Fish & Shellfish Immunology 70 (2017) 164–173

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Effects of organic acids and essential oils blend on growth, gut microbiota, immune response and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio parahaemolyticus*

Wangquan He [1], Samad Rahimnejad [1], Ling Wang, Kai Song, Kangle Lu, Chunxiao Zhang *

Xiamen Key Laboratory for Feed Quality Testing and Safety Evaluation, Fisheries College, Jimei University, Xiamen 361021, China

ARTICLE INFO

Article history:
Received 5 July 2017
Received in revised form
23 August 2017
Accepted 1 September 2017
Available online 4 September 2017

Keywords:
Litopenaeus vannamei
Organic acids
Essential oils
Gut microbiota
Immune response
Disease resistance

ABSTRACT

An 8-week feeding trial was undertaken to evaluate supplemental effects of AviPlus® (AP), a blend of organic acids [citric acid, 25%; sorbic acid, 16.7%] and essential oils [thymol, 1.7%; vanillin, 1.0%], on growth, gut microbiota, innate immunity and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio parahaemolyticus*. A basal experimental diet was formulated and supplemented with 0, 0.3, 0.6, 0.9 and 1.2 g kg^{-1} AP to produce five test diets (Con, AP0.3, AP0.6, AP0.9 and AP1.2). Each diet was fed to triplicate groups of shrimp (0.2 ± 0.01 g, mean ± SE) to apparent satiation three times daily. Growth performance and survival rate were not significantly influenced by AP supplementation ($P > 0.05$). Significantly ($P < 0.05$) higher serum total protein was found in groups fed ≥ 0.6 g kg^{-1} AP compared to control. Serum alkaline phosphatase and phenoloxidase activities were significantly increased in AP0.9 and AP1.2 groups. Also, the group received AP0.6 diet showed significantly higher glutathione peroxidase activity than control. Expression of gut pro-inflammatory genes including TNF-α, LITAF and RAB6A were down-regulated by AP administration. Gut microbiota analysis showed the significant enhancement of the operational taxonomic unit (OTU) diversity and richness indices by AP application. AP supplementation led to increased abundance of *Firmicutes* and a reduction in abundance of *Proteobacteria*. Also, dietary inclusion of 1.2 g kg^{-1} AP led to a significant increase in the abundance of *Lactobacillus* in shrimp gut. The group offered AP0.3 diet showed significantly higher disease resistance than control group. Furthermore, AP application significantly enhanced relative expression of immune related genes including lysozyme, penaeidin and catalase at 48 h post challenge. In conclusion, these findings show that the tested organic acids and essential oils mixture beneficially affects intestinal microflora and improves immune response and disease resistance of *L. vannamei*.

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is widely cultured all around the world and its annual aquaculture production accounts for roughly two-third of the global shrimp production [1]. Diseases caused by various bacterial, fungal, parasitic and viral species are a significant constraint to productivity of aquaculture industry imposing substantial losses, and shrimp culture is not certainly an exception [2]. It is necessary to develop environment-friendly strategies for controlling shrimp disease which is one of the major problems facing the large-scale shrimp farming. Antibiotics have long been used for promoting growth and controlling disease in animals feed. However, nowadays their application is restricted due to their numerous adverse effects on the environment and the human food chain such as occurrence of antibiotic-resistant bacteria, accumulation of antibiotic residues in aquaculture products and suppression of aquatic animal immune systems [3–6]. Taking these into account, it is imperative to identify potential alternatives for antibiotics. Probiotics [7,8], prebiotics [9], immunostimulants [10,11], and acidifiers [5,12,13] have been identified as promising alternatives to antibiotics. These candidate compounds enhance the immune function mainly through altering the gut microbiota by selectively stimulating beneficial bacterial strains and reducing the number of potential pathogens [14,15].

Acidifiers are defined as inorganic or organic acids and their

* Corresponding author. Fisheries College, Jimei University, No. 43 Yindou Road, Xiamen 361021, China.
E-mail address: cxzhang@jmu.edu.cn (C. Zhang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fsi.2017.09.007
1050-4648/

salts. Among the dietary supplements tested in aquafeeds so far, organic acids are increasingly gaining interest because of their strong antimicrobial and prophylactic properties against various pathogenic bacteria [16–18]. Organic acids are generally composed of short-chain fatty acids (C1-C7), volatile fatty acids and weak carboxylic acids with one or more carboxyl groups in their structure. Currently, there is an increasing tendency of using organic acids in commercial aquafeeds both for controlling disease and enhancing growth performance. Improved growth, feed utilization and disease resistance have been reported in several fish species offered diets supplemented with organic acids, their salts or mixtures [13,19,20]. Organic acids inhibit microbial growth and the uptake of pathogens and their metabolites [5,21,22]. They also reduce the gastrointestinal pH thereby inhibiting the growth of pathogenic gram-negative bacteria [12]. The main antimicrobial activity of organic acids is attributed to altering the cell cytoplasm pH of bacteria thereby inhibiting sensitive bacteria to such changes [23].

Essential oils are concentrated hydrophobic liquid containing volatile aroma compounds isolated from secondary metabolites of plants [24]. Aromatic plants and the essential oils extracted from them have been suggested as potent prophylactic agents because of their anti-microbial activity and stimulating effects on animal digestive system. Currently, essential oils from aromatic plants are extensively used in medicine and food industry [25]. They are obtained from many plant materials such as flowers, buds, seeds, leaves and fruits [26]. Dietary supplementation of essential oils in animals' feed has been reported to increase antioxidant activity [27], enhance immune responses [28], and alter gut microbiota [29].

