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The effects of galactooligosaccharide on systemic and mucosal immune response, growth performance and appetite related gene transcript in goldfish (*Carassius auratus gibelio*)

Hamed Kolangi Miandare [a, *], Shoeib Farvardin [a], Ali Shabani [a], Seyed Hossein Hoseinifar [a], Seyyede Sanaz Ramezanpour [b]

[a] Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[b] Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Iran

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ABSTRACT

The present study investigates the effects of supplementation of goldfish (*Carassius auratus gibelio*) diet with galactooligosaccharide (GOS) on serum immune response, mucosal immune parameters as well as appetite-related (Ghrelin) and immune-related (TNF-1α and TNF-2α) genes expression. One hundred and eighty fish with an average weight of 4.88 ± 0.28 g were stocked in twelve 500-L fiberglass tank assigned to four treatments repeated in triplicates. Fish were fed on experimental diets contain 0.5, 1 and 2% GOS for 6 weeks. Supplementation of diet with GOS had no remarkable effect on goldfish growth performance ($P > 0.05$). Evaluation of serum innate immune parameters revealed that supplementation of diet with GOS significantly elevated total protein, Albumin, Globulins, Lysozyme and Alkaline phosphatase activity as well as agglutination compared to control group in a dose dependent manner ($P < 0.0.5$). Also, Fish fed 2% GOS supplemented diet showed increased skin mucus immune response (total protein and lysozyme activity) compared other groups ($P < 0.0.5$); except in case of ALP activity. Molecular studies on appetite (ghrelin) and inflammatory cytokine (TNF-1α and TNF-2α) genes expression revealed remarkably decrease and increase, respectively in GOS fed fish ($P < 0.0.5$). These results showed immunomodulatory effects of dietary GOS on serum and skin mucus response as well as expression of inflammatory cytokines in goldfish, though this supplement decreased appetite gene expression and had no effect on growth performance.

1. Introduction

Ornamental fish are among economically important species, which received increasing attention as a result of local and global demands [1]. The latest statistics of the UN Food and Agriculture Organization (FAO) shows that the ornamental fish industry estimated to be worth around US$15 billion [2]. Goldfish, *Carassius auratus* is one of the most commercially important species gaining value in the export trade [3]. Nowadays, improving the health status, elevation of immune response and disease resistance in ornamental fish culture plan has been practiced using various feed additives include vitamins, minerals, and pro- and prebiotics and synbiotics [4]. Prebiotics are non-digestible dietary supplement which has beneficial effects on host through modulation of gut microbiota toward potentially beneficial populations (e.g. Lactic acid bacteria) [5]. Galactooligosaccharide (GOS) is one of the promising prebiotic which has improved growth performance, immune response, stress resistance and disease resistance in various fish species [6,7]. In spite of extensive researches on administration of GOS in edible cultured fish [8–16], to the best of our knowledge there was no published study the effects of GOS on performance and immune response of ornamental fish. In case of prebiotics, the results of previous studies revealed that several parameters include, prebiotics type, dosage, species etc. can affect the functionality of prebiotics. Furthermore, contradictory results have been reported regarding the effects of prebiotics on growth performance and immune response in the same condition. Therefore, several studies should be performed for determination of optimum prebiotic and dosage for improving growth performance. Therefore, the present study was performed to determine the

* Corresponding author.
E-mail address: hkolangi@gau.ac.ir (H.K. Miandare).

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effects of dietary administration of different levels of GOS on serum immune response, mucosal immune parameters as well as expression of appetite-related (Ghrelin) and immune-related (TNF-1α and TNF-2α) genes of goldfish.

2. Material and methods

2.1. Fish and experimental conditions

The present study was conducted at Aquaculture Lab of Department of Fisheries of Gorgan University of agricultural sciences and natural resources. Goldfish (*Carassius auratus gibelio*) (n = 180) were supplied from a private farm of ornamental fish (Guilan province, Iran) and transferred to Aquaculture Lab and acclimated for one week. Thereafter, goldfishes (4.88 ± 0.28) were randomly stocked into 12 fiberglass tanks (500 L) at a density of 15 fish per tank. Treatments were investigated under static aerated water conditions with a 70% water change every day. Fish were fed for 6 weeks with experimental diets at rate of 4% of BW twice a day (08:00 and 15:00). The feeding ration was corrected every 10-days following a 24-h starvation period and batch weighing.

2.2. Prebiotic

Vivinal-GOS® which was kindly supplied by Friesland Foods Domo Company (Zwolle, The Netherlands) used as the source of galactooligosaccharide (GOS). The product is rich in GOS that was obtained through the enzymatic conversion of lactose.

