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Feed supplementation with quorum quenching derived from vinasse improved immune responses and disease resistance against *Vibrio parahaemolyticus* causing AHPND in white shrimp (*Litopenaeus vannamei*)

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Abstract The effects of ethanolic extract of vinasse exhibiting quorum quenching (QQ), defined as yeast-QQ, was tested *in vivo* in the juvenile white shrimp. Supplementation of this extract at the concentration of 0.1% and 0.2% showed positive effects on the shrimp health. It demonstrated that the yeast-QQ extract at the test concentrations could enhance the shrimp immunity against the infection of *Vibrio parahaemolyticus* that causes acute hepatopancreatic necrosis disease (AHPND) or *Vp*$_{AHPND}$. The number of total *Vibrio* in the hepatopancreas of the extract-treated shrimp was significantly lower than that of the control groups (no *Vp*$_{AHPND}$ infection) ($P<0.05$). In addition, histopathological analysis revealed the recovery of the hepatopancreas disorder affected by *Vp*$_{AHPND}$ infection in the extract-treated groups and the recovery trended to be a dose-dependent manner. Therefore, the yeast-QQ extract provides an alternative bioproduct for the management of shrimp diseases caused by *Vibrio* spp.

Keywords: Quorum quenching, *Vibrio parahaemolyticus*, Acute hepatopancreatic necrosis disease (AHPND), Immune response, Hepatopancreas histology

Introduction

Litopenaeus vannamei, a commercially important aquaculture species with a sabstaintial global market, has faced a risk of infectious disease outbreaks. Bacteria are one of the most important pathogens in aquaculture that can cause significant economic losses by reducing shrimp productivity (Delphino *et al*., 2019; Huang and Nitin, 2019). In the *Vibrio* genus, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. harveyi*, *V. anguillarum*, and *V. campbelli*, have been reported as pathogens affecting shrimp (Chatterjee and Haldar, 2012;

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Aguiera-Rivera *et al*., 2019). Acute hepatopancreatic necrosis disease (AHPND), previously known as early mortality syndrome (EMS) disease, is a significant threat causing devastating losss in shrimp industry, particularly *L. vannamei*, for almost a decade (Tran *et al*., 2013; Peña-Navarro *et al*., 2020). This disease is caused by a special strain of *V. parahaemolyticus* (*Vp*$_{AHPND}$) that contains a 70-kbp plasmid (pVA1) carrying Pir A and Pir B genes encoding the Photorhabdus insect-related toxins (Dangtip *et al*., 2015; Sirikharin *et al*., 2015; Xiao *et al*., 2017).

Application antibiotics and chemical substances has been utilized to deal with bacterial diseases, however, this approach has led to increase the risk of antibiotic-resistant bacteria as well as cause adverse effects on human health (Suzuki *et al*., 2019). Thereafter, several alternative measures by utilization of natural subtances including medicinal herbal extracts and immunostimulants as well as probiotics, have been focused and developed (Soltani *et al*., 2019; Citarasu *et al*., 2022). There has been demonstrated that the pathogenicity of *Vibrio* spp. including *Vp*$_{AHPND}$ is associated with quorum sensing (QS) or its cell-to-cell communication. Numerous studies have reported the role of QS in regulating various physiological, phenotypic behaviors, and survival mechanisms that enable organisms to adapt and resist to challenging environmental conditions (Henke and Bassier, 2004; Liu *et al*., 2018; Prescott and Decho, 2020). Nowadays, control or inhibition the QS signaling pathway, known as quorum quenching (QQ), of the pathogenic strains of *Vibrio* has been more attractive (Ye *et al*., 2008; Gode-Potratz and McCarter, 2011; Kalburge *et al*., 2017; Zhong *et al*., 2021).

