics (*Enterococcus faecalis* and *Pediococcus acidilactici*) and synbiotics for 126 days on the immune responses, hemolymph indices, antioxidant enzymes, and biological responses after a 48-hour *Aeromonas hydrophila* exposure of sub-adult crayfish (11.45 ± 1.87 g). Most antibacterial activities were observed in the shell mucus of crayfish fed a diet containing xyloooligosaccharide + *E. faecalis* and mannanoligosaccharide + *Pediococcus acidilactici* against *Nocardiia Brasilience* and *Vibrio harveyi* (p < 0.05). Feeding crayfish a xyloooligosaccharide + *E. faecalis* diet increased protein levels and the activities of alkaline phosphatase and lysozyme in the shell mucus after the feeding trial and 48 h after the *A. hydrophila*-injection challenge (p < 0.05). The highest ratio of the lactobacillus count to the total viable count was observed in synbiotic diets (p < 0.05). Feeding crayfish a xyloooligosaccharide + *E. faecalis* diet increased the growth rate and the resistance to the *A. hydrophila*-injection challenge (p < 0.05). These results revealed that feeding crayfish with synbiotic diets was more effective than a single administration with prebiotics and probiotics.

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1. Introduction

The gills of decapod crustaceans have the potential to remove invasive microorganisms through two mechanisms—phagocytosis or hemocyte encapsulation—which is started by the prophenoloxidase (proPO) activating system [1]. Live parasites and their cell-wall composition, including 1, 3-glucan in bacteria and lipo-polysaccharide and peptidoglycan in fungal species, are regarded as stimulators of the proPO system [2]. Tegmental glands play a key role in the life cycle of crustacean species, including epicuticle secretion, tanning of the integument, mucus production for feeding lubrication or food entanglement, cement production for egg attachment, and production of a bacteriostatic and anti-fouling agent [3–8]. These glands with unicellular and multicellular structures produce acidic sulphated and carboxylated mucopolysaccharides [7,8]. To protect crustacean species similar to the finfish, the mucosal surface is considered as the first physical barrier against opportunistic pathogenic organisms [7–9]. Innate immune components in the finfish mucus skin (lysozyme, protease, lectins, protease, and C-reactive proteins) were reported in the literature [9,10]. Microbicidal activity in the hemolymph of tiger shrimp (*Penaeus monodon*) immersed in three immunostimulants (heat-killed cells of *Vibrio vulnificus*, β-1,3-1,6-extracted from cell walls of *Saccharomyces cerevisiae*, and β-1,3-glucan-protein-lipid compound extracted from cell wall of *S. cerevisiae*) showed that the hemolymph could eliminate the invading bacteria within three hours [1]. A. monodon injection with heat-killed *V. alginolyticus* removed the majority of stressful agents within four hours [11]. While evaluating bactericidal effects in shellfish species, it is important to consider the form (viable and non-viable), virulence of pathogen, type of test animals (shrimp and prawn), experimental protocols (immersion, injection and feeding), rearing conditions (e.g. salinity content), and measuring methods (e.g. enzyme immunoassay) [1,2,12–14]. However, the effects of feeding protocols and dietary additives did not reveal that microbicidal effects of the mucosal surface in decapod crustaceans.

Recently, dietary manipulation using feed additives (nucleotides, organic salts, prebiotics, probiotics, parabiotics, synbiotics, and phyto-products) had been considered as one of the important strategies to enhance immune responses, scale up performance,
and increase the survival rate in the crayfish culture (astaciculture) industry [14–18]. Symbiotics, the combined forms of probiotics and prebiotics, are regarded as potential feed additives with growing interests and worries about achieving a sustainable aquaculture industry [13,19–21]. The synergistic effects of symbiotics on biological indices and the stress resistance of cultivable aquatic species were reported in previous studies [13,19,22,23].

The successful inclusion of two types of gram-positive cocci (Enterococcus faecalis, Pedicoccus acidilactici) [24] in finfish and shrimp aquafeeds have been confirmed [14,22,25–29]. Mannanoligosaccharide (glucosamnoprotein-hydrolyzed fungi cell wall; MOS) and xylooligosaccharide (chemical and enzymatically-hydrolyzed lignocellulosic materials; XOS) as potential prebiotics have positive effects on the biological indices of Atlantic salmon (Salmo salar), common carp (Cyprinus carpio), Caspian roach (Rutilus rutilus), red drum (Sciaenops ocellatus), and freshwater crayfish (Astacus leptodactylus leptodactylus) [16,30–33]. The beneficial effects of enriched artemia with symbiotics (P. acidilactici and fructooligosaccharide) in the diet of angelfish (Pterophyllum scalare) on the mucosal immunity was seen [36]. The ability of symbiotics—the best combination between probiotic and a special prebiotic as the substrate—to maximize the population of beneficial gut microbiota after ceasing treatment, has been critically evaluated in the aquafeed industry [20,37].

