

# Evaluation of Antimicrobial Activity of *Cymbopogon citratus* Essential Oil Alone and in Combination with *Origanum majorana* and *Caryophyllus aromaticus* Essential Oils against Some Foodborne Bacteria



Razieh Partovi<sup>1\*</sup>, Fazeleh Talebi<sup>2</sup>, Zahra Boluki<sup>3</sup>, Aghil Sharifzadeh<sup>4,5</sup>

<sup>1</sup>Department of Food Hygiene, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran

<sup>2</sup>Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

<sup>3</sup>Knowledge Utilization Research Center (KURC), Tehran University of Medical Sciences, Tehran, Iran

<sup>4</sup>Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

<sup>5</sup>Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

## \*Corresponding Author:

Razieh Partovi, Amol University of Special Modern Technologies, Haraz Street, Aftab 24, P.O. Box 46168-49767, Amol, Iran.  
Tel: +98 11 44271057;  
Fax: +98 1144271054;  
Email: r.partovi@ausmt.ac.ir

Published Online March 10, 2019

**Keywords:** Antimicrobial activity, *Origanum majorana*, *Cymbopogon citratus*, *Caryophyllus aromaticus*, Essential oils, Chemical composition



## Abstract

**Background:** Food spoilage and foodborne diseases are two important problems in the food industry. On the other hand, consumers' tendency to use natural additives is increasing. Hence, plant essential oils (EOs) can be safe alternatives in this regard.

**Objective:** The objectives were to determine the chemical composition and to evaluate the antimicrobial activity of *Cymbopogon citratus* EO against some foodborne bacteria alone and in combination with *Origanum majorana* and *Caryophyllus aromaticus* EOs.

**Materials and Methods:** Chemical composition of *C. citratus* EO was analyzed by gas chromatography-mass spectrometry. Further, antibacterial activity of the EO against foodborne bacteria was assessed using disk diffusion method. In addition, the minimum inhibitory concentration of the EO was determined by microdilution broth method and then the minimum bactericidal concentration value was determined. Checkerboard synergy testing was also performed to determine the fractional inhibitory concentration index. Finally, time-kill curves were drawn based on the bacterial population (CFU/mL) against time (h).

**Results:** The major compounds of *C. citratus* EO were isothymol, thymol, trans-caryophyllene, and cymene. The most and the least sensitive foodborne bacteria to *C. citratus* EO were *Staphylococcus aureus* and *Bacillus subtilis*, respectively. The minimum inhibitory concentration (MIC) values of *C. citratus* EO against all the evaluated bacteria were 0.1% and The minimum bactericidal concentration (MBC) values ranged between 0.1 and >2% (v/v). The combination of *C. citratus* and *O. majorana* EOs showed a synergistic activity against *Salmonella typhimurium* and partial synergism against *B. subtilis*, *Escherichia coli* O157:H7, *S. aureus*, and *Listeria monocytogenes*. Moreover, the combination of *C. citratus* and *C. aromaticus* EOs demonstrated partial synergism against *S. aureus* and *L. monocytogenes*, and additive interaction against *S. typhimurium*; however, the combination was indifferent against *E. coli* O157:H7 and *B. subtilis*. Furthermore, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs showed a bactericidal effect against *S. typhimurium* after 24 hours in the time-kill assay.

**Conclusion:** In general, the synergism, partial synergism, and additive effects of *C. citratus* in combination with *C. aromaticus* and *O. majorana* EOs strengthen the antimicrobial activity, expand the spectrum of activity, reduce the concentrations required, decrease the side effects, and prevent the alteration of organoleptic properties of food.

Received August 7, 2018; Revised February 25, 2019; Accepted February 27, 2019

## Background

Foodborne diseases are among the causes of hospitalization and/or deaths all over the world every year.<sup>1,2</sup> In the meanwhile, foodborne bacteria can shorten the shelf life of food.<sup>3</sup> Food industry is seeking for natural, safe, and

effective alternatives to synthetic food additives because of their side effects.<sup>4,5</sup> Essential oils (EOs) are the secondary plant metabolites, which are liquid, volatile, and soluble in fat,<sup>6</sup> and are reported to do an antimicrobial activity, as well.<sup>7-11</sup> Further, they are hydrophobic compounds, which

accumulate in the lipid layer of bacterial cell membrane and cause membrane damage.<sup>12-15</sup> Thymol, carvacrol, and eugenol as the major compounds of some plant EOs affect the function and permeability of cell membranes.<sup>14-16</sup> A number of studies have assessed the antimicrobial activities of EOs in combination.<sup>12,17</sup> Using EOs alone in food industry necessitates high concentrations of EOs and have some adverse effects on food sensory acceptability such as alteration in the taste, color, odor, and texture of food. However, using EOs in combination reduces the required concentration of each EO.<sup>18,19</sup>

*Cymbopogon citratus* (lemon grass) belongs to Poaceae family. *C. citratus* is native to tropical and subtropical areas of the world especially India and Sri Lanka.<sup>20,21</sup> Lemon grass is also cultivated in some other regions like Jiruft, Dezfool, Sari, and Masjed Soleiman in Iran.<sup>22,23</sup> It is used as diuretic, sedative, antispasmodic, and antibacterial, as well as being used in the treatment of neurological and gastrointestinal disorders, acne, and pimples.<sup>20,21</sup> Furthermore, lemon grass has antiamebic, antidiarrheal, antifilarial, antifungal, and anti-inflammatory effects.<sup>11,24-26</sup> The major compounds of *C. citratus* EO are geranial, neral, myrcene, and  $\beta$ -pinene.<sup>27</sup> The antimicrobial and antifungal activities of *C. citratus* EO alone and in combination with other EOs have been proved in some studies.<sup>11,25,26,28,29</sup> *Origanum majorana* originates from eastern Mediterranean region and grows in the north and northwest of Iran.<sup>30</sup> This plant has been used as tonic, diuretic, sedative, and antiseptic, as well as being used in wound healing.<sup>31</sup> *Caryophyllus aromaticus* is native to tropical areas especially Indonesia and India.<sup>32,33</sup> The most important usage of *C. aromaticus* in traditional medicine is the treatment of toothache and gingivitis with its antibacterial effect against oral bacteria.<sup>34,35</sup>

The objectives of this study were to assess chemical compositions and to evaluate antimicrobial activity of *C. citratus* EO against some gram positive (*Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 65138, *Bacillus subtilis* ATCC 11778) and gram negative (*Escherichia coli* O157:H7 ATCC 43895 and *Salmonella typhimurium* ATCC 14028) foodborne bacteria alone and in combination with *O. majorana* and *C. aromaticus* EOs.

