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ORIGINAL RESEARCH

Dietary supplement with dihydroquercetin and arabinogalactan affects growth performance, intracellular protease activities and muscle-specific gene expression in bacterially infected *Oncorhynchus mykiss*

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Abstract This feeding trial aimed to evaluate the effect of a natural dietary supplement with proposed immunostimulant and related biological activities on growth performance and muscle growth markers in rainbow trout, *Oncorhynchus mykiss*. Fish grown in cages were fed either a commercial diet (control) or a diet supplemented with dihydroquercetin and arabinogalactan at a dosage of 25 and 50 mg/kg of feed, respectively, during the summer-autumn season. Unplanned infectious disease did not interfere with the scheme of the experiment since both groups were infected and then treated with antibiotics. Significant differences in growth parameters between the control and experimental groups were shown only in a few cases; however, their body weight and length, Fulton's condition factor, and relative growth rate tended to be higher in the fish fed the diet with supplements. Bacterial infection influenced muscle growth mechanisms and protein turnover through partial suppression of myosin heavy chain (*MyHC*) expression and proteasome activity. In fish fed the experimental diet, *MyHC* expression was higher throughout the experiment and was more readily restored during the post-infection period. In the experimental group, the system of protein quality control involving proteasome activity was less perturbed by infection. Moreover, the suppression of the protein-degrading capacity of calpains found in the muscles of fish fed the experimental diet probably demonstrates a mechanism of growth acceleration during the post-infection period. Therefore, in infected rainbow trout, the experimental diet diminished the effects of the disease and substantially promoted post-infection repair, improving survival and infection tolerance and affecting growth mechanisms in fish.

Keywords Nature supplement . Rainbow trout . Growth . Bacterial infection

Introduction

Rainbow trout (*Oncorhynchus mykiss*) is one of the most widely cultured fish species worldwide, and its growth and survival are constantly threatened by a prevalence of infectious diseases, resulting from high rearing density, low water exchange, increase in water temperature, low dissolved oxygen, handling, etc. (Ackerman et al. 2000, 2001; Jørgensen and Buchmann 2007; Stoltze and Buchmann 2001). To optimize fish aquaculture production, various dietary supplements including antimicrobial agents are commonly used, but the use of antibiotics to control diseases may lead to their accumulation in fish tissues as well as to the development of antibiotic-resistant pathogens and environmental pollution (Romero et al. 2012). The use of natural prophylactic dietary supplements instead of chemotherapeutics in aquaculture has received a

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great deal of attention in recent times (Heidarieh et al. 2012). For example, it has been shown that feeding rainbow trout fingerlings ajwain and marjoram extracts as dietary supplements improves growth, lysozyme activity, and survival rate (Ali et al. 2018); dietary garlic supplementation promotes fish growth performance and induces changes in their intestinal microbiota, conferring beneficial effects to the host (Büyükdeveci et al. 2018); dietary loquat leaf extract supplementation upregulates immune-related genes in the intestine and improves innate immune responses in common carp (Hoseinifar et al. 2018); dietary myrtle improves non-specific immune parameters and bactericidal activity of skin mucus in rainbow trout (Taee et al. 2017). Dihydroquercetin (DHQ), also known as taxifolin, is a flavonoid compound commonly found in many plants. Dihydroquercetin is associated with many biological effects including antioxidant, anti-inflammatory, anti-tumor and antiviral activities (Weidmann 2012). Arabinogalactan (AG) is a plant polysaccharide with anti-inflammatory, gastro-protective, membranotropic, and immune-modulating activities (Ma et al. 2016). Larch raw material is used for the isolation of these two valuable bioactive compounds (Liu et al. 2018). To the best of our knowledge, there is very little information regarding the possible effects of dihydroquercetin and arabinogalactan in aquaculture. However, it was shown that dihydroquercetin as a food supplement is able to improve the immune status of gilthead seabream (Awad et al. 2015).

In aquaculture, the fillet of reared fish is the main product sold to consumers. In fish, skeletal muscles comprise the larger part of the body (about 60% by weight) and, therefore, they determine total body growth and have great metabolic value (Houlihan et al. 1993). Skeletal muscle growth reflects the results of the opposing processes of protein synthesis and degradation (Johnston et al. 2011). It was revealed that the expression of myosin mRNA, which codes for the most abundant muscle protein, can be a useful marker for evaluating protein accretion as well as whole body growth in fish (Hevroy et al. 2006). It was shown that myosin heavy chain (*MyHC*) mRNA levels correlate with somatic growth rate in rainbow trout (Overturf and Hardy 2001), wolfish (Imsland et al. 2006), and walleye (Dhillon et al. 2009).

