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Growth parameters, innate immune response and resistance to *Listonella* (*Vibrio*) *anguillarum* of *Dicentrarchus labrax* fed carvacrol supplemented diets

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Abstract

The research was aimed to assess the effect of dietary carvacrol (0.025% and 0.05%) on sea bass (*Dicentrarchus labrax*) growth, immune response and resistance to *Listonella anguillarum*. Fish (69.2 ± 0.22 g) were fed the experimental diets for 9 weeks. Dietary carvacrol did not negatively affect fish survival, growth performance, feed intake and feed conversion ratio ($P > 0.05$) nor carcass yield and viscerosomatic, hepatosomatic and mesenteric fat index ($P > 0.05$). Serum and head kidney leucocytes were collected after 1, 4 and 8 weeks of feeding. Carvacrol significantly reduced serum proteins, immunoglobulins and lysozyme activity ($P < 0.01$) and moderately increased phagocytosis and pinocytosis of head kidney macrophages. The release of reactive oxygen species by leucocytes was reduced in carvacrol-fed fish, even if significantly ($P < 0.05$) only in those fed 0.05% carvacrol for 1 week. Dietary carvacrol did not significantly affect the aspecific immune response, although a potential antioxidant activity might be speculated. Moreover, feeding carvacrol provided an appreciable resistance to a challenge with *L. anguillarum*, when a bacterial dose lower than the Lethal $Dose_{50}$ was used. Cumulative mortality in fish fed 0.025% carvacrol was significantly lower than that of untreated controls (75% Relative Per cent Survival).

Keywords: *Dicentrarchus labrax*, carvacrol, phytotherapy, immune response, *Listonella anguillarum*, disease resistance

Introduction

The increasing need to guarantee food safety is promoting research directed towards the use of natural products to replace chemically synthesized antibiotics and growth enhancers. As in other fields of animal-rearing, phytoadditives obtained from medicinal herbs are also of potential use in aquaculture. Plants could represent a promising alternative to traditional drugs, as they provide bioactive molecules and at the same time are commercially available, inexpensive and biocompatible (Mohamad & Abasali 2010). Although they have a large spectrum of use, their mechanisms of action are still not fully understood and their possible use in aquaculture has only recently gained attention (Jeney, Yin, Ardò & Jeney 2009; HariKrishnan, Balasundaram & Heo 2011).

Among the possible biomolecules that are available, carvacrol (2-methyl-5-(1-methylethyl)-phenol), a major component (70–80%) of the essential oils of oregano and thyme (family *Labiatae*) (Burt 2004; De Vincenzi, Stammati, De Vincenzi & Silano 2004), represents one interesting candidate.

Generally recognized as a legally registered food additive by the Council of Europe (2000), the FAO/WHO Committee on Food Additives (2001) and the U.S. Food and Drug Administration (2010), carvacrol is used as a flavouring agent in baked goods, sweets and beverages (Kiskó & Roller 2005; Xu, Zhou, Ji, Pei & Xu 2008)

The dietary addition of carvacrol-rich oregano oils to the diets of farmed animals (CRINA® Poultry and Pigs; DSM Nutritional Products Ltd, Basel,

Switzerland) has been proposed to improve their growth performances and health (Baser 2008). Carvacrol-containing essential oils are biostatic and/or biocidal against many bacteria, yeast and fungi *in vitro,* and consequently have attracted considerable attention as potential food preservatives (Burt 2004; Zhou, Ji, Zhang, Jiang, Yang, Li & Yan 2007). The biocidal action of carvacrol on bacteria is similar to that of other phenolic compounds and occurs via membrane damage, resulting in an increase in membrane permeability to protons and potassium ions, depletion of the intracellular ATP pool and disruption of the proton-motive force (Helander, Alakomi, Latva-Kala, Mattila-Sandholm, Pol, Smid, Gorris & Wright 1998; Ultee, Kets & Smid 1999; Ultee, Bennik & Moezelaar 2002).

Like other phenolic compounds, carvacrol exhibits a range of biological activities, demonstrated by *in vitro* or *in vivo* (animal model) experiments, as recently reviewed by Baser (2008). It inhibits the release of reactive nitrogen and oxygen species that are toxic to the animal, it reduces platelet aggregation, it acts as an anti-inflammatory by inhibiting COX-1 and COX-2, and it is an anti-mutagenic and anti-tumoral agent.

The information concerning its role as an immunostimulant is rather controversial. Experiments conducted in swine underline that dietary carvacrol promotes the proliferation of $CD4^+$, $CD8^+$ and MHC-II^+ cells (Walter & Bilkei 2004), whereas other authors observed that swine circulating leucocytes are apparently unaffected by carvacrol treatment (Nofrarias, Manzanilla, Pujol, Gilbert, Majo, Segales & Gasa 2006; Bimczock, Rau, Sewekow, Janczyk, Souffrant & Rothkotter 2008).

Until now, carvacrol administration in fish species has been poorly documented. When a 0.05% carvacrol-based diet was fed to catfish for 8 weeks, it promoted fish growth, dismutase and catalase antioxidant activity in plasma, as well as fish resistance to a challenge with *Aeromonas hydrophila* (Zheng, Tan, Liu, Zhou, Xiang & Wang 2009). Dietary carvacrol supplementation, when fed prophylactically for 2 weeks (0.02% level) to tilapia, decreased the mortality of fish challenged with *Edwardsiella tarda* (Rattanachaikunsopon & Phumkhachorn 2010). These preliminary findings suggest that carvacrol could be a useful dietary supplement to promote growth and disease resistance in fish, but further work is necessary to understand its role as an immunostimulant and/or antioxidant in fish.

In this study, different dietary carvacrol levels (0.025% and 0.05%) were administered to European sea bass (*Dicentrarchus labrax*), an important species in Mediterranean marine aquaculture. Key factors of growth performance and immune response were measured during the experiment. Subsequently, fish were intraperitoneally challenged with *Listonella anguillarum* to determine disease resistance.