## Materials and Methods

### Plant Material and Extraction Procedure

Leaves of *C. citratus* were purchased from Pakan Bazar Company (Isfahan, Iran). The plant was taxonomically identified at the Pharmacognosy Department, Faculty of Pharmacy, University of Tehran, Tehran, Iran. The plant was submitted to hydrodistillation in a Clevenger-type apparatus at 100°C for 5 hours. The EO was isolated and dried over anhydrous sodium sulfate and then stored in dark glass bottles at 4°C until required. *O. majorana* and *C. aromaticus* EOs were provided from the previous study.<sup>36</sup>

### Gas Chromatography-Mass Spectrometry Analysis

*C. citratus* EO was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (Thermoquest 2000, Manchester, UK). The chromatograph was equipped with DB5 capillary column (30 m  $\times$  0.25 mm ID  $\times$  0.25  $\mu$ m film thickness) and the data were acquired under the following conditions: initial temperature 50°C, program rate 2.5°C per minute, final temperature 265°C, and injector temperature 250°C. The carrier gas was helium and the split ratio was 1:120. The mass spectrum (MS) was run in the electron ionization mode, using an ionization energy of 70 eV. The components of *C. citratus* EO were identified tentatively by comparing their retention indices and mass spectra with those of Wiley 275 Registry of Mass Spectral Data and literature citations.<sup>37,38</sup> The chemical composition of *O. majorana* and *C. aromaticus* EOs have been determined in the previous study.<sup>36</sup>

### Bacterial Strain and Inoculum Preparation

Standard strains of Gram positive (*L. monocytogenes* ATCC 7644, *S. aureus* ATCC 65138, *B. subtilis* ATCC 11778) and Gram negative (*E. coli* O157:H7 ATCC 43895 and *S. typhimurium* ATCC 14028) foodborne bacteria were supplied from Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran. Bacterial strains were refreshed twice in sterile brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) at 37°C for 18 hours. The bacterial broth culture was placed in sterile cuvette and its optical density (OD) was adjusted to the absorbance at 600 nm of 0.1, using T80+ UV/VIS Spectronic spectrophotometer (PG Instruments Ltd, Leicestershire, UK). The number of cells in the suspension was estimated by duplicate plating from 10-fold serial dilutions on BHI agar (Merck, Darmstadt, Germany) and counting the colonies after incubation at 37°C for 18 hours.<sup>39</sup>

### Disk Diffusion Assay

Antibacterial activity of *C. citratus* EO against foodborne bacteria was assessed using disk diffusion method. One hundred  $\mu$ L of bacterial suspension containing  $1 \times 10^5$  colony forming units per mL (CFU/mL) was spread onto BHI agar containing 10% dimethyl sulfoxide (DMSO). The inoculated plates were put at room temperature for 5 minutes to dry. Then sterile paper disks inoculated with 10  $\mu$ L of the EO were placed on BHI plates with chloramphenicol and streptomycin disks as positive controls and blank disks as negative controls. The plates were left for 15 minutes at room temperature to allow the diffusion of the EO, and were incubated at 37°C for 24 hours. At the end of the period, the diameter of the clear zone around each disk was measured with a ruler and expressed in millimeters as its antimicrobial activity. The EO would have antimicrobial activity if inhibition zone was more than 12 mm in diameter.<sup>40,41</sup>

### Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) of *C. citratus* EO was determined by microdilution broth method based on the document M7-A6 of CLSI (CLAI, 2015)<sup>42</sup> against foodborne bacteria. Sterile 96-well microplates were used for the assay. Dilution series of the EOs were prepared from 0.0031% to 1% (v/v) in BHI broth. The stock solutions of the EOs contained 10% (v/v) DMSO. Two hundred microliters of each dilution was transferred into 96-well microtiter plates, followed by the addition of 20 µL of respected standardized microorganism suspension containing  $1 \times 10^5$  CFU/mL. Growth control consisted of BHI broth, 10% (v/v) DMSO, and bacterial suspension. After incubation at 37°C for 24 hours, the lowest concentrations without visible growth were defined as the concentrations that completely inhibited bacterial growth (MICs). The minimum bactericidal concentration (MBC) of the EO was determined according to the MIC values, based on Celiktas et al.<sup>43</sup> Ten microliters of each well that showed complete absence of growth was transferred to BHI agar plates and incubated at 37°C for 24 hours. The lowest concentrations of the EO where no viable bacteria were identified were recognized as the MBCs.

