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Secondary Modeling and Strain Variability of *Listeria monocytogenes* Isolated from Seafood and Clinical Samples at Various Environmental Conditions Using High-Throughput Turbidity Method

Esmail Abdollahzadeh[1], Hedayat Hosseini[2,3 *], Seyed Mahdi Ojagh[4], Hamid Salari Joo[5], Mohammad Reza Koushki[2], Lila Ansari Moghaddam[2]

1-International Sturgeon Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rasht, Iran

2-Deptartment of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

3- Food Safety Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

4-Department of Fisheries, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

5-Department of Fisheries, Faculty of Natural Resources, University of Kurdistan, Sanandaj, Iran

Abstract

Background and Objective: Variability of foodborne pathogens plays important roles in microbial risk assessment. In the present study, kinetic behaviors of seven *Listeria monocytogenes* and two *Listeria innocua* strains from various sources were assessed at various pH (4.5, 5.0, 5.5, 6.8 and 7.0) and salinity (0.5, 2.5, 3.5, 5, 7 and 10% NaCl).

Material and Methods: Maximum specific growth rates (μ_{max}) were assessed using Bioscreen C (time-to-detection method). Nearly 1500 curves were produced for *Listeria monocytogenes* and *Listeria innocua* strains and secondary models were developed for the bacteria as a function of NaCl, pH, temperature and undissociated lactic acid concentrations.

Results and Conclusion: Variability of μ_{max} enhanced as the growth environment became unfavorable. Coefficient of variation of μ_{max} in *Listeria monocytogenes* strains were 6.5 and 20% at 0.5 and 7.5% NaCl (pH 7.26), respectively. After selecting the most robust strain, models demonstrating relationships between the growth rate and environmental conditions (NaCl, pH and undissociated form of lactic acid) were generated using Origin 2018 Software and polynomial and nonlinear surface fitting. In conclusion, intraspecies variability of the growth kinetic behaviors and developed models based on the worst case (e.g., the most robust *Listeria monocytogenes* strain) include important uses in food industries.

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*Corresponding author:
Hedayat Hosseini,
Department of Food Science and Technology, Faculty of Nutrition Sciences, Food Science and Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Food Safety Research Center, Shahid Beheshti University of Medical Sciences,Tehran, Iran. Food Safety Research Center, Shahid Beheshti University of Medical Sciences,Tehran, Iran

Tel (Fax): 98-21-22086347
E-mail:
hedayat.s.hosseini@gmail.com

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1. Introduction

Listeria (*L.*) *monocytogenes* is a foodborne pathogen responsible for an extremely severe infection with high mortality rate [1]. The *L. monocytogenes* is more deadly (10–30%) than other common foodborne bacteria such as *Vibrio* (mortality rate of 0.005-0.01%), *Salmonella enteritidis* (0.38%) and *Campylobacter* (0.02-0.1%) [1,2]. This

pathogen is responsible for the foodborne disease of listeriosis associated with outbreaks from the consumption of various contaminated foods such as vegetables, dairies, seafood and ready-to-eat foods [1,2]. Differences in growth kinetic behaviors in bacterial strains produce significant variability sources in quantitative microbial risk assessments (QMRA) and predictive microbiology studies [3]. Therefore, findings of microbiological studies on a bacterial strain cannot be safe extended to other strains [4-7]. Strain selection is an important step when designing studies to assess growth dynamics of the pathogens in food products or culture media. Use of a cocktail of at least 3-5 strains of the target pathogen is recommended in food safety and challenge studies. Therefore, microbial growth variability in strains of a single species is studied [3,5]. However, challenge studies may be carried out using strains with robust growth or inactivation behaviors (e.g., resistance of strains to inactivation process) [4-7]. When assessing mechanisms of resistance to environmental conditions in basic investigations, a single strain with described phenotypic characteristics is more appropriate. Hence, characterization of intraspecies variability with regards to phenotypic responses under various cultural conditions can help select appropriate strains for challenge studies [4].

Variability of *L. monocytogenes* strains has been investigated in several studies [8-11]. For example, De Jesus and Whiting [9] investigated behaviors of 21 *L. monocytogenes* strains and reported significant strain and intralineage variations. Similarly, Uyttendaele et al. [10] studied various suboptimal growth conditions (temperature, pH and a_w) and showed that *L. monocytogenes* behaviors in suboptimal growth environments were strain-dependent. More recently, Bannenberg et al. [12] investigated variability of sub-lethally heat-injured lag-phase *L. monocytogenes* strains (n = 23) in half Fraser enrichment broth. Results showed significant variations in lag phase of 4.7-15.8 h in the strains. In a study by Lianou et al. [13], effects of heat and lactic acid stress responses were assessed in 25 *L. monocytogenes* strains. Study revealed extensive variations in stress resistance in the serotypes. Most of previous studies on variability of growth kinetics have focused on primary modelling, which are able to estimate microbial growth parameters (e.g., growth rate and lag phase). In studies on secondary modelling of *L. monocytogenes*, investigating effects of environmental conditions on parameters estimated by the primary modelling, no screening steps are described for the strain selection.

Predictive microbiology models, explaining bacterial behaviors, are categorized as primary, secondary and tertiary models. Several data collection methods are available for using in predictive microbiology models, including viable plate counting, flow cytometry and turbidimetric methods. From these methods, viable plate count is the most popular method to estimate growth parameters. However, this method is extremely laborious, especially when the growth kinetics of several bacterial strains in various matrices are studied. Therefore, fast, inexpensive and high-throughput methods such as time-to-detection (TTD), which is based on the OD method, can be used to estimate growth parameters. The TTD method allows estimation of growth rate and lag time and can be used as an alternative of primary models when OD data are available [14]. Using TTD method (Bioscreen C), intra-species diversity of *L. monocytogenes* (n = 388) was reported by Aalto-Araneda et al. [15] at 9.0% NaCl concentration. The primary models were used without secondary modeling to estimate the bacterial growth rates. To the best of the authors' knowledge, various primary and secondary models are available to describe *L. monocytegenes* growth parameters; however, a limited number of studies are available to include the worst-case scenarios (e.g., the most robust *L. monocytogenes* strain) in secondary modeling [16-19]. Therefore, the aim of the present study was to investigate variability of growth rates in seven strains of *L. monocytogenes* and two strains of *L. innocua* using TTD method. Furthermore, a bacterial strain with robust growth kinetic was chosen and secondary models were developed as a function of NaCl, pH, temperature and undissociated lactic acid concentrations.

