Effects of the prebiotic mannan-oligosaccharide on the stress response of feed deprived zebrafish (Danio rerio)

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A R T I C L E   I N F O

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A B S T R A C T

Feed deprivation has deleterious effects on fish behavior and stress physiology which may susceptible them to disease outbreak. Functional ingredients in diets may substantially impact the physiology and stress responses of host organisms. Here, we hypothesized that the administration of a dietary prebiotic might attenuate the negative influences of feed deprivation on the behavioral profile of anxiety and physiological responses to stress in zebrafish (Danio rerio). Fish were fed with either basal or mannan-oligosaccharide supplemented (0.4% MOS/kg diet) diets, once per day (normal-control: CN, and normal-prebiotic: PN) or once every other day (starved-control: CS, and starved-prebiotic: PS) for 8 weeks. Afterwards, fish were subjected to a novel tank test to measure anxiety. Fish from the CS treatment exhibited more pronounced bottom-dwelling behavior than the other treatments. The number of transitions from the bottom to the top third of the novel tank was significantly higher in PN fish than the CS specimens. No significant differences were found between the CN and PS treatments in all of the anxiety behaviors. CS fish showed higher baseline cortisol levels than the other treatments, which was in line with higher expression of CRH gene in fish subjected to this treatment. Cortisol levels and CRH gene expression of the subjects were also measured after induction of two routine aquaculture stressors. CN and PS fish exhibited similar patterns of cortisol responses at most of the sampling times after stress, and PN specimens showed a significantly lower concentration of cortisol than the other treatments in most cases. Expression of the CRH gene was higher in feed deprived fish immediately after stress induction. Overall, the results show that feed deprivation in some cases influenced anxiety-like behaviors and elevated stress response in zebrafish juveniles; however, the addition of MOS to the diet helped deprived fish exhibit behaviors more typical of normally fed animals.

1. Introduction

Various stressors are inevitable in commercial fish farming, and may influence the physiology, behavior, and welfare of fish [1]. Routine manipulations of fish in a farm, such as handling, tank/container cleaning and live transport have all been shown to promote stress in fish [2]. Fish respond to external stimuli via modulation of the endocrine system and altering their behaviors [3]. In fish, the perception of a stressful signal activates a neuroendocrine cascade response that culminates in secretion and release of corticosteroids (cortisol in teleosts) that ultimately function to mobilize energy resources necessary for flight, fight, or coping [4]. Cortisol, as well as upstream factors in the pathway, hypothalamic corticotropin-releasing hormone (CRH), and the adrenocorticotropic hormone (ACTH), are all suitable indicators of the endocrine response to acute stress [5].

Despite a large number of studies on the physiological and behavioral effects of myriad stressors on fish species, relatively few of them have addressed the impact of starvation on the stress response. Feed deprivation is a phenomenon that occurs both in the wild and in aquaculture settings [6]. It may be a chronic condition, as individuals are not able to meet their nutritional requirements for extended periods of time. It is worth noting that nutritional status may have far-reaching effects on metabolic, hormonal and behavioral pathways in fish [7–9]. Interestingly, it has been reported that feed deprivation elevated the cortisol response in jundiá (Rhamdia quelen; [10]), Senegalese sole (Solea senegalensis; [11]), and red porgy (Psetta pagrus; [12]), but had no effects on cortisol production in sunshine bass (Morone chrysoptos × Morone saxatilis; [13]). However, because a limited form of feed deprivation could be employed to help reduce operational costs, there is a great deal of interest in exploring whether this practice could be
a few chronic stress and mood disorders such as anxiety is well established in a few fish species [3,15]. In this regard Chakravarty et al. [16] found that a variety of chronic stressors induced anxiety in zebrafish. Therefore, it is expected that disturbances in homeostatic equilibrium, i.e. a chronically stressful condition, might influence the anxiety responses of individuals. In zebrafish, for instance, anxiety can be simply induced by exposing animals to a novel environment [3,17]. The hallmark anxiety behaviors, such as bottom-swimming, a longer latency to enter the upper half of the water column, and freezing/immobility are useful indicators of anxiety in zebrafish (reviewed in [18]). However, we are not aware if stress arising from the negative nutritional status (i.e. starvation) can induce anxiety-like behaviors in fish. Some evidence shows that there is an increase in physical activity levels after a short term caloric restriction [9], but behaviors associated with anxiety are beyond a simple change in the levels of activity.

