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THE INFLUENCE OF SYNBIOTIC ON GROWTH AND EXPRESSION OF *GH, GHR1* AND *IGF-I* GENES IN *Oreochromis niloticus* L FINGERLINGS

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Abstract- A combination of probiotics and prebiotics as synbiotics allows assessing their synergistic effects. This study evaluated the effect of synbiotic (*Lactobacillus acidophilus* and fructooligosaccharides+mannan oligosaccharides, FMOS) on growth, hematological parameters, plasma hormonal, and genes expression in *Oreochromis niloticus*. A total of 600 *O. niloticus* of an average initial weight ranged from (4.96 to 5.96 g) was divided into four experimental groups for 84 days. Four basal diets Diet 1 (control), Diet 2, Diet 3 and Diet 4 were formulated to contain four levels of *L. acidophilus* (0.00, 0.42×10^7, 0.84×10^7 and 1.35×10^7CFU g^{-1}) respectively, each level supplemented with 1% FMOS except of the control diet. *O. niloticus* fed diet supplemented with synbiotic showed significant ($P<0.05$) increases in growth and feed utilization. The highest final body weight, best feed conversion ratio, protein efficiency ratio and best chemical composition were obtained by the fish fed synbiotic Diet 3. Supplementation with synbiotic significantly increased in hematological parameters, growth hormone (GH) and insulin-like growth factor-I (IGF-I). The highest expression of *GH* and *GHR1* were detected in liver of fish fed Diet 3. However, *IGF-I* was down regulated in liver of fish fed Diet 2 and Diet 4 whereas, *IGF-I* mRNA level in liver of fish fed Diet 3 up regulated and its expression was parallel with *GH* and *GHR1* expression in liver of fish fed Diet 3. The expression of *GH* and *GHR1* genes in spleen down regulated in all fish fed Diet 2, Diet 3 and Diet 4. On contrary, the expression level of *IGF-I* in spleen of fish received either Diet 3 or Diet 4 was slightly up regulated, but *IGF-I* mRNA level was down regulated in fish fed Diet 2 than other treatments. The expression level of *GH, IGF-I* and *GHR1* genes were down regulated in intestine of fish fed synbiotic than other control diet.

Keywords- Synbiotic, growth, gene expression, *Oreochromis niloticus*

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Introduction

Tilapia is a worldwide fish of great commercial importance and it is recognized as one of the most important aquaculture species of the 21st century. The world's total tilapia production in 2010 was 3.49 million tons [1]. Unfortunately, intensive aqua-farming is accompanied by several problems where the disease infection is a limiting factor for the production through the negative impact on growth. One of the main challenges to achieve productive, feasible and sustainable aquaculture is to develop alternative preventive practices that may help to maintain high animal welfare standards as well as healthy environment, resulting in a better production and higher profits. A novel approach to achieve the above mentioned goals is an application of probiotics and prebiotics in the fish farming industry [2-4]. In a practical sense, probiotics are defined as live microorganisms that are used as dietary supplementations in aquaculture and could enhance the growth and health of the host [5,6]. Gibson and Robefroid [7] defined prebiotics as 'nondigestible food ingredients that beneficially affects the host by selectively stimulating growth and/or activity of one or a limited number of bacteria in the colon' through the combination of probiotics and prebiotics in so-called synbiotics. Also they reported that the use of the synbiotics concept may give the benefit of both pre- and probiotics on fish growth. The synergistic effect may improve the survival of the probiotic organism, where the simultaneous presence of probiotic and prebiotic reward the host in a proper manner [8]. Few data are available regarding the application of synbiotics in aquaculture [9-11]. The research on the effects of synbiotic on levels of growth hormone (GH), insulin like growth factor I (IGF-I) and their gene expression in fish is very limited. In fish, growth is under the control of GH secretion from the pituitary, regulating somatic growth, organ and tissue growth and metabolic processes [12]. Most of these biological functions are mediated by plasma IGF-I, released from the liver in response to circulating GH [13]. Indeed, the hypothesis that the GH/IGF-I axis could be used as a marker of growth performance and nutritional status in aquaculture has already been suggested [14]. Thus, there is obviously a need to excess our knowledge of the effective preparation and safety valuation of synbiotics. Hence, this trial aimed to assess the effect of synbiotic with

different levels on growth performance, feed utilization, hematological parameters, plasma hormonal level of GH and IGF-I and *GH, GHR1* and *IGF-I* genes expression in Nile tilapia (*Oreochromis niloticus*) fingerlings.

