Original Article

Methylation levels of lysozyme gene in rainbow trout (*Oncorhynchus mykiss*) fed by commercial immunogen probiotic

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Abstract: In the present study, we evaluated the effect of commercial immunogenic prebiotic on the rate of methylation of type-C gene. A total of 120 rainbow trout were divided into two treatments, including a control group and another one with 2.0% commercial prebiotic immunogen each in three replicates. On the first, 15 and 45 days, DNA of adrenal tissue was extracted and treated with bisulfite. Samples were amplified by polymerase chain reaction (PCR) and sequenced. Based on the results, there was no significant difference ($P<0.05$) between the 1st day (R), 15th day of control (C), 15th day of immunogen (I) and 40th day of control (CS). However, there was a significant difference ($P<0.05$) between the 45th day immunogenicity (IS) and other samples, i.e. the CpG islet methylation rate in the IS samples was lower leading to increase in the expression of the lysozyme gene.

Introduction

Development of intensive aquaculture systems have been caused problems such as disease and physiological disturbances following the increase of stress. The most common way to treat fish diseases is application of chemicals and antibiotics, which are led many health and social problems, e.g. the antibiotic bioaccumulation and producing antibiotic-resistant strains. Therefore, application of these substances for treatment should be seriously monitored (Austin and Austin, 2016). In this regard, many researches have focused to maximize aquaculture productivity in the short time, with minimal cost and side effects using additives with no health and environmental consequences (Khurana, 2005). Hence, the use of various dietary supplements, including probiotics, prebiotics and their combinations (synbiotics) has received much attention and interest to elicit the immune response and enhance immunity of aquatic species (Huynh et al., 2017).

Prebiotics are indigestible nutrients (carbohydrates) that have beneficial effects on the host and improve their health by growing or activating one or a limited number of bacterial species in the gut. Therefore, they improve and balance the intestinal microflora enhancing the host defense mechanism (Fooks and Gibson, 2002). In recent years, nutrition has been shown to exert drastic changes in gene expression by affecting transcription mechanisms and subsequent steps (Waterland el al., 2004). Nutrition also has a permanent effect on the gene expression pathway by epigenetic mechanisms, which can be inherited (Waterland and Michels, 2007).

Immunogen is a commercial prebiotic consisting several stimulants, such as beta-glucan and mannaligosaccharide (Salze et al., 2008). It can prevent diarrhea, increase immunogenicity, stimulate growth and absorb mycotoxin. This dietary compound also prevents the accumulation of pathogenic bacteria such as *Salmonella*, *Clostridium*, and *Escherichia coli* in the intestine and increases the secretion of IgA, IgG, and IgM immunoglobulins, which impedes the attachment of bacteria or toxins to the intestinal epithelium (Sado et al., 2008). Supplementation of immunogen as a prebiotic to rainbow trout increases the expression of lysozyme type-C gene resulting in

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Table 1. Forward and reverse primers designed by express primer methyl software for lysozyme type-C gene.

Forward primer	5' GTATGTTTTTGAGTTTTAAAAATATTTT 3'
Reverse Primer	5' CACATTTCTTATCAAAAATAATACTCTA 3'

increased resistance to environmental stress and disease (Hashim, 2016).

Epigenetic changes are reversible in gene expression, which can be inherited through mitosis, meiosis, or both, which does not include changes in DNA sequences (Novik et al., 2002). Epigenetic changes can be classified into three major categories, including changes in covalent histones, non-coding RNAs and DNA methylation (Zargar and Rabbani, 2000, 2002; Herceg, 2007). In DNA methylation process, a methyl group, in a covalent form, binds to a cytosine base (at carbon number 5) by the action of DNA methyl transferase. DNA methylation usually occurs at or near the CpG islands located within or near the gene promoter (Lętowska-Andrzejewicz et al., 2011) which highly effects the gene expression level.

Bisulfite sequencing is a method of DNA methylation analysis in which bisulfite treatment of DNA is used before routine sequencing to determine the pattern of methylation (Li and Tollefsbol, 2011). Changes in global methylation rate in different nutritional and environmental circumstances have been investigated in some aquatic species (Blouin et al., 2010; Morán and Pérez-Figueroa, 2011; Xiao et al., 2013; Anastasiadi et al., 2017; Fan et al., 2019). The present study aimed to investigate the relationship between increased specific gene expression and methylation rate of the lysozyme gene in *Oncorhynchus mykiss* treated with commercial immunogenic prebiotic is investigated.