Materials and methods

Diets

A fish meal-based complete feed including organic ingredients was prepared to be used as a control (Diet CTR) using MSC fish meal (Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, 27472 Cuxhaven, Germany) as the major protein source, wheat gluten meal [100 g kg^{-1}; Sacchetto SpA, 12030 Lagnasco (CN), Italy], soybean meal (80 g kg^{-1}; F.A. ZOO Mangimi srl 61020 Chiusa di Ginestreto, Pesaro, Italy), wheat meal [120 g kg^{-1}; Molino Di Giusto & C. S.n.c., Reana del Rojale (UD), Italy] and fish oil (Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG). The composition and proximate composition analyses of the experimental diet are shown in Table 1 (i.e. crude protein 51.3% DM, crude lipid 16.8% DM).

Carvacrol (5-isopropyl-2-methylphenol) (cod. 282197; Sigma-Aldrich, Milan, Italy) was diluted in fish oil and added to the basal CTR diet at two different levels 0.025% and 0.05%. The levels were based on experimental trials in catfish (Zheng *et al.* 2009), tilapia (Rattanachaikunsopon & Phumkhachorn 2010) and gilthead sea bream (J. Malvisi, unpubl. res.). However, the calculated total ingested amount was lower than the LD_{50} of carvacrol reported for guinea pig (810 mg kg^{-1}; Sigma-Aldrich safety sheet) at the end of the trial. All diets were pelleted through a 4.0 mm dye, dried overnight at 35°C in a fluid bed drier, then stored at 3°C until used.

The diets were analysed for proximate composition according to AOAC (1998). Moisture was determined by oven-drying at 105°C until constant weight (method 934.01); crude protein was determined as Kjeldahl N x 6.25 (method 940.25) using an Auto Kjeldhal distillation System [Buchi K-355; BÜCHI Italia s.r.l 20090 Assago (MI) Italy] after acid digestion (Buchi K-424, BÜCHI Italia s.r.l); total lipid content was determined using ether

Table 1 Composition and proximate analysis of the basal diet (CTR)

Composition (g kg^{-1})	
MSC Fish meal	548
Wheat gluten meal	100
Soybean meal mech. extracted	90
Wheat Meal	135
MSC Fish oil	104
Mineral mix*	10
Vitamin mix†	10
Binder (CMC)	3
Proximate analysis (% DM)	
Moisture	8.5
Crude protein	51.3
Crude lipid	16.8
Ash	15.9

*Composition of the mineral mix (% Mix): $CaHPO_4 \cdot 2H_2O$, 78.9; NaCl, 17.65; MgO, 2.725; $FeCO_3$, 0.335; KI, 0.005; $ZnSO_4 \cdot H_2O$, 0.197; $MnSO_4 \cdot H_2O$, 0.094; $CuSO_4 \cdot 5H_2O$, 0.027; Na Selenite, 0.067.

†Composition of the vitamin mix (g kg^{-1} diet): Tiamine HCl Vit B1, 20; Riboflavine Vit B2, 26; Piridoxine HCl Vit B6, 20; Cianocobalamine B12, 5.2; Niacine Vit PP, 156; Pantotenate Ca, 26; Folic Acid, 3; Biotine Vit H, 26; Myoinositol, 258; stay C Roche, 295; α-tocoferol Vit E, 103; Menadione Vit K3, 58; Vit A (2500 UI kg^{-1} diet) 1.3; Vit D3 (2400 UI kg^{-1} diet) 2.5; choline clorure, 8.

extraction using a Soxtec System Ser 148/6 (VELP Scientifica srl, 20040 Usmate, MB, Italy) after acidic hydrolysis of the sample with hydrochloric acid (method 948.15) and ash content was measured after samples combustion in a muffle furnace at 515°C until constant weight (method 938.08).

Fish and experimental conditions

European sea bass (*D. labrax*) juveniles, obtained from a commercial hatchery (Agroittica Toscana spa, Piombino, LI, Italy), selected to be uniform in size and free from grossly discernible anatomical malformations, were randomly allotted in 250-L fibreglass tanks being part of an indoor partially recirculating marine water system (14 m^3 total volume, 5% daily water renewal, 12 h artificial daylength, 400 lux light intensity), provided with thermostatic control of water temperature, mechanical sand filter, bio-filter and UV lamp apparatus.

Fish were fed Diet CTR and adapted over 3 weeks to the experimental conditions. At the end of this preliminary period, under or oversized specimens were removed to limit the size variation within fish group. Nine fish groups of 21 fish per tank (average individual weight, 69.2 ± 0.22 g) were assigned to the three diets according to a complete random design with triplicate units per dietary treatment. Seventy fish per diet were maintained under the same experimental conditions to be submitted to the challenge test. Fish were kept under veterinary control during the trial and any clinical sign of disease was registered. The fish were sedated during handling (i.e. for sampling and challenge) and the rearing/experimental procedures were performed in such a way so as to minimize suffering and pain. The number of fish used was kept to a minimum where possible.

The feeding trial lasted 9 weeks and the fish were hand-fed to satiation in two daily meals. Feed intake and mortality per group were recorded on a daily basis. Fish were group-weighed every 3 weeks, after a 24-h fasting and under moderate anaesthesia (0.03 g L^{-1} benzocaine; Sigma-Aldrich). Water quality was monitored twice a week throughout the experiment and all parameters were maintained at optimal values for sea bass (temperature, 20.4 ± 1.6°C, salinity, 27.6 ± 3.3 ‰, dissolved oxygen, 6.1 ± 0.5 mg L^{-1}, pH, 7.3 ± 0.3, total ammonia nitrogen, 0.11 ± 0.08 mg L^{-1}, nitrite nitrogen, 0.09 ± 0.1 mg L^{-1}).