Material and Methods

Experimental Design and Culture Technique

Nile tilapia (*Oreochromis niloticus*) with an average initial body weight of (5.91±0.04 g) was obtained from Abbassa, Abo-Hammad, Sharkia Governorate, Egypt. The fish were acclimated for two weeks at fish research station, El-Kanater El-Khayria, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt. During this period, fish were fed a commercial tilapia diet twice a day. The feeding experiment was conducted in 12 concrete ponds (0.5 m^3 and 1m depth). The ponds were supplied with fresh water from the Darawa irrigation branch, Kalubiya, Governorate using a pump machine and putting a fine net in inlet of each pond. Each pond was stocked with 50 fish. Three replicate units were randomly assigned to each treatment, prior to the start of the experiment and each pond was considered as an experimental unit. During the experiment, all fish were hand-fed their respective diets at a level of 3% of body weight 6 days a week. The daily ration was divided into three equal amounts and offered three times a day (09:00, 12:00 and 15:00 hours). A random sample of fish from each treatment was weighed biweekly and the amount of daily diet was adjusted accordingly. Freshwater in each pond was renewed 30% by the outlet at the bottom of the pond daily, before feeding. They were provided with continuous aeration to maintain the dissolved oxygen level near saturation, and fish were held under natural light.

Water temperature and dissolved oxygen were measured every other day using a YSI model 58 oxygen meter (YSI Company, Yellow Springs Instrument, Yellow Springs, Ohio, USA). Total ammonia was measured twice weekly using a DREL, 2000 spectrophotometer (Hash Company, Loveland, CO, USA). pH was monitored twice weekly using a pH meter (Orion pH meter, Abilene, Texas, USA). During the feeding trial, the water-quality parameters averaged (±SD): water temperature was 26.37±0.3: dissolved oxygen, 5.9±0.8 mg/ L: total ammonia, 0.18±0.12 mg/ L and pH 8.52±0.3. All tested water quality criteria were suitable and within the acceptable limits for rearing Nile tilapia fingerlings [15].

Preparation Inoculum of *L. acidophilus*

L. acidophilus culture was prepared by adding 10 g of dried form (Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams Univ., Egypt) to 100 ml of prepared medium containing (gl^{-1}): (peptone 5.0; beef extract, 3.0) broth and adjusted pH at 7.0 incubation was done at 37°C. After 24 h, 1 ml was inoculated into 100 ml fresh prepared medium broth that was incubated for a further 48 h at 37°C. After incubation, the cells were harvested by centrifugation (2000 g for 15 min), washed twice with phosphate buffered saline (PBS; pH 7.3; Oxoid) and re-suspended in PBS for addition to the basal diet.

Experimental Diets

The basal diet was formulated to contain approximately 30% crude protein and gross energy (19.41KJ g^{-1}) which have been shown to be sufficient to support the optimal growth of *O. niloticus*. The basal diet was divided into four groups (Diet 1 (control), Diet 2, Diet 3 and Diet 4). Washed cells of *Lactobacillus acidophilus* were added dropwise into the basal diet mixture prior to pellet after to produce the probiotic diet with three levels 0.42×10^7, $0.84\times10^7$and 1.35×10^7 respectively CFU g^{-1} [16]. The same volume of PBS (*Lactobacillus acidophilus*) was added to the basal mixture for the control. Each diet was supplemented with 1% (fructo-oligosaccharides and mannan oligosaccharides (FMOS) mixture which prepared with ratio 1:1, except diet1 (control). Fructo-oligosaccharides was purchased from (Encore Technologies, Plymouth, MN, USA.) and mannan oligosaccharides (Bio-Mos) purchased from (Alltech Inc., Nicholasville, KY, USA) [Table-1]. The ingredients were ground into fine powder through 200 µm mesh. All dry ingredients were thoroughly mixed with soybean oil, and then passing the mixed feed through a laboratory pellet mill (2-mm die) in National Institute of Oceanography and Fisheries, Cairo Governorate, Egypt (a California Pellet Mill, San Francisco, CA, USA), and stored at -20°C until use.

Table 1- Composition and proximate analysis of the experimental diets (% dry matter)

Ingredients %	Diet NO. (*L. acidophulus cfu* g^{-1}/fructooligosaccharide and mannoligosacchrids (FMOS) %)			
	Diet 1 (0/0)	Diet 2 (0.42×10^7/1)	Diet 3 (0.84×10^7/1)	Diet 4 (1.35×10^7/1)
Fish meal	10	10	10	10
Soybean meal	46	46	46	46
Yellow corn	29.5	29	29	29
Wheat bran	10	9.4	9.35	9.3
soybean oil	3	3	3	3
Vit. & mineral[1]	1.5	1.5	1.5	1.5
L. acidophulus[2]	0	0.1	0.15	0.2
FMOS[3]	0	1	1	1
Proximate analysis				
Crude protein %	30.05	29.92	29.91	29.9
Lipids %	5.69	5.65	5.65	5.64
Ash %	5.43	5.49	5.54	5.57
Total carbohydrate[4] %	58.83	58.94	58.9	58.89
Gross energy (KJ g^{-1})[6]	19.45	19.42	19.41	19.4

