Effect of Partial Replacement of Fish Meal by *Bacillus* sp-Fermented Soybean Meal on Growth Performance, Immunity, Hepatopancreas Microbiota and Disease Resistance in Pacific White Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

This study examined the effect of substituting a diet containing 20% fish meal (FM) and 10% soy bean meal (SBM) with *Bacillus*-fermented soybean meal (FSBM) on growth, immune response, microbial community in the hepatopancreas, and disease prevention in Pacific white shrimp. Shrimp were fed diets with four levels of FSBM (0%, 15%, 20% and 25%) to replace FM and SBM. After 60 days of the feeding trial, no difference was found among treatments for specific growth rate (1.62-1.66 g), survival rate (88.08-93.64 %), feed conversion ratio (FCR) (1.31-1.41), or protein efficiency ratio (88.08-93.46). However, shrimp fed 25% FSBM showed a significant improvement in total hemocyte count (THC), phagocytic activity, and phenoloxidase activity (PO) compared with other groups. In the hepatopancreatic microbiota, we identified six phyla, of which Bacteroidetes was the most dominant phylum in the three groups fed with FSBM. In contrast, Firmicutes was dominant in the control group. After a challenge test with *Vibrio parahaemolyticus*, shrimp fed 25% FSBM had a significantly higher average survival rate compared with other experimental groups infected with *V. parahaemolyticus*. The histopathology of the hepatopancreas of shrimp from this group showed fewer signs of bacterial infection than other groups infected with *V. parahaemolyticus*. This study indicates that FSBM at the concentration of 25% can enhance immune response and tolerance to pathogenic *V. parahaemolyticus* in the Pacific white shrimp.

Keywords: *Bacillus*-fermented soybean meal (FSBM), Pacific white shrimp (*Litopenaeus vannamei*), *Vibrio parahaemolyticus*

INTRODUCTION

Fish meal (FM) has been utilized as a major protein source for farm-reared shrimp owing to its high nutritive value. The use of FM has become more limited in recent years due to its high cost and environmental concerns regarding the sustainable use of marine resources (Tacon and Metian, 2008; Nguyen *et al*., 2017). Alternatives to FM using sustainable and more economical protein resources for shrimp are being sought and researched. Among other feed resources, soybean meal (SBM) is an promising candidate for FM replacement because of its acceptable amino acid content, consistent quality, and relatively low cost (Amaya *et al*., 2007). Aside from these favorable characteristics,

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however, high levels of SBM use may negatively influence animals' health. SBM includes several anti-nutritional aspects, including an imbalance in amino acids and low protein content compared to FM (Cheng *et al*., 2013; Sookying *et al*., 2013). However, additional processing of soybean meal by fermentation with *Lactobacillus* sp. and *Bacillus* sp. can remove anti-nutritional factors, decrease peptide size, enhance nutrient status of the diet, and reduce abnormalities in the digestive tract of aquatic animals (Hong *et al*., 2004; Yamamoto *et al*., 2010). Shao *et al*. (2018) reported that an acceptable degree of FSBM as FM replacement in the Pacific white shrimp diet is 20%-30%. However, our study differs from Shao *et al*. (2018) in that we replaced both FM and SBM with FSBM in the shrimp feed. Replacement of FM with fermented soybean meal (*Bacillus*-FSBM) in diets has been shown to improve digestibility and protein content (Kiers *et al*., 2000), reduce immune reactions, and increase overall amino acids (Song *et al*., 2008). *Bacillus* spp. is used as a probiotic in aquaculture, and several reports have demonstrated the advantages of utilizing *Bacillus* spp. to enhance growth performance and disease resistance in aquatic animals (Rengpipat *et al*., 2000; Vaseeharan and Ramasamt, 2003). In this study, the effects of partial replacement of FM and SBM with FSBM on growth, survival, immune response, hepatopancreatic microbiota, and disease resistance of white shrimp are assessed.