Sampling

At the end of the trial, three fish were randomly sampled from each group/tank, sacrificed with an excess of anaesthetic (Benzocaine; Sigma-Aldrich) and were subjected to linear biometry: total weight (TW) and, after dissection, viscera, liver, mesenteric fat, gutted carcass weights were recorded to calculate dressing out parameters.

At the end of the trial, growth performance was calculated as follows:

$$\text{Weight gain (WG)} = [\text{final body weight (FBW) (g)} - \text{initial body weight (IBW) (g)}] / \text{initial body weight (g)} \times 100$$

$$\text{Specific growth rate (SGR)} = [\ln(\text{final weight (g)}) - \ln(\text{initial weight (g)})] / \text{days of experiment} \times 100$$

$$\text{Feed conversion ratio (FCR)} = \text{feed intake (g)} / \text{weight gain (g)} \times 100$$

The dressing out parameters of the fish subjected to the dietary treatments were calculated as follows:

$$\text{Viscerosomatic index (VSI)} = \text{viscera weight (g)} / \text{whole body weight (g)} \times 100$$

$$\text{Hepatosomatic index (HSI)} = \text{liver weight (g)} / \text{whole body weight (g)} \times 100$$

$$\text{Mesenteric fat index (MFI)} = \text{mesenteric fat weight (g)} / \text{whole body weight (g)} \times 100$$

$$\text{Carcass yield} = \text{gutted carcass weight (g)} / \text{whole body weight (g)} \times 100$$

After 1, 4 and 8 weeks of feeding the experimental diets, serum was sampled from 10 fish per group/dietary treatment. Fish were anaesthetised with benzocaine (0.03 g L^{-1}), bled from the caudal vein and sacrificed by anaesthetic overdose. Blood was allowed to clot overnight at 4°C and centrifuged (2000 × *g* for 15 min). Sera were aliquoted and stored at −80°C for further analysis. Head kidneys were then aseptically removed ($N = 5$ per group; at 1, 4 and 8 weeks) from the same individuals previously submitted to blood sampling. Throughout the sampling session (about 1 h), organs were maintained at 4°C in sea bass-isosmolar Hank's balanced salt solution (HBSS) to carry out simultaneous purifications of leucocytes from individuals.

Serum total proteins and immunoglobulins

Serum proteins were determined using the method of Bradford (1976). Forty microlitres per well of serially diluted serum were distributed in 96-well microtitre plates and 200 μL per well of Bradford solution (Sigma-Aldrich) added. The absorbance was read at 595 nm. Bovine serum albumin was used as standard.

Immunoglobulins concentration was measured using the method of Klein and Siminovitch (1986) modified as follows. Immunoglobulins were precipitated with 10 000 kDa polyethylene glycol (PEG; Sigma-Aldrich). Serum (100 μL) was mixed with an equal volume of 12% PEG solution for 2 h at 20°C under constant shaking. After centrifugation at 5000 × *g* for 15 min, the supernatant was collected and the concentration of proteins determined as described above. The total immunoglobulins concentration was calculated by subtracting this value from the total serum proteins concentration.

Serum lysozyme activity

Serum lysozyme activity was measured using the turbidimetric method described by Parry, Chandan and Shahani (1965) adapted to microplates. As substrate, a 0.2 mg mL^{-1} suspension of lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich) in 0.067 M phosphate buffered saline (PBS pH 6.3) was used. Each serum sample (10 μL $well^{-1}$) was incubated in duplicate with 200 μL $well^{-1}$ of bacterial suspension and the decrease of turbidity was assessed throughout an incubation time of 1 h at 10-min intervals (540 nm). A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1}.

Leucocyte purification

Head kidney (HK) leucocytes were purified according to Galeotti, Volpatti, D'Angelo and Rusvai (1996). The tissues were gently pressed in HBSS and the resulting suspension, deprived from tissue debris, was enriched with 0.25% sodium salt heparin (5 KU mL^{-1}, Sigma-Aldrich, cod. H3149) and layered over a sea bass-isosmolar discontinuous Histopaque® (Sigma Aldrich, Milano, Italy) gradient (densities 1.119 and 1.077). After centrifugation (480 × *g* for 25 min at 4°C), cells layered between the densities mentioned above were collected, washed twice with HBSS, counted using Trypan blue exclusion method and suspended at the final concentration of 10^7 viable cells mL^{-1}.

Phagocytosis activity

This assay was based on the methods described by Seeley, Gillespie and Weeks (1990) and Jeney, Galeotti, Volpatti, Jeney and Anderson (1997). In duplicate, 250 μL of cell suspension (1×10^7 cells mL^{-1}) in HBSS were incubated with 500 μL of congo red stained yeast cells (2×10^8 cells mL^{-1}), for 1.5 h at 20°C. The phagocytosis activity was stopped by adding to the vial 1 mL of ice-cold HBSS. The cell/yeast suspension was layered onto 1 mL of Histopaque® 1.119, then the vials were centrifuged at 800 × *g* for 5 min at 4°C to separate phagocytes from free yeast cells. The phagocytes were harvested from the Histopaque-media interface and washed

with HBSS using centrifugation at 400 × *g*, 4°C, for 5 min. Pelleted cells containing yeasts were treated with 200 μL of 10× Trypsin-EDTA (Sigma-Aldrich, cod. T4174) overnight at 37°C, then the absorbance of the samples was determined by transferring the suspension in a microplate, and by reading at 510 nm (Sunrise, TECAN s.r.l. Milan, Italy) against a blank composed of an equal volume of 10× Trypsin-EDTA.