Materials and Methods

A total of 120 rainbow trout (with mean weight of 81±0.05 g) were obtained from Karaj fish farm and transported to the Aquaculture lab of Fisheries Department, University of Tehran. For adaptation, fish were divided into two 1000-liter tanks equipped with a central aeration system and kept for 48 hours without feeding. The water replacement was 50%

daily. They were adapted to lab conditions for 10 days prior to the experiment.

The experiment was conducted as a completely randomized design in two treatments and each with three replications. Treatments were a group fed with the commercial immunogenic prebiotic supplement (2 g/kg) and control group with no dietary supplement. The group were randomly assigned to six tanks of 1000 fiberglass tanks (20 fish per tank).

Food preparation and feeding: The commercial immunogenic prebiotic product was obtained from ICC Company, USA, and added with a ratio of 0.2% to Rainbow Trout commercial diet from Behparvar Company at mixing step before pelleting. During the experiment period, feeding was performed manually based on 2% of live biomass weights in the tank twice a day. Biomass was measured at 14 days intervals.

Tissue sampling: The methylation rate of the lysozyme gene was measured from the anterior part of the kidney obtained from three fish from each treatment at the first, 15th and 45th days of the experiment. The sampling was performed in a sterile condition and the fish were anesthetized with clove powder prior to the sampling. The samples were kept in -80°C before DNA extraction.

DNA extraction, bisulfite treatment and amplification: Express Primer Methyl software (V 1.0) was used for primer design, and the primers were manufactured and shipped by Macrogen Company, Korea (Table 1). DNA extraction was performed by modifying the protocol of Tierling et al. (2007). Nanodrop Model 7.V3.1000 was used to evaluate the quantity and quality of the extracted DNA. Bisulfite treatment was performed by modifying the protocol of Fernandez et al. (2007). For PCR amplification, TOPAZ PCR Mix Master with 1x final concentration, primers with 0.4µM and DNA template with 50-150 ng concentration were used. The PCR amplification was performed with 33 cycles with 94°C for 3 min for initialization step, 95°c for 45s for denaturation step,
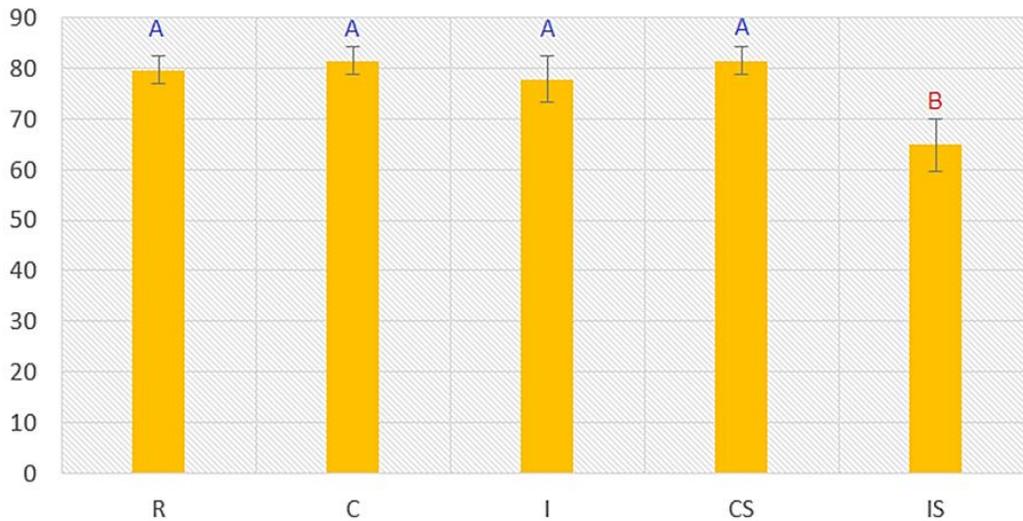
Figure 1. Methylation percentage in each group in the experiment.

55°C for 40s for annealing step, 72°C for 25s for extension/elongation step and 72°C for 10 min final elongation step (Table 1).

After amplification of DNA fragments, Nanodrap Model 7.V3.1000 was used to evaluate the quantity and quality of the fragments and bands obtained from 1.5% agar electrophoresis were prepared according to the protocol from Macrogen Company, Korea and sent for sequencing. Flanking bases at the two ends of each sequence with low quality were removed by UGENE Unipro software and sequences were aligned.

Statistical analysis: Changes in methylation levels of CpG islets in each treatment were measured by BiQ Analyzer software. Data were analyzed using lollipop diagrams and bars. In order to compare the mean methylation rate in different groups, SPSS software (V. 17) was used at a significant level of 5%.

