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Research Article

Bacillus spp. Fermented Plant Protein Mix as a Potential Fishmeal Substitute in the Diet of *Penaeus monodon*: Emphasis on Nitrogen Metabolism

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A feeding experiment (60 days) was conducted to evaluate the effect of *Bacillus* spp. fermented plant protein mix in *Penaeus monodon*. A control diet (CNT) contains 25% fishmeal, of which 50% was substituted (w/w) with *Bacillus* spp. fermented plant protein mix at two different concentrations of 0.25 and 0.50% (PP-0.25 and PP-0.50, respectively), while the diet prepared with untreated ingredients served as a negative control (PP-0). The daily growth coefficient (DGC) did not differ in the groups fed CNT, PP-0.25, and PP-0.50 diets. The feed conversion ratio (FCR) was low in the CNT and PP-0.50 diets, while (protein efficiency ratio (PER) and apparent protein utilization (APU) were high in the PP-0.50-fed group. The dietary change did not affect survival and whole-body composition, while free amino acids varied among the treatments. Increased nitrogen intake and oxygen consumption were observed in subadults fed with CNT diet. Increasing *Bacillus* spp. concentrations significantly lowered ammonia-N excretion by 2.09-11.69%. Regression analysis revealed higher coefficients for oxygen consumption ($R^2 = 0.8625$) and O : N ratio ($R^2 = 0.7791$), while it was 0.5894 for ammonia-N excretion. The dietary change significantly influenced hemolymph indices and chitinase, glutamate dehydrogenase (GDH), and arginase activity. Results conclude that *Bacillus* spp. fermented plant protein mix could be a viable fishmeal substitute in shrimp feed.

1. Introduction

Shrimp farming is growing steadily year by year, and its global production is expected to increase at least 8.9% by 2021 compared to 2020 [1]. In crustacean aquaculture, black tiger shrimp, *Penaeus monodon* is one of the important shrimp species, accounting 68% of the total global shrimp output in the 1990s [2]. However, its global production significantly declined in the past two decades due to various factors, mainly severe disease outbreaks, the introduction of specific pathogen-free and fast-growing whiteleg shrimp, *Penaeus vannamei* production. Of late, the situation is slowly changing in favor of *P. monodon* and the culture of this species is again picking up globally, especially in Asian countries (Bangladesh, Viet Nam, Thailand, Indonesia, and Malaysia) because of the development of specific pathogen-free (SPF) juveniles [3]. The intensive and semi-intensive culture of *P. monodon* is more efficient on a nutritionally balanced diet that generally contains a higher content of marine proteins, in particular fishmeal. However, diminishing availability and increased cost reduced the inclusion level of fishmeal by incorporating various alternate protein sources. The nutrient utilization of various plant proteins and their by-products has been evaluated as a fishmeal

substitute, as they are easily available, reasonably priced, and to a certain extent, have a desirable nutrient content [4]. However, their utilization is not picking pace even after including them in combination [5] due to the presence of antinutrients that hinders digestion, absorption, and nutrient utilization.

Microbial fermentation plays a vital role in degrading antinutrients and consequently to which they enhance the nutritional quality of the ingredients/diets. There are numerous microbial species employed in the fermentation process; however, *Bacillus* spp. have been considered in recent times due to their safety, capability for producing various hydrolyzing enzymes, survival ability and resistance to extreme temperature, pH, and UV radiation [6]. In general, a single microbial species is naturally used for the fermentation process and has been well documented in earlier studies [5, 7–9]. Though several microbial species are commercially available for fermentation, the *Bacillus* spp. are widely used in recent times as they are saprophytic, spore-forming bacterium and classified under Gram-positive, nonpathogenic category. Inclusion of *Bacillus* fermented plant proteins not only had a positive influence on the digestibility and growth of the farmed species but also decreased the formulation cost by reducing the inclusion level of dietary fishmeal. More recently, a design of a mixed culture with two or more microbial species was employed in the fermentation process because of the potential synergetic effects between the microbial consortia that would enhance the diverse quality criteria with a single process [10]. The present study is therefore aimed at evaluating the effect of fermented plant protein mix with a conglomeration of microorganisms containing five different *Bacillus* spp. on the growth and nutrient utilization in *P. monodon* juveniles fed partially fishmeal substituted diets. Most of the earlier studies are restricted to growth parameters, nutrient utilization, digestibility, and carcass composition, but in our study, nitrogen metabolism studies were also carried out in the subadult group to evaluate the quality of diets. This baseline data thus generated would give an idea about the utilization and limitation of a *Bacillus* spp. fermented plant protein mix in formulating cost-effective commercial feed for shrimp in the future.

2. Materials and Methods

2.1. Preparation of Bacillus Conglomeration. A conglomeration of five different *Bacillus* strains viz., *B. subtilis*, *B. licheniformis*, *B. polymyxa*, *B. megaterium*, and *B. pumilus* was used in the study. These strains that are being maintained in our laboratory at ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India, have been characterized earlier and were selected based on the production of various extracellular enzymes viz. amylase, protease, cellulase, xylanase, and chitinase. Prior to use, the strains were cultured separately in an Erlenmeyer flask (500 ml) containing nutrient broth (200 ml) (M1383, Himedia, India) at 200 rpm with 37°C for 48 h according to Boonthai et al. [11]. Bacterial suspensions were centrifuged at 10000 g at 4°C for 5 min for obtaining the cell pellets. They were washed thrice with sterile phosphate-buffered saline (pH 7.2) and their cell density was adjusted to 5×10^7 CFU/g using serial dilution. All individual bacterial suspensions made were mixed in the ratio of 1 : 1 : 1 : 1 : 1 to prepare a conglomeration that was used to enhance the plant protein mix.

2.2. Bacillus spp. Fermented Plant Protein Mix. Four different plant protein sources viz., groundnut oil cake, rapeseed meal, sesame oil cake, and corn gluten meal purchased ($n = 6$) at local markets (Chennai, India) were ground to fine particles (<250 μm). The ground materials were mixed in the ratio of 1 : 1 : 1 : 3 to prepare a plant protein mix as per our earlier study [12]. They were further hydrated using deionized water to obtain the appropriate moisture content and then autoclaved at 121°C for 15 min. The cooled autoclaved sample was inoculated with a *Bacillus* conglomeration at two different concentrations (0.25 and 0.50%) and incubated at 37°C for four days. Prior to *Bacillus* spp. fermentation, the required conditions were optimized with different factors including moisture (20, 25, 30, 35, and 40%), temperature (30, 33, 35, 37, and 40°C), pH (5.0, 5.5, 6.0, 6.5, and 7.0), and incubation period (1, 2, 3, 4, and 5 days). The optimized conditions were further used to ferment the plant protein mix with six sets of replications for each concentration of the *Bacillus* conglomeration. All six replicates were individually analyzed for nutrient composition and the average values were reported in Table 1. As all replicates had an almost similar composition, they were pooled together as a representative sample prior to including into the test formulas.

