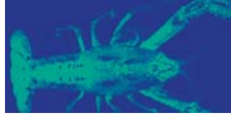

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Effect of *Thymus vulgaris* essential oil on intestinal bacterial microbiota of rainbow trout, *Oncorhynchus mykiss* (Walbaum) and bacterial isolates

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Abstract

The application of natural and innocuous compounds has potential in aquaculture as an alternative to antibiotics. We evaluated the effect of diet supplementation with *Thymus vulgaris* essential oil (TVEO) on the allochthonous microbial composition of rainbow trout. DNA was extracted directly from the intestinal contents, and the V3-V4 regions of the 16S rRNA genes were amplified by PCR. The bacterial composition was analysed using temporal temperature gradient electrophoresis (TTGE). No significant changes ($P > 0.05$) were detected in the TTGE profiles of TVEO-treated trout compared with the controls. The Dice similarity index revealed a high stability ($C_s > 70\%$) of the intestinal microbiota in both groups during the 5-week period. Sequence analyses of the TTGE bands revealed the same bacterial composition in both groups, with most bacteria belonging to the *Proteobacteria* and *Firmicutes* phyla. The *in vitro* antibacterial activity of TVEO was assessed using a range of normal intestinal isolates and fish pathogens. The inhibitory concentrations for all the tested bacteria were higher than the TVEO levels used in trout, which may explain the *in vivo* results.

Keywords: microbiota, PCR–TTGE, 16S rRNA gene, *Thymus vulgaris*, essential oil, *Oncorhynchus mykiss* (Walbaum)

Introduction

Chile is currently one of the leading salmon producers worldwide (Vielka, Morales & Moreno 2006). However, intensive fish farming in Chile has promoted the growth of several bacterial diseases, leading to an elevated use of antimicrobials (Miranda & Zemelman 2002; Miranda & Rojas 2007). Concerns regarding antibiotic resistance in bacteria associated with salmon as well as the dissemination of bacterial resistance among environmental microbiota have increased. Recently, commensal bacterial populations of the gastrointestinal tract have been proposed as reservoirs for antibiotic resistance determinants that could disperse resistance via horizontal gene transfer (Salyers, Gupta & Wang 2004; Salyers & Shoemaker 2006). In the Atlantic salmon (*Salmo salar*) gut, antibiotic treatment has been reported to reduce bacterial diversity and lead to the development of opportunistic *Aeromonas* harbouring antibiotic resistance (Navarrete, Mardones, Opazo, Espejo & Romero 2008). These opportunistic bacteria are normally present in low numbers, and therefore, they do not present a significant risk; however, they can grow and reach significant numbers after antibiotic treatment due to the reduction in bacterial diversity, at which time, they may occupy previously unavailable ecological niches. Concerns about bacterial resistance and antibiotic residues have contributed to increased caution in the use of antibiotics in animal production and especially in aquaculture (Cabello 2006; Moffitt & Mobin 2006; Benchaar, Calsamiglia, Chaves, Fraser, Colombatto, McAllister & Beauchemin 2008; Navarrete *et al.* 2008) and have encouraged research for alternatives to antibiotics.

Essential oils (EOs) are natural components of plants that are generally recognized as safe substances (GRAS) (http://www.cfsan.fda.gov/ ~ dms/

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eafus.html). Because of their antimicrobial properties, these oils may constitute viable alternatives to antibiotics as prophylactic and therapeutic agents in animal production and aquaculture systems. These antibacterial properties have been examined in several studies (Kim, Marshall, Cornell, Preston & Wei 1995; Lambert, Skandamis, Coote & Nychas 2001; Delaquis, Stanich, Girard & Massa 2002; Mejlholm & Dalgaard 2002; Mourey & Canillac 2002; Burt 2004). However, most of the research has been conducted to extend the shelf-life of different foods, and so these reports have focused on the *in vitro* evaluation of EO effectiveness against food-borne pathogens (Nascimento, Locatelli, Freitas & Silva 2000) and spoilage microorganisms (Mejlholm & Dalgaard 2002; Mahmoud, Yamazaki, Miyashita, Kawai, Shin & Suzuki 2006). Thymol is the principal component of the *Thymus vulgaris* essential oil (TVEO), and it has the widest spectrum of antimicrobial activity (Dorman & Deans 2000; Burt 2004). Nevertheless, there is limited information about the *in vivo* effects of the TVEO.

The basis for the antibacterial action of EOs is poorly understood. It has been suggested that they can disturb the permeability of the bacterial cell membrane, leading to a disruption of the proton motive force, electron flow and active transport (Conner & Beuchat 1984; Cox, Gustafson, Mann, Markham, Liew, Hartland, Bell, Warmington & Wyllie 1998; Helander, Alakomi, Latva-Kala, Mattila-Sandholm, Pol, Smid & von Wright 1998; Lambert *et al.* 2001; Ultee, Bennik & Moezelaar 2002). Other potential mechanisms are related to the coagulation of cell contents (Sikkema, De Bont & Poolman 1995; Lambert *et al.* 2001) and/or the inactivation of the genetic material (Kim, Marshall & Wei 1995).