[1]Vitamin and mineral mix (mg or g / Kg diet): MnSO4, 40 mg; MgO, 10 mg; K2SO4, 40 mg; ZnCO3, 60 mg; KI, 0.4 mg; CuSO4, 12 mg; Ferric citrate, 250 mg; Na2SeO3, 0.24 mg; Co, 0.2 mg; retinol, 40000 IU; cholecalciferol, 4000 IU; α-tocopherolacetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 mcg; ;nicotinic acid, 300 mg; folic acid, 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg.

[2]*Lactobacillus acidophilus* was prepared to obtain (1.0×10^{10} CFU g^{-1} approximately, Microbiological Resources centre (MIRCEN), Faculty of Agriculture, Ain Shams Univ., Cairo, Egypt.).

[3](FMOS): 0.1% (fructo-oligosaccharide and mannan oligosaccharides) mixture which prepared with ratio 1:1. Fructooligosaccharides (Inulin) purchased from (Encore Technologies, Plymouth, MN, USA.) and mannanoligosaccharides (Bio -Mos) purchased from (Alltech Inc., Nicholasville, KY, USA).

[4]: (Total carbohydrate) =100-(crude protein + lipid + ash).

[4]Calculated using gross calorific values of 23.63, 39.52 and 17.15 kJ g^{-1} for protein, fat and carbohydrate, respectively according to Brett [45].

Growth Parameters

Growth performance and feed conversion were measured in terms of final body weight (g), weight gain (WG), specific growth rate (SGR, % day^{-1}) feed conversion ratio (FCR), Protein efficiency ratio (PER) and feed intake. Growth response parameters were calculated on dry matter as follows:

Weight gain (WG) = final body weight (g) – initial body weight (g); Specific growth rate (SGR) =100× ((Ln (W2)-Ln (W1))/T) Where: Ln = the natural log; W1 = initial body weight; W2 = final body weight and T= period of study (12 weeks); Feed conversion ratio (FCR) =Feed intake (FI) (g)/WG (g); Protein efficiency ratio (PER) = WG (g)/Protein intake (g).

Hematological Parameters and Hormonal Levels

At the end of the experimental feeding, ten fish were randomly collected from each treatment. Whole blood in each treatment was collected in Eppendorf tubes with anticoagulant (heparin 15 unit ml^{-1}) from the caudal vein of each fish. The blood sample was divided into two portions. The first one was used to determine hematocrit (Htc), haemoglobin (Hb), erthyrocyte counts (RBCs) and total count of white blood cells (WBCs) according to standard methods as described elsewhere [17]. The second portion was centrifuged at 1000 × g for 5 min to separate the plasma. Plasma GH was measured by a radioimmunoassay (RIA) kit for Tianjin Nine Tripods Medical and Bioengineering Co., Ltd. (Tianjin, China), following the manufacturer's protocol. Plasma IGF-I levels were determined in undiluted samples by RIA after SepPak C18 chromatography (Waters Corp., Milford, MA, USA), as described earlier for mammals [18].

Total RNA Extraction and Complementary Deoxyribonucleic Acid (cDNA) Synthesis

Liver, spleen and intestine samples were dissected from fish fed different diets and frozen at -80°C immediately until use. Tissue from each sample was ground by Tissue Lyser LT apparatus (QIAGEN GmbH, QIAGEN Strasse 1, Hilden, Nordrhein-Westfalen-40724, and Germany) then total RNA was extracted from the suspension of cells using RNeasy® Mini kit (Qiagen) following the manufacturer's protocol. DNase treatment was carried out to ensure that RNA samples were genomic DNA free. Then re-suspended in RNAse-free water and quantified using Thermo Scientific Nano Drop 1000 Spectrophotometer (Thermo Scientific, USA). Single stranded cDNA was synthesized from 1000 ng of total RNA according to manufacturer's protocol of High Capacity cDNA Reverse Transcription Kits (Applied Bio systems, Catalog number 4368813). Cycling conditions were: 25°C for 10 min, 37°C for 120 min and 85° C for 5 min. Then total RNA and cDNA samples were stored at -80° C until use.