2.3. Experimental Diets. A control diet (CNT) was formulated with 25 g/100 g fishmeal to have 40% crude protein [13]. Prior to preparation, all solid ingredients (Table 2) were ground and passed through a sieve of 250 μm. To the fine ground materials, dry additives of vitamin-mineral mix, vitamin C as an antioxidant, binder, and chromic oxide as an inert marker were added. After a hand mix, fish oil and soy lecithin were further added and then homogenized in an electric blender for 20 min. About 500 ml deionized water per kg mash was added to the homogenized mash to make them like soft dough, and then, it was steamed at atmospheric pressure for 5 min. The semimoist material was pelletized in a table-top pelletizer with 0.8 and 1.4 mm dies and dried at 60°C. Following that, three more test diets were formulated by replacing 50% dietary fishmeal with a plant protein mix. Test diets were prepared as mentioned earlier with the ingredients listed in Table 2; however, the diet PP-0 contained untreated plant protein mix, while the diets PP-0.25 and PP-0.50 were prepared using fermented plant protein mix with 0.25 and 0.5% *Bacillus* conglomeration, respectively. All experimental diets were air-dried and stored in a plastic container at 4°C until use. The proximate and essential amino acid composition of the experimental diets is given in Table 3.

2.4. Experimental Shrimp. Postlarvae of *P. monodon* (15 days old) were acquired from a certified hatchery (Govt. of India), Chennai, India. They were placed in a clean oxygen-filled polythene container to transport them to the experimental

Table 1: Nutrient composition of fishmeal and test ingredients (untreated and *Bacillus* spp. fermented plant protein mix) used in the present study.

Particulars	Fishmeal	Plant protein mix		
		Untreated	PPM@0.25[1]	PPM@0.50[2]
Proximate composition (% dry weight basis)				
Crude protein	63.74	55.19	58.41	59.02
Ether extract	10.51	1.79	1.47	1.43
Crude fiber	0.53	7.10	7.07	6.99
Nitrogen free extract[3]	6.27	31.37	28.18	27.64
Total ash	18.95	4.55	4.87	4.92
Essential amino acids (g/16 g N)				
Arg	6.86	5.55	5.60	5.59
His	2.65	2.28	2.71	2.84
Ile	4.64	3.86	4.10	4.22
Leu	7.97	11.15	11.52	11.37
Lys	8.30	5.19	5.56	5.58
Met	2.98	2.05	2.11	2.16
Phe	4.31	5.29	5.65	5.53
Thr	4.53	3.37	3.45	3.51
Trp	1.11	0.77	0.77	0.79
Val	5.41	4.37	4.86	4.79
Antinutrients (mg/100 g dry weight basis)				
Trypsin inhibitor	—	153.36	BDL[4]	BDL[4]
Phytic acid	—	1708.55	780.46	764.16
Saponin	—	266.69	101.35	98.16
Tannin	—	488.43	291.37	278.91
Glucosinolates	—	51.68	42.39	39.37

[1]Plant protein mix contains groundnut oil cake, rapeseed meal, sesame oil cake, and corn gluten meal (1 : 1 : 1 : 3) that were fermented using 0.25% of *Bacillus* conglomeration. [2]Plant protein mix contains groundnut oil cake, rapeseed meal, sesame oil cake, and corn gluten meal (1 : 1 : 1 : 3) that were fermented using 0.50% of *Bacillus* conglomeration. [3]Calculated by a difference: 100 − (crude protein + ether extract + crude fiber + total ash). [4]Below deductible level.

facility (Muttukadu Experimental Station of ICAR- CIBA, Chennai, India). Prior to the experiment, the procured post-larvae were reared in a net cage (2 m × 2 m × 1 m) built in the lagoon of the institute with the feed containing 40% crude protein. Once the shrimp reached ≈0.5 g size, a part was used for a feeding trial, whereas the remaining shrimp were kept in the cage itself for growing them to subadults (>10 g) for carrying out a further study of nitrogen metabolism. The feed given was monitored continuously and adjusted according to the body size and intake. Prior to transfer to the indoor laboratory from the lagoon, the health status of shrimp was checked and ensured for the absence of OIE-listed pathogens to rule out the risk of disease.

2.5. Feeding Trial. A week prior to the experiment, the transferred shrimp were acclimatized to indoor experimental conditions in a 1000 l flat-base (43.3-inch bottom diameter) circular fiberglass reinforced plastic (FRP) tank with the

Table 2: Ingredient composition of experimental diets (% as fed basis) containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Particulars	Experimental diets			
	CNT	PP-0	PP-0.25	PP-0.50
Fishmeal[1]	25.0	12.5	12.5	12.5
Plant protein mix[2]	—	12.5	12.5	12.5
Acetes[3]	12.0	12.0	12.0	12.0
Squilla meal[4]	11.4	11.4	11.4	11.4
Squid meal[5]	5.0	5.0	5.0	5.0
Soybean meal	20.0	20.0	20.0	20.0
Wheat gluten meal	—	5.4	5.4	5.4
Wheat flour	20.0	14.6	14.6	14.6
Fish oil[1]	2.0	2.0	2.0	2.0
Soy lecithin[6]	1.0	1.0	1.0	1.0
Vit-min mix[7]	2.0	2.0	2.0	2.0
Vitamin C	0.1	0.1	0.1	0.1
Binder[8]	1.0	1.0	1.0	1.0
Chromic oxide[9]	0.5	0.5	0.5	0.5

[1]Bismi Fisheries, Mayiladuthurai, Tamil Nadu, India. [2]Plant protein mix contains groundnut oil cake, rapeseed meal, sesame oil cake and corn gluten meal (1 : 1 : 1 : 3). [3]Om-Sai Aqua, Dandi, Gujarat, India. [4]Blueline Foods India Pvt. Ltd., Mangalore, Karnataka. [5]Khaja Mohammed Store, Chennai, Tamil Nadu, India. [6]Real Soy Enterprises, Indore, Madhya Pradesh, India. [7]Thiamine hydrochloride (25.50 g), riboflavin (25.00 g), prydoxine hydrochloride (50.00 g), cyanogobalamine (0.10 g), menadione (5.00 g), all-trans tocopherol acetate (99.00 g), retinyl acetate (10.00 g), vitamin D (50 g), nicotinic acid (101.00 g), D-Ca-pantothenate (61.00 g), biotin (25.00 g), folic acid (6.25 g), inositol (153.06 g), ferric citrate (13.70 g), $ZnSO_4{\cdot}7H_2O$ (28.28 g), $MgSO_4{\cdot}7H_2O$ (0.12 g), $MnSO_4{\cdot}H_2O$ (12.43 g), $CuSO_4{\cdot}5\ H_2O$ (19.84 g), $CoC1_2{\cdot}6H_2O$ (4.07 g), KIO_4 (0.03 g), KCl (15.33 g), and Na_2SeO_3 (0.02 g). [8]Pegabind, Bentoli Agri-Nutrition Asia Pvt. Ltd., Singapore. [9]Sigma-Aldrich, Missouri, USA. All other ingredients are purchased in local markets.

