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Research article

Protective and therapeutic efficacy of mangosteen extract feed additive against *Vibrio parahaemolyticus* AHPND strain in Pacific white shrimp (*Litopenaeus vannamei*)

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Abstract

Importance of the work: Shrimp aquaculture has faced severe losses due to an epizootic bacterial disease caused by the *Vibrio parahaemolyticus* AHPND (VpAHPND) strain, characterized by pale-to-white color with atrophy of the hepatopancreas and migh mortality. This study highlights the potential of MEX1™, a phytobiotic from mangosteen pericarp extract, as a natural, prophylactic feed additive to enhance health and AHPND resistance in Pacific white shrimp.

Objectives: To assess the effect of 0.05% (weight per weight) MEX1™ supplement on growth, immune response and resistance to VpAHPND.

Materials and Methods: Juvenile shrimp were fed a MEX1™-supplemented diet and performance was evaluated through growth indices, hematological profiles, enzyme activities and immersion-based AHPND challenge trials mimicking natural infection.

Results: MEX1™ significantly improved growth performance (% weight gain, Fulton's condition factor, average daily gain, specific growth rate, % feed efficiency and feed conversion ratio) and survival rate, which increased from 58.30% in controls to 91.42% in the MEX1™-treated group. The extract enhanced protein digestive enzymes (trypsin and chymotrypsin) and hematological parameters (total hemocyte count, differential hemocyte count and superoxide dismutase). In the VpAHPND resistance test, of the three experimental groups evaluated: controls, Tre-MEX (as the VpAHPND-treated group and Pre-MEX (as the VpAHPND-protected group), the Pre-MEX group had significantly elevated cellular immune parameters and the lowest pathogen loads in the hemolymph, hepatopancreas and intestine compared to both the Tre-MEX and control groups.

Main finding: VpAHPND primarily targeted and damaged hyalinocytes, while MEX1™ stimulated hematopoiesis, particularly granulocyte production, in response to infection. The mangosteen extract functioned as a growth promoter and an immunostimulant, boosting shrimp resilience against VpAHPND. These results supported the application of MEX1™ as a natural, effective feed additive for disease prevention and performance enhancement in shrimp aquaculture.

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Introduction

Shrimp is an important economic aquatic species, as it is highly popular for consumption both domestically and internationally. In Thailand, Fisheries Statistics Group (2024) reported that most shrimp production comes from intensive farming—from 2018 to 2023, the cultured shrimp production exceeded 390,000 t/yr, consisting of 90% Pacific white shrimp (*Litopenaeus vannamei*) and 9% black tiger shrimp (*Penaeus monodon*). However, the main loss during shrimp production is from epizootic diseases, especially during temperature fluctuations, flooding and a lower salinity level in the rainy season.

Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome, is one of the major bacterial disease responsible for mass mortality in shrimp farms affecting economically important shrimp species cultured in brackish water, including white shrimp (*L. vannamei*) and black tiger shrimp (*P. monodon*) (OIE, 2019). The early stages of shrimp are more susceptible to infection, mainly targeting the hepatopancreas with clinical signs of pale-to-white coloration and severe atrophy in the acute stage. AHPND is mainly caused by *Vibrio* species harboring the virulent pVA1 plasmid (63–70 kb), which encodes the $PirA^{VP}$/$PirB^{VP}$ binary toxins, homologues of *Photorhabdus luminescens* toxins (Thitamadee et al., 2016; Kumar et al., 2021). Key species include *V. parahaemolyticus*, *V. punensis*, *V. harveyi*, *V. owensii* and *V. campbelli*. In addition, the pVA1 plasmid contains genes for conjugative transfer, allowing potential spread to other bacteria (OIE, 2019). The $PirA^{VP}$/$PirB^{VP}$ toxin genes have also been detected in *Shewanella* spp. (Kumar et al., 2021).

Clinical signs associated with AHPND include links to white feces syndrome due to aggregated transformed microvilli (Thitamadee et al., 2016; Keetanon et al., 2021) and greater susceptibility has been observed in shrimp co-infected with *Enterocytozoon hepatopenaei* (EHP), which causes hepatopancreatic cell sloughing (Aranguren et al., 2017). VpAHPND isolates vary in colony morphology, genetic profiles and antibiotic resistance (Han et al., 2015; Hossain et al., 2020; Lam et al., 2023; Zhang et al., 2024; Thi et al., 2025).

Transmission of VpAHPND to shrimp broodstock occurs through contaminated live feeds such as clams, oysters, polychaetes and squid (Thitamadee et al., 2016) and co-infections with viral pathogens have been documented (Chowdhury et al., 2024). Polymerase chain reaction (PCR) detection targeting $PirA^{VP}$/$PirB^{VP}$ genes is the recommended diagnostic method (Thitamadee et al., 2016; OIE, 2019).

Several strategies inhibiting VpAHPND have been investigated, including using natural substances such as a mix of supplements (formic acid, β-carotene and vitamin E) (Yowaphui et al., 2016), blended essential oils (Jha et al., 2016), single essential oil (Lam et al., 2023), biomolecules such as IgY-specific antibody from hen as feed supplement (Keetanon et al., 2021) and probiotic-fermented feed (Cherdkeattipol et al., 2021). Phytobiotics and products have been reported to be effective at inhibiting *Vibrio* spp. such as seaweed (*Chetomorpha antennina*) extract (Thanigaivel et al., 2014) and spent hops and yeast cell wall and grape pomace (Chuchird et al., 2017). Probiotics, prebiotics, biofloc and phage therapy have been investigated to control VpAHPND in shrimp (Kumar et al., 2021, Hassan et al., 2025). In addition, metallic nanoparticles were considered to prevent AHPND in shrimp aquaculture (Hassan et al., 2025).

Medicinal plants have been considered for the prevention of AHPND due to their effective bioactive compounds and safe application. Mangosteen (*Garcinia mangostana*), one of commercial fruits of Thailand, is known for its medicinal properties, especially in the pericarp, which is rich in xanthones (Pedraza-Chaverri et al., 2008). Xanthones, a group of oxygen-containing heterocyclic compounds, are secondary metabolites and important bioactive compounds, including a-, b- and g-mangostins, garcinone E, 8-deoxygartanin and gartanin (Yuvanatemiya et al., 2022).

The bioactivity of α-mangostin, the major natural xanthonoid, and of other xanthones in the crude extract of mangosteen pericarp has been reported to have pharmacological benefits, including antioxidant, anti-inflammation, anti-apoptosis, antiproliferation, wound healing, cytotoxic and anti-cancer, anti-bacterial, antifungal, antiviral and antimalarial properties (Sultan et al., 2022). Pongsawat et al. (2017) reported extramedullary hematopoiesis in the rat spleen. Rats injected intraperitoneally with 100 mg/5 mL/kg of a-mangostin pericarp extract, 5 d/wk for 1 mth, showed increased platelet formation, myeloid progenitor cells and megakaryocytes in the spleen. Immunohistochemical analysis of the spleen confirmed that the newly formed blood cells originated from myeloid and erythroid precursor cells. Notably, Sultan et al. (2022) conducted a meta-analysis encompassing 30 studies on the antibacterial efficacy of α-mangostin; they reported no significant difference in effectiveness compared to conventional antibiotics.

