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Comparative Epigenomic Profiling and Gene Expression Patterns of Zebrafish, *Danio rerio*, Administrated by Dietary Agrimos®

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ABSTRACT

The different aspects of using dietary supplements such as prebiotics in aquaculture and their effects on innate immune response, and especially their vertical transmission, are of a grave importance. To address such issues in both horizontal and vertical transmission of boosting immune system, the present study was designed to investigate the effect of different levels of dietary Agrimos® on the innate immune-related gene expression [Lysozyme (Lyz) and Tumor Necrosis Factor alpha (TNFα)], DNA methylation, and three Histone MethylTransferase [HMTs (H3K4, H3K9, H3K27)] activities as well as growth performance in zebrafish (*Danio rerio*). Three hundred and sixty healthy 24-days-old zebrafish were randomly distributed in twelve aquaria assigned to four groups. Zebrafish were fed with either control diet or a diet supplemented by different levels (0.2, 0.4, and 0.8%) of Agrimos® for 90 days. The offspring of each treatment was assessed to find the potential of vertical transmission of immunity by using this dietary prebiotic. At the end of the experiment, gene expression studies revealed significant up-regulation (P< 0.05) of *TNFα* and *Lyz* genes in 0.2 and 0.4% Agrimos® fed fish compared with the control group. Although our findings showed that supplemented diet reduced DNA methylation (P< 0.05) in Agrimos® treatments compared with the control, there was no significant change in all three HMTs' activities among experimental groups (P> 0.05). The result shows the successful transmission of *Lyz* gene expression as an innate immune response to the offspring of the treated adults and supports a direct role of DNA demethylation in the regulation of these candidate gene expressions, suggesting possible role of diet on regulating the epigenetic processes.

Keywords: Epigenetic, Innate immunity, Methylation, Prebiotic.

INTRODUTION

The food industry and aquaculture research sector have encouraged the development of dietary supplements with prebiotic properties in pursue of health promotion, growth improvement, and disease prevention (Akhter *et al*., 2015; Ulloa *et al.*, 2014). These indigestible food ingredients can shift the microbial community to one dominated by beneficial bacteria, such as *Lactobacillus* sp. (Ganguly *et al.*, 2010; Tarnecki *et al*., 2017; Hoseinifar *et al*., 2019).

Innate immune response can recognize non-self-molecules like MannanOligoSaccharides (MOS) prebiotic, through receptors (Yarahmadi *et al.*, 2014; Yuji-Sado *et al.*, 2015). Among innate immunity parameters, lysozyme is one of the most important ones, involved in the lysis of the bacterial cell wall peptidoglycans (Cerezuela *et al.*, 2011; Wang and Zhang, 2010). Lysozyme often works simultaneously with other mechanism of the non-specific immune system like Tumor

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Necrosis Factor alpha (TNFα) from the Cytokines (Akhter *et al.,* 2015; Sullivan *et al.*, 2007).

The epigenetic processes are crucial in regulating gene expression and have an essential impact on growth and development of an organism by leading to cell differentiation (Massicotte *et al.,* 2011; Davidovic *et al.,* 2014; Paul *et al.,* 2015). Therefore, in this study, we focused on the first level of transcription process of innate immune response by investigating the effects of prebiotics on methylation and its effect on gene expression. In addition, histone methylation that cooperates with DNA methylation (Anderson *et al.,* 2012) was anaLyzed by examining Histone MethylTransferases (HMTs) activity. Methylation of lysine (K) 4 of histone H3 (H3K4) is linked to transcriptional activation, whereas methylation of lysine 9 and 27 on H3 (H3K9 and H3K27) causes gene silencing and transcription inhibition (Saleem *et al.*, 2015). Additionally, it has recently been established that epigenetic effects can be inherited from one generation to the next; so, it is possible to program the development of the fish by tightly controlling various components of the environment including the fish diet at different stages of development (Moghadam *et al.,* 2015; Triantaphyllopoulos *et al.*, 2016).

Since the zebrafish, *Danio rerio*, offers many advantages over other models (Ulloa *et al.*, 2014; Vaz *et al.,* 2015), and shares many of the same characteristics with numerous fish species of economic interest (Ribas and Piferrer, 2013), it was selected for this study.

To move one step forward and investigate if the candidate immune response can be transferred to the next generation, in this study, we focused on evaluating the effect of dietary Agrimos® on genetic [two candidate immune-related (Lyz and TNFα) gene expressions] and epigenetic (DNA methylation and HMTs activity) changes of immature, mature, and offspring of zebrafish as an animal model for high-throughput testing of experimental diets in aquaculture.

MATERIALS AND METHODS

Animals and Housing

Three hundred and sixty of ten-day old zebrafish larvae were procured from the local zebrafish hatchery of ornamented fish farm in Karaj, Iran, transferred to the fish laboratory of the University of Tehran, and acclimated to the experimental conditions for 14 days. Water temperature was 27±1ºC and the photoperiod was regulated to provide a 14 hour light: 10 hour dark cycle, with lights on at 0800 hours. The aquariums were continuously aerated by a central pump equipped with sponge filter to maintain the water parameters within the following condition: Dissolved O_2= 6.1-6.5 mg L^{-1}, pH= 7.2-7.6, Hardness= 160-180 mg L^{-1} $CaCO_3$, monitored daily.

A commercially available extruded granular pellet (BioMars proximate composition of 35% crude protein, 12% total lipid, 10% ash, 4% moisture, and 20.5 kj g^{-1} energy) was used to feed fish ad libitum twice a day. The food particle size for juvenile stage of zebrafish was 600-800 μm. Fish with an initial body weight of 40.12- 49.67 mg (20-days old) were distributed into 12 aquarium (30×30×30 cm) of 25 cm water height. Each treatment contained 30 juvenile fish in triplicate (90 fish per treatment). All experimental fish in this study were conducted in accordance with legal regulations (EU, 2010).

Prebiotics and Diet Preparation

The prebiotic Agrimos® mannan-oligosaccharides (Agrimos® MOS) was used in the present experiment. It is a specific combination of MOS and β- glucans extracted from the yeast cell walls of *Saccharomyces cerevisiae* (Lallemand Animal Nutrition, France).

Four experimental diets were prepared for this study based on literature review: the basal

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diets as the control plus three prebiotic test diet group supplemented at levels of 0.2, 0.4, and 0.8% (Forsatkar *et al*., 2018). Diets were prepared by diluting the appropriate amount of Agrimos® MOS (2, 4 and 8 g kg^{-1} food weight) in distilled water and gently mixed with the crushed BioMar food to make a paste that was then spread on a plastic sheet, air dried, and slightly ground and sieved to produce a suitable crumble size of 600-800 µm (control diet had no prebiotic). The duration of feeding period was chosen to be 90 days since the fish had to reach maturity to be prepared for the reproduction. Subjects were fed twice a day at 1,000 and 1,600 hours throughout the experiment.

Sampling

After acclimation, nine fish were collected from each treatment (3 from each triplicate) at days 10, 30, 60, and 90 starting from treatments. After 65 days from the beginning of the test, adult zebrafish (2 males'×4 females, for each treatment) were treated for 3 weeks in another aquarium at 28.5°C to collect the first generation for further analysis. RNA was extracted from a pool of the eggs taken from each group 96 hpf.

