

## Modeling of *Salmonella typhimurium* growth under the effects of *Carum copticum* essential oil, temperature, pH and inoculum size

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Article Info	Abstract
<b>Article history:</b> Received: 15 April 2016 Accepted: 19 July 2016 Available online: 15 March 2017	The purpose of this study was to elucidate some factors affecting the growth of <i>Salmonella typhimurium</i> . These factors included <i>Carum copticum</i> essential oil (0%, 0.015%, 0.03% and 0.06%), temperature (25 °C and 35 °C), pH (5, 6 and 7) and inoculum size (10 <sup>3</sup> and 10 <sup>5</sup> CFU mL <sup>-1</sup> ). Brain heart infusion broth was used as the growth medium. There were 48 treatment combinations and the experiment was carried out in triplicate. Growth was monitored by visible turbidity over a 30 days period. A parametric survival model based on the log-normal distribution was used to estimate the most influential factors on the time to detection of <i>Salmonella</i> growth. According to our results, the selected factors significantly affected the growth of <i>Salmonella typhimurium</i> . Furthermore, the final graph demonstrated good agreement between the values predicted by predictive model and the results which were observed in this study. So that a parametric survival model can be a useful and practical tool to predict how the parameters will influence the bacterial growth.
<b>Key words:</b> <i>Carum copticum</i> Essential oil Modeling <i>Salmonella typhimurium</i>	

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### مدل سازی رشد باکتری *سالمونلا تیفی موریم* تحت تاثیر اسانس زنیان، دما، pH و دوز تلقیح

#### چکیده

هدف این مطالعه بررسی برخی فاکتورهای اثرگذار بر روی رشد باکتری *سالمونلا تیفی موریم* بود. این فاکتورها شامل اسانس زنیان (صفر، ۰/۰۱۵، ۰/۰۳ و ۰/۰۶ درصد)، دما (۲۵ و ۳۵ درجه سانتیگراد)، pH (۵، ۶ و ۷) و دوز تلقیح (کلونی بر میلی لیتر ۱۰<sup>۳</sup> و ۱۰<sup>۵</sup>) بود. برات عصاره مغز و قلب به عنوان محیط کشت رشد به کار گرفته شد. ۴۸ ترکیب تیماری مختلف برای رشد باکتری در نظر گرفته شد و برای هر حالت سه تکرار نیز انجام پذیرفت. رشد به واسطه مشاهده کدورت در یک دوره ۳۰ روزه مورد بررسی قرار گرفت. مدل بقای پارامتریک با توزیع لگ - نرمال، جهت بررسی تاثیرگذارترین متغیرها بر زمان شروع رشد *سالمونلا* استفاده شد. با توجه به نتایج این مطالعه متغیرهای انتخابی به صورت معنی داری بر رشد باکتری *سالمونلا تیفی موریم* تاثیرگذار بودند. همچنین، بر طبق نمودار نهایی، ارتباط مناسبی بین نتایج حاصل از این مطالعه و مقادیر پیشگویی شده توسط مدل پیشگو مشاهده شد. بر این اساس مدل بقای پارامتریک می تواند ابزاری کاربردی جهت تخمین اثر فراسنجه ها بر رشد باکتریایی باشد.

**واژه های کلیدی:** اسانس، زنیان، *سالمونلا تیفی موریم*، مدل سازی

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## Introduction

*Salmonella* species have been considered as one of the most important foodborne pathogens all around the world.<sup>1,2</sup> Animals are the principal reservoir of this pathogen.<sup>3</sup> *Salmonella enterica* serovar *typhimurium* and *Salmonella enterica* serovar *enteritidis* are the most frequently isolated serovars from foodborne outbreaks throughout the world.<sup>4</sup>

The use of natural antimicrobial compounds is of utmost interest in the food industry.<sup>5</sup> In this regard, the antimicrobial properties of plant essential oil (EO) are investigated in different studies.<sup>6,7</sup> The combination of different preservation methods have been already described as hurdle technology concept in food safety by Leistner.<sup>8</sup>