A new report by Yeh, Shiu, Shei, Cheng, Huang, Lin and Liu (2009) led to a new perspective on the use of natural extracts in aquaculture. This study reported that extracts from *Cinnamomum kanehirae* (stout camphor tree) showed antibacterial effects against different pathogens of aquatic animals. Shrimp treated with this extract exhibited an enhanced disease resistance to *Vibrio alginolyticus* (Yeh *et al.* 2009)

Oral administration of antibacterial chemicals may produce an alteration in the gut microbiota or may even facilitate the establishment of opportunistic pathogenic bacteria (Navarrete *et al.* 2008). Recent studies have revealed that, depending on the kind of bacteria present, gut microbiota induce several important host responses related to nutrient metabolism, the innate immune system and gut differentiation (Rawls, Samuel & Gordon 2004; Bates, Mittg, Kuhlman, Baden, Cheesman & Guidulain 2006; Gomez & Balcazar 2008).

The objective of this study was to evaluate the effect of a diet supplemented with TVEO on the composition of rainbow trout intestinal microbiota using molecular profiling methods based on 16S rRNA gene analysis [restriction fragment length polymorphism (RFLP)] and PCR–temporal temperature gradient electrophoresis (PCR–TTGE). In addition, *in vitro* determination of the antibacterial activity of TVEO was performed using several bacteria isolated from the gut of healthy trout as well as some pathogens.

Materials and methods

Diets, fish and sample collection

To test the effect of TVEO on trout microbiota, four diets containing 0, 5, 10 and 20 mg TVEO kg^{-1} feed were prepared using a base of commercial pellets from EWOS Aquaculture International. The composition of this basic feed is presented in Table 1; proximal analysis was performed in the food laboratory of the Universidad Católica de Valparaíso. The composition of the TVEO used in this study was determined by gas chromatography in the analytical laboratory of Universidad Federico Santa María (Table 2). The amount of TVEO was selected based on a previous toxicological experiment performed in trout (Stroh, Wan, Isman & Moul 1998).

Commercial pellets were ground in a lab mixer (450 W) and then milled using an IKA-Werke A11 ba-

Table 1 Diet composition: components and proximal analysis of pelleted feed used as a base for TVEO supplementation diet

Ingredients	g kg^{-1} feed
Fish meal	480
Poultry by-products	115
Starch sources	107
Wheat gluten	16
Corn gluten meal	78
Oil	172
Others (vitamins, minerals, amino acids)	32
Proximal analysis	
Crude protein	500
Fat	220
Humidity	90
Ash	85
Crude fibre	10
Nitrogen-free extract	95

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Table 2 Major component of *Thymus vulgaris* essential oil (TVEO) used in this study

Chemical component	g kg^{-1}
Thymol	400
p-Cymene	130
Linalool	39
Carvacrol	22
Borneol	8
α-Pinene	7
Eucaliptol	5
β-Pinene	2

sic analytical mill (3 mm size) (IKA Werke GmbH, Staufen, KG, Germany). The resultant powder was separated into portions of equal weight before mixing with the TVEO suspensions. The TVEO suspensions were prepared in phosphate-buffered saline (PBS) to obtain the different final concentrations. These suspensions were mixed with the powdered basic feed and then homogenized. The mixtures were pelleted and dried at room temperature.

One hundred and twenty *Oncorhynchus mykiss* (Walbaum) rainbow trouts with an average weight of 25 ± 5 g were used in this study. They were randomly and equally distributed into eight tanks. The fish were fed to visual satiation twice a day for 5 weeks using the experimental diets (0, 5, 10 and 20 mg TVEO kg^{-1} feed). Each diet was assessed in duplicate using two tanks containing freshwater (50 L); the temperature and pH were set at 14 ± 2 °C and 8.0 ± 0.5 respectively. The fish were monitored for their health status, specific growth rates and condition factors (Sveier, Whathne & Lied 1999). The microbiota was analysed every week; 15 trout per tank were anaesthetized and the intestinal contents were collected by squeezing the abdomens. The intestinal contents of three to four individuals per tank were pooled before DNA extraction (Romero & Navarrete 2006; Navarrete, Espejo & Romero 2009). Three samples per tank were analysed, and the intestinal contents were maintained at − 20 °C until use.