Postnatal muscle growth in fish occurs by both hyperplasia (generation of new myotubes) and hypertrophy (increase in myotube size), which are controlled by the sequential expression of certain transcription factors. The main role in myogenesis regulation belongs to specific myogenic regulatory factors (MRFs), including MyoD, Myf5, myogenin, and MRF4 (Watabe 2001). A negative regulator of myogenesis is myostatin (MSTN), a member of the transforming growth factor-β (TGF-β) family (Gabillard et al. 2013). Protein degradation occurs mainly through three distinct pathways: intralysosomal digestion by cathepsins, calcium-dependent proteolysis by calpains, and degradation by the ubiquitin-proteasome system. The calcium-dependent proteolytic pathway may be a major pathway for regulating muscle turnover in fish (Overturf and Gaylord 2009; Salem et al. 2005, 2006, 2007), while ubiquitin-targeted protein digestion by the proteasome is primarily responsible for bulk protein degradation (Seiliez et al. 2008).

Therefore, this study evaluated the effects of dihydroquercetin and arabinogalactan as dietary supplements on the growth performance, muscle-specific gene expression and protease activities in caged rainbow trout, *O. mykiss*, infected with natural bacterial disease.

Materials and methods

Ethical procedures

All animal handling procedures were approved by the Ethics and Animal Care Committee of the Institute of Biology, Karelian Research Centre of the Russian Academy of Sciences, following EU-established norms and procedures.

Fish culture and feed composition

The feeding trial was carried out on a commercial trout farm (Republic of Karelia, Russia) using floating net cages under natural conditions with ambient photoperiod and temperature (Fig. 1). Since the water temperature in the lake did not rise above 19 ºC, the oxygen level remained high (7-10 mg L^{-1}) and stable throughout the studied season. In June, each cage was stocked with rainbow trout individuals (age 1+, 100.1±2.3 g initial body weight) with a density of 2.1 kg/m^3, and the fish were acclimatized for two weeks.

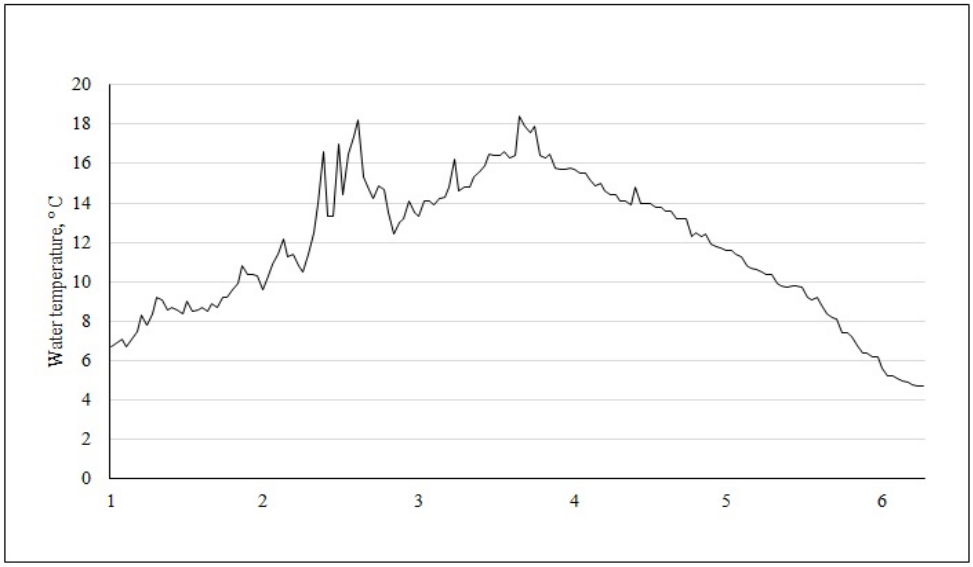

Fig. 1 Daily water temperature in the cages of rainbow trout (1 – Jun 1; 2 – Jul 1; 3 – Aug 1; 4 – Sep 1; 5 – Oct 1; 6 – Nov 1)

During acclimatization, they were given a basal diet of Efico alpha 717 (BioMar, Denmark) containing 21-24% lipid, 39-42% protein, 19-22% carbohydrate, 4-5.9% fiber, 0.8% total P, 4-7% ash, and 21-24 MJ/kg total energy. The feeding trial was carried out from July to October. Fish were fed one of two diets in duplicate: a basal diet without any supplements (control diet) or a basal diet supplemented with dihydroquercetin and arabinogalactan (DHQ+AG; at a dosage of 25 and 50 mg kg^{-1} of feed, respectively) according to the recommendations of the manufacturer (Ametist, Russia; quality and safety certificate no. 396-08.17). The feeding level and diet pellet size were equal for experimental and control groups and depended on the body weight of fish and water temperature. An unplanned infectious disease outbreak on the trout farm, manifested in late July, did not disturb the scheme of the experiment since both fish groups were infected and then treated by antibiotic therapy with enrofloxacin from 10 to 15 August.