CNT diet. A total of 240 shrimp weighing an average body-weight of 0.52 g were placed at random in twelve oval-shaped 500 l (1.31 × 0.64 × 0.73 m) FRP tanks (three tanks/diet and twenty shrimp/tank). In the experimental tanks, filtered seawater and continuous aeration was used, which were covered with fiber sheets to prevent escapees and reduce disruption. The shrimp were fed the experimental diet (8% of total biomass) three times a day (7:30 am, 12:30 pm, and 5:30 pm) by splitting the entire meal into 40, 30, and 30%, respectively, and as the experiment progressed, we followed the ad libitum feeding, i.e., feed to satiety. After an hour of feeding, uneaten feed particles (if any) were collected using a clean Falcon tube. They were rinsed with deionized water and dried (60°C) in a hot air oven to calculate a daily feed intake [14]. Meanwhile, the bottoms of the experimental tanks were kept clean so that the freshly defecated feces could be collected using a clean Falcon tube for determining the digestibility parameters. They were rinsed in deionized water and transferred to filter paper with clean forceps, dried, and frozen immediately at -20°C until analysis. Aeration was stopped during the fecal collection to prevent the leach of nutrients. An apparent digestibility coefficient of dry matter, crude protein, and essential amino

Table 3: Proximate and essential amino acid composition of experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Particulars	Experimental diets				R^1
	CNT	PP-0	PP-0.25	PP-0.50	
Proximate composition (% as fed basis)					
Moisture	9.70	8.20	7.98	8.54	
Crude protein	40.94	40.78	40.45	40.01	
Ether extract	7.99	6.99	7.12	7.86	
Crude fiber	3.22	5.21	4.85	4.17	
Nitrogen free extract[2]	20.73	24.37	24.85	25.04	
Total ash	17.42	14.45	14.75	14.38	
Essential amino acid composition (g/16 g N)					
Arg	6.06	5.81	5.88	6.07	5.3
His	2.25	2.50	2.45	2.60	2.2
Ile	3.64	4.05	4.10	4.02	2.7
Leu	7.60	8.61	8.88	8.37	4.3
Lys	6.45	5.05	5.29	5.20	5.2
Met	2.54	2.04	2.30	2.12	2.4
Phe	4.13	4.46	4.62	4.57	3.7
Thr	3.59	4.02	4.18	4.27	3.5
Trp	0.90	0.83	0.87	0.87	0.5
Val	4.30	4.61	4.72	4.75	3.7

[1]Dietary essential amino acid requirements of *P. monodon* [13]. [2]Calculated by a difference (100 – (%of moisture + crude protein + ether extract + crude fiber + total ash)).

acids were determined after Jannathulla et al. [15]. The growth performance in terms of daily growth coefficient (DGC), feed conversion ratio (FCR), protein efficiency ratio (PER), apparent protein utilization (APU), and survival was determined at the end of the experiment as follows:

$$\mathrm{DGC}\left(\frac{\%}{\mathrm{d}}\right) = 100 \times \frac{\text{Final weight (g)}^{1/3} - \text{Initial weight (g)}^{1/3}}{\text{No.of days}}, \quad (1)$$

$$\mathrm{FCR} = \frac{\text{Dry feed consumed (g)}}{\text{Wet weight gain (g)}}, \quad (2)$$

$$\mathrm{PER} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}, \quad (3)$$

$$\mathrm{APU}\,(\%) = \frac{\text{Protein gain (g)}}{\text{Protein intake (g)}} \times 100, \quad (4)$$

$$\text{Survival}\,(\%) = \frac{\text{Final number} - \text{Initial number}}{\text{Initial number}} \times 100. \quad (5)$$

2.6. Nitrogen Metabolism Study. In addition to growth, a three-week study was carried out to elucidate the effect of *Bacillus* spp. fermented plant protein mix on nitrogen metabolism of subadult tiger shrimp (10.48 ± 0.51 g). A triplicate group of shrimp on the diet CNT, PP-0.25, and PP-0.50 were kept singly in oval-shaped 100 l ($0.26 \times 0.13 \times 0.17$ m) FRP tanks. Diets containing pellets with a diameter of 1.4 mm were used at the rate of 4% of the biomass in this experiment. Diet was manually given once a day in the morning (7:30 am), and shrimp were allowed to feed for 15-20 min or until apparent satiety. The shrimp that did not have any deformities were then placed in a sealed respirometry metabolic system (5-inch bottom diameter) to evaluate both oxygen consumption and ammonia-N excretion on a daily basis [3]. Simultaneously, fresh seawater was collected from the animals' free respirometry chambers to serve as a blank. The measurement of daily intake was carried out as mentioned above in the feeding trial.

2.7. Sample Collection. 1 ml syringe with a 26-gauge hypodermic needle (2 mm thickness) containing 0.3 ml anticoagulant solution was used to collect hemolymph samples from the ventral sinus portion located at the first abdominal segment of subadult shrimp. After hemolymph collection, the same shrimp were further dissected to obtain the hepatopancreas and gills. The preweighed dissected hepatopancreas was homogenized with an equal amount of acetate buffer (pH 5) for a minute using a tissue homogenizer. It was then centrifuged at 10000 g in a cold (4°C) environment, and the resultant supernatant was used as an enzyme source for the assay of digestive enzyme activity. The activity of glutamate dehydrogenase (GDH) and arginase was measured using gill homogenate.

2.8. Water Quality Parameters. After filtering via a 5 μm cartridge filter, ultraviolet-treated water was used in both trials. In the growth experiment, about 80% of the water was exchanged daily. The water quality parameters were

Table 4: Mean value of water quality parameters analyzed during the experimental periods of 60 days.

Particulars	
Temperature (°C)	27.28 ± 1.53
Salinity	24.43 ± 1.16
pH	8.46 ± 0.37
Dissolved oxygen (mg/l)	7.64 ± 0.71
Turbidity (NTU)	17.32 ± 2.01
Total alkalinity (mg/l as $CaCO_3$)	153.16 ± 5.19
Total hardness (mg/l as $CaCO_3$)	2128.14 ± 63.48
Total ammonia (mg/l)	0.08 ± 0.01
Nitrite-N (mg/l)	0.03 ± 0.01
Nitrate-N (mg/l)	0.04 ± 0.01
Phosphate (mg/l)	0.14 ± 0.04

monitored at frequent intervals and the analyzed [16] mean values are presented in Table 4. The photoperiods in both trails were kept at their natural levels.

2.9. Biochemical Analysis. The AOAC [17] standard method was used to determine the proximate compositions of experimental diets and shrimp carcasses. For moisture, samples were oven-dried at 105°C until obtaining a constant weight and were incinerated at 540°C for estimating total ash. Total nitrogen was determined using a Tecator Kjeltec system (KjeltecTM-8100) and was multiplied with a common empirical factor (6.25) to obtain crude protein. The ether extract (Scocs Plus-SCS:6) and crude fiber (FOSS-2022-TecatorTM) were determined using a standard protocol. A difference [100 − (percentage of moisture + crude protein + crude fat + crude fiber + total ash)] was used to determine the level of nitrogen-free extract (NFE). Using a UV visible spectrophotometer (UV-1800, Shimadzu, Japan), the level of chromium in the diet and feces was determined according to Furukawa [18]. The HPLC model LC-10A (Shimadzu Corp, Japan) was used to determine the amino acid composition after hydrolyzing the samples with 6-N hydrochloric acid. Individual amino acids were separated in a Shimpack column (ISC-07/S1504 Na) that packed with a strong acidic Na^+ type cation exchange resin (styrene-divinyl benzene copolymer with sulfinic group). Separation was carried out using a combination of sodium citrate-perchloric acid as buffer-A (pH: 3.2) and boric acid sodium citrate-sodium hydroxide as buffer-B (pH: 10.0) at a flow rate of 0.3 ml/min under gradient elution. After postcolumn derivatization with O-pthaladehyde and 2-mercaptoethanol, amino acids were qualified and quantified using a fluorescence detector (FLD-6A). A fluorescence detecting amino acid standard obtained from Sigma-aldrich Inc., USA was used as an external standard [13]. As tryptophan is labile to acid hydrolysis, it was quantified using a spectrophotometric technique at 500 nm after alkali hydrolysis [19]. Antinutrients such as trypsin inhibitors [20], phytic acid [21], saponins [22], glucosinolates [23], and tannins [24] were analyzed using the respective standard methods.