### Checkerboard Assay

Checkerboard synergy testing was performed to determine the fractional inhibitory concentration index (FICI). Checkerboard assay was done by the microdilution broth method. In brief, serial double dilutions of *C. citratus*, *O. majorana*, and *C. aromaticus* EOs from 2 MIC to 1/64 MIC were prepared. The MIC of *O. majorana* EO was 0.1% against all tested bacteria except for *B. subtilis* (0.3%) and the MIC of *C. aromaticus* EO was 0.1% against all tested bacteria.<sup>36</sup> One hundred microliters of *C. citratus* dilutions were added to the rows of a 96-well microtiter plate in diminishing concentrations and 100 µL of *O. majorana* dilutions were also added to the columns in diminishing concentrations. Moreover, 100 µL of *C. citratus* dilutions were added to the rows of another 96-well microtiter plate in diminishing concentrations and 100 µL of *C. aromaticus* dilutions were also added to the columns in diminishing concentrations. A 20-µL suspension of the bacterial strains adjusted to  $1 \times 10^5$  CFU/mL was added to each well and incubated at 37°C for 24 hours, with shaking at 125 rpm. The MIC of *C. citratus* EO in combination was determined as described above. Each experiment was repeated two times. FICI was calculated as follows<sup>44</sup>:

$$\text{FIC of } C. \text{ citratus} = \frac{\text{MIC of } C. \text{ citratus in combination}}{\text{MIC of } C. \text{ citratus alone}}$$

$$\text{FIC of } O. \text{ majorana} = \frac{\text{MIC of } O. \text{ majorana in combination}}{\text{MIC of } O. \text{ majorana alone}}$$

$$\text{FICI} = \text{FIC of } C. \text{ citratus} + \text{FIC of } O. \text{ majorana}$$

$$\text{FIC of } C. \text{ citratus} = \frac{\text{MIC of } C. \text{ citratus in combination}}{\text{MIC of } C. \text{ citratus alone}}$$

$$\text{FIC of } C. \text{ aromaticus} = \frac{\text{MIC of } C. \text{ aromaticus in combination}}{\text{MIC of } C. \text{ aromaticus alone}}$$

$$\text{FICI} = \text{FIC of } C. \text{ citratus} + \text{FIC of } C. \text{ aromaticus}$$

FICI was interpreted as follows: synergism,  $\text{FICI} \leq 0.5$ ; partial synergism,  $1.0 > \text{FICI} > 0.5$ ; additive effect,  $\text{FICI} = 1.0$ ; indifference,  $1.0 < \text{FICI} \leq 4.0$ ; and antagonism,  $\text{FICI} > 4.0$ .

### Time-Kill Assay

The EOs used in the time-kill assay had concentrations equivalent to  $1 \times \text{MIC}$ . The final concentration of the bacterial suspension in BHI tubes was adjusted to  $1 \times 10^5$  CFU/mL. A growth control without EO was included. The suspensions were incubated at 37°C for 24 hours, with shaking at 125 rpm. Each suspension was cultured on BHI agar after incubation for 0, 3, 6, and 24 hours and was then incubated at 37°C for 24 hours. Time-kill curves were drawn based on the bacterial population (CFU/mL) against time (h).<sup>17</sup> Experiments were carried out in duplicate.

### Statistical Analysis

Data from disk diffusion assay were subjected to Kruskal-Wallis test using SPSS statistical package, version 22.0. For comparison of MIC and MBC of *C. citratus* EO on the evaluated bacteria, the Kruskal-Wallis test was used. For all analyses,  $P < 0.05$  was considered statistically significant.

## Results

### Chemical Composition of the EO

The main constituents of *C. citratus* EO are presented in Table 1. *C. citratus* EO consisted of 15 compounds representing 99.03% of the EO. The major compounds of *C. citratus* EO were isothymol (59.42%), thymol (15.23%), trans-caryophyllene (10.18%), and cymene (5.82%).

### Agar Disk Diffusion Assay

Antimicrobial activity of *C. citratus* EO was evaluated by disk diffusion method (Table 2). The evaluated EO had remarkable antimicrobial effect (inhibition zone  $> 12$  mm). Based on this evaluation, the most and the least sensitive foodborne bacteria to *C. citratus* EO were *S. aureus* and *B. subtilis*, respectively. Moreover, the inhibition zone of *C. citratus* EO against all the tested bacteria except for *B. subtilis* were even greater than that of streptomycin ( $P > 0.05$ ). Furthermore, the inhibition zone of *C. citratus* EO against *E. coli* O157:H7 and *S. typhimurium* was greater than that of chloramphenicol ( $P > 0.05$ ).

### Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC and MBC values of *C. citratus* EO against foodborne bacteria are shown in Table 3. The MIC values

**Table 1.** Chemical Composition (%) of *Cymbopogon citratus* EO Determined by GC-MS

No.	Component	Quantity (%)	Retention Time (min)
1	2-beta-Pinene	0.53	7.61
2	Cymene	5.82	9.57
3	gamma-Terpinene	2.70	10.99
4	Thymol	15.23	22.56
5	alpha-Cubebene	0.09	24.54
6	Copaene	0.38	25.57
7	trans-Caryophyllene	10.18	27.36
8	alpha-Humulene	1.41	28.48
9	delta-Cadinene	0.16	30.90
10	1S alpha-Pinene	0.13	6.15
11	beta-Phellandrene	0.22	9.69
12	trans-Anethole	0.72	21.66
13	Isothymol	59.42	23.58
14	Eugenol	1.79	25.12
15	(-)-Caryophylleneoxide	0.25	32.76
Total		99.03	

of *C. citratus* EO against all the evaluated bacteria were 0.1% and the MBC values ranged between 0.1 and >2% (v/v).

### Checkerboard Assay

The results of checkerboard analyses for *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs against foodborne bacteria are shown in Tables 4 and 5, respectively. FICI values of *C. citratus* plus *O. majorana* EOs against foodborne bacteria ranged from 0.500 to 0.750. The combination of *C. citratus* and *O. majorana* EOs showed a synergistic interaction (FICI≤0.5) against *S. typhimurium*. According to the analysis, the MIC of *C. citratus* and *O. majorana* EOs alone against *S. typhimurium*

was lowered from 0.100 to 0.025 (% v/v) in combination. Moreover, the combination of *C. citratus* and *O. majorana* EOs showed partial synergism (1.0>FICI>0.5) against *B. subtilis*, *E. coli* O157:H7, *S. aureus*, and *L. monocytogenes*. Likewise, FICI values for *C. citratus* plus *C. aromaticus* EOs ranged from 0.75 to 1.25 against foodborne bacteria. The combination of *C. citratus* and *C. aromaticus* EOs showed partial synergism (1.0 >FICI >0.5) against *S. aureus* and *L. monocytogenes* and an additive interaction (FICI= 1.0) against *S. typhimurium*. While, the combination of *C. aromaticus* and *C. citratus* EOs was indifferent against *E. coli* O157:H7 and *B. subtilis*. Finally, no antagonistic effect was observed for *C. citratus*.