The sometimes stressful conditions experienced by fish in aquaculture may have profound effects on their physiology and behavior, making them more susceptible to disease outbreaks (e.g. [19]). Stress induced by feed deprivation may reduce innate resistance to pathogens as reported in previous studies [20,21]. The search for nutraceutical products that can be used as alternatives to antibiotics in aquaculture has demonstrated the promise of prebiotics to improve fish health and resistance to disease (e.g. [22]). Prebiotics, which are non-digestible food ingredients that selectively stimulate the growth of health-promoting bacteria within the intestine [23,24], have the potential to improve the well-being of the host and counteract the negative impacts of stressors experienced in culture.

Mannan-oligosaccharides (MOS) are one of the well-studied prebiotics in several cultured fish species. Improved growth, feed conversion, stress resistance, and immune function are the main effects of MOS in fish [24–26]. In addition, gut development, stimulation of intestinal microbiota, and increased stamina and survival during stress are all the effects of MOS in fish species (reviewed in [24]). It is therefore not unreasonable to theorize that the inclusion of MOS in the diet may make fish more resistant to starvation stress.

The aim of the present study was to examine i) how feed deprivation impacts the anxiety and stress response of zebrafish (Danio rerio), and ii) whether or not the inclusion of MOS in the diet would alter those responses. Zebrafish is a good model to study behavior and stress, as the stress response and anxiety like behaviors are extremely well defined in this species [18]. Furthermore, the zebrafish has excellent utility as a model for finfish aquaculture, as it shares many of the same characteristics with numerous fish species of economic interest [27]. Therefore, the results of the present study are expected to have relevance to other fish species.

2. Materials and methods

2.1. Animals and housing

In a local zebrafish hatchery (Karaj, Iran; where zebrafish are produced for ornamental fish industry), 300 twenty day old long-fin albino zebrafish were sorted and subsequently transported to laboratory. Fish were kept in two 100 l glass rectangular tanks for 10 days to allow them to acclimate. Water temperature was 27 ± 1 °C and the photoperiod was regulated to provide a 14 h light: 10 h dark cycle, with lights on at 0800H. The tanks were continuously aerated by a central pump equipped with a sponge filter to maintain the water parameters within the following range: dissolved O2: 6.9–8.2 mg/L; pH: 7.3–7.7; hardness: 150–180 mg/L as CaCO3. A commercially available extruded granular pellet (BioMar group: proximate composition of 35% crude protein, 12% total lipid, 10% ash, 4% moisture; 20.5 kJ/g energy) was used to feed fish twice daily to apparent satiation. This feed was ground down to a particle size such that it would suitable to feed juvenile stage zebrafish (600–800 μm). This feed, prepared in the same way, was used in the following main experiments.

2.2. Prebiotic and diet preparation

The prebiotic Agrimos® mannan-oligosaccharides (Agrimos® MOS) was used in the present experiments. Agrimos® MOS is a specific combination of mannan-oligosaccharides and glucose (B-glucans) extracted from the yeast cell walls of Saccharomyces cerevisiae (Lallemand Animal Nutrition, France). According to the manufacturer, Agrimos® MOS is obtained by the autolysis of yeast cells at high temperature and a controlled pH. After yeast autolysis is completed, cell wall and yeast extracts are separated by centrifugation, and the cell wall is spray dried.

Diet preparation in this study was after Akrami et al. [28]. Diets were prepared weekly, by diluting the appropriate amount of Agrimos® MOS in distilled water and gently mixing it with the crushed BioMar food to make a paste that was then spread on a plastic sheet, air dried, slightly ground and sieved to produce a suitable crumble size of 800 μm. The same procedure was used to prepare the control diet, except that no prebiotic was added.

2.3. Experimental design

In a pre-test experiment, among the concentrations of 0, 0.2, 0.4, 0.6 and 0.8% MOS/kg diet for 6 weeks, we found that inclusion of 0.4% Agrimos® MOS results in higher final body weight, food intake, and specific growth rate of juvenile zebrafish [29]. Accordingly, we developed four treatments to assess the stress response of the subjects under starvation conditions:

i) normal control (CN): fed daily with the control diet,
ii) starved control (CS): fed every other day with the control diet,
iii) normal prebiotic (PN): fed daily with prebiotic supplemented diet (0.4%),
iv) starved prebiotic (PS): fed every other day with the prebiotic supplemented diet (0.4%).

