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# Evaluation of dietary yeast derived mannan oligosaccharide for pacific whiteleg shrimp *Penaeus vannamei*: Effects on growth performance, immune response, hepatopancreas morphology and resilience to infection against *Vibrio parahaemolyticus*

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ABSTRACT

With trends towards low fishmeal and higher plant ingredient-based diets for shrimp, there is consequently greater demand on feed additives to bolster the protein and nutrient profile quality for achieving more efficiency. Also, there is a need to enhance innate immunity and resilience to infection under intensive shrimp production scenarios. This investigation tested incremental levels 0, 0.2; 0.4; 0.6 and 0.8 % of a commercial yeast cell wall extract mainly comprising β-glucan and mannan oligosaccharides (MOS) (YM, YeaMOS, Angel Yeast, Yichang, Hubei, China) for post-larval shrimp *Penaeus vannamei* in isonitrogenous and iso-lipidic diets with proteinaceous plant ingredients amounting to 50 % contribution of the diet formulation. After 60 d, growth and feed utilization performance were not significantly affected in shrimp in terms of weight gain or FCR, but due to much higher survival, total biomass in groups of shrimps fed with dietary YM was higher compared to the control treatment. A sequential specific pathogen challenge test with *Vibrio parahaemolyticus* at final dose of $10^4$ CFU $mL^{-1}$ resulted in a marked improvement in survival against the control group with much enhancement at the inclusion level of 0.4 % YM ($p < 0.05$). Before challenged, the total haemocyte count (THC) and the relative gene expression of Prophenoloxidase (ProPO) were higher in the group of shrimps fed with YM. Likewise, after infection, the amount of THC and ProPO remained better in the group of shrimps fed with YM. However, the 0.8 % YM treatment did not significantly differ from the control for phagocytic activity and index. The histomorphology condition of the hepatopancreas of shrimp before the infection for all treatments appears normal without significant numbers of hepatopancreatic tubules (T) and epithelial cell vacuoles (V). After pathogen challenge, all shrimp exhibited severe necrotic cell damage and massive sloughing of hepatopancreatic tubule epithelial cells into the lumen. Overall, giving YM as feed additives can improve the shrimp biomass and health, with the use of 0.4 % inclusion levels in practical diets for shrimp *P. vannamei* become the optimum dose to develop more efficient aquafeed production.

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## 1. Introduction

Farmed production of Pacific white shrimp *Penaeus vannamei* serves as one of the fastest-growing commodities in the aquaculture industry (Asmild et al., 2024; Boudad-Reantaso et al., 2012; Emerenciano et al., 2022). Globally, the production number has increased from 155 thousand tonnes in 2000–5.8 million tonnes in 2020, with the main driving factors being the growth in population and income (Asmild et al., 2024). However, disease outbreaks caused by viruses, bacteria, and microsporidians are becoming one of the primary factors that could hamper shrimp production and trade (Kumar et al., 2023), resulting in serious economic losses of around US$ 6 billion worldwide and US$ 20 billion over a decade in Asian countries (Shinn et al., 2018). Although antibiotics is quite popular to treat diseases, the frequent use of antibiotics has allowed for the development of drug-resistant strains leading to antimicrobial resistance (Defoirdt et al., 2008; Seethalakshmi et al., 2021). Therefore, preventive approaches to stimulate and enhance immune responses are essential in the shrimp industry and to mitigate the serious issue of antimicrobial resistance.

Optimization of the immune response through the administration of a functional diet that provides health benefits beyond essential nutrition is often necessary for shrimp (Hernández-Cabanyero et al., 2023), especially considering the primitive immune condition in shrimp and mostly dependent on their innate immune factors as they lack a true adaptive immune response mechanism like fish and higher vertebrates (Chen and He, 2019; Fajardo et al., 2022; Kulkarni et al., 2021; Mansour et al., 2022). Several research investigations has been concluded and detailed the successful activation of immune like response through the administration of functional feed additives such as administrating probiotics (Olmos et al., 2011); nucleotides (Novriadi et al., 2022); β glucan and yeast based additives (Chen et al., 2020; Chuchird et al., 2023; Jin et al., 2018; Li et al., 2019); seaweed polysaccharides (Abbas et al., 2023), enzymes and functional immunostimulants (Chuchird et al., 2023). Among these, β glucan and yeast based additives have emerged as promising candidates due to their stable nutritional content and immunomodulatory properties.

Yeast is also rich in high-quality protein (Agboola et al., 2021), making it a valuable dietary supplement for shrimp supplying a balanced source of essential amino acids. A study by Zhao et al. (2017) demonstrated that the supplementation of yeast extract for replacing dietary fish meal in the presence of supplemental fish oil, phosphorus and calcium resulted in similar weight gain and survival of shrimp. Moreover, the presence of polysaccharides (mannan and β-glucans) contained in the yeast cell wall could inhibit the pathogen adherence, modulate the bacterial growth (Smith et al., 2020), and improve the sensitivity of the immune response mechanism (Faustino et al., 2021; Liu et al., 2021). Furthermore, the hydrolysis process of mannan to produce mannan oligosaccharide (MOS) received significant attention in aquaculture as a prebiotic and immune modulator by triggering the activation of the innate immune system in response to a non-self-substance (Torrecillas et al., 2014). Interestingly, the combination of MOS with β glucan even prolonged the high levels of immune indices compared with MOS or β-glucan supplementation alone (Gu et al., 2011).

In aquaculture, dietary β-glucans have been shown to stimulate the immune system of many species by binding specific receptors on immune cells, activating them to defend against pathogens (Hadiuzzaman et al., 2022; Kim et al., 2011; Rodrigues et al., 2020). Research on fish has shown that dietary supplementation with β-glucans from yeast enhances immune responses (Machuca et al., 2022). Meanwhile in shrimp, the use of β-glucan could be recognized by the specific pattern recognition receptors (PRR) and potentiate the humoral innate immune response, activate the immune-related genes, including prophenoloxidase (proPO) (Cerenius et al., 2008; Ernesto Ceseña et al., 2021; Zhao et al., 2013), increase the production of anti-microbial peptides, and improve the agglutination process (Guluarte et al., 2023).

The adoption of MOS and β-glucan yeast-based supplements in shrimp farming can contribute to sustainable and efficient aquaculture practices while reducing the reliance on fishmeal. We know that high plant-based feeds with low animal or marine ingredients may compromise performance, health condition and resilience of aquatic organisms. For these reasons, a study was implemented to ascertain further evidence for the commercial yeast cell wall extract mainly comprising β glucan and mannan oligosaccharides, MOS (YM, YeaMOS, Angel Yeast Co. Ltd, Yichang, Hubei, China) product as a partial functional ingredient for *P. Vannamei* under experimental conditions. Therefore, this research was conducted to evaluate the effect of YM supplementing the low fish meal-based diet at 0.2; 0.4; 0.6 and 0.8 % in Vannamei diets by assessing the growth performance and feed utilisation. Additionally, we conducted a subsequent pathological challenge trial to evaluate the effect of YM on the survival, immune responses and hepatopancreatic histomorphological condition of shrimp before and after being challenged *by Vibrio parahaemolyticus* under controlled exposure. The findings from this research might provide useful information on the formulation of economical and functional diets for *P. vannamei*.