Gene Expression

Total RNA Extraction and cDNA Synthesis

RNA extraction from zebrafish whole body samples were carried out by TRIzol™ (Invitrogen) reagent and ethanol precipitation with some laboratory modifications. The quantity and quality of RNA were measured by Nanodrop spectrophotometer (Thermo 2000c, USA) at 260/280 nm and 1% agarose gel electrophoresis. Three µg of total RNA was used to DNase I treatment (Thermo Scientific, USA) and the first-strand cDNA was synthesized in total volume of 20 uL containing 200 Unit RevertAid reverse transcriptase (Fermentas, USA), 50 uM oligodT primer, 100 uM random primer and 1ug of treated RNA.

Real-Time PCR

The primers for target and reference genes (Lyz, TNFα and β-actin) were designed based on the conserved regions using PRIMER3.0 program (Table 1). Real-time PCR analysis was carried out using a StepOne real-time PCR (ABI, USA) in triplicate reactions for all samples. The real time PCR reaction with a total volume of 15 µL containing 7.5 uL of 2X SYBR Premix Ex Taq II (Takara-Clontech, Japan), 0.4 uM each primers' add 20 ng of cDNA. Thermo-cycling program include 30 seconds at 95°C, 40 cycles of 15 seconds at 94°C, 20 seconds at 59°C, 30 seconds at 72°C and final melt curve analysis. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

DNA Methylation Analysis

Genomic DNA extraction was performed by Exgene CellSV kit (GeneAll, South Korea) according to manufacturing instruction. DNA was eluted in 50 uL DNase free water and solved for 15 minutes at 40°C and under the quality control in 260

Table 1. Sequences of oligonucleotide primers and the conditions used for real-time PCR.

Primer name	qPCR Primers (Forward/Reverse)	Tm	Accession No	Amplicon (bp)
TNFα	CGGGTGTATGGAGGGTGTTTGG	60	NM_212859.2	147
	TGCCTTGTGAAATGCGATCTCTC	59		
Lyz	GGCGTGGATGTCCTCGTGTG	60	BC162644.1	150
	CGGTGGGTCTTAAACCTGCTTTC	59		

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nm (Thermo 2000c, USA). Bisulfite treatment of extracted DNA was performed by using the EZ DNA Methylation Direct Kit (Zymo research). The specific Primers for PCR amplification of target genes (Lyz and TNFα) were designed in CpG containing segments located in the upstream of start codon of genes using methyl primer 1.0 (Table 2). PCR products were evaluated in 1.5 percent agarose gel electrophoresis and purified by using PCR purification combo kit (Invitrogen). PCR amplicons were sequenced directly in ABI 3730xl capillary sequencer implemented by Macrogen Inc, sequencing services. The genes alignments were implemented by bioedit software.

HMTs Activity Assay

Three histone methyltransferase enzymatic activity/inhibition kits from Epigentek were used to measure the relative levels of total histone methylation activity on three histone H3 moieties; Histone H3K4 [Epigentek; Catalog #. P-3002-2 (96 assay)], histone H3K9 [Epigentek; Catalog #. P-3003-2(96 assay)], and histone H3K27 [Epigentek; Catalog #. P-3005-96 (96 assay)]. The assays were performed according to manufacturer's instructions using total soluble protein extracts. Briefly, a standard curve was prepared for each of the three assays using the HKMT standard (supplied with the kits) at concentrations ranging from 0.1, 0.2, 0.5, 1, 2, and 5 ng μL^{-1} along with negative and positive controls. The test samples were set up by adding the protein extracts, histone assay buffer, S-Adenosyl Methionine (SAM) substrate (supplied with the kit), and biotinylated substrate at 25 µg mL^{-1} (supplied with the kit). The strip wells were covered with aluminum foil and incubated on a plate shaker (50-100 rpm) at 37ºC for 90 minutes. After incubation, the strip wells were aspirated and washed three times with 150 µL of 1X wash buffer. Each strip well was then incubated with 50 µL of 1 µg mL^{-1} of capture antibody at room temperature for 60 minutes on plate shaker (50-100 rpm). Next, the strip wells were aspirated and washed five times with 150 µL of 1X wash buffer. Subsequently, 50 µL of 0.2 µg mL^{-1} detection antibody was added to each strip well and incubated at room temperature for 30 minutes on a plate shaker (50-100 rpm). The strip wells were then aspirated and washed five times with 150 µL of 1X wash buffer. 100 µL of the developing solution was added to each strip well and incubated at room temperature for 10 min away from the light. Lastly, 50 µL of stop solution was added to each strip well to stop the enzyme reactions and the absorbance was measured every 5 minutes using a microplate reader (Synergy HT Multi-Mode) at 450 nm. The total HKMT activity was calculated using the following formula;

$$Activity\left(\frac{\frac{ng}{h}}{mg}\right) = \left[\frac{OD(sample - blank)}{protein\ amount\ (\mu g) \times h \times slope}\right] \times 1000$$

Where, protein amount is the total soluble protein (µg) added to the test sample wells, h is the incubation time at 37ºC, slope is the slope of the line of the standard curve created from concentrations ranging from 0.1, 0.2, 0.5, 1, 2, and 5 ng μL^{-1}.

Statistical Analysis

Data were anaLyzed by one-way ANOVA, followed by Duncen's multiple range tests using SPSS statistical package

Table 2. Sequences of methylation specific PCR primers and the conditions used for real-time PCR.

Primer name	qPCR Primers (Forward/Reverse)	Tm	Accession No	Amplicon (bp)
TNFα	GGGGGTTTTTTGGTGATATATT (Fwd)	61	NM_212859.2	435
	TCCTCCTAAACACTATCACCAAC (Rev)	60		
Lyz	ATTAAGATTAGTGGAGGTGTATGG (Fwd)	60	BC162644.1	269
	TAATAAACAATTTCTCCATCATAAACT (Rev)	58		

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version 25.0 (IBM SPSS statistics) at 5% probability to evaluate the effect of Agrimos® on selected factors. Data are presented as mean values±SD. Prior to statistical analysis, normality, and homogeneity of variance were checked by Kolmogorov-Smirnov and Levene tests, respectively.

RESULTS

Body Weight and Mean Age of First Egg Laid

The results of the body weight of the zebrafish that fed with diet supplemented with 0.2, 0.4, and 0.8% prebiotic Agrimos® are presented in Table 3.

No significant differences (P< 0.05) were observed in body weight after 30, 60, and 90 days feeding on 0.4 and 0.8% supplemented diets. However, body weight of 0.4 and 0.8% were significantly higher (P< 0.05) than the control group. Also, after 90 days from the experiment, results revealed that feeding on 0.2% dietary Agrimos® significantly (P< 0.05) increased the body weight compared to the control group.

Gene Expression

The effect of Agrimos® on the expression of immune related genes (Lyz and TNFα) of zebrafish are shown in Figures 1 and 2, respectively. The present study revealed that feeding on 0.4 and 0.8% dietary Agrimos® (after 30, 60, and 90 days) significantly (P< 0.05) increased the expression of *Lyz* compared to the 0.2% Agrimos® and control group. The expression of *TNFα* gene showed no significant (P< 0.05) differences between 0.4 and 0.8% Agrimos® after 30, 60, and 90 days feeding on experimental diets. However significantly higher expression was observed in fish that received 0.4 and 0.8% prebiotic compared to the 0.2% dietary prebiotic and the control group.

