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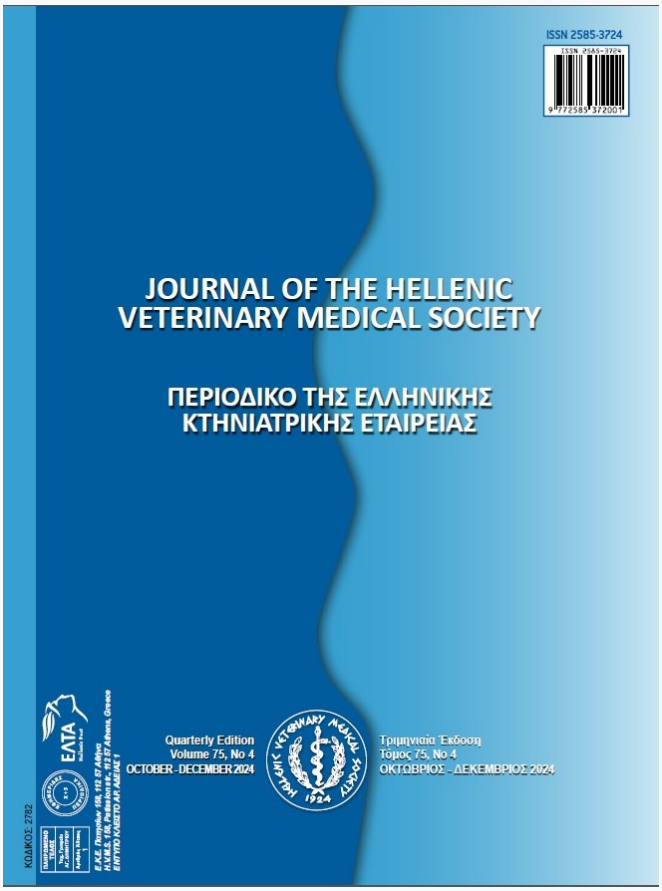

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Research article
Ερευνητικό άρθρο

Effect of fermented feed on the growth performance, health condition and immune response of white leg shrimp *Litopenaeus vannamei*

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ABSTRACT: The objective of this study was to evaluate the effects of rice bran fermented with commercial probiotic bacteria in relation to the culture density, with a focus on its impacts on the performance, health, microbiota, immune and transcriptomic responses of *Litopenaeus vannamei* as well as water quality. The white leg shrimp, *L. vannamei* (PL12) with an average weight of 0.34± 0.041 g was obtained from Egypt's shrimp hatchery at Ghallioun project and cultivated in a stocking density of 150 and 200 animals/ m^3. The shrimp were divided into three groups: three replicate each. Group I (G-I) was fed only on formulated feed (Control group), Group II (G-II) was fed on 50 % formulated feed and 50 % fermented feed and Group III (G-III) was fed on 100 % fermented feed base /ingredient. The results revealed that, the feed modification only considerably affected water temperature, but the stocking density significantly affected both the concentration of dissolved oxygen and the total ammonia nitrogen ($P<0.05$). The stocking density and feed fermentation had an impact on the individual / liter of the copepod presence in the culture pond. With 100% meal replacement, the maximum number of copepods was seen. High stocking density has resulted in a decrease in the number of copepods in the water. The phytoplankton decreased significantly as the food replacement increased. The highest growth performance was reported in shrimp larvae fed 100% formulated feed followed by 50% formulated feed and 50 % fermented feed then 100% fermented feed. The carbohydrate and the total ash percentage have increased while the crude protein content and fat content has decreased following the application of fermented feed. The application of fermented feed reduced the antioxidant capacity of *L. vannamei* while decreasing the expression of immune response genes and factors. In conclusion, using fermented feed may reduce the overall use of formulated feed and improve the health and immunity of shrimp larvae.

Keywords: *Litopenaeus vannamei; feed fermentation; growth performance; immunity; microbiota*

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INTRODUCTION

In 2020, global aquaculture production reached a new high score of 122.6 million tonnes, with a value reached USD 281.5 billion (FAO, 2022). Among all the cultivated species, the Pacific white shrimp (*Litopenaeus vannamei*) is the most commercial species in shrimp farming, accounting for more than 70% of total global production (Bardera *et al.,* 2021). This species' significance arose because of its traits and competency. It can withstand a variety of environmental conditions as well as stocking densities of up to 400 animal/m^3 (Bondad-Reantaso *et al.,* 2012; Ray *et al.,* 2010). Shrimp aquaculture has become one of the most rapidly increasing sectors of the aquaculture industry. This advancement, however, has been associated with increases in health-related issues and deterioration of environmental circumstances (Abdella *et al.,* 2023; Lauria *et al.,* 2018). It is known that the quantity and quality of feeds have a significant impact on aquaculture production (Sarker, 2023).

