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Research Article
Effect of Dietary Phytobiotics Products on Growth, Immune Responses and Vibriosis Resistance in *Litopenaeus vannamei*

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Abstract

Background and Objective: Phytobiotic products have been found to have anti-bacterial activity, improved immune responses and the survival rate of some aquatic organisms. This study aimed to explore the effects of phytobiotics products from spent hops and yeast cell wall (HY) and Grape Pomace (GP), which are polyphenol-rich feed additives, on the health and tolerance to *Vibrio* infection of Pacific white shrimp under laboratory conditions. **Materials and Methods:** Shrimp post-larvae 12 were stocked in 24×500 L fiberglass tank (50 shrimp/tank) and salinity were maintained at 20-25 ppt. The shrimps were randomly distributed into four groups (six replicates/treatment) and fed four times daily with six experimental diets contained 400 ppm HY, 400 ppm GP, 800 ppm GP, or none of these supplements (control diet). After 60 days, 30 shrimp from each tank were sampled and stocked in new 24 tanks. *Vibrio parahaemolyticus* was added into each tank to obtain a final concentration of 10^4 CFU mL^{-1}. Immune parameters and survival rate after *Vibrio* challenged test were compared using one-way ANOVA model followed by SPSS (Version 20). **Results:** The body weights of shrimp raised on 400 and 800 ppm, GP were significantly higher than the control group $p<0.05$. Shrimp fed with 400 ppm HY- and 800 ppm GP had significantly higher survival rates than the control group $p<0.05$. The shrimp fed with 400 ppm HY also had the highest immune responses. **Conclusion:** The present study indicated that both phytobiotics feed additives tested had positive effects on shrimp health.

Key words: Phytobiotics, growth, immune responses, survival, *Litopenaeus vannamei*, vibriosis , hops, yeast cell wall, grape pomace

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei*), which are native to the Pacific coasts of Central and South America, is the primary shrimp species cultured in China, Taiwan and Thailand[1]. Since 2012, shrimp farmers in Thailand have experienced Early Mortality Syndrome (EMS), which has caused major economic losses in many cultivation areas throughout the country. Affected shrimp shows signs of a pale coloration due to pigment loss as well as hepatopancreas atrophy[2]. These signs may become apparent as early as four days after stocking. *Vibrio parahaemolyticus* was reported to be a suspected agent that has caused mass mortality[3]. As a prevention method, many scientists have attempted to solve this problem using probiotic bacteria or organic acids to reduce the pathogenic bacteria in the gut of the shrimp[4-6]. The use of phytogenic compounds is one of the most promising solutions because they have a good ability to inhibit pathogens as well as having antioxidant properties and the ability to enhance the activities of digestive enzymes and increase nutrient absorption, which causes better growth[7-10]. Polyphenols are a major group of plant secondary metabolites that have one or several phenolic hydroxyl groups. They are widespread in many plant foods and beverages such as fruits, vegetables, cereals, legumes, tea, wine and beer[11-13]. Their many biological effects include antioxidant, anti-inflammatory and antimicrobial activities and make polyphenols useful in health promotion[14-16]. From this aspect, polyphenol-rich plants extracts are among the best candidates for use as a feed additive because of the many previous reports showing the positive effects of polyphenols on health performance in both humans and animals[17-19]. This study is useful to find a new alternative product to replace antibiotics in shrimp culture. Therefore, the objective of this study was to evaluate the effects of two dietary polyphenol-rich feed additives, namely natural products from spent hops and yeast cell wall (HY) and Grape Pomace (GP) on growth, survival, intestinal bacteria counts and tolerance to *Vibrio parahaemolyticus* infection in Pacific white shrimp under laboratory conditions.

MATERIALS AND METHODS

This study was conducted from December, 2015 to May, 2016.

Experiment 1-effects of spent hops and yeast cell wall (HY) and Grape Pomace (GP) on the growth and survival of Pacific white shrimp post-larvae.

Experimental diets trials: The commercial grade of spent hops and yeast cell wall (HY) and Grape Pomace (GP) used in this study were Anta®Phyt Aqua (Dr. Eckel GmbH, Niederzissen, Germany) and Anta®Ox FlavoSyn (Dr. Eckel GmbH, Niederzissen, Germany), respectively. Four experimental diets were formulated: group I none of these supplements (control diet), group II 400 ppm HY, group III 400 ppm GP or group IV 800 ppm GP. Both substances were applied by spraying and mixing with commercial pellet feed containing 36% crude protein and 6% lipid from Charoen Pokphand, Thailand.