Fish with an initial body weight of 43.27–51.83 mg (One-way ANOVA: p > 0.05) were distributed into 18 L glass rectangular tanks (30 cm × 20 cm × 25 cm water height). Each treatment contained 25 juvenile fish in triplicate (75 fish per treatment). The duration of feeding period was 8 weeks, as proposed by Torrecillas et al. [24]. Subjects were fed twice daily (3% body weight) at 1000 h and 1600 h throughout the experiment, and the laboratory conditions were similar to what the fish experienced during the acclimation period (water temperature: 27 ± 1 °C, photoperiod: 14 h light: 10 h dark cycle, dissolved O2: 6.9–8.2 mg/L; pH: 7.3–7.7; hardness: 150–180 mg/L as CaCO3).

2.4. Intestinal microbiota analysis

Autochthonous lactic acid bacteria (LAB) levels were determined at the start and end of the trial of 9 fish per treatment (3 per replicate). Samples preparation was performed after Hoseinifar et al. [30] and the resulted homogenates were spread in triplicate onto deMan, Rogosa and Sharpe (MRS) agar media (Merck, Germany). For 5 days, plates were incubated at 25 °C, and colony forming units (CFU/g) were calculated from statistically viable plates (i.e. plates containing 30–300 colonies; [30]).

2.5. Behavioral assay

Anxiety-like behaviors of 8 fish in each treatment (2–3 per replicate) were assessed a day after the eight weeks of the feeding trials, i.e. on

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day 57 of the experiment. Fish were not fed on the day of the behavioral measurements. We used the novel tank test to assess the anxiety-like behaviors in zebrafish of different treatments [3]. The test tank was rectangular glass aquarium (22 cm length × 7 cm width × 20 cm height), filled with dechlorinated tap water to height of 15 cm (2.3 L) and divided to three equal horizontal zones by two lines marking the outside walls (Fig. 1). The tank was illuminated from above by a 40 W lamp, and the back and two sides were covered with white cardboards.

By using two similar test tanks we could test 2 fish simultaneously. Fish were netted and individually transferred to the test tank and their behaviors were recorded over a 6 min period by a camera located 60 cm in front of the tank. The videos were then converted to pictures (1 picture: 1 s), and the following behaviors were scored: time (s) spent in the bottom zone, latency to leave (s) the bottom zone, the number of transitions to bottom zone, time (s) spent in the middle zone, latency to inter (s) the upper zone, time (s) spent in the upper zone, the number of transitions to upper zone, number of freezing bouts, and freezing duration (s). Zebrafish tend to spend in more time the bottom, have a higher latency to leave the bottom and initiate top exploration, and display fewer transitions to the bottom and top and more freezing bouts when experiencing stressful conditions [3,18]. After each trial, the tank water was changed. All the behavioral recordings of 8 fish in each treatment were completely performed maximally within 30 min of each other.

2.6. Stress response

At the end of the feeding trial, two different methods were used to simulate stressful conditions for the subjects. The two stress methods were administered independent of each other. For each method, just before the induction of the stressor, six fish for each treatment (2 per replicate) were sampled to determine baseline cortisol levels.

2.6.1. Simulated transport

Simulated transport stress events were performed after Sang et al. [26]. From each dietary treatment, thirty one zebrafish (10–11 fish per replicate) were netted, packed in plastic bags (54 × 23.5 cm), and put in a polystyrene box. The box was then covered by a lid, sealed, and was left undisturbed in the laboratory. After 36 h in the box, fish were placed back into the holding tanks and their whole-body cortisol was sampled at 0, 30, 60 and 240 min after the stress. The point “0 min after the stress” was exactly the time when fish were returned to their home tanks.

2.6.2. Simulated tank cleaning

Simulated tank cleaning stress events were performed after Rubio et al. [31]. One day after separation of the fish for the transport stress, the simulated cleaning stress was performed on the remaining 10–11 fish in the tanks (31 per treatment). The treatment comprised of 1) removal of ~2/3 of total volume of water in the tank, 2) brush-scrubbing of tank walls, and 3) refilling of the tanks with fresh dechlorinated water. This was completed within a 15 min period. Fish were then sampled for whole-body cortisol at 0, 30, 60 and 240 min after tanks were refilled with fresh water.