The first generation gene expression results are presented in Table 4. No significant differences (P< 0.05) were observed in the *TNFα* gene expression in the fish fed dietary Agrimos® as compared to the control group. Significant increases in *Lyz* gene expression were shown in the offspring of all supplemented groups in comparison to the control group. Among the supplemented diet groups, 0.4% showed significantly (P< 0.05) the highest *Lyz* gene expression. However, a significant increase (P< 0.05) was shown in 0.8% compared to the control and 0.2% treatment.

Methylation Percentage and HMTs Activity

Constitutive changes in percent of C methylation during development between 30, 60, and 90 days are shown in Figures 3 and 4 (for Lyz and TNFα respectively). C methylation percent decreased significantly during the experiment period (from the first day to 90 days after trial) in both *Lyz* and *TNFα* in zebrafish. Overall, due to the low content of CG, C methylation was

Table 3. Body weight (mg) of zebrafish *Danio rerio* fed 0, 0.2, 0.4 and 0.8% dietary Agrimos®.

Body weight (mg)	Agrimos® (%)			
	0	0.2	0.4	0.8
0	46.53±2.70[a] [a]	73.35±2.63[a]	172.03±2.02[a]	277.73±25.00[a]
30	45.36±3.01[a]	77.90±2.42[ab]	187.17±3.26[a]	320.05±5.27[b]
60	43.08±2.35[a]	82.85±3.25[bc]	236.83±16.14[b]	372.76±10.57[c]
90	44.57±3.57[a]	84.67±3.19[c]	230.16±17.23[b]	358.03±12.72[c]

[a] Different alphabet denote significant difference between treatments (P< 0.05). Values are presented as mean±SD (N=9).

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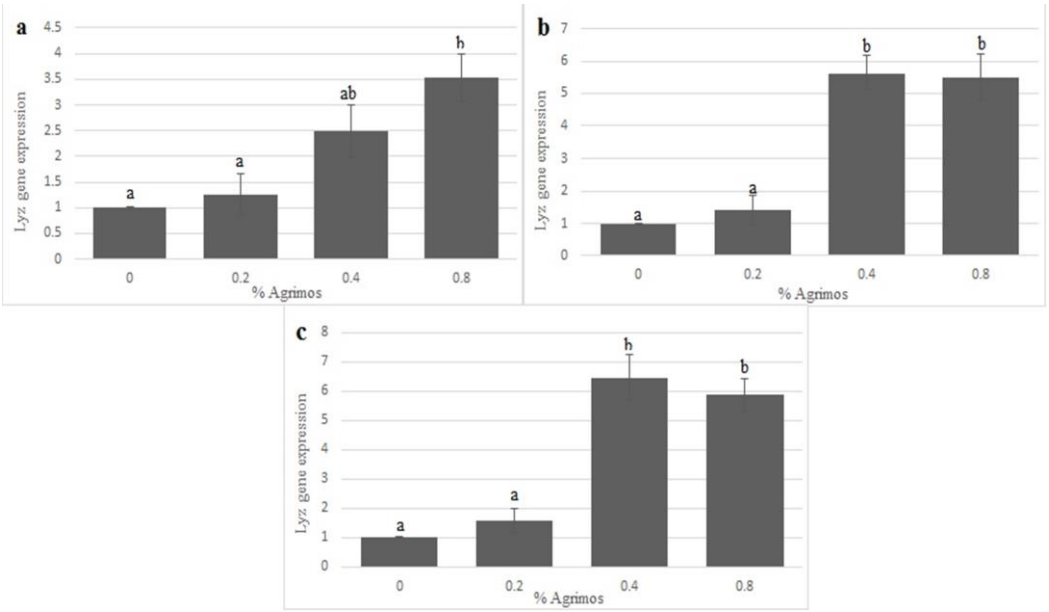

Figure 1. The relative expression of *Lyz* gene in zebrafish fed 0, 0.2, 0.4 and 0.8% prebiotic Agrimos®. (a) First sampling (30 days after start of experiment), (b) Second sampling time (60 days) and (c) Third sampling time (90 days). The mean±SD for the first day (start experiment) was 0.81±0.04. Data are expressed as the mean±SD. The bar in each treatment marked with different alphabet shows significant difference (P< 0.05).

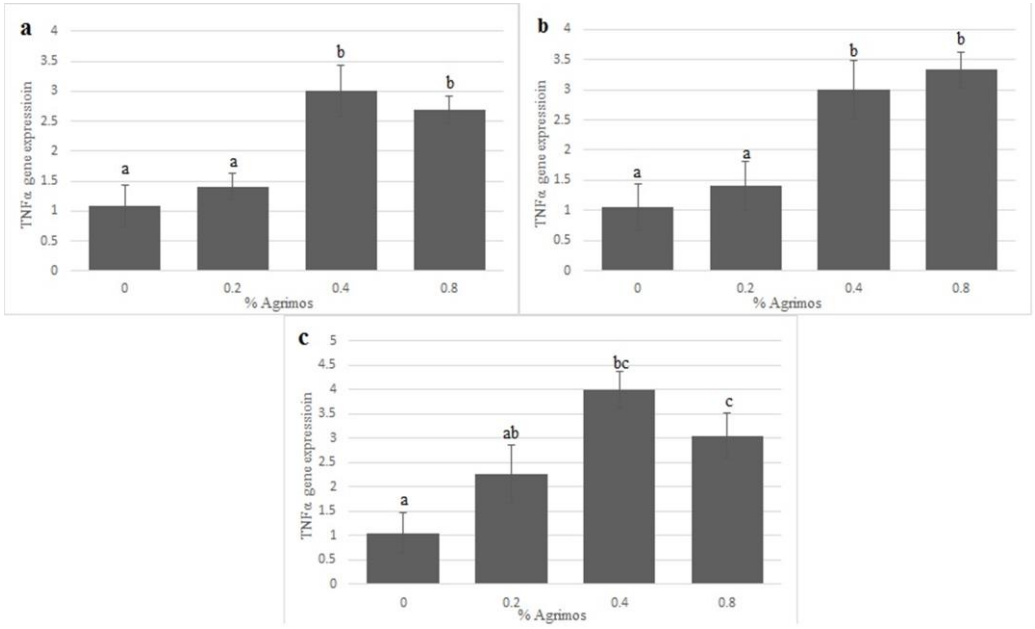

Figure 2. The relative expression of *TNFα* gene in zebrafish fed 0, 0.2, 0.4 and 0.8% prebiotic Agrimos®. (a) First sampling (30 days after experiment), (b) Second sampling time (60 days) and (c) Third sampling time (90 days). The mean±SD for the first day (start experiment) was 0.92±0.17. Data are expressed as the mean±SD. The bar in each treatment marked with different alphabet shows significant difference (P< 0.05).

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Table 4. F_1 (the offspring of each treatment) gene expression.

