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Bio-functions of carvacrol-supplemented feeds on lipopolysaccharide-induced rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792)

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Abstract

To investigate the effects of carvacrol supplementation in rainbow trout induced by lipopolysaccharide (LPS), relative gene expressions levels of some pro-inflammatory cytokines and apoptosis markers in kidney and liver were measured. Fish with a mean weight of 44.71±1.33 g were studied using four different treatments with three replicates each. Inflammation and apoptosis were performed using LPS of *Escherichia coli* (25 µg ml^{-1}) except in the control group and only carvacrol (100 µg ml^{-1} diet) containing diets were fed to this group (CAR). The last group was the infected fish fed carvacrol supplemented diet (+CAR). Kidney and liver tissues were removed 3 days after to determine the levels of interleukin-1β (IL-1β), interferon gamma (INF-γ), caspase 3 (Cas 3), caspase 8 (Cas 8) using Real-Time PCR analyses. IL-1β expressions of both kidney and liver was significantly decreased (12.9 and 2.14 fold, respectively) in LPS treated cells (p<0.05). While IFN-γ expression was up regulated in kidney, it had down regulation in liver. LPS decreased both Cas 3 and 8 expressions in kidney but increased in liver. +CAR increased expressions of IL-1β and IFN-γ compared with the control (up to 2-3 fold) in all tissues except caspase gene expressions that were similar in the control. In all tissues, IL-1β and IFN-γ expressions increased in +CAR group, expect IFN-γ in the kidney. However, Cas 3 and 8 expressions including apoptosis was induced by up-down regulations in all tissues compared to LPS-injected fish. The results showed that carvacrol had pro-inflammatory and apoptotic effects especially on liver tissue of LPS-induced inflammation model of *Oncorhynchus mykiss*.

Keywords: Carvacrol, Caspases, Inflammatory cytokines, LPS, Rainbow trout

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Introduction

Diseases caused by pathogenic microorganisms in fish culture have limited the quality of production. Antibiotics have been used for many years to effectively treat bacterial diseases in fish. However, because of the limitations to antibiotic use (antimicrobial-resistant bacteria and resistance genes and antimicrobial residues), scientists have recently focused on natural antibacterial and antioxidants (e.g.: medicinal herb oil) as alternatives to antibiotic treatments (Akhtar *et al*., 2014; Kucukgul *et al*., 2013a, b).

Carvacrol [2-methyl- 5-(1-methylethyl) phenol] and thymol (2-isopropyl-5- methylphenol) are major components of oregano and thyme essential oils from medicinal herbs. Several studies have reported antimicrobial activities of carvacrol in many infected fish (Hellander *et al*., 1998; Rattanachaikunsopon and Phumkhachorn, 2010; Giannenas *et al*., 2012). Bacterial lipopolysaccharide (LPS) is one of the most common molecular structures of pathogenic microorganisms. It is the major constituent of the external layer of the outer membrane of Gram-negative bacteria and is often involved in disease processes (Paterson and Fryer, 1974; Baba *et al.*, 1988).

Inflammation is a complex process initiated by several factors ranging from bacterial infection and chemical injury to environmental pollution that result in cell injury or death (O'Byrne *et al*., 2000; O'Byrne and Dalgleish, 2001). Tissue injury induced by this trauma results in the release of inflammatory mediators including the cytokines and tumor necrosis factor (TNF-α), interleukin-1 (IL-1) from leukocytes, monocytes and macrophages (Paterson *et al*., 2003). IL-1β been identified in a number of fish species such as rainbow trout (Zou *et al*., 1999), carp (Fujiki *et al*., 2000), sea bass (Scapigliati *et al*., 2001). IL-1β is one of the earliest expressed pro-inflammatory cytokines and plays an important role in bacterial and viral infections (Blum and Miller, 2000). IL-1β and IFN-γ can be stimulated by regulations in response to various stimuli, such as LPS (Dao *et al*., 1998; Zou *et al*., 1999, Scapigliati *et al*., 2001; Lee *et al*., 2006). In addition, pro-inflammatory cytokines can cause diverse host responses including apoptosis. Apoptosis known as programmed cell death is a highly conserved process used by multicellular organisms to eliminate those cells that are either unnecessary or potentially injurious to the host (Jacobson *et al*., 1997). Apoptosis can occur both under physiological and pathological events, also in acute inflammation. Previously published data show the up- or down-regulations of apoptotic caspase expressions studied in a number of fish species (Sepulcre *et al*., 2007; Mary Lini *et al*., 2013).