Respiratory burst activity

The production of reactive oxygen species (ROS) was measured using a microplate luminol based assay (Coteur, Warnau, Jangoux & Dubois 2002) partially modified. Ninety-six-well black microplates were coated (100 μL well^{-1}) with 20 μg mL^{-1} phorbol-12-myristate-13-acetate (PMA) in isosomolar HBSS. Control wells did not contain any stimulant. Subsequently, 2 mM luminol in HBSS was added (50 μL well^{-1}), followed by the purified leucocytes (50 μL well^{-1}, 10^7 cells mL^{-1}). Each sample was evaluated in triplicate, and the reading started immediately after the addition of luminol to the plates. The luminescent response was measured at 5-min intervals (T = 25°C), throughout a 60-min incubation time, using the luminometer Sunrise TECAN (integration time = 0.5 s, photomultiplier gain = 180). The ROS production was analysed in terms of both cumulative and kinetic response [relative luminescence units (RLU)/10^7 cells mL^{-1}].

Leucocyte peroxidase content

The peroxidase activity was assessed using a spectrophotometric method described by Salinas, Abelli, Bertoni, Picchietti, Roque, Furones, Cuesta, Mesenguer and Esteban (2008) in *Sparus aurata*, adapted by the authors to *D. labrax*. Briefly, 870 μL of cell suspension (1 × 10^6 cells mL^{-1}) in HBSS was incubated with 30 μL of 0,06% cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich, cod. 52369) in HBSS, for 5 min at room temperature. Each sample was evaluated in duplicate. Vials were then centrifuged at 400 × *g* for 10 min to separate cell debris from supernatant containing peroxidases. The supernatant was collected and transferred to microplate wells (150 μL/well). Twenty-five microlitre per well of tetramethylbenzidine-hydrocloride (TMB) solution and 25 μL well^{-1} 30% H_2O_2 were added to the microplate containing the supernatants under investigation. After an incubation of 1 min, the measurement of optical densities were preformed at 450 nm using Sunrise TECAN reader.

Pinocytosis activity

Pinocytosis was determined using the method of Mathews, Warinner and Weeks (1990) adapted to microplates by Skouras, Broeg, Dizer, Westernhagen, Hansen and Steinhagen (2003). Plates were filled with 75 μL well^{-1} neutral red solution in HBSS (23 mg L^{-1}) and 100 μL well^{-1} of cell suspension in HBSS (1 × 10^7 cells mL^{-1}). All samples were evaluated in triplicate. Control wells were included as blanks, composed of neutral red solution and HBSS without cells. After an incubation of 2.5 h at room temperature, plates were washed two times with 125 μL well^{-1} phosphate buffered saline (PBS). After removing the PBS, the cells were air-dried overnight. For spectrophotometric reading, the cells were lysed with 100 μL well^{-1} acid ethanol (3% HCl in 95% ethanol) and mixed with 100 μL well^{-1} PBS. Optical densities were recorded at 492 nm using a Sunrise TECAN reader.

Challenge with *Listonella (Vibrio) anguillarum*

At the end of the feeding period, fish were submitted to challenge experiments to evaluate their resistance to vibriosis. The serotype O1 *Listonella* (*Vibrio) anguillarum* strain 77/I03 (kindly provided by Dr Manfrin, IZS Venezie, Italy) was chosen due to its *in vitro* susceptibility to carvacrol, as preliminarily tested by the authors. The strain was cultivated in TSB 2% NaCl at 23°C until constant exponential growth. Bacteria were washed and suspended in sterile PBS at the desired concentration, determined using spectrophotometry and confirmed (1 day after the infection) using solid plate counting. Lethal Doses $_{50}$ and $_{70}$ (LD_{50} and LD_{70}) were assessed using preliminary *in vivo* trials (European Pharmacopoeia 2001) and adopted as reference doses for the experimental intraperitoneal (i.p.) infections. In the first experiment, each fish ($N = 25$ per diet) was challenged against a bacterial suspension containing 2 × 10^6 CFU mL^{-1} (200 μL fish^{-1}). In the second experiment, each fish ($N = 25$ per diet) was challenged against a bacterial suspension of 6 × 10^6 CFU mL^{-1} (200 μL fish^{-1}). In all instances,

mortality was recorded for 15 days (Nordmo 1997) and expressed as cumulative percentage. Protection was expressed as relative percentage of survival (RPS), calculated as follows (Amend 1981; European Pharmacopoeia 2001): [1 − (% mortality in vaccinated group / % mortality in control group)] × 100. Died subjects were submitted to necropsy and microbiological tests to confirm the diagnosis of vibriosis.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test differences among means for each variable according to a completely randomized design. Differences were considered statistically significant at $P < 0.05$. If appropriate, Duncan test was applied to test significant differences among means. The results of the *in vivo* bacterial challenge were analysed using Fisher's exact test (two-tailed). All statistical analyses were performed using SPSS-PC statistical software (Release 17.0; SPSS, Chicago, IL, USA).

Results

Growth performance and biometric traits

Feeding the experimental diets did not affect fish survival, as no mortality was recorded by the end of the experimental period nor any adverse effects on behaviour and external appearance or on palatability expressed as feed intake (1.36 g fish^{-1} day^{-1}) were observed. Growth performance and feed utilization by sea bass fed the experimental diets for 9 weeks are presented in Table 2. The inclusion of carvacrol did not significantly affect either the growth performance expressed as weight gain (69.1%) and specific growth rate (0.83%) or the feed conversion ratio (1.04) of sea bass fed the experimental diets ($P > 0.05$).

As shown in Table 3, feeding the experimental diets for 9 weeks did not result in significant differences in carcass yield (88.59%), viscerosomatic (10.59%), hepatosomatic (1.63%) and mesenteric fat (4.47%) indexes ($P > 0.05$).

Serum total proteins and immunoglobulins

In fish fed the CTR diet, the average serum protein concentrations ranged from 28 to 46 mg mL^{-1} with a progressive increase throughout the trial. After 1 week being fed the experimental diets, fish fed the 0.025% carvacrol diet exhibited a significantly higher level of total proteins when compared with the other groups ($P < 0.01$). Feeding carvacrol resulted in a progressive lowering of the total serum protein concentrations throughout the following 7 weeks. A significant decrease ($P < 0.01$) was observed after 4 and 8 weeks in fish fed the higher level of dietary carvacrol (0.05%) and after 8 weeks in fish fed the lower dietary carvacrol level (0.025%) (Fig. 1a).