Sampling

Eight fish from each cage were randomly captured twice a month (sampling dates Jun 25, Jul 14, Jul 26, Aug 12, Aug 28, Sept 12, and Nov 16). Fish were killed with a blow to the head and the brain was subsequently destroyed with a lancet. Then, fish were subjected to length and weight measurements as well as tissue sampling. Morphological and inner organ anomalies were assessed along with the presence of exogenous and endogenous parasites. Skeletal muscle pieces were then frozen in liquid nitrogen, transported to the laboratory, and maintained at –80 °C.

Biometric calculations

Growth performance was assessed using the following equations: percent weight gain (%) = (final weight – initial weight) × 100 / initial weight; relative growth rate (% body mass per day) = 100 × [(final weight – initial weight) / initial weight] / days of treatment; mortality rate (%) = mean number of fish that died during the period / initial mean number of fish × 100; feed conversion ratio (FCR) = dry weight of given feed / body weight gain. Fulton's condition factor (CF) was calculated from the formula: CF = 100 × body weight / body $length^3$.

Reagents and equipment

Chemical reagents, protease inhibitors, and protease substrates, all of analytical grade, were purchased from Sigma-Aldrich (USA), DNAse was purchased from Sileks (Russia), and primers and reagents were

purchased from Evrogen (Russia). Technical equipment of the Core Facility of the Karelian Research Centre of the Russian Academy of Sciences was used, including a freezing chamber, UF 240-86 E (Snijders Scientific, the Netherlands), a Tissue Lyser LT homogenizer (Qiagen, Germany), an Allegra 64R centrifuge (Beckman Coulter, USA), a CLARIOstar microplate reader (BMG LABTECH, Germany), an Implen NanoPhotometer C spectrophotometer (Implen, Germany), and a CFX96 Touch system (BioRad, USA).

Extraction of intracellular proteases

Muscle samples were homogenized for 1 min at 50 Hz with 10 volumes of 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 20 mM dithiothreitol, 1 mM ATP, 5 mM $MgCl_2$, and 0.1% Triton X-100. Homogenates were centrifuged at 20,000 g for 30 min. The resulting supernatants (enzyme-containing fraction) contained a pooled fraction of cytoplasmic and organelle proteins. All procedures were carried out in ice or at 4 °C.

Calpain activity assay

Calcium-dependent proteolytic activity was quantified using a microplate assay and casein as a substrate (Enns and Belcastro 2006). A reaction mixture with 500 mL total volume was composed of 0.4% alkali-denatured casein, 20 mM dithiothreitol, 50 mM Tris-HCl (pH 7.5), 5.0 mM Ca^{2+} (as $CaCl_2$, calcium-dependent activity) or 5.0 mM EDTA (a calcium chelator, as a negative control), and the enzyme-containing fraction. Following incubation at 28 °C for 30 min, the remaining protein was quantified by Bradford assay (1976). We defined a unit of calpain activity (AU) as the amount of enzyme needed to cause an increase in optical absorption at 595 nm by 0.1 OU during the reaction under the above conditions. The specific activity of calpains was calculated per 1 mg of protein in the corresponding fraction.

Proteasome activity assay

The chymotrypsin-like activity of the proteasome was determined in the enzyme-containing fraction using a fluorescence assay (Rodgers and Dean 2003). Peptidase activity against a synthetic oligopeptide substrate was measured in a reaction mixture containing 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ATP, 30 µM Suc-LLVY-AMC as the substrate, and 20 mM Tris-HCl (pH 7.5) in the absence or presence of 5 µM specific inhibitor MG132. Following incubation at 37 °C for 30 min, proteasome activity was calculated as the difference in fluorescence intensity between the samples with and without inhibitor at excitation and emission wavelengths 380 nm and 440 nm, respectively. The change in proteasome activity was normalized to sample protein concentration and expressed as relative fluorescence fold change (FU).

