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THE COMBINED EFFECT OF TEMPERATURE, pH AND LACTOSE CONCENTRATION ON THE GROWTH PROBABILITY OF *LISTERIA INNOCUA*

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ABSTRACT

Growth response data of *Listeria innocua* in broth were monitored at different combinations of temperature (4, 8 and 12 °C), pH (4.0, 4.5, 5.0, 5.5 and 6.0) and lactose concentration [0, 1, 2, 3, 4 and 5 (w/v %)] for 35 days. Especially low levels of temperature and pH were effective to prevent the growth of *L. innocua* whereas lactose concentration should be at least ≥ 4% at low temperature and pH values to obtain no growth response. Logistic regression model was developed to describe and further predict the growth boundaries of *L. innocua* under different combinations of the conditions. Goodness-of-fit indices and measures of predictive power such as percent concordant revealed that the fitted equation described the growth limits of *L. innocua* successfully. The proposed model could be also used to predict growth limits or probabilities of *Listeria* in whey at different temperature levels since pH and lactose concentration of whey is similar to the conditions tested and this was demonstrated for both sweet whey and acid whey. This study indicated that a quick estimate of growth probability of *L. innocua* is possible at different combinations of temperature, pH and lactose concentration.

Keywords: Predictive microbiology, probabilistic models, growth/no growth, *Listeria monocytogenes*

INTRODUCTION

Listeriae are gram-positive, nonsporulating, psychrotrophic and facultative anaerobic bacteria, and can grow over a wide range of temperature (optimum growth temperature is between 30 and 37 °C) and pH (4.1-9.6). Due to their ability to form biofilms on various surfaces, Listeriae can be commonly found in food processing facilities and equipment (**Ferreira *et al.*, 2016**). *Listeria monocytogenes* is the specie responsible for listeriosis in humans which is a health concern throughout the world.

Different names such as growth/no growth interface, growth boundary models have been attributed to the probabilistic models which are used to define and predict the growth limits of microorganisms under various environmental conditions (**Haberbeck *et al.*, 2015**). Predictive growth/no growth modeling have been successfully applied during the last 25 years, and logistic regression technique is generally used to define the growth boundaries of microorganisms although other techniques such as artificial neural networks (**Hajmeer and Basheer, 2003**) and growth/no growth model derived from the Gamma model (**Polese *et al.*, 2011**) were also applied. This type of models could be very beneficial to food industry by giving a quick estimate of chance of growth under a set of environmental factors such as temperature as well as intrinsic factors such as pH and water activity of the food.

Production of different cheese varieties such as Salamoura (Turkey), Feta (Greece), Telemea (Greece, Rumania), and Domiati (Egypt) from raw milk is a common practice in some countries (**Papageorgiou and Marth, 1989**). Although these cheeses are brined and matured for several months to prevent the pathogen survival, pathogens may pass through whey during production of cheese. Dairy foods, especially cheeses are the major causes of listeriosis outbreaks (**Rogers *et al.*, 2018**).

Studies on growth/no growth modeling of different bacteria including *L. monocytogenes* are abundant in literature; however, to the best our knowledge there is no study on the combined effect of temperature, pH and lactose concentration. Lactose and pH levels may be important for the growth of *Listeria* in dairy products such as whey. Therefore, the objective of this study was to develop a probabilistic model to define the growth limits of *L. innocua* which is a non-pathogenic bacterium and a surrogate for *L. monocytogenes* due to its genetic similarity (**Sheng *et al.*, 2020**).

MATERIALS AND METHODS

Bacterial strain and inoculum preparation

L. innocua strain ATCC 33090 was used in this study. The strain was stored at -80 °C in tryptic soy broth (TSB; 7164, Acumedia) supplemented with 0.6% yeast extract (YE; 7184A, Acumedia) and 15-20% glycerol. Frozen culture was activated in TSBYE at 30 °C for 24 h, and a subculture was taken twice and incubated in TSBYE for 24 h at 30 °C. An inocula (100 µL) from the activated culture, which was at the stationary phase (21 h at 30 °C), was transferred to 10 mL of TSBYE to obtain 10^8-10^9 CFU/mL.

