Modeling the effects of *Bunium persicum* (Black Zira) essential oil, pH, inoculums size and temperature on the growth of *Listeria monocytogenes*

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Summary

Listeriosis is acknowledged as a major foodborne disease throughout the world caused by *Listeria monocytogenes*. Different factors can affect the growth of food borne microbial pathogens. The aim of this study was to investigate the combined effects of different concentrations of *Bunium persicum* essential oil (EO) (0%, 0.08%, 0.16%, 0.24%), three incubation temperatures (35ºC, 25ºC, 4ºC), three levels of pH (5, 6, 7) and two inoculum sizes (10^3 and 10^5 cfu ml^{-1}) on the growth of *Listeria monocytogenes* in brain heart infusion (BHI) broth. To evaluate effects of explanatory variable on time to detection (TTD) of bacterial growth, parametric survival models based on the log normal distribution were used. All explanatory variables had significant association with TTD ($P<0.05$). The final model accurately predicted the growth initiation and inhibition of *L. monocytogenes*.

Key words: *Listeria monocytogenes*, Black Zira essential oil, Time to detection, Modeling

Introduction

Listeria monocytogenes is a gram-positive, psychrotrophic and opportunistic foodborne pathogen. The disease caused by this bacterium, listeriosis, is acquired by ingesting contaminated food products and mainly affects immunocompromised individuals, pregnant women and newborns. Listeriosis can cause death in 25-30% of cases (Hamon *et al*., 2006).

The bacterium can survive in a wide range of conditions such as low or high temperatures, low pH, reduced water activity and high salt content (Campos *et al*., 2011). *Listeria monocytogenes* can grow in temperature conditions (0°C to 42°C), and the pH range for growth is between 4.5 and 9.6 (Zhu *et al*., 2005).

Studying the effects of environmental conditions on the growth of foodborne pathogens is important in order to limit and control their risks (Jamshidi *et al*., 2008).

Predictive microbiology is used with the aim of developing and applying mathematical models to predict the response of microorganisms to specified environmental variables (McDonald *et al*., 1999). In predictive microbiology, microbial responses are measured under controlled and defined conditions (Ross *et al*., 1994).

Given the increased public awareness and rising concerns about the use of chemicals and synthetic additives in foods, scientists were encouraged to investigate the antimicrobial activity of natural food additives (Gutierrez *et al*., 2009).

In recent years, natural preservatives such as plant essential oils (EOs) have been used as additives instead of chemical preservatives (Oonmetta *et al*., 2006).

Essential oils are oily liquid compounds of plant secondary metabolites and are obtained from different parts of aromatic plants (Campos *et al*., 2011). Although different studies have shown the antifungal, antioxidant and antibacterial activity of EOs (Baratta *et al*., 1998; Celikel *et al*., 2008), the organoleptic properties of EOs are among the most important factors limiting the usage of EOs in food. Therefore, determining the minimum concentration of EOs is of crucial importance as it can inhibit the growth of pathogens (Burt, 2004).

Black cumin or Zireh Kuhi is a plant species in the family Apiaceae whose scientific name is *Bunium persicum*. It is native to the Middle East, particularly Iran (Moghtader *et al*., 2009). In traditional medicine, *B. persicum* is useful as a stimulant and carminative and also in the management of dyspepsia and diarrhea (Jalilzadeh *et al*., 2011).

The main objective of this study was to assess the behavior of *L. monocytogenes* in the growth/no growth domain as a function of four different factors: inoculum size, temperature, pH and concentrations of plant EOs of *B. persicum*.

Materials and Methods

Experimental design

To assess the effects of *B. persicum* EO, pH (adjusted

by HCl), inoculum size and temperature on growth initiation of *L. monocytogenes*, the experiment was arranged in a factorial design. This design ($4 \times 3 \times 2 \times 3 \times 3$ equal to 216 combinations) included four concentrations of the EO (0, 0.08, 0.16 and 0.24%), three levels of pH (5, 6 and 7), two inoculums size (10^3 and 10^5 cfu ml^{-1}), three incubation temperatures (35, 25 and 4°C), three replicates of all combinations and repeated observations (daily) for growth in brain heart infusion (BHI) broth for up to 30 days.

