Int. J. Aquat. Biol. (2020) 8(2): 91-97
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Effects of dietary olive leaf extract on intestinal immune-related gene expressions in common carp, *Cyprinus carpio*

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Abstract: This study aimed to investigate the effects of dietary olive leaf extract (OLE) on intestinal immune-related genes expression of tumor necrosis factor alpha (TNFa), interleukin 1 beta (IL1b), lysozyme (LYS), and mucin2 (MUC2). For this purpose, common carp (~15 g) were fed with 0 (control), 0.1 (OLE-0.1), 0.5 (OLE-0.5) and 1 (OLE-1) % OLE diets for eight weeks. The fish were sampled after one and eight weeks to study intestinal TNFa, IL1b, LYS, and MUC2 gene expressions. The results showed that dietary OLE administration significantly up-regulated intestinal TNFa gene expression after one (all OLE-treated groups) and eight (OLE-0.5) weeks. Moreover, OLE-0.1 and OLE-1 groups showed up-regulated intestinal IL1b expression, after one week, all the OLE-treated fish had significantly higher intestinal IL1b expression, after eight weeks. OLE had no significant effects on LYS gene expression after one week, but OLE-0.1 and OLE-0.5 had significantly higher gene expressions after eight weeks. OLE-0.1 and OLE-1 had significantly lower MUC2 gene expression after one week, but all OLE-treated fish had significantly higher MUC2 gene expression after eight weeks. In conclusion, dietary 0.1-0.5% OLE supplementation is suitable to support common carp intestinal health.

Article history:
Received 31 January 2020
Accepted 17 March 2020
Available online 25 April 2020

Keywords:
Common carp
Olive leaf
Intestine
Health

Introduction

Aquaculture is an important industry supplying human foods and Common carp, *Cyprinus carpio*, is one of the most common aquaculture reared species (Yousefi et al., 2019). One of the critical issues in aquaculture is fish health deterioration under artificial conditions, which leads to fish susceptibility to diseases. Therefore, several researches have been conducted to find methods for boosting fish health. In this case, dietary supplementation with herbal materials have gained great attention, because the herbal materials are natural antioxidant and most of them stimulate fish immune system (Chakraborty and Hancz, 2011; Chakraborty et al., 2014).

There are several studies on common carp, showing dietary herbal material supplementation, including basil, *Ocimum basilicum* extract, palm fruit extract and ginger, significantly increased fish immune and health (Amirkhani and Firouzbakhsh, 2015; Hoseinifar et al., 2015, 2017; Fazelan et al., 2020). Among the herbal extracts, olive leaf extract (OLE) has recently gained attention as feed additives in aquaculture. Administration of OLE to fish diet significantly up-regulated tumor necrosis factor alpha (TNFa) and interleukin 1 beta (IL1b) along with higher resistance against bacterial challenge (Baba et al., 2018; Zemheri-Navruz et al., 2019). Therefore, it might be used as feed supplements in common carp to boost the fish health.

Fish intestine is an important organ, involving in both nutrient absorption and fish health. Fish intestine is directly connected to ambient water, thus is under the threat of ambient pathogens (Jutfelt, 2011). Therefore, fish must have healthy and high-immunity intestine. There are several studies showing herbal materials are capable to augment fish intestinal health. For example, dietary supplementation with guava, *Psidium guajava*, leaf extract (Giri et al., 2015),

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DOI: https://doi.org/10.22034/ijab.v8i2.839

Table 1. Composition of diets (%) used in this study.

	Control	OLE0.1	OLE0.5	OLE1
Soybean meal	17	17	17	17
Fish meal	16	16	16	16
Poultry meal	15	15	15	15
Wheat meal	38.1	38	37.6	37.1
Wheat gluten	10	10	10	10
Fish oil	1	1	1	1
Soybean oil	1	1	1	1
Phytase	0.5	0.5	0.5	0.5
Lysine	0.6	0.6	0.6	0.6
Methionine	0.3	0.3	0.3	0.3
Mineral mix	0.25	0.25	0.25	0.25
Vitamin mix	0.25	0.25	0.25	0.25
OLE	0	0.1	0.5	1
Dry matter	90.8	91	90.6	91.1
Protein	39.3	39.2	39.1	39.2
Lipid	8.87	8.81	8.78	8.91
Ash	6.21	6.22	6.18	6.20
Energy (kCal/kg)	3831	3831	3831	3831

jujube, *Ziziphus jujube*, extract (Hoseinifar et al., 2018a), raffinose (Karimi et al., 2020), beta-glucan (van der Marel et al., 2012) and pectin (Edirisinghe et al., 2019) were found to augment intestinal TNFa, IL1b, lysozyme (LYS), and mucin (MUC) gene expression. Accordingly, the aim of the present study was to investigate the effects of dietary OLE administration on intestinal gene expression in common carp. For this, the expression of TNFa, IL1b, LYS, and MUC2 were studied, as they are important genes in immune responses in fish (Karimi et al., 2020).