However, only a few studies have investigated the studies relative to pro-inflammatory infected fish. The aim of the present study is to assess the effects of carvacrol supplementation diets on mRNA *gene* expression of rainbow trout induced by LPS.

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Materials and methods

Animals

Rainbow trout, *O. mykiss* (Walbaum, 1792) was used for this study. The fish were obtained from a local fish farm near Tunceli Province, Turkey. Experimental fish were 44.71±1.33 g in mean weight±SD. During the study, water temperature, pH, and dissolved oxygen values were maintained at 15.5 °C, 8.01, 9.57 mg L^{-1}, respectively.

The experimental diets and design

100 µg mL^{-1} of carvacrol was sprayed on 1 kg of commercial diet of trout to prepare the experimental diet (Zheng *et al*., 2009; Hashiem and Abd El-Galil, 2012). The study included four trial groups, the first group was the control, the second group included fish-infected with *E. coli* LPS (25 µg mL^{-1}) that fed a control diet (commercial trout diet). While in the third group fish were only fed with the experimental diet, and the last group was created with infected fish fed the experimental diet for 3 days (Harun *et al*., 2008; Raida and Buchmann, 2009).

Total RNA isolation, cDNA synthesis and real-time PCR

QRT-PCR analysis was performed in a qPCR system (Bio-RAD, CFX96 Touch Real- Time PCR, South Korea). Total RNA from fish tissues were extracted by using TRIZOL reagent (Sigma, USA) according to the manufacturer's instructions. 50 ng of total RNA was taken from each sample and cDNA synthesis and PCR analysis were performed by using Verso Sybr Green 1 step qRTPCR kit (Thermo Sci. Coo. /EU). One microliter of each cDNA was used as templates for amplification using SYBER Green PCR amplification reagent and gene-specific primers. Temperature was maintained at 50 °C for 15 min and then at 95°C for 15 s for cDNA synthesis. PCR 40 cycles were completed at 95 °C (15 s), 60 °C (30 s) and 72 °C (30s) and then melt curve analysis was carried out to confirm a single PCR product per reaction. The fish primer sets were obtained from Ella Biotech Gmbh (USA): The specific primers for IL-1β, IFN-γ, Caspase 3 and 8 gene designs and GenBank IDs are shown in Table 1.

Table 1: QPCR primers and GenBank IDs.

Target genes	1) Forward primer (5'-3') 2) Reverse primer (5'-3')	GenBank ID or reference
IL-1β	CCG ACT CCA ACT CCA ACA CTA TTG CTG GAG AGT GCT GTG GAA GAA	AY617117
IFN-γ	TCA CTG TCC TCA AAC GTG GCT GTT CAA CGG AAA ACC TGT TT	AJ841811
Cas-3	TTT GGG AGT AGA TTG CAG GG TGC ACA TCC ACG ATT TGA TT	TC172513
Cas-8	CAG CAT AGA GAA GCA AGG GG TGA CTG AGG GGA GCT GAG TT	TC172513
GAPDH	TCC TCG ATG CCG AAG TTG TCG ATG TCA GAC CTC TGT GTT GG	AF027130

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Data analysis

The threshold cycle (Ct) values for the transcripts are normalized to **GAPDH** by subtracting the average Ct value for each treatment (Pfaffi *et al*., 2002). Each PCR reaction was performed in triplicate. mRNA transcription values were determined as down- or up regulated for each gene.

Results

In the kidney, expression of IL-1β was significantly decreased (by 12.9 fold) in LPS treated as compared with the control. However, +CAR caused an increase in IL-1β expression by 6.66 fold in comparison to LPS. While IFN-γ expression was increased by 4.82 fold for the LPS-treated group, it decreased by 3.13 fold with CAR application as compared with the control. +CAR group compared to LPS reduced the expression of IFN-γ by 120.5 fold. LPS treatment resulted in downregulation of Cas 3 and 8 genes by 3.24 and 4.11 fold, respectively as compared with the control group. +CAR decreased the expression of Cas 3 by 2.38 fold and increased Cas 8 expression by 3.37 fold in comparison to LPS (Fig. 1).