Test organism

Listeria monocytogenes ATCC 7644 was purchased from Mast International Inc-England and used as the test organism in this study.

Preparation of inocula

The reference bacterium was prepared through transferring cells from working cultures to tubes of BHI broth and was incubated at 37°C for 24 h. A second subculture was prepared by incubation for 24 h at 37°C, in a 13×100 mm sterile cuvette. The broth culture was adjusted to absorbance of 0.48 at 600 nm using a spectrophotometer. This adjustment resulted in a cell concentration of 7.5×10^6 cfu ml^{-1}. The number of cells in the suspension was estimated using duplicate plating from 10-fold serial dilutions on BHI agar and counting the colonies after 24 h of incubation at 37°C.

Essential oil

Essential oil of *B. persicum* was obtained from the department of Horticulture, Faculty of Agriculture, Ferdowsi University of Mashhad (FUM). The EO was extracted by steam distillation using a Clevenger-type apparatus and their composition was determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

According to the provider, the GC-MS apparatus was a varian GC-MS spectrometer consisting of a varian star 3400 GC equipped with a fused-silica column (DB-5, 30 m $\times$ 0.25 mm i.d., film thickness 0.25 μm), interfaced with a mass spectrometric detector (varian saturn 3). The operating conditions were as follows: oven temperature 60-240°C with a rate of 3°C/min, injector temperature 280°C, injector mode: split injection, with carrier gas, He, flow rate 2 ml/min, mass spectra: electronic impact, ionisation potential 70 eV, ion source temperature 250°C, ionization current 1000 lA, resolution 1000 and mass range 40-300 u.

Determination of minimum inhibitory concentration

To evaluate minimum inhibitory concentration (MIC) of the EO, standard tube dilution (Macrobroth dilution) technique was used (Chandrasekaran and Venkatesalu, 2004). 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% (10 μl/ml) using dimethylsulfoxide, then serially diluted to achieve 5, 2.5, 1.25, 0.625, 0.312,

0.156, 0.078, 0.04, 0.02 and 0.01 μl ml^{-1} (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. Both sets of tubes (tests and controls) were incubated at 37°C for 24 h. The tube containing 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

Brain heart infusion powder (3.7 g) was dissolved in 90 ml distilled water in a 250 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), the method explained by Mann and Markham (1998) was used with some modifications. Briefly, 5% (v/v) dimethylsulfoxide (DMSO, Merck Germany) as an emulsifier and 0.05% (w/v) agar agar (Merck, Germany) as a stabilizer were added 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 contents 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 *L. monocytogenes* (10^5 and 10^3 cfu ml^{-1}). For each combination, the inoculated tubes were incubated at 35, 25 and 4°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 was recorded. For each combination, a negative control (uninoculated tube) was used. All experiments were conducted in independent triplicate. The total numbers of combinations were 216 ($4 \times 3 \times 3 \times 2 \times 3$).

Statistical analysis

The statistical analysis was performed using SAS statistical software (version 8.2). The outcome variable taken into consideration in the present study was the time to visible growth. Given that some combinations did not grow until the end of the study (30 days), standard regression methods were realized inappropriate. Instead, event-time (survival) analysis was employed which was able to use all the experimental data irrespective of whether or not growth occurred. Kaplan-Meier survival

curves for each level of an explanatory variable were plotted and the homogeneity of the curves between the levels was tested utilizing the log rank statistics. Explanatory variables that revealed an association with time to detection (TTD) of bacterial growth (that is, a difference in the Kaplan-Meier survival curves that was significant at P<0.20) were selected for inclusion in the multivariate analysis.

Parametric survival model based on accelerated failure time (AFT) approach (Kleinbaum and Klein, 2005) was employed with the aim of quantifying the effect of each of the prescribed explanatory variables on TTD of bacterial growth. The general form of the AFT model is:

$$\log(t) = \left(\alpha + \beta_1 \chi_{1i} + ... + \beta_m \chi_{mi}\right) + \log(\tau) \qquad \text{Equation 1}$$

where,

$\log(t)$: The natural logarithm of the time to 'failure' (growth)
α: An intercept term
$\beta_1 x_{1i} + ... + \beta_m x_{mi}$: A linear combination of the m explanatory variables and their regression coefficients
$\log(\tau)$: An error term

Using this approach the AFT coefficients represent the expected change in $\log(t)$ for changes in the predictor levels.