MATERIALS AND METHODS

Experimental diets

The control diet contained 20 percent fish meal and 10 percent soybean meal. Three experimental iso-nitrogenous diets were formulated to replace the SBM and a portion of the FM with 15, 20, and 25% FSBM (Table 1). All ingredients of each diet were blended with an electric mixer. This mixture was then autoclaved at 95±2 °C for 20 min and made into dry pellets using a pelleting machine at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University. Pelleted feed was further dried in a hot air oven at 60 °C for 3 h and until the moisture was lower than 10 %.

Table 1. Experimental feed formulation and proximate composition of feed (%) used in this study.

Ingredients	**Control**	**FSBM 15%**	**FSBM 20%**	**FSBM 25%**
Fish meal 60%	20.35	15.35	11.60	7.63
Soybean meal	10.00	-	-	-
Fermented soybean meal	-	15.00	20.00	25.00
Poultry Meal 64%	22.25	22.25	22.25	22.25
Wheat Gluten	1.25	1.25	1.25	1.25
Corn Protein Concentrate	3.75	4.50	4.50	4.50
Wheat Flour	25.00	25.00	25.00	25.00
Rice Bran	3.03	2.28	1.28	-
Premix	2.10	2.10	2.10	2.10
Preservative	4.53	4.53	4.53	4.53
Attractant	7.75	7.75	7.75	7.75
Chemical composition				
Ash	15.44	14.49	13.74	13.11
Carbohydrate	28.36	29.77	30.82	31.70
Lipid	8.00	7.60	7.23	6.84
Moisture	10.12	9.70	9.68	9.66
Protein	38.08	38.44	38.53	38.69

Experimental shrimp

Postlarvae-12 (0.002-0.003 g body weight) were acquired from a hatchery in Chachoengsao Province, Thailand. Shrimp were randomly assigned into 16 (500 L) fiberglass tanks at a density of 60 shrimp·tank^{-1}. Each treatment comprised four replicates. The shrimp were fed with one of the four experimental diets four times per day (08:00 am, 11:00 am, 02:00 pm, 05:00 pm) for 60 days. The water salinity for rearing shrimp was maintained at 25 psu. Water was exchanged daily to eliminate uneaten feed. Water quality, dissolved oxygen, temperature, pH, total ammonia, and nitrite was measured weekly. After the 60-day feeding trial, the final weight (shrimp weighed individually), weight gain, specific growth rate, survival rate, feed conversion ratio (FCR), and protein efficiency ratio were recorded.

$$\text{Specific growth rate} = \frac{\text{final weight-initial weight}}{\text{time (experiment period)}} \times 100$$

Immunology analysis

Following the 60-day feeding trial, 40 shrimp from each experimental group were randomly collected for immunological study. A hemolymph sample of 0.5 mL was withdrawn from the ventral sinus of each shrimp using a syringe containing 1.5 mL anticoagulant (K-199+5% L-cysteine).

Total hemocyte

Hemocytes were counted using a hemocytometer, and the number of blood cells per volume was calculated (total hemocytes per cubic millimeter).

Phagocytic activity

Phagocytic activity was determined following Itami *et al*. (1994).

Phenoloxidase activity test

Phenoloxidase activity was determined following Nonwachai *et al*. (2010).

Superoxide dismutase activity test

Superoxide dismutase activity was determined using a superoxide dismutase activity assay kit (Ransod), from Randox Company.

Microbiota analysis

Total genomic DNA was extracted from the hepatopancreas of five shrimp sampled from each treatment group using the DNeasy PowerSoil Kit (Qiagen, Inc., Hilden, Germany) according to manufacturer protocols. The NanoDrop spectrophotometer (A260 and A260/A280) was used to measure DNA concentration and quality. Agarose gel electrophoresis was used to confirm the size and quality of DNA fragments. The 16s rRNA gene was amplified using 341F (TCGTCG GCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCWGCAG) and 805R(GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAGG ACTACHVGGGTATCTAATCC), targeting V3-V4 variable regions and sparQ HiFi PCR master mix (Quanta bio, USA). The amplification conditions included an initial denaturation step at 94 ºC for 3 min, followed by 25 cycles at 98 ºC for 20 s, annealing at 55 ºC for 30 s, followed by a single final extension step at 72 ºC for 5 min. Also, the internal transcribed spacer of nuclear ribosomal DNA was amplified using the primers ITS-1F and ITS-2R with the following PCR conditions: an initial denaturation step at 94 ºC for 3 min followed by 25 cycles at 98 ºC for 20 s, annealing at 60 ºC for 30 s, extension at 72 ºC for 30 s, and finishing with a single final extension step at 72 ºC for 5 min. Subsequently, both metagenomic marker amplicons were purified using AMPure XP beads and indexed using 5 µL of each Nextera XT index primer in a 50 µL PCR reaction, followed by 8-10 cycles of PCR with conditions as above. The final PCR products were cleaned, pooled, and diluted to