Out previous works have demonstrated the inhibitory QQ effect of Beta-Sac Plus®, which is the yeast extract product derived from the bioethanol distillation vinasse byproduct and the ethanolic extract of vinasse to suppress the growth, virulence gene expression and biofilm formation of *Vp*$_{AHPND}$ (Tep-Ubon *et al*., 2020; Damayanti *et al*., 2023; U-taynapun *et al*., 2023). We found that with the same aspect of QQ inhibiting *Vp*$_{AHPND}$, concentration of the ethanolic vinasse extract is approximately 10-time lower than that of the vinasse. However, the previous works have only been studied for the QQ effects of the crude vinasse extract against *Vp*$_{AHPND}$ *in vitro*. This present work aimed to investigate the *in vivo* effects of the ethanolic vinasse extract exhibiting QQ in the experimental shrimp. The shrimp immunity and the hepatopancreas histology was evaluated to compare between the shrimp fed with/without supplementation of yeast QQ substance and infection/non-infection with *Vp*$_{AHPND}$.

Materials and methods

Preparation of ethanolic vinasse extract

The vinasse is sourced by the private Thai company that extracted using ethanol with a ratio of 1:4 (w/v) as described by U-taynapun *et al*. (2023). The mixture was filtered using a filter paper (Whatman No. 1). The supernatant was separated and ethanol was removed by evaporation. The obtained crude extract, named yeast-QQ, was dried, weighed and kept at -20ºC until used. For further study, a 2.0% ethanolic vinasse stock was prepared. The stock solution was subsequently filtered through a 0.45 µm membrane filter (Whatman) and kept at -20°C.

Experimental shrimp

Juveniles *L. vannamei* (mean weight 9±0.2 g) were gained from a commercial farm in Nakhon Si Thammarat Province. After acclimation in a 2,000 L sea water (10 ppt) in plastic aquarium tank under the controlled laboratory condition, shrimps were fed apparent satiation 4 times daily. After a week of acclimation, shrimps with an average body weight of 10±0.3 g were randomly separated into 24 of 200 L plastic tanks (20 shrimps/tank) for further study.

V. parahaemolyticus and growth condition

The strain of *V. parahaemolyticus* isolated and characterized from the AHPND-diseased shrimp in Nakhon Si Thammarat Province, Thailand was used throughout this study. Pure culture stock of AHPND-causing *V. parahaemolyticus* strain or Vp_{AHPND} was maintained at -80°C in a house culture collection (Aquatic Animal Health Management Research Unit, Department of Agricultural Science, Faculty of Agriculture, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat Campus**)** according to the method of Bunserm *et al*. (2022). The bacterial culture was grown in tryptic soy broth (TSB, Difco) supplemented with 1.5% (w/v) NaCl (TSB^+) at 37°C with shaking at 150 rpm. After 18-24 h, the bacteria culture was centrifuged at 8,000 x g for 10 min. The obtained bacterial cells were resuspended in sterile 1.5% (w/v) NaCl and adjusted to a concentration of 10^7 CFU/ml (OD_{600} ~ 0.5) for further experiments.

Experimental design and sampling

The experiment was conducted using completely randomized design (CRD) with 6 experimental treatments (4 replicates/treatment). There were 2 groups of treatment, the first group (T1-T3) was not infected by Vp_{AHPND} while another group (T4-T6) was orally administrative infection with Vp_{AHPND} (10^4 CFU/g diet). All treatments included; Treatment 1 (T1): Control (fed with basal diet), Treatment 2 (T2): Shrimp fed with diet supplemented with 0.1% yeast-QQ extract, Treatment 3 (T3): Shrimp fed with diet supplemented with 0.2% yeast-QQ extract, Treatment 4 (T4): Shrimp fed with basal diet + $Vp_{AHPND,}$ Treatment 5 (T5): Shrimp fed with diet supplemented with 0.1% yeast-QQ extract + $Vp_{AHPND,}$ and Treatment 6 (T6): Shrimp fed with diet supplemented with 0.2% yeast-QQ extract + $Vp_{AHPND.}$

After 4 and 7 days, hepatopancreas tissue was collected from 2 shrimps/tank and pooled for each treatment for the analysis of total *Vibrio* and gene expression while histological change was determined in the samples collected at 7 days.

Total Vibrio in shrimp hepatopancreas

The collected hepatopancreatic tissue (approximately 0.1 g/sample) was ground in 1.5% (w/v) NaCl (0.9 ml) to make 10-fold dilution. Afterwards, the serial 10-fold dilution was conducted to the desired concentrations, then spread onto Thiosulfate-Citrate-Bile-Sucrose (TCBS, Difco) agar. After 18-24 h of incubation at 37°C, bacterial counts and total *Vibrio* concentrations (CFU/g) were determined.