Owing to the economic importance of astaciculture and the health and welfare of cultured crayfish in farm conditions, we will identify the potential dietary additives in order to obtain sustainable production. The aim of the study was to evaluate the effects of two selected symbiotics on the immunity, bacteriocidal responses, and disease resistance of juvenile narrow-clawed crayfish (Astacus leptodactylus leptodactylus).

2. Material and methods

2.1. Experimental diets

A basal diet was formulated (Table 1) as described previously [16]. To prepare experimental diets, mannanoligosaccharide (MOS; International Enterprise Corporation Co., USA; DP: 6) and xylooligosaccharide (XOS; Shandong Longlives Biotechnology Co., China; DP: 3) as prebiotics as well as E. faecalis (Nichi Nichi Pharmaceutical Co., Ltd, Japan; 7.59 log CFU g⁻¹) and P. acidilactici (Bactocell®, Lallemand Inc., Montreal, QC, Canada; 7.59 log CFU g⁻¹) as probiotics were used. Both the probiotic strains used in the present study were 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 used. Fresh colonies of the probiotics were obtained after re-culturing on MRS agar (Merck, UK). Bacterial numbers were estimated by serial dilutions being plated in triplicate on MRS agar plates and counted after 24 h of incubation at 30 °C for E. faecalis and 37 °C for P. acidilactici. The experimental diets were prepared as the following: (1) control; (2) MOS (10 g kg⁻¹); (3) XOS (10 g kg⁻¹); (4) E. faecalis (EnF; 7.86 log CFU g⁻¹); (5) P. acidilactici (PeA; 7.86 log CFU g⁻¹); (6) MOS (10 g kg⁻¹) + EnF (7.86 log CFU g⁻¹); (7) XOS (10 g kg⁻¹) + EnF (7.86 log CFU g⁻¹); (8) MOS (10 g kg⁻¹) + PeA (7.86 log CFU g⁻¹); (9) MOS (10 g kg⁻¹) + PeA (7.86 log CFU g⁻¹).

2.2. Crayfish and sample collection

Five hundred and forty healthy sub-adult crayfish (11.45 ± 1.87 g) were obtained from a local reservoir and stocked at a density of 20 crayfish per 1000-L tank (2 × 1 × 0.5 m) in a semi-recirculating system with a daily water exchange rate of 35% at three replicates for each experimental diet. Each tank was fitted with 20 plastic tubes (4 cm diameter and 12 cm length), which served as hiding places for the animals. The water temperature was maintained at 25.5 °C throughout the feeding trial. DO (6.68 ± 0.36 mg l⁻¹), pH (7.18 ± 0.64), hardness (145 ± 4.1 mg l⁻¹ as CaCO₃), unionized ammonia (<0.06 mg l⁻¹), and nitrite contents (<0.6 mg l⁻¹) were evaluated every week. The animals were held under L:D 14:10 h. Briefly, each diet was randomly assigned to a tank of crayfish and they were fed 4% of their body weight thrice daily (8 a.m., 2 p.m., and 8 p.m.) for 126 days. Biometry was done during the first and last days of the experiment.

2.3. Evaluation of 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 ingested feed. The growth parameters and the survival rate were calculated as follows [16,38]:

Specific growth rate (SGR; % day⁻¹) = [(lnWfi − lnWfi)/t] × 100

Feed conversion ratio (FCR) = (Feed consumed/W gain)

Survival rate (%) = (Final individual numbers/Initial individual numbers) × 100

In the above equations, Wi, Wf, W gain, t, and Feed consumed are initial weight, final weight, weight increment (g), time period (day) and feed consumed (g), respectively.

2.4. Biochemical analyses

2.4.1. Hemolymph indices

On the 126th day, five crayfish from each tank (15 crayfish per
treatment) were killed after 24 h of feeding. All assays were done one by one at three replicates. According to the protocol previously described [16], the hemolymph was obtained from the ventral sinus with a needle (25G), pooled, and stored via two following methods: (1) one milliliter microtube without an anticoagulant agent and (2) one milliliter microtube containing 0.4 ml Alsever as an anticoagulant and then divided into two parts. Briefly, one part (125 µl) was used to measure the following hemolymph indices: THC with hemocytometer cell (Beco, Hamburg, Germany) [39], hyaline count (HC), semi and large granular count (SGC and LGC, respectively) via hemolymph extension at room temperature (25 °C), fixation at methanol for one minute, staining with the May-Grünwald-Giemsa method and then counting with the light microscope [40]. Total plasma protein was estimated using the biuret procedure.