a final loading concentration of 6 pM. Cluster generation and 250-bp paired-end read sequencing were performed on an Illumina MiSeq at Omic Sciences and Bioinformatics Center (Tanrattanapitak and Pairohakul, 2018). Sequencing read quality was examined using FASTQC software. Overlapping paired-end reads were assembled using PEAR. FASTX-Toolkit was used to filter out assembled reads that did not have a quality score of 30 for at least 90 % of bases; also, reads that were shorter than 300 bp were removed. Chimeras were removed by the UCHIME method (Edgar *et al*., 2011) as implemented in vsearch 1.1.1 (Rognes *et al*., 2016) using –uchime ref option against chimera free the Gold RDP database. OTU picking was performed with the *pick_open_reference_otus.py* command in QIIME 1.9.0, specifying SortMeRNA for reference picking, and taxonomic assignments were conducted against Greengenes 97 % database.

Challenge test with Vibrio parahaemolyticus

After the feeding trial, 30 healthy shrimp out of each replication were used in bacterial challenge tests. The challenge bacteria (*Vibrio parahaemolyticus*) was obtained from Aquaculture Business Research Center, Faculty of Fisheries, Kasetsart University, Thailand. This bacterium was grown in Tryptic Soy Broth with 1.5% NaCl at 37 ºC for 24 h. The experimental infection was performed by immersion of shrimp into the bacterial solution at a concentration of 105 $CFU \cdot mL^{-1}$ for 14 days. Two control groups were used, including a positive control group, which was challenged with *Vibrio parahaemolyticus*, and a negative control group without *Vibrio parahaemolyticus*. The survival of shrimp was recorded at the end of the trial.

Histopathological study

At the end of the challenge trial, ten shrimp from each group were fixed in Davison's fixative and processed for histological study, as described by Bell and Lighter (1998), to investigate the condition of the hepatopancreas and intestine.

Statistical analysis

All data from this research were analyzed with one-way ANOVA, and means were compared using Duncan's New Multiple Range Test.

RESULTS AND DISCUSSION

The growth performance, survival rate, FCR, and protein efficiency ratio after 60 days of the feeding trial are presented in Table 2. There was no difference between the growth performance, survival rate, FCR, or protein efficiency ratio among the groups of shrimp fed with different diets. Shiu *et al*. (2013) reported that the fermentation of SBM with *Bacillus subtilis* could decrease anti-nutritional factors in SBM and improve the nutrition value and utilization of FSBM in shrimp. Ding *et al*. (2015) suggested that FSBM can replace 25% of FM in the diet of *Macrobrachium nipponense* with no negative impact on shrimp health. Similarly, our results demonstrate that up to 25% of FM and 10% of SBM can be replaced with FSBM in Pacific white shrimp's diet without a negative influence on growth performance or survival rate of shrimp under experimental conditions (Table 1).

