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Growth, immune responses, and resistance of vannamei shrimp fed with *Lactobacillus paracasei* probiotic and paraprobiotic and infected with *Vibrio parahaemolyticus*

Pertumbuhan, respons imun, dan resistansi udang vannamei diberi probiotik dan paraprobiotik *Lactobacillus paracasei* dan diinfeksi *Vibrio parahaemolyticus*

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ABSTRACT

Vannamei shrimp is one of the most economically valuable aquaculture commodities in Indonesia. One of the pathogenic bacteria that is often found in vannamei shrimp farming is *Vibrio parahaemolyticus*. This study aimed to analyze the effectivity of *Lactobacillus paracasei* probiotics and paraprobiotics through feed with different cell densities on growth, and immune responses infected with *V. parahaemolyticus*. Vannamei shrimp of size 0.63 ± 0.01 were reared in containers with a stocking density of 15 shrimp per container and supplemented feed for 30 days. The research design consisted of six treatments, each with three replicates, namely (K-) feeding without supplements and injected with phosphate-buffered saline (PBS), (K+) feeding without supplements and infected with *V. parahaemolyticus* (10^4 CFU/mL), (PRI) feeding with 1% (v/w) probiotic *L. paracasei* with cell density of 10^6 CFU/mL, (PRII) 1% (v/w) probiotic *L. paracasei* cell density 10^9 CFU/mL, (PAI) 1% (v/w) paraprobiotic *L. paracasei* cell density 10^6 CFU/mL, (PAII) 1% (v/w) paraprobiotic *L. paracasei* cell density 10^9 CFU/mL. All treatments, except K-, were infected with *V. parahaemolyticus* (10^4 CFU/mL). Vannamei shrimp rearing was continued post the challenge test with *V. parahaemolyticus* which was conducted up to 6 days post injection. The results showed that feeding both probiotic and paraprobiotic *L. paracasei* through feed has improved growth, immune response, protein fat retention, and digestive enzyme activity of vannamei shrimp better than those of control. As the recommendation for the disease control of *V. parahaemolyticus* is feed supplementation with 1% (v/w) probiotic *L. paracasei* with cell density of 10^9 CFU/mL.

Keywords: *Lactobacillus paracasei*, paraprobiotic, probiotic, vannamei shrimp, *Vibrio parahaemolyticus*

ABSTRAK

Udang vaname merupakan salah satu komoditas akuakultur yang bernilai ekonomis tinggi di Indonesia. Salah satu bakteri patogen yang sering ditemukan dalam budidaya udang vaname ialah bakteri *Vibrio parahaemolyticus*. Penelitian ini bertujuan untuk menganalisis pemberian probiotik dan paraprobiotik *Lactobacillus paracasei* melalui pakan dengan kepadatan sel berbeda terhadap pertumbuhan, dan respons imunitas yang diinfeksi *V. parahaemolyticus*. Udang vaname dengan ukuran 0,63 ± 0,01 gr dipelihara di dalam kontainer dengan padat tebar 15 ekor per wadah dan pemberian pakan bersuplemen selama 30 hari. Rancangan penelitian terdiri dari enam perlakuan, tiga ulangan, yaitu (K-) pemberian pakan tanpa suplemen dan diinjeksi PBS, (K+) pemberian pakan tanpa suplemen dan diinfeksi *V. parahaemolyticus* (10^4 CFU/mL), (PRI) pemberian pakan dengan probiotik *L. paracasei* kepadatan sel 10^6 CFU/mL dosis 1% (v/w), (PRII) probiotik *L. paracasei* kepadatan sel 10^9 CFU/mL dosis 1% (v/w), (PAI) paraprobiotik *L. paracasei* kepadatan sel 10^6 CFU/mL dosis 1% (v/w), (PAII) paraprobiotik *L. paracasei* kepadatan sel 10^9 CFU/mL dosis 1% (v/w), dan masing-masing diinfeksi *V. parahaemolyticus* (10^4 CFU/mL). Pemeliharaan udang vaname dilanjutkan setelah uji tantang dengan *V. parahaemolyticus* yang dilakukan hingga 6 hari pasca injeksi. Hasil penelitian menunjukkan bahwa pemberian probiotik maupun paraprobiotik *L. paracasei* melalui pakan telah meningkatkan pertumbuhan, respons imun, retensi lemak protein, dan aktivitas enzim pencernaan udang vaname lebih baik dibandingkan kontrol. Untuk pengendalian *V. parahaemolyticus* diperoleh hasil terbaik dengan aplikasi probiotik *L. paracasei* dosis 1% (v/w) dengan kepadatan sel 10^9 CFU/mL.

