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Synergistic effects of *Bacillus pumilus* and exogenous protease on Nile tilapia (*Oreochromis niloticus*) growth, gut microbes, immune response and gene expression fed plant protein diet

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ABSTRACT

The synergistic effects of probiotic (*Bacillus pumilus*) and/or exogenous enzyme (protease) on growth, immunity, serum parameters, gene expression and gut bacteria of Nile tilapia (3.62 ± 0.06 g) were investigated. Plant protein-based diets were formulated as the control diet, probiotic (pro, 1.85×10^5 *B. pumilus* CFU kg^{-1}), protease enzyme (enzy, 0.5 g protease kg^{-1}), and their mixture of 1.85×10^5 *B. pumilus* CFU kg^{-1} + 0.5 g protease kg^{-1} (pro-enzy), respectively. After 84 days, the results revealed that fish fed pro-enzy diet resulted in better ($P < 0.05$) growth performance and feed utilization, including highest goblet cells, thickness of musclaris, mucosal folds and enterocytes. In addition, the highest values of hematocrit (Htc), hemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs), immunoglobulin M (IgM), lysozyme and phagocytic and serum parameters, including albumin, globulin and total protein were detected in the pro-enzy treatment ($P < 0.05$). The best levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were found in group fed pro-enzy diet. The highest relative levels of growth hormone (GH) were recorded in brain and liver of fish fed the control diet, while, insulin like growth factor (IGF-I) was higher in fish fed pro-enzy diet. Culture-independent molecular analyses of 16S ribosomal ribonucleic acid (16S rRNA) gene of gut bacteria showed that treatments directed intestinal bacteria towards health of fish, through inhibition of the growth of some hydrocarbon-degrading bacteria, such as *Pseudomonas indica*, and pathogenic bacteria, such as *Citrobacter koseri*. There were four genotypes, which homologized with mostly gas-fixating bacteria, were not affected by treatments, suggesting core gut bacteria. In conclusion, the addition of pro-enzy improved the growth performance, intestinal histological morphometric, immune

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; *B. pumilus*, *Bacillus pumilus* (CFU); enzy, protease enzyme; GH, growth hormone; Hb, hemoglobin; Htc, hematocrit; IGF-I, insulin like growth factor; IgM, immunoglobulin M; pro, probiotic; pro-enzy, *B. pumilus* + protease; RBCs, red blood cells; 16S, rRNA 16S ribosomal ribonucleic acid; WBCs, white blood cells.

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response, hematological, biochemical blood, metabolic gene expression and intestinal bacterial flora of Nile tilapia fed diet free fish meal.

1. Introduction

In the last decade, aquaculture sector grows higher than other sectors of food production (FAO, 2018), furthermore, the total growth exceeding human population growth. Therefore, intensive aquaculture should be supported by an equivalent increase in artificial feed (El-Sayed et al., 2015). Fish meal (FM) is the key fundamental in aquafeed formulation, and most aquafeed still depended on fish meal (≥100 g/kg diet) (Hassaan et al., 2018a, 2021). On the other hand, FM production during the last decades has declined. Accordingly, there is an obvious gap between the shortage of the FM production and the needs of an increasingly growing aquaculture sector. In the global situation of competition for crops, finding suitable plant protein sources instead of FM are now receiving greater attention, for economic aquaculture production, especially in the developing countries (Hassaan et al., 2018b). In recent years, several studies have investigated the replacement of FM with different sources of plant protein in Nile tilapia diets (Hassaan et al., 2017, 2018b; Khalifa et al., 2018; Ribeiro et al., 2016). But, the high inclusion of plant protein directly decreased the growth due to the high level of antinutritional factors (ANFs). Consequently, it is necessary to inhibit the ANFs in plant protein-based aquatic feed.

Recently, addition of exogenous enzymes can help to inactivate ANFs and release some specific compounds, and consequently, increasing the amount of plant protein in feeds without any negative effects (Adeoye et al., 2016; Hassaan et al., 2018a, b; Hlophe-Ginindza et al., 2016; Jiang et al., 2014). Furthermore, addition of exogenous enzymes as protease in feed containing high level of plant protein could hydrolyze the protein into amino acids as well as increase the bioavailability of some nutrients (Hassaan et al., 2019a, b; Liu et al., 2018). The activity of exogenous enzymes is influenced by several factors such as their type, quality and pH of the diet (ali Zamini et al., 2014; Huan et al., 2018). In this context, Liu et al. (2018) reported that the supplemental diets containing high level of plant protein with exogenous protease enzyme could increase the efficiency of feed nutrient. Dietary protease has a beneficial effect on secretion of digestive enzymes (Huan et al., 2018; Song et al., 2017), improved the digestibility of plant protein (Hassaan et al., 2018b). Furthermore, growth performance of *Oreochromis niloticus* (Huan et al., 2018; Liu et al., 2016), crucian carp (Shi et al., 2016) were improved by protease supplementation in high plant protein diets.

Probiotic as eco-feed supplement have been used in tilapia feed to enhance the growth performance (Elsabagh et al., 2018; Huan et al., 2018). The mode of action, providing probiotic as safe alternative to antibiotics are competitive inhibition of pathogenic bacteria

Table 1
Composition and proximate analysis of the basal diet (g/kg).

Ingredient	g/kg
Soybean meal	450
Corn gluten	60
Yellow corn	170
Wheat bran	150
Rice polishing	100
Fish oil	40
Vitamin & Mineral mix[a]	25
Vitamin C	5
Chemical analysis (g/kg)	
Dry matter	895.3
Protein	293.2
Lipid	60.6
Neutral detergent fiber (NDF)	161
Acid detergent fiber (ADF)	96
Ash	50.7
Metabolizable energy (MJ kg^{-1}feed)[b]	13.40

Soybean meal, corn gluten, yellow corn, wheat bran and rice polishing containing 440 g, 650 g, 120 g, 140 and 145 g crude protein per kg, respectively.

[a] Vitamin and mineral mix (per kg of diet): MnSO4, 40 mg; MgO, 10 mg; K_2SO_4, 40 mg; $ZnCO_3$, 60 mg; KI, 0.4 mg; $CuSO_4$, 12 mg; Ferric citrate, 250 mg; Na_2SeO_3, 0.24 mg; Co, 0.2 mg; retinol, 40,000 IU; cholecalciferol, 4000 IU; α-tocopherolacetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 mg; nicotinic acid, 300 mg; folic acid, 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg.

[b] Metabolizable energy was calculated based on the standard physiological values of 18.8 kJ g^{-1} protein, 13.8 kJ g^{-1} carbohydrate, and 33.5 kJ g^{-1} lipid (Brett and Groves, 1979).