Primer Design

Primers used in this study [Table-2] were created for *GH, IGF-I* and *GHR1* genes and designed using the software GenScript Online PCR Primers Designs Tool based on *Oreochromis niloticus* and *O. mossambicus* mRNA sequences deposited in GenBank. The specificity of the primers was checked by alignments with the original GenBank sequences using the standard nucleotide-nucleotide BLAST (blastn; provided online by NCBI). In this study *18s rRNA* used as a reference gene whereas the used primers matched with Shved [19], whose used two references genes, *EF1ά* and *18S rRNA* of which comparison revealed that 18S rRNA was the most stably expressed across their experimental groups and therefore was selected as the reference gene for qPCR data normalization in the present study.

Quantitative Real Time PCR (qRT-PCR)

Triplicate PCR reactions were carried out for each analyzed sample. Each PCR reaction consisted of, 2.5µl of 1µg/µl cDNA, 12.5 µl SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen), 0.3 µM of each forward and reverse primer and double distilled water to a final volume of 25 µl. Reactions were then analyzed on an Applied Biosystem 7500 Real time PCR Detection system (Applied Bio systems) under the following conditions: 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. The fluorescence monitoring occurred at the end of each cycle. 18s rRNA gene was used as reference gene for qPCR data normalization according to Shved [19].

Table 2 - List of real time qPCR assays used in this work

Gene	Primers	Amplicon (bp)	GenBank ID
18s rRNA	F: GGTTGCAAAGCTGAAACTTAAAGG R: TTCCCGTGTTGAGTCAAATTAAGC	85	AF497908.1
IGF-I	F: GTTTGTCTGTGGAGAGCGAGG R: GAAGCAGCACTCGTCCACG	97	Y10830.1
GH	F: TCGACAAACACGAGACGCA R: CCCAGGACTCAACCAGTCCA	75	M2916
GHR	F: CAGACTTCTACGCTCAGGTC R: CTGGATTCTGAGTTGCTGTC	80	AY973232.1

F: Forward primer; R: Reverse primer

Statistical Analysis of Treatment Effects

All experimentally induced changes in *GH, IGF-I* and *GHR1* expression are presented as n-fold changes (graphically depicted in %) relative to the corresponding controls set as 1 (100%). The comparative threshold cycle ($\Delta\Delta C_T$) method of Livak and Schmittgen [20] was used to calculate relative gene expression ratios as previously described [21]. Prior to analysis, qPCR assays were validated by plotting CT values against the logarithms of the dilution factors. Relative gene expression ratios (R) between treated and control groups were calculated using the formula: $R = 2^{-\Delta\Delta C_T}$ with $\Delta C_T = C_T$ (target gene) $-C_T$ (reference gene), with $\Delta\Delta C_T = \Delta C_T$ (treated group) $- \Delta C_T$ (untreated control). All data are presented as means ± standard error (SE) and were analyzed using one way ANOVA, followed by Duncan's [22] multiple range tests was used to compare differences among individual means, with statistical software SAS ANOVA procedure (statistical analysis system, 1993). A probability of 0.05 was utilized to account for the statistical difference between the means.

Results

Growth, Nutrient Utilization and Chemical Composition Indices

No mortality occurred during the entire experimental period. The indicators of growth performance and feed utilization were higher in *O. niloticus* fed synbiotic compared with control diet and the statistical analysis demonstrated significant differences ($P<0.05$) in growth performance and feed utilization. The greatest means of final body weight, WG and SGR were observed in fish fed Diet 3 [Table-3]. Significant enhancement ($P<0.05$) in feed intake (FI), protein efficiency ratio (PER) and feed conversion ratio (FCR) were recorded by fish fed the dietary synbiotic, in particularly with fish fed Diet 3 recorded the best indices [Table-3].

Concerning the influence of different dietary synbiotic levels on chemical proximate analysis of whole body fish [Table-4], dry matter, lipid, crude protein and ash contents of *O. niloticus* were significantly ($P<0.05$) influenced by the different treatments. Fish fed either Diet 3 or Diet 4 showed the highest lipid and crude protein, while fish fed Diet 3 was recorded higher ash content than other diet.

Table 3- Growth performance and nutrient utilization of *O. niloticus* after 84 days of feeding synbiotic-supplemented diets

	Experimental diet				
	Diet1 (0/0)	Diet2 (0.42×10^7/1)	Diet3 (0.84×10^7/1)	Diet4 (1.35×10^7/1)	±SE
Initial body weight (g/ fish)	5.91	5.83	5.95	4.96	0.04
Final body weight (g/ fish)	34.70^{d}	41.37^{c}	44.10^{a}	42.57^{b}	0.145
Weight gain (g/ fish)	28.79^{d}	35.53^{c}	38.11^{a}	36.61^{b}	0.157
Specific growth rate (%/day)	1.97^{c}	2.18^{b}	2.22^{a}	2.19^{b}	0.008
Feed intake (g/ fish/ period)	52.97^{b}	56.32^{a}	55.57^{a}	56.53^{a}	0.232
Feed conversion ratio	1.84^{a}	1.95^{b}	1.46^{d}	1.54^{c}	0.008
Protein efficiency ratio	1.84^{c}	2.13^{c}	2.32^{a}	2.20^{b}	0.013

-Values (± SE, N= 3). Means in within same row sharing the same superscript are not significantly different (P>0.05).

