Effect of pH and Water Activity on the Growth Limits of *Listeria monocytogenes* in a Cheese Matrix at Two Contamination Levels

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ABSTRACT

*Listeria monocytogenes* can proliferate at the beginning of cheesemaking as the conditions favor growth. The objective of this study was to establish the growth limits of *L. monocytogenes* in a cheese matrix, in case of potential contamination of the milk prior to cheese manufacture. A semisoft laboratory scale model cheese system was made at different initial pH and water activity (aw) levels with a mix of two strains of *L. monocytogenes*. A factorial design of five pH values (5.6 to 6.5), four aw values (0.938 to 0.96), and two *L. monocytogenes* inoculation levels (1 to 20 CFU/ml and 500 to 1,000 CFU/ml) was carried out. Each combination was evaluated in six independent replicates. In order to determine if there was a dominant strain, isolated colonies from the cheeses were analyzed by pulsed-field gel electrophoresis. The data relating to growth initiation were fitted to a logistic regression model. The aw of milk influenced the probability of growth initiation of *L. monocytogenes* at both low and high contamination levels. The pH, at the concentrations tested, had a lower effect on the probability of growth initiation. At pH 6.5 and aw of 0.99 for low contamination levels and pH 6.5 and aw of 0.97 for high contamination levels, increases in population of up to 4 and 2 log were observed at low and high contamination levels, respectively. This shows that if conditions are favorable for growth initiation at the early stages of the cheesemaking process, contamination of milk, even with low numbers, could lead to *L. monocytogenes* populations that exceed the European Union’s microbiological limit of 100 CFU/g of cheese.

*Listeria monocytogenes* is a well-recognized foodborne pathogen widely distributed in nature. Infection by this organism causes listeriosis, affecting mainly pregnant, neonatal, and immunocompromised people. It was responsible for several foodborne outbreaks of listeriosis reported in Europe, the United States, and Japan (6, 7, 31, 45) with an average case-fatality rate of 20 to 30%, despite correct antimicrobial treatment (45). *L. monocytogenes* has been isolated from foods such as milk (13), milk derivatives (17), chicken (43), meat (41), meat products (46), seafood (9), fish (34, 35), ready-to-eat foods (16, 26), and vegetables (28).

Among these foods, milk and milk-based products have been confirmed as responsible for bacterial outbreaks in industrialized countries (8). The published literature reflects that *L. monocytogenes* has been isolated from cheeses made either from raw milk (2, 8, 27, 29) or from pasteurized milk (40). In this regard, lack of attention to detail in hygiene at high-risk production areas in the cheese factories (which may allow transfer of microorganisms to the milk or cheese) and physical adaptations of the strains such as improved attachment to surfaces and subsequent biofilm formation (18) or resistance to sanitizers that may lead to increased survival of strains play an important role and may therefore lead to postpasteurization contamination scenarios.

The European Union regulation 2073/2005 (11) as amended by regulation 1441/2007 (12) on the microbiological criteria for foodstuffs has placed stringent criteria on ready-to-eat foods that are able to support the growth of *Listeria*. This includes many farm-produced speciality foods such as cheese and dairy products. The legislation requires absence of *L. monocytogenes* in 5 samples of 25 g (with none positive) before the food has left the immediate control of the producer, and <100 CFU/g on products placed on the market during their shelf life. The identification and quantification of factors that limit the growth of *L. monocytogenes* during cheese manufacture are therefore important for cheese safety.

Probability models in predictive microbiology take into account whether the expected response (initiation of growth in this study) is observed under the experimental conditions or not at a specific period of time, from a few hours to several months, depending on the product-process conditions that the model refers to (39). Even though probability models have been developed for *L. monocytogenes* in laboratory media (24, 47, 49), there are relatively few studies that have been carried out directly in food products and systems (3, 19, 50). Predictions from models based on laboratory media may deviate markedly from those developed with food systems. Recently, Hwang (19) studied...
the probability of growth of *L. monocytogenes* in cooked salmon and laboratory media by using a logistic regression model and found that there were significant differences in the output models depending on the medium used.

The technology used for cheese production presents different environmental scenarios for bacteria (pH and water activity \( a_w \) variations); how *L. monocytogenes* behaves during the cheesemaking process has not been studied before, and the actual limiting conditions that would allow control of *Listeria* populations in a cheesemaking scenario are unknown to date.

