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# Dietary Use of Monoglycerides Blend in Plant-Based Diets Influences Growth Performance, Hepatopancreatic Histology, Immunity and Disease Resistance of Pacific White Shrimp *Penaeus vannamei* Against *Vibrio harveyi*

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## Abstract

Feeding trials were conducted to evaluate the efficacy of commercially available optimized mixtures of monoglyceride blend (MG, as BalanGut® AQ L supplied by BASF, Germany) on growth performance and health condition of Pacific white shrimp *Penaeus vannamei.* A basal diet was formulated without MG, and four other diets were prepared by supplementing the powder forms of MG at inclusion levels of 0.5, 0.75, 1,0, and 1,5%. In a growth trial, eight replicate groups of shrimps were hand-fed with one of the diets four times daily for 60 days and observed for growth, total hemocyte count (THC), lysozyme activity, and histomorphological conditions of the hepatopancreas of the shrimp. In a challenge test, diseases challenged with *Vibrio harveyi* infection at a dose of $1 \times 10^5$ CFU $mL^{-1}$ intramuscularly were conducted and then followed with the observation of the cumulative survival rate after 96 h post-challenge, phenoloxidase, the mean percentage of phagocytic activity and phagocytic index on the blood of the shrimp. In the growth trial, shrimps with inclusion levels of 0,75 up to 1,5% of MG showed significantly higher growth performance than the control treatment. The number of THC and lysozyme activity showed an increasing trend with the inclusion of MG at 0.5 up to 1.0% and then declined with the inclusion of 1.5%. The inclusion of MG did not cause any significant changes in the histomorphology of the shrimp's hepatopancreas. In the challenge test, the lowest survival rate after being infected with *Vibrio harveyi* was found in the control treatment, and the highest survival rate was found in the group of shrimps fed with 1 MG. The phenoloxidase (PO) activity, phagocytic activity, and index were higher in the group of shrimps fed with MG compared to the control treatment. The results of these studies indicate that the dietary supplementation of 0.75 – 1.0 % of MG can improve the growth, health condition, and disease resistance of *P. vannamei*.

## Introduction

Pacific white shrimp *Penaeus vannamei* farming industry is challenged by the optimization of feed formulation (Al Eissa et al., 2022; Rahman et al., 2017) and the presence of diseases (Delphino et al., 2022; Geetha et al., 2022) that could affect the sustainability, efficiency, profitability, and productivity of the production system. Feed formula optimization is important since shrimp require specific levels of nutrients for healthy growth at different life stages including during the larvae, post-larvae, juvenile, sub-adults, and adults' stage (Fox et al., 2001). Traditionally, to achieve optimal growth, fish meal (FM) is used to fulfill the specific nutritional requirements of shrimp, which provide a relatively high level of energy per unit weight (Fox et al., 2004; Miles & Chapman, 2006), as an excellent sources of digestible essential amino and fatty acids (Zinn et al., 2009) as well as vitamins and minerals (Nguyen et al., 2009). Due to its balanced nature, FM is the most widely used ingredient in aquafeed formulation, including for shrimp (Abo-Taleb et al., 2021; Tacon, 1993). However, high inclusion of FM is challenging from the perspectives of cost, availability, legislation (Gatlin III et al., 2007) and the sustainability of the environments. Therefore, sustainable feed needs to consider a proper blending of a variety of plant-based ingredients, novel feed ingredients, and animal by-products to substitute the use of FM in the feed formulation (Kok et al., 2020).

Factors to consider in selecting alternative proteins include the quality and economic value of ingredients, sustainability, availability of appropriate nutrients, and attractability or palatability to the shrimp (Novriadi et al., 2019; Rahman et al., 2017). It is also essential to consider limitations on the inclusion level for alternative feed ingredients. A study from Lim and Dominy (1990) showed that as soybean meal (SBM) exceeded 42%, replacing FM in the diet formulation negatively affected the weight gain, feed intake and body phosphorus percentage of the shrimp. An additional study with other alternative ingredients, such as dietary dried distiller's grains with solubles (DDGS), demonstrated that the inclusion of DDGS up to 12% to replace dietary FM also caused adverse effects on the growth performance of shrimp *P. vannamei* (Gyan et al., 2021). These studies indicate that alternative proteins' direct replacement of FM can be challenging. However, proper combination with other ingredients supplemented with additives might provide beneficial impacts to reduce the detrimental effects (Novriadi et al., 2022; Xie et al., 2018)

Other than growth performance, the use of alternative ingredients in combination with novel and functional ingredients can be designed to stimulate and enhance the non-specific immune system of shrimp (Ayiku et al., 2020; Novriadi et al., 2023). The presence of diseases due to parasites, bacteria and viruses continues to cause substantial economic losses worldwide (Jithendran et al., 2021; López-Téllez et al., 2020; Novriadi, 2016; Shinn et al., 2018). The inclusion of several functional ingredients or additives in the diet formulation could become an essential mitigation approach against pathogens, including the use of yeast (Jin et al., 2018), hydrolyzable tannins (Novriadi et al., 2021), algae (Niu et al., 2019) and bile acids (Niu et al., 2019). However, only a few studies have looked into the evaluation on the efficacy of optimized mixtures of monobutyrin, glycerides, and glycerol as a functional ingredient to support the growth and the non-specific immune system response of shrimp *P. vannamei* (Bruno Corrêa da Silva et al., 2016; Bruno Correa da Silva et al., 2016). Therefore, this study was conducted to investigated the effects dietary blend of commercial monoglycerides (MG, as BalanGut® AQ L supplied by BASF, Germany) supplemented into shrimp feed formulation on the growth performance, feed conversion ratio (FCR), total hemocyte count (THC), lysozyme activity and histomorphological condition of the hepatopancreas. In addition, a challenge test was performed to evaluate the efficacy of MG to support the optimal survival rate of shrimp against infection with *Vibrio harveyi* for 96 hours, followed by the analysis of phenoloxidase activity, phagocytic activity and phagocytic index of the Pacific white shrimp *P. vannamei* at day-5 post-infection.