Gartanin, a natural xanthone compound, is the second most abundant after α-mangostin in mangosteen that has biological benefits, including antioxidant, anti-inflammatory, antibacterial activity, antifungal effect, antiviral influenza, anti-cancer, neuroprotective and anti-neoplastic properties (Pedraza-Chaverri et al., 2008; Yuvanatemiya et al., 2022). Gartanin induced

an autophagic response in various cancer cells (Kim et al., 2015) and suppressed migration in human glioma cells (Gao et al., 2016).

There have been studies on mangosteen extract and its xanthone compounds regarding cell regeneration and hematopoiesis. Arundina et al. (2018) reported that mangosteen methanol extract increased the proliferation and differentiation of mesenchymal stem cells (MSCs) into osteoblasts. Nurani et al. (2021) reviewed MSC cultures and reported that α-mangostin enhanced the expression of transforming growth factor-β1, reduced the expression of platelet-derived growth factor-β, increased the expression of fibroblast growth factor-2 and elevated the expression of vascular endothelial growth factor-A. MSCs have the ability to self-renew and demonstrate considerable angiogenic potential. Angiogenesis is a key process in tissue healing, responsible for supplying nutrients to the repaired tissue (Pettet et al., 1996).

Acute toxicity of mangosteen extract was demonstrated by Fatimawali et al. (2013) in brine shrimp (*Artemia salina* Leach) with 24 hr, with a half maximal inhibitory concentration (LC_{50}) value of 418 parts per million. Similarly, Kitipaspallop et al. (2021) reported that purified α-mangostin, at concentrations up to 15 mmol/L had a toxic effect in developing zebrafish (*Danio rerio*) embryos. Observed abnormalities included truncated bodies, bent tails, blood clots and pericardial and yolk edema, as well as malformations in body shape and tail morphology. furthermore, high concentration of α-mangostin affected the transcriptional expression levels of genes involved in oxidative stress, inflammation, apoptosis and hematopoiesis.

Studies in aquaculture research have reported bioactivity of mangosteen pericarp extract in inhibiting pathogens, including protozoa (*Zoothamnium* sp.; Daengroj and Sanothong, 2017), saprolegniasis fungi (*Achlya bisexualis*, *Aphanomyces invadans* and *Saprolegnia diclina*) in both the mycelial and zoospore stages (Jaichuen et al, 2017), *Flavobacterium columnare* from channel catfish (*Ictalurus punctatus*; Meepagala and Schrader, 2018), *Aeromonas veronii* in Nile tilapia (*Oreochromis niloticus*; Yostawonkul et al., 2023), *Aeromonas hydrophila* in seabass fingerlings (*Lates calcarifer*; Thiankham et al., 2024), *Lactococcus garvieae* in giant freshwater prawn (*Macrobrachium rosenbergii*; Kitikiew et al., 2023; Kuo et al., 2023) and *Vibrio parahaemolyticus* in black tiger shrimp (*Penaeus monodon*; Salamah et al., 2024).

Although published studies on the effects of mangosteen on shrimp are still limited, recent research has demonstrated promising outcomes. For example, Kuo et al. (2023) evaluated various mangosteen pericarp powder, boiled powder and extract (MPE) products in the diets of giant freshwater prawns (*M. rosenbergii*), reporting significant improvements in growth performance indicators [weight gain, specific growth rate (SGR), feed efficiency (FE), feed conversion ratio (FCR) and survival rate] and enhanced immune responses, such as total and differential hemocyte counts, phenoloxidase activity, respiratory burst, phagocytic activity and pathogen clearance, against *Lactococcus garvieae*. Similarly, Kitikiew et al. (2023) found that injecting 20–40 μg of hot-water mangosteen pericarp extracts boosted hemocyte levels and modulated neurohormonal responses under hypothermal stress, including increases in dopamine, norepinephrine, tyramine and octopamine. These changes were accompanied by enhanced carbohydrate metabolism, as evidenced by elevated glucose and lactate levels in the hemolymph.

Complementing these findings, Salamah et al. (2024) investigated the use of MPE as a dietary additive in black tiger shrimp (*P. monodon*) over 33 d, followed by a challenge with VpAHPND. Their results showed that shrimp receiving 10–14% MPE had significantly better growth, survival rates (up to 98%) and resistance to infection compared to the controls. Higher MPE concentrations led to minimal infection based on clinical signs, rapid recovery and healthier internal organs post-challenge. These studies collectively highlighted the potential of mangosteen-derived products as effective natural alternatives to synthetic antibiotics, enhancing both growth performance and disease resistance in shrimp aquaculture.

Given the limited number of *in vivo* studies and the lack of detailed investigation into hemocyte responses to mangosteen extract, the present study aimed to evaluate the bioactivity of a mangosteen extract product (MEX1™) as a feed additive, with a focus on its effects on growth performance, hematological parameters and its potential benefits to mitigating VpAHPND infection in juvenile Pacific white shrimp (*L. vannamei*). The infection challenge was performed via immersion using a sublethal concentration (1/10 of the 96 hr LC_{50} dose), representing a lower pathogen concentration than that used in acute exposure models. This method was designed to facilitate the evaluation of host immune responses through cellular indicators. This approach closely simulates natural infection conditions in shrimp and could offer a practical and reliable model for developing effective disease prevention and treatment strategies applicable to pond culture systems.

Materials and Methods

Preparation of mangosteen extract

The phytochemical MEX1™(mainly containing mangosteen pericarp extract) was prepared by Biotechnology Industry

(Thailand) Co., Ltd. The bioactive compounds were analyzed using high performance liquid chromatography, which indicated that MEX1™contains two major xanthones: α-mangostin at a concentration of 141.9 mg/g and gartanin at 65.2 mg/g (Jaichuen et al., 2017).

Preparation of experimental feed pellets

The commercially available nursery feed (INTEQC 113S), containing 40% crude protein, was selected as the basal feed. The feed is manufactured by INTEQC Feed Co., Ltd., Thailand. To incorporate the extract into the feed, the extract solution was uniformly sprayed onto the surface of the pellets, followed by air-drying at room temperature. Then, the treated pellets were weighed according to the experimental design, sealed in airtight plastic bags and stored at 4 °C until feeding.

A preliminary experiment was conducted to evaluate the effects of MEX1™ as a feed additive at concentrations of 0.0%, 0.25%, 0.5% and 1.0% (all weight per weight, w/w) on the growth of juvenile Pacific white shrimp. The 0.5% (w/w) supplementation resulted in the highest growth performance (data not shown). Based on this result, the control feed was the standard feed, whereas the treatment feed comprised the same standard feed coated with 0.5% (w/w) MEX1™ and was called MEX-feed.

Preparation of bacterial pathogen

The *Vibrio parahaemolyticus* AHPND strain (VpAHPND) was a single isolate from the Songkhla Aquatic Animal Health Research and Development Center, Department of Fisheries, Songkhla, Thailand. The bacteria were cultured in thiosulphate citrate bile-salt sucrose (TCBS) agar for single colony isolation; then, they were cultured in tryptic soy broth + 2 % NaCl and incubated at 35°C for 24 hr. The VpAHPND bacterial cells were collected using centrifugation at 5,000 revolutions per minute for 5 min, washed twice with normal saline solution (NSS, 0.85% NaCl) and then diluted in NSS. The bacterial concentration was evaluated using the correlation between absorbances at an optical density of 600 nm (OD_{600}) and the number of bacterial colonies was recorded based on the plate count method.