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

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

where,

n: The number of observations
ρ: The number of parameters to be estimated

To select those explanatory variables that best explained TTD a backward stepwise approach was utilized. 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 *B. persicum* Boiss. EO

The components of oil were determined by GC-MS analysis. The constituents of *B. persicum* EO, were accompanied by their retention index and percent, as listed in Table 1. GC-MS analysis resulted in the identification of 35 components representing 95.5% of the total oil. The main constituents were γ-terpinene (44.2%), cuminaldehyde (16.9%), γ-terpinen-7-al (10.5%), and ρ-cymene (8%) (Table 1).

The *in vitro* antibacterial activities of *B. persicum* EO were assessed through standard tube dilution technique against *L. monocytogenes*.

Table 1: EO composition of *B. persicum* identified by GC-MS

No.	Phytochemicals	RI[a]	%
1	α-thujene	925	0.4
2	α-pinene	932	1.0
3	Camphene	946	0.1
4	Sabinene	970	1.2
5	ß-pinene	975	1.6
6	Myrcene	990	1.0
7	δ-2-carene	1002	tr[b]
8	Isosylvestrene	1013	0.3
9	ρ-cymene	1019	8.0
10	Limonene	1025	2.0
11	1,8-cineole	1032	2.9
12	Z-ß-ocimene	1037	0.1
13	γ-terpinene	1055	44.2
14	3-methylbenzaldehyde	1059	tr
15	Cis-sabinene hydrate	1061	tr
16	Terpinolene	1085	0.7
17	Linalool	1093	0.1
18	Trans-sabinene hydrate	1095	0.1
19	Borneol	1162	0.1
20	Terpinen-4-ol	1170	0.4
21	α-terpineol	1189	tr
22	Meta-cuminol	1217	tr
23	ρ-cuminaldehyde	1231	16.9
24	trans-ρ-menth-2-en-7-ol	1261	0.2
25	Perillaldehyde	1265	0.2
26	Bornyl acetate	1280	2.9
27	α-terpinen-7-al	1281	0.4
28	γ-terpinen-7-al	1287	10.5
29	Thymol	1289	0.1
30	9-epi-ß-caryophyllene	1413	tr
31	ar-curcumene	1474	tr
32	Germacrene D	1476	0.1
33	α-zingiberene	1490	tr
34	EE-α-farnesene	1503	tr
35	ß-sesquiphellandrene	1518	0.1
	Total identified		95.5

[a] Retention index relative to n-alkane series on the DB-5 column. [b] Trace (<0.05%)

Antibacterial activity was expressed as MIC value. The MIC of the EO was 5 μl ml^{-1}. In this study, four different concentrations of EO lower than MIC were selected as test concentrations.

About 75.5% of combinations grew during the study period and 24.5% of combinations (53 out of 216) did not grow and were taken into consideration as censored observations. On the basis of MSE value, the log normal distribution provided the best fit to the data. The MSE value of the log normal model was 39.9, while the MSE values were 44.4, 119.2, and 134.5 for log logistic, weibull and exponential models, respectively. Median TTD of bacterial growth was 3 days. Kaplan-Meier survival curve for different levels of explanatory variables is presented in Figs. 1a, b, c and d.

All of the explanatory variables showed an association with TTD of bacterial growth at P<0.20 and were included in the final model. The final model showed that all explanatory variables had significant association with TTD (Table 2). On average, TTD for combinations with 0.08%, 0.16% and 0.24% of EO was 2.64, 4.49 and 5.83 times greater than those without it,

respectively.