DNA extraction and PCR amplification

DNA from the trout intestinal contents was obtained from the pooled samples using the Power Soil DNA kit according to the manufacturer's instructions (MOBIO Laboratories, Carlsbad, CA, USA). To obtain a rapid overview of the effect of TVEO on the microbiota profiles of different samples, the 16S rRNA gene was PCR amplified with the bacteria-specific primers 27F and 1492R (De Long 1992). Analysis of the RFLP using *Alu*I, *Rsa* I and *Hae*III (Invitrogen) was performed to examine the 16S rRNA gene as described previously (Romero & Navarrete 2006). Amplification of the V3-V4 region of the 16S rRNA gene was carried out as described previously (Magne, Abely, Boyer, Morville, Pochart & Suau 2006) using the conserved bacterial domain-specific primers 341F (5′-CCTACGGGAGGCAGCAG-3′ with a GC clamp at the 5′ end; McCracken, Simpson, Mackle & Gaskins 2001) and 788R (5′- GGACTACCAGGGTATCTAA-3′; Magne *et al*. 2006). TTGE was performed according to Magne *et al*. (2006). Each gel included standards containing PCR amplicons of known bacterial sequences (%GC) to validate comparisons between gels. The TTGE profiles were analysed using GELCOMPAR II software (Applied Maths, Sint-Martens-Latem, Belgium) and by applying the Dice similarity index (C_s).

Statistical analyses were performed using the non-parametric Kruskal–Wallis test. The pairwise similarity coefficient (C_s) is a similarity index used to compare the bacterial compositions of different samples (McCracken *et al*. 2001). Two identical profiles generate a $C_s = 100\%$, whereas completely different profiles generate a $C_s = 0\%$. Each sample can be compared with every other sample; therefore, the mean percentage similarities (C_s values) can be used to compare each diet/treatment group with itself and with all the other groups (McCracken *et al*. 2001). For intragroup comparisons (i.e., fish under the same treatment), the Dice index was calculated using profiles derived from samples collected at the same time and from duplicate tanks. For intergroup comparisons, C_s values were calculated using profiles derived from samples collected at the same time but corresponding to different treatments (with TVEO versus without TVEO). To estimate the stability of the microbiota composition during the experiment, the C_s values were calculated for each treatment group using the microbial profiles obtained at the beginning of the trial (time = 0 weeks) and those obtained in subsequent weeks.

Identification of bacterial components in the TTGE profiles

All bands visually recognized in each TTGE pattern were excised from the gel and eluted overnight in 50 μL of MilliQ water (Millipore, Bedford, MA, USA). All samples (1 μL) were reamplified, as described above, except by using the forward primer without the GC clamp.

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The 16S rRNA sequences from the reamplified bands were sent to the Macrogen Sequencing Service Center (Seoul, Korea) for purification and sequencing with the 788R primer. The retrieved sequences were deposited in GenBank (EU861368–EU861387, EU888865–EU888879) and aligned with reference sequences using Sequence Match from the Ribosomal Database Project II (RDP II) website (Cole, Chai, Farris, Wang, Kulam-Syed-Mohideen, McGarrell, Bandela, Cardenas, Garrity & Tiedje 2007).

Bacterial strains and isolates

Pathogenic collection strains *Vibrio anguillarum* ATCC19264, *Flavobacterium psychrophilum* ATCC49418, *Vibrio ordalii* ATCC33509 and *Vibrio parahaemolyticus* RIMD 2210633 were obtained from the ATCC and RIMD collections. Pathogenic isolates (ThV-1, ThV-2, ThV-5, ThV-6) were kindly provided by a diagnostic laboratory in Puerto Montt, Chile. These pathogenic isolates were obtained from sick fish using different tissues and media depending on the bacterial type; *Vibrio* were isolated in TCBS and *Streptococcus* and *Lactococcus* in blood agar (Valdés, Jaureguiberry, Romalde, Toranzo, Magariños & Avendaño-Herrera 2009). Indigenous bacterial isolates were obtained previously from the intestinal contents of healthy rainbow trout after culture in tryptic soy agar and incubation at 17 °C. The indigenous bacteria A8P1-8, A8P1-9, B8P3-1, ThV-A, ThV-E, ThV-F, ThV-G, ThV-H and ThV-I were isolated in a previous study (Navarrete, Magne, Mardones, Riveros, Opazo, Suau, Pochart & Romero 2010); I8 and P1 were obtained from healthy *Salmo salar* gut (Navarrete *et al*. 2009). Molecular identification of pathogenic and indigenous isolates was carried out by 16S rDNA sequencing.

Assessment of TVEO *in vitro* antibacterial activity

The antibacterial activity of TVEO was assessed using several isolates obtained from faecal samples of rainbow trout and pathogenic bacteria from a veterinary laboratory. Minimum inhibitory concentrations (MICs) of TVEO were determined using a previously described broth microdilution method (Cosentino, Tuberoso, Pisano, Satta, Mascia, Arzedi & Palmas 1999). Briefly, serial doubling dilutions of TVEO were performed in a 96-well microtitre plate (Nunc, Copenhagen, Denmark) over a range of 2.5–1280 μg mL^{-1} (ppm). The final concentration of each bacterial inoculum was 5.0×10^5 CFU mL^{-1} in each well. Positive and negative growth controls were included with every bacterial strain tested; all analyses were performed in triplicate. *Vibrio ordalii* (Vo), *Vibrio anguillarum* (Va), *Vibrio parahaemolyticus* (Vp) and *Flavobacterium psychrophilum* (Fp) were tested in Luria–Bertani (LB) broth with 1% NaCl, tryptic soy broth (TSB) with 1% NaCl, LB with 3% NaCl and nutrient broth with 0.5% tryptone, 0.05% yeast extract, 0.02% sodium acetate and 0.02% meat extract respectively. All indigenous bacteria were grown in TSB at 30 °C for 24 h. Pathogenic bacteria (Vp, Vo, Va and Fp) were grown in TSB at 17 °C for 48 h. These cultures were then used to inoculate plates supplemented with 0.5% Tween 80. All plates were incubated aerobically at 17 °C for 48 h.

