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The synergistic *Lactobacillus plantarum* L20 and *Sargassum polycystum* -added diet for improvement of Black tiger shrimp, *Penaeus monodon* 's growth, immune responses, bacterial profiles, and resistance against *Vibrio parahaemolyticus* associated Acute hepatopancreatic necrosis disease (AHPND) infection

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) has inflicted massive economic losses and posed a considerable threat to the development of penaeid shrimp aquaculture in Malaysia. The restriction on antibiotic uses have necessitated exploring alternatives preventive measures against AHPND outbreak, such as the use beneficial bacteria and nutritional additives. In this study, diets which were fed to *Penaeus monodon* postlarvae (PL15) were fed with diets containing probiotic (*L. plantarum* at 10^8 CFU/mL), prebiotic (*S. polycystum* at 2%) and synbiotic diets (combination of probiotic and prebiotic) formulations. These diets were administered in triplicate for 35 days, followed by an immersion challenge with 6×10^5 CFU/mL AHPND-causing *V. parahaemolyticus* S2–4. Sampling of shrimp's cephalothorax and abdomen from the feeding trial and the immersion challenge were conducted for gene expression and histopathology analyses. Results indicated that the synbiotic-fed group displayed the most significant weight gain, specific growth rate, and protein conversion ratio among the tested groups. In addition, shrimp cephalothorax from this group displayed a significant immune response, with expression of LGBP, peroxinectin, prophenoloxidase during post-feeding trial, and expression of prophenoloxidase, toll-like receptor, penaeidin, during post-challenge trial. Furthermore, the highest *L. plantarum* concentration in abdomen, lowest *V. parahaemolyticus* S2–4 concentration in cephalothorax and highest percent survival of shrimp during post-challenge were observed in this group. Therefore, this study highlighted the positive effect of synbiotic-supplemented diet on growth, immune response, and disease resistance of shrimp, offering a promising and sustainable solution to alleviate substantial production losses in shrimp farming.

1. Introduction

Penaeid shrimp aquaculture industry in the countries of Latin America, East Asia, and Southeast Asia are the main contributors for the global production of *P. vannamei* and *P. monodon* in the year 2020. Together, they accounted for a production of 5.8 million tonnes

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(*P. vannamei*) and 0.7 million tonnes (*P. monodon*), valued at USD 42.1 billion and USD 5.2 billion, respectively (FAO, 2022). In Malaysia, local penaeid shrimp culture practice primarily involved *P. vannamei* (80%) and *P. monodon* (15%) between the years 2017 and 2021, these two species contributed about 70% (approximately 85000 tonnes) of total aquaculture production worldwide (FAO, 2023).

However, acute hepatopancreatic necrosis disease (AHPND) is a major bacterial disease that causes massive loss for penaeid shrimp aquaculture since 2009 (Lai et al., 2015). This disease currently poses a significant threat to *P. monodon* culture, mainly because *P. monodon* is more susceptible to *Vibrio*-related shrimp diseases, as compared with *P. vannamei*. Notably, genetically-improved lines of AHPND-tolerant *P. vannamei* were developed in 2017–2018 (Aranguren et al., 2020). This disease can cause sudden mass mortalities of up to 100% within the initial 30–35 days after stocking *P. monodon* in their postlarvae to early-stage juvenile. As they progress to the late-stage juveniles, mortalities can still reach 40–60% within 56–94 stocking days (Peña et al., 2015). For the pathogenesis in shrimp, AHPND-positive *Vibrio parahaemolyticus* and other *Vibrio* sp. first colonise the digestive tract including the stomach of penaeid shrimp species, where upon reaching the hepatopancreas, the pathogen would induce the sloughing of tubule epithelial cells via the production of *pirAB* toxin, ultimately leading to shrimp mortality (Kumar et al., 2020). Our previous study (Chin et al., 2024) revealed that *L. plantarum* L20 had a high inhibition activity against *Vibrio* spp. and higher survival rate of *P. monodon* postlarvae compared to other *L. plantarum* strains during *V. parahaemolyticus* immersion challenge. Additionally, our research demonstrated that incorporating prebiotic *S. polycystum* powder (Patent No: PI2013702168) into feeds improved the growth performance of Asian seabass, *Lates calcarifer* (Nazarudin et al., 2020). Hence, combining prebiotic and probiotic is anticipated to yield more promising results.

Studies by Huynh et al. (2018) and Huang et al. (2023) reported the beneficial effects of synbiotic diets (*L. plantarum* 7–40 with galactooligosaccharide and *Leuconostoc mesenteroide* B4 with dextran) on shrimp growth performance and disease resistance against vibriosis for *P. vannamei*. Consequently, in this study, synbiotic was formulated by combining probiotic *L. plantarum* L20 with nutrient rich-prebiotic *S. polycystum* powder, integrated into shrimp feeds This formulation is hypothesized to yield synergistic effects, enhancing growth performance, immune response, and disease resistance for penaeid shrimp farming. However, no study has described the synergistic potential of *L. plantarum* L20 and *S. polycystum* as an effective synbiotic for improving *P. monodon*' s growth performance, bacterial profiles, immune responses of digestive system and disease resistance against Malaysia-prevalent *V. parahaemolyticus* isolate, associated with AHPND infection. In addition, no study has also described *L. plantarum* L20 enumeration in shrimp by using RT-qPCR technique for quantitative bacteria profiling. Therefore, this study would elucidate the effects of three different supplemented feeds which are probiotic, prebiotic and synbiotics on the humoral immune responses of shrimp digestive system, and defense against pathogen, AHPND-causing *V. parahaemolyticus* S2–4.

2. Materials and Methods

2.1. Bacteria culture

Lactobacillus plantarum L20 was inoculated on MRS agar (Himedia, India) supplemented with 1.5% NaCl and incubated at 30 °C for 48 h. Repeated subculturing was performed until single colony was found. Each single colony was then inoculated into the MRS broth (Himedia, India) supplemented with 1.5% NaCl and incubated at 30 °C for 48 h. AHPND-causing *V. parahaemolyticus* S2–4 that was previously isolated from diseased shrimp by Wan Haifa-Haryani et al. (2022), was selected for the challenge test. The pathogen was grown on tryptic soy broth (TSB; Oxoid, Hampshire, England) supplemented with 1.5% NaCl for 24 h at 27 °C and harvested by centrifugation for 10 min, at 1000 rpm and 4 °C. The pellet was washed and re-dissolved in sterile autoclaved filtrated seawater and adjusted to 10^8 CFU/mL as stock by using total plate count technique.

2.2. Preparation of Lactobacillus plantarum L20 and Sargassum polycystum powder supplemented feed

Four experimental diets including a control diet without probiotic and prebiotic supplement, and three experimental diets consisting of probiotic diet (PRO) containing 10^8 CFU/g *L. plantarum* L20 (Chin et al., 2024), prebiotic diet (PRE) containing 2% (w/w) *S. polycystum* powder (Patent No: PI2013702168; Nazarudin et al., 2020), and synbiotic diet (SYN) containing 10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder were prepared. The cultured broth of *L. plantarum* L20 was centrifuged to obtain pellets. After the supernatant was discarded, the bacterial pellet was resuspended with cool autoclaved PBS saline water. Before diet preparation, the basal feed (Star Feedmill, Beranang, Malaysia; crude protein 40%, crude fat 5%, crude fiber 3%, moisture 12%) was ground to pass through a 60-mesh screen. The diet mixed with blank phosphate buffered saline (PBS) water (Sigma-Aldrich, Missouri, USA) only was assigned as control diet; the diet mixed with *L. plantarum* L20 suspension as a probiotic diet; the diet mixed with 2% *S. polycystum* powder and PBS water (Sigma-Aldrich, Missouri, USA) as a prebiotic diet; and the diet mixed with *L. plantarum* L20 suspension and 2% *S. polycystum* powder as a synbiotic diet. The ratio of feed powder with bacterial suspension or PBS water (Sigma-Aldrich, Missouri, USA) was 1:1. The four mixtures were placed into a stirring machine and slowly added with water until a stiff dough was formed. The dough was then passed through a mincer to form spaghetti-like strings, and was cut into pellets of approximately 2 mm in diameter and length. The pellets were dried by air-conditioner at 20 °C. The pellets were ground into powder form. The experimental diets were individually preserved in transparent sterile plastic bags and kept at 4 °C before use. The viabilities of *L. plantarum* in the experimental diets were assessed by total plate count technique with MRS agar (Himedia, Mumbai, India) supplemented with 1.5% NaCl and incubated at 30 °C for 48 h, before the feeding trial experiment.

