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Effects of bioactive plant extracts on the immune-related gene expression of common carp (*Cyprinus carpio*)

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SUMMARY

In recent years, intensive fish farming has led to an outbreak of several diseases, and the health status of fish can affect the economy of aquaculture. Since fish health and intestinal health are in correlation, it may also have an impact on immunity. Accordingly, many natural feed additives are being used to improve immune functions. In our study, carotenoids, oligosaccharides, and anthocyanins were applied at 1 m/m% in feed to investigate their effects on cytokines, such as interleukin-1β (IL-1β), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), and interferon regulatory factor-1 (IRF-1) in spleen and mid-intestine of 6 months old carp. Gene expression analysis was carried out to examine IL-1β, IL-8, TNF-α, and IRF-1 mRNA levels in fish spleen and mid-intestine. The gene expression level of pro-inflammatory IL-1β decreased in the mid-intestine of carotenoid-fed carp compared to anthocyanin supplemented group, but the effects of the bioactive plant extracts were not observed on the examined cytokines compared to control fish.

Keywords: *carp, gene expression, immunological parameters, natural compounds, plant extracts*

INTRODUCTION

Due to the higher need of animal fish farms, the aquaculture industry has developed rapidly in recent years (FAO, 2016). Therefore, culture systems have become more complex and farmers increased the density and the production level as well (Dawood and Koshio, 2016; Zhou et al., 2009). However, intensification of pond cultures has resulted in the appearance of many diseases in fish (Cerezuela et al., 2012; Bondad-Reantaso et al., 2005), therefore it can affect the health status of the fish and the economical profit of the aquaculture industry as well (Harikrishnan et al., 2011; Miest et al., 2012). The health of the fish is tightly associated with the health status of the gastrointestinal tract, which is also correlated to the immune status (Feng et al., 2015). Therefore, dietary supplementations are often applied in these days to increase the intestinal immunity of the fish (Hoseinifar et al., 2016; Hoseinifar et al., 2017; Chen et al., 2005; Shi et al., 2016).

Carotenoids have been reported as potential regulators of cell-mediated, and humoral immune responses, such as phagocytosis, non-specific cytotoxicity, serum lysozyme activity, and serum complement activity in fish. These also have a role in resistance against diseases (Amar et al., 2004; Tachibana et al., 1997; Torrissen, 1984; Amar et al., 2000; Amar et al., 2001; Yanar et al., 2007).

Oligosaccharides, such as inulin, fructo-, mannan- or galactooligosaccharides are also applied as feed additives in fish feed since prebiotics can boost specific immunity (Das et al., 2017) and affect the microflora positively, which can enhance the immune status of the host as well (Bailey et al., 1991).

Previous studies in fish also reported anthocyanins as they could enhance immunological parameters, such as respiratory burst activity, phagocytic activity, phagocytic index, lysozyme activity, myeloperoxidase activity, and total immunoglobulin levels (Yilmaz, 2019a; Yilmaz, 2019b). Besides, anthocyanins could alter the gene expression levels of some cytokines, such as interleukin-1β (*IL-1β*) interleukin-8 (*IL-8*), tumor necrosis factor-α (*TNF-α*), and interferon-γ (*IFN-γ*) (Yilmaz, 2019a).

Cytokines are small proteins part of the innate and acquired immunity, through they have a role in signaling processes between cells and protect the host against infections (Gomez and Balcazar, 2008; Kaiser and Stäheli, 2008; Gonzalez et al., 2007). In terms of function, they can modulate the innate-, the acquired immune response and stimulate hematopoiesis (Gomez and Balcazar, 2008). Cytokines involve chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors (Secombles et al., 2001). Pro-inflammatory cytokines, such as interleukin-1β (*IL-1β*) and tumor necrosis factor-α (*TNF-α*) are the most extensively characterized cytokines in fish (Saeij, 2003; Savan et al., 2003). They have been reported as multifunctional cytokines since they affect gene expressions under inflammation (Sigh et al., 2004). *IL-1β* and *TNF-α* take part in the first line of immune response and accrete leukocytes to the inflammation site (Huising et al., 2003). *IL-1β* and *TNF-α* are also the most important cytokines since they are the signals of numerous types of interactions between cells and take