Analysis of gene expression

Total RNA was isolated from shrimp hepatopancreas by using Total RNA Mini Kit (Tissue) (Geneaid) followed by quality and quantity assessment using spectrophotometer (BioDrop). Total RNA (1 µg) was used as a template for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) in T100TM Thermal Cycler (BioRad). Quatitative PCR (qPCR) was performed to analyze the relative mRNA expression level of 3 immune-related genes (*proPO, Toll* and *HSP70*) using *β-actin* as a reference gene. qPCR reactions (total 20 µl) were performed in triplicate in CFX96 Touch™ Real-Time PCR (Bio-Rad) using HOT FIREPol® EvaGreen® (Solis Biodyne), primers (Table 1) and DEPC-treated water. Cycling conditions were as follows: 95°C for 12 min followed by 40 cycles of 15 s at 95°C, 20 s at 60-

65ºC (depending on the primer pairs) and 20 s at 72ºC. Melting curve analysis was also carried on for all DNA fragment products. The relative expressions of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1. Primer used for qRT-PCR analysis in this study

Target gene	Primer sequence	References
β-actin	Forward: 5'-CCACGAGACCACCTACAAC-3'	Chen *et al*., 2020
	Reverse: 5'-AGCGAGGGCAGTGATTTC-3'	
proPO	Forward: 5'-CACGGAAGGAGGCGTATCAT-3'	Wang *et al*., 2020
	Reverse: 5'-CAATGACCAGCAGCGTCTTC-3'	
Toll	Forward: 5'-CTCCATCACTGGCGCACTTA-3'	Wang *et al*., 2020
	Reverse: 5'-GGTCCTCAGCCTTGGAGA-3'	
HSP70	Forward:5'-CCTTCTTGTCGAGGCCG-3'	Wang *et al*., 2020
	Reverse: 5'-TCCTCAGCCTTGGAGA-3'	

Determination of histolological changes and histopathology

Shrimp hepatopancreas was fixed in Davidson's AFA fixative (DAFA) for 24 h, stored in 70% ethanol, and then dehydrated through a graded ethanol series, 70%, 80%, 90% and 100%, and cleared with xylene. The processed tissue was embedded in paraffin and sectioned at 5 µm. Histologocal analysis was performed using haematoxylin and eosin (H&E) staining according to Goldner (1938). Microscopic examination for the histopathology and histological changes were conducted (Nikon, Japan) and recorded.

Statistical analysis

Statistical differences in total *Vibrio* and the expression levels of immune-related genes were evaluated by one-way analysis of variance (ANOVA) with the SPSS Statistics Software version 16.0 (SPSS Inc.). Duncan's Multiple Range Test (DMRT) was employed to state significant differences among treatments. A p-value of less than 0.05 ($P<0.05$) indicated statistically significant.

Results

Total Vibrio in experimental shrimp

The effects of yeast-QQ extract on the disease resistance against the pathogenic Vp_{AHPND} was determined by comparative analysis of the number of *Vibrio* in hepatopancreas among the treatments of infected shrimps (T4-T6). We

found that *Vibrio* concentration in the hepatopancreas of shrimp in T4 was the highest, whereas shrimps fed with yeast-QQ supplementation exhibited a significant reduction in *Vibrio* levels in 4 and 7 days ($P<0.05$). However, the numbers of *Vibrio* were not statistically different ($P>0.05$) among T5 and T6 which fed with 0.1% and 0.2% yeast-QQ, respectively. The number of total *Vibrio* was reduced from day 4 to day 7 in T5 and T6 which fed with yeast-QQ extract while it seemed stable in the no extract treatment (T4). As expected, *Vibrio* was rarely detected, lower than limit of detection via spread plate technique, in the uninfected shrimps (T1-T3) (Table 2).

