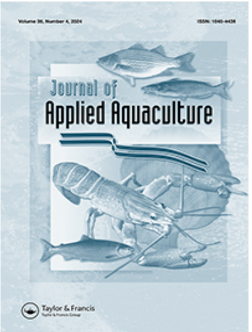

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Effects of dietary synbiotic supplementation on digestive enzyme, total *Vibrio* count, and hepatopancreas of Pacific white shrimp, *Penaeus vannamei* challenged with *Vibrio parahaemolyticus*

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ABSTRACT
This study evaluated the effects of dietary synbiotics on enhancing digestive enzymes, reducing the overall bacterial count of *Vibrio*, and minimizing hepatopancreatic histological damage caused by *V. parahaemolyticus* in shrimp (*Penaeus vanamei*). The experiments involved administering different treatments, namely *Bacillus* NP5 (SBM), *Pseudoalteromonas piscicida*1Ub (SPM), and *Bacillus* NP5 and *P. piscicida* 1Ub (SBPM), incombination with the prebiotic mannan oligosaccharide (MOS). This study was conducted for 60 days, followed by a challenge test with *Vibrio parahaemolyticus* for 7 days. The results of the experiments showed that dietary synbiotic supplementation demonstrated better digestive enzyme activity and histology of the hepatopancreas compared to controls ($p < .05$). After the challenge test, it was found that the damage to the hepatopancreatic tissue of shrimps was less severe and the total *vibrio* count was lower in the synbiotic treatment, indicating a protective effect compared to the positive controls ($p < .05$). In conclusion, the use of dietary synbiotics had the potential to enhance digestive enzyme function and provide disease protection for *Penaeus vannamei* shrimp.

KEYWORDS
Aquaculture; Pacific white shrimp; *Penaeus vannamei*; Sustainable Fisheries; Synbiotic; *Vibrio parahaemolyticus*

Introduction

Pacific white shrimp scientifically known as *Penaeus vannamei* is a lucrative aquaculture item that is becoming increasingly popular worldwide. Their output has been steadily increasing. Pacific white shrimp is an important global aquaculture commodity with a consistent increase in production every year (FAO 2024). However, the occurrence of vibriosis

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infection in white shrimp poses a significant challenge, leading to substantial mortality rates and hindering the progress of *P. vannamei* cultivation (Ananda Raja et al. 2017). The Pacific white shrimp culture faces a significant challenge despite its increasing production, primarily due to the prevalence of infectious diseases. Among these diseases, vibriosis caused by *Vibrio* bacteria, particularly *Vibrio parahaemolyticus*, is a notable threat.

Vibriosis results in extensive mortality, production setbacks, and substantial economic losses in shrimp farming globally (Austin and Austin 2016). One alternative to control or prevent infectious diseases in shrimp is through using dietary synbiotics, a combined product of probiotics and prebiotics, which are expected to increase their benefits (Merrifield et al. 2010). Dietary probiotic bacteria can increase the digestive enzymes of shrimp. According to the literature, Pacific white shrimp supplemented with probiotics could increase digestive enzyme activity (Huynh et al. 2017; Merrifield et al. 2010). Probiotics *Bacillus* sp. NP5 incorporated with honey (prebiotics) as synbiotics can increase the activity of amylase, protease, and lipase enzymes (Hasyimi, Widanarni, and Yuhana 2020; Munaeni, Yuhana, and Widanarni 2014). In addition, the administration of probiotics can improve the immune system in shrimp, including reducing damage to the hepatopancreas (HP) in shrimp.