2. Materials and Methods

2.1. Bacterial strains

Originally, *L. monocytogenes* and *L. innocua* were isolated from clinical and seafood samples, including fresh fish and shrimp samples. Lineage II strains (serotypes 1/2a, 1/2c and 3c) are common in food products, commonly isolated from listeriosis cases. Serotypes of Lineage II were used in this study (Table 1).

Table 1. *Listeria monocytogenes* and *Listeria innocua* strains used in the present study

No.	Nomenclature	Strain	PCR serogroup	Source
1	6F	*L. monocytogenes*	IIa	Seafood (rainbow trout)
2	21B	*L. monocytogenes*	IIc	Clinical
3	22B	*L. monocytogenes*	IIc	Clinical
4	23F	*L. innocua*	-	Seafood (Caspian tyulka)
5	24F	*L. monocytogenes*	IIa	Seafood (Caspian tyulka)
6	28F	*L. innocua*	-	Seafood (Caspian tyulka)
7	28M	*L. monocytogenes*	IIc	Milk
8	66B	*L. monocytogenes*	IIc	Clinical
9	42F	*L. monocytogenes*	IIb	Seafood (tilapia fillet)

L; Listeria; PCR, polymerase chain reaction

Strains were cultured in TSB at 37 °C for 24 h. From the stock cultures, streaks were prepared onto Mueller-Hinton agar (Liofilchem, Italy) and incubated at 37 °C for 24 h. Then, strains were inoculated into 10 ml of TSB at 37 °C for 24 h. Bacterial pellets were separated from the culture media using three times of centrifugation at 9000 rpm for 5 min. At each centrifugation, supernatant was replaced with physiological saline serum (٠,9% w v^{-1} NaCl). Inocula were diluted in physiological serum to achieve 0.08-0.1 OD (optical density; ca. 8 log CFU ml^{-1}) at 600 nm [20]. This value (8 log CFU ml^{-1}) was verified and standardized using direct plate count. Diluted inocula were used for the inoculation of all treatments.

2.2. Media preparation and estimation of the growth rate as a function of pH, NaCl, temperature and strain

Briefly, TTD method was used to estimate *Listeria* growth rate. To estimate the growth rates of *L. monocytogenes* (seven strains) and *L. innocua* (two strains), various concentrations of NaCl (0.5, 2.5, 3.5, 5, 7 and 10%) and pH (4.5, 5.0, 5.5, 6.8 and 7.0 adjusted with HCl) in 10 ml of TSB were prepared and used for studying kinetics of the bacterial growth. Inocula with concentration of 8 log CFU ml^{-1} were diluted to similar levels of NaCl or pH to prepare 6-2 log CFU ml^{-1}. These final bacterial concentrations were enumerated using pour plate method (Mueller-Hinton agar) and the values were used for the calculation of bacterial kinetics. Aliquots of 400 µl of each bacterial dilution (6-2 log CFU ml^{-1}) were transferred to 96-well honeycomb plates. Two wells were used for each bacterial dilution. Non-inoculated media were used as negative control. Honeycomb plates were incubated using automatic turbidimetric system (Bioscreen C, FB-1100-C; MBR Company, Helsinki, Finland) [14]. Plates were agitated at medium intensity. Bioscreen C was used to monitor the bacterial growth by reading OD at 600 nm at regular time intervals (15 min) up to a maximum of seven days depending on treatments. Growth rates were estimated at 25 °C (as abuse ambient storage temperature) and 37 °C (as optimum growth temperature) from turbidity growth curves using TTD method. This method was described as the time to produce an OD of 0.2 [14]. Then, the growth rate (1 h^{-1}) was calculated as shown in Fig. 1.

2.3. Secondary modeling for the growth rate as a function of NaCl concentration

In general, the experimental conditions included strains of six *L. monocytogenes* strains; NaCl concentrations of 0.5, 2.5, 3.5, 5.0, 7.0 and 10%; temperature of 37 °C; pH 7.26; and media of TSB. Validation of the model was carried out using one strain of *L. monocytogenes* (42F) and two strains of *L. innocua*.

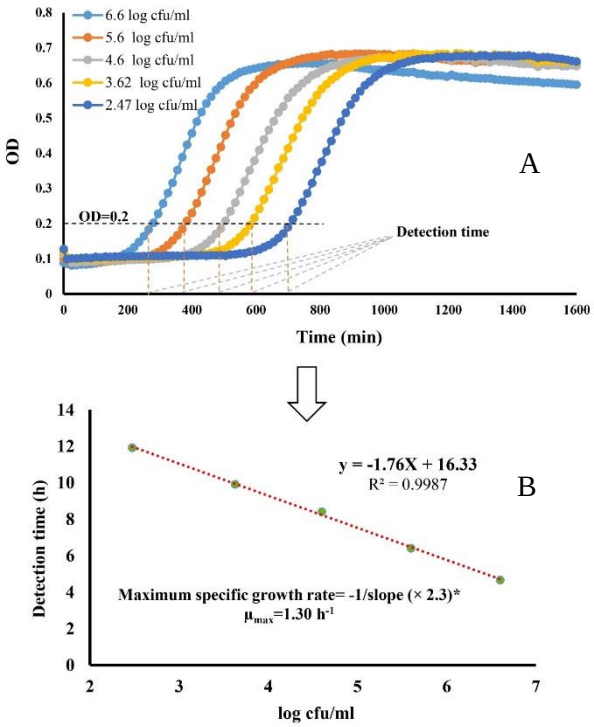

Figure 1. Schematic of time-to-detection method used for estimating growth rate of *L. monocytogenes*. Coefficient of ×2.3 was used to convert log CFU ml^{-1} to ln CFU ml^{-1}