### Time-Kill Assay

Time-kill assay was used to analyze the killing rate of *C. citratus* EO alone and in combination with *O. majorana* and *C. aromaticus* EOs against foodborne bacteria. The time-kill curves of *C. citratus* EO alone and in combination with *O. majorana* and *C. aromaticus* EOs (at MIC values) against foodborne bacteria are shown in Figure 1. Bactericidal effects of EOs are concluded when a three or more reduction in bacterial counts is observed in time-kill curves and the bacteriostatic effect when EO inhibits the bacterial growth.<sup>45,46</sup> *C. citratus* EO showed a bacteriostatic effect against foodborne bacteria. The combination of *C. citratus* and *C. aromaticus* EOs reduced the bacterial colony count of *E. coli* O157:H7 in comparison to *C. citratus* EO by 2 log after 6 hours. *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs showed bactericidal effects against *S. typhimurium* after 24 hours. Furthermore, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs reduced the bacterial colony count of *S. typhimurium* in comparison to *C. citratus* EO by 3 log after 24 hours. In

**Table 2.** Antimicrobial Activity (mm) of *Cymbopogon citratus* EO Against Foodborne Bacteria as Detected by Agar Disk Diffusion Assay

EO	Inhibition Diameter (mm)					P value
	<i>E. coli</i> O157:H7	<i>S. typhimurium</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>L. monocytogenes</i>	
<i>C. citratus</i>	20.16±0.28 <sup>bcD*</sup>	20.16±0.57 <sup>bcD</sup>	20.66±0.28 <sup>bc</sup>	18.66±0.57 <sup>cD</sup>	19.33±0.76 <sup>bcCAB</sup>	0.033
Streptomycin	12.33±0.57 <sup>bcC</sup>	13.66±0.57 <sup>bcdeC</sup>	12.00±0.00 <sup>bcC</sup>	23.00±0.00 <sup>ceCD</sup>	14.00±0.00 <sup>bcdeCA</sup>	0.011
Chloramphenicol	17.66±0.57 <sup>cCD</sup>	19.00±0.00 <sup>cbCD</sup>	24.66±0.57 <sup>cbC</sup>	29.00±0.00 <sup>bc</sup>	22.66±0.57 <sup>cbB</sup>	0.008
P value	0.025	0.023	0.023	0.020	0.023	

\*Inhibition area including 6 mm disk diameter.

Results are mean ± SD of 3 replicates.

Within the columns, significant differences are represented by different superscript capital letters ( $P < 0.05$ ). Within the rows, significant differences are represented by different superscript small letters ( $P < 0.05$ ).

**Table 3.** MIC and MBC values (% v/v) of *Cymbopogon citratus* EO Against Foodborne Bacteria Determined by Microdilution Broth Method

	Foodborne Bacteria					P Value
	<i>E. coli</i> O157:H7	<i>S. typhimurium</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>L. monocytogenes</i>	
MIC	0.1 <sup>a</sup>	0.1 <sup>a</sup>	0.1 <sup>a</sup>	0.1 <sup>a</sup>	0.1 <sup>a</sup>	1.000
MBC	0.1 <sup>a</sup>	0.1 <sup>a</sup>	0.1 <sup>a</sup>	>2 <sup>b</sup>	>2 <sup>b</sup>	0.061

Note: Within the rows, significant differences are represented by different superscript small letters ( $P < 0.05$ ).

**Table 4.** Effects of 2 EO Combinations (*C. citratus* and *O. majorana*) Against Foodborne Bacteria Using Checkerboard Assay

Microorganism	MIC (%v/v) of each EO in combination		FIC (%)		FICI	Outcome
	<i>C. citratus</i>	<i>O. majorana</i>	<i>C. citratus</i>	<i>O. majorana</i>		
<i>E. coli</i> O157:H7	0.025	0.050	0.250	0.500	0.750	Partial synergism
<i>S. typhimurium</i>	0.025	0.025	0.250	0.250	0.500	Synergism
<i>S. aureus</i>	0.025	0.050	0.250	0.500	0.750	Partial synergism
<i>B. subtilis</i>	0.050	0.012	0.500	0.041	0.541	Partial synergism
<i>L. monocytogenes</i>	0.050	0.025	0.500	0.250	0.750	Partial synergism

**Table 5.** Effects of 2 EO Combinations (*C. citratus* and *C. aromaticus*) Against Foodborne Bacteria Using Checkerboard Assay

Microorganism	MIC (%v/v) of Each EO in Combination		FIC (%)		FICI	Outcome
	<i>C. citratus</i>	<i>O. majorana</i>	<i>C. citratus</i>	<i>O. majorana</i>		
<i>E. coli</i> O157:H7	0.025	0.1	0.25	1	1.25	Indifference
<i>S. typhimurium</i>	0.05	0.05	0.5	0.5	1	Additive
<i>S. aureus</i>	0.025	0.05	0.25	0.5	0.75	Partial synergism
<i>B. subtilis</i>	0.0124	0.1	0.041	1	1.041	Indifference
<i>L. monocytogenes</i>	0.025	0.05	0.25	0.5	0.75	Partial synergism