Preparation of experimental shrimp

The juvenile shrimp (each 2–4 g) were purchased from a hatchery farm (Khaopong Farm, Ban Pho; Chachoengsao, Thailand). Nine diseases were detected using quantitative PCR (qPCR) for white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis (IHHNV), necrotizing hepatopancreatitis bacterium (NHPB), shrimp hemocyte iridescent virus (SHIV), *Enterocytozoon hepatopenaei* (EHP) and VpAHPND and using reverse transcriptase-qPCR for infectious myonecrosis virus (IMNV), yellow head virus (YHV) and Taura syndrome virus (TSV), according to Songkhla Aquatic Animal Health Research and Development Center (2018).

The juvenile shrimp were cultured in 500 L fiberglass tanks, each equipped with a heater to maintain the water temperature at 29 ± 1°C, with salinity at 20–25 parts per trillion and acclimatization for 7 d. Water quality parameters (pH, dissolved oxygen (DO), ammonia, nitrite and nitrate) were measured daily using commercial test kits (V UNIQUE; Thailand) throughout the experimental period.

Growth performance experiment

The shrimp were cultured in 500 L fiberglass tanks, for two experimental treatments: 1) the control group, fed commercial pellet feed; and 2) the MEX group, fed MEX1-feed. Each tank contained 200 shrimp, with three replicate tanks per treatment. Each week, 10 shrimp were sampled randomly in triplicate from each tank to determine the average body weight. The shrimp were fed at 5% of body weight, divided into five feeding times daily for 4 wk. Shrimp mortality was recorded daily and at the end of the experiment, with the data used to calculate survival rates each week. Each week, 20 shrimp were sampled randomly from each tank and measured individually to assess growth parameters, consisting of initial weight, final weight, final weight, % weight gain (WG), Fulton's condition factor (K), average daily gain (ADG), specific growth rate (SGR), % feed efficiency (FE) and feed conversion ratio (FCR). Cellular and humoral innate parameters and protein digestive enzymes, trypsin and chymotryptin, were monitored.

Determination of cellular and humoral innate parameters

Hemolymph was drawn from the ventral sinus at the base of the first abdominal segment using a sterile pre-chilled syringe preloaded with anticoagulant (0.114 M trisodium citrate, 450 mM NaCl, 10 mM KCl and 10 mM HEPES at pH 7.4), according to Nonwachai et al. (2010). The total hemocyte count (THC) and differential hemocyte count (DHC), were determined according to Sritunyalucksana et al. (2005). The production of the superoxide anion (O_2^-) was measured by the reduction of nitroblue tetrazolium (NBT), based on the ability of the superoxide dismutase (SOD), superoxide anion enzyme, to convert NBT into formazan at OD_{620}, according to Munoz et al. (2000).

Specific activity assay of protein digestive enzymes

Trypsin and chymotrypsin activities were determined according to Thongprajukaew *et al*. (2013) using BAPNA (*N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide HCl) and SAPNA (*N*-succinyl-*L*-ala-*L*-ala-*L*-pro-*L*-phe-*p*-nitroanilide) as specific substrates, respectively. The value of trypsin-to-chymotrypsin ratio (T/C ratio) was evaluated.

Bacterial challenge test

An immersion challenge was conducted to simulate field-relevant exposure conditions. A 96 hr LC_{50} immersion assay was performed using juvenile shrimp that were exposed to VpAHPND at concentrations in the range 1×10^4–1×10^8 colony-forming units (CFU)/mL, prepared based on 10-fold serial dilutions. Groups of shrimp (n = 20 per replicate, in triplicate) were immersed in each concentration for 6 hr, then transferred to clean, aerated seawater for post-exposure monitoring. Mortality was recorded at 24 hr intervals during 96 hr and the cumulative percentage mortality at each exposure interval was plotted against the logarithm of test concentrations to determine the LC_{50} value (OECD, 2019). The 96 hr LC_{50}value of the VpAHPND strain in the present study was 1.58×10^6 CFU/ml. Subsequently, a sublethal concentration of VpAHPND, equivalent to one-tenth of this LC_{50} value, was prepared and used in further challenge experiments.

For the bacterial challenge test, shrimp from the previous growth performance experiment that had been reared for 4 wk, were subjected to challenge with VpAHPND. There were three experimental treatments: 1) the control group; 2) the Tre-MEX group (MEX1™ feeding post challenge); and 3) the Pre-MEX group (MEX1™ feeding before and post challenge). The juvenile shrimp, continuing from the growth experiment, were allocated randomly to glass aquariums, with 30 shrimps/tank and five replicate tanks per treatment. During acclimation, the control and Tre-MEX groups were fed with commercial feed while the Pre-MEX group was fed with MEX-feed. Shrimp mortality in each tank was observed for 3 d before challenging. Then, shrimp were challenged by immersion in VpAHPND concentration of 1.58×10^5 CFU/ml in water for 6 hr.

Following the challenge, shrimp were returned to their respective glass aquaria. The control group was fed with commercial feed. The shrimp in the Tre-MEX and Pre-MEX groups were fed with MEX-feed. One replicate from each group was observed for the survival rate. For the other four replicates, sampling was conducted post-infection with 5 shrimp/tank at 0 hr, 24 hr, 48 hr, 72 hr and 96 hr post-infection (hpi). Hemolymph was collected and cellular parameters of THC and DHC were determined. The VpAHPND amounts in the hemolymph, hepatopancreas and intestine were examined based on a total plate count using the spread plate method (Buck and Cleverdon, 1960).

Bacteria concentration in shrimp organs

Organs were collected from sampled shrimp (hemolymph, hepatopancreas and intestine). The hepatopancreas and intestine samples were weighed to 100 µg and added with 900 ml of 0.85% NaCl and then homogenized thoroughly. Each tissue homogenate was subjected to serial 10-fold dilutions. For the hemolymph sample, 50 µl was added with 450 ml of 0.85% NaCl. Then, 100 µl of each sample was used to determine the total *Vibrio* load using the spread plate method with TCBS agar, with counting within the range 30–300 CFU/plate, with triplicates per sample.

To confirm the absence of VpAHPND before the challenge test, juvenile shrimp were sampled randomly and checked using qPCR to ensure a negative result. Samples of the hemolymph, hepatopancreas and intestinal tissues were collected and analyzed for concentration of total *Vibrio* spp. using the spread plate method. The result showed there almost all white colonies were at <10 CFU/mg tissue, indicating that the experimental shrimp were free from VpAHPND infection at the beginning of the trial. Therefore, any subsequent detection of *Vibrio* spp. in the challenged groups was attributed to the experimental VpAHPND inoculation.

Statistical analysis

Numerical data of the growth parameters, hemocyte count and SOD were analyzed using Student's t test, while data from the bacterial challenge test and the hemocyte ratio were analyzed using one-way ANOVA, followed by Duncan's new multiple range test for pairwise comparisons of treatment means. The data were presented as mean ± SD values and statistical significance was determined at $p < 0.05$. All statistical analyses were performed using the SPSS software (IBM SPSS Statistics for Windows (Version 28; IBM Corp.; Armonk, NY, USA).

Ethics statements

All experimental procedures involving Pacific white shrimp (*Litopenaeus vannamei*) were performed in accordance with the Ethical Principles and Guidelines for the Use of Animals (2015) issued by the National Research Council of Thailand.

The shrimp were handled gently to minimize stress and their health and behavior were monitored daily throughout the experiment. This study was approved by the Ethics Committee of Maejo University (MACUC 054F/2565).