Shrimps and experimental protocol: The experiments were carried out at the Aquaculture Business Research Center Laboratory, Faculty of Fisheries, Kasetsart University, Thailand. Postlarvae-9 (PL-9) of Pacific white shrimp were obtained from a hatchery in Chachoengsao Province, Thailand. After 3 days of acclimation, shrimps (PL-12) were randomly distributed into 24×500-L fiberglass tanks (six replicate tanks per treatment). Each tank was stocked with 50 shrimp. Each treatment group was fed with one of the four diets four times daily to satiation for 60 days. Salinity throughout the experiment was maintained at 20-25 ppt, dissolved oxygen above 4 ppm and water temperature at 29±1°C. Leftover feed and feces were siphoned daily and 10% of the water was exchanged every 3 days. The average body weight and survival rate of shrimp were recorded after a 60 day experimental period. Ten shrimps from each tank were randomized and weighted individually by two-decimal point balance (METTLER TOLEDO, Greifensee, Switzerland).

Experiment 2-effects of spent hops and yeast cell wall (HY) and Grape Pomace (GP) on growth, survival and intestinal bacteria of Pacific white shrimp challenged with Vibrio parahaemolyticus.

Shrimps and experimental protocol: Shrimps from each tank in experiment 1 were randomly distributed into new 24×500 L fiberglass tanks (six replicate tanks per treatment). The stocking density was 30 shrimps per tank. At the beginning of this experiment (0 days) and 14 days after stocking, *Vibrio parahaemolyticus* was added into each tank to obtain a final concentration of 10^4 colony-forming units CFU mL^{-1}, which is the normal concentration of *Vibrio* in the water of shrimp farm[20,21]. *Vibrio parahaemolyticus* used for immersion challenge test in this study was collected from the EMS farm in Thailand according to Joshi *et al.*[22]. Each treatment group received the same diet as in Experiment 1 four times

daily for another 30 days. Salinity, dissolved oxygen and water temperature were maintained as in Experiment 1. Leftover feed and feces were siphoned every 2 days.

Growth and survival study: The weight of shrimp from each treatment was measured and their survival rate was recorded on the 30th day after being challenged with *V. parahaemolyticus* at 10^4 CFU mL^{-1}.

Intestinal bacterial study: Five shrimp from each group were randomized and their intestines collected on the 10th, 20th and 30th day. The intestine of each shrimp was homogenized and spread on TCBS (selective media for *Vibrio* spp. culture) or NA (general media for most bacterial cultures) by the spread plate technique, then incubated at 37°C for 24 h. Finally, all colonies of bacteria were counted and calculated as CFU g^{-1} unit.

Immune parameters study: The immune parameters were measured at the end of the feeding trial. Ten shrimp per treatment were used for immunological tests. A hemolymph sample of 250 µL from each shrimp was withdrawn from the base of the 3rd walking leg using a syringe containing 750 µL of precooled (4°C) anticoagulant (0.114 M trisodium citrate, 450 mM NaCl, 10 mM KCl, 10 mM HEPES at pH 7.4) according to Nonwachai *et al.*[23]. The hemolymph-anticoagulant mixture was used to measure Total Hemocyte Count (THC), phagocytosis activity, Phenoloxidase (PO) activity, Superoxide Dismutase (SOD) activity and bactericidal activity.

Total hemocyte count: After collecting hemolymph, hemocytes were counted using a hemocytometer (Bright-Line™, Buffalo, NY, USA) and calculated as THC (cells mL^{-1}) = Count $\times 10^4 \times$ dilution factor according to Itami *et al.*[24].

Phagocytosis activity: Phagocytotic activity was determined[24]. Collected shrimp hemocytes were rinsed with shrimp saline (a solution of NaCl 28.4 g, $MgCl_2 \cdot 6H_2O$ 1.0 g, $MgSO4 \cdot 7H_2O$ 2.0 g, $CaCl_2 \cdot 2H_2O$ 2.25 g, KCl 0.7 g, glucose 1.0 g and HEPES 2.38 g L^{-1}) and the viable cell number adjusted to 1×10^6 cells mL^{-1}. The cell suspension (200 µL) was inoculated onto a cover slip. After 20 min, the cell suspension was removed and rinsed with shrimp saline 3 times. Heat-killed yeast preparation (2 mL) was added and incubated for 2 h. Next, the heat-killed yeast preparation was removed and the cell suspension rinsed with shrimp saline 5 times to reach a concentration of 5×10^8 cells mL^{-1} and fixed with 100% methanol. Then, the cover slip was stained with Giemsa stain and mounted with Permount slide mounting fluid. Two hundred hemocytes were counted for each sample. Phagocytic activity, defined as percentage phagocytosis was expressed as according to Itami *et al.*[24]:

$$\text{Phagocytosis (\%)} = \frac{\text{Phagocytic hemocytes}}{\text{Total hemocytes}} \times 100$$