The immune response parameters of shrimp fed with different diets are presented in Table 3. Shrimp fed FSBM 25% had significantly higher ($p<0.05$) total hemocyte count (THC) compared to other groups, whereas the groups fed FSBM 20%, FSBM 15%, and the control group were not different from each other. Likewise, the shrimp fed FSBM 25% had significantly higher ($p<0.05$) percentage phagocytic activity than other groups. No difference was found in percentage phagocytic activity among groups fed FSBM 20%, FSBM 15%, and the control group. Shrimp fed FSBM 25% demonstrated significantly higher ($p<0.05$) phenoloxidase activity in comparison to other groups. There was no difference between the phenoloxidase activity of shrimp fed FSBM 20% and FSBM 15%, while shrimp from the control group

had considerably lower ($p<0.05$) phenoloxidase activity than all other groups. Shrimp fed with FSBM 25% had the highest superoxide dismutase activity, followed by the group fed FSBM 20%, FSBM 15%, and the control group. There was no difference between the superoxide dismutase activity of shrimp fed FSBM 25% and FSBM 20%. Shrimp fed FSBM 25% had considerably higher ($p<0.05$) superoxide dismutase activity compared to the group fed FSBM 15% and the control group. No difference was detected in superoxide dismutase activity among shrimp fed FSBM 20%, FSBM 15%, and the control group. This study suggests that the shrimp diet with FSBM 25% provides the highest immune response in comparison with other diets. The explanation for these results might be that fermentation of SBM by *Bacillus* spp. could inhibit the immunosuppression caused by SBM. Lin and Mui (2017) reported that bioactive peptides from the fermentation process might act as an immunostimulant for the Pacific white shrimp. Furthermore, the probiotic effects of *Bacillus* sp. used in this trial are also responsible or the immune-stimulatory ability. Previous reports mentioned that *Lactobacillus* sp. and *Bacillus* sp. can act as probiotics in shrimp culture, and that they can stimulate shrimp immunity (Rengpipat *et al*., 2000; Nayak *et al*., 2012; Zokaeifar *et al*., 2012; Wangsoontorn *et al*., 2018). Because shrimp lack adaptive immunity and rely upon their innate immune system, addition of *Bacillus*-FSBM in the shrimp feed can improve immune responses of shrimp (Farzanfar, 2006). As suggested by several reports, *Bacillus*-FSBM can stimulate the innate immune system of *Fennerpenaeus chinensis*, *Macrobranchium rosenbergii*, and *Litopenaeus vannamei* (Kim *et al*., 2015; Miao *et al*., 2017; Hamidoghli *et al*., 2020). These reports are consistent with our research, in that the replacement of FM and soybean meal (SBM) with 25% FSBM enhanced the shrimp's immune response.

Table 2. Growth performance of Pacific white shrimp fed different diets for 60 days.

Parameters	Control	FSBM 15%	FSBM 20%	FSBM 25%
Final weight (g)	6.27 ± 0.29^{a}	6.25 ± 0.11^{a}	6.27 ± 0.54^{a}	6.34 ± 0.34^{a}
Weight gain (%)	436.66 ± 53.85^{a}	454.99 ± 72.42^{a}	453.45 ± 44.91^{a}	460.81 ± 34.91^{a}
Specific growth rate (%·day^{-1})	1.62 ± 0.10^{a}	1.65 ± 0.13^{a}	1.65 ± 0.08^{a}	1.66 ± 0.06^{a}
Survival rate (%)	88.85 ± 4.76^{a}	88.08 ± 4.59^{a}	93.08 ± 1.99^{a}	93.46 ± 2.31^{a}
Feed conversion ratio (FCR)	1.4 ± 0.06^{a}	1.41 ± 0.07^{a}	1.34 ± 0.09^{a}	1.31 ± 0.03^{a}
Protein efficiency ratio	1.53 ± 0.03^{a}	1.51 ± 0.10^{a}	1.59 ± 0.13^{a}	1.62 ± 0.05^{a}

Note: Data are presented as mean±SD; Means in the same row with different superscripts are significantly different ($p<0.05$).

Table 3. Immune response of Pacific white shrimp fed different diets for 60 days.

