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Effect of fungal fermented sunflower oil cake on digestive enzymes activity and immune responses of Whiteleg shrimp, *Penaeus vannamei* (Boone, 1931) reared at indoor laboratory conditions

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Abstract
A 45-days trial was performed to assess the effect of fermented sunflower oil cake (FSFC) on digestive enzymes activity and immune responses in *Penaeus vannamei.* Five iso-nitrogenous diets were formulated with FSFC (0, 25, 50, 75 and 100 g/kg) by substituting fishmeal (w/w). A total of 300 juveniles (3.08±0.07 g) were randomly distributed to the experimental tanks (twenty shrimp per tank) with three replications for each treatment. Results revealed that protease activity was decreased (P<0.05) with increasing the inclusion level of FSFC. Shrimp fed with FSFC 75 diets had a higher (P<0.05) amylase activity (0.816 U/mg of protein) than others. Lipase activity did not vary significantly among the treatments. Total haemocyte count was 11.24-18.06x10^6 cells/ml. Control group has shown the highest activity of phenoloxidase (2.85 dopachrome/ml). Results concluded that fishmeal could be partially substituted with FSFC without having any negative impact on health status of *P. vannamei*.

Keywords: *Aspergillus niger,* digestive enzyme, fermentation, immune response, sunflower oil cake, *Penaeus vannamei*

Introduction
Pacific whiteleg shrimp (*Penaeus vannamei*) is a commercially important species worldwide and it is nowadays, most extensively cultured in India due to its ability of higher tolerance to salinity, temperature and the availability of specific pathogen free post larvae [1]. Its increased market demands forced the aquafeed industry to formulate an improved feed with high quality protein sources. Though fishmeal is being used as a good protein sources traditionally, its demand and higher cost is created a need for suitable alternatives. The field of solid state fermentation is emerged in recent years to maximize the utilization of plant proteins by substituting a considerable quantity of fishmeal to formulate the cost-effective feeds. Fermented plant proteins were incorporated in shrimp feed with varying degree of success in earlier studies [2-6]. However, complete fishmeal replacement by using the same is a quite challenging task to the researchers. Le Moullac and Haffiner [7] stated that having deeper knowledge of the suitability of any fishmeal alternative on the biological performance of shrimp, in particular digestive enzymes activity and their distributed patterns would be helpful in formulating the most effective feeds. In addition, there is no perceptible signs due to dietary changes, however it led a severe impact on the health status of organisms that would not emerge from nutritional parameters in general. Rumsey *et al.* [8] observed serological and non-specific defence mechanisms in Rainbow trout fed diets containing graded level of soybean meal by substituting fishmeal but the same was absent in those fed control diet (no fishmeal replacement). Barros *et al.* [9] reported that immune parameters of Channel catfish was affected due to the supplementation of cottonseed meal and iron and the result was corroborated with the findings of Krogbahl *et al.* [10] in Atlantic salmon. However, available reports related to fermented ingredients on the immune responses of the shrimp is scarce. Hence, it is aimed to investigate the effect of inclusion of fungus, *Aspergillus niger* fermented sunflower oil cake on three digestive enzymes activity (protease, lipase and amylase) and two immune responses (total haemocyte count and phenoloxidase activity) in *P. vannamei* in the present study.

The output from this study would help to explore the usage and limitation of fermented ingredients in producing good quality shrimp.