Several production outputs, such as feed conversion ratios, growth and survival rates, are jeopardized at densities greater than 100 shrimp per cubic meter (Bardera *et al.,* 2021). As aquaculture production intensifies, appropriate management measures must be employed. The carbon source may have an impact on both the water quality and growth performance of the growing species (Romano *et al.,* 2018). This is happening as a result of various unfavorable effects such as waste accumulation, deterioration of water quality, and cannibalism caused by heterogeneity in feed management and inadequate feed accessibility (Arnold *et al.,* 2006; Ruiz-Velazco *et al.,* 2010). The expense of feed is seen as the principal source of high market pricing in marine farmed species, along with a global supply scarcity of fish meal (Chen & Gao, 2023). Moreover, Sarker, (2023) stated that by 2050, there may be an estimated 0.4 to 1.32 million metric tonnes of fishmeal shortage, which would have a considerable negative impact on the aquaculture industry's growth. Because of the depletion of fishery stocks, alternative replacements for fish meal in fish feed are becoming increasingly important (Kokou & Fountoulaki, 2018). To give appropriate protein and reduce the release of nitrogenous compounds to the culture medium, which is significantly impacted by stocking density, shrimp, like any other aquatic species, requires a balanced and precise calculation of feed (Mehrim & Refaey, 2023). Moreover, the shift in carbon source may have an impact on growth performance and water quality (Romano *et al.,* 2018). Rice bran fermentation, as an example for the shift in carbon source, may improve digestibility while decreasing anti-nutritional content in aquatic animals' diet (Liñan-Vidriales *et al.,* 2021).

Furthermore, the use of plant-based protein sources in the fish diet increases presence of anti-nutritional factors, resulting in fewer advantages from the proteins available (Francis *et al.,* 2001; Magbanua & Ragaza, 2022). Because of its beneficial effects, the fermented diet has piqued the interest of nutritionists. Some of these advantages are anti-nutritional elements are decomposed during the fermentation process, whereas beneficial components such as short peptides, organic acids, probiotics, and flavonoids are also produced or boosted (Mukherjee *et al.,* 2015; H. Yang *et al.,* 2022). Different probiotic strains have been utilized in the fermentation of soybean meal to improve digestibility and its nutritional value (J. Huang *et al.,* 2023). Moreover, the fermentation has mitigated the negative effect of soybean's anti-nutritional factors and has improved the non-specific immune response of *Litopenaeus vannamei* (Lin & Mui, 2017).

Rice bran contains a protein content ranging from 10 to 15 % (Fabian & Ju, 2011). Previous research has shown that fermenting rice bran can enhance its protein content up to 23% (Chinma *et al.,* 2014). The increased protein content by fermentation makes it a prospective candidate for partial replacement of fish meal in shrimp diets. Furthermore, there is scarcity of information about the performance of the white leg shrimp *L. vannamei* in terms of its stocking density and feeding on a diet made up of fermented agricultural waste. Thus, the main goal of this study was to evaluate the effects of rice bran fermented with commercial probiotic bacteria in relation to the culture density, with a focus on its impacts on the performance, health, microbiota, immune and transcriptomic responses of *L. vannamei.*

MATERIAL AND METHODS

Shrimp husbandry and acclimation period

The research was conducted in a private marine farm at Borg-El Arab city, Alexandria, Egypt, from May to September 2021. The white leg shrimp, *Litopenaeus vannamei* (PL12) with an average weight of 0.34± 0.041 g was obtained from Egypt's shrimp hatchery at Ghallioun project. The shrimp were given the control diet contains 40 % crude protein (CP) and it was acclimatized to the culture environment for two weeks in oxygenated seawater at 15‰ salinity prior to the experiment. The acclimatization was carried out

using the protocol provided by Wabete *et al.,* (2006).

Feed fermentation

The fermented feed was prepared by the fermentation of the ingredients as follow: 20 kg of rice bran, 100 kg of molasses and100 g of commercial probiotics (Sanolife PRO-W ® contain a mixture of *Bacillus* species, INVE Company Belgium which contains 10^{11} CFU/gram) were mixed in one tonne of water and pH was adjusted to 7.9 using sodium bicarbonate. Then the mixture was aerated for 48 hours then added 500 liters/Fadden every day divided equally on feeding time and the quantity was increased based on the weekly growth monitoring.