Gene expression	Agrimos® (%)			
	0	0.2	0.4	0.8
TNFα	1.11±0.03	1.14±0.07	1.13±0.00	1.16±0.08
Lyz	0.98 ±0.00[a]	1.08±0.08[a]	1.73 ±0.06[c]	1.61±0.06[b]

[a-c] Different alphabet denote significant difference between treatments (P< 0.05). Values are presented as mean±SD (N=9).

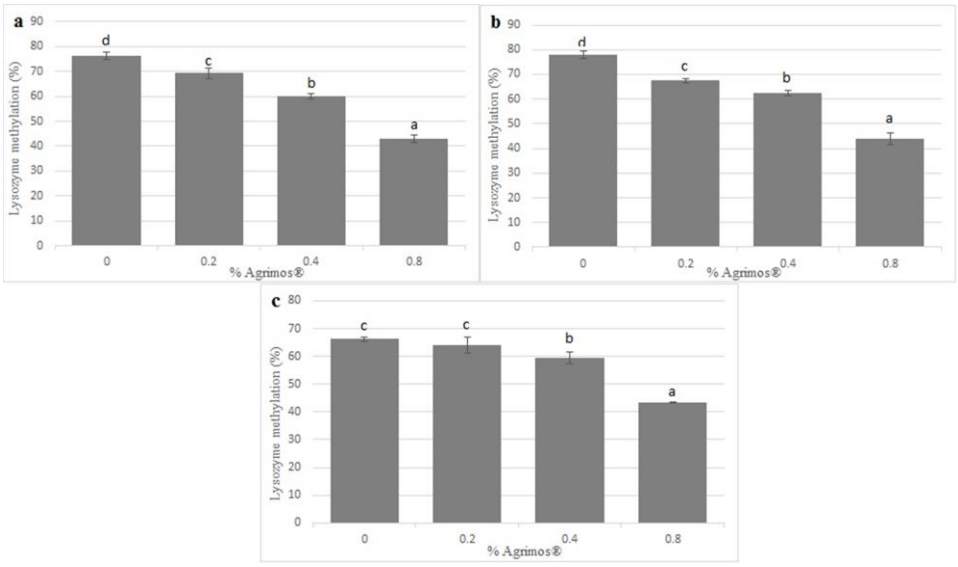

Figure 3. Methylation percent of *Lyz* gene. (a) First sampling (30 days after experiment), (b) Second sampling time (60 days) and (c) Third sampling time (90 days). The mean±SD for the first day (start experiment) was 84.97±1.08. Data are expressed as the mean±SD. The bar in each treatments marked with different alphabet shows significant difference (P< 0.05).

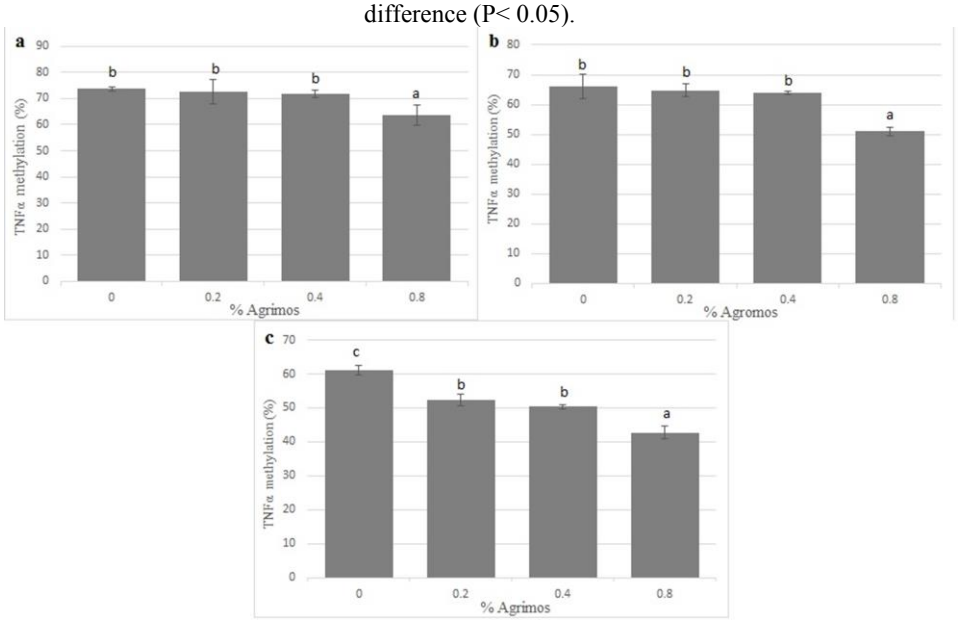

Figure 4. Methylation percent of *TNFα* gene. (a) First sampling (30 days after experiment), (b) Second sampling time (60 days) and (c) Third sampling time (90 days). The mean±SD for the first day (start experiment) was 82.83±0.55. Data are expressed as the mean±SD. The bar in each treatments marked with different alphabet shows significant difference (P< 0.05).

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anaLyzed. The result showed methylation was the highest at C in both *Lyz* and *TNFα*. Percent of C methylation was the highest in the control group in comparison with all other groups of dietary supplemented by Agrimos®. The results of C methylation percent indicated a significant decrease (P< 0.05) in fish fed dietary Agrimos® compared to the control group and the group containing 0.2 % Agrimos®. *TNFα* C methylation percent in 0.2 and 0.4% were not affected 30 and 60 days after treatment with Agrimos® (P< 0.05). Ninety days after feeding with dietary prebiotic, all the groups treated by Agrimos® showed significant decrease in C methylation percent.

The effect of dietary Agrimos® on C methylation percent of the first generation of zebrafish fed by supplemented diet is displayed in Table 5. There were no significant differences in the C methylation percent of *TNFα* of the offspring in the fish treated by dietary Agrimos® and the control group. In contrast, the C methylation percent of *Lyz* decreased significantly in 0.8% compared to other groups.

Figures 5, 6, and 7 and Table 6, respectively. There were no significant (P< 0.05) changes in all three HMTs activities assayed between the experimental groups (Figures 5-7). H3K4 showed a significant increase (P< 0.05) in 0.4%, but there were no significant differences between 0.8 and 0.4%. There was no significant (P< 0.05) difference in H3K9 and H3K27 methyltransferase activities assayed between experimental groups.

DISCUSSION

Food and beverage companies are highly focusing on research and development activities to enhance the nutritional and multifunctional profile of food products that offer superb health benefits. Prebiotics have greater visibility in the health supplement market, which should have a positive spillover effect into the mass consumer market (Beikzadeh *et al*., 2017). The rising demand for fishmeal with natural ingredients, which promotes a wide range of

Table 5. F_1 (the offspring of each treatment) methylation percent.

Methylation %	Agrimos® (%)			
	0	**0.2**	**0.4**	**0.8**
TNFα	81.83 ± 1.83	83.03 ± 1.76	81.93 ± 2.31	82.70 ± 0.95
Lyz	82.30 ± 1.90^{bc}	84.30 ± 2.60^{c}	79.56 ± 2.04^{b}	74.46 ± 2.82^{a}

a-c Different alphabet denote significant difference between treatments (P< 0.05). Values are presented as mean±SD (N=9).

