Optimizing dietary inclusion levels of fermented soybean meal with *Bacillus coagulans* for enhanced growth performance and feed efficiency in Nile tilapia (*Oreochromis niloticus*)

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Abstract. Nile tilapia (*Oreochromis niloticus*) has emerged as one of the most important and widely cultivated fish species in global aquaculture. The present study aimed to examine the effects of incorporating fermented soybean meal with *Bacillus coagulans* on growth performance and feed utilization in Nile tilapia's diets. Juvenile *O. niloticus*, each starting with an average initial weight of 25.22±0.45 g per fish were fed test diets containing varying levels of fermented soybean meal (0, 25, 50, 75, and 100%), while ensuring a consistent protein level of 32% and a digestible energy level of 285 kcal 100 g^{-1}. The experiment was conducted over a 60-day period. Prior to initiating the feeding trial, the protein digestibility of the experimental diets was evaluated. The study revealed that there was no statistically significant difference in protein digestibility ($p>0.05$). At the end of the 60-day feeding trail, it was noted that *O. niloticus* fed diets containing 50, 75, and 100% fermented soybean meal exhibited better growth performance in comparison to those fed diets with 0% and 25% fermented soybean meal, with a significant difference ($p<0.05$). Related to the percentage weight gain and specific growth rate, there were no statistically significant differences ($p>0.05$) observed in protein efficiency ratio, feed conversion ratio, and survival rate. The highest protein production value in *O. niloticus* fed diets containing 75 and 100% fermented soybean meal was found to be of 43.124±4.99% and 43.61±4.98%, respectively, which were significantly different ($p<0.05$) from those in the other experimental groups. Liver enzymes analysis indicated that fish fed diets with conventional soybean meal had the highest ALT levels (14.47±0.92 U L^{-1}), which were significantly different from other treatments ($p<0.05$), while AST and ALP levels showed no significant differences across groups ($p>0.05$). Histopathological examinations of liver and intestinal tissues revealed no abnormalities, indicating good health among the fish throughout the study. Overall, these findings suggest that enhancing the quality of soybean meal through fermentation with *B. coagulans* substantially improved its chemical properties and protein digestibility. Fermented soybean meal proves to be a viable protein source in diets at levels of 50% or higher, without adversely impacting growth performance and feed utilization in *O. niloticus*. Moreover, this approach may also have broader applications in aquafeed for other aquatic species.
Key Words: microbial fermentation, soybean meal, nutritive value, growth parameters.

Introduction. The global aquaculture industry is presently undergoing significant expansion. Experts anticipate that by 2030, the sector will need to produce an additional 106 million tonnes to satisfy worldwide consumption requirements (Zhang et al 2023a). This expansion has led to increased demand for aquafeed, with global production reaching 52.9 million tonnes in 2023. Nile tilapia (*Oreochromis niloticus*) has emerged as one of the most important and widely cultivated fish species in global aquaculture, with a production reaching approximately 6.31 million tonnes in 2023, accounting for 45% of total farmed tilapia production worldwide (El-Sayed & Fitzsimmons 2023). However, the industry faces challenges in sourcing sustainable protein ingredients, particularly fishmeal, which serves as the primary protein source and has seen rising prices and supply constraints. Consequently, aquafeed researchers have intensified their efforts to identify alternative raw materials that could effectively replace fishmeal as another alternative protein source, including plant-based proteins, insect meal, single-cell proteins, and other novel ingredients. Current investigative endeavors primarily thus target plant-based protein sources, such as soybean meal. In this regard, utilizing high levels of soybean meal in aquafeed presents certain limitations in its efficacy, however. A plurality of anti-nutritional factors (ANFs), including trypsin inhibitors, phytates, and hemagglutinin, have been identified as barriers to its optimal utilization. These ANFs significantly impede the digestibility and nutrient absorption of soybean meal, thereby limiting its applicability in certain aquatic species.

Enhancing the quality of soybean meal through microbial fermentation is a method to increase its utility in aquafeed. This involves fermenting soybean meal using various methods. For example, fermenting with bacteria such as *Lactobacillus plantarum*, *Bacillus subtilis*, *Bacillus licheniformis* and *Pediococcus acidilactici*, and also with yeasts like *Saccharomyces cerevisiae* and *Candida utilis*, as well as with the fungus *Aspergillus oryzae* (Chiu et al 2015; Ding et al 2015; Kim et al 2009; Lee et al 2016; Sharawy et al 2016; Shiu et al 2013; Wang et al 2016; Zhou et al 2011). This fermentation method can reduce anti-nutritional factors and enhance the efficiency of nutrient absorption and utilization.

Fermentation with *Bacillus coagulans* is an effective method for enhancing the quality of plant-based animal feed ingredients such as cottonseed meal, okara (soy pulp), and soybean meal. This technique optimizes the potential of these ingredients by improving their nutritional value and suitability for use in aquafeed. This process significantly improves the digestibility and nutritional value of soybean meal and other plant ingredients by reducing anti-nutritional factors, breakdown of complex carbohydrates and proteins into more bioavailable forms, and increasing essential amino acid content (Keong et al 2023; Shudong et al 2022). These combined benefits make *B. coagulans* fermentation a superior choice for improving the quality and nutritional value of plant-based ingredients in aquafeed, ultimately leading to enhanced fish growth, health, and production efficiency. Therefore, this study aimed to evaluate the effects of varying fermented soybean meal inclusion on protein digestibility, growth performance, feed utilization, and liver function-related enzymes in Nile tilapia. The findings will provide valuable insights to guide future aquafeed formulations, potentially enhancing the efficiency and sustainability of tilapia production.