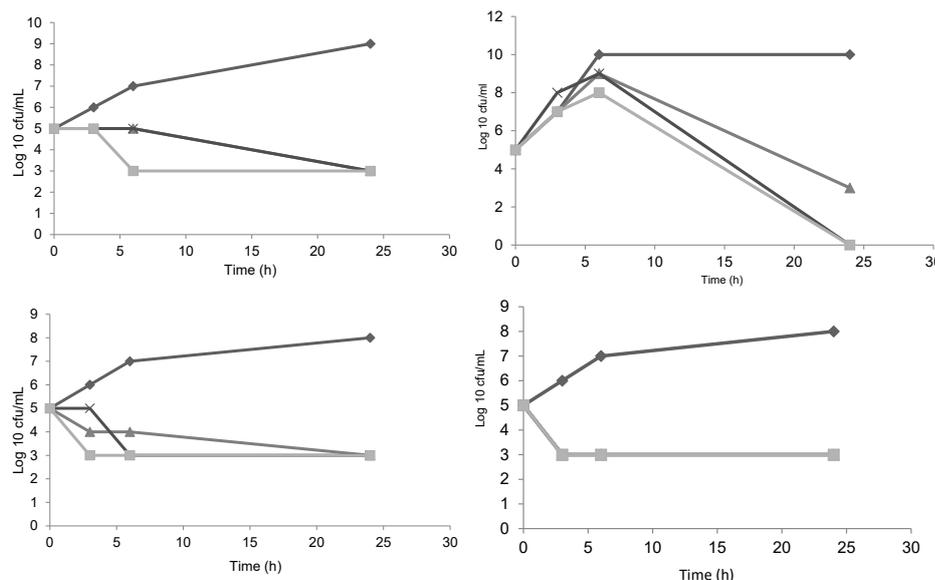
addition, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs reduced the bacterial colony count of *S. aureus* in comparison to *C. Citratus* EO by 1 log after 6 hours. It was also found that the combination of *C. citratus* and *C. aromaticus* EOs reduced the bacterial colony count of *L. monocytogenes* in comparison to *C. Citratus* EO by 1 log after 6 hours.

**Discussion**

In the present study, the main components of the EO of *C. citratus* leaves were found to be isothymol, thymol, trans-caryophyllene, and cymene. The major components of whole plant of *C. citratus* collected from Kenya were geranial, neral, myrcene, and geraniol.<sup>47</sup> Oliveira et al

reported that the main components of EO of *C. citratus* fresh leaves collected from Brazil were geranial, neral, and myrcene.<sup>48</sup> The factors such as characteristics of plant species, plant part used for extraction, and extraction technique, as well as environmental, seasonal, and geographical conditions are the reasons for the differences in the chemical composition of plant EOs.<sup>49,50</sup> Thymol causes a distortion of the membrane physical structure and increases the microbial cytoplasmic membrane permeability.<sup>51</sup>

It was found that *C. citratus* EO had a remarkable antimicrobial effect (inhibition zone > 12 mm). *S. aureus* and *B. subtilis* were the most and the least sensitive bacteria to *C. citratus* EO, respectively. The inhibition



**Figure 1.** Time-kill Curves of Control (◆), *C. citratus* EO (▲), *C. citratus* Plus *O. majorana* EOs (×) and *C. citratus* Plus *C. aromaticus* EOs (■) (at MIC Value) Against Foodborne Bacteria (a=*E. coli* O157:H7, b= *S. typhimurium*, c=*S. aureus*, d= *B. subtilis* and e=*L. monocytogenes*).

zone of *C. citratus* EO against all the tested bacteria except for *B. subtilis* were even greater than that of streptomycin ( $P > 0.05$ ). And the inhibition zone of *C. citratus* EO against *E. coli* O157:H7 and *S. typhimurium* was greater than that of chloramphenicol ( $P > 0.05$ ). Bassol et al showed the antimicrobial effect of *C. citratus* EO against *E. faecalis*, *S. aureus*, *L. monocytogenes*, *E. aerogenes*, *E. coli*, *S. typhimurium*, *S. dysenteriae*, and *P. aeruginosa*, and reported larger inhibition zone for *C. citratus* EO against other microorganisms.<sup>28</sup> This difference can be attributed to the main components of the EO which were geraniol and nerol. Naik et al studied the antimicrobial effect of *C. citratus* EO against *S. aureus*, *Bacillus cereus*, *B. subtilis*, *E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* by disk diffusion method. *S. aureus* and *P. aeruginosa* were the most and the least sensitive bacteria, respectively. Akin to the results of the present study, zone of inhibition (mm) of *C. citratus* EO (30% (v/v)) against *S. aureus* was 29.66 mm.<sup>52</sup> EOs exert their antimicrobial effects through a number of mechanisms including inhibition of nucleic acid synthesis, disturbance in the cytoplasmic membrane properties and energy metabolism.<sup>45</sup> EOs attack cell membrane phospholipids and increase the permeability of the cell wall and cause cytoplasmic leakage.<sup>12-15</sup>

The MIC values of *C. citratus* EO against all foodborne bacteria were 0.1% (v/v). Likewise, the MBC values of *C. citratus* EO against foodborne bacteria were 0.1% except for *B. subtilis* and *L. monocytogenes* (MBC >2%). This result was proved by disk diffusion assay which showed the least inhibition zones for *B. subtilis* and *L. monocytogenes*. Bassolé et al found the MIC values of *C. citratus* EO, ranged from 0.1% for *Enterococcus faecalis* to 8% for *P. aeruginosa*.<sup>28</sup> Chaftar et al also found the MIC value of *C. citratus* EO against *E. coli* O157:H7 and *S. typhimurium* (>0.4%).<sup>53</sup>