(Hassaan et al., 2018a), and improvement of digestive intestinal enzyme activities (Dawood et al., 2017; Martínez Cruz et al., 2012). Several investigations have showed that *Bacillus* sp. isolates are promising probiotic applicants for aquafeed production (Banerjee et al., 2017; Dawood et al., 2019).

To enhance performance, digestibility and immunity of fish fed feed free-fish meal, functional feed additives should be used, but their use may cause synergistic improvements for fish. Therefore, this study was designed to examine the combined effect of dietary *Bacillus pumilus* and exogenous protease on performance of growth, feed utilization, intestinal morphology, immunity and associated gene expression of growth hormone and insulin-like growth factor and gut microbes.

2. Materials and methods

2.1. Preparation of probiotic supplement and source of exogenous protease

The *Bacillus pumilus* (*B. pumilus*) (1486^T) strain was brought from microbiological resources center, Ain Shams Univ., Cairo, Egypt. Briefly, the obtained *B. pumilus* was inoculated into 5.0 *g* L^{-1} of peptone + 3.0 *g* L^{-1} of beef extract medium at pH 7.0 and incubated at 37 °C. The overnight-grown culture was used as an inoculum according to Hassaan et al. (2017). After 24 h, 1 mL was inoculated into 100 mL fresh specific media broth that was incubated for a further 48 h at 37 °C. After cultivation, the cells were collected by centrifugation (3000 *g* for 10 min) in powdered form according to methods of Lee et al. (2018). The probiotic of *B. pumilus* (pro) was included into the control prepared diet to obtain 1.85×10^5 colony forming unit (CFU) kg^{-1} diet and used in the feeding experiment of Nile tilapia, *Oreochromis niloticus*.

Exogenous protease (EC3.4.23.18), produced by the fermentation of Humicola sp.L8, was a neutral protease obtained from SunHY Biology Co. Ltd., China. The highest activity was at a pH of 8.0 and the relative enzyme activity of the protease was higher than 60 % in the range of 20 °C ~ 70 °C. The enzyme activity was 13,830 U/g. One unit of this protease activity was defined as the amount of enzyme that hydrolyzes 10 g/L casein solution to yield 1 μg of tyrosine per minute at 40 °C at a pH of 8.0.

2.2. Experimental design and diets

The basal plant-based protein diet, without fish meal, was formulated (Table 1). Separately, *Bacillus pumilus* (*B. pumilus*) at 1.85×10^5 *B. pumilus* (CFU kg^{-1}) (probiotic; pro) or 0.5 g (2500 U) protease kg^{-1} diet (enzyme; enzy), and their mixed 1.85×10^5 *B. pumilus* (CFU kg^{-1} diet) + 0.5 g protease kg $^{-1}$ diet (pro-enzyme; pro-enzy) were supplemented to basal diet. All experimental diets were prepared according to Hassaan et al. (2018) using laboratory pellet mill (2-mm die), and stored at 4 °C until used. The basal diet was supplemented drop wise with the collected cells of *B. pumilus* before pelting diets to obtain 1.85×10^5 (CFU) kg^{-1}, respectively according to Hassaan et al. (2018). The dry matter, ash, crude protein and crude lipid were determined according to AOAC (1995) method 930.15, method number 942.05, method number 984.13 and method number 920.39, respectively. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) of experimental diets were estimated by Van Soest et al. (1991). Metabolize energy of experimental diets was calculated according to Brett and Groves (1979).

2.3. Culture technique

Experiment was subjected to ethical reviewed and approved by the National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt through the Animal and Welfare Ethical Review Body. Nile tilapia, *Oreochromis niloticus* were acclimated in concrete pond (30 m^3) after collected in farm of NIOF for 15-day in two concrete ponds (4 m^{-3}) at National institute of Oceanography and Fisheries (NIOF), Cairo, Egypt. Fish were fed a commercial feed three times daily at 09:00, 11.00 and 15:00 h for two weeks. A total of 300 fish (3.62 ± 0.06 g) were stocked into 12 concrete ponds (1 × 1×0.5 m^3 each) at a density of 25 per pond representing the four diet treatment groups in three replicates (n = 3). Freshwater in each pond was renewed 30 % by the outlet at the bottom of the pond daily,

Table 2
Growth performance and feed utilization of Nile tilapia fed the experimental diets for 84 days.

Parameters	Control	*B. pumilus*	Protease	*B. pumilus* + Protease	SEM	P-values
Initial body weight (g)	3.62	3.75	3.70	3.65	0.521	0.624
Final body weight gain (g)	32.40^c	38.12^b	38.44^b	40.59^a	1.312	0.014
Weight gain (g)	28.78^c	34.37^b	34.74^b	37.40^a	1.621	0.012
Specific growth rate (% /day)	2.60^c	2.77^b	2.79^b	2.87^a	0.545	0.001
Feed intake (g /fish)	48.06^b	51.90^a	52.80^a	53.86^a	1.564	0.001
Feed conversion ratio	1.67^a	1.51^b	1.52^b	1.44^c	0.252	0.003
Protein efficiency ratio	1.99^b	2.21^a	2.19^a	2.31^a	0. 521	0.001

Means followed by different letters in the same row are significantly different ($P < 0.05$).
Weight gain (WG) = final weight (g) – initial weight (g); Specific growth rate (SGR) = 100× (LnW_2 - LnW_1)/t (days), Where, Ln = the natural log; W_1= initial fish weight, W_2 = the final fish weight in grams and t = Period in days; Feed conversion ratio (FCR) was calculated according to the equation: FCR = Feed intake (g)/weight gain (g); Protein efficiency ratio (PER) = Weight gain (g)/protein ingested (g); number of replicates / treatment = 3 ponds; *Bacillus pumilus* (*B. pumilus*) at 1.85×10^5 CFU kg^{-1}diet (probiotic; pro); 0.5 g protease kg $^{-1}$ diet (enzyme; enzy); 1.85×10^5 *B. pumilus* (CFU kg^{-1}diet) + 0.5 g protease kg $^{-1}$ diet (pro-enzyme; pro-enzy).

before feeding. During the 84 days, fish were offered their diets three times daily (9:00, 11:00 and 15.00 h), at rate of 3 % of the total biomass daily. Every 15-day, fish were weighted to adjust the amount of respective feed during the experiment. Water temperature was recorded daily with a mercury thermometer suspended at 15-cm depth. pH was determined by using a pH meter (Orion pH meter, Abilene, Texas, USA). While dissolved oxygen (mg/L) was measured using YSI model 56 oxygen meter (YSI Company, Yellow Springs Instrument, Yellow Springs, Ohio, USA). Total ammonia was measured using DREL/2 HACH kits (HACH Co., Loveland, Co. USA). During the feeding trial, the water quality parameters averaged (±standard deviation): Water temperature 26.8 ± 0.3 °C; dissolved oxygen 5.98 ± 0.22; pH values 8.17 ± 0.35; total ammonia 0.16 ± 0.01 mg/L, all tested water quality criteria (temperature, dissolved oxygen, pH value and total ammonia) and were within the permissible range fish farming according to Boyd (1990).