### 2.4.2. 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 plasma and the supernatant fluid was used for plasma determinations [16,41]. All activities of enzymes were standardized based on the protein concentration. Phenoloxidase activity (PO) was assayed spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) at a final reading of 490 nm [16,42]. The superoxide dismutase (SOD) activity was measured by observing the inhibition of ferricytochrome C reduction at the final reading 550 nm [43]. The lysozyme (LYZ) activity was determined with a decrease in the absorbance comparison to Micrococcus lysodeikticus suspension without plasma at final reading of 530 nm [44]. Nitric oxide synthase (NOS) activity was measured with the assay kit (Nanjing Jiancheng Bioengineering Institute, China) [45].

### 2.5. Shell mucus parameters

#### 2.5.1. Mucus collection

At the end of the feeding trial (126 days), the shell mucus samples (20 crayfish per treatment) were collected from previously starved crayfish for 24 h [10]. Briefly, the tested crayfish were transferred into polyethylene bags containing NaCl (50 mM; 5 ml/g crayfish). The crayfish were removed from the bags after gently shaking (for five minutes) and returned to recovery tanks. The mucus samples were immediately transferred to 15 ml sterile centrifuge tubes, centrifuged (5810R Eppendorf, Engelsdorf, Germany) (1500 g, 10 min, 4 °C) and the supernatants were stored in centrifuge tubes (2 ml) at −80 °C.

#### 2.5.2. Alkaline phosphatase (ALP) and lysozyme (LYZ)

The soluble protein concentration of shell mucus samples was measured after a 126-day feeding trial at a final reading of 750 nm by spectrophotometer (Biochrom, Libra S12). Bovine serum albumin (Sigma Aldrich) was used as the standard [46]. The ALP activity of the mucus was measured using a commercial kit (Pars Azmoun Co, Iran). The LYZ activity was determined at a final reading of 530 nm, according to the method previously described in Section 2.4.2 [44].

#### 2.5.3. Mucus bactericidal activity

The bactericidal activity of shell mucus samples was measured using diffusion disc plates on agar media [47]. In order to evaluate bactericidal activity, five bacterial strains, including Aeromonas hydrophila (ATCC 7966), Nocardia brasiliensis (PTCC 1422), Sphrumpalma mirum (ATCC 29335), Vibrio harveyi (PTCC 1755), and Vibrio mimicus (ATCC 33654) were selected based on previous reports [48].

After culturing bacteria (24 h at 37 °C) in the nutrient broth medium (Merck, Germany), aliquots (0.1 ml) of each broth culture medium (1.5 × 10² CFU ml⁻¹; OD₆₀₀) were cultured on nutrient agar (Merck, Germany). Paper discs (6 mm diameter) were inoculated with 150 ml of the mucus sample and kept for 20 min to absorb the mucus [37], placed in the medium, and incubated (37 °C for 24 h). Finally, the discs were checked and the diameter of the growth-inhibition zone was measured with a ruler. A clear zone enveloping the discs was considered as bactericidal activity.

### 2.6. Bacteriological analysis

At the start of the feeding trial, the total viable counts (TVC) of heterotrophic aerobic bacteria and presumptive lactic acid bacteria (LAB) levels in hepatopancreas were determined by a random sampling of 20 crayfish from the stock. As described previously [16], at the end of the experiment, crayfish (12 individuals per treatment) were transported alive to the laboratory, anesthetized with ice, rinsed with benzalkolium chloride (0.1% for 60 min) and dissected with a scalpel. Then the hepatopancreas was removed, homogenized with sodium chloride (0.9 w/v) using a homogenizer (DI 18 Disperser), and the homogenate was centrifuged at 5000 × g for five minutes. One hundred microliters from the samples prepared were by plate count agar (PCA; Merck Co) and de Man, Rogosa, and Sharpe media (MRS; Merck Co) at three replicates to determine the TVC and the LAB, respectively. The plates were incubated at room temperature (25 °C) for five days and colony-forming units (CFU) g⁻¹ were calculated from plates containing 30–300 colonies [49].