Kata kunci: *Lactobacillus paracasei*, paraprobiotik, probiotik, udang vaname, *Vibrio parahaemolyticus*

INTRODUCTION

Vannamei shrimp (*Litopenaeus vannamei*) is one of the aquaculture commodities with high economic value in Indonesia. Vannamei shrimp has a number of advantages, including being more resistant to disease, can live at a fairly high stocking density (Renanda *et al.,* 2019), vannamei shrimp production volume continues to show an increase from 2.64 million tons in 2010 to 5.81 million tons in 2020 (FAO, 2022). One of the pathogenic bacteria that is often found in shrimp farming is *V. parahaemolyticus,* which causes acute hepatopancreatic necrosis disease (AHPND) possessing toxins encoded by *pirA* and *pirB* genes (Yuhana & Afiff, 2023). Bacteria that cause AHPND attack the digestive gland (hepatopancreas) and damage hepatopancreatic cells causing massive dysfunction and death in shrimp (Dong *et al.,* 2017). AHPND-affected shrimp exhibit lethargy, anorexia, slow growth, empty digestive tract, and pale hepatopancreas (Hong *et al.,* 2016). As a result of the pathogen, cultured vannamei shrimp experienced mass mortality, causing very high economic losses (Kaligis, 2015).

One way to overcome vibriosis disease besides feeding with a balanced nutrient composition is by feed supplementation with probiotics (Yuhana *et al.,* 2022). Probiotics are live microbes that applied in adequate quantities will have beneficial effect to the host because they can modify microbial communities, improve nutritional value, balance bacteria in the host gut, improve host responses to disease and improve environmental quality (Sukmawati & Badaruddin, 2019). Bacteria that can be used as probiotics include *Lactobacillus* sp. *Lactobacillus* sp. is classified as a group of lactic acid bacteria that are safe for host digestion (Eliyani *et al.,* 2013). The advantages of *Lactobacillus* sp. bacteria are able to survive in low pH, and can colonize in the host intestine. In addition, the enzymes contained in *Lactobacillus* sp. able to improve the immune system and balance and normalize the number of bacteria in the digestive tract (Andriyanto *et al.,* 2020).

Probiotics application to shrimp can achieve optimal growth, increase immunity and resistance to stress and disease (Yuhana, 2010; Sukenda *et al.,* 2016; Yuhana *et al.,* 2021). The survival of probiotic microorganisms during the process of making and storing feed is an obstacle in the utilization of probiotics. According to Thy *et al*. (2017) the number of probiotic cells in feed decreased by about 10% after three weeks of storage. This is because probiotic cells are living microorganisms that are easily damaged or die due to various production process factors (Zorriehzahra *et al.,* 2016). It was evaluated to find a solution with the application of dead probiotic bacteria. Dead or inactive probiotic cells are called paraprobiotics (De Almada *et al.,* 2016).

The concept of paraprobiotics is the utilization of *non-viable* probiotics that can provide certain benefits to the host. Paraprobiotics are derived from good microorganisms that lose their viability after exposure to factors that alter the microbial cell structure such as DNA filament breaks, cell membrane disruption or mechanical damage to the cell envelope (De Almada *et al.,* 2016). Paraprobiotics can be stored without the use of refrigeration and have a longer shelf life (Wang *et al.,* 2022). So far, the application of paraprobiotics is mostly given to fish and shrimp through artificial feed (Mulyadin *et al.,* 2021; Widanarni *et al.,* 2022; Noventri *et al.,* 2023). *L. paracasei* belongs to the lactic acid bacteria (LAB) group and characterized as facultative anaerobic bacteria containing a high peptidoglycans polysaccharides (Zivkovic *et al.,* 2016).

Research related to the use of *L. paracasei* probiotics include, *L. paracasei* probiotics are able to increase the innate immunity of vannamei shrimp, resistant to *V. parahaemolyticus* infection (Huang *et al.,* 2022). In addition, according to Doan *et al*. (2021) that *L. paracasei* can improve growth, survival, feed efficiency, and increase resistance to *Streptococcus agalactiae* infection. Based on the explanation above, it is expected that the application of *L. paracasei* paraprobiotics through feed can be an alternative in controlling *V. parahaemolyticus* disease in vannamei shrimp.

MATERIALS AND METHODS

Research design

The administration of probiotics and paraprobiotics *L. paracasei* to vannamei shrimp using a completely randomized design (CRD) consisted of 6 treatments and three replicates, namely (K-) feed without probiotics and paraprobiotics and injection with *Phosphate-Buffered Saline* (PBS); (K+) feed with probiotics and paraprobiotics and challenged with 10^4 CFU/mL cell suspension *V. parahaemolyticus*; (PRI) diet with probiotic *L. paracasei with a* cell density of 10^6 CFU/mL at 1% (v/w), and

challenged with 10^4 CFU/mL cell suspension of V. *parahaemolyticus*; (PRII) feed supplemented with probiotic *L. paracasei with a* cell density of 10^9 CFU/mL at 1% (v/w), and challenged with 10^4 CFU/mL cell suspension of *V. parahaemolyticus*; (PAI) feed with paraprobiotic *L. paracasei* with a cell density of 10^6 CFU/mL at 1% (v/w), and tested against 10^4 CFU/mL *V. parahaemolyticus cell suspension;* (PAII) Feed with paraprobiotic L. *paracasei with a* cell density of 10^9 CFU/mL at 1% (v/w), and tested against 10^4 *CFU/mL V. parahaemolyticus* cell suspension.