Results

Effectiveness of mangosteen extract on growth performance

The shrimp were negative for the nine pathogens (WSSV, IHHNV, IMNV, NHPB, SHIV, YHV, TSV, EHP and VpAHPND). At the end of shrimp nursing for 4 wk, the values of growth performance of the MEX group were significantly better than for the control group (Table 1). The values of final weight, ADG and SGR of the MEX group were higher than those of the control group at $p < 0.05$, whereas the values of WG, K, FE and FCR were higher at $p < 0.01$. Notably, the FE and FCR values of the MEX group were 3.33 times greater than those of the control group, while the survival rate of the MEX group was 33.12% higher than that of the control group. The WG, survival rates, ADG and SGR were evaluated every week (Fig. 1),

Table 1 Comparison of growth performance and growth involving digestive enzymes of juvenile Pacific white shrimp fed with control feed and MEX-feed at end of 4 wk experiment

Parameter	Experimental group	
	Control	MEX
Growth		
Initial body weight (g)	2.63±0.16^{a}	2.62±0.06^{a}
Final body weight (g)	6.15±0.74^{a}	8.47±0.77^{b}
Weight gain (%)	133.22±14.44^{A}	222.98±22.26^{B}
Condition factor (K)	0.46±0.10^{A}	0.66±0.06^{B}
ADG (g/day)	0.13±0.02^{a}	0.21±0.03^{b}
SGR (%/day)	3.02±0.22^{a}	4.18±0.25^{b}
FE (%)	24.41±1.29^{A}	81.42±1.21^{B}
FCR	4.10±0.22^{B}	1.23±0.12^{A}
Survival rate (%)	58.30±6.16^{A}	91.42±4.31^{B}
Protein digestive enzymes		
Trypsin (T)*	2.74±0.57^{a}	3.62±0.31^{b}
Chymotrypsin (C)*	14.60±1.55^{a}	17.75±0.98^{b}
T/C ratio	0.19±0.06	0.20±0.04

Values are presented as mean ± SD. Means in same row with different lowercase superscripts are significantly different at $p < 0.05$ and with different capital superscripts are significantly different at $p < 0.01$. Growth parameters are values per shrimp, with * values presented as micro moles of p-nitroaniline per hour per milligram of protein.

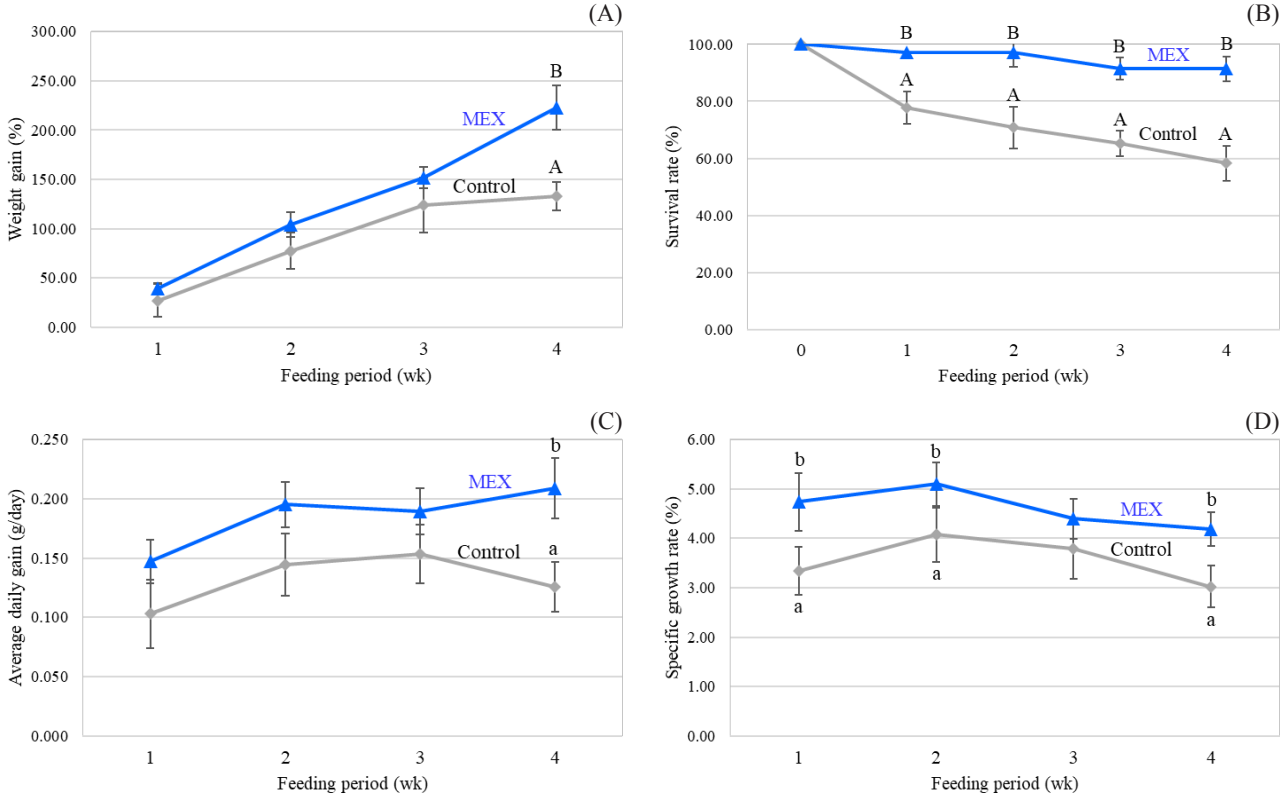

Fig. 1 Weekly growth parameters of juvenile Pacific white shrimp fed with control feed and MEX feed: (A) weight gain; (B), survival rate; (C) average daily gain; (D) specific growth rate, where values are presented as mean ± SD and different lowercase and capital letters indicate significant differences between groups in each period at $p < 0.05$ and $p < 0.01$, respectively

with the survival rate of the control group gradually decreasing and was significantly lower than that of the MEX group during weeks 1–4. Although WG and ADG of the MEX group were significantly higher than those of the control group only at week 4, these values in the MEX group tended to be higher than those in the control group. The SGR values of both groups were only slightly different ($p < 0.05$).

The specific activity levels of the protein digestive enzymes trypsin and chymotrypsin were investigated due to their important role in growth performance. The enzyme activity levels of the MEX group were significantly higher than those of the control group; however, there was no significant difference between groups for the T/C ratio.

At the end of the growth experiment, most of the cellular parameters in the MEX group were significantly different from those in the control group (Table 2). The THC value of the MEX group was significantly higher than the value for the control group. For differential hemocytes, based on the analysis of the MEX and control groups, the granulocyte ratio was higher ($p < 0.05$), the semi-granulocyte ratio was lower ($p < 0.01$) and there was no significant difference in the hyalinocyte ratio.

Table 2 Hematological parameters of juvenile Pacific white shrimp fed with control feed and MEX-feed at end of 4 wk experiment

Parameter	Experimental group	
	Control	MEX
THC (10^7 cells/ml)	1.49±0.11^{A}	2.26±0.41^{B}
Granulocyte		
- ratio (%)	23.34±0.47^{a}	26.33±9.00^{b}
- concentration ($\times10^6$ cells/ml)	3.48±0.31^{A}	6.21±2.80^{B}
Semi-granulocyte		
- ratio (%)	6.84±1.18^{B}	2.50±0.17^{A}
- concentration ($\times10^6$ cells/ml)	1.01±0.05^{B}	0.56±0.04^{A}
Hyalinocyte		
- ratio (%)	69.83±0.71^{a}	71.17±8.83^{a}
- concentration ($\times10^6$ cells/ml)	10.40±0.85^{A}	15.81±0.08^{B}
SOD (unit/ml)	11.92±0.98^{a}	17.35±1.91^{b}

Values are presented as mean ± SD. Means in same row with different lowercase superscripts are significantly different at $p < 0.05$ and with different capital superscripts are significantly different at $p < 0.01$.