Table 4- Chemical composition of the whole carcass of *O. niloticus* after 84 days of feeding synbiotic-supplemented diets

	Experimental diet				
	Diet1 (0/0)	Diet2 (0.42×10^7/1)	Diet3 (0.84×10^7/1)	Diet4 (1.35×10^7/1)	±SE
Dry matter	24.66^{b}	24.29^{c}	26.32^{a}	24.50^{bc}	0.081
Crude protein %	55.29^{b}	55.78^{ab}	56.35^{a}	56.22^{a}	0.166
Lipid %	14.48^{b}	15.29^{a}	15.65^{a}	15.45^{a}	0.123
Ash %	14.20^{c}	14.67^{ab}	14.85^{a}	14.43^{bc}	0.102

-Values (± SE, N= 3). Means in within same row sharing the same superscript are not significantly different (P>0.05).

Hematological Parameters and Hormonal Levels

The effect of synbiotic on hematological and hormonal parameters is displayed in [Table-5]. Htc, Hb, RBC and WBCs in fish fed with different levels of synbiotic were significantly (P< 0.05) higher than the control. The highest values in Htc, Hb, RBCs and WBCs were showed in Fish fed Diet 3.

Hormonal level for *O. niloticus* feeding different level of synbiotic showed significant rise (P<0.05) in growth hormone (GH) and insulin like-growth hormone factor-I (IGF-I) in fish fed Diet 3 and Diet 4 and the highest values of GH and IGF-I were detected in fish fed Diet 3.

Table 5- Hematological parameters, GH and IGF-I of *O. niloticus* after 84 days of feeding synbiotic-supplemented diets

	Experimental diet				
	Diet1 (0/0)	Diet2 (0.42×10^7/1)	Diet3 (0.84×10^7/1)	Diet4 (1.35×10^7/1)	±SE
Hct (%)	25.14b	25.23b	25.39a	25.35a	0.006
Hb (g/ L)	10.80b	10.94b	11.14a	10.90b	0.041
WBCs ($\times10^4$/ mm^3)	36.33c	37.13b	38.26a	36.57c	0.073
RBCs ($\times10^4$/ mm^3)	1.79b	1.84b	1.95a	1.81b	0.013
GH (ng/ ml)	0.73c	0.75c	0.81a	0.78b	0.006
IGF-I (ng/ ml)	9.14c	9.78b	10.29a	9.84b	0.024

-Values (± SE, N= 3). Means in within same row sharing the same superscript are not significantly different (P>0.05).

Expression of *IGF-I, GH* and *GHR1* Genes

Melting curves is an approach for validation of real-time PCR analysis and distinguishing between DNA fragments. During this study, the used primers gave a specific PCR product and there is no non-specific amplification as revealed from melting curve analysis. Also melting curves show that no contaminating products are present in this reaction, contaminating DNA or primer dimers would show up as an additional peak separate from the desired amplicon peak. The effect of synbiotic with different levels on differential expression and regulation of *IGF-I, GH* and *GHR1* genes in different organs, liver, spleen and intestine in Nile tilapia, by SYBR-Green real-time PCR assay showed in [Fig-1], [Fig-2] & [Fig-3].

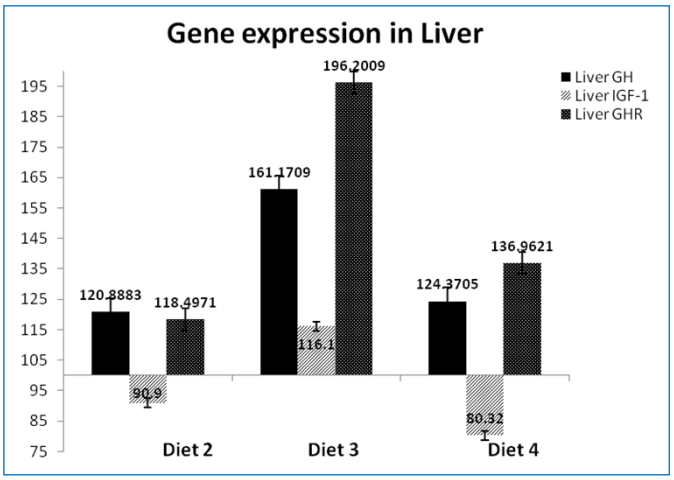

Fig. 1- Effects of Synbiotic on liver *GH*, *IGF-I* and *GHR1* mRNA expression levels measured by real-time qPCR. Y-axis shows data represented as fold change (%) between treated group and control group (set as 100%) ±SEM.