Table 2. The number of total *Vibrio* in shrimp treated/non-treated with Vp_{AHPND} and fed with the diet supplementation with 0%, 0.1% and 0.2% yeast-QQ extract at 4 and 7 days

Treatment	**Total *Vibrio* in terms of Log CFU/g (mean ± SD)**[1]	
	4 Days	**7 Days**
T1 (no extract)	ND	ND
T2 (0.1% extract)	ND	ND
T3 (0.2% extract)	ND	ND
T4 (no extract + Vp_{AHPND})	5.21 ± 0.04^{b}	5.09 ± 0.04^{b}
T5 (0.1% extract + Vp_{AHPND})	3.81 ± 0.05^{a}	3.72 ± 0.02^{a}
T6 (0.2% extract + Vp_{AHPND})	3.69 ± 0.05^{a}	3.59 ± 0.05^{a}

[1/] Determined by spread plate technique and ND means not detected (The number of *Vibrio* was lower than the limit of detection since the number of bacterial colonies was lower than 30 in the dilution of 10^{-1}). Different letters in each column indicate a statistically significant difference ($P<0.05$).

Expression of immune-related genes

The comparative analysis of 3 immune-related gene expression including *proPO, Toll* and *HSP70* was determined to investigate the effects of yeast-QQ and Vp_{AHPND} alone and in combination on shrimps. The results are shown in Figure 1. We found that all the studied genes expressed statistically higher in the Vp_{AHPND}-infected shrimps (T4-T6) than that of non-treated shrimps (T1-T3) ($P<0.05$). The constant level of these genes was demonstrated in the non-treated shrimps (T1-T3). In addition, the expression level of all 3 genes were declined from day 4 to day 7 after treatment. This may be due to shrimp responses was reduced in concordance of the reduced number of bacterial pathogens shown in Table 2.