2.3. Feeding management for Black tiger shrimp Penaeus monodon

Apparently healthy black tiger shrimp (*P. monodon*) postlarvae approximately 10 mg were selected randomly from the hatchery of Teluk Senangin, Perak, Malaysia. Six hundred shrimps were brought to the hatchery of Institute of Biosciences, Universiti Putra Malaysia, Serdang, Malaysia. During acclimation, 200 shrimps were randomly assigned to three fiberglass tanks (500 L) each tank. They were fed three times daily with the control diet and 4% of feed per times according to their body weight and cultured for 7 days with 30 ppt of UV treated and filtered natural seawater. The tanks were equipped with air stones for aeration, covered with black plastic cloth for preventing light and a heater for controlling the temperature at approximately 27 °C. All tanks were set within the same space and area of the hatchery to maintain the same environmental condition. Water quality was monitored by using YSI professional plus multiparameter meter (YSI, Ohio, United states) and maintained by water exchange with 1/3 of total tank water volume once per 2–3 days.

After acclimation, 600 shrimps were distributed into 12 fiberglass tanks (90 cm × 60 cm × 30 cm) of 100 L (50 shrimps/tank × 4 groups × 3 replicates). The groups were control, probiotic (PRO), prebiotic (PRE) and synbiotic (SYN) groups. All tanks were set within the same space and area of the hatchery to maintain the same environmental condition. Shrimps were fed three times daily for 35 days with the experimental diets, corresponding to 4% of their body weight. In the growth trial, weight was measured at the beginning of the trial, at 7 days interval and after 35 days. The tanks were equipped with air stones for aeration,

artificial shrimp shelters, covered with black plastic cloth for preventing light and a heater for controlling the temperature at approximately 27 °C. All tanks were set within the same space and area of the hatchery to maintain the same environmental condition. Water quality was monitored by using YSI professional plus multiparameter meter (YSI, Ohio, United states) and maintained by water exchange with 1/3 of total tank water volume once per 2–3 days. Uneaten feed was collected daily, dried in an oven, and weighed to determine the food conversion ratio (FCR). Prior to morning feeding, feces were removed daily. Shrimp survival was monitored throughout the trial period. At 35th day, measurements including final length and weight of the shrimp, as well as the dry weight of uneaten feed, were recorded to calculate growth related parameters. Nine shrimps were sampled from each group for qPCR study upon RNA extraction and cDNA synthesis as described in Sections 2.6 to 2.7 while three shrimp were sampled from each group for histopathology study as described in Section 2.8. Fig. 1 shows the timeline of feeding trial regime and immersion challenge.

The parameters of growth performance were calculated as follows:

1. Survival = (final shrimp number/initial shrimp number) × 100%;.
2. Weight gain = (final weight – initial weight)/initial weight × 100%;.
3. Specific growth rate = (ln final weight – ln initial weight)/days × 100%;.
4. Feed conversion ratio = Total dry feed intake (g)/Total wet weight gain (g).
5. Protein efficiency ratio = Total fresh flesh gain (g)/Total protein intake (g).

2.4. Immersion challenge test

For the immersion challenge experiment, shrimps at 35th day feeding trial were chosen (4 treatment groups × 20 shrimps/tank × 4 replicates), where they were challenged with LC_{50} concentration of 6×10^5 CFU/mL *V. parahaemolyticus* S2–4 by mixing the calculated volume of bacterial suspension with UV treated seawater in the aquarium (Chin et al., 2024). During the immersion challenge period, all aquariums were maintained with 30 ppt of the seawater, equipped with air stones for aeration, artificial shrimp shelters, covered with black plastic cloth for preventing light and a heater for controlling the temperature at 27 °C. No water exchange occurred during the immersion challenge test. Four replicates of each group were used for survival observation, qPCR and histopathological study. Mortality and clinical signs were observed for 7 days. At 3rd day (60 h post challenge), nine shrimps were sampled from each group for qPCR study while three shrimp was sampled from each group for histopathological study.

2.5. RNA extraction and cDNA synthesis

Nine shrimps were sampled at end of the feeding trial, while another nine shrimps were sampled at 60 h post challenge from each group. All shrimps from each treatment group (pre- and post-challenge) were kept in RNAlater (Invitrogen, Massachusetts, USA) after anaesthetised with 300 ppm pH buffered tricaine methanesulphonate (MS-222; Sigma-Aldrich, Missouri, USA). Then, shrimp shells were removed and shrimp were cut into cephalothorax portion and abdomen portion separately after being dried from RNAlater (Invitrogen, Massachusetts, USA). Cephalothorax portion consisted of shrimp hepatopancreas while the abdomen portion consisted of the shrimp intestinal tract. Three cephalothorax portions were assembled as one replicate, while three abdomen portions were assembled as one replicate. Three replicates were obtained from the four treatment groups from the feeding trial and post challenge. The samples of the four treatments were ground using electronic homogeniser. RNA was extracted using Trizol (Invitrogen, Massachusetts, USA) for each homogenised sample according to the manufacturer's manual. The steps were repeated for other treatment groups. The integrity of the RNA was validated by using 1.0% agarose of gel electrophoresis.

The RNA samples were treated with the RapidOut DNA Removal Kit (Thermo Fischer Scientific, Waltham, USA) according to the manufacturer's manual. The concentration and quality of the total RNA was checked based on its optical density at 260 and 280 nm as measured by GENOVA-NANO Spectrophotometer (Jenway, Staffordshire, UK). The concentration of total RNA was adjusted into 1 μg/μL by using nuclease-free water. The RNA was added with 4 μL of OneScript® reverse-transcription buffer (Applied Biological Materials, Richmond, Canada) and 1 μL of OneScript ® reverse-transcription enzyme (Applied Biological Materials, Richmond, Canada), 1 μL of dNTP, 1 μL of random primer, 1 μL of oligo (dT) and up to 20 μL of nuclease-free water. The final mixture was incubated at 25 °C for 10 min and 60 °C for 30 min, followed by transcription enzyme inactivation for 5 min at 85 °C. The first-strand complementary DNA (cDNA) was subjected for gene expression analysis.

2.6. Gene expression by real time-quantitative PCR

Quantitative PCR analysis was performed using QuantiNova® SYBR Green real-time PCR mix (Qiagen, Hilden, Germany) following the manufacturer's instructions. About 1 μL of cDNA sample was added with

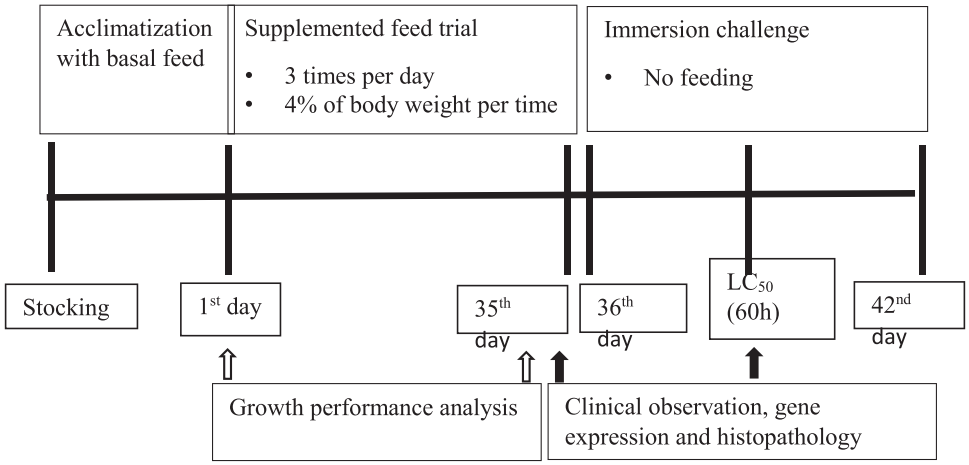

Fig. 1. Experimental timeline of feeding trial regime and immersion challenge. Growth performance was recorded along the experiment. Survival, clinical observation, gene expression and histopathology assessments were performed at end of feeding trial (35th day) and immersion challenge test with LC_{50} of 6×10^5 CFU/mL AHPND-causing *V. parahaemolyticus* S2–4 (3rd day, 60 h).

10 μL of 2x SYBR Green PCR Master mix, 1 μL of forward primer, 1 μL of reverse primer and 7 μL of nuclease-free water into a PCR tube. Each cDNA sample was performed in triplicate. The samples were run by using Rotor Gene Q Real-time PCR machine (Qiagen, Hilden, Germany). No template control (NTC) and no reverse transcriptase control (NRT) were added also in each run. The CT value was analysed and recorded through Rotor Gene Q software (Qiagen, Hilden, Germany). The primers used in RT-qPCR and their efficiency for each gene were listed in Table 1. The primer pairs were efficiently determined by performing serial dilutions of reference cDNA and used to quantify cDNA concentration. The standard cycling parameters included an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/elongation at 60 °C for 30 s. Melting curve analysis was performed at the end of each PCR run. A melting curve profile was obtained by heating the mixture to 95 °C, cooling to 60 °C for each 10 s. Primer specificity was evaluated by determining single peak in all melting curves and single targeted band on 1.0% agarose gel electrophoresis of all RT-PCR amplicons indicating absence of primer-dimer and optimal performance of the primers. The mean CT value was used for gene expression analysis.