## 2. Materials and methods

### 2.1. Experimental diets

Experimental diets, including control treatment, were designed to be iso-nitrogenous and iso-lipidic (35 % protein and 7 % lipid). The control diet contained 10 % fish meal (FM), 44,1 % de-hulled solvent extracted soybean meal (SBM), and 8 % corn protein concentrate (CPC) as the dietary protein sources. The series of five diets were formulated to contain 0, 0.2; 0.4; 0.6; and 0.8 % of commercial β-glucan and MOS (YM, YeaMOS, Angel Yeast Co. Ltd, Yichang, Hubei Province, China) as an effective broad-spectrum product with >20 % purity for β-glucan and >20 % purity for MOS, designated as 0.2 % YM, 0.4 % YM, 0.6 % YM, and 0.8 % YM, respectively (Table 1). Prior to the production of experimental diets, the yeast was mixed with micro ingredients, including the mineral premix, vitamin premix, choline chloride, Vitamin C and CaP-dibasic. All micro-ingredients then mixed with macro-ingredients homogenously in a 100 kg batch followed by grinding to a particle size of <200 μm using a disk mill (Jinan Shengrun, China). The mixed ingredients were conditioned in a steam injection conditioner for 12 s prior to pelleting. The pellet temperature just after pelleting was 80 – 82°C. After the drying process in an oven, the pellets (3-mm diameter) were sampled for homogeneity and stored in sealed plastic bags until further use. Proximate composition and amino acid profile of the diets were analyzed at the Saraswanti Indo Genetech Laboratory, Bogor, West Java, Indonesia and summarized in Table 2.

### 2.2. Experimental design

The growth trials were conducted at the Batam shrimp research development (Batam, Kepulauan Riau, Indonesia). Shrimp *P. vannamei* post larvae (PL) were obtained from PT. Maju Tambak Sumur (Kalianda, Lampung, Indonesia) and nursed in a semi-indoor recirculating system. The PL were fed with a commercial feed (Evergreen Feed, Lampung, Indonesia) for three weeks until they reached the suitable size. Shrimp (2.02±0.02 g initial mean weight) were stocked into 70 ×35 x 40 cm (98 L) aquaria tank with 15 shrimp per tank or equal with 150 shrimp $m^{-2}$. Six replicate groups of shrimps were administered different types of experimental diets using nutrition research standard protocols for 60 days and fed by hand four times daily, at 07:00, 11:00, 15:00 and 19:00 h. Based on our historic results, daily feed inputs were pre-programmed assuming the normal growth of shrimp and feed conversion ratio of 1.5. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality records.

The sequential challenge test followed by the assessment of health

**Table 1**
Formulation of experimental diets (%, *as is*) used to evaluate the effects of various inclusion levels of yeast cell wall extract to the shrimp *P. vannamei*.

| Ingredient (*As is* % inclusion) | Dietary treatments | | | | |
|---|---|---|---|---|---|
| | Control | 0.2 % YM | 0.4 % YM | 0.6 % YM | 0.8 % YM |
| Menhaden fishmeal[a] | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Soybean meal[a] | 44.10 | 44.10 | 44.10 | 44.10 | 44.10 |
| Corn protein concentrate[b] | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 |
| Commercial yeast (β-glucan and mannan oligosaccharides)[c] | 0.00 | 0.20 | 0.40 | 0.60 | 0.80 |
| Menhaden fish oil[a] | 5.57 | 5.56 | 5.55 | 5.54 | 5.52 |
| Lecithin (soy)[d] | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Cholesterol[d] | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Corn Starch[d] | 1.78 | 1.59 | 1.40 | 1.21 | 1.03 |
| Whole wheat[e] | 28.00 | 28.00 | 28.00 | 28.00 | 28.00 |
| Mineral premix (shrimp)[f] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Vitamin premix (shrimp)[f] | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| Choline chloride (0.2 % all diets)[d] | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Rovimix Stay-C 35 %[f] | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| CaP-dibasic[f] | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |

[7]Trace mineral premix (g/100 g premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.550; ferrous sulfate, 2.000; magnesium sulfate anhydrous, 13.862; manganese sulfate monohydrate, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193; alpha-cellulose, 69.664.
[8]Vitamin premix (g/kg premix): thiamin·HCL, 4.95; riboflavin, 3.83; pyridoxine·HCL, 4.00; Ca-pantothenate, 10.00; nicotinic acid, 10.00; biotin, 0.50; folic acid, 4.00; cyanocobalamin, 0.05; inositol, 25.00; vitamin A acetate (500,000 IU/g), 0.32; vitamin D3 (1000,000 IU/g), 80.00; menadione, 0.50; alpha-cellulose, 856.81

[a] PT FKS Multi Agro, Tbk. Jakarta, Indonesia
[b] Empyreal[75], Cargill starches, sweeteners and texturizers, Blair, NE, USA
[c] Angel yeast, Fubon Nutrition, China
[d] PT Rajawali Mitra Pakanindo, Banten, Indonesia
[e] PT Pundi Kencana, Cilegon, Banten, Indonesia
[f] PT Fenanza Putra Perkasa, Jakarta, DKI Jakarta, Indonesia

condition of the shrimp were performed at the Department of Aquaculture, Faculty of Agriculture, Gadjah Mada University, Indonesia after 14 days of a feeding period with yeast-containing feed. Just before and after the challenge test, blood and tissue sampling inventories were conducted to determine the immune status system in shrimp, including the total haemocyte count, phagocytosis index and activity analysis, and the gene expression for the phenoloxidase. All the analyses were also supported with the histomorphological scrutiny of the hepatopancreas of the shrimp before and post infection.

