DIETARY FULVIC ACID EFFECTS ON SURVIVAL AND EXPRESSION OF IMMUNE-RELATED GENES IN *Litopenaeus vannamei* CHALLENGED WITH WSSV AND *Vibrio parahaemolyticus*

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Introduction

The most important diseases of cultured penaeid shrimp, in terms of economic impact, have viral and bacterial agents as their cause. Among the virus and bacteria-caused diseases, the White Spot Disease (WSD), caused by the White Spot Syndrome Virus (WSSV), and the Acute Hepatopancreatic Necrosis Disease (AHND), caused by *Vibrio parahaemolyticus*, are considered a serious concern to shrimp culture. Therefore, prevention of disease outbreak and enhancement of immunity are of primary concern.

This study was undertaken to examine the effect of fulvic acid (FA) on survival and the expression of immune-related genes in *L. vannamei* challenged with WSSV and *V. parahaemolyticus* IPNGS16.

Materials and methods

The powdered FA was incorporated to commercial feed (CF). Shrimp (weighing 80±5 mg, 10 shrimp/tank) were placed in glass tanks with 4 L of seawater (bioassay 1). Shrimp (weighing 2.84±0.3 g, 60 shrimp/tank) were placed in 120-L plastic tank with 100 L of seawater (bioassay 2). Treatments were conducted in triplicate. Physicochemical parameters (temperature, salinity, pH, and dissolved oxygen) were optimal. Animals were fed *ad libitum* twice a day. Uneaten food and waste matter were removed every three days and water was recovered. After infection tanks were not cleaned. **Bioassay 1** was conducted for 11 days with treatments as follow: **I)** Control group: CF + cellulose (2 g/kg feed); **II)** CF-cellulose + *Vibrio* CL_{50} (7 x 10^4 cells/mL) + WSSV (500 mg of infected tissue/tank); **III)** CF + FA (2 g/kg feed) daily + *Vibrio* CL_{50} (7 x 10^4 cells/mL) + WSSV (500 mg of infected tissue/tank); **IV)** CF + FA (2 g/kg feed) every 2 days + *Vibrio* CL_{50} (7 x 10^4 cells/mL) + WSSV (500 mg of infected tissue/tank); **V)** CF + FA (2 g/kg feed) every 3 days + *Vibrio* CL_{50} (7 x 10^4 cells/mL) + WSSV (500 mg of infected tissue/tank). At the 7^{th} day of the bioassay, shrimp were infected with pathogens at the same time. **Bioassay 2** was conducted for 5 days. Animals (60) were put in a tank and haemolymph and hepatopancreas of six shrimp were sampled at 0, 6, 12, 24, 48, 72, and 96 h. Tuberculin syringes put on ice were loaded with anticoagulant solution for haemolymph sampling. After taking the samples (haemolymph and hepatopancreas) at time zero, shrimp were fed with CF + FA (2 g/kg feed) twice a day during the first day. Haemolymph was centrifuged (800 x g, 10 min) to obtain haemocyte pellet. Each sample was put in 250 µL of precooled Trizol®. The total RNA isolation and cDNA synthesis were performed. The expression of four immune-related genes (Superoxide dismutase [SOD], toll receptor [LvToll], translationally controlled tumor protein [TCTP], and heat shock protein 70 [Hsp70]) were determined by real time RT-PCR (EvaGreen), using β-actin, EF1α, and L-21 as reference genes (Wang et al., 2010; Wu et al., 2013). TCTP, SOD, and LvToll genes were studied in haemocytes. Hsp70 gene was studied in hepatopancreas. The relative expression of each gene was obtained according to Vandesompele et al. (2002). Data obtained were subjected to one-way ANOVA followed by a Tukey HSD test. A probability value of less than 0.05 was considered as statistically significant.

Results

Survival (mean ± SE) in treatments of bioassay 1 was: I) 100 ± 00%, II) 36.6 ± 0.88%, III) 50 ± 1.52%, IV) 70 ± 0.58%, and 23 ± 1.45%. In bioassay 2, the expression of SOD, TCTP, and hsp70 gene was

modulated but not in LvToll. SOD and hsp70 gene expression was down-regulated while TCTP gene expression was up-regulated (Table 1).

Discussion

Fulvic acid protects shrimp infected with vibrio and WSSV when they are fed every two days because survival was 70% as compared with treatment II (36.6%). Fulvic acid is known by its properties such as antioxidant and anti-inflammatory (Vucskits et al., 2019). It is possible that the above mentioned properties of FA protect shrimp proteins from damage by oxidizing substances, therefore, the expression of SOD and Hsp70 genes was down-regulated. SOD is an antioxidant enzyme and Hsp70 is very important in protein repair and folding and transport of nascent proteins. Fulvic acid increases the expression of TCTP gene which plays important roles in cell growth, cell cycle progression, and anti-apoptotic activity. Furthermore, TCTP is involved in the immune response of *L. vannamei* against virus infection (Wu et al., 2013). The LvToll gene expression did not change with time in shrimp fed with FA.

Conclusions

Fulvic acid has protective effects on the shrimp challenged with vibrio and WSSV. Also, FA modulates the expression of three immune-related genes.

References

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Table 1. Immune-related gene expression in haemocytes and hepatopancreas of *L. vannamei* fed with fulvic acid. The results are expressed as means ± SE. Different letters in the same column indicate significant differences.

Time (h)	SOD	LvToll	TCTP	Hsp70
0	1.6 ± 0.3^{ab}	0.9 ± 0.2	0.6 ± 0.0^{a}	1.6 ± 0.2^{bcd}
6	2.3 ± 0.3^{b}	1.1 ± 0.2	0.7 ± 0.1^{a}	1.8 ± 0.4^{cd}
12	1.6 ± 0.5^{ab}	1.0 ± 0.3	0.9 ± 0.1^{a}	1.7 ± 0.3^{bcd}
24	2.0 ± 0.4^{b}	1.8 ± 0.3	1.1 ± 0.1^{a}	2.5 ± 0.3^{d}
48	1.5 ± 0.1^{ab}	1.2 ± 0.3	1.3 ± 0.1^{a}	1.0 ± 0.2^{abc}
72	0.6 ± 0.1^{a}	1.8 ± 0.3	1.4 ± 0.3^{ab}	0.6 ± 0.1^{ab}
96	0.3 ± 0.1^{a}	1.1 ± 0.2	2.1 ± 0.3^{b}	0.4 ± 0.1^{a}