Similarly, serum total immunoglobulins in fish fed the CTR diet increased throughout the feeding period. Fish fed 0.025% carvacrol for 1 week exhibited an increase in the level of immunoglobulins, but this parameter was significantly lower when compared with the controls after 8 weeks ($P < 0.01$). Feeding the diet containing 0.05% carvacrol compared with the CTR diet resulted in a significant decrease ($P < 0.01$) of the immunoglobulins after 4 and 8 weeks (Fig. 1b).

Serum lysozyme activity

The lysozyme activity of fish fed the CTR diet ranged from 550 to 586 U mL^{-1} and did not vary throughout the experimental period, as shown in Fig. 1c. Compared to the fish fed the CTR diet, fish fed the 0.05% carvacrol diet for 4 weeks exhibited a significant decrease in lysozyme activity ($P < 0.01$). A significant decrease in the lysozyme

Table 2 Weight gain (% IBW), specific growth rate (SGR), feed intake (g fish^{-1} d^{-1}) and feed conversion ratio of sea bass fed the experimental diets

	Final weight (g)	Weight gain (%)	SGR (%)	Feed intake (g fish^{-1} d^{-1})	FCR
control	117.7	69.5	0.84	1.04	1.36
0.025% carvacrol	115.7	66.1	0.81	1.00	1.36
0.05% carvacrol	119.6	71.8	0.86	1.07	1.35
ESM (d.f. = 6)	6.688	16.076	0.001	0.004	0.005

Table 3 Carcass yield, viscerosomatic index (VSI), hepatosomatic index (HSI) and mesenteric fat index (MFI) of sea bass fed the experimental diets for 9 weeks

	Carcass yield (%)	IVS (%)	HSI (%)	MFI (%)
control	88.96	10.09	1.84	4.08
0.025% carvacrol	88.51	10.89	1.46	5.07
0.05% carvacrol	88.29	10.79	1.59	4.27
ESM (d.f. = 24)	1.484	1.611	0.172	1.532

activity in fish fed the 0.025% carvacrol diet was observed after 8 weeks ($P < 0.01$).

Phagocytosis activity

Phagocytosis activity increased throughout the experiment in sea bass fed the CTR diet. The feeding of both doses of dietary carvacrol for 8 weeks resulted in an increase in phagocytosis, but due to the high individual variability, the differences were not statistically significant ($P > 0.05$) (Fig. 2a).

Leucocyte peroxidase content

This parameter, expressed in terms of $O.D._{450nm}$, remained relatively constant (1.3 ± 0.2) in fish fed the CTR diet throughout the survey period and was not significantly affected by the inclusion of dietary carvacrol ($P > 0.05$). Data not shown.

Respiratory burst activity

Results are expressed in terms of cumulative chemiluminescence response, intended as the sum of microplate single readings performed every 5 min for 1 h (Fig. 2b). In general, carvacrol induced a decrease in the respiratory burst activity throughout the experiment. After 1 week, feeding the respiratory burst in the group fed the 0.05% carvacrol diet was significantly lower than that recorded in fish fed the 0.025% carvacrol diet and the CTR diet ($P < 0.05$).

Pinocytosis activity

After 1 week, feeding the three test diets yielded similar pinocytosis activities ($P > 0.05$). Carvacrol administration, however, significantly enhanced the pinocytosis activity in fish groups fed the 0.05% level compared with the other groups after 4 weeks ($P < 0.05$). No significant differences were detectable among the groups after 8 weeks of feeding the test diets ($P > 0.05$) (Fig. 2c).

Challenge test with *Listonella* (*Vibrio*) *anguillarum*

The cumulative mortalities induced by the experimental infections of sea bass with two doses of *L. anguillarum* O1, 2×10^6 and 6×10^6 CFU mL^{-1} (200 μL $fish^{-1}$) are presented in Fig. 3a and b respectively. The mortality rate in the experiments was shown to be repeatable and suggested that the oral administration of carvacrol can notably reduce the disease severity and the mortality due to the infection. The highest bacterial dose determined a high cumulative mortality both in control and carvacrol-fed fish. In the challenge with 2×10^6 CFU mL^{-1} (Fig. 3a), mortality started 2 days postinfection (dpi) in the control group, 3 dpi in the group fed 0.025% carvacrol and 4 dpi in the group fed 0.05% carvacrol. In the case of fish fed 0.025% carvacrol, the cumulative mortality was significantly lower than that for the control fish starting from the third days postinfection, until the end of the experiment. In the case of fish fed 0.05% carvacrol, the cumulative mortality was significantly lower than that observed in untreated fish (8% versus 27%) at 3 dpi, whereas at the end of the survey (15 dpi), the difference was statistically negligible. In synthesis, feeding 0.025% carvacrol determined a 75% RPS while feeding 0.05% carvacrol gave a 31% RPS. In the challenge performed using the 6×10^6 CFU mL^{-1} bacterial dose (Fig. 3b), mortality started 2 dpi in all experimental groups. On days 3 and 4 dpi, it was significantly lower in the control than in fish fed 0.025% carvacrol. As far as the group receiving 0.05% dietary carvacrol was concerned, no significant differences were detected compared with control group throughout the trial.