### 2.3. Growth sampling and water quality analysis

For the growth trials, pH, dissolved oxygen (DO), water temperature and salinity were measured four times daily using the Aqua TROLL 500 Multiparameter Sonde instrument and connected to AquaEasy apps (Bosch, Singapore) for data monitoring and recording. Ammonium ($NH_4$), Nitrate ($NO_3$), Nitrite ($NO_2$), and Phosphate ($PO_4$) were measured once per week by using absorption spectrophotometry (DR890, HACH, USA). At the end of the feeding period, the shrimp in each hapa net were counted and weighed to calculate the final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR) and thermal unit growth coefficient (TGC) as follows:

$$PWG = \frac{(average\ individual\ final\ weight - average\ individual\ initial\ weight)}{(average\ individual\ initial\ weight)} {}^{*}100$$

$$FCR = \frac{feed\ given(g)}{alive\ weigh\ gain(g)}$$

$$SR = \frac{final\ number\ of\ shrimp}{initial\ number\ of\ shrimp} {}^{*}100$$

$$TGC = \frac{FBW^{1/3} - IBW^{1/3}}{\sum TD} {}^{*}100$$

**Table 2**
Proximate and amino acid (AA) composition (% *as is, dry matter basis*) of experimental diets.

| Composition | Unit | Control | 0.2 %YM* | 0.4 %YM | 0.6 %YM | 0.8 % YM |
|---|---|---|---|---|---|---|
| **Proximate analysis[1]** | | | | | | |
| Protein Content | % | 35.04 | 35.03 | 35.27 | 35.10 | 35.16 |
| Total Fat | % | 7.33 | 7.47 | 7.49 | 7.51 | 7.55 |
| Total Calories | Kcal/100 g | 370.79 | 379.37 | 381.65 | 379.07 | 381.33 |
| Moisture Content | % | 8.59 | 7.26 | 7.76 | 7.44 | 7.98 |
| Ash Content | % | 7.88 | 8.23 | 8.45 | 8.56 | 7.88 |
| **Amino acid profile[1]** | | | | | | |
| L-Alanine | % | 1.50 | 1.57 | 1.55 | 1.55 | 1.56 |
| L-Arginine | % | 2.13 | 2.29 | 2.02 | 2.40 | 2.12 |
| L-Aspartic Acid | % | 2.50 | 2.72 | 2.63 | 2.57 | 2.61 |
| Glycine | % | 1.50 | 1.53 | 1.48 | 1.63 | 1.60 |
| L-Glutamic Acid | % | 5.23 | 5.57 | 5.44 | 5.37 | 5.38 |
| L-Histidine | % | 0.89 | 1.02 | 0.84 | 1.03 | 0.90 |
| L-Isoleucine | % | 1.43 | 1.46 | 1.45 | 1.50 | 1.54 |
| L-Cysteine | % | 1.90 | 1.81 | 1.47 | 1.55 | 1.55 |
| L-Leucine | % | 2.68 | 2.74 | 2.72 | 2.83 | 2.79 |
| L-Lysine | % | 1.70 | 1.68 | 1.76 | 1.63 | 1.69 |
| L-Methionine | % | 0.59 | 0.60 | 0.59 | 0.60 | 0.60 |
| L-Tryptophan | % | 0.31 | 0.33 | 0.34 | 0.34 | 0.35 |
| L-Valine | % | 1.57 | 1.61 | 1.59 | 1.65 | 1.63 |
| L-Phenylalanine | % | 1.86 | 2.21 | 1.77 | 2.18 | 2.03 |
| L-Proline | % | 1.72 | 1.75 | 1.75 | 1.79 | 1.78 |
| L-Serine | % | 1.79 | 1.87 | 1.77 | 1.98 | 1.99 |
| L-Threonine | % | 1.50 | 1.58 | 1.46 | 1.66 | 1.65 |
| L-Tyrosine | % | 1.07 | 1.20 | 0.95 | 1.25 | 1.23 |

[1]Analysis conducted by the Saraswanti Indo Genetech Laboratory, Bogor, West Java, Indonesia. Website www.siglaboratory.com
[YM]Commercial yeast cell wall extracts mainly comprising β-glucan and mannan oligosaccharides (MOS) (YM, YeaMOS, Angel Yeast, Yichang, Hubei, China)

### 2.4. Challenge test

The challenge tests were conducted according to a slightly modified version of the bacteria immersion protocols previously described by Tran et al. (2013). Briefly, after the bacterial strain (*Vibrio parahaemolyticus*) was cultured overnight in TSB medium containing 2 % NaCl at 30 °C, aliquots of the cultures were transferred into ~100 mL of fresh TSB medium plus 2 % NaCl to a bacterial density of OD 600 = 0.1. Bacterial suspension was then mixed with artificial sea water to give an intermediate bacterial density of approximately $10^4$ CFU $mL^{-1}$. Thirty six individual shrimp in each treatment were divided equally and randomly in three tanks containing sea water. Cumulative mortality (dead shrimp) was recorded at 0, 6, 12, 18, 24, 48, 72 and 96 h post challenge. Dead shrimp were removed from the experimental tanks immediately.

### 2.5. Immune analysis in shrimp

#### 2.5.1. Total haemocyte count

Before and at the 96-h post immersion challenge test, haemolymph aliquots were taken from the first abdominal segment of shrimp using a 1 mL sterile syringe (26-gauge needle) that has been moistened using a 10 % Na citrate solution as an anticoagulant. Haemolymph samples were then transferred into microtubes that had been moistened with anticoagulants and labelled according to the sample treatment. During the sampling and travel process, the samples in the microtube were stored at cold temperatures in a cool box to avoid damage to the sample. Samples were stored in a −20 °C freezer before the analysis of immune parameters. Calculation of Total Haemocyte Counts (THC) was done by mixing 20 μL of hemolymph and 80 μL of Phosphate Buffer Saline (5 times dilution). Next, 20 μL from the mixture was put into a hemocytometer (Hausser Scientific, USA) and observed under a 40x magnification microscope. THC was determined as followed:

$$THC = The\ number\ of\ haemocytes\ per\ block \times \frac{1}{5} \times 25 \times dilution\ rate \times 10^4 cells \Big/ mL.$$

#### 2.5.2. Phagocytosis activity – phagocytosis index (AF-IF)

Phagocytosis activity test was carried out by diluting 20 μL of haemolymph in 10 μL Phosphate Buffer Saline, with the addition of 30 μL Formalin Killed-Vibrio cells (the density of $1\times10^8$ cells $mL^{-1}$) and then incubated at room temperature for 30 min. In the next step, a 5 μL from the mixture was smeared on the glass object and fixed using 2.5 % glutaraldehyde for 20 min, followed by rinsing in 0.85 % NaCl to remove cells that are not sticking, then air-dried. The samples were stained using a 10 % Wright stain for 20 mins, rinsed in tap water, and air-dried. The samples were observed under a microscope with a magnification of 100x. The AF-IF was calculated based on the following equation:

$$AF(\%) = \frac{number\ of\ engulfing\ phagocytes}{number\ of\ observed\ phagocytes} \times 100\%$$

$$IF = \frac{number\ of\ engulfing\ particles}{number\ of\ engulfing\ phagocytes}$$

The samples for Phagocytosis activity – phagocytosis index (AF-IF) analysis also obtained before and after challenge after immersed with pathogen for 96-h.