### 2.7. Bacterial exposure challenge

The challenge test was initiated on day 127 of the feeding trial. Twelve crayfish from each test diet-tank were injected with 1 × 10⁶ cells ml⁻¹ Aeromonas hydrophila, ATCC 49141 through the base of the fifth thoracic leg with 20 ml bacteria stock suspension [16,50]. The injected crayfish were marked before being released back into their original tanks to avoid repeat-sampling. The infected crayfish were monitored for survival rate, PO, SOD, LYZ, and NOS after 48 h of the injection.

### 2.8. Statistical analysis

All percentage data was transformed using the arcsine method. After confirming the homogeneity of variance and the normality of the data using the Leven and the Kolmogorov–Smirnov tests [51], respectively, an ANOVA was used to compare the treatments at four replicates. The Duncan test was also used to compare significant differences among the treatments (p < 0.05) with SPSS™ version 19. All results were given as mean ± SD.

### 3. Results

#### 3.1. Mucus bactericidal activity

The bactericidal activity of crayfish shell mucus after the 126-day feeding trial on the test diets showed significant differences (p < 0.05) among the control diet and the diets containing probiotics, prebiotics, and symbiotics (Fig. 1). The highest growth-inhibition zone was observed in shell mucus of crayfish in the diets containing XOS + EnF and MOS + PeA against N. brasiliensis and V. harveyi (p < 0.05).
3.2. Total plasma protein level and activities of ALP and LYZ

The protein levels and activities of ALP and LYZ in the shell mucus of crayfish fed in the control diet group were lower ($p < 0.05$) than in other test diets (Table 2). After the 126-day feeding trial, the highest protein level (1.59 mg ml$^{-1}$), and the highest activities of ALP (1.17 IU l$^{-1}$) and LYZ (5.95 U min$^{-1}$) were observed in crayfish that were given the XOS$^+\text{EnF}$ diet ($p < 0.05$). Also, similar trends were observed after 24 h after the challenge test. In this regard, the protein levels and the activities of ALP and LYZ in crayfish fed the diet containing MOS$^+\text{PeA}$ were the second-highest after those fed with XOS$^+\text{EnF}$ diet. The probiotic diets containing EnF and PeA showed higher ($p < 0.05$) activities of ALP and LYZ compared to those fed the prebiotic diets (MOS and XOS) (Table 2).

3.3. Bacteriological analysis

The results of the LAB count to TVC ratio (%) of hepatopancreas extracted from crayfish after 126 days feeding are shown in Fig. 2. The significantly ($p < 0.05$) highest LAB/TVC ratio (91%) was in the case of crayfish fed the XOS$^+\text{EnF}$ diet. The crayfish fed the EnF and PeA diets (71.33–75.33%) showed higher ($p < 0.05$) LAB/TVC ratios than those fed the MOS and XOS diets (60.67–65.33%) (Fig. 2).

3.4. Hemolymph indices and bacterial exposure challenge

As shown in Table 3, crayfish fed the XOS$^+\text{EnF}$ diet had the highest values for THC, HC, SGC, and LGC. Feeding crayfish with the diets containing prebiotics (MOS and XOS) resulted in a fall ($p < 0.05$) in hemocyte indices (THC, HC, SGC, and LGC) compared...
to those fed probiotic diets (EnF and PeA) (Table 3). After the feeding trial, crayfish fed the control diet had the lowest activities (p < 0.05) for PO, SOD, LYZ, and NOS (Table 4). However, the higher activities (p < 0.05) of PO, SOD, LYZ, and NOS were observed in crayfish fed the diets containing XOS + EnF and MOS + PeA (Table 4).

Forty-eight hours after the challenge with the A. hydrophila injection, the survival rate of crayfish fed the XOS + EnF diet (91%) was higher (p < 0.05) than those fed the control diet (83.3%) and those given probiotics (61.3%–65.3%) and prebiotics (51.3%–57%) (Fig. 3). In this regard, the trend of the survival rate against the challenge in the 48-hour period was shown in Fig. 4. Feeding crayfish the XOS + EnF diet enhanced their resistance compared to the ones that were fed other test diets (Fig. 4). The dietary administration of XOS + EnF increased the activities of PO, SOD, LYZ, and NOS after 48 h post challenge compared to control and

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**Table 2**
The mean (±SD) of protein levels (mg ml⁻¹) and activities of alkaline phosphatase (ALP; IU L⁻¹) and lysozyme (LYZ; U min⁻¹) in crayfish shell mucus fed the experimental diets after 126-day feeding trial and 48 h post challenge (n = 3).