2.7. Cortisol assay

Whole-body cortisol levels were assayed after Sink et al. [32]. Fish were euthanized with 500 mg/L of clove powder, decapitated, and frozen at −20 °C. Each fish was then weighed, sliced into small sections, and a pool of two fish were minced and placed into a 15 mL screw top test tube including 0.5 mL PBS (pH 7.4). They were entirely crushed and homogenized with a glass rod, and the rod was then washed with another 0.5 mL PBS in the tube. Then, 5 mL of laboratory grade diethyl ether was added to the tube. The tube was vortexed for 1 min followed by centrifuging at 7000 rpm for 15 min. The supernatant was then separated and centrifuged for another 15 min. The final supernatant was then immediately frozen at −20 °C for 2 h, and the unfrozen portion was decanted into a fresh 15 mL test tube. The ether was evaporated under a flow of air, and cortisol was reconstituted in 1 mL phosphate buffer. The extracts were stored at −80 °C until the ELISA was conducted using commercial cortisol kit (AccuBind™ microplate ELISA, Monobind Inc., USA). The inter- and intra-assay coefficient of variance was 6.7% and 4.5%, respectively which was determined by measuring 3 known concentrations with 5 replications. The amount of cortisol was measured in duplicate samples of tissue extract in a single plate.

2.8. CRH mRNA expression

For the gene expression experiments, head of the subjects used for the cortisol assay before (baseline) and after (0 and 4 h) each stressor were sampled to remove the whole brain. The pool of 3 brains (as one replicate) was immediately transferred into a 1.5 mL micro-tube containing 0.5 mL RNA-Later (Behnogen Co. Tehran, Iran), and kept at 4 °C until RNA extraction. RNA extraction was performed according to Ghisleni et al. [5]. In accordance with the manufacturer’s instructions, TRIzol reagent (Invitrogen, USA) was used for total RNA extraction. Spectrophotometric (NanoDrop™ 1000 Spectrophotometer, Thermo Scientific) and electrophoresis (2% agarose gel and staining with ethidium bromide) assays were used to investigate the quantity and quality of the extracted RNA, respectively. Total RNA was then treated with 1.5 μL DNase I (MBI Fermentas) at 37 °C for 30 min. A total amount of 2 μg of RNA was used for the first strand cDNA synthesis using Fermentas cDNA Synthesis Kit (Fermentas; USA), following the manufacturer protocol.

Quantitative real-time PCR (qPCR) analysis was carried out using an iCycler (BioRad) with Fermentas Maxima SYBR Green qPCR Master Mix (2 ×) kit (Fermentas), and all primers (100 nM) in a total of 25 μL per well, using standard protocol (initial 40 s denaturation step at 95 °C, 15 s at 95 °C, 30 s annealing step at 62 °C, 20 s extension step at 72 °C for 40 cycles and a final 10 min extension at 72 °C). All reactions were run in duplicate. Primers were generated using the Oligo7 software and Primer3 website. Primers used for CRH were CCGCGGTATAAGTTAGACGACATC (forward) and GGAGAGGATCT GGTTTTCTGGTG (reverse). For β-actin, the primers used were TGTCTCTGTTGCTTCTGTGT (forward) and AAGTTCAGACGG AGGATGG (reverse). The mRNA expression levels of genes were recorded as Ct values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. Baseline and threshold for Ct calculation were set manually. Standard
curves were constructed from dilution series of pooled cDNA (including 7 dilutions from 1:10 to 1:1000), and the mRNA levels of the gene was normalized based on the relative expression of β-actin.

2.9. Ethical note

Currently there are no laws regarding animal research in Iran; however protocols were approved by a scientific committee within the Department of Fisheries of the University of Tehran. Members of this group include a veterinarian, a statistician, and eight other academics. The proposal of the present work was reviewed and approved by this committee. Animal handling and testing techniques were designed using guidance from the Association for the Study of Animal Behavior and the Animal Behavior Society (ASAB/ABS 2012).

2.10. Statistics

We used two-way ANOVA analysis with the nutritional status and dietary inclusion of MOS as fixed factors and anxiety-like behaviors of the subjects as dependent variables followed by Duncan’s multiple range tests. The same analyses were performed to investigate if the fixed factors had effects on the LAB levels, cortisol responses and CRH mRNA expression of the subjects after the different stress induction methods. Statistical analyses were performed by SPSS (IBM Statistics, version 22.0), and significant difference was attributed to p values < 0.05, unless otherwise stated.