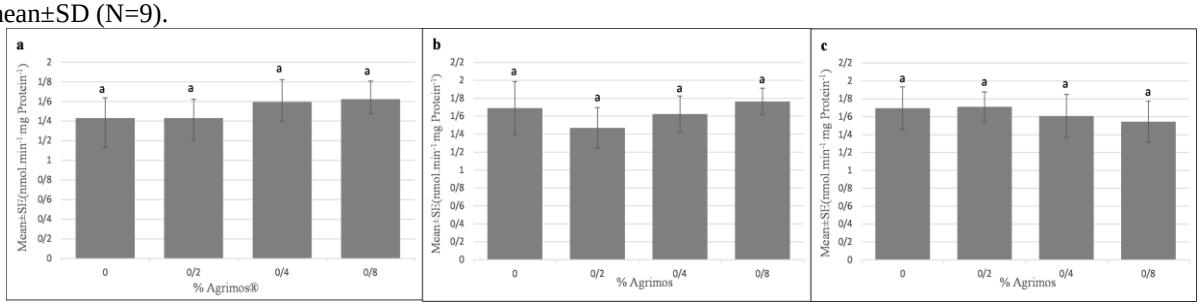

Figure 5. H3K4 methyltransferase activity. (a) Sampling (30 days after experiment), (b) Second sampling time (60 days) and (c) Third sampling time (90 days). The mean±SD for the first day (start experiment) was 1.81±0.07. Data are expressed as the mean±SD. The bar in each treatments marked with different alphabet shows significant difference (P< 0.05).

The results of HMTs activity in treated zebrafish and their offspring is shown in

health benefits, is expected to result in significantly high sales of prebiotic based

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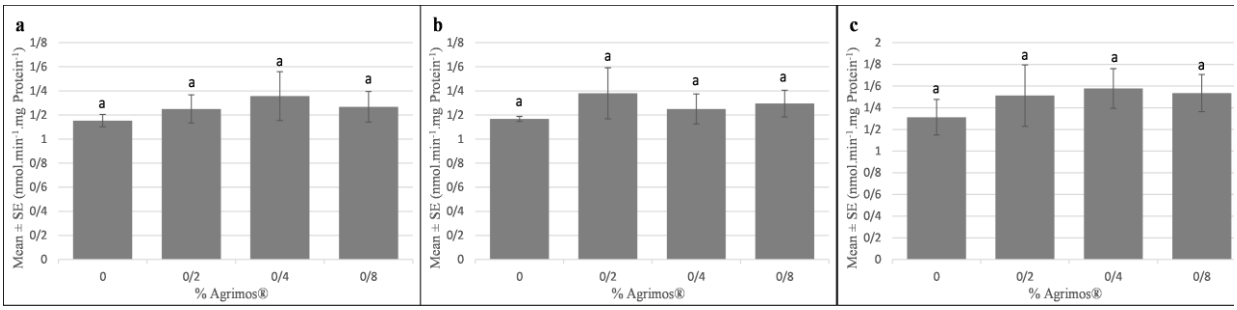

Figure 6. H3K9 methyltransferase activity. (a) First sampling (30 days after experiment), (b) Second sampling time (60 days) and (c) Third sampling time (90 days). The mean±SD for the first day (start experiment) was 1.62±0.23. Data are expressed as the mean±SD. The bar in each treatments marked with different alphabet shows significant difference (P< 0.05).

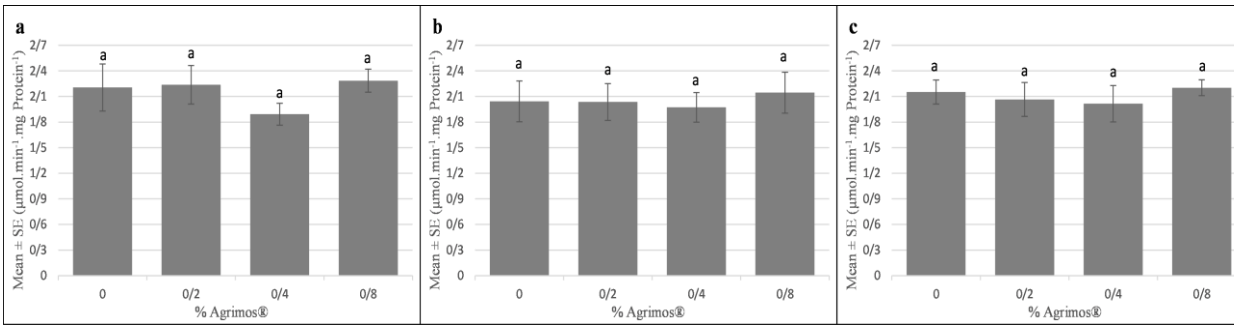

Figure 7. H3K27 methyltransferase activity. (a) First sampling (30 days after experiment), (b) Second sampling time (60 days) and (c) Third sampling time (90 days). The mean±SD for the first day (start experiment) was 2.34±0.20. Data are expressed as the mean±SD. The bar in each treatments marked with different alphabet shows significant difference (P< 0.05).

Table 6. F_1 (the offspring of each treatment) HMTs activity.

HMTs activity	Agrimos® (%)			
	0	0.2	0.4	0.8
H3K4	1.49 ± 0.24[a]	1.47 ± 0.36[a]	1.87 ± 0.08[b]	1.79 ± 0.07[ab]
H3K9	1.53 ± 0.25	1.38 ± 0.07	1.46 ± 0.26	1.25 ± 0.13
H3K27	2.24 ± 0.38	2.36 ± 0.20	2.49 ± 0.23	2.57 ± 0.26

a-b Different alphabet denote significant difference between treatments (P< 0.05). Values are presented as mean±SD (N=9).

products. Owing to its wide range of preventive therapeutic possibilities, prebiotics research in aquaculture industry is of the utmost importance (Rolim, 2015; Hoseinifar *et al*., 2019).

The growth capacity of fish is contingent upon several factors including nutrient digestion, assimilation rate or feed conversion efficiency (Torrecillas *et al.*, 2014). As Opazo *et al.* (2017) stated, the most likely explanation of the key growth gene expression patterns results could be associated with the larvae nutritional status. In this study, utilization of dietary Agrimos® started from juvenile stage until adult, and the results in the mature and offsprings indicated that the administration of dietary 0.4% and 0.8 % Agrimos® could significantly enhance the growth performance of zebrafish. Similarly, some previous studies confirmed that the growth were improved by MOS supplemented diets in fish and shrimp (Torrecillas *et al.*, 2013; Mohamed *et al.*, 2017) and regulated by

[DOR: 20.1001.1.16807073.2020.22.6.12.8]

nutritional status, or the glucose level. However, other studies reported a lack of effect on the feed efficiency and growth rate (Mehrad *et al.,* 2012; Piccolo *et al.,* 2013; Torrecillas *et al.,* 2014; Mohamed *et al.,* 2017), which in part could be explained due to the structural differences of the MOS used, dose of supplementation, treatment duration, culture conditions, fish species, or age (Torrecillas *et al.,* 2014). Moreover, MOS provides mannose substrate upon which pathogenic gut bacteria selectively attach, impairing the adhesion to enterocytes, leading to better gut health, and villi integrity, and diet nutrients' uptake (Yuji-Sado *et al.,* 2015); therefore, it can improve weight gain efficiency and food conversion ratio.