Synbiotics are a combination of probiotics and prebiotics and therefore increase the benefits for cultivated shrimp/fish. Based on the research results of Zubaidah, Yuhana, and Widanarni (2015), the administration of synbiotics (*Bacillus* sp. NP5 and oligosaccharides) was able to provide a better immune response in white shrimp and resistance to *V. harveyi* infection. In addition, the administration of the synbiotic galactooligosaccharide (GOS) and the probiotic *Lactobacillus plantarum* can increase the immune response, gene expression, and disease resistance to *V. alginolyticus* in vannamei shrimp (Huynh et al. 2018). Another application of synbiotics is using mannan oligosaccharides (MOS) and *Bacillus lincheniformis*, which are known to improve intestinal health and immune responses in vannamei shrimp (Chen et al. 2020). Application of synbiotics *P. piscicida* 1Ub and *Bacillus* NP5 was able to increase total hemocyte count, PO activity, RB activity, phagocytosis activity and survival rate after being challenged with *V. parahaemolyticus* (Pardede et al. 2023). However, studies on synbiotics in vannamei shrimp are still limited, especially with different probiotics. This lack of research hinders the understanding of optimal probiotic combinations for enchancing shrimp health and growth, potentially leading to suboptimal aquaculture practices and economic losses. This study aimed to evaluate the effects of dietary synbiotics on the enhancement of digestive enzymes, histological analysis of the hepatopancreas, and total *Vibrio* count in white shrimp after *V. parahaemolyticus* infection.

Materials and methods

Synbiotics treatment preparation

The probiotics used in this study were isolated from white shrimp nauplii and subsequently identified as *P. piscicida* 1Ub (Widanarni et al. 2009). *Bacillus* NP5 was isolated from the digestive tract of tilapia (Putra and Widanarni 2015). *Bacillus* NP5 and *P. piscicida* 1Ub were cultured using sea water complete (SWC) media and given *rifampicin resistant marker* ($^{Rf^{®}}$) as a molecular marker with doses of 50 mg L^{-1}. To prepare synbiotic microcapsules, maltodextrin 10% (w/v) and 0.4 ml L^{-1} of whey protein were mixed with the probiotics (probiotics and MOS in a 1:1 (v/v) ratio). All ingredients were homogenized using a stirrer plate for 30 min prior to spray drying using a Mini Buchi 190 (Buchi Corporation, United States) with an output temperature of 55 to 58°C, and an inlet temperature of 100 to 110°C. The synbiotic microcapsules were subsequently tested for cell viability. Samples with a cell density of 10^8 CFU g^{-1}, were then kept until use in a refrigerator at −20°C.

Feeding experimental formulation

This experiment was performed by administering synbiotic microcapsules containing different probiotics, i.e., 1% (w/w) *Bacillus* NP5 (SBM), 1% (w/w) *P. piscicida* 1Ub (SPM), and a combination of the two probiotic strains, 0.5% (w/w) *Bacillus* NP5 and 0.5% (w/w) *P. piscicida* 1Ub (SBPM) (Munaeni, Yuhana, and Widanarni 2014; Putra and Widanarni 2015; Ramadhani, Widanarni, and Sukenda 2019). The control treatments consisted of a negative control group (NC) and a positive control group (PC) infected with *V. parahaemolyticus*$^{Rf^{®}}$. The experiments were performed using five treatments with three replications.

Shrimp feeding trials

The Pacific white shrimp post larvae were fed a commercial diet (Shrimp Grower STP, Indonesia) containing 32% crude protein, 6% lipid, and 4% fiber. The treatment feed was given 5 times a day (06.00, 10.00, 14.00, 18.00 and 22.00 WIB) with a feeding rate of 12% and decreasing to 5% according to the shrimp's weight (w/w). The treatment container consisted of 15 units of aquariums filled with 15 L of filtered seawater with 15 shrimps per tank. After that, the shrimp were raised for 60 days. Commercial feed was given 5 times a day. Water quality parameters were checked and maintained throughout the experiment, including temperature (28 ± 1 °C), dissolved oxygen (DO) (>6.0 mg L^{-1}), pH (7.8 ± 0.5), salinity (29 g L^{-1}), ammonia (<0.01 mg L^{-1}), nitrite (<0.1 mg L^{-1}), and nitrate (0.4 ± 0.1 mg L^{-1}).

Enzyme performance in digestion

Digestive enzyme activities (i.e., protease, amylase, and lipase) were determined at the end of the feeding trials. The white shrimp digestive tract sample (0.1 g sample/shrimp) was removed and added with Tris buffer solution (20 mm Tris HCl, 1 mm EDTA, and 10 mm $CaCl_2$ pH 7.5) at a ratio of 10% (w/v). Shrimp with a size of 5.22 ± 0.02 cm were used in the experiment. The sample was then centrifuged for 10 min at a speed of 12,000 rpm at 4°C. The enzymatic activities of protease and amylase in the gut samples were determined based on methods described by (Borlongan 1990); Hasyimi, Widanarni, and Yuhana (2020). The lipase enzymatic activity was measured using the method described by Amoah et al. (2019). These previously established protocols were followed to ensure accurate and consistent results in the analysis of the enzymatic activities.