Experimental population and treatments

With an initial weight of 0.34±0.041 g and a stocking density of 150 and 200 animals/ m^3, the acclimatized shrimp were divided into three groups (Table 1); three replicate each (one faddan and water capacity 4000 m^3). Group I (G-I) was fed only formulated feed (Table 2) with CP=40 % (control group), Group II (G-II) was fed on 50 % formulated feed with CP=40 % and 50 % fermented feed and Group III (G-III) was fed on 100 % fermented feed. The shrimps were fed the experimental diets up to apparent satiation at 8:00, 10:00, 12:00, 14:00, 16:00, 20:00 and 22:00 h for three weeks.

On weekly basis, 100 individuals were taken from each pond (100 animals/ pond) and transported to the laboratory for analysis of the various parameters.

Physico-chemical analyses of water

OxyGuard Handy Gamma was used to record temperature and dissolved oxygen twice daily (08:00 am and 3:00 pm). The pH and salinity were measured once each day (08:00 am) using a pH meter (Hach Lange HQ 40D). Total ammonia nitrogen (NH4+-N) was measured daily using a spectrophotometric technique using a portable photometer (Martini MI 405).

Phytoplankton and zooplanktons monitoring

During the experiment, the densities of phytoplankton, copepods, and zooplankton in general were taken into consideration by employing a 150-micron net to gather the various samples and examining them under a microscope. Water sampling for phytoplankton and water analysis were done weekly at 6-8 am. Composite samples were taken from four different points in each pond and stored in one-liter acid-washed polyethylene containers for analysis. Zooplankton abundance and profile were assessed at three different depths, aliquots (10 L) were taken from the corners and center of each pond (0.1, 0.5, and 1.0 m). The zooplankton were then characterized and counted using a stereoscope in a Sedgwick-Rafter chamber (Wildlife Supply Co. Buffalo, NY, USA). Microscopy was used to identify smaller organisms (10 X and 40 X).

Table 1: Experimental design of the trial with different stocking densities

Group	Stocking density 150/1-m^3	Stocking density 200/1-m^3
G-I	600000 shrimp/ faddan	800000 shrimp/ faddan
G-II	600000 shrimp/ faddan	800000 shrimp/ faddan
G-III	600000 shrimp/ faddan	800000 shrimp/ faddan

Table 2: Ingredients and proximate chemical composition (g/kg on dry matter basis) of the control diet

Ingredients	g/kg diet	Analysis	%
Fish meal (72% protein)	300	Dry matter	90
Soybean meal (45% protein)	300	Crude protein	40
Corn gluten (60% protein)	100	Ether extract	9
Corn grain	215	Crude fiber	3.3
Vegetable oil	40	Ash	12.7
Vitamins mixture	10	Nitrogen free extract[1]	35
Minerals mixture	10		
Di-Calcium phosphate	15	Gross energy (kcal/kg diet)[2]	4587.5
Anti-aflatoxin	5		
Vitamin C	5		
Total	1000		

[1]Nitrogen-Free Extract (calculated by difference) = 100 - (protein % + lipid% + ash% + fiber %). [2]GE (gross energy) was calculated according to NRC (1993) by factors of 5.65, 9.45 and 4.22 kcal per gram of protein, Lipid and carbohydrate, respectively.

Vibrio spp. and total bacterial count monitoring

During the experiment, water samples were obtained at a weekly basis. Villamil *et al.,* (2002) protocols were used for samples examination. The total bacterial count (TBC) was determined using nutrient agar (NA), and the total *Vibrio* count (TVC) was determined using trypticase soy agar (TSA) medium supplemented with 7 % NaCl, as the number of and the results were calculated as CFU/mL of water sample.

Growth performance measurements

During the experimental work the shrimp samples were weighted, and the body weight gain (BGW) was calculated in all groups at the end of the experiment. The values were calculated using the following equations according to Abdel-Rahim *et al.,* (2021); BWG= W2-W1 and the specific growth rate (SGR) = 100 x [(ln W2- ln W1)/T], where W2 = final weight; W1 = Initial weight; T = experiment period (days); ln = Natural logarithms. The proximate composition of the shrimp body was analyzed according to the standard methods (n=10) (AOAC, 1995).

Immunological responses

Lysozyme (LSZ) activities turbidimetric technique using *Micrococcus lysodekticus* ATCC No. 4698 (Sigma M 3770) as substrate following Leaño *et al.,* (1998) method. Phenoloxidase activity was measured spectrophotometrically L-dihydroxyphenylalanine (L-DOPA, Sigma) as substrate following the procedure of Hernández-López *et al.,* (1996).