When the THC was calculated as the DHC (%) to hemocyte concentration (cells/ml), the granulocyte concentration was higher ($p < 0.01$) in the MEX group than in the control group, while the semi-granulocyte concentration was lower ($p < 0.01$). Notably, the hyalinocyte concentration in the MEX group was significantly higher ($p < 0.01$), due to the high ratio for the control group (69.83 ± 0.71%) and for the MEX group (71.17 ± 8.83%). The calculation of hemocyte concentration is based on the number of hemocyte cells per 1 ml of hemolymph. Therefore, the calculated concentrations of each hemocyte type are associated with the significant THC values.

Effectiveness of mangosteen extract against VpAHPND infection

The shrimp from the growth experiment were used in the challenge experiment.

Based on observations, the control group had a reduction in feed intake of approximately 25%, whereas the Tre-MEX and Pre-MEX groups maintained normal feeding behavior. No mortality was observed in any group up to 72 hpi. In the control group, the shrimp showed signs of poor health at 72 hpi and some mortality was observed at 96 hpi, resulting in a survival rate of 93.33 ± 9.43%. In contrast, both the Tre-MEX and Pre-MEX groups had 100% survival rates.

Post VpAHPND challenge, the THC of the control and Tre-MEX groups clearly decreased while the value of the Pre-MEX group only slightly decreased (Fig. 2). The THC in the control group continuously decreased over 96 hpi, while THC in the Tre-MEX group decreased until 48 hpi, then notably increased slightly at 72 hpi and 96 hpi. In contrast, the THC of the Pre-MEX group, which was initially about two-folds higher than that of the control and Tre-MEX groups, slightly decreased until 72 hpi and then increased at 96 hpi. At the end of the VpAHPND challenge experiment (96 hpi), there was a significant difference among the three groups,

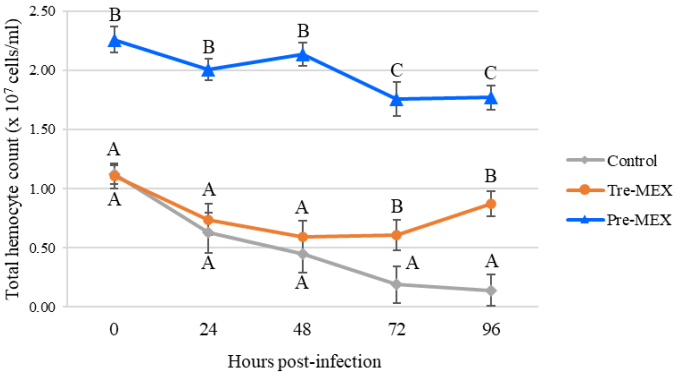

Fig. 2 Total hemocyte counts of juvenile shrimp post VpAHPND challenge in different treatment groups for standard feed (control), MEX-feed administered only post challenge (Tre-MEX) and MEX-feed administered both before and post challenge (Pre-MEX) measured at 96 hr post infection, where values are mean ± SD and 0 hr post infection represents the before-challenge event and different capital letters indicate significant differences between groups in same period at $p < 0.01$

with the control group having the lowest THC ($p < 0.01$). The ratiogram of the DHC post VpAHPND challenge had patterns across the groups (Fig. 3). In both the control and Tre-MEX groups, the hyalinocyte ratio decreased sharply from 24 hpi to 48 hpi. Concurrently, the granulocyte ratio of both groups increased due to the reduction in hyalinocytes. During 72–96 hpi, the hyalinocyte ratio began to rise again, with the hyalinocyte and semi-granulocyte ratios in the Tre-MEX group being significantly higher than in the control group at 96 hpi. In contrast, for the Pre-MEX group, the hyalinocyte ratio decreased slightly from 24 hpi to 96 hpi, while both the granulocyte and semi-granulocyte ratios increased

Based on the THC-to-DHC ratio of cell concentrations, the amounts of all hemocyte types in the Pre-MEX group were significantly higher than those in the control and Tre-MEX groups throughout the 0–96 hpi period (Fig. 4).

For the Pre-MEX group, the hyalinocyte concentration decreased slightly at 24 hpi, 48 hpi, 72 hpi and 96 hpi (74.85%, 65.76%, 45.45% and 33.04%, respectively), while the granulocyte concentration increased at 48 hpi, 72 hpi and 96 hpi (125.65%, 145.02% and 162.21%, respectively) and the semi-granulocyte increased greatly at 24 hpi, 48 hpi, 72 hpi and 96 hpi (372.89%, 586.14%, 296.15% and 485.93%, respectively).

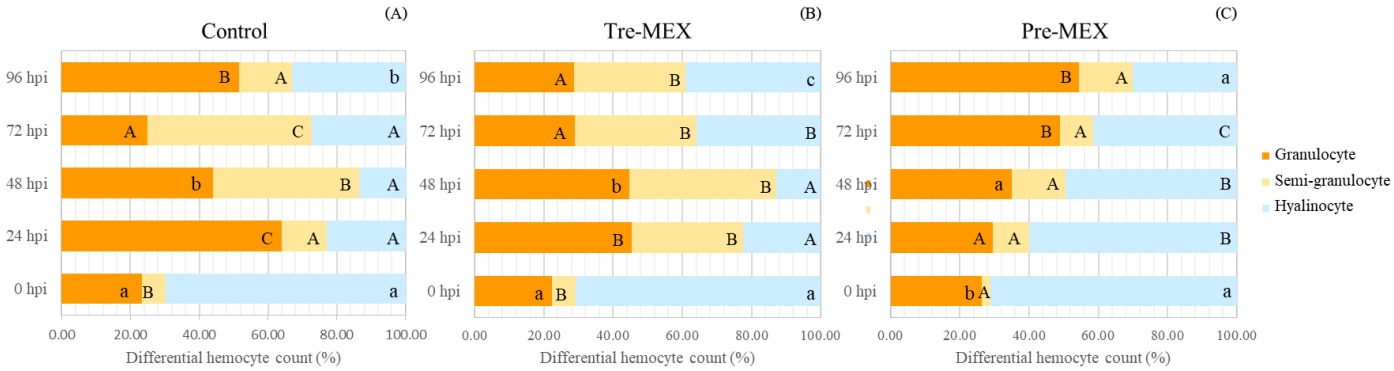

Fig. 3 Ratio of differential hemocyte count of juvenile shrimp post VpAHPND challenge in different treatment groups: (A) standard feed (control); (B) MEX-feed administered only post challenge (Tre-MEX); (C) MEX-feed administered both before and post challenge (Pre-MEX) measured at 96 hr post infection, where values are mean ± SD, 0 hr post infection represents before-challenge event and different lowercase and capital letters at the end of bars in columns indicate significant differences at $p < 0.01$and $p < 0.05$, respectively