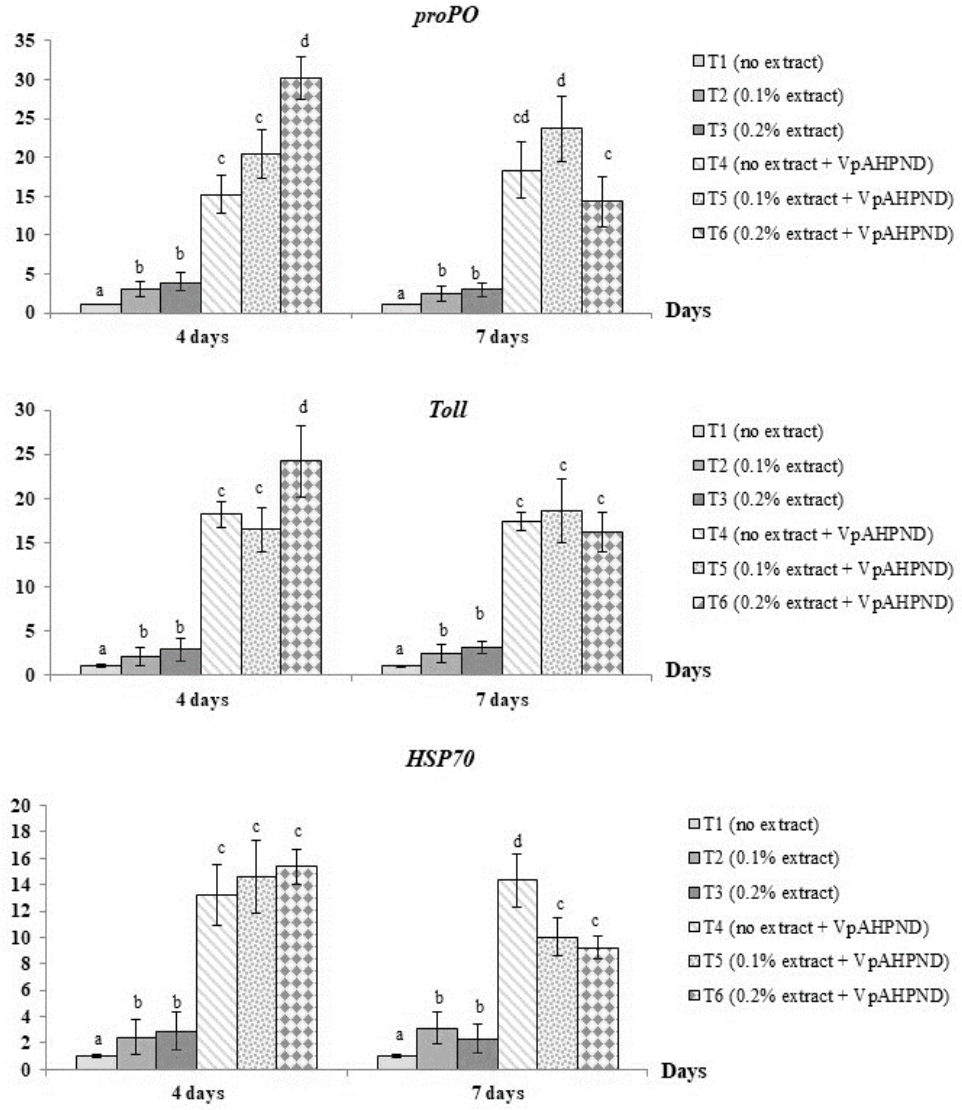

Figure 1. The expression level of immune-related genes (*proPO, Toll* and *HSP70*) in hepatopancreas of shrimp treated/non-treated with Vp_{AHPND} fed with the diet supplementation with 0%, 0.1% and 0.2% yeast-QQ extract at 4 and 7 days. T1: Control (fed with basal diet), T2: 0.1% yeast-QQ extract, T3: 0.2% yeast-QQ extract, T4: Basal diet + Vp_{AHPND}, T5: 0.1% yeast-QQ extract + Vp_{AHPND}, and T6: 0.2% yeast-QQ extract + Vp_{AHPND}. Data are presented as mean ± SD. Statistically significant differences ($P<0.05$) among groups are indicated by different letters