Antioxidant capacity

The antioxidant capacity of the homogenized larvae was estimated spectro-photometrically. Catalase (CAT) (U/mg protein) activity assayed by the method described by Claiborne, (1985). Superoxide dismutase (SOD) activity assayed according to Payá *et al.,* (1992) with minor modifications made by Peixoto *et al.,* (2006). The total antioxidant capacity of larval extract was determined using ferric reducing antioxidant power (FRAP) method (Benzie & Strain, 1996). Reduced Glutathione (GSH) activity was determined according to the method reported by Maran *et al.,* (2009). Glutathione peroxidase (GPx) activity was measured following the method of Flohé & Günzler, (1984). Finally, Lipid peroxidase (Malondialdehyde assays): Peroxidative damage of lipids was determined according to the method of Utley *et al.,* (1967).

Cytokines and antioxidant gene expression

Cytokine gene expression, total RNA was extracted from larvae homogenate using TRIzol reagents (Invitrogen, UK) according to the manufacturer's instructions. The reverse transcription (RT-PCR) was

Table 3: Primers sequences for the cytokine genes expression

Genes	Primer sequence	Reference
IL-4	Forward; 5`CTATTAATGGGTCTCACCTCCCAACT`3 Reverse; 5`CATAATCGTCTTTAGCCTTTCCAAG`3	(Moustafa *et al.,* 2020)
IL-12	Forward; 5`CAGCCTTGCAGAAAAGAGAGC`3 Reverse; 5`CCAGTAAGGCCAGGCAACAT`3	(Moustafa *et al.,* 2020)
HSP	Forward; 5`GTGACGCGAAGATGGACAAGTC`3 Reverse; 5`CACCGTAAGCTACAGCCTCGTC`3	(Moustafa *et al.,* 2020)
β-actin	Forward; 5`GCCCATCTACGAGGGATA`3 Reverse; 5`GGTGGTCGTGAAGGTGTAA`3	(Huang *et al.,* 2017)

IL-4 =interleukin 4, IL-12 = interleukin 12, HSP = heat shock protein, β-actin = Beta actin (a housekeeping gene)

Table 4: Primers sequences for the antioxidant genes expression

Gene name	Primer sequence (5'-3')	Reference
cMn-SOD	F: AATTGGAGTGAAAGGCTCTGGCT R: ACGGAGGTTCTTGTACTGAAGGT	(Jian *et al.,* 2013)
CAT	(F): GCCCGTACAAGGAACTACCA (R): TGACGTTCTGCCTCATTCAG	(Zhang *et al.,* 2013)
GPx	(F): AGGGACTTCCACCAGATG (R): CAACAACTCCCCTTCGGTA	(S. Yang *et al.,* 2022)
β-actin	(F): GCCCATCTACGAGGGATA (R): GGTGGTCGTGAAGGTGTAA	(Jian *et al.,* 2013)

cMn-SOD = Cytosolic manganese superoxide dismutase; CAT = Catalase; GPx = Glutathione peroxidase; β-actin = Beta actin (a housekeeping gene).

performed as described by Chi *et al.,* (2014) using SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad). The reactions were set on a 96-well plate by mixing 1 μL of diluted (1/20) cDNA, 5 μL of 2x concentrated iQ^{TM} SYBR Green Supermix (Bio-Rad), 0.3 μM forward primer and 0.3 μM of reverse primer. The sequences of specific primers used for interleukin 4 (IL-4), interleukin 12 (IL-12) and heat shock protein (HSP) respectively are listed in table 3. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20s at 95 °C, 20s at 60 °C and 20s at 72 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included as an internal reference for normalization of gene expression data. Data analysis was conducted using the 2–ΔΔCT method (Livak & Schmittgen, 2001).

The genes expression of cytosolic manganese superoxide dismutase (*cMn-SOD*), catalase (*CAT*), and glutathione peroxidase (*GPx*) were tested at the end of experiment. The sequences of specific primers used in the present study of each gene were designed based on the published *L. vannamei* cDNA sequence using Primer 3 software as presented in Table 4. The *β-actin* was used as the housekeeping gene. The real-time PCR program was adjusted at 95 ℃ for 1 min, followed by 40 cycles at 95 ℃ for 15 s, 60 ℃ for 15 s, 72 ℃ for 45 s, and one step of 95 ℃ for 10 s. To obtain the melting curves, the temperature increased from 65 to 95 ℃ (0.5 ℃ /s) to denature the double-stranded DNA. The relative mRNA expression levels of genes were calculated with the comparative *C*T method ($2^{-\Delta\Delta CT}$), and the values mean n-fold difference was compared with the control (Livak & Schmittgen, 2001).