Some investigated parameters have indicated that the dietary MOS increases some immune-related gene expressions (Yarahmadi *et al.,* 2014; Torrecillas *et al.,* 2014) in fish (Buentello *et al.,* 2010; Torrecillas *et al.,* 2011; Yar Ahmadi *et al.,* 2014) such as *Lyz* and *TNFα*. However, in some other studies these factors remained unaffected (Peterson *et al.,* 2010; Welker *et al.,* 2011). Of these, two selected innate immune factors, lysozyme plays a key role in lysis of both Gram positive and negative bacteria and *TNFα* is necessary for proinflammatory activity (Yarahmadi *et al.,* 2014). Similar to the results of the present study, the dietary administration of probiotic elevated cytokine gene expression level such as tnfα (Kim and Austin, 2006), and using dietary prebiotics such as Immunogen®, which is similar to Agrimos®, mainly includes *β*-glucan and MOS (Yar Ahmadi *et al.,* 2014), and galacto oligosaccharide (Yousefi *et al.,* 2018), significantly raised the expression of these two genes. To answer the questions about vertical transmission of immune response in gene expression level and since the major component of maternal antibacterial immunity, lysozyme has been detected in oocytes, fertilized eggs, and larval stages of several fish species including coho salmon, sea bass and tilapia (Wang and Zhang, 2010), adult zebrafish in this study with mature gonads, and their offspring compared. The results has shown a significant augmentation in Lyz expression at 90 days post treatment in comparison with the initiation day. Similarly in this study it was founded that maternal lysozyme was present in the offspring of D. rerio that has provided the evidence for a maternal transfer of lysozyme in zebrafish; as Wang and Zhang (2010) discussed the egg cytosol is able to lyse the cell wall of bacteria and can prevent the vertical transmission of some bacterial pathogens and the resulting diseases. Lysozyme is found in many areas, such as mucus, serum, intestines, and eggs of marine animals (Akhter *et al.,* 2015), so, the results of this study support the hypothesis that using dietary additives has positive effects on triggering innate immune response like lysozyme and TNFα. Therefore, using dietary additives is important in aquaculture for improvement of their immunity against pathogens. Although TNFα plays a pivotal role in coordinating host defense against infection and is a crucial contributor to the pathophysiology of severing systemic inflammation and septicemia (Gazzar *et al.,* 2007), the regulation of *TNFα* expression has been recognized as being complex (Akbari *et al*., 2017). And as Sullivan *et al*. (2007) investigated, although epigenetic mechanisms such as histone methylation, acetylation, and DNA are not more correlates of transcriptional competence, but they are mechanistically important. Additional studies are needed to confirm the exact effects and reasons for these results in different species.

Despite the scarcity of CpG in both *TNFα* and *Lyz* genes and promoter sequence in zebrafish, in this study, it was tried to assess the influence of dietary prebiotic, especially at C nucleotides and CG dinucleotides methylation in zebrafish. The results driven from this study strongly support the idea that epigenetic affects gene expression and highlights the importance of nutrition and how it is linked to epigenetic alteration.

[DOR: 20.1001.1.16807073.2020.22.6.12.8]

Previous studies indicated that the DNA methylation causes induction of immune gene transcription (Chernyavskaya *et al.*, 2017) and is influenced by genetic and epigenetic changes that are often stably inherited (Zhang *et al.*, 2016). A decrease in C methylation and increase in gene expression were observed in 0.4% and 0.8% Agrimos® treated fish because the DNA methylation on cytosine in vertebrates, such as zebrafish, through the recruitment of repressive chromatin machinery, serves to silence gene expression by interfering with the binding of certain transcription factors (Wu *et al.*, 2011). As reported previously, the C methylation is chemically stable and heritable through the germline from one generation to the next (Wu *et al.*, 2011; Triantaphyllopoulos *et al.*, 2016). The result of this study verified the presence of the same methylation pattern that was inherited to their offspring after using Agrimos® as a food supplement.

Vertebrate shows development proceeds from a cascade of gene activation and repression events in response to extracellular signals and local determinants (Lindeman *et al.*, 2010). The DNA methylation can start histone modification and vice versa (Saleem *et al.*, 2015). Changes in chromatin nucleosome structure due to histone modifications regulate transcription of many genes like inflammatory genes such as *TNFα* (Gazzar *et al.*, 2007). In this study, the upregulated transcription activation of *TNFα* and *Lyz* expressions is found during 90 days after using Agrimos®. The DNA methylation data was associated with gene expression in 0.4 and 0.8% Agrimos® treatment as Anderson *et al.* (2012) and Saleem *et al.*, (2015) reached the same result. Active gene associates when CpG site is in unmethylated form. When methylation occurs at lysine 4 on histamine (H3K4), there is histone acetylation, and chromatin is openly configured (Saleem *et al.*, 2015). In this study, the result is somewhat surprising, as there were no significant differences recorded in all three HMTs activities.

In this study, *TNFα* and *Lyz* gene expressions showed a significant increase in their expression, and there was a clear correlation between the immune system candidate gene expression and the epigenetic selected factors. The importance of the contribution of the zebrafish is highlighted in this study as it can be used in the field of nutritional genomics and the nutritional immunity. In accordance with these findings, Huidobro *et al.* (2013) investigated zebrafish as a model that has demonstrated which diet regulates gene expression through changes in the DNA methylation.

In conclusion, the results of this study revealed that inclusion of dietary prebiotic Agrimos® in the diet of zebrafish could be considered as a beneficial dietary supplement for the upregulation of immune related genes and the growth. The diets containing 0.4% and 0.8% of Agrimos® were found to be more optimal for zebrafish. Additional experiments are needed on the effects of dietary Agrimos® and its beneficial dose of action on the methylation and gene expression in immune related genes. Considering that the zebrafish has been clearly established as an important vertebrate model for biomedical research, these results support the potential of Agrimos® as a prebiotic used in aquaculture practice to improve innate immune system and growth. Especially, the findings of this study suggest the successful transmission of innate immune response to offspring of the treated adults with the same characteristics in genomic levels. Further research that can pay more attention to the addition of other prebiotics in the feed to increase the growth and health factors are also necessary and recommended in specific species and rearing conditions to obtain the expected responses in fish.