2.4. Growth indices

Growth and feed efficiency indices were estimated in the current trail and the equation of these parameters was noted in the footnote of Table 2.

2.5. Hematological and serum biochemical parameters

After 84-day, blood of experiment fish was sampled in two parts to detriment the hematological and biochemical indices from caudal vessels of five fish (n. of fish / replicate = 5; 15 fish/ treatment), after fish anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Sigma-Aldrich, St. Louis, MO, USA) at 100 mg L^{-1}. First part of blood sample was collected by using anticoagulant 10 % Ethyl enediaminetetraacetate (EDTA) to estimate hematocrit (Htc) and white blood cells (WBCs) according the method of Brown (1988) and Svesbodora et al. (1991), respectively. Furthermore, hemoglobin (Hb) and red blood cells (RBCs) was determined using the standardized cyanomethemoglobin procedure with Hb kits. Other portion was centrifuged at 3000 × g for 15 min to obtain serum (Hassaan et al., 2019c). After collected the serum, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as described the method of Reitman and Frankel (1957). While serum protein, albumin and globulin were determined according to Doumas et al. (1981).

2.6. Histological techniques

By the end of experiment, mid-sections of five fish intestine from each treatment (n. of fish/replicate = 5; 15 fish/treatment) were removed after fish anesthetized by MS-222 at 100 mg/L (Sigma-Aldrich, St. Louis, MO, USA). The samples were fixed in Bouin's fluid for 24 h, then prepared according to the method of Hassaan et al. (2019b). The measurements of mid-intestine; goblet cells, musclaris thickness, mucosal folds and enterocytes were measured as described by Wassef et al. (2016).

2.7. Molecular analyses

2.7.1. Gene expression

After fish anesthetized by using MS-222 at 100 mg/L (Sigma-Aldrich, St. Louis, MO, USA), liver and brain samples were removed from each treatment (n. of fish/ replicate = 5; 15 fish/ treatment) were homogenized by tissue homogenizer (QIAGEN GmbH, QIAGEN Strasse 1, Hilden, Nordrhein-Westfalen-40724, Germany). Total ribonucleic acid (RNA) was extracted from the tissues, using RNeasy® Mini kit (Qiagen, Cat No. 74,104), based on manufacturer's protocol provided in the kit. cDNA was synthesized from 1000 ng of total RNA using the protocol of High Capacity cDNA Reverse Transcription Kit (Applied Bio systems, Cat# no.4368813). RT-PCR condition was 25 °C for 10 min, followed by 37 °C for 120 min and finally 85 °C for 5 min. cDNAs were stored at −80 °C for further molecular analyses.

Primers for amplifications of the gene, which encodes insulin-like growth factor-I (IGF-I) and 18S ribosomal RNA (18S rRNA) gene, as reference gene, were designed based on multiple alignments of the targeted gene sequences from *O. niloticus*, deposited in DNA database, using software GenScript Online polymerase chain reaction (PCR) Primers Design Tool, https://www.genscript.com/tools/pcr-primers-designer. The obtained IGF primers were IGF-If, 5/-GTTTGTCTGTGGAGAGCGAGG-3/ and IGF-Ir, 5/-GAAGCAG-CACTCGTCCACG-3/. Primers for amplification of 18S rRNA gene were 18S-F, 5/- GGTTGCAAAGCTGAAACTTAAAGG-3/, and 18S-R, 5/- TTCCCGTGTTGAGTCAAATTAAGC-3/. The specificity of the primers was checked by both of BLAST homology and *in silico* PCR tool, https://genome.ucsc.edu/cgi-bin/hgPcr. Quantitative PCR reaction contained 2.5 µl of 1 µg/µl cDNA, 12.5 µl SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen), 0.3 µM of each of forward and reverse primers and double distilled water to a final volume of 25 µl. Reaction was run on an Applied Biosystem 7500 Real time PCR Detection system (Applied Bio systems) under the conditions of 95 °C for 10 min and 45 cycles of 95 °C for 20 s followed by 60 °C for 20 s and 72 °C for 20 s.

2.7.2. Molecular analyses of fish gut bacteria

Microbial metagenomic DNA was extracted from foregut contents of treated fishes, using DNA Isolation Kit (MO BIO Laboratories, 12888−50, Carlsbad, CA, USA) according to the manufacture's protocol with modifications of Elsaied et al. (2019). First, the gut contents were lysed with a mixture of 5 M guanidine thiocyanate, and 10 % sodium dodecyl sulfate, SDS, at 75 °C for 40 min with strong shaking. The metagenomic DNA was purified from the crude lysate, using a Sephadex column, provided in the Kit and visualized by Gel Doc™ XR + imager (BIO-RAD).

PCR amplification of 16S rRNA gene, from the purified metagenomic DNAs, was carried out, using conserved bacteria domain-

specific primers, 341 F with GC clump, 5′- CgC CCg CCg CgC CCC gCg CCC gTC CCg CCg CCC CCg CCC g CCT ACg ggA ggC AgC Ag -3′ and 907R, 5′−CCg TCA ATT CMT TTg AgT TT -3′ (Nubel et al., 1996). PCR mixture, 25 μL, contained 2.5 mM dNTP each, 10X Ex Taq™ buffer (Mg^{2+} free), 25 mM $MgCl_2$, 0.25 μM of each primer, 250 U Takara Ex-Taq™ Polymerase (Takara, Japan) and 500 ng DNA template. Touchup PCR was conducted with an initial denaturation step of 3 min at 95 °C, followed by 30 cycles of denaturation for 50 s at 95 °C, annealing at 50 °C, with an increasing 0.5 °C every 10 cycles, and extension for 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. The 16S rRNA gene PCR amplicons were run through Denatured Gradient Gel Electrophoresis, DGGE, using 6 % polyacrylamide gel in 1 × TAE running buffer (Tris–acetate 0.04 M, EDTA 0.002 M, pH 8.5) and denaturing gradients, of urea and deionized formamide, according to Muyzer et al. (1993). Electrophoresis was run for 17 h at 120 V under a constant temperature of 60 °C in a D-Code system (Bio-Rad, UK). The gel was stained with 10 mg/ ml ethidium bromide and visualized under UV light by Gel Doc™ XR + imager (BIO-RAD). The designation of the band-classes was based on their position on the gel patterns. The denaturing gradient gel electrophoresis (DGGE) fingerprinting was manually scored by the presence or absence of co-migrating bands, independent of intensity. The recovered bands were purified using the QIAquick® PCR purification kit (Catalog no. 28104, Qiagen, Germany) and analyzed by sequencing. Sequencing was done by the Applied Biosystems 3500 Genetic analyzer sequencer (Hitachi, Japan). Band sequences were analyzed by FASTA homology screening to determine their similarity to the known sequences in the DNA database (http://www.ebi.ac.uk/Tools/sss/fasta/), and consequently, identify gut bacteria species fluctuations under dietary treatments.