Calculations and statistical analysis

Data were examined for homoscedasticity and normalcy. Following that, data were evaluated using two-way analysis of variance (ANOVA) and Tukey's test for post hoc comparisons using Graph Pad Prism 7 (Graph Pad Prism v7.0, San Diego, CA, USA). For all statistical studies, a *P*-value < 0.05 was considered as a significant value. The findings were presented as the mean and standard error of the mean (SEM) of the replicates (n=3).

RESULTS AND DISCUSSION

Physico-chemical analyses of water

A closer examination of the data (Table 5) revealed that the feed modification only considerably affected temperature, but the stocking density significantly affected both the concentration of dissolved oxygen and the TAN. However, the necessary optimal range of dissolved oxygen (5.0 mg/L) was still present. The ammonia started to develop in high stocking density and the highest replacement. Through the breakdown of larger molecules, microbial activity may be to blame for this as well as the stocking density. Despite not being statistically significant, the pH value indicated a modest decrease in both the high density and high replacement treatment. This might be caused by the buildup of organic acids generated during fermentation processes via microbial activity. The fermented soybean meal intended to partial replace of

Table 5: Parameters of water quality during larval rearing feeding on fermented rice bran

Items	Parameters			
	Dissolved oxygen (mg/L)	pH	Temperature (°C)	TAN (mg/L)
Stocking Density				
150/m^3	5.978^a	8.111	30.89	0.030^b
200/ m^3	5.567^b	7.956	31.11	0.120^a
Feed treatments				
G-I	5.900	8.150	30.33^b	0.033
G-II	5.783	8.050	31.00^{ab}	0.067
G-III	5.633	7.900	31.67^a	0.133
SEM	0.133	0.094	0.471	0.041
P-value				
Stocking Density	0.003**	0.066	0.574	0.021*
Feed treatments	0.177	0.061	0.047*	0.082
Interaction	0.990	0.484	0.723	0.021

Means within a column and effect that lack common superscripts letters (a,b.c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*$p \leq 0.05$). SEM= standard error of the mean.

the fish meal in goldfish similarly showed a pH decline (da Cunha *et al.,* 2022). The change in temperature caused by the change in diet might be attributed to the increase of the microbial metabolism due to the 100 % replacement of the diet with high amount. This might be attributed to the increase of metabolic rate and respiration of the bond that has received high amount of rice bran and fermentation probiotics. The increase metabolic rate might add to water temperature as same happens in response to global warming (Shi *et al.,* 2022). In other words, microbial respiration release carbon dioxide (CO2) and heat. CO2 is a greenhouse gas that contributes to climate change, and heat can have a variety of effects on aquatic ecosystems, including increasing the growth of algae and other harmful organisms. This claim is backed up by Jacobson who discovered that increased CO2 levels might raise local temperatures through extensive air modeling (Jacobson, 2011).

Phytoplankton and zooplanktons monitoring

In fishpond habitats where zooplankton grazes, phytoplankton is the main primary producer (Sadique *et al.,* 2018). According to the findings in Table 6, the stocking density and feed replenishment had a substantial impact on the individual/liter of the copepod presence in the culture bond. With 100% meal replacement, the maximum number of copepods was seen. High stocking density has resulted in a decrease in the number of copepods in the water. The phytoplankton decreased significantly as the food replacement increased.

***Vibrio* spp. and total bacterial count monitoring**

The *Vibrio* spp. is a predominant pathogen present in marine water and can cause disease for fish and shellfish (Abdella *et al.,* 2017; El-Wazzan *et al.,* 2020; Mahmoud *et al.,* 2022). The effects of diet changes and increased stocking density on total and *Vibrio* spp. counts were not statistically significant. However, when the diet replacement ratio increases, the overall viable bacterial count rises while the *Vibrio* count falls. The obtained result makes it abundantly evident that, of the three treatments, the TBC had the greatest value in the G-III group in both densities, but the TVC displayed the reverse pattern, with the G-III tends to be the lowest in the TVC despite the count being not substantially different (Figure 1a and 1b). This could be as a result of the probiotic strain being employed producing an anti-*Vibrio* substance that controls *Vibrio* spp. Similar evidence of *Bacillus subtilis*' capacity to inhibit *Vibrio* spp. was shown in shrimp culture (Vaseeharan & Ramasamy, 2003; Wang *et al.,* 2020).