Materials and Methods

OLE preparation: OLE extraction was performed according to Sarhadi et al. (2020) with some modifications. Olive leaves were dried against a fan blow for one week. After pulverizing, the leaves were mixed with 70% ethanol with 1:5 proportion and remained at room temperature for a week. Then, the mixture was passed through a filter and filtrates were concentrated by a freeze-drier (Beta LDpluse, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) 72 h (-50ºC).

Experimental protocol: Four diets were used in this experiment, namely control, OLE-0.1, OLE-0.5 and OLE-1 according to Fazelan et al. (2020) (Table 1). Common carp (~15 g) were stocked in 12 tanks (100 L) at a density of 10 fish per tank. The fish were fed the control diet for one week to acclimate with the experimental conditions. Then the fish were fed control, OLE-0.1, OLE-0.5 or OLE-1 diets for eight weeks based on 2% of biomass per day. The fish intestine samples were collected after one and eight weeks of rearing for intestinal gene expression analysis. Water temperature, dissolved oxygen, pH and ammonia were measured by Hach HQ40d (Loveland, Colorado, USA) and Wagtech photometer (7100, Berkshire, UK), being 22.9±0.55°C, 6.21±0.78 mg/L, 7.15±0.24 and 0.28±0.03 mg/L, respectively.

For intestine sampling, two fish were caught from each tank and anesthetized by eugenol (100 mg/L) (Yousefi et al., 2018). Then the fish were euthanized by spinal cord cutting, followed by cutting the posterior part of the intestine. The samples were immediately frozen in liquid nitrogen and transferred to -70ºC freezer.

The RNX-plus extraction kit (Sinagene, Iran) was used for total RNA extraction from the kidney samples according to the kit instructions. The primer sets for quantification of mRNA levels of the selected genes were designed based on the common carp sequences found in Gen Bank (Table 2). The Oligo 7 program was used for designing the primers. The SYBR green

Table 2. Sequences of the used primers in this study.

Gene name	Primer sequences	Accession number/Reference
Beta-actin	F: CCTGTATGCCAACACCGTGCTG R: CTTCATGGTGGAGGGAGCAAGG	JQ619774.1
IL1b	F: ACCAGCTGGATTTGTCAGAAG R: ACATACTGAATTGAACTTTG	AB010701.1
TNFa	F: GGTGATGGTGTCGAGGAGGAA R: TGGAAAGACACCTGGCTGTA	AJ311800.1
LYS	F: GTGTCTGATGTGGCTGTGCT R: TTCCCCAGGTATCCCATGAT	AB027305
MUC2	F: TGACTGCCAAAGCCTCATTC R: CCATTGACTACGACCTGTTTCTC	van der Marel et al. (2012)