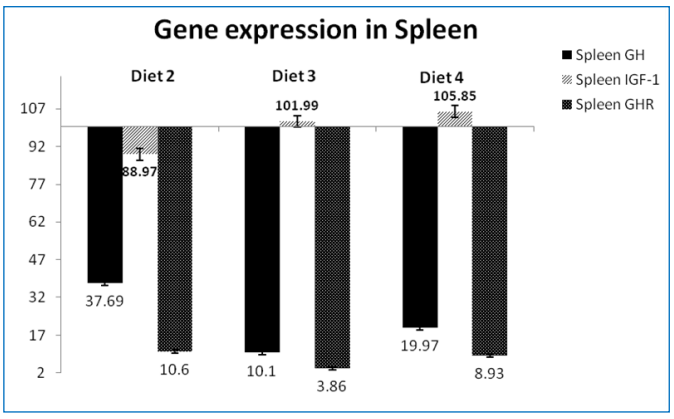

Fig. 2- Effects of Synbiotic on spleen *GH*, *IGF-I* and *GHR1* mRNA expression levels measured by real-time qPCR. Y-axis shows data represented as fold change (%) between treated group and control group (set as 100%) ±SEM.

GH and *GHR1* were parallel up regulated in liver of fish fed different levels of synbiotic and the highest significant were detected in fish fed Diet 3. However, *IGF-I* was down regulated in liver of fish fed Diet 2 and Diet 4 whereas, *IGF-I* mRNA level in liver of fish fed Diet 3 up regulated and its expression was parallel with *GH* and *GHR1* expression in liver of fish fed Diet 3 [Fig-1]. The expression of *GH* and *GHR1* genes in spleen down regulated in all fish fed Diet 2, Diet 3 and Diet 4. On contrary, the expression level of *IGF-I* in spleen of fish received either Diet 3 or Diet 4 was slightly up regulated, but *IGF-I* mRNA level was down regulated in fish fed Diet 2 than other treatments [Fig-2]. The expression level of *GH, IGF-I* and *GHR1* genes were down regulated in intestine of fish fed synbiotic than other control diet [Fig-3].

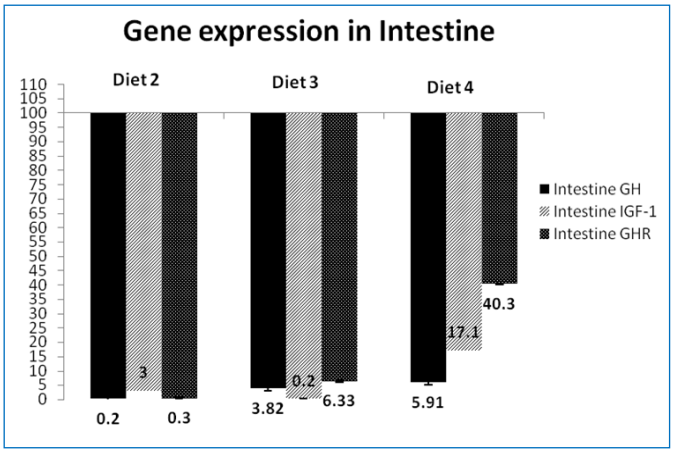

Fig. 3- Effects of Synbiotic on intestine *GH*, *IGF-I* and *GHR1* mRNA expression levels measured by real-time qPCR. Y-axis shows data represented as fold change (%) between treated group and control group (set as 100%) ±SEM.

Discussion

Manipulation of gastrointestinal tract microbiota through probiotic and/or prebiotics dietary supplementations is a novel approach from nutritional point of view and an alternative for antibiotics and immunity promotion. Recently, probiotics and prebiotics have become an integral parts of the aquaculture practices for improving the growth performance [23,24].

Synbiotics, the combined application of probiotics and prebiotics, is based on the principle of providing probionts with a competitive advantage over competing endogenous populations; thus, effectively improving the survival and implantation of the live microbial dietary supplement in the gastrointestinal tract of the host [7]. The use of synbiotics it may be possible to produce greater benefits than the application of individual probionts [25].

