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# Inhibition of verocytotoxigenic *Escherichia coli* in model broth and rumen systems by carvacrol and thymol

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ABSTRACT

The antimicrobial activities of thymol and carvacrol were assessed against a selection of verocytotoxigenic *Escherichia coli* (VTEC) strains ($n=11$) and other bacterial species and spoilage bacteria ($n=7$) using a model broth system. The effects of pH, temperature, water activity, sodium chloride concentrations, inoculum size and the presence of competing microflora on the activities of thymol and carvacrol against *E. coli* O157:H7 strain 380-94 were also determined. The minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively) and numbers of surviving *E. coli* O157:H7 were determined following incubation. The mean numbers of VTEC surviving exposure to thymol or carvacrol at concentrations of ≥500 μg/ml were between 2.0 and 7.8 log cfu/ml less than the numbers in the corresponding controls. The susceptibility of *E. coli* O157:H7 to carvacrol or thymol was found to increase with decreasing storage temperature, water activity, pH and *E. coli* O157:H7 inoculum size. Sodium chloride (0.5–2.5%) and the presence of a microflora cocktail did not significantly ($p>0.05$) affect the antimicrobial activities of thymol or carvacrol against *E. coli* O157:H7. The antimicrobial activity of carvacrol against *E. coli* O157:H7 was also tested in a model rumen system. A MIC of 500 μg/ml carvacrol reduced *E. coli* O157:H7 inoculated at levels of $10^3$ and $10^6$ cfu/ml to undetectable levels in the system after 24 h incubation. This concentration of carvacrol significantly ($p<0.05$) decreased the total gas production and volatile fatty acid concentrations in the model rumen assay.



## 1. Introduction

Many strains of verocytotoxigenic *Escherichia coli* (VTEC) are recognised as human pathogens and are a food safety concern. The VTEC serotype O157:H7, as well as other clinically important serotypes such as O26 and O111, are associated with public health risks and outbreaks of VTEC illness worldwide (European Food Safety Authority, 2007). Although most VTEC infections are associated with the consumption of contaminated meat products, other foods such as fruits and vegetables and dairy products have also been implicated in VTEC infection and outbreaks (Strachan et al., 2006; Erickson and Doyle, 2007; Franz and van Bruggen, 2008). Controlling the numbers and growth of VTEC remains an important objective for the food industry in general and the beef industry in particular. In addition, the use of antibiotics and the expanding resistance to antibiotics of some pathogens that are associated with foodborne illness have caused a growing demand for more natural antimicrobials to improve the quality and safety of food.

Thymol and carvacrol are the two major components of oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) essential oils (EOs) and are generally recognised as safe by the United States Food and Drug Administration (FDA). Although there are numerous studies reporting the antimicrobial activities of EOs or their components including carvacrol and thymol against *E. coli*, many studies utilise a limited number of *E. coli* strains and usually target the serotype O157 (Kim et al., 1995; Cosentino et al., 1999; Friedman et al., 2002; Burt and Reinders, 2003; Fisher and Phillips, 2006). As other serotypes such as O26 and O111 are also clinically significant, it is of interest to determine whether there is a variation in the susceptibility profiles of different VTEC strains and serotypes to thymol and carvacrol.

The use of EOs and their components in food products is often limited because effective antimicrobial concentrations may exceed the acceptable sensory levels in food (Burt, 2004). Determining the minimum inhibitory and bactericidal concentrations of these EO components and their potential use in combination with other environmental factors is important to establish a balance between the efficacy of the antimicrobial and desirable organoleptic properties of food products (Santiesteban-Lopez et al., 2007). Furthermore, the use of these antimicrobials for the control of foodborne pathogens in ruminant animals prior to slaughter requires study, as many reports to

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date have investigated their use for the purpose of improving animal production only (Hart et al., 2008; Evans and Martin, 2000; Cardozo et al., 2005; Castillejos et al., 2008). Therefore, the first objective of the present study was to use a model broth system to determine the antimicrobial activities of carvacrol and thymol against a selection of VTEC strains, other bacterial pathogens and food spoilage bacteria, and to determine the antimicrobial activities of carvacrol and thymol against *E. coli* O157:H7 under different conditions typical of a food matrix. The second objective was to determine the antimicrobial activity of carvacrol against *E. coli* O157:H7 in a model rumen system.

## 2. Materials and methods

### 2.1. Bacterial cultures and reagents

Eleven strains of *E. coli* consisting of mainly VTEC strains were included in this study. Most of the strains were *E. coli* O157:H7 but other pathogenic serotypes were also included (Table 1). Strains were screened for the presence of virulence factors including *vt* 1 and *vt* 2, *eae,* and *hly*A (Paton and Paton, 2002). In addition, nine bacterial strains representing other foodborne pathogens and microflora in meat were also tested (Table 1). For the experiments, all bacterial cultures were prepared from protect beads stored at −20 °C and inoculated into 10 ml tryptone soya broth (TSB; Merck, Whitehouse Station, NJ, USA) and incubated overnight at 37 °C without shaking. The exceptions were the *Lactobacillus sakei* and *Brochothrix thermosphacta* strains which were grown in 10 ml de Man Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, U.K.) or brain heart infusion (BHI) broth (Oxoid), respectively and were incubated overnight at 30 °C without shaking. Carvacrol was purchased as a liquid and thymol as a solid from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Model broth system experiments

