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Corn fermented protein in production diets for pacific white legged shrimp *Litopenaeus vannamei*: Improved growth performance, health status and resistance to infection

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ABSTRACT

Two separate trials were conducted to evaluate the use of a novel Corn Fermented Protein (CFP) on growth performance and feed utilization of Pacific white legged shrimp *Litopenaeus vannamei* in out-door pond conditions as well an independent pathogen challenge (*Vibrio harveyi*) test under controlled conditions following an internal feeding trial with varying inclusions of CFP. In out-door ponds, the 12% inclusion level of CFP to completely replace the use of corn gluten meal (CGM) was shown to support shrimp growth in the pond that was historically heavily infected with pathogen, including white spot syndrome virus (WSSV) and acute hepatopancreatic necrosis disease (AHPND). In an indoor growth trial parallel with the pond challenge study, CFP at the level of 6%, 12% and 18% at the expense of fish meal (FM), soybean meal (SBM) and CGM were also able to improve growth and several related parameters such as final body weight, percentage weight gain and thermal growth coefficient (TGC). The feed conversion ratios (FCR) for shrimp fed with CFP were also lower compared to the shrimp fed without CFP. Numerically, despite no statistical difference, survivability, and total haemocyte count were also elevated for shrimp receiving CFP. The challenge test results showed that the mean cumulative survival rate of shrimp injected with *V. harveyi* at the dose of 5×10^4 CFU shrimp^{-1} were higher in the group of shrimp fed CFP compared to the control treatment. Values for mean phagocytosis activity were superior for fermented corn-fed shrimp. Meanwhile, haemocyte profiles remained uniform across treatments and phagocytic index was significantly reduced at 18% inclusion level of CFP. Phenoloxidase activity post-challenge with *V. harveyi* was elevated in shrimp fed CFP at all levels. These findings suggest that 6 – 12% inclusion of CFP can be utilized as a novel ingredient for shrimp feed. Advocating for functional ingredients with characteristics that can support health and welfare of intensively farmed shrimp will be of strategic importance in the future for sustainable production of shrimp under intensive conditions.

1. Introduction

The balance of nutrients in modern compounded aquafeed able to meet the specific nutritional requirements of aquatic production to optimize their growth and health condition becomes one of the essential elements to support the rapid growth of aquaculture production worldwide (Li et al., 2009; Nates, 2015; Novriadi and Davis, 2018; Novriadi et al., 2019b). In the midst of efforts to reduce the inclusion of fish meal (FM) in the diet formulation, the nutrient supply could be achieved by proper blending of several alternative protein sources to produce sufficient protein, digestible amino acids, and energy levels (Hodar et al., 2020; Kaushik et al., 2004; Luo et al., 2006; Novriadi et al., 2019a). Several alternative protein sources have been evaluated to be used for complementation in the diet formulation, including soybean meal, cotton seed meal, and corn protein (Lim, 1996; Novriadi, 2018; Novriadi et al., 2019b; Novriadi et al., 2017). Among the ingredients,

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products derived from corn are some of the most promising because of their excellent protein-richness (Molina-Poveda et al., 2015), price, favorable protein and amino acid composition (Novriadi et al., 2022b) and absence of anti-nutritional factors (Hisano et al., 2016).

Newly developed ingredients derived from the dry-mill ethanol production process are now available in the market and can be used to formulate the shrimp feed (Galkanda-Arachchige et al., 2021; Novriadi et al., 2022a; Novriadi et al., 2022c). Based on our previous results (Novriadi et al., 2022c), the inclusion of 12% CFP to partially replace SBM and completely replace CGM were able to enhance the growth of shrimp *Litopenaeus vannamei* compared to the control group. In addition, the use of 12% corn fermented protein (CFP) could improve the biomass, final mean weight and better feed conversion ratio (FCR) compared to the control treatment (Novriadi et al., 2022c). The work of Guo et al. (2019) further demonstrated that the inclusion of CFP could be used up to 20% to effectively replace corn protein concentrate (CPC) or up to 15% to replace the use of FM in a typical diet formulation for shrimp *L. vannamei*. The superior crude protein level contained in CFP obtained from a corn post-distillation process could explain the better growth of shrimp during the culture system (Novriadi et al., 2022c).

Another advantage on the use of CFP in aqua feed formulation is the presence of yeast, up to 25%, and those microorganisms have shown the potential to produces several bioactive substances, such as glucans, enzymes, and vitamins to enhance immunity, growth and protection against pathogen infection (Ernesto Ceseña et al., 2021; Lara-Flores et al., 2003; Li and Gatlin III, 2003; Sarkar and Rao, 2016). Specifically in shrimp, supplementing yeast into feed has shown to improve growth, intestinal microbiota and immune response against *Vibrio harveyi* infection (Ayiku et al., 2020). Functional roles of yeast have also been observed to increase the total haemocyte count (THC) level at pre- and post-infection with White Spot Syndrome Virus (WSSV) (Sajeevan et al., 2006) and elevate phenoloxidase (PO) and lysozyme activities which indicates potential protection against invading pathogens (Ernesto Ceseña et al., 2021; González et al., 2009).