Preparation and maintenance of container

This experiment used 18 containers each with the volume of 15 L. Clean containers was filled with 10 L of seawater with a salinity ranging approximately of 30 g/L and equipped with aeration. The test animals used were vannamei shrimp with an average weight of 0.63 ± 0.01 g and reared with a stocking density of 15 shrimp/L. Supplementation with probiotics and paraprobiotics in feed was carried out for 30 days. Residual feed and feces were cleaned using a siphon. Shrimp feeding was done four times a day (07:00; 11:00; 15:00; 19:00).

Preparation of paraprobiotic bacteria

The probiotic bacteria used in this study were *L. paracasei* from Gajah Mada University from the Japanese culture collection number IFO 3074, which has been exposed in a 50 µg/mL *Ciprofloxacin* as the antibiotic resistance marker. Probiotic biomass production of *L. paracasei* is bacteria that has been cultured in *Man Ragosa Sharpe Broth* (MRSB) media. The culture process was carried out in an incubator at 34-35°C for 48 hours. The process of preparing *L. paracasei* paraprobiotics is by centrifugation to harvest probiotic cell cultures that have been grown in MRSB media at 10,000 rpm for 10 minutes. The cell pellet was washed twice with sterile phosphate-buffered saline (PBS) and a cell density of 10^9 CFU/mL was obtained. To inactivate the probiotics, the bacterial cell suspension was heated for 60 minutes in a water bath at 95°C. Checking the viability of the inactivated bacterial cells was done by spreading the cells on man ragosa sharpe agar (MRSA) media and incubating for 24 hours at 37°C, if there was no cell growth then it was ready to be used as a paraprobiotic supplement.

Preparation of supplemented shrimp feed

The feed used was a commercial pellet branded Feng Li with a protein content of 35%. Spraying of cell suspensions containing probiotics and paraprobiotics as supplements to the test feed was carried out with PBS for the control treatment, each bacteria according to the treatment dose was diluted with distilled water at 100 ml/kg. Egg white liquid as much as 2% (v/w) was sprayed onto the test feed and control feed which was used as a *binder*. Furthermore, cell suspensions containing probiotics or paraprobiotics according to each treatment were sprayed with a *syringe* evenly into the feed. The probiotic or paraprobiotic supplemented feed was then dried, put into airtight plastic bags, labeled, and stored in a refrigerator at 4°C. The treatment feed was made every three times a week.

Challenge test with pathogenic *V. parahaemolyticus*

The challenge test was conducted on day 31 of rearing, after 30 days of feed supplementation treatment. *Vibrio parahaemolyticus* bacteria were cultured on solid *Sea Water Complete* (SWC) agar media. The growing colonies were taken with an ose needle inoculated in liquid SWC media and then incubated for 24 hours. The challenge test was carried out by injection method at a cell concentration of 10^4 CFU/mL with a volume of 0.1 mL per shrimp. The challenge test was carried out for all supplementation treatments as well as the positive control treatment, while the negative control shrimp were only injected with PBS solution with a volume of 0.1 mL per shrimp. Post the challenge test, shrimp in all treatments were fed with commercial pellets that were not given probiotics or paraprobiotics. Observations post the challenge test were carried out for 7 days and survival was calculated at the end of the challenge test.

Experimental parameters

Shrimp immune response

Parameters of shrimp immune response include survival rate (SR), daily growth rate (DGR) (Aalimahmoudi *et al.,* 2016), feed conversion ratio (Ho *et al.,* 2017), total hemocyte count (Nabi *et al.,* 2022), phagocytosis activity (Anderson & Siwicki, 1995), respiratory burst (Nabi *et al.,* 2022), phenoloxidase activity (Hsieh *et al.,* 2008).

Nutritional test parameters

Nutritional test parameters include protein fat retention test (Putri *et al.,* 2017), amylase enzyme activity (Putri *et al.,* 2017), protease and lipase (Sembiring *et al.,* 2022).

Microbiological analysis

Calculation of bacterial abundance was carried out at the beginning, end of treatment, and post the challenge test. The observations made were bacterial abundance/total bacterial count (TBC), and total plate count (TPC).

Data analysis

All data obtained include data on survival rate, daily growth rate, total haemocyte count (THC), phenoloxidase activity (PO), phagocytosis activity (AF), respiratory burst (RB), digestive enzyme activity, fat and protein retention in shrimp, an abundance of gut bacteria tested by normality and homogeneity tests first. If found to be significantly different, then further tests were carried out using the Tukey test with a 95% confidence interval.