In the present study, growth performance and feed utilization of *O. niloticus* were enhanced significantly by synbiotic (0.84×10^7 $CFUg^{-1}$ and 1%) and (1.35×10^7 $CFUg^{-1}$ and 1%) supplementation. This result may be attributed with Gibson and Robefroid [7] they concluded that a combination between probiotic and prebiotic could improve the survival of the probiotic organism because fermentation can be implemented more effectively as its required specific substrate is readily available. Simultaneous presence of probiotic and prebiotic, therefore, benefits the host in a proper manner [8]. Furthermore, the obtained results may be due to the effect of synbiotic that inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and space, and alteration of the microbial metabolism. It also improves the nutrition by detoxifying the potentially harmful compounds in feeds, by producing vitamins such as biotin and vitamin B12 ([26], and by stimulating host immunity [27]. Another possible explanation for increased growth performance with added probiotic is the improvement in digestibility, which may in turn explain the better growth and feed efficiency observed with the supplemented diets. Otherwise, probiotics influence digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity; improving the intestinal microbial balance, consequently improving the digestibility and absorption of food and feed utilization [28]. Recently, Firouzbakhsh [29] reported that *O. mykiss* fingerlings fed diets containing *Enterococcus fascism* as probiotic, and Fructooligosaccharide (FOS) as prebiotic significantly showed higher WG and SGR. Rodriguez-Estrada [10] showed that rainbow trout fed diets supplemented with mannan oligosaccharides (MOS), (*Enterococcus* and MOS) and (*Enterococcus*, MOS and polyhydroxybutyrate acid, PHB) recorded significantly higher WG and SGR than those of the rest experimental groups. Ye [30] reported that, Japanese flounder fed diet supplemented with (fructo-oligosaccharides (FOS), MOS and *Bacillus clausii*) increased FBW and WG than other diets. Also, Ai [31] showed that at each dietary FOS level, dietary supplementation of 1.35×10^7 $CFUg^{-1}$ *B. subtilis* significantly increased the SGR compared with the groups without *B. subtilis* supplementation in juvenile large yellow croaker, *Larimichthys crocea*. Similarly, Mehrabi [32] reported that, rainbow trout (*Oncorhynchus mykiss*) fingerlings fed diets supplemented with different levels of synbiotic (*Entercoccus faecium*/ FOS) showed increase in growth performance in comparison with the control group. Montajami [33] reported that Texas cichlid larvae (*Herichthys cyanoguttatus*) fed the synbiotic had significantly increased final body weight in comparison to control treatment ($P<0.05$). The minimal FCR of the fish in this study was detected with a dietary synbiotic of (0.84×10^7 $CFUg^{-1}$ and 1%). This may suggest that *O. niloticus* is able to utilize food efficiently while receiving relatively medium level of synbiotic, which, in turn, would be more beneficial, also, synbiotic may serve in this case as a co-feeding of inert feed and may help to maximize the diets efficiency through stimulating digestive tract. Co-feeding not only stimulates the ingestion of feed particles, but also promotes digestion and assimilation of diets by fish [34]. Feed conversion ratio is considered to be one of the economic benefits of aquaculture because, in addition to reduction in feeding costs due to decreased feeding, it prevents deteriorating of the cultivation media and, as a result, degradation of water quality eventually leading to increased profits [35].

Dietary synbiotic significantly ($P<0.05$) affected on chemical proximate analysis of whole fish *O. niloticus* dry matter, lipid, crude protein and ash contents. The increase in protein contents in the present study may be due to this fact that by application of synbiotics, the ingested food was converted more effectively into the structural protein and subsequently was resulted more muscle as it is a desirable aspect in fish farming. Also, Mehrabi [32] indicated that higher body protein content in the rainbow trout (*Oncorhynchus mykiss*) fingerlings fed synbiotic. However, application of synbiotic in trout fingerlings diet did not have any significant effect on lipid content. In Japanese flounder there was an increase in body protein content in fish fed synbiotic (FOS, MOS and/or *Bacillus clausii)* in comparison with control, body lipid content demonstrated an opposite trend to body protein content, where fish fed diets *B. clausii*, ((MOS/ *B. clausii*) and (FOS/MOS/*B. clausii)*) presented with significantly lower levels than fish fed the control diet. Neither body moisture nor ash content was affected by any dietary treatments Ye [30]. Accordingly, Bagheri [36] reported that application of 3.8×10^9 $CFUg^{-1}$ of *Bacillus spp*. probiotic in diet of rainbow trout fries made a significant increase in body protein content compared to the control group.

In this study, the administration of synbiotic (*L. acidophilus* and FMOS) levels appeared to be safe for the experimented fish based on the slightly significant increase recorded in the Htc, Hb and RBC values compared with control fish. Fortunately, the increase in hematological indices in the diet 3 and Diet 4 help to increase the oxygen carrying for fish fed the synbiotic. In this respect, Firouzbakhsh [29] reported that a high metabolism caused by synbiotic increased oxygen requirements in rainbow trout fingerlings fed 1.0 g kg^{-1} synbiotic leading to greater total number of their RBC than the control. Accordingly, the increased number of RBC multi-

plies the concentration of hemoglobin ultimately resulting in a high capacity for oxygen carrying in the probiotic-fed fish. Rodriguez-Estrada [10] found that Htc value was higher in the (*Enterococcus* and MOS) and (*Enterococcus*, MOS and PHB) groups than the other groups.