3. Results

3.1. Intestinal lactic acid bacteria

The intestinal microbiota analyses of zebrafish juvenile fed Agrimos® MOS showed changes over the course of the experiment (Fig. 2). At the beginning of the experiment, differences in LAB levels were insignificant between the treatments; however, feeding status (F1,32 = 10.95; p < 0.01), dietary inclusion of MOS (F1,32 = 119.60; p < 0.001), or their interaction (F1,32 = 5.32; p < 0.05) were significant on the levels of LAB. PN specimens showed significantly higher LAB levels than the other treatments at the end (F3,35 = 45.29, p < 0.001). The levels of LAB in PS fish were also significantly higher than that of CN and CS treatments (Fig. 2).

3.2. Anxiety-like behaviors

The multivariate analyses of behaviors showed that starvation status had significant effects on both the time spent in the bottom (F1,28 = 6.98, p < 0.05) and time spent in the upper zone (F1,28 = 4.20, p < 0.05) in subjects at the end of the feeding trials (Fig. 3). Dietary inclusion of MOS significantly decreased time spent in the bottom zone (F1,28 = 5.70, p < 0.05) and time spent in the upper zones (F1,28 = 4.66, p < 0.05). MOS inclusion also increased the number of transitions to the bottom or upper zones of the test chambers (F1,28 = 3.04, p < 0.05; F1,28 = 3.12, p < 0.05, respectively; Fig. 3). The interactions of starvation status and dietary MOS were insignificant for all of the anxiety behaviors.

3.3. Plasma cortisol concentrations

Both of the starvation status and dietary inclusion of MOS had significant effects on cortisol levels of fish exposed to simulated transport stress in all of the sampling times (Fig. 4A). Baseline whole-body cortisol levels of CS specimens (mean ± SE; 2.86 ± 0.16) were higher than that of the other treatments (CN: 2.11 ± 0.19; PS: 2.28 ± 0.21). Fish in the PN treatment showed a lower cortisol response at the 0 and 30 min after stress timepoints than the other treatments. Furthermore, CN and PS subjects had similar pattern of cortisol response before (baseline) and after stress induction (Fig. 4A). The interaction of the fixed factors was only significant on the fish cortisol concentration at 60 min after stress (F1,20 = 3.07; p < 0.05).

Baseline cortisol levels of fish were significantly different before the simulated tank cleaning stress (Fig. 4B). Starvation status (F1,20 = 12.29; p < 0.01) but not dietary MOS (F1,20 = 3.50; p = 0.076) caused CS fish to have higher baseline cortisol responses (mean ± SE; 2.75 ± 0.36) than the other treatments (CN: 1.64 ± 0.19; PN: 1.37 ± 0.16; PS: 2.06 ± 0.26). Despite the significant effects of starvation on cortisol levels in all sampling times, dietary MOS also did not influence cortisol immediately after exposure, i.e. 0 min (F1,20 = 1.56; p = 0.22; Fig. 4B). The interaction effect of starvation status and dietary inclusion of MOS was only significant on cortisol levels at 60 min after stress exposure (F1,20 = 5.88; p < 0.05).

3.4. Gene expression

The expression of CRH gene within the whole brain of subjects were determined at the end of feeding trials, as the baseline CRH expression, and at 0 and 240 min after stress induction procedures. Baseline levels showed a significant effects of starvation status (F1,8 = 10.30; p < 0.05) but not dietary MOS (F1,8 = 4.96; p = 0.05) on CRH expression, in which the CS subjects showed higher expression of CRH gene (Fig. 5). The interaction of the factors was insignificant (F1,8 = 1.19; p = 0.30).

Simulated live transport had significant influences on the expression of CRH gene. Both of the starvation status and dietary MOS, and also their interactions significantly changed CRH expression at 0 min and 240 min after stress in the subjects of different treatment (p < 0.001; Fig. 6A). Starvation status showed significant effects on CRH expression at both the sampling time after simulated tank cleaning stress (p < 0.001); however, and unlike to 240 min after the stressor (F1,8 = 52.02; p < 0.001), dietary MOS had not effect on CRH expression at 0 min (F1,8 = 0.23; p = 0.64; Fig. 6B). The interaction of starvation status and dietary MOS was also insignificant for the gene expression at 0 min after the tank cleaning stress.

