Comparison of Probabilistic and Deterministic Predictions of Time to Growth of *Listeria monocytogenes* as Affected by pH and Temperature in Food

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Abstract

Stochastic models are useful for estimating the risk of foodborne illness and they can be integrated, besides other sources of variability, into microbial risk assessment. A stochastic approach to evaluate growth of two strains of *Listeria monocytogenes* influenced by different factors affecting microbial growth (pH and storage temperature) was performed. An individual-based approach of growth through optical density measurements was used. From results obtained, histograms of the lag phase were generated and distributions were fitted. Histograms presented increased variation when the factors applied were suboptimal for *L. monocytogenes* and they were combined. The extreme value distribution was ranked as the best one in most cases, whereas normal was the poorest fitting distribution. To evaluate the influence of pH and storage temperature on *L. monocytogenes* CECT 5672 in real food, commercial samples of courgette and carrot soup were inoculated with this pathogen. It was able to grow in both soups at storage temperatures from 4°C to 20°C. Using the distributions adjusted, predictions of time to growth (10^2 cfu/g) of *L. monocytogenes* were established by Monte Carlo simulation and they were compared with deterministic predictions and observations in foods.

Introduction

PREDICTIVE MICROBIOLOGY is an important tool in the food industry to predict the behavior of microorganisms (Baranyi and Roberts, 1995). The main objective is to use mathematical models to describe the evolution of microorganisms present in food under the influence of intrinsic environmental factors (pH, a_w) and extrinsic factors (temperature, gaseous atmosphere) (Zwietering *et al.*, 1991). At present, most of the models predicting the microbial load are deterministic: at a certain time instant, the model predicts a single value for the microbial load. However, the microbial quality and safety may be characterized by a high level of variation in its behavior at a population level. Typical examples of variation are changes in growth and pregrowth conditions (e.g., temperature, pH, and water activity), measurement of uncertainty, and variation among strains. There is an urgent need for stochastic predictive microbiology. Models should incorporate uncertainty at the level of measurements and model parameters and predict the microbial load by a probability mass function (Nicolai and Van Impe, 1996). As such, stochastic

predictive microbiology is a key element for performing statistically founded hazard analysis and critical control points (HACCP) and risk analysis studies (Baranyi, 1998; Franco *et al.*, 2006).

To produce stochastic data, it is necessary to generate high numbers of replicates of growth curves parameters (maximum specific growth rate and lag time). To do so, absorbance data can be very useful and it has been shown that they compare well with viable counts (Dalgaard and Koutsoumanis, 2001). There are probabilistic models in the literature for *Listeria monocytogenes*, describing its behavior in different food substrates and under dynamic conditions (Koutsoumanis and Sofos, 2005; Xanthiakos *et al.*, 2006; Kagkli *et al.*, 2009), and for spoilage bacteria (Koutsoumanis *et al.*, 2006).

Risk analysis is divided into three related but distinct components: risk assessment, risk management, and risk communication. Predictive microbiology, together with a dose-response relationship, should be the key element to perform quantitative microbial risk assessments of foods (Buchanan and Whiting, 1998). Currently, the Food and Agriculture Organization (FAO)/World Health Organization

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(WHO) framework of risk analysis is being implemented worldwide (Schlundt, 2000).

Monte Carlo analysis is a general method that deals with stochastic models. Examples in the field of predictive microbiology can be found in the papers by Cassin *et al.* (1998), Coleman and Marks (1999), Nauta (2000), Nicolai and Van Impe (1996), and Poschet *et al.* (2003). Monte Carlo simulation has been proposed as a tool to establish the probability of growth or inactivation of microorganisms under certain conditions (Poschet *et al.*, 2003; Ferrer *et al.*, 2007).

The aim of the present study was to compare the inhibitory effects of combined treatments of pH and growth temperature on the distributions of the lag times of two strains of *L. monocytogenes* and to establish the time before a certain level of growth was achieved. To achieve this, a Monte Carlo simulation was carried out. Results were compared with those obtained for two food substrates (courgette and carrot soup).