Phenoloxidase activity: Phenoloxidase activity was measured spectrophotometrically (Thermo Spectronic, Waltham, MA, USA) by recording the formation of dopachrome produced from l-dihydroxyphenylalanine, following a modification of a published protocol[25]. The hemolymph-anticoagulant mixture was washed 3 times with shrimp saline and centrifuged at 1000 rpm and 4°C for 10 min. Hemocyte lysate was prepared from hemocytes in cacodylate buffer (pH 7.4; 0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride and 0.26 M magnesium chloride; pH 7.0) by using a sonicator at 30 amplitude for 5 sec and the suspension was then centrifuged at 10,000 rpm at 4°C for 20 min and the supernatant collected. Then, 200 µL of 0.25% trypsin in cacodylate buffer was mixed into the 200 µL of hemocyte lysate followed by 200 µL of l-dihydroxyphenylalanine at 4 mg mL^{-1} as substrate. Enzyme activity was measured as the absorbance of dopachrome at 490 nm wavelength. The protein content in hemocyte lysate was measured following a published protocol[23]. The phenoloxidase activity was calculated as the increase in optimum density min^{-1} mg^{-1} of protein.

Superoxide dismutase activity: SOD activity was measured by its ability to inhibit superoxide radical-dependent reactions using a Ransod Kit (Randox, Crumlin, UK). This method is based on the formation of red formazan during a reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) and superoxide radical, which is assayed in a spectrophotometer (Thermo Spectronic, Waltham, MA, USA) at 505 nm. The reaction mixture (1.7 mL) contains 0.05 mM xanthine and 0.025 mM INT dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase, superoxide and uric acid are produced from the xanthine. The superoxide radicals then react with INT to produce a red formazan dye. The hemolymph-anticoagulant mixture was centrifuged at 3000 rpm at 4°C for 10 min. Plasma was removed and the pellet was resuspended in 3 mL of 0.9% NaCl

and centrifuged again. The supernatant was discarded and the pellet was resuspended in 2 mL of triple distilled water at 4°C. A 50 µL aliquot of resuspended hemocytes was placed in each well of a 96-well plate that contained 200 µL of the reaction mixture. Fifty microliters of xanthine oxidase solution was added to each well and the absorbance measured at 505 nm at 37°C. The rate of reaction was estimated from the absorbance readings of 0.5 and 3 min after adding xanthine oxidase. A reference standard of SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. The specific activity was expressed as SOD units mL^{-1}.

Bactericidal activity: Bactericidal activity was determined according to Supamattaya *et al.*[25]. The results were recorded from a dilution that could decrease 50% of *Vibrio harveyi* compared with the control.

Statistical analysis: Results are presented as the Mean±SD. One way analysis of variance (One-way ANOVA) and Duncan's New Multiple Range test were used to compare data among treatments[25]. Differences were considered significant if $p<0.05$. The SPSS statistical software version 20 was used for these statistical analyses.

RESULTS

Experiment 1: The effects of spent hops and yeast cell wall (HY) and Grape Pomace (GP) on the growth and survival of Pacific white shrimp post-larvae.

After 60 days of dietary administration, the average body weight of shrimp from these groups were significantly higher than the group fed with 400 ppm HY and the control group ($p<0.05$). However, the average survival rate of the shrimp in all of the experimental groups was not significantly different from each other, being in the range of 86-90% (Table 1).

Experiment 2 effects of spent hops and yeast cell wall (HY) and Grape Pomace (GP) on growth, survival and intestinal bacteria of Pacific white shrimp challenged with Vibrio parahaemolyticus.

At the end of experiment 2, there was no significant difference in weight gain among any of experimental groups, although the highest weight gain was in the 800 ppm GP-fed group. Nevertheless, the survival rate of the 400 ppm HY and 800 ppm GP groups was significantly higher ($p<0.05$) than the 400 ppm GP and control groups (Table 2).

