African Journal of Microbiology Research Vol.6(28), pp. 5736-5744, 26 July, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.506
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Growth response and modeling the effects of *Carum copticum* essential oil, pH, inoculum level and temperature on *Escherichia coli* O157:H7

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Accepted 9 June, 2012

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is an important verotoxin producing *E. coli* (VTEC). It is associated with food and water borne infections. This study was designed to carefully examine the effects of four different factors on the growth of *E. coli* O157:H7 in the brain heart infusion broth. These factors included four concentrations of *Carum copticum* (Zenyan in Persian) essential oil (0, 0.015, 0.03 and 0.06%), with the major components of thymol (57.18%), p-cymene (22.55%), and γ-terpinene (13.07%), two incubation temperatures (35 and 25°C), three levels of pH (5, 6 and 7) and two inoculum size (10^3 and 10^5 cfu ml^{-1}). The experiment was carried out in triplicate. Growth was monitored by visible turbidity during a 30-day period. To evaluate the effects of explanatory variable on time to detection of bacterial growth, parametric survival model based on the weibull distribution was used. All explanatory variables had significant association with time to detection (P < 0.05). The models accurately predicted the growth initiation and inhibition of *E. coli* O157: H7.*

Key words: *Escherichia coli* O157:H7, *Carum copticum* essential oil, modeling, predictive microbiology.

INTRODUCTION

Escherichia coli O157:H7 has emerged as an important zoonotic pathogen posing a tremendous challenge to public health, because of the severity of infection and low infectious dose (Cagney et al., 2004). Illnesses caused by *E. coli* O157: H7 can range from mild, watery diarrhea to life-threatening conditions, such as hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC) and thrombotic thrombocytopenic purpura (TTP) (Coia, 1998). *E. coli* O157:H7 contaminates beef carcasses by a direct transmission from hides and feces (Arthur et al., 2010; Chapman et al., 1997). Predictive microbiology is a subcategory of food microbiology. One of the main aims of this area is the prediction of microbial growth during a limited time (Ferrer et al., 2009). Mathematical models are the summaries of microbial growth responses to environmental factors (McMeekin et al., 1997). There has been an enormous increase in research on the development of mathematical models that describe how microorganisms behave in foods (Buchanan, 1993). Microbial responses to environmental conditions are reproducible and this phenomenon is the premise of predictive microbiology. We can predict the behavior of same organism at the similar environmental condition based on previous observations of microbial growth (Ross and McMeekin, 1994; McMeekin et al., 2002).

Increased consumer awareness, raised a high concern about the use of synthetic chemical additives in food, so scientists encouraged examining natural food additives with a broad spectrum of antimicrobial activity (Gutierrez et al., 2009). In this regard, the antimicrobial properties of plant essential oil are investigated in different studies (Oussalah et al., 2006; Cao et al., 2009; Hanamanthagouda et al., 2010). Essential oils

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concentrated in various parts of the plant such as leaves, bark or fruit. Terpenoids and phenolic compounds such as thymol, carvacrol and eugenol are responsible for their antimicrobial activity (Burt, 2004).

Although, essential oils (EOs) are generally recognized as safe (GRAS), but their organoleptic properties is one of the most important factors that limits their usage in food. So, it is important to determine the minimum concentration of EO that can inhibit the growth of pathogens without affecting the organoleptic properties of the food (Burt, 2004). The control of food-borne pathogens or food spoilage bacteria by the use of essential oil needs the examination of their activity within food or food modeled systems. Some food components may reduce the effective potential function of many natural antimicrobials. So, successful application of EOs in food require the primary studies in representative food model media to determine potential interactions between EOs and food components (Gutierrez et al., 2009; Burt, 2004).

Ajwain (*Carum copticum* Benth. and Hook, syn: *Trachyspermum ammi*) is an aromatic, grassy, annual plant, belonging to the Apiaceae family. This plant grows in Iran, India, Pakistan and Egypt. It has white flowers and small brownish fruits. In Persian folk medicine, the fruits of *C. copticum* (Zenyan in Persian) were used as a diuretic, anti-vomiting, carminative and antihelmentic agent (Zargari, 1988).