Therefore, this research was conducted to evaluate the effect of CFP (NexPro™. POET Bioproducts. Sioux Falls, SD. USA) on growth performance of shrimp cultured in a heavily infected pond scenario while replacing portions of costly ingredients in *L. vannamei* diets. A separate feeding trial followed by challenge infection under laboratory conditions with *V. harveyi* allowed for data validation of diet effects in isolation. The findings of this study might provide useful information on the development of functional feed type ingredients for dual purposes in supporting better growth performance whilst mitigating infection and disease in intensively farm raised shrimp.

2. Materials and methods

2.1. Diet preparation

2.1.1. Diet preparation for commercial trial

For the commercial trial, shrimp were fed one of two diets: (Diet 1) 12% CFP (NexPro™. POET Bioproducts. Sioux Falls, SD. USA) which completely replaced the corn gluten meal (CGM) component based on study from Novriadi et al. (2022c) or (Diet 2) a commercial shrimp feed with protein level between 34% and 36% (CJ Feed code SA, East Java, Indonesia). For 12% CFP, cooking-extrusion process were employed to produce the experimental diet in the form of powder, crumble, and pelleted diet with size of 0.2 – 0.8 mm and 0.8 – 1.2 mm. The temperature degree in the conditioner was 98–100°C and the conditioner retention time was 120 – 150 s. Feed pellets were air-dried to approximately 10% moisture, sealed in vacuum-packed plastic bags. For the commercial feed, cooking-extrusion processes were also utilized to produce the crumbles, and pelleted diet with size of 0.2 – 0.8 mm and 0.8 – 1.4 mm. All experimental and commercial feed were stored in a temperature-controlled room until further use.

2.1.2. Diet preparation for controlled feeding trial

For the growth trial using a controlled environment, the experimental diets were formulated like the previous study described by Novriadi et al. (2022) where the control diet was designed based on a similar approach to the Indonesian shrimp feed market by utilizing 10% FM, 47.2% SBM, 8.0% corn gluten meal (CGM) and 17% wheat flour (WF) (Table 1). Three experimental diets were formulated to utilize titrated inclusion levels of corn fermented protein (CFP, NexPro™, POET Bioproducts, SD, USA) to be added into the basal diet at 6% and 12%. The fourth diet was designed to partially replace the use of FM and completely replace the use of CGM with 18% inclusion level of CFP. All experimental diets were produced at the Main Center of Mariculture Development of Lampung (Lampung, Indonesia) using cooking-extrusion process. Briefly, all ingredients were mixed in a paddle mixer (Marion Mixers, Inc., Marion, IA, USA) in a 100 Kg batch followed by grinding to a particle size of < 200 μm using a disk mill (Jinan Shengrun, China). The cooking-extrusion diets exposed to an average of 100–110 °C for approximately 14 s in five-barrel sections and the last section was maintained at 62 °C. Pressure at the die head was approximately 50 bars, and screw speed was maintained at 423 rpm. Experimental feed than produced through 1 and 2 mm die to produce 1.5- and 2.5 mm diet particles. Diets were dried in a pulse bed dryer (Jinan Shengrun, China) until moisture readings were below 10%. Pellets were dried at approximately 107 °C with an upper limit outflow air temperature of approximately 88 °C for 8 – 10 h. All finished diets were

Table 1
Composition (% *as is*) of diets containing corn fermented protein (CFP) into the basal diet and fed to *L. vannamei* for 60 days under controlled-environmental condition.

Ingredients (% *as is*)	Diet code			
	Control	6% CFP	12% CFP	18% CFP
Menhaden Fishmeal[a]	10.00	10.00	10.00	7.50
Soybean meal[b]	47.20	44.80	45.20	42.20
Corn Gluten Meal[b]	8.00	5.00	0.00	0.00
Corn Fermented Protein[c]	0.00	6.00	12.00	18.00
Menhaden fish oil[d]	5.22	5.14	5.05	5.10
Corn Starch[b]	6.38	5.86	4.55	4.00
Wheat products[d]	17.00	17.00	17.00	17.00
Mineral premix[e]	0.50	0.50	0.50	0.50
Vitamin premix[f]	1.80	1.80	1.80	1.80
Choline chloride[b]	0.20	0.20	0.20	0.20
Stay-C 35%[g]	0.10	0.10	0.10	0.10
Soy-lecitihin[b]	1.00	1.00	1.00	1.00
Cholesterol[h]	0.10	0.10	0.10	0.10
KP dibasic[h]	2.50	2.50	2.50	2.50
Proximate analysis (% *as is*)[g]				
Crude protein	37.13	38.58	38.49	38.56
Lysine	2.01	1.97	1.98	1.98
Methionine	0.82	0.80	0.82	0.78
Moisture	7.68	7.82	7.51	7.59
Crude Fat	8.13	8.38	8.72	8.95
Crude Fiber	3.56	3.49	3.63	3.76
Ash	6.15	6.90	5.64	6.30

[a] High protein fish meal (Peru) supplied by Agri Permata Asia, Jakarta, Indonesia
[b] FKS Multi Agro, Jakarta, Indonesia
[c] NexPro™, POET Bioproducts, Sioux Falls, SD, USA
[d] PT Pundi Kencana, Cilegon, Banten, Indonesia
[e] Trace mineral premix (g/100 g premix): cobalt chloride, 0004; cupric sulfate pentahydrate, 0550; ferrous sulfate, 2000; magnesium sulfate anhydrous, 13,862; manganese sulfate monohydrate, 0650; potassium iodide, 0067; sodium selenite, 0010; zinc sulfate heptahydrate, 13,193; alphacellulose, 69,664,
[f] Vitamin premix (g/kg premix): thiamin·HCL, 4,95; riboflavin, 3,83; pyridoxine·HCL, 4,00; Ca-pantothenate, 10,00; nicotinic acid, 10,00; biotin, 0,50; folic acid, 4,00; cyanocobalamin, 0,05; inositol, 25,00; vitamin A acetate (500,000 IU/g), 0,32; vitamin D3 (1000,000 IU/g), 80,00; menadione, 0,50; alpha-cellulose, 856,81
[g] DSM Nutritional products
[h] Supplied by Bogor Ingredients, Indonesia

bagged and stored in a temperature-controlled room until further use. Proximate analyses of the diets were analyzed at the SUA Integrated Fish Farm. Bogor Agricultural University. Bogor, West Java. Indonesia.