2.6.1. Measurement of the expression of immune related genes in Penaeus monodon

The immune related genes included lipopolysaccharide and β-1,3-glucan-binding protein (LGBP), peroxinectin, prophenoloxidase, toll-like receptor, crustin, penaeidin, lysozyme, and heat shock protein (HSP70) (Table 1). By using three computational programs included geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) for analysing stability of housekeeping genes, the most stable elongation factor 1-alpha (EF1-α) was chosen as control gene among other housekeeping genes (β-actin, GAPDH) for the immune gene expression study of the shrimp cephalothorax and abdomen. Relative quantification was applied for determining expression of immune-related genes in gastrointestinal digestion system of shrimp (Silveira et al., 2018). The relative expression levels of shrimp immune genes were calculated by using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6.2. Quantitative intestinal bacterial profiling of Penaeus monodon

For quantifying *L. plantarum*, specific conserved protein-encoding gene, GHKL domain containing protein gene was used as indicator (Table 1) because they can distinguish closely related species and strains of *Lactobacillus* sp. strains (Huang et al., 2018; Kim et al., 2020). For quantifying AHPND-causing *V. parahaemolyticus*, Photorhabdus insect related (*pir*) toxin A (*pirA*) gene was used as indicator (Table 1) because it is a crucial toxin genes of AHPND-causing *V. parahaemolyticus* (Aranguren Caro et al., 2020; Han et al., 2020).

Absolute quantification was applied for detecting and quantifying bacterial protein genes in gastrointestinal digestion system of shrimp (Zhang et al., 2020). The absolute concentrations of the bacterial protein genes were calculated by using the linear regression of their standard curve (Bustin, 2000). Standard curves were generated by the amplification of a 10-fold dilution series of the targeted protein genes in the presence of the bacterial cDNA. The coefficient of correlation (R^2) between the Threshold value (CT) and targeted cDNA concentration was between 0.990–0.997, while the PCR efficiency was between 90% and 110%. Zero concentration of bacteria was determined if CT value was not available or more than 40 (McCall et al., 2014). Table 1 shows the primers used in RT-qPCR.

2.7. Histopathological observation

Three shrimp from all treatment groups were chosen for histopathological analysis. The shrimp shells were removed and whole shrimp were fixed in 10% neutral buffered formalin for 24 h after anaesthetised with 300 ppm tricaine methanesulphonate, known as MS-222 (Sigma-Aldrich, Missouri, USA). The samples were transferred to 70% ethanol and dehydrated using series of increasing concentration of ethanol (50–100%). The samples were embedded in paraffin wax, cut into 4 μm thickness and stained with haematoxylin and eosin. The samples were observed under microscope and microphotographed with magnification 100X and 400X under the Olympus BX51 digital light microscope (Olympus, Tokyo, Japan). The hepatopancreas and gut of the shrimps were observed histologically.

2.8. Data analyses

Normality of the variance was evaluated using Shapiro–Wilk test. Then, differences in data were analysed by one-way analysis of variance (ANOVA) following post-hoc Duncan test. All statistical analyses were

Table 1
The primer sequence that used for Reverse transcriptase Quantitative PCR (RT-qPCR) add info on each selected genes.

Gene	Function	Primers	Sequences	Efficiency	Reference
	Shrimp immune related gene				
lipopolysaccharide and β-1,3-glucan-binding protein (LGBP)	Pathogen recognition	Forward	CATGTCCAACTTCGCTTTCAGA	100%	Mai et al. (2021)
		Reverse	ATCACCGCGTGGCATCTT		
Peroxinectin	Immune signalling transduction	Forward	ATCCAGCAGCCAGGTATG	98%	Lin et al. (2012)
		Reverse	CAGACTCATCAGATCCATTCC		
Prophenoloxidase	Main component of Prophenoloxidase cascade	Forward	ACCACTGGCACTGGCACCTCGTCTA	100%	Lin et al. (2012)
		Reverse	TCGCCAGTTCTCGAGCTTCTGCAC		
Toll-like receptor	Main component of Toll signalling pathway	Forward	CTTAGCCTTGGAGACAAC	98%	Deris et al. (2020)
		Reverse	GATGCTTAACAGCTCCTC		
Crustin	Cationic antimicrobial peptides	Forward	AGCGACTGCAGGTATTGGTG	97%	Mai et al. (2021)
		Reverse	TCGTTGGAACAGGTTGTGG		
Penaeidin	Cationic antimicrobial peptides	Forward	TCGTGGTCTGCCTGGTCTT	102%	Mai et al. (2021)
		Reverse	CAGGTCTGAACGGTGGTCTTC		
Lysozyme	Cationic antimicrobial peptides	Forward	TGGTGTGGCAGCGATTATG	102%	Deris et al. (2020)
		Reverse	GATCGAGGTCGCGATTCTTAC		
Heat shock protein 70 (HSP70)	Stress and immunostimulant protein	Forward	CCTCCTACGTCGCCTTCACAGACA	96%	Lin et al. (2012)
		Reverse	GGGGTAGAAGGTCTTCTTGTCTCCC		
Elongation factor 1α (EF1-α)	house-keeping gene	Forward	TCGCCGAACTGCTGACCAAGA	104%	Mai et al. (2021)
		Reverse	CCGGCTTCCAGTTCCTTACC		
	Bacterial protein gene				
GHKL domain containing protein	Specific conserved protein-encoding gene, act as *L. plantarum* indicator	Forward	GCGGTATCGATTCGATTGGT	99%	Kim et al. (2020)
		Reverse	TGATGTCAATCGCCTCTTGG		
Photorhabdus insect-related toxins A (*pirA*)	Toxin of AHPND, act as *V. parahaemolyticus* AHPND indicator	Forward	CAAACGGAGGCGTCACAGA	100%	Mai et al. (2021)
		Reverse	GACCGACTTCCGGGATGAT		

performed using IBM SPSS Statistics® version 22.0. The values were considered significantly different if $p < 0.05$.

3. Results

3.1. Growth performance

The final weight and weight gain were significantly higher in the group PRO, group PRE and group SYN ($p < 0.05$) than those in the control group but not significantly different ($p > 0.05$) between group PRO, group PRE and group SYN (Table 2). The specific growth rate and protein conversion ratio were slightly higher ($p < 0.05$) in the group PRO and PRE than those in the control group, but those in the group SYN was significantly highest ($p < 0.05$) among the groups (Table 2). The feed conversion ratio was slightly lower in the group PRO and PRE ($p < 0.05$) than those in the control group, but those in group SYN was significantly lowest ($p < 0.05$) among the groups (Table 2). No significant ($p > 0.05$) different for survival among all groups (Table 2). All representive shrimp shows normal colour of hepatopancreas from Group PRO, PRE, SYN and control (Supplemental ^{1}A–^{1}D).

3.2. Survival of post-challenge

The survival rates of the group PRE was significantly ($p < 0.05$) higher than those in the control group, but the survival rates of the group PRO and SYN were significantly highest ($p < 0.05$) among all groups (Fig. 2). Supplemental 1 shows normal hepatopancreas (HP) and pale hepatopancreas (PHP). All representive shrimp shows normal colour of hepatopancreas from Group PRO, PRE, SYN but pale hepatopancreas from control group (Supplemental 1E-1 H).

3.3. Immune gene expression of Penaeus monodon

For shrimp cephalothorax in the feeding trial (Fig. 3A), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP), peroxinectin, prophenoloxidase and lysozyme were significantly up-regulated ($p < 0.05$) but toll-like receptor, crustin, penaeidin and HSP70 were significantly down-regulated ($p < 0.05$) from the Group PRO and SYN compared with control group. LGBP, peroxinectin, toll-like receptor and HSP70 were significantly down-regulated ($p < 0.05$), crustin, penaeidin and lysozyme were not significantly difference ($p > 0.05$), prophenoloxidase were significantly up-regulated ($p < 0.05$) from the Group PRE compared with control group.