The main objective of this study was to assess the behavior of *E. coli* O157:H7 in the growth/no growth domain as a function of four different factors: inoculum levels, temperatures, pH and concentrations of plant essential oil "*C. copticum*".

MATERIALS AND METHODS

Experimental design

To assess the effects of *C. copticum* essential oil, pH (adjusted by HCl), inoculum level and temperature on growth initiation of *E. coli* O157:H7, the experiment was arranged in a factorial design. This design included four concentrations of the essential oil (0, 0.015, 0.03 and 0.06%), three levels of pH (5, 6 and 7), two inoculums (10^3 and 10^5 cfu ml^{-1}), two incubation temperatures (35 and 25°C) and repeated observations (daily) for growth in brain heart infusion (BHI) broth for up to 30 days.

Test organism

E. coli O157:H7 ATCC 35150 was purchased from Mast International Inc and used as the test organism in this study.

Preparation of inocula

The reference bacteria were plated on MacConkey-Agar plates and incubated at 37°C for 24 h. Inoculums were prepared by transferring a loop full of the bacterial colonies to isotonic saline solution in a sterile cuvette to adjust the absorbance of 0.669 at 600 nm using a spectrophotometer (Jenway 6105, Essex, England).

This adjustment gave a cell concentration of 5×10^8 cfu ml^{-1}. The number of cells in the suspension was estimated by duplicate plating from 10-fold serial dilution on BHI agar and counting the colonies after 24 h of incubation at 37°C. For inoculation, 10-fold serial dilution (10^7, 10^6, 10^5 cfu ml^{-1}) was prepared from the adjusted suspension. After that, for combinations with inoculum of 10^5 cfu ml^{-1}, 30 µl from 10^7 and for those with inoculum of 10^3 cfu ml^{-1}, 30 µl from 10^5 diluted tubes transferred to the test tube combinations. Before this, the same volume of inoculation was discarded from the test tube combinations.

Plant material

The pure *C. copticum* essential oil (plant origin: central provinces of Iran. steam distillation extraction) was purchased from the Nader-Co® (Company of Agro-Industry, Mashhad- Iran).

Gas chromatography-mass spectrometry (GC-MS) analyses

The gas chromatography-Mass spectrometry (GC-MS) analyses were carried out using an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) equipped with a HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with electron impact ionization (70 eV).

Oven temperature was maintained at 50°C for 5 min initially, and then raised at the rate of 3°C/min to 240°C and finally raised to 290°C at 15°C/min then held isothermally for 3 min. Helium was used as carrier gas at a flow rate of 0.8 ml/min, samples of 1.0 µl were injected manually in the splitless mode.

Peaks area percents were used for obtaining quantitative data. Retention indices were calculated for all components using a homologous series of n-alkanes injected in conditions equal to samples ones.

The components of the oil were identified by comparing their retention indices relative to (C8 to C22) n-alkanes with those of literature or with those of authentic compounds available in our laboratory and confirmed by matching their mass spectra with those of a computer library of the GC–MS data system and other published mass spectra (Adams, 2001; Safaei-Ghomi et al., 2009; Hammami et al., 2011).

Determination of minimum inhibitory concentration

To evaluate minimum inhibitory concentration (MIC) of the essential oil, standard tube dilution technique was used (Chandrasekaran and Venkatesalu, 2004). Briefly, the experiment was performed by preparing twofold serial dilutions of EO in BHI broth. The essential oil were emulsified into BHI broth medium to get a concentration of 1% (100 µl/10ml) Using dimethylsulfoxide, then serially diluted to achieve 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.4, 0.2 and 0.1 µl/10 ml (from 1 to 0.001%), respectively. Amount of 10^6 cfu ml^{-1} of test organism was transferred to the test tubes which had different concentrations of EO. The control tubes, contained different concentrations of EO without inoculation of the bacteria. Then, both sets of tubes (tests and controls) were incubated at 37°C for 24 h. The tube contained the lowest concentration of EO which had no visible bacterial growth (no turbidity in the tube), and was determined as MIC. In this study, four different concentrations of EO lower than MIC were selected as test concentrations.