Secondary models were adjusted to the growth rate data as a function of NaCl (0.5, 2.5, 3.5, 5.0, 7.0 and 10%) for the six *L. monocytogenes* strains. Model fitting perfo-rmances were compared using goodness-of-fit test (chi-square test) and adjusted R-squared. Origin 2018 Software (Origin Lab Corp., USA) was used to fit the secondary model to data. Residual sum of squares and R-squared were used to check the model performance.

$$RSS = \sum(Xobs - Xprd)^2 \qquad \text{Eq. 1}$$

Where, X_{obs} was the observed data value and X_{prd} was the predicted value from the fit. A value close to 0 showed that the model included a smaller error and was more useful for the prediction. Various strains of *L. monocytogenes* included variations in their kinetic behaviors. To achieve a global model, describing effects of NaCl on μ_{max}, the six *L. monocytogenes* strains were used in developing the model. To validate the model for other strains, one *L. monocytogenes* (42F) with no uses in modeling process was used to assess the model. Therefore, bias and accuracy factors were used for the validation step (eqs. 2 and 3). Moreover, *L. innocua*, an attractive non-pathogenic bacterium, could be used in the modeling process as an alternative of *L. monocytogenes.* To assess usefulness of the final model for non-pathogenic bacteria, bias and accuracy factors were calculated for the two strains of *L. innocua* (23F and 28F) (Table 1).

For the generalization and validation of the model as well as testing its performance, one *L. monocytogenes* strain (42F) and two strains of *L. innocua* were used.

$$Bias\ factor = 10^{(\Sigma \log(\mu\ predicted/\mu\ observed)\ /\ n)} \quad \text{Eq. 2}$$

$$Accuracy\ factor = 10^{(\Sigma \left|\log\left(\mu \frac{predicted}{\mu} observed\right)\right| / n)} \quad \text{Eq. 3}$$

2.4. Growth rates of Listeria strains at two levels of pH

General experiment conditions included strains of six *L. monocytogenes*; temperature of 37 °C; pH 6.64 and 5.77; and media of TSB. To find the maximum growth rate in *L. monocytogenes* strains, growth rates were compared at two levels of pH, 5.77 and 6.64 at optimum temperature (37 °C) in TSB. Strain with the maximum growth rate was selected for further studies.

2.5. Secondary modeling for the growth rate as a function of pH and NaCl

Experimental conditions included strain of 28M *L. monocytogenes*; NaCl concentrations of 0.5, 2.5, 3.5 and 5%; temperature of 37 and 25 °C; pH 4.5, 5.0, 5.5, 6 and 7.0; and media of TSB. The same strain, 28M strain (the most robust *L. monocytogenes* strain), was used for the validation based on the results from the previous study (see growth rate of *Listeria* strains at two levels of pH). A secondary model was developed for the estimated growth rate at four levels of NaCl (0.5, 2.5, 3.5 and 5%) and five levels of pH (4.5, 5.0, 5.5, 6 and 7.0) at 37 and 25 °C. Origin 2018 Software (Origin Lab Corp., USA) was used for the modeling. Residual sum of squares (eq. 1) and R-squared were calculated to check the model performance. Validation of the model was carried out using 28M train under various conditions.

2.6. Effects of undissociated forms of lactic acid on growth rates

Experimental conditions included strain of 28M *L. monocytogenes*; 15 various concentrations of lactic acid (0-1.34 mM l^{-1}); temperature of 25 °C; pH 5.5; and media of TSB. In this experiment, 15 various concentrations (0-1.34 mM l^{-1}) of lactic acid (90%; Sigma-Aldrich, Germany) in its undissociated form were used. The strain of 28M was used in the experiment. Growth study was carried out in TSB at 25 °C using Bioscreen C (FB-1100-C; MBR Company, Helsinki, Finland). Ratios between the dissociated and undissociated forms of lactic acid at pH 5.5 were calculated using Henderson-Hasselbalch equation (eq. 4) [21].

$$[\text{Hlac}] = \left[\frac{[\text{Lactot}]}{1+10^{(pH-pKa)}}\right] \quad \text{Eq. 4}$$

Where, pH of TSB was 5.5, pK_a (acid dissociation constant) was the value of lactic acid at 25 °C (3.86), Hlact was the concentration of undissociated acid and Lactot was the total concentration of lactic acid. A spreadsheet was developed to facilitate the calculation. Eleven and four concentrations of undissociated acid were used to develop the secondary model and model validation, respectively. Bias and accuracy factors were used for the validation step with the same strain (28M) at various conditions.

3. Results and Discussion

In this study, effects of various environmental conditions (NaCl, pH, temperature and lactic acid) on the behaviors of *Listeria* strains were studied using turbidimetric method, which produced more than 1500 growth curves used to estimate μ_{max} in TTD method. The estimated growth rates were used to develop the secondary model. Moreover, the lag phase was calculated using TTD method (Supplementary 1).