For shrimp abdomen in the feeding trial (Fig. 3B), LGBP, toll-like receptor, HSP70 were significantly down-regulated ($p < 0.05$), but significantly up-regulated ($p < 0.05$) from the Group PRO, PRE and SYN compared with control group. Crustin and penaeidin were significantly down-regulated ($p < 0.05$) from the Group PRO and SYN, but not significantly difference ($p > 0.05$) compared with control group. Peroxinectin was significantly up-regulated ($p < 0.05$) from Group PRO

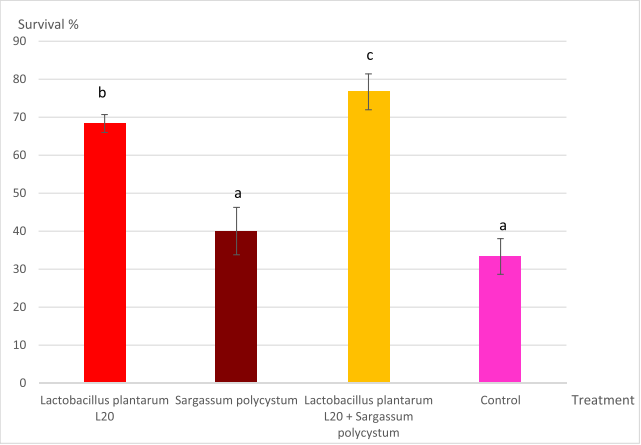

Fig. 2. Survival percentage of different diet administered *Penaeus monodon* after immersion challenge of 6 × 10^5 CFU/mL AHPND-causing *Vibrio parahaemolyticus* S2–4 at 3rd day (60 h) post challenge. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group (PRO) that fed with 1 × 10^8 CFU/g *L. plantarum* L20 supplemented feed; Prebiotic group (PRE) that fed with *2% S. polycystum* powder supplemented feed; Synbiotic group (SYN) that fed with 1 × 10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed; Control group that fed with basal feed.

while prophenoloxidase was significantly down-regulated ($p < 0.05$) from Group PRE, but the rest of groups were not significantly different ($p > 0.05$) for the two genes compared with control group.

For shrimp cephalothorax in the challenge test (Fig. 4A), LGBP, peroxinectin and HSP70 were significantly down-regulated ($p < 0.05$), crustin was not significant difference ($p > 0.05$) but toll-like receptor and penaeidin were significantly up-regulated ($p < 0.05$) from the Group PRO, PRE and SYN compared with control group. Prophenoloxidase and lysozyme were significantly up-regulated ($p < 0.05$) from Group PRO but significantly down-regulated ($p < 0.05$) from Group PRE compared with control group. Group SYN exhibited significantly up-regulated prophenoloxidase ($p < 0.05$) but significantly down-regulated lysozyme ($p < 0.05$) compared with control group.

For shrimp abdomen in the challenge test (Fig. 4B), LGBP and crustin were not significant difference ($p > 0.05$), peroxinectin was significantly down-regulated ($p < 0.05$) but toll-like receptor was significantly up-regulated ($p < 0.05$) from Group PRO, PRE and SYN compared with control group. Prophenoloxidase was significantly up-regulated ($p < 0.05$) whereas penaeidin was significantly down-regulated ($p < 0.05$) from Group PRO and SYN but the Group PRE were not significantly difference ($p > 0.05$) for the prophenoloxidase and significantly up-regulated for penaeidin compared with control group. Lysozyme and HSP70 were not significantly difference ($p > 0.05$) from Group PRO and PRE but significantly down-regulated ($p < 0.05$) from

Table 2
Parameters of growth performance for supplemented diets administrated *Penaeus monodon*.

	Probiotic (PRO: 10^8 CFU/g of *Lactobacillus plantarum* L20)	Prebiotic (PRE: 2% of *Sargassum polycystum*)	Synbiotic (SYN: 10^8 CFU/g of *Lactobacillus plantarum* L20 with 2% of *Sargassum polycystum*)	Control (PBS water only)
Initial weight (g)	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}
Final weight (g)	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}	0.04 ± 0.00^{a}
Weight gain%	332.03 ± 77.11^{b}	326.95 ± 68.17^{b}	387.55 ± 66.07^{b}	179.15 ± 24.78^{a}
Specific growth rat %	3.61 ± 0.48^{ab}	3.60 ± 0.40^{ab}	3.94 ± 0.33^{b}	2.56 ± 0.23^{a}
Feed conversion ratio	2.66 ± 0.62^{ab}	2.66 ± 0.53^{ab}	2.20 ± 0.29^{b}	3.63 ± 0.41^{a}
Protein conversion ratio	0.03 ± 0.01^{ab}	0.03 ± 0.01^{ab}	0.03 ± 0.00^{b}	0.02 ± 0.00^{a}
Survival%	98.00 ± 1.63^{a}	96.67 ± 2.49^{a}	97.33 ± 0.94^{a}	99.33 ± 0.94^{a}

Different superscript letter indicates significant differences ($p < 0.05$) of the same row.

Fig. 3. Immune gene expression in cephalothorax and abdomen of *Penaeus monodon* fed with different supplemented feed during at 35th day of feeding trial. 3 A is cephalothorax part while 3B is abdomen part. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group (PRO) that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; Prebiotic group (PRE) that fed with 2% *S. polycystum* powder supplemented feed; Synbiotic group (SYN) that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed. Control group fed with control feed.

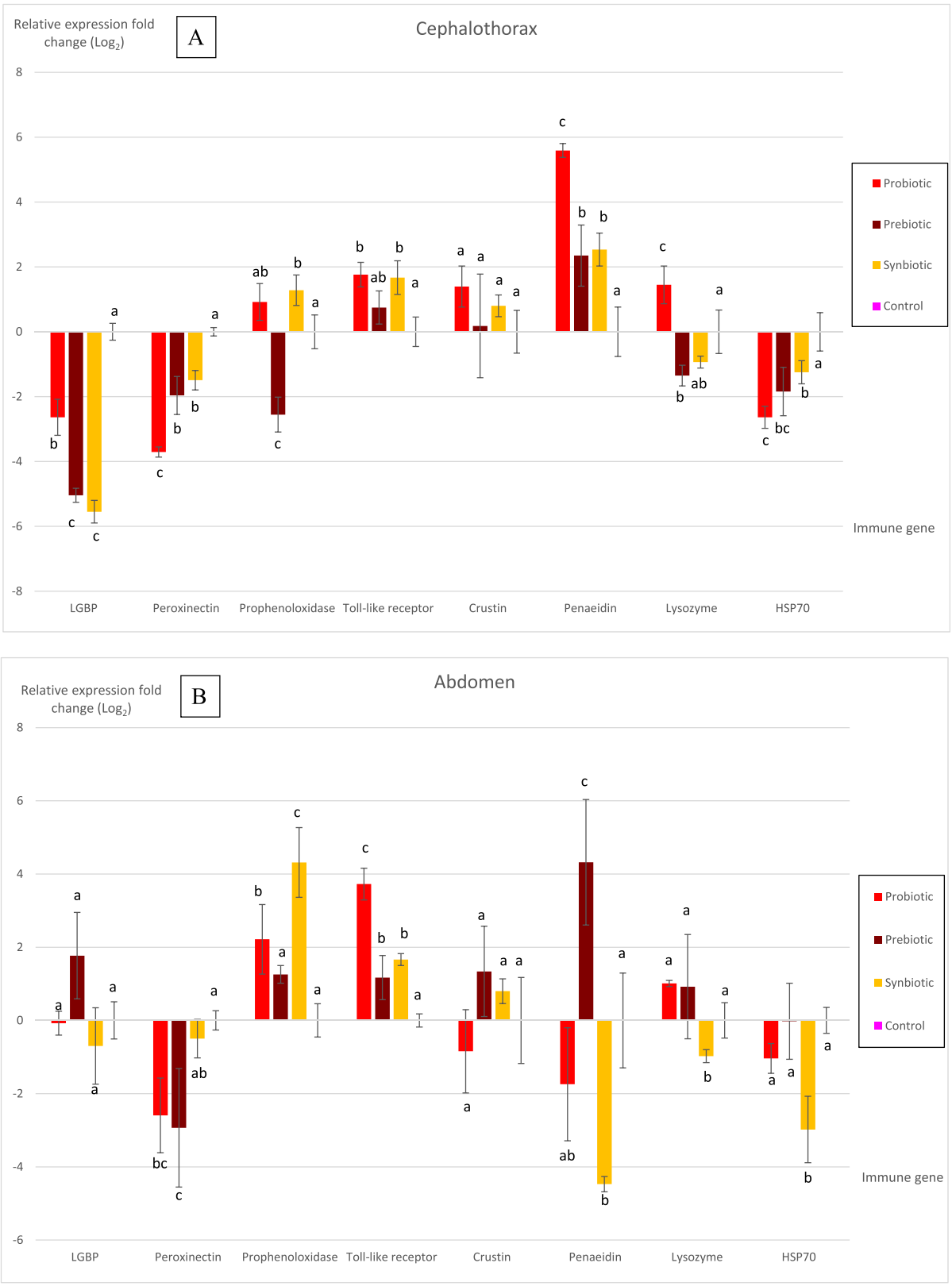

Fig. 4. Immune gene expression in cephalothorax and abdomen of *Penaeus monodon* fed with different supplemented feed during at 60 h of AHPND-causing *Vibrio parahaemolyticus* S2–4 immersion challenge. 4 A is cephalothorax part while 4B is abdomen part. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group (PRO) that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; Prebiotic group (PRE) that fed with 2% *S. polycystum* powder supplemented feed; Synbiotic group (SYN) that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed. Control group fed with control feed.