Materials and Method

Fermentation methodology

Commercial solvent extracted sunflower oil cake was purchased from various local markets (n=6), Chennai, India. De-ionized water was added to a ground sample (<500 µm) to adjust the moisture content of 60-65%. The hydrated samples were autoclaved for sterilization at 121 °C (105 kPa) for 15 min. The cooled sterilized samples were inoculated with 5% *A. niger* suspension (10^7 spores/ml). The ATCC culture of the fungus used in the present study was purchased from Himedia Laboratory, India, which is listed under GRAS notifications (Generally Recognized As Safe) by FDA (GRAS Notice No. 35, 2010). Prior to inoculation, the ATCC culture was grown on potato dextrose agar (PDA) for five days at 35±1 °C in an incubator and fungal spores were harvested using 0.1% Tween-80 and the suspension was approximately adjusted to 10^7 spores/ml. fermentation was carried out in Erlenmeyer flasks (500 ml) for three days at 35±1 °C in an incubator [11-12]. The flasks were plugged with cotton to facilitate air transfer. At the end of the fermentation process, all the samples were dried (50 °C) for 48 h to bring down the moisture content below 10% and then stored at 4 °C after grinding to fine particles of <250 µm.

Experimental diets

A control diet was formulated with locally available ingredients based on the nutritional requirements recommended for *P. vannamei* which contained 250 g/kg of fishmeal as a major protein source. In experimental diets, FSFC was included at the rate of 0 (control), 25, 50, 75 and 100 g/kg by replacing fishmeal (w/w). The experimental diets were prepared according to Dayal *et al.* [13], briefly, all the listed dry ingredients (Table 1) were ground to a particle size of <250 µm using a micro pulverizer. Pre-mix including, vitamin-mineral mixture, binder and butylated hydroxytoluene (BHT as an antioxidant) were added to the ground materials. After 2 to 3 min of the manual mixing, oil sources (fish oil, palm oil and soy-lecithin) were added and blended in an electric blender for 20 min. The homogenized mash was then hydrated with water (500 ml/kg) and kneaded into the dough. The dough was then steamed at atmospheric pressure for 5 min and pelleted in a table top pelletizer having a 2 mm diameter die. The pellets were dried in a forced-air oven at 60 ºC for 12 h, sealed in a plastic container and stored at 4 ºC until further use. The proximate composition of experimental diets is given in Table 1.

Table 1: Ingredient and proximate composition of experimental diets containing graded levels of fermented soybean meal and sunflower oil cake (g/kg as fed basis)

Particulars	Experimental diets				
	FSFC 0	FSFC 25	FSFC 50	FSFC 75	FSFC 100
Fishmeal[1]	250	225	200	175	150
FSFC[2]	-	25	50	75	100
Acetes[3]	80	80	80	80	80
Squid meal	15	15	15	15	15
Soybean meal	200	200	200	200	200
Corn gluten	20	27	34	42	50
Sesame cake	50	50	50	50	50
Wheat flour	324	315	306	296	286
Fish oil[1]	20	20	20	20	20
Palm oil	-	2	4	6	8
Lecithin	10	10	10	10	10
Pre-mix[4]	20	20	20	20	20
Binder[5]	10	10	10	10	10
BHT[6]	1	1	1	1	1
Proximate composition					
Moisture	87.6	88.2	87	84.5	86.7
Crude protein	374.4	372.6	371.4	374.3	381.7
Ether extract	67.6	69.8	71.1	71.3	70.5
Crude fiber	29.8	29.8	32.6	34.4	40.4
NFE	297.1	305.4	315.1	305	292.8
Total ash	143.5	134.2	122.8	130.6	127.9

[1]Bismi Fisheries, Mayiladuthurai, Tamil Nadu, India

[2]Fermented sunflower oil cake

[3]Mantis shrimp used as a protein source

[4]Pre-mix (g kg^{-1}): Thiamine hydrochloride (25.50 g), riboflavin (25.00 g), prydoxine hydrochloride (50.00 g), cyanocobalamin (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), $ZnSO_4.7H_2O$ (28.28 g), $MgSO_4.7H_2O$ (0.12 g), MnSO4.H2O (12.43 g), $CuSO_4.5\ H_2O$ (19.84 g), $CoC_{12}.6H_2O$ (4.07 g), KIO_4 (0.03 g), KCl (15.33 g), Na_2SeO_3 (0.02 g)