2.3. Preparation of diets

A commercial diet (Sanyu Fish Foods, Protein 32%, Fat 3%, Fibre 4%, Moisture 10%) which was routinely used for goldfish was supplemented by different levels of prebiotic GOS (0, control or non-supplemented diet, 0.5, 1 and 2%) according to the protocol described elsewhere [17]. The prepared experimental diets were stored in plastic bags at 4 °C until use.

2.4. Evaluation of skin mucus immune responses

Mucus samples were collected at the end of the feeding trial (6th week), based on the method suggested by Subramanian et al. [18] as described elsewhere [9]. The mucus samples were immediately transferred to 15 ml sterile centrifuge tubes, centrifuged (5810R Eppendorf, Engelsdorf, Germany) (1500 × g for 10 min at 4 °C) and the obtained supernatant were stored in 2 ml tubes at −80 °C for future analysis.

Total protein: The protocol suggested by Lowry et al. [19] was used for determination of the total protein concentration of skin mucus. Bovine serum albumin was used as standard and the absorbance was read using spectrophotometer (Biochrom, Libra S12) at 750 nm.

Lysozyme activity: Skin mucus Lysozyme activity was measured by the turbidimetric method as described by Esteban et al. [20]. The reduction in absorbance at 450 nm was measured for 15 min at 25 °C on a microplate reader (Benchmark, BioRad, USA).

Alkaline phosphatase activity (*ALP*): ALP activity of the mucus was measured using a commercial kit (Pars Azmoun Co., Iran) [21,22]. Samples were prepared according to the manufacturer protocol, and the absorbance was read at 405 nm.

2.5. Evaluation of humeral immune parameters

Blood sampling: Three goldfish per tanks were sampled at the end of the feeding trial, anesthetized using clove powder (250 mg L^{-1}) and blood samples were taken. Blood samples were collected into non-heparinized tubes and left to clot for 12 h (at 4 °C), prior to centrifugation (5000 g, 5 min) in a clinical centrifuge (International Model CL, International Equipment Co., Needham, MA, USA). Isolated serum samples were stored at −80 °C until further analysis.

Serum lysozyme activity: The serum lysozyme activity was determined according to the method described above in case of lysozyme activity of skin mucus.

Alternative complement activity (ACH50): The serum alternative complement activity (ACH50) was determined using sheep red blood cells (SRBC) as described by Ortuno et al. [23]. The volume of serum yielding 50% hemolysis was determined and used to calculate the complement activity of the sample.

Agglutination antibody titer: The agglutination antibody titer was conducted in round bottomed 'U' shaped micro titer plates according to the modified method of Swain et al. [31] as described by Yarahmadi et al. [24].

Total protein and Alkaline phosphatase activity (ALP): Serum total protein and ALP activity was determined according to the method described above in case of total protein and ALP activity of skin mucus.

2.6. RNA extraction and relative mRNA expression of appetite and immune-related genes

Three fish were sampled per each replicate, intestine and head kidney samples were taken and immediately immersed in liquid nitrogen and then transferred to −80 °C until analysis. Total RNA was extracted from 100 mg of all homogenized body tissue by using 1 ml Biozol Rigent (Bio flux; China) and prepared as previously described [25]. The quantity and quality of RNA were measured by nanodrop spectrophotometer at 260/280 nm and 1% agarose gel and staining with ethidium bromide. RNAs were stored at −80 °C until used. The first strand cDNA was synthesized by SuPrime Script RT Premix (2X) cDNA Synthesis Kit (GeNet BIO Inc; Daejeon, South Korea) for RT-PCR, following the protocol suggested by company. For real-time-PCR analysis, an iQ5 system (Bio Rad, USA) with 1× SYBR Green PCR Master Mix (SYBR biopars, GUASNR, Iran) 100 nM

Table 1
Primers sequences for the study of selected immune related genes expression in goldfish.

Gene	Accession Number	qPCR primers, forward/reverse	Amplicon	Efficiency (%)
Ghrelin	AF454390	TTCATGATGAGTGCTCCGTTC GTCAGAATTCAAGTGGCGAATC	124	98
TNF1-α	EU069817.1	CATTCCTACGGATGGCATTTACTT CCTCAGGAATGTCAGTCTTGCAT	88	95
TNF2-α	EU069818.1	TCATTCCTTACGACGGCATTT CAGTCACGTCAGCCTTGCAG	85	97
β- actin	AB039726	ACTGCACAGCCAAGAGAGTTCA GTTATTAAAGCGGCCGATATGC	188	96