<table>
<thead>
<tr>
<th>Protein levels (mg ml⁻¹)</th>
<th>ALkaline phosphatase activity (IU L⁻¹)</th>
<th>Lysozyme activity (U min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After feeding trial</td>
<td>48 h post challenge</td>
</tr>
<tr>
<td>Control</td>
<td>0.22 ± 0.02ᵃ, A</td>
<td>0.10 ± 0.02ᵃ, A</td>
</tr>
<tr>
<td>MOS</td>
<td>0.44 ± 0.03ᵇ, A</td>
<td>0.34 ± 0.03ᵇ, A</td>
</tr>
<tr>
<td>XOS</td>
<td>0.46 ± 0.05ᶜ, A</td>
<td>0.44 ± 0.02ᶜ, A</td>
</tr>
<tr>
<td>EnF</td>
<td>0.59 ± 0.05ᶜ, A</td>
<td>0.67 ± 0.01ᶜ, A</td>
</tr>
<tr>
<td>PeA</td>
<td>0.61 ± 0.07ᶜ, A</td>
<td>0.66 ± 0.03ᶜ, A</td>
</tr>
<tr>
<td>MOS + EnF</td>
<td>0.95 ± 0.06ᶜ, A</td>
<td>0.93 ± 0.01ᶜ, A</td>
</tr>
<tr>
<td>MOS + PeA</td>
<td>1.32 ± 0.09ᶜ, A</td>
<td>1.08 ± 0.01ᶜ, A</td>
</tr>
<tr>
<td>XOS + EnF</td>
<td>1.59 ± 0.09ᶜ, A</td>
<td>1.17 ± 0.01ᶜ, A</td>
</tr>
<tr>
<td>XOS + PeA</td>
<td>0.78 ± 0.09ᶜ, A</td>
<td>0.83 ± 0.02ᶜ, A</td>
</tr>
</tbody>
</table>

ᵃ Standard deviation.
ᵇ Bars assigned with the different small letters in columns are significantly different among experimental treatments and with the different capital letters in row are significantly differed between groups before and after challenge differences at p < 0.05 (MOS: mannanoligosaccharide; XOS: xylooligosaccharide; EnF: E. faecalis; PeA: P. acidilactici).

**Table 3**
The mean (±SD) of total haemocyte count (THC, × 10⁵ cell ml⁻¹), hyaline count (HC, × 10⁵ cell ml⁻¹), semi-granular count (SGC, × 10⁵ cell ml⁻¹) and large-granular count (LGC, × 10⁵ cell ml⁻¹) in crayfish fed the experimental diets after 126 days (n = 3).

<table>
<thead>
<tr>
<th>Total haemocyte count</th>
<th>Hyaline count</th>
<th>Semi-granular count</th>
<th>Large-granular count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>94.67 ± 2.36ᵃ</td>
<td>45.67 ± 2.46ᵃ</td>
<td>21.67 ± 1.96ᵃ</td>
</tr>
<tr>
<td>MOS</td>
<td>132.67 ± 3.78ᵇ</td>
<td>70.67 ± 3.18ᵇ</td>
<td>25.33 ± 2.28ᵇ</td>
</tr>
<tr>
<td>XOS</td>
<td>131.33 ± 3.33ᵇ</td>
<td>76.67 ± 3.53ᵇ</td>
<td>27.33 ± 2.50ᵇ</td>
</tr>
<tr>
<td>EnF</td>
<td>167.67 ± 2.58ᵇ</td>
<td>89.33 ± 2.28ᵇ</td>
<td>33.67 ± 2.20ᵇ</td>
</tr>
<tr>
<td>PeA</td>
<td>159.33 ± 3.06ᶜ</td>
<td>85.33 ± 3.16ᶜ</td>
<td>31.33 ± 3.66ᶜ</td>
</tr>
<tr>
<td>MOS + EnF</td>
<td>196.66 ± 2.75ᶜ</td>
<td>101.33 ± 2.65ᶜ</td>
<td>41.00 ± 2.61ᶜ</td>
</tr>
<tr>
<td>MOS + PeA</td>
<td>214.01 ± 4.16ᶜ</td>
<td>108.67 ± 5.26ᶜ</td>
<td>46.67 ± 4.66ᶜ</td>
</tr>
<tr>
<td>XOS + EnF</td>
<td>224.67 ± 2.46ᶜ</td>
<td>113.33 ± 3.66ᶜ</td>
<td>50.67 ± 2.43ᶜ</td>
</tr>
<tr>
<td>XOS + PeA</td>
<td>185.37 ± 3.26ᶜ</td>
<td>96.67 ± 2.86ᶜ</td>
<td>37.37 ± 2.80ᶜ</td>
</tr>
</tbody>
</table>