RESULTS AND DISCUSSION

Results

Shrimp growth performance prior the challenge test

The growth performance of vannamei shrimp post probiotic and paraprobiotic *L. paracasei* treatment was not significantly different (P>0.05) between treatments, LPH in the PRII treatment did not different significantly from the PRI and PAII treatments (P>0.05), but different significantly from the PAI and control treatments (P<0.05). FCR was lowest in the PRII treatment and did not different significantly from the PRI treatment (P>0.05) but different significant from PAI, PAII, and control treatment (P<0.05).

Shrimp digestive enzyme activities

Digestive enzyme activities including amylase, protease and lipase enzyme activities after 30 days of rearing are presented in Table 2. The effectivity of probiotics and paraprobiotics *L. paracasei was* able to increase the activity of amylase, lipase and protease enzymes in vannamei shrimp.

Table 1. Results of growth tests on vannamei shrimp fed with probiotics and paraprobiotics *L. paracasei* supplementation after 30 days of rearing.

Parameters	Treatment				
	K	PRI	PRII	PAI	PAII
Prior weight (g)	0.63 ± 0.01^{a}	0.63 ± 0.01^{a}	0.63 ± 0.01^{a}	0.63 ± 0.01^{a}	0.63 ± 0.01^{a}
Post weight (g)	1.70 ± 0.02^{a}	1.94 ± 0.04^{c}	1.96 ± 0.02^{c}	1.85 ± 0.03^{b}	1.93 ± 0.03^{c}
FCR	1.18 ± 0.02^{b}	1.12 ± 0.03^{a}	1.10 ± 0.02^{a}	1.17 ± 0.03^{b}	1.17 ± 0.02^{b}
SR (%)	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
DGR (%/day)	3.55 ± 0.07^{a}	4.36 ± 0.13^{c}	4.42 ± 0.07^{c}	4.06 ± 0.11^{b}	4.33 ± 0.10^{c}

Note: FCR (feed conversion ratio), SR (survival rate), DGR (daily growth rate). The numbers in the same column followed by the same letter are not significantly different at the 5% test level (Tukey test). Control (K), 10^{6} CFU/mL probiotic *L. paracasei* (PRI), 10^{9} CFU/mL probiotic L. *paracasei* (PRII), 10^{6} CFU/mL paraprobiotic *L. paracasei* (PAI), 10^{9} CFU/mL paraprobiotic L. *paracasei* (PAII).

Table 2. Results of digestive enzyme activity test in vannamei shrimp fed with probiotic and paraprobiotic *L. paracasei* after 30 days of rearing.

Enzymatic Parameters	Treatments				
	K	PRI	PRII	PAI	PAII
Amylase (IU/mL)	1.35 ± 0.02^{a}	1.44 ± 0.06^{b}	1.54 ± 0.04^{c}	1.38 ± 0.05^{ab}	1.40 ± 0.02^{b}
Lipase (IU/mL)	0.06 ± 0.00^{a}	0.09 ± 0.00^{c}	0.10 ± 0.00^{d}	0.08 ± 0.00^{b}	0.08 ± 0.00^{b}
Protease (IU/mL)	0.04 ± 0.00^{a}	0.05 ± 0.00^{b}	0.07 ± 0.00^{c}	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}

Note: The numbers in the same column followed by the same letter are not significantly different at the 5% test level (Tukey test). Control (K), 10^{6} CFU/mL probiotic *L. paracasei* (PRI), 10^{9} CFU/mL probiotic L. *paracasei* (PRII), 10^{6} CFU/mL paraprobiotic *L. paracasei* (PAI), 10^{9} CFU/mL paraprobiotic L. *paracasei* (PAII).

Amylase, lipase, and protease enzyme activity test parameters in the PRII treatment obtained significantly different results from the control as well as PRI, PAI, and PAII treatments. The highest value of each enzyme activity is found in the PRII treatment.

Fat and protein retention of shrimp

Protein and fat retention after 30 days of maintenance are presented in Table 3. For protein retention, the PRII treatment is significantly higher retention than the other treatments and did not different significantly from the PRI and PAII treatments (P>0.05). Fat retention in the PRII treatment was significantly different from all other treatments and the control (P<0.05).

Shrimp immune response

Total hemocyte count (THC), phagocytosis activity (AF), respiratory burst (RB), phenoloxidase activity (PO) are shown in Figure 1. The results of immune response parameters after 30 days of rearing showed that probiotic and paraprobiotic *L. paracasei* treatments significant increase compared to the control. Post the *V. parahaemolyticus* challenge test, each parameter obtained different results, in THC, AF, and RB each treatment decreased. PO experienced an increase in each treatment. The highest values in THC, RB, PO, and AF post the challenge test were in the PRII treatment.

Prior challenge test in THC parameters showed that in KP, KN, and PAI treatments were lower and significantly different from those in PRI, PRII, and PAII treatments (P<0.05). Post challenge test, THC levels in KN, PRI, PRII, and PAII treatments were not significantly different (P>0.05) but was significantly different from PRI and KP (P<0.05). Prior challenge of AF parameters in PRI and PRII treatments not significantly different (P>0.05) but were significantly different from PAI, PAII, and control treatments (P<0.05). Post challenge test, AF parameters in shrimp given PRII treatment was higher and significantly different from PRI, PAI, PAII, and control treatments (P<0.05).