## Materials and Methods

### Experimental Diets

Five experimental diets for growth trial and challenge test were formulated according to a typical commercial formulation for Pacific white shrimp *Penaeus vannamei* in Indonesia. The control diet was formulated to contain a mixture of 41% soybean meal (SBM), 12% Poultry meal (PM) and 10% fish meal (FM) as the sources of protein in the diet formulation without monoglycerides (MG). Four experimental diets were formulated to contain increasing levels of MG (0.5%; 0.75%; 1%; and 1.5%) to partially replaced the wheat flour as the filler in diet formulation, and labeled with 0.5; 0.75; 1.0; and 1.5 MG, respectively (Table 1). All ingredients were grounded and passed through < 200 mesh sieve (Jinan Shengrun, China). All dry ingredients were carefully weighed and mixed in a paddle mixer (Marion Mixers, Inc., Marion, IA, USA) in a 100 kg batch, followed by grinding to a particle size of <200 µm using a disk mill (Jinan Shengrun China). Fish oil was then gradually added and mixed constantly. A twin extruder (Jinan shengrun, China) was used to extrude the feed through a 2 mm die at temperature gradient of 62ºC, 80ºC and 110 ºC in three zones of extruder barrel and the die head, respectively. Diets were air-dried in a pulse bed dryer (Jinan Shengrun, China) and stored at 4°C in sealed containers until further use. Proximate and amino acid profiles of the experimental diets were analyzed at Saraswanti Indo Genetech Laboratory, Bogor, West Java, Indonesia and summarized in Table 2.

**Growth Trials**

Growth trials were carried out at the Faculty of Fisheries and Marine Science, Diponegoro University (Semarang, Central Java, Indonesia). The juvenile stage of the shrimp was tested free from pathogens prior to transport and was nursed in the faculty facilities by utilizing 2000-L tanks with seawater under controlled conditions (Temperature 28 – 29°C, salinity 25 ppt and dissolved oxygen above 4 mg $L^{-1}$). At the start of the trial, Shrimp (1.06±0.01 g initial mean weight) were randomly distributed into 40 aquaria tank (70 x 35 x 40 cm per aquaria tank). Eight replicate groups of shrimps per dietary treatment were administered different types of experimental diets for 60 days and fed by hand four times daily at 07:00, 11:00, 15:00, and 20:00h. Based on our historic results, 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 shrimp counts, and mortality. During the 60 days of the feeding trial, water quality parameters, including pH, dissolved oxygen (DO), water temperature, total dissolved solid and salinity were measured four times daily using sensors (Aqua TROLL 500 Multiparameter Sonde instrument) and the data were stored to an application (AquaEasy apps, Bosch, Singapore) for data traceability and recording. Meanwhile, total ammonia-nitrogen (TAN), alkalinity, nitrate, nitrite, ammonia and phosphate were measured once a week using absorption spectrophotometry (DR890, HACH, USA). At the termination of the feeding period, shrimp in each aquaria tank were group-counted and weighed to calculate the final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR), and percentage (%) thermal unit growth coefficient (TGC) as follows:

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

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

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

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

Where FBW is final body weight, IBW is initial body weight, T is water temperature ($^0$C) and D is number of trial days

**Total Haemocyte Count**

At the end of the growth trial, hemolymph was sampled from one shrimp per aquaria tank or eight shrimp per dietary treatment to determine the total hemocyte count. Hemolymph (100 µL) of individual shrimps was withdrawn from the pleopod base of the second abdominal segment with a sterile 1-mL syringe (25 G × 13 mm needle). Before hemolymph extraction, the syringe was loaded with a precooled (4°C) solution (10%-EDTA, Na) used as an anticoagulant. The

**Table 1.** Ingredient composition (%, *as is*) of experimental diets containing several inclusion levels of monoglycerides mixture (BalanGut®, BASF, Germany)

| Ingredient | Control | 0.5 MG | 0.75 MG | 1.0 MG | 1.5 MG |
|---|---|---|---|---|---|
| Soybean meal[1] | 41.00 | 41.00 | 41.00 | 41.00 | 41.00 |
| Poultry Meal (pet food grade)[2] | 12.00 | 12.00 | 12.00 | 12.00 | 12.00 |
| **Monoglyceride[3]** | 0.00 | 0.50 | 0.75 | 1.00 | 1.50 |
| Fish Meal Chile[2] | 10.00 | 10.0 | 10.0 | 10.0 | 10.0 |
| Soya Lecithin[4] | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Fish Oil[2] | 3.20 | 3.20 | 3.20 | 3.20 | 3.20 |
| MCP[2] | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 |
| Cholesterol[4] | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Wheat flour[5] | 29.79 | 29.29 | 29.04 | 28.79 | 28.29 |
| L-Lysine HCl[4] | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| DL-Methionine[4] | 0.35 | 0.35 | 0.35 | 0.35 | 0.35 |
| L-Threonine[4] | 0.18 | 0.18 | 0.18 | 0.18 | 0.18 |
| Mineral premix[6] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Vitamin premix[7] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Choline Chloride[4] | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Anti-Mold[4] | 0.12 | 0.12 | 0.12 | 0.12 | 0.12 |
| Stay C – 35[4] | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Sum | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |

[1] De-hulled solvent extract soybean meal, PT FKS Multi Agro, Tbk. Jakarta, Indonesia [2]

[2] PT FKS Multi Agro, Tbk. Jakarta, Indonesia

[3] BalanGut, AQ L, BASF, Germany

[4] PT Fenanza Putra Perkasa, Jakarta, Indonesia

[5] PT Pundi Kencana, Cilegon, Banten, Indonesia

[6] 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.

[7] 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 (1,000,000 IU/g), 80.00; menadione, 0.50; alpha-cellulose, 856.81

hemolymph with an anticoagulant solution was diluted in 150 µL of formaldehyde (4%), and then 20 µL was placed on a hemocytometer (Neubauer) to determine THC using an optical microscope (Olympus, DP72).

**Lysozyme Activity Analysis**

Eight shrimps per dietary treatment were collected at the end of the growth trials to analyze the lysozyme activity levels after 60 days of the feeding period. Commercial kits (Sigma-Aldrich, Cat. No. LY0100) were used to measure the activity in shrimp, and the determination procedures came from the instruction manual. The lysis of the Micrococcus lysodeikticus cells defined the results of lysozyme activity. The reactions were conducted at room temperature, and the absorbance of samples at 450 nm was measured using an ultraviolet/visible spectrophotometer (Perkin Elmer, Lambda XLS, USA)

$$\text{Lysozyme activity } \left(\frac{\text{Units}}{\text{mL}}\right) = \frac{(\Delta A450/\min Test - \Delta A450/\min Blank)\ (df)}{(0.001)(0.03)}$$

df = dilution factor
0.001 = $\Delta A_{450}$ as per the unit definition
0.03 = Volume (in mL) of enzyme solution

**Histomorphological Condition of Hepatopancreas**

Upon termination of growth trials, the hepatopancreas sections from four shrimps per dietary treatment were immediately preserved in Davison's fixative solution for 48 h at room temperature (Bell and Lightner, 1988) and then transferred to 50% 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). Double-blinded evaluation with a grading scale of 1 to 5 was used for estimations. Score 1 was considered the normal condition; subsequent scores accounted for increasing levels of histopathological alteration compared to the normal condition. Images were acquired using a digital imaging microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan).