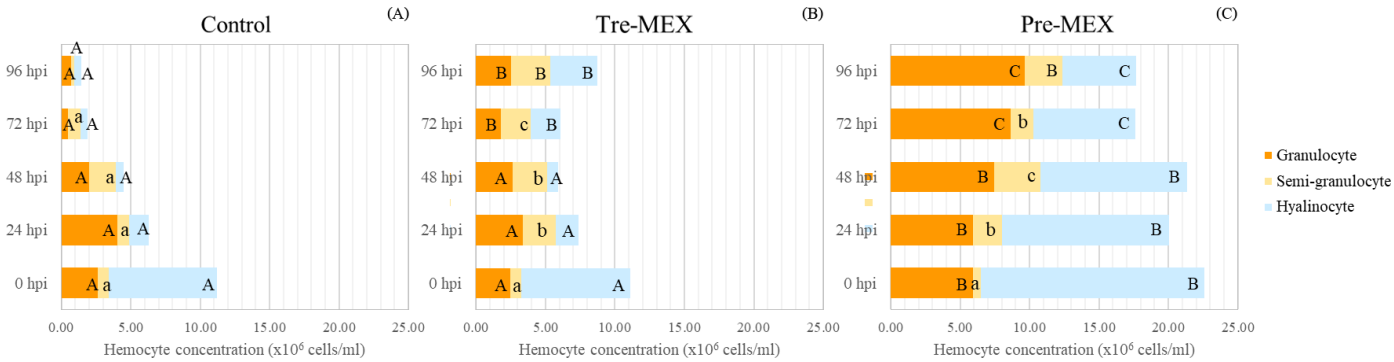

Fig. 4 Concentration of each different hemocyte type of juvenile shrimp post VpAHPND challenge in different treatment groups: (A) standard feed (control); (B) MEX-feed administered only post challenge (Tre-MEX); (C) MEX-feed administered both before and post challenge (Pre-MEX), measured at 96 hr post infection. Values are mean ± SD.; The 0 hr post-infection time point represents the pre-challenge condition. Different letters at the end of each bar indicate significant differences in each hemocyte type among treatments at the same time point (lowercase letters: $p < 0.05$; uppercase letters: $p < 0.01$).

All hemocyte types in the control group declined continuously from 24 hpi to 96 hpi and shrimp mortality began to occur without any apparent clinical signs. In contrast, the hemocyte concentration in the Tre-MEX group initially decreased at 24 hpi and 48 hpi, followed by slight increases at 72 hpi and 96 hpi.

Notably, the concentrations of all hemocyte types in both the Tre-MEX and Pre-MEX groups were significantly higher ($p < 0.01$) compared to the control group. In contrast, each hemocyte concentration in the control group gradually declined throughout the 96 hpi of observation.

The patterns for the ratio and concentration of granulocyte and semi-granulocyte in the Pre-MEX group had higher values than for the Tre-MEX group.

The VpAHPND concentrations in the hemolymph, hepatopancreas and intestine of each shrimp in the samples were measured in triplicate using the spread plate method (Fig. 5). Based on these results, there was a clear correlation for bacterial numbers in each organ of each group. In the control group (not fed MEX1™ before or post the challenge), the VpAHPND concentrations in the three organs increased steadily from 24 hpi to 96 hpi, with bacterial concentrations in the hepatopancreas being approximately 2-folds higher than in the hemolymph and intestine.

In the Tre-MEX group, the VpAHPND concentrations in the three organs followed a similar pattern to the control group, but were significantly lower from 48 hpi to 72 hpi. However, at 96 hpi, the bacterial concentration in the hepatopancreas was significantly lower than that of control group ($p < 0.01$).

Notably, in the Pre-MEX group, the VpAHPND concentrations in the three organs increased slightly but remained the lowest values compared to the control and Tre-MEX groups. The bacterial concentrations of the Pre-MEX group across each organ were within similar levels and were not as high as those in the other experimental groups.

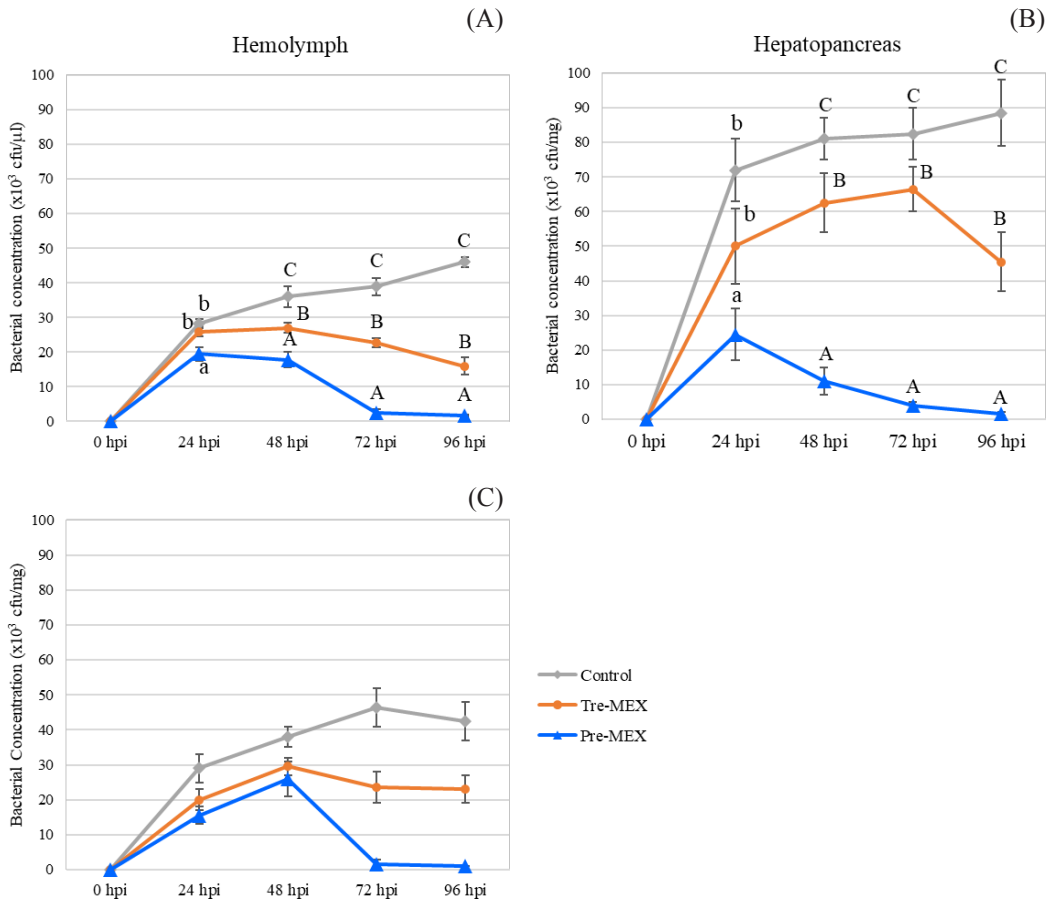

Fig. 5 Total *Vibrio* spp. concentration post challenge of juvenile shrimp in different treatment groups of standard feed (control), MEX-feed administered only post challenge (Tre-MEX) and MEX-feed administered both before and post challenge (Pre-MEX) measured at 96 hr post infection: (A) hemolymph; (B) hepatopancreas; (C) intestine. Values are mean ± SD.; The 0 hr post-infection time point represents the pre-challenge condition. Different letters at each time point indicate significant differences among treatments (lowercase letters: $p < 0.05$; uppercase letters: $p < 0.01$).