Materials and Methods

Bacterial strain

The strains used in the experiments were *L. monocytogenes* CECT 5672 and 4031 (from Spanish Type Culture Collection, CECT, Valencia, Spain). Both were stored on tryptic soy agar (TSA; Scharlau, Barcelona, Spain) slants at 6°C and subcultures were grown in tryptic soy broth (TSB; Scharlau) with addition of 0.6% yeast extract (TSBYE; pH 7.3 ± 0.2).

Growth conditions

L. monocytogenes CECT 5672 and 4031 were subcultured twice in TSBYE for 24 h at 37°C before being inoculated in a microtiter plate. Growth was investigated at five different

incubation temperatures (37°C, 20°C, 12°C, 8°C, and 4°C) combined with four different pH values (pH 7.4, 6.5, 5.5, and 4.5). The pH of TSBYE was modified using HCl solution (Panreac Quimica, Barcelona, Spain). All experiments performed are listed in Table 1.

Growth curves and determination of the lag phase

The dilution procedure of Francois *et al.* (2003) was applied to obtain single *L. monocytogenes* cells in the wells of a microtiter plate. Serial 10- and twofold dilutions of bacterial cultures were made in TSBYE modified at different pH, and aliquots (400 µL) were added into the wells of a microwell plate to achieve a cell concentration of 1×10^3 to 1×10^0 . To estimate bacterial growth parameters, a numerical method was used by means of detection times generated by different initial counts. The observed detection times were subjected to a transformation involving the maximum specific growth rate and the ratios between the different inoculum sizes and the constant detectable level of counts (Baranyi and Pin, 1999). The plates were incubated in the Bioscreen C automatic reader (Labsystems, Helsinki, Finland) at different incubation temperatures, and optical density (OD) was measured at 600 nm.

At predetermined intervals of time, samples were removed from wells, diluted, and plated onto TSA for viable counts. The OD of the well was recorded immediately before the sample was taken. OD values were plotted against viable counts, and calibration curves were obtained for each environmental condition.

The kinetic parameters of the single cells were estimated by the method described by McKellar and Knight (2000). The lag times of *L. monocytogenes* cells were obtained through the time of detection (t_d), which is the time required for the microbial population to generate a 0.15 increase in OD from the start of

TABLE 1. BEST-FIT DISTRIBUTION OF *LISTERIA MONOCYTOGENES* CECT 5672 AND CECT 4031 UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

T (°C)	pH	L. monocytogenes CECT 5672				L. monocytogenes CECT 4031			
		Lag (h)	SD	Distribution	p-Value (χ^2)	Lag (h)	SD	Distribution	p-Value (χ^2)
37	7.4	1.77 ^b	0.383	Logistic	0.779	1.30 ^a	0.669	Exponential	0.485
37	6.5	2.68 ^b	0.423	Normal	0.819	1.35 ^a	0.837	Normal	0.774
37	5.5	5.19 ^b	0.348	Extreme value	1.000	2.94 ^a	0.785	Extreme value	0.485
37	4.5	26.13 ^b	4.625	Extreme value	0.368	9.64 ^a	3.696	Exponential	0.248
20	7.4	3.66 ^a	1.074	Exponential	0.051	4.78 ^b	1.322	Exponential	0.485
20	6.5	4.62 ^b	1.331	Extreme value	0.286	3.33 ^a	0.943	Extreme value	0.572
20	5.5	6.15 ^b	2.592	Exponential	0.751	4.58 ^a	0.221	Extreme value	0.116
20	4.5	9.98 ^a	5.564	Normal	0.778	21.88 ^b	5.216	Exponential	0.412
12	7.4	38.35 ^b	11.776	Extreme value	0.779	21.27 ^a	11.033	Exponential	0.881
12	6.5	52.83 ^a	11.640	Extreme value	0.931	44.35 ^a	12.327	Logistic	0.774
12	5.5	63.41 ^a	11.548	Extreme value	0.735	55.69 ^a	22.356	Extreme value	0.881
12	4.5	148.15 ^b	37.135	Extreme value	0.881	71.83 ^a	29.897	Extreme value	0.485
8	7.4	99.01 ^b	26.879	Logistic	0.881	75.83 ^a	12.305	Exponential	0.881
8	6.5	125.86 ^b	29.509	Extreme value	1.000	100.44 ^a	39.417	Exponential	0.774
8	5.5	185.07 ^b	29.549	Logistic	0.248	96.29 ^a	42.414	Exponential	0.881
8	4.5	273.60 ^b	40.765	Extreme value	0.751	190.89 ^a	56.267	Exponential	0.881
4	7.4	200.20 ^b	25.122	Normal	0.010	129.23 ^a	22.472	Extreme value	0.369
4	6.5	140.56 ^a	44.040	Logistic	0.818	143.14 ^a	37.319	Exponential	0.564
4	5.5	192.37 ^b	71.484	Exponential	0.238	138.22 ^a	38.540	Extreme value	0.751
4	4.5	296.12 ^b	52.708	Extreme value	1.000	217.05 ^a	63.614	Extreme value	0.485