#### 2.5.3. Gene expression analysis for prophenoloxidase

Three shrimp per dietary treatment were used for sampled analysis before and after challenged (96-h post immersion). Total RNA was extracted from haemolymph of shrimp using TRIzol® reagent (Thermo Scientific) kit following the manufacturer's instructions (which includes DNAse treatment), after which the concentration and purity of the RNA was quantified spectrophotometrically with a nanodrop (Thermo Fisher Scientific. Waltham, MA, USA). The Reverse Transcriptase PCR (RT-qPCR) were carried out as follows: primer annealing at 25 °C for 10 minutes; reverse transcription at 42 °C for 15 minutes; inactivation at 85 °C for 5 minutes and hold at 4 °C. The cDNA isolate was then stored at −20°C. The gene expression in this study were performed following the method described by Yudiati et al. (2016), where the expression level of each gene was analysed quantitatively using a real time thermocycler machine (Biorad CFX96). For the qPCR process, a 2.4 μL of cDNA was added with 17.6 μL of PCR Mix (SensiFAST SYBR No ROX Mix – BIOLINE, consisting of: 10 μL 2x SensiFAST SYBR No ROX Mix; 0.8 μL forward primer (10 μM); 0.8 μL reverse primer (10 μM); 6 μL $H_2O$ PCR grade) in the PCR tube. Then the primer and target genes were used as described in Wang et al. (2012) and Subaidah et al. (2012) and as shown in Table 3.The qPCR condition consisted of initial denaturation at 95 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 5 seconds, annealing at 60 °C for 30 s and elongation at 68 °C for 50 s, ending with additional elongation step at 72 °C for 7 min. Finally, relative quantification of target gene transcripts with a chosen reference gene transcript was done following the ΔΔCT method described by Livak and Schmittgen (2001).

### 2.6. Hepatopancreas histology

Ten samples of shrimp per dietary treatment were randomly selected and individually anesthetized in a solution of Tricaine S (MS 222, tricaine methane sulfonate salt, Western Chemical, Inc., Ferndale, WA, USA) for histomorphological appraisal of the hepatopancreas of the shrimp before and after 96 h post immersion challenge test. The section of hepatopancreas were immediately preserved in Davison's fixative solution for 24 h at room temperature and then transferred to 70 % ethanol solution (VWR, Radnor, PA, USA) until processed by standard histological analysis procedures. Samples were dehydrated through a standard ethanol series to 100 %, embedded in paraffin wax, and sectioned at 4 μm intervals for staining with Hematoxylin-Eosin (H&E) stain (Merck, Darmstadt, Germany). For estimations, double blinded evaluation with a grading scale of 1–5 was used. Score 1 was considered as the normal condition and subsequent scores accounted for increasing levels of histopathological alteration compared to the normal condition. Images were acquired by using a digital imaging microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan).

### 2.7. Statistical analysis

Growth parameters, total haemocyte counts, Phagocytosis activity – phagocytosis index (AF-IF) and phenoloxidase activity were analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests to determine the difference between treatment means among the treatments. Score data for histomorphological condition of the hepatopancreas of the shrimp as well as the score for organoleptic analysis were treated as categorical data, tested for normality and homoscedasticity, and subsequently analyzed using a linear regression model. Statistical analyses for hapa net were conducted using SAS system (V9.4. SAS Institute, Cary, NC, USA).

**Table 3**
Primers used to quantify the relative gene expression of prophenoloxidase.

| Primers | Sense | Sequences |
|---|---|---|
| Prophenoloxidase (AY723296.1) | F | 5' – TTCAACGGTAGACCCGTGATTCTTC – 3' |
| | R | 5' – TCTTGCCGGGTTTAAGGTGAACAGT – 3' |
| B-Actin lybac (AF100986) | F | 5' – CCTCCACCATGAAGATCAAGATCAT – 3' |
| | R | 5' – CACTCCTGTGAACAATTGATGGTC – 3' |

## 3. Results

### 3.1. Growth trial

#### 3.1.1. Water quality analysis

Water quality data (mean ± standard deviation), including temperature, D.O., salinity, pH, Ammonium ($NH_4$), Nitrate ($NO_3$) and Nitrite ($NO_2$) are presented in Table 4. For physical parameters, dissolved oxygen (DO), temperature, salinity and pH were in the range of 4.25 ± 0.66 mg $L^{-1}$; 26,89 ± 1.44 °C; 26.22 ± 2.11 g $L^{-1}$; and 7.85 ± 0.22, respectively. For the chemical parameters, during the 60 days of the observation period, Ammonium ($NH_4$); Nitrate ($NO_3$); and Nitrite ($NO_2$) were in the range of 0.075 ± 0.012; 14.561 ± 1.472; and 0.019 ± 0.018 mg $L^{-1}$, respectively.

#### 3.1.2. Growth Performance and survival of shrimp

Based on the growth performance and survival of Pacific white shrimp *P. vannamei* fed with the experimental diet presented in Table 5, the final body weight (FBW), feed conversion ratio (FCR), thermal growth efficient (TGC), and percentage weight gain (PWG) were not affected by the treatments ($P > 0.05$). Meanwhile, for the survival rate (SR) of the shrimp were significantly affected by the inclusion of YM, where the SR of the shrimp fed with YM were higher compared to the control treatment ($p < 0.0001$). This in turn also affect the total biomass of the productivity. Higher SR in the group of shrimps were significantly increase the biomass yield during 60 days of the observation period ($p < 0.0001$).

### 3.2. Challenge and health condition of the shrimp

#### 3.2.1. Challenge test

Mortality of shrimp occurred starting from 24 h post-infection with signs of weakness, displaying passive swimming on the surface, milky white abdominal muscles, anorexia, reddish-yellow coloration of the hepatopancreas, and reduced growth rate. Shrimp that was fed with 0.4 % YM had the greatest ($P < 0.05$) survival rate with 0.8 % and 0.6 % YM being intermediate, and control together with 0.2 % YM having the lowest out of the five experimental treatments (Fig. 1).

#### 3.2.2. Total haemocyte counts

Before infection, the lowest total haemocyte count (THC) was found in control group (7.1791 ± 0.0051 cells $mL^{-1}$) and the highest THC were found in the group of shrimps fed with 0.4 % YM (Fig. 2). Moderate THC number were found in the group of shrimps fed with 0.2; 0.6; and 0.8 % of YM. After infection, the lowest THC number still found in the control group compared to other dietary treatment. Meanwhile, the highest THC number were found in the group of shrimps fed with 0.4 and 0.6 % of YM. Moderate THC number were found in the group of shrimps fed with 0.2 and 0.8 % of YM (Fig. 2).

#### 3.2.3. Phagocytic index and activity

Before infection, the lowest phagocytic index (PI) and phagocytic activity (PA) were found in the control group ($P < 0.001$) (Table 6). The highest PI and PA were found in the group of shrimps fed with 0.2 and 0.4 % YM. After infection, the highest PI and PA also found in the group of shrimps fed with 0.2 and 0.4 % YM (Table 6). Interestingly, as the inclusion of YM in the diet increased, the PI and PA of the shrimp tended to decrease significantly ($P < 0.05$).