Group SYN.

3.4. Quantitative profiling of Lactobacillus plantarum in Penaeus monodon

The cycle threshold value (CT value) of the GHKL domain-containing protein gene indicated the concentration of *L. plantarum* by referencing the standard curve (Supplemental 2 A), revealed viable *L. plantarum* with specified GHKL domain containing protein gene in shrimps (Fig. 5). On the 35th day of the feeding trial, shrimp from groups PRO, PRE, and SYN exhibited the presence of *L. plantarum*, in contrast to the control group where *L. plantarum* was absent. Shrimp from group PRO and SYN exhibited significantly ($p < 0.05$) highest concentration of *L. plantarum* in the shrimp cephalothorax which 2.413 ± 0.316 $\log_{10}$ CFU/mL and

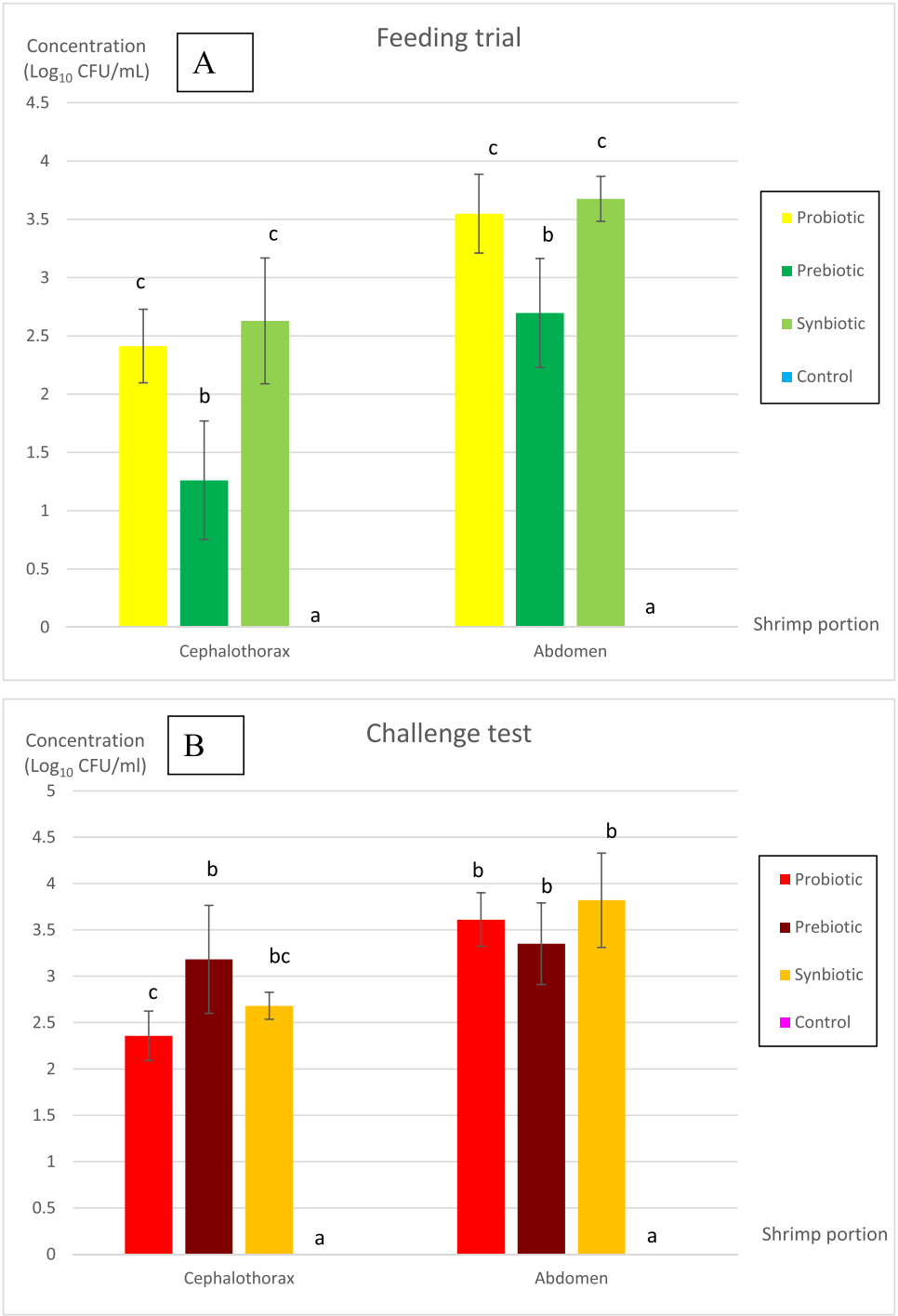

Fig. 5. Comparative quantification of *Lactobacillus plantarum* by using specified GHKL domain containing protein gene in cephalothorax and abdomen of *Penaeus monodon* fed with different supplemented feed. 5 A is 35th day of the feeding trial while 5B is challenge test is 60 h of 6×10^5 CFU/mL of AHPND-causing *Vibrio parahaemolyticus* S2–4 immersion challenge. No GHKL domain-containing protein gene was detected in cephalothorax and abdomen of control group. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group (PRO) that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; Prebiotic group (PRE) that fed with 2% *S. polycystum* powder supplemented feed; Synbiotic group (SYN) that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed. Control group fed with control feed.

2.629 ± 0.539 $\log_{10}$ CFU/mL respectively, as well as highest concentration of *L. plantarum* which 3.549 ± 0.338 $\log_{10}$ CFU/mL and 3.676 ± 0.193 $\log_{10}$ CFU/mL in the shrimp abdomen group PRO and SYN respectively compared to other groups.

Similarly, there were presence of *L. plantarum* in shrimp from group PRO, PRE and SYN, in contrast with control group in 60 h post-immersion challenge with 6×10^5 CFU/mL of *V. parahaemolyticus* S2–4. The concentration of *L. plantarum* was 3.182 ± 0.267 Log_{10} CFU/mL in the shrimp cephalothorax from group PRE which was significantly ($p > 0.05$) highest compared with control group. However, *L. plantarum* concentration were 3.611 ± 0.290 Log_{10} CFU/mL, 3.351 ± 0.440 Log_{10} CFU/mL, 3.820 ± 0.509 Log_{10} CFU/mL in the abdomen of shrimp from group PRO, PRE and SYN respectively which not significantly different ($p > 0.05$) with each other.

3.5. Quantitative profiling of AHPND-causing Vibrio parahaemolyticus in Penaeus monodon

The CT value of *pirA* toxin gene indicated concentration of AHPND-causing *V. parahaemolyticus* by referring the standard curve (Supplemental 2B), revealed viable AHPND-causing *V. parahaemolyticus* including strain S2–4 in shrimp (Fig. 6).

No concentration of AHPND-causing *V. parahaemolyticus* was determined since no *pirA* gene was detected in cephalothorax and abdomen of shrimp from all groups in 35th day of feeding trial. In 60 h post-immersion challenge with 6×10^5 CFU/mL of *V. parahaemolyticus* S2–4, the concentration of AHPND-causing *V. parahaemolyticus* which 6.410 ± 0.291 Log_{10} CFU/mL and 4.936 ± 0.099 Log_{10} CFU/mL were exhibited significantly ($p < 0.05$) highest in cephalothorax and abdomen respectively of shrimp from control groups compared with other groups. However, lowest concentration of *AHPND-causing V. parahaemolyticus* was exhibited significantly ($p < 0.05$) by shrimp from probiotic and synbiotic group which 4.244 ± 0.409 Log_{10} CFU/mL and 4.504 ± 0.203 Log_{10} CFU/mL respectively in cephalothorax, 3.713 ± 0.246 Log_{10} CFU/mL and 4.128 ± 0.331 Log_{10} CFU/mL respectively in abdomen.