[5]Pegabind, Bentoli AgriNutrition Asia Pvt Ltd, Singapore

[6]Butylated hydroxytoluene: Sigma Aldrich (Cat. No: PHR1117)

Experimental conditions

A 45-days' indoor laboratory trial was performed at the Muttukadu Experimental Station of ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India with the juveniles of *P. vannamei* procured from the local farm. Prior to the experiment, juveniles were acclimatized to the indoor laboratory conditions and fed with a control diet having 374.46 g/kg of crude protein and 67.6 g/kg of ether extract for two weeks. Post acclimatization, a total of 300 healthy shrimps with an average weight of 3.08±0.07 g were

randomly distributed to 15 numbers of 500 L (1.31 x 0.64 x 0.73 m) oval-shaped fiberglass reinforced plastics (FRP) tanks at the rate of twenty shrimps per tank with three replicates for each treatment. All the tanks were equipped with aquaculture flow-through system (1.5 ml/min) and covered with a fiber mat to prevent the light intensity and escapees. Shrimps were fed the respective diets (6% of the biomass) three times at 7.00 AM, 12.00 PM and 5.30 PM and the feed given was adjusted according to body weight, survival and consumption. The uneaten feed particles (if any) were siphoned out from the experimental tanks after an hour of feeding by using a clean Falcon tube and dried at 60°C in a hot air oven overnight to measure the feed intake on a daily basis. During the experimental periods, ultraviolet treated water was used after filtering through a 5 µm cartridge filter. The water quality parameters *viz*., salinity (20 g/L), temperature (26.5 to 28.5 °C), dissolved oxygen (5.8 to 7.8 mg/L), pH (8 to 8.5) and total ammonia-nitrogen (<0.1 mg/L) were found to be within the normal range.

Digestive enzymes

At the end of the experiment, a total of 15 shrimp in a treatment (5 shrimp per replication) were collected and washed with de-ionized water to remove the adhering contaminations. Shrimps were dissected to remove the hepatopancreas and was rinsed again with de-ionized water. Hepatopancreas was homogenized with ice-cold de-ionized water (1:10) at 4 °C and the extract was centrifuge at 5000 g for 10 min. The supernatant was collected in an Eppendorf vial and used as a crude enzyme. The enzyme extract was kept at -80 °C until being analyzed. The total soluble protein was determined by the Lowry *et al.* [14] method using bovine serum albumin (Sigma-Aldrich, USA) as a standard in a UV-Visible spectrophotometer (UV-1800, Shimadzu). All the digestive enzymes, including protease, lipase and amylase studied in the present investigation was determined by the methods described in Moreno-Arias *et al.* [15] with slight modifications and their activity was expressed as U/mg of protein.

The protease activity was analyzed by adding 10 µl of enzyme extract to 250 µl of Tris buffer (50 mM, pH 7.5). They were mixed with 2% azocasein (substrate) that previously kept at 4°C and allowed to incubate for 10 min. After incubation the enzymatic reaction was stopped by adding 500 µl of 20% trichloroacetic acid. The content was centrifuged at 2000 g for 10 min. The upper phase was collected using a pipette and its absorbance was read at 440 nm in a UV-Visible spectrophotometer. Lipase activity was determined using 4-Nitrophenyl stearate as a substrate. To the 10 µl of enzyme extract, 100 µl of sodium taurocholate (100 mM) and 1900 µl of Tris-HCl buffer (50 mM, pH 7.2). The mixer was incubated at room temperature for 30 min after adding 20 µl of substrate (200 mM). A solution contains 20 µl of Fast Blue (100 mM), 200 µl of trichloroacetic acid (0.72 N), 2.71 ml of ethanol: ethyl acetate (1:1) was added to arrest the enzymatic reaction. After incubation, absorbance was read at 540 nm in a UV-Visible spectrophotometer. In estimating the amylase activity, the mixer of 5 µl of enzyme extract, 500 µl of Tris-HCl buffer (50 mM, pH 7.5) and 500 µl of soluble starch (1%) was kept at room temperature. After 10 min of incubation, the enzymatic reaction was arrested by adding 200 µl of sodium carbonate (2 N) and 1.5 ml of dinitrosalicylic acid. The mixture was agitated and kept in a boiling water bath for 10 min. The content was cooled, made up to 10 ml using de-ionized water and read at 550 nm in a UV-Visible spectrophotometer.