The objective of this study was to identify the combinations of pH and \( a_w \) that prevent *L. monocytogenes* from initiating growth during the early stages of simulated cheesemaking (since this could establish safety limits for the manufacturing of cheese). The data generated were used to model the probability of growth initiation of *L. monocytogenes* under simulated cheesemaking conditions and therefore to identify growth limits by determining which conditions lead to no growth initiation during simulated cheesemaking.

### MATERIALS AND METHODS

**Culture preparation.** The strains used (strain 6179 serotype 1/2a and strain C5 serotype 4b) are natural isolates from cheese and the environment, respectively. For each experiment, cultures were prepared by overnight growth in tryptic soy broth (TSB; BD, Franklin Lakes, NJ) at 37 °C, from a stock culture kept on a slope at 4 °C. The cultures were mixed equally and diluted for each experiment to achieve the desired inoculum level of 1 to 20 CFU/ml (low population) or 500 to 1,000 CFU/ml (high population).

**Manufacture of the cheese matrix.** A laboratory cheese model was made from pasteurized milk (pH 6.8; protein, 3.4%; fat, 3.7%; and lactose, 4.68%) in 800-ml volumes with the addition of lactic acid to control the pH, rather than a starter culture. In this way, inconsistencies within replicates associated to dynamic conditions (from starter culture), competing microflora (that may be present in raw milk), and different changing rates in pH could be reduced to minimum values. This was necessary for analytical purposes, since dynamic pH through cheesemaking would have been detrimental in the determination of growth initiation limits. Pasteurized milk, with 8% (wt/vol) low-heat skim milk powder (Tipperary Co-Operative, Tipperary, Ireland) added, was adjusted to the target pHs of 6.5, 6.1, 5.9, and 5.6 by adding sterile 10% (wt/vol) lactic acid (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) or 10% (wt/vol) sodium hydroxide (BDH Chemicals, Poole, UK); sodium hydroxide was necessary when the addition of sodium chloride to the milk decreased the pH to below 6.5. The addition of sodium chloride to the milk was used to achieve different \( a_w \) levels (all wt/vol): 0% NaCl (\( a_w \), 0.990 to 0.996); 3% NaCl (\( a_w \), 0.972 to 0.979); 4.5% NaCl (\( a_w \), 0.962 to 0.972); 6% NaCl (\( a_w \), 0.952 to 0.964); and 8% NaCl (\( a_w \), 0.938 to 0.955). Addition of low-heat skim milk powder was essential to increase the protein content and aid coagulation at low \( a_w \) and pH values. Therefore, for consistency, the powder was added to all cheesemaking experiments. In preliminary experiments, it was shown that there was no significant difference \( P > 0.05 \) between growth of *L. monocytogenes* with and without powder added. The milk was then heated to 30 °C. Following inoculation with *L. monocytogenes* at target levels of 1 to 30 CFU/ml (low contamination level, 0.62 to 2.64 log CFU/g dry weight [gdw]) or 400 to 1,000 CFU/ml (high contamination level, 3.25 to 3.81 log CFU/gdw) and addition of rennet (~0.18 ml/liter diluted 10-fold in sterile distilled water, depending on protein concentration; CHY-MAX PLUS Fermentation Produced Chymosin, CHR HANSEN, Horsholm, Denmark), the curd was cut in cubes after being tested for firmness (visually). The curd was left to settle for 30 min and then cooked to 36 °C at a temperature increase rate of 1 °C every 10 min, stirring continuously. The curd was then molded in plastic molds (top diameter, 89 mm; bottom diameter, 82 mm; height, 83 mm; Moorlands Cheesemakers Limited, Castle Cary, UK) and turned every 30 min until sampling. Molding was undertaken at room temperature (20 °C) for approximately 3 h. The time from inoculation to final sampling was 8 h, as this is the typical time taken to complete the initial cheesemaking process. The pH was monitored with a probe (Orion 420A pH meter).

A partial factorial design of the four pH values, five \( a_w \) values, and two contamination levels was undertaken (Table 1), and six independent replicate cheeses were manufactured under each condition tested.