ᵃ Standard deviation.
ᵇ Bars assigned with the different small letters in columns are significantly different among experimental treatments and with the different capital letters in row are significantly differed between groups before and after challenge differences at p > 0.05 (MOS: mannanoligosaccharide; XOS: xylooligosaccharide; EnF: E. faecalis; PeA: P. acidilactici).
Table 4

<table>
<thead>
<tr>
<th></th>
<th>Phenoloxidase activity (U min(^{-1}))</th>
<th>Superoxide dismutase activity (U min(^{-1}))</th>
<th>Lysozyme activity (U min(^{-1}))</th>
<th>Nitric oxide synthase activity (U min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After feeding trial 48 h post challenge</td>
<td>After feeding trial 48 h post challenge</td>
<td>After feeding trial 48 h post challenge</td>
<td>After feeding trial 48 h post challenge</td>
</tr>
<tr>
<td>Control</td>
<td>1.93 ± 0.53(^b)</td>
<td>1.90 ± 0.43(^b)</td>
<td>0.86 ± 0.14(^b)</td>
<td>0.54 ± 0.15(^b)</td>
</tr>
<tr>
<td>MOS</td>
<td>4.07 ± 0.65(^a)</td>
<td>5.39 ± 0.35(^h)</td>
<td>2.21 ± 0.32(^h)</td>
<td>4.65 ± 0.37(^h)</td>
</tr>
<tr>
<td>XOS</td>
<td>4.47 ± 0.43(^c)</td>
<td>6.54 ± 0.53(^c)</td>
<td>2.96 ± 0.28(^c)</td>
<td>5.96 ± 0.36(^c)</td>
</tr>
<tr>
<td>EnF</td>
<td>5.87 ± 0.63(^e)</td>
<td>9.94 ± 0.73(^e)</td>
<td>4.97 ± 0.16(^e)</td>
<td>10.05 ± 0.42(^e)</td>
</tr>
<tr>
<td>PeA</td>
<td>5.07 ± 0.74(^f)</td>
<td>8.54 ± 0.84(^f)</td>
<td>4.36 ± 0.14(^f)</td>
<td>8.56 ± 0.34(^f)</td>
</tr>
<tr>
<td>MOS + EnF</td>
<td>6.87 ± 0.95(^g)</td>
<td>13.32 ± 0.85(^g)</td>
<td>7.35 ± 0.41(^g)</td>
<td>14.75 ± 0.32(^g)</td>
</tr>
<tr>
<td>MOS + PeA</td>
<td>7.33 ± 0.87(^h)</td>
<td>15.19 ± 0.97(^h)</td>
<td>8.75 ± 0.32(^h)</td>
<td>17.67 ± 0.37(^h)</td>
</tr>
<tr>
<td>XOS + EnF</td>
<td>7.60 ± 0.86(^i)</td>
<td>16.30 ± 1.16(^i)</td>
<td>9.59 ± 0.32(^i)</td>
<td>19.47 ± 0.45(^i)</td>
</tr>
<tr>
<td>XOS + PeA</td>
<td>6.37 ± 0.93(^j)</td>
<td>11.89 ± 1.23(^j)</td>
<td>6.41 ± 0.32(^j)</td>
<td>13.01 ± 0.49(^j)</td>
</tr>
</tbody>
</table>

* Standard deviation.

b Bars assigned with the different small letters in columns are significantly differed among experimental treatments and with the different capital letters in row are significantly differed between groups before and after challenge differences at p > 0.05 (MOS: mannanoligosaccharide; XOS: xylooligosaccharide; EnF: E. faecalis; PeA: P. acidilactici).

Fig. 3. The mean (±SD) of survival rate of *Aeromonas hydrophila* injected crayfish fed the test diets after 48 h post challenge test. Bars assigned different letters are significantly different (p < 0.05) at three replicates (MOS: Mannanoligosaccharide; XOS: Xylooligosaccharide; EnF: E. faecalis; PeA: P. acidilactici).

Fig. 4. Survival rate (%) of injected crayfish with *Aeromonas hydrophila* during 48 h post challenge fed the experimental diets (MOS: Mannanoligosaccharide; XOS: Xylooligosaccharide; EnF: E. faecalis; PeA: P. acidilactici) at three replicates.
other test diets (Table 4).