Growth performance measurements

The initial body weight (IBW), final body weight (FBW), weight gain (WG), daily weight gain (DWG) and the SGR of shrimp are listed in. **Among the three different treatments, the G-I group has responded in both densities with the highest body weight. The conclusion of results boosts that the formulated**

Table 6: Phyto- and Zooplankton during larval rearing (Nursery Stage) feeding on fermented rice bran (21 days)

Items	Parameters	
	Copepods (individual /L)	Phytoplankton x 10^5 (cells /mL)
Stocking density		
150/ m^3	71.22^a	5.960
200/ m^3	58.00^b	5.770
Feed treatments		
G-I	23.33^c	6.267^a
G-II	47.67^b	5.750^{ab}
G-III	122.8^a	5.567^b
SEM	6.572	0.241
P-value		
Stocking density	0.0298*	0.356
Feed treatments	0.0001***	0.034*
Interaction	0.052	0.782

Means within a column and effect that lack common superscripts letters (a,b.c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*p≤ 0.05). SEM= standard error of the mean.

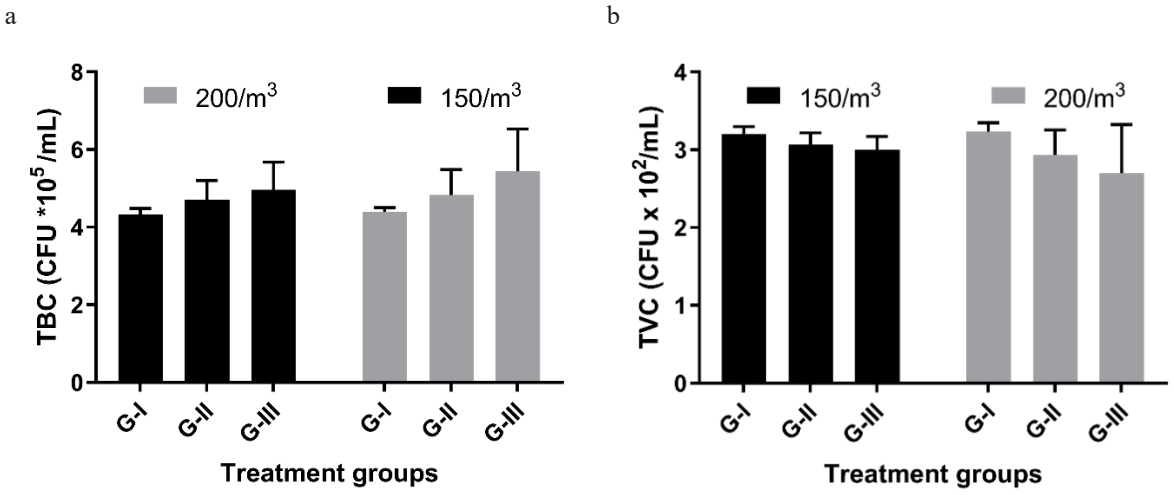

Figure 1: Total bacterial counts (a) and Total Vibrio spp. count (b) of the rearing water. Error bares represent the SEM, n=3.

diet is the best. The G-I group had the highest weight gain, followed by the G-II group, and then, the G-III group had the lowest weight gain, as can be seen by taking a closer look at the numbers in the table. Comparing G-I to the other groups, the average daily gain (ADG) and SGR were also significantly the highest. The 150/m^3 FBW, WG, ADG, and SGR were significantly greater than in the 200/m^3 stocking density when considering the effect of stocking density. The effect of experimental treatments on the body weight of shrimp was demonstrated in. Among the three different treatments, the G-I group has responded in both densities with the highest body weight. The conclusion of results boosts that the formulated diet is the best.

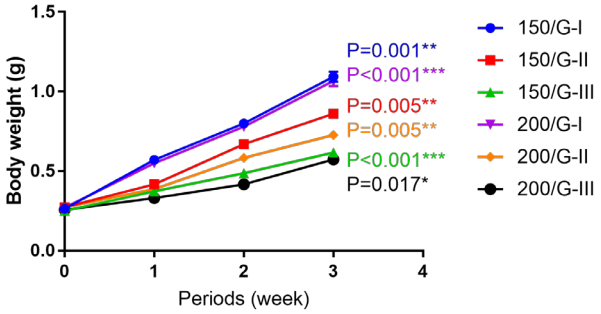

Figure 2: Weekly development of the average body weight of *L. vannamei* cultivated in two different stocking densities and fed on 3 different formulated diets.

Table 7: Growth performance of *L. vannamei* in the treatments of the nursery periods 21 days experimental period

	IBW (g)	FBW	WG (g)	ADG (g)	SGR (%/ day)
Stocking Density					
150/1-m^3	0.262	0.857^a	0.594^a	0.028^a	5.643^a
200/1-m^3	0.267	0.789^b	0.522^b	0.025^b	5.160^b
Feed					
G-I	0.267	1.08^a	0.813^a	0.039^a	6.655^a
G-II	0.273	0.793^b	0.520^b	0.025^b	5.078^b
G-III	0.253	0.595^c	0.342^c	0.016^c	4.072^c
SEM	0.009	0.021	0.021	0.006	0.099
P-value					
Stocking Density	0.539	0.0001***	<0.0001***	<0.0001***	<0.0001***
Feed	0.101	0.002**	0.001**	0.001**	0.001**
Interaction	0.906	0.054	0.073	0.073	0.073

Means within a column and effect that lack common superscripts letters (a,b.c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*$p \leq 0.05$). SEM= standard error of the mean.

