Fish & Shellfish Immunology 64 (2017) 392–400

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

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

Omid Safari [a, *], Marina Paolucci [b], Hamidreza Ahmadnia Motlagh [a]

[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

Article history:
Received 24 October 2016
Received in revised form
16 March 2017
Accepted 26 March 2017
Available online 29 March 2017

Keywords:
Crayfish
Synbiotic
Immunity
Disease resistance
Challenge

ABSTRACT

The aim of this study was to evaluate the effects of prebiotics (mannanoligosaccharide and xylooligosaccharide), 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 xylooligosaccharide + *E. faecalis* and mannanoligosaccharide + *Pediococcus acidilactici* against *Nocardia brasilience* and *Vibrio harveyi* ($p < 0.05$). Feeding crayfish a xylooligosaccharide + *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 xylooligosaccharide + *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.

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 lipopolysaccharide and peptidoglycan in fungal species, are regarded as stimulators of the proPO system [2]. Tegumental glands playa 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 *P. 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,

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

http://dx.doi.org/10.1016/j.fsi.2017.03.049
1050-4648/

and increase the survival rate in the crayfish culture (astaciculture) industry [14–18]. Synbiotics, 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 synbiotics 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*, *Pediococcus acidilactici*) [24] in finfish and shellfish aquafeeds have been confirmed [14,22,25–29]. Mannanoligosaccharide (glucomannoprotein-hydrolyzed fungi cell wall; MOS) and xylooligosaccharide (chemical and enzymatic-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*) [16,30–35]. The beneficial effects of enriched artemia with synbiotics (*P. acidilactici* and fructooligosaccharide) in the diet of angelfish (*Pterophyllum scalare*) on the mucosal immunity was seen [36]. The ability of synbiotics—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 synbiotics 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 Commerce Corporation Co., USA; DP: 6) and xylooligosaccharide (XOS; Shandong Longlive Bio-Technology 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 probionts 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 20crayfish 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 $CaCO_3$), 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.

Table 1
Composition (g kg^{-1} dry matter) of the control diet fed juvenile crayfish (11.45 ± 1.87 g).

Ingredient	g kg^{-1} (dry-weight basis)
Menhaden fish meal[a]	120
Soybean meal[a]	182
Corn gluten[a]	112
Wheat flour[a]	299
Corn starch[b]	69
Fish oil[a]	47
Canola oil[a]	46
Soy lecithin[a]	50
Cholesterol[d]	5
Glucosamine[c]	10
Choline chloride[4] (70%)[d]	15
Vitamin C (stay)[d]	10
Vitamin premix[d,e]	20
Mineral premix[d,e]	15
Carboxymethyl cellulose[c]	17.9
Ytterbium oxide[c]	0.1
Chemical composition	
Dry matter	870.3
Crude protein	320.1
Crude fat	163.8
Crude fiber	43.9
Nitrogen free extract	300.6
Ash	41.9
Gross energy (Mj kg^{-1})	15.18

[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.

2.3. 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]:

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

$$\text{Feed conversion ratio (FCR)} = (\text{Feed}_{\text{consumed}}/W_{\text{gain}})$$

$$\text{Survival rate (\%)} = (\text{Final individual numbers/Initial individual numbers}) \times 100$$

In the above equations, W_i, W_f, 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