Results

Effect of TVEO dietary inclusion on fish growth

During the 5-week trial, the fish were evaluated based on growth parameters. No significant differences were observed in these parameters between untreated fish and those treated with TVEO. In the untreated fish, the average specific growth rate (SGR) was 2.70 ± 0.22 and the average condition factor (*K*) was 1.27 ± 0.05. In the TVEO-treated fish, SGR was 2.70 ± 0.25 and K was 1.25 ± 0.07. During the treatment, the fish displayed normal behaviour and no signs of illness; thus, no particular therapy was provided. No mortality was observed during the experiment.

Effect of TVEO dietary inclusion on the composition of the microbiota

To obtain a rapid overview of the effect of TVEO on bacterial populations, the microbiota profiles from all groups (0, 5, 10 and 20 mg TVEO kg^{-1} feed) were obtained by digesting the PCR-amplified 16S rRNA gene with *Alu*I, *Rsa*I and *Hae*III. The RFLP profiles revealed a mixture of bands, indicating the coexistence of different kinds of microorganisms. The bacterial profiles of untreated and TVEO-treated groups remained highly similar (Fig. 1).

To identify the bacterial components of the microbiota and to evaluate the stability as well as the intra- and intergroup variabilities, PCR–TTGE from the 0 and 20 mg TVEO kg^{-1} feed groups were analysed.

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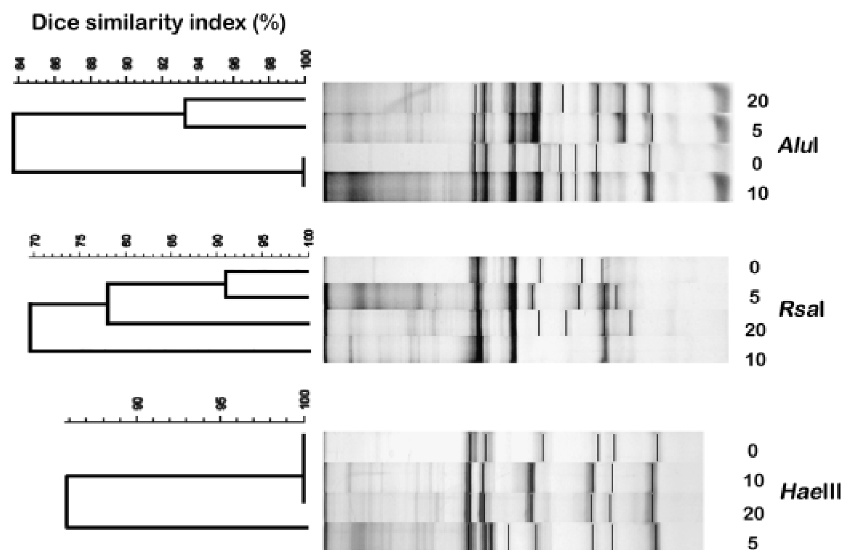

Figure 1 Dice's similarity indices (C_s) were calculated to compare the 16SrRNA RFLP patterns obtained using *Alu*I, *Rsa*I and *Hae*III. Each profile was derived from the pooled intestinal contents of three to four fish, which were obtained after 5 weeks of treatment (0, 5, 10 and 20 mg TVEO kg^{-1} feed). Bands marked with artificial lines were included in the comparison. Silver stain reveals single DNA strands like red bands that have limited migration and remain on the top of the gel. These bands were not included in the analysis.

The variabilities of the microbiota profiles in the replicates (intragroup) for the control and the TVEO-treated groups showed high coincidences, with values ranging from 70% to 90%. These results indicated that the microbiota was homogenous enough to allow diet and temporal comparisons (Fig. 2a, intragroup comparison). Comparison of the microbiota profiles using the Dice index (C_s) showed high similarities (>71%) between the 20 mg TVEO kg^{-1} feed-treated and -untreated trout. No statistical differences were observed between the TVEO-treated and the untreated fish ($P>0.05$, Fig. 2b, intergroup comparison). Thus, for these concentrations, TVEO induced no changes in the profiles of the intestinal microbiota of the fish. These results are in accordance with the similarity of the microbiota profiles observed in the RFLP analysis described above.

When the TTGE profiles within the same groups (treated or untreated) were compared throughout the collection period, several common bands were observed. These bands were persistent throughout the trial, indicative of the stability of the microbiota composition in both TVEO-treated and -untreated fish. The stability of the TTGE pattern over time was revealed by the Dice index (C_s), which showed average values >65% for both TVEO-treated and -untreated trout (Fig. 2c).