The oxygen concentration and ammonia-N was determined after Winkler [25] and Chaney and Marbach [26], respectively. Both the measured values were given as μg/g of DM/h as oxygen consumption and ammonia-N excretion by shrimp. The O : N ratio was subsequently computed using the abovementioned values. The activity of protease, amylase, cellulase, xylanase, and chitinase was assayed by the methods described by Rajaram [12]. The GDH activity was assayed on the homogenates of gill tissue using King et al. [27] method. Arginase was measured in a UV spectrophotometer (UV-1800-Shimadzu Corp. Japan) according to the manufacturer's instructions using a commercial enzyme assay kit (Sigma-Aldrich, Code No: MAK112). The hemolymph indices were also quantified using Sigma-Aldrich kits (Code No: GAHK20, TP0100, AA0100, MAK006).

2.10. Statistical Analysis. To assess the means, a one-way ANOVA (SPSS ver.16.0 for Windows) was deployed. Tukey's test was used to compare the difference between the means at a probability of $p < 0.05$. A regression analysis was performed to determine the correlation between oxygen consumption, ammonia-N excretion, and the O : N ratio with nitrogen intake.. The data were examined for homogeneity of variance after ascertaining the normal distribution prior to statistical evaluation. The data were presented as a mean of three replicates with p values, pooled SEM (±) and CV (%).

3. Results

3.1. Digestibility Parameters. Shrimp fed on PP-0.50 diet showed significantly ($p < 0.05$) higher apparent dry matter digestibility (ADMD) and the group reared on PP-0 diet showed the least value, while no significant difference was observed between the groups fed on CNT and PP-0.25 diets (Table 5). The apparent crude protein digestibility (ACPD) significantly ($p < 0.05$) increased by 2.5% in the group fed on the PP-0.50 diet compared to those fed on PP-0.25. The ACPD was significantly ($p < 0.05$) low in PP-0 compared to all other diets. The apparent energy digestibility (ADEAA) significantly ($p < 0.05$) increased with enhanced *Bacillus* spp. concentration used to ferment the plant protein mix except for Thr, which was significantly high ($p < 0.05$) in shrimp fed with CNT diet. Among the EAA, the digestibility of Met was found to be high in the control group. The Arg and Thr, in addition to Met exhibited >90% digestibility, whereas >90% digestibility was found only for Arg in shrimp fed with PP-0 diet and Phe in shrimp fed with PP-0.25 diet. However, except His, Thr, and Trp, all other EAA had >90% digestibility with PP-0.50 diet, in which the predominant one was Arg, followed by Ile and Phe. No significant difference was observed for the digestibility of His between PP-0.25 and PP-0.50 diets, and it was His, Met, and Trp between CNT and PP-0.50 diets. The lowest digestibility parameters including dry matter, crude protein, and EAA were observed in shrimp fed with PP-0 diet compared to the control, and the reduction was in the range of

Table 5: Apparent digestibility coefficients (%) of dry mater, crude protein, and essential amino acids of experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal in *P. monodon* juveniles.

Particulars	Experimental diets				SEM (±)	CV (%)	p value
	CNT	PP-0	PP-0.25	PP-0.50			
ADMD[1]	79.14^{b}	77.59^{c}	79.54^{b}	81.22^{a}	0.236	0.805	0.003
ACPD[2]	87.14^{ab}	83.47^{c}	86.83^{b}	89.29^{a}	0.791	1.350	0.005
Arg	90.77^{b}	90.01^{bc}	88.94^{c}	94.08^{a}	0.395	0.910	0.001
His	88.00^{a}	85.80^{b}	87.58^{a}	88.79^{a}	0.288	0.807	0.010
Ile	88.25^{b}	86.03^{c}	88.82^{b}	93.62^{a}	0.177	0.620	<0.001
Leu	88.47^{b}	84.98^{d}	85.88^{c}	91.70^{a}	0.049	0.333	<0.001
Lys	88.83^{b}	83.83^{d}	87.08^{c}	90.92^{a}	0.088	0.444	<0.001
Met	92.08^{a}	82.59^{c}	84.10^{b}	92.64^{a}	0.066	0.386	<0.001
Phe	86.12^{c}	84.02^{d}	90.52^{b}	93.28^{a}	0.241	0.730	<0.001
Thr	91.06^{a}	79.57^{d}	82.62^{c}	87.05^{b}	0.179	0.654	<0.001
Trp	89.14^{a}	85.47^{c}	88.17^{b}	89.47^{a}	0.090	0.449	<0.001
Val	89.32^{b}	80.59^{d}	86.01^{c}	91.75^{a}	0.064	0.384	<0.001

All the values are mean of three replications. Mean bearing the same superscript within the row do not differ significantly ($p > 0.05$). [1]Apparent dry mater digestibility. [2]Apparent crude protein digestibility.

Table 6: Growth performance of *P. monodon* juveniles fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Particulars	Experimental diets				SEM (±)	CV (%)	p value
	CNT	PP-0	PP-0.25	PP-0.50			
Initial wt (g)	0.52^{a}	0.49^{a}	0.53^{a}	0.51^{a}	0.001	3.756	0.182
Final wt (g)	4.86^{a}	4.16^{b}	4.73^{a}	4.62^{a}	0.011	3.064	0.004
DGC (%/d)[1]	1.48^{a}	1.36^{b}	1.44^{a}	1.44^{a}	0.001	1.442	0.002
FCR[2]	1.73^{c}	1.98^{a}	1.77^{b}	1.73^{c}	0.001	0.961	<0.001
PER[3]	1.54^{b}	1.38^{c}	1.54^{b}	1.61^{a}	0.001	0.659	<0.001
APU (%)[4]	28.97^{c}	25.87^{d}	29.75^{b}	30.91^{a}	0.017	0.597	<0.001
Survival (%)	95.56^{a}	95.56^{a}	95.56^{a}	97.78^{a}	0.948	1.334	0.183

All the values are mean of three replications. Mean bearing the same superscript within the row do not differ significantly ($p > 0.05$). [1]Daily growth coefficient. [2]Feed conversion ratio. [3]Protein efficiency ratio. [4]Apparent protein utilization.