Proximate composition of *L. vannamei*

The data of the proximate analysis of shrimp body weight along the experiment shows that, the replacement of diet significantly affected the proximate composition. The carbohydrate and the total ash percentage has increased while the CP content and fat content has decreased following the application of fermented rice bran as a 100 percent substitute. The stocking density showed a small effect on the proximate composition analysis (Table 8).

Immunological responses

The lysozyme and phenol oxidase activity of the post larva homogenate after the experiment are shown in **However, the increased percentage of diet replacement has reduced the activity of LSZ significantly. The drop in LSZ activity might be attributed to a diet deficient in protein or heavy in carbohydrates or lipids, which can impair lysozyme activity (MuÑoz et al., 1981). The 50% substitution of fermented food had no effect on LSZ activity, whereas population density exhibited a drop in LSZ activity, which might be attributable to stress from crowding.**. The results show insignificant differences between the treated groups with respect to PO activities. However, the increased percentage of diet replacement has reduced the activity of LSZ significantly. The drop in LSZ activity might

Table 8: The proximate composition of *L. vannamei* at the end of the nursery periods 21 days experimental period

Items	Proximate Composition				
	Crude Protein (%)	Crude lipid (%)	Moisture (%)	Carbohydrate (%)	Total Ash (%)
Stocking density					
150/ m^3	16.32^{b}	3.349^{a}	74.69	2.809^{b}	2.829^{a}
200/ m^3	16.99^{a}	3.140^{b}	74.64	2.943^{a}	2.276^{b}
Feed treatment					
G-I	17.17^{a}	3.723^{a}	74.65^{ab}	2.650^{c}	1.808^{b}
G-II	17.16^{a}	3.368^{b}	74.76^{a}	2.838^{b}	1.881^{b}
G-III	15.65^{b}	2.642^{c}	74.60^{b}	3.140^{a}	3.967^{a}
SEM	0.071	0.041	0.050	0.043	0.113
P-value					
Stocking density	0.0001***	0.0001***	0.280	0.002**	0.0001***
Feed treatment	0.0001***	0.0001***	0.026*	0.0001***	0.0001***
Interaction	0.0001***	0.1025	0.154	0.240	0.0001***

Means within a column and effect that lack common superscript letters (a,b.c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*p≤ 0.05). SEM= standard error of the mean.

Table 9: Immune parameters of *L. vannamei* of the nursery periods 21 days experimental period

Items	LSZ (UmL_{-1})	PO ($Umg_{-1}prot$)
Stocking density		
150/ m^3	1553^{a}	2.786
200/ m^3	1535^{b}	2.528
Feed treatment		
G-I	1562^{a}	2.698
G-II	1551^{a}	2.797
G-III	1518^{b}	2.475
SEM	9.789	0.162
P-value		
Stocking density	0.045*	0.075
Feed treatment	0.002**	0.168
Interaction	0.415	0.545

Means within a column and effect that lack common superscript letters (a,b.c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*p≤ 0.05). SEM= standard error of the mean.

be attributed to a diet deficient in protein or heavy in carbohydrates or lipids, which can impair lysozyme activity (MuÑoz *et al.,* 1981). The 50% substitution of fermented food had no effect on LSZ activity, whereas population density exhibited a drop in LSZ activity, which might be attributable to stress from crowding.

In general, all the studied antioxidant factors have not changed much as diet replacement has increased. Only GSR and TAC were lowered with complete replacement. However, as compared to the control group, it did not alter much in GII (Table 10).