Total RNA isolation and reverse transcription

Total RNA was isolated from the dorsal white muscle samples using a kit with RNA-extran, an analog of TRIzol according to the manufacturer protocol. Then, total RNA was treated with DNase. RNA integrity and quality were assessed by 1% agarose gel electrophoresis and spectrophotometrically with the 260/280 nm absorbance ratio. RNA was reverse transcribed using MMLV-reverse transcriptase and random hexamer primers.

Real-time PCR

The primers for *MyHC*, *myogenin*, *MyoD1b*, *MSTN-1a*, and elongation factor-1 (*Ef-1α*) were selected using the Beacon Designer 5.0 software (Premier Biosoft, USA). The primer sequences are given in Table 1. Amplification of 2 µl complementary DNA (cDNA; 1:5 dilution of RT reaction) was carried out using 5 µl qPCRmix-HS SYBR Green 5× (Evrogen, Russia) and 500 nM primers in a final volume of 25 µl. The real-time conditions were as follows: DNA denaturation for 5 min at 95 °C; repeat cycles (40): denaturation for 20 s at 95 °C, annealing for 30 s at 60 °C, and DNA elongation for 30 s at 72 °C. Melt curve analysis verified that the primer sets for each qPCR assay generated one single product and no primer-dimer artifacts.

Table 1 Oligonucleotide primers used for RT-qPCR amplification

Gene	Sequence 5′-3′	Size of amplified fragment (bp)	GenBank accession no.
EF-1a	F: GGTGGTGTGGGTGAGTTTGAG R: AACCGCTTCTGGCTGTAGGG	149	NM_001124339.1
MyHC	F: AGAATGTTCGCCAGGTCAATG R: TCCTCAATCGCCCTCTTCAG	169	Z48794
Myogenin	F: TGAACGAGGCATTCGAGGC R: AGTGCCTGCAGCCTCTCAA	115	NM_001124727.1
MyoD1b	F: ATTTCGTTCCCTGTCACCTCTG R: TCGTCTTCGTTGTAATGG	146	NM_001124728.1
MSTN-1a	F: CGGAAACCCAAGTGTTGCTTATTC R: GGCATCAGGCGGGAGATTTG	137	NM_001124282.1

Note: Forward and reverse primer sequences (5'-3'), bp – base pairs; *EF1a* – elongation factor 1a; *MyHC* – myosin heavy chain; *MyoD1b* – myoblast determination protein 1b; *MSTN-1a* – myostatin 1a.

Standard curves corresponding to 5-fold dilution series of mixed cDNA from all samples were used to calculate the PCR efficiency. Each sample was run in triplicate on a single plate. The relative expression levels of genes were determined by the cycle threshold (Ct) method and normalized against Ef-1α using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analyses

Data are expressed as the mean ± SD. Raw data were initially checked for normality of distribution and homogeneity of variances using the Kolmogorov-Smirnov and Levene's tests, respectively, and then analyzed with two-way analysis of variance (ANOVA). Differences among means were detected using Tukey's honest significant difference test ($p \leq 0.05$).

Results

Growth, survival, and welfare of rainbow trout

Growth performance of rainbow trout fed control and experimental diets is shown in Table 2. In both groups, weight and length of fish steadily increased with the growing season, and a significant difference was detected between the sampling dates. Significant differences in growth parameters between the control and experimental groups were not shown with the exception of fish length and body weight gain in the middle of the observation period. Although non-significant, diet-related differences, fish body weight and length, CF, relative growth rate, and growth increments, tended to be higher in the individuals fed the diet supplemented with DHQ and AG than in those fed the control diet. Mortality rate was equal in both groups at the beginning of the studied season (0.95 vs 0.96% in the experimental and control groups, respectively). During the studied season, welfare of both groups of rainbow trout was disturbed by an unexpected infectious agent. In late July, manifestations of the disease state included side-swimming and food refusal in fish, in addition to liver pathology, including abnormal color, shape, and turgor of the organ and hemorrhages in the liver capsule, which was revealed at autopsy. By the peak of the infection (late July to early August), the mortality rate increased in both groups with fewer losses in the group fed the experimental diet (1.62±0.21% vs 3.14±0.11% in the control, $p = 0.052$). After the identification of pathogenic bacteria as *Pseudomonas putida* and *Cytophaga psychrophile* (act no. 939/22, the Kovalenko Research Institute for Experimental Veterinary Medicine, Moscow, Russia), both groups of rainbow trout were treated with a standard antibiotic therapy of enrofloxacin mixed with feed at a dosage of 25 mg kg^{-1} of fish from August 10 to 15. By November, the end of the studied season, the percentage of total mortality in the experimental and control groups differed significantly reaching 2.55±0.66% vs 6.68±0.84%, respectively ($p \leq 0.05$). The feed conversion ratios (FCR) by the end of observations averaged 1.07±0.14 vs 1.13±0.17 in the experimental and control groups, respectively.