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part in host defense mechanisms and inflammation pathological development (Boudjellab et al., 2000). TNF-α is secreted by macrophages and enhances cells to other cellular factors (De and Mukherjee, 2009). IL-1β has a role in secondary cytokine production (Markus and Susetta, 2011). Interleukin-8 (*IL-8*) is classified as a CXC chemokine, that contains a glutamate-leucine-arginine (ELR) motif before the CXC sequence and it has a role in attracting neutrophils (Chen et al., 2005). Finally, interferon-regulatory factor 1 (*IRF-1*) is a member of the interferon (IFN) regulatory factor family, those family of transcription factors, which have a role during viral infections or other types of cell stress (Tamura et al., 2008; Barnes et al., 2002). *IRF-1* participates in antiviral processes against viruses, such as Newcastle disease virus (NDV), encephalomyocarditis virus (EMCV), and Hepatitis C virus (HCV) (Wyllie et al., 1980; Fujimoto et al., 2000; Kanazawa et al., 2004).

Based on previous studies, carotenoids, oligosaccharides, and anthocyanins can be potential compounds to enhance immune responses in fish, therefore the aim of the study was to examine the gene expression levels of cytokines, such as IL-1β, IL-6, TNF-α, IRF-1 in common carp (*Cyprinus carpio*).

MATERIALS AND METHODS

Animal ethics

Experiments were confirmed by the University of Debrecen Committee of Animal Welfare, Hungary (Permit number: 15/2019/DEMÁB).

Preparation of Extracts

For the preparation of the experimental diet, Hungarian red sweet pepper powder was applied to extract carotenoids. Extraction was carried out with high-performance liquid chromatography (HPLC) as described earlier (Nagy et al., 2017). Diode Array Detector (DAD) detection Determination of carotenoid compounds was applied on 460 nm and 350 nm. HPLC profile and main carotenoid compounds with the greatest areas were identified in a previous study (Csernus et al., 2020), which were the following: capsanthin, cis-capsanthin, β-carotene, zeaxanthin.

Hungarian red sweet pepper retained from industrial food waste was used to extract oligosaccharides with high arabino-galactose content to gain natural prebiotics. An HP 5890 Gas chromatograph with SP-2380 capillary column was used and Flame Ionization Detector (FID) detection was applied to determine the monomer units of oligosaccharides, which were the glucose, arabinose, xylose, galactose, mannose (Csernus et al., 2020).

Hungarian sour cherry was used to extract anthocyanins with a VWR-Hitachi ChromasterUltraRs UHPLC using a Phenomenex Kinetex® column (Nemes et al., 2018). The main anthocyanin compounds were cyanidin-3-O-glucosyl-rutinoside, cyanidin-3-O-rutinoside, and cyanidin-3-O-monoglucoside (Homoki et al., 2016).

Fish and Feeding trial

The experiment was carried out at the Laboratory of Fish Biology of the University of Debrecen, Faculty of Agricultural, Food Science, and Environmental Management. A total of 132 common carp (*Cyprinus carpio*) juveniles were used from artificial propagation and kept in a water recirculation system provided with mechanical and aerated biofilter and UV lamp. Carp juveniles were randomly assigned to 3 experimental groups (3 tanks/treatment, 11 fish/tank), and a control group. Each circular plastic tank has a water volume of 350 L. Oxygen saturation was set at 85 ± 0.9% by aeration stones and the temperature was kept at 23.5 ± 0.5 °C. The fish were exposed to light as follows: 12 h light and 12 h dark. Water temperature, pH, total dissolved solids (TDS, Hanna HI98130), dissolved oxygen (DO, Hach HQ30d), NO_2^-, NO_3^- and NH_4^+ concentration (HACH DR3900) were checked daily. The experiment was started at 6 months of age with an initial body weight of 123.45 ± 0.37 g and lasted through 6 weeks. During this period fish were fed up to 3 percent of the total biomass three times (08:00, 12:00, 16:00) a day. Uneaten feed and feces were removed daily. The feeding trial consisted of the control group (basal diet) and supplementation of carotenoids, oligosaccharides, or anthocyanins. Each treatment included 1 m/m% bioactive compounds. The composition of the experimental diet is presented in *Table 1*. Calculated energy and nutrient content are shown in *Table 2*.

Sample collection

Eight carps were randomly selected from each treatment and control group for tissue sampling at the end of the feeding trial (6^{th} week). Fish were euthanized with clove oil solution and the whole spleen and 10-mm segments from the middle part of the mid intestine were collected and kept at -80 °C until analysis.