PO test parameters in prior challenge test showed that the PRII treatment was higher than the control and other treatments (P<0.05), and post challenge test which showed that the PRII treatment was significantly different from the control and other treatments (P<0.05). RB parameters of vannamei shrimp post challenge test showed that there was no significant difference between the PRI and PRII treatment groups (P>0.05). Both treatments were higher and significantly different from the PAI, PAII, and control treatments (P<0.05). After the challenge test, the RB parameter in the PRII treatment was higher and significantly different from the other treatments (P<0.05).

Intestinal bacterial population monitoring

The results of the calculation of total bacterial cells in the shrimp intestine after 30 days of rearing showed that PRII was significantly different from PRI, PAI, PAII, and the control (P<0.05). Probiotic bacteria *L. paracasei* were only found in the probiotic supplementation treatment. The results of the calculation of total bacterial cells and probiotic cells of *L. paracasei* in the intestine during rearing are presented in Table 4.

Hepatopancreatic V. parahaemolyticus population monitoring

The bacteria of *V. parahaemolyticus* in the hepatopancreas organ in the PRII and KN treatments was not significantly different (P>0.05) but was significantly different from PRI, PAI, PAII, and KP (P<0.05).

Table 3. Fat and protein retention of vannamei shrimp after administration of probiotic and paraprobiotic *L. paracasei.*

Parameters	Treatment				
	K	PRI	PRII	PAI	PAII
Protein Retention (%)	34.94 ± 1.69^{a}	42.97 ± 0.94^{bc}	44.36 ± 1.22^{c}	40.54 ± 1.57^{b}	42.50 ± 1.06^{bc}
Fat Retention (%)	17.11 ± 0.77^{a}	30.10 ± 2.99^{bc}	37.73 ± 1.79^{d}	26.10 ± 1.58^{b}	29.11 ± 1.12^{c}

Note: The numbers in the same column followed by the same letter are not significantly different at the 5% test level (Tukey test). Control (K), 10^{6} CFU/mL probiotic *L. paracasei* (PRI), 10^{9} CFU/mL probiotic L. *paracasei* (PRII), 10^{6} CFU/mL paraprobiotic *L. paracasei* (PAI), 10^{9} CFU/mL paraprobiotic L. *paracasei* (PAII).