Table 2 Growth performance of rainbow trout fed control (1) or experimental (2) diets

	Jul 14	Jul 26	Aug 12	Aug 28	Sep 12	Nov 16
				Body weight (g)		
1	143.3± 8.7	187.3± 12.9^{a}	235.3± 29.3^{a}	324.8± 36.1^{ab}	478.3± 46.5^{abc}	702.3± 41.2^{abcde}
2	134.8± 5.4	185.1± 10.8^{a}	283.3± 14.9^{ab}	378.9± 22.8^{abc}	527.1± 16.5^{abcd}	743.1± 53.5^{abcde}
				Body length (cm)		
1	21.1± 0.9	22.9± 1.3^{a}	22.6± 3.3	25.6± 3.1^{abc}	28.4± 2.7^{abc}	32.9± 1.7^{abcde}
2	20.9± 1.2	22.4± 1.2^{a}	25.6± 1.1^{ab*}	26.8± 1.1^{ab}	30.7± 1.4^{abcd}	33.6± 2.7^{abcde}
				CF		
1	1.5± 0.1	1.5± 0.1	1.9± 0.4^{ab}	1.9± 0.3^{ab}	2.1± 0.3^{ab}	1.9± 0.2^{ab}
2	1.5± 0.1	1.7± 0.1	1.6± 0.2	2.0± 0.2^{a}	1.8± 0.1^{ab}	2.0± 0.1^{abc}
				Body weight gain (%)		
1	28.1± 4.9	44.8± 3.9^{a}	51.8± 6.8^{a}	64.9± 5.7^{ab}	77.7± 6.1^{abc}	86.1± 2.8^{abcde}
2	25.0± 3.1	44.8± 2.9^{a}	63.9± 1.9^{ab*}	72.9± 1.7^{abc}	80.9± 1.8^{abcd}	85.4± 2.5^{abcde}
				Relative growth rate (% body mass per day)		
1	2.16± 1.22	2.73± 0.4	2.76± 1.4	3.45± 1.57	4.3± 1.4^{a}	4.4± 0.9^{ab}
2	1.74± 0.77	2.66± 0.96	3.73± 0.86^{ab}	4.29± 0.99^{ab}	5.3± 0.6^{abcd}	4.2± 0.7^{ab}

* – differences are significant compared with control, $p \leq 0.05$
a – differences are significant compared with the same group from the sampling date 14 Jul, $p \leq 0.05$.
b – differences are significant compared with the same group from the sampling date 26 Jul, $p \leq 0.05$.
c – differences are significant compared with the same group from the sampling date 12 Aug, $p \leq 0.05$.
d – differences are significant compared with the same group from the sampling date 28 Aug, $p \leq 0.05$.
e – differences are significant compared with the same group from the sampling date 12 Sep, $p \leq 0.05$.

Muscle-specific gene expression

Muscle-specific gene expression changed throughout the period of observation, and some molecular markers significantly responded to bacterial infection, food supplementation or both. The expression of the *MyHC* gene (Figure 2A) was substantially influenced by the infection with a significant decrease in the *MyHC* mRNA levels in infected fish (sampling Jul 26) and a sharp increase during the post-infection period (Aug 12, Aug 28). The *MSTN-1a* mRNA levels significantly increased after antibiotic therapy without any dietary supplementation response (Figure 2D). The expression levels of *myogenin* (Figure 2C) and *MyoD1b* (Figure 2B) significantly increased in infected fish (Jul 14), particularly in those fed with supplements, and then readily restored to pre-infection levels in both groups.

Protease activities

In the control group, muscle protease activities decreased in infected fish (Jul 14, Jul 26) compared to healthy individuals (Jun 25) with a significant response in proteasome activity; however, there were no infection-induced changes in calpain activity levels in fish fed the experimental diet (Fig. 3A, B). After antibiotic therapy (from Aug 12 through the end of observation; Fig. 3A), calpain activity decreased in skeletal muscle with more significant changes in fish fed the experimental diet. Proteasome activity in fish muscle demonstrated season-related decline from June to November with significantly higher levels in fish fed the supplemented diet (Jul 14, Aug 12, Sep 12; Fig. 3B).