Hepatopancreas tissue structure analysis

The Hepatopancreas tissues in the tested shrimps with three tails and a weight of 0.1 g per shrimp with a size of 5.22 ± 0.02 cm were used in the experiment) were examined through histological techniques described by Bell and Lightner (1988). The process involved various steps, including fixation, dehydration, clearing, impregnation, embedding, blocking, trimming, tissue sectioning, staining agent (*haematoxylin-eosin*), and tissue mounting using a mounting agent (xylene). The tissue preparation procedures were conducted at the Aquatic Organism Health Laboratory (Laboratorium Kesehatan Organisme Akuatik; LKOA), Department of Aquaculture, Institut Pertanian Bogor. The slide samples were then analyzed using a microscope (400 × magnification). The Hepatopancreas tissues were then identified under a microscope and labeled for further data analysis.

In vivo Vibrio *challenging test*

Following a 60-day period of feeding trials, the Pacific white shrimps were subjected to a multispecies synbiotic treatment. Subsequently, the tested organisms were exposed to a challenge involving intramuscular injection of 100 µl *V. parahaemolyticus* at a concentration of 10^6 CFU mL^{-1}, targeting the second and third segments of the shrimps (Ananda Raja et al. 2017).

Total Vibrio *count in the intestinal samples*

A total of 0.1 g intestinal samples of 2 shrimps with a size of 5.22 ± 0.02 cm were used in the experiment in each treatment were sampled and homogenized in 0.9 ml of PBS solution. The supernatant of the mixtures was spread on (Thiosulfate Citrate Bile Salt Sucrose) TCBS-specific agar media containing rifampicin 50 mg L^{-1} for the observation of *V. parahaemolyticus* $^{Rf^{®}}$ count and on TCBS agars without antibiotics for the total *Vibrio* count. The total *Vibrio* count in the intestinal samples was determined on days 63, 65, and 67 after the challenging test.

Data analysis

The samples of this research were analyzed based on normalized data. Enzyme activity analysis were performed using Microsoft Excel 2010 and Total *Vibrio* Count was analyzed by Analysis of Variance (ANOVA) using Statistical Product and Service Solutions (SPSS) ver. 25 software IBM, 2017 in US. If the obtained results were significantly different ($p < .05$), further tests were carried out using Tukey's test.

Results

Enzymatic activity

Synbiotic treatments increased the activities of amylase and protease enzymes compared to the controls. The SBPM treatment resulted in higher lipase enzyme activity than other treatments and controls (Table 1). The SBPM treatment achieved the highest increase in digestive enzyme activity, with amylase, protease, and lipase enzymes at values of 0.93 ± 0.01, 0.08 ± 0.01, and 0.04 ± 0.01 IU ml^{-1}, respectively ($p < .05$).

Histopathology of hepatopancreas

Histological examination showed that Hepatopancreas tissues had an increase formation of the distal HP's tubule containing B cells, R cells,

Table 1. Effects of different levels of dietary microcapsule synbiotic on intestinal digestive enzymes of white shrimp (*Penaeus vannamei*).

Parameters (IU mL^{-1})	SBM	SPM	SBPM	C
Amylase	0.92 ± 0.01^{c}	0.79 ± 0.01^{b}	0.93 ± 0.01^{c}	0.59 ± 0.01^{a}
Protease	0.08 ± 0.01^{b}	0.08 ± 0.01^{b}	0.08 ± 0.01^{b}	0.04 ± 0.01^{a}
Lipase	0.03 ± 0.03^{a}	0.03 ± 0.02^{a}	0.04 ± 0.03^{b}	0.03 ± 0.03^{a}

Data are expressed as the mean ± SD of triplicate samples. [a,b,c] Different letters represent significant difference ($p < .05$). SBM = 1% *Bacillus* NP5 and MOS; SPM = 1% P. *piscicida* 1Ub and MOS; SBPM = 0.5% *Bacillus* NP5 and 0.5% P. *piscicida* 1Ub and MOS.