Parameters	Control	FSBM 15%	FSBM 20%	FSBM 25%
THC (10^{6} cells·mL^{-1})	12.90 ± 2.47^{b}	10.93 ± 2.42^{b}	12.06 ± 0.98^{b}	15.38 ± 2.95^{a}
Phagocytic activity (%)	59.25 ± 3.30^{b}	60.25 ± 1.50^{b}	59.25 ± 0.96^{b}	64.25 ± 1.71^{a}
Phenoloxidase activity (PO units·min^{-1}·mg^{-1} protein)	242.80 ± 5.80^{c}	253.96 ± 5.37^{b}	256.93 ± 3.92^{b}	283.51 ± 5.75^{a}
Superoxide dismutase activity (SOD units·mL^{-1})	57.11 ± 1.08^{b}	57.18 ± 1.33^{b}	59.57 ± 4.04^{ab}	63.60 ± 3.85^{a}

Note: Data are presented as mean±SD; Means in the same row with different superscript are significantly different ($p<0.05$).

The cumulative mortality rates after challenge tests with *Vibrio parahaemolyticus* are presented in Figure 1. Shrimp in the negative control group had a significantly lower cumulative mortality rate compared with other groups. They were followed by the group fed FSBM 25%, which had a significantly lower ($p<0.05$) mortality rate compared to the positive control group, the group fed FSBM 15%, and the group fed FSBM 20%. There was no difference in mortality rate among groups fed FSBM 15%, FSBM 20%, and the positive control group after challenge with *V. parahaemolyticus*. Several previous studies have described curative properties of bioactive peptides in FSBM, including their antioxidant and antimicrobial properties (Liu *et al*., 2012; Sanjukta *et al*., 2015; Chen *et al*., 2017). Moreover, the protective ability of *Bacillus*-FSBM against Vibriosis in shrimp has been demonstrated in many studies (Decamp *et al*., 2008; Zokaeifar *et al*., 2012; Krummenauera *et al*., 2014). Our results are in agreement with these reports.

The bacterial diversity in the hepatopancreas of shrimp from each group is presented in Figure 2. More than 5,000 OUT (Operational Taxonomic Units) were analyzed and identified among six phyla, including Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Patescibacteria, and Actinobacteria. Bacteroidetes was the most dominant phylum in the hepatopancreas of the three groups fed with FSBM. In comparison, Firmicutes was dominant in the control group. Zhanga *et al*. (2014) reported that bacteria of the group Bacteroidetes are usually dominant in animals' gut and are involved with the degradation of fiber in the diet. The abundance of this phylum could be due to the higher fiber content in FSBM diets than the FM diet; we found a higher percentage of this bacteria in the group fed a higher content of FSBM. Fan *et al*. (2017) reported that Firmicutes was found abundantly in the intestine of mammals. In comparison, Proteobacteria was found in abundance in the intestine of many fishes and crustaceans (Wu *et al*., 2010; 2012; Rungrassamee *et al*., 2014; Miao *et al*., 2017). Since the effects of feed ingredients on the microbiota of the hepatopancreas of shrimp has rarely been studied, a comprehensive study using different replacement levels of FM with other terrestrial resources could provide valuable insights for shrimp nutrition and health.

Histology of the hepatopancreas of the shrimp from all experimental groups after the challenge test with *V. parahaemolyticus* is illustrated in Figure 3. Shrimp in the negative control group (not infected with *Vibrio*) showed signs of a normal hepatopancreas, with a high amount of lipid accumulation (R-cells); no sign of bacterial infection was found (Figure 3a, 3b). The hepatopancreas of shrimp fed with 25% FSBM and infected with *Vibrio* showed some signs of sloughing off of hepatopancreas cells (Figure 3i, 3j). A higher degree of sloughing off of cells was found in the groups fed 20% FSBM (Figure 3g, 3h) and 15% FSBM (Figure 3e, 3f). Shrimp fed the control diet and infected with *Vibrio* (positive control) showed signs of severe sloughing off of hepatopancreas cells (Figure 3c, 3d). These results show that the replacement of FM and SBM with 25% FSBM results in a better condition of the hepatopancreas than other diets after infection with *Vibrio*. This result is similar to the report by Yamamoto *et al*. (2010), which revealed that rainbow trout fed with a high level of FSBM had fewer effects on the histology of digestive tissues than the group fed with SBM. Likewise, Yao *et al*. (2019) reported that *L. vannamei* fed 33% FSBM had fewer effects on midgut histology compared with groups with less FSBM in the diet. The benefits of FSBM diets on the histopathology of shrimp in our study may be due to the probiotic effect and strong antioxidant activity of FSBM, which is in accordance with a previous study by Yatip et al. (2018), who reported that shrimp fed with *Bacillus*-FSBM extract had fewer signs of bacterial infection in the hepatopancreas and lower mortality after challenge with *V. harveyi*.