Identification of TTGE bands by sequencing

Sequencing was used to identify the bands that were visually detected in TTGE from the untreated and the TVEO-treated groups. Partial sequences of the 16S rRNA gene (400 bp) were compared with sequences available in RDP II, and their identities are shown in Table 3. Our results indicated that microorganisms represented by the main bands belong to the phyla *Proteobacteria* and *Firmicutes*. Specifically, they were related to Gram-negative bacteria from the genera *Moraxella, Vibrio, Butiauxella* and *Legionella* and to Gram-positive organisms such as *Streptococcus* and *Alicyclobacillus*. An additional weak band corresponding to *Streptomyces* of the *Actinobacteria* phylum was also observed. The most frequent bands observed in both the untreated and the TVEO-treated groups correspond to *Moraxella, Vibrio* and *Legionella* (Table 4).

Antibacterial activity of TVEO on bacterial fish pathogens and bacteria isolated from the salmonid gut

Table 5 summarizes the identification of bacterial isolates by partial sequencing of the 16S rRNA gene and the antibacterial activity of TVEO. The MIC range

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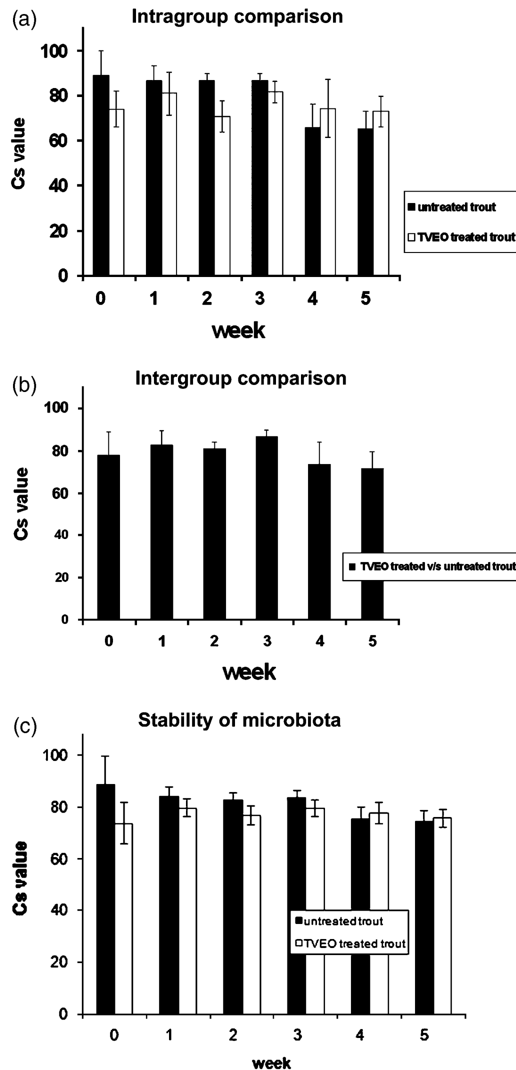

Figure 2 Dice's similarity indices (C_s) were calculated to compare TTGE patterns. Values represent the average similarities and SEMs for three groups of pooled intestinal contents from three to four fish in each experimental treatment from weeks 0, 1, 2, 3, 4 and 5. (a) Intragroup comparisons (replicate): 20 mg TVEO kg^{-1} feed-treated and untreated groups. (b) Intergroup comparison: untreated versus TVEO 20 mg TVEO kg^{-1} feed-treated groups. (c) Stability considering the TTGE profile of week 0 as a reference. The C_s values did not differ significantly (ANOVA, $P > 0.05$).

of TVEO against all bacteria was 80–1280 mg L^{-1}. In general, bacterial isolates from the intestine of healthy fish were more resistant than pathogenic isolates to TVEO with higher MIC values (640–1280 mg L^{-1}). The most potent activity of TVEO was demonstrated against *Vibrio anguillarum*, with an MIC of 80 mg L^{-1}.

Discussion

In the present study, we evaluated the *in vivo* effect of 20 mg TVEO kg^{-1}feed (8 mg thymol kg^{-1} feed) introduced into the diet on the intestinal microbiota of rainbow trout. The level of TVEO was selected based on a previous study that reported an *in vivo* toxic effect on rainbow trout and salmon at concentrations above 20 mg kg^{-1} (Stroh *et al.* 1998). The effect of EO inclusion on the microbiota composition was evaluated using PCR–TTGE. This method has been useful for detecting changes in the composition of microbiota after treatment with antibacterial agents in several hosts such as mice, humans and rabbits (McCracken *et al.* 2001; De la Cochetière, Durand, Lepage, Bourreille, Galmiche & Doré 2005; Abecia, Fondevila, Balcells, Lobley & McEwan 2007). For this study, we focused on bacteria populations that could be exposed to feed ingredients (i.e., TVEO). Allochthonous microbiota is likely to be more affected by these ingredients. Because bacterial communities were extracted from the intestinal contents of trout, the microbiota analysed comprised mainly allochthonous bacteria (transient or associated with digesta; Salinas, Myklebust, Esteban, Olsen, Meseguer & Ringø 2008).