Microbial responses to environmental conditions are reproducible and this phenomenon is the premise of predictive microbiology. We can predict the behavior of the same organism at the similar environmental condition based on previous observations of microbial growth.<sup>9,10</sup>

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.<sup>11</sup> Among traditional potential herbs used as spice in day to day life, Ajwain (*Trachyspermum ammi* L. or *Carum copticum* Benth. and Hook) belonging to *Apiaceae* family, is widely used for curing various diseases in both humans and animals. In Iran, the fruits of *C. copticum* is known as Zenyan. *Trachyspermum ammi* is a grassy, annual plant which grows in the east of India, Iran and Egypt with a white flower and small, brownish seeds.<sup>12</sup>

*Carum copticum* fruit, due to favorite its taste, is commonly used in Iran as common spice and food preservative, especially for traditional meat products.<sup>13-16</sup>

Overall, according to many prior studies, the *C. copticum* EO exhibits potent antimicrobial and antioxidant activities supporting its potential use for perishable and high fatty foods.<sup>17-19</sup>

The objective of this study was to assess the effects of four different factors: inoculum size, temperature, pH and different concentrations of *C. copticum* EO on the growth/no growth domain of *S. typhimurium*.

## Materials and Methods

**Experimental design.** To assess the combined effects of *C. copticum* EO, pH (adjusted by normal solution of HCl), inoculum size and temperature on growth initiation of *S. typhimurium*, the experiment was arranged in a factorial design. This design included four concentrations of the EO (0, 0.015, 0.03 and 0.06%), three levels of pH (5, 6 and 7), two inoculum levels ( $10^3$  and  $10^5$  CFU mL<sup>-1</sup>), two incubation temperatures (25 °C and 35 °C) and repeated observations (daily) for growth initiation in brain heart infusion (BHI,

Merck, Darmstadt, Germany) broth for up to 30 days. *S. typhimurium* ATCC 14028 (Mast International Ltd., Berkshire, UK) was used as the test organism in this study.

**Preparation of inocula.** The reference bacterium was plated on MacConkey-Agar plates (Merck) and incubated at 37 °C for 24 hr. 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 0.133 at 600 nm using a spectrophotometer (Model 6105; Jenway, Essex, UK). This adjustment gave a cell concentration of  $1.50 \times 10^8$  CFU mL<sup>-1</sup> (equal to No: 0.50, McFarland standards). Tenfold serial dilution was prepared from the adjusted suspension.

**Plant material.** The pure *C. copticum* EO was extracted by steam distillation at the Nader-Co® (Agro-Industry Co., Mashhad, Iran).

**Gas chromatography-mass spectrometry (GC-MS) analyses.** The GC-MS analyses were carried out using an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, USA) with HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) equipped with an HP 5973 mass spectrometer (Agilent Technologies) with electron impact ionization (70 eV). The oven temperature was maintained at 50 °C for 5 min initially, raised at the rate of 3°C per min to 240 °C, finally raised to 290 °C at 15 °C per min and then held isothermal for 3 min. Helium was used as carrier gas at a flow rate of 0.80 mL per min, samples of 1.00 μL were injected manually in the split less mode. Peak area percent 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.<sup>20</sup>

**Determination of minimum inhibitory concentration (MIC).** To evaluate MIC of the EO, standard tube dilution (macrobroth dilution) technique was used.<sup>21</sup> Briefly, the experiment was performed by preparing twofold serial dilutions of EO in BHI broth. The EO was emulsified into BHI broth medium to get a concentration of 1% (100 μL 10 mL<sup>-1</sup>) using (DMSO, Merck, Hohenbrunn, Germany) 5% (v/v), then serially diluted to achieve 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.40, 0.20 and 0.10 μL 10 mL<sup>-1</sup> (from 1% to 0.001%), respectively. Amount of  $10^6$  CFU mL<sup>-1</sup> of test organism was transferred to the test tubes which had different concentrations of EO. The negative control tubes contained different concentrations of EO without inoculation of the bacteria. Both sets of tubes (tests and controls) were incubated at 35 °C for 24 hr. The tube with the lowest concentration of EO which had no visible

bacterial growth (no turbidity in the tube) was determined as MIC. In this study four different concentrations of EO lower than MIC were selected as test concentrations.