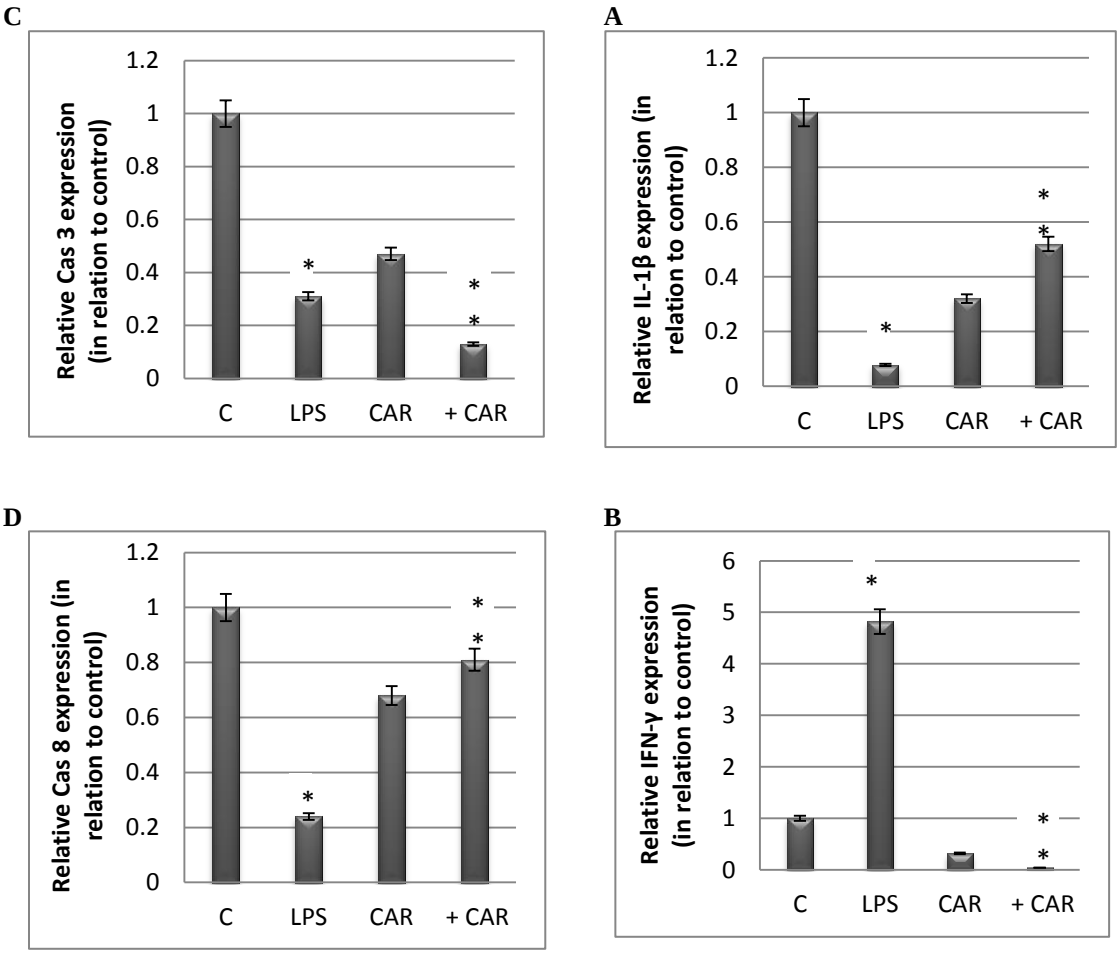

Figure 1: Relative to pro-inflammatory (IFN-γ and IL-1β, A-B) and apoptosis *g*ene expressions (Cas 3 and 8, C-D) of kidney. Shown the bars as *: versus control, ** versus LPS groups.

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In the liver, the LPS-treatment reduced IL-1β expression by 2.14 fold. However, CAR up regulated this gene expression by 2.01 fold as compared with the control group. +CAR increased IL-1β expression by 3.93 fold as compared with the LPS-infection group. IFN-γ expression was down regulated by LPS treatment by 2.87 fold and CAR increased the expression of IFN-γ by 3.22 fold as compared with the control group. +CAR increased IFN-γ expression by 1.53 fold compared with the LPS group. LPS increased both Cas 3 and Cas 8 levels by 4.0 and 12.81 fold, respectively in relation to the control group. CAR alone increased the expression of Cas 3 (6.19 fold), but it decreased Cas 8 expression (1.45 fold) as compared with the control. While +CAR increased the expression of Cas 3 by 1.06 fold, it reduced Cas 8 expression by 3.01 fold in comparison with LPS (Fig. 2).

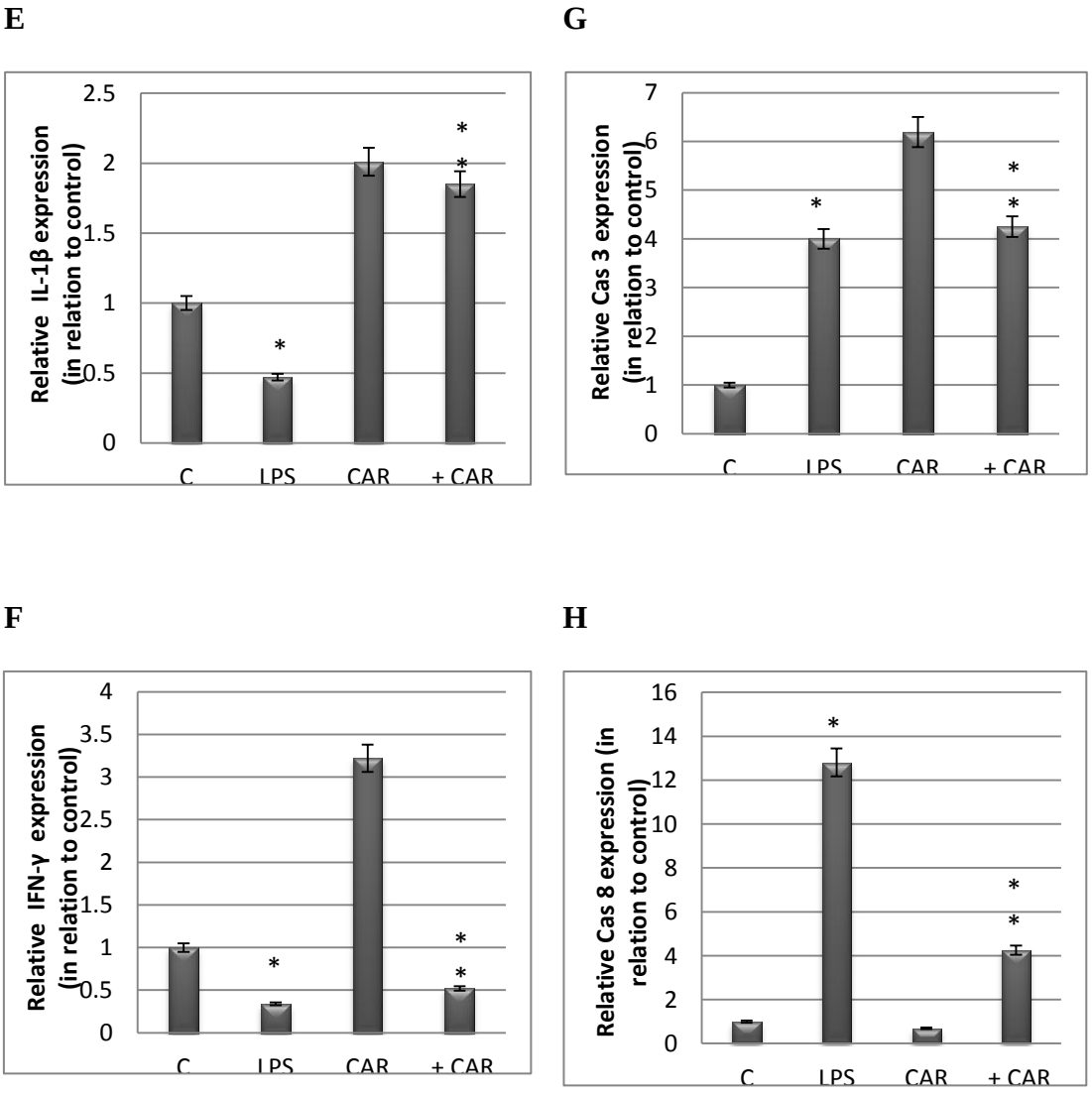

Figure 2: Relative to pro-inflammatory (IFN-γ and IL-1β, E-F) and apoptosis *g*ene expressions (Cas 3 and 8, G-H) of liver. Shown the bars as *: versus control, ** versus LPS groups.