**Table 4**
Water quality of the culture environment during 60 d of the growth trial using aquaria tank. Data were presented as mean± standard deviation (range).

| Parameters | Unit | Results of analysis |
|---|---|---|
| Dissolved oxygen | mg $L^{-1}$ | 4.25 ± 0.66 |
| Temperature | °C | 26,89 ± 1.44 |
| Salinity | g $L^{-1}$ | 26.22 ± 2.11 |
| pH | - | 7.85 ± 0.22 |
| Ammonium ($NH_4$) | mg $L^{-1}$ | 0.075 ± 0.012 |
| Nitrate ($NO_3$) | mg $L^{-1}$ | 14.561 ± 1.472 |
| Nitrite ($NO_2$) | mg $L^{-1}$ | 0.019 ± 0.018 |

**Table 5**
Growth performance and survival rate of Pacific white shrimp *P. vannamei* fed with experimental diet for 60 d using aquaria tanks.

| Diet Code | FBW[a] (g) | FCR[b] | TGC[c] | PWG[d] (%) | SR[e] (%) | Biomass |
|---|---|---|---|---|---|---|
| Control | 10.08 | 1.44 | 0.5164 | 401.04 | 80.00[c] | 120.94[c] |
| 0.2 % YM | 10.17 | 1.43 | 0.5199 | 405.30 | 91.11[b] | 138.89[b] |
| 0.4 % YM | 10.33 | 1.40 | 0.5259 | 411.93 | 95.56[a] | 148.09[a] |
| 0.6 % YM | 10.22 | 1.42 | 0.5198 | 403.35 | 94.44[ab] | 144.75[ab] |
| 0.8 % YM | 10.17 | 1.43 | 0.5204 | 406.73 | 92.22[ab] | 140.52[b] |
| P-value | 0.3712 | 0.3895 | 0.5390 | 0.6452 | <0.0001 | <0.0001 |
| PSE[f] | 0.1184 | 0.0201 | 0.0049 | 6.4554 | 1.3700 | 1.7253 |

Note:
[a] FBW = Final Body Weight.
[b] FCR= Feed conversion ratio.
[c] TGC = Thermal growth coefficient.
[d] PWG = Percentage weight gain.
[e] SR = Survival rate.
[f] PSE = Pooled Standard Error.

#### 3.2.4. Relative expression analysis for prophenoloxidase

Before infection, the lowest immune-related genes prophenoloxidase (ProPO) activity was obtained in the control group, while the highest expression of ProPO were found in group of shrimps fed with YM based diets. After infection with *Vibrio parahaemolyticus* at a dose of $10^4$ CFU $mL^{-1}$ under a controlled situation, the relative gene expression of ProPO were still lower in the control group and the highest relative expression of ProPO were found in the group of shrimps fed with 0.4 % YM. The moderate expression level of ProPO were found in the group of shrimps fed with 0.2; 0.6 and 0.8 % YM (Fig. 3).

#### 3.2.5. Histomorphological condition of the hepatopancreas before and after infection

In our study, after being fed with dietary treatments and before the infection, shrimp in the control group exhibited minor histological alterations in the hepatopancreas with some vacuolation (V). Vacuolated cells can be regarded as reliable biomarkers of toxic injury and disruptive to the detoxification functions of the hepatopancreas. Histological observations also showed that the hepatopancreas of shrimp fed with YM-supplemented diets appeared normal in terms of the hepatopancreatic tubules (T) and epithelial cell vacuoles (V) prior to the infection test. Following infection, all surviving shrimp in all treatments had hepatopancreatic tissue destruction, which was typified by severe necrotic cells (N) and massive sloughing of hepatopancreatic tubule epithelial cells into the lumen (MS). The histology conditions following infection did not exhibit any discernible variation amongst the treatments at the stage of assessment (Fig. 4).

## 4. Discussion

Our investigation targeted the application of a commercial yeast cell wall extract rich in mannan oligosaccharides (MOS) and β-glucan with powerful biotic effects confirmed in wider animal studies. There has been a paucity of information for shrimp species and certainly few studies relating to disease and prophylactic intervention through dietary inclusion of these additives. There are many versions globally of such products that vary considerably in their potency and level of purity. We evaluated the product of YeaMos (YM) manufactured by Angel Yeast Co. Ltd (Yichang, Hubei Province, China) due to its consistent and high specification. The attributes of MOS and glucan supplementation have been noted in numerous studies such as in Nile tilapia *Oreochromis*