Experimental design

A full factorial design with three temperatures (4, 8 and 12 °C), four pH levels (4.5, 5.0, 5.5 and 6.0) and six lactose concentrations [0, 1, 2, 3, 4 and 5 % (w/v)] was used. Each treatment was tested twice, resulting in a total of 144 data points (3 temperatures × 4 pH × 6 lactose × 2 replication). The pH and lactose levels were selected to simulate whey properties.

For each combination, TSBYE (10 mL) was inoculated to about 10^6 CFU/mL by diluting the previously prepared inocula using sterile peptone water (LAB 104, Lab M). The pH of the TSBYE was adjusted with appropriate values with 1 N HCl. At first, lactose concentration was adjusted to 5% for each TSBYE with different pH. Broth solutions were filter-sterilized with 0.2 µm membrane filter system under vacuum (10040-464, VWR). After sterilization, solutions were serially diluted to 4, 3, 2 and 1 % of lactose. Uninoculated samples (with adjusted pH and containing lactose) were served as negative controls and TSBYE (pH = 7.2 with no lactose) inoculated with 10^6 CFU/mL bacteria was used as the positive control.

Growth assessment

Microbial growth i.e., presence/absence of growth was monitored weekly by measuring the optical density of the media at 600 nm using a microplate reader (BTH1M, Biotek™) for 35 days. Media in the glass tubes with all combinations and inoculated with *L. innocua* were shaken manually, and 200 µL from each tube was transferred into 96-wells plate with lid. Positive and negative controls were also transferred. Before reading the optical density linear mixing was applied for 10 s. A tube was considered as showing growth if the optical density

had increased by at least 0.1 above the optical density at time zero (**Khanipour *et al.*, 2016a**). Growth was also verified by spread plating onto a tryptone soy agar (TSA; LAB 011, Lab M) supplemented with 0.6% yeast extract and *Listeria*-selective (Palcam) agar (LAB 148, Lab M).

Model development

Growth or no growth responses of *L. innocua* were scored as 1 or 0, respectively. Data were fitted to a logistic regression model using SPSS (Version 22, Chicago, IL, USA). Two models were proposed:

$$\text{logit}(p) = a_0 + a_1 \cdot T + a_2 \cdot pH + a_3 \cdot LC + a_4 \cdot T \cdot pH + a_5 \cdot T \cdot LC + a_6 \cdot pH \cdot LC + a_7 \cdot T \cdot pH \cdot LC \quad (1)$$

where logit(p) is ln[p/(1 – p)], p is the probability of growth in the range of 0 to 1, a_i are the coefficients to be estimated, T (°C) is temperature, pH is the initial pH of the medium and LC is the lactose concentration (w/v %).

Ratkowsky and Ross (1995) proposed to use logarithmic terms for temperature, pH and water activity on the right hand side of the logistic equation and therefore as a second model:

$$\text{logit}(p) = b_0 + b_1 \cdot \ln(T - T_{min}) + b_2 \cdot \ln(pH - pH_{min}) + b_3 \cdot LC + b_4 \cdot \ln(T - T_{min}) \cdot \ln(pH - pH_{min}) + b_5 \cdot \ln(T - T_{min}) \cdot LC + b_6 \cdot \ln(pH - pH_{min}) \cdot LC + b_7 \cdot \ln(T - T_{min}) \cdot \ln(pH - pH_{min}) \cdot LC \quad (2)$$

where T_{min} and pH_{min} are the minimum notional temperature and pH, respectively; and b_i are the coefficients to be estimated. Minimum lactose concentration (LC_{min}) were not placed in Eq.(2) because lactose is not an essential component for *Listeria* to grow. Moreover, although lactose concentration may affect the water activity of an aqeous solution the concentration range (0-5%) that we used had no impact on the water activity. Both T_{min} and pH_{min} can be estimated from regression or can be treated as fixed constants (**Ratkowsky, 2002**). **Le Marc *et al.* (2002)** obtained T_{min} as −4.5 °C and pH_{min} 4.21 for the same *L. innocua* strain (ATCC 33090). Frozen temperature as the minimum growth temperature of *L.innocua* may seem odd; however, substantial growth of *L. monocytogenes* was reported at subzero temperatures: −2.2 °C in broth (**Bajard *et al.*, 1996**) and −0.4 °C in chicken broth (**Walker *et al.*, 1990**).