Results

Methylation levels in day 1 (R), control treatment on day 15 (C), immunogen treatment on day 15 (I), control treatment on day 45 (CS) and immunogen treatment on day 45 (IS) were compared with the original gene version. There was no significant difference ($P<0.05$) between control on day 15 and 45 and immunogen treatments on day 15 and first-day samples, while there was a significant difference ($P<0.05$) between the methylation percentage of the immunogen treated samples on day 45 to all other

samples (Fig. 1).

Based on the results, no significant relationship was observed between methylated and unmethylated CpG dinucleotides, and each sample exhibited a different response in different locations of the CpG dinucleotides (Fig. 2), but the regions with two or three CpGs were more susceptible to methylation.

Discussions

In bisulfite sequencing technique, the number of treated bases should be in the range of 300 to 400 bp so that all the bases can be exposed to bisulfite during treatment, which in this way the rate of cleavage and breakage of DNA would be minimal (Tierling et al., 2007). In the present study, the primers were designed to amplify 309 bp of the lysozyme gene close to the promoter and 18 CG sites were measured. More investigations on other regions of the lysozyme gene can highly elucidate the relation between methylation and gene expression. Our findings showed that methylation in CGs increases in regions closer to the promotor and incidentally the CGs near the end of the 5' gene play an important role in the expression of the gene. In the IS group, CG dinucleotides that were closer to the 5' end of the gene were more demethylated. 45 days of commercial immunogenic prebiotic use was caused partial dimethylation of the type-C lysozyme gene, which increased the expression of this gene. Epigenetic traits, e.g. genetic traits, are transmitted to the next generation known as

a)

Methylation

| | 33 bp | | 0 bp | | 43 bp | | 24 bp | | 6 bp | | 1 bp | | 7 bp | | 26 bp | | 12 bp | | 1 bp | | 2 bp | |

CpG Position	1	2	3	4	5	6	7	8	9	10	11
Unmethylated	3.0	3.0	2.0	3.0	2.0	2.0	3.0	1.0	0.0	0.0	0.0
Methylated	0.0	0.0	1.0	0.0	1.0	1.0	0.0	2.0	3.0	3.0	3.0
Not present	0	0	0	0	0	0	0	0	0	0	0

Methylation

| | 5 bp | | 11 bp | | 11 bp | | 7 bp | | 16 bp | | 8 bp | | 0 bp | | 4 bp | |

CpG Position	12	13	14	15	16	17	18
Unmethylated	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Methylated	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Not present	0	0	0	0	0	0	0

Legend: ■ unmethylated □ methylated ▣ not present

b)

Methylation

| | 33 bp | | 0 bp | | 43 bp | | 24 bp | | 6 bp | | 1 bp | | 7 bp | | 26 bp | | 12 bp | | 1 bp | | 2 bp | |

CpG Position	1	2	3	4	5	6	7	8	9	10	11
Unmethylated	2.0	1.0	2.0	1.0	1.0	1.0	1.0	0.0	1.0	0.0	0.0
Methylated	1.0	2.0	1.0	2.0	2.0	2.0	2.0	3.0	2.0	3.0	3.0
Not present	0	0	0	0	0	0	0	0	0	0	0

Methylation

| | 5 bp | | 11 bp | | 11 bp | | 7 bp | | 16 bp | | 8 bp | | 0 bp | | 4 bp | |

CpG Position	12	13	14	15	16	17	18
Unmethylated	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Methylated	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Not present	0	0	0	0	0	0	0