0.8-12.6%. Dry matter and EAA like Ile and Phe of shrimp with PP-0.25 diet had higher digestibility by 0.5, 0.6, and 5.1%, whereas, except Thr (-4.4%), the digestibility of all parameters were found to be high in shrimp reared on PP-0.50 diet (0.4-8.3%) compared to the CNT diet.

3.2. Growth Performance. The growth performance of *P. monodon* juveniles fed various experimental diets is presented in Table 6. Results of the final body weight differed significantly ($p < 0.05$) at the end of the experiment. The DGC was not significantly different in the groups fed fishmeal substituted diets using *Bacillus* spp. fermented plant protein mix, whereas, shrimp fed on the PP-0 diet had the significantly lowest DGC among the dietary groups. The FCR was significantly ($p < 0.05$) high in shrimp fed PP-0.25 and PP-0 diets than CNT and PP-0.50 diets. The group fed PP-0.50 diet showed significantly ($p < 0.05$) higher PER and APU and they were low in the PP-0 fed group. However, CNT and PP-0.25 diets showed no significant difference in PER, whereas the PP-0.25 diet performed better in APU than the CNT. The dietary change had no effect on survival and was in the range of 95.56-97.78% among the treatments.

3.3. Body Composition. There was no significant difference in carcass composition (Table 7) but the dietary change influenced the quantity of free amino acids (FAA) in the tail muscle (Table 8). The Arg was the most abundant one among the essential FAA and was substantially higher in shrimp reared on the CNT diet. Similarly, His, Ile, and Lys were high ($p < 0.05$) in the same treatment compared to the other three dietary groups. However, shrimp fed with PP-0.50 diet had significantly ($p < 0.05$) higher values in other EAA, including Leu, Met, Phe, Thr, and Val. The level of Leu and Met in the tail muscle of *P. monodon* juveniles fed CNT and PP-0 diets did not differ significantly. The Ile between PP-0 and PP-0.25 and Met between CNT and PP-0.25 diets was not affected significantly. The dietary change had no influence on Trp levels among the treatments. The Gly was the predominant non-essential amino acid (NAA), accounting for more than half of the total, while Tyr had

Table 7: Whole-body composition (% dry weight basis) of *P. monodon* juveniles fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Particulars	Experimental diets				SEM (±)	CV (%)	*p* value
	CNT	PP-0	PP-0.25	PP-0.50			
Crude protein	73.24	72.92	73.50	73.53	0.162	0.723	0.512
Ether extract	8.09	8.37	8.06	8.07	0.012	1.751	0.101
Total ash	13.34	13.91	13.29	13.30	0.071	2.609	0.186
Arg	4.56	4.74	4.75	4.82	0.012	3.099	0.247
His	1.31	1.32	1.22	1.30	0.004	6.234	0.521
Ile	2.82	2.76	2.70	2.75	0.022	7.065	0.905
Leu	5.93	6.09	6.32	5.92	0.082	6.213	0.570
Lys	4.24	4.04	4.06	4.02	0.042	6.561	0.723
Met	1.75	1.86	1.63	1.81	0.009	7.252	0.266
Phe	2.99	3.20	3.08	2.90	0.022	6.391	0.349
Thr	2.57	2.65	2.74	2.76	0.102	15.674	0.939
Trp	0.58	0.58	0.52	0.62	0.004	13.591	0.501
Val	3.18	3.30	3.08	3.06	0.059	10.126	0.775

All the values are mean of three replications. No significant difference observed among the treatments.

Table 8: Free amino acid levels (mg/g of tissue) of tail muscle in *P. monodon* juveniles fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Particulars	Experimental diets				SEM (±)	CV (%)	*p* value
	CNT	PP-0	PP-0.25	PP-0.50			
Essential amino acids (EAA)							
Arg	8.33^{a}	6.93^{d}	7.34^{c}	8.18^{b}	0.001	0.176	<0.001
His	0.60^{a}	0.52^{c}	0.50^{d}	0.59^{b}	0.001	0.231	<0.001
Ile	0.68^{a}	0.55^{c}	0.55^{c}	0.59^{b}	0.003	0.372	<0.001
Leu	1.67^{b}	1.66^{b}	1.47^{c}	1.80^{a}	0.001	0.519	<0.001
Lys	1.47^{a}	1.37^{b}	1.26^{d}	1.32^{c}	0.002	0.332	<0.001
Met	0.65^{bc}	0.66^{b}	0.65^{c}	0.75^{a}	0.002	0.747	<0.001
Phe	0.36^{c}	0.40^{b}	0.35^{d}	0.43^{a}	0.003	0.241	<0.001
Thr	0.96^{d}	1.10^{b}	1.07^{c}	1.13^{a}	0.001	0.140	<0.001
Trp	0.28^{a}	0.21^{a}	0.27^{a}	0.28^{a}	0.001	14.574	0.211
Val	1.14^{b}	1.11^{c}	1.07^{d}	1.40^{a}	0.003	0.344	<0.001
Nonessential amino acids (NAA)							
Ala	2.80^{b}	2.76^{c}	3.28^{a}	2.76^{c}	0.002	0.194	<0.001
Asp	0.53^{a}	0.45^{c}	0.53^{a}	0.47^{b}	0.001	0.216	<0.001
Glu	2.15^{c}	2.02^{d}	2.17^{b}	2.46^{a}	0.002	0.380	<0.001
Gly	5.40^{c}	5.65^{b}	5.24^{d}	6.32^{a}	0.002	0.983	<0.001
Pro	1.31^{d}	1.38^{c}	1.45^{b}	1.47^{a}	0.002	0.154	<0.001
Ser	0.61^{c}	0.65^{b}	0.65^{a}	0.57^{d}	0.003	0.217	<0.001
Tyr	0.45^{b}	0.40^{c}	0.45^{a}	0.39^{d}	0.003	0.551	<0.001
∑EAA	16.18^{b}	14.55^{c}	14.57^{c}	16.52^{a}	0.002	0.378	<0.001
∑NAA	13.29^{c}	13.33^{c}	13.81^{b}	14.47^{a}	0.002	0.459	<0.001
∑TAA	29.47^{b}	27.88^{d}	28.38^{c}	30.99^{a}	0.006	0.36	<0.001

All the values are mean of three replications. Mean bearing the same superscript within the row do not differ significantly ($p > 0.05$).

the lowest value. When compared to other dietary groups, shrimp reared on PP-0.50 had the highest ($p < 0.05$) values for Glu, Gly, and Pro and the lowest ($p < 0.05$) values for Ser and Tyr, whereas Ala, Asp, Ser, and Tyr were significantly ($p < 0.05$) higher in the tail muscle of *P. monodon* juveniles fed PP-0.25 diet. The level of Ala and Asp did not differ significantly between PP-0 and PP-0.50 diets and between CNT and PP-0.25 diets, respectively.

Table 9: Nitrogen metabolism in sub-adult *P. monodon* fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Particulars	Experimental diets			SEM (±)	CV (%)	p value
	CNT	PP-0.25	PP-0.50			
N intake (mg/shrimp/meal)	1.72[a]	1.50[b]	1.52[b]	0.001	1.675	0.001
O consumption (μg/g of DM/h)	600.25[a]	536.31[b]	508.99[b]	149.271	2.931	0.005
N excretion (μg/g of DM/h)	19.58[a]	19.17[b]	17.29[c]	0.001	0.264	<0.001
O:N ratio	30.57[a]	27.94[b]	29.35[ab]	0.304	2.477	0.028

All the values are mean of three replications. Mean bearing the same superscript within the row do not differ significantly ($p > 0.05$).