In this study, the antimicrobial effect of *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs against foodborne bacteria were studied for the first time. The combination of *C. citratus* and *O. majorana* EOs showed a synergistic effect against *S. typhimurium*. Furthermore, the combination of two EOs (*C. citratus* and *O. majorana*) showed partial synergism against *B. subtilis*, *E. coli* O157:H7, *S. aureus*, and *L. monocytogenes*. The combination of *C. citratus* and *C. aromaticus* EOs also showed partial synergism against *S. aureus* and *L. monocytogenes* and an additive interaction against *S. typhimurium*. In addition, the combination of two EOs (*C. citratus* and *C. aromaticus*) was indifferent against *E. coli* O157:H7 and *B. subtilis*. Yet no antagonistic effect was observed for *C. citratus*. Bassolé et al showed that the combination of *C. citratus* and *Cymbopogon giganteus* EOs had a synergistic effect against *S. aureus*, *L. monocytogenes*, *S. typhimurium*, and *E. aerogenes*.<sup>28</sup> Gutierrez et al reported that the combination of *O. majorana* and *Origanum vulgare* EOs were indifferent against *L.*

*monocytogenes* and *P. aeruginosa*; while an additive effect was seen against *B. cereus* and *E. coli* O157:H7.<sup>19</sup> Tserennadmid et al reported that the combination of *O. majorana* and *Juniperus communis* EOs had a synergistic effect against *E. coli*.<sup>54</sup> *C. aromaticus* plus *Rosmarinus officinalis* EOs had additive antimicrobial effects against *S. epidermidis*, *S. aureus*, *B. subtilis*, *E. coli* O<sub>157</sub>H<sub>7</sub>, *P. vulgaris*, and *P. aeruginosa*.<sup>17</sup> Using the combinations of EOs with synergistic or additive effects may decrease the need for chemical additives, limit their adverse effects and antibiotic resistance, may reduce required doses, and expand the spectrum of activity.<sup>18,44</sup> Furthermore, by using EOs in combination, the microorganisms were inhibited through the simultaneous effects of various chemical compounds, thereby improving antimicrobial properties.<sup>46</sup>

In the current study, *C. citratus* EO alone did not show bactericidal effect against foodborne bacteria in the time-kill assay. While, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs showed a bactericidal effect against *S. typhimurium* after 24 hours. Furthermore, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs reduced the bacterial colony count of *S. typhimurium* in comparison to *C. citratus* EO by 3 log after 24 hours. Khan and Ahmad studied the antimicrobial effect of *C. citratus* and *Syzygium aromaticum* EOs against *Candida albicans* by time-kill assay and reported that more than 60% reduction in viable count of *C. albicans* was exhibited by *C. citratus* EO after 10–12 hours which was more effective than amphotericin B and fluconazole.<sup>29</sup> Oliveira et al studied the antimicrobial effects of *Origanum vulgare* and *O. majorana* EOs at the MIC values against *S. aureus*, *Proteus* spp., and *Klebsiella* spp. by the time-kill assay. The most potent inhibitory effect was shown by *O. majorana* EO against *Proteus* spp. which killed the initial inoculum after 4 hours.<sup>51</sup> The results of time-kill assay of the current study verified the abovementioned results on the synergistic effect of *C. citratus* and *O. majorana* EOs and additive effect of *C. citratus* and *C. aromaticus* EOs against *S. typhimurium*.

## Conclusion

In general, the current study showed that *C. citratus* EO had an antimicrobial activity against the most important foodborne bacteria. Therefore, the combination of *C. citratus* EO with *O. majorana* and *C. aromaticus* EOs can be used as an alternative for synthetic additives to reduce their side effects and also to decrease antibiotic resistance. The combination of these EOs, depending on the corresponding microorganism, exhibited additive, synergistic, and partial synergistic, as well as indifferent interaction. These interactions strengthen the antimicrobial activity, expand the spectrum of activity, reduce the concentrations required, decrease the side effects, and prevent the alteration of organoleptic

properties of food. To the best of our knowledge, this is the first study on the antimicrobial effects of *C. citratus* in combination with *O. majorana* and *C. aromaticus* EOs. Further studies on the interaction of these EOs with food ingredients, their modes of action, and their components' mechanisms of action are required.

#### Authors' Contributions

RP: Designing the study, writing the manuscript; FT: Drafting the manuscript; ZB: Conducting the statistical analyses; AS: Obtaining the samples.

#### Ethical Approval

All procedures performed in this study were in accordance with the ethical standards of the National Research Committee.

#### Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

#### Financial Support

This study was supported by a grant from Amol University of Special Modern Technologies.