**Performing the experiment.** 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 with some modifications.<sup>22</sup> Briefly, 5% (v/v) DMSO (Merck) was added as an emulsifier and 0.05% (w/v) agar-agar (Merck) as stabilizer was introduced to the broth substrate. The same amounts of DMSO and agar agar were also added to the combinations with no EO (0.00%) to consider any likely effects on the growth of test organism. The final volume was brought to 100 mL with additional distilled water. After preparation of BHI broth, pH was adjusted using a pH meter (Jenway Ltd.) and normal solution of HCl as acidulant. The content of each flask was autoclaved at 121 °C for 15 min. After cooling, the pH of each combination was measured and adjusted again to the considered pH using 1 N filter sterilized HCl (or NaOH; Merck). 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 capped tubes (16 × 100 mm; Becton Dickinson, Durham, USA). The tubes were inoculated with *S. typhimurium* (10<sup>5</sup> and 10<sup>3</sup> CFU mL<sup>-1</sup>). For each combination, the inoculated tubes were incubated at 35 °C and 25 °C for up to 30 days. During the period of incubation, all tubes were observed for visible growth (turbidity) daily. The number of tubes (combinations) showing growth at a particular observation was recorded. For each combination, a negative control (uninoculated tube) was used. All experiments were conducted in independent triplicate. This design (4 × 3 × 2 × 2 equal to 48 combinations) with three replicates, to prepare 144 treatment tubes, has been evaluated in this study.

**Statistical analysis.** The outcome variable for consideration in this study was the time to visible growth. Since some combinations did not grow until the end of the study, standard regression methods were considered inappropriate. Instead, event-time (survival) analysis was employed which was able to use all experimental data irrespective of whether or not growth occurred.

Parametric survival model based on accelerated failure time (AFT) approach<sup>16,23</sup> was used to quantify the effect of each of prescribed explanatory variables on detection time of bacterial growth. The general form of AFT model is:

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

where,  $\log(t)$  is the natural logarithm of the time to failure (growth),  $\alpha$  is an intercept term,  $\beta_1 x_{1i} + \dots + \beta_m x_{mi}$  are linear combinations of the  $m$  explanatory variables and their regression coefficients and  $\log(\tau)$  is the error term. Using this approach, the AFT coefficients represent the expected change in  $\log(t)$  for changes in the predictor levels.

In present study, we evaluated the exponential, Weibull, log-normal and log-logistic distributions that can be interpreted in the AFT metric. To evaluate the fitness of candidate distribution to the current data, the mean square error (MSE) was compared. The smaller MSE value indicates a better fit.

$$MSE = \frac{\sum (\text{Predicted} - \text{Observed})^2}{(n - p)}$$

where,  $n$  is the number of observations and  $p$  is the number of parameters to be estimated.

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 less than 0.05.

## Results

**Chemical composition of *C. copticum* EO.** The components of oil were determined by GC-MS analysis. The constituents of *C. copticum* EO, accompanied by retention time and percent, are listed in Table 1. The GC-MS analysis resulted in identification of 16 components representing 98.88% of total oil. The main constituents were thymol (57.18%),  $\rho$ -cymene (22.55%),  $\gamma$ -terpinene (13.07%) and trans-anethole (1.70%).

**MIC assay.** *In vitro* antibacterial activities of *C. copticum* EO were assessed by standard tube dilution technique against *S. typhimurium*. Antibacterial activity was expressed as MIC value. In this study, the MIC of the EO was 1.25  $\mu\text{L mL}^{-1}$ . Then, four different concentrations of EO lower than MIC were selected as test concentrations.

