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Research paper

Dietary sodium propionate affects mucosal immune parameters, growth and appetite related genes expression: Insights from zebrafish model

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ABSTRACT

Propionate is a short-chain fatty acid (SCFA) that improves physiological and pathophysiological properties. However, there is limited information available about the effects of SCFAs on mucosal immune parameters as well as growth and appetite related genes expression. The aim of the present study was to evaluate the effect of sodium propionate (SP) intake on the mucosal immune parameters, growth and appetite related genes expression using zebrafish (*Danio rerio*) as model organism. Zebrafish fed control or diet supplemented with different levels (0.5, 1 and 2%) of SP for 8 weeks. At the end of feeding trial, the expression of the key genes related to growth and appetite (GH, IGF1, MYSTN and Ghrl) was evaluated. Also, mucosal immune parameters (Total Ig, lysozyme and protease activity) were studied in skin mucus of zebrafish. The results showed that dietary administration of SP significantly ($P < 0.05$) up-regulated the expression of GH, IGF1 and down-regulated MYSTN gene. Also, feeding zebrafish with SP supplemented diet significantly increased appetite related gene expression ($P < 0.05$) with a more pronounced effect in higher inclusion levels. Compared with control group, the expression of appetite related gene (Ghrl) was remarkably ($P < 0.05$) higher in SP fed zebrafish. Also, elevated mucosal immune parameters was observed in zebrafish fed SP supplemented diet. The present results revealed beneficial effects of dietary SP on mucosal immune response and growth and appetite related genes expression. These results also highlighted the potential use of SP as additive in human diets.

1. Introduction

The link between feed intake and physiology is well-known and in this regards feed supplements have substantial effects on immune responses and diseases resistance (Genovese et al., 2012; Faggio et al., 2015; Guardiola et al., 2016). Short-chain fatty acids (SCFAs) are produced in bacterial fermentation of fiber, resistant starches and other low digestible polysaccharides. They have beneficial effects on human and animals physiological and pathophysiological responses (Venter et al., 1990; Hijova and Chmelarova, 2007; Hosseini et al., 2011; Romano et al., 2015). Acetic acid, propionic acid and butyric acid are well-known SCFAs and preferred energy source for the intestinal epithelium (Scheppach, 1994; Lim et al., 2015; Ng and Koh, 2016). Besides, immunomodulatory feature of prebiotics were attributed to SCFAs production following microbial fermentation (Hoseinifar et al., 2015; Carbone and Faggio, 2016). It has been reported that SCFAs improves immune responses through binding to G protein coupled receptor; GPR43 (Maslowski and Mackay, 2011). Furthermore, acetate and propionate are known ligands for GPR43. Also, dietary fiber intake has shown to lessen intestinal inflammation (Kanauchi et al., 2006; Maslowski and Mackay, 2011). Propionic acid, also reduces inhibitory activity on cyclooxygenase, a major enzyme involved in producing proinflammatory eicosanoids (Bos et al., 2004; Liu et al., 2014). About practical use of SP in fish nutrition, Hoseinifar et al. (2016b) showed beneficial effects of sodium propionate (SP) on mucosal immune response and growth performance of Caspian sea white fish. Also, dietary administration of SP up-regulated immune and antioxidant related genes in zebrafish (Safari et al., 2016a). However, inclusion of SP in Arctic charr diet negatively affected weight gain (Ringø, 1991). The literature review revealed contradictory results and gap of existing knowledge about SP effects on growth and appetite related genes expression in fish. Therefore, the present study was performed to

Abbreviations: SCFAs, short-chain fatty acids; SP, sodium propionate; GH, growth hormone; IGF1, insulin-like growth factor; MYSTN, myostatin; Ghrl, ghrelin; SCFAB, short-chain fatty acids blend.

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investigate the effects of SP on the mucosal immune parameters as well as growth and appetite related genes expression using zebrafish (*Danio rerio*) as model organism.

2. Materials & methods

2.1. Fish husbandry and experimental diets

Six hundred healthy zebrafish were supplied from a private sector farm (Golestan province, Iran). Fish were acclimatized to the experimental conditions for two weeks. During the acclimatization period fish were hand-fed three times a day with a commercial diet (Biomar, France). Then, the zebrafish (0.42 ± 0.06 g) were randomly stocked in 12 aquaria (100 L) at a density of 50 fish in each aquarium. During the feeding trial (8 weeks), fish were fed to apparent satiation three times a day. Water temperature, pH and dissolved oxygen were monitored daily and maintained at 25 ± 2 °C, 7 ± 0.2 and 7.9 ± 0.1 mg L^{-1}.

2.2. Preparation of experimental diets

A commercial diet (BioMar SAS, Nersac, France) was used as basal diet and experimental diets were prepared by supplementation of basal diet with different levels of SP (Sigma, UK) (0 [control], 0.5, 1 and 2%). The details on the proximate composition and preparation of experimental diets were described in our previous publication (Safari et al., 2016a). The experimental diets stored in plastic bags at the 4 °C until use.