For the intestinal bacterial study, neither *Vibrio* spp., nor the total bacterial count was significantly different among all of the experimental groups throughout the feeding trial. At the 30 day point, the lowest number of *Vibrio* spp., and total bacteria were observed in the 400 ppm HY group (Table 3 and 4).

An immunological study revealed the immunostimulatory effect of HY. Shrimp fed 400 ppm HY had a significantly higher immune response ($p<0.05$), including Total Hemocyte Count (THC), phagocytosis and phenoloxidase (PO), Superoxide Dismutase (SOD) and bactericidal activity, compared with the control group. Shrimp fed 800 ppm GP also had higher PO and bactericidal activity than the control shrimp but less pronounced than the HY group. Nonetheless, the SOD activity was not affected by HY or GP (Table 5).

Table 1: Body weight and survival rate of pacific white shrimp after 60 days of feeding with four different diets

Experimental groups	Body weight (g)	Survival rate (%)
Group I	2.64 ± 0.43^{b}	86.67 ± 3.72^{a}
Group II	3.04 ± 0.14^{b}	89.67 ± 2.94^{a}
Group III	3.42 ± 0.22^{a}	89.33 ± 2.42^{a}
Group IV	3.48 ± 0.18^{a}	90.33 ± 2.66^{a}

Data are presented as the Mean±SD. Means in the same column with different superscripts are significantly different from each other ($p<0.05$)

Table 2: Weight gain and survival rate of pacific white shrimp fed with four different diets for 30 days after being challenged with *V. parahaemolyticus* at 10^4 CFU mL^{-1}

Experimental groups	Weight gain (g)	Survival rate (%)
Group I	2.15 ± 0.61^{a}	64.17 ± 1.67^{b}
Group II	2.17 ± 0.67^{a}	78.33 ± 1.92^{a}
Group III	2.00 ± 0.37^{a}	67.50 ± 1.67^{b}
Group IV	2.27 ± 0.47^{a}	78.33 ± 1.92^{a}

Data are presented as the Mean±SD. Means in the same column with different superscripts are significantly different from each other ($p<0.05$)

Table 3: Total number of *Vibrio* spp., (10^6 CFU g^{-1}) in the intestine of pacific white shrimp after being challenged with *V. parahaemolyticus* at 10^4 CFU mL^{-1} and feeding with four different diets for 30 days

Experimental groups	10th day	20th day	30th day
Group I	3.51 ± 3.33^{a}	8.99 ± 5.64^{a}	8.02 ± 7.82^{a}
Group II	2.27 ± 2.10^{a}	7.18 ± 5.32^{a}	4.89 ± 3.44^{a}
Group III	3.05 ± 1.85^{a}	8.65 ± 3.76^{a}	7.00 ± 3.00^{a}
Group IV	2.87 ± 1.56^{a}	7.68 ± 2.67^{a}	5.75 ± 2.29^{a}

Data are presented as the Mean±SD. Means in the same column with different superscripts are significantly different from each other ($p<0.05$)

Table 4: Bacterial load (10^6 CFU g^{-1}) in the intestine of Pacific white shrimp during 30 days after being challenged with *V. parahaemolyticus* at 10^4 CFU mL^{-1}

Experimental groups	10th day	20th day	30th day
Group I	7.22 ± 6.98^{a}	17.48 ± 9.80^{a}	15.74 ± 9.94^{a}
Group II	5.11 ± 3.88^{a}	14.40 ± 10.57^{a}	10.89 ± 4.34^{a}
Group III	6.51 ± 3.84^{a}	16.88 ± 7.57^{a}	14.94 ± 6.17^{a}
Group IV	5.70 ± 2.57^{a}	15.33 ± 6.04^{a}	12.92 ± 7.28^{a}

Data are presented as the Mean±SD. Means in the same column with different superscripts are significantly different from each other ($p<0.05$)

Table 5: Immune parameters of white shrimp after being challenged with *V. parahaemolyticus* at 10^4 CFU mL^{-1} feeding with four different diets for 30 days