The relative gene expression of a few antioxidant genes, as indicated in **Table** supports this. When compared to the control diet, the expression of cMn-SOD, CAT, and GPx were downregulated in response to 100 % replacement in G-III. While 50% replacement resulted in minor downregulation of cMn-SOD and GPx, CAT still displays considerable downregulation as compared to the control. Since the purpose of anti-oxidative enzymes is to shield macromolecules and organelles from oxidation brought on by free radicals and extremely reactive oxygen species (Ighodaro & Akinloye, 2018). The low diet protein represented by 100% replacement might be the reason why the

Table 10: Antioxidant parameters of *L. vannamei*) at the end of the nursery periods 21 days experimental period

Items	SOD Umg_1 protein	CAT Umg_1 protein	MDA Umg_1 protein	GSR mmol/g wet tissue	TAC mML-1/g wet tissue	GPx, U/g tissue
Stocking density						
150/ m^3	22.38	1.568	5.283	5.732	1.818	17.33
200/ m^3	21.89	1.478	5.389	5.600	1.727	17.49
Feed treatment						
G-I	23.62	1.650	5.292	5.835^{a}	1.945^{a}	17.24
G-II	21.96	1.527	5.650	5.722^{ab}	1.758^{ab}	17.39
G-III	20.83	1.392	5.067	5.442^{b}	1.613^{b}	17.61
SEM	1.031	0.109	0.249	0.105	0.075	0.311
P-value						
Stocking density	0.574	0.335	0.614	0.149	0.164	0.548
Feed treatment	0.056	0.103	0.102	0.008**	0.003**	0.509
Interaction	0.850	0.954	0.999	0.872	0.959	0.933

Means within a column and effect that lack common superscript letters (a,b.c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*p≤ 0.05). SEM= standard error of the mean

Table 11: Relative genes expression of antioxidants enzymes expression in larval extract of *L. vannamei*

Items	Relative expression of genes in larval extract of *L. vannamei*		
	cMn-SOD	***CAT***	***GPx***
Stocking density			
150/m^3	1.424	1.497^{a}	1.589^{a}
200/m^3	1.328	1.407^{b}	1.703^{b}
Feed treatment			
G-I	1.433^{a}	1.645^{a}	1.560^{a}
G-II	1.432^{ab}	1.442^{b}	1.602^{ab}
G-III	1.263^{b}	1.268^{c}	1.777^{b}
SEM	0.032	0.023	0.018
P-value			
Stocking density	0.061	0.001***	0.0001***
Feed treatment	0.017*	0.0001***	0.0001***
Interaction	0.921	0.5369	0.765

Means within a column and effect that lack common superscript letters (a,b.c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*p≤ 0.05). SEM= standard error of the mean.

Table 12: Relative expression of genes of cytokine genes expression in larval extract of *L. vannamei*

Items	Relative expression of genes in larval extract of *L. vannamei*		
	IL-4	IL-12	HSP
Stocking density			
150/m^3	1.563^a	1.521^a	1.399^a
200/m^3	1.410^b	1.374^b	1.313^b
Feed treatment			
G-I	1.718^a	2.698^a	1.507^a
G-II	1.460^b	2.797^b	1.338^b
G-III	1.282^c	2.475^b	1.223^c
SEM	0.032	0.162	0.026
P-value			
Stocking density	0.0001***	0.002**	0.002**
Feed treatment	0.0001***	0.0001***	0.0001***
Interaction	0.540	0.248	0.903

Means within a column and effect that lack common superscript letters (a,b,c…) differ significantly (Tukey's multiple comparison test, $P \leq 0.05$). Asterisks indicate significant differences between groups (two-way ANOVA*p≤ 0.05). SEM= standard error of the mean.

antioxidant expression is downregulated. In another explanation, the decrease in such enzyme activity and gene expression might be interpreted as a sign that the fermented rice bran contains an antioxidant component. This is also supported by the antioxidant properties of fermented rice bran produced by mixed bacteria fermentation in zebra fish (Liu *et al.,* 2022). The population density has affected the expression of CAT and GPx significantly.

Cytokines genes expression

The relative gene expression of cytokines such as interleukin 4 (IL-4), interleukin 12 (IL-12), and HSP in *L. vannamei* were extracted at the end of the 21-day nursery phase. Both an increase in dietary replacement % and population density have reduced the expression of IL-4, IL-12, and HSP (). In the investigated genes, the proportion of 100% replacement indicated a considerable drop. The fermented rice bran diet reduced the expression of cytokines and immune genes in shrimp compared to the control diet. This implies that a replacement diet might weaken shrimp's immune systems, leaving them more prone to infection.

CONCLUSION

To summarize, the current study found that fermented rice bran, when used as a dietary replacement for a formulated diet, reduced the antioxidant capacity of *L. vannamei* while decreasing the expression of immune response genes and factors. Furthermore, it has no effect on the water quality indicators in cultivation ponds. However, after 21 days of feeding, the highest FBW was reported in shrimp larvae fed 100% formulated feed followed by 50% formulated feed and 50 % fermented feed then 100% fermented feed. Conclusively, using of fermented feed may reduce the overall use of formulated feed and improve the health and immunity of shrimp larvae.

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CONFLICT OF INTEREST

The authors declare that they have NO conflict of interest.

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