3.1. Growth rates of *Listeria monocytogenes* and *Listeria innocua* strains

When investigating variations of μ_{max} in bacterial strains, it is important to assess a large set of strains at short interval times (e.g., less than 60 min). To address this need, classic viable count methods may not be feasible because such methods could be time consuming. To solve this problem, turbidimetric methods can be used. Various methods for the estimation of μ_{max} from absorbance datasets have been suggested [24, 26-28] and frequently used in studies on strain variability. In the present study, TTD method was used for the estimation of growth rates under various environmental conditions. Table 2 shows the μ_{max} of seven *L. monocytogenes* and two *L. innocua* strains at various NaCl concentrations (10, 7.5, 5, 2.5 and 0.5 %). The minimum and the maximum growth rates of *L. monocytogenes* were 0 and 1.25 ln 1 h^{-1}, respectively. Moreover, NaCl at 10% concentration included inhibitory effects on all the strains with no visible growth. The great means of the six strains at 7.5, 5, 2.5 and 0.5% NaCl were 0.27, 0.73, 1.08 and 1.12 1 h^{-1}, respectively. The intraspecies variability of μ_{max} in *L. monocytogenes* strains was shown using coefficient of variation (CV = 100 × standard deviation/mean). The CVs of 19.9, 15.3, 9 and 6.5% of μ_{max} in seven *L. monocytogenes* strains included 7.5, 5, 2.5 and 0.5% NaCl (pH 7.26), respectively. In culture media (0.5% NaCl), the average μ_{max} (h^{-1}) ranged 1.03-1.23 at pH 7.26 for *L. monocytogenes* and 1.32–1.35 at pH 7.26 for *L. innocua* strains. Important variability of μ_{max} was reported under the highlighted conditions of this study (Table 2, Fig. 1). Studies have shown the strain variability of foodborne pathogens, including *Escherichia coli* [7,22], *Staphylococcus aureus* [23,24], *L. monocytogenes* [9,11,25] and *S. enterica* [4].

Table 2. Growth rates (ln 1 h^{-1} = log 1 h^{-1} * 2.3) of seven *Listeria monocytogenes* and two *Listeria innocua* strains in various NaCl concentrations (pH 7.26) at 37 °C in TSB media calculated by time-to-detection method. A total of ten replications (*n* =10) of 96-well honeycomb plates were used for each strain (five bacterial dilutions 6–2 log CFU ml^{-1} in duplicate)

Strain	*Listeria* spp.	NaCl (%)	Average growth rate (ln CFU h^{-1})	SD
6F	*L. monocytogenes*	10	NG*	NG
6F	*L. monocytogenes*	7.5	0.27	0.02
6F	*L. monocytogenes*	5	0.70	0.02
6F	*L. monocytogenes*	2.5	1.03	3.929E-05
6F	*L. monocytogenes*	0.5	1.05	0.02
22B	*L. monocytogenes*	10	NG	NG
22B	*L. monocytogenes*	7.5	0.34	0.02
22B	*L. monocytogenes*	5	0.69	0.05
22B	*L. monocytogenes*	2.5	1.04	0.09
22B	*L. monocytogenes*	0.5	1.03	0.02
66B	*L. monocytogenes*	10	NG	NG
66B	*L. monocytogenes*	7.5	0.31	0.0005
66B	*L. monocytogenes*	5	0.67	0.04
66B	*L. monocytogenes*	2.5	0.96	0.03
66B	*L. monocytogenes*	0.5	1.15	0.04
28M	*L. monocytogenes*	10	NG	NG
28M	*L. monocytogenes*	7.5	0.31	0.10
28M	*L. monocytogenes*	5	0.98	NG
28M	*L. monocytogenes*	2.5	1.26	5.742E-05
28M	*L. monocytogenes*	0.5	1.15	0.03
21B	*L. monocytogenes*	10	NG	NG
21B	*L. monocytogenes*	7.5	0.23	0.02
21B	*L. monocytogenes*	5	0.71	0.005
21B	*L. monocytogenes*	2.5	1.15	0.01
21B	*L. monocytogenes*	0.5	1.24	0.01
24F	*L. monocytogenes*	10	NG	NG
24F	*L. monocytogenes*	7.5	0.19	0.01
24F	*L. monocytogenes*	5	0.64	0.04
24F	*L. monocytogenes*	2.5	1.07	0.04
24F	*L. monocytogenes*	0.5	1.14	0.05
42F	*L. monocytogenes*	10	NG	NG
42F	*L. monocytogenes*	7.5	0.34	0.03
42F	*L. monocytogenes*	5	0.71	0.01
42F	*L. monocytogenes*	2.5	1.04	0.02
42F	*L. monocytogenes*	0.5	1.21	0.02
28F	*L. innocua*	10	NG	NG
28F	*L. innocua*	7.5	0.42	0.02
28F	*L. innocua*	5	0.82	0.08
28F	*L. innocua*	2.5	1.22	0.06
28F	*L. innocua*	0.5	1.36	0.05
23F	*L. innocua*	10	NG	NG
23F	*L. innocua*	7.5	0.28	0.01
23F	*L. innocua*	5	0.84	0.003
23F	*L. innocua*	2.5	1.21	0.02
23F	*L. innocua*	0.5	1.32	0.08

SD, standard deviation; NG, no growth; *L, Listeria*

As shown in Fig. 2 a, b and c, growth of *L. monocytogenes* strains in certain NaCl concentrations was strain-dependent. For example, populations of 22B and 21B strains after 12 h of storage in 0.5% NaCl (pH 7.26) were respectively predicted to increase by 6.9 and 7.7 log CFU ml^{-1} using Baranyi model and estimated μ in TTD method. However, the variations rise at less favorable environments. Populations of 24F and 22B strains after 36 h of storage in 7.5% NaCl (pH 7.26) were predicted to increase by 4.57 and 6.89 log CFU mL^{-1}, respectively. The estimated μ_{max} values at 7.5% NaCl were 4-6 times lower than that at 0.5% NaCl. At 7.5% NaCl, the corresponding CV was approximately 20%. The greater variability of μ_{max} in non-optimal growth environments has been reported by other studies. These results were similar to those by Lianou et al. [4], who studied growth rate of *S. enterica* strains and showed that strain variability of the estimated μ_{max} increased as the growth conditions became more stressful. Uyttendaele et al. [10] reported a CV of 25% for the estimated μ_{max} values of 11 *L. monocytogenes* strains in culture media at suboptimal conditions. Equation (5) was developed to predict the

growth rate of several *L. monocytogenes* strains as a function of NaCl concentrations (Fig. 3).

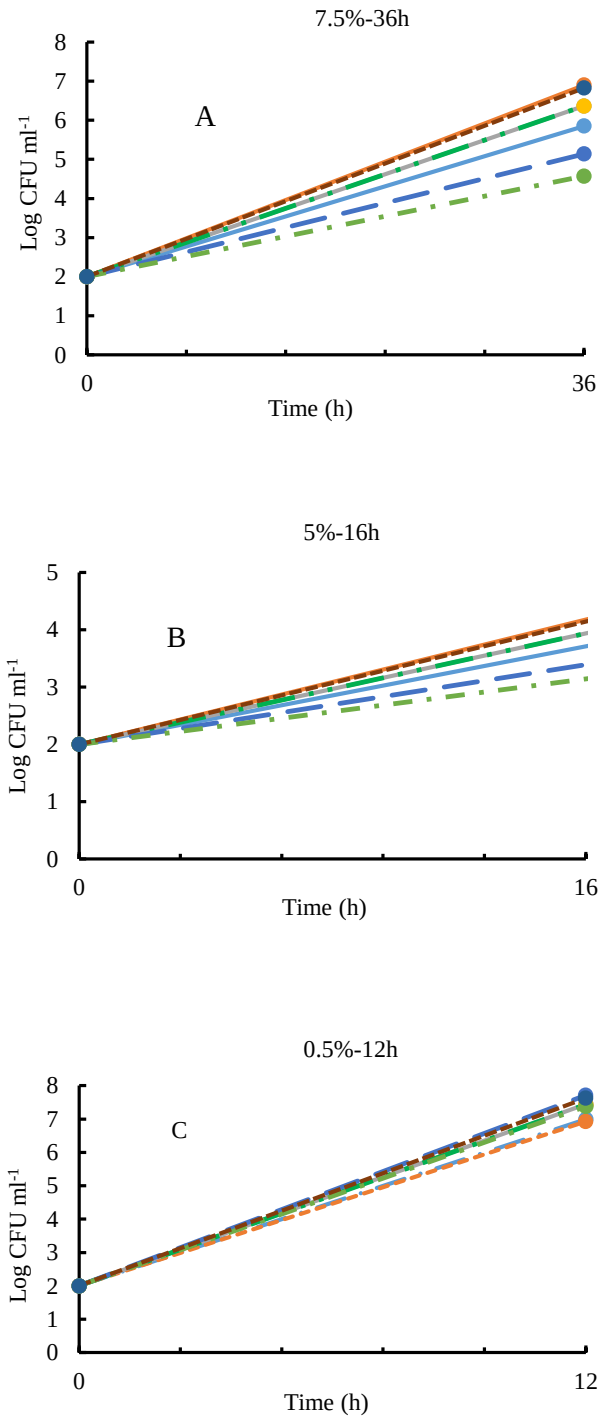

Figure 2. Growth curves for seven *L. monocytogenes* strains (lines with various colors) based on the estimated growth rate of the strains in TSB at pH 7.26 (a, 7.5% NaCl; b, 5% NaCl; c, 0.5% NaCl). Curves were plotted using Baranyi and Pin [22] model and the following conditions were assumed for all strains: (i) physiological state (h_0) = 1; (ii) maximum bacterial population (N_{max}) = 8 log CFU ml^{-1}; and (iii) initial bacterial population (N_0) = 2 log CFU ml^{-1}

$$y = A2 + (A1 - A2) / \left(1 + \left(\frac{x}{x0}\right)\right)^{p} \quad \text{Eq. 5}$$

Where, y was the growth rate (1 h^{-1}), x was NaCl (%), A1 = 1.130 ±0.0061, A2 = -0.257 ±0.0358, x0 = 6.528 ±0.1187 and p was 3.460 ±0.1433. To validate the secondary model, Eq. (5) was used to predict growth rates of one *L. monocytogenes* stain (42F) and two strains of *L. innocua*, not used in model preparation. Bias and accuracy factors were 0.96 and 1.05 for *L. monocytogenes* (42F) strains, respectively. These factors were 0.84-0.91 (bias) and 1.11-1.03 (accuracy) for two strains of *L. innocua* (23F and 28F). Results of the testing model are shown in Supplementary 2. Results showed that slope of the regression line of all strains was greater than 0.86 (R-squared ≥ 0.98). Moreover, results showed that the secondary model could successfully predict the growth rate of *L. monocytogenes* and *L. innocua* at sub-minimal inhibitory (sub-MIC) NaCl concentrations. The current results demonstrated that the strain variability increased under non-optimal growth conditions (Fig. 2), possibly affecting the secondary modeling. Importance of the strain variability depends on the aim of model developer. For example, a worst case scenario can be used for the aim of building models for food safety purposes. However, predictive models based on worst case scenarios may result in risk overestimation. When models are used for the exposure assessment in QMRA, the strain variability is important and the stochastic models is necessary for showing the variability, known as biological variability [29]. For QMRA, uncertainty in bacterial behaviors is however linked to incorrect experiments or lack of knowledge, which can be dismissed by additional measurements. Findings of the current study are useful in strain selection for use in predictive models and challenge studies. In this study, a secondary model was developed by analyzing growth responses of six *L. monocytogenes* strains and 95% confidence interval was used to show the strain variability. A similar methodology was used in Membre et al. [30] study.

In general, *L. innocua* can be used as an alternate of *L. monocytogenes* because of its genetic similarities [31,32]. Regarding safety risks to researchers, the former strain has been used as a non-pathogenic substitute in various studies [33-36]. However, results have shown strain variability in *L. innocua* strains [37]. In addition to its strain variability, appropriateness of *L. innocua* as a *L. monocytogenes* substitute needs further assessments for phenotypic responses to various environmental stresses. Due to phenotypic differences, a review by Milillo et al. [32] highlighted that similar responses of *L. monocytogenes* and *L. innocua* are necessary before using *L. innocua* as *L. monocytogenes* substitute.