Material and Method

Experimental design and diet preparation. Soybean meal was subjected to solid-state fermentation using *B. coagulans*, following the methods of Kim et al (2009), Ding et al (2015), Shiu et al (2013), and Wang et al (2016). The strain used was isolated from Thua Nao (a traditional fermented soybean product) and obtained from the Microbial Culture Collection, Thailand Institute of Scientific and Technological Research. The *B. coagulans* culture was propagated by cultivation in nutrient broth (NB) using an incubate shaker at 250 rpm, at 40°C for 24 hours. Subsequently, the obtained bacterial culture was centrifuged and washed. The bacteria were diluted with 0.85% NaCl to achieve a concentration of approximately 1×10^7 cfu mL^{-1} in a volume of 250 mL. The obtained bacterial solution was mixed with 500 g of ground soybean meal that had been steamed in an autoclave at 100°C for 10 minutes and dried at 60°C. Fermentation was conducted in plastic bags with a moisture content of 50% at 40°C for 48 hours.

After fermentation, the fermented soybean meal was used as a raw material to prepare the experimental diets by following these steps: the feed formulations were calculated to meet the requirements of Nile tilapia according to NRC (2011), with a protein level of 32% and an average digestible energy level of 285 kcal 100 g^{-1}. Using fermented soybean meal at various levels in the diets, the experiment used a Completely Randomized Design with five treatments and three replicates each, as follows (Table 1):

Treatment 1: Fermented soybean meal with *B. coagulans* 0% (Control; FSBM 0)
Treatment 2: Fermented soybean meal with *B. coagulans* 25% (FSBM 25)
Treatment 3: Fermented soybean meal with *B. coagulans* 50% (FSBM 50)
Treatment 4: Fermented soybean meal with *B. coagulans* 75% (FSBM 75)
Treatment 5: Fermented soybean meal with *B. coagulans* 100% (FSBM 100)

As a subsequent step, each feed ingredient was finely ground. The ground feed was then processed through an extruder at a temperature not exceeding 100°C, with a length and diameter of 5.0 mm. The extruded feed was air dried to a moisture content of no more than 10% and stored at a temperature of 4°C.

Table 1

Formulation and proximate composition in experimental diets using fermented soybean meal with *Bacillus coagulans* at various levels

Ingredients (%)	*Levels of fermented soybean meal (%)*				
	FSBM 0	*FSBM 25*	*FSBM 50*	*FSBM 75*	*FSBM 100*
Fish meal	7	7	7	7	7
Soybean meal	40	30	20	10	0
Fermented soybean meal	0	10	20	30	40
Poultry by-product meal	11	10.31	9.62	9.02	8.32
Corn	15	15	15	15	15
Tapioca starch	18.9	19.42	19.97	20.45	21.07
Vegetable oil	2.5	2.55	2.6	2.64	2.68
Fish oil	2.5	2.55	2.6	2.64	2.68
Dicalcium phosphate	1	1	1	1	1
Vitamin premix[1]	1	1	1	1	1
Mineral premix[2]	1	1	1	1	1
DL-methionine	0.1	0.13	0.13	0.13	0.13
Lysine	0	0.04	0.08	0.12	0.17
Proximate composition by analysis (%)					
Protein	32.07±0.42	32.70±0.55	31.99±0.56	31.94±0.39	31.92±0.59
Lipid	6.81±0.45	6.83±0.65	6.85±0.82	6.85±0.67	6.85±0.64
Fiber	2.45±0.19	2.46±0.44	2.49±0.34	2.51±0.36	2.50±0.82
Ash	4.99±0.19	5.02±0.65	5.09±0.65	5.07±0.52	5.10±0.62
NEF[3]	37.38±1.01	37.33±1.03	37.30±0.98	37.22±0.89	37.2±0.95
Digestible energy (DE)[4] (Kcal g^{-1})	295.99	295.94	295.98	295.86	295.92

[1] Vitamin mixture (mg or IU kg^{-1} diet): A 5,000 IU; D3 1,000 IU; E 5,000 mg; K 2,000; B1 2,500 mg; B2 1,000 mg; B6 1,000 mg; B12 10 mg; inositol 1,000 mg; pantothenic acid 3,000 mg; niacin acid 3,000 mg; C 10,000 mg; folic acid 300 mg; biotin 10 mg; [2] Mineral mixture (g kg^{-1} feed); calcium phosphate, 80; calcium lactate, 100; ferrous sulphate, 1.24; potassium chloride, 0.23; potassium iodine,0.23; copper sulphate, 1.2; manganese oxide, 1.2; cobalt carbonate, 0.2; zinc oxide, 1.6; Magnesium chloride, 2.16; sodium selenite, 0.10 ; [3] NFE = 100% (Crude protein - Crude lipid - Crude fiber-total ash moisture) (NRC 2011); [4] DE: Digestible energy calculated form digestible energy of ingredients as protein (4 kcal g^{-1}), lipid (8 kcal g^{-1}) and carbohydrate (2.5 kcal g^{-1}) in the diets; Kcal 100 g^{-1}.

In vitro protein digestibility. The protein digestibility of the experimental diets was studied using a modified method from Rungruangsak-Torrissen et al (2006). Enzymes extracted from Nile tilapia weighing approximately 30 g were used to digest the experimental diets. The free amino group from the digestion was measured using the TNBS method and compared with a standard curve of DL-Alanine. The results were expressed as μmol DL-Alanine equivalent feed per Trypsin activity, relatively to the trypsin enzyme activity.

Chemical composition. The chemical composition of the experimental diets and fish body were analyzed through proximate analysis following the standardized methods of AOAC (1990). from a sample of each experimental diets there were determined protein, fat, fiber, ash, and moisture contents. Gross energy (GE) was analyzed according to AOAC (1990) using an IKA bomb calorimeter C5000 from Germany. The non-fiber carbohydrate (NFE) content was calculated by difference, following the method described by NRC (2011).

Trypsin inhibitor activity. The inhibition of trypsin activity in the experimental diets was studied using a modified method from Kumar et al (2006) and Kumar et al (2019). BAPNA (benzoyl-DL-arginine-p-nitroanilide hydrochloride) was used as the substrate. The trypsin inhibitor in the sample was allowed to react with trypsin. BAPNA was then used as the substrate to measure the remaining trypsin by detecting absorbance at 410 nm.

Experimental fish and rearing conditions. Sex-reversed Nile tilapia juveniles were obtained from private farms in Sakon Nakhon Province, Thailand. The fish were acclimated in circular fiberglass tanks with a diameter of 150 cm and a height of 100 cm for 7 days prior to the study. During this period, they were fed a control diet (FSBM0) three times a day at 08:00, 12:00, and 17:00 for seven days. The experiment was conducted in fish with an average initial weight of 25.22±0.45 g per fish. The fiberglass tanks had a total capacity of 2,000 liters each and it contained 1,000 liters of water stocked with 30 fish and density of 30 fish per tank was maintained. Fish were fed the experimental diet until apparent satiation for eight weeks. Water quality parameters were monitored throughout the experiment, including temperature (30.41±2.94°C), pH (7.22±0.65), ammonia (0.14±0.08 mg N L^{-1}), and dissolved oxygen (5.32±0.58 mg L^{-1}). To ensure optimal rearing conditions, water quality parameters were monitored throughout the 60-day experimental period. Water temperature was measured twice daily, in the morning and afternoon. Dissolved oxygen (DO) levels were measured once daily to ensure they remained above 5 ppm. The pH of the water was measured every two days. DO was measured using a portable DO meter (YSI, 550A), pH was measured with a portable pH meter (Eutech, pH 450), and ammonia levels were checked using a portable ammonia meter (HANNA, HI96733). Water exchanges of 30–50% were performed every 7 days to maintain water quality.

Growth performance and feed utilization. At the end of the 60-day feeding period, all fish were counted and weighed to determine the growth performance, feed utilization parameters and survival rate. The obtained values were used to calculate the following: percentage of weight gain (WG, %), specific growth rate (SGR, % day^{-1}), protein efficiency ratio (PER), feed conversion ratio (FCR) and survival rate (SR, %) according to the following equations (NRC 2011):

WG = [(Final weight (g) – Initial weight (g) x 100]/Initial weight (g)
SGR = [(ln Final weight (g) – Initial weight (g)) x 100]/Duration of the experiment (days)
PER = Weight gain of fish (g)/Amount of protein consumed (g)
FCR = Feed intake (g)/[Final weight (g)-Initial weight (g)]
SR = [Number of fish at the end of the experiment/Initial number of fish]x100

Liver enzymes. At the end of the experiment, blood samples were collected from five fish per replicate. The sampling procedure involved puncturing the caudal peduncle of Nile tilapia specimens. Blood was collected into 1.5 mL Eppendorf tubes containing an anticoagulant. The blood samples were allowed to coagulate at room temperature. Following coagulation, the plasma was carefully separated for subsequent analysis of liver function-related enzymes. The activity of liver enzymes related was determined by measuring the levels of Aspartate transaminase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP). These enzymes were quantified in the plasma samples using an Automatic Chemical Analyzer (PKL, Italy).

The quantity of bacteria residing in the intestines of Nile tilapia and the histopathological changes in the intestine. To quantify the intestinal bacterial population in Nile tilapia, five fish per replicate were randomly sampled using aseptic techniques. The intestines were weighed and diluted with 0.85% NaCl solution. They were then homogenized using a homogenizer (IKA, T 25 Digital), and the intestinal homogenate was serially diluted tenfold with 0.85% NaCl solution. The diluted samples were used for microbial enumeration using the spread plate method on nutrient agar (Difco, USA), and the cultures were incubated at 30°C for 24-48 hours.

At the end of the experiment, intestinal tissue samples were collected from three randomly selected fish per replicate for histopathological analysis. Intestinal sections, of approximately 1–2 cm, were collected and preserved in 10% formalin for at least 24 hours. Following that, samples were dehydrated using an automatic tissue processor and embedded in paraffin to create tissue blocks. The tissue blocks were then sectioned at a thickness of approximately 3–5 μm. The slides of the samples were stained with hematoxylin and eosin (H&E), according to the method of Humason (1979), to examine histopathological changes under a microscope. The study focused on assessing alterations in the dimensions of intestinal villi, measuring both their width and height following consumption of experimental diets. The measurements were conducted in accordance with the methodology described by Hedemann et al (2005), allowing for a comparative analysis of villi width and height across different experimental groups.

Statistical analysis. All experimental data were presented as mean±SD. Statistical analysis was performed using statistical software (SAS 2009). All data were subjected to one-way Analyses of Variance (ANOVA). If significant differences were detected ($P<0.05$), the Tukey's multiple range test was used for determining the statistical significance among groups.

Results. The present study examines the fermented soybean meal by *B. coagulans* substituting soybean meal for Nile tilapia diets at varying levels: 0% (FSBM 0), 25% (FSBM 25), 50% (FSBM 50), 75% (FSBM 75), and 100% (FSBM 100) over a 60-day period. The results indicated that the protein digestibility remained consistent across the different groups, with no statistically significant differences observed ($P>0.05$), as presented in Table 2. The protein digestibility of the diets reached 0.83±0.03, 0.85±0.03, 0.87±0.03, 0.85±0.04, and 0.86±0.03 μmol Dl-Alanine 100 μg feed^{-1}, respectively. Regarding the trypsin inhibitor levels in the experimental diets, it was found that diets containing fermented soybean meal at 0, 25, and 50% had the highest trypsin inhibitor values of 0.21±0.012, 0.19±0.001, and 0.19±0.002 mg g^{-1}, respectively. These values were significantly different ($P<0.01$) from the diet with 75% fermented soybean meal, which had a value of 0.10±0.001 mg g^{-1}. In the experimental diet at FSBM 100 (100% fermented soybean meal), no trypsin inhibitor was detected, as shown in Table 2.

Table 2
In vitro protein digestibility and trypsin inhibitor levels in test diets of fermented soybean meal by *Bacillus coagulans* substituting soybean meal at various levels (mean±SD)

Parameter	*Levels of fermented soybean meal (%)*					*p-value*
	FSBM 0	*FSBM 25*	*FSBM 50*	*FSBM 75*	*FSBM 100*	
In vitro digestibility (μmol Dl-Alanine 100 μg feed^{-1})	0.83±0.03	0.85±0.03	0.87±0.03	0.85±0.04	0.86±0.03	0.954
Trypsin inhibitor (mg g^{-1} dry basis)	0.21±0.012^{a}	0.19±0.001^{a}	0.19±002^{a}	0.10±0.001^{b}	ND*	0.002

Means within the same row with different superscripts are significantly different at the 95% confidence level; *Not detected.

At the end of the experiment, the percentage weight gains in Nile tilapia fed diets containing fermented soybean meal at levels of 100, 75, and 50% was increased ($P<0.05$), compared to those of the groups fed diets containing 0% and 25% fermented soybean meal. Similarly, the specific growth rates (SGR) of fish fed diets containing 100, 75, and 50% of the fermented soybean meal were the highest ($P<0.05$). No significant differences were observed in protein efficiency ratio (PER), feed conversion ratio (FCR), and survival rate (SR). The observed PER values ranged from 3.22±0.04 to 3.26±0.06, while the FCR varied between 1.48±0.99 and 1.59±0.89 (Table 3). In terms of water quality during the experiment, all measured values remained within the acceptable range for aquaculture. The pH values ranged from 6.9 to 7.5, the temperature ranged from 27.2 to 33.8°C, and ammonia (NH_3) levels were less than 0.02 ppm.

Table 3

Growth performance and feed utilization of Nile tilapia fed test diets of fermented soybean meal by *Bacillus coagulans* substituting soybean meal at various levels for 60 days (mean±SD)

Parameter	*Levels of fermented soybean meal (%)*					*P-value*
	FSBM 0	*FSBM 25*	*FSBM 50*	*FSBM 75*	*FSBM 100*	
IW (g fish^{-1})[1]	25.47±0.34	25.43±0.94	25.21±0.94	25.06±1.01	25.13±0.92	0.71
FW (g fish^{-1})[2]	151.23±4.00^a	151.27±2.37^a	158.24±2.55^b	158.69±1.56^b	159.72±4.02^b	<0.01
WG (%)[3]	493.74±7.86^a	499.31±4.69^a	527.69±5.05^b	533.25±3.12^b	535.83±5.28^b	<0.01
SGR (% day^{-1})[4]	2.97±0.04^a	2.98±0.03^a	3.06±0.03^b	3.08±0.07^b	3.08±0.04^b	<0.01
PER[5]	3.26±0.06	3.24±0.04	3.26±0.06	3.23±0.03	3.22±0.04	0.624
FCR[6]	1.48±0.99	1.50±1.01	1.53±1.04	1.55±0.36	1.59±0.89	0.711
SR (%)[7]	95.56±1.92	97.78±1.92	97.78±1.92	96.67±1.92	95.56±3.33	0.691

Means within the same row with different superscripts are significantly different at the 95% confidence level.
[1] IW-Initial body weight (g fish^{-1}), [2] FW-Final body weight (g fish^{-1}), [3]WG-Weight gain (%), [4]SGR-Specific growth rate (% day^{-1}), [5] PER-Protein efficiency ratio, [6] FCR-Feed conversion ratio, and [7]SR-Survival rate (%).

Histopathological analysis following the 60-day experiment revealed no evidence of cellular degeneration across any experimental groups, with a significant distribution of goblet cells observed throughout the intestinal epithelium. Assessment of distal intestine integrity showed that Nile tilapia fed diets containing fermented soybean meal at levels of 50, 75, and 100% exhibited significantly greater villus height and width compared to those in the groups with 0 and 25% fermented soybean meal (P<0.05), as shown in Table 4. Villus height for fish fed diets containing fermented soybean meal at levels of 50, 75, and 100% groups were 342. 44±89. 86, 343. 66±79. 11, and 345. 44±88. 14 μm, respectively, while villus widths were 78.16±10.15, 78.66±13.48, and 77.33±10.82 μm, respectively.

Table 4

Intestinal villi morphology of Nile tilapia fed test diets of fermented soybean meal by *Bacillus coagulans* substituting soybean meal at various levels for 60 days (mean±SD)

Intestinal villi morphology	*Levels of fermented soybean meal (%)*					*P-value*
	FSBM 0	*FSBM 25*	*FSBM 50*	*FSBM 75*	*FSBM 100*	
Villi height (μm)	307.40± 39.05^a	308.33± 59.86^a	342.44± 89.86^b	343.66± 79.11^b	345.44± 88.14^b	0.001
Villi width (μm)	57.33± 9.79^a	56.59± 10.09^a	78.16± 10.15^b	78.66± 13.48^b	77.33± 10.82^b	<0.001

Means within the same row with different superscripts are significantly different at the 95% confidence level

The total microbial count in the gut showed no statistically significant differences (P>0.05) among all experimental groups. The total bacterial counts were (2.34±0.03)x10^7, (2.44±0.04)x10^7, (2.41±0.04)x10^7, (2.33±0.04)x10^7, and (2.41±0.04)x10^7 cfu g^{-1}, respectively (Figure 1).

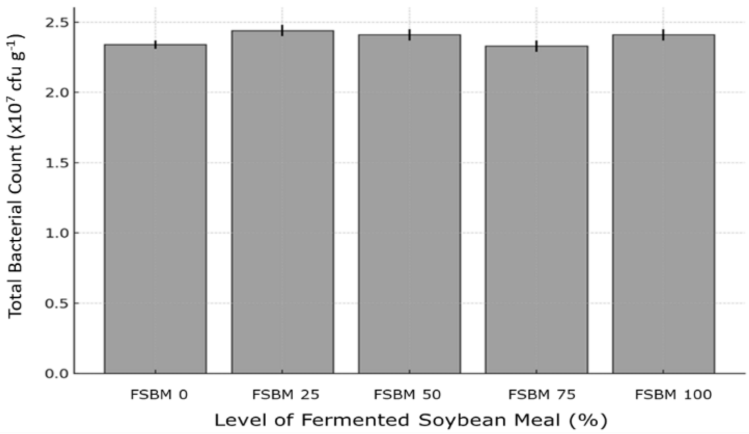

Figure 1. Total bacterial count in the gut of Nile tilapia fed test diets of fermented soybean meal by *Bacillus coagulans* substituting soybean meal at various levels for 60 days.

This experiment measured three enzymes: Aspartate transaminase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP). The results showed that AST levels in Nile tilapia-fed diets containing 0 and 25% fermented soybean meal were the highest, with values of 72.78±2.53 U L^{-1} and 70.60±1.37 U L^{-1}, respectively. These values were significantly different from those observed in the group that received 100% fermented soybean meal. The group that received 100% fermented soybean meal had the lowest AST level, at 67.63±3.32 U L^{-1}. However, the groups fed diets with 50% and 75% fermented soybean meal showed no statistically significant differences (P>0.05) from the other experimental groups, with values of 68.64±2.27 and 68.03±1.49 U L^{-1}, respectively.

The level of Aspartate aminotransferase (ALT) in Nile tilapia fed a diet with 100% fermented soybean meal was the highest, with a value of 14.47±0.92 U L^{-1}. This value showed a statistically significant difference (P<0.05) compared to all other experimental groups. The groups with the lowest enzyme levels, which were significantly different from the other experimental groups, were those fed diets with 50, 75, and 100% fermented soybean meal, with values of 11.33±0.67, 11.13±1.21, and 11.56±1.32 U L^{-1}. In contrast, Alkaline phosphatase (ALP) is an enzyme that transports phosphate groups, is involved in the lysosome production process of macrophage cells, and serves as a marker for immune system activity. In this experiment, there were no statistically significant differences (P>0.05) in ALP levels among all experimental groups, with values of 49.45±1.22, 49.97±0.94, 48.64±1.12, 48.69±0.98, and 48.44±0.92 U L^{-1}, respectively. The results are summarized in Table 5.

Table 5

Liver enzyme of Nile tilapia fed test diets of fermented soybean meal by *Bacillus coagulans* substituting soybean meal at various levels for 60 days (mean±SD)

Parameter	*Levels of fermented soybean meal (%)*					*P-value*
	FSBM 0	*FSBM 25*	*FSBM 50*	*FSBM 75*	*FSBM 100*	
AST[1] (U L^{-1})	72.78±2.53 ^{a}	70.60±1.37 ^{a}	68.64±2.27 ^{ab}	68.03±1.49 ^{ab}	67.63±3.32 ^{b}	0.04
ALT[2] (U L^{-1})	14.47±0.92 ^{a}	13.40±1.02 ^{b}	11.33±0.67 ^{c}	11.13±1.21 ^{c}	11.56±1.32 ^{c}	<0.01
ALP[3] (U L^{-1})	49.45±1.22	49.97±0.94	48.64±1.12	48.69±0.98	48.44±0.92	0.768

Means within the same row with different superscripts are significantly different at the 95% confidence level.
[1]AST-Aspartate transaminase, [2]ALT-Alanine aminotransferase, [3]ALP-Alkaline phosphatase.

The histopathological study of liver tissues is displayed in Figure 2. The results found that in all experimental groups there were revealed abnormalities with varying characteristics. These changes included congestion in hepatic sinusoids distributed throughout the tissue and fat accumulation in the hepatocyte, causing the nuclei to be displaced to the cell margins.

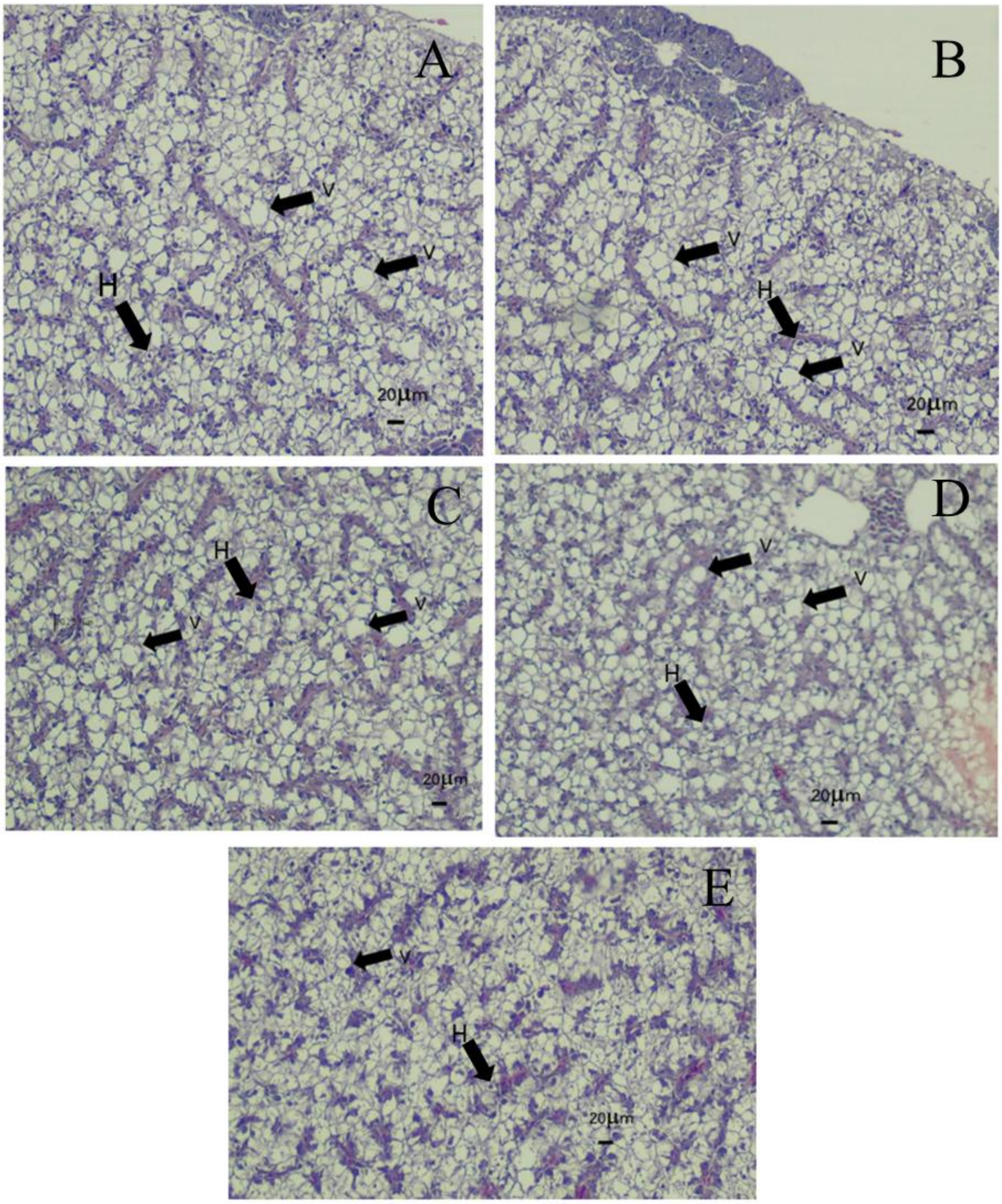

Figure 2. Histological examination of liver tissue of Nile tilapia fed test diets of fermented soybean meal by *Bacillus coagulans* substituting soybean meal for Nile tilapia diets at varying levels: 0% [A], 25% [B], 50% [C], 75% [D] and 100% [E] at various levels for 60 days. V=Vacuole, H=Hepatocyte (scale bar=20 µm).

Discussion. Given its status as a high-quality and cost-effective protein source, current research on soybean meal continues to focus on optimizing its efficiency and feasibility for broader application across various animal species. This focus is partly driven by challenges such as the rising price of fishmeal and its decreasing production capacity. Soybean meal is widely used in animal feed production, primarily as a by-product of soybean oil extraction process. It typically contains 44-50% protein and offers a relatively high content of essential amino acids. However, the use of plant proteins, particularly soybean meal, in fish feed has limitations despite being a sustainable alternative to fishmeal. It lacks essential amino acids, is less digestible, and contains anti-nutritional factors like trypsin inhibitors and phytic acid, which reduce nutrient absorption and protein digestion. Additionally, it is less palatable for some fish, especially carnivorous species, and raises environmental concerns, such as deforestation and phosphorus pollution. Processing soybean in order to address these issues adds costs, but solutions like amino acid supplementation, enzyme additives, and alternative proteins are being developed. Fermented soybean meal (FSBM) has emerged as a superior alternative to conventional soybean meal (SBM) in animal feed, offering enhanced nutritional value and reduced anti-nutritional factors. Through microbial fermentation, ordinary SBM undergoes significant improvements in its nutrient profile and digestibility.

Fermenting soybean meal with microorganisms is a process used to enhance the quality of animal feed ingredients. In this study, fermented soybean meal was used to replace regular soybean meal at five different levels (0, 25, 50, 75, and 100%) over a 60-day trial period, with sex-reversed Nile tilapia with an average initial weight of 25.22±0.45 g per fish. The experimental results showed that when considering overall growth, based on percentage weight gain and specific growth rate at the end of the trial, it was evident that Nile tilapia fed diets with 100, 75, and 50% fermented soybean meal exhibited the highest growth, with a statistically significant difference ($P<0.05$) compared to the groups fed 25 and 0% fermented soybean meal. The results show a trend in utilization similar to that observed in studies on various aquatic species that have benefited from fermented soybean meal with microorganisms. This improvement is attributed to several factors. Fermentation reduces anti-nutritional factors (e.g., trypsin inhibitors and lectins), enhancing the digestibility of proteins and nutrients. Additionally, fermentation increases the availability of essential nutrients, such as amino acids, vitamins, and bioactive peptides, which support better nutrient absorption and utilization. The above-mentioned species include white shrimp (*Litopenaeus vannamei*), barred knifejaw (*Oplegnathus fasciatus*), Indian prawn (*Fenneropenaeus indicus*), rohu (*Labeo rohita*), and rainbow trout (*Oncorhynchus mykiss*) (Dan et al 2017; Kim et al 2009; Lin & Mui 2017; Sharawy et al 2016; Yamamoto et al 2010).

Histopathological studies of intestinal tissues revealed that groups fed diets containing 25 and 0% fermented soybean meal had shorter and narrower villi compared to groups receiving higher levels of fermented soybean meal. The experimental groups fed diets with 50, 75, and 100% fermented soybean meal showed significantly better villus growth, which is consistent with findings from Ismail et al (2019). Their study investigated intestinal development and gene expression in fish fed diets in which soybean meal served as the primary protein source, compared to fishmeal-based diets, with soybean meal inclusion levels as high as 56.5%. The test results indicated that the villus length in the jejunum and ileum was shorter, and the number of goblet cells was significantly lower in the groups fed diets with soybean meal compared to those where fishmeal was the primary protein source ($P<0.05$). The intestinal health of fish is a critical factor in aquaculture, with well-developed villi and abundant goblet cells serving as key indicators of optimal digestive function. Additionally, shorter villi are associated with reduced absorptive surface area, potentially compromising nutrient uptake (Ellis 2001; Samanya & Yamauchi 2002).

The levels of liver enzymes released into the bloodstream can measured as indicators of hepatocellular damage which may result from diet or toxins. In this experiment, the levels of three enzymes were measured: Aspartate transaminase (AST) and Alanine aminotransferase (ALT) and Alkaline Phosphatase (ALP). These three enzymes are indicators of hepatocellular damage, as ALP is associated with the biliary system, and elevated levels of ALP frequently suggest issues within the bile ducts, including obstruction. The analysis of these enzymes facilitates the monitoring of pollution effects and health management within aquaculture systems (Lehmann-Werman et al 2018; Yousafzai & Shakoori 2011). The results showed that AST levels in Nile tilapia fed diets containing 50, 75, and 100% fermented soybean meal were lower compared to fish fed diets containing 0 and 25% fermented soybean meal. AST levels demonstrated a decline from 72.78±2.53 U L^{-1} in the control group to 67.63±3.32 U L^{-1} in the group receiving 100% fermented soybean meal (FSBM100). Suggestion that fermented soybean meal may mitigate liver or muscle damage in fish is plausible based on several mechanisms and research findings. Fermentation reduces anti-nutritional factors, such as trypsin inhibitors, lectins, and saponins, which can stress the liver and impair nutrient digestion and absorption (Chen et al 2023; Pang et al 2023). Additionally, fermentation enhances the bioavailability of essential nutrients, such as amino acids, vitamins, and bioactive peptides, which play vital roles in tissue repair and maintenance (Zhang et al 2023a). Furthermore, studies on various aquatic species have shown that fermented soybean meal can reduce oxidative stress and improve immune responses, which may contribute to minimizing organ damage (Zhang et al 2023b). However, while these mechanisms and findings are promising, further research is necessary to confirm the extent of these benefits across different fish species and dietary conditions. The ALT levels were lowest in the experimental groups of fish fed diets containing 50, 75, and 100% fermented soybean meal. The ALT levels were lower in these groups compared to fish fed diets containing 0 and 25% fermented soybean meal. Measurements of both enzymes revealed a consistent trend: Nile tilapia in the group fed a diet with 0% fermented soybean meal had the highest AST and ALT levels. This indicates that fermented soybean meal did not significantly affect the biliary system or mineral metabolism in fish. In comparison to the standard enzyme ranges documented in literature, the AST and ALT levels observed in this study fell within the typical range for freshwater fish, specifically *O. niloticus*, with AST levels ranging from 65.33 to 103.67 U L^{-1} and ALT levels from 18.67 to 21.33 U L^{-1} (Hastuti & Subandiyono 2020). In this study, ALP levels were approximately 49 U L^{-1}, aligning with the established normal range for freshwater fish, which is 31.48-91.62 U L^{-1} depending on environmental conditions (Alaryani et al 2024). This agreement establishes that fermented soybean meal does not have a significant impact on ALP-related processes in Nile tilapia. Banaee et al (2020) indicated that ALP levels may vary with age and bone development in juvenile fish, potentially accounting for the absence of significant variation in ALP levels among the groups in this study. The findings indicate that fermented feed ingredients, including soybean meal, may improve nutritional value by mitigating liver cell damage and oxidative stress in fish (Li et al 2024; Shimul et al 2024). The notable decrease in AST and ALT levels underscores the efficacy of fermented soybean meal in enhancing fish health. Utilizing these enzymes as biomarkers for evaluating fish health is advantageous for the future development of safe and efficient aquafeed formulations.

Histopathological analysis revealed liver tissue abnormalities across all experimental groups, primarily hepatic steatosis, characterized by the accumulation of fat within hepatocytes. This condition caused the compression of hepatocyte nuclei toward the cell periphery. The pathological mechanism is likely associated with imbalances in the synthesis, transport, and metabolism of fats within liver cells. Contributing factors may include consumption of an unbalanced diet, a diet excessively high in fat, or an inappropriately high protein content. These imbalances can result in increased fat accumulation in the liver and histopathological alterations, resembling those observed in terrestrial animals (Shimul et al 2024). Groups receiving diets with 0, 25, and 50% fermented soybean meal showed widespread blood cell congestion in blood vessels and fat accumulation in liver cells. Groups fed 75 and 100% fermented soybean meal exhibited fat accumulation in liver cells but no blood congestion, resulting in lower AST and ALT levels. Considering the levels of AST, ALT, and ALP, it can be concluded that fermented soybean meal with *B. coagulans* at levels of 50% or higher can be used in Nile tilapia diets. The normal enzyme levels observed indicate proper liver function, suggesting that the liver and internal organs of the fish are in good condition when fed these diets.

The structure of fish intestinal villi plays a crucial role in both digestion and nutrient absorption. Denser and longer villi increase the surface area available for absorbing nutrients, promoting growth and overall well-being. Changes in villi structure can indicate gut health, making them essential for assessing the impact of dietary and environmental factors on fish growth and immune function (Samanya & Yamauchi 2002). Fermented soybean meal (FSBM) has been shown to significantly improve the intestinal morphology of *O. niloticus*, with enhanced villi width and height observed at substitution levels of 50% or greater. This morphological development can positively impact the general health and growth performance of fish, indicating improved nutrient absorption capacity.

Recent studies have consistently demonstrated the benefits of feeding fish diets containing fermented plant materials. For instance, fermenting plant-based diets with *Lactobacillus acidophilus* enhanced the intestinal health of young Nile tilapia, as evidenced by taller villi and increased goblet cells (Neves et al 2024). Similarly, Li et al (2024) found that fermented rapeseed meal substitution in young tilapia diets showed that gut health and growth performance were unaffected at replacement levels up to 50%. Furthermore, studies on mixed-culture microorganisms added to fermented soybean meal revealed improved feed efficiency and increased intestinal villi height in young Nile tilapia (Picoli et al 2022). Fermentation not only adds beneficial bacteria to the feed but also enhances nutrient absorption, improving intestinal health and growth performance (Lian et al 2024). However, it is crucial to determine optimal FSBM inclusion levels to maximize positive effects while minimizing potential negative impacts, as excessive incorporation may decrease palatability and cause nutrient imbalances (Li et al 2024).

Conclusions. According to the findings from the study on the optimal level of incorporating soybean meal fermented with *B. coagulans* in Nile tilapia diets conducted over a 60-day period on fish weighing approximately 25 g, it was concluded that incorporating 50% fermented soybean meal with *B. coagulans* in the diet significantly improves growth, feed utilization, and the overall health of Nile tilapia. This optimal level of fermented soybean meal inclusion not only enhanced the performance of Nile tilapia but also demonstrated potential for application in other aquatic species. The implementation of this dietary strategy could lead to reduced production costs and promote more sustainable aquaculture practices. Further research is warranted to explore the long-term effects and broader applicability of this approach across various aquaculture systems and species.

Conflict of interest. The authors declare no conflict of interest.

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