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Stochastic modelling of *Listeria monocytogenes* single cell growth in cottage cheese with mesophilic lactic acid bacteria from aroma producing cultures

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ABSTRACT

A stochastic model was developed for simultaneous growth of low numbers of *Listeria monocytogenes* and populations of lactic acid bacteria from the aroma producing cultures applied in cottage cheese. During more than two years, different batches of cottage cheese with aroma culture were analysed for pH, lactic acid concentration and initial concentration of lactic acid bacteria. These data and bootstrap sampling were used to represent product variability in the stochastic model. Lag time data were estimated from observed growth data (lactic acid bacteria) and from literature on *L. monocytogenes* single cells. These lag time data were expressed as relative lag times and included in growth models. A stochastic model was developed from an existing deterministic growth model including the effect of five environmental factors and inter-bacterial interaction [Østergaard, N.B, Eklöw, A and Dalgaard, P. 2014. Modelling the effect of lactic acid bacteria from starter- and aroma culture on growth of *Listeria monocytogenes* in cottage cheese. International Journal of Food Microbiology. 188, 15-25]. Growth of *L. monocytogenes* single cells, using lag time distributions corresponding to three different stress levels, was simulated. The simulated growth was subsequently compared to growth of low concentrations (0.4-1.0 CFU/g) of *L. monocytogenes* in cottage cheese, exposed to similar stresses, and in general a good agreement was observed. In addition, growth simulations were performed using population relative lag time distributions for *L. monocytogenes* as reported in literature. Comparably good predictions were obtained as for the simulations performed using lag time data for individual cells of *L. monocytogenes*. Therefore, when lag time data for individual cells are not available, it was suggested that relative lag time distributions for *L. monocytogenes* can be used as a qualified default assumption when simulating growth of low concentrations of *L. monocytogenes*.

1. Introduction

Listeria monocytogenes is a well-known food borne pathogenic bacterium. The organism has received extensive attention in relation to (i) growth response in different food products (Beumer et al., 1996; Bolton and Frank, 1999; Jørgensen and Huss, 1998), (ii) development of deterministic- and stochastic predictive growth- and growth boundary models (Augustin et al., 2005; Mejlholm et al., 2015), and (iii) in studies dealing with risk assessment and risk management (McLauchlin et al., 2004; Tenenhaus-Aziza et al., 2014). A range of deterministic- and stochastic growth models are available and their use, as a supplement to traditional testing in risk assessment and product evaluation, has been recognised by the European Union (EC, 2005). These well founded predictive models are based on population data which represent an average growth response of a bacterial population (Pin and Baranyi, 2006). However, contamination of food products with *L. monocytogenes* often occurs with low cell numbers (EFSA, 2013; Kozak et al., 1996) and individual cell behaviour seems relevant when predicting growth of *L. monocytogenes* in naturally contaminated food products. The time until growth initiated by each individual cell is determined by the physiological state of the cell in combination with the environment it is transferred to (Standaert et al., 2007). This lag time has been shown to vary considerably between cells (Francois et al., 2006a) and with increasing variability observed for injured cells (Guillier et al., 2005). Naturally contaminated batches of cottage cheese are very rarely available and this makes model validation more complicated. As an alternative to naturally contaminated products, samples inoculated with low cell numbers have been used in previous studies to evaluate growth of individual cells in food. For these data sets the inoculum has been prepared under controlled laboratory conditions and variability in the physiological state is likely to be limited (Augustin et al., 2015; Ferrier et al., 2013; Francois et al., 2006b; Manios et al., 2013). A stochastic modelling approach facilitates inclusion of e.g. variable product characteristics, lag

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time variability, and variability between strains and enables simulation of all possible outcomes related to the observed or estimated variability (Couvert et al., 2010; Koutsoumanis et al., 2010). As previously demonstrated, accurate predictions of *L. monocytogenes* growth in fermented dairy products should also include the inter-bacterial interaction and associated inhibition caused by growth of lactic acid bacteria (LAB) from added LAB cultures (Guillier et al., 2008; Le Marc et al., 2009; Østergaard et al., 2014). Few previous *L. monocytogenes* single cell or low inoculum stochastic models included inter-bacterial interactions (Mejlholm et al., 2015; Pouillot et al., 2007) and this type of models has not been evaluated and validated for cottage cheese.

The aim of the present study was to quantify growth of low *L. monocytogenes* cell numbers in cottage cheese with a high concentration of LAB from added cultures. The effect of the pre-history of the *L. monocytogenes* inoculum was investigated, using three different pre-cultures to reflect conditions of potential routes of contamination during cottage cheese processing. In order to include the effect of variability of (i) product characteristics, (ii) initial LAB concentration, and (iii) lag time duration in the predictions of growth, a stochastic modelling approach was applied for both LAB populations and individual cells of *L. monocytogenes*. Validated secondary growth models for LAB and *L. monocytogenes* in cottage cheese were used in combination with input data represented by theoretical- or empirical distributions. The variability in product characteristics (pH, and lactic acid) and initial- and final concentrations of LAB was modelled by using observed data (100 datasets) and bootstrap sampling. Variability in lag time of LAB was modelled using distributions fitted to observed relative lag times (*RLT*) data. For *L. monocytogenes* lag time and *RLT* distributions extracted from literature were used. The observed growth of low initial *L. monocytogenes* concentrations as determined in the present study was then compared with simulated growth. Finally, the developed stochastic model was applied for three different scenarios to evaluate *L. monocytogenes* growth and safety of cottage cheese.

2. Materials and methods

2.1. Collection of variable input data

During a period of more than two years, 23 growth kinetics of LAB populations were determined in different experiments between 5 °C and 15 °C and for four independent batches of cottage cheese with added aroma culture. 19 kinetics originated from Østergaard et al. (2014) and 4 kinetics were from Østergaard (2014). These data were used to determine the lag time duration for the LAB aroma culture in cottage cheese. Microbiological analyses were performed as described by Østergaard et al. (2014). In brief, the logistic growth model with delay (Eq. (1); see e.g. Dalgaard, 2009) was fitted to the 23 LAB growth curves to obtain estimates of initial concentrations (log CFU/g), lag time duration, and maximum population densities (log CFU/g) at different storage temperatures using GraphPad Prism ver. 4.03 for Windows (GraphPad Software, San Diego California, USA).

$$\begin{aligned} \log(N_t) &= \log(N_0) && \text{if } t < t_{lag} \\ \log(N_t) &= \log\left(\frac{N_{max}}{\left(1+\left(\left(\frac{N_{max}}{N_0}\right)-1\right)\cdot\exp\left(-\mu_{max}\cdot\left(t-t_{lag}\right)\right)\right)}\right) && \text{if } t \geq t_{lag} \end{aligned} \quad (1)$$

where t is the time of storage and t_{lag} the lag time, N_t, N_0 and N_{max} are the cell concentrations (CFU/g) at time t and zero and the maximum asymptotic cell concentration, respectively. μ_{max} is the maximum specific growth rate (h^{-1}). The lag time estimates (t_{lag}) were used to calculate relative lag time (*RLT*) values using Eq. (2) (Ross and Dalgaard, 2004).

$$RLT = \frac{t_{lag}\cdot\mu_{max}}{\ln(2)} \quad (2)$$

2.1.1. Variability of lag time for lactic acid bacteria

Determination of the most appropriate distribution (normal, exponential, logistic, gamma, or weibull) to describe variability of the collected *RLT*-values was performed using the *fitdistrplus* package in R (R Core Team, 2014) with the Bayesian Information Criterion (BIC) as a measure for goodness-of-fit (Guillier and Augustin, 2006; Vose, 2010).

2.1.2. Variability in product characteristics, initial and final population densities of lactic acid bacteria

During previous studies (Østergaard et al., 2014; Østergaard, 2014) and in the present study, initial product pH and lactic acid concentration (ppm in the water phase) of six individual batches of cottage cheese were determined (includes the four batches referred to in Section 2.1). Corresponding initial- and final concentrations of LAB were also recorded and a total of 100 coupled observations of pH, lactic acid, LAB N_0 and LAB N_{max} were compiled. This dataset was used for bootstrap sampling during simulation in order to maintain a relevant relationship between product characteristics (pH and lactic acid) and initial and final LAB concentrations (Appendix A).

2.1.3. Lag time data for individual L. monocytogenes cells

Data from four studies (Francois et al., 2005, 2006a; Guillier and Augustin, 2006; Guillier et al., 2005) were assessed to represent potential responses of *L. monocytogenes* in cottage cheese inoculated with the pre-cultures used in the present study (see Section 2.2.1), based on the different pre-treatments cells were exposed to. In the available literature variability in *L. monocytogenes* lag times has been represented by Weibull, Extreme Value type I and II, and shifted Gamma distributions (Table 1). The distributions represented either lag times (Francois et al., 2005; Guillier and Augustin, 2006; Standaert et al., 2007) or standardised detection times (Guillier et al., 2005) which were related to lag times by Eq. (3) (Baranyi and Pin, 1999).

$$lag = T_d - \frac{(\ln(N_d) - \ln(N_0))}{\mu_{max}} \quad (3)$$

where T_d is the detection time from absorbance measurements, N_d is the bacterial concentration at T_d (estimated to 1.8×10^7 *L. monocytogenes* cells/well (Guillier et al., 2005)), N_0 is the initial number of cells, assumed to be one and μ_{max} is the maximum specific growth rate at exponential growth as reported in the studies (Francois et al., 2005, 2006a; Guillier and Augustin, 2006; Guillier et al., 2005). Sampled lag times and lag times calculated from recorded detection times were transformed into *RLT*-values by applying Eq. (2). It was assumed that *RLT*-values were temperature independent within the applied temperature range although these values may increase near the lower temperature limit of growth (Hereu et al., 2014). Subsequently, the *RLT*-values were used in combination with predicted growth rates (see Section 2.3) to estimate the lag time (Eq. (2)) associated with the simulated growth conditions. In addition, growth was simulated using the 283 *RLT*-values for *L. monocytogenes* populations in food collected and reported by Ross (1999). These *RLT*-values were represented by fitting a suitable distribution using *fitdistrplus* in R (Table 1). Simulated growth was compared to pooled growth data of pre-culture 1, 2 and 3 (see Section 2.2.1), and the initial *L. monocytogenes* concentration was represented by a Poisson distribution fitted to all (n = 24) estimated initial concentrations in inoculated cottage cheese. The pooled data were intended to mimic unknown pre-history of contaminating cells.

2.2. Challenge tests with cottage cheese inoculated with low concentrations of L. monocytogenes

2.2.1. Preparation of inoculum and inoculation of products

L. monocytogenes isolate SLU-92 from a dairy production environment was provided by Arla Strategic Innovation Centre (ASIC). This