#### References

- Newell D. G, Koopmans M, Verhoef L, et al. Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *Int J Food Microbiol.* 2010;139: S3-S15. doi:10.1016/j.ijfoodmicro.2010.01.021
- Thorns CJ. Bacterial food-borne zoonoses. *Rev Sci Tech.* 2000;19(1):226-239.
- Klein G, Rüben C, Upmann M. Antimicrobial activity of essential oil components against potential food spoilage microorganisms. *Curr Microbiol.* 2013;67(2):200-208. doi:10.1007/s00284-013-0354-1
- Quintavalla S, Vicini L. Antimicrobial food packaging in meat industry. *Meat science.* 2002;62(3):373-380. doi:10.1016/S0309-1740(02)00121-3
- Partovi R, Khanjari A, Abbaszadeh S, Sharifzadeh A. Chemical composition and antimicrobial effect of five essential oils on pathogenic and non-pathogenic vibrio parahaemolyticus. *Nutr Food Sci Res.* 2017;4(2):43-52. doi: 10.18869/acadpub.nfsr.4.2.6
- Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils—a review. *Food Chem Toxicol.* 2008; 46(2): 446-75. doi:10.1016/j.fct.2007.09.106
- Aliyannis N, Kalpoutzakis E, Mitaku S, Chinou IB. Composition and antimicrobial activity of the essential oils of two *Origanum* species. *J Agric Food Chem.* 2001;49(9):4168-4170. doi: 10.1021/jf001494m
- Benkeblia N. Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *LWT-food science and technology.* 2004;37(2):263-268. doi:10.1016/j.lwt.2003.09.001
- Carson C, Riley T. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. *J Appl Microbiol.* 1995;78(3):264-269. doi:10.1111/j.1365-2672.1995.tb05025.x
- Farag R, Daw Z, Hewedi F, El-Baroty G. Antimicrobial activity of some Egyptian spice essential oils. *J Food Prot.* 1989;52(9): 665-667. doi:10.4315/0362-028X-52.9.665
- Hammer K. A, Carson C, Riley, T. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol.* 1999;86(6):985-990. doi:10.1046/j.1365-2672.1999.00780.x
- Goni P, López P, Sánchez C, Gómez-Lus R, Becerril R, Nerín C. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chem.* 2009;116(4): 982-989. doi:10.1016/j.foodchem.2009.03.058
- Ahmad M, Benjakul S, Prodpran T, Agustini T. W. Physico-mechanical and antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated with essential oils. *Food Hydrocolloids.* 2012;28(1):189-199. doi:10.1016/j.foodhyd.2011.12.003
- Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int J Food Microbiol.* 2004;94(3): 223-253. doi:10.1016/j.ijfoodmicro.2004.03.022
- Gill AO, Holley RA. Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Appl Environ Microbiol.* 2004;70(10): 5750-5755. doi: 10.1128/AEM.70.10.5750-5755.2004
- Xu J, Zhou F, Ji BP, Pei RS, Xu N. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. *Lett Appl Microbiol.* 2008;47(3):174-179. doi:10.1111/j.1472-765X.2008.02407.x
- Fu Y, Zu Y, Chen L, et al. Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytother Res.* 2007;21(10): 989-994. doi:10.1002/ptr.2179
- Bag A, Chattopadhyay RR. Evaluation of synergistic antibacterial and antioxidant efficacy of essential oils of spices and herbs in combination. *PLoS One.* 2015;10(7):e0131321. doi:10.1371/journal.pone.0131321
- Gutierrez J, Barry-Ryan C, Bourke P. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int J Food Microbiol.* 2008;124(1): 91-97. doi:10.1016/j.ijfoodmicro.2008.02.028
- Jafari B, Ebadi A, Aghdam B. M, Hassanzade Z. Antibacterial activities of Lemongrass methanol extract and essence on pathogenic bacteria. *American-Eurasian J Agric Environ Sci.* 2012;12(8):1042-1046. doi: 10.5829/idosi.ajeaes.2012.12.08.6551
- Balakrishnan B, Paramasivam S, Arulkumar A. Evaluation of the lemongrass plant (*Cymbopogon citratus*) extracted in different solvents for antioxidant and antibacterial activity against human pathogens. *Asian Pac J Trop Dis.* 2014;4: S134-S139. doi:10.1016/S2222-1808(14)60428-X
- Mirjalili M, Fakhr Tabataba'ie M, Omidbeigi R. Study of compatibility and evaluation of the essential function in the indigenous biomass of the Iranian lemongrass. *Mag Agric Sci.* 2005;36(1): 33-41.
- Yazdani D, Rezazadeh S, Shahabi NA. Volatile oil constituents of Lemon grass (*Cymbopogon citratus* Stapf) cultivated in north of Iran. *J Med Plants.* 2004;1(9): 69-74.
- Shah G, Shri R, Panchal V, Sharma N, Singh B, Mann AS. Scientific basis for the therapeutic use of *Cymbopogon citratus*, stapf (Lemon grass). *J Adv Pharm Technol Res.* 2011;2(1): 3. doi: 10.4103/2231-4040.79796
- Paranagama PA, Abeysekera KH, Abeywickrama K, Nugaliyadde L. Fungicidal and anti-aflatoxigenic effects of the essential oil of *Cymbopogon citratus* (DC.) Stapf. (lemongrass) against *Aspergillus flavus* Link. isolated from stored rice. *Lett Appl Microbiol.* 2003;37(1):86-90. doi:10.1046/j.1472-765X.2003.01351.x
- Wilson CL, Solar JM, El Ghaouth A, Wisniewski ME. Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. *Plant Dis.* 1997;81(2): 204-210. doi:10.1094/PDIS.1997.81.2.204
- Tchoumboungang F, Zollo P. A, Dagne E, Mekonnen, Y. In vivo antimalarial activity of essential oils from *Cymbopogon citratus* and *Ocimum gratissimum* on mice infected with *Plasmodium berghei*. *Planta Med.* 2005;71(01):20-23. doi: 10.1055/s-2005-837745