Also, this time for combinations with inoculum level of 10^3 was 2.55 times greater than combinations with inoculum level of 10^5. Furthermore, this period for combinations with incubation temperature of 25°C and 4°C was 1.27 and 10.03 times greater than combinations with incubation temperature of 35°C. Time to detection

a

b

c

d
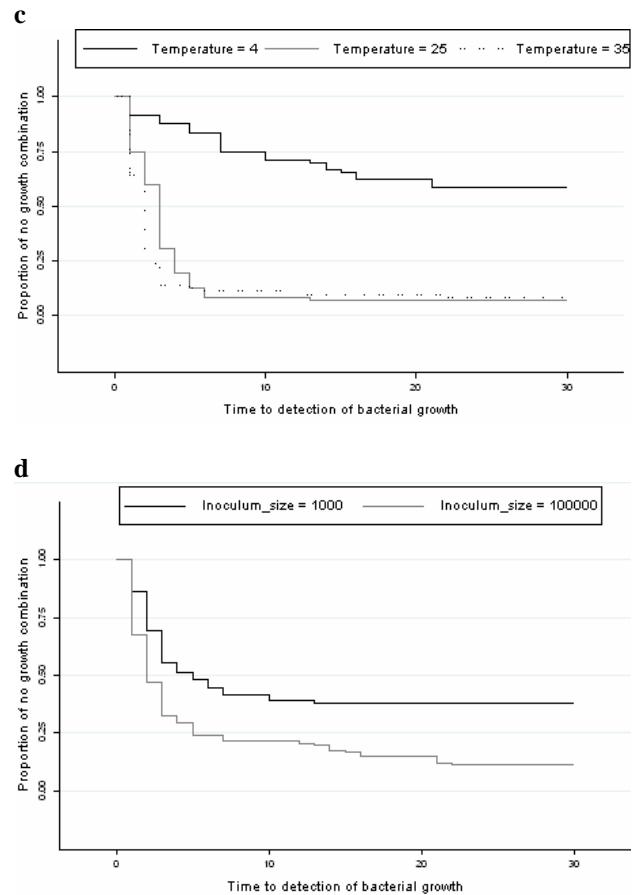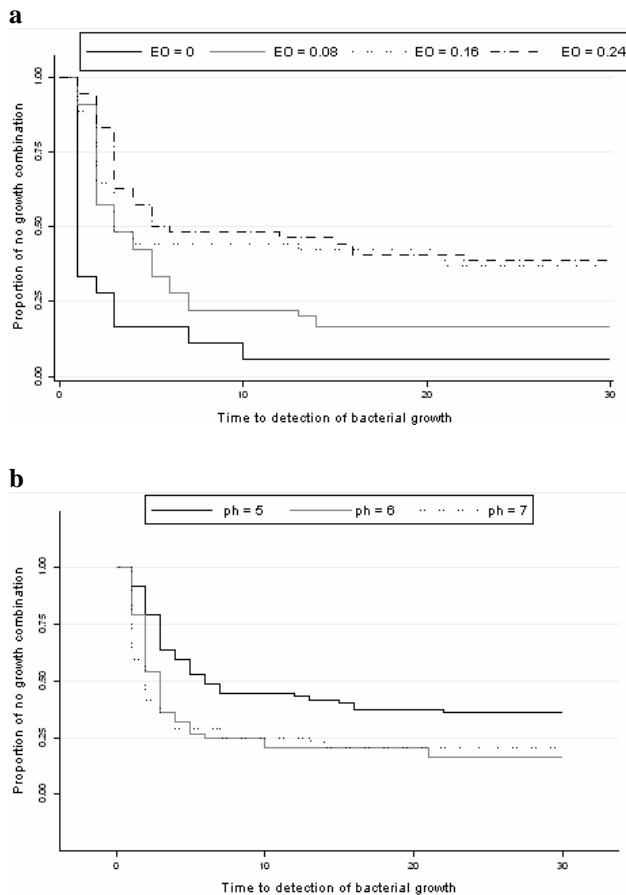
Fig. 1: Kaplan-Meier survival curves showing the proportion of no growth combinations for different levels of EO (a), pH (b), temperature (c), and inoculation (d)

for those combinations with pH levels of 5 was 2.75 times greater than those with pH level of 7. pH level of 6 did not affect TTD of bacterial growth significantly.