**Challenge Test**

In parallel with the growth trial, a separate study explored the resistance of *P. vannamei* fed with MG against pathogenic strain of *Vibrio harveyi* ATCC 14126 and the immune system characteristics post-challenge. For this purpose, 80 shrimp per dietary treatment (1.06±0.01 g initial mean weight) were challenged by intramuscular injection with an LD50 dose of $10^5$ CFU of *V. harveyi* shrimp$^{-1}$ after receiving the experimental diet for 30 days. The concentration was based on previous work from Novriadi et al. (2022) and shrimp mortality was monitored every 12 – 96 h for a period of a maximum of 5 days. The protective effects of bacteria were evaluated based on the following relative percentage survival (RPS) value (Amend, 1981). In this challenge test, the group of shrimps fed with a control diet without challenge were also included as the negative control group

**Table 2.** Detail composition of proximate and amino acid (AA) (% as is, dry matter basis) of experimental diets utilized in this trial

| | Diet code | | | | | Methods |
|---|---|---|---|---|---|---|
| | Control | 0,5 MG | 0,75 MG | 1 MG | 1,5 MG | |
| **Proximate analyses (%, *as is*)*** | | | | | | |
| Ash content | 10.68 | 9.58 | 9.73 | 9.92 | 9.93 | Incineration |
| Fat content | 6.68 | 6.71 | 6.74 | 6.75 | 6.68 | Soxhlet extraction |
| Moisture content | 7.43 | 7.88 | 7.50 | 7.18 | 7.95 | Drying method |
| Protein content | 37.76 | 37.78 | 37.75 | 37.84 | 37.85 | Kjeldahl *method* |
| **Amino acid profile (%. *as is*)*** | | | | | | |
| L-Serine | 1.45 | 1.49 | 1.46 | 1.55 | 1.49 | *UPLC* Photodiode Array (*PDA*) |
| L-Glutamic acid | 4.45 | 4.45 | 3.36 | 4.47 | 4.44 | *UPLC* Photodiode Array (*PDA*) |
| L-Phenylalanine | 1.75 | 1.73 | 1.20 | 1.88 | 1.82 | *UPLC* Photodiode Array (*PDA*) |
| L-Isoleusine | 1.46 | 1.63 | 0.99 | 1.54 | 1.47 | *UPLC* Photodiode Array (*PDA*) |
| L-Valine | 1.64 | 1.65 | 1.23 | 1.67 | 1.73 | *UPLC* Photodiode Array (*PDA*) |
| L-Alanine | 2.26 | 2.29 | 1.28 | 2.28 | 2.27 | *UPLC* Photodiode Array (*PDA*) |
| L-Arginine | 2.76 | 2.89 | 1.71 | 2.83 | 2.76 | *UPLC* Photodiode Array (*PDA*) |
| Glycine | 2.80 | 2.73 | 1.75 | 2.80 | 2.79 | *UPLC* Photodiode Array (*PDA*) |
| L-Lysine | 1.57 | 1.64 | 1.59 | 1.59 | 1.59 | *UPLC* Photodiode Array (*PDA*) |
| L-Aspartic Acid | 2.93 | 2.94 | 2.94 | 2.94 | 2.89 | *UPLC* Photodiode Array (*PDA*) |
| L-Leusine | 2.65 | 2.68 | 2.67 | 2.86 | 2.80 | *UPLC* Photodiode Array (*PDA*) |
| L-Tyrosine | 1.27 | 1.34 | 1.38 | 1.76 | 1.27 | *UPLC* Photodiode Array (*PDA*) |
| L-Proline | 1.99 | 2.02 | 1.99 | 1.97 | 1.99 | *UPLC* Photodiode Array (*PDA*) |
| L-Threonine | 1.67 | 1.69 | 1.74 | 1.72 | 1.73 | *UPLC* Photodiode Array (*PDA*) |
| L-Histidine | 0.73 | 0.82 | 0.82 | 0.75 | 0.75 | *UPLC* Photodiode Array (*PDA*) |
| L-Tryptophan | 0.20 | 0.26 | 0.19 | 0.22 | 0.20 | *UPLC* Photodiode Array (*PDA*) |
| L-Systine | 2.14 | 2.17 | 2.42 | 2.33 | 2.44 | *UPLC* Photodiode Array (*PDA*) |
| L-Methionine | 1.09 | 1.13 | 1.12 | 1.09 | 1.12 | *UPLC* Photodiode Array (*PDA*) |

*Analysis conducted by the Saraswanti Indo Genetech Laboratory, Bogor, West Java, Indonesia. Website www.siglaboratory.com

**Phenoloxidase Activity, Phagocytic Activity and Index**

Upon termination of the challenge test, the phenoloxidase (PO) activity of shrimp was measured spectrophotometrically, recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Huang et al., 2010). For this purpose, A total of 100 µL of hemolymph was centrifuged at 700 g (4$^{o}$C) for 20 min. The supernatant was removed, while the remaining pellets was diluted in100 µL of cacodylate citrate buffer. A total of 100 µL of the mixture was transferred to a 96 wells-microplate, and added with 50 µL Trypsin (Sigma Aldrich), incubated at room temperature for 10 min, and then added with a 50 µL of L-DOPA. PO activities measurement was made using a microplate reader (R-Biopharm Well Reader, Germany) at 490 nm

Phagocytic activity (PA) and index (PI) were measured using ten shrimp per dietary treatment following the protocols described by Romi (2023). The PA and PI evaluation were described as follows:

$$PA\ (\%) = \frac{number\ of\ engulfing\ phagocytes}{number\ of\ observed\ phagocytes}\ x\ 100\%$$

$$PI\ (\%) = \frac{number\ of\ engulfing\ particles}{number\ of\ engulfing\ phagocytes}\ x\ 100\%$$

**Statistical Analysis**

Data for growth parameters, total hemocyte counts, lysozyme activity, challenge test, phenoloxidase activity, and phagocytic activity and index were analyzed using regression and one-way analysis of variance (ANOVA) to determine significant differences among treatments followed by Tukey's multiple comparison tests to determine the difference between treatment means among the treatments. Score data for the histomorphological condition of the hepatopancreas of shrimp were treated as categorical data, tested for normality and homoscedasticity, and subsequently analyzed using a linear regression model. All statistical analyses were conducted using the SAS system (V9.4. SAS Institute, Cary, NC, USA).

## Results

**Water Quality Parameters**

Data for water quality analysis were presented in Table 3. During the growth trial, water quality parameters including pH, dissolved oxygen (DO), temperature and salinity were in the range of 7.71±0.18; 5.76±0.24 mg $L^{-1}$; 27.78±0.28°C; and 27.99±0.18‰, respectively. Additionally, ammonia ($NH_3$-N), nitrite ($NO_2$-N), nitrate ($NO_3$-N) and phosphate ($PO_4$-N) were 0.02±0.04 mg $L^{-1}$; 0.011±0.005 mg $L^{-1}$; 4.077±0.622 mg $L^{-1;}$ and 0.011±0.005 mg $L^{-1}$, respectively. Overall, all water quality remained within the acceptable ranges for shrimp growth under outdoor pond conditions.