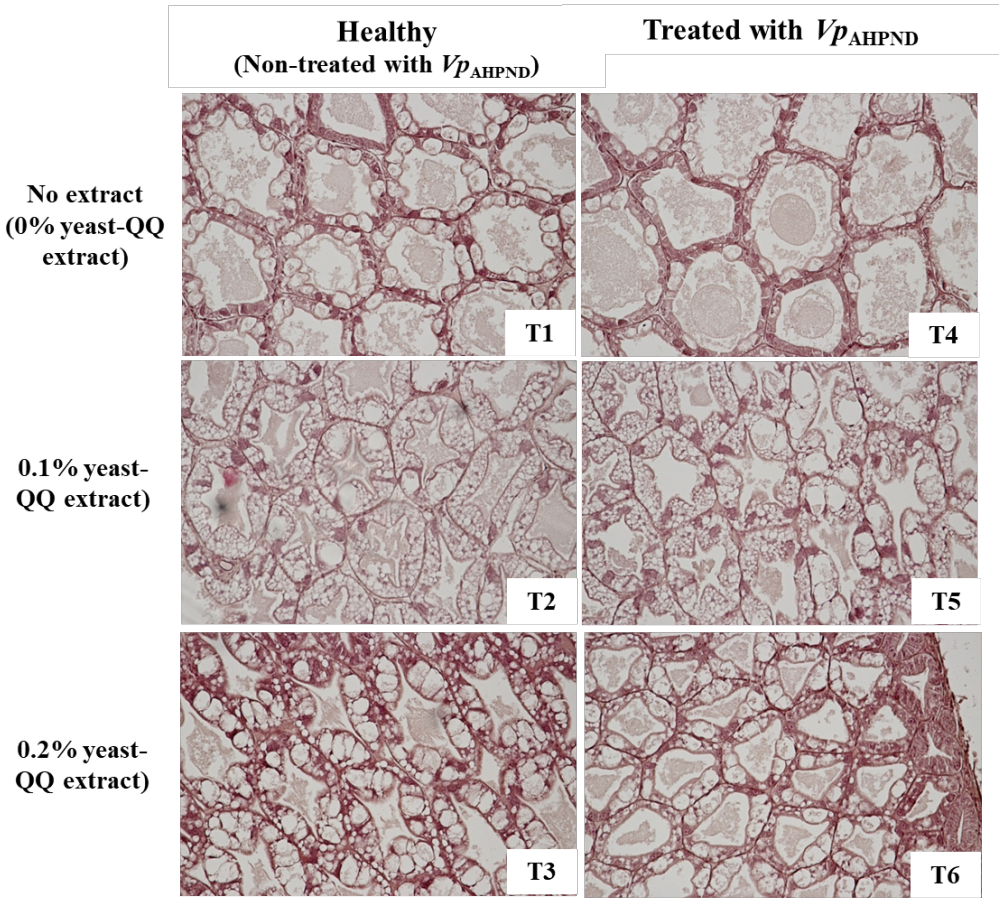

Figure 2. Histological sections of hepatopancreas of white shrimp *L. vannamei* (Magnification: 400X): Shrimps were fed with diet supplementation with 0%, 0.1% and 0.2% yeast-QQ extract treated or non-treated orally with Vp_{AHPND} for 7 days. T1: Control (fed with basal diet), T2: 0.1% yeast-QQ extract, T3: 0.2% yeast-QQ extract, T4: Basal diet + Vp_{AHPND}, T5: 0.1% yeast-QQ extract + Vp_{AHPND}, and T6: 0.2% yeast-QQ extract + Vp_{AHPND}. Sections were stained with haematoxylin and eosin (H&E)

Histological changes of shrimp hepatopancreas

The results of histological analysis of *L. vannamei* fed with diet supplementation with yeast-QQ extract showed no negative effects. The cells in T2 and T3, shrimps fed with 0.1% and 0.2% yeast extract, was similar as those of the control group (T1). No histopathology of the hepatopancreatic cells in T1, T2 and T3 which were non-infected or healthy shrimp (Figure 2). This finding

indicated that the yeast-QQ extract had no toxicity on the shrimp hepatopancreas. Conversely, histology sections of the shrimp treated with *Vp*$_{AHPND}$, particularly T4 with no yeast-QQ supplementation, showed delamination, atrophy, and cell sloughing in hepatopancreas, and decrease of the B-cell height responsible for the normal characters of AHPND (Figure 2). The recovery refering to the lower numbers of damage hepatopancreatic cells and bigger B-cell was found in T5 and T6 fed with 0.1% and 0.2% yeast-QQ extract, respectively. Although the shrimp in T6 was orally administration of *Vp*$_{AHPND}$, the density and size of B-cell was similar to that observed in the healthy (non-treated with *Vp*$_{AHPND}$) groups of T1, T2 and T3. This finding suggested that 0.2% yeast extract might effectively recovered the shrimp hepatopancreas.

Discussion

Recently, many researchers have discovered the procedures and natural substances to control *Vp*$_{AHPND}$, the causative agent of AHPND. On the other hand, the measures of improvement of the shrimp immunity have been developed in parallel. Not only bioactive compounds exhibiting antibacterial activity but also the substances targeting quorum sensing (QS), a bacterial communication mechanism crucial for survival, virulence, and pathogenecity have gained significant interest. Numerous strategies focused on modulating or disrupting QS have been explored to overcome pathogenic bacteria. By reducing the threats postured to bacteria, these strategies also lessen the resistance development (Tay and Yew, 2013). Consequently, development of natural substances exhibiting quorum quenching (QQ) or QS inhibition has become a focal area of research. Indeed, the ability of *Vibrio* spp. to produce certain virulence factors, such as biofilm formation, has been demonstrated to link to their pathogenicity (Yildiz and Visick, 2009; Gode-Potratz and McCarter, 2011, Kalburge *et al*., 2017).

Our previous works have shown that Beta-Sac Plus®, the yeast extract product, and the ethanolic extract of vinasse are able to suppress the growth and reduce the number of viable *Vp*$_{AHPND}$ via QQ effect at the concentration of 2% and a range between 2% and 0.2% (Tep-Ubon *et al*., 2020; Damayanti *et al.*, 2023; U-taynapun *et al*., 2023). Moreover, the ethanolic vinasse extract or yeast-QQ extract at the test concentrations inhibit the expression of vilurence-related and QS-related genes and biofilm formation of the *Vp*$_{AHPND}$. Therefore, in this present study we established the *in vivo* experiment to test the inhibitory effect of the yeast-QQ extract on *Vp*$_{AHPND}$ control in the shrimp. The diet was supplementation with 0.1% and 0.2% of yeast-QQ extract and fed 2 groups of shrimps; oral administration of *Vp*$_{AHPND}$ and non-infected or healthy shrimp. As expected, comparison within the *Vp*$_{AHPND}$ infected group, total *Vibrio* in the