RNA isolation and cDNA synthesis

Total RNA from the spleen and intestinal tissues from the middle part of mid-intestine was purified with Direct-zol™ RNA MiniPrep (Zymo Research, Orange, CA, USA) following the manufacturer's protocol. RNA purification included the DNA digestion step. RNA concentration and the purity of each sample were determined by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was checked by 1% agarose gel electrophoresis. 400 ng of the purified RNA was applied to obtain cDNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) in 20 µl final volume. Each mixture involved RNA, oligo d(T) primers, dNTPs, reverse transcription buffer, reverse transcriptase enzyme, RNase inhibitor, and nuclease-free water. The conditions consisted of incubation at 65 °C for 30 min and termination at 85 °C for 5 minutes. cDNA samples were diluted 10 fold and kept at -20 °C.

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Table 1

Components of the diets

Ingredients (g/100 g diet)	Control diet	Carotenoid treatment	Oligosaccharide treatment	Anthocyanin treatment
Poultry by-products meal	20	20	20	20
Blood meal (porcine hemoglobin)	2	2	2	2
JPC 56 soy protein concentrate	10	10	10	10
Fish meal, wild fish	15	15	15	15
Vitamin and mineral premix*	2	2	2	2
Zeolite	2	2	2	2
Glucose	1	1	1	1
Fish oil**	2	2	2	2
Experimental additive	0	1	1	1
Wheat meal	46	45	45	45
Total (100 g)	100	100	100	100

*1 kg of vitamin and mineral premix contains: Vitamin A (retinyl acetate), 9000000IU; Vitamin D_3 (cholecalciferol), 7200000IU; Vitamin E, 5400 mg/kg; Vitamin K_3 (MSB), 9600 mg/kg; Vitamin B1 (thiamin-HCL), 1000 mg/kg; Vitamin B2 (riboflavin), 9600 mg/kg; Vitamin B3 (niacin), 45000 mg/kg; Vitamin B5 (calcium d-pantothenate), 15000 mg/kg; Vitamin B6 (pyridoxine–HCL), 5400 mg/kg; D-Biotin, 100 mg/kg; Folic acid, 1200 mg/kg; Vitamin B12 (cyanocobalamin), 27 mg/kg; Vitamin C, 4000 mg/kg; Choline chloride, 1500 mg/kg;
**Anchovy fish oil

Table 2

Calculated energy and nutrient content

	Control diet	Carotenoid treatment	Oligosaccharide treatment	Anthocyanin treatment
Digestible Energy (MJ/kg)	15.04	14.92	14.92	14.92
Dry matter (DM)	90.26	90.39	90.39	90.39
Crude protein	33.5	33.39	33.39	33.39
Crude fat	6.91	6.90	6.90	6.90
Crude fiber	1.32	1.30	1.30	1.30
Ash	6.09	6.08	6.08	6.08

Gene expression analysis of cytokines

For evaluation of selected immune-related gene expression (*IL-1β, IL-8*, *TNF-α,* and *IRF-1*) oligonucleotide primers were designed on the available sequences for carp in genebank by using Oligo 7 software. Primers were checked for target identity by National Center for Biotechnology Information (NCBI) Primer Blast (Ye et al., 2012). The relative expression of immune-related genes was determined by LightCycler 480 Instrument II (Roche Life Science, Penzberg, Germany). Reactions were run in triplicates using 384-well microplates (4titude, Surrey, UK). Each reaction contained: 4 ng cDNA template, 5x HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia), 200 nM of each primer, and distilled water in 12 µl final volume. No template controls were involved for each primer. Conditions of quantitative PCR were the following: initial activation at 95 °C for 12 min, 40 cycles of denaturation at 95 °C for 15 sec, primer annealing at 60 °C for 20 sec and chain elongation at 72 °C for 20 sec. Ct values and mean reaction efficiencies were identified by LinReg PCR 2017.0 software with linear regression analysis on each amplification curve. Stability of common carp reference genes, as β-cytoskeletal actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and gene of 40S ribosomal protein (*40S*) were determined by 3 methods (Best Keeper, NormFinder, deltaCt). The *40S* for the spleen and *GAPDH* for the intestine was defined as the most stable reference gene for normalization. Expression of the immune-related genes was calculated with the Pfaffl method (Pfaffl, 2001) and target genes were normalized to the reference gene. Results were given in fold changes as the expression of the target gene in treatment groups compared to the control group.