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REFERENCES

1. Akbari, M., Taghizadeh, V., Heidarieh, M. and Hajimoradloo, A. 2017. The Key Role of Tumor Necrosis Factor Alpha (TNF-α) in Vaccinated Rainbow Trout via Irradiated Ichthyophthirius multifiliis Trophont. *Vet. Arh.*, **87(2):** 229-237.
2. Akhter, N., Wu, B., Memon, A. M. and Mohsin, M. 2015. Probiotics and Prebiotics Associated with Aquaculture: A Review. *Fish Shellfish Immun.*, **45**: 733-741.
3. Anderson, I., Reiner, A. H., Aanes, H., Alestrom, P. and Collas, P. 2012. Developmental Features of DNA Methylation during Activation of the Embryonic Zebrafish Genome. *Genome Biol.*, **13:** 65-78.
4. Beikzadeh, M., Peighambardoust, S.H., Beikzadeh, S. and Homayoun-Rad, A. 2017. Effects of Inulin, Oligofroctose and Oligofroctose-Enriched Inulin on Physiochemical, Staling and Sensory Properties of Prebiotic Cake. *J. Agr. Sci. Tech.*, **19:** 1241-1252.
5. Buentello, J. A., Neill, W. H. and Gatlin, D. M. 2010. Effects of Dietary Prebiotics on the Growth, Feed Efficiency and Non-Specific Immunity of Juvenile Red Drum *Sciaenops ocellatus* Fed Soybean-Based Diets. *Aquac. Res.*, **41:** 411-418.
6. Cerezuela, R., Meseguer, J. and Esteban, A. 2011. Current Knowledge in Synbiotic Use for Fish Aquaculture: A Review. *J. Aquac. Res. Dev.*, https://doi.org/10.4172/2155-9546, S1-008
7. Chernyavskaya, Y., Mudbhary, R., Zhang, C., Tokarz, D., Jacob, V., Gopinath, S., Sun, X. and Wang, S. 2017. Loss of DNA Methylation in Zebrafish Embryos Activates Retrotransposons to Trigger Antiviral Signaling. *Development*, **144:** 2925-2939.
8. Davidovic, R. S., Bozovic, A. M., Mandusic, V. L. and Krajnovic, M. M. 2014. Methylation-Specific PCR: Four Steps in Primer Design. *Cent. Eur. J. Biol.*, **9:** 1127-1139.
9. EU. 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. *O. J.*, **276:** 33-79.
10. Forsatkar, M. N., Nematollahi, M. A., Rafiee, G., Farahmand, H. and Lawrence, C. 2018. Effects of the Prebiotic Mannan-Oligosaccharide on Feed Deprived Zebrafish: Growth and Reproduction. *Aquac. Res.*, **49(8):** 2822-2832.
11. Ganguly, S., Paul, I. and Mukhopadhayay, S. K. 2010. Application and Effectiveness of Immunostimulants, Probiotics, and Prebiotics in Aquaculture: A Review. *Isr. J. Aquac.*, **62:** 130-138.
12. Gazzar, M., Yoza, B. K., Hu, J., Cousart, S. L. and McCall, C. E. 2007. Epigenetics Silencing of Tumor Necrosis Factor α during Endotoxin Tolerance. *J. Biol. Chem.*, **282:** 26857-26864.
13. Hoseinifar, S. H., Van Doan, H. and Ashouri, G. 2019. Galactooligosaccharide Effects as Prebiotic on Intestinal Microbiota of Different Fish Species. *Agro. J.*, **14(3):** 266-278.
14. Huidobro, C., Fernandez, A. F. and Fraga, M. F. 2013. Aging Epigenetics: Causes and Consequences. *Mol. Aspects Med.*, **34:** 765-781.
15. Kim, D. and Austin, B. 2006. Innate Immune Responses in Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) Induced by Probiotics. *Fish Shellfish Immun.*, **21:** 513-524.
16. Lindeman, L. C., Winata, C. L., Aanes, H., Mathavan, S., Alestrom, P. and Collas, P. 2010. Chromatin States of Developmentally-Regulated Genes Revealed by DNA and Histone Methylation Patterns in Zebrafish Embryos. *Int. J. Dev. Biol.*, **54:** 803-813.
17. Livak, K. J. and Schmittgen, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*, **25:** 402-408.
18. Massicotte, R., Whitelaw, E. and Angers, B. 2011. DNA Methylation: A Source of Random Variation in Natural Populations. *Epigenetics*, **6:** 421-427.
19. Mehrad, B., Jafaryan, H. and Taati, M. 2012. Assessment of the Effects of Dietary Vitamin E on Growth Performance and Reproduction of Zebrafish, *Danio rerio* (Pisces, Cyprinidae). *Oceanogr. Mar. Sci.*, **3(1):** 1-7.
20. Moghadam, H., Morkore, T. and Robinson, N. 2015. Epigenetics-Potential for Programming

[DOR: 20.1001.1.16807073.2020.22.6.12.8]

Fish for Aquaculture? *J. Mar. Sci. Eng.*, **3:** 175-192.
21. Mohamed, K., Megahed, M. E. and Ali, M. A. M. 2017. Effect of Dietary Supplementation of Agrimos® on Growth Performance, Feed Utilization and Immunological Parameters of *Macrobrachium rosenbergii* Juveniles. *Aquacult. Int.*, **25:** 1441-1452.
22. Opazo, R., Valladares, L. and Romero, J. 2017. Comparison of Gene Expression Patterns of Key Growth Genes between Different Rate Growths in Zebrafish (*Danio rerio*) Siblings. *Lat. Am. J. Aquat. Res.*, **45:** 766-775.
23. Paul, B., Barnes, S., Demark-Wahnefried, W., Morrow, C., Salvador, C., Skibola, C. H. and Tollefsbol, T. O. 2015. Influence of Diet and the Gut Microbiome on Epigenetic Modulation in Cancer and Other Diseases. *Clin. Epigenetics*, **7:** 112-123.
24. Peterson, B. C., Bramble, T. C. and Manning, B. B. 2010. Effects of Bio-Mos® on Growth and Survival of Channel Catfish Challenged with *Edwardsiella ictaluri*. *J. World Aquacult. Soc.*, **41**: 149-155.
25. Piccolo, G., Centodocati, G., Bovera, F., Marrone, R. and Nizza, A. 2013. Effects of Mannan Oligosaccharide and Inulin on Sharpsnout Seabream (*Diplodus puntazzo*) in the Context of Partial Fish Meal Substitution by Soybean Meal. *Ital. J. Anim. Sci.*, **12:** 133-138.
26. Ribas, L. and Piferrer, F. 2013. The Zebrafish (*Danio rerio*) as a Model Organism, with Emphasis on Applications for Finish Aquaculture Research. *Rev. Aquacult.*, **5:** 1-32.
27. Rolim, P. M. 2015. Development of Prebiotic Food Products and Health Benefits. *J. Food Sci. Technol.*, **35**: 3-10.
28. Saleem, M., Abbas, K., Manan, M., Ijaz, H., Ahmed, B., Ali, M., Hanif, M., Farooqi, A. A. and Qadir, M. I. 2015. Epigenetic Therapy for Cancer. *Pak. J. Pharm. Sci.*, **28:** 1023-1032.
29. Sullivan, K. E., Reddy, A. B. M., Dietzmann, K., Suriano, R., Kocieda, V. P., Stewart, M. and Bhatia, M. 2007. Epigenetics Regulation of Tumor Necrosis Factor Alpha. *Mol. Cell. Biol.*, **27:** 5147-5160.
30. Tarnecki, A. M., Burgos, F.A., Ray, C. L. and Arias, C. R. 2017. Fish Intestinal Microbiome: Diversity and Symbiosis Unraveled by Metagenomics. *J. Appl. Microbiol.*, **123**: 2-17.
31. Torrecillas, S., Makol, A., Caballero, M. J., Montero, D., Gines, R. and Sweetman, J. 2011. Improved Feed Utilization, Intestinal Mucus Production and Immune Parameters in Sea Bass (*Dicentrarchus labrax*) Fed Mannan Oligosaccharides (MOS). *Aquac. Nutr.*, **17:** 223-233.
32. Torrecillas, S., Makol, A., Betancor, M. B., Montero, D., Caballero, M. J. and Sweetman, J. 2013. Enhanced Intestinal Epithelial Barrier Health Status on European Sea Bass (*Dicentrarchus labrax*) Fed Mannan Oligosaccharides. *Fish Shellfish Immun.*, **34:** 1485-1495.
33. Torrecillas, S., Montero, D. and Izquierdo, M. 2014. Improved Health and Growth of Fish Fed Mannan Oligosaccharides: Potential Mode of Action. *Fish Shellfish Immun.*, **36:** 525-544.
34. Triantaphyllopoulos, K. A., Ikonomopoulos, I. and Bannister, A. 2016. Epigenetics and Inheritance of Phenotype Variation in Livestock. *Epigenetics Chromatin*, **9:** 31-49.
35. Ulloa, P. E., Medrano, J. F. and Feijoo, C. G. 2014. Zebrafish as Animal Model for Aquaculture Nutrition Research. *Front Genet.*, **5:** 313-318.
36. Vaz, C., Wee, C. W., Lee, G. P., Ingham, P. W. and Tanavde, V. 2015. Deep Sequencing of Small RNA Facilitates Tissue and Sex Associated MicroRNA Discovery in Zebrafish. *BMC Genom.*, **16:** 950-966.
37. Wang, Zh. and Zhang, Sh. 2010. The Role of Lysozyme and Complement in the Antibacterial Activity of Zebrafish (*Danio rerio*) Egg Cytosol. *Fish Shellfish Immun.*, **29:** 773-777.
38. Welker, T. L., Lim, C., Yildrim-Aksoy, M. and Klesius, P. H. 2011. Effect of Short-Term Feeding duration of Diets Containing Commercial Whole-Cell Yeast or Yeast Subcomponents on Immune Function and Disease Resistance in Channel Catfish, *Ictalurus punctatus*. *J. Anim. Physiol. Anim. Nutr.*, **96:** 159-171.
39. Wu, S. F., Zhang, H., Hammoud, S. S., Potok, M., Nix, D. A., Jones, D. A. and Cairns, B. R. 2011. DNA methylation profiling in Zebrafish. *Method Cell Biol.*, **104:** 327-339.
40. Yar Ahmadi, P., Farahmand, H., Kolangi Miandare, H., Mirvaghefi, A. and Hoseinifar, S. H. 2014. The Effects of Dietary Immunogen® on Innate Immune Response, Immune Related Genes Expression and Disease Resistance of Rainbow Trout (*Oncorhynchus mykiss*). *Fish Shellfish Immun.*, **37**: 209-214.
41. Yarahmadi, P., Kolangi Miandare, H., Farahmand, H., Mirvaghefi, A. and Hoseinifar,