**Microbiological and chemical analyses.** Analyses for *L. monocytogenes*, \( a_w \), pH, and moisture were carried out at the time of inoculation (0 h) and from the fresh cheeses (8 h). *L. monocytogenes* organisms were enumerated in milk by spreading 1 ml onto 20-cm-diameter petri dishes prepared with Agosti and Ottaviani Listeria Agar (LAB M, Lancashire, UK) and incubated at 37 °C for 48 h. Cheeses were sampled according to the International Dairy Federation (IDF) Standards 50B (21) and 122B (23) for sampling and microbiological analysis. In order to compare the populations of *L. monocytogenes* in milk (in milliliters) and in cheese (in grams), the counts were expressed per gram dry weight. The counts per gram dry weight were calculated by determining first the total solid content of each sample. The total solids content in cheese was determined according to IDF Standard 4A (20) and IDF Standard 21B for milk (22). The number of cells counted in x grams of dry sample (milk or cheese) was then transformed to counts per gram of dry sample with a “rule of three” calculation. For \( a_w \), samples of milk (5 ml) and cheese (as directed by the manufacturer’s instructions) were placed in a sampling cup, and the \( a_w \) was determined with an AquaLab Monitor Series 3T equipment (Labcell, Hampshire, UK). The pH of milk was adjusted as described above and measured by an Orion pH meter model 420A. Standard BS 770:5:1976 (44) was followed for the measurement of pH in cheese. The amount of \( l \)-lactic acid was measured with an \( l \)-lactic acid kit (Boehringer Mannheim, Darmstadt, Germany).

**Determination of a dominant strain.** For the contamination of the milk with *L. monocytogenes*, an equal mix of two strains was used. In order to determine if one of the strains dominated during the cheesemaking process, one or two colonies per replicate from each experimental case where growth or survival was observed were picked randomly and stored in TSB plus glycerol at −20 °C. A final set of 191 isolates were analyzed by pulsed-field gel electrophoresis (PFGE) (15).

**Evaluation of data on probability of growth initiation during cheesemaking.** The cheesemaking experiments were carried out in a partial factorial design (Table 1) based on the following assumptions: if 100% of the cases showed growth initiation at any tested combination with a low contamination level, growth would be initiated in 100% of the cases with a higher contamination level; if growth was not initiated in 100% of the cases at any tested combination with a high contamination level,
growth would not be initiated in 100% of the cases with a lower contamination level.

Since the milk (time 0) and cheeses (8 h after inoculation) were analyzed by the plate count enumeration method, the contamination level of the milk and the log increase observed in growth were determined for each replicate. More specifically, the difference between the population of *L. monocytogenes* in cheese and in milk for each replicate, expressed in log CFU per gram dry weight, was known. In order to transform the amount of growth observed into dichotomous data, i.e., growth was initiated or not, the data were classified as “growth” when a statistically significant difference (determined by the *t* test, *P* < 0.05) of at least 0.5 log CFU/gdw was observed between the two samplings. The remaining cases were considered “no growth.” The 0.5-log-increase criterion was taken from Bolton and Frank (3), Koutsoumanis and Sofos (25), and Skandamis et al. (42).

**Modeling the probability of growth initiation during cheesemaking with the ordinary logistic regression model.**

Logistic regression is a mathematical modeling procedure used in the analysis of dichotomous data (for example, growth initiated during the cheesemaking or not). The procedure used by the SAS software to estimate the unknown parameters is the maximum likelihood method, which yields estimators that maximize the probability of obtaining the observed set of data. Data for growth initiated or growth not initiated for each experimental case replicate were classified as 1 or 0, respectively, based on the aforementioned criteria for growth of *L. monocytogenes*, and fitted to a logistic model by means of logistic regression using the PROC LOGISTIC procedure in SAS 9.1 software (*P* < 0.05; SAS Institute Inc., Cary, NC). In this way, for example, given the experimental case at high contamination levels, pH 6.1, and aw of 0.96, where three of six replicates presented growth initiation, three replicates were classified as 1 and the other three as 0 for those conditions. As the population of *L. monocytogenes* was enumerated in each replicate, all the data for low and high milk contamination levels were analyzed collectively, including the contamination level as a variable in the model. Therefore, the probability of growth of each replicate was modeled as a function of aw, pH, and contamination level, according to the following equation:

\[
\text{Logit}(P) = \ln \left( \frac{P}{1-P} \right) = a_0 + a_2 \text{aw} + a_2 \text{pH} + a_2 \text{cont} + a_1 \text{aw} \text{pH} + a_1 \text{aw} \text{cont} + a_1 \text{pH} \text{cont} + a_2 \text{aw}^2 + a_2 \text{pH}^2 + a_2 \text{cont}^2
\]