**Table 1.** Essential oil composition of *Carum copticum* identified by gas chromatography-mass spectrometry.

Phytochemicals	Percent	Retention index (RI)
$\alpha$ -Pinene	0.29	11.35
$\beta$ -Pinene	0.43	13.45
$\beta$ -Myrcene	0.34	14.28
$\alpha$ -Phellandrene	0.06	14.89
$\alpha$ -Terpinene	0.31	15.54
$\rho$ -Cymene	22.55	16.21
$\beta$ -Phellandrene	0.54	16.29
$\gamma$ -Terpinene	13.07	17.93
$\alpha$ -Terpinolene	0.09	19.18
$\alpha$ -Terpineol	0.15	24.92
L-Carvone	0.90	27.97
Trans-Anethole	1.70	28.68
Thymol	57.18	29.73
Carvacrol	0.52	29.84
3-Dodecen-1-Al	0.16	36.51
Apiol	0.56	42.73
Total	98.88	-

**Description of growth/no growth.** About 85.40% of the treatment tubes (123 out of 144) showed growth during the study period. On the basis of MSE value, the log-normal model provided the best fit to the data. The MSE value of the log-normal model was 62.06, while the MSE values were 119.62, 63.03 and 190.21 for weibull, log-logistic and exponential models, respectively.

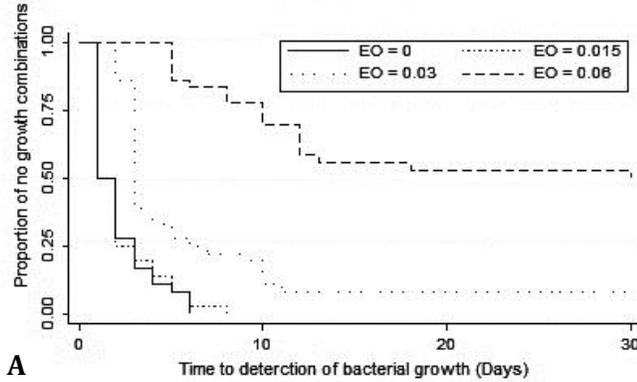
**Evaluation of time to detect the bacterial growth.**

Median time to detection of bacterial growth was 3 days. Kaplan-Meier survival curve for explanatory variables is presented in Figure 1. The final model equation as below:

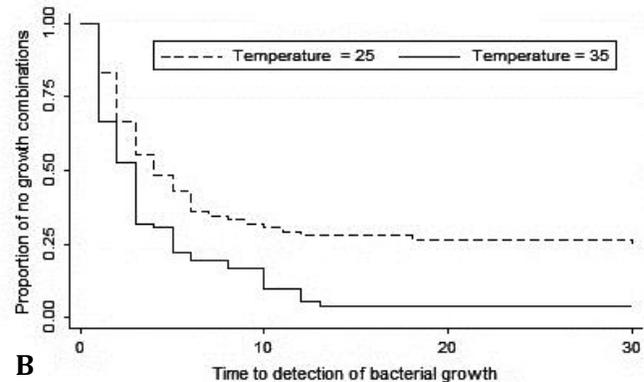
$$TTD = e^{-0.43 + 0.72T + 0.36IL + 0.01EO + 0.015 + 2.46EO + 1.14PH + 0.17pH^2}$$

where, in this equation *TTD* is time to detection, *e* is a mathematical constant approximately equal to 2.718281828, *T* is temperature, *IL* is inoculum size and *EO* is essential oil.

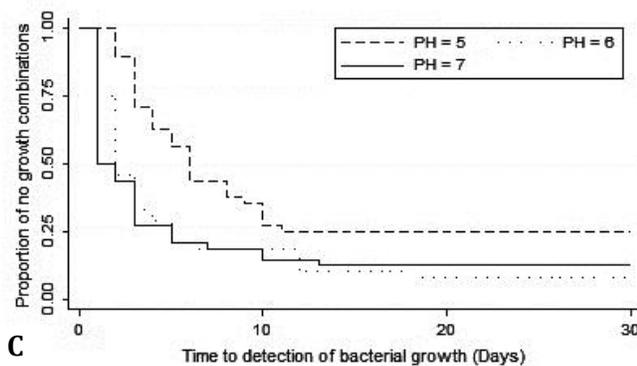
This model showed that all explanatory variables had significant association with *TTD* (Table 2). On average, *TTD* for treatment combinations with 0.03% and 0.06% of *C. copticum* EO was 2.59 and 11.70 times greater than those without it, respectively but 0.015% of EO didn't influence the *TTD* significantly. The *TTD* for those combinations with pH levels of 6 and 5 was 1.18 and 3.13 times greater than those with pH level of 7, respectively.