Our results showed that the TVEO concentration did not significantly alter the bacterial populations of trout intestines, as assessed by PCR–TTGE. Sequencing of the TTGE bands showed that the intestinal microbiota of trout was composed of three phyla: *Proteobacteria*, *Firmicutes* and *Actinobacteria* (Table 3). These taxa have been reported previously in salmonids, and they represent the abundant bacterial populations present in the gut of these fish (Holben, Williams, Gilbert, Saarinen, Sarkilahti. & Apajalahti 2002; Huber, Spanggaard, Appel, Rossen, Nielsen & Gram 2004; Romero & Navarrete 2006; Skrodenyte-Arbaciauskiene, Sruoga & Butkauskas 2006). In our previous investigations, we consistently found the predominance of a few bacterial groups in the salmonid guts within Chilean farms (Romero & Navarrete 2006; Navarrete *et al.* 2009, 2010), in accordance with the observation by Holben *et al.* (2002). Previous investigations have reported that the genera *Lactococcus* (Navarrete *et al.* 2010), *Streptococcus* (Ringø, Bendiksen, Wesmajervi, Olsen, Jansen & Mikkelsen 2000), *Streptomyces* (Merrifield, Dimitroglou, Bradley, Baker & Davies 2009), *Buttiauxella* (Kim, Brunt & Austin 2007; Navarrete *et al.* 2010), *Kluyvera* (Kim *et al.* 2007; Navarrete *et al.* 2010), *Hafnia* (Kim *et al.* 2007; Navarrete *et al.* 2010) and *Citrobacter*

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Table 3 Nearest-match identification of 16S rDNA sequences obtained with PCR-TTGE approach from rainbow trout, *Oncorhynchus mykiss* (Walbaum), to known sequences in the RDP II database

Band name	Accession number	% identity	Affiliation phylum/class	Closest sequence	Frequency of band detection in replicates	
					0 mg TVEO kg^{-1} feed	20 mg TVEO kg^{-1} feed
P 0–6	EU861369	90.7	Firmicutes/clostridia	*Acetanaerobacterium elongatum* (AY487928)	1/6	1/6
P 0–7	EU861370	99.5	Firmicutes/bacilli	*Streptococcus bovis* (AY442813)	1/6	0/6
P 1–5	EU861368	100	Proteobacteria/γ-proteobacteria	*Vibrio sp.* (EU854882)	1/6	0/6
P 2–1	EU861371	88	Proteobacteria/γ-proteobacteria	*Legionella brunensis* (Z32636)	3/6	2/6
P 2–4	EU861372	99.7	Proteobacteria/γ-proteobacteria	*Vibrio* sp. (AJ316187)	1/6	2/6
P 3–2	EU861373	89.7	Proteobacteria/γ-proteobacteria	*Legionella* sp. (X97365)	6/6	6/6
P 5–2	EU872321	87.6	Proteobacteria/γ-proteobacteria	Uncultured bacterium (AY661981)	6/6	5/6
		75.1		*xMoraxella caprae* (DQ156148)		
P 5–3	EU861374	96.2	Proteobacteria/γ-proteobacteria	*Legionella* sp. (X97359)	1/6	1/6
P 5–5	EU861375	95.5	Proteobacteria/γ-proteobacteria	*Legionella quateirensis* (Z49732)	2/6	3/6
P 5–7	EU861376	97.7	Proteobacteria/γ-proteobacteria	*Legionella rubrilucens* (Z32643)	5/6	5/6
P 5–8	EU861377	96.4	Proteobacteria/γ-proteobacteria	*Legionella worsleiensis* (Z49739)	1/6	0/6
Q 1–4	EU861378	100	Proteobacteria/γ-proteobacteria	*Vibrio* sp. (AY542526)	5/6	4/6
Q 1–8	EU861379	99.5	Actinobacteria/actinobacteria	*Streptomyces* sp. (AB052845)	1/6	2/6
Q 2–2	EU861380	99.5	Proteobacteria/γ-proteobacteria	*Moraxella* sp.(X95304)	2/6	3/6
Q 2–7	EU861381	99.7	Proteobacteria/α-proteobacteria	*Paracoccus* sp. (AY515424)	0/6	1/6
Q 2–8	EU861382	100	Proteobacteria/γ-proteobacteria	*Buttiauxella* sp. (AJ293683)	1/6	1/6
		100		*Pantoea* sp. (AF227860)		
		100		*Kluyvera intermedia* (AF310217)		
Q 4–6	EU861383	98.9	Proteobacteria/γ-proteobacteria	*Vibrio* sp. (AJ316187)	1/6	2/6
Q 4–7	EU861384	99.7	Proteobacteria/γ-proteobacteria	*Vibrio* sp. (AJ316187)	5/6	4/6
Q 4–8	EU861385	92	Proteobacteria/γ-proteobacteria	*Legionella erythra* (Z32638)	0/6	1/6
Q 4–15	EU861386	92	Proteobacteria/γ-proteobacteria	*Legionella birminghamensis* (Z49717)	1/6	1/6
Q 4–16	EU861387	98.7	Firmicutes/bacilli	*Alicyclobacillus pohliae* (AJ564766)	0/6	1/6

PCR-TTGE profiles were obtained from DNA extracted from the intestinal content of trout fed after 5 week with a diet supplemented with 0 and 20 mg TVEO kg^{-1} feed.