Individual cell lag time mean values in each row not followed by the same superscript letter are significantly different ($p \leq 0.05$). T, temperature; Lag, individual cell lag time mean; SD, standard deviation.

incubation. When t_d values are plotted against the corresponding inoculum size (calculated as $\ln$ cfu/mL), μ can be calculated as the negative reciprocal of the slope of the regression line (Cuppers and Smelt, 1993), using the following formula:

$$\mu_{\max} = -1/\text{slope} \quad (1)$$

The individual cell lag times (λ) were calculated from the following formula (Baranyi and Pin, 1999):

$$\lambda = t_d [\ln(N_d) - \ln(N_0)] / \mu \quad (2)$$

where N_d is the bacterial number at t_d obtained by means of calibration curves and N_0 is the number of cells initiating growth in the considered well.

Statistical data processing, distribution fitting, and Monte Carlo analysis

Statistical data processing was performed using Statgraphics Plus 5.1 software (Statistical Graphics Corp., Rockville, MD). Histograms were made from every set of conditions showing the distribution of the lag phases. From each histogram, the most common statistical parameters (mean value, standard deviation, etc.) were determined.

Probability distributions were fitted to individual cell lag times and growth rate data using @RISK 4.5.2 Professional Edition (Palisade Corporation, Newfield, NY). Distributions were ranked using the χ^2 goodness-of-fit statistics (BestFit Users Guide, 2002; Palisade Corporation) as previously described by Li *et al.* (2006). The probability (p) is a measure of confidence that the fitted distribution could have generated the original dataset and ranges from 0 to 1, with 1 being the highest confidence.

Monte Carlo simulation was performed to predict the time to growth to a certain microbial concentration (10^2 cfu/mL, in this case). A risk assessment of *L. monocytogenes* in ready-to-eat foods has stated that this level of contamination (considered as low) has a low probability of causing illness (FAO/WHO, 2004). This is an important aspect to set food safety criteria when a certain level of growth or counts is recommended in a product, as part of a risk assessment study.

Eq. (2) was used to analyze the dependence of N_0 and λ on the times to reach 100 cfu/mL. The individual cell lag times were assumed to follow a gamma distribution of shape parameter β and scale parameter α . The parameters of the gamma distribution and the growth rate, μ , in each environmental condition were calculated from the detection times. The mean and the variance of λ are $\alpha\beta$ and $\alpha\beta^2$, respectively.

On the other hand, the initial number of cells in a well followed a Poisson distribution, with an average of one cell per well, according to Metris *et al.* (2006). This distribution is widely used to represent the initial numbers of a microbial population. Hence, the following equation was used:

$$P(N=0) = e^{-m} \quad (3)$$

where $P(N=0)$ is the probability of having no cells in a well and is estimated as the proportion of wells without growth of the 100 wells inoculated, and m is the average number of cells per well. The number of wells inoculated with exactly one cell was approximated from the following equation: $P(N=1) = me^{-m}$.

Food experiments

The study of previous experiments in a food matrix was developed in courgette and carrot soup. Refrigerated courgette and carrot soup were purchased from a local supermarket. These foods were chosen for their pH values (pH 6.43 and 5.38, respectively), similar to those used in the initial experiments. Triplicate independent experiments per condition tested were performed. For these studies, aliquots (100 mL) of courgette and carrot soup were inoculated with *L. monocytogenes* CECT 5672 (10^2 cfu/mL) and stored at 20°C, 12°C, 8°C, and 4°C. Tests revealed that the contaminating flora in the soups was very low (below 1 cfu/100 mL). Everyday the samples were plated out on TSA.