hepatopancreas of shrimp fed with the basal diet (no yeast-QQ extract) was statistically higher ($P<0.05$) with around 1.3-1.5 log more than that detected in the yeast-QQ treated groups. In addition, total *Vibrio* was continually increased in the basal diet fed group while the constant level of *Vibrio* was recorded from day 4 to day 7. This finding is corresponding with the QQ effect not the antibacterial or bactericidal effect. The QQ has been demonstrated to inhibit the growth not kill the bacteria. This is typically observed as a decreased log phase and an accelerated entry into the stationary phase compared to the control group (Leguina *et al.*, 2018; Christwardana *et al.*, 2019; Damayanti *et al.*, 2023; U-taynapun *et al*., 2023). The result in this present work therefore indicated the QQ effect of the yeast-QQ *in vivo* in the experimental shrimp.

Yeast extract containing massive bioactive substances, for example, β-glucan, nucleotides, and secondary metabolites, has been documented as the immunostimulant activate the immune responses of aquatic organisms. Numerous studies on enhancing immune responses of yeast and its constituents in crustaceans including shrimp have been reported (Suphantharika *et al*., 2003; Chang *et al*., 2013; Chirapongsatonkul *et al*., 2019; Ayiku *et al*., 2020; Luan *et al*., 2021). Here, we determined the effects of yeast-QQ extract administration on the expression of 3 immune-related genes, prophenol oxidase (*proPO*), Toll-like receptor (*Toll*) and heat shock protein 70 (*HSP70*) of *L. vannamei*. Dynamic change of these 3 genes were observed from day 4 to day 7. Among the groups of *Vp*$_{\text{AHPND}}$ treatment, administration of 0,1% and 0.2% yeast-QQ extract induced higher level of *proPO* and *Toll* than that of only *Vp*$_{\text{AHPND}}$ at day 4 and then the expression was declined. The proPO system in shrimp plays a crucial role in antibacterial immunity through producing melanin and cytotoxic compounds for bacterial sequestration. As a key enzyme in the melanization cascade, proPO is involved in cuticle hardening, wound repair, and pathogen elimination (Charoensapsri *et al*., 2014). Toll pathway is one of the important mechanisms underlying the immune responses against bacterial and viral infection in shrimp. Up-regulation of *proPO* and *Toll-like receptor* has been reported to be associated with the *Vp*$_{\text{AHPND}}$ infection (Boonchuen *et al*., 2021) and administration of yeast β-glucan (Luan *et al*., 2021). The expression of *HSP70* revealed that yeast-QQ extract could induce shrimp immune responses. Jumprung *et al*. (2019) has also demonstrated that *L. vannamei* HSP70 enhances the shrimp resistances to *Vp*$_{\text{AHPND}}$. The induction of the gene expression of *proPO, Toll* and *HSP70* might be due to the component in the yeast-QQ extract used in this study.

Hepatopancreas histological analysis revealed the unique histopathological characteristics of *Vp*$_{\text{AHPND}}$ infection corresponding with other studies (Tran *et al*., 2013; Dong *et al*., 2017). We found the lesser damage in hepatopancreas of the

shrimp fed with the yeast-QQ extract compared to control group that infected *Vp*$_{AHPND}$. This might be affected by the lower number of *Vp*$_{AHPND}$ associated with the QQ effect described above. In addition, the recovery of hepatopancreas of the *Vp*$_{AHPND}$-infected shrimp fed with yeast-QQ extract supplementation diet might be due to the effect of yeast constituents correlated to the researches recently described (Rairat *et al*., 2022; Wei *et al*., 2023).

In conclusion, this work presents the first evidence of the beneficial effects of byproduct vinasse extracts, yeast-QQ extract, on the inhibition and control of AHPND-associated *V. parahaemolyticus* (*Vp*$_{AHPND}$) *in vivo*. Moreover, the yeast-QQ extract notably enhance the shrimp immune responses and capable to recover the hepatopancreas of *Vp*$_{AHPND}$-infected shrimp. Our findings align with strategies that aim to reduce bacterial numbers or inactive bacterial growth while simultaneously improving shrimp health. Therefore, the potent yeast-QQ extract derived from the byproduct vinasse could be a promising candidate for bacterial control in aquaculture, contributing to environmental sustainability.

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