Statistical analysis

The statistical analysis of the results was performed by One-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test after confirmation of the assumption of data normality by Kolmogorov–Smirnov test. Outliers were determined by GraphPad Outlier Calculator at the significance level of Alpha = 0.05. GraphPad Prism 8.4.2 software was applied for statistical analysis and differences among treatments were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Relative mRNA expressions of *IL-1β*, *IL-8*, *TNF-α,* and *IRF-1* in the spleen of carp are shown in *Figure 1*. Ct values were: *IL-1β*, 26.457 ± 1.494; *IL-8*, 26.945 ± 0.985; *TNF-α*, 35.209 ± 1.157; *IRF-1*, 24.743 ± 1.150, indicating constitutive expression of mRNA in the spleen of the control group and the treatments. Relative mRNA expression levels show that none of the carotenoids, oligosaccharides, and anthocyanins

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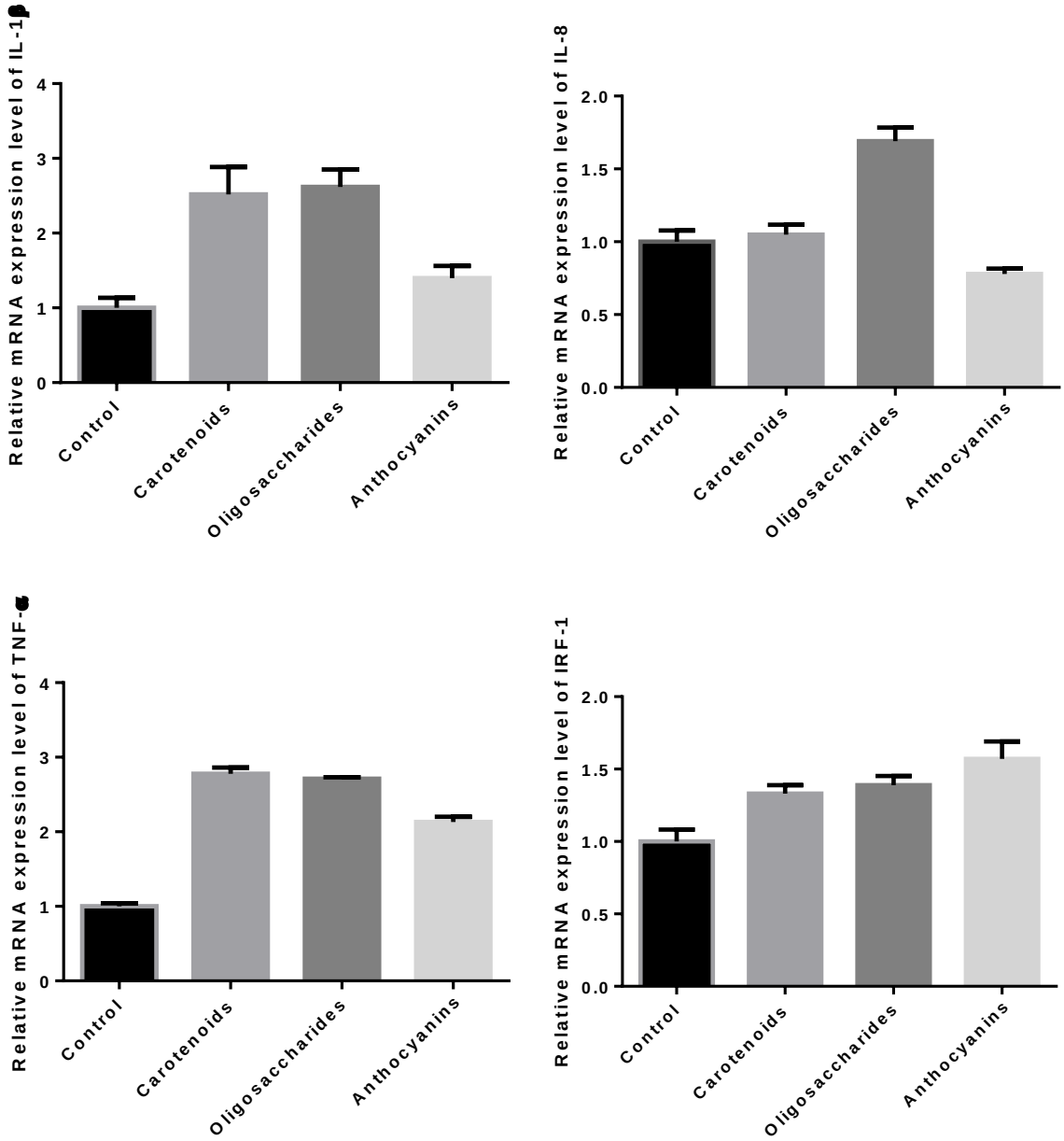
applied in this study could alter gene expression levels of the examined cytokines, significantly.