Results and Discussion

Effect of pH and temperature on individual cell lag phase

The effect of combinations of the environmental factors temperature (4°C–37°C) and pH (4.5–7.4) on individual cell lag phase was investigated for two strains of *L. monocytogenes* (CECT 5672 and CECT 4031).

The mean lag phase duration for both strains increased dramatically when factors affecting microbial growth were applied, a trend that can be confirmed for temperature and pH (Table 1). Also the standard deviations were calculated and increases were found with the application of various factors, indicating that more variability in the adaptation time occurred under those conditions. The duration of the lag phase of *L. monocytogenes* CECT 5672 increased significantly with the decrease of the incubation temperature, for example, the mean individual cell lag phase was increasing from 1.77 h over 99.01 h to 200.20 h when temperature decreased from 37°C to 8°C and 4°C, respectively, and at pH 7.4. The effect of decreasing pH was also significant as the mean individual lag phase was increasing from 99.01 h over 185.07 h to 273.60 h when the pH was lowered from 7.4 to 5.5 and 4.5 at a growth temperature of 8°C. On the other hand, values of individual cell lag phase for *L. monocytogenes* CECT 4031 were significantly ($p \leq 0.05$) lower than the corresponding values for *L. monocytogenes* CECT 5672, but the effects of the factors affecting microbial growth conditions were similar (Table 1). Individual cell lag times of strain CECT 4031 at pH 7.4 increased from 1.30 to 129.23 h when the growth temperature was decreased to 4°C.

The largest increase was found with combinations of acid pH (4.5) and growth temperature of 4°C, reaching lag values of 296.12 and 217.05 h for *L. monocytogenes* CECT 5672 and 4031, respectively.

Histograms of the lag values for different treatments are shown in Figures 1 and 2 for *L. monocytogenes* CECT 5672 and CECT 4031, respectively. Increases in the variability of histograms (due to a wider spread of the observations) representing the lag phase of individual cells (where 10- and twofold dilution procedures were used to achieve single cells per well, there were some wells where growth was not achieved) were observed.

For *L. monocytogenes* CECT 5672, grown at 12°C, significant increases in lag times and in standard deviations were observed when lower pHs were applied, which are reflected in the shift of the histogram to the right. "This meant that the

FIG. 1. Distributions of individual cell lag times of *Listeria monocytogenes* CECT 5672 grown at (A) 12°C and (B) 8°C in broth at pH 7.4 (■), 6.5 (▨), 5.5 (▤), and 4.5 (▧).

adaptation period in acidic conditions became much longer.” Similar effects can be observed for all growth temperatures and for both strains (Figs. 1 and 2). It can also be observed that the variance of the lag time is generally higher with longer lag times, because of the need for an adaptation response (Table 1). A similar observation was made by Smelt *et al.* (2002), who studied the lag times of individual cells of *Lactobacillus plantarum* after sublethal injury.

Also the width of histograms varied depending on the level of factors affecting microbial growth during the growth of the cells. There was less dispersion with optimal conditions than at lower temperatures or pHs (Fig. 2A, B).

The shape of the histogram changed when levels of factors tested increased. The highest density of observations in the curve (skewness) was situated at the left side for moderate stress levels, whereas it shifted to the right one for more severe conditions, meaning that a longer adaptation period in most of the population took place.

Several authors studied the effect of the environmental factors on the individual lag phase of *L. monocytogenes*. A similar behavior with pH has been described in *L. monocytogenes* (Francois *et al.*, 2005) and for the individual cell lag

phase of *L. innocua* at 30°C (Metris *et al.*, 2002). The importance of the inoculum size on growth boundary limits has also been evidenced (Koutsoumanis and Sofos, 2005). When two or more factors are combined (in our case, temperature and pH), there was a trend toward broader histograms and distributions that were shifting toward the right, as it has been also pointed out by Francois *et al.* (2007) in *L. monocytogenes* exposed to combinations of low incubation temperature, pH, and a_w . This is opposed to an adaptation phenomenon as, for example, growth at low temperatures, where bacteria start growing faster, which gives a positive skew to the distributions (Francois *et al.*, 2007).