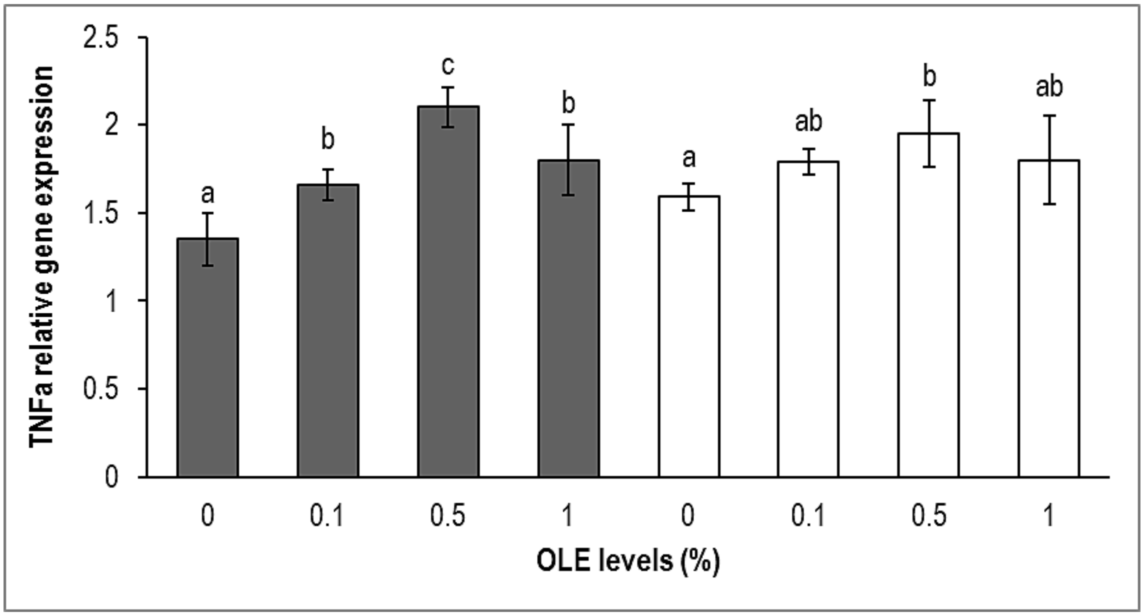

Figure 1. Effects of different levels of dietary OLE supplementation on intestinal TNFa gene expression, after one (gray bars) and eight (white bars) weeks. Different letters above the bars show significant difference among the treatments at each sampling time.

method was followed for determination of relative expression of the selected genes using Real-time PCR analysis as described by Karimi et al. (2020).

Statistical analysis: Data normal distribution and variance homogeneity were confirmed by Shapiro-Wilk and Levene tests (Abtahi et al., 2013). Then, the data were analyzed by two-way ANOVA (sampling time and OLE levels as factors); as there were interaction effects of the factors on tested parameters, the data were reanalyzed by one-way ANOVA and Tukey tests. All data were analyzed in SPSS v.22 and expressed as mean±SD.

Results

All OLE-treated fish showed up-regulated TNFa expression after one week, with the highest value in OLE-0.5 treatment. However, after eight weeks, only OLE-0.5 treatment had significantly higher gene expression compared to the control treatment (Fig. 1). Compared to the control group, the OLE-0.1 and OLE-1 showed up-regulated IL1b expression after one week. All OLE-treated fish showed up-regulated IL1b expression after eight weeks, and the highest value was recorded in the OLE-0.5 (Fig. 2).

There was no significant difference in LYS gene expression among the treatments after one week. Whereas OLE-0.1 and OLE-0.5 treatments had a significantly higher LYS gene expression compared to the control treatment after eight weeks (Fig. 3). Those of OLE-0.1 and OLE-1 had significantly lower MUC2 expression compared to the control treatment after one week. The OLE-treated fish had significantly higher

