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# Dietary values of *Forsythia suspensa* extract in *Penaeus monodon* under normal rearing and *Vibrio parahaemolyticus* 3HP ($VP_{3HP}$) challenge conditions: Effect on growth, intestinal barrier function, immune response and immune related gene expression

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## ABSTRACT

Two trials were conducted to determine the effects of dietary *Forsythia suspensa* extract (FSE) on shrimp, *Penaeus monodon*, first on growth performance, second on the immune response and immune related gene expression of shrimp. In trial 1, shrimp (mean initial wet weight about 3.02 g) were fed with five diets containing 0% (basal diet), 0.01%, 0.02%, 0.04% and 0.06% FSE in triplicate for 60 days. Growth performance (final body wet weight, FBW; weight gain, WG; biomass gain, BG) of shrimp fed FSE diets were higher ($P < 0.05$) than that of shrimp fed the basal diet. The survival among all the diets treatments were above 90% and no significant difference was revealed among them ($P > 0.05$). The antioxidant capacity (total antioxidant status, TAS; glutathione peroxidase, GSH-Px) appears in the trend of firstly increasing then decreasing with the increasing of dietary FSE levels. The highest value of TAS and GSH-Px were found in shrimp fed 0.02% FSE diet and were significantly higher than that of shrimp fed the basal and 0.06% FSE diets ($P < 0.05$). Hepatopancreas malondialdehyde (MDA) of shrimp fed FSE diets were lower ($P < 0.05$) than that of shrimp fed the basal diet. Total haemocyte count of shrimp fed the basal diet was lower ($P < 0.05$) than that of shrimp fed FSE diets. Haemolymph clotting time of shrimp had the opposite trend with the total haemocyte count of shrimp. No significant differences were found in haemolymph biomarkers of intestinal permeability (endotoxin and diamine oxidase) and in molecular gene expression profiles of heat shock protein 70 (Hsp 70) mRNA and hypoxia inducible factor-1α (HIF-1α) mRNA in haemolymph of shrimp among all diet treatments ($P > 0.05$). In trial 2, a pathogenic strain of *Vibrio parahaemolyticus* 3HP ($VP_{3HP}$) injection challenge test was conducted for 6-day after the rearing trial and shrimp survival were also compared among treatments. Survival of shrimp fed diets supplemented with 0.01%–0.02% FSE were higher than that of shrimp fed the basal and 0.06% FSE diets ($P < 0.05$). Dietary FSE supplementation produced stronger hepatopancreas antioxidant capacity (TAS, GSH-Px) ($P < 0.05$) and higher glutathione (GSH) level ($P < 0.05$), lower superoxide dismutase activity (SOD) ($P < 0.05$), higher total haemocyte count ($P < 0.05$), lower haemolymph clotting time ($P < 0.05$), lower MDA and carbonyl protein concentration ($P < 0.05$), lower haemolymph biomarkers of intestinal permeability (endotoxin and diamine oxidase) ($P < 0.05$), generated lower molecular gene expression profiles of HSP 70 mRNA and higher HIF-1α mRNA ($P < 0.05$) than the basal diet. The immune response were characterized by lower TAS and higher antioxidant enzyme activities (SOD, GSH-Px) and higher oxidative stress level (MDA and carbonyl protein) and higher haemolymph biomarkers of intestinal permeability (endotoxin and diamine oxidase) compared to levels found in trail 1. However, the total haemocyte counts and haemolymph clotting times were not changed in 0.01%–0.02% FSE diets treatments between trial 1 and trial 2 ($P > 0.05$). The molecular gene expression profile of Hsp 70

*Abbreviations:* BG, biomass gain; FBW, final body wet weight; FCR, feed conversion ratio; FSE, *Forsythia suspense* extract; GSH, glutathione content; GSH-Px, glutathione peroxidase; hypoxia inducible factor-1α mRNA, HIF-1α mRNA; Hsp 70 mRNA, heat shock protein 70 mRNA; IBW, initial body wet weight; MDA, malondialdehyde; SOD, superoxide dismutase; TAS, total antioxidant status; WG, weight gain

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mRNA was increased while HIF-1α mRNA was decreased when compared to trial 1. In conclusion, results suggested that dietary intake containing FSE could enhance the growth performance and antioxidant capacity of *P. monodon* and furthermore reduce oxidative stress and immune depression challenged by a pathogenic strain of *Vibrio parahaemolyticus* stress. Considering the effect of FSE on both growth performance and immune response of *P. monodon*, the level of FSE supplemented in the diet should be between 0.01% and 0.02%.