Performing the experiment

BHI powder (3.7 g) was dissolved in 90 ml distilled water in a 250

Table 1. Essential oil composition of *C. copticum* identified by GC-MS.

No.	Phytochemical	Percent	Retention index (RI)
1	*α*-Pinene	0.29	11.35
2	*ß*-Pinene	0.43	13.45
3	*ß*-Myrcene	0.34	14.28
4	*α*-Phellandrene	0.065	14.89
5	*α*-Terpinen	0.311	15.54
6	ρ-Cymene	22.55	16.21
7	β-Phellandrene	0.541	16.29
8	*γ*-Terpinene	13.07	17.93
9	*α*-Terpinolene	0.095	19.18
10	*α*-Terpineol	0.155	24.92
11	L-Carvone	0.908	27.97
12	*trans* -anethole	1.7	28.68
13	Thymol	57.18	29.73
14	Carvacrol	0.524	29.84
15	3-Dodecen-1-al	0.161	36.51
16	Apiol	0.566	42.73
	Total identified	98.886	

ml flask by mild heating. In order to produce and maintain a stable oil–water emulsion in broth substrate during the period of study (30 days), we used the method explained by Mann and Markham, (1998) with some modifications. Briefly, we added 5% (v/v) dimethylsulfoxide (DMSO, Merck Schuchardt OHG, Hohenbrunn, Germany) as an emulsifier and 0.05% (w/v) agar (Merck, KGaA Germany) as a stabilizer to the broth substrate. The same amounts of DMSO and agar were also added to the combinations with no EO (0.0%) to consider any likely effects of them on the growth of test organisms. The final volume was brought to 100 ml with additional distilled water. After the preparation of BHI broth, pH was adjusted using a pH meter (Jenway Ltd.,UK) and normal solution of hydrochloric acid (HCl) as acidulant.

The content of each flask was autoclaved at 121°C for 15 min. After cooling, the pH of each combination in broth medium was measured and adjusted again to the considered pH using 1 N filter sterilized HCl (or NaOH). Then filter sterilized EO was added in different amounts to satisfy the experimental design. The content of flask containing sterile BHI broth was dispensed in portions of 3 ml into sterile caped tubes (Becton Dickinson 16 × 100 mm). The tubes were inoculated with *E. coli* O157:H7 (10^5 and 10^3 cfu ml^{-1}). For each combination, the inoculated tubes were incubated at 35 and 25°C for up to 30 days. During the period of incubation, all the tubes were observed for visible growth (turbidity) daily up to 30-days. The number of tubes (combinations) showing growth at a particular observation were recorded. For each combination, a negative control (uninoculated tube) was used. All experiments were conducted in independent triplicate. The total numbers of combinations were 144 (4 × 3 × 2 × 2 × 3).

Statistical analysis

To evaluate the effects of explanatory variables on time to detection of bacterial growth, survival analysis was used. We used parametric accelerated failure time (AFT) approach (Kleinbaum and Klein, 2005) based on the weibull distribution to quantify the effects of each of the prescribed explanatory variables on time to detection. The general form of the accelerated failure time model is:

$$\log(t)=(\alpha+\beta_1 x_{1i}+...+\beta_m x_{mi})+\log(\tau) \quad (1)$$

where log (*t*) is the natural logarithm of the time to 'failure' (growth), an intercept term, $\beta_1 x_{1i}$ + … + $\beta_m x_{mi}$ is a linear combination of the *m* explanatory variables and their regression coefficients, and log (τ) is an error term. Using this approach the accelerated failure time coefficients represent the expected change in log (*t*) for one unit changes in the predictor.

To select those explanatory variables that best explained time to detection, a backward stepwise approach was used. Explanatory variables that were not statistically significant were removed from the model one at a time, beginning with the least significant, until the estimated regression coefficients for all retained variables were significant at an alpha level of <0.05.

RESULTS

Chemical composition of *C. copticum* essential oil

The components of oil were determined by GC-MS analysis. The constituents of *C. copticum* EO, were accompanied by their retention time and percent, as listed in Table 1. GC-MS analysis resulted in the identification of 16 components representing 98.88% of total oil. The main constituents were thymol (57.18%), ρ-cymene (22.55%), γ-terpinene (13.07%) and trans-anethole (1.7%) (Table 1).