3.4. Nitrogen Metabolism. Nitrogen intake and oxygen consumption were significantly ($p < 0.05$) high in the CNT diet compared to the diets of PP-0.25 and PP-0.50 (Table 9). Ammonia-N excretion was significantly ($p < 0.05$) decreased by 2.09 and 11.69% in the groups fed PP-0.25 and PP-0.50 diets compared to CNT. Shrimp reared on CNT and PP-0.25 diets significantly ($p < 0.05$) differed in O : N ratio, but both diets had no significant variation with the PP-0.50 diet. Regression of oxygen consumption, ammonia-N excretion, and O : N ratio (Figure 1) on nitrogen intake demonstrated that oxygen consumption ($R^2 = 0.8625$) and O : N ratio ($R^2 = 0.7791$) had higher coefficients of determination than the ammonia-N excretion ($R^2 = 0.5894$).

3.5. Enzyme Activities. The activity of hepatopancreatic digestive enzymes in subadult shrimp-fed experimental diets revealed that the activity of protease was influenced by the *Bacillus* spp. fermentation but not by their concentration, as shown in Figure 2. In comparison to the control group, shrimp fed with the diets PP-0.25 and PP-0.50 had significantly ($p < 0.05$) higher protease activity and the trend was similar for amylase and cellulase. The activity of xylanase was increased ($p < 0.05$) in the groups fed PP-0.25 and PP-0.50 diets compared to CNT, whereas no significant difference was observed in chitinase activity among the treatments. Similarly, *Bacillus* spp. fermentation had no effect on the activity of enzymes (GDH and arginase) analyzed in the gills of subadults (Figure 3).

3.6. Hemolymph Indices. The results of hemolymph indices of subadult shrimp-fed experimental diets (Table 10) revealed that shrimp on the PP-0.25 diet showed significantly ($p < 0.05$) higher hemolymph protein compared to other diets. There was no difference between CNT and PP-0.25 diets in lipid and glucose content, while it was significantly ($p < 0.05$) reduced in the PP-0.50-fed group. Both hemocyanin and ammonia increased ($p < 0.05$) with increasing the *Bacillus* spp. concentration used in our study whereas the reverse was true for urea.

4. Discussion

In our study, a conglomeration of *Bacillus* spp. was used to ferment the plant protein mix as it is more effective than the individual strains [28]. We optimized fermentation conditions using a variety of variables such as moisture, pH, temperature, and time. The diets containing *Bacillus* spp. fermented plant protein mix (PP-0.25 and PP-0.50), even after substituting 50% with fishmeal, resulted in a comparable growth in terms of final body weight and DGC as in control (no fishmeal substitution). The beneficial effects of *Bacillus* have earlier been reported in various shrimp species, in particular, *P. indicus* [29], *P. vannamei* [30], and *P. monodon* [11]. On the other hand, shrimp reared on a PP-0 diet containing untreated plant protein mix performed poorly compared to all other dietary groups. This could be attributed to the substitution of 50% dietary fishmeal. It is reported that the inclusion of plant proteins in shrimp feed, either individually or in combination, is a quite a challenge due to various factors, the most notable of which is the existence of antinutrients. The plant protein mix used in our study had 153.36 mg/100 g of trypsin inhibitor, 1708.55 mg/100 g of phytic acid, 266.69 mg/100 g of saponin, 488.43 mg/100 g of tannin, and 51.68 mg/100 g of glucosinolates.

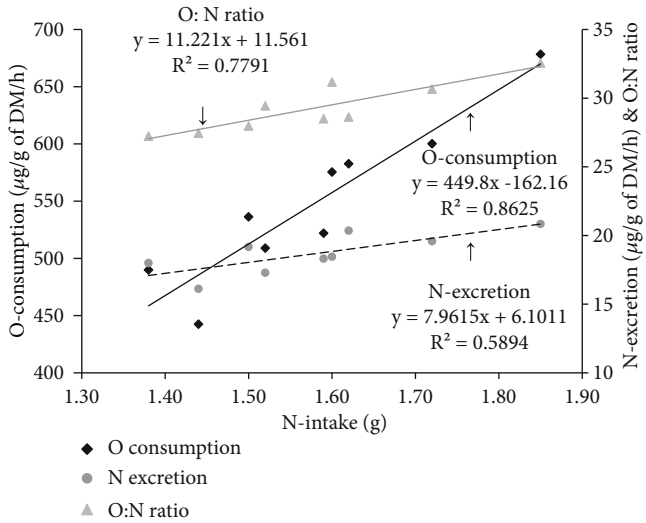

Figure 1: Regression analysis on oxygen consumption, ammonia-N excretion, and O : N ratio against nitrogen intake in subadult *P. monodon* fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

The negative effects of the abovementioned antinutrients have already been reported in various aquatic species. Makkar et al. [31] reported that tannin forms an indigestible protein complex by inhibiting the protease enzyme, which hinders protein digestibility. A similar effect was also

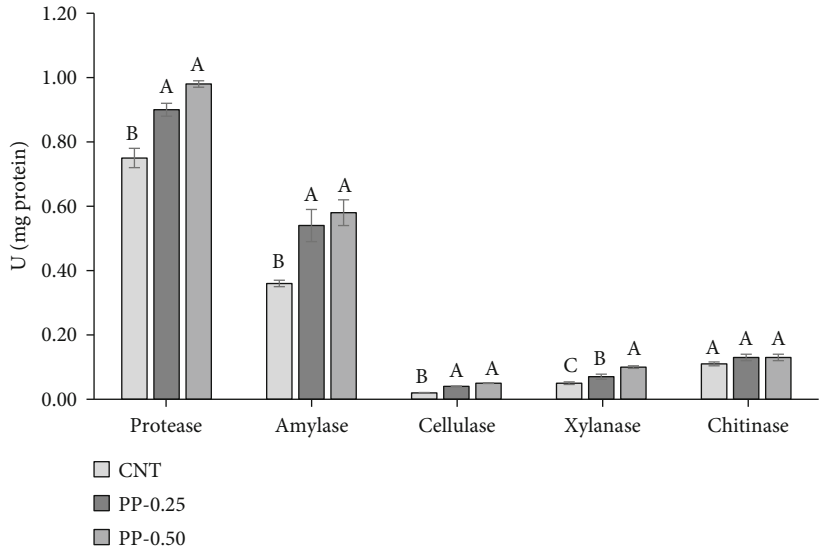

Figure 2: Digestive enzymes activity of hepatopancreas in subadult *P. monodon* fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