The VpAHPND concentrations in the hepatopancreas were much lower than those in the control and Tre-MEX groups from 24 hpi to 96 hpi ($p < 0.01$). The highest concentrations in all three organs were at 24 hpi (hemolymph, 19.50×10^3 cells/mL; hepatopancreas, 24.50×10^3 cells/mg; and intestine, 26.00×10^3 cells/mg). The VpAHPND concentrations in the three organs of the Pre-MEX group at 24 and 48 hpi were significantly lower than in the control and Tre-MEX groups. However, at 72 hpi and 96 hpi, the VpAHPND concentrations reduced further to the significantly lowest values of 1.75×10^3 cells/mL in the hemolymph, 1.50×10^3 cells/mg in the hepatopancreas and 1.00×10^3 cells/mg in the intestine ($p < 0.01$).

Discussion

Mangosteen extract promotion of growth and hematological profiles

The assessment at 4 wk of the MEX1™ feed additive demonstrated that mangosteen extract significantly enhanced growth performance as well as both the cellular and humoral components of the innate immune parameters in juvenile shrimp.

Compared to the control group, all growth-related parameters significantly improved in the MEX group. Shrimp receiving the MEX1™-supplemented diet had enhanced growth performance, with increases in final body weight, average daily gain and specific growth rate by 1.38-, 1.66- and 1.38-folds, respectively ($p < 0.05$). Additionally, significant improvements were observed in weight gain, condition factor, feed conversion ratio and survival rate (increases of 1.67-, 1.42-, 3.34- and 1.57-folds, respectively, ($p < 0.01$). Furthermore, the activity levels were significantly elevated for the protein digestive enzymes in the intestines of the shrimp in the MEX group for trypsin and chymotrypsin. Based on these findings, mangosteen extract, as incorporated in MEX1™, could be an effective dietary supplement to promote growth and health performance in juvenile shrimp.

In terms of hematological parameters, the shrimp fed the MEX-supplemented diet had enhanced hematopoietic and immune responses, as evidenced by significant increases in the THC, granulocyte concentration, the semi-granulocyte ratio and concentration and the hyalinocyte concentration ($p < 0.01$). Furthermore, the ratios of granulocytes and hyalinocytes, as well as the SOD activity, were significantly altered ($p < 0.05$).

These results were consistent with other studies that reported the positive effects of MPE on the growth performance and survival rate of giant freshwater prawns (*M. rosenbergii*; Kuo et al., 2023) and of black tiger shrimp (*P. monodon*; Salamah et al., 2024).

The protein digestive enzyme, trypsin—an endoprotease—plays an important role in growth and various physiological pathways in aquatic animals, including the breakdown of peptides into amino acid building blocks via a hydrolysis reaction. Chymotrypsin contributes to protein digestion by cleaving internal peptide bonds within peptides and proteins, particularly at sites where the carboxyl-donating amino acid residue possessed a hydrophobic side chain. The trypsin-to-chymotrypsin (T/C ratio) has been associated with growth performance. Both trypsin specific activity and the T/C ratio are considered important indicators of digestion efficiency and growth potential (Rungruangsak-Torrissen et al., 2006).

In the blue swimming crab (*Portunus pelagicus*), the T/C ratio was reported to be positively correlated with growth indicators such as body weight and carapace width, where the highest T/C ratio was observed during the pre-molt stage of the molting cycle, corresponding to increased feeding activity (Chamchuen et al., 2014).

In the present study, the activities of both trypsin and chymotrypsin were upregulated, resulting in no significant change in the T/C ratio. However, overall growth performance parameters (WG, K, FE, FCR and survival rate) were significantly improved ($p < 0.01$). These findings suggested that the growth-promoting effects may not be solely attributed to enhanced digestive enzyme activities, but could also involve other physiological systems, such as the circulatory (hemolymph) system.

Hemocytes in shrimp play a vital role in oxygen transport and innate immunity via both cellular and humoral mechanisms (Söderhäll and Söderhäll, 2022). Among these, granulocytes and semi-granulocytes are primarily involved in phagocytosis, nodulation and encapsulation of foreign materials and non-self entities (protozoa, bacteria and fungi). These hemocyte types contain granules that store immune-related biomolecules, including prophenoloxidase (proPO), antimicrobial peptides and clotting enzymes, with a high concentration of hemocytes perhaps indicating increased oxygen availability at the cellular level and enhanced capacity for immune defense capacity.

In the present study, the concentrations of granulocytes and semi-granulocytes were significantly elevated ($p < 0.01$), which was consistent with the other research on the giant freshwater prawn (*M. rosenbergii*) reported by Kuo et al. (2023).

Additionally, the SOD activity in the MEX group was significantly higher than in the control group, suggesting that SOD, a key humoral immune parameter, was also stimulated by bioactive compounds in the mangosteen pericarp extract. SOD is an enzymatic antioxidant and a critical component of the antioxidant defense systems (Storey, 1996). It mitigates oxidative stress by catalyzing the dismutation of superoxide ions into oxygen and hydrogen peroxide. SOD activity is widely used as an indicator of oxidative status and immune function and was revealed to be enhanced by β-glucan and sulfated polysaccharides (Campa-Córdova et al., 2002). Furthermore, other humoral immune parameters, such as PO activity and respiratory burst (RB), were reported to be stimulated by mangosteen pericarp extract (Kuo et al., 2023).

The results of the present hematological analysis were consistent with the findings of Arundina et al. (2018), who demonstrated that mangosteen pericarp extract supported stem cell proliferation in mammalian systems. Their study highlighted the biological activity of xanthone compounds, particularly α-mangostin, which act as potent agents in promoting stem cell growth and offering protection against oxidative stress. Similarly, Nurani et al. (2021) reviewed the role of α-mangostin in promoting liver regeneration during early-stage cirrhosis by enhancing the angiogenic potential of mesenchymal stem cells. Collectively, these studies underscore the regenerative and protective capabilities of xanthones, particularly in environments characterized by cellular stress or tissue damage.

In shrimp, a similar mechanism may be at play within the hematopoietic tissue (HPT), the primary site for hemocyte production. The antioxidant and anti-inflammatory properties of α-mangostin and gartanin may help create a favorable microenvironment within the HPT, stimulating the proliferation of precursor cells and their differentiation into hyaline, semi-granular and granular hemocytes. The regenerative and angiogenic properties observed in mammalian models suggest that these xanthones could also enhance cellular renewal and immune function in invertebrates.

Based on the present results, mangosteen extract had strong potential as a natural immunostimulant in shrimp aquaculture. By enhancing hemocyte synthesis and improving hematological parameters, it may contribute to increased disease resistance and overall health in Pacific white shrimp, offering a sustainable alternative to synthetic or antibiotic-based immune enhancers.

Therefore, it is proposed that mangosteen-derived xanthones (α-mangostin and gartanin) may further contribute to improved shrimp health by enhancing the expression of digestive enzymes, stimulating hematopoiesis and upregulating antioxidant defense genes. These combined effects likely support better nutrient absorption, immune function and oxidative stress resilience, ultimately leading to improved growth performance and physiological condition in juvenile shrimp.

Prophylactic effects of mangosteen extract against VpAHPND infection

The results from VP-infected shrimp exposed to a lower concentration (1/10 of the sublethal concentration based on the 96 hr LC_{50}), reflecting field-relevant conditions, indicated that MEX1™ was effective for both treatment and prevention.