Description of growth/no growth

About 95.8% of combinations (138 out of 144) showed growth during the study period and 4.2% of combinations (6 out of 144) did not grow until the end of the study, and were considered as censored observations.

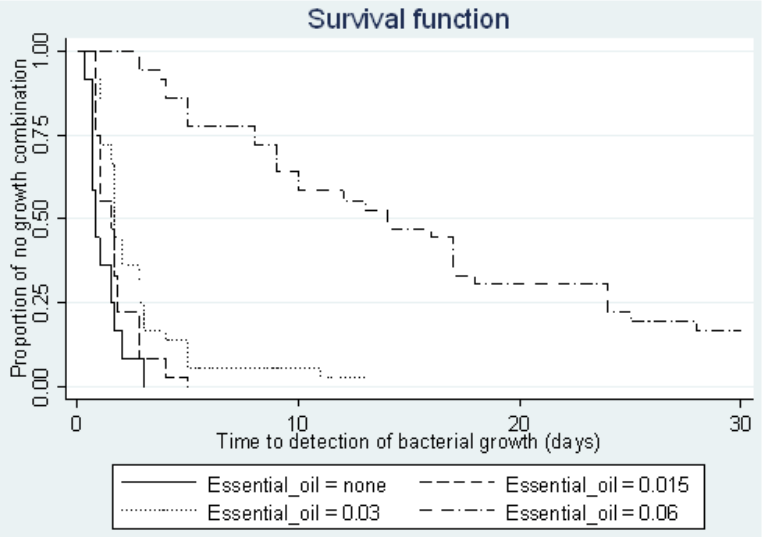

Figure 1a. Kaplan-meier survival curves showing the proportion of no growth combinations for different levels of essential oil.

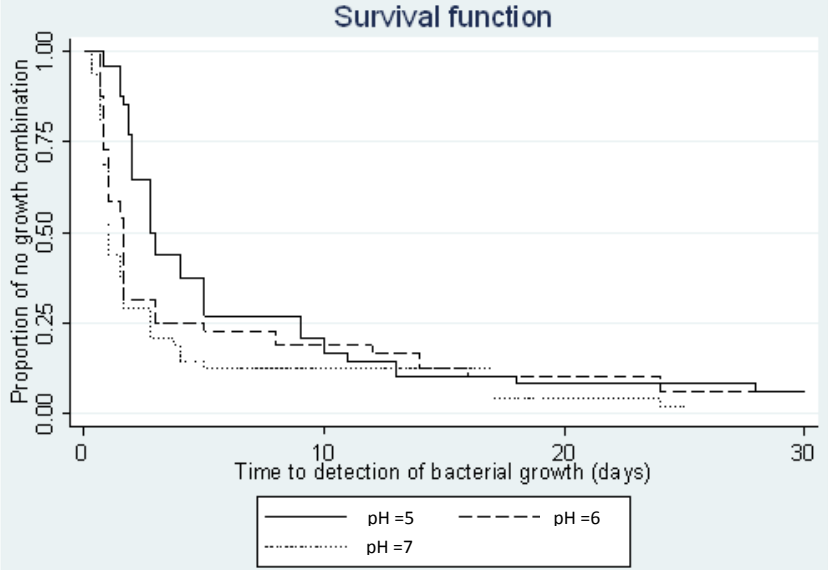

Figure 1b. Kaplan-meier survival curves showing the proportion of no growth combinations for different levels of pH.

Evaluation of time to detection of bacterial growth

Median of time to detection of bacterial growth was 1.66 days. Kaplan-Meier survival curve for different levels of explanatory variables is presented in Figure 1a, b, c and d.

All main effects were included in the model. The final model showed that all explanatory variables had significant association with time to detection (Table 2). Controlling for the effects of temperature, pH and inoculums size, time to detection for combinations which contained 0.015, 0.03 and 0.06% of *C. copticum* essential oil was 1.49, 2.17 and 16.25 times greater than those without it, respectively.