where *P* is the probability of growth of each replicate, receiving a value of 1 or 0 for growth initiation and no growth initiation, respectively; *b*aw is a transformation of aw, proposed by Gibson et al. (14), in order to homogenize the variance of aw and enhance the fitting procedure [baw = √(1 − aw)]; cont is the initial population level of *L. monocytogenes* in milk (log CFU per gram dry weight), and a1 through a0 are coefficients to be estimated by the ordinary logistic regression procedure. Significant parameters were selected by means of a backward elimination method in SAS, using the option “selection = backward.” The statistical index used to measure the goodness of fit of the model was the percent concordance (3, 19, 47).

**Validation of the ComBase database.** The ComBase Initiative is a collaboration between the Food Standards Agency and the Institute of Food Research from the United Kingdom, the U.S. Department of Agriculture Agricultural Research Service and its Eastern Regional Research Center from the United States, and the University of Tasmania Food Safety Centre in Australia. The information within the ComBase predictor is “quantitative microbiological” data since it describes how the concentration of microorganisms (both spoilage organisms and pathogens) changes over the course of time. The ComBase database comprises thousands of microbial growth and survival curves that have been collated in research establishments and from publications (5).

The sampling method used in this study resulted in data on counts at the inoculation level (in milk) and from the cheese samples; information on the actual log increase or amount of growth for each experimental case was extracted from the data. Given that for each experimental case the temperature, pH,
inoculation level, $a_w$ (or NaCl added), and lactic acid amount were known, predictions of the amount of growth for 8 h were possible with the ComBase Modelling Toolbox (5). The growth predicted by the ComBase Modelling Toolbox was compared with the observed data and to the predictions of the ordinary logistic regression model used. The goodness of fit of the ComBase Modelling Toolbox was measured by using the predicted and the observed outcomes with the coefficient of determination, $R^2$.

**Model validation.** An independent set of data was obtained where cheeses were made with pasteurized milk at $a_w$-pH combinations of 0.939-6.38, 0.963-6.43, 0.967-5.84, 0.969-6.44, and 0.982-6.34 at high or low contamination levels. Pasteurized milk was prepared and inoculated with L. monocytogenes for each $a_w$-pH combination and sampled for initial counts: the milk was then distributed into 10 aliquots of 200 ml each, and cheeses were made following the same procedure detailed previously. After 8 h of cheesemaking, L. monocytogenes organisms were counted from the cheeses. An increase of $\geq 0.5$ log CFU/gdw was considered to indicate growth being initiated; below 0.5 log CFU/gdw, L. monocytogenes was considered not to have initiated growth. By following these criteria, percentages of growth were obtained for each case, and the data were used to validate the logistic regression model predictions. The capability of the model to predict well future outcomes was measured with the coefficient of determination, $R^2$.

**RESULTS**

**Factors affecting growth.** At low contamination levels, an $a_w$ value above 0.975 always resulted in growth initiation. Below 0.975, the behavior of L. monocytogenes was variable (Fig. 1A). At high contamination levels, the critical $a_w$ was $\approx 0.965$ (Fig. 1B). Complete inactivation (no colonies recovered) of L. monocytogenes was only observed at low contamination levels, and at an $a_w$ level where growth could be observed with higher contamination (Table 1). When growth occurred, the log increase observed was $a_w$ and contamination level dependent. The correlation between $a_w$ values and log increase in numbers of bacteria was 0.74 and 0.63 for low and high contamination levels, respectively. No positive correlation was found between pH and log increase ($-0.03$ and $-0.08$ for low and high contamination levels, respectively).

The assumptions (that if 100% growth was observed at low inoculums, there would be 100% growth at high inoculums and vice versa) were justified by the following results. (i) At low contamination levels, growth was always observed with a 1.57- to 3.96-log CFU/gdw increase, at $a_w$ 0.972 to 0.996 (0 to 3% NaCl). For these cases, growth was assumed to occur at higher contamination levels. (ii) In experiments undertaken with high contamination levels, growth was not initiated (<0.5-log increase) at $a_w$ values between 0.938 and 0.957 in 93% of the replicates; it was assumed that growth would not be initiated at the same $a_w$ values with lower contamination levels.