**Growth Performance**

Table 4 shows the growth performance of shrimp fed with graded levels of MG. After 60 days of the feeding trial, final biomass (FB), final mean weight (FMW), weight gain (WG) and feed conversion ratio (FCR) of shrimp fed with 0.75%, 1% and 1.5% MG were significantly improved compared to the control treatment ($P<0.05$). Meanwhile, no significant differences were observed on survival and thermal growth coefficient (TGC) of shrimp fed with the experimental diets.

**Total Haemocyte Counts (THC) and Lysozyme Activity**

Data for THC and lysozyme activity are presented in Figure 1 and 2. Numerically, THC and lysozyme activity were higher in the group of shrimps fed with 0.75 and 1.0 of MG. However, there were a decreasing trend as the inclusion of MG increased in the diet formulation.

**Histomorphological Condition of Hepatopancreas of Shrimp**

Figure 3 shows the hepatopancreas of the shrimp *P. vannamei* after feeding the shrimp with experimental diets for 60-days. The results of the histopathological

**Table 3.** Water quality data during the controlled feeding trials for 60 days in aquaria tank. Data were presented as mean± standard deviation (range)

| Parameters | Unit | Analysis results |
|---|---|---|
| Parameters | Unit | Results |
| Dissolved oxygen | mg $L^{-1}$ | 5.76±0.24 |
| Temperature | $^{0}$ C | 27.78±0.28 |
| Salinity | ‰ | 27.99±0.18 |
| pH | | 7.71±0.18 |
| Ammonia ($NH_3$-N) | mg $L^{-1}$ | 0.02±0.04 |
| Nitrite ($NO_2$-N) | mg $L^{-1}$ | 0.011±0.005 |
| Nitrate ($NO_3$-N) | mg $L^{-1}$ | 4.077±0.622 |
| Posphate ($PO_4$-N) | mg $L^{-1}$ | 0.011±0.005 |

analysis showed that the addition of MG in the range of 0.5 – 1.0 % MG had a normal structure in the presence of B, R, and F cels compared to control and 1.5% MG. In all dietary treatments, numbers of vacuoles appeared in the tubular epithelial cells of the hepatopancreas of the shrimp.

**Challenge Test**

After 24 h infection by *Vibrio harveyi*, the tested shrimp showed apathetic behavior near the culture tank, slow movement, experienced damage in the antennae and rostrum. At the end of the challenge test, the cumulative survival rate of shrimp fed with MG was significantly higher compared to the control treatment (Figures 4A and 4B).

**Phenoloxidase (PO) Activity and Analysis of Phagocytic Activity and Index**

Phenoloxidase (PO) activity at day-5 post injection with *Vibrio harveyi* were displayed in Figure 5. The PO activity was higher in all the group of shrimps fed with MG compared to the control treatment (P<0.05). The mean percentage of phagocytic activity (PA) and phagocytic index (PI) of post challenged shrimp can be viewed in Figure 6. Shrimp fed diets containing MG, at any level, had significantly (P<0.01) higher phagocytic activity compared to the control treatment. In detail for PA, the shrimp fed with 0.75 and 1% MG had the highest PA activity.

## Discussion

The current study used a specific blend of monoglyceride (MG) commercially available as BalanGut AQ L (BASF, Germany). The product is a synergistic combination of short (propionic and butyric) to medium (caproic, caprylic, capric and lauric acid) chain-length fatty acids. The main active ingredient is Monobutyrin, which could improve epithelium integrity, host immune defense, microbiota balance and reduce liver cholesterol (Lee et al., 2021; Nguyen et al., 2019). The results of the present study showed that dietary supplementation of MG with inclusion levels of 0.75; 1.0 and 1.5% into shrimp feed could increase the final biomass, final mean weight (FMW), and weight gain (WG) as well as to increase the production efficiency by reducing the feed conversion ratio (FCR) compared to control treatment. However, as the inclusion levels of MG increases, there is a tendency for a declining trend on the growth performance of the shrimp. Interestingly, there is no difference in TGC among the dietary treatments used as a model to estimate the growth of

**Table 4.** Growth performance of pacific white shrimp *Penaeus vannamei* (Mean initial weight 1.06 ± 0.01 g) fed experimental diets for 60 d. Values represent the mean of six replicates. Results in the same columns with different superscript letter are significantly different (P<0.05) based on analysis of variance followed by Tukey's multiple comparison test.

| Diet code | Final Biomass (g) | Final Mean Weight (g) | Survival (%) | WG[1] (%) | FCR[2] | TGC[3] |
|---|---|---|---|---|---|---|
| Control | 79.07[b] | 8.54[b] | 92.50 | 8.18[b] | 1.30[a] | 0.0780 |
| 0.5 MG | 75.25[b] | 8.65[b] | 87.50 | 8.30[b] | 1.29[a] | 0.0786 |
| 0.75 MG | 93.91[a] | 9.63[a] | 97.50 | 9.22[a] | 1.15[b] | 0.0809 |
| 1.0 MG | 90.53[a] | 9.41[a] | 96.25 | 9.02[a] | 1.18[b] | 0.0806 |
| 1.5 MG | 90.15[a] | 9.38[a] | 96.25 | 9.01[a] | 1.18[b] | 0.0811 |
| *p-value* | <0.0001 | <0.0001 | 0.1012 | 0.0001 | <0.0001 | 0.5189 |
| PSE[4] | 3.5552 | 0.2375 | 3.9866 | 0.2355 | 0.0325 | 0.0022 |

Note: [1] WG = Weight gain; [2] FCR= Feed conversion ratio; [3] TGC = Thermal growth coefficient; [4] PSE = Pooled standard error