Controlling for the effects of temperature, *C. copticum* essential oil and inoculums size, time to detection (TTD) for those combinations with pH values of 6 and 5 was 1.32 and 2.74 times greater than those with the pH values of 7, respectively. Also, controlling for the effects of other explanatory variables, this period for

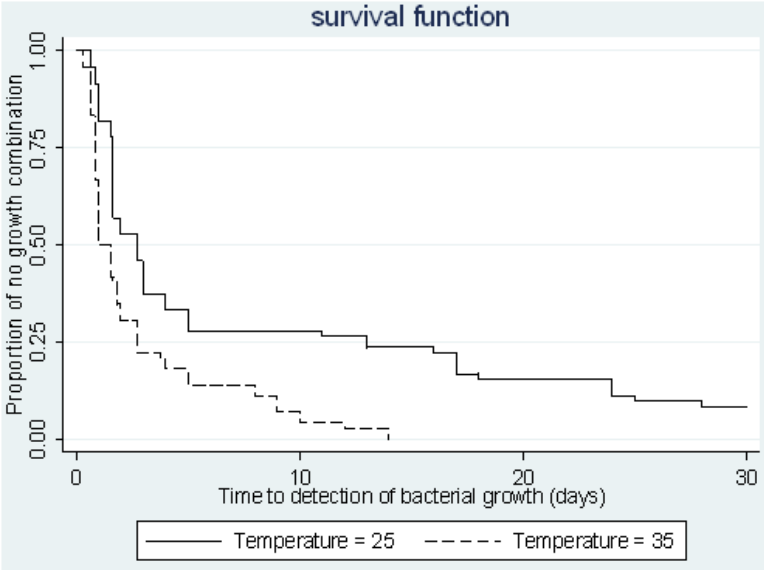

Figure 1c. Kaplan-meier survival curves showing the proportion of no growth combinations for different levels of temperature.

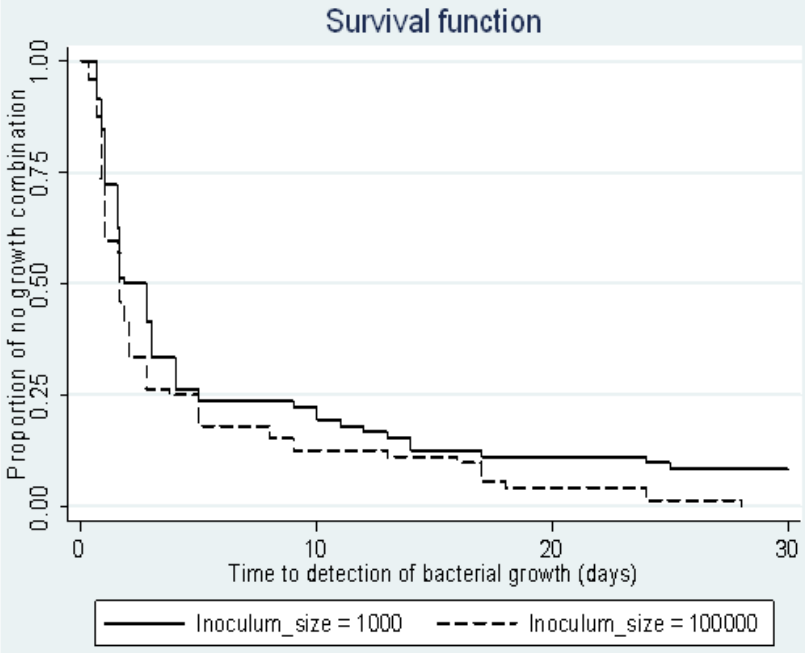

Figure 1d. Kaplan-meier survival curves showing the proportion of no growth combinations for different level of inoculation.

combinations with inoculum size of 10^3 was 1.43 times greater than combinations with inoculum size of 10^5. Furthermore, this period for combinations with incubation temperature of 25°C was 2.14 times greater than those by incubation temperature of 35°C.

The final model equation is shown:

$$TTD = \left[-\ln S(t)\right]^{1/p} \times e^{-0.92+0.76T25+0.35IL1000+0.4EO0.015+0.77EO0.03+2.78EO0.06+1PH5+0.28PH6}$$

Table 2. Accelerated failure time model of factors influencing time to detection of bacterial growth.