The final model equation is as follows:

Table 2: AFT model of factors influencing TTD of bacterial growth

Variables	β (SE)	P-value	Time ratio (95% CI)
Intercept	-1.097 (0.146)		
Essential oil			
Essential oil 0	0		1
Essential oil 0.08	0.97 (0.135)	<0.01	2.64 (2.03-3.43)
Essential oil 0.16	1.50 (0.139)	<0.01	4.49 (3.42-5.89)
Essential oil 0.24	1.76 (0.140)	<0.01	5.83 (4.44-7.67)
pH			
pH 7	0		1
pH 6	0.16 (0.118)	<0.175	1.17 (0.93-1.48)
pH 5	1.01 (0.122)	<0.01	2.75 (2.17-3.49)
Inoculum level			
Inoculum level 10^5	0		1
Inoculum level 10^3	0.94 (0.099)	<0.01	2.55 (2.09-3.10)
Temperature			
Temperature 35	0		1
Temperature 25	0.24 (0.115)	0.034	1.27 (1.01-1.60)
Temperature 4	2.30 (0.128)	<0.01	10.03 (7.81-12.89)
Sigma	0.68 (0.039)		

$$TTD = e^{-1.097+0.24T_{25}+2.3T_4+0.94IL_{1000}+0.97EO_{0.08}+1.50EO_{0.16}+1.76EO_{0.24}+1.01PH_5+0.16PH_6}$$

where,

TTD: Time to detection

E: A mathematical constant approximately equal to 2.718281828

T: Temperature

IL: Inoculums size

EO: Essential oil

The model predicts the value of TTD and describes the growth of *L. monocytogenes*, as environmental factors change. Resorting to these models, the values of predicted TTD can be calculated from any combination of EO, T, pH and IL with the limits studied.

Figure 2 shows the observed TTD of bacterial growth and value that was predicted by model for TTD of *L. monocytogenes* in designated combinations.
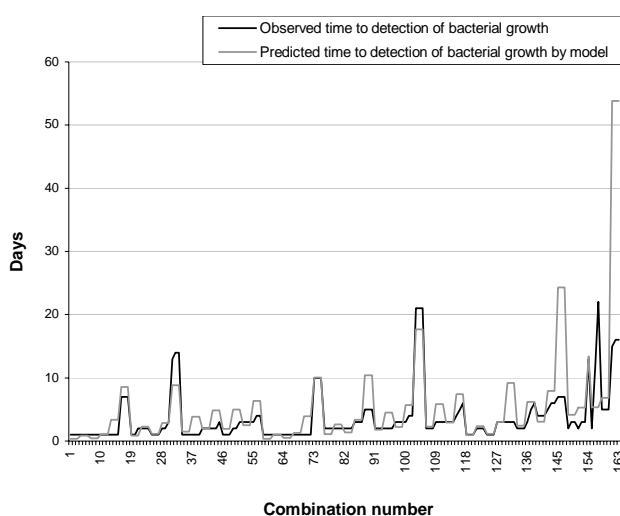
Fig. 2: Observed and predicted days needed for growth initiation of *L. monocytogenes* (TTD) according to the log normal model

Discussion

In this paper, the effects of *B. persicum* EO, pH, temperature, and inoculum size have been studied on the growth responses of *L. monocytogenes* in BHI broth. Further, log normal model was proposed to predict these growth responses.

Plant EOs are potentially useful sources of antimicrobial compounds (Bagamboula *et al.*, 2004). *In vitro* studies have demonstrated antibacterial activity of EOs against different bacteria at levels between 0.2-10 µl ml^{-1}. Essential oils containing phenolic compounds, e.g. thymol, carvacrol, terpinene and ρ-cymene, are widely reported to possess high levels of antibacterial activity and may have applications in controlling pathogens in food (Valero and Salmeron, 2003; Burt, 2004; Akhondzadeh Basti *et al.*, 2007; Khanzadi *et al.*, 2010; Oroojalian *et al.*, 2010).

In the present study, γ-terpinene (44.2%), cuminaldehyde (16.9%) and ρ-cymene (8%) were the major components of *B. persicum* EO. Several researchers have investigated antibacterial and antifungal effect of *B. persicum* EO (Sekine *et al.*, 2007; Moghtader *et al.*, 2009; Oroojalian *et al.*, 2010). Some studies have converged to the conclusion that there is a correlation between the chemical structures of the most abundant compounds in the EOs and their antibacterial activity (Campos *et al.*, 2011; Zeinali *et al.*, 2012).