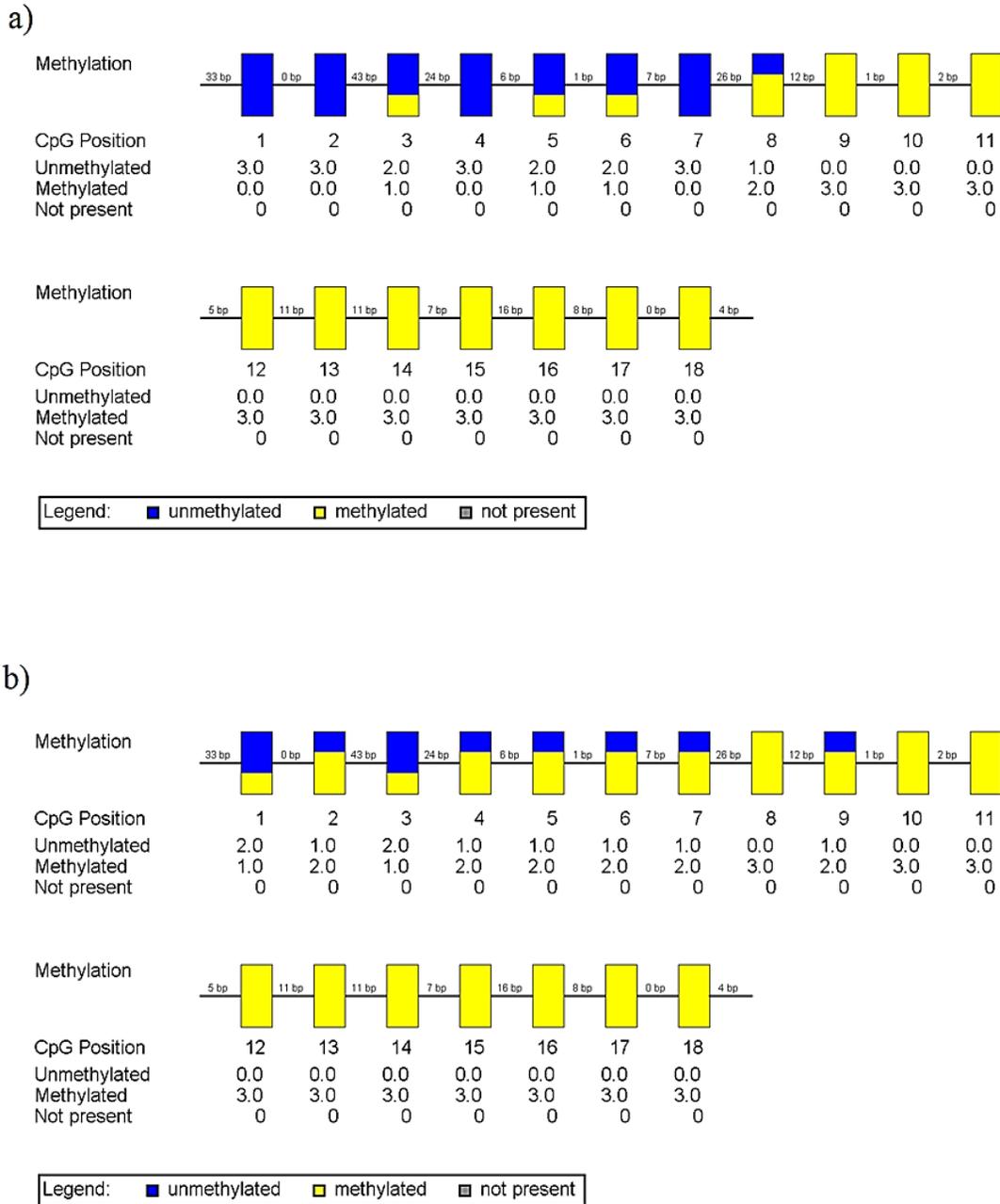Legend: ■ unmethylated □ methylated ▣ not present

Figure 2. (a) Bar graphs immunogen treated samples and (b) control group on day 45th.

vertical transmission. Therefore, the positive changes in the epigenome will be permanent and transferable organisms (Portela and Esteller, 2010). Reinforcing the innate immune system is critical to improving disease resistance in aquatic species (Liu et al., 2012).

Most epigenetic studies are based on global methylation in a single generation and investigated the percentage of methylated CpG in the whole genome of an organism. There are also few works on global CpG methylation after consuming a dietary treatment and its comparison with a control treatment without aiming at a specific gene or trait. Although there have been many studies of DNA methylation in mammals in recent years, there are very few studies on aquatic species to compare with our findings.

Global methylation levels in mature rats, which were fed by prebiotics, were significantly decreased (Zheng et al., 2014), which is consistent with our results. In another work, feeding mice with vitamin B12 had significantly increased dimethylation rates in the GR1 and PPAR genes, which are genes related to resistant to stress (Lee, 2015). As immunogen

prebiotic is rich in vitamins and antioxidants that are not digestible, the fish takes advantage of these substances with the help of bacterial flora in their intestine. Furthermore, a study on Daphnia showed that exposing it to high levels of NaCl is led higher overall methylation rate, with the greater amount of methylation being returned to immunity and stress control and the overall methylation status. The downside is the function of the body's immune system, which was inconsistent with our results (Asselman et al., 2015). However, in this study, no dietary supplements were used.

Based on our results, it can be concluded that commercial immunogenic prebiotic can affect the methylation rate. The current work examined one gene that has been proven to increase expression by commercial immunogenic prebiotics.

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