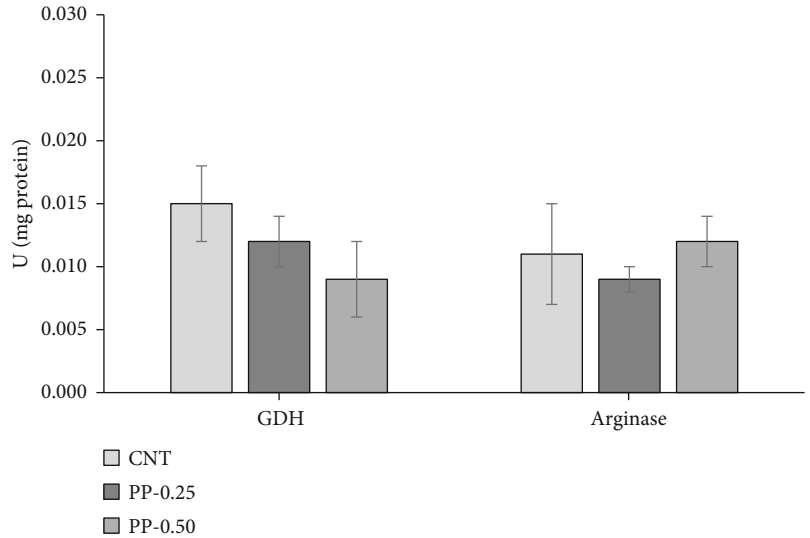

Figure 3: Glutamate dehydrogenase (GDH) and arginase activity in gill of subadult *P. monodon* fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Table 10: Hemolymph indices of subadult *P. monodon* fed experimental diets containing *Bacillus* spp. fermented plant protein mix by replacing fishmeal.

Particulars	Experimental diets			SEM (±)	CV (%)	*p* value
	CNT	PP-0.25	PP-0.50			
Protein (mg/ml)	23.88^{c}	29.87^{a}	27.64^{b}	0.044	1.015	<0.001
Lipid (mg/dl)	122.22^{a}	123.59^{a}	106.19^{b}	0.295	0.609	<0.001
Glucose (ml/dl)	1.45^{a}	1.44^{a}	1.27^{b}	0.001	1.102	<0.001
Hemocyanin (mM)	189.74^{b}	184.83^{c}	201.46^{a}	1.181	0.745	<0.001
Ammonia (mg/l)	2.11^{c}	2.54^{b}	3.05^{a}	0.001	0.225	<0.001
Urea (mg/l)	11.32^{a}	8.22^{b}	7.98^{c}	0.001	0.327	<0.001

All the values are mean of three replications. Mean bearing the same superscript within the row do not differ significantly ($p > 0.05$).

observed due to the presence of trypsin inhibitors [4]. Hossain and Jauncey [32] observed severe adverse effects in common carp when reared on a diet containing 1.14% of tannins than those reared on 0.57%. In addition, a lower feed intake was reported in salmonids due to poor palatability that was attributed to the bitter taste of tannins [33]. Minsalan and Chiu [34] reported that the inclusion of tea seed cake that is rich in saponins showed a poor growth rate in *P. monodon* than those fed a diet without tea seed cake. A similar result was reported in *P. japonicus* when exposed to >0.1 ppm of saponins [35]. Jannathulla et al. [4] reported that around 80% of the phosphorus exists as a phytate-bound form in plant proteins. Due to the lack of phytase enzyme in monogastric animals, in particular shrimp, it is not readily accessible to them, thereby retarding the growth by forming a complex of phytate proteins. Notwithstanding, Liener [36] stated that phytic acid can reduce the availability of major elements, including calcium, magnesium, and potassium. Likewise, the toxic effects of glucosinolates were reported earlier in fish reared on semipurified practical diets containing a higher content of either rapeseed meal or mustard seed meal [37]. The presence of these multiple antinutrients would be possible reasons for poor performance in the group fed PP-0 diet in our study.

The experimental diets of PP-0.25 and PP-0.50 were also formulated by substituting 50% dietary fishmeal with a plant protein mix. Although the abovementioned issues persist in these diets (PP-0.25 and PP-0.50) also, the shrimp performed better as in the CNT diet. This could be due to the beneficial effects of *Bacillus* spp. Cheng et al. [38] documented that *B. subtilis*-treated soybean meal could substitute 15% of dietary fishmeal in *P. vannamei.* The findings of Hamidoghli et al. [39] revealed an improvement in the nutrient utilization of plant proteins due to fermentation, which could substitute 30% of fishmeal in *P. vannamei.* Shiu et al. [9] reported that soybean meal could substitute 37.4% fishmeal in the diet of *P. vannamei*, which increased significantly after treatment with *B. subtilis.* This result is in agreement with the findings of Sharawy et al. [8], who found 28.6% fishmeal substitution with untreated soybean meal, which increased to 50% after treating with *Bacillus* spp. A similar observation was reported in *P. monodon* fed diets containing *B. coagulans*-treated oil cake mix [40]. The authors suggested that the improved performance of shrimp obtained with *Bacillus* spp. fermented ingredients could be due to the detoxification and/or elimination of antinutrients. In our study, *Bacillus* spp. fermentation enhanced the nutritional quality of plant protein mix by significantly ($p < 0.05$) reducing trypsin inhibitor, phytic acid, saponin, tannin, and glucosinolates by 100%, 54.0-55.3%, 61.9-63.2%, 40.3-42.8%, and 18.1-23.8%, respectively (Table 1) irrespective of the *Bacillus* spp. concentrations (0.25 and 0.50%) used for the fermentation process. These results are consistent with the earlier studies of Das and Ghosh [41], Jannathulla et al. [4], and Hamidoghli et al. [39]. The significant reduction in antinutrients could have contributed to obtaining better performance with the *Bacillus* spp. fermented plant protein mix than the untreated one.

Wang et al. [42] found that *Bacillus* spp. along with photosynthetic bacteria, rather than using them individually, resulted in a higher growth rate in common carp, and concluded that a conglomeration of *Bacillus* spp. yields better performance than using an individual one alone. Similarly, using five different *Bacillus* spp. together increased the weight gain of *P. monodon* from 890.09% (control) to 1016.82% [11]. *P. latisulcatus* fed with *P. synxantha* and *P. aeruginosa* separately had the SGR of 1.02 and 1.01, respectively, after 28 days according to Hai et al. [43] and was enhanced to 1.14 when both were used together. These results are in agreement in *P. vannamei* fed on diets containing both *B. subtilis* and *B. licheniformis* spp. (Sadat Hoseini Madani et al., 2018). The authors suggested that the improved growth with a conglomeration of microorganisms' could be owing to improved digestive ability mediated by increased digestive enzyme activity. For instance, the group fed PP-0.25 and PP-0.50 diets showed increased ($p < 0.05$) protease activity (0.90-0.98 U/mg protein) in the hepatopancreas, while it was 0.75 U/mg protein in shrimp fed CNT diet. A similar pattern was observed almost in all other enzymes reported in our study. However, the enzyme activities were not influenced by the concentration of *Bacillus* conglomeration. The results of digestibility parameters were higher in the groups fed diets containing *Bacillus* spp. fermented plant proteins (PP-0.25 and PP-0.50) compared to the one that had untreated plant protein mix (PP-0), which is almost identical to the findings of the feeding trial. A significant decrease in FCR was observed in juvenile shrimp fed with diets of PP-0.25 and PP-0.50 compared to the PP-0 diet. This result is in agreement with the findings in *P. monodon* [11] and *P. vannamei* [44] fed diets containing a mix of *Bacillus* spp. Similarly, a feed containing a mix of *B. subtilis*, *B. licheniformis*, and *E. faecium* decreased FCR in rainbow trout [45]. The FCR is greatly reduced nowadays compared to the earlier days, even the commercial formulation made with low-fishmeal showed FCR of <1.2. However, the values of FCR observed in our study were slightly higher than the previous reports. There are numerous factors that influence the FCR in addition to the quality of feeds, mainly environmental conditions, physiology of animals, genetic make-up of animals, feeding space, management practices, etc. This might be a reason for obtaining a slightly higher FCR in the present work. However, no parameters were studied to relate this concern during our work. However, these effects (if any) might be nullified in our study as all four treatments were carried out simultaneously in the same conditions. Both PER and APU were comparable in shrimp fed PP-0.25 diet to the control group and were significantly higher in PP-0.50 diet, indicating an increased efficiency of protein utilization, as a result of increased protease activity due to *Bacillus* spp. fermentation. Improved protein quality of the test ingredients due to fermentation could also be a reason for better protein utilization.