Variable	*B* (SE)	P-value	Time ratio (95% CI)
Intercept	-0.922(0.062)	< 0.01	
Essential oil			
0	0		1
0.015	0.405(0.064)	< 0.01	1.49 (1.32 - 1.70)
0.03	0.776(0.067)	< 0.01	2.17 (1.90 - 2.48)
0.06	2.78(0.075)	< 0.01	16.25 (14.03 - 18.83)
pH			
7	0		1
6	0.276(0.057)	< 0.01	1.32 (1.18 - 1.47)
5	1.008(0.059)	< 0.01	2.74 (2.44 - 3.08)
Inoculum level			
10^5	0		1
10^3	0.355(0.048)	< 0.01	1.43 (1.30 - 1.57)
Temperature			
35°C	0		1
25°C	0.763(0.053)	< 0.01	2.14 (1.93 - 2.38)
p	3.69 (0.24)		
1/p	0.27 (.017)		

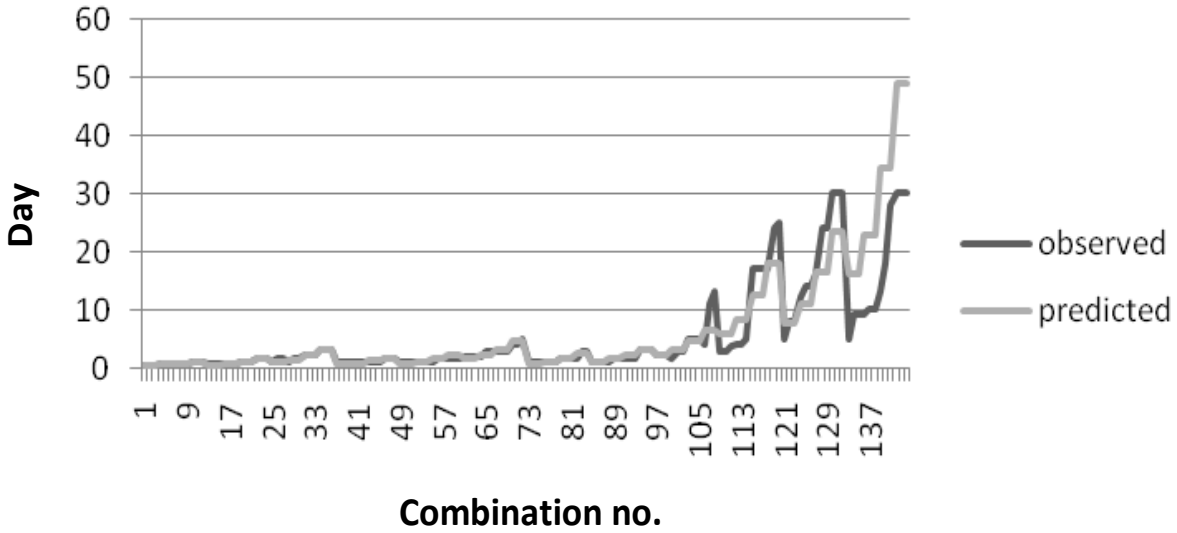

Figure 2. Observed and predicted days needed for growth initiation of *E .coli* O157:H7 (TTD) according to the weibull model (total numbers of combinations were 144).

where "*TTD*" is time to detection, "*ln*" is the natural logarithm (logarithm to the base *e*), "*p*" is the shape parameter of weibull distribution, "*e*" is a mathematical constant approximately equal to 2.718281828, "*T*" is temperature,"*IL*" is inoculum size, and "*EO*" is essential oil.

Figure 2 shows the observed time to detection of bacterial growth and value that was predicted by Weibull model for time to detection of *E.coli* O157:H7 in designated combinations.

DISCUSSION

Evolution of predictive food microbiology during the last 30 to 35 years changed it to a new paradigm in food microbiology. Most of the industries and regulatory authorities prefer it to predict the shelf life and safety of foods (McMeekin et al., 2008).