2.8. Statistical analysis

All the data were analyzed with SAS version 6.12 software (SAS, 1993). One-way ANOVA analysis was used to show the different significant variation among the treatments using version 6.12 of SAS program with a pond of fish being the experimental unit. When overall differences were found, differences between means were tested by Duncan (1955) new multiple range test. All differences were considered significant at $P < 0.05$ and the results are presented as means with standard error of the mean.

3. Results

3.1. Growth

Diets supplemented with probiotic (*Bacillus pumilus* 1.85×10^5 CFU kg^{-1} diet), protease (2500 U kg^{-1} diet) and pro-enzy (probiotic + protease) significantly ($P < 0.05$) enhanced the growth indices (Table 2). Fish fed pro-enzy had the highest final body weight (FW), weight gain (WG), specific growth rate (SGR), feed intake (FI), protein efficiency rate (PER) and best feed conversion ratio (FCR). No significant ($P > 0.05$) differences were found in growth performance and feed utilization between fish fed *B. pumilus* or protease individually.

3.2. Histological morphometric

Goblet cells, musclaris thickness, height of mucosal folds and enterocytes in middle intestine were significantly improved ($P < 0.05$) in fish fed pro (probiotic), enzy (protease) and combination, pro-enzy (probiotic + protease) with the control diet (Table 3 and Fig. 1). The highest significant ($P < 0.05$) records of above indices were found in fish fed diet supplemented with pro-enzyme, compared with other diets.

3.3. Hematological indices

Diets supplemented with pro, enzy and pro-enzy significantly improved the hematological parameters (Table 4). Higher hematocrit (Htc), hemoglobin (Hb), red blood cells (RBCs) and white blood cells (WBCs) counts were detected in the pro-enzyme treatment ($P < 0.05$) compared with other treatments (Table 4). On the other hand, Htc, Hb, RBCs and WBCs indices did not affect significantly ($P > 0.05$) by probiotic or protease supplementation individually.

Table 3
Histological morphometric of intestine of Nile tilapia fed experimental diets for 84 days.

Parameters	Control	*B. pumilus*	Protease	*B. pumilus* + Protease	SEM	P-values
Goblet cell (per 100 μm)	1.15^{c}	1.96^{b}	2.06^{b}	2.69^{a}	0.021	0.012
Musclaris mucosa thickness (μm)	65.23^{c}	73.36^{b}	77.12^{b}	89.00^{a}	0.820	0.023
Height of mucosal folds (μm)	262.16^{b}	283.03^{a}	289.29^{a}	312.19^{a}	3.011	0.001
Height of enterocytes (μm)	23.16^{c}	27.23^{b}	28.19^{b}	32.16^{a}	0.032	0.023

Means followed by different letters in the same row are significantly different ($P < 0.05$); number of replicates / treatment = 3 ponds. *Bacillus pumilus* (*B. pumilus*) at 1.85×10^5 CFU kg^{-1}diet (probiotic; pro); 0.5 g protease kg^{-1} diet (enzyme; enzy); 1.85×10^5 *B. pumilus* (CFU kg^{-1}diet) + 0.5 g protease kg^{-1} diet (pro-enzyme; pro-enzy).

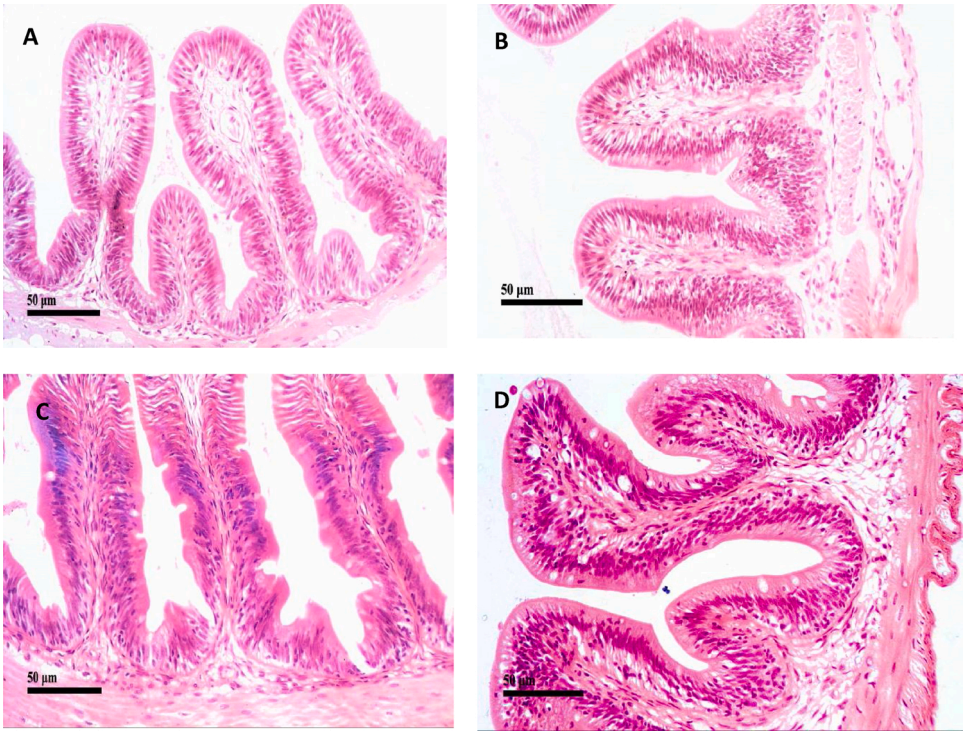

Fig. 1. Histoarchitecture of intestine tissue of Nile tilapia fed the experimental diets; control (A), *B. pumilus* (B), Protease (C) and *B. pumilus* + Protease (D).

Table 4
Hematological parameters and biochemical parameters of Nile tilapia fed the experimental diets for 84 days.