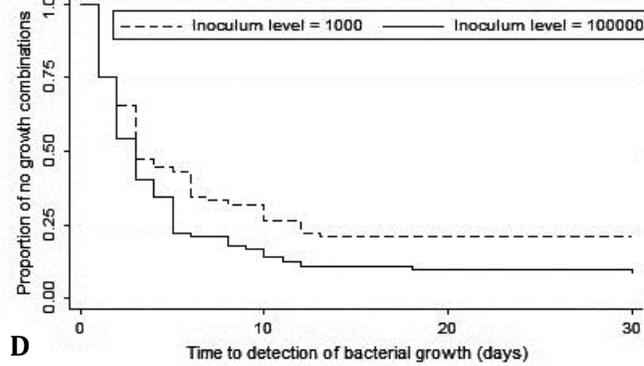
A



B



C

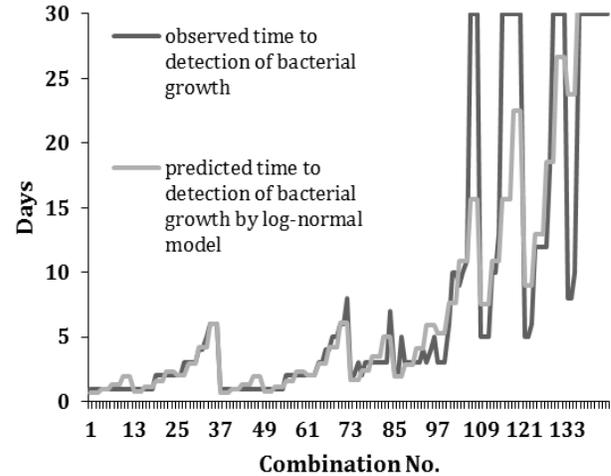


D

**Fig. 1.** Kaplan-meier survival curves showing the proportion of no growth combinations; **A)** concentrations of essential oil, **B)** temperature, **C)** pH, and **D)** inoculum size.

Also, this period for combinations with inoculum size of  $10^3$  CFU  $\text{mL}^{-1}$  was 1.44 times greater than combinations with inoculum size of  $10^5$  CFU  $\text{mL}^{-1}$ . Furthermore, this period for combinations with incubation temperature of 25 °C was 2.06 times greater than combinations with incubation temperature of 35 °C.

Observed and predicted *TTD*s of bacterial growth for each combination are presented in Figure 2.



**Fig. 2.** Observed and predicted days needed for growth initiation of *S. typhimurium* according to the log-normal model.

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

Variables	Measure	$\beta$ (SE)	p-value	TTD ratio (95% CI)
Intercept	-	-0.43 (0.09)	< 0.01	-
	0	0	-	1
Essential oil (%)	0.01	0.01 (0.09)	0.90	1.01 (0.85-1.20)
	0.03	0.95 (0.09)	< 0.01	2.59 (2.17-3.10)
	0.06	2.45 (0.09)	< 0.01	11.70 (9.65-14.19)
pH	7	0	-	1
	6	0.17 (0.07)	0.03	1.18 (1.02-1.38)
	5	1.14 (0.08)	< 0.01	3.13 (2.66-3.68)
Inoculum size (CFU mL <sup>-1</sup> )	10 <sup>5</sup>	0	-	1
	10 <sup>3</sup>	0.36 (0.06)	< 0.01	1.44 (1.26-1.64)
Temperature (°C)	35	0	-	1
	25	0.72 (0.06)	< 0.01	2.06 (1.81-2.35)
Sigma	-	0.38 (0.02)	-	-

$\beta$ : Regression coefficient; SE: Standard error; CI: Confidence interval; TTD: Time to detection.