2.3. Evaluation of immune, appetite and growth related gene expression

2.3.1. Sample collection

At the end of the experiment, twenty specimens were randomly sampled from each aquarium. Fish were rapidly anesthetized by using clove powder (0.5 g l^{-1}). Thereafter, the intestine, brain, liver and muscle samples were taken, immediately deep-frozen in liquid nitrogen and stored at −80 °C freezer until future analysis.

2.3.2. RNA isolation, cDNA synthesis and real-time PCR

Relative gene expression was investigated in the intestine, brain, liver and muscle of twenty fish (24-h fasted) per aquarium using Real time PCR as described previously (Safari et al., 2016b). Four replicates (obtained by pooling from five fish) were considered for each organ. RNA isolation performed following BIOZOL Reagent protocol (Bioflux-Bioer, China). The concentration of RNA was determined by Nanophotometer (IMPLEN-P100) reading at 260/280 nm and the integrity verified by evaluation of 28 S and 18 S ribosomal RNA (rRNA) using agarose gel (1.5%). The DNA of the samples was eliminated by treating with DNase I (Fermentas, France). cDNA synthesis was performed according to the Fermentase protocol. Briefly, 1 μg RNA was denatured and annealed at 70 °C for 5 min with 0.2 μl of oligo (dT). After chilling on ice, 4 μl 5X reaction buffer, 1 μl Ribo Lock Ribonuclease inhibitor (20U μl^{-1}) and 2 μl 10 Mm dNTP were mixed and incubated at 37 °C for 5 min. This mixture was added to 1 μl M-MuLV RT (200 U μl^{-1}) in a final volume of 20 μl and incubated at 37 °C for 10 min, 60 min at 42 °C and finally at 70 °C to stop the reaction (Fermentase, France). Real-time PCR analysis was performed using Fermentase Maxima SYBR Green qPCR Master Mix (1x) in an iCycler (BioRad, USA). After the amplification phase, a melt curve analysis performed to confirm the specificity of the amplification reaction. The obtained data were analyzed using the iQ5 optical system software version 2.0 (BioRad). The fold change in MYSTN, IGF1, GH and Ghrl relative mRNA expression were calculated by the $2^{-\Delta\Delta Ct}$ method. In all cases, each PCR was performed in triplicates.

2.3.3. Primer design

The qPCR primers for MYSTN, IGF1, GH and Ghrl were designed based on the sequences available in GenBank. The β-actin gene was used as an internal control gene to normalize the expression of the target genes. The sequences of primers, melting temperatures and accession numbers are listed in Table 1.

2.3.4. Skin mucus collection

Nine fish were sampled from each aquarium, anaesthetized using clove powder (500 mg L^{-1}) and transferred into a polyethylene bag containing 5 mL of 50 mM NaCl (Sigma, Steinheim, Germany). Skin mucus was collected as described previously (Hoseinifar et al., 2016b) by gently rubbing the fish inside the plastic for 1–2 min. Thereafter, the samples were immediately transferred to sterile 15 mL-tubes and centrifuged at 1500×*g* at 4 °C for 10 min (5810R Eppendorf, Engelsdorf, Germany). The supernatant was collected and kept at −80 °C until analysis.

2.4. Skin mucus total Ig

The skin mucus total Ig was measured as described in our previous work (Hoseinifar et al., 2016a). Briefly, the total protein level of mucus samples were determined using a 12% solution of polyethylene glycol (Sigma) before and after precipitating down the immunoglobulin molecules. The difference in protein content was measured as the total Ig content of skin mucus.

2.5. Skin mucus lysozyme activity

The lysozyme activity of samples was measured by the turbidimetric method assay using a lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (Sigma) as described in previous work (Safari et al., 2016b). Briefly, equal amount of mucus sample (50 μL) with lyophilized *Micrococcus luteus* (Sigma) suspension were added to 96 well plate, incubated for 15 min (at 30 °C) and the absorbance was monitored for 50 min. A unit of activity defined as the amount of enzyme decreases the absorbance 0.001 min^{-1} at 450 nm.

2.6. Skin mucus protease activity

The protease activity of mucus samples was measured according to the protocol suggested by Palaksha et al. (2008). Briefly, 100 μl of mucus sample was mixed with 0.7% azocasein solution (Sigma) and incubated for 19 h at 30 °C with constant agitation. Then, the reaction was stopped by adding trichloroacetic acid (4.5% final concentration), the mixture was centrifuged at 15000*g* for 5 min to obtain the supernatant. The supernatant was pipetted to a 96-well flat bottom plate containing 100 mL 1 N sodium hydroxide (NaOH). The optical density (OD) was measured at 450 nm.

2.7. Statistical analysis

All data were subjected to One way ANOVA followed by Duncan's multiple-range test, to determine differences among all