Parameters	Control	*B. pumilus*	Protease	*B. pumilus* + Protease	SEM	P-values
Hematocrit (%)	26.13^{c}	27.33^{b}	28.01^{b}	30.12^{a}	1.123	0.001
Hemoglobin (g/L)	12.13^{c}	14.48^{b}	15.02^{b}	17.09^{a}	0.821	0.012
RBCs[1] ($\times 10^4$ mm^{-1})	1.82^{c}	1.98^{b}	2.02^{b}	2.52^{a}	0.252	0.015
WBCs[2] ($\times 10^4$ mm^{-1})	35.25^{c}	37.59^{b}	36.51^{b}	40.17^{a}	1.721	0.001
ALT[3] (U/L)	39.09^{a}	30.12^{b}	28.01^{b}	26.31^{c}	1.601	0.091
AST[4] (U/L)	21.13^{a}	14.48^{b}	15.02^{b}	12.09^{c}	0.901	0.022
Total Protein (g/L)	2.15^{c}	2.86^{b}	2.93^{b}	3.32^{a}	0.032	0.043
Albumin (g/L)	1.45^{c}	1.79^{b}	1.82^{b}	1.92^{a}	0.031	0.013
Globulin (g/L)	0.7^{c}	1.07^{b}	1.11^{b}	1.40^{a}	0.404	0.025

Means followed by different letters in the same row are significantly different ($P < 0.05$).
[1] RBCs, Red blood cell count.
[2] WBCs, White blood cell count.
[3] ALT, Alanine aminotransferase.
[4] AST, Aspartate aminotransferase; number of replicates / treatment = 3 ponds. *Bacillus pumilus* (*B. pumilus*) at 1.85×10^5 CFU kg^{-1}diet (probiotic; pro); 0.5 g protease kg $^{-1}$ diet (enzyme; enzy); 1.85×10^5 *B. pumilus* (CFU kg^{-1}diet) + 0.5 g protease kg $^{-1}$ diet (pro-enzyme; pro-enzy).

Table 5
Immunoglobulin M (IgM), lysozyme activity and phagocytic activity of Nile tilapia fed the experimental diets for 84 days.

Parameters	Control	*B. pumilus*	Protease	*B. pumilus* + Protease	SEM	P-values
Immunoglobulin M (IgM, µg/mL)	22.71^{c}	35.00^{b}	33.00^{b}	45.31^{a}	1.121	0.001
Lysozyme (u/mL)	134.70^{c}	204.71^{b}	200.31^{b}	235.70^{a}	0.821	0.012
Phagocytic activity (%)	85.70^{c}	119.72^{b}	118.31^{b}	152.72^{a}	0.223	0.015

Means followed by different letters in the same row are significantly different ($P < 0.05$), number of replicates / treatment = 3 ponds. *Bacillus pumilus* (*B. pumilus*) at 1.85×10^5 CFU kg^{-1}diet (probiotic; pro); 0.5 g protease kg $^{-1}$ diet (enzyme; enzy); 1.85×10^5 *B. pumilus* (CFU kg^{-1}diet) + 0.5 g protease kg $^{-1}$ diet (pro-enzyme; pro-enzy).

3.4. Serum biochemical parameters

Serum of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes were decreased in fish fed diets supplemented with pro, enzy and pro-enzy (Table 4). But, the lowest significant ($P < 0.05$) values of ALT and AST were found in fish fed pro-enzy diet. Values of serum total protein, globulin and albumin were higher ($P < 0.05$) in fish fed diet containing pro-enzyme in comparison to other diets (Table 4). The highest values of total protein, globulin and albumin were found in fish fed diet containing pro-enzy.

3.5. Innate immunity

Diets supplemented with pro, enzy and pro-enzy, improved the activities of immune globulin M (Ig M), lysozyme and phagocytic, compared with the control diet (Table 5). The highest activities of Ig M, lysozyme and phagocytic were detected in fish received pro-enzy diet.

3.6. Gene expression

Expression of growth hormone (GH) gene was significantly down-regulated in brain and liver (Fig. 2) in fish fed diet containing pro, enzy, and pro-enzy. The highest relative growth hormone (GH) was detected in the control diet. The highest expression of insulin-like growth factor (IGF-I) gene were detected in samples of liver and brain of tilapia fed diet supplemented with pro-enzyme (Fig. 3).

3.7. Molecular analyses of intestinal bacteria

16S Ribosomal ribonucleic acid (16S rRNA)/ polyacrylamide gel electrophoresis-based (PAGE) genotyping of intestinal bacterial flora produced total of 16 bands, representing 16 bacterial genotypes, from guts of fish, which fed on both of the control diet and treated diets (Fig. 4). There were 4 bacterial genotypes did not affect by treatments, represented by 4 bands, which occurred in all PAGE profiles, the control diet and treatments (Fig. 4 and Table 6). These four genotypes had homology with those of species *Oryzobacter terrae*, *Orbus hercynius*, *Hydrogenophaga pseudoflava*, *Shinella daejeonensis* (Table 6). Six genotypes were displayed on the gel of control, but disappeared in profiles of all treatments, and showing homologies with those of *Pseudomonas indica*, *Brevibacterium ammoniilyticum*, *Citrobacter koseri*, and Uncultured Chloroflexi (Fig. 4 and Table 6). Two genotypes, that were absent in the gel profile of control diet, but displayed on the gel profile of the other treated diets and were related to those of *Anaerolineaceae* and uncultured alkane-degrading bacterium (Fig. 4 and Table 6). Control diet, pro, enzy and pro-enzy were characterized by occurrence of genotypes that are similar with *Alkalilimnicola* sp., uncultured Gammaproteobacterium, and *Simiduia* sp., respectively (Fig. 4 and Table 6).

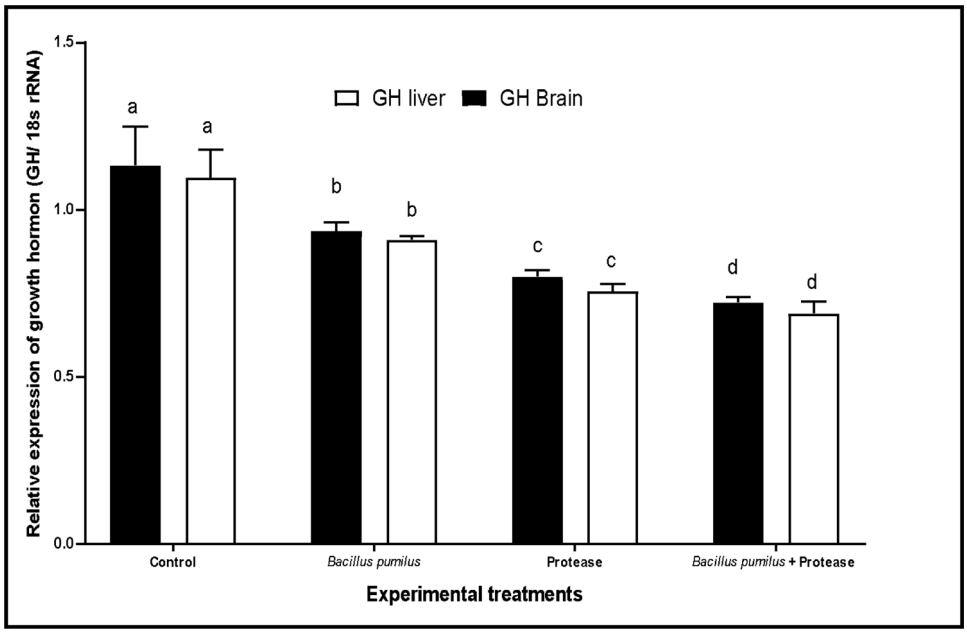

Fig. 2. Gene expression chart of growth hormone (GH) gene from liver and brain of Nile tilapia. Different letters in columns indicate significant differences among treatments ($P < 0.05$).