Immune parameters	Control	HY 400 ppm	GP 400 ppm	GP 800 ppm
THC (10^5 cells mL^{-1})	19.00±2.63^b	44.00±9.26^a	23.75±8.23^{ab}	26.50±8.23^{ab}
Phagocytosis (%)	17.33±1.15^b	23.33±3.06^a	20.00±2.00^{ab}	21.33±4.16^{ab}
Phenoloxidase activities (unit min^{-1} mg^{-1} protein)	106.06±3.20^c	120.72±3.20^a	110.25±3.20^{bc}	114.44±2.42^b
Superoxide dismutase activity (SOD unit mL^{-1})	51.06±2.40^a	54.59±1.90^a	52.52±1.96^a	53.22±1.85^{ab}
Bactericidal activity	1:4	1:8	1:4	1:8

Data are presented as the Mean±SD. Means in the same row with different superscripts are significantly different from each other ($p<0.05$)

DISCUSSION

The present study showed that dietary GP supplementation significantly increased ($p<0.05$) the average body weight of shrimp. There are many reports about the positive effects of the polyphenol-rich feed additive extracted from grape pomace (*Vitis vinifera*) or, to a lesser extent, spent hops (*Humulus lupulus*) on the health of pigs and chickens[26-30]. Niewold[31] reported that polyphenols have distinct anti-inflammatory effects and this activity may be responsible for growth promotion in many animals. It is likely that the growth-enhancing effects of GP shown in this study may be the result of the anti-inflammation property of grape seed and grape marc polyphenols. These properties have been shown to include suppressing NF-κB activity and their target genes are involved in the inflammation in the duodenal mucosa of pigs and Caco-2 intestinal cells[28,29]. Grape skin is rich in flavanols (e.g., quercetin, myricetin and kaempferol), flavanols (e.g., catechin, epicatechin and procyanidin), hydroxycinnamic acid, anthocyanins and resveratrol, while flavanols (e.g., catechin, epicatechin and procyanidin) and hydroxybenzoic acid can be found in abundant amounts in grape seeds[15,32]. One or several of these polyphenols of grapes may account for many biological effects, including antimicrobial and anti-inflammatory activities, even if the identity of the active substances and their precise mechanisms of action have yet to be investigated. Although HY also showed a growth-improving effect, its extent was lower than GP. However, this result was not a surprising outcome because the compositions of these two feed additives were different and there is a great variation in the quality and quantity of the polyphenol content between plant species and environmental conditions[11,12]. Despite several studies showing the antimicrobial effect of polyphenols[33,34] no difference in both *Vibrio* counts and total bacteria counts were found in this study. These indicated that the antimicrobial property of polyphenols was not relevant mechanism.

The immunostimulatory effect of HY was also observed in the present study; the Total Hemocyte Count (THC), phagocytosis activity, Phenoloxidase (PO) activity and bactericidal activity of the HY-fed shrimp improved compared with the GP-fed group and the control group. This result is believed to be an effect of a hop polyphenol that is rich in catechin and proanthocyanidin[34] and the yeast cell wall which contains a high amount of β-glucans (BGs), a potent immunostimulant[35,36]. Polyphenols exert their immune-enhancing effects mostly by acting against oxidative stress or, in other words, by antioxidant-mediated immune modulation mechanisms[14]. The immunostimulatory activity of BGs is initiated after shrimp BG Binding Protein (BGBP) reacts with BGs to form a BGBP-BG complex, which in turn leads to the release of hemocytic granules and the activation of prophenoloxidase (proPO)[37,38]. The immunostimulatory effect of BGs in shrimp has been reported in many studies[6,38,39]. From this aspect, the survival rates of shrimp infected with *Vibrio parahaemolyticus* observed in the 400 ppm HY- and 800 ppm GP-fed groups were higher compared with the control group. Further study on the addition of GP and HY to the commercial diets to confirm the function of the two products is recommended.

CONCLUSION

This study revealed the beneficial results of using polyphenol-rich feed additives in shrimp aquaculture. Grape Pomace (GP) (800 ppm) exhibited a growth-promoting effect in uninfected PL shrimp and improved the survival rate in *V. parahaemolyticus*-infected juvenile shrimp. The survival rate of *V. parahaemolyticus*-infected juvenile shrimp fed with spent hops and yeast cell wall (HY) was also increased in addition to several immunological parameters. Therefore, this study conclude that polyphenol-rich feed additive products can be useful in shrimp farming.

SIGNIFICANCE STATEMENT

This study discovers the use of phytobiotic products as an alternative to antibiotics in shrimp culture that can be beneficial for farmers and consumers. This study will help the researchers to investigate new methods to improve shrimp aquaculture without using antibiotics.

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