#### 2.2.1. Microtitre plate assay to determine the minimum inhibitory and bactericidal concentrations

The minimal inhibitory concentrations (MIC) of carvacrol and thymol were determined using the micro-broth dilution assay outlined by Anderson and Yu (2005) with modifications. The same medium and temperature used to initially grow each bacterial culture (Section 2.1) was used for the microtitre plate assay. Carvacrol was added to the growth medium with 0.05% bacteriological agar (Oxoid) to make a working solution of 4 mg/ml. Thymol was initially dissolved in 95% (v/v) ethanol prior to adding it to the growth medium with 0.05% (w/v) bacteriological agar. The agar acted as a stabiliser for carvacrol and thymol as outlined by Burt et al. (2005). Polypropylene microtitre plates with 96 wells (Sigma-Aldrich) were used for the assay. Carvacrol and thymol were diluted across the rows in two-fold dilutions from 2000 to 3.9 μg/ml so that each well contained 100 μl of carvacrol or thymol in growth medium with 0.05% agar. Bacterial cultures were diluted to $10^6$–$10^7$ cfu/ml in the growth medium with 0.05% agar, and 100 μl of the diluted culture was added to each well. A control consisting of the bacterial strain inoculated in the growth medium with 0.05% agar but without carvacrol or thymol (0 μg/ml) was included in all assays. For thymol, the control also included 2.6 μl of 95% (v/v) ethanol which represented the maximum amount of ethanol used in the assay. This amount of ethanol was previously found to have no affect on *E. coli* O157:H7 growth in the broth (data not shown).

The plates were incubated for 16–18 h at 37 °C or at 30 °C for *L. sakei* and *B. thermosphacta*. Following incubation the optical absorbance at 595 nm ($A_{595}$) of each well was recorded using a Genios microtitre plate reader (Tecan, Männedorf, Switzerland). A well with growth medium with 0.05% agar only was included in each plate as a blank for the $A_{595}$ readings. The MIC was defined as the concentration of the carvacrol or thymol in the last well in which culture growth was not detected following incubation. The numbers of cells surviving exposure to different carvacrol or thymol concentrations were determined by serial dilution into minimal recovery diluent (MRD, Merck) and plating onto tryptone soya agar (TSA, Merck) followed by overnight incubation at 37 °C. For *L. sakei* and *B. thermosphacta*, diluted samples were plated onto MRS or BHI agar, respectively and incubated overnight at 30 °C. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of carvacrol or thymol that resulted in significantly ($p<0.05$) less counts compared to the initial inoculum level. All assays were replicated at least three times.

#### 2.2.2. Determination of carvacrol and thymol activity against E. coli O157:H7 incubated under various test conditions

The antimicrobial activities of thymol and carvacrol against a strain of *E. coli* O157:H7 (380-94) were evaluated with the microtitre plate assay outline above, under the following conditions. A control for each test condition was included in each assay and consisted of *E. coli* O157:H7 in the same TSB with 0.05% agar used for each test condition but without carvacrol or thymol. The results were also compared to standard broth conditions which consisted of TSB with 0.05% agar which was pH 7, $a_w$ 1.00, NaCl concentration of 0.5%. The standard

**Table 1**
Bacterial strains used in study.

| Bacterial strain/Isolate no. | Serotype | Source | *vt1* | *vt2* | *eae* | *hlyA* |
|---|---|---|---|---|---|---|
| 380-94 | *E. coli* O157:H7 | Food isolate | + | + | + | + |
| J21 | *E. coli* O157:H7 | Food isolate | − | + | + | + |
| CO1 | *E. coli* O157:H7 | Human clinical | + | + | + | + |
| CO2 | *E. coli* O157:H7 | Human clinical | − | + | + | + |
| VC47 | *E. coli* O157:H7 | Bovine | − | + | + | + |
| DPC6054 | *E. coli* O157:H7 | Dairy products collection | − | − | + | + |
| DPC6055 | *E. coli* O157:H7 | Dairy products collection | − | − | + | + |
| 361 | *E. coli* O26 | Clinical isolate | + | − | + | + |
| 378 | *E. coli* O111 | Food isolate | − | + | + | − |
| MB2652 | *E. coli* O103:H2 | Clinical isolate | + | − | + | + |
| MB2692 | *E. coli* O145:H- | Clinical isolate | + | − | + | + |
| DPC6053 (JM109) | *E. coli* | Dairy products collection | − | − | − | − |
| *Salmonella* Typhimurium | | DT104 | | | | |
| *Listeria monocytogenes* | | NCTC 11994 | | | | |
| *Hafnia alvei* | | DSMZ 30163 | | | | |
| *Staphylococcus aureus* | | NCTC 7428 | | | | |
| *Lactobacillus sakei* | | UC7012 | | | | |
| *Pseudomonas putida* | | ATCC 49128 | | | | |
| *Brochothrix thermosphacta* | | DSMZ 20171 | | | | |

NCTC, National Collection of Type Culture; ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkuturen.