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Evaluation of fermented soybean meal by *Bacillus subtilis* as an alternative to fishmeal on the growth, and physiological status of Nile tilapia *Oreochromis niloticus* fingerlings

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ABSTRACT

A feeding trial was conducted to investigate the effect of fermented soybean meal with *Bacillus subtilis* bacteria on growth performance, feed utilization, carcass composition, and hematological, and histological section of the liver and intestine of Nile tilapia *Oreochromis niloticus* fingerlings. Commercial soybean meal (SBM) containing 44% Crude Protein (CP) was fermented using the solid-state fermentation method which depended on autoclaving of SBM, then bacterial treatment injection by *Bacillus subtilis*, and finally incubation at 40C for 72 h then autoclaved to stop the growth of bacteria. Five isonitrogenous (25% crude protein) and isocaloric (4.4 kcal\g gross energy) experimental fish meal-free diets were formulated to compare with a common control diet containing fishmeal and unfermented soybean meal. Diets without fish meal contain fermented soybean meal (FSM) as a sole protein, FSM with corn gluten (CG), FSM with free amino acid methionine (Meth), FSM with corn gluten and methionine, and unfermented soybean meal. Eighteen glass aquaria, 80-L net volume, were used to stock 10 fingerlings (10.0 ± 0.1 g/fish) in each aquarium in the replicates group. The feed amount was given three times daily, six days a week throughout the 98 days experimental period. Fish were weighed biweekly and feed amounts were adjusted based on the new fish weight. Bacterial fermentation enhanced the protein content of commercial soybean meals by 6%. The crude protein of fermented soybean meal increased from 43.44% to 50.67%. Used of FSM as a sole dietary protein source resulted in a decrease in growth rate and feed utilization. However, the incorporation of FSM with corn gluten, and/or methionine amino acid led to an improvement in the performance of fish. Finally, the best final body weight, weight gain, specific growth rate, protein efficiency ratio, and protein productive value were recorded by a fish-fed mixed plant protein diet (FSM + CG + Meth). Also, Hematocrit and red blood cells were not significantly affected including the FSM.

1. Introduction

The rapid expansion of fish culture in recent years has created a demand for alternative supply resources that reduce the cost of

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nutrition in aquaculture, which can be 50–80% of total fish production costs [1,2].

The majority of such costs are due to high prices and shortages of fishmeal, the main ingredient used in the diets, and no strategies for increasing fishmeal production currently exist [3], making its future use unfeasible. Plant proteins appear to be the most suitable fishmeal alternatives [4].

The efficiency of the various alternative protein sources, including sunflower meal [5], linseed meal [6], and canola meal [7] as either partial or complete replacements for fishmeal that have been individually evaluated in fish diets.

Soybean meal (SBM) is considered an alternative to fish meal however; it contains many anti-nutritional factors, such as protease inhibitors, phytic acid, saponins, antivitamins, and allergens. These anti-nutritional factors primarily inhibit the ability of animals to digest proteins and carbohydrates [8]. Therefore, it is generally difficult to replace fish meals beyond a certain level with SBM without compromising the growth of crustaceans [9]. Therefore, it is useful to ferment plant proteins by solid-state fermentation technology that expands with increasing importance for the production of high-value-added products, for instance, enzymes, from agro-industrial by-products [10].

Solid-state fermentation technology involves the growth of microorganisms on moist solid substrates in the absence of free-flowing water. It has gained considerable attention of late due to several advantages over submerged fermentation [11]. According to studies [12], the fermentation of soybeans caused proteins to break down into tiny peptides and water-soluble molecules, increasing the nutrients' nutritional value and availability of amino acids. Additionally, Ref. [13] demonstrated that fermented soybean meal including *Bacillus, Lactobacillus* spp., and *Saccharomyces cerevisiae* can boost antioxidant activity and metal-chelating capacity, mostly as a result of elevated amounts of phenolic compounds and bioactive peptides. The olive flounder (*Paralichthys olivaceus*) may also exhibit an increase in nonspecific immune responses as a result of FSM, according to Ref. [14].

Therefore, the present study aimed to assess the effect of fermented soybean meal by *Bacillus subtilis* on nutritive values, growth performance, feed utilization, hematological, biochemical blood parameters and histological section of Nile tilapia, *Oreochromis niloticus.*

2. Materials and methods

2.1. Ethical approval

The proposed of this study was conducted with the strict recommendations and approval of the National Institute of oceanography and fisheries (NIOF, Egypt) Committee for ethical care and use of animals/aquatic animals (NIOF-IACUC, Code: NIOF–FI5–F-23-R-009).

2.2. Fish resources and site of experiment

Nile tilapia (*O. niloticus*) fingerlings were obtained from a commercial hatchery, EL-kantara East, Ismailia Governorate, Egypt. They were transferred to Fish Nutrition Lab., Faculty of Fish Resources, Suez University, Egypt. Using the bags filled with oxygen by inserting the oxygen supply pipe in the water from oxygen cylinder. Prior to beginning of the experiment, fish were acclimatized to the experimental conditions and fed by hand commercial diet (25% protein) twice daily to apparent satiation for two weeks.

Table 1
Proximate composition of the experiment diets (g/kg diet).

Experimental Diets						
Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fish meal[1]	12	0	0	0	0	0
Soybean meal 44%	25	0	0	0	0	24
Fermented soy meal	0	37	22	38	24	0
corn gluten	0	0	12	0	12	12
Yellow corn	10	10	10	10	10	10
Wheat milling by-products	34	34	37	32	34	35
wheat bran	10	10	10	10	10	10
Corn oil	5	5	5	5	5	5
Methionine	0	0	0	1	1	0
Vitamin & Minerals mixture[2]	3.7	3.7	3.7	3.7	3.7	3.7
Oscrabic acid	0.3	0.3	0.3	0.3	0.3	0.3
Proximate analysis, % On DM basis						
Crude protein	25.54	25.36	25.43	25.11	25.56	25.00
Ether extract	7.47	7.12	7.35	7.04	7.24	7.27
Crude Fiber	2.64	2.84	2.1	2.82	2.13	2.59
Nitrogen free extract[3]	48.28	50.09	51.12	48.46	49.06	51.46
Ash	8.06	6.57	6.01	6.52	5.99	5.98
Gross energy (kcal/g)[4]	4.46	4.49	4.55	4.48	4.55	4.62
Digestible energy (kcal/g)[5]	3.95	3.98	4.04	3.98	4.04	4.02

2.3. Experimental fish and conditions

After acclimatization, fingerlings, with an initial body weight of 10 ± 0.1 g, were stocked into eighteen glass aquarium (70 × 30 × 40 cm, 60 L each). Three replicate aquaria were randomly assigned to each treatment representing the sex experimental treatments; each aquarium was stocked with 10 fish (*O. niloticus*). All treatments were tested in triplicate groups where each aquarium was considered as an experimental unit. The glass aquaria were supplied with compressed air for oxygen requirement from 1 hp air blower (JAD, Model ACQ-909, Raoping, Guangdong, China). About one-third of the water volume in each aquarium was replaced by new fresh water (chlorine free) after cleaning and removing of the waste feed and accumulated excreta every two day. A photoperiod of a 12-h light, 12-h dark cycle was provided. Fish were fed their respective diets manually for 98 days. The fish fed two times daily at (9:00 and 15.00 h). All fish in each aquarium were weighed biweekly.

2.4. Preparation of the tested diets

Four isonitrogenous (25% crude protein) and isocaloric (4.4 kcal/g gross energy) experimental diets were formulated without fishmeal as fishmeal free diets. FSM was used as sole plant protein (diet 2) or incorporated with corn gluten (diet 3) or methionine (diet 4) or corn gluten and methionine (diet 5). All the above experimental diets were compared with a control diet containing fishmeal and unfermented soybean meal (diet 1). The proximate chemical composition of the experimental diets is presented in Table 1.

The first is the control diet which contained 12% FM. In the second diet, FM protein was replaced with FSM. In the third diet, FM protein was replaced with FSM and corn gluten to cover the shortfall in the amino acid "methionine" of FSM. In the diet (4) FM protein was replaced with FSM and methionine. The least diet FM protein was replaced with FSM, corn gluten, and methionine. All dietary ingredients of the fish meal, soybean meal, fermented soybean meal, yellow corn, corn gluten, and wheat bran were ground and mixed with vitamin–mineral premix and corn oil for 5 min. Water was added until a desirable paste–like consistency was reached then the mix was pelleted through 1 mm holes in a meat grinder (MOULINEX, ME605131 meat grinder, Moulinex, French) and dried in the sun. The dry pellet is stored in a freezer at −20 °C in polythene bags until use.

2.4.1. Bacterial strains and medium

Bacillus subtilis ATCC35854 was maintained on nutrient agar slopes. The strain of bateria was inoculated into brain heart infusion broth and incubated for 18 h at 40 °C. The culture was diluted in sterile distilled water with 0.85% sodium chloride NaCl and 0.1% peptone (PPS) to approximately 10^7 colony forming units (cfu)/ml as described by Ref. [15].

2.4.2. Solid-state fermentation of soya beans meal

Fermented soybean meal was produced as described by Ref. [16]. Five hundred grams of defatted SBM, with moisture content 50% (adjusted with distilled water) was autoclaved at 121 °C for 20 min in 2-L glass beakers covered with aluminum foil and then cooled to room temperature. Thereafter, 5 ml of *B. subtilis* (~10^7 colony forming units (cfu) mL^1) was used to inoculate the SBM. After even mixing with a glass rod, fermentation was carried out in an incubator at 40 °C which is the optimal growth temperature for *B. subtilis* for 72 h after that the fermented soyabean meal was autoclaved at 121 °C for 20 min to stop fermentation process [16]. Chemical composition of Soy meal, fermentation soybean is presented in Table 2.

2.5. Water quality indicators

Water temperature, dissolved oxygen, pH, and total ammonia were monitored during the study, to maintain water quality at optimal range for the Nile tilapia. Water temperature was adjusted using electrical heaters (model RS308-C, RS Electrical, Guangdong, China) in each aquarium. Dissolved oxygen (DO) was measured daily at 07.00 h using (DO meter) model (HI 9146 Company, HANA Instrument, Guangdong, China) and pH was recorded daily at 09.00 h using a pH meter (pH meter Adwa, AD8000, Texas, USA). Total ammonia was measured three times a week according to Ref. [17].

2.6. Growth performance and biological parameters

Records of initial body weight (IBW) and final body weight (FBW) of each individual fish were measured in all fish for each

Table 2
A compare the fermented soybean meal and non-fermented soybean meal in chemical composition on the basis of dry matter (%).

Items	FSM[2]	SBM[1]
Crude protein	50.67	43.44
Crude lipid	1.00	0.90
Crude fiber	5.40	7.07
Ash	4.80	4.45
Nitrogen-free extract[3]	38.13	43.14

Data are presented as means (n = 3).

aquarium. Weight gain (WG), specific growth rate (SGR %), feed conversion ratio (FCR), protein efficiency ratio (PER) and protein productive value (PPV), condition factor (K), Relative intestine index (RIL) and Hepatosomatic index (HI) were calculated according the following equations.

Weight gain (WG, g) = Final body weight (FBW, g) − initial body weight (IBW, g).
Specific growth rate (SGR, % day^{-1}) = [(Ln FBW − Ln IBW × 100)/time, days].
Feed intake (FI, g fish-1) = consumed feed of each pond/NO. of fish.
Feed conversion ratio (FCR) = Feed given (g)/Weight gain (g).
Protein efficiency ratio (PER) = WG, g/protein intake, g.
Protein productive value (PPV) = (Retained protein, g/Protein intake, g) × 100
Condition factor (K) = (FBW, g/Final $length^3$, cm^3) × 100.
Hepatosomatic index (HI) = (liver weight/body weight) × 100
Relative intestine index (RIL) = (Gut length / total length) × 100

2.7. Chemical analysis of experimental feeds and fishes

Dry matter, ash, crude protein, total lipids and crude fiber were performed on the individual dietary ingredients and diets, and at the beginning and end of the experimental fish, using standard [18] methodology. Nitrogen-free extract was computed by taking the sum of values for crude protein, crude lipid, crude fiber, ash then subtracting this sum from 100.

2.7.1. Amino acids analyses

The essential amino acid and non-essential amino acid profiles (g 100 protein− 1) of soybean meal, fermented soyabean meal, corn gluten and fish meal were determined by applying the [19] (Table 3). Three samples of each ingredient were dried and the pretreated ground sample (approximately 0.2 g) was digested in a closed chamber with 6 N HCL (10 ml) for 24 h at 100 °C. The mixture was brought to a final volume of 25 ml with de-ionized water. Then filtering, 5 ml of the solution was vaporized until clear of HCL vapor. The residue was dissolved in a diluted buffer (0.2 M sodium citrate buffer solution of pH 2.2.) and injected into an amino acids analyzer [20].

2.8. Blood sampling

At the end of the trial period, three fish from each replicate tank were anesthetized using MS-222 (tricaine methanesulfonate, 0.1 g L^{-1}, Sigma-Aldrich). Blood samples were collected from the caudal vein of all treatments and were divided into two portions. The first portion was collected in a small plastic tube containing heparin solution (0.2 ml/ml blood) as anticoagulant to determine the hematocrit (Htc), hemoglobin (Hb), erythrocyte counts (RBCs), total count of white blood cells (WBCs), platelet count (PLT) according to standard methods as described by Ref. [21]. The second portion of the blood samples were collected into vacationer containing sodium fluoride prior to centrifugation at Electric Centrifuge (Model: 80-2, Shantou, Guangdong, China) for 20 min at 3000 rpm to separate serum. The fresh serum was subjected to determined Serum glucose [21].

Table 3
Analytical amino acid composition of commercial soybean meal, fermented soybean meal, fish meal and corn gluten.

Item	SBM	FSM	Fish meal	Corn gluten
Essential amino acid				
Argnine	3.11	3.21	3.87	1.76
Histidine	1.23	1.62	1.24	1.27
Lysine	2.56	3.34	4.08	1.05
Methionine	0.60	0.60	1.82	1.43
Leucine	3.02	3.80	3.88	8.11
Isoleucine	1.76	2.20	2.18	2.12
Threonine	1.62	2.00	2.38	1.79
Valine	2.07	2.50	2.97	2.83
Phenylalanine	2.30	2.80	2.03	3.53
None-essential amino acid				
Glutamic	8.06	10.04	8.27	12.66
Tyrosine	1.93	2.33	1.81	3.03
Aspartic	4.55	5.00	5.08	3.55
Cystine	0.84	1.03	1.32	1.85
Serine	1.96	2.06	2.12	2.39
Proline	2.35	2.64	8.54	5.22
Glycine	1.99	2.39	5.15	1.71
Alanine	1.91	2.31	4.12	4.69

(SBM) Commercial soybean meal. (FSM) fermented soybean meal.

2.9. Histological examinations of liver and intestine

Segments of liver and distal intestine for histological observations were fixed in 10% phosphate-buffered formalin, After the fixation, the samples were dehydrated in alcohol series, embedded in paraffin wax and cut in 3–5 μm-thick sagittal sections using a Leica RM 2125 rotary microtome. Sectioned and stained with Haematoxylin and Eosin (HE). The sections were examined microscopically as described in Ref. [22].

2.10. Statistical analysis

Growth, hematology, and blood chemistry data were analyzed using one-way ANOVA, followed by Duncan's multiple range tests which were used to compare differences among individual means, with statistical software SAS ANOVA procedure. A probability of 0.05 was utilized to account for the statistical difference between the means.

3. Results

3.1. Water quality

During the period of the feeding trial, the water-quality parameters were {averaged (±SD)}: Water temperature was 25.17 ± 0.8 °C: dissolved oxygen, 5.6 ± 0.8 mg L^{-1}, pH 7.52 ± 0.3and total ammonia 0.18 ± 0.012 mg L^{-1}. All tested water quality criteria were suitable and within the acceptable limits for rearing the Nile tilapia, O. *niloticus* fingerlings [23,24].

3.2. Amino acid content of commercial soybean meal, fermented soybean meal, fish meal and corn gluten

As describe in Table (3), after solid-state fermentation the total amino acid of FSM increased by 19.66% compared to CSBM. Essential amino acids, including arginine, histidine, lysine, leucine, isoleucine, threonine, valine and phenylalanine in FSBM increased by 3.22%, 31.71%, 30.47%, 25.83%, 25%, 23.46%, 20.77%, and 21.74%, respectively. Moreover, non-essential amino acid, glutamic, tyrosine, aspartate, cysteine, serine, proline, glycine and alanine in FSBM increased by 24.57%, 20.73%, 14.73%, 22.62%, 5.10%, 12.34%, 20.10%, and 20.94%, respectively.

3.3. Growth performance and feed utilization

All tilapia fed fishmeal free diets (diets No 2–5) were significantly inferior ($P \leq 0.05$) in growth performance to those fed diet fishmeal, except diet No (5) that had gluten and methionine (Table 4). Use FSM as a sole of protein source or with corn gluten meal did not appear any improvement of growth rate of tilapia. Moreover, addition of methionine to plant protein mixture (FSM + CG) was enhanced final weight, weight gain and specific growth performance. The same trend was observed with FCR. The FCR of fish fed diet No. (3) (FSM + CG) and diet No (4) (FSM + Meth.) were not significantly ($P \leq 0.05$) different from each other. But these parameters were significantly improved compared to those of fish fed diet FSM. However, they were slightly but significantly ($P \leq 0.05$) lower than fish fed diet FM. Survival rates were not significantly different among the treatments. With regard to biological parameters, the hepatosomatic index of fish fed diet SBM was significantly lower than that of fish fed diet FM (Table 4).

Table 4
Growth performance of Nile tilapia, *Oreochromis niloticus* fed fermented soybean meal by Bacillus subtilis as plant protein sources in fish meal free diets.

Item	Experimental diets					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Growth performance						
Initial weight (g)	10.07^{a}	10.03^{a}	10.04^{a}	10.00^{a}	10.06^{a}	10.14^{a}
Final weight (g)	34.81^{a}	29.73^{b}	30.43^{b}	31.61^{b}	33.78^{a}	27.83^{c}
Weight gain (g/day)	24.57^{a}	19.68^{b}	20.33^{b}	21.67^{b}	23.62^{a}	17.69^{c}
Specific growth rate (%/day)	1.27^{a}	1.11^{c}	1.13^{c}	1.18^{bc}	1.23^{a}	1.03^{d}
Nutritional efficiency						
Feed intake (g/fish)	74.82^{a}	66.35^{b}	67.91^{b}	69.14^{b}	72.68^{a}	66.68^{c}
Feed conversion ratio	3.03^{a}	3.37^{b}	3.33^{b}	3.20^{ab}	3.06^{a}	3.77^{c}
Protein efficiency ratio (g/g)	1.30^{a}	1.16^{b}	1.18^{b}	1.25^{ab}	1.28^{a}	0.98^{c}
Protein productive value (%)	19.38^{a}	16.71^{b}	16.69^{b}	18.46^{ab}	19.00^{a}	13.27^{c}
Biologial parameters						
Condtion factor, %	1.90^{b}	2.10^{b}	2.10^{b}	2.00^{b}	1.70^{a}	2.20^{b}
Hepatosomatic index, %	1.10^{a}	1.20^{a}	1.60^{a}	1.28^{a}	1.13^{a}	2.60^{b}
Relative intestine length, %	2.20^{c}	3.10^{a}	2.90^{ab}	2.26^{bc}	2.22^{b}	2.30^{c}

Diets 1, 2.3, 4, 5 and 6 are control, fermented soybean meal FSM, FSM + corn gluten, FSM + Meth., and unfermented soy bean meal respectively. Values are means ± SD (n = 3) Values with the same superscript within the same row are not significantly different ($P < 0.05$).

3.4. Proximate analysis of whole body fish

Whole body crude protein contents were significantly ($P \leq 0.05$) lower in fish fed diet FSM than fish fed diet FM (Table 5). Moisture, crude fat and ash contents showed not significant ($P \leq 0.05$) differences were observed among the treatments.

3.5. Hematology and biochemical blood parameters

The hemoglobin concentrations of the four fermented soybean meal diet groups were not significantly different from fish fed diet FM. No significant differences were observed in other measured plasma nutrient concentrations among the treatments **(**Table 6**).**

3.6. Histological section of liver and intestine for Nile tilapia fed the experimental diets

3.6.1. Histological section of liver

The nuclei and cytoplasm of hepatocytes in fish fed FSM "fish meal free diets" were deeply stained, the hepatic cell cords were disordered, and the cytoplasm had few vacuoles (Fig. 1c, d). The presence of granules in the cytoplasm was shown (Fig. 1c, d). These abnormalities were Improved in fish fed diet FSM + CG + Meth (Fig. 1e) showing similar features to fish fed diet FM (Fig. 1a).

3.6.2. Histological section of intestine

The epithelial cells of mucosal folds in the intestine showed induced morphological changes such as disintegration of microvilli, absence of granulated pinocytotic vacuoles and presence of vacuoles (Fig. 2b–d) larger than absorptive vacuoles observed in fish fed diet FM (Fig. 2a, e). The epithelial cells of mucosal folds in fish fed diet FSM + CG + Meth (Fig. 2e)., showed similar features to those of fish fed diet FM (Fig. 2a).

The lamina propria of mucosal folds in the distal intestine of fish fed the fish meal free diets (Fig. 2b–d) showed moderate edema not seen in fish fed diet FM (Fig. 2a). In some of the fish fed diet FSM, a similar abnormality to fish fed diet FSM + CG was observed (Fig. 2b–d), however, the other fish showed normal features (Fig. 2e) as shown in fish fed diet FM (Fig. 2a).

The sub-mucosa in the intestine of fish fed diet FSM were widened with increased amounts of connective tissues (Fig. 2b–d). The sub-mucosa of fish fed diet FSM + CG + Meth (Fig. 2e) were similar to fish fed diet FM (Fig. 2a).

4. Discussion

As it is known, FSM is a microbial-treated form of SBM and is rich in protein (56%) and low in anti-nutrition levels. Solid-state fermentation has been applied in the production of metabolites, such as enzymes, antibiotics, and other value-added products, such as organic compounds [25]. The current study found a significant increase in the crude protein content of FSM may be due to the elevation in the level of amino acids during the process of fermentation, and this is in agreement with other previous studies [26]. The crude protein content increased for FSM as shown by Ref. [27] in which the percentages of crude and soluble proteins in FSM increased by 8.61% and 63.11% with *B. subtilis* BS102 and by 8.42%, 19.4% with *Aspergillus oryzae* AO3042 respectively. These increases in crude protein were also associated with increases in the overall content of amino acids in FSM. Fermentation allows microorganisms, such as *Bacillus subtilis*, to degrade protein macromolecules to a large extent water-soluble low molecular weight compounds [15]. Whereas, *B. subtilis* was reported to have great proteolysis activity. In the present study, results showed that fish-fed fish meal-free diets have lower significance ($P < 0.05$) in growth, FCR, PER, and PPV. This attributes to plant protein fermented soybean meal (diet 2) and fermented soybean meal corn gluten (diet 3) had an imbalance in essential amino acids that present high in fish meal. Another reason to lower growth rate and feed nutrient efficiency decrease feed intake with the replacement of fishmeal. It refers to poor palatability.

Recently works confirmed that fermentation of SBM could improve the content of acid-soluble, water-soluble, and macromolecular protein which could improve the digestibility and nutritional value of SBM as well as provide EAA. Increasing micro-molecular protein leads to increase protease activity and hence improves the PER contributing to the deposition of protein in animal muscles [28,29]. Similar works detected that fermentation of soybean leads to bio-modification substrate, whereas fermentation with microorganisms leads to reduce trypsin inhibitor in SBM from 2.7 to 0.42 mg/g [30].

Besides, all amino acid composition increased after the fermentation of SBM by *Bacillus subtilis*. The increase in total essential amino acids (20.80%) was higher than the increase of (18.78%) in the total non-essential amino acids in FSM which may be attributed to the

Table 5
Whole body proximate composition of Nile tilapia fed the experimental diets, % on dry matter basis.

Item	Experimental Diets					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Dry matter %	26.60^{a}	25.43^{a}	25.05^{a}	25.59^{a}	25.57^{a}	22.72^{b}
Crude Protein %	58.40^{a}	57.50^{ab}	56.70^{b}	58.05^{ab}	58.24^{a}	54.2^{c}
Lipid %	18.80^{a}	18.78^{a}	19.12^{a}	18.48^{a}	18.74^{a}	18.22^{a}
Ash %	22.20^{a}	22.47^{a}	21.33^{a}	21.60^{a}	22.11^{a}	28.57^{b}

Diets 1, 2.3, 4, 5 and 6 are control, fermented soybean meal FSM, FSM + corn gluten, FSM + Meth, and unfermented soy bean meal respectively. -Values are means ± SD (n = 3). Values with the same superscript within the same row are not significantly different ($P \leq 0.05$).

Table 6
Blood hemoglobin and plasma nutrient concentration of Nile tilapia fed the experimental diets.

Items	Experimental Diets					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Hemoglobin (Hb) g/dl	10.70^{b}	10.20^{b}	10.05^{b}	11.15^{a}	11.00^{a}	9.90^{c}
Hematocrit (Htc) (%)	21.00^{b}	24.60^{a}	21.70^{ab}	28.10^{a}	17.10^{c}	23.50^{a}
Erythrocyte counts ($\times$ 106 mm^{-3})	3.20^{a}	3.80^{b}	3.40^{a}	3.20^{a}	3.25^{a}	3.30^{a}
White blood cells ($\times$ 104 mm^{-3})	1.50^{b}	1.90^{a}	1.90^{a}	1.80^{a}	1.60^{a}	1.30^{b}
Plated count ($\times$ 104 mm^{-3})	10.30^{ab}	9.20^{a}	11.00^{b}	10.10^{ab}	9.00^{a}	9.00^{a}
Glucose, mg/dl	75.00^{a}	61.00^{c}	60.00^{c}	70.00^{b}	77.00^{a}	70b

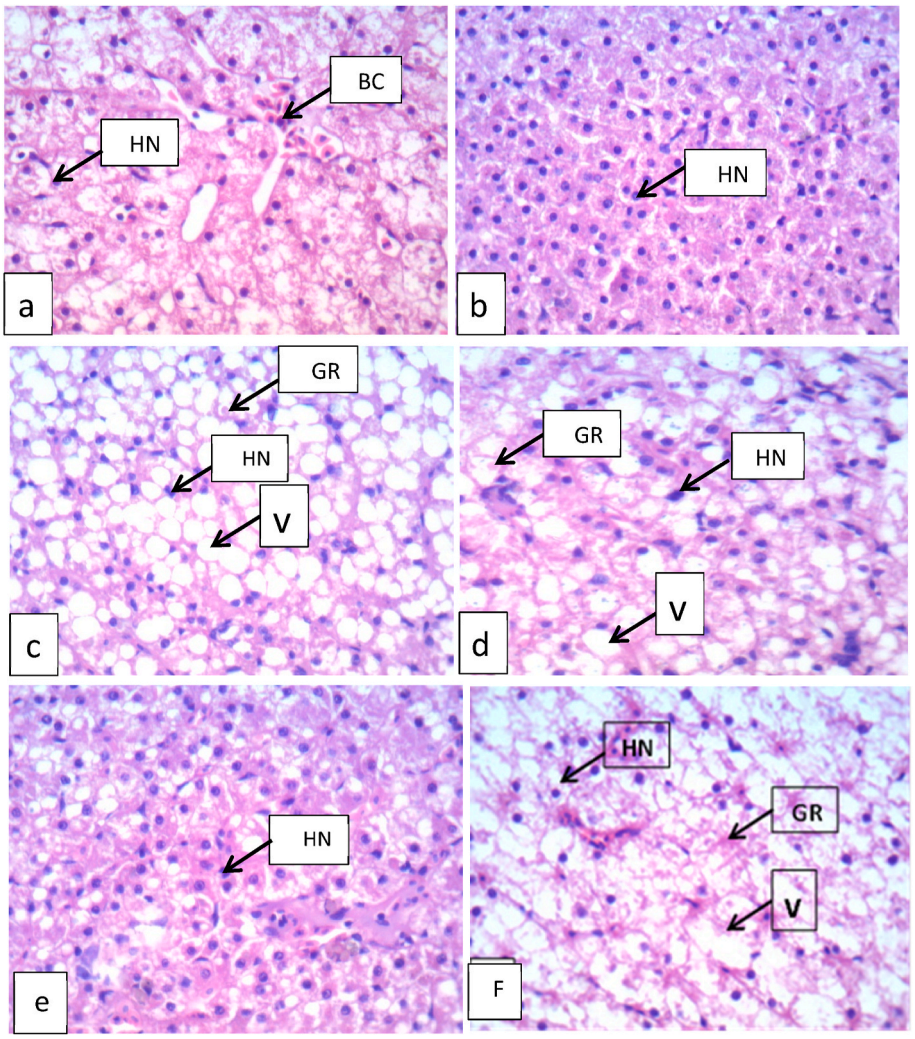

Fig. 1. Histological sections of the liver of Nile tilapia fed the experimental diets. **a** fish fed FM; **b** fish fed FSBM; **c** fish fed FSM + CG; **d** fish fed FSBM + M; **e** fish fed FSBM + CG + M.**F** fish fed unfermented soybean meal **BC** blood cell**, HN** hepatocyte nucleus**, V** vacuole**, GR** granule.

high production of the cell mass of bacteria and consequently the production of protein within the bacteria population. Processing of fermentation soybean with *Bacillus subtilis* led to a 60-fold increase in free amino acids which accounted for approximately 26% of the total amino acid content [31]. The other reason of due to the abrasion of bacteria *Bacillus subtilis* as a probiotic *Bacillus subtilis* plays a vital role to enhance of performance aquatic animals e. g tilapia [32,33] and rabbitfish and shrimp [34,35].

Several studies have reported that FSM induced higher growth and feed efficiency compared to non-fermented SBM in diets of Yellow tail [16]. Also, Ref. [36] reported that the improvement in fish performance when fed dietary FSM is attributed to improving palatability and digestibility by reducing exposed to ant nutrition factors of the used dietary plant protein in their diets. Furthermore, Ref. [37] recommended the replacement of FSB on diet Nile tilapia fingerlings at 100% levels.

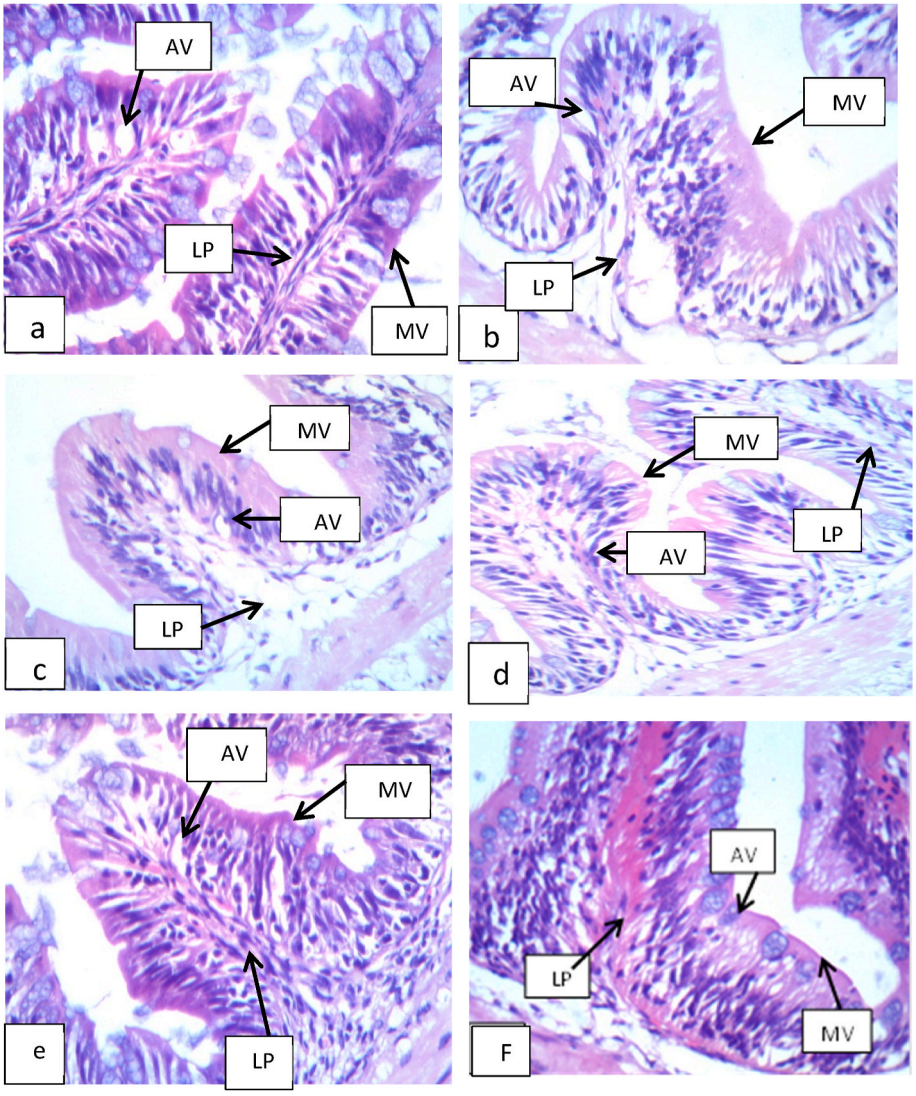

Fig. 2. Histological sections of mucosal folds of the intestine of Nile tilapia fed the experimental diets. (a) Fish fed FM; (b) fish fed FSM; (c) fish fed FSM + CG; (d) fish fed FSBM + M; e fish fed FSM + CG + Meth. F fish fed unfermented soybean meal: AV absorptive vacuole; LP lamina propria; MV microvilli; PV pinocytotic vacuole.

The above results from Table (4) confirmed insignificant differences in FBW, SGR, and FCR between fish fed with diet 2 and that fed diet 5 (FSM + CG + Meth). This is due to diversifying protein sources in diet 5 and then increasing the balance of amino acids in the diet leading to improve protein synthesis. Recorded data of [38] showed that protein synthesis and accretion in fish required all amino acids to be present simultaneously at the synthesis sites. Moreover, dietary methionine has a vital role in the synthesis of choline hence phosphatidylcholine and acetylcholine or other phospholipid accordingly it is essential for improving the growth and feed efficiency of fish.

As it is known Meth., is the first limiting EAA in the SBM-based diets, and it can not be produced by fish and must be provided by the diets it supplies sulfur and other compounds required by fish for normal metabolism [39]. In the same context, many researchers were supported these findings such as [40,41].

Regarding whole body composition at the end of the experimental trial, Table 5 showed insignificant differences among groups in lipid and ash content but protein content did not significantly differ among fish fed dietary fish meal and those fed dietary FSM + Meth., or FSM + CG + Meth., and these groups had significantly higher protein content than other treatments. These results were completely in agreement with [36,42] who found insignificant differences among experiment groups in the body with finding significant differences in protein content which was increased as increasing the FSM level. In a similar observation by Ref. [29] protein content of whole-carb muscles was higher in groups that fed dietary FSM than the control group. They added that the amino acids composition and protein content of the fish's body represents its ability to utilize the dietary protein.

Regarding, Blood indicators are the reflection of fish health and immunological status, hematological parameters showed

improvements in Hct, Hb, and WBCs with groups that fed dietary FSM alone or with Meth or CG. As it is known, Hb quantity increased significantly with increasing the growth rate of fish as a result of increasing the oxygen demand of fish, and then erythrocytes synthesize more Hg to maintain the optimum oxygen level in blood as reported by Ref. [43]. In the same trend, [44] the more active fishes tend to have higher Hb and Hct than sedentary ones.

WBCs are the defensive cells of animal bodies, and they increase as a result of increased leukocyte synthesis from the hematopoietic tissues of the kidneys and possibly the spleen. This may occur through non-specific immune stimulation [45].

Results of the WBCs count affirmed the validity of our interpretations, whereas an increase in the utilization of dietary protein leads to an increase WBCs. Similarly, [46] found that fish fed a diet containing high protein levels had higher macrophage migration compared with those fed a low dietary protein.

As it is known, glucose can be an immune suppressor in fish this is because when fish are stressed they need a lot of energy to adapt to stress. The high energy needed for maintaining life will stimulate the mobilization of glucose into their blood [47]. Moreover, [48] a high level of plasma glucose reflects a higher stress status in fish. But our results of high levels of blood glucose did not mean exposing the fish to stressful conditions but increasing blood glucose may be related to improving the feed quality. In view of the blood glucose levels in Table 6, it can be noted that the highest glucose was recorded with fish fed with dietary fish meal and those fed with the diet containing FSM + CG + Meth. This is due to the both diets 1 and 5 were balanced in their content of nutritional compounds and more palatability and feeding utilization of their components were higher than the other diets. Also, the positive effects of fermentation of SBM on non-starch polysaccharides in feeds led to the release of some sugars leading to increased glucose in fish blood as reported by Refs. [49,50].

Results of histological examination showed that tissue specimens from the intestines, and liver of fish-fed diets containing FM or FSM in special diets 1 and 5 (FSM + CG + Meth) were less negatively affected compared fish fed dietary SBM. The height and width of the intestinal villus determine the contact area between the mucosal epithelial cells and the chyme, which is of great importance for digestion and absorption [51]. Subsequently, several authors reported that microbial fermentation of SBM could enhance the bioavailability of potential antioxidants, and the production of enzymes, carbohydrase, and proteinase, leading to improved digestibility and health condition of fish [52,53].

The fermentation process could be an important factor for the improvement of the physiological condition in the liver and distal intestine of pompano, the major ANFs in SBM, is β-conglycinin has been demonstrated to induce inflammation and oxidation, and cause dysfunction of intestinal digestion and absorption in Jian carp [54]. Moreover, methionine can affect the balance of gut microflora and improve the morphology of intestinal [55,56]. In the same trend, [57] the better liver condition of fish fed with FSM 100 may be attributable to the reduction in ANFs found in the fermented product or other beneficial factors obtained from the microbial fermentation process.

Noteworthy, beneficial bacteria *Bacillus subtilis* that are used in fermentation processes have a big role in improving the digestive activity in fish guts by synthesis of vitamins and digestive enzymatic. Also, they have a role in replacing depressive microbial agents which hinder growth [35,58]. Furthermore, the competitive exclusion mechanism, based on the substitution of pathogens by the beneficial population, has been considered important.

The growth and feed efficiency of Nile tilapia fed the fermented soybean meal-based diet supplemented with methionine and corn gluten (diet 5, FSM + Meth + CG) were not different from fish fed the diet supplemented with fish meal.

5. Conclusion

In conclusion, the present results suggest that supplementation of methionine and corn gluten to a fermented soybean meal based diet for Nile tilapia produces the same effect as diet containing fish meal.

Contribution statement

Ashraf Y. El-Dakar: Conceived and designed the experiments, performed the experiments, and wrote the paper. Amin, A. Elgamal: Conceived and designed the experiments, contributed reagents, materials, analysis tools or data, and wrote the paper. Mohamed Abdel Baky Amer: Performed the experiments, analyzed and interpreted the data, and wrote the paper. Aala S. Mohammed: Conceived and designed the experiments, analyzed and interpreted the data, contributed reagents, materials, analysis tools or data, and wrote the paper. Mohamed F. Abdel-Aziz: Performed the experiments, analyzed and interpreted the data, contributed reagents, materials, analysis tools or data, and wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1- Herring
2- Vitamin and mineral mixture kg^{-1}
of mixture contains: 40*105 I.U. Vit A, 10*105 IU cholecalciferol (vit. D), 13.3 g Vit E, 2 g Vit K, 10 mg Vit B12, 5.0 g Vit B2, 2 g Vit B6, 1 g Vit B1, 6.0 g Pantothenic acid, 20 g Nicotinic acid, 1000 mg Folic acid, 100 mg Biotin, 200 gm Choline, 4 g Copper, 0.4 g Iodine, 12 g Iron, 22 g Manganese, 22 g Zinc, 0.04 g Selenium. Folic acid, 1.2 mg; niacin, 12 mg; d-calcium pantothenate, 26 mg;

pyridoxine. HCl, 6 mg; riboflavin, 7.2 mg; thiamin. HCl, 1.2 mg; sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; ferrous sulfate (FeSO4·7H2O, 20% Fe), 65 mg; manganese sulfate (MnSO4, 36% Mn), 89 mg; zinc sulfate (ZnSO4·7H2O, 40% Zn), 150 mg; copper sulfate (CuSO4·5H2O, 25% Cu), 28 mg; potassium iodide (KI, 24%K, 76% I).

3- Nitrogen free extract = 1- (%lipid + %moisture + %protein + %fiber + %ash).
4- Gross energy calculated using gross calorific values of 23.63, 39.52 and 17.15 Kcal\g of protein, lipid and carbohydrate, respectively [59].
5- Digestible energy calculated from standard physiological fuel values as 4,4 and 9 kcal\g of protein, carbohydrate and lipid respectively [60].

1 (SBM) Commercial soybean meal.
2- (FSM) fermented soybean meal.
3- Nitrogen-free extract = 100− (crude protein + crude lipid + ash + fiber).

- Diets 1, 2.3, 4, 5 and 6 are control, fermented soybean meal FSM, FSM + corn gluten, FSM + methionine, and unfermented soy bean meal respectively.
- Values are means ± SD (n = 3). Values with the same superscript within the same row are not significantly different ($P < 0.05$).

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