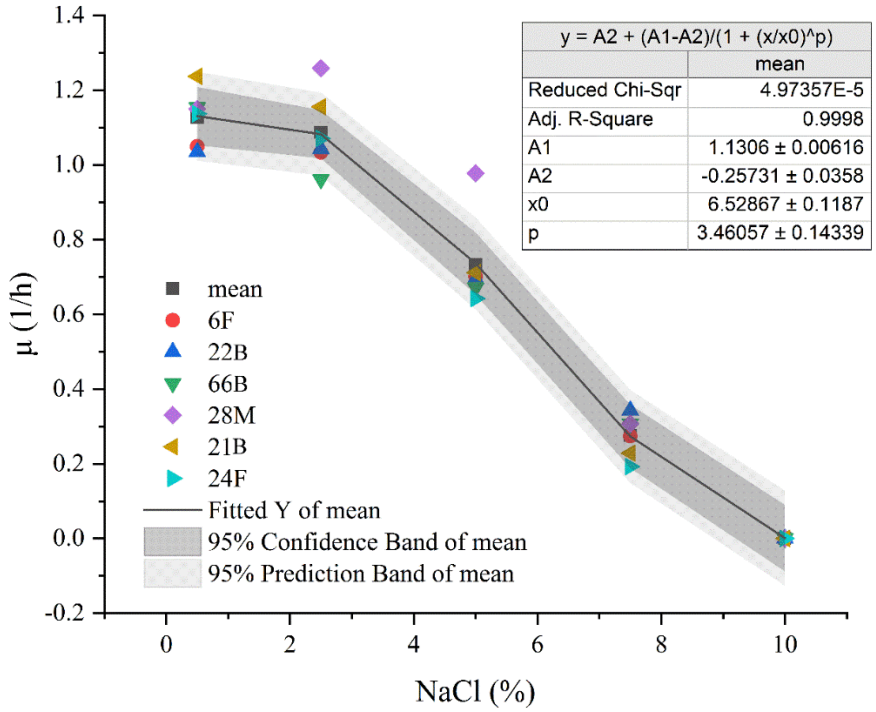

Figure 3. Results of modeling growth rate (ln 1 h^{-1}) of six *L. monocytogenes* strains at five NaCl (%) concentrations with 95% confidence interval for the response prediction

Results of the present study showed that the secondary model developed for *L. monocytogenes* strains could successfully predict (R-square = 0.99) the growth rates of two *L. innocua* strains at sub-MIC NaCl concentrations. These findings also demonstrated suitability of *L. innocua* as an alternative of *L. monocytogenes* when assessing effects of NaCl on the μ_{max} of *L. monocytogenes*.

3.2. Growth rates of Listeria strains at two levels of pH

The growth rates of nine *Listeria* strains at two levels of pH (5.77 and 6.64) were assessed and results showed that the 28M strain included the highest growth rate at pH 5.77 (1.81 l h^{-1}) and 6.64 (1.94 l h^{-1}).

3.3. Secondary modelling for the growth rate as a function of pH and NaCl concentration

Based on its high growth rate, the 28M strain was selected as the worst case and secondary models were developed for μ_{max} of this strain. These models were developed and validated at four concentrations of NaCl (0.5, 2.5, 3.5 and 5) and five levels of pH (4.5, 5.0, 5.5, 6 and 7.0) at 37 and 25 °C. Equation (6) was developed to estimate the bacterial growth rate (1 h^{-1}) at various NaCl concentrations and pH levels at 37 °C (Fig. 4a).

$$\mu = z0 + a \times NaCl + b \times pH + c \times NaCl^2 + d \times pH^2$$

(for NaCl < 5%) Eq. 6

Where, z0, a, b, c and d were -7.83 ±1.37, -0.035 ±0.067, 2.69 ±0.487, -0.0019 ±0.015 and -0.193 ±0.042, respectively. The sum of squared deviations for differences between the current μ_{max} and predicted μ_{max} was 0.265. The model bias and accuracy factors were 0.96 and 1.21 for 28M strain, respectively. Equation (7) represents results of modeling at 25 °C for the estimation of growth rates (l h^{-1}) at four levels of NaCl and five levels of pH (Fig. 4b).

$$\mu = z0 + a \times NaCl + b \times pH + c \times NaCl^2 + d \times pH^2$$

(For NaCl < 5%) Eq. 7

Where, z0, a, b, c and d were equal to -6.724 ±1.023, -0.013 ±0.05, 2.36 ±0.357, -0.005 ±0.0089 and -0.184 ±0.03, respectively. The great mean (±SD) of all environmental conditions (four NaCl concentrations and five pH levels) for one strain (28M) was 0.941 ±0.49 and 0.486 ±0.27 at 37 and 25 °C, respectively. Regarding μ_{max} at 37 °C, the minimum growth requirements of *L. monocytogenes* was at pH 4.5 and 5% NaCl concentration. Tienungoon et al. [38] reported a minimum pH of 4.3 in HCl-acidified media for the growth of *L. monocytogenes*.

Moreover, these observations have been reported in previous studies [39,40]. Van der Veen et al. [39] studied the growth limits of 138 *L. monocytogenes* strains at low pH and high salt concentrations in BHI broth. They reported that almost all strains could not grow at 30 °C and pH 4.4.