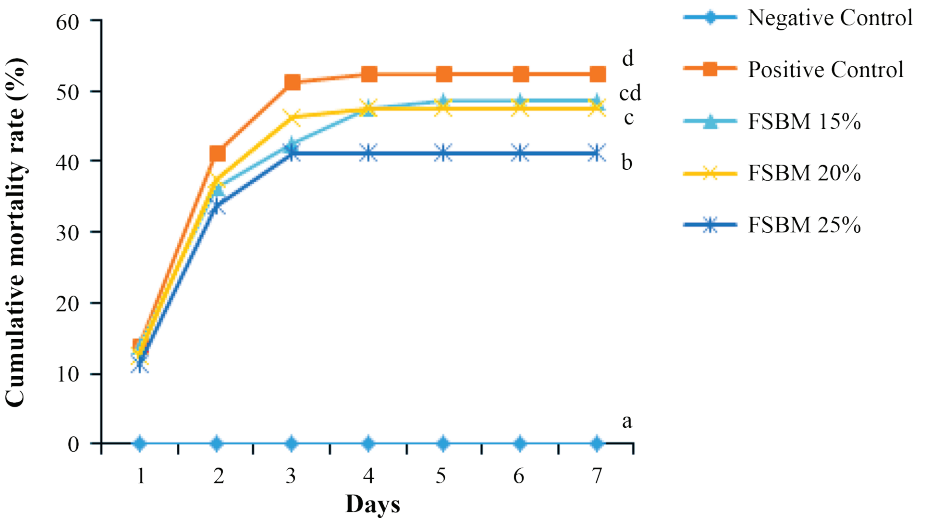

Figure 1. Cumulative mortality rate (%) of Pacific white shrimp after challenge with *Vibrio parahaemolyticus*; different letters at day 7th denote significant differences between means.

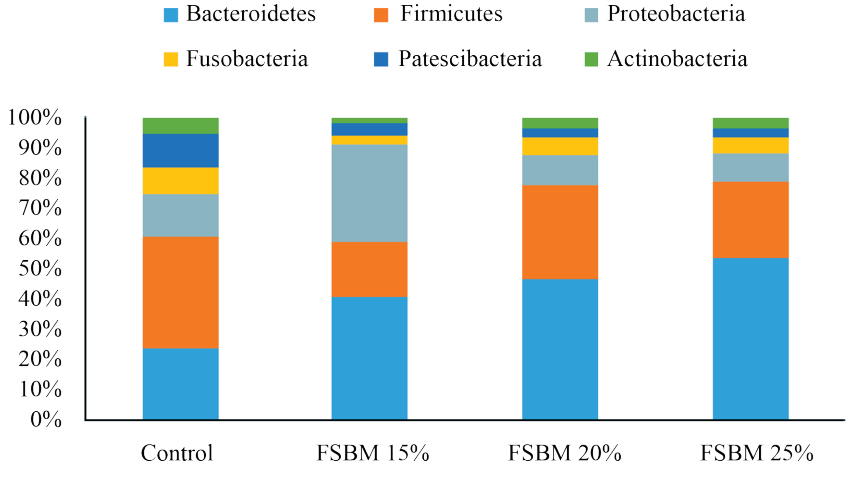

Figure 2. Proportions of dominant phyla in the hepatopancreas of *Litopenaeus vannamei* fed four experimental diets.