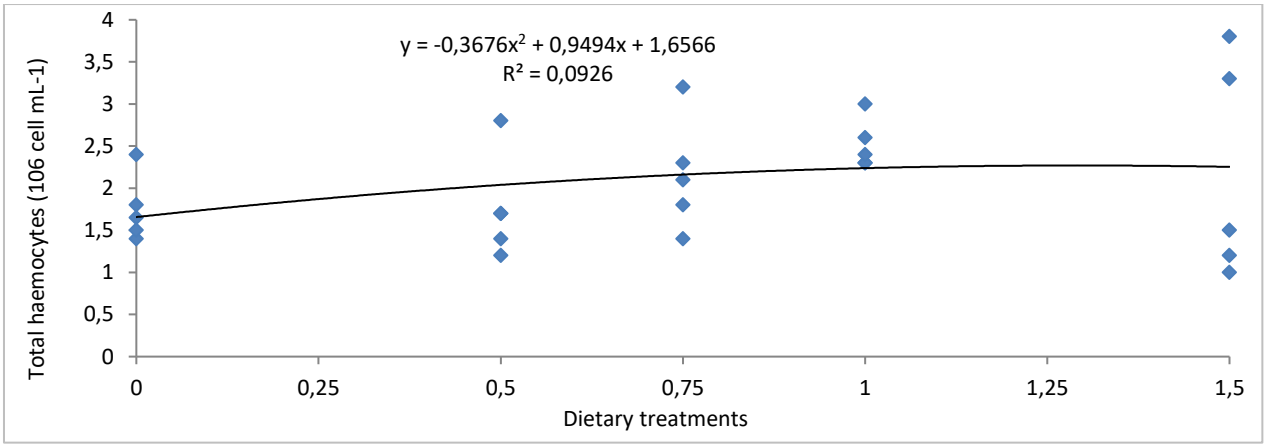


**Figure 1.** Total hemocyte count of Pacific white shrimp *Penaeus Vannamei* ($10^6$ cell $mL^{-1}$) at the end of feeding trial. Values represent the mean of five replicates (P-value = 0,457).

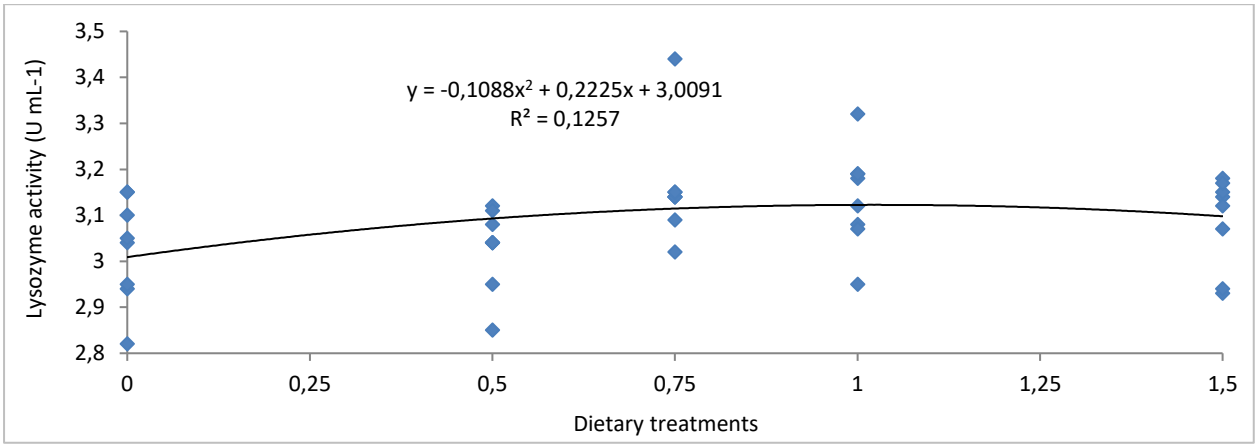


**Figure 2.** Lysozyme activity of Pacific white shrimp *Penaeus Vannamei* (U $mL^{-1}$) at the end of feeding trial. Values represent the mean of eight replicates (P-value = 0,055).

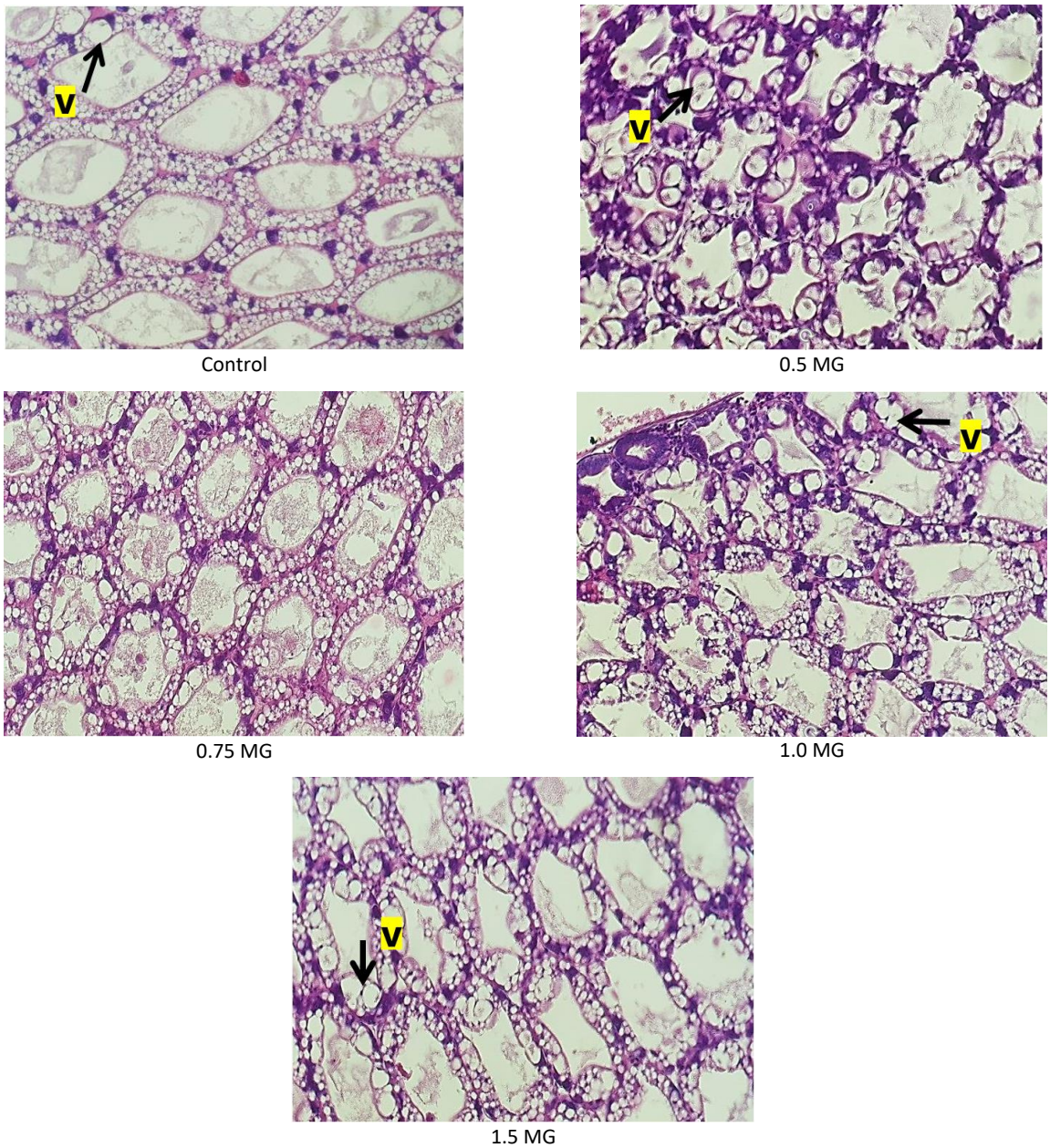


**Figure 3**. Representative histopathological images of hematoxylin and eosin-stained sections of hepatopancreas of shrimp after feeding with the experimental diets for 60-days