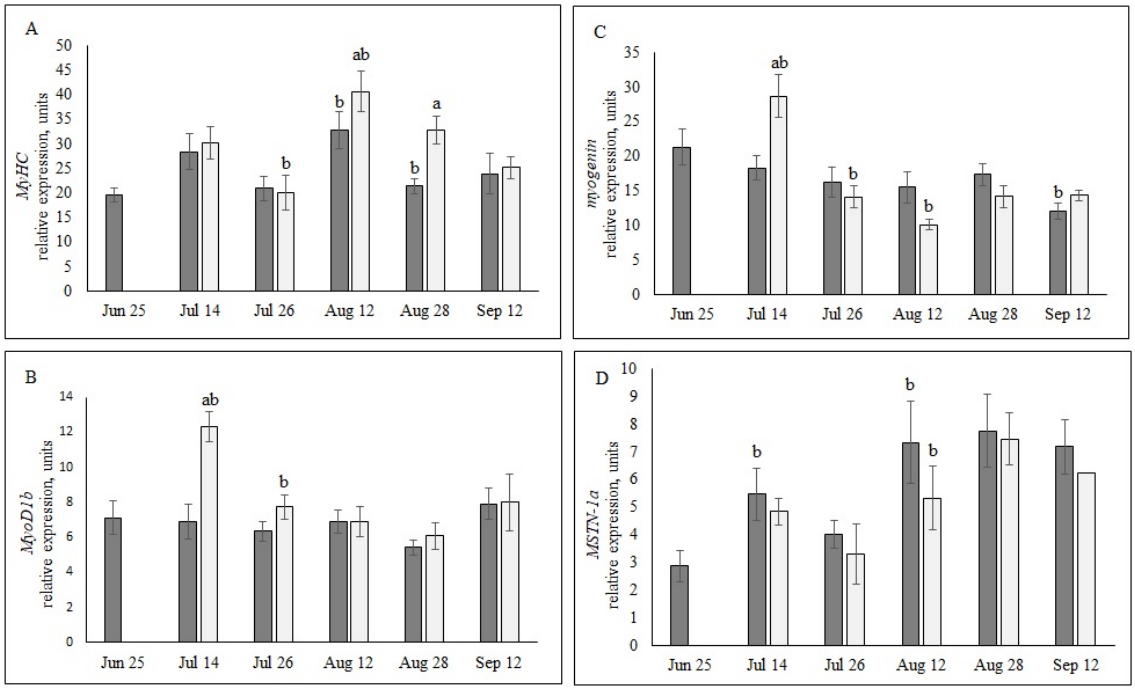

Fig. 2 Expression of muscle-specific genes *MyHC* (A), *MyoD1b* (B), *myogenin* (C), and *MSTN-1a* (D) in rainbow trout fed control (dark columns) or experimental (light columns) diets. (a – differences are significant compared with control, $p \leq 0.05$; b – differences are significant compared with the same group in the previous sampling date, $p \leq 0.05$).

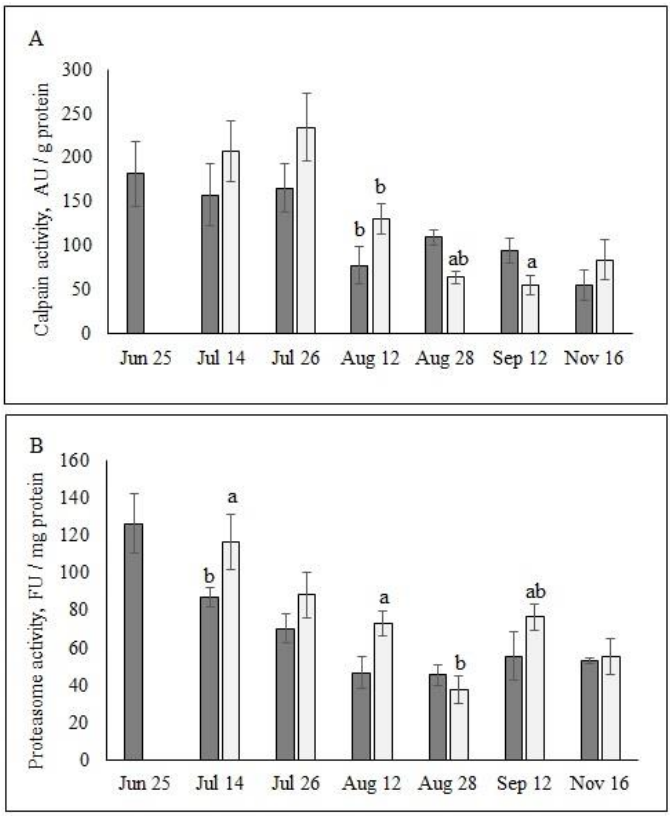

Fig. 3. Calpain (A) and proteasome (B) activities in the skeletal muscle of rainbow trout fed control (dark columns) or experimental (light columns) diets.