28. Bassolé IH, Lamien-Meda A, Bayala BO, et al. Chemical composition and antimicrobial activity of *Cymbopogon citratus* and *Cymbopogon giganteus* essential oils alone and in combination. *Phytomedicine*. 2011;18(12):1070-1074. doi:10.1016/j.phymed.2011.05.009
29. Khan MS, Ahmad I. Biofilm inhibition by *Cymbopogon citratus* and *Syzygium aromaticum* essential oils in the strains of *Candida albicans*. *J Ethnopharmacol*. 2012;140(2):416-423. doi:10.1016/j.jep.2012.01.045
30. Mozaffarian VA. Dictionary of Iranian plants names. Tehran: Farhange Moaser; 1998.
31. Zargari A. Iranian Medicinal plants. Tehran: Tehran University press; 1987.
32. Paoli SD, Giani TS, Presta GA, et al. Effects of clove (*Caryophyllus aromaticus* L.) on the labeling of blood constituents with technetium-99m and on the morphology of red blood cells. *Braz Arch Biol Technol*. 2007;50:175-182. doi:10.1590/S1516-89132007000600022
33. Atawodi SE, Atawodi JC, Pfundstein B, Spiegelhalter B, Bartsch H, Owen R. Assessment of the polyphenol components and in vitro antioxidant properties of *Syzygium aromaticum* (L.) Merr. & Perry. *Electron J Environ Agric Food Chem*. 2011;10(3): 1970-1978.
34. Halder S, Mehta AK, Kar R, Mustafa M, Mediratta PK, Sharma KK. Clove oil reverses learning and memory deficits in scopolamine-treated mice. *Planta Medica*. 2011;77(08): 830-834. doi: 10.1055/s-0030-1250605
35. Khodadoust K, Ardalan M, Pourabbas R, Abdollahi M. Dental and oral diseases in Medieval Persia, lessons from Hedayat Akhawayni. *J Med Ethics Hist Med*. 2013;6: 9.
36. Partovi R, Talebi F, Sharifzadeh A. Antimicrobial efficacy and chemical properties of *Caryophyllus aromaticus* and *Origanum majorana* Essential Oils against Food-borne Bacteria Alone and in Combination. *Int J Enteric Pathog*. 2018;6(4):95-103. doi:10.15171/ijep.2018.25
37. Mirzaei-Najafgholi H, Tarighi S, Golmohammadi M, Taheri P. The effect of Citrus essential oils and their constituents on growth of *Xanthomonascitri* subsp. *citri*. *Molecules*. 2017;22(4):591. doi:10.3390/molecules22040591
38. Massada, Y. Analysis of essential oil by gas chromatography and spectrometry. New York: Wiley; 1976.
39. Khanjari A, Misaghi A, Basti AA, et al. Effects of *Zataria multiflora* Boiss. Essential Oil, Nisin, pH and Temperature on *Vibrio parahaemolyticus* ATCC 43996 and Its Thermostable Direct Hemolysin Production. *J Food Saf*. 2013;33(3):340-347. doi:10.1111/jfs.12058
40. Djenane D, Aïder M, Yangüela J, Idir L, Gómez D, Roncalés P. Antioxidant and antibacterial effects of Lavandula and Mentha essential oils in minced beef inoculated with *E. coli* O157:H7 O157: H7 and *S. aureus* during storage at abuse refrigeration temperature. *Meat Sci*. 2012;92(4): 667-674. doi:10.1016/j.meatsci.2012.06.019
41. Kurekci C, Padmanabha J, Bishop-Hurley SL, Hassan E, Al Jassim RA, McSweeney CS. Antimicrobial activity of essential oils and five terpenoid compounds against *Campylobacter jejuni* in pure and mixed culture experiments. *Int J Food Microbiol*. 2013;166(3): 450-457. doi:10.1016/j.ijfoodmicro.2013.08.014
42. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard- M07-A06, 10th ed. Wayne, PA: Clinical and Laboratory Standards Institute, CLSI; 2015.
43. Celiktas OY, Kocabas EH, Bedir E, Sukan FV, Ozek T, Baser KH. Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations. *Food Chem*. 2007;100(2):553-559.
44. Sharifzadeh A, Khosravi A. R, Shokri H, Tari PS. Synergistic anticandidal activity of menthol in combination with itraconazole and nystatin against clinical *Candida glabrata* and *Candida krusei* isolates. *Microb Pathog*. 2017;107: 390-396. doi:10.1016/j.micpath.2017.04.021
45. Barbosa LN, da Silva Probst I, Andrade BF, et al. In vitro antibacterial and chemical properties of essential oils including native plants from Brazil against pathogenic and resistant bacteria. *J Oleo Sci*. 2015;64(3): 289-298.
46. Probst IS, Sforcin JM, VLM R, Fernandes AAH, Fernandes Júnior A. Antimicrobial activity of propolis and essential oils and synergism between these natural products. *J Venom Anim Toxins Incl Trop Dis*. 2011;17(2):159-167. doi:10.1590/S1678-91992011000200006
47. Matasyoh JC, Wagara IN, Nakavuma JL. Chemical composition of *Cymbopogon citratus* essential oil and its effect on mycotoxigenic *Aspergillus* species. *African Journal of Food Science*. 2011;5(3):138-142.
48. Oliveira VC, Moura DM, Lopes JA, de Andrade PP, da Silva NH, Figueiredo RC. Effects of essential oils from *Cymbopogon citratus* (DC) Stapf., *Lippia sidoides* Cham., and *Ocimum gratissimum* L. on growth and ultrastructure of Leishmania chagasi promastigotes. *Parasitol Res*. 2009;104(5):1053-1059. doi:10.1007/s00436-008-1288-6
49. Chamorro ER, Zambón SN, Morales WG, Sequeira AF, Velasco GA. Study of the chemical composition of essential oils by gas chromatography. Nat. Tech University, Argentina. 2012;15:307-324. doi: 10.5772/33201
50. Ghnaya A. B, Hanana M, Amri I, et al. Chemical composition of *Eucalyptus erythrocorys* essential oils and evaluation of their herbicidal and antifungal activities. *J Pest Sci*. 2013;86(3): 571-577. doi:10.1007/s10340-013-0501-2
51. Oliveira JL, Diniz MD, Lima ED, Souza EL, Trajano VN, Santos BH. Effectiveness of *Origanum vulgare* L. and *Origanum majorana* L. essential oils in inhibiting the growth of bacterial strains isolated from the patients with conjunctivitis. *Braz Arch Biol Technol*. 2009;52(1): 45-50. doi:10.1590/S1516-89132009000100006
52. Naik MI, Fomda BA, Jaykumar E, Bhat JA. Antibacterial activity of lemongrass (*Cymbopogon citratus*) oil against some selected pathogenic bacterias. *Asian Pac J Trop Med*. 2010;3(7): 535-538. doi:10.1016/S1995-7645(10)60129-0
53. Chaftar N, Girardot M, Labanowski J, et al. Comparative evaluation of the antimicrobial activity of 19 essential oils. In: Donelli G, ed. *Advances in microbiology, infectious diseases and public health*. Cham: Springer; 2015:1-15. doi:10.1007/5584\_2015\_5011
54. Tserennadmid R, Takó M, Galgóczy L, et al. Anti yeast activities of some essential oils in growth medium, fruit juices and milk. *Int J Food Microbiol*. 2011;144(3):480-486. doi:10.1016/j.ijfoodmicro.2010.11.004