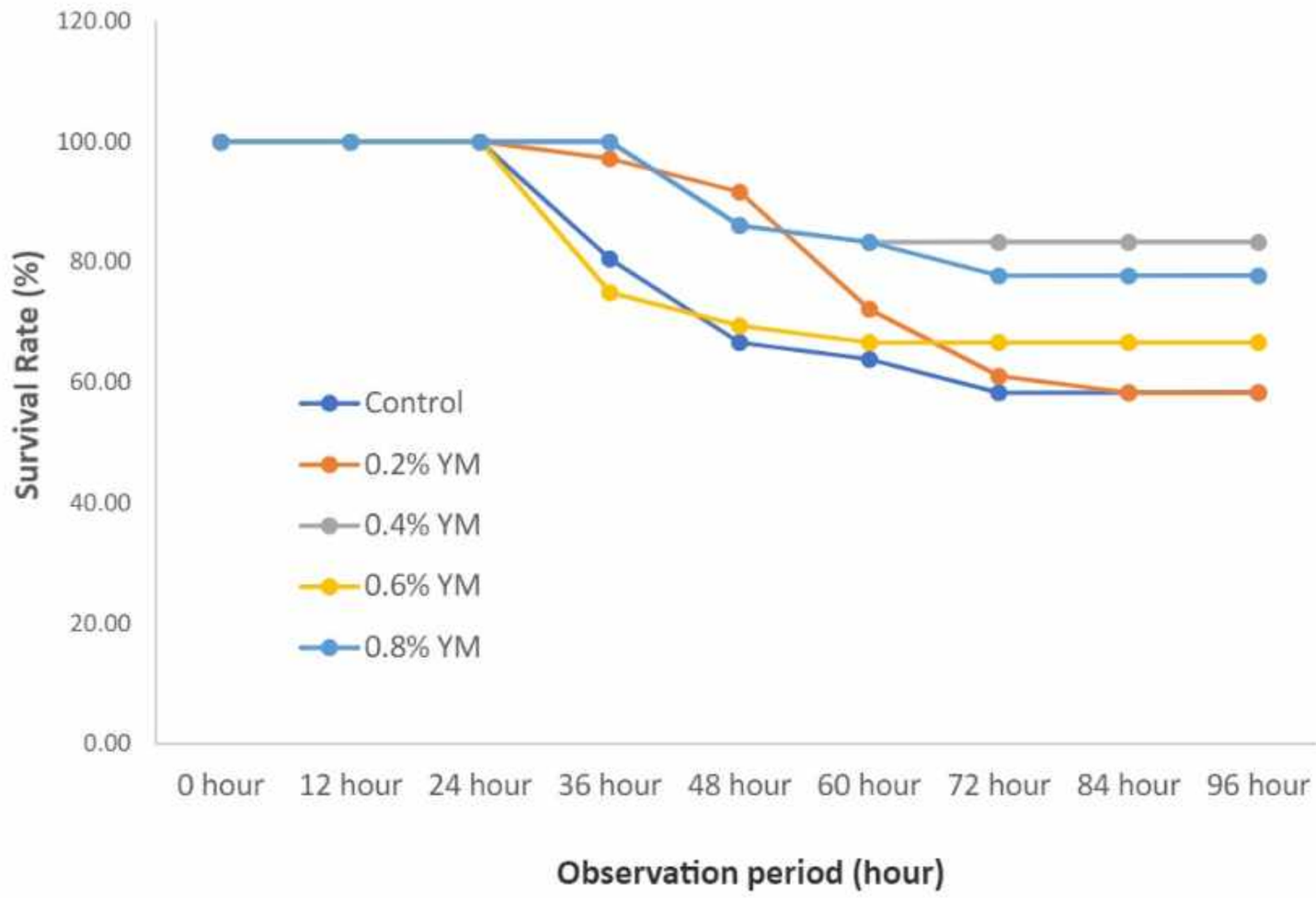
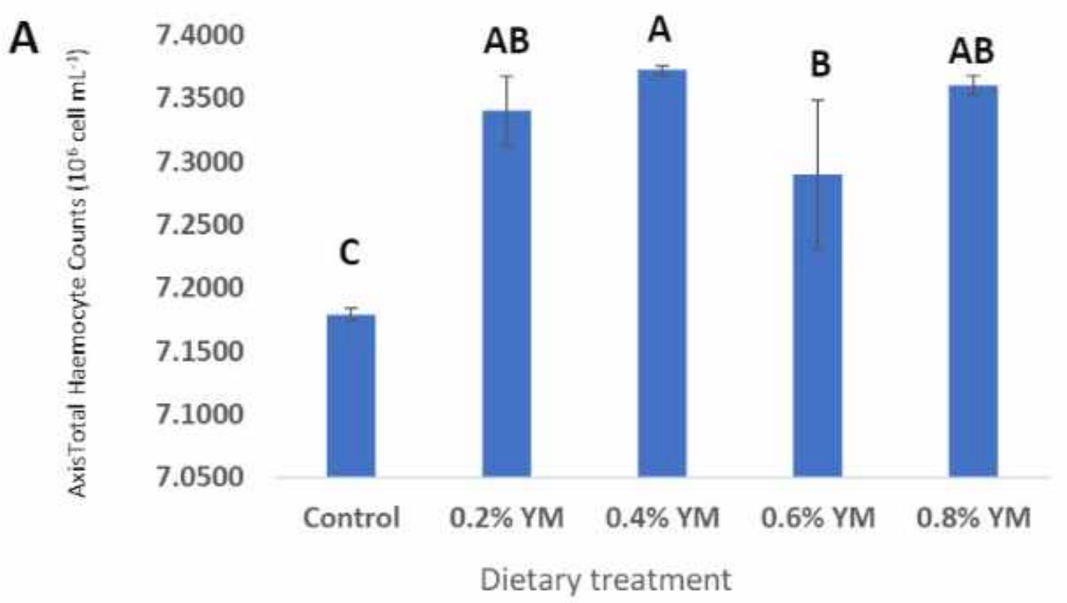



**Fig. 1.** Survival of shrimp *P. vannamei* after challenged by *Vibrio parahaemolyticus* at final dose of $10^4$ CFU $mL^{-1}$ and observed for 96 h post-challenged.



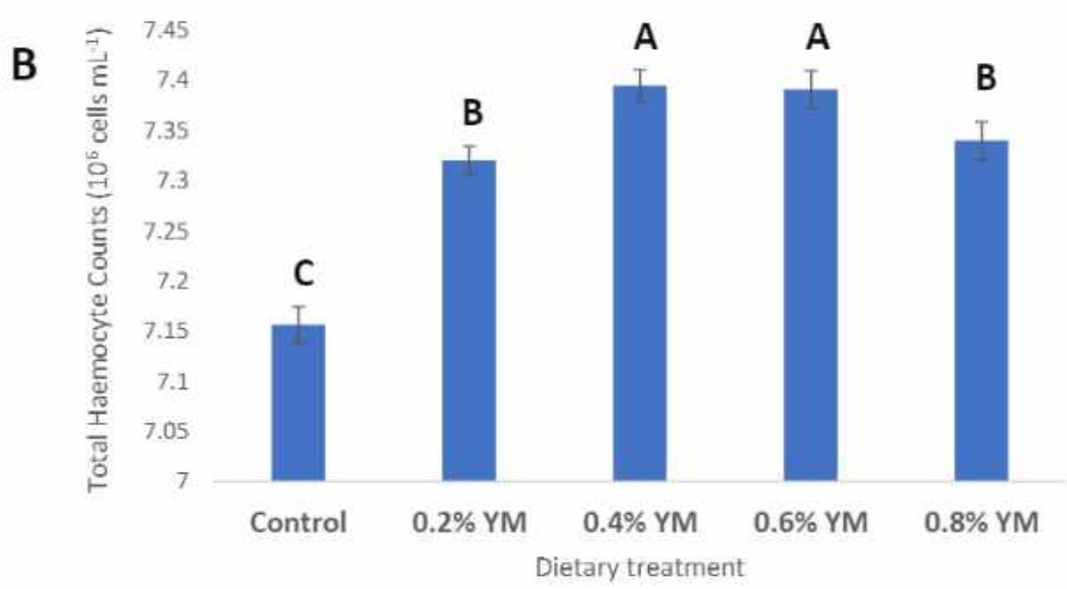




**Fig. 2.** Total haemocyte counts of shrimp *Penaeus vannamei* before and after challenged with *Vibrio parahaemolyticus* at final dose of $10^4$ CFU $mL^{-1}$. Different letters indicate statistically significant differences within the treatment of before (A) and after infection (B) ($p<0.05$).

*niloticus* (Liranço et al., 2013; Selim and Reda, 2015); common carp *Cyprinus carpio* (Magouz et al., 2021); Starry Flounder *Platichthys stellatus* (Schmidt et al., 2017); and Pacu *Piaractus mesopotamicus* (Hisano et al., 2018). Most investigations have shown positive results leading to improved growth rates and feed utilisation efficiency with superior health indicators and often increased survivability either in feed trials or post-trial infection studies. Our results agree with other scientific findings measuring similar parameters and indices of health status in farmed shrimp. Numerically, the FBW, TGC, PWG and FBW were better in the group of *P. vannamei* shrimps fed with YM compared to the control treatment despite there being no statistical differences detected. Moreover, the survival rate and biomass in the group of shrimps fed with YM were deemed to be significantly raised compared to the shrimp fed with control diet.