AviPlus® (AP) is a blend of organic acids (citric acid and sorbic acid) and essential oils (thymol and vanillin) that has shown promising as a dietary supplement in poultry and pigs feed [30,31]. To date, numerous studies have been conducted on individual application of organic acids and essential oils as dietary additives for aquatic animals, however, to the best of our knowledge there is no available report on the use of organic acids and essential oils mixture in shrimp feed. The aim of the present study was to examine the effects of dietary supplementation of AP on growth performance, gut microbiota, innate immunity and resistance of Pacific white shrimp to *Vibrio parahaemolyticus* challenge.

2. Materials and methods

2.1. Experimental diets

A basal experimental diet was formulated using fish meal and soybean meal as protein sources; and fish oil and soybean oil as lipid sources (Table 1). The blend of organic acids and essential oils tested in this study consisted of citric acid (25%), sorbic acid (16.7%), thymol (1.7%), vanillin (1.0%), and hydrogenated palm oil (55.6%) as a carrier (Aviplus-S®, VetAgro SPA, 42100 Reggio Emilia, Italy). Totally five experimental diets were prepared by supplementing the basal diet with 0, 0.3, 0.6, 0.9 and 1.2 g kg^{-1} AP designated as Con, AP0.3, AP0.6, AP0.9 and AP1.2, respectively. AP was supplemented in the diets at the expense of wheat flour. Squid visceral paste was used as palatability enhancer in all the diets. All dry ingredients were finely ground using a hammer mill and then passed through a 180 μm mesh. Different levels of AP were added to the dietary ingredients mixture together with fish oil, soybean oil and soybean lecithin. Then an appropriate amount of water was added and pellets were produced by passing the mash through a 1- and 2- mm die, using multifunctional spiral extrusion machinery (CD4XITS, South China University of Technology, Guangzhou, China). The pellets were dried at 35 °C in a dry oven overnight and stored at −20 °C in airtight polyethylene bags until use. Proximate composition of the experimental diets was analyzed according to standard methods [32].

Table 1
Formulation and proximate composition of the experimental diets (g kg^{-1}dry matter).

Ingredients	Diets				
	Con	AP0.3	AP0.6	AP0.9	AP1.2
Fish meal[a]	240	240	240	240	240
Soybean meal[b]	250	250	250	250	250
Shrimp meal	50	50	50	50	50
Squid visceral paste	15	15	15	15	15
Wheat flour	369	368.7	368.4	368.1	367.8
Fish oil	20	20	20	20	20
Soybean oil	10	10	10	10	10
Lecithin	10	10	10	10	10
Choline chloride	5	5	5	5	5
Monocalcium phosphate	20	20	20	20	20
Vitamin premix[c]	3	3	3	3	3
Mineral premix[d]	5	5	5	5	5
L-Ascorbate-2-phosphate	1	1	1	1	1
Ethoxyquin	0.5	0.5	0.5	0.5	0.5
AviPlus®	0	0.3	0.6	0.9	1.2
Proximate composition					
Dry matter	903	909	901	896	910
Crude protein	390	393	392	390	395
Crude lipid	74.2	73.6	75.0	76.8	74.6
Ash	82	81	84	83	86

[a] Xiamen ITG group Corp., Ltd., Xiamen, China, imported from Peru (crude protein:65.3%, crude lipid:8.65%).
[b] Soybean meal, obtained from Quanzhou Fuhai cereals and oils industry Co., Ltd. (crude protein: 46.3%, crude lipid:1.0%).
[c] Vitamin premix (mg or g kg^{-1} diet): thiamin, 10 mg; riboflavin, 8 mg; pyridoxine HCl, 10 mg; vitamin B12, 0.2 mg, vitamin K3, 10 mg; inositol, 100 mg; pantothenic acid, 20 mg; niacin acid, 50 mg; folic acid, 2 mg; biotin, 2 mg; retinol acetate, 400 mg; cholecalciferol, 5 mg; alpha-tocopherol, 100 mg; ethoxyquin, 150 mg; wheat middling, 1.1328 g.
[d] Mineral premix (mg or g kg^{-1} diet): Na F, 2 mg; KI,0.8 mg; Co $Cl_2 \cdot 6H2O$ (1%), 50 mg; Cu $SO_4 \cdot 5H_2O$, 10 mg; $FeSO_4 \cdot H_2O$, 80 mg; $ZnSO_4 \cdot H_2O$, 50 mg; Mn SO4·H2O, 25 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; Zoelite, 4.582 g.

2.2. Experimental shrimp and feeding trial

The feeding trial was conducted at the experimental station of Jimei University (Xiamen, China). Pacific white shrimp (specific pathogen free; SPF) post-larvae (PL-12) were purchased from a private hatchery in Zhangzhou (Fujian, China). The shrimp were stocked into three 1000-L round-shaped fiberglass tanks and fed a commercial diet (Dabeinong Technology Group Co., Ltd) for two weeks to adapt them to the experimental conditions. At the end of the acclimation period, 35 healthy shrimp (0.2 ± 0.01 g; mean ± SE) were stocked into each of 15 indoor circular fiberglass tanks (300 L) in a recirculating system. The recirculating system consisted of a reservoir with a biological filter, a circulation pump and an automatic temperature control device supplied with aerated water. The experiment was performed in triplicates and shrimp were hand fed with the experimental diets to apparent satiation three times daily (07:00, 13:00 and 19:00) for eight weeks. Uneaten diets and feces matter were siphoned out and 10% of water was exchanged after each feeding. During the experimental period, water temperature, salinity and pH were measured daily and the values were 28 ± 1 °C (mean ± SE), 26 g L^{-1} and 8.0 respectively. Dissolved oxygen was not less than 6 mg L^{-1} and the photoperiod was natural. Feeding was stopped 24 h prior to handling and sampling to minimize the stress on shrimp.

2.3. Sample collection and analyses

At the end of the feeding trial, all the shrimp in each tank were