Gene expression levels of *IL-1β*, *IL-8*, *TNF-α,* and *IRF-1* in the intestine of carp are shown in *Figure 2*. Ct values were *IL-1β*, 35.773 ± 1.504; *IL-8*, 25.498 ± 0.698; *TNF-α,* 33.313 ± 1.038; *IRF-1*, 24.337 ± 2.278 indicating mRNA expressions of the cytokines in the control and treated fish. Similarly to the spleen, none of carotenoids, oligosaccharides, or anthocyanins could affect mRNA levels of cytokines compared to the control treatment. In the intestine, carotenoids showed a decreased mRNA level of *IL-1β* ($p < 0.0404$) compared to the anthocyanin treatment.

The effects of three natural plant extracts were examined on gene expression levels of pro-inflammatory cytokines, such as *IL-1β*, *IL-8*, *TNF-α,* and *IRF-1* in the spleen and intestine of common carp (*Cyprinus carpio*).

Figure 1: **Relative interleukin-1β, interleukin-8, tumor necrosis factor-α, and interferon regulatory factor-1**



mRNA expression in the spleen of carp fed the control diet, and diets supplemented with 1% carotenoids-, oligosaccharides- or anthocyanins (n = 8/treatment). Error bars represent means ± standard errors of the mean. No significant differences were observed among treatment groups at the 0.05 level.

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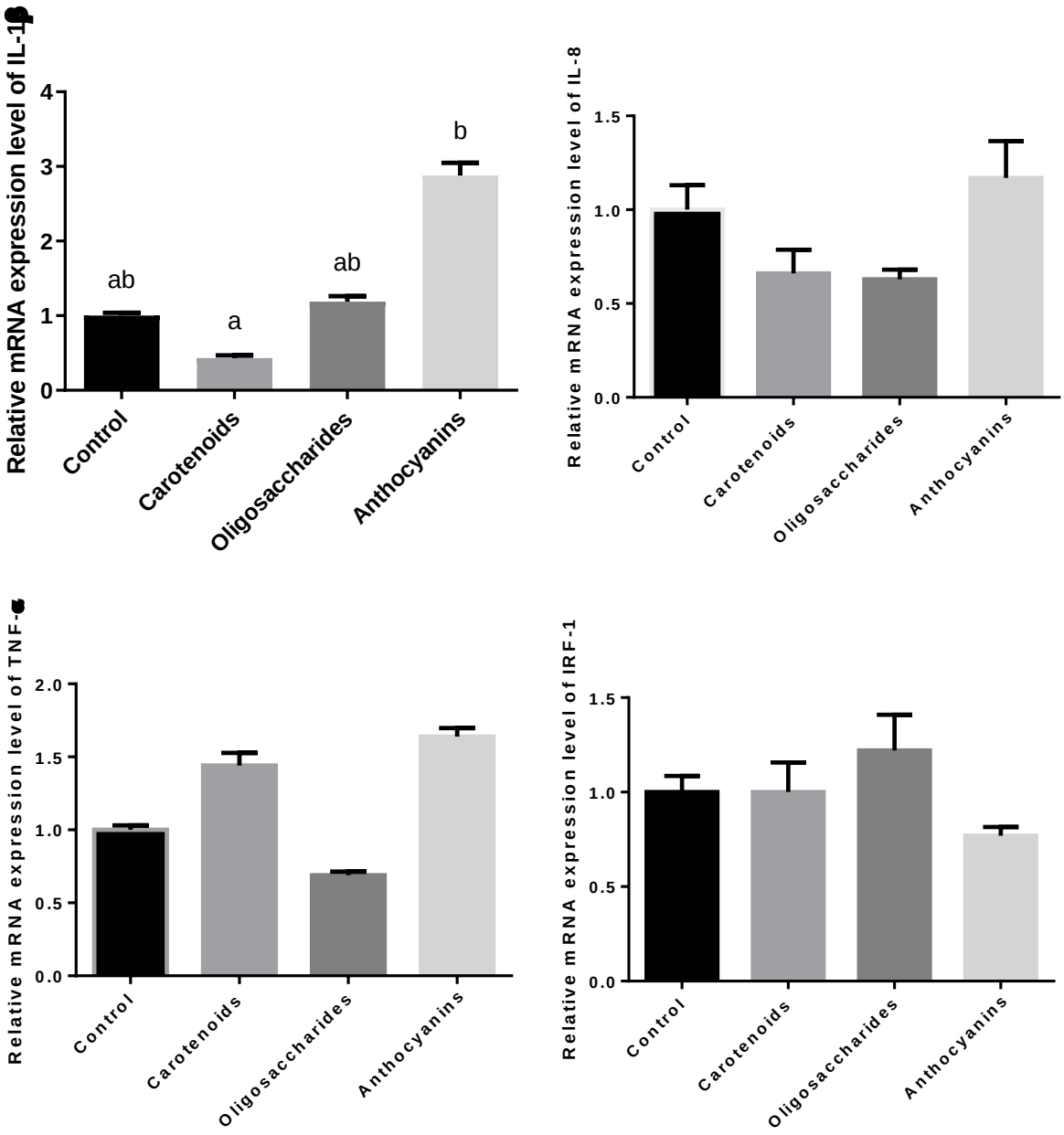
Figure 2: **Relative interleukin-1β, interleukin-8, tumor necrosis factor-α, and interferon regulatory factor-1**