## Discussion

In the present study, we determined different combinations which can decrease/stop *S. typhimurium* growth initiation. Using predictive microbiology, we designed a model which can predict growth/no growth of *S. typhimurium* in different conditions.

An important characteristic of EOs and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death.<sup>24,25</sup>

In general, EOs containing phenolic compounds, e.g. thymol, carvacrol, terpinene and p-cymene, are widely reported to possess high levels of antibacterial activity and may have applications in controlling pathogens in food.<sup>11,26,27</sup> In the present study, the phytochemical components of the EO of *C. copticum* have been analyzed by GC-MS and thymol (57.18%), p-cymene (22.55%) and  $\gamma$ -terpinene (13.07%) were the major components of the EO. In some studies, amount of thymol has been reported greater than which we analyzed,<sup>28</sup> while other reports indicated that thymol is the major constituent of *C. copticum* EO but mostly less than 50.00% of all components.<sup>29-31</sup> It is believed that differences in essential oil profile in a certain plant are resulted from various cultivation situations and locations, time of cultivation and also different extracting method.<sup>32</sup>

Antimicrobial activity of *C. copticum* which could be attributed to high content of compounds such as thymol, has been reported by large numbers of studies.<sup>15,16,30</sup>

The inhibitory effects of EOs increased with increasing their concentration.<sup>33</sup> In the present study, similar results were obtained and TTD for combinations with 0.03% and 0.06% of *C. copticum* EO was 2.59 and 11.70 times greater than those without EO, respectively but 0.015% of EO didn't impact the TTD significantly.

Singh *et al.* suggested that the use of EOs in conjunction

with other preservation techniques such as chemical preservatives or low temperature could develop a synergistic alternative to the current method.<sup>34</sup> Tassou *et al.* 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*) EO, by decreasing the storage temperature from 35 to 10 °C. It may be due to the lower metabolic activity at lower temperature.<sup>35</sup> Based on our results, decreasing 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 2.06 times greater than those with the same parameters, but incubation temperature of 35 °C.

The combined bacteriostatic effect of both low pH and low temperature has been proved to be very effective against *Salmonella* species.<sup>36</sup> The effect of pH can be attributed either to the direct effect of pH or to the better dissolving of the EO in the lipid phase of the bacterial membrane at the lower pH.<sup>37</sup> Lowering the pH of a food product or beverage has been used as a preserving method.<sup>38</sup> According to our results, decreasing the pH of broth medium had a significant effect on growth initiation of inoculated bacteria.

There are some reports about the effect of inoculum size on bacterial growth.<sup>39</sup> It has also been reported that by higher inoculum size, growth can be initiated at the lower pH.<sup>40,41</sup> In this regard, Skandamis *et al.* modeled the effect of inoculum size on growth interface of *E. coli* O157:H7. They found that at high inoculum size, the intensity of additional environmental inhibitory factors should be higher, which could be explained by the fact that at high inoculum size, there will be more probability of finding cells in the proper physiological state to initiate growth.<sup>42</sup> According to our results, *S. typhimurium* growth was significantly influenced by inoculum size significantly. Initiation of visible growth period for combinations with inoculum size of 10<sup>3</sup> CFU mL<sup>-1</sup> was 1.44 times greater than those with the same parameters but with inoculum size of 10<sup>5</sup>.

Combined data from growth response of a given bacteria in different environmental conditions could be explained in a model. The model offers a cost-effective approach to understand the microbial growth response in foods and forms a data-base against which other controlling factors could be evaluated.<sup>43</sup>

Based on our results, EO, pH, temperature and inoculum size had significant effects on TTD of *S. typhimurium*. Furthermore, the log-normal model which has used in this study can be a useful and practical tool to predict how the parameters will influence the bacterial growth. 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. Samira Khajenasiri for her technical assistance.

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