At combinations of pH 5.6 and $a_w$ of 0.97 or 0.95, milk did not coagulate in any of the six replicates, and it was therefore not possible to obtain a cheese matrix. The experiments at those critical conditions of pH and $a_w$ were repeated three times in order to reject a possible milk composition effect, but coagulation was not achieved in any repetition. This (unavoidable) gap in the data set at pH 5.6 and $a_w$ of 0.97 or 0.95 has a possible effect on the results of the logistic regression.

The PFGE patterns of the two strains used (6179 and C5) were determined. Of the 191 colonies isolated from the cheeses, 157 (82%) had the same PFGE pattern as strain C5 (data not shown).

**Limits and probability of growth.** The three variables tested, i.e., pH, $a_w$, and contamination level, affected the probabilities of growth initiation during the cheesemaking simulation. There were increased probabilities of growth initiation by L. monocytogenes at high $a_w$ and high contamination levels. For instance, for an $a_w$ value of <0.97 and pH 5.6, growth is expected to initiate with a 0.1 probability for a contamination level of 0.6 log CFU/gdw. At the same pH and $a_w$ combination, the probabilities that populations of 2 and 3 log CFU/gdw will initiate growth are 0.3 and 0.8, respectively. The probability of getting growth initiation given different $a_w$ values, at pH 6.5 for instance, varies depending on the contamination level (Fig. 2), and in the same way, probabilities of growth initiation at different $a_w$ values, given a contamination level, vary depending on pH (Fig. 3).

**Model performance.** Of the 224 observations obtained (including 66 assumed values), 138 corresponded to growth initiation and 86 to no growth initiation. Parameters for pH ($a_2$), contamination level ($a_3$), $b_w \times$ pH ($a_4$), and $b_w^2$ ($a_7$)
we were significant. The estimates of the ordinary logistic regression model, standard errors, and $P$ values are shown in Table 2. The association between predicted probabilities and observed responses showed a percent concordance of 94.3%.

An independent set of data was obtained in order to validate the predictions of the model. Table 3 shows the predicted values from the ordinary logistic regression model and the observed data. The approximation of the predicted data by the ordinary logistic regression model to the observed data had a coefficient of determination, $R^2$, equal to 0.94. Overall, the model had a good fit.

**ComBase predictions.** The ComBase Modelling Toolbox predictions on the log increase had a poor fit ($R^2 = 0.37$). Examples of the deviation encountered between ComBase predictions and observations on the amount of growth are shown in Figure 4A and 4B. ComBase underestimated the observed amount of growth in 70% of the cases at $a_w$ values of 0.99 and 0.98 (data not shown). In contrast, overestimation occurred in cases where the $a_w$ values were $\sim 0.96$ to 0.97 (data not shown). When the predictions obtained from ComBase were classified as growth or no growth (based on a statistically significant difference of 0.5 log CFU/gdw), ComBase correctly predicted growth initiation in 41% of the cases in which growth was actually observed to be initiated. Some examples of the ComBase outcome and the ordinary logistic regression model outcome for given values are shown in Table 4.

**DISCUSSION**

There are multitudes of different cheesemaking protocols that result in the many different cheese varieties that are produced, many having pH values within the range tested, particularly soft cheeses (for example, queso fresco). The type of cultures and acidification (10), the salting method, the time and temperature of the make procedure, and the ripening conditions influence the characteristics of the cheese (1). However, there are certain common steps followed by all manufacturers in order to produce cheese, namely, acidification of the milk, coagulum formation, cutting or breaking of the gel, cooking, molding or pressing of the curd, and ripening. A generic simulated cheesemaking...