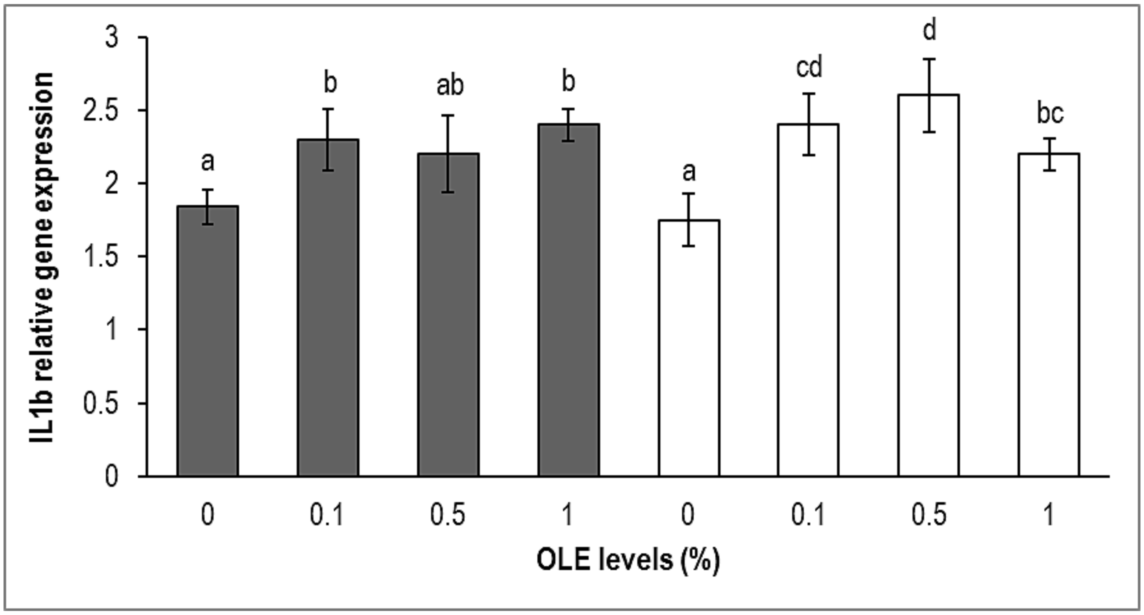

Figure 2. Effects of different levels of dietary OLE supplementation on intestinal IL1b gene expression, after one (gray bars) and eight (white bars) weeks. Different letters above the bars show significant difference among the treatments at each sampling time.

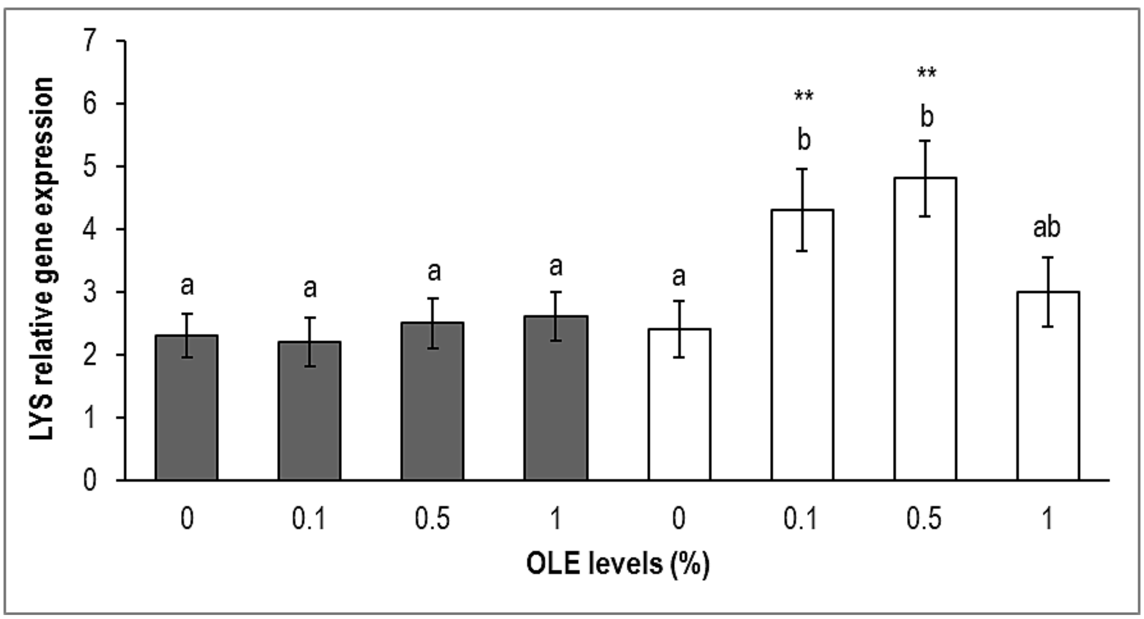

Figure 3. Effects of different levels of dietary OLE supplementation on intestinal LYS gene expression, after one (gray bars) and eight (white bars) weeks. Different letters above the bars show significant difference among the treatments at each sampling time. Asterisks show significant difference between the sampling times.

MUC2 expressions after eight weeks (Fig. 4).