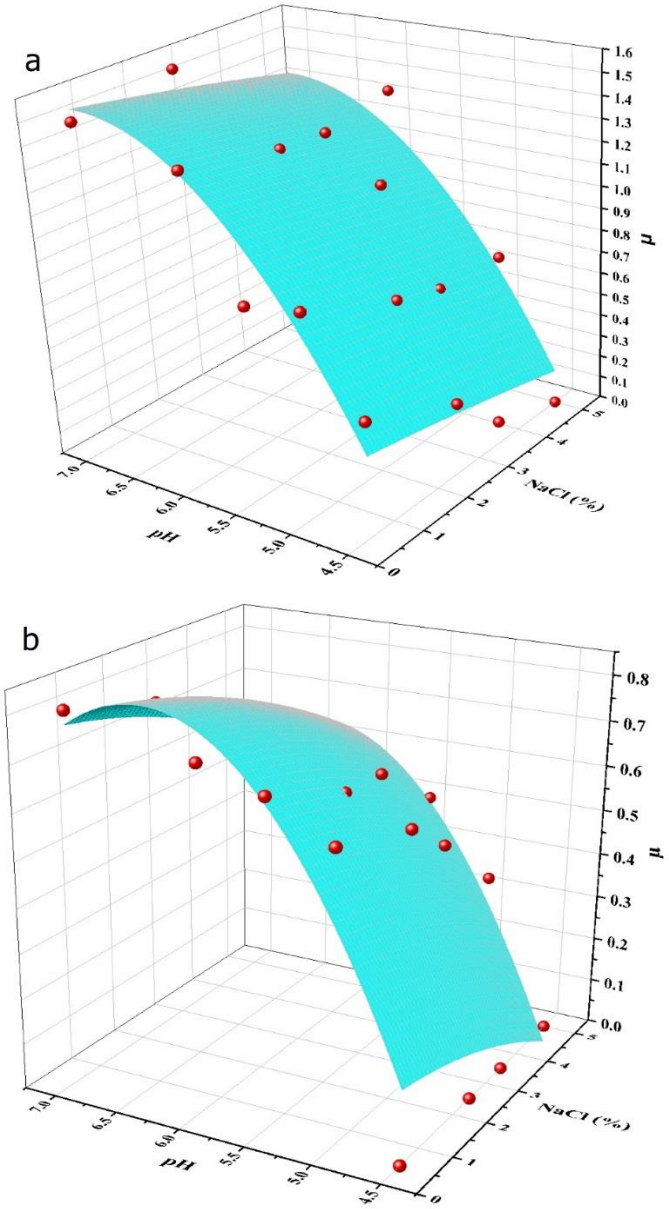

Figure 4. Secondary model for the estimated growth rate of four levels of NaCl and five levels of pH in TSB at 37 °C (a) and 25 °C (b)

Moreover, 87% of the strains showed growth inhibition at 5.8% NaCl concentrations and pH 4.7.

3.4. Modeling effects of the undissociated form of lactic acid on growth rate

Figure 5 shows results of modeling the undissociated form of lactic acid (0–1.34 mM l^{-1}) in TSB at pH 5.5 and 25 °C. As the undissociated lactic acid level increased, the maximum growth rate decreased. The best fit to the data was described by the high R-square (0.98). Equation (8) could be used to predict the growth rate under various concentrations of the undissociated forms of lactic acid at 25 °C.

$$\mu = a - b / (1 + c \times x)^{\frac{1}{d}} \quad \text{Eq. 8}$$

Where, a, b, c and d were 0.47 ±0.46, 0.157 ±0.454, -0.681 ±0.134 and 2.26 ±5.879, respectively. Results revealed a good model performance (R^2 = 0.988) against the test data, which were not used in the modeling procedure (data not shown). Bias and accuracy factors of the model were 0.97 and 1.02 for 28M strain, respectively. Tienungoon et al. [38] reported that organic acids and undissociated acid concentrations increased the minimum pH level; at which, bacterial growth was investigated. Suggested model for the μ_{max} ofb *L. Monocytogenes* in response to undissociated form of lactic acid showed a good description of the data (Fig. 5). In previous studies, various secondary models were provided for fitting the growth rate as a function of pH, temperature and undissociated lactic acid (Table 3). However, bias factors for all the developed models of the present study were in the range of 0.75-1.25, which suggested as a criterion for the successful validation of bacterial models.

Table 3. Summary of secondary models for fitting the growth rate as a function of pH, temperature and undissociated lactic acid

No.	Model	No. of parameters	Reference
1	$\mu_{max} = \alpha_{pH}^2(pH-pH_{min})$	2	Adams et al. [41]
2	$\mu_{max} = \mu_{opt}(1-10^{pHmin-pH})$	2	Presser et al. [42]
3	$\mu_{max} = \mu_{opt}(1-10^{pHmin-pH})(1-10^{pH-pHmax})$	3	Biesta-Peters et al. [43], Presser et al. [42]
4	$\mu_{max} = \mu_{opt}(1-\alpha_{pH}^{(pHmin-pH)})$	3	Aryani et al. [44]
5	$\sqrt{\mu_{max}} = \alpha_T(T-T_{min})$	2	Ratkowsky et al. [45]
6	$\mu_{max} = \mu_{opt}(1-\sqrt{[HA]/[HA]_{max}})$	2	Le Marc et al. [46]
7	$\mu_{max} = \mu_{opt}\exp(-\alpha_{[HA]}[HA] - [HA_{max}])$	3	Yeh et al. [47]