Discussion

Essential oils or plant extracts, as well as other plant-derived substances, are able to promote several non-specific immune defence mechanisms and, to a lesser extent, some specific defence

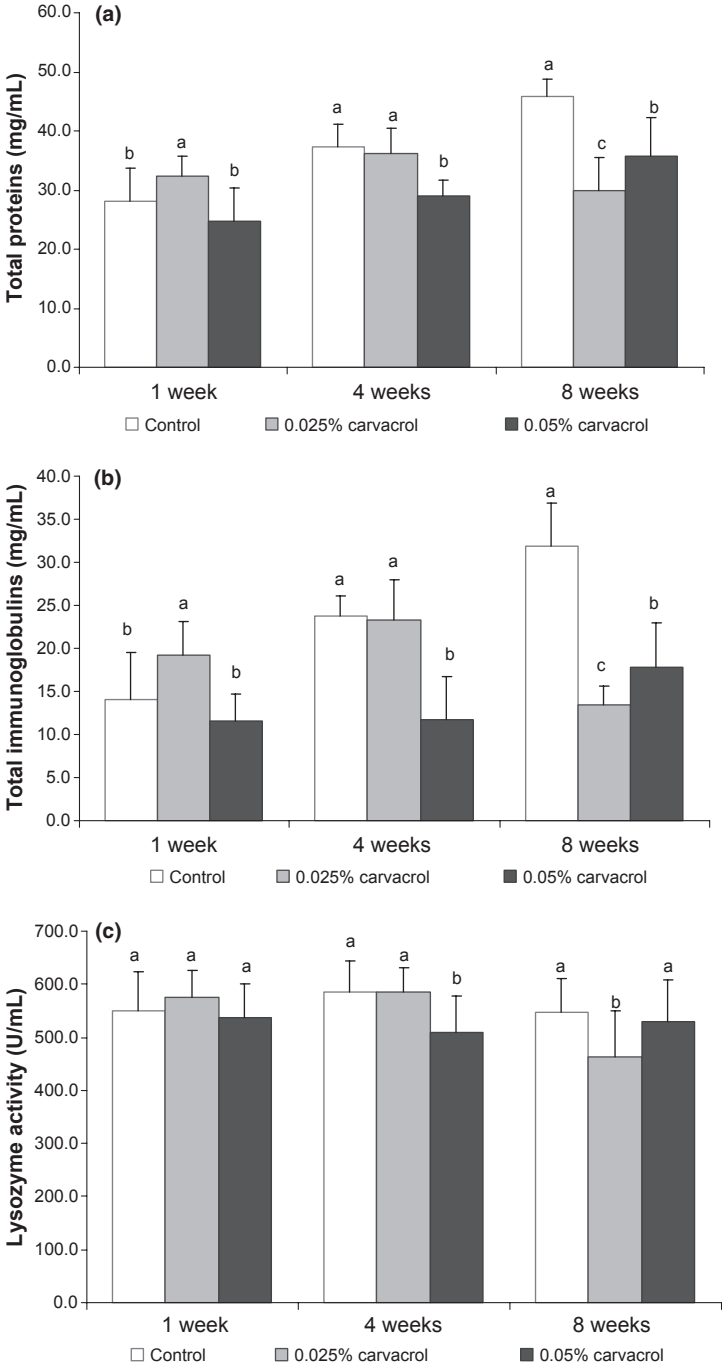

Figure 1 Effect of the dietary carvacrol level on: (a) serum total proteins (mg mL^{-1}), (b) serum total immunoglobulins (mg mL^{-1}), (c) serum lysozyme activity (U mL^{-1}) after 1, 4 and 8 weeks of feeding the test diets. Vertical bars indicate average values + standard deviation ($n = 10$). Statistical differences among groups within each sampling time are indicated with different letters ($P < 0.01$).

responses, increasing the resistance of fish species to infectious agents (Jeney *et al.* 2009; HariKrishnan *et al.* 2011).

In the present trial, carvacrol, a major component of the essential oils of oregano and thyme, was evaluated in sea bass as a possible feed addi-