2.2. Feeding trial

2.2.1. Commercial feeding trial

The commercial field trial was conducted in four open-pond systems with dimensions of 50 × 40 × 1.5 m per pond at the CV Horas Tambak Sanjaya (Langkat, North Sumatera, Indonesia) and labeled as C1, C2, C3 and C4. The ponds used in this study were known to be previously heavily infected with pathogens. Among the ponds, two ponds never gave a good production yield due to disease infection, namely pond C2 and C4 and were utilized to challenge the experimental diet containing 12% CFP. The other ponds, C1 and C3, having a less severe condition were used to culture the shrimp fed with the commercial diet (CJ Feed code SA, East Java, Indonesia). The density used in this study was 100 PL m^{-3} and considered as an intensive culture system. The two replicate treatment shrimp groups were fed by hand four times per day at 7:00; 11:00; 15:00; and 20:00 h following nutrition research standard protocol. Based on our historic results, feed inputs were pre-programmed assuming the normal growth of shrimp and feed conversion ratios of 1.5. Daily allowances of feed were adjusted based on observed feed consumption and weekly counts of shrimp mortality.

2.2.2. Controlled feeding trial

The controlled feeding trial and subsequent challenge test were conducted in the Fish and Environmental Health laboratory Department (Gadjah Mada University, Yogyakarta, Indonesia) and Sundak Mariculture Facilities (Yogyakarta, Indonesia), and maintain according to the animal care policy. Pacific white shrimp post larvae (PL) were obtained from a private commercial shrimp hatchery PT. Maju Tambak Sumur (Kalianda, Lampung, Indonesia) and nursed in a semi-indoor recirculating system. Quality of water was maintained by recirculation through vertical sand filter (Dab Pumps S.p.A., Mestrino, Italy). Dissolved oxygen was maintained near saturation using air stones in each culture tank and temperature was maintained in the range of 20–30 °C. Post-larvae were fed a commercial feed (Evergreen Feed, Lampung, Indonesia) for three weeks until reach the suitable size. Shrimp (1.04 ± 0.05 g initial mean weight) were stocked into 70 × 35 × 40 cm^3 (98 L) tanks with 15 shrimp per tank. Five replicate shrimp groups were offered experimental diets using nutrition research standard protocol for 60 days. Based on our historic results (Novriadi et al., 2022c), feed inputs were pre-programmed assuming the normal growth of shrimp and employing a standard feed conversion ratio of 1.5. Daily allowances of feed were adjusted based on observed feed consumption and weekly shrimp counts.

2.2.3. Water quality and growth sampling

For all feeding trials, parameters of pH, dissolved oxygen (DO), water temperature and salinity were measured four times daily using Aqua TROLL 500 Multiparameter Sonde instrument and connected to Aqua-Easy apps (Bosch, Singapore) for data monitoring and recording system. Total ammonia-nitrogen (TAN), nitrate and nitrite were measured weekly by using absorption spectrophotometry (DR890, HACH, USA). At the end of their respective feeding periods, the shrimp in the commercial pond study were group weighed and for the controlled study, shrimp were grouped and individually weighted to calculate final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), survival rate (SR), and thermal unit growth coefficient (TGC) as follows:

$$\mathrm{PWG} = \frac{(averageindividualfinalweight - averageindividualinitialweight)}{(averageindividualinitialweight)} \times 100$$

$$\mathrm{FCR} = \frac{feedgiven(g)}{aliveweightgain(g)}$$

$$\mathrm{SR} = \frac{finalnumberofshrimp}{initialnumberofshrimp} \times 100$$

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

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

2.3. Challenge test trial

In parallel with controlled feeding trial, a challenge test was performed by using 20 shrimps per aquaria tank that had been acclimatized to laboratory conditions. Prior to challenge, shrimp with initial mean weight of 4.01 ± 0.04 g were randomly distributed into 20 units of glass aquarium with size of 70 × 35 × 40 cm^3 (98 L per aquaria tank) and fed with experimental diets following the nutrition research standard protocol for 30 days. Shrimp were fed by hand four times daily at 07:00, 11:00, 15:00 and 20:00 h. On the 30th day of dietary treatment, the challenge test was carried out by injecting *V. harveyi* suspension at a final cells density of LD_{50} (5×10^4 CFU $shrimp^{-1}$). Mortality was monitored for 96 h after the challenge. Protective effects of experimental feed were evaluated based on the following relative percentage survival (RPS) value (Amend, 1981). Immune-related parameters were immediately examined after the termination of the challenge test.

$$\text{Relative percentage survival} = \left(1 - \frac{\%treated\ mortality}{\%control\ mortality}\right) \times 100$$

2.4. Shrimp immune response observation

Immune parameters were **e**xamined for two observation schemes: (1) measurement of THC at the end of 60-day controlled growth trial and (2) measurement of THC, phagocytosis activity, phagocytic index, and phenoloxidase activity at the end of challenge test.