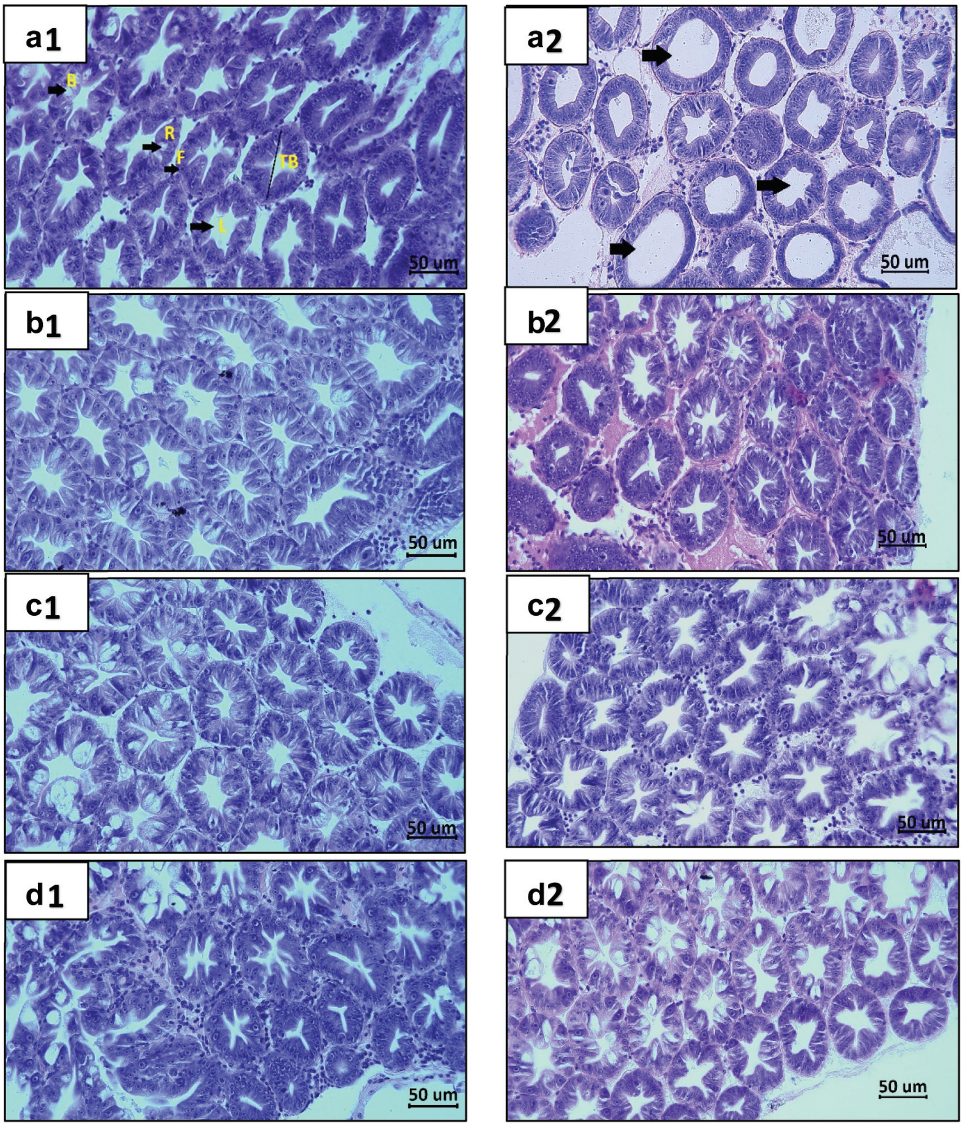

Figure 1. Histopathology of white shrimp hepatopancreas (HP) before treatment (a1) control (b1) SBM (c1) SPM (d1) SBPM, and HP after challenged test with *V. parahaemolyticus* (a2) positive control (b2) SBM (c2) SPM (D2) SBPM.

and F cells, with no damage to these cells after synbiotics administration (Figure 1). The shrimps exhibited less tubular epithelial cell sloughing after synbiotics treatment when challenged with *V. parahaemolyticus* compared to the positive control. The positive control group exhibited necrosis of R, B, and F cells, as well as an enlarged lumen size (Figure 1).