The dietary modification had no effect on carcass proximate composition and EAA. Adel et al. [46] found that the carcass composition of moisture, crude protein, crude lipid, and total ash content in *P. vannamei* fed diets containing *P. pentosaceus* at various concentrations (1×10^6, 1×10^7,

and 1×10^8 CFU/g) did not differ significantly. Similarly, *P. vannamei* fed fermented oil cakes had no effect on carcass composition [5]. However, increased crude protein content was reported in *P. vannamei* when reared on a mix of *B. subtilis* and *B. licheniformis* [44], which might be due to increased protein deposition that was attributed to the higher digestive enzyme activity. Although increased enzyme activities have been observed in our study, *Bacillus* spp. fermentation has not shown any protein deposition and had no effect on the whole-body composition. However, a significant difference was observed in the tail muscle composition of *P. monodon* juveniles among the dietary groups. In EAA, Leu, Met, Phe, Thr, and Val were found to be high in shrimp fed on a PP-0.50 diet and others like Arg, His, Ile, and Lys were high in the CNT diet-fed group, while there was no significant effect on the level of Trp among the treatments. Mente et al. [47] observed that *P. vannamei* fed an unbalanced diet had a lower FAA, which could be a reason for obtaining a lower value in most of the FAA in shrimp reared on a PP-0 diet in our study. The Arg was the most prevalent EAA regardless of treatment, which could be related to its crucial metabolic role as a precursor to the phosphagen phosphoarginine. The Gly was dominated in NAA. Although there were minor differences in individual FAA, the total remained consistent across all experimental groups, suggesting that intracellular amino acid pools are regulated by a mode of the active transmembrane transport system rather than the passive flow of amino acids [47].

A result of a three-week experiment performed in subadult *P. monodon* consequent to the feeding trial revealed that shrimp fed on PP-0.25 and PP-0.50 diets had a lower nitrogen intake than those reared with the CNT diet, which could be due to the substitution of dietary fishmeal. This lower intake consequently reduced the postprandial oxygen consumption rate by decreasing the metabolic rate in PP-0.25- and PP-0.50-fed groups. Various environmental factors influence the metabolic rate of cultured species by affecting the rate of oxygen consumption. Although all parameters have been kept at a constant level (Table 4) in our study, the rate of oxygen consumption rate was significantly increased in sub-adult shrimp fed a CNT diet, indicating that a diet high in fishmeal increased the metabolic rate of shrimp. Postprandial ammonia-N excretion was reduced in a group fed on PP-0.50, although all experimental diets were formulated to be iso-nitrogenous. The lower ammonia-N excretion in the PP-0.50 diet could be due to an improved protein quality that can be attributed to fermentation with *Bacillus* spp. Lovett and Felder [48] stated that the ability of aquatic species, in particular, shrimp, in utilizing protein as an energy source is closely related to the enzyme activity of digestive glands and is identified by measuring the O : N ratio. The O : N ratio was significantly lower in the PP-0.25 diet and was comparable to the PP-0.50 diet with the CNT diet, which indicates that *Bacillus* enhances the feed quality as in control and exploits the same as a structural component for growth.

The activity of GDH was almost similar among the experimental groups, indicating that *Bacillus* spp. fermentation would be effective in maintaining the protein quality of the diet even after replacing 50% of fishmeal. This trend was almost similar in the activity of arginase as in GDH. It is documented that hemolymph protein is an important assessment, as it plays a vital role in the regulation of inflammatory responses and response to infections and in addition, shrimp depend on the innate immune system [49]. An increased hemolymph protein with *Bacillus* spp. fermented plant proteins indicates that shrimp reared on both PP-0.25 and PP-0.50 diets were healthier. Notwithstanding, a higher nutritional status that can be attributed to *Bacillus* spp. would also be a reason for increasing hemolymph protein, which has already been reported in *H. gammarus* [50]. Almost a similar trend was noticed in hemocyanin content. In general, a high-quality feed slows the hemocyanin catabolism, which could be a reason for having high hemocyanin content in shrimp fed with the PP-0.50 diet. Yu et al. [51] found a similar increase in *P. vannamei*. Meshkini and Tafi [52] stated that hemolymph glucose is a stress indicator and was low in the groups fed on diets with *Bacillus* spp. fermented ingredients (PP-0.25 and PP-0.50) probably indicating that shrimp reared on these diets had higher resistance against common stress. Hemolymph lipid is a metabolic characteristic that varies according to the ability of the shrimp, digestibility of the diet and dietary sources. Although all extrinsic factors in our study were kept in a constant range (Table 4) among the treatments, the changes observed in hemolymph ammonia might be due to the dietary effect.

5. Conclusion

Fermenting the plant protein mix with a conglomeration of *Bacillus* spp. (*B. subtilis*, *B. licheniformis*, *B. polymyxa*, *B. megaterium*, and *B. pumilus*) improved the growth and digestibility of *P. monodon* juveniles and could be used as a promising fishmeal substitute in shrimp feed. *Bacillus* spp. fermented plant protein mix could replace 50% of dietary fishmeal without having any deleterious effects on shrimp in terms of growth performance, feed utilization, and whole-body composition. Similarly, *Bacillus* spp. fermentation had significant benefits and advantages on nitrogen metabolism in subadult tiger shrimp. However, the positive effect was more pronounced for certain parameters with a higher dose of 0.5% than the lower dose of 0.25%. Therefore, further studies with different microbial flora in various combinations and concentrations are required for commercial applications in the future.

Data Availability

The data that support the findings of this study are available based on a reasonable request from the corresponding author.

Ethical Approval

This study was undertaken with the approval of the Institute Animal Ethics Committee (IAEC), ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India.

Conflicts of Interest

There are no conflicts of interest declared by the authors.

Authors' Contributions

Dr. V. Rajaram was involved in the study design, conducted the experiment, and analyzed all parameters. Dr. R. Jannathulla wrote the draft of the manuscript. Dr. R. Rajendran assisted in conducting experiments and analysis. Dr. K. Ambasankar K did the statistical analysis. Dr. A. Panigrahi procured the experimental animals and created experimental facilities. Dr. J. Syama Dayal drafted a fair draft and coordinated overall.

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