(a – differences are significant compared with control, $p \leq 0.05$; b – differences are significant compared with the same group in the previous sampling date, $p \leq 0.05$).

Discussion

Seasonal variations in fish growth

Besides the trophic factor, the seasonal variations in photoperiod and water temperature influence growth and welfare of fish growing in cages. Rainbow trout, like other salmonids, are adapted to relatively low water temperatures (Janhunen et al. 2016; Jobling 1981). It was shown that rainbow trout had lower feed intake, faster growth and a better feed conversion ratio at lower (14 °C) than at higher (20 °C) rearing temperatures (Janhunen et al. 2016). Because water temperatures in summer 2017, the period of observation, were unusually low, trout temperature tolerance, growth, appetite, and behavior were not perturbed by heat, based on a standard curve describing the season-related dynamics of weight and linear growth.

Muscle growth represents the balance between catabolic and anabolic processes in protein metabolism. Intense fish growth is associated with excessive synthesis and accumulation of the main contractile protein, myosin, under the control of the protein synthetic machinery and myostatin. *MSTN* is thought to be expressed in response to high *MyHC* expression attenuating both hyperplasia and hypertrophy and regulating muscle growth (Johansen and Overturf 2005, 2006). Similar to studies of other fish species (Dhillon et al. 2009; Imsland et al. 2006; Overturf and Hardy 2001), myosin expression in rainbow trout correlates with the growth rate as well as with protein accretion (Hevroy et al. 2006). The correlation of *MyHC* mRNA levels with growth rate depends on fish size, feed content, and protein synthesis and degradation rates in skeletal muscle (Alami-Durante et al. 2010; Koedijk et al. 2010; Talukdar et al. 2019). Muscle growth involves the recruitment of myosin and other myofibrillar proteins to myofibrils through a system that includes both transcription factors MyoD, responsible for myoblast determination, and myogenin, involved in the differentiation and fusion of myoblasts to form myofibers (Watabe 2001).

Our data support the observations that *MyoD* activation leads to robust expression of target genes, including *myogenin* and those encoding myosin heavy and light chains (Berkes and Tapscott 2005). Similarly orchestrated changes in *MyoD* and *myogenin* expression were also described in growing rainbow trout (Johansen and Overturf 2005), pacu (Almeida et al. 2010), brown trout (Churova et al. 2017a) and Atlantic salmon (Churova et al. 2017b). In our study, we showed the coordinate changes in muscle-specific gene expression and protein-degrading enzymes in growing rainbow trout. Protein degradation in fish skeletal muscle relies on calcium-dependent proteolysis contributing to myofibrillar disintegration (Goll et al. 2008), an initial step in the routine turnover of myofibrillar proteins. On the other hand, maximum calpain activity corresponds with excessive myosin production and muscle growth. As stated by Overturf and Gaylord (2009), muscle protein degradation management acts as the checkpoint in directing the regulation of protein turnover, muscle deposition, and growth.

Infection-induced effects on fish growth

In infected fish, the disease state is accompanied by decreased gene expression of myosin heavy chain, *MyHC*, and *myostatin,* a negative regulator of muscle growth, while an increased expression of both genes was seen during the post-infectious period. The simultaneous change of *MyHC* and *myostatin* expression has been shown in brown trout (Churova et al. 2017a) and in Atlantic salmon (Churova et al. 2017b) in groups of different ages and life stages. One potential explanation for the correlation is that *MSTN* is expressed in response to excess *MyHC* mRNA as a regulatory mechanism to attenuate hyperplasia and hypertrophy and to control muscle growth (Johansen and Overturf 2005, 2006). The patterns of *myogenin* and *MyoD* expression in rainbow trout muscle during the observation period were similar. It is known that *MyoD* activates *myogenin* expression by directly binding to its regulatory elements (Berkes and Tapskot 2005).