Modeling was done as follows: (i) regression was applied with all the coefficients in the model, (ii) if any coefficient (or coefficients) was insignificant ($P \geq 0.05$), then it was removed from the model and regression was repeated without it. Only one insignificant coefficient was removed before each regression and this was repeated until there were no insignificant coefficients left in the model i.e., remaining coefficients in the model were all significant ($P < 0.05$). The predicted growth/no growth interfaces for different growth probabilities were plotted by using Microsoft® Excel Solver.

Evaluation of the model performance

Proposed models were assessed by –2·ln L with L the likehood in its optimum, Hosmer-Lemeshow (H-L) statistic, maximum rescaled R^2 statistic and percent concordant. The First statistic (–2·ln L) can be used to rank the models according to their goodness-of-fit, but does not give an idea about the adequacy of the model fit (**Dang *et al.*, 2010**). Small values of –2·ln L correspond to better fitting models (**Gysemans *et al.*, 2007**). The H-L P value indicates how well the logistic regression equation fits the data by comparing the number of individuals with each outcome with the number expected based on the logistic equation. If H-L statistic takes a small value or its corresponding P value is high, then the model fits the data adequately. The maximum rescaled R^2 indicates how useful the independent variables are in describing the response variable (**Bewick *et al.*, 2005**) and percent concordance reflects the correspondence between observed and fitted values (**Dang *et al.*, 2010**).

RESULTS AND DISCUSSION

Growth response data (1: growth, 0: no growth) of *L. innocua* were monitored at different temperature, pH and lactose concentrations for 5 weeks (35 days), and no increase in OD values of negative controls during this period was observed indicating that there was no contaimination during the preparation of the liquid media. On the other hand, OD values of the positive control increased as expected.

Change of growth/no growth responses at different temperature, pH and lactose concentration with respect to time

Fig. 1 shows the growth/no growth responses of *L. innocua* in broth containing 5 % lactose at different temperature and pH values. Growth was observed at 12 °C for all pH values except the pH 4.5 at the 5th day (Fig. 1b) and growth at 12 °C could not be prevented even at the lowest pH value (pH = 4.5) at day 10 i.e., at 12 °C all growth responses were 1 for all pH levels (Fig. 1c). Note that there were no changes in growth/no growth responses between the days 16 and 35. Growth was not observed at 8 °C, pH = 4.5, and at 4 °C, pH = 4.5 and 5.0 (Fig 1d).

It is known that *L. monocytogenes* can grow well even at refrigeration temperatures (< 5 °C) or at low pH values (4.4) (**Montiel et al., 2020**); however, combining both conditions i.e., low temperature + low pH may prevent the growth of *Listeria* (Fig. 1). Among the three conditions studied lactose was the least effective one. High concentrations of lactose reduce the water activity of a solution and therefore may prevent the growth of bacteria; however, even at 5 % (w/w) lactose concentration water activity of an aqueous solution was still higher than 0.99 (**Miracco *et al.*, 1981**). It seemed that lactose to a extend did not effect the growth since *L. monocytogenes* can grow even at lower water activity values (**Vermeulen *et al.*, 2007**). Lower temperature (< 8 °C) and pH (< 5.0) values had an impact on the growth of *L. innocua* regardless of lactose concentration in broth, whereas high lactose concentration (≥ 4%) was only effective at 4 °C, pH = 4.5 and 5.0 – results not shown. **McKellar and Lu (2001)** also observed that sucrose up to 8 % had no impact on the growth of *Escherichia coli* O157:H7; however, temperature, pH, salt and acetic acid had significant effect.