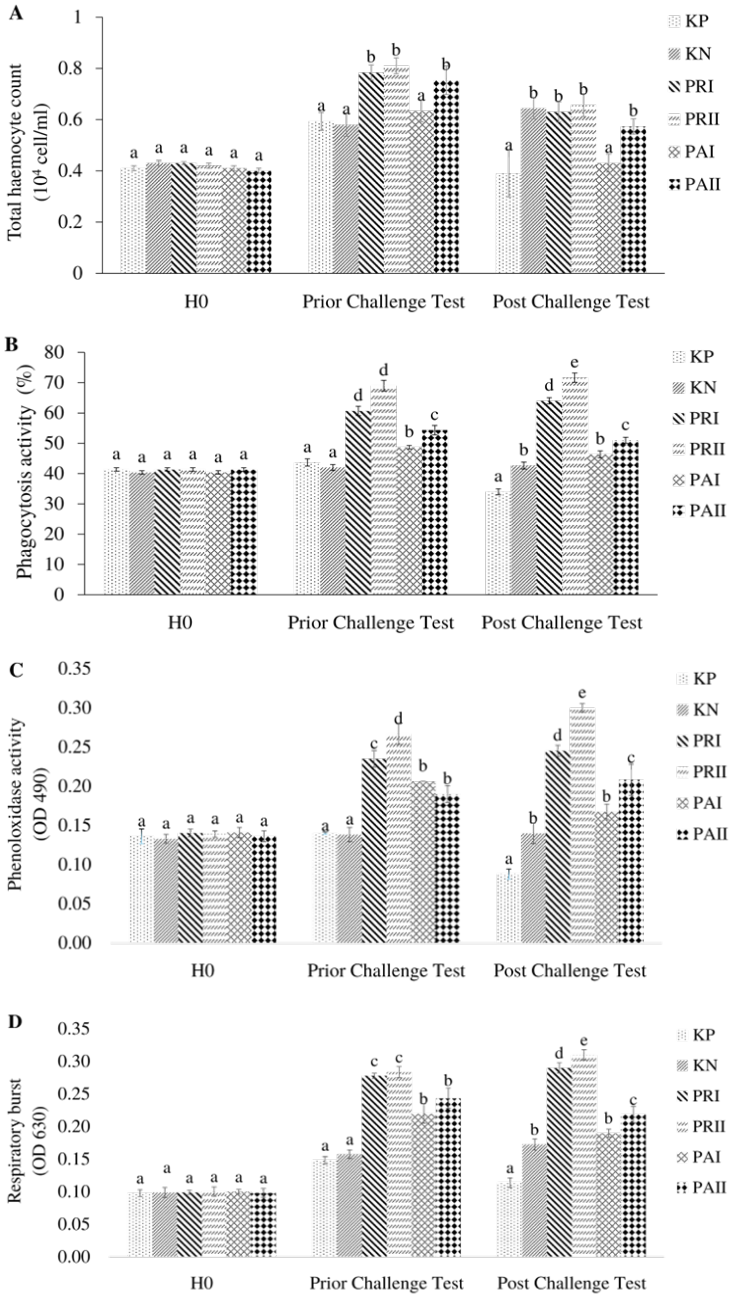

Figure 1. Total haemocyte count (A), phagocytosis activity (B), phenoloxidase (C), and respiratory burst (D) of vannamei shrimp prior and post challenge with *V. parahaemolyticus*. The numbers in the same column followed by the same letter are not significantly different at the 5% test level (Tukey test). Positive control (KP), negative control (KN), 10^6 CFU/mL probiotic *L. paracasei* (PRI), 10^9 CFU/mL probiotic L. *paracasei (*PRII), 10^6 CFU/mL paraprobiotic *L. paracasei* (PAI), 10^9 CFU/mL paraprobiotic L. *paracasei* (PAII).

Table 4. Total bacterial count of probiotic and paraprobiotic *L. paracasei* in vannamei shrimp.

Parameters	Day	Treatment				
		K	PRI	PRII	PAI	PAII
Total bacteria	0	3.0 ± 0.1^{a}	3.0 ± 0.1^{a}	3.0 ± 0.1^{a}	3.0 ± 0.1^{a}	3.0 ± 0.1^{a}
(10^6 CFU/mL)	30	4.9 ± 0.1^{a}	7.9 ± 0.2^{d}	7.1 ± 0.3^{c}	5.7 ± 0.5^{b}	5.8 ± 0.6^{b}
L. paracasei	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
(10^4 CFU/mL)	30	0.00 ± 0.00^{a}	1.25 ± 0.06^{b}	2.45 ± 0.01^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

The numbers in the same column followed by the same letter are not significantly different at the 5% test level (Tukey test). Control (K), 10^6 CFU/mL probiotic *L. paracasei* (PRI), 10^9 CFU/mL probiotic *L. paracasei* (PRII), 10^6 CFU/mL paraprobiotic *L. paracasei* (PAI), 10^9 CFU/mL paraprobiotic *L. paracasei* (PAII).

Shrimp survival rate post-challenge test

The survival rate of vannamei shrimp in K- was significantly different from the positive control and other treatments (P<0.05). The highest SR was in the negative control (K-).

Discussion

The production of vannamei shrimp fed with probiotic and paraprobiotic *L. paracasei* treatment resulted in better performance compared to the control. Shrimp fed with probiotic *L. paracasei* cell supplementation at a dose of 10^9 CFU/mL was able to produce a better daily growth rate compared to other treatments with a value of 4.42 ± 0.07. Probiotics can increase shrimp growth with a positive contribution from digestive enzymes (Yan & Charles, 2018). Giving probiotics can increase the production of lysozyme in shrimp. Lysozyme hydrolyze and break glycoside bonds in bacterial cell walls, thereby inhibiting pathogenic bacteria from infecting shrimp, then lysozyme increase aspartate aminotransferase and alanine aminotransferase which are indicators of natural immunity in shrimp, and increase other defense cells in shrimp (Du *et al.,* 2022).

Hemocytes play a critical role in the immune response of crustaceans, including phagocytosis, mediation of cytotoxicity, encapsulation, and nodule formation (Yuhana *et al.,* 2022). However, post the challenge test the results in each treatment decreased. According to Muharrama *et al*. (2020), the decrease in THC value can be caused by foreign bodies that enter the shrimp body will be recognized by hemocyte cells and then responded to through the stages of mechanisms and various immune responses to pathogens. The presence of foreign bodies can cause hemocyte cells to migrate from the shrimp's circulatory system and infect cells (Widanarni *et al.,* 2020). The number of hemocyte counts decreased due to the effects of the operation of the body's defense mechanisms such as infiltration of hemocytes in