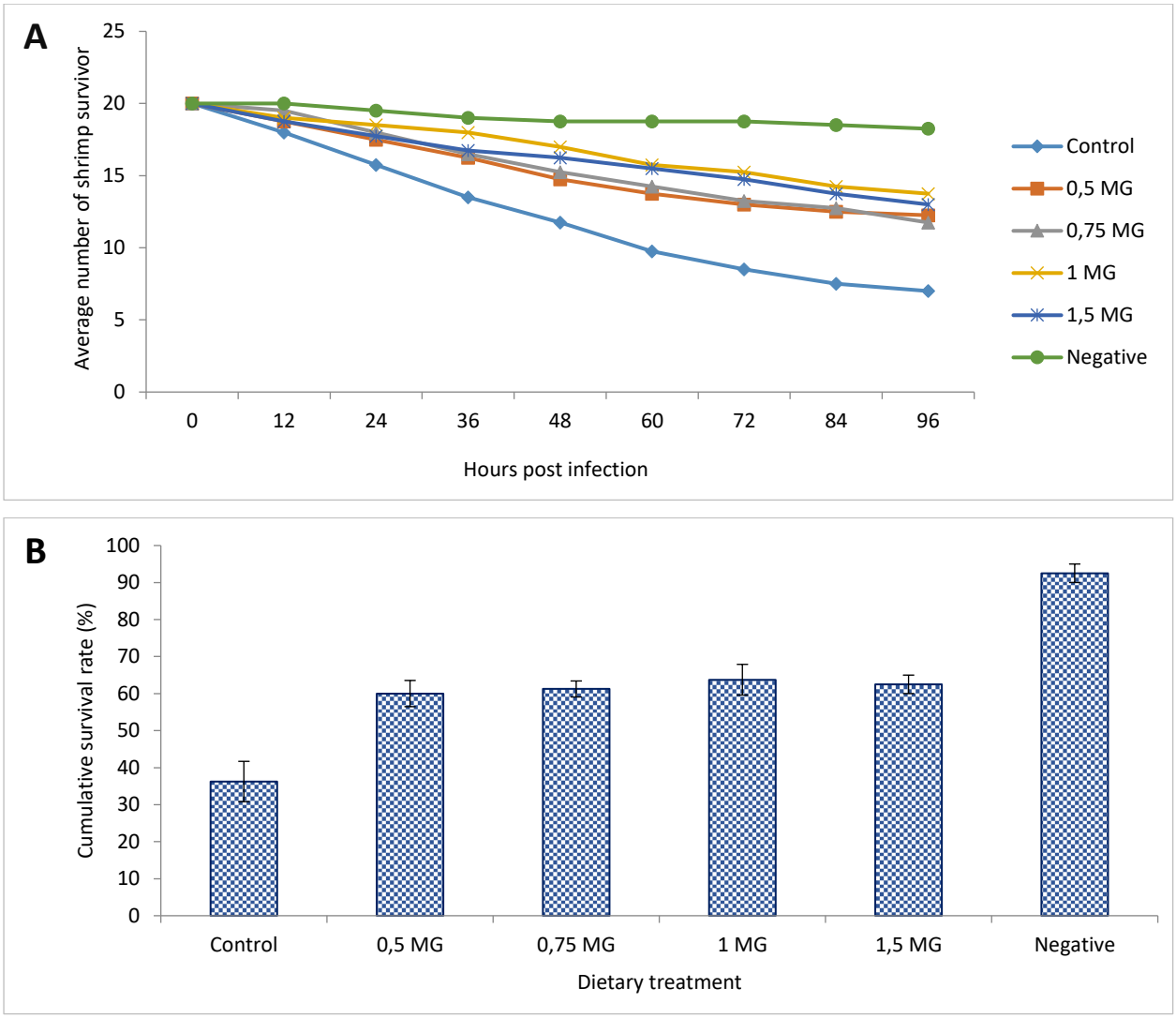


**Figure 4.** (A) Survival trend over time after challenged with *Vibrio harveyi* infection at dose of 1 x $10^5$ CFU $mL^{-1}$, intramuscularly. (B) cumulative mean survival rate of shrimp at the end of challenge test. Different letters indicate statistically significant differences (P<0.05) among the dietary treatment.

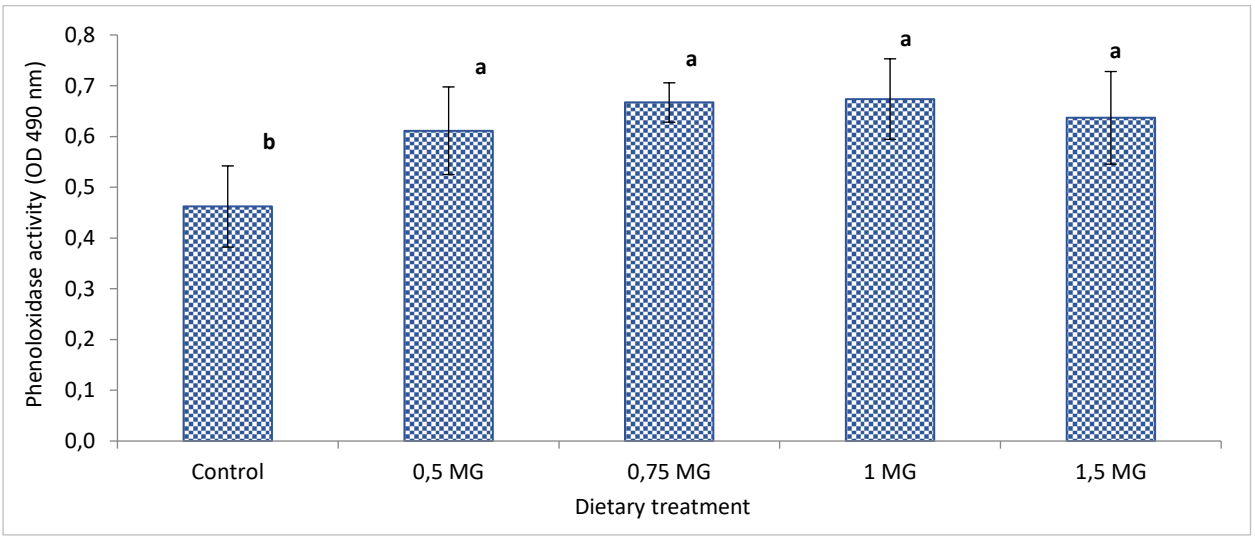


**Figure 5.** Phenoloxidase activity of experimental shrimp survivor at day-5 post injection with *Vibrio harveyi* at the dose of 1 x $10^5$ CFU $shrimp^{-1}$. Different letters indicate statistically significant differences (P<0.05) among the dietary treatment.