Essential oils contain various compounds which have antimicrobial activities such as antifungal and antibacterial properties. The antibacterial activity of essential oils demonstrated against some bacteria, at levels of EO between 0.2 and 10 µl/ml (Cosentino et al., 1999). There is a relationship between the chemical structures of the most abundant compounds in the EOs and their antibacterial activity (Burt, 2004). EOs containing phenolic compounds, e.g. thymol, carvacrol, c-terpinene, and p-cymene, are widely reported to possess high levels of antibacterial activity (Burt, 2004; Khanzadi et al., 2010; Akhondzadeh-Basti et al., 2007; Oroojalian et al., 2010).

In the present study, thymol (57.18%), p-cymene (22.55%), and γ-terpinene (13.07%) were the major components of *C. copticum* essential oil. Similar to previous studies, thymol was found to be the major constituent of the oil (Mohagheghzadeh et al., 2007; Thangam and Dhananjayan, 2003; Behravan et al., 2007). In two other reports major components of the oil were reported as thymol (35.4 and 49.0%), c -terpinene (28.6 and 3 0.8%) and p-cymene (29.2 and 15.7%) with no carvacrol (Khajeh et al., 2004; Lucchesi et al., 2004).

It has been shown that thymol and its precursors, p-cymene and terpinene, have strong antimicrobial activities. Based on previous studies, the antimicrobial role of thymol may induce through the alteration of the plasma membrane permeability. Its alteration leads to leakage of intracellular material (Burt, 2004). P-cymene is the second major compound identified in *C. copticum* oil, is a hydrophobic molecule and causes swelling of the cytoplasmic membrane (Burt, 2004). Using p-cymene alone did not show any effective antibacterial activity (Juven et al., 1994; Dorman and Deans, 2000), however, in combination with other phenolic compounds such as carvacrol, it has shown a great antimicrobial activity by incorporating cymene in the lipid bilayer of bacteria, facilitating the transport of phenolic monoterpenes of EOs across the cytoplasmic membrane (Juliano et al., 2000). Some studies have shown that the use of whole EO has more antibacterial activity. It shows the important synergistic effect of minor components of EO (Burt, 2004; Karami-Osboo et al., 2010).

Oroojalian et al. (2010) compared the MICs of the three EOs: *C. copticum*, *Bunium persicum* and *Cuminum cyminum*. The results showed that *C. copticum* EO was more active against all pathogenic bacteria than other two EOs. Singh et al. (2002) compared the antibacterial activities of EOs from seven Apiaceae species against *Corynebacterium diphtheriae, Staphylococcus aureus, Streptococcus haemolyticus, E. coli, Klebsiella* spp., and *Proteus vulgaris*, and reported that *C. copticum* EO was the most effective. The higher antibacterial activity of *C. copticum* than other species, possibly relates to higher amount of thymol and γ-terpinene which have been reported to possess antibacterial properties (Oroojalian et al., 2010).

Goudarzi et al. (2011) studied antibacterial activity of *C. copticum* against *S. aureus*, *Pseudomonas aeruginosa*, *E. coli*, and *Salmonella typhimurium* by agar diffusion and broth dilution methods. In agar diffusion assay, *C. copticum* showed inhibitory effects against all the examined species except *P. aeuroginosa.* Also, in the present study, the inhibitory effects of *C. copticum* EO against *E. coli* O157:H7 was observed. In agreement to these results, several other studies showed that the antimicrobial properties of plant EO are dose-dependent (Burt, 2004; Sagdic, 2003).

Outbreaks of *E. coli* O157:H7 infections mainly originated from foods that were previously considered too acidic to support the survival of enteric pathogens. These outbreaks from acidic food have challenged the safety of current practices in the food industry. Some bacteria, including pathogenic *E. coli*, have been found to have a high tolerance to low pH. This tolerance describes the reason of outbreaks of *E. coli* infections caused by certain acidic foods occur, and tolerance to low pH is believed to be a virulence factor for such food-borne pathogens (that is, pathogens that are able to survive the gastric acidity barrier) (Presser et al., 1998).