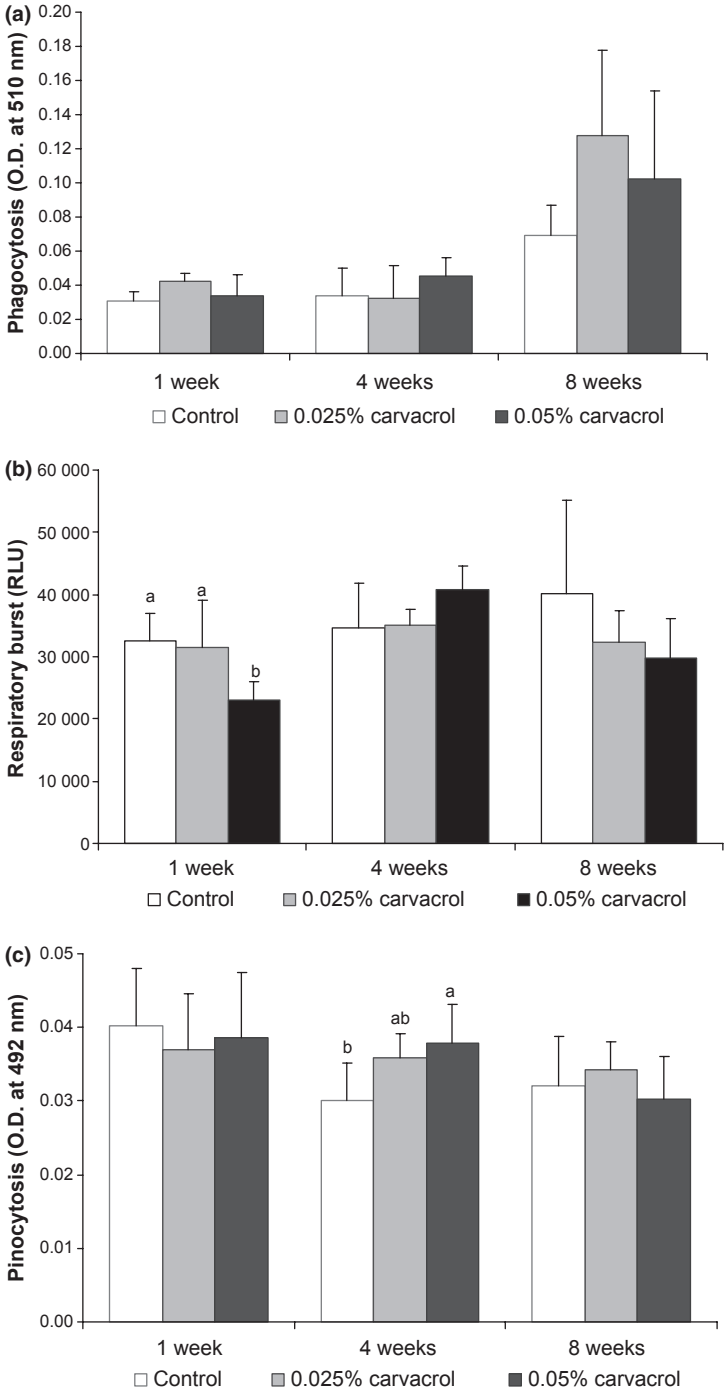

Figure 2 Effect of the dietary carvacrol level on HK leucocytes: (a) phagocytosis (O.D. at 510 nm); (b) respiratory burst (RLU, 1 h cumulative response); (c) pinocytosis (O.D. at 492 nm) after 1, 4 and 8 weeks of feeding the test diets. Vertical bars indicate average values + standard deviation (n = 5).

tive. Dietary carvacrol induced a progressive decrease in serum total proteins and immunoglobulins, which was already significantly different after 4 weeks of administration in subjects fed 0.05% carvacrol and after 8 weeks in fish fed 0.025% carvacrol. A possible reason could be the

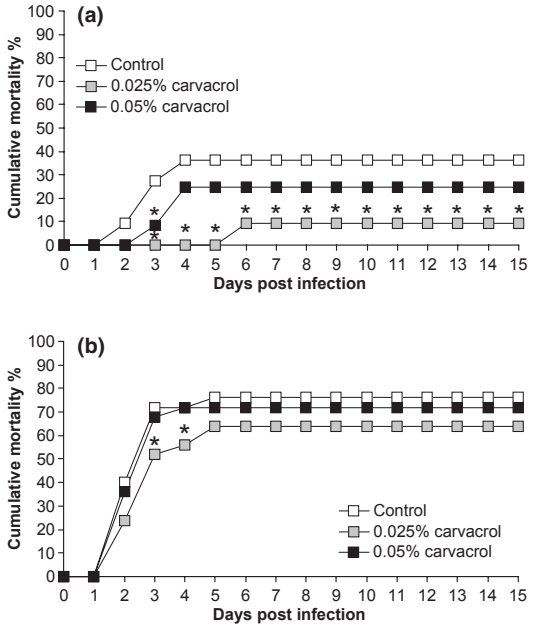

Figure 3 Cumulative mortality due to intraperitoneal infection with *L. anguillarum*. (a) Dose 2×10^6 CFU mL^{-1} (200 μL $fish^{-1}$) ($n = 25$); (b) Dose 6×10^6 CFU mL^{-1} (200 μL $fish^{-1}$) $n = 25$. *Significant differences compared to control group ($P < 0.01$).

decrease in the absorption activity of the intestinal mucosa, which is modulated in response to dietary carvacrol, as observed in swine (Michielis, Missotten, Fremaut, De Smet & Dierick 2007; Michiels, Missotten, Dierick, Fremaut, Maene & De Smet 2008; Michielis, Missotten, Van Hoorick, Ovyn, Fremaut, De Smet & Dierick 2010). On the other hand, Soltani, Sheikhzadeh, Ebrahimzadeh-Mousavi and Zargar (2010), in a similar trial, did not observe significant changes of this serum parameter in carp (*Cyprinus carpio*) fed for 8 weeks, although the medicinal plant *Zataria multiflora* (fam. *Labiatae*), containing carvacrol as 59% of the oily extract, was used in their trial. Lysozyme is a non-specific humoral defence molecule present in blood, mucus and various organs. It is synthesized by monocytes and neutrophils and its main roles are to induce the lysis of bacteria, the complement activation and to promote phagocytosis (as opsonin) (Magnadóttir 2006; Saurabh & Sahoo 2008). Lysozyme activity was significantly reduced in sea bass fed 0.05% carvacrol for 4 weeks compared to the control group and in sea bass fed for 8 weeks with 0.025% carvacrol. These data suggest that carvacrol may interfere with the secretion or activity of lysozyme and that its modulation depends on the dose, as well as the period of administration. A decrease in circulating lysozyme, although not significant, was previously described in catfish (*Ictalurus punctatus*) after an experimental trial, in which subjects were fed a diet supplemented with 0.05% carvacrol for 8 weeks (Zheng *et al.* 2009). On the other hand, plants rich in carvacrol showed varying effects on fish lysozyme. The administration of *Zataria multiflora* (fam. *Labiatae)* had no effect on serum lysozyme activity in carp (Soltani *et al.* 2010), whereas the use of oregano (*Origanum minitiflorum,* fam. *Labiatae*) enhanced the activity of this enzyme in *Diplodus puntazzo* (Karagouni, Athanassopoulou, Lytra, Komis & Dotsika 2005). Phagocytosis and reactive oxygen and nitrogen species (ROS and NOS) production are the most important mechanisms of the innate immune response in fish (Neumann, Stafford, Barreda, Ainsworth & Belosevic 2001). During the oxidative respiratory burst, neutrophils also release myeloperoxidases (MPO) from the azurophilic granules, enzymes that contribute to their antimicrobial activity. In our investigation, the oral administration of carvacrol induced an increase in the phagocytosis of HK leucocytes compared with that found in controls, but the differences observed were not statistically significant. The information regarding the effect of this compound on phagocyte activity in mammals or fish (either *in vivo* or *in vitro* assessments) are lacking. However, the results of Karagouni *et al.* (2005) can be considered by way of comparison. These authors did not observe changes in the number and activity of phagocytic cells collected from *D. puntazzo* infected by *Myxobolus* sp. and treated with dietary oregano. In this study, the activity of respiratory burst was quantified after *in vitro* stimulation with PMA, using a chemiluminescence assay (Coteur *et al.* 2002). In general, the production of ROS in sea bass fed carvacrol was found to be lower than that of control subjects, although the chemiluminescence response of leucocytes was significantly different only in fish treated for 7 days with the 0.05% dose. These findings suggest that *in vivo* carvacrol may act as an antioxidant, in agreement with what has been reported by other authors for resveratrol, another phenolic compound contained in the roots of *Veratrum grandiflorum* (Brisdelli, D'Andrea & Bozzi 2009). Resveratrol caused a significant decrease in ROS production by turbot (*Psetta maxima*) HK