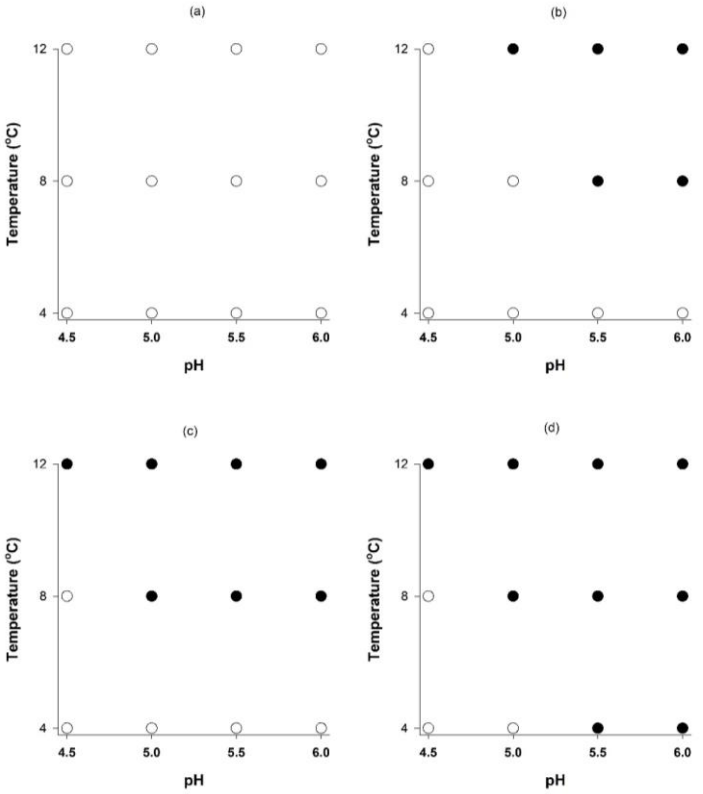

Figure 1 Growth (black circles) and no growth (white circles) data of *Listeria innocua* (10^6 CFU/mL) in broth containing 5 % lactose at different temperature and pH levels with respect to time: 0th day (a), 5th day (b), 10th day (c), and days between 16 and 35 (d).

Model identification

Following logistic regression models [Eqs. (3) and (4)] were obtained:

$$\text{logit}(p) = -4.222 + 0.192 \cdot T \cdot pH - 0.155 \cdot T \cdot LC + 0.155 \cdot pH \cdot LC \quad (3)$$

$$\text{logit}(p) = 8.218 + 2.797 \cdot \ln(T - T_{min}) \cdot \ln(pH - pH_{min}) + 46.428 \cdot \ln(pH - pH_{min}) \cdot LC - 17.663 \cdot \ln(T - T_{min}) \cdot \ln(pH - pH_{min}) \cdot LC \quad (4)$$

where T_{min} = −4.5 °C and pH_{min} = 4.21.

The coefficients of the models with their standard error and P values are given in Table 1. It can be seen that second model i.e., model with logarithmic terms [Eq.(4)] had better fit than the first model [Eq.(3)] because it had lower –2·ln L value, smaller H-L statistic and higher P value, much higher maximum rescaled R^2 statistic and percent concordant. In fact, first model was completely useless because its maximum rescaled R^2 was 0.430 and its concordance was 84.7 %. Therefore, Eq.(4) was further used to plot the predicted growth/no growth interfaces of *L. innocua*. Boundaries for likely to grow probability (p = 0.9), equal probability of growth and no growth (p = 0.5) and unlikely to grow probability (p = 0.1) are displayed in Fig. 2.

Table 1 Coefficients of the models together with their standard error (S.E.) and P values, and goodness-of-fit indices of the models

Model 1 [Eq.(1)]				Model 2 [Eq.(2)]			
Coefficient	Value	S.E.	P value	Coefficient	Value	S.E.	P value
a_0	− 4.222	1.159	< 0.001	b_0	8.218	2.716	0.002
a_1	—[a]	—	—	b_1	—	—	—
a_2	—	—	—	b_2	—	—	—
a_3	—	—	—	b_3	—	—	—
a_4	0.192	0.045	< 0.001	b_4	2.797	0.952	0.003
a_5	− 0.155	0.049	0.001	b_5	—	—	—
a_6	0.155	0.056	0.006	b_6	46.428	16.226	0.004
a_7	—	—	—	b_7	− 17.663	6.263	0.005
$-2\cdot\ln L$[b]	96.583			$-2\cdot\ln L$	17.213		
H-L[c]	11.256 (P = 0.188)			H-L	0.197 (P = 1.000)		
max rescaled R^2	0.431			max rescaled R^2	0.924		
concordant	84.7 %			concordant	97.2 %		