3.5. Growth performance and survival rate

The results of the growth performance and the survival rate of crayfish fed the experimental diets are presented in Table 5. The results revealed that the dietary administration of XOS + EnF improved final weight, SGR, and FCR compared to those given control and other treatments (Table 5). In terms of growth enhancement, the diets containing probiotics (EnF and PeA) were better than those fed the prebiotic (MOS and XOS) diets.

4. Discussion

4.1. Mucus bactericidal activity and immune responses (ALP and LYZ)

The results of the present study showed that a single administration of prebiotics, probiotics and their combination (synbiotics) in crayfish modulated the immune responses (higher ALP and LYZ activities) of the shell mucus. Additionally, the stress resistance in the crayfish fed the synbiotic diets was also higher than those fed the prebiotics and probiotics diets. To the best of our knowledge, there was no information to determine the efficiency of prebiotic, probiotic, and synergistic included in the diets on the immune responses of crayfish shell mucus. Higher lysozyme activity, total immunoglobulin, and protease of skin mucus were measured in angefish fed enriched artemia with synbiotics (fructooligosaccharide + P. acidilactici) compared to those given a single administration [36]. In spite of existing tegumental glands in decapods with different roles in mucus production and bactericidal reaction [3–8], there is no information about the shell mucus of shrimp and prawn species. A dose-dependent response was reported in the skin mucus of black swordtail (Xiphophorus helleri) when using Lactobacillus acidophilus in the diet [52]. The results of the present trial showed that feeding crayfish synbiotic diets (XOS + EnF, MOS + PeA) increased the protein levels and the bactericidal activity of the shell mucus against important pathogens (mainly, N. brasiliensis, V. harveyi and A. hydrophila) in astaciculture. A rise in antibacterial activity of skin mucus was reported in some fish species using dietary supplements, including S. cerevisiae, Vit C and xyloooligosaccharide [53–55]. This can be related to nitrogenous materials existing in the skin mucus (e.g., lysozyme, proteases, immunoglobulins, mucins, and lectins) [9]. This was in accordance with the findings of other researchers [36,52], who found that an enhancement in skin indicators (mucus protein level and antibacterial activity) indicates an improvement of the immunological responses. Further studies need to elucidate the composition of the shell mucus and the metabolic pathways from synbiotic feeding on shell mucus immunity. However, the effects of dietary manipulations and feeding practices on the immune responses of crustacean species, especially crayfish, need further investigation.

4.2. Bacteriological analysis

The crayfish fed synbiotic diets (XOS + EnF and MOS + PeA) showed the highest LAB to TVC ratio (87.33–91%) and the lowest ones were for crayfish fed the control diet (22%). This was in accordance with the findings of other researchers [19,36,56,57], who observed that the selective fermentation of synbiotics can alter the composition of intestinal microbiota by stimulating bifidobacteria and Lactobacilli. In the present study, the administration of E. faecalis and P. acidilactici modulated the hepatopancreas of crayfish towards potentially-suitable community. Considering the properties of prebiotics (origin, structure, and degree of polymerization) and the test animals (species, initial weight, feeding duration and supplement dose) is necessary to describe the results [16,58]. However, further studies on potential substrate-probiotic interactions, as well as molecular and histological aspects, are needed.

4.3. Hemolymph indices and bacterial exposure challenge

Biological stressors such as pathogen injection are commonly used in nutritional research in order to evaluate the potential of feed additives against the harsh conditions of rearing systems [16,50,59]. In the present study, dietary synbiotics (XOS + EnF and MOS + PeA) reduced the mortality rate of the crayfish challenged by A. hydrophila and modulated the intestinal microbiota, as well as stimulated immunity, antioxidant responses, and the hemocyte counts of crayfish. This was in accordance with the findings of other researchers [16,60,61], who revealed the promoting effects of prebiotics and probiotics on the immune responses and resistance of finfish and shellfish species on different stressors. A likely explanation of enhanced resistance and non-specific immune responses may be attributed to an increase in the mechanism of pathogen inhibition in the gastrointestinal tract (GIT) [62], the competition for space in the GIT, the reduction in pH, the release of natural antibiotics from beneficial microbial populations, and the increase in the concentration of short-chain fatty acids as by-products of fermentation in the GIT [63]. However, further investigation into gene expression related to immunity and physiological cascade pathways is needed.