**Table 6**
Phagocytic index and activity of shrimp *P. vannamei* before and after challenge test (P-value < 0.005). Values represent the mean of three replicates. Results in the same columns with different superscript letter are significantly different ($P<0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

| Diet Code | Phagocytic index | | Phagocytic activity | |
|---|---|---|---|---|
| | Before Infection | After Infection | Before Infection | After Infection |
| Control | $2.0775^{c}$ | $2.5725^{ab}$ | $0.3800^{c}$ | $0.4600^{b}$ |
| 0.2 % YM | $3.1650^{a}$ | $2.6475^{a}$ | $0.5250^{a}$ | $0.5300^{a}$ |
| 0.4 % YM | $3.5025^{a}$ | $2.7175^{a}$ | $0.5500^{a}$ | $0.5325^{a}$ |
| 0.6 % YM | $2.4800^{bc}$ | $2.8025^{a}$ | $0.4575^{b}$ | $0.4875^{b}$ |
| 0.8 % YM | $2.6600^{b}$ | $2.2750^{b}$ | $0.3650^{c}$ | $0.3550^{c}$ |
| P-value | <0.0001 | 0.0050 | <0.0001 | <0.0001 |
| PSE[6] | 0.1079 | 0.08390 | 0.0074 | 0.0086 |

Mannan oligosaccharides (MOS) derived from yeast cell walls have gained significant attention as a dietary supplement in aquaculture due to their potential to improve gut health, modulate the intestinal microbiome, enhance the innate immune response, and promote growth in fish and for shrimp (Gainza and Romero, 2020). MOS in the diet of fish and shrimp can effectively act as prebiotics, promoting the growth and activity of beneficial gut bacteria such as *Lactobacillus and Bifidobacterium* (Guerreiro et al., 2018; Levy-Pereira et al., 2018; Momeni-Moghaddam et al., 2015). These microorganisms help establish and maintain a balanced gut microbiome, which is essential for nutrient absorption and overall gut health. The promotion of beneficial bacteria can lead to the production of short-chain fatty acids (SCFAs) like butyrate, which can strengthen the gut mucosal barrier (Guerreiro et al., 2018; Harikrishnan et al., 2023). MOS may have a role in modulating

**Fig. 3.** Relative gene expression of prophenoloxidase (proPO) in shrimp *P. vannamei* before and after challenged with *Vibrio parahaemolyticus* at final dose of $10^4$ CFU mL$^{-1}$. Different letters indicate statistically significant differences within the treatment of before (A) and after infection (B) ($p<0.05$).

the innate immune response in fish and shrimp and bind to pathogenic bacteria and prevent their adhesion to the gut epithelium. This reduces the colonization of harmful microorganisms in the intestinal tract, which can decrease the incidence of infections (Guerreiro et al., 2018). Additionally, MOS may stimulate the activity of immune cells such as macrophages and neutrophils. In combination with probiotics, MOS could act synergistically to enhance the commensal bacterial colonisation to greater effect in shrimp although not determined in the current investigation.

MOS and β-glucan have been shown to enhance the survival rate of shrimp after challenged with a specific pathogen. A study from E.J.G. Mameloco and R.F.M. Traifalgar (2020) suggest that the supplementation of MOS and β-glucan could enhance the immune responses and improve the resistance of *L. vannamei* against *V. parahaemolyticus* infection. Moreover, Solidum et al. (2016) revealed that the administration of MOS and β-glucan at a dose of less or equal to 0.4 % have beneficial effects in enhancing survival rate against Vibriosis. The results of the present study indicate that shrimp fed with 0.4 % YM had the higher survival rate, while the treatment with 0.6 and 0.8 % YM being intermediate compared to the control treatment. If we observe the pattern, shrimp treated with an optimum dose of 0.4 % YM could maintain the survival rate for 100 % up to 36 h post immersion. After that, mortality occurred with increasing signs of weakness, passive swimming on the surface, milky white abdominal muscles, anorexia, and poor feeding responses. This is in line with the results from Solidum et al. (2016) who demonstrated that using MOS + β-glucan less than or equal to 0.4 % activated the immune responses and resistance against vibriosis also in *P. Vannamei.*

It should be noted that shrimp survived from bacterial infections by relying on the non-specific immune mechanism instead of any pre-existing highly evolved adaptive immunity (Amparyup et al., 2013). Among the immune mechanisms of invertebrates, cellular melanotic encapsulation that requires the combination of circulating haemocytes and several associated proteins of the prophenoloxidase (ProPO) system become one of the most effective components that is responsible for wound healing and parasite entrapment as well as for microbe killing (Amparyup et al., 2013; Cerenius and Söderhäll, 2004; Kanost and Gorman, 2008; Nappi and Christensen, 2005). In this current study with *P. Vannamei*, before and after pathogenic challenge, relative expression of immune related genes of prophenoloxidase (ProPO) were higher in the group of shrimps treated with YM compared to the control treatment. In concurrence with our results, a study from E. J. Mameloco and R. F. Traifalgar (2020) showed that the supplementation of combined β-glucan and MOS could enhance the activation of proPO gene in shrimp *P. vannamei*. According to Amparyup et al. (2013), activation of the Pro-PO system is triggered upon recognition of the microbial-derived pathogen-associated molecular patterns (PAMPs), including β-glucan. Further work should also be done to examine the proteomic and even metabolomic profiles associated with the innate immune system of shrimp for a more holistic comprehension of their mechanism to combat