4. Discussion

In the present study, we evaluated the potential role of the probiotic Agrimos® MOS to reduce feed deprivation-induced stress responses in zebrafish. Our results showed that feed deprivation significantly influenced cortisol levels and expression of CRH gene at the end of the feeding trials, and also after the administration of two routine “aquaculture” stressors. These changes were in some cases; most notably the baseline and most of the times at which cortisol concentrations were sampled, prevented in fish fed the prebiotic supplemented diet. Furthermore, we found that starvation status, along with the use of
MOS in diet, had no outstanding effects on anxiety-like behaviors of the subjects in the novel test tank, although feed deprivation, per se, did result in significant increased bottom-dwelling. Also, fish fed MOS showed higher levels of LAB in the intestine. Prebiotics and their target microorganisms (e.g. probiotics) are used in fish nutrition to modulate intestinal microbiota and increase feed efficiency [23]. Beside their use as a growth promoter and pathogen colonization blocker, both the prebiotics and probiotics (separate or in combination) have also been used to help improve resistance to different stressors (e.g. [33,34]). However, data on the effects of these supplements on zebrafish are scarce (but see [35,36]), and to our knowledge, there is no study that evaluates behavior and stress responses of juvenile zebrafish subjected to feed deprivation with or without administration of a dietary prebiotic.

The bidirectional relationships between the intestinal microbiota and behavior have been recently investigated in higher vertebrates. There is some evidence in mammals that suggests changes in microbiota can induce changes in behavior (reviewed in [37]), and

Fig. 3. Behavioral effects of 8 weeks exposure to feed deprivation protocol and/or dietary MOS supplementation on juvenile zebrafish tested in the novel tank. Data are given as mean ± SEM. Different superscripts are significantly different (p < 0.05).

Fig. 4. Whole-body cortisol levels of juvenile zebrafish after exposure to the simulated live transport (A), or simulated cleaning tank (B) stressors at the end of the 8 weeks feed deprivation protocol and/or dietary MOS supplementation. CN: fed every day with basal diet; CS: fed 1 day, starved 1 day with basal diet; PN: fed every day with MOS supplemented diet), and PS: fed 1 day, starved 1 day with MOS supplemented diet for 8 weeks. Data are given as mean ± SEM, and were compared in each sampling time between the treatments. Different superscripts are significantly different (p < 0.05).

Fig. 5. The mRNA levels of CRH in brain of zebrafish after 8 weeks exposure to feed deprivation protocol and/or dietary MOS supplementation. CN: fed every day with basal diet, CS: fed 1 day, starved 1 day with basal diet, PN: fed every day with MOS supplemented diet), and PS: fed 1 day, starved 1 day with MOS supplemented diet for 8 weeks. Data are given as mean ± SEM. Different superscripts are significantly different (p < 0.05).
conversely, the behavioral environment may alter the structure of the intestinal microbiota (e.g. [38]). For example, germ-free (GF) adult male mice showed elevated basal level of *CRF* gene expression and over-reacted to an acute restraint stress by a hyper-secretion of ACTH and corticosterone [39]. Higher anxiety-like behavior and lower turnover rates of monoamines in several brain regions of GF mice were also reported elsewhere [40]. In an example of one of the few studies over-reacted to an acute restraint stress by a hyper-secretion of ACTH of zebrafish [47], and jundí [10]. This is not surprising as the hypothalamic-pituitary-interrenal (HPI) axis that controls the secretion of cortisol may activate to mobilize energy during feed deprivation [12]. However, contradictory studies also show reduction [48], or no changes [13] in cortisol after starvation. This may be attributable to differences in feeding habits, feed restriction cycles, and life stage, as well as fish species. We also found that feed deprivation induced a significant increase in baseline *CRH* gene expression of CS treatment, but had no effects on PS specimens in comparison to CN fish. This was concomitant with high baseline cortisol responses of the CS subjects, and similar cortisol levels in the CN and PS fish. It has been reported that *CRH* mediated physiological and behavioral responses to stress in vertebrates [49] like fish [5]. It is therefore reasonable to suggest that feed deprivation resulted in increased *CRH* mRNA expression and subsequent high cortisol levels. Further, dietary inclusion of MOS appeared to modulate these responses.