3.6. Histological observation of Penaeus monodon

The histopathological changes of hepatopancreas in the representative *P. monodon* by referring Sukor et al. (2016). For the feeding trial, the normal structures of different hepatocytes including, resorptive cell (R-cell: r), blister-like cell (B-cell: b), fibrillar cell (F-cell: f), hepatic lumen (lu) were shown in representative shrimp from the group PRO (Fig. 7A), group PRE (Fig. 7B), group SYN (Fig. 7C) and control group (Fig. 7D). Moreover, Group PRO (Fig. 7A), group PRE (Fig. 7B) and group SYN (Fig. 7C) exhibited hemocytes in the shrimp hepatopancreas. For the challenge test, B-cell (b), hepatic lumen (lu), mild-vacuolated R-cell (mvr), mild-vacuolated F-cell (mvf) and hemocytes (h) were showed in representative shrimp from the group PRO (Fig. 7E) and group SYN (Fig. 7G). Hepatic lumen (lu), vacuolated R-cell (vr), vacuolated F-cell (vf) and reptured B-cell (eb) and hemocytes (h) were showed in representative shrimp from the group PRE (Fig. 7F). However, ruptured karyomegaly R-cell (ekr), ruptured karyomegaly F-cell (ekf), ruptured B-cell (eb), sloughed hepatic lumen (slu) and hemocytes inflation (ih) were observed in control group (Fig. 7H).

The histopathological changes of intestine in the representative *P. monodon* by referring Duan et al. (2017). For the feeding trial, the normal structure of brush border (b), intestinal epithelium (e) and intestinal lumen (lu) were shown in representative shrimp from group PRO (Fig. 8A), group PRE (Figure 9B), group SYN (Fig. 8C) and control group (Fig. 8D). Moreover, Group PRO (Fig. 8A), group PRE (Fig. 8B)

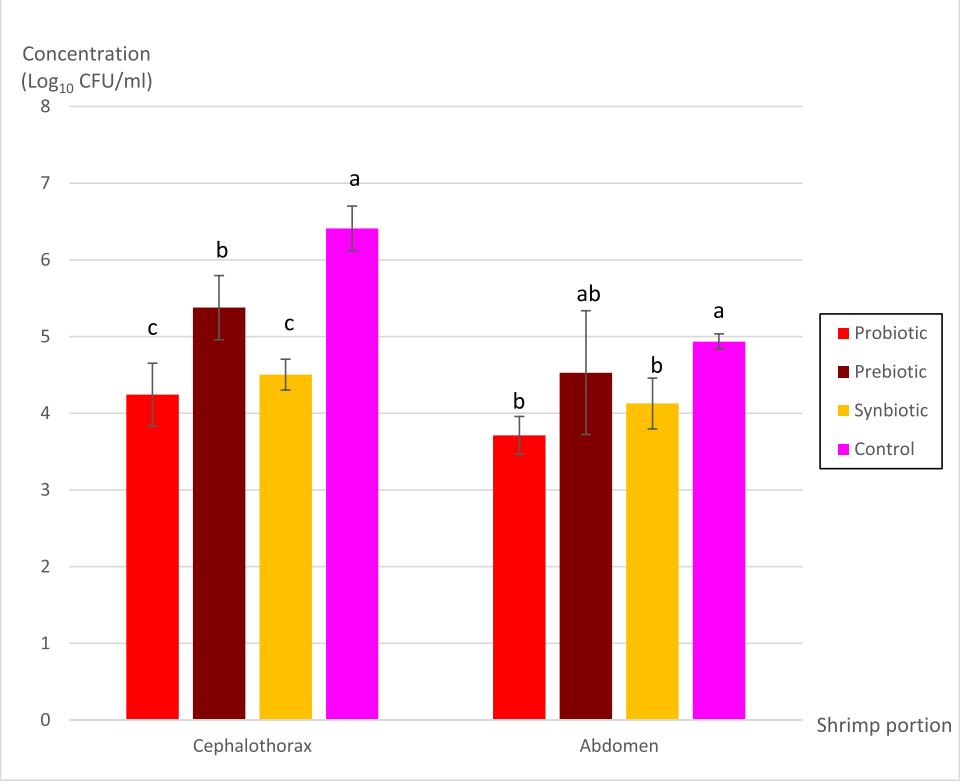

Fig. 6. Comparative quantification of AHPND-causing *Vibrio parahaemolyticus* by using specified *pirA* toxin gene in cephalothorax and abdomen of *Penaeus monodon* were fed with different supplemented feed in 60 h of 6×10^5 CFU/mL AHPND-causing *V. parahaemolyticus* S2–4 immersion challenge. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group (PRO) that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; Prebiotic group (PRE) that fed with 2% *S. polycystum* powder supplemented feed; Synbiotic group (SYN) that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed. Control group fed with control feed.

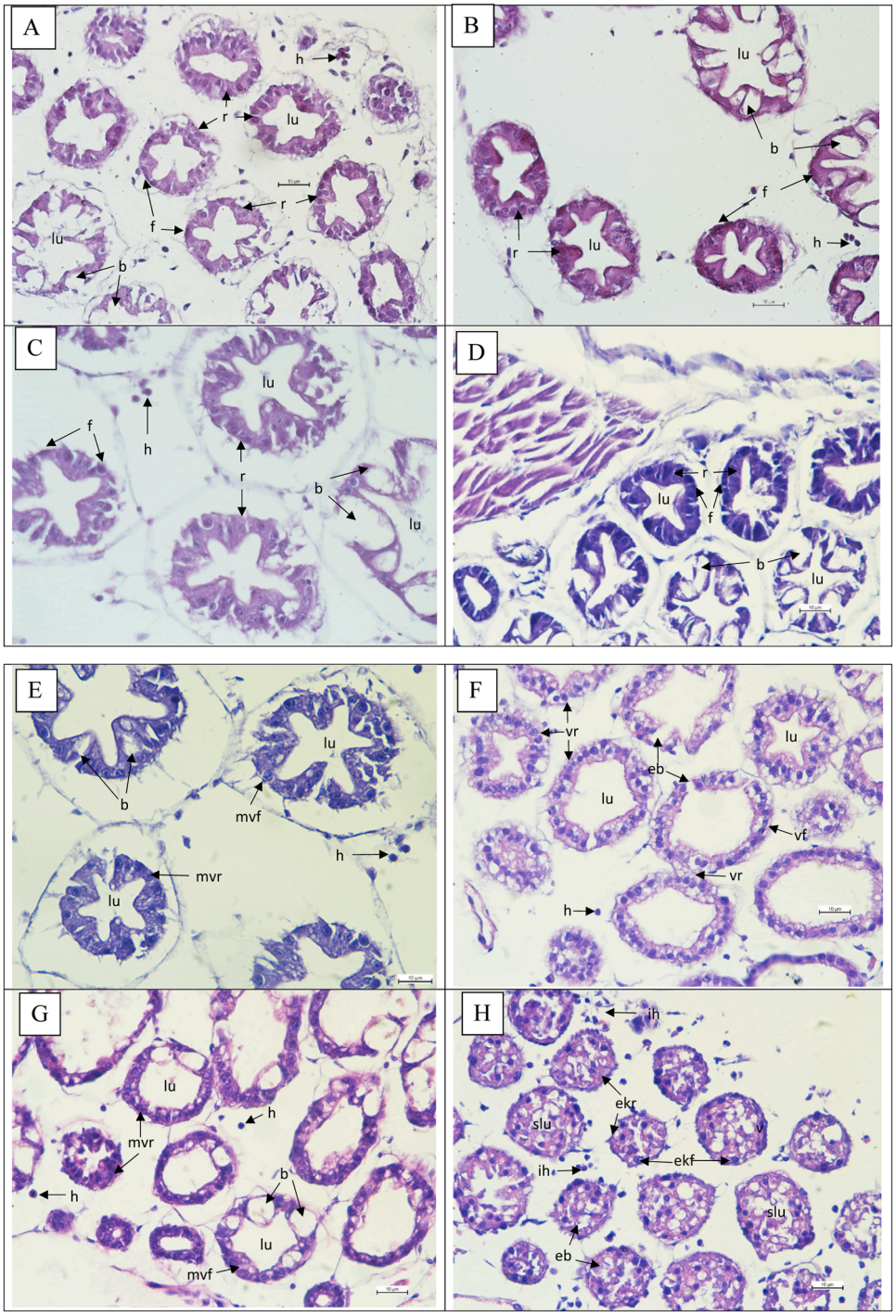

Fig. 7. Histopathology for hepatopancreas in cephalothorax of *Penaeus monodon* fed with different supplemented feeds. Figs. 7A, 7B, 7C and 7D are 35th day of feeding trial while Fig. 7E. 7 F, 7 G and 7 H are 60 h of 6×10^5 CFU/mL AHPND-causing *V. parahaemolyticus* S2–4 immersion challenge. Figs. 7A and 7E are probiotic group (PRO) which fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; Figs. 7B and 7F are prebiotic group (PRE) which fed 2% *S. polycystum* powder supplemented feed; Figs. 7C and 7G are synbiotic group (SYN) which fed 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed; Figs. 7D and 7H are control group which fed with control feed. Fig. 7A-7D show resorptive cell (R-cell: r), blister-like cell (B-cell: b), fibrillar cell (F-cell: f), hepatic lumen (lu) (× 400). Fig. 7A-7 C show hemocyte (h) (× 400). Figs. 7E and 7G show blister-like cell (B-cell: b), hepatic lumen (lu), mild-vacuolated R-cell (mvr), mild-vacuolated F-cell (mvf) and hemocyte (h) (× 400). Fig. 7F shows hepatic lumen (lu), vacuolated R-cell (vr), vacuolated F-cell (vf) and reptured B-cell (eb) and hemocyte (h) (× 400). Fig. 7H shows ruptured karyomegaly R-cell (ekr), ruptured karyomegaly F-cell (ekf), ruptured B-cell (eb), sloughed hepatic lumen (slu) and hemocyte infiltration (ih) (× 400).