(Kim *et al.* 2007; Navarrete *et al.* 2010) are indigenous microbiota of healthy salmonids. Moreover, *Legionella*, *Moraxella* and *Paracoccus* have been retrieved from the guts of healthy zebrafish (Rawls *et al.* 2004). In contrast, to our knowledge, *Acetanaerobacterium elongatum* and *Alicyclobacillus pohliae* have never been described as fish gut microbiota. However, the latter has been identified in the guts of termites (Yang, Schmitt-Wagner, Stingl & Brune 2005), chickens (Gong, Si, Forster, Huang, Yu, Yin, Yang & Han

Table 4 Bacteria present in the intestinal microbiota of trout fed after 5 weeks with a diet supplemented with 0 and 20 mg TVEO kg $^{-1}$ feed

	mg TVEO kg $^{-1}$ feed											
	0						20					
	Tank 1			Tank 2			Tank 1			Tank 2		
Bacterial group	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
Legionella spp.	x	x	x	x	x	x	x	x	x	x	x	x
Vibrio sp.	x	x	x	x	x			x	x	x	x	
Moraxella sp.	x	x	x	x	x	x	x	x	x		x	x
Paracoccus sp.									x			
Streptococcus sp.	x											
Streptomyces sp.						x	x	x				
Butiauxella/*Pantoea*/*Kluyvera intermedia*			x						x			
Acetanaerobacterium elongatum	x								x			
Alicyclobacillus pohliae									x			

Each diet was assessed in duplicate using two tanks (15 trout per tank) and the intestinal contents of three to four individuals per tank were pooled (P1, P2 and P3) before DNA extraction.

2006) and humans (Kassinen, Krogius-Kurikka, Mäkivuokko, Rinttilä, Paulin, Corander, Malinen, Apajalahti & Palva 2007).

Despite an interest in developing new alternatives to antibiotics in aquaculture, our work is the first study to address the *in vivo* effect of EOs on the bacterial composition of fish. Although some studies have reported the effect of different dietary oils on the gut microbiota (Ringø, Bendiksen, Gausen, Sundsfjord & Olsen 1998; Ringø, Lødemel, Myklebust, Jensen, Lund, Mayhew & Olsen 2002), EOs have only been used as preserving agents in seafood. For example, dipping carp fillets into a solution containing both carvacrol and thymol led to reduced growth and numbers of bacteria, consequently extending the shelf-life of the fillets (Kim, Marshall, Cornell *et al.* 1995; Mejlholm & Dalgaard 2002; Mahmoud *et al.* 2006). In aquaculture systems, examples for *in vivo* use of EOs are rare but promising. Yeh *et al.* (2009) showed that shrimp treated with an extract of *C. kanekirae* exhibited enhanced disease resistance to *V. alginolyticus*.

The results of the *in vivo* studies of EOs have been contradictory, and these studies were mostly performed in cows, chickens and pigs. In cows, viable bacteria, cellulolytic bacteria and protozoa were not influenced by a mixture of EO supplementation, including thymol (Benchaar *et al.* 2008). In broiler digesta, a diet supplemented with EOs (thymol at 50 mg kg $^{-1}$ feed) induced a decrease in the *E. coli* CFU counts, whereas the *Lactobacillus* counts were not affected (Jang, Ko, Kang & Lee 2007). However, chickens fed with a herbal mix (100 g oil kg $^{-1}$ herb, including thyme) showed no changes in the viable counts of several bacterial groups, including lactic acid bacteria, coliforms and *Clostridium perfringens* (Cross, McDevitt, Hillman & Acamovic 2007). In contrast, Janczyk, Trevisi, Souffrant and Bosi (2008) showed that the inclusion of thymol [1% (w/w)] in the pig diet caused clear changes in the small intestine microbial community, notably decreasing the *Actinobacillus* to undetectable levels. These results suggest that the effect of dietary inclusion of EO on the microbiota may depend on the susceptibility of the bacterial group.