In our study, a coordinate suppression of calpain and proteasome proteolytic pathways was detected throughout the post-infection period. This was thought to be a compensatory response to the previous growth retardation that resulted from infection. Calpain and proteasome probably respond to common stimuli or regulatory mechanisms, and as a result, their activities change in an orchestrated manner in the muscle of rainbow trout, as they do in other fish species (Kantserova et al. 2017; Lysenko et al. 2017). Besides contributing to physiological protein turnover, proteases, particularly, those in the ubiquitin-

proteasome pathway, contribute to protein quality control, eliminating abnormal proteins (Ciechanover 2013). Apparently, infectious agents and possibly antibacterial drugs led to oxidative stress in the fish (based on the data by Sukhovskaya et al. unpublished observations) resulting in the accumulation of oxidized and misfolded proteins and a subsequent excessive load on the protein quality control system, involving proteasome activity.

Diet-related variations in fish growth and tolerance

Although the investigated dietary mix of plant components, dihydroquercetin and arabinogalactan, has little effect on fish growth performance, it improves the physiological responses of fish to multiple stressors. Besides the previously discussed effects of season-related environmental variations and physiological status on protein turnover in rainbow trout skeletal muscle, it is also modified by dietary supplementation. The upregulation of muscle-specific genes, including myogenic factors such as *myogenin* and *MyoD*, detected in fish fed the experimental diet during the first month of observations reveals the pro-myogenic activity of the supplements, stimulating molecular mechanisms of muscle growth. Our data have shown that in fish fed the experimental diet, *MyHC* expression is higher throughout the observation period, and their disease-associated decrease is more readily overcome during the post-infection period. The suppression of the calpain-dependent proteolytic pathway probably accelerated fish growth since curing the infection with antibiotics was found to be more effective in fish fed a diet with natural supplements. Unlike calpains, the proteasome activity suppression induced by the infection and subsequent antibacterial chemotherapy was less pronounced in supplement-fed fish. Despite coordinate suppression in both proteolytic systems, less proteasome inactivation in supplement-fed fish emphasizes a lesser load on the proteasome system contributing to abnormal (mainly oxidized) protein elimination. These data indicate that the dietary mix probably realizes its biological activity by promoting molecular mechanisms of protein turnover in muscles to maintain growth and post-infection repair processes in fish. Based on the data showing a lower mortality rate in the experimental fish group from the start of early disease manifestations through the end of the studied season and on the facts that the biochemical responses of those fish were less perturbed by the bacterial infection and more readily improved in the post-infection period, we can assume that the DHQ and AG mix in the fish diet stimulates both inherent fish resistance to infection and likely tolerance to other stress-inducing factors, such as water temperature variation, fish-farming, and handling. Versatile welfare- and growth-related effects of the natural supplement, such as supporting survival, stimulating muscle growth, and promoting repair, demonstrate its antioxidant and prebiotic activities. By diminishing cellular component oxidation, food supplementation can reduce energy utilized on the antioxidant system response (Sukhovskaya et al. unpublished observations) and quality control machinery response. In addition, fish immunity appears to be improved by natural supplements through promoting immune system constituents such as the gut microbiota (Parshukov et al. 2018). The improvement of microbiota status with the use of plant extracts was supported by lower FCR values, which correspond to a higher degree of feed energy converted to mass gain, in the experimental trout group; this promising result has the potential to increase the profitability of fish production.

Our data on the molecular mechanisms of growth in rainbow trout fed natural prophylactic dietary supplements correspond with data in the literature describing the effects of other related compounds on the welfare of and growth performance in fish. For example, the naturally occurring antioxidant resveratrol has positive effects on the growth and skeletal muscle physiology of juvenile southern flounder (Wilson et al. 2015). There is very little information regarding the possible effects or effective dose of DHQ and AG in aquaculture (Awad et al. 2015) with no conclusions on its effects on fish growth performance.

In conclusion, physiological indices and muscle growth-associated molecular markers in rainbow trout, *O. mykiss*, respond to multiple factors, including seasonal dynamics of environmental variables, physiological growth process, natural infection, and diet supplementation. Independently, on the control diet, muscle growth and protein turnover are substantially modified in infected and repairing fish, while the experimental diet substantially increased survival and promoted post-infection repair in fish. These promising results on the biological activities of the experimental supplements appear to be supported by the mechanistic experiments done under controlled conditions to reveal the individual effects of DHQ and AG even when accounting for seasonal variables. Based on our observations, we recommend exploring the

use of this dietary mix in fish production to improve fish tolerance to fluctuations in the nature environment and to stress-inducing factors.

Conflict of Interest The authors declare no conflicts of interest.

Author's contributions Conceived and designed the experiments: LAL IVS. Performed the experiments: LAL IVS. Performed the biochemical and molecular-genetic analysis: NPK MVC EDT IVS: Analyzed the data: NPK LAL MVC IVS NNN. Wrote the paper: NPK LAL.

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