and group SYN (Fig. 8C) exhibited hemocytes infiltration (ih) in the submucosa. Control group exhibited a smaller number of hemocytes (h) in the submucosa. For the challenge test, brush border (b), mild-vacuolated intestinal epithelium (mve), intestinal lumen (lu) and hemocytes infiltration (h) showed in intestine of representative shrimp from group PRO (Fig. 8E), PRE (Fig. 8F) and group SYN (Fig. 8G). However, (db) degenerated brush border, (ve) vacuolated intestinal epithelium, (de) disintegrated intestinal epithelium and (lu) intestinal lumen were examined in control group (Fig. 8H).

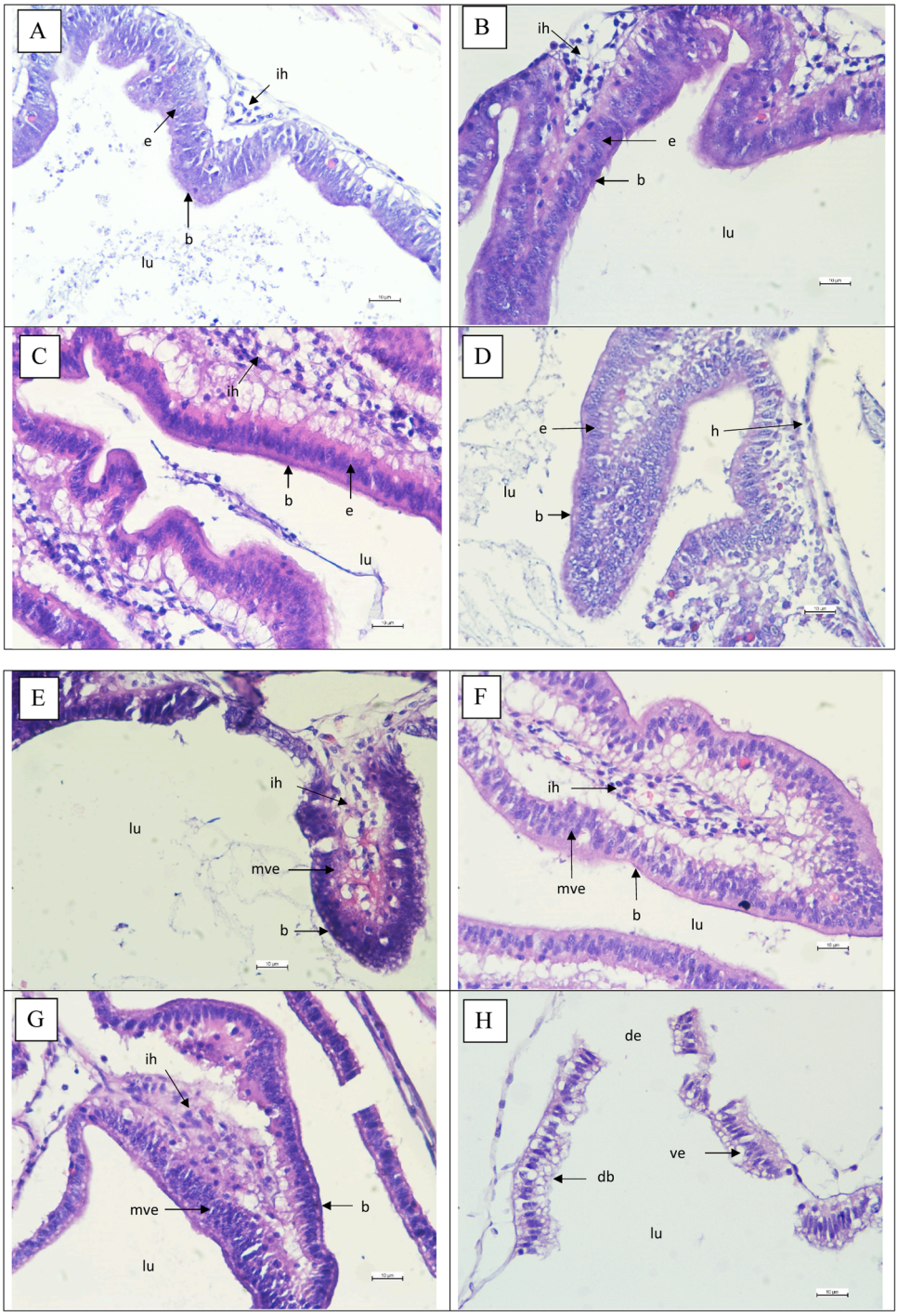

Fig. 8. Histopathology for intestinal epithelium in abdomen of *Penaeus monodon* fed with different supplemented feed. Figs. 8A, 8B, 8C and 8D are 35th day of feeding trial while Figs. 8E, 8F, 8G and 8H are 60 h of 6×10^5 CFU/mL AHPND-causing *V. parahaemolyticus* S2–4 immersion challenge. Figs. 8A and 8E are probiotic group (PRO) which fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; Figs. 8B and 8F are prebiotic group (PRE) which fed 2% *S. polycystum* powder supplemented feed; Figs. 8C and 8G are synbiotic group (SYN) which fed 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed; Figs. 8D and 8H are control group which fed with normal feed. Fig. 8A-8D show brush border (b), intestinal epithelium (e), intestinal lumen (lu) (× 400). Fig. 8A-8 C show submucosa hemocytes infiltration (ih) (× 400). Fig. 8D shows submucosa hemocyte (h) (× 400). Fig. 8E-8 G show brush border (b), mild-vacuolated intestinal epithelium (mve), intestinal lumen (lu) and submucosa hemocytes infiltration (ih) (× 400). Fig. 8H shows degenerated brush border (db), vacuolated intestinal epithelium (ve), disintegrated intestinal epithelium (de) and intestinal lumen (lu) (× 400).

4. Discussion

4.1. Influence of PRO, PRE and SYN for Penaeus monodon in the feeding trial

4.1.1. Growth performance and Lactobacillus plantarum profile of the feeding trial

Group PRO, PRE and SYN demonstrated effectiveness of *L. plantarum* L20 and *S. polycystum* as PRO and PRE for improving growth performance of shrimp. Previous studies have reported many varieties of synbiotic feeds by using different types of extracted polysaccharides (galactooligosaccharide, mannan oligosaccharide, chitosan) and gut beneficial bacteria (*Lactobacillus* spp., *Bacillus* spp.) for improving penaeid shrimp's growth performance (Huynh et al., 2018; Chen et al., 2020; Kewcharoen et al., 2022). However, *S. polycystum* were recommended as prebiotic because of its higher nutritional contents (minerals and vitamins) and cheapest market price (USD 0.50 to 1.08 per kg in year 2021) in Malaysia (Shannon and Abu-Ghannam, 2019; Asri et al., 2021). The highest SGR, PER and best FCR of shrimp from Group SYN indicated synergistic effect of combined *L. plantarum* L20 and *S. polycystum*.

Moreover, a notably high abundance of *L. plantarum* was observed in shrimp from Group PRO and SYN, indicating effective colonization of administrated *L. plantarum* within the shrimp gastrointestinal tract (Zheng et al., 2018). We suggested that shrimp hepatopancreas was stimulated by PRO to enhance digestive enzymes secretion (protease, lipase, amylase), thus improved food digestion and nutrient absorption in shrimp intestine (Wang et al., 2020). Similarly, the presence of *L. plantarum* was detected in shrimp from Group PRE, suggesting that the dietary *S. polyscytum* improved the proliferation of indigenous *Lactobacillus* spp. in the gastrointestinal tract of the host (Nazarudin et al., 2020). The finding supports the idea that the polysaccharides present in PRE (fructooligosaccharide, galactooligosaccharide, mannanoligosaccharide) positively impacted shrimp gastrointestinal microbiota, promoting the production of short-chain fatty acids utilized by the shrimp intestine for improved nutrient absorption (Nazarudin et al., 2021; Zhou et al., 2023). In synergistic effect of SYN, we suggested that PRO-induced shrimp protease hydrolysed PRE proteins to release additional peptides and free essential amino acids, that could be readily absorbed by the shrimp intestine (Xie et al., 2012; Lee and Lee, 2018; Raveschot et al., 2018).