Discussions

TNFa is a pro-inflammatory cytokine with important role in cell proliferation and differentiation (Hoseinifar et al., 2019). It stimulates IL1b production, which acts as cell proliferator and apoptotic (Yarahmadi et al., 2016). These two cytokines have remarkable role in immune response signaling. Up-regulation of these genes indicates boosted immune strength in the present study. The present results are in line with previous studies on OLE administration to fish. Baba et al. (2018) reported elevated TNFa and IL1b gene expressions in rainbow trout spleen, along with higher resistance against yersiniosis. Moreover, intestinal transcriptome modulation by other herbal materials has also been reported. Giri et al. (2015) reported that dietary

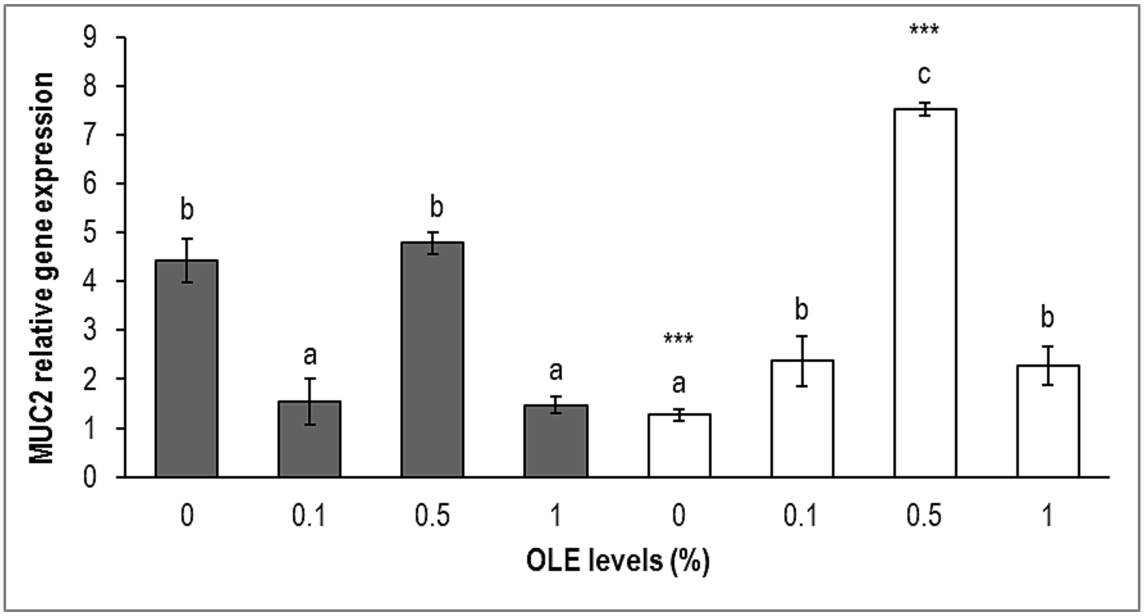

Figure 4. Effects of different levels of dietary OLE supplementation on intestinal MUC2 gene expression, after one (gray bars) and eight (white bars) weeks. Different letters above the bars show significant difference among the treatments at each sampling time. Asterisks show significant difference between the sampling times.

supplementation with guava leaf extract significantly up-regulated TNFa and IL1b expression in intestine of rohu carp, *Labeo rohita*. Hoseinifar et al. (2018a) found that dietary supplementation with jujube fruit extract significantly up-regulated the cytokines gene expression in common carp intestine.

LYS is a bactericidal enzyme, effective against gram-positive bacteria (Saurabh and Sahoo, 2008). Elevation of LYS benefits the host to better react to bacterial infections. The present results indicated that intestinal LYS is not sensitive to short-tern-OLE administration; however, long-term administration improves LYS gene expression. There are several studies reporting up-regulation of intestinal LYS gene expression by dietary herbal material administration. Karimi et al. (2020) reported that dietary raffinose administration significantly up-regulated intestinal LYS gene in common carp. Similar results were observed in common carp, treated with jujube fruit extract (Hoseinifar et al., 2018a).

Intestinal mucus is a complex fluid and MUC is its main component. MUC is filamentous and highly-glycosylated glycoprotein, with high adherence capacity that play important roles in intestinal defense (Edirisinghe et al., 2019). The short-term OLE treatments significantly down-regulated intestinal MUC2 gene expression, which needs further studies to find the exact reasons. However, long-term OLE administration clearly up-regulated MUC2 gene expression, which is indication of higher immune strength of the fish intestine. Previous study on common carp showed that dietary beta-glucan administration significantly up-regulated MUC5, but not MUC2, gene expression in the fish gill (van der Marel et al., 2012). Moreover, dietary pectin administration significantly up-regulated whole body MUC5, but not MUC2, gene expression in zebrafish, *Danio rerio* (Edirisinghe et al., 2019). Such differences among the studies might be due to different studies tissues, which need further studies for clarification.

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