[a] Insignificant ($P \geq 0.05$)
[b] L: likelihood
[c] H-L: Hosmer-Lemeshow statistic

Note that main effects (*T*, *pH* and *L*) were not significant ($P \geq 0.05$) and only interaction terms existed in both models i.e., Eq.(3) and Eq.(4) (Table 1). Normally, in logistic regression models main effects (temperature, pH and lactose concentration in our case) are forced to stay in the model (**Buzrul, 2019**; **Daelman *et al.*, 2013**); however, these effects had high P values ($P \geq 0.98$) so that standard errors of the coefficients were even higher than the coefficients themselves. Therefore, main effects were removed from the model and regression was repeated until a model with significant terms obtained. To the best of our knowledge, this study was the first where the interaction terms stayed in the model while main effects were removed from the model.

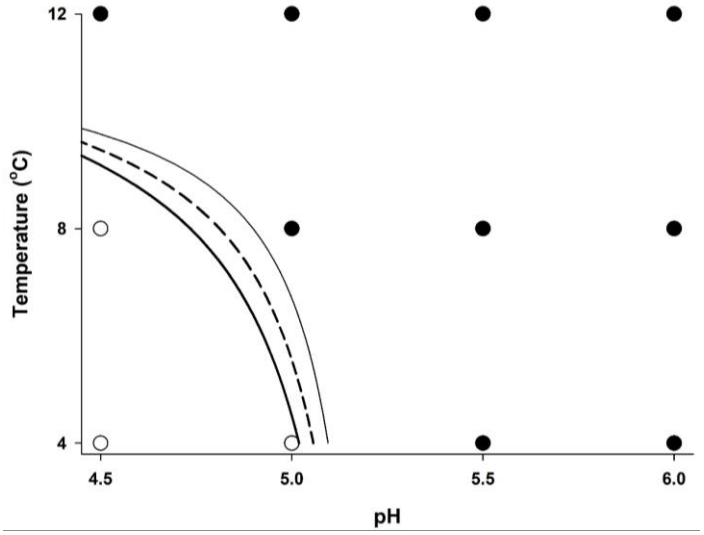

Figure 2 Growth (black circles)/no growth (white circles) interface of *Listeria innocua* (10^6 CFU/mL) in broth containing 5 % lactose at different temperature and pH levels at the end of 5 weeks. Thin solid line, dashed line and thick solid line represent the growth probabilities of 0.9, 0.5 and 0.1, respectively according to Eq.(4).

The growth/no growth interfaces were consistent with the experimental data (Fig. 2). It could be also possible to predict the outcome of other conditions than the experimental levels within the extrapolation region by using Eq.(4). For example, when *T* = 6.2 °C, *pH* = 4.8 and *LC* = 4.7% in Eq.(4) *p* = 0.0014 indicating the growth probability is very low and one may consider that under these conditions *L. innocua* and most probably *L. monocytogenes* would not grow. On the other hand, when *T* = 9 °C, *pH* = 5.2 and *LC* = 2.5% in Eq.(4) *p* = 0.999, so these conditions favor the growth of *Listeria*.

Fig. 3 shows the growth probability of *L. innocua* in liquid media which have 4.6% lactose (w/v) and pH = 6.0 (sweet whey analogue), and 4.6% lactose (w/v) and pH = 4.7 (acid whey analogue), respectively with respect to temperature.