aquatic organisms in temperature range conditions. A study with short-chain fatty acid glycerides (propionate and butyrate) showed that the supplementation of these substances was able to improve the final weight and weekly weight gain of shrimp *P. vannamei* after 47 days of the cultivation period (Bruno Correa da Silva et al., 2016). Furthermore, the author highlighted that better feed efficiency, survival, and yield were obtained using 2% inclusion levels of butyrate (Bruno Correa da Silva et al., 2016). In addition, another study revealed that the inclusion of a 2% mixture of organic acids, such as propionate, butyrate, fumarate, succinate, and citrate salts, was able to improve the growth performance and feed efficiency in shrimp (Bruno Corrêa da Silva et al., 2016). However, the inclusion of 0.5% commercial products of short-and-medium fatty acid in the diet did not significantly improve the growth of gilthead sea bream *Sparus aurata* stocked with about 60 g mean initial body and cultured in 2 $m^3$ of fiberglass tank (Rimoldi et al., 2018). Recent research in shrimp *P. vannamei* showed that after feeding for 53 days with a diet supplemented with a mixture of glycerol esters of short-chain fatty acid (SCFAs) and medium-chain fatty acids (MCFAs), the growth of shrimp fed with 0.07 of these mixtures (SMCFAM) are lower compared to the growth of shrimp fed with 0.035 and 0.055 % of SMCFAM, and then spiked again with the inclusion of 0.11 % SMCFAM (Shin et al., 2023). In addition to the amount of MG included in the diet, further research to evaluate the inclusion effect of MG on the growth of shrimp cultured in an outdoor pond will be required to validate the results.

The total hemocytes in shrimp are vital as the first defense mechanism. In the present study, despite no statistically significant impact on the total hemocyte count (THC), we can see the increasing number of THC as the inclusion of MG increases from 0.5 to 1.0% in the diet formulation. Interesting to observe is that the trend for THC decreases as the inclusion levels go up to 1.5%. Recently, there was also no notable increase of THC in shrimp *P. vannamei* fed with several inclusion levels of propionate and butyrate from 0.5 to 2.0% for 47 days

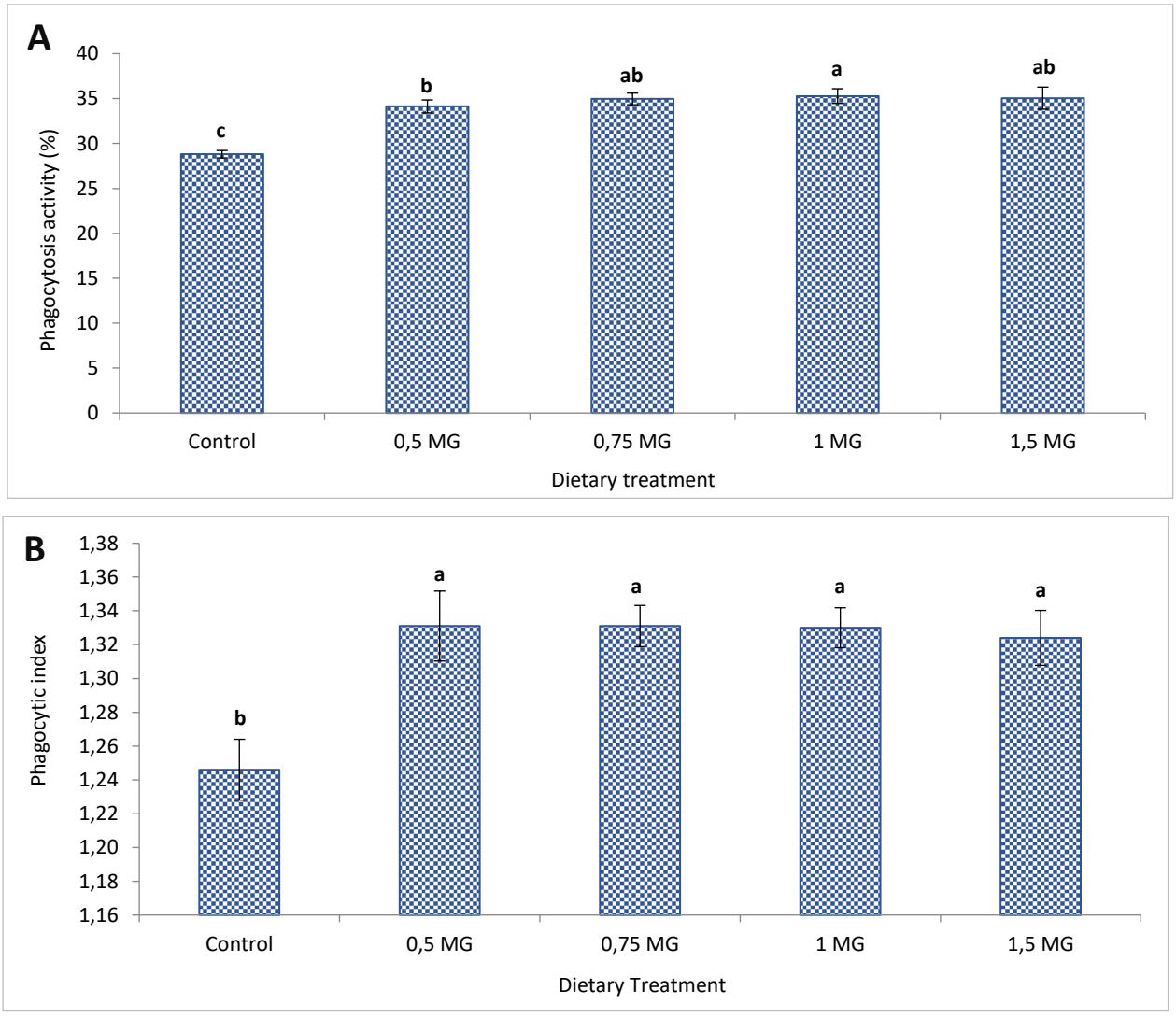


**Figure 6.** Mean percentage of phagocytic activity (A) and phagocytic index (B) in shrimp blood cells after challenge test with *vibrio harveyi* at the dose of 1 x$10^5$ CFU $shrimp^{-1}$. Different letters indicate statistically significant differences (P=0.0187) among the dietary treatment.