The significant (P<0.05) increasing in the WBCs of fish which fed synbiotic (0.84×10^7/1%) in the present study compared with control. Similar findings were reported by Firouzbakhsh [29] who showed that the highest (P < 0.05) WBC was recorded by the fish fed a diet of 1 g/ kg symbiotic for two months. Reinforcement of non-specific immune system as a result of probiotic consumption can be a possible explanation of the elevated number of WBC [23]. Also, Irianto and Austin [2] found in rainbow trout *(Oncorhynchus mykiss*) and Firouzbakhsh [37] found in Oscar (*Astronotus ocellatus*) that WBC was increased especially lymphocytes following the use of probiotic.

In fish, growth is under the control of growth hormone (GH) secretion from the pituitary, regulating somatic growth, organ and tissue growth and metabolic processes that influence somatic growth [12]. Most of these biological functions are mediated by plasma insulin-like growth factor I (IGF-I), released from the liver in response to circulating GH [13]. Indeed, the hypothesis that the GH/IGF-I axis could be used as a marker of growth performance and nutritional status in aquaculture has already been suggested [14]. Also GH may be acting in an endocrine and paracrine fashion within and between neighboring cells to stimulate IGF-I which may in turn act in an autocrine or paracrine manner to stimulate growth [38,39]. In the present study, the dietary of synbiotic was enhanced and regulated physiological status of the experimented fish based on the significant change records in the Plasma GH and IGF-I levels compared with control fish. Growth hormone (GH) initiates many of its growth-promoting actions by binding to GH receptors (GHRs) and stimulating the synthesis and secretion of insulin-like growth factor-I (IGF-I) from the liver and other sites [40]. At the same time, at the molecular level, the expression of genes involved in muscular growth was also positively affected by bacterial integrators confirming a beneficial role of synbiotic on the whole metabolism. Furthermore, the higher expression level of *GH, IGF-I* and *GHR1* genes in liver tissues obtained from fish fed Diet 3 than other organ. This indicated that spleen and intestine tissues are not specific organs to express *GH* and *GHR1*, but, the specific organ to express *IGF-I* gene is liver followed by spleen tissues. Carnevali [41] showed that sea bass juveniles (*Dicentrarchus labrax*) fed on probiotics showed significantly higher *IGF-I* expression with respect to control group. *IGF-I* is extremely important for the regulation of the establishment and the maintenance of differentiated cell functions via endocrine and paracrine, autocrine signaling [42], as well as the promotion of cellular proliferation and differentiation in many systems [43]. An explanation for this apparent gene expression level in the liver under our treatments remains elusive. The extent of feeding might affect *GHR* expression [44]. Such studies are vital for understanding the differential regulation of expression of these growth factors under the studied treatments and would help us to delineate the biological significance of these growth factors in *O. niloticus*.

Conclusions

Considering the low cost of production of *L. acidophilus* for aquaculture which offer convenience and cost benefits to farm operators and on the basis of the data here obtained, we suggest that (synbiotic) composed by *L. acidophilus*, fructo-oligosaccharides and mannan oligosaccharides as a valuable feed additive *in O. niloticus* L fingerlings. Specially, (0.84×10^7CFU g^{-1}/1%) and (1.35×10^7CFU g^{-1}/1%) enhance significantly growth parameters, feed utilization, Plasma GH and IGF-I levels and the expression level of *GH*, *GHR* and *IGF-I* genes in liver and spleen. The results of this study provided new insight for emerging synbiotic biotechnology, for further increase of productivity and competitiveness of the aquaculture industry.

Abbreviations

GH: Growth hormone

GHR1: Growth hormone receptor-1

IGF-I: Insulin like growth factor- I

FMOS: fructooligosaccharides+mannan oligosaccharides

CFU: colony-forming unit

mRNA: Messenger Ribonucleic Aid

FAO: Food and Agriculture Organization

WG: weight gain

SGR: specific growth rate

FCR: feed conversion ratio

PER: Protein efficiency ratio

FI: Feed Intak

Htc: hematocrit

Hb: haemoglobin

RBCs: erthyrocyte counts

WBCs: total count of white blood cells

cDNA: DNA complementary to RNA

Ct: threshold cycle

rRNA: ribosomal RNA

DNase: deoxyribonuclease

FBW: final body weight

qRT-PCR: Quantitative real time PCR

Conflicts of Interest: None declared.

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