Distribution fitting

A total of eight distributions were fitted to the 20 datasets of lag values of *L. monocytogenes* CECT 5672 and CECT 4031. Distribution functions were ranked according to the χ^2 statistic. The best-fit distribution to the pooled data of each treatment is listed in Table 1. The χ^2 statistic showed that in most cases the listed distributions fitted better the data. Probability (p) values were generally >0.1 .

FIG. 2. Distributions of individual cell lag times of *L. monocytogenes* CECT 4031 grown at (A) 37°C and (B) 8°C in broth at pH 7.4 (■), 6.5 (▨), 5.5 (▤), and 4.5 (▩).

Overall, the extreme value distribution was fitted highest, being 10 times the best fit for individual cell lag times of *L. monocytogenes* CECT 5672 and eight times for CECT 4031. An exponential distribution was fitted by two times of the 20 datasets of *L. monocytogenes* CECT 5672; however, this distribution was the best fit by 10 times in individual cell lag times of *L. monocytogenes* CECT 4031. Logistic distribution was the best fit in only 5 of 20 conditions, being four times for CECT 5672 and one time for CECT 4031. Normal was the poorest fitting distribution, being only three times the best fit for CECT 5672 and one time for CECT 4031.

A small number of studies have examined the impact of factors affecting microbial growth on individual lag time distributions; some of them are in agreement with our results. The extreme value distribution was mentioned by Smelt *et al.* (2002) to describe the individual cell lag phase of *Lactobacillus plantarum* in MRS broth at 15°C. This distribution was also observed by Guillier and Augustin (2006), who investigated the individual lag times of *L. monocytogenes* for 54 combinations of initial physiological states, growth conditions, and strains, finding extreme value type II distribution as the most effective to describe the 54 observed distributions.

The differences in the conditions of growth allowed us to observe variability in the distributions between experiments.

In general, when more stringent conditions were applied, our results were fitted with an extreme value distribution, for both *L. monocytogenes* strains. These differences were also observed by other authors, namely Francois *et al.* (2005), who investigated the individual lag times of *L. monocytogenes* cells in 26 different growth conditions, the best distributions being exponential, gamma, and Weibull when growth conditions were optimal, intermediate, and severe, respectively.

The applied distributions differ from author to author: Metris *et al.* (2002) and Kotalik *et al.* (2005a and 2005b) used a gamma distribution, whereas Wu *et al.* (2000) used a normal distribution. This discrepancy could be explained by different reasons: the kind of strain and its physiological states, the use of different primary models (Francois *et al.*, 2005), or the environmental factors used.

Effects of pH and growth temperature on the lag time of L. monocytogenes in real systems

To evaluate the activity of pH and storage temperature on *L. monocytogenes* CECT 5672 in real food, commercial samples of courgette and carrot soup were inoculated with this pathogen. The soups were chosen by their pHs (pH 6.43 and 5.38 in courgette and carrot soup, respectively). The evolution of

FIG. 3. Effect of different storage temperatures on *L. monocytogenes* CECT 5672 inoculated in courgette soup. (◆) 20°C; (▲) 12°C; (■) 8°C; (●) 4°C. Solid lines represent the data predicted by the model. Growth data are presented as ln.

FIG. 4. Effect of different storage temperatures on *L. monocytogenes* CECT 5672 inoculated in carrot soup. (◆) 20°C; (▲) 12°C; (■) 8°C; (●) 4°C. Solid lines represent the data predicted by the model. Growth data are presented as ln.

L. monocytogenes population was monitored over time during storage at 20°C, 12°C, 8°C, and 4°C (Figs. 3 and 4) and the $\ln(N_d)$ were successively fitted by means of the Baranyi equation.

L. monocytogenes CECT 5672 was able to grow in courgette and carrot soup at storage temperatures of 4°C, 8°C, 12°C, and 20°C. When *L. monocytogenes* was inoculated in courgette soup and was exposed to low growth temperature, a progressive delay in the onset of growth was observed but did not affect the maximum population density (Fig. 3).