mRNA expression in the intestine of carp fed the control diet, and diets supplemented with 1% carotenoids-, oligosaccharides- or anthocyanins (n = 8/treatment). Error bars represent means ± standard errors of the mean. Different superscripts indicate significant differences among treatment groups ($P < 0.05$).

In this study, no significant differences were observed in gene expression levels of splenic and intestinal *IL-1β*, *IL-8*, *TNF-α,* and *IRF-1* when carotenoids were applied as feed additives compared to the control treatment. However, the gene expression level of intestinal *IL-1β* could decrease in carotenoid fed carp compared to anthocyanin fed ones. Li et al. (2019) reported decreased gene expression levels of pro-inflammatory cytokines, such as *IL-1β* and *TNF-α*, when astaxanthin (carotenoid compound) was applied at 50, 100, or 200 mg/kg of body weight in snakehead (*Channa argus*) under LPS induced inflammation. Consequently, the authors discussed astaxanthin reduced the inflammatory responses, since inhibition of the inflammatory cytokines was observed (Li et al., 2019). In our study, oligosaccharides could not alter the relative mRNA levels of examined cytokines. Yousefi et al. (2018) investigated the effect of galactooligosaccharides at 0.5, 1, and 2% in feed on innate immune parameters in Zebrafish (*Danio rerio*). Similarly to our results, the authors found no significant differences in *IL-1β* mRNA level when galactooligosaccharides were applied at 1% and 2% in feed, and gene expression level of *TNF-α* was not

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altered when galactooligosaccharides were used at 2%, either. In contrast, the authors defined significantly decreased *IL-1β* gene expression levels when treatment involved galactooligosaccharides at 0.5%. Also, significantly increased *TNF-α* gene expression levels were identified in treatments that involved galactooligosaccharides at 0.5 and 1%. The authors explained that prebiotics may impact the immune parameters and immune-related gene expression and suggest a possible immunomodulatory effect of galactooligosaccharides at the molecular level (Yousefi et al., 2018). The effect of anthocyanins was also examined on carp cytokines in this study. However, the mentioned bioactive extract did not change significantly the gene expression levels of cytokines, either. Similarly to our results, the gene expression level of splenic *IL-8* was not changed, when blackberry syrup with high anthocyanin content was added to Nile tilapia (*Oreochromis niloticus*) diet at 7.5, 15, and 30 g/kg. In contrast, the other immune-related genes expression levels, such as *IL-1β* and *TNF-α* were increased in the spleen when blackberry syrup was used at 7.5 g/kg and discussed as blackberry syrup could produce more innate components and improved the immune parameters (Yilmaz, 2019b). In another study, Yilmaz (2019a) also reported an increased gene expression level of *IL-1β* in the spleen of tilapia after anthocyanins were applied at 40, 80, and 160 mg/kg in the feed. Additionally, using anthocyanins at 20, 40, 80, 160 mg/kg did result in higher mRNA levels of *IL-8* and *TNF-α*. None of the examined compounds influenced gene expression levels of IRF-1 neither in the spleen nor in the intestine. In agreement, another study found *IRF-1* was not changed significantly in the liver and the intestine of European Sea Bass (*Dicentrarchus Labrax*) between the control and the treatment (Terova et al., 2016).

CONCLUSIONS

In conclusion, the effects of carotenoids, oligosaccharides, and anthocyanins were not observed in our study. In other studies, results are contradictory, thereby further experiments are suggested to identify the effects of carotenoids, oligosaccharides, and anthocyanins on interleukins, tumor necrosis factor, and interferons of common carp, such as different concentrations of plant extracts, or the alterations to different fish pathogens.

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