4.1.2. Innate immune responses and histological changes in the feeding trial

Shrimp cephalothorax of Group PRO and SYN expressed consequential cellular innate immune responses and exhibited constant hepatopancreas structure and hemocyte histologically. We assumed that peptidoglycan of PRO's bacterial cell wall was recognized by shrimp hemocytes via LGBP (Tseng et al., 2023). LGBP activated peroxinectin and prophenoloxidase system cascade in shrimp hemocyte (Lin et al., 2015). Activated peroxinectin enhanced hemocyte degranulation and facilitated hemocyte phagocytosis (Cai et al., 2020). Activated prophenoloxidase in prophenoloxidase system cascade of hemocytes triggered the production of the melanin and toxic reactive intermediates as immune defends (Amparyup et al., 2013). We assumed the PRE's fucoidan induced also prophenoloxidase system cascade without recognized by LGBP of shrimp from Group PRE (Sivagnanavelmurugan et al., 2014). The series of cellular innate immunity were suppressed in shrimp abdomen from Group PRO, PRE and SYN. Moreover, humoral innate immune responses were suppressed by hemocyte in the shrimp cephalothorax and abdomen from Group PRO, PRE and SYN. We postulated component of PRO, PRE and SYN were not activated pro-Spätzle that crucial for toll-like signaling pathways in the shrimp (Liu et al., 2022). However, immune-activated hemocyte associated releasing of lysozyme despite its mechanism behind still unknown (Morales-Covarrubias et al., 2023). HSP70 induction could indicated oxidative stress response which related with high reactive oxygen species (ROS) level from respiratory burst upon overwhelming of shrimp hemocyte phagocytosis activities (Bautista-Covarrubias et al., 2020; Janewanthanakul et al., 2020). Therefore, absence of oxidative stress was indicated by suppression of HSP70 in shrimp cephalothorax and abdomen from Group PRP, PRE and SYN.

Shrimp cephalothorax of Group PRO, PRE and SYN exhibited histologically normal structure of hepatic and intestinal epithelial cells. We postulated that PRO, PRE and SYN improved structural integrity, mucus components and immune molecules of shrimp hepatopancreas and intestine (Duan et al., 2019). It exhibited histologically presence and increasing hemocytes in the shrimp hepatopancreas and intestine. This could be PRO, PRE and SYN had immunostimulant properties to increase circulating hemocytes for enhancing shrimp innate immunity (Saptiani et al., 2020).

4.2. Influence of PRO, PRE and SYN for Penaeus monodon in the immersion challenge

4.2.1. Shrimp survival, Lactobacillus plantarum and Vibrio spp. positive AHPND profile of the immersion challenge

Highest survival of shrimp from Group PRO and SYN were aligned with similar study, synergistic combination of *Bacillus* sp. B2 and red seaweed, *Gracilaria* spp. supplemented diet reduced the mortality of Pacific white shrimp, *P. vannamei* post-challenge with the AHPND-causing *V. parahaemolyticus* strain (Lim et al., 2020). High abundance of *L. plantarum* were found in shrimp from Group PRO, PRE and SYN compared with control group. We suggested PRO and enhanced indigenous *Lactobacillus* spp. produces various antimicrobial compounds, including organic acids (especially lactic acid and acetic acid), hydrogen peroxide and antimicrobial peptides (Hu et al., 2019). *Sargassum* spp. have antibacterial agents (n-hexane, ethyl acetate, ethanol, alkaloids, flavonoids) inhibited *Vibrio* sp. (Ahmed et al., 2022). Since the deadly *pirAB* toxin was released by *V. parahaemolyticus* via quorum sensing when its population reaches 10^6 CFU/mL (Hong et al., 2015), the decreasing *V. parahaemolyticus* S2–4 in survival shrimp of Group PRO, PRE and SYN demonstrated the *V. parahaemolyticus* S2–4 was eliminated by the antimicrobial peptides and phenol oxidase enzymes of the stimulated humoral innate immune system (Kumar et al., 2022). A study described cell wall lipoteichoic acid (LTA) of the *L. plantarum* prevented adhesion and internalization of *Vibrio* spp. on host intestinal epithelial cells (FAO et al., 2016).

4.2.2. Innate immune responses and histological alternation in the immersion challenge

Suppression of LGBP and peroxinectin in shrimp cephalothorax and abdomen from Group PRO, PRE and SYN upon *V. parahaemolyticus* S2–4 immersion challenge. Subsequently, prophenoloxidase and toll-like receptor were highly expressed in shrimp abdomen and cephalothorax from Group PRO and SYN except Group PRE which expressed toll-like receptor only. We assumed C-type lectins (CTLs) was highly expressed instead of LGBP in shrimp. Evidence shows CTLs recognized *V. parahemolyticus* lipopolysaccharide to evoke subsequential of shrimp innate immune responses and had strong growth inhibition against *V. parahaemolyticus* (Huang et al., 2022). However, the reason of prophenoloxidase suppression in Group PRE is yet to investigated. PRO, PRE and SYN boosted expression of penaeidin as effective antimicrobial peptide against *V. parahaemolyticus* S2–4. Evidence shows prophenoloxidase cascade and toll-like pathways were highly expressed in shrimp for melanisation and antimicrobial peptide production respectively during *Vibrio* spp. AHPND infection (Boonchuen et al., 2020). The penaeidins penetrated superficial cell membrane to bind bacterial DNA, thereby eliminate *V. parahaemolyticus* (Xiao et al., 2021). However, suppression of penaeidin was determined in shrimp abdomen from Group PRO and SYN. We postulated Anti-lipopolysaccharide factors were possible expressed against *V. parahaemolyticus* S2–4 and broad spectrum of opportunistic Gram-negative bacteria to prevent

co-infection or secondary infection in shrimp intestine (Supungul et al., 2015).

The natural AHPND-affected shrimp are divided into three phases: initial phase (tissue deterioration), acute phase (tissue necrosis) and terminal phase (tissue sloughing, hemocyte infiltration) (Kumar et al., 2021). Obviously, AHPND initial stage was determined in the Group PRO and SYN; acute stage was determined in the Group PRE; and terminal stage was determind in the control group. Administration of PRO, PRE and SYB delayed the progressive infection of AHPND (Pinoargote et al., 2018). Histological vacuolation with tissue necrosis of intestinal epithelium of the shrimp samples from control group suggested the lipopolysaccharide of *V. parahaemolyticus* can damage the intestine mucosal barrier by decreasing the viability of epithelial cells, disrupting tight junctions, and disordering the stability of mucosal mucins of shrimp gastrointestinal tract (Duan et al., 2018). Interestingly, intact histologically structure of intestinal epithelium from Group PRE and SYN demonstrated that *S. polycystum* feed administration strengthens the structure integrity of the intestinal epithelium (Abdel-Rahim et al., 2021).

5. Conclusion

Our study reported that feed supplemented with synbiotic containing *L. plantarum* L20 and *S. polycystum* synergistically improved the growth performance, innate immune responses, and enhanced survival of *P. monodon* compared probiotic and prebiotic. In perspective, synbiotic-supplemented feed can be used potentially against AHPND in aquaculture to prevent income loss and generate additional income for global aquaculture practices.

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Ethical statement

All procedures in this study involving animals were approved by the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee, approval number: UPM/IACUC/AUP-R061/2022.

CRediT authorship contribution statement

Salwany Ina: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – review & editing. **Chin Yong Kit:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation. **Haifa-Haryani Wan Omar:** Data curation, Project administration. **Nazarudin Muhammad Farhan:** Investigation, Project administration, Resources. **Ahmad Mohd Ihsanuddin:** Investigation, Resources. **Azzam-Sayuti Mohamad:** Investigation, Software, Validation, Visualization, Writing – review & editing. **Mohamad Aslah:** Project administration. **Ida-Muryany Md Yasin:** Resources. **Karim Murni:** Conceptualization, Methodology, Supervision. **Salleh Annas:** Data curation, Investigation, Supervision. **Amal Mohammad Noor Azmai:** Conceptualization, Methodology, Supervision. **Norhariani Mohd Nor:** Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101903.

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