The antibacterial activity of TVEO was assessed *in vitro* using fish pathogens and common bacteria isolated from healthy salmonids. The MICs were similar to those reported for food-borne pathogens (Cosentino *et al.* 1999; Burt 2004). Noticeably, the pathogens seemed to be more susceptible than the indigenous bacterial microbiota. This difference may be related to membrane permeability; however, there is no clear mechanism of TVEO action. Notably, the pathogenic *Lactococcus piscium* was clearly more susceptible to TVEO than those isolates belonging to indigenous microbiota. The cell-surface components of this bacterial group showed considerable diversity within several lactococcal strains, with multiple differences observed between many of the strain pairs (Crow, Gopal & Wicken 1995; Giaouris, Chapot-Chartier & Briandet 2009), such as hydrophobicity, extracellular lipoteichoic acid concentration, molecular weight profile of proteins and amount of protein. These dif-

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Table 5 Minimum inhibitory concentration of TVEO on bacteria isolated from salmon

Isolate name	Accession number	% identity	Affiliation phylum/class	Closest sequence	Gram	MIC (mg L^{-1}) pH 7.0
Pathogenic isolates						
ThV-1	EU888876	100	Firmicutes/Bacilli	*Lactococcus piscium* (DQ343754)	+	320
ThV-2	EU888877	100	Firmicutes/Bacilli	*Lactococcus piscium* (DQ343754)	+	320
ThV-5	EU888878	97	Firmicutes/Bacilli	*Streptococcus phocae* (EF599165)	+	640
ThV-6	EU888879	100	Firmicutes/Bacilli	*Streptococcus phocae* (EF599165)	+	640
ATCC 49418				*Flavobacterium psychrophilum*	–	320
ATCC 33509			Proteobacteria/γ-proteobacteria	*Vibrio ordalii*	–	320
ATCC 19264			Proteobacteria/γ-proteobacteria	*Vibrio anguillarum*	–	80
RIMD 2210633			Proteobacteria/γ-proteobacteria	*Vibrio parahaemolyticus*	–	320
Microbiota isolates						
I8	EU888874	99.3	Proteobacteria/γ-proteobacteria	*Shewanella baltica* (AB205578)	–	640
P1	EU888875	99.9	Proteobacteria/γ-proteobacteria	*Pseudomonas* sp. (AY456701)	–	640
A8P1-8	EU888871	99.7	Proteobacteria/γ-proteobacteria	*Kluyvera intermedia* (AF047187)	–	1280
A8P1-9	EU888872	100	Proteobacteria/γ-proteobacteria	*Citrobacter gillenii* (AF025367)	–	1280
B8P3-1	EU888873	99.9	Proteobacteria/γ-proteobacteria	*Hafnia alvei* (DQ412565)	–	1280
ThV-A	EU888865	100	Proteobacteria/γ-proteobacteria	*Psychrobacter* sp.(DQ337539)	–	1280
ThV-E	EU888866	100	Firmicutes/Bacilli	*Lactococcus lactis* (AJ488176)	+	1280
ThV-F	EU888867	100	Firmicutes/Bacilli	*Lactococcus lactis* subsp. *lactis* (DQ173744)	+	1280
ThV-G	EU888868	100	Firmicutes/Bacilli	*Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* (AB100805)	+	1280
ThV-H	EU888869	99.7	Actinobacteria/Actinobacteria	*Arthrobacter* sp. (EU034524)	+	1280
ThV-I	EU888870	100	Actinobacteria/Actinobacteria	*Arthrobacter* sp. (AJ786821)	+	1280

Pathogenic isolates were obtained from different tissues of sick fish. Microbiota isolates were obtained from faeces of healthy fish. Nearest-match identification of 16S rDNA sequences obtained to known sequences in the RDP II database.

ferences have important biological effects and could explain the susceptibility to TVEO. It should be noticed that the level of TVEO required to inhibit bacterial pathogen (>80 mg L^{-1}) is higher than the level used in the *in vivo* study (20 mg TVEO kg^{-1} feed). Therefore, more *in vivo* studies are needed to evaluate the effect of a higher TVEO concentration in gut bacteria. Also, it would be necessary to evaluate whether these higher concentrations can alter feed flavour or induce toxic responses in the fish (Stroh *et al.* 1998). The encapsulation of EOs could be a plausible alternative to deliver active EOs into the fish gut, as this would reduce interactions with the food matrix and possibly reduce toxic effects (Wang, Gong, Huang, Yu & Xue 2009).

Some authors have suggested that the preservation of a diverse microbial community that includes innocuous and beneficial bacteria is key to managing a successful hatchery (Schulze, Alabi, Tattersall-Sheldrake & Miller 2006), which is important to consider in terms of antibiotic use and abuse. Antibiotic treatments can eradicate susceptible microorganisms, which may promote colonization by resistant opportunist bacteria (Moffitt & Mobin 2006; Navarrete *et al.* 2008). Because TVEO is effective with pathogens and permissive with indigenous microbiota, we suggest that natural EOs could be used as alternatives for managing bacterial populations and avoiding bacterial resistance. However, more studies are needed to evaluate the *in vivo* effects of TVEO in fish.

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In conclusion, TVEO feed supplementation had no toxic effects on trout and did not significantly alter the bacterial populations of trout intestines, as assessed by PCR–TTGE. However, TVEO demonstrated *in vitro* antibacterial activity against fish bacterial pathogens, and it can potentially be used as a protective agent in fish. Nevertheless, more detailed studies using infected fish are needed.

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