Both of the simulated live transport and tank cleaning stress methods resulted in elevation of whole-body cortisol levels in all treatments. Cortisol responses of the fish returned to low levels, but not necessarily to the baseline levels, 240 min after incidence of the stressors. Our results are in accordance with the previous studies on the zebrafish [50,51] or other fish species [2,52] which showed highest levels of cortisol expression upon initial exposure to a stressor. Like to the initial sampling time (0 min) CS fish represented highest cortisol levels at 240 min after the both stressors. Unsurprisingly, *CRH* mRNA expression patterns were similar to that of cortisol levels; however, the expression of the gene was significantly influenced by nutritional status immediately after the both stressors. These results mirror the classic endocrine response to stress in fish; i.e. the HPI axis, and also represent the link that exists between stress response and nutritional status (e.g. [31]), along with the effects of prebiotics on HPI. In the simulated transport test, cortisol levels of CN and PS fish did not differ at all of the sampling times. This is promising as the dietary inclusion of MOS caused improvement in stress response of the feed deprived fish. Information regarding cortisol response of fish to prebiotics is scarce; however, some recent studies showed higher stress response in fish fed with a prebiotic supplemented diet (e.g. [25,26,33,34]). PS fish had lower cortisol levels than the CS specimens at 30 min after cleaning tank stress onward; however, they showed higher stress response at 0 and 60 min after stress than the CN fish. This was concomitant with higher *CRH* mRNA expression of PS fish than that of CN specimens. These results indicate that dietary MOS may not always overcome negative feed deprivation effects. Varying responses to different stressful conditions in marron (*Cherax tenuimanus*) were reported in another study that used MOS (Bio-Mos®) as the dietary supplement [26]. In total, our results suggest that dietary administration of MOS might have the potential to be beneficial in aquaculture conditions, based on cortisol responses of feed deprived zebrafish that were subjected to two routine stressors.

In conclusion, the results of this study showed for the first time that inclusion of the prebiotic Agrimos® MOS in the diet of zebrafish reduce some anxiety-like behaviors and cortisol production when feed deprivation cycles are performed. This may, in part, be the results of alteration in intestinal microbiota. What is clear from our results is that modulation of gut microbiota by MOS play a role in the stress reactivity of zebrafish. Additional experiments need to be conducted, both on evaluation of LAB cannot give a complete view of the fish microbiome, thus the study of total bacteria and the other beneficial microbial populations is also important. In total, the results of our novel tank tests suggest that the starvation protocol and the prebiotic that we used here had at best only a limited effect on the anxiety-like behaviors of juvenile zebrafish.

In the present study CS fish exhibited higher baseline cortisol responses after completion of feeding trials. This suggests that feed deprivation is a stressful condition in zebrafish culture. Similar results on the stress effects of starvation have been reported in other fish species like rainbow trout (*Oncorhynchus mykiss*; [47]), and jundí [10]. This is not surprising as the hypothalamic-pituitary-interrenal (HPI) axis that controls the secretion of cortisol may activate to mobilize energy during feed deprivation [12]. However, contradictory studies also show reduction [48], or no changes [13] in cortisol after starvation. This may be attributable to differences in feeding habits, feed restriction cycles, and life stage, as well as fish species. We also found that feed deprivation induced a significant increase in baseline *CRH* gene expression of CS treatment, but had no effects on PS specimens in comparison to CN fish. This was concomitant with high baseline cortisol responses of the CS subjects, and similar cortisol levels in the CN and PS fish. It has been reported that *CRH* mediated physiological and behavioral responses to stress in vertebrates [49] like fish [5]. It is therefore reasonable to suggest that feed deprivation resulted in increased *CRH* mRNA expression and subsequent high cortisol levels. Further, dietary inclusion of MOS appeared to modulate these responses.

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In conclusion, the results of this study showed for the first time that inclusion of the prebiotic Agrimos® MOS in the diet of zebrafish reduce some anxiety-like behaviors and cortisol production when feed deprivation cycles are performed. This may, in part, be the results of alteration in intestinal microbiota. What is clear from our results is that modulation of gut microbiota by MOS play a role in the stress reactivity of zebrafish. Additional experiments need to be conducted, both on
zebrafish and ultimately on target commercial species, to further elucidate the relationship between feed deprivation and intestinal microbiota.

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Conflict of interest

All of the authors declare that they have no conflict of interest.

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