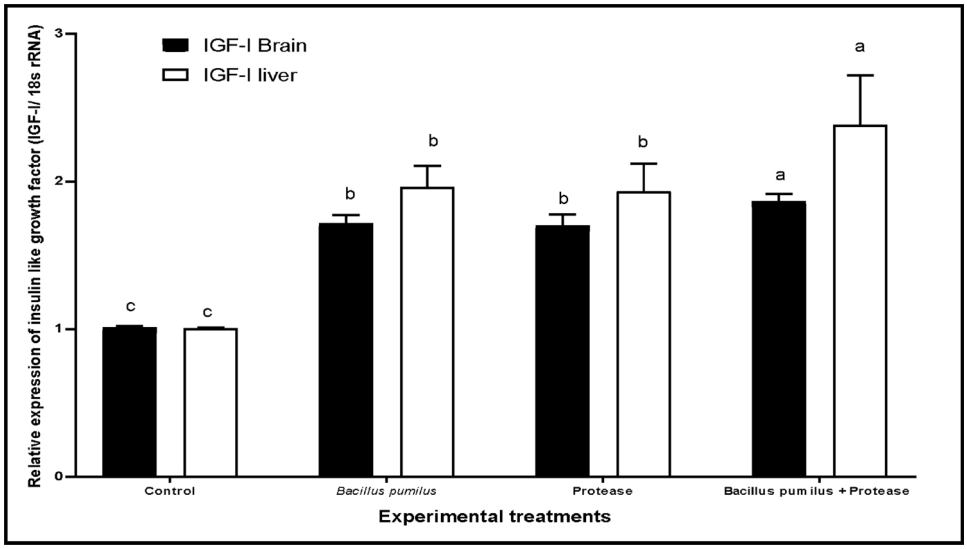

Fig. 3. Gene expression chart of insulin like growth factor (IGF-I) gene from liver and brain of Nile tilapia. Different letters in columns indicate significant differences among treatments ($P < 0.05$).

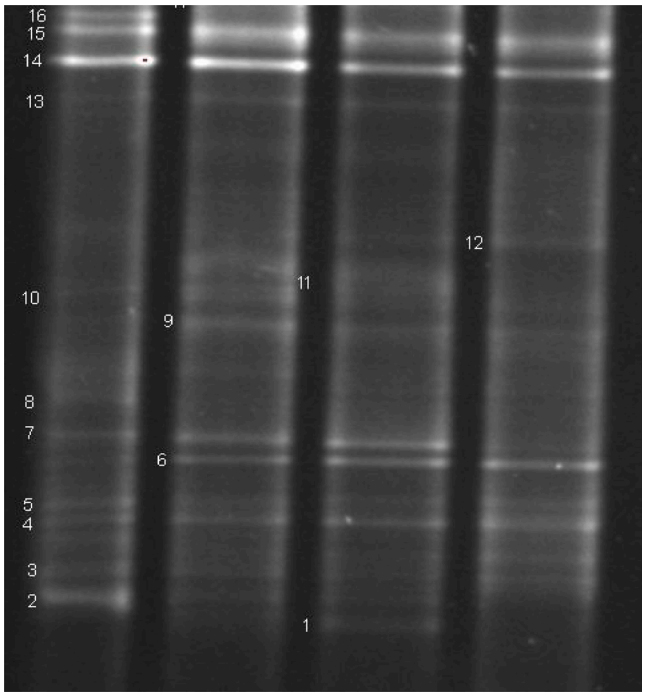

Fig. 4. DGGE image, lane one: control, lane two: (*B. pumilus*), lane three: (protease) and lane four: (*B. pumilus* + protease).

4. Discussion

4.1. Growth and feed efficiency

Many reasons have obligated to use plant protein sources instead of high cost fish meal in fish feed industry. However, the main reasons for limiting inclusion of plant protein in aquafeed are imbalanced amino acid profile and presence of antinutritional factors. In the present study, the highest growth indices were obtained for diet of probiotic (*Bacillus pumilus*) and exogenous protease (pro-enzy). However, the individual supplementation of *B. pumilus* or exogenous protease also improved nutrient efficiency. The potential reasons for superiority of the combination between probiotic and enzymes than other treatments may be due to the benefit of exogenous

Table 6
Molecular analyses of intestinal bacteria of Nile tilapia fed the experimental diets for 84 days.

Band (genotype) no.	Sequence accession no.	Basal diet	*B. pumilus*	Protease	*B. pumilus* + Protease	Best Homology Organism match	Homology Reference.
1	MN822129.1	–	–	+	–	Uncultured Cellulose-degrading gamma proteobacterium	(Ishii et al., 2008)
2	MN821573.1	+	–	–	–	*Pseudomonas indica* Hydrocarbon-degrading bacterium	(Pandey et al., 2002)
3	MN821574.1	+	–	–	–	*Brevibacterium ammoniilyticum* Nitrogenous compound-degrading bacterium	(Kim et al., 2013)
4	MN821575.1	+	+	+	+	*Oryzobacter terrae* Methylotrophic bacterium	(Kim et al., 2015)
5	MN821576.1	+	–	–	–	*Citrobacter koseri* opportunistic pathogen	(Kämpfer et al., 2014)
6	MN822131.1	–	+	+	+	Uncultured Carbohydrate- degrading bacterium	(Kim et al., 2007)
7	MN821578.1	+	+	+	–	Uncultured gut bacterium from zebrafish	(Rawls et al., 2006)
8	MN821577.1	+	–	–	–	*Citrobacter koseri* Opportunistic pathogen	(Kämpfer et al., 2014)
9	MN822130.1	–	+	+	+	Uncultured Anaerolinaceae bacterium, Anaerobic alkanes-degrading bacterium	(Ramos-Padrón et al., 2011)
10	MN822128.1	+	–	–	–	Uncultured Chloroflexi bacterium, Anaerobic hydrocarbon degrading bacterium	(Kolukirik et al., 2011)
11	MN822132.1	–	+	–	–	Uncultured *Alkalilimnicola* sp. Halogenated hydrocarbon-degrading bacterium	(Núñez et al., 2012)
12	MN821579.1	–	–	–	+	*Simiduia* sp. Carbohydrate-degrading bacterium	(Tawara et al., 2015)
13	MN821580.1	+	+	+	+	Uncultured Enterobacteriaceae bacterium	(Chandler et al., 2011)
14	MN821581.1	+	+	+	+	*Hydrogenophaga pseudoflava* Hydrogen-Oxidizing bacterium	(Wen et al., 1999)
15	MN821582.1	+	+	+	+	*Shinella daejeonensis* Nitrate-reducing bacterium	(Lee et al., 2011)
16	MN821583.1	+	–	–	–	*Humihabitans oryzae* Metal-reducing bacterium	(Hamada et al., 2009)