Immune responses

Two immune parameters *viz*., total haemocyte count (THC) and phenoloxidase activity were assayed in the present study. For that, haemolymph samples were acquired from five shrimps in each replication of a treatment (fifteen shrimp per treatment) through the ventral sinus in the first abdominal segment using a 26-gauge hypodermic needle on a one ml syringe containing an equal volume of fixative solution (10% formalin in 0.45 M sodium chloride). The THC was determined by a method of Sritunyalucksana *et al.* [16] with slight modification. Briefly, a known volume of fixed haemocyte suspension was mixed with an equal volume of Rose Bengal Solution (1.2% Rose Bengal in 50% ethanol) and incubate at a temperature between 27 and 35 °C. Haemocytes were counted in a haemocytometer (Neubauer, Marienfeld, Germany) in five out of 25 squares. The volume of each square is 0.2x0.2x0.1 mm^3 and the THC was reported as 10^6 cells/ml of haemolymph.

The activity of phenoloxidase was assayed using a method of Cheng and Chen [17]. The collected haemolymph was centrifuged at 300 g at 4 °C for 10 min. The settled pellet was collected after discarding the supernatant and was re-suspended in a ml of cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride and 0.10 M trisodium citrate, pH 7.0). the content was mixed and centrifuged again in a similar condition mentioned above. After centrifugation, the supernatant fluid was discarded and the pellet was again re-suspended in 200 µl of cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride 0.01 M calcium chloride and 0.26 M magnesium chloride, pH 7.0). To the 100 µl of aliquot, 50 µl of elictor solution (mg/ml of trypsin) was added and kept at 25 °C for 10 min. To which, 50 µl of substrate (L-dihydroxy phenylalanine) was added and made up to a ml using cacodylate buffer after 5 min. A blank was also treated simultaneously without trypsin and absorbance was measured at 490 nm in a UV-Visible spectrophotometer (UV-1800, Simadzu). The activity of phenoloxidase was expressed as dopachrome formation per ml of haemolymph.

Biochemical composition

The method of AOAC [18] was used to analyse the proximate composition of experimental diets. Briefly, the samples were dried at 105 °C in a hot air oven overnight to determine the moisture content. The crude protein was computed by multiplying the analyzed nitrogen (Kjeltec™-8100, Tecator™ Line) with a common empirical factor (6.25). Crude lipid was extracted using petroleum ether (60 to 80 °C) in a Soxhlet extraction unit (Scocs Plus-SCS 6). The crude fiber was analyzed in FOSS-2022 (Tecator™) using a fiber cap after digesting the sample in 1.25% of acid (sulphuric acid) and alkali (sodium hydroxide). The sample was incinerated at 540 °C in a muffle furnace for 6 h to estimate the total ash content. Nitrogen-free extract (NFE) was calculated by a difference of 1000 – (g/kg of moisture + crude protein + ether extract + crude fiber + total ash).

Statistical analysis

Experimental data were subjected to one-way analysis of variance (ANOVA) to assess the effect of the fermented ingredient on *P. vannamei* and multiple comparisons of treatments were done using Tukey's test to find significant

differences between the treatments. Regression analysis was performed to assess the relationship between the inclusion level and THC. Prior to statistical evaluation, data were checked for ascertaining a normal distribution and then determining the homogeneity of variance. The entire data were analyzed using SPSS version 16.0 and statistical tests were evaluated at 5% significance (p<0.05).