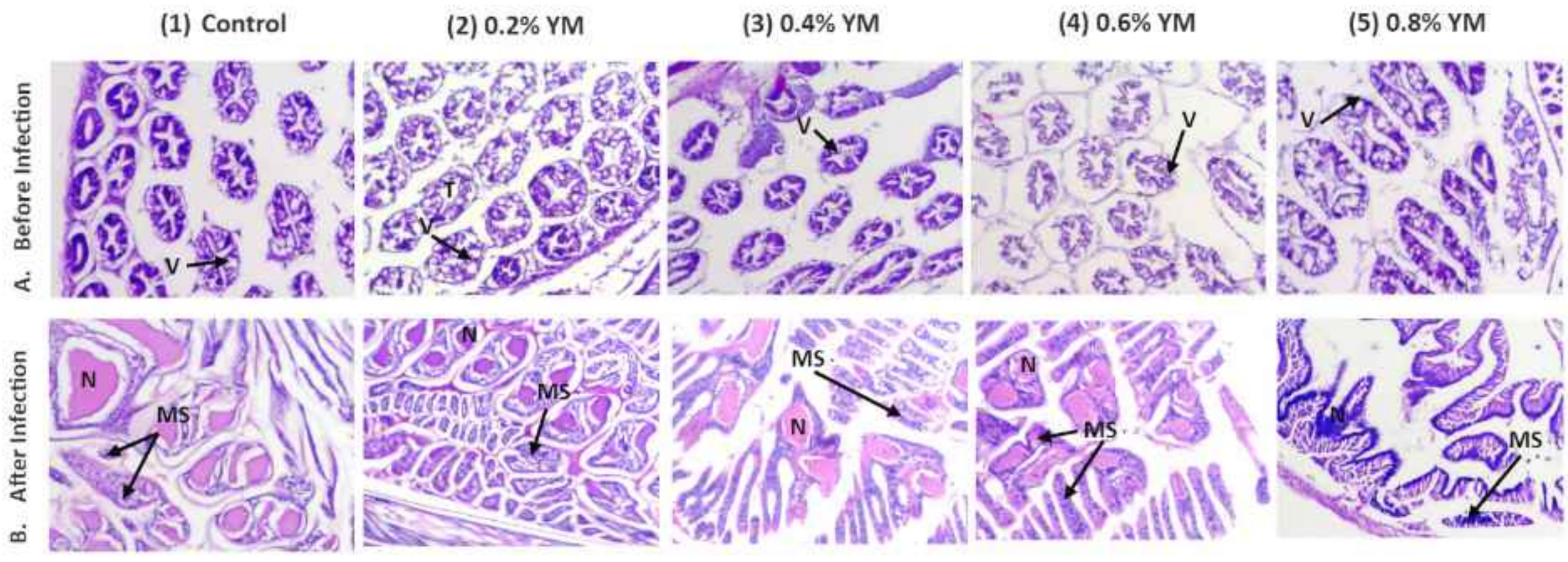



**Fig. 4.** Representative histopathological images of hematoxylin and eosin-stained sections of hepatopancreas of shrimp *P. vannamei* (A) before and (B) after being infected with *Vibrio parahaemolyticus* at final dose of $10^4$ CFU mL$^{-1}$ treated with (1) control; (2) 0.2 % YM, (3) 0.4 % YM, (4) 0.6 % YM, and (5) 0.8 % YM (n=10). Note that V = Epithelial cell vacuoles, T = hepatopancreatic tubules, MS = mass sloughing of hepatopancreatic tubule epithelial cells into the lumen, and N = severe necrotic cells.

infection. The production of antimicrobial peptides is a first-line host defence mechanism of innate immunity in shrimp. However, in spite of the importance of infectious diseases in crustaceans, few molecules displaying antimicrobial activities have been fully characterized in these invertebrates. The identification of a family of antimicrobial peptides (AMPs), named penaeidins, in the shrimp *Penaeus vannamei* have led to important mechanisms of pathogen mitigation being identified (Bachère, 2000). Future investigations should be directed to test the role of functional feed ingredients like yeasts and their cell wall constituents on AMPs and measure their gene expressions and direct protein activation within the hepatopancreas.

In our investigation, phagocytosis analysis was also determined as another crucial assessment of the shrimp's innate immune response. Haemocytes, which are the primary immune cells in shrimp, are responsible for engulfing and digesting foreign particles, including pathogens. The phagocytic index is a measure of the efficiency of this process. In this research, before and after infection, the lowest PO, THC as well as the Phagocytic index and activities were found in the control group. Meanwhile, the use of YM have been shown to enhance the immune system of shrimp, with the inclusion of 0.4 % becoming established as the optimum dose obtained from this research to optimize the activation of the immune-related genes, suggesting an enhancement of the immune response in shrimp *P. vannamei*.

In shrimp, absorption and storage of nutrients as well as the secretion of digestive enzymes, antimicrobial peptides, and immune related molecules mainly occurs in the hepatopancreas (Novriadi et al., 2023; Tzuc et al., 2014; Wang et al., 2014). The supplementation of yeast and its extracted components in the diet of shrimp has been a topic of considerable research interest in recent years due to its potential benefits on the hepatopancreas. However, few studies have indicated the structural changes of hepatopancreas before and after infection. In this research, all shrimps receiving YM treatment and before the infection with *V. parahaemolyticus* at final dose of $10^4$ cells $mL^{-1}$ seem to have a normal condition compared to the control treatment. However, after being infected, all shrimp exhibited severe necrotic cells and massive sloughing of hepatopancreatic tubule epithelial cells into the lumen. In line with our study, Zhang et al. (2022) reported that the use of yeast culture in the range of 5.25 – 10.52 % to replace the inclusion of FM from 4 % to 8 % in the diet formulation had no significant effects on the hepatopancreas of shrimp. However, when shrimp are infected with *V. parahaemolyticus*, the hepatopancreas will undergo symptoms characterized by shedding of epithelial cells within the hepatopancreas, followed by necrosis of epithelial cells, and hemocytic infiltration at later stages (Soto-Rodriguez et al., 2015). Furthermore, a challenged study performed by Velazquez Lizarraga et al. (2019) revealed that after 12 h post infection, sloughing of epithelial cells were observed from the tubule of shrimp. In addition, necrosis, epithelial cells sloughing, atrophy, and massive hemocyte infiltration will become more evident in shrimp from 48 h post infection with *V. parahaemolyticus* onwards (Velazquez-Lizarraga et al., 2019). Our current histopathological results suggest that the dietary treatment of YM were able to maintain the integrity of the hepatopancreas within an ideal condition period. Meanwhile, shrimp showed severe structural damage after massive exposure to *V. parahaemolyticus* at a later evaluation time where infection has become more established in tissues and organs and beyond dietary intervention.

## 5. Conclusion

In conclusion, this current study with *P. vannamei* and scientific evidence supports incorporating yeast-based products into shrimp diets, improve the biomass yield and immune function against *Vibrio parahaemolyticus*. The inclusion level of 0.4 % could be considered as an optimum dose as it provides the optimum responses for biomass gain, survival rate, phagocytic activity and index, survival rate of shrimp after challenged with *V. parahaemolyticus*, haemocyte count and relative gene expression of prophenoloxidase (proPO) before and after infection. This will be very important in feeds containing reduced fishmeal and higher plant by product inclusions that can impede shrimp resilience under stressful intensive production systems.

## CRediT authorship contribution statement

**Zhang Yan:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Ren Tao:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Yuan Honggao:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Romi Novriadi:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gong Fayuan:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simon Davies:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Indah Istiqomah:** Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Alim Isnansetyo:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Mochammad Farkan:** Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Dai Jinjun:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Yi Jianhua:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Huang Xin:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

## Declaration of Competing Interest

Gong Fayuan, Dai Jinjun, Yi Jianhua, Huang Xin, Zhang Yan, Ren Tao and Yuan Hongguo employed by Angel Yeast Co. Ltd (Hubei Province, China). The remaining authors state no conflict of interest

## Data Availability

Data will be made available on request.

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