(+) present, (−) Absent.

enzymes and probiotic together than when it was used individually (Avella et al., 2010). Moreover, exogenous enzyme always enhances the probiotic function through offer appropriate substrate for intestinal biota action (Bedford and Cowieson, 2012). Thus, the combination of the *B. pumilus* and the protease could exert a beneficial effect on growth and feed efficacy, as presented in this study. However, a combination between *Bacillus amyloliquefaciens* and the exogenous multienzyme complex did not have any significant effect in growth and feed efficiency of snakehead, *Channa argus* (Dai et al., 2019). The irregularity in these findings may be due to variety of enzymes, type of probiotic, fish species, ingredients of diets and rearing conditions, further works to establish the benefit of synergistic effect between probiotic and exogenous enzymes are still needed. Nevertheless, several studies have assessed the effectiveness of supplementing aquafeeds with different either probiotic bacteria (Dai et al., 2019; Hamdan et al., 2016; Hassaan et al., 2014, 2018a; Ramos et al., 2017) or exogenous enzymes (Adeoye et al., 2016; Hassaan et al., 2019a, b; Hassaan et al., 2020). A significant growth promoting of probiotic in the present study may have be due to the stimulation of appetite (Hamdan et al., 2016), inhibitor/destroy any toxic compounds or production of beneficial metabolites and/or intestinal microbes, which may improve the nutrient efficiency (El-Rhman et al., 2009; Sankar et al., 2017) or alter the beneficial microbiota (Irianto and Austin, 2002; Zhang et al., 2019). Several investigations noted that the improvement of nutrient utilization of fish fed dietary exogenous enzymes may be due to solubilize various or inhibit anti-nutrition factors to improve the absorbance of nutrient (Cowieson, 2005; Hassaan et al., 2019a, b). (Ghobadi et al., 2009) have observed that plant protein diet supplemented with exogenous protease, xylanase and amylase could improve feed efficiency of rainbow trout (*Oncorhynchus mykiss*), also decrease the negative effect of plant diet. Addition of exogenous protease in plant-based diet could enhance digestibility and feed utilization of tilapia by the degradation of complex protein in plant cell wall into simple form of free amino acids and peptidase (Hassaan et al., 2019a; Ray et al., 2012, 2010).

4.2. Histological morphometric

In the current study, either probiotic or exogenous enzyme enhanced the histological morphometric of intestine including (goblet cell, musclaris mucosa thickness, enterocyte and mucosal folds) with special reference to the highest improvement recorded for pro-enzyme treatment compared to other treatments. The synergistic effects between probiotic and exogenous enzymes have a beneficial effect than it was used separately. The current enhancement in the intestinal morphology herein was regarded to increasing rates of digestion and absorption of nutrient for fish fed diet supplemented with pro-enzyme. Hence, the thickness and fluidity of the intestinal

brush-border membrane have effects on the digestion, absorption and intestinal barrier function, and the functional of mucosa (Einarsson et al., 1997). Also, the function of the muscularis mucosa is to promote intestine peristalsis; a thinner muscularis decreased the rate of peristalsis, and the time of feed remaining in the small intestine is increased, thus increasing the time for absorption of nutrients (Liu et al., 2018). The mucus layer that covered the enterocytes of intestine are secreted by goblet cells throughout the gastrointestinal tract (Johansson et al., 2011). In a previous study, Adeoye et al. (2016) showed improvement in enterocyte, goblet cells perimeter ratio and micro villi of tilapia fed diet supplemented with combined of mix of exogenous and probiotic. Also, mucosal folds of paddlefish (*Polyodon spathula*) improved especially in foregut and mid gut for fish fed dietary probiotic (Fang et al., 2015). Similarly, addition of probiotic improved musclaris mucosa thickness, microvilli density and absorptive surface area of rainbow trout (Ramos et al., 2015), tilapia (Elsabagh et al., 2018; Ramos et al., 2017; Standen et al., 2015), revealing that the beneficial effect of probiotic probably depends on the period of feeding. Beneficial effects of exogenous enzyme on the intestinal morphology of tilapia have been indicated through improving of musclaris mucosa thickness, microvilli density and absorptive surface area towards the absorbance of nutrition (Hassaan et al., 2019a; Wallace, 2015). More studies are wanted to clear the effect of combined effect of probiotic and exogenous enzymes in fish intestine dynamics.

4.3. Hematological indices

Monitoring of hematological and blood biochemical parameters are useful to show the protective effects of feed additives on health status of fish (Dawood et al., 2019; Hassaan et al., 2018a, a). Hemoglobin (Hb), hematocrit (Htc), red blood cells (RBCs) and white blood cells (WBCs) in the current study were improved by combination of probiotic and exogenous enzyme or individually supplementation. Several studies assumed that probiotics supplementation could be enhanced the hematological indices of fish (Dawood et al., 2017; Hassaan et al., 2014; Rodriguez-Estrada et al., 2013). However, Hb, Htc, RBC and WBC of tilapia did not affect by dietary probiotics, external enzymes, individually or in combination (Adeoye et al., 2016). Furthermore, using exogenous protease in diets containing high level of cotton seed meal enhanced Hb, Htc and RBCs of tilapia compared to un-supplemented diets (Hassaan et al., 2019b). In addition, WBCs of Salmon (*Salmo truttacaspius*) increased with dietary supplemented with commercial multi-enzyme (Ali Zamini et al., 2014). From the above, the obvious evidence to show the relationship between the hematological parameters and pro-enzyme supplementation not found, thus further studies should be needed.

4.4. Serum biochemical parameters

Regarding serum biochemical indices were significantly improved by dietary pro-enzymes than other treatments. Improvement of these parameters may be due to dietary probiotic could prevent the liver from damage through reducing the lipid peroxidation (Adawi et al., 2001; Lin and Chang, 2000). This finding was similar to those of Hassaan et al. (2014; 2018a; 2019a) who reported that *Bacillus licheniformis* resulted in improved the serum biochemical. Also, transferase enzymes of tilapia fed diet supplemented with probiotic were significantly improved (Dawood et al., 2019). Similarly, levels of serum protein are increased by probiotic supplementation in red sea bream (Dawood et al., 2015). Exogenous multienzymes complex (amylase, acid protease and papain) significantly could increase serum total protein in snakehead, *Channa argus* (Dai et al., 2019). This increase in serum protein is responsible for the innate immune response, and higher levels of this provide stronger responses (Sahu et al., 2007). Plant protein diets supplemented with exogenous proteases could be enhanced the metabolic rate of tilapia's liver (Hassaan et al., 2019a) which agreement with present results.