2.4.1. Total haemocyte count (THC)

Total haemocyte count (THC) was directly determined after the 60 days of feeding trial under controlled environment and at immediately after the termination of challenge test. For THC determination after 60 days feeding trial, 2 shrimp per aquaria tank or ten shrimps per dietary treatment were randomly collected, while for THC determination at the end of challenge test, as much as four shrimp per tank or sixteen shrimp per dietary treatment were collected. Calculation of total haemocyte counts (THC) was completed by mixing 20 μL of hemolymph and 80 μL of phosphate buffer saline (PBS, 5 times dilution). After mixing, 20 μL from the mixture was put into a hemocytometer (Hausser Scientific, USA) and observed under a 40x magnification microscope (Campa-Cordova Modification, 2002). Total haemocyte count was determined as followed:

THC = number of haemocyte (1/5 × 25 × dilution rate × 10^4 cells mL^{-1}.

2.4.2. Phagocytic activity (PA) – phagocytic index (PI)

Phagocytic activity test was carried out by diluting 20 μL of hemolymph in 10 μL phosphate buffer saline (PBS), with the addition of 30 μL formalin killed-Vibrio cells (the density of 1 ×10^8 cells mL^{-1}). The sample was then incubated at room temperature for 30 min. Post incubation, 5 μL from the mixture was smeared on the object glass, fixed using 2.5% glutaraldehyde for 20 min, followed by rinsing in 0.85% NaCl to remove non-sticking cells, and then allowed to air-dry overnight and stored air in the dark. The samples were stained using a 10% wright stain (Chotigeat et al., 2004) for 20 mins, rinsed in tap water, and air-dried. Samples were then observed under a microscope with

100 × magnification to calculate the number of engulfing phagocytes per observed phagocytes. The phagocytic index, which determined the average number of engulfing particles per phagocytes, was also calculated according to the formula:

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

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

2.4.3. Phenoloxidase (PO) activity

Phenoloxidase (PO) activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA). A total of 100 μL of hemolymph was diluted in PBS (1:1), and centrifuged at 700 *g* at 4 °C for 20 min. The supernatant was removed while the remaining pellet was diluted in 100 μL of cacodylate citrate buffer (0.1 M sodium cacodylate trihydrate; 0.45 M NaCl, and 0.01 M sodium citrate), and centrifuged at 700 *g* at 4 °C for 20 min. The supernatant was then discharged and the pellet was diluted again with 100 μL cacodylate buffer (0,01 M sodium cacodylate trihydrate; 0.45 M NaCl; 0.01 M $CaCl_2$0.2 H_2O; 0.26 M MgCl 0.6 H_2O). The supernatant was transferred into a 96well micro plate with the addition of 100 μL of Trypsin (Sigma Aldrich), re-suspended, and incubated at room temperature for 10 min. Post incubation, fifty μL of L-DOPA was added to the well and the absorbance was measured using the microplate reader (R-Biopharm Well Reader, Germany) at 490 nm.

2.5. Statistical analysis

Growth performance, total haemocyte count, survival of shrimp after challenge test and immune-related parameters were analyzed using one-way analysis of variance (ANOVA) to determine significant differences among treatments followed by Tukey's multiple comparison tests to determine the difference between treatment means. All statistical analyses were conducted using SAS system (V9.4. SAS Institute, Cary, NC, USA). Differences considered significant at a *P*-value of 0.05 with tendencies considered significant or insignificant for confidence interval of 95%. All Errors bars indicate standard deviation.

3. Results

3.1. Water quality parameters

The results for water quality parameters during the commercial and controlled-environment feeding trials are shown in Tables 2 and 3, respectively. The measured water quality at the commercial feeding trials demonstrated that parameters such as dissolved oxygen (D.O), temperature, salinity and pH remain within acceptable range for shrimp culture. Ammonia (NH_3-N) in 12% CFP were higher compared to the control ponds. In addition, level of nitrite (NO_2-N) remains higher in 12% CFP pond compared to control pond. Other parameters, such as nitrate (NO_3-N), phosphate (PO_4-N), alkalinity, calcium and magnesium fluctuated among the culture ponds. With regards to the controlled-environment feeding trial, the measured water quality in term of D.O; temperature; salinity; pH; NH_3-N; NO_2-N; NO_3-N; and PO_4 still within the optimum range for shrimp culture.

3.2. Feeding trial

3.2.1. Commercial feeding trial

For the very first time, the key farmer participating in this research was able to successfully harvest shrimp from pond C-2 and C-4 that were heavily infected by pathogens with survival rate more than 70%. In this growth trial, it was observed that the average individual weights were improved in shrimp fed 12% CFP (13.16 – 13.51 g) compared to the commercial group (12.99 – 13.06 g; Table 4). The lowest FCR was also observed in the group of shrimp fed 12% CFP compared to the commercial diet. There is a consistency in terms of survival rate in the group of shrimp fed with 12% CFP with comparable amount of biomass among the dietary treatment.

Table 2,
Water quality data during the commercial feeding trial for 85 – 86 days in the out-door ponds, Data were presented as mean± standard deviation (range).