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**TABLE 2. Estimated coefficients obtained from the logistic regression analysis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>Wald chi-square</th>
<th>Pr $&gt;\text{chi-square}^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_0$</td>
<td>$-53.2942$</td>
<td>$\pm 17.7002$</td>
<td>$9.0658$</td>
<td>$0.0026$</td>
</tr>
<tr>
<td>$a_1$</td>
<td>NS $^b$</td>
<td>NS</td>
<td>$0.0092$</td>
<td>$0.9235$</td>
</tr>
<tr>
<td>$a_2$</td>
<td>$17.4885$</td>
<td>$\pm 5.6939$</td>
<td>$9.4339$</td>
<td>$0.0021$</td>
</tr>
<tr>
<td>$a_3$</td>
<td>$1.2425$</td>
<td>$\pm 0.2736$</td>
<td>$20.6275$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>$a_4$</td>
<td>$-81.7109$</td>
<td>$\pm 28.4933$</td>
<td>$8.2238$</td>
<td>$0.0041$</td>
</tr>
<tr>
<td>$a_5$</td>
<td>NS</td>
<td>NS</td>
<td>$2.6346$</td>
<td>$0.1046$</td>
</tr>
<tr>
<td>$a_6$</td>
<td>NS</td>
<td>NS</td>
<td>$0.4261$</td>
<td>$0.5139$</td>
</tr>
<tr>
<td>$a_7$</td>
<td>$1.0613$</td>
<td>$\pm 431$</td>
<td>$6.0643$</td>
<td>$0.0138$</td>
</tr>
<tr>
<td>$a_8$</td>
<td>NS</td>
<td>NS</td>
<td>$0.0083$</td>
<td>$0.9722$</td>
</tr>
<tr>
<td>$a_9$</td>
<td>NS</td>
<td>NS</td>
<td>$2.2209$</td>
<td>$0.1362$</td>
</tr>
</tbody>
</table>

$^a$ Pr, probability.

$^b$ NS, not significant.

**TABLE 3. Validation of the ordinary logistic regression model using an observed data set obtained independently from the data used to generate the model**

<table>
<thead>
<tr>
<th>$a_w$</th>
<th>pH</th>
<th>CL$^a$</th>
<th>OGI (%)$^b$</th>
<th>OLRM (%)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.982</td>
<td>6.43</td>
<td>3.41 (430)</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>0.969</td>
<td>6.44</td>
<td>3.71 (850)</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>0.959</td>
<td>6.38</td>
<td>3.38 (400)</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td>0.963</td>
<td>6.34</td>
<td>2.29 (31)</td>
<td>40</td>
<td>51</td>
</tr>
<tr>
<td>0.967</td>
<td>5.84</td>
<td>2.29 (31)</td>
<td>44</td>
<td>50</td>
</tr>
</tbody>
</table>

Overall adjusted $R^2$ of the model = 0.94

$^a$ CL, contamination level, expressed in log CFU per gram dry weight. Values in parentheses are the equivalents in CFU per milliliter.

$^b$ OGI, observed growth initiation (percentage of cases where growth initiation was observed).

$^c$ OLRM, ordinary logistic regression model predicted probability of growth initiation (expressed as a percentage).
ing procedure was adopted in the present study, including
the first five steps. The purpose was to model the probability
of growth initiation of L. monocytogenes during the early
stages of cheesemaking. From the point of view of growth,
the first few hours of cheesemaking are important because
the growth-inhibitory effects of cheese (such as pH, lactic
acid production, and decreased \( a_w \)) are not fully developed
and temperature is more optimal than during ripening.

Kinetic models are adequate when dealing with
spoilage microorganisms or certain pathogens for which
some growth may be expected. However, generally, when
foodborne pathogens are concerned, quantitative information
on growth limits may be more valuable than describing
the rates of growth. Logistic regression is a useful model
aimed at facilitating prediction of the probability of growth
under specific conditions within a given time. The use of
logistic regression as a tool for modeling the boundaries
between growth and no growth for bacterial strains was
proposed by Ratkowsky and Ross (38) by transforming a
kinetic model into a probabilistic model. McMeekin et al.
(33) used this approach to model the effects (additive or
synergistic) of various hurdles on the probability of growth.
Logistic models have been used to predict growth limits of,
for example, Escherichia coli (37, 42) or Shigella flexneri
(38). However, most studies modeling the behavior of L.
monocytogenes have been undertaken in laboratory media
because their use has many advantages, including the
homogeneity of substrate, the constant intrinsic factors, the
use of optical density for analysis, and the limited labor
required compared to experiments with foods. All these
have contributed greatly to the widespread use of laboratory
media in the development of predictive models.

The ordinary logistic regression model has been used to
model the probability of growth of L. monocytogenes in
foods such as cooked salmon (19) and Mexican-style cheese
during ripening (3). When used in food systems, this model
has a lower concordance rate than when it is applied to
laboratory media experiments. Most of the growth boundary
studies carried out with broth reported percent concordance
rates higher than 0.97 (4, 24, 42, 48) in contrast to the 0.7
and 0.91 values previously reported in food experiments
(3, 19). In both these cases, the use of the ordinary logistic
regression model for food systems was considered adequate,
despite the higher variability associated with dynamic food
systems, as reflected in the poorer fit of the model.