Results and Discussion

Digestive enzyme activities of *P. vannamei* fed different experimental diets containing graded levels of FSFC is depicted in Table 2. Of all the analyzed enzymes, the highest activity corresponded to amylase (0.494-0.816 U/mg of protein) and the lowest to lipase (0.025-0.027 U/mg of protein). Protease activity was high in the control group (0.281 U/mg of protein) and was gradually (P<0.05) decreased with increasing the inclusion level of FSFC. This result is corroborated with the findings of Moreno-Arias *et al.* [15] when replacing fishmeal with vegetable mix in the diet of *P. vannamei.* However, no significant difference was observed in shrimp fed with the diets containing FSFC up to 50 g/kg compared to control in our study. Amylase activity was significantly (P<0.05) high in test groups fed diets containing FSFC compared to those fed control diet (FSFC 0). It has been suggested that the suitability of fermented ingredient usage was more in the diet of *P. vannamei* and is in agreement by Jannathulla *et al.* [5-6]. Among the experimental groups, the highest activity of amylase was found to be in a group reared with FSFC 75 diet (0.816 U/mg of protein). However, a non-clear correlation between the inclusion of FSFC and amylase activity was observed in the present study. Becerra-Dorame *et al.* [19] suggested that an influence of certain extrinsic and intrinsic factors would be a reason for obtaining a non-clear tendency among fishmeal replacement and digestive enzyme activities. The range of lipase activity in our results (0.025-0.028 U/mg of protein) was slightly higher in *P. vannamei* fed *Spirulina platensis* as a fishmeal substitute [20], but it was similar to those reported by Moreno-Arias *et al.* [15] for the same species. However, the activity of lipase was not significantly affected due to the dietary change in the present study as in the result of Pakravan *et al.* [20]. But, in contrast a significant difference was noticed by Moreno-Arias *et al.* [15] in the activity of lipase in *P. vannamei*.

Table 2: Digestive enzymes activity (U/mg of protein) of *Penaeus vannamei* fed experimental diets having graded levels of fermented sunflower oil cake (FSFC)

Particulars	Digestive enzymes activity		
	Protease	Lipase	Amylase
FSFC 0	0.281^{a}±0.041	0.027^{a}±0.002	0.494^{e}±0.028
FSFC 25	0.253^{a}±0.061	0.027^{a}±0.004	0.671^{b}±0.027
FSFC 50	0.252^{a}±0.008	0.025^{a}±0.006	0.537^{d}±0.061
FSFC 75	0.197^{b}±0.049	0.028^{a}±0.007	0.816^{a}±0.054
FSFC 100	0.173^{b}±0.052	0.026^{a}±0.005	0.619^{c}±0.042

All the values are mean±SD of three replications

Values with the same superscript letters in the same column within the category did not significantly different (p>0.05)

The THC is an important indicator in assessing the shrimp health as it is associated with cellular mechanisms and was shown in Fig. 1. Dietary change had a significant (P<0.05) variation in THC and was varied from 11.24 to 18.06 x 10^6 cells/ml. The values obtained in the present study were almost similar to those reported in *Macrobrachium nipponense* [21], but were slightly lower compared to *P. monodon* [22]. The difference between the studies would probably due to the variation in adopting capability of species to the culturing environment [7]. The effect of various extrinsic factors, in particular temperature on THC have already been reported in different shrimp species earlier [7, 23]. THC (11.24 x 10^6 cells/ml) was found to be low (P<0.05) in shrimp reared with control diet (FSFC 0) and was significantly (P<0.05) increased with increasing the inclusion level of FSFC in the present investigation. The analysis of regression between the inclusion level and THC was $y = -0.1736x^2 + 2.8704 + 8.222$ and found to be highly correlated among the experimental groups (r = 0.9716).