Parameters	Unit	Analysis results			
		C-1	C-2	C-3	C-4
Dissolved oxygen	mg L^{-1}	6.85 ± 0.82	6.53 ± 0.34	6.78 ± 0.84	7.07 ± 0.94
Temperature	^{0} C	31.19 ± 0.98	31.93 ± 0.98	31.18 ± 0.82	31.10 ± 0.73
Salinity	‰	25.35 ± 1.22	25.96 ± 0.08	26.35 ± 0.06	26.21 ± 0.04
pH		7.99 ± 0.16	7.99 ± 0.09	8.03 ± 0.11	8.02 ± 0.09
Ammonia (NH_3-N)	mg L^{-1}	0.10 ± 0.10	0.19 ± 0.15	0.11 ± 0.11	0.23 ± 0.15
Nitrite (NO_2-N)	mg L^{-1}	1.66 ± 1.03	2.35 ± 1.99	1.12 ± 1.64	1.89 ± 1.87
Nitrate (NO_3-N)	mg L^{-1}	27.15 ± 42.13	22.92 ± 33.64	12.85 ± 31.11	20.84 ± 37.85
Phosphate (PO_4-N)	mg L^{-1}	2.92 ± 0.63	2.56 ± 0.67	3.05 ± 0.49	2.12 ± 0.39
Alkalinity	mg L^{-1}	169.69 ± 27.11	164.54 ± 25.97	174.31 ± 17.99	168.12 ± 17.39
Calcium	mg L^{-1}	243.00 ± 10.21	254.69 ± 14.82	295.46 ± 169.02	256.85 ± 18.95
Magnesium	mg L^{-1}	951.15 ± 98.54	944.92 ± 84.66	933.34 ± 284.47	978.23 ± 67.71

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

Parameters	Unit	Analysis results
Parameters	Unit	Results
Dissolved oxygen	mg L^{-1}	5.56 ± 0.32
Temperature	^{0} C	29.87 ± 0.42
Salinity	‰	26.11 ± 0.33
pH		7.75 ± 0.22
Ammonia (NH_3-N)	mg L^{-1}	0.02 ± 0.09
Nitrit (NO_2-N)	mg L^{-1}	0.02 ± 0.04
Nitrat (NO_3-N)	mg L^{-1}	3.29 ± 0.81
Posfat (PO_4-N)	mg L^{-1}	0.01 ± 0.01

3.2.2. Growth trial under controlled environment

For the controlled growth trial, it was observed that the shrimp fed with 12% CFP had significantly ($P = 0.03$) greater final biomass compared to the control with the 6% and 18% treatments being intermediate (Table 5). Final mean weight (FMW) was statistically ($P < 0.01$) greater for shrimp fed all diets containing CFP compared to the control. There was no difference ($P = 0.63$) in shrimp survivability between

Table 4,
Growth performance of Pacific white shrimp fed either commercial diet (pond C-1 and C-3) or 12% CFP-containing diet (pond C-2 and C-4) over a 85 – 86 days in pond system.

Pond	C-1	C-2	C-3	C-4
Day of culture (DOC)	85	85	85	86
Number of post larvae (PL)	200.000	200.000	200.000	200.000
Final Biomass, kg	1.870	1.924	1.984	1.975
Average individual weight, g	12.99	13.16	13.06	13.51
Survival rate, %	72.01	73.11	76.00	73.08
Total Feed, kg	2.737	2.646	3.038	2.800
FCR	1.46	1.38	1.53	1.42

Table 5,
Growth performance of Pacific white shrimp (mean initial weight 1.04 ± 0.05 g) fed the experimental diets for 60 d, Results in the same row with different superscript letter are significantly different (P < 0.05) based on analysis of variance followed by the Tukey's multiple comparison test,.

Diet code	Final Biomass (g)	Final Mean Weight (g)	Survival (%)	WG[a] (%)	FCR[2]	TGC[3]
Control	137.57[b]	10.67[b]	87.78	922.19[b]	1.44[a]	0.0685[b]
CFP 6%	160.21[ab]	11.61[a]	92.22	1001.09[a]	1.30[b]	0.0723[a]
CFP 12%	165.64[a]	11.88[a]	92.22	1023.14[a]	1.27[b]	0.0733[a]
CFP 18%	163.86[ab]	11.69[a]	93.33	1054.48[a]	1.28[b]	0.0736[a]
P-value	0.0258	< 0.0001	0.6270	< 0.0001	< 0.0001	< 0.0001
PSE[4]	10.1622	0.1930	5.1400	0.2316	0.0280	0.0011

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

treatments, however, shrimp fed diets containing CFP had a 5.48% greater survivability rate compared to shrimp fed the control diet (average 92.59 versus 87.78, respectively). Similarly to FMW, shrimp fed any inclusion (6%, 12% or 18%) of CFP had greater weight gains, improved FCR, and higher thermal growth coefficients ($P < 0.01$, respectively) compared to shrimp fed with the control treatment. At the end of growth trial, there was a significant improvement on growth performance when 12% CFP was used to replace the utilization of SBM and corn gluten meal in diet composition. (Fig. 1).