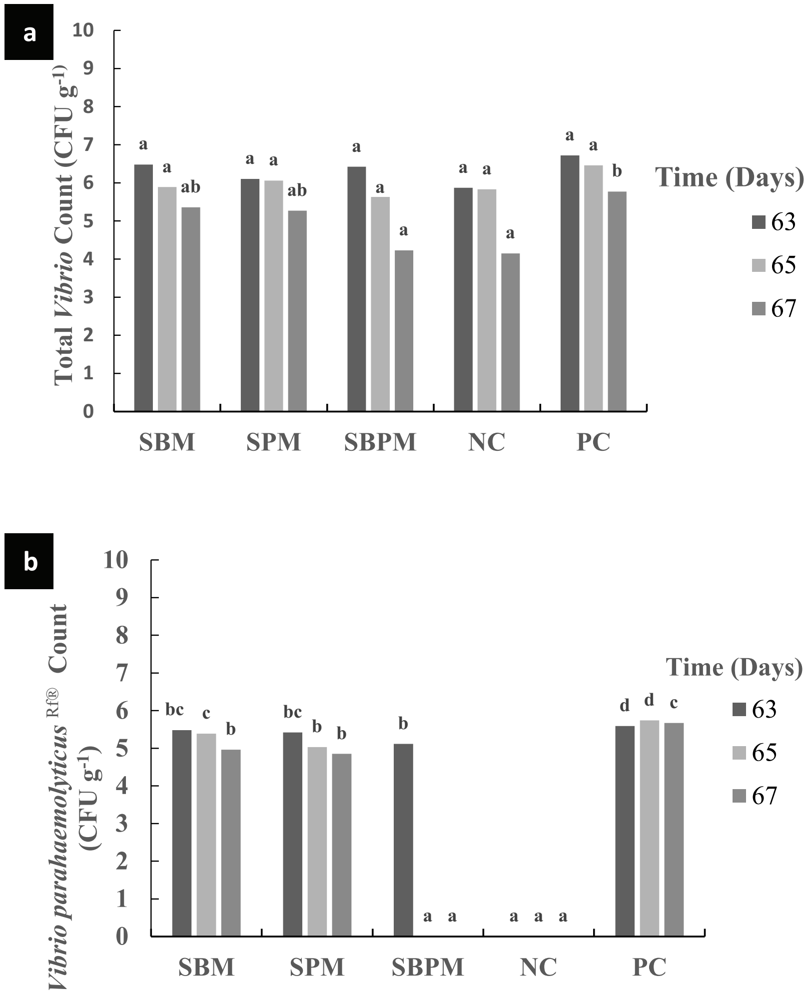

Figure 2. (a) Total *Vibrio* Count and *Vibrio parahaemolyticus* Rf® Count (b) In intestines of Pacific white shrimp on days 63, 65, and 67 after challenged with *V. parahaemolyticus*. (NC) negative control, (PC) positive control, SBM (*bacillus* NP5 and MOS), SPM (*P. piscicida* 1Ub and MOS), SBPM (*bacillus* NP5, *P. piscicida* 1Ub, and MOS). Different letters over each treatment bar (mean ± standard error) indicate a significant difference (Tukey $p < .05$).

Total Vibrio *count*

The SBM, SPM, and SBPM treatment groups showed a lower density of total *Vibrio* after day 65 of the challenge test compared to the positive control group ($p < .05$). The synbiotic treatment groups exhibited lower and significantly different value of *V. parahaemolyticus* Rf® on days 65 and 67 compared to the positive control group ($p < .05$) from the positive control group (Figure 2). On the day 65, the SPM synbiotic treatment group had a lower value of *V. parahaemolyticus* Rf® (log 5.03 ± 0.03) was than the SBM treatment group (log 5.39 ± 0.02 log CFU g^{-1}) ($p < .05$).