Phenolic compounds such as carvacrol, thymol, γ-terpinene and ρ-cymene are responsible for antibacterial activities of EOs (Burt, 2004). The effectiveness of γ-terpinene might be the result of its phenolic structure which interferes with the lipid bilayer of the outer membranes (Oyedemi *et al.*, 2009).

Essential oil components may cause crucial changes in cell wall, cytoplasmic membrane as well as proteins of membrane. Hence, these components result in an increase in the permeability and leakage of vital intracellular constituents and subsequently the destruction of bacterial respiratory systems (Celikel *et al.*, 2008; Campos *et al.*, 2011).

Comparing different EOs obtained from three Apiaceae species against *L. monocytogenes*, *Staphylococcus aureus*, *Basillus cereus*, *Escherichia coli* and *Salmonella enteritidis* the results reveal *B. persicum* EO against *B. cereus* as that most efficient antibacterial activity followed by *S. aureus* and *L. monocytogenes*. The main components were identified in the EO of *B. persicum* including cumin aldehyde, γ-terpinene and ρ-cymene (Oroojalian *et al.*, 2010).

Moghtader *et al.* (2009) studied antibacterial activity of *B. persicum* against gram-positive and gram-negative bacteria using disk diffusion. They reported that the antibacterial activity of *B. persicum*, possibly relates to high amount of cuminaldehyde and γ-terpinene.

The control of *L. monocytogenes* in food systems is difficult since this pathogen can survive a wide range of adverse conditions, including acidic pH, low temperatures, and high sodium chloride concentrations (Campos *et al.*, 2011).

According to the present study's results, decreasing the pH of broth medium had a significant effect on growth initiation of inoculated bacteria. Akhondzade Basti *et al.* (2007) demonstrated that *Zataria multiflora* Boiss. essential oil had an inhibitory effect on the growth initiation of *S. typhimurium* and *S. aureus* enhanced by decreasing the pH value at each defined temperature.

This can be attributed to the fact that the EO becomes more hydrophobic at low pH and thus can be dissolved better in the lipid phase of the bacterial membrane (Campos *et al.*, 2011).

Koutsoumanis *et al.* (2004) reported that pH and temperature synergistically affect the growth rate of *L. monocytogenes*. As was shown, by decreasing temperature below 15°C, the pH required for growth rises.

Regarding Marc *et al.* (2002), the pH range over which *Listeria* can grow is narrower at low temperatures, thus the growth rate can be reduced to zero via applying specific combinations of temperature and pH, which separately would not be capable of bacterial inhibition.

Temperature is the most common method used to control microbial growth. Our results showed that,

decreasing the incubation temperature also had a significant effect on the growth initiation of inoculated bacteria. Carrasco *et al.* (2006) modeled the effect of temperatures and acids on the growth of *L. monocytogenes*. The results revealed that the maximum bacterial growth occurs at the optimum temperature. Despite the fact that *Listeria* is a psychrophilic microorganism, low temperature is the primary factor in controlling bacterial growth.

Results of the present study showed that increasing the inoculum size had a significant effect on growth initiation. Koutsoumanis *et al.* (2005) studied the effects of inoculums size on the growth of *L. monocytogenes*. The results indicated the importance of inoculum size for microbial growth initiation; the results provided further quantitative data that illustrated high inoculum size could protect the pathogen against other inhibitory factors.

On the whole, in this study, the values of TTD were higher at low levels of temperature and pH, but had high concentrations of EO. It should also be highlighted that the TTD was markedly influenced by the inoculum size. The same results have been reported by Akhondzadeh Basti *et al.* (2007) and Jamshidi *et al.* (2008).

Figure 2 shows the observed TTD of bacterial growth and value predicted by log normal model for TTD of *L. monocytogenes* in designated combinations.

In the present study, designated models adequately predicted the growth initiation time and growth inhibition conditions of *L. monocytogenes* as affected by different levels of pH, temperature, *B. persicum* EO and inoculum size.

Also, the results showed that EO, pH, temperature and inoculums size had significant effects on TTD of *L. monocytogenes*. 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

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