3.3. Challenge test

Mortality of shrimp occurred between 12 h and 36 h post-infection with signs of weakness, passive swimming on the surface, milky white abdominal muscles, anorexia, poor feeding, reddish-yellow coloration of the hepatopancreas, and reduced growth rate. Shrimp that were fed with 12% CFP had the greatest ($P < 0.05$) survival rate with 6% and 18% CFP being intermediate, and the positive control having the lowest out of the four experimental treatments (Fig. 2).

3.4. Immune parameters of shrimp

3.4.1. Total haemocyte counts at the end of 60-days feeding trial and challenge test

Dietary treatments did not significantly affect the total haemocytes count after 60-day of feeding trial (Fig. 1) and at the termination of challenge test (Fig. 3). However, numerically, the THC in the group of shrimp fed with 12% CFP after 60 days of feeding trial were higher compared to other dietary treatment. At the termination of challenge test, all shrimp fed with CFP showed increasing number of THC compared to the control treatment (Fig. 3).

3.4.2. Phagocytic activity (PA) – phagocytic index (PI)

The phagocytic activity and phagocytic index of post challenged shrimp can be viewed in Figs. 4 and 5, respectively. Shrimp fed diets containing 6% and 12% CFP had significantly ($P < 0.01$) higher phagocytic activity with 18% CFP being intermediate and the control treatment being the lowest. The phagocytic indexes of experimental shrimp post challenge were significantly higher in shrimp fed with 12% CFP, intermediate with 6% CFP and the control, and lowest for the 18% CFP treatment ($P = 0.0187$).

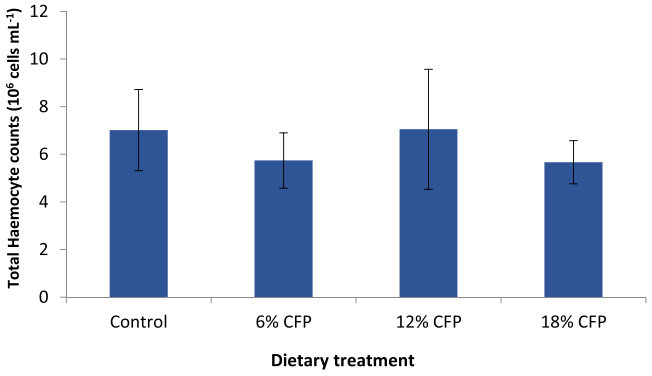

Fig. 1,. Total hemocyte counts (THC) of Pacific white shrimp *Litopenaeus vannamei* (10^6 cells mL^{-1}) at the end of 60-day growth trial (controlled condition), No differences were observed between treatments (*P-value*: 0.5028). Errors bars indicate standard deviation.

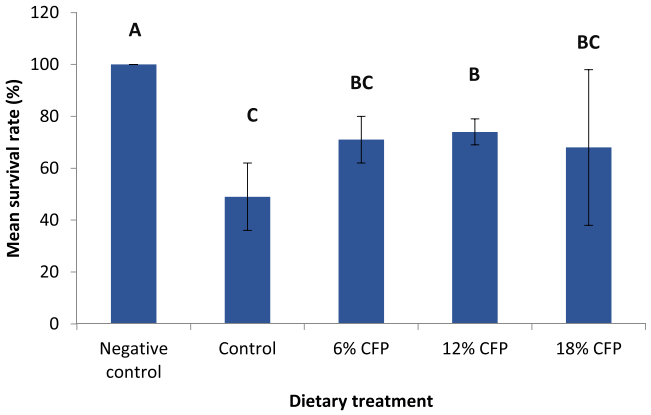

Fig. 2,. Mean survival rate at the end of challenge test infected with *Vibrio harveyi* at the dose of 5×10^4 CFU $shrimp^{-1}$, Different letters indicate statistically significant differences ($p < 0.05$) among the dietary treatment. Errors bars indicate standard deviation.

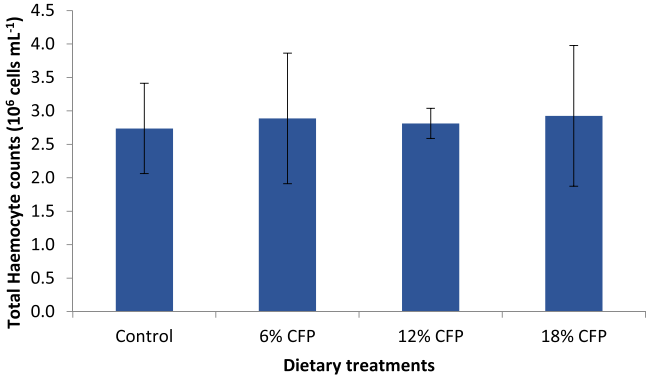

Fig. 3,. Total hemocyte counts (THC) of experimental shrimp *Litopenaeus vannamei* (10^6 cells mL^{-1}) at the termination of challenge test. Errors bars indicate standard deviation.

3.4.3. Phenoloxidase (PO) activity

The PO activity of post challenged shrimp can be observed in Fig. 6. The highest PO activity was shown in the group of shrimp fed with 6% CFP, intermediate with 12% and 18% CFP and the lowest for the control treatment ($P < 0,05$).