When *L. monocytogenes* was grown in carrot soup at refrigeration temperatures (8°C and 4°C), the maximum population density was reduced. In this case, the growth rate decreased drastically from 20°C to 12°C (Fig. 4), which is likely due to lower pH value of carrot soup.

Monte Carlo simulation to estimate time to a certain growth

Monte Carlo simulations were carried out, considering an initial contamination of 1 cfu/mL to achieve a final concentration of 100 cfu/mL of *L. monocytogenes* CECT 5672 under different treatments.

Predicted times to an increase from 1 to 100 cells of *L. monocytogenes*, obtained from a Monte Carlo simulation, in different conditions of temperature and pH are shown in

Table 2. They were compared with the values obtained from deterministic fit, and as can be seen, the values of the output parameters generated by both analyses were very close. Results were also compared with growth curves obtained for courgette and carrot soup. There was variability when comparing predictions obtained with real food systems (soups), as in some cases the growth in soups was faster than that predicted by both deterministic and stochastic models. This indicates the need to perform tests in the real conditions of the food, because predictive microbiology can give an estimate from observations in synthetic media, but there are many other factors present in foods that cannot be taken into account. In pasteurized milk, a probabilistic model revealed that *L. monocytogenes* could grow in the distribution and storage conditions in Greece (Koutsoumanis *et al.*, 2010).

Changes in environmental factors produced important changes in the time to increase *L. monocytogenes* to 10^2 cfu/mL. Monte Carlo analysis allows quantification of the probability distributions of the model parameters (Poschet *et al.*, 2003) and, in our case, gives information of the variability and distribution over the time of the predictions. When the risk of growth beyond a certain level of a foodborne pathogen is considered, an average value is not a valid information and it needs to be replaced by a probabilistic one. From this setting, it could be predicted, for example, with a probability level of 95% the time before growth to a certain level could occur.

TABLE 2. PREDICTED TIME TO REACH 100 CFU/ML BY *LISTERIA MONOCYTOGENES* EXPOSED TO THE CONDITIONS INDICATED, OBTAINED FROM A MONTE CARLO SIMULATION, DETERMINISTIC FIT, AND DETERMINISTIC CALCULATIONS IN REAL FOOD

Conditions		<i>L. monocytogenes</i> CECT 5672				<i>L. monocytogenes</i> CECT 4031	
Temperature (°C)	pH	Deterministic fit	Monte Carlo	Courgette soup	Carrot soup	Deterministic fit	Monte Carlo
20	6.5	7.43 ± 0.97 ^a	7.09 ± 1.35	10.11 ± 1.13		7.44 ± 1.28	6.98 ± 0.97
20	5.5	16.31 ± 3.41	15.10 ± 2.88		6.27 ± 0.138	8.74 ± 0.77	8.27 ± 0.48
12	6.5	174.90 ± 17.32	108.65 ± 12.70	84.49 ± 3.75		94.99 ± 24.06	81.39 ± 12.28
12	5.5	108.70 ± 29.06	95.43 ± 12.42		129.65 ± 8.27	151.27 ± 52.69	116.39 ± 23.44
8	6.5	250.56 ± 82.69	220.95 ± 30.86	93.19 ± 4.02		192.08 ± 43.23	181.10 ± 41.23
8	5.5	315.02 ± 65.70	291.64 ± 32.26		218.81 ± 18.17	194.41 ± 31.86	173.57 ± 41.42
4	6.5	285.34 ± 76.63	256.16 ± 46.52	136.78 ± 4.54		238.96 ± 46.47	228.46 ± 37.59
4	5.5	629.02 ± 76.63	380.87 ± 74.91		256.98 ± 8.28	1071.04 ± 124.45	332.87 ± 44.69

^aMean ± standard deviation.

With this information, a food processor can decide the best processing conditions according to the level of risk it is ready to accept in the product. Monte Carlo simulation has been also used to establish the influence of parameters on microbial load by pulsed electric fields (Ferrer *et al.*, 2007) and it is a useful tool for implementation of microbiological data in microbial risk assessment.

This is an important aspect to set food safety criteria when a certain level of growth or counts is recommended in a product, as part of a risk assessment study.

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Disclosure Statement

No competing financial interests exist.

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