leucocytes stimulated *in vitro* with PMA, findings similar to that described in mammals. It was hypothesized that resveratrol penetrates cells and inhibits ROS production or acts as a 'scavenger', limiting oxidative stress (Castro, Lamas, Morais, Sanmartin, Orallo & Leiro 2008). Moreover, our results are consistent with those observed in catfish (*I. punctatus*) fed the same dose of carvacrol (0.05%). Fish fed carvacrol showed a slight increase in both plasmatic superoxide dismutase and catalase activity, two enzymes involved in antioxidant cellular mechanisms (Zheng *et al.* 2009). The MPO content of leucocytes decreased in both groups of sea bass fed carvacrol (even if the difference was not significant), supporting the results obtained regarding the oxidative burst, as peroxidase activity is closely correlated to ROS production. The inhibition of intracellular and extracellular MPO was also observed in turbot leucocytes treated with resveratrol *in vitro* (Castro *et al.* 2008). Pinocytosis is another major activity of macrophages. This mechanism is essential for their effectiveness, as it allows the absorption and degradation of macromolecules, but may also play a role in the uptake and processing of antigens (Weeks, Keisler, Warinner & Mathews 1987). During the present trial, feeding carvacrol resulted in an increase in pinocytosis in purified HK leucocytes, starting from the 30th day of treatment, with significant differences in sea bass fed the highest dose.

The effect of carvacrol as a growth enhancer, as proposed in poultry and pig studies (Baser 2008) and observed in catfish (Zheng *et al.* 2009), could not be demonstrated either in sea bass after 9 weeks of carvacrol administration (present trial) or in *Oreochromis niloticus* (Rattanachaikunsopon & Phumkhachorn 2010) and it was not coupled with modification in the somatic indices as has been shown in *I. punctatus* fed carvacrol and OregoStim® (Zheng *et al.* 2009).

The assessment of disease resistance against a virulent strain of *L. anguillarum* serotype O1, after 8 weeks of feeding, demonstrated that carvacrol is able to affect the fish response to a bacterial infection. When the test was performed with a bacterial dose of 2×10^6 CFU mL^{-1} (200 μL $fish^{-1}$), lower than the LD_{50} and comparable to those causing spontaneous infections, the cumulative mortality rate in the group of fish fed 0.025% carvacrol was significantly lower than that of untreated controls, with an RPS of 75%. Moreover, a significant delay in the onset of mortality in the treated groups versus the control was evident. The 0.05% dose of carvacrol was also demonstrated to be protective, although to a lesser extent (RPS = 31%). The submission of fish to the highest infectious dose (6×10^6 CFU mL^{-1}; 200 μL $fish^{-1}$), close to the LD_{80}, revealed, as expected, that both levels of carvacrol were unable to protect the fish from developing the disease.

In conclusion, carvacrol did not significantly affect the immune response, growth performance or quality traits in sea bass when administered for 8 weeks at the reference doses of 0.025% and 0.05%; although under these experimental conditions, there is evidence for a potential antioxidant activity. Anyway, a protective effect against bacterial infections could be evidenced as a result of the appreciable resistance to *L. anguillarum,* demonstrated at the end of the *in vivo* challenge performed with a dose lower than LD_{50}.

It could be argued that the protection conferred by carvacrol might primarily depend upon its partial 'accumulation' throughout the pre-infection phase and its consequent *in vivo* antibacterial action, rather than the promotion of an effective immune response in the fish. The highest level of carvacrol given to fish (0.05%) may have negatively interfered with the ROS-based microbicidal activity of sea bass HK leucocytes, and, as a result, have proved less protective than the 0.025% level.

From the literature, we know that the supplementation of feed with carvacrol in broilers (for 42 days) retards lipid oxidation in meat. This provides indirect evidence that this antioxidant may be absorbed and enter the systemic circulatory system after ingestion (Luna, Lábaque, Zygadlo & Marin 2010). Lee, Everts and Beynen (2004) mention that accumulation of essential oils in the body is unlikely due to their fast metabolic conversion and excretion, but if chickens are continuously fed, diets containing essential oil constituents these compounds may be deposited in various tissues. Botsoglou, Florou-Paneri, Christaki, Lunn, Thomas, Best, Spiegelhalter and Fletouris Spais (2002) demonstrated a dose-dependent deposition of essential oils and a similar mechanism of processing and tissue deposition of carvacrol could therefore also be hypothesized in teleosts. The approach applied in this study, on an animal model such as sea bass, seems to be a useful tool for studying the biological activity of phytochemicals proposed as prophylactics or therapeutics in veterinary medicine,

in particular when their use is strongly recommended within organic rearing protocols (COMMISSION REGULATION EU 710/09). With this in mind, it would be desirable to investigate the integration of diets with plant or vegetable extracts particularly rich in carvacrol.

Future experimental improvements should perhaps focus on the evaluation of the effects of carvacrol on gastric/enteric mucosa to clarify the kinetics of absorption of this substance and to exclude possible damage to cell membranes, as well as the suitability of this class of compounds in modulating/selecting 'positive' bacterial populations colonizing the gastrointestinal tract of teleosts and blocking the colonization by potentially pathogenic species or strains, as suggested in swine (Michielis *et al.* 2007; Michiels *et al.* 2008).

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