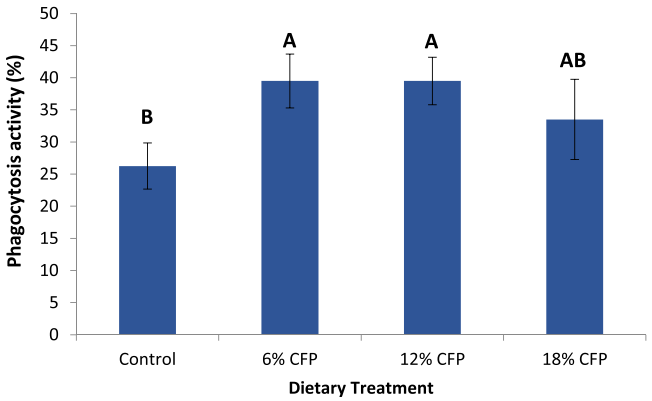

Fig. 4,. Mean percentage phagocytosis activity in shrimp blood cells after challenge test with *vibrio harveyi* at the dose of 5×10^4 CFU shrimp^{-1}, Different letters indicate statistically significant differences (P=0.0041) among the dietary treatment. Errors bars indicate standard deviation.

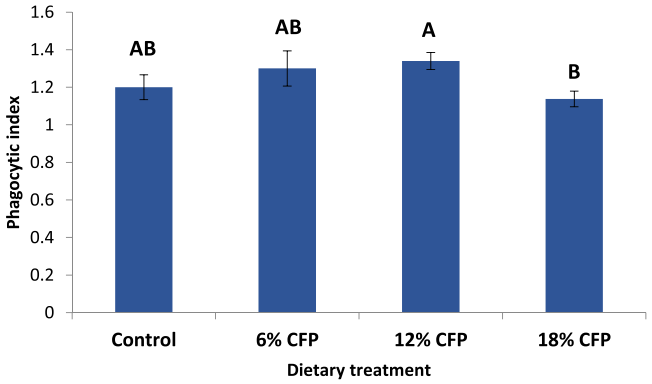

Fig. 5,. Phagocytic index in shrimp blood cells after challenge test with *Vibrio harveyi* at the dose of 5×10^4 CFU shrimp^{-1}, Different letters indicate statistically significant differences (P=0.0187) among the dietary treatment. Errors bars indicate standard deviation.

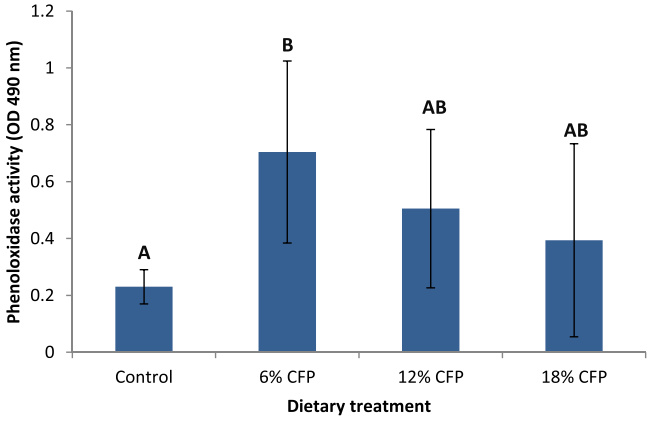

Fig. 6,. Phenoloxidase activity of experimental shrimp survivor after challenge test with *Vibrio harveyi* at the dose of 5×10^4 CFU shrimp^{-1}, Different letters indicate statistically significant differences ($P < 0.05$) among the dietary treatment. Errors bars indicate standard deviation.

4. Discussion

The current investigation has demonstrated that corn fermented protein (CFP) can be an effective contributor to the feed formulation of Pacific white shrimp *L. Vannamei*. Growth performance and feed utilization efficiency of shrimp fed with CFP cultured in ponds that were heavily infected with pathogen, including white spot syndrome virus (WSSV) and acute hepatopancreatic necrosis disease (AHPND) showed similar performance with shrimp cultured in normal ponds and fed with common commercial feeds used routinely in Indonesia. Across Asia, the shrimp cultured in AHPND-regions has dropped considerably (to ~60%) and caused massive economic losses (Kua et al., 2016; Kumar et al., 2019; Lee et al., 2015; Shinn et al., 2018). In addition, the WSSV infection can cause 100% accumulative mortality in 2 – 10 days (Wu et al., 2005). In our research, despite receive similar water sources, shrimp cultured in pond C-2 and C-4 (12% CFP) also experienced higher level of NH_3-N and NO_2-N during the culture period compared to the pond loaded with commercial diet. The successful production of shrimp reaching consumption size could be due to the availability of yeast in the CFP product stimulating immune response to the shrimp against pathogen and environmental stressors. Previous studies from McLean et al. (2006) revealed that the use of organically certifiable yeast-based protein which replaced the FM component on a unit protein basis using commercial ponds were able to achieve equivalent growth to that of *L. vannamei* fed the traditional, commercial shrimp diet. Furthermore, study from Chen et al. (2020) demonstrated that the inclusion of hydrolyzed yeast provide positive impacts to increase the ammonia resistance of *L. Vannamei* during the culture period. Thus, the present investigations illustrates that the use of yeast in combination with low-cost alternative ingredients was able to enhance the growth and survival of shrimp and attained similar performance or better with shrimp fed with marine or high-cost ingredients.

Under a controlled environment, growth performance and feed utilization efficiency as measured by weight gain and FCR was typical for shrimp under experimental conditions. Previous work has shown clearly that shrimp show superior overall performance when diets are inclusive of up to 18% corn fermented protein (Novriadi et al., 2022; Davies et al., 2021; Davies et al., 2022). Many similar such studies have been reported to assess the biological value of DDGS and more lately advanced corn product, such CFP, in both fish and shrimp. Previously, Deng et al. (2012) had investigated the effects of a yeast-based additive on growth and immune responses of white shrimp showing favorable effects on performance such as biomass increase and feed conversion.