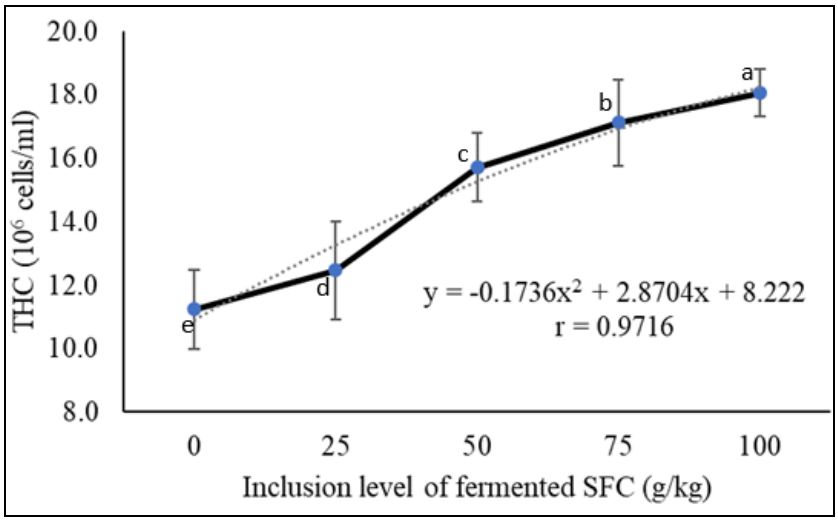

Fig 1: Total haemocyte count (THC) of *Penaeus vannamei* fed experimental diets with graded levels of fermented sunflower oil cake

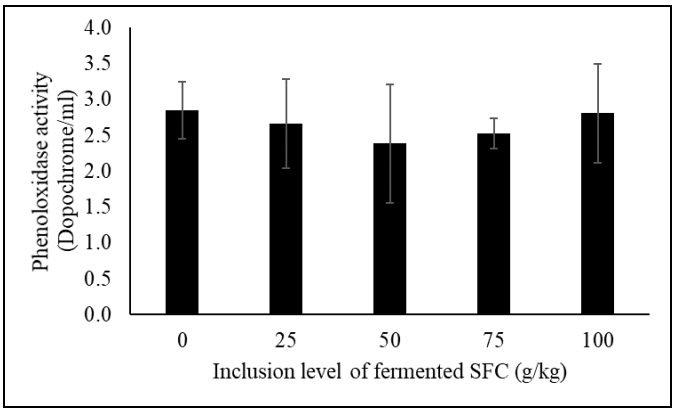

Fig 2: Phenoloxidase activity of *Penaeus vannamei* fed experimental diets with graded levels of fermented sunflower oil cake

The important role of phenoloxidase on defence mechanism has largely been discussed earlier [24]. The activity of phenoloxidase is influenced by various factors majorly by certain cations, in particular calcium and magnesium. An enhanced phenoloxidase activity by calcium and magnesium was reported in *P. paulensis* [25] and *P. monodon* [26]. Fishmeal contained higher calcium content than the fermented ingredients tested [4-5]. Phenoloxidase activity was in the range of 2.38-2.85 dopochrome/ml in FSFC fed groups (Fig. 2). Though control group has shown a highest activity of phenoloxidase (2.85 dopochrome/ml), it did not significantly differ from other treatments due to dietary change, which indicates that the partial substitution of fishmeal using fermented ingredients had no negative impact on the health status of *P. vannamei.*

Conclusion

It can be concluded that *P. vannamei* could be successfully formed with a feed containing minimum inclusion of fishmeal, without having a significant negative impact on immune response. Notwithstanding, an enhanced digestive enzymatic activity of shrimp was observed with fermented plant proteins. In conclusion, the analyzed parameters of *P. vannamei* in this study exhibited satisfactory results with respect to the inclusion of fermented sunflower oil cake (FSFC) in substituting fishmeal. Hence, fermented plant proteins could be considered as a sustainable ingredient in the shrimp feed industry, with great potential to reduce the pressure on fishmeal demand.

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