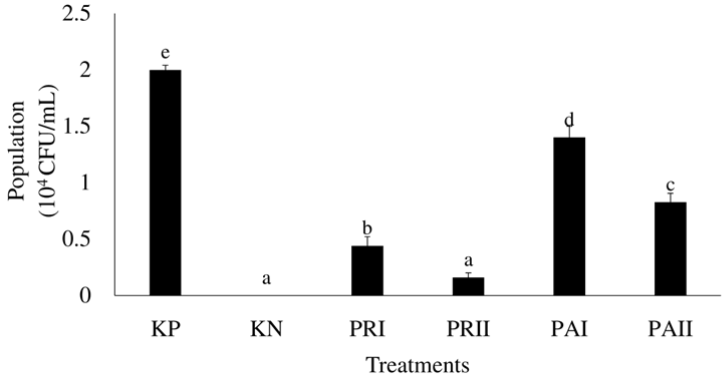

Figure 2. Total *V. parahaemolyticus* in vannamei shrimp post challenge test. The numbers in the same column followed by the same letter are not significantly different at the 5% test level (Tukey test). Positive control (KP), negative control (KN), 10^6 CFU/mL probiotic *L. paracasei* (PRI), 10^9 CFU/mL probiotic L. *paracasei* (PRII), 10^6 CFU/mL paraprobiotic *L. paracasei* (PAI), 10^9 CFU/mL paraprobiotic L. *paracasei* (PAII).

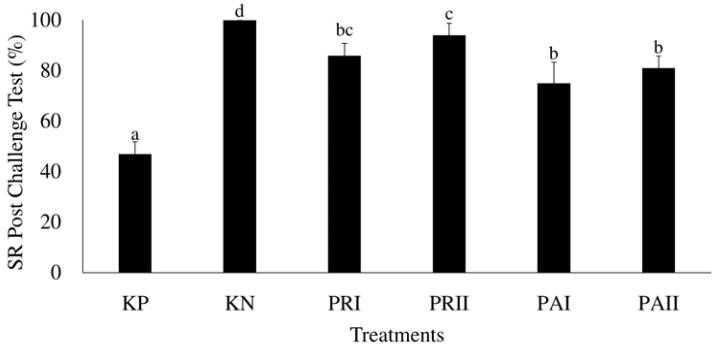

Figure 3. Survival rate post challenge test of *V. parahaemolyticus*. The numbers in the same column followed by the same letter are not significantly different at the 5% test level (Tukey test). Positive control (KP), negative control (KN), 10^6 CFU/mL probiotic *L. paracasei* (PRI), 10^9 CFU/mL probiotic *L. paracasei* (PRII), 10^6 CFU/mL paraprobiotic *L. paracasei* (PAI), 10^9 CFU/mL paraprobiotic *L. paracasei* (PAII).

infected tissues, and hemocyte cell death due to apoptosis (Hamsah *et al.,* 2019).

Probiotics as immunostimulants mainly by stimulating phagocytosis (Butt *et al.,* 2021). Phagocytosis is a mechanism of multicellular organisms to eliminate microorganisms, foreign particles, and cells that die due to apoptosis. Phagocytosis activity occurs through several stages, namely recognition, internalization, and degradation (Smith *et al.,* 2010). During phagocytosis, reactive oxygen is produced such as superoxide anion and is microbicidal (Khodary *et al.,* 2018). Pathogens in the shrimp will be recognized by receptors on the surface of hemocytes in the form of lectins, scavenger receptors (SRs), immunoglobulin-related proteins, and fibrinogen-related proteins (FREPs). Phagocytosis in vannamei shrimp can be influenced by several factors such as antimicrobial peptides (AMPs), and two neuroendocrines namely crustacean hyperglycemic hormone (CHH) and dopamine.

Antimicrobial peptides (AMPs) function for defense from a spectrum of microorganisms that are expressed and released in the hemolymph (Liu *et al.,* 2020). Meanwhile, CHH functions to regulate phagocytosis through the activation of nuclear factor kappa B (NF-KB) and dopamine acts to inhibit phagocytosis (Zhang *et al.,* 2018). AF values prior to and post the challenge tests in shrimp fed probiotic and paraprobiotic feed increased. The increase in AF occurs because when foreign bodies such as pathogenic bacteria enter the fish body, the bacteria will be phagocytized by macrophages or monocytes, macrophages destroy antigens by phagocytizing and sending signals to lymphocytes to form specific antibodies, and the antibodies formed will reduce toxicity and weaken pathogenic bacteria so as not to spread so that phagocytic cells will easily attack pathogens (Estrada *et al.,* 2013).

Increased phagocytosis can result from the presence of various compounds derived from probiotics in the form of polysaccharide compounds. Increased phagocytosis activity through the administration of *L. paracasei* in vannamei shrimp has been studied by Huang *et al*. (2022). PO is an immune response associated with phagocytosis, encapsulation, and melanization of foreign bodies (Hamsah *et al.,* 2019). Phenoloxidase (PO) is an enzyme that catalyzes the oxidation of *monophenols* to *o-diphenols* and is converted non-enzymatically to melanin. The enzyme is exocytosed by semigranular cells and granular cells of shrimp through degranulation.. This process produces *antimicrobial peptides* (AMPs) and *peroxinectin* (PXN).