In the present investigation with *L. vannamei*, the performance parameters all confirmed reported studies for this species within the expected thresholds. Additionally, after challenge with the specific pathogen, *V. harveyi,* we observed strong evidence that this product can be effective in the enhancement of general immune and defensive mechanisms in shrimp against infection. It was pertinent to observe that numerically, the increase in total hemocyte counts in shrimp fed with several inclusion levels of CFP after subjection to the pathogenic challenge. This was possibly indicative of the degree of tolerance induced by the functionality of key functional components in the commercial product thereby able to trigger the increasing need for hemocyte activity due to the suppression of the disease effects on inflammatory requirements.

In the current study, the degree of phagocytosis in the hemolymph of shrimp was elevated appreciably in groups fed the CFP suggesting a direct mediation of innate cell activity concomitant with an overall higher phagocytic index in group of shrimp fed with CFP compared to the control treatment. This could be interpreted as the ability of CFP to enhance the cellular defense reactions in shrimp. Study from Rairat et al. (2022) demonstrated that the phagocytic activity of *L. vannamei* significantly increase in the group of shrimp fed with diet supplemented with several inclusion level of yeast-derived nucleotide and yeast-derived RNA compared to the basal diet. The results of the present study also concur with the observation conducted by Pope et al. (2011) where the haemocytes from *V. harveyi*-injected shrimp showed elevated levels of phagocytosis, these situations resulting in the possibility of intracellular killings of pathogen in shrimp *L. vannamei.*

The increased activity of PO is an interesting observation. The great

majority of PO activity (more than 90%) is in shrimp hemocytes. The enzyme activity can be primed by selective components of microorganism cell walls, such as lipo-polysaccharides (LPSs) and β-1,3-glucans, suggesting its involvement in non-self-recognition. We know that fermented grain such as corn will also contain yeast and yeast cell wall components especially galactomannans and β-glucans like those found structurally in bacterium types (Soltanian et al., 2009). Thus, similar mechanisms may exist to illicit a general increase in immune related activities. We also see stimulation of PO enzyme activity in *L. vannamei* receiving all diets containing CFP. Similar findings have been reported for PO by other investigators evaluating various feed supplements for shrimp. The stimulation of immune and related response has been observed in White legged shrimp by Xu et al. (2021). The influence of dietary β-1,3-glucan on growth performance, feed utilization, antioxidative and immune status of Pacific white shrimp, *L. vannamei* was earlier reported by Vargas-Albores and Yepiz-Plascencia (2000). It was stated by these authors that β-glucan binding protein (BGBP) in shrimp can react with β-glucans and the glucan–BGBP complexes. This can cause the induction degranulation and the activation of prophenoloxidase (proPO). It should be cautioned however that Huang et al. (2000) criticized the measurement of phenoloxidase as a marker of immune capacity in shrimp due to the variations and inconsistency of response to dietary stimulants. Therefore, reconsideration of phenoloxidase activity determination in white shrimp *L.vannamei* has been suggested by these investigators and research is needed to validate more consistent methods for antioxidant mechanisms for shrimp.

In conclusion we have demonstrated in this investigation the ability of a novel protein rich ingredient to effectively replace several major traditional feed ingredients in formulated diets for *L. vannamei* and support to global challenge to meet the protein gap with a sustainable alternative. In addition, the benefits of fermentation derived co-products such as CFP to improve shrimp health is a vital cost-effective gain in the quest to reduce the need for chemotherapeutics and antibiotics that can be environmentally challenging as well as conferring antimicrobial resistance (AMR) that may be becoming of global concern. Utilization of bioethanol co-product ingredients meets the circular bioeconomy and ethical aquaculture production of shrimp. Finally in terms of economic gain, it would be important to undertake a cost benefit exercise to evaluate the increased return and profit on shrimp fed the CFP product up to 18% dietary inclusion with an emphasis on biomass gain, feed conversion efficiency, feed costs and overall survivability as integral factors. This would be of paramount importance under practical situations where a chronic disease is present and where special functional feed ingredients like CFP may support shrimp resistance to infection and improve welfare.

CRediT authorship contribution statement

Romi Novriadi: Research conceptualization, Methodology, Validation, Formal analysis, Investigation, Draft creation, Supervision and Writing the original draft, **Indah Istiqomah:** Research conceptualization, Methodology, Validation, Formal analysis, Investigation, Draft creation, Supervision and Writing the original draft, **Alim Isnansetyo:** Research conceptualization, Methodology, Validation, Formal analysis, Investigation, Draft creation, Supervision and Writing the original draft, **Derek Balk:** Research conceptualization, Methodology, Validation, Formal analysis, Investigation, Draft creation, and Visualization. **Melissa Jolly-Breithaupt:** Research conceptualization, Methodology, Validation, Formal analysis, Investigation, and Funding acquisition. **Simon Davies:** Research conceptualization, Methodology, Validation, Formal analysis, Investigation, Draft creation, Supervision and Writing the original draft.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Ethical statement

All procedures and handling process in the present study were approved by the recommendations in the Guide for the Use of experimental Animals of the Jakarta Technical University of Fisheries.

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