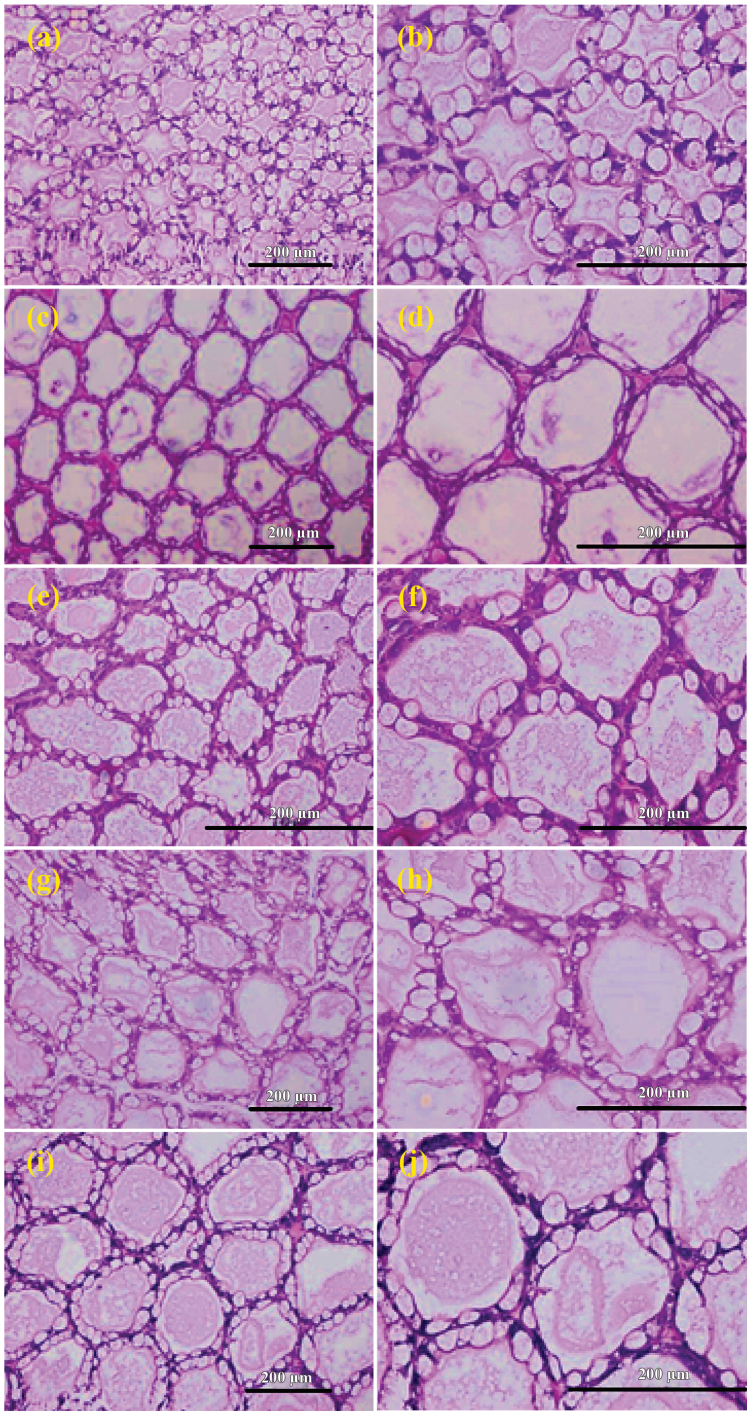

Figure 3. Histopathology of the hepatopancreas of *Litopenaeus vannamei* fed with different diets and challenged with *Vibrio parahaemolyticus* (H&E staining). Hepatopancreas of shrimp from the negative control group (without *Vibrio* infection), showing normal hepatopancreas cells (a, b). Hepatopancreas of shrimp fed the control diet and challenged with *Vibrio*, showing severe sloughing off of hepatopancreas cells (c, d). Hepatopancreas of shrimp fed 15% (e, f), 20% (g, h) and, 25% FSBM (i, j) challenged with

CONCLUSION

This study reveals that replacing FM (20%) and SBM (10%) with 25% *Bacillus*-FSBM in the diet of Pacific white shrimp has no effect on growth performance. *Bacillus*-FSBM (25%) significantly improves the immune response and makes shrimp more resistant to *Vibrio parahaemolyticus*. Bacteroidetes were dominant in the gut of shrimp provided with FSBM, while Firmicutes were prevalent in the hepatopancreas of shrimp from the FM group.

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