Regarding Akhondzade et al. (2007), the inhibitory action of the *Zataria multiflora Boiss*. EO on the growth initiation of *S. typhimurium* and *S. aureus* was enhanced by decreasing the pH value at each defined temperature. According to our results, decreasing the pH of broth medium had a significant effect on growth initiation of inoculated bacteria. This can be attributed either to the direct effect of pH or to the better dissolving of the essential oil in the lipid phase of the bacterial membrane at the lower pH (Koutsoumanis et al., 1999). So, it could be concluded that the use of EO along with decreased pH can reduce the risk of acidic foods.

Based on our results, decreasing the incubation temperature had also a significant effect on growth initiation of inoculated bacteria. Initiation of visible growth period for combinations with incubation temperature of 25°C was 2.14 times greater than those with the same parameters, but incubation temperature of 35°C.

In another study, Tassou et al. (2000) showed a significant extension of detection time of *S. aureus* and *S. enteritidis* growth in a nutrient broth medium at a given concentration of mint (Laminaceae) essential oil, by decreasing the storage temperature from 35 to 10°C. It may be due to the lower metabolic activity at the lower temperature.

Presser et al. (1998) reported that pH and temperature synergistically affect the growth rate of *E. coli*. They

showed when the temperature is lower, the minimum pH for growth rises slightly, and growth was not observed at any pH at 10°C. Regarding Valero et al. (2010) study, at low temperatures high values of pH were required to enable growth of *E. coli*. Also, Skandamis et al. (2007) showed that, as temperature decreased, the minimum pH allowing growth of *E. coli* within 60 days increased.

In relation to bacterial population, there are evidences that indicate the increased bacterial numbers in the environment can protect the pathogen against the inhibitory effects of the acid (Duffy et al., 1999).

Inoculum size has a very significant effect on the growth initiation of the bacteria at the lower pH, and by the higher inoculum size, growth can be initiated at the lower pH (Valero et al., 2010; Koutsoumanis et al., 2004b). Skandamis et al. (2007) modeled the effect of inoculum level on growth interface of *E. coli* O157:H7. They concluded that at high inoculum levels, the intensity of additional environmental inhibitory factors should be higher, which could be explained by the fact that at high inoculum levels, there will be more probability of finding cells in the proper physiological state to initiate growth. According to our results, increasing the inoculum size had a significant effect on growth initiation.

This conclusion has also been reported for other microorganisms, such as *Brochothrix thermosphacta* (Masana and Baranyi, 2000) and *Listeria monocytogenes* (Koutsoumanis et al., 2004b; Koutsoumanis and Sofos, 2005). Such results may be explained based on the hypothesis that the minimum levels of environmental factors that allow the growth of individual cells within a population follow a distribution, which becomes wider as the population size increases (Francois et al., 2005, 2006; Koutsoumanis and Sofos, 2005).

Overall, in our study, the values of TTD were higher at low levels of temperature and pH, but high level of EO. It should be also highlighted that the TTD was markedly influenced by the inoculum level. The same results have been reported by Valero et al. (2010) and Jamshidi et al. (2008).

It should be noted that prolonged survival of a bacteria under suboptimal conditions may be due to the fact that suboptimal growth factors impose a stress on the pathogen and thus triggers a general stress response system in the bacteria leading to increased resistance and thus enhancing survival of the pathogens. The RpoS sigma factor is the master regulator of the general stress response in *E. coli* and other enteric bacteria when environmental stresses like as acid shock or temperature shifts are encountered (Abee and Wouters, 1999).

These evidences showed that application of hurdle technology for food preservation might be inhibited outgrowth but probably induces prolonged survival of *E. coli* O157:H7 in minimal processed foods (Uyttendaele et al., 2001).

Our designated models adequately predicted the growth initiation, and inhibition conditions of *E. coli* O157:H7 as affected by different values of pH, temperature, *C. copticum* EO, and inoculums levels.

The predicted values may not match with whatever would occur in any special food system. This means that the model must be validated before use in any interested food.

ACKNOWLEDGEMENTS

The authors express their sincere appreciation and thanks for the financial support received from Faculty of Veterinary Medicine, Ferdowsi University of Mashhad for conducting this research (Grant no: 17485/3). We also thank Mrs. S. Khajenasiri for her technical assistance.

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