## 1. Introduction

*Penaeus monodon* is another important aquaculture shrimp species except for *Litopenaeus vannamei* in China. However, disease is a primary constraint to the culture of many aquatic species including shrimp, impeding both economic and social development in many countries including China. Shrimp aquaculture is continuously affected by the outbreak of ecosystem factors (such as dissolved oxygen), viral or bacterial diseases [1]. A recently emerging disease called acute hepatopancreatic necrosis disease (AHPND) is particularly susceptible to stress, which is one of the causes of Early Mortality Syndrome (EMS), resulting in mass mortalities of commercially cultivated *litopenaeus vannamei* and *Penaeus monodon* during the first 20–30 days of pond rearing [2]. The first outbreak was reported in Vietnam and southern China in 2010 [3]. Outbreaks also appeared in Malaysia since 2011, in Thailand since 2012, in Mexico since 2013 and in the Philippines since 2014 and are still a major problem for shrimp culture at present [4–7]. The causative agent of AHPND-positive shrimp was identified as *Vibrio parahaemolyticus* [8]. AHPND mainly affects the hepatopancreas and stomach of the infected shrimp. AHPND has a characteristic histopathology in shrimp which also includes (1) acute progressive degeneration and dysfunction of the hepatopancreas, (2) necrosis and sloughing of tubule epithelial cells of the hepatopancreas and (3) hemocytic infiltration, necrotic and sloughed hepatopancreatic tubules. Additionally, infected shrimp have a white hepatopancreas, an atrophy of hepatopancreas, soft shells and white feces or guts with no contents [9]. However, no effective solutions were found till now. To help shrimp cope with these problems, antibiotics are often applied in the field of animal production and medicine to overcome farming problems. However, awareness of the adverse side effects of antibiotics has motivated researchers to find alternative. Furthermore, now consumer demand for farming shrimp has increasingly stressed quality and safety, and the absence of concomitant pollutants and antibiotics. Therefore, the rearing strategy needs to hasten the search to identify and develop safe dietary supplements and additives that enhance the life activity, health and immune systems of farming shrimp. The addition of natural antioxidants and immunostimulants to feed has been considered a potential non-antibiotic means to improve health status and performance [2].

The restriction or even prohibition on the use of antibiotics as feed additives has driven nutritionists and feed manufacturers to develop alternatives such as organic acids, feed enzymes, and pro- or pre-biotics. These substances are well established in animal nutrition. In contrast, plant extracts, are a new class of feed additives and knowledge regarding their modes of action and aspects of application are still rather rudimentary [10]. *Forsythia suspensa* Vahl (Oleaceae) is widely distributed in China, Japan, Korea and many European countries and its fruit extract is a famous traditional Chinese medicine for the treatment of infections, such as acute nephritis, erysipelas, pharyngitis, tonsillitis, ulcers and pyrexia [11]. Moreover, the major active components of *F. suspensa* are phenylethyl alcohol glycoside, lignin, pentacyclic triterpenoids and volatile oil, several experiments showed *Forsythia fructus* has antioxidant effects in rats [12], in vitro anti-inflammatory effects [13], in vitro prevention of unfavorable microorganisms [14], effects against allergic reactions to soybean in weaned pigs [15], and can regulate blood pressure in rabbits [16].

In recent years, *Forsythia suspense* extract has attracted increased attention from the swine and poultry industries. However, the use of supplemented FSE in the diet of *P. monodon* to improve the health status and immunity is still unknown and there is no record on the use of FSE in the diet of *P. monodon*. Therefore, the aim of the present study was to evaluate, firstly, the possible effect of dietary FSE supplementation on the growth performance of *P. monodon*. Secondly, the possible effect of dietary FSE on the immune response and immune related gene expression profiles in *P. monodon*, which was evaluated in two different situations: normal rearing shrimp and shrimp under a pathogenic strain of *Vibrio parahaemolyticus* 3HP ($VP_{3HP}$) injection challenge test. Hepatopancreas antioxidant parameters (TAS, SOD, GSH-Px and GSH levels), blood parameters (total haemocyte counts and haemolymph clotting times), hepatopancreas oxidative stress parameters (MDA and carbonyl protein) and haemolymph biomarkers of intestinal permeability (endotoxin and diamine oxidase) were used as tools to evaluate the immune responses of *P. monodon*. Moreover, we also looked at the immune related gene expression profiles of Hsp 70 mRNA and HIF-1α mRNA to evaluate the oxidative stress status, which is an important part of the cell's machinery for protein folding, and help to protect cells from stress.

## 2. Materials and methods

### 2.1. Preparation of FSE

*Forsythia suspensa* extract is derived from a climbing plant widely distributed in China, Japan, and Korea. The FSE was prepared using the methods described by Wang et al. [17]. In brief, dried fruits of *Forsythia suspensa* were purchased from the Tong Ren Tang Company (Beijing, China). The fruits were ground to powder (100 g), extracted with 500 mL of 80% methanol, sonicated for 3 h, filtered, and extracted twice (500 mL each time). The filtrates were combined and dried by rotary vaporization (Büchi, Rotavapor R-124, Flawil, Switzerland). Lu et al. [12] identified 3 compounds (forsythoside A, forythialan A, phillygenin and phillyrin) in FSE, which have been demonstrated to be the major antioxidant constituents in FSE.

### 2.2. Experimental diets

Dietary formula and proximate composition are given in Table 1 and Table 2. The powdered FSE was supplemented at the levels of 0, 0.01, 0.02, 0.04 and 0.06 g–100 g of basal diet. The method of diet preparation was the same as described by Niu et al. [18]. Briefly, all the dry ingredients of the experimental diets were grounded into a fine powder and were weighed, combined and thoroughly mixed until homogenous in a Hobart-type mixer. The oil mix was then added to the Hobart mixer slowly while mixing was still continuing. Deionized water (40% dry ingredients mixture) was added and mixed for another 5 min. The wet mixture was placed in a monoscrew extruder (Institute of Chemical Engineering, South China University of Technology, Guangzhou, P.R. China) and extruded through a 1.2-mm die. The resulting pellets were dried at 25 °C with the aid of an air conditioner and an electrical fan. All the diets were stored at −20 °C until used.

### 2.3. Shrimp and experimental set up-trial 1

In trial 1, juvenile *P. monodon* were obtained from a semi-intensive culture pond near Hongsha Bay, Sanya, Hainan province, China. Shrimp were acclimated to the experimental conditions and fed the basal diet