(Bruno Corrêa da Silva et al., 2016; Bruno Correa da Silva et al., 2016). However, studies by Bruno Corrêa da Silva et al. (2016) showed that the supplementation of butyrate and polyhydroxybutyrate as much as 2.0 % for 42 days enhances the number of THC in shrimp. Another study by Bolívar Ramírez et al. (2017) revealed that including 2.0 % of butyrate fed to shrimp for 28 days could enhance the THC in shrimp compared to the control treatment. These results show dynamics in THC numbers at different sampling points. Therefore, it is essential to have different sampling points when using a single or mixture of short- and medium-chain length fatty acids.

Lysozyme is an enzyme that is part of the innate immune system and protects shrimp from the invasion of bacterial pathogens (Hikima et al., 2003; Jollès & Jollès, 1984). Lysozyme in shrimp can be modulated; one way is by using feed additives and supplements. (Deng et al., 2013; Novriadi et al., 2021). As shown in Figure 2, despite statistical insignificance, an increasing number of lysozyme activity is observed as the inclusion levels increase from 0.5% to 1.0%. However, a corresponding decrease was observed as the inclusion of MG increased to 1.5%.

In shrimp, hepatopancreas is known to be very sensitive to dietary changes due to differences in ingredient sources and production management practices (Chen et al., 2019; Prathomya et al., 2019; Wang et al., 2016). The hepatopancreas mainly consists of branched tubules and different types of epithelial cells lining the tubules (Wu et al., 2008). In our research, all shrimp, including those within the control treatment, showed that several vacuoles appeared in the tubular epithelial cells of the hepatopancreas of the shrimp. A study by Bruno Correa da Silva et al. (2016) indicated that shrimp fed with a sodium butyrate-supplemented diet had good integrity of intestinal mucous membranes, as reflected by few vacuoles, intact and well-defined cells and intercellular cells. Furthermore, a study by Silva et al. (2016) demonstrated that including PHB and butyrate as dietary supplements did not cause any damage to shrimp's intestinal structure. In this study, including MG up to 1.5 % did not cause any morphological changes to the hepatopancreas of the shrimp.

It was reported that Vibrio's have been shown to cause severe mortalities in the aquaculture industry worldwide (Novriadi, 2016; Toranzo & Barja, 1990), including in shrimp (Kewcharoen & Srisapoome, 2019; Supono et al., 2019). Among the various *Vibrio* spp., *Vibrio harveyi* represents the most frequent pathogen causing substantial production losses to the penaeid industry (Peeralil et al., 2020; Soto-Rodriguez et al., 2012). In this study, the survival trend of the shrimp over time after being challenged with *V. harveyi* intramuscularly at a dose of 1 x $10^5$ CFU mL-1 was significantly higher in the group of shrimps fed with MG compared to the control treatment. After the challenge test, we found that the phenoloxidase (PO) as well as phagocytic activity (PA) and phagocytic index (PI) of shrimp fed with MG were significantly higher than the control treatment. Shrimp depends mostly on the innate immune systems and may have some form of adaptive immunity in defending against pathogens (Aguirre-Guzman et al., 2009; Roth & Kurtz, 2009). Subsequently, after the first defense mechanisms cannot control pathogens' access, cellular component complements with humoral responses are deployed to initiate the protective mechanisms (Kulkarni et al., 2021). Hemocytes are essential parameters involved in cellular immune responses like phagocytosis, nodule formation and encapsulation, while the prophenoloxidase (proPO) cascades and releases antimicrobial peptides and lysozyme for self-protection are two important components of humoral immune reactions in shrimp (Chen et al., 2014). The higher number of PA, PI, and PO activities post-challenge, together with the higher lysozyme activity at the end of the growth trial, indicate that MG could induce the activation of the immune system in shrimp. Study from Romano et al. (2015) indicate that the increase of PO activity with the addition of organic acid blend in diets at 10 – 20 g $Kg^{-1}$ of feed linearly increases the resistance of *P. vannamei* against *V. harveyi*. In addition, the inclusion of 2 g $Kg^{-1}$ tributyrin can significantly enhance the non-specific immune system of *P. vannamei* against *V. harveyi* during the challenge test (Lee et al., 2021).

Monoglycerides are amphipathic molecules and thus can penetrate bacterial cell membranes and alter their permeability, causing cell lysis. In addition, aquaglyceroporins in bacterial cell walls play an essential role since they enable the entry of glycerol, which is naturally used as an energy source by the bacteria. Once inside, bacterial lipases hydrolyze the ester bonds and release the fatty acid, which will then act as any other organic acid, disrupting the electron transport chain and uncoupling oxidative phosphorylation, inhibiting membrane enzymatic activities and nutrient uptake (Kabara et al., 1972; Namkung et al., 2011; Yoon et al., 2018). Therefore, it is assumed that the synergistic effect of aminobutyric, other glycerides, and glycerol in MG enhanced the non-specific immune system and the ability to disrupt the bacterial cell membrane, resulting in adequate protection and a better survival rate for shrimp against the pathogen.

The present study shows that dietary supplementation of optimized mixtures of monobutyrin, other glycerides, and glycerol (mono-, di- and triglycerides of short- and medium-chain organic acids) in relatively low inclusion levels can improve growth performance, disease resistance against *V. harveyi*, phenoloxidase activity, phagocytic index and activity as well as the health condition in shrimp. The results further show that MG could be a potential supplement in developing functional feed for shrimp. The optimum dietary level for *P. vannamei* will likely be 0.75 – 1.0% MG in the diet formulation.

## Ethical Statement

All procedures were conducted in accordance with the Indonesian Animal (Scientific Procedures) SNI 01-7252-2006 and SNI 8008:2014, approved by institutional ethical review committees (Politeknik Ahli Usaha Perikanan)

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BASF provided monoglycerides in the form of BalanGut® AQ L for the production of experimental diet as well as funding for this study.

## Author Contribution

RN: Research conceptualization, Methodology, Validation, formal analysis, investigation, Draft creation, supervision and writing the original draft.

NK: Research conceptualization, Methodology, Validation, formal analysis, investigation, Draft creation, supervision and writing the original draft.

ASK: Research conceptualization, Methodology, Validation, formal analysis, investigation, Draft creation, supervision and writing the original draft.

VEH: Research conceptualization, Methodology, Validation, formal analysis, investigation, Draft creation, supervision and writing the original draft.

SBP: Research conceptualization, Methodology, Validation, formal analysis, investigation, Draft creation, and visualization.

SW: Research conceptualization, Methodology, Validation, formal analysis, investigation, Draft creation, and visualization.

CM: Research conceptualization, Methodology, Validation, formal analysis, investigation, and funding acquisition.

AB: Research conceptualization and funding acquisition.

PS: Research conceptualization and funding acquisition.

## Conflict of Interest

Dr. Chi Man and Peer Staehler was employed by BASF and Dr. Adriana Barri still under BASF. The remaining authors state no conflict of interest.

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