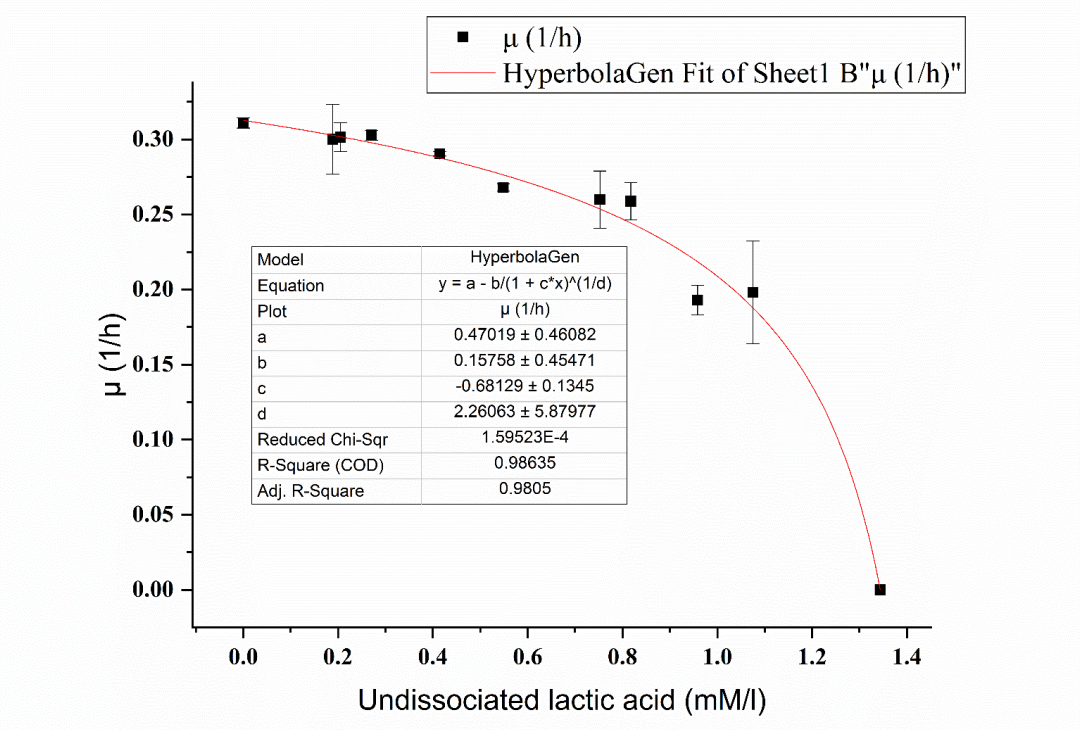

Figure 5. Modeling growth rates (strain 28M) under various concentrations of undissociated forms of lactic acid

In general, results of this study demonstrated that variability of the μ_{max} in *L. monocytogenes* and *L. innocua* strains was affected by various NaCl and pH conditions. Models developed in this study can help food industries produce safer foods.

4. Conclusion

The effects of various environmental conditions on the behaviors of Listeria strains revealed the amount of μmax in *L. monocytogenes* and *L. innocua* strains is affecting by changes of NaCl and undissociated lactic concentration, temperature and pH conditions. Developed secondary models base of findings of current study can help for prediction of growth kinetic behaviors of these pathogens for prevention listerial foodborne diseases for producing safe foods.

5. Acknowledgements

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6. Conflict of Interest

The authors report no conflicts of interest.

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مدل‌سازی ثانویه و تغییرات بین سویه‌ای *لیستریا مونوسیتوژنز* جداسازه شده از نمونه‌های شیلاتی و کلینیکی در شرایط محیطی گوناگون با استفاده از روش کدورت سنجی با گذرداد[1] بالا

اسماعیل عبداله زاده[1]، هدایت حسینی[2و3]، سید مهدی اجاق[4]، حمید سالاری جو[5]، محمد رضا کوشکی[2]، لیلا انصاری

۱-موسسه تحقیقات بین المللی تاس ماهیان دریای خزر، سازمان تحقیقات، آموزش و ترویج کشاورزی (AREEO)، رشت، ایران
۲- انستیتو تحقیقات تغذیه‌ای و صنایع غذایی کشور، دانشکده علوم تغذیه و صنایع غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران.
۳- مرکز تحقیقات ایمنی مواد غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران.
۴-دانشکده شیلات و منابع طبیعی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران.
۵-گروه شیلات، دانشکده منابع طبیعی، دانشگاه کردستان، سنندج، ایران.

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- سدیم کلرید
- لاکتیک اسید تفکیک‌نشده
- نرخ رشد
- بایواسکرین
- *لیستریا اینوکا*

***نویسنده مسئول**
هدایت حسینی
گروه علوم و صنایع غذایی، دانشکده علوم تغذیه و صنایع غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران.
تلفن: ۲۲۰۸۶۳۴۷ -۲۱-۹۸+
دورنگار: ۲۲۰۸۶۳۴۷ -۲۱-۹۸+
پست الکترونیک:
hedayat.s.hosseini@gmail.com

چکیده

سابقه و هدف: گوناگونی بیماری‌زاهای غذازاد[2] نقش مهمی در ارزیابی خطر میکروبی ایفا می‌کند. در مطالعه حاضر، رفتار کینتیکی هفت سویه *لیستریا مونوسیتوژنز* و دو سویه *لیستریا اینوکا* جداسازی شده از منابع گوناگون در شرایط مختلف pH (۴/۵، ۵، ۵/۵، ۶/۸ و ۷) و شوری (۰/۵، ۲/۵، ۳/۵، ۵، ۷ و ۱۰ درصد) مورد ارزیابی قرار گرفت.

مواد و روش ها: مقادیر بیشینه نرخ ویژه رشد باکتری (μ_{max}) با استفاده از زمان رسیدن بایواسکرین C (روش زمان رسیدن به کدورت) ارزیابی شد. حدود ۱۵۰۰ نمودار جذب نوری برای سویه‌های *لیستریا مونوسیتوژنز* و *لیستریا اینوکا* تولید شده و مدل‌های ثانویه برای باکتری‌ها به‌عنوان تابع غلظت‌های سدیم کلرید، pH، درجه حرارت و لاکتیک اسید تفکیک نشده توسعه داده شدند.

یافته‌ها و نتیجه‌گیری: میزان تغییرات μ_{max} به موازات نامساعد شدن شرایط رشد محیطی بیشتر شد. ضریب تغییرات (CV) نرخ رشد در بیان سویه‌های *لیستریا مونوسیتوژنز* در سطوح ۰/۵ درصد نمک طعام (pH =۷/۲۶) و ۷/۵ درصد نمک طعام (pH =۷/۲۶) به‌ترتیب ۶/۵ و ۲۰ درصد بود. پس از انتخاب مقاوم‌ترین سویه، مدل‌هایی که ارتباط بین نرخ رشد و متغییرهای شرایط محیطی (سدیم کلرید، pH و لاکتیک اسید تفکیک نشده) را توصیف می‌کردند، با استفاده از نرم‌افزار Origin 2018 و با روش‌های برازش چند جمله‌ای و مدل‌سازی غیرخطی توسعه داده شدند. در نتیجه، تنوع بین‌سویه‌ای رفتارهای کینتیکی رشد و مدل‌های توسعه داده شده بر اساس بدبینانه ترین سناریو (به‌عنوان مثال، سویه *لیستریا مونوسیتوژنز* بسیار مقاوم) کاربرد مهمی در صنایع غذایی دارند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] Throughput مقدار اطلاعاتی که از جایی به جای دیگر منتقل یا در مدتی معین پردازش می‌شود

[2] Foodborne pathogens عاملی بیماری‌زا و عموماً باکتریایی که ازطریق غذا منتقل می‌شود