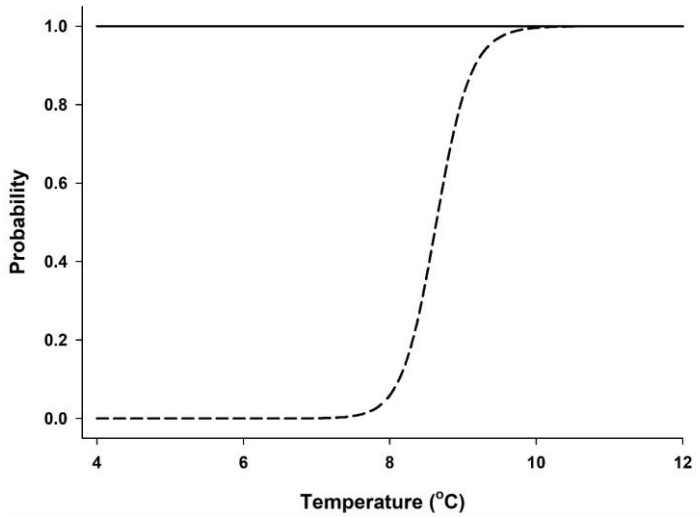

Figure 3 Predicted growth probabilities of *Listeria innocua* according to Eq.(4) with respect to temperature: solid line represents a liquid medium containing 4.6 % lactose (w/v) and pH 6.0 (sweet whey analogue) and dashed line represents a liquid medium containing 4.6 % lactose (w/v) and pH 4.7 (acid whey analogue).

While proposed model [Eq.(4)] could be also used to predict the growth probability of *Listeria* in whey since pH and lactose concentration of whey is similar to the conditions tested in this study, it should be noted that the inoculum size (10^6 CFU/mL) was higher than the expected contamination of whey and therefore it may be supposed that growth probability under realistic conditions would be less (**Khanipour *et al.*, 2016b**). Moreover, liquid whey has some other components than lactose such as amino acids and minerals which may also effect the growth of bacteria. Sweet whey has pH values of 6.0-7.0 and lactose concentrations of 4.6-5.2% (w/v) while acid whey has pH < 5.0 and lactose concentrations of 4.4-4.6% (w/v) (**Panesar and Kennedy, 2012**). Considering those limits one can plot the probability of growth of *L. innocua* with respect to any condition such as Fig. 3 by using Eq.(4). It is apparent that *Listeria* could grow in sweet whey (pH = 6.0) whatever the temperature value was between 4-12 °C; however, in acid whey (pH = 4.7) growth probability was equal to or almost zero up to 8 °C. Furthermore, growth probability increased drastically between 8 and 9 °C indicating that low pH (< 5.0) in combination with refrigeration temperature (< 8 °C) could be used to avoid *Listeria* growth in acid whey (Fig. 3). Note that the results were extended from broth to whey. However, there may be deviations from the results obtained in the laboratory medium compared to a real food system.

Growth/no growth modeling has been applied since the mid-90s and there are numerous researches available on different bacteria including *L. monocytogenes*. Temperature, pH and water activity (generally adjusted with NaCl) are the most popular conditions to determine the growth boundaries; however, inoculum size (**Koutsoumanis and Sofos, 2005**), acetic acid (**Vermeulen *et al.*, 2007**), lactic acid (**Tienungoon *et al.*, 2000**; **Yoon et al., 2009**), $NaNO_2$ (**Gwak et al., 2015**), citric and ascorbic acids (**Valero et al., 2006**), and sodium lactate and sodium acetate (**Skandamis *et al.*, 2007**) were also studied together with those factors to define the growth limits of *L. monocytogenes*.

There are limited number of studies on the growth of *Listeria* in whey in literature. **Ryser and Marth (1988)** found that two out of four strains of *L. monocytogenes* (Scott A, V7, CA and OH) can grow in whey (obtained from Camembert cheese and pH was adjusted to 5.0-6.8) at 6 °C and below pH 5.4. **von Staszewski and Jagus (2008)** observed immediate growth of *L. innocua* in liquid cheese whey system at 7 °C with pH adjusted to 5.5. These studies also confirmed the results obtained here (Figs. 1 and 2).

CONCLUSION

This study revealed that growth probability of *L. innocua* in broth supplemented with lactose (0-5%) at different pH (4.5-6.0) and temperature (4-12 °C) could be successfully described by logistic regression model. Further prediction of growth boundaries could also be possible (Fig. 2). Because the whey has a pH range of 4.6 to 7.0, and lactose (4.4 to 5.2%), protein source and minerals in it, it could be a suitable environment for the growth of *L. monocytogenes*, especially if the cheese is produced from raw milk. However, storage temperature of whey is crucial and a rapid growth would not be surprising at temperatures > 8 °C. We believed that the obtained results are important because *Listeria* can grow or survive under harsh environmetal conditions such as refrigeration temperatures, high salt levels and acidic pH range in the presence or absence of oxygen.

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