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Discussion

Antibiotics have long been used in aquaculture to treat fish diseases. However, the use of antibiotics is becoming limited due to potential side effects on animals. Therefore, phytogenic compounds have been widely recognized as potential alternatives to antibiotics. Carvacrol and thymol are the two main active components of oregano essential oils. Some phytogenic compounds are known to have antimicrobial, antiviral, antifungal and anti-oxidative properties (Reichling *et al*., 2009). Recently, there has been an increased interest in fish fed diets containing compounds such as carvacrol or thymol (Giannenas *et al*., 2012; Volpatti *et al*., 2012; Kucukgul *et al*., 2013a; Kucukgul *et al*., 2013b). Our data indicated that carvacrol has a strong antimicrobial activity as mentioned in other findings.

Pro-inflammatory fish cytokines like interleukins (IL) and interferons (INFs) are induced in the presence of LPS and gram-negative bacteria that play an important role in diverse host responses, including cell proliferation, differentiation, necrosis and apoptosis (Secombes *et al*., 2001; Peddie *et al*., 2002). Rainbow trout and carp IFNγ have functional properties including the ability to enhance phagocytosis of bacteria in macrophages (Zou *et al*., 2005; Arts *et al*., 2010). Many of the effector roles of IL-1β are mediated through the up- or down-regulation of the expression of other cytokines and chemokines (Dinarello, 1997). Ocaña and Reglero (2012) studied inflammatory effects of thyme extracts from three different species on cytokine production and gene expression. They reported that TNF-α, IL-1B, IL-6, and IL-10 gene expression changes were dose dependent and thyme extracts had anti-inflammatory effects. In a previous study, we found downregulations in levels of IL-1ß and TNF-α expression on *Yersinia ruckeri*-infected fish after 3 days, but upregulation in essential oil application of injected fish (Kucukgul *et al*., 2013a). The results of the present study are in concordance with the data observed previously. The observations of Furnes *et al*. (2009) suggest that *IFN-γ* gene expression up-regulates in cod head kidney 24 h after injection of *Vibrio anguillarum*-inactivated formalin. The upregulation of *IFN-γ* in the kidney observed in our study was later (3 days after), but similar to the work reported by Furnes *et al*. (2009) although the time was different. The later upregulation might be because of dose differences or that the bacterium itself might have mechanisms to down-regulate or at least delay the gene expression.

Apoptosis is a well-known mechanism of programmed cell death. Several proteins produced by pathogenic bacteria or bacterial components, such as lipopolysaccharide (LPS), are capable of specifically initiating apoptosis in macrophages that play an important role to activate pro-apoptotic signaling pathways indicating caspase activation (Navarre and Zychlinsky, 2000; Fukui *et al*., 2008). In a study reported by Mary Lini *et al*. (2013) who indicated that an increase in the expression of caspase 3 from 0 to 6

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h in the gills, liver and kidney of infected *Labeo rohita* and *Aeromonas hydrophila* and returned to its initial level after 24 h. A similar observation was reported by Sepulcre *et al*. (2007) who found that *V. anguillarium* evades the immune response of the bony fish sea bass (*Dicentrarchus labrax*) through the down regulation of apoptotic caspases. Our data obtained indicated that infection of LPS resulted in the down regulation of the apoptotic caspases (Cas 3 and 8) in the kidney, but an upregulation in the liver. Feeding carvacrol to LPS-injected fish (+CAR) showed decreases in caspases in the kidney. We observed that there was a decrease only in caspase-3 levels of +CAR in the liver. Compared to the data reported by Mary Lini *et al* (2013), up-regulation of caspases in the liver was similar to our results. This suggests that apoptosis induction in LPS-infected tissues could be linked to the activation of caspases.

In conclusion, fully understanding the modulation of the immune system (including inflammation, apoptosis and infection) of diet supplementation in infected fish is important to develop alternatives to antibiotics. The data obtained indicated that both infection of LPS and carvacrol supplementation significantly stimulated the expression of all genes, which had both pro-inflammatory and apoptotic effects on tissue of *O. mykiss*.

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