In the context of VpAHPND infection in shrimp, the present findings were consistent with another study that demonstrated that mangosteen pericarp extract enhanced immune responses in giant freshwater prawns, with reported improvements in cellular (THC and DHC) and humoral (RB, PO and phagocytic activities) hematological parameters, as well as antibacterial activity to inhibit *Lactococcus* spp. (Kuo et al., 2023).

A protein known as LvAPN1, located on shrimp hemocytes has been identified as a receptor for the VpAHPND toxin, where it facilitated the entry of the toxin into hemocytes and played a key role in the pathogenesis of AHPND (Luangtrakul et al., 2021). In the present study, the VpAHPND challenge test revealed significant damage to hyalinocytes following infection, likely attributed to their lack of granules as the organelles responsible for storing pre-expressed immune defense and antibacterial molecules.

The THC of the Pre-MEX group was higher than that of the other two groups due to the elevated levels sustained from the prior growth experiment. Although the THC levels of the Pre-MEX group decreased continuously from 0 to 96 hpi, the value was still 2-folds higher than those of the control and Ter-MEX groups.

The granulocyte ratios in all experimental groups were higher at 24 hpi due to the decrease in hyalinocytes. Notably, the granulocyte concentrations in the Pre-MEX group were significantly elevated at 48–96 hpi compared to 0 hpi, suggesting that mangosteen extract may stimulate granulocyte hematopoiesis following bacterial infection in the shrimp. Furthermore, this granulocyte level was inversely proportional to the VpAHPND concentration in the examined organs, perhaps indicating a potential protective role of granulocytes against the bacterial infection. The VpAHPND concentrations in the hemolymph, hepatopancreas and intestine significantly decreased in both Tre-MEX and Pre-MEX groups.

Additionally, the bacterial pathogens in the Pre-MEX group were significantly lower than in the Tre-MEX group. Notably, the increased hemocyte levels in the Tre-MEX and Pre-MEX groups, compared to the control group, were associated with a reduction in the VpAHPND levels in the examined organs.

Although no mortality was observed in the VpAHPND-treated shrimp (Tre-MEX group) and the VpAHPND-protected shrimp (Pre-MEX group) at 96 hpi, shrimp that received the mangosteen extract before infection, as protected shrimp, had healthier behavior, including improved swimming, feeding and coloration.

Notably, in the control group, shrimp began to die when the THC dropped below 1×10^7 cells/mL during 72–96 hpi, coinciding with an increase in the VpAHPND concentrations in the hemolymph and hepatopancreas. This observation may indicate the immune system's failure to effectively combat the bacterial pathogen.

In the Tre-MEX group, consisting of MEX1™-treated shrimp, the THC levels increased during 72–96 hpi, while the VpAHPND concentrations in the hemolymph and hepatopancreas decreased, suggesting that mangosteen extract may play a role in restoring hemolymph function and enhancing immune responses in shrimp infected with VpAHPND.

Notably, there was a notable reduction in hemocyte damage post-infection in shrimp fed with the MEX1™ additive, along with a recovery in the hemocyte concentration, suggesting a protective effect of the extract. This effect was likely associated with the immunostimulatory properties of the bioactive compounds present in the mangosteen, such as xanthones and flavonoids, which are known to enhance antioxidant capacity, modulate immune signaling pathways and stabilize cellular structures under pathogenic stress (Yuvanatemiya et al., 2022). These findings suggested that dietary supplementation with mangosteen extract may mitigate toxin-induced cytotoxicity and help to preserve the functional integrity of immune cells during VpAHPND infection.

Mangosteen crude extract has demonstrated antimicrobial activity against various bacterial and fungal pathogens. Additionally, it was reported that mangostanaxanthone I and α-mangostin, the 2 bioactives isolated from mangosteen pericarp, had quorum-sensing inhibitory activity against *Chromobacterium violaceum* ATCC 12472 with minimum inhibitory concentration values of 2 µg/mL and 3 µg/mL, respectively. The proposed mechanism involved suppressing the production of virulence-associated compounds such as violacein and pyocyanin (Mohamed et al., 2014). In the present study, the *in vivo* protective effect of MEX1™ against VpAHPND suggested that the α-mangostin within the extract may similarly inhibit quorum sensing in this bacterial pathogen.

In summary, the proposed mechanism of mangosteen-derived xanthones, particularly α-mangostin and gartanin, may involve a multifaceted mode of action, including the enhancement of innate immune responses, stimulation of hemocyte proliferation and upregulation of antioxidant enzymes. Together, these effects contribute to mitigating toxin-induced cellular damage and reducing pathogen burden in shrimp challenged with VpAHPND.

Conclusion

Dietary supplementation with mangosteen extract MEX1™ significantly enhanced proteolytic enzyme activity, hemolymph quality and growth performance in the juvenile shrimp. Notably, MEX1™ had both therapeutic and prophylactic effects against VpAHPND infection, potentially through its antibacterial properties and stimulation of hematopoiesis. The shrimp pre-treated with MEX1™ prior to VpAHPND challenge had markedly improved hemolymph parameters and immune competence compared to both the untreated control shrimp and those treated post-infection.

The infection model applied in this study simulated sublethal exposure levels similar to those typically observed in shrimp farming ponds, characterized by a lower and more gradual pathogen pressure than acute challenge models. This approach allows for the possibility of timely therapeutic intervention and prophylaxis. Clinical signs in the untreated control group began to appear on day 3 post-infection, with mortality commencing on day 4. The observations supported the potential of MEX1™ to enhance shrimp resilience and facilitate recovery under naturally progression scenarios.

A distinctive strength of this study was the monitoring of cellular parameters regarding both growth performance and VpAHPND infection, providing a comprehensive assessment of shrimp health and disease resistance.

Overall, MEX1™ supplementation appeared to have great potential as a practical, eco-friendly feed additive to mitigate AHPND-related mortality and enhance shrimp productivity. These findings suggested that mangosteen-derived phytobiotics could serve as a viable alternative to antibiotics in shrimp aquaculture, contributing to improved food safety and sustainable production practices.

Implications for Future Studies

Further investigations are warranted to explore the bioactivity levels of mangosteen xanthones, particularly

αmangostin and gartanin, in modulating physiological processes related to growth, immunity and stress responses in shrimp.

Due to the high cost of mangosteen extract, further studies are needed to determine the most suitable duration for its administration as a feed additive. In addition, it is of interest whether MEX1™ inhibits other AHPND-causing bacteria and different VP_{AHPND} isolates.

The efficacy of MEX1™ against various bacterial pathogens consisting of the PirA/PirB toxin gene should be evaluated thoroughly prior to its application in shrimp farms. In addition, the potential toxicity of both crude and purified mangosteen extract should be assessed in shrimp and fish species of aquaculture.

From a practical standpoint, the large-scale application of MEX1™ in shrimp farming should be investigated to evaluate its efficacy under natural farming conditions, as its protective effects may vary with environmental factors such as temperature, water quality and feed quality. Key production parameters, including yield, cost-effectiveness and the feasibility of long-term supplementation in commercial-scale operations, should also be assessed thoroughly in future studies. Additionally, other scientific aspects should be explored, such as the influence on gut microbiomes and its potential effects on other economically important shrimp species.

Conflict of Interest

The authors declare that there are no conflicts of interest. There was no input to the measurement, data analysis or conclusions by any representative of the co-funder Phataveerote Ltd., Part., Ban Pho, Chachoengsao, Thailand.

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