[DOR: 20.1001.1.16807073.2020.22.6.12.8]

S. H. 2014. Dietary Fermentable Fiber Upregulated Immune Related Genes Expression, Increased Innate Immune Response and Resistance of Rainbow Trout (*Oncorhynchus mykiss*) against *Aeromonas hydrophila*. *Fish Shellfish Immun.*, **41:** 326-331.

42. Yousefi, S., Hoseinifar, S. H., Paknejad, H. and Hajimoradloo, A. 2018. The Effects of Dietary Supplement of Galactooligosaccharide on Innate Immunity, Immune Related Genes Expression and Growth Performance in Zebrafish (*Danio rerio*). *Fish Shellfish Immun.*, **73:** 192-196.
43. Yuji-Sado, R., Raulino-Domanski, F., Freitas, P. F. and Baioco-Sales, F. 2015. Growth, Immune Status and Intestinal Morphology of *Nile tilapia* Fed Dietary Prebiotics (mannan Oligosaccharides-MOS). *Lat. Am. J. Aquacult. Res.*, **43:** 944-952.
44. Zhang, N., Luo, G., Tan, H., Liu, W. and Hou, Z. 2016. Growth, Digestive Enzyme Activity and Welfare of Tilapia Reared in a Biofloc-Based System with Poly-β-Hydroxybutyric as a Carbon Source. *Aquaculture*, **464:** 710-717.

مقایسه پروفیل اپی‌ژنتیکی و الگوهای بیان ژن ماهی گورخری (*Danio rerio*) تغذیه شده با مکمل غذایی اگریموس

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چکیده

یکی از سوالات مهم در تکثیر و پرورش آبزیان، بررسی جنبه های مختلف استفاده از مکمل‌های غذایی مانند پربیوتیک‌ها و اثرات آن بر پاسخ‌های ایمنی اولیه و نیز امکان انتقال عمودی آن می‌باشد. مطالعه حاضر برای پاسخ به این سوال در زمینه انتقال عمودی فاکتورهای سیستم ایمنی اولیه طراحی شد تا اثر سطوح مختلف مکمل غذایی اگریموس را بر بیان ژن‌های انتخابی مرتبط با سیستم ایمنی (لیزوزیم (*Lyz*) و تومورنکروزیز فاکتور آلفا (*tnfα*))، متیلاسیون DNA، فعالیت ۳ آنزیم هیستون متیل ترانسفراز (H3K4, H3K9, H3K27) و نیز عملکرد رشد ماهی گورخری (*Danio rerio*) را بررسی نماید. به این منظور ۳۶۰ قطعه ماهی گورخری ۲۴ روزه به طور تصادفی در ۱۲ آکواریوم و چهار تیمار تقسیم شدند. ماهیان گورخری با غذای کنترل و یا غذای دارای مکمل (با سطوح ۰.۲٪، ۰.۴٪ و ۰.۸٪) برای ۹۰ روز تغذیه شدند. نتاج هر تیمار برای بررسی پتانسیل انتقال عمودی فاکتورهای ایمنی از طریق تغذیه والدین با مکمل غذایی اگریموس، مورد بررسی قرار گرفت. در انتهای آزمایش، افزایش معنی‌دار ($P<0.05$) بیان ژن‌های *Lyz* و *tnfα* در ماهیان تغذیه شده با تیمار ۰.۲٪ و ۰.۴٪ اگریموس در مقایسه با ماهیان تیمار کنترل نشان داده‌شد. اگرچه نتایج این مطالعه نشان دهنده کاهش نرخ متیلاسیون به وسیله استفاده از مکمل اگریموس ($P<0.05$) در مقایسه با تیمارکنترل بود، تفاوت معنی‌داری در فعالیت آنزیم‌های HMT گروه‌های مختلف مشاهده نشد. این مطالعه به طور کل نشان‌دهنده انتقال عمودی موفق بیان ژن *Lyz* به عنوان پاسخ سیستم ایمنی اولیه به نتاج بوده و مؤید نقش مستقیم دیمتیلاسیون DNA در تنظیم بیان ژن‌های انتخابی بود، همچنین این نتایج نشانگر نقش احتمالی تغذیه در تنظیم فرایندهای اپی‌ژنتیک می باشد.

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