Discussion

The formulation of probiotics and prebiotics into synbiotics mainly depends on the mechanisms of action of the prebiotics in the host (Mazzola et al. 2015). This is also supported by the results of the activity of digestive enzymes; the administration of synbiotics can increase the activity of the digestive enzymes of white shrimp. Synbiotic treatments increased amylase and protease enzyme levels compared to the controls. These enzymes are vital for increasing the growth and catabolism of nutrients. As for the activity of the lipase enzyme, the SBPM treatment group showed a higher value than the control. Several prior studies have found that the administration of synbiotics and probiotics can improve digestive enzymes in shrimp (Hien et al. 2022; Wang et al. 2007; Yao et al. 2021).

The addition of synbiotics can increase the activity of digestive enzymes in white shrimp and encourage shrimp growth by releasing extracellular bacterial enzymes and bioactive products from metabolic processes. The synbiotics treatment can activates the host's digestive enzyme precursors and enhances nutrient absorption, thereby increasing feed utilization (De et al. 2015; Huynh et al. 2018). The present study suggests that synbiotic treatment has biological effects on the shrimp's HP. The morphology of the HP improved dramatically when synbiotics were added to the diet. synbiotics treatment can improve the digestive system and biological functions of the HP and midgut, restrict the formation of pathogenic bacteria, and reduce the occurrence of illnesses (Duan et al. 2017; Yao et al. 2021).

The 60-day feeding trials revealed no disparity in the HP histology of Pacific white shrimp between the synbiotic treatment groups and the negative controls. However, within the treatment cohorts (SBM, SPM, and SBPM) challenged with *V. parahaemolyticus*, HP damage was notably milder compared to the positive control group. In the positive control group, the HP tissue of white shrimp exhibited severe damage, characterized by tubule necrosis (TB), HP cell sloughing, and tubular lumen widening, all attributed to *V. parahaemolyticus* infection. The study demonstrates that synbiotic treatment mitigates lysis and damage to epithelial cells within HP tubules when compared to positive controls. The extensive damage observed in the HP tissue of white shrimp in the positive control group correlated with a higher mortality rate during subsequent challenging tests compared to other groups, as documented in prior research (Pardede et al. 2023). This agrees with previous studies showing that shrimp infected with *V. parahaemolyticus* experienced interstitial hemolytic infiltration, sloughing of tubular epithelial cells, and necrosis in the HP's tubules after 24 h of *Vibrio* infection, causing R-cells and B-cells to malfunction (Muharrama et al. 2021; Yao et al. 2021), as well as widening or sloughing of the lumen in the HP (Tran et al. 2013). *V. parahaemolyticus* infection can cause gastroenteritis, wound infection, and septicemia (Broberg, Calder, and Orth 2011). It is important to note that the decrease in *V. parahaemolyticus* $^{Rf^{®}}$ count and total *Vibrio* count that occurred in the treatment groups of this study was suspected to be influenced by the administration of synbiotics.

Probiotic bacteria have the capacity to produce antibacterial compounds like bacteriocins, which directly impede the growth of other bacteria, actively combat infections, and hinder the colonization of pathogenic bacteria on the intestinal epithelium, thus bolstering nonspecific immune responses (Cerezuela, Meseguer, and Esteban 2011). In this study, synbiotic treatment demonstrated a notable enhancement in resistance against *V. parahaemolyticus* in Pacific white shrimp. Feeding Pacific white shrimp with synbiotics such as *Bacillus* NP5 and *P. piscicida* 1Ub proved beneficial in boosting disease resilience within shrimp farming. The findings underscore the potential of synbiotics, such as *Bacillus* NP5 and *P. piscicida* 1 Ub, to fortify disease resistance in Pacific white shrimp, offering promising prospects for shrimp farming practices. Based on the study's finding, shrimp farmers should integrate synbiotics into shrimp diets to enhance digestive efficiency, improve health and increase resilience againts pathogens *Vibrio parahaemolyticus*.

Conclusion

Supplementation of synbiotics in feed has been shown to improve digestive enzyme activity, enhance hepatopancreatic health, and increase disease resistance against *V. parahaemolyticus*. Future studies should focus on understanding the long-term effectiveness of synbiotic supplementation and exploring the synergistic effects of different synbiotic combinations to optimize these health benefits.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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