4.5. Innate immunity

The immune system plays main role in immune response of fish (Ibrahem et al., 2010). Therefore, diet must supplement with feed additives as immunostimulants to increase disease resistance of fish (Dawood et al., 2019; Rodriguez-Estrada et al., 2013). In the present study, probiotic, exogenous enzyme individually or in combination increased Immunoglobulin M (IgM), phagocytic and lysozymes activity of tilapia. Phagocytic and lysozyme activities play an important role in immune system of fish against pathogens (MacARTHUR and Fletcher, 1985). In the present results, the enhancement of immune response associated with dietary probiotic and/or exogenous enzymes supplementation could be due to their inhibitory effects against the pathogenic microorganisms throughout the gastrointestinal tract. Similar to the present data, Díaz-Rosales et al. (2006) and Salinas et al. (2005) found that the diet supplemented with different species of probiotic individually or mixed, increased the phagocytic activity of sea bream (*Sparus aurata* L.). Also, lysozyme and IgM were improved by supplementation with probiotics, *lactobacillus* in Nile tilapia (Dawood et al., 2019). Immunoglobulins play main role in humoral immune system, and it was detected in skin, gills, mucus of gut and bile (Uribe et al., 2011). The mode of action of probiotics supplementation which improve the immunity of aquatic animals may be due to that probiotic enhance secretion of enzymes and protein in blood (Ramos et al., 2017). Furthermore, exogenous enzymes may be contributed in enhancing proliferation of macrophages and monocytes and resultant cytokine production resulted in improved the immune response of fish (Ehsani and Torki, 2010). Recently, total Immunoglobulin and metabolic activity of spleen macrophages of Sterlet were improved by bromelain supplementation in the diets (Wiszniewski et al., 2019).

4.6. Gene expression

Dietary administration of probiotics and exogenous protease has positive effect of gene expression related growth of fish (Dawood et al., 2019; Hassaan et al., 2019a; Zhang et al., 2019). In the present study, relative expression of IGF-I gene was upregulated in tilapia

received probiotic, exogenous enzyme individually or their mix (pro-enzy). This finding showed that diets which have upregulated of insulin growth factor (IGF-I) expression increased growth also, and this agrees with Carnevali et al. (2006). Several studies noted that administration of probiotic have significantly positive effect on IGF-I expression of fish (Avella et al., 2010; Eissa, 2014). However, expression of growth hormone (GH) was decreased in fish fed diet supplemented with probiotic, exogenous enzyme individually or in combination (pro-enzy) in the present study. A similar study reported that diet supplemented with *Lactobacillus delbrueckii* 1×10^6 CFU g^{-1} obtained lower expression of GH than without supplementation. The negative correlation between GH and IGF-I was found in the current study supported by Ayson and Takemura (2006) and Beckman et al. (2004). The reports clarified the influence of combination of probiotic and exogenous enzyme on growth-related genes was not found. Thus, further studies in this context needed to show the synergistic effects between probiotic and exogenous enzyme.

4.7. Molecular analyses of intestinal bacteria

Gut microorganisms have been considered as biosensors for nutritional health of the fish (Adeoye et al., 2016), through helping in absorption (Ray et al., 2012) and improve the immune system (Niu et al., 2019; Zhang et al., 2019). The diversity of gut microbiota influenced by many factors, such as fish diet supplements (Liu et al., 2016). The predictive genomic function showed dietary probiotic potential on enhancing metabolisms with respect to plant protein sources (Fan et al., 2017; Niu et al., 2019).

In the current study, addition of probiotic and exogenous protease to fish diet shaped the gut bacteria in three forms. First, the treatments prohibited the growth of nitrogenous hydrocarbon-degrading bacteria, which occurred in guts of fishes, which feed control diet (Table 6) (Kim et al., 2013; Pandey et al., 2002). This observation implicated that addition of probiotic and exogenous protease individually or combined may helped in complete digestion of protein diet, giving no chance for leaving any nitrogenous hydrocarbon compounds, like protein, the main substrate of those bacteria. The second microbial form was obtained through addition of probiotic *B. pumilus*, which could inhibit the growth of pathogenic bacterium, *Citrobacter koseri,* with genotype accession numbers, Acc.no., MN821576 and MN821577, improving the health of the fish (Table 6) (Kämpfer et al., 2014). Since all experimental diets in the current study were plant-based protein, pro-enzy diet, could direct gut bacteria towards proliferation of carbohydrate degrading-bacteria, which had genotype Acc.no. MN822131, MN822129 and MN821579 (Table 6), for production of energy from current diet plant source carbohydrate (Ishii et al., 2008). Generally, the used probiotic and exogenous protease could improve the gut flora toward health of fishes. On the other hand, there were four genotypes did not affect by diet treatments, implicating intestinal core bacteria (Table 6), representing the third form. These genotypes were belonged to gas, methane-, hydrogen- and ammonia-fixating bacteria (Kim et al., 2015; Wen et al., 1999), a beneficial character for fish health. Previous study revealed that dietary *Bacillus* species could shift the community of the microbiota in the black tiger shrimp, *Penaeus monodon* (Hill, 2009). Also, using *B. amyloliquefaciens* may be reduced the adverse effects of non-digestible carbohydrate and anti-nutritional factors in plant protein sources via increase glycolysis and gluconeogenesis (Chi and Cho, 2016). Dai et al. (2019) reported that composition of gut microorganisms of snakeheads fed diet containing mix of multienzyme and probiotic performed the function of catabolizing a variety of amino acids and carbohydrates. However, the biota intestine in tilapia received probiotic or mixture of exogenous enzymes may be contributed to improve fish performance (Adeoye et al., 2016). The beneficial effect of exogenous enzymes supplementation on the community of the microbiota in intestine are noted by Jiang et al. (2014). Further studies are needed to clarify the relationship between *B. pumilus*, exogenous protease or combination and diversity of microbiome.

5. Conclusions

The diet supplemented with mixture of 1.85×10^5 *Bacillus pumilus* CFU kg^{-1} diet+ 0.5 g protease kg^{-1}diet (pro-enzy) improved performance of growth, immune response, serum biochemical parameters, related gene expression and intestinal bacterial flora of Nile tilapia.

Ethics statements

Experiment was subjected to ethical reviewed and approved by the National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt through the Animal and Welfare Ethical Review Body.

Authors statements

All authors declared that the present article is not submitted or published in any other journal. All authors declared that the roles of the authors are equal.

Declaration of Competing Interest

All the authors declared that they have no any conflict of interest.

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