AMPs play a role in microbial activity and PXN with melanin plays a role in cell adhesion, opsonization, and encapsulation. RB is one of the most important parameters to evaluate the defense system mechanism of vannamei shrimp. When foreign bodies are ingested by phagosomes, it involves the release of degradative enzymes that produce *reactive oxygen intermediates* (ROI) known as RB (Rodriguez & Moullac, 2000). The release of radical oxygen compounds or RB activity is closely related to oxidative enzyme activity that produces superoxide and oxidizes H_2O_2 compounds into other reactive compounds. Reactive compounds such as superoxide, hypochlorous acid, *hydroxyl radical*, *peroxynitrite,* and nitrile chloride will kill pathogenic compounds in the *phagolysosome* (Smith *et al.,* 2010).

According to Effendi (2016) RB is one of the shrimp immune parameters related to phagocytosis reactions which are the most common reactions in shrimp cellular defense. Probiotics are an important source of nutrients and can produce enzymes, such as amylase, protease, and cellulose, to improve nutrient utilization and growth performance (El-Saadony *et al.,* 2021). Giving probiotics and paraprobiotics *L. paracasei* can increase the activity of digestive enzymes in vannamei shrimp. This is in accordance with the statement of Huynh *et al*. (2017) that the addition of probiotics to feed can increase the activity of digestive enzymes, the diversity of microbiota of the digestive tract, intestinal microvilli, and is able to absorb nutrients in cultured organisms. Probiotics can increase digestive enzyme activity because they can produce exogenous enzymes including lipase and protease.

According to Sewaka *et al*. (2019) digestive enzymes produced by shrimp include protease, amylase, and lipase which play a role in digestion and assimilation of feed. If enzyme activity increases, the overall body metabolism can increase. Zheng *et al*. (2019) stated that microorganisms and exoenzymes have a role in the digestive process by increasing the activity of intestinal enzymes and stimulating the production of endoenzymes that can improve food digestibility and nutrient utilization. Protein retention value illustrates the utilization of feed nutrients digested by the shrimp body and will be absorbed and stored to produce energy (Dahlan *et al.,* 2017). The low value of protein retention in

the control treatment is due to the absence of the addition of probiotics to the feed, so the availability of protease enzyme-producing bacteria in the digestive tract is limited. This limitation causes the lack of absorption of feed protein which is not assisted by the presence of probiotic bacteria so the absorption of feed protein is not optimal.

Meanwhile, the low-fat content in the control treatment of shrimp meat states that the fat that has been absorbed from the digestive process is used by shrimp as a source of energy and metabolic processes. The lipids are transported to several organs and tissues during a certain time (Fahrudin and Subandiyono 2023). Fat is used for energy and maximizes protein for growth (Boonyaratpalin, 1996). Total *V. parahaemolyticus* in the target organs tended to be in the treatment of probiotics and paraprobiotic *L. paracasei* showed lower values than the positive control. This is in accordance with the statement of Saiz *et al*. (2019) that lactic acid bacteria can inhibit pathogens by producing inhibitory compounds, preventing adhesion, competing for nutrients, and modulating the host system. Wold (2001) also said that probiotics enhance the immune system by inducing IgA formation, macrophage activation, proinflammatory cytokines, and antioxidants.

The overall results showed that this study gave better results in both probiotic and paraprobiotic treatments. According to Borrero *et al*. (2018) antibacterials in probiotics can produce hydrogen peroxide, lactic, acetic, and other organic acids, synthesize lysozyme and bacteriocins with a broad spectrum of action (lactococci, enterosin, sublansin, aureosin, gassericin, closticin, thurisin, subtilisin), these antibacterials can reduce intestinal wall permeability disorders caused by bacterial and viral infections, reduce intestinal epithelial apoptosis, and help maintain cytoskeleton integrity (Roman *et al.,* 2014). In addition, probiotics can increase IgA, stimulate local interferon release, which facilitates antigen transport to lymphoid cells underlying the intestinal wall and promotes phagocytosis, and can contribute to the suppression of pro-inflammatory cytokines and lower IgE levels (Araujo *et al.,* 2016). Meanwhile, paraprobiotics are derived from microorganisms that lose their viability after exposure to factors that alter the microbial cell structure such as DNA filament breaks, cell membrane disruption or mechanical damage to the cell envelope. In addition, viability can be lost due to changes in microbial physiological functions, such as inactivation of key enzymes or deactivation of membrane selectivity (Barros *et al.,* 2021). In general, mechanisms of inactivation by heat can result in membrane damage, loss of nutrients and ions, ribosome aggregation, DNA filament rupture, inactivation of essential enzymes and protein coagulation (Cebrian *et al.,* 2017).

CONCLUSIONS

Feeding probiotics and paraprobiotics *L. paracasei* is effective in improving protein fat retention, digestive enzyme activity, growth performance, immune response, and resistance of vannamei shrimp to *Vibrio parahaemolyticus* infection. Feed supplementation with 1% (v/w) probiotic *L. paracasei* with cell density of 10^9 CFU/mL was the most effective treatment for the disease control of *V. parahaemolyticus* in vannamei shrimp.

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