<p>| Table 5 | The mean (±SD) of initial weight (g), final weight (g), specific growth rate (% day⁻¹), feed conversion ratio and survival rate (%) of crayfish fed the experimental diets after 126 days (n = 3).^a^ |
|---------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Specific growth rate (% day⁻¹)</th>
<th>Feed conversion ratio</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.43 ± 1.75</td>
<td>30.67 ± 2.16^a</td>
<td>0.78 ± 0.36^a</td>
<td>3.83 ± 0.86^a</td>
<td>44.00 ± 3.56^a</td>
</tr>
<tr>
<td>MOS</td>
<td>11.43 ± 1.55</td>
<td>44.67 ± 2.18^b</td>
<td>1.09 ± 0.28^b</td>
<td>3.57 ± 0.88^b</td>
<td>62.67 ± 1.38^b</td>
</tr>
<tr>
<td>XOS</td>
<td>11.43 ± 1.45</td>
<td>49.33 ± 2.37^c</td>
<td>1.16 ± 0.47^c</td>
<td>3.33 ± 0.64^c</td>
<td>69.33 ± 2.54^c</td>
</tr>
<tr>
<td>EnF</td>
<td>11.43 ± 1.55</td>
<td>61.33 ± 2.25^d</td>
<td>1.33 ± 0.50^d</td>
<td>2.87 ± 0.49^d</td>
<td>83.33 ± 3.56^d</td>
</tr>
<tr>
<td>PeA</td>
<td>11.42 ± 1.65</td>
<td>54.67 ± 2.39^e</td>
<td>1.24 ± 0.69^e</td>
<td>3.07 ± 0.76^e</td>
<td>76.67 ± 3.65^e</td>
</tr>
<tr>
<td>MOS + EnF</td>
<td>11.42 ± 1.45</td>
<td>70.33 ± 1.87^f</td>
<td>1.44 ± 0.97^f</td>
<td>2.53 ± 0.48^f</td>
<td>89.67 ± 3.87^f</td>
</tr>
<tr>
<td>MOS + PeA</td>
<td>11.41 ± 1.75</td>
<td>74.67 ± 2.95^g</td>
<td>1.49 ± 0.85^g</td>
<td>2.37 ± 0.93^g</td>
<td>92.67 ± 3.68^g</td>
</tr>
<tr>
<td>XOS + EnF</td>
<td>11.43 ± 1.45</td>
<td>78.33 ± 2.43^h</td>
<td>1.53 ± 0.53^h</td>
<td>2.13 ± 0.59^h</td>
<td>95.67 ± 3.87^h</td>
</tr>
<tr>
<td>XOS + PeA</td>
<td>11.42 ± 1.25</td>
<td>67.33 ± 2.45^i</td>
<td>1.40 ± 0.75^i</td>
<td>2.67 ± 0.49^i</td>
<td>86.67 ± 3.55^i</td>
</tr>
</tbody>
</table>

^a^ Standard deviation.

^b^ Bars assigned with the different letters are significantly differed among experimental treatments at p > 0.05 (MOS: mannanooligosaccharide; XOS: xyloooligosaccharide; EnF: E. faecalis; PeA: P. acidilactici).
4.4. Growth performance and survival rate

The beneficial effects of dietary synbiotics on the performance and the survival rate of finfish [36,37,49,58,64] and shellfish [41] species have been reported in the literature. During the past decade, the low survival rate of astaciculture led to a focus on the use of feed additives. To the best of our knowledge, there is no information about the effects of synbiotics on the mucosal immunity, immune and antioxidant responses, and stress resistance of the juvenile narrow-clawed crayfish.

Feeding crayfish with synbiotics (MOS + EnF, MOS + PeA, XOS + EnF and XOS + PeA) gave a boost to the growth performance compared to those fed prebiotics (MOS and XOS), probiotics (EnF and PeA), and control treatments. Feeding test crayfish with the diet containing XOS + EnF showed better performance (final weight, SGR, and survival rate) and lower FCR. Growth enhancement, as a result of using prebiotics [16], probiotics [25,28] and synbiotics [36,37,49,58,64], has been seen in several finfish and shellfish species in literature. It may be related to an increase in digestive enzyme activities, voluntary feed intake, the production of some metabolites (short-chain fatty acids and vitamins), the hydrolysis of non-digestible substrate, and adaptive responses in the morphology of the digestive tract [14,16,37,49,58,64].

In conclusion, feeding sub-adult crayfish synbiotic diets (XOS + EnF, MOS + PeA) improved growth performance, accelerated shell mucosal and antioxidant responses and stress resistance against A. hydrophilica injection, and modulated hepatopancreatic microbiota. The present study may open up avenues in astaciculture to improve survival rate and performance.

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References