Some of the combinations tested in this work for low or
high contamination levels induced what may be considered
as an extended lag time, which under cheesemaking
conditions is considered a no-growth scenario; at low
contamination levels, populations of Listeria at \( a_w \) values
below 0.975 were unable to survive, and inactivation was
observed. In these cases, the term “extended lag phase”
would be incorrect. The term “growth initiation” is
adequate even when growth is detected in very small
amounts; but when cells could not be recovered after 8 h of
cheesemaking, no growth is observed in absolute terms.
Thus, models constructed from broth conditions and relating
to cheese would be inaccurate (they would predict growth in
cases where growth was extremely unlikely in practice) and

![FIGURE 4. Comparison of the ComBase Modelling Toolbox
predictions (○) and observed data on amount of growth (●)
against \( a_w \) (A) and pH (B).](http://meridian.allenpress.com/jfp/article-pdf/74/11/1805/1682856/0362-028x_jfp-11-102.pdf)

### TABLE 4. Comparison of the ComBase Modelling Toolbox predictions against the observed data on the amount of growth/no growth

<table>
<thead>
<tr>
<th>pH</th>
<th>NaCl</th>
<th>Lact</th>
<th>Inoc</th>
<th>Obs log ( i^1 )</th>
<th>CMT log ( i^2 )</th>
<th>Difference (Obs − CMT)</th>
<th>Obs G/NG</th>
<th>CMT G/NG</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>0</td>
<td>1.31</td>
<td>0.75</td>
<td>3.96</td>
<td>0.92</td>
<td>3.03</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5.6</td>
<td>0</td>
<td>13</td>
<td>0.72</td>
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\( ^a \) Amount of l-lactic acid expressed (parts per million).

\( ^b \) Contamination level (log CFU per gram dry weight).

\( ^c \) Observed log increase (log CFU per gram dry weight).

\( ^d \) ComBase Modelling Toolbox predicted log increase (log CFU per gram).

\( ^e \) Classification of amount of growth as 1 (growth initiated) or 0 (growth not initiated) following the criteria described in the text according to observed data.

\( ^f \) Classification of amount of growth as 1 (growth initiated) or 0 (growth not initiated) following the criteria described in the text according to ComBase Modelling Toolbox predictions.
are too stringent for cheesemaking conditions where intrinsic inhibitory factors contribute to more no-growth scenarios. This is important in the context of food safety. Whether growth is initiated or not during cheesemaking, other factors such as temperature, $a_w$, and other environmental conditions during ripening will determine the evolution of the pathogen. Typical $a_w$ values in cheeses at the selling point are 0.98 for cottage and soft cheese, 0.95 to 0.97 for semisoft cheese, 0.917 for Parmesan cheese, and 0.86 to 0.97 for hard cheese (30); hence, soft and semisoft cheeses (mimicked in this study) not subjected to long ripening periods are very likely to support survival or growth of cells that survive the cheesemaking process.

The results show that the probability of $L$. monocytogenes growing during cheesemaking will depend on pH, $a_w$, and contamination level. The pH and $a_w$ combinations at which growth is expected to be initiated with a probability lower than 0.1 depends therefore on the contamination levels of the milk. The values that control the growth initiation of $L$. monocytogenes were different for low or high contamination levels of the milk; the higher the contamination, the greater the probability of growth initiation. Milk contaminated with at least 120 CFU/ml ($10^3$ CFU/gdw) has an increased chance of promoting bacterial proliferation as the probability of getting growth is 0.46 at very stringent conditions ($a_w$, 0.95) and 1 at optimum conditions ($a_w$, 0.99). The minimum $a_w$ values measured ($a_w$, 0.938 to 0.955) at 8% NaCl are above the minimum $a_w$ values known for growth of $L$. monocytogenes ($a_w$, 0.92) (36), but growth was not initiated at these levels of $a_w$, probably because the cells required adaptation or because they were inactivated.

The duration of the cheesemaking (8 h) probably limited the growth of $L$. monocytogenes populations that may be in suboptimal conditions at certain pH, $a_w$, and contamination level combinations. This work suggests that a reduction in the $a_w$ value of the milk to 0.97 would reduce the probability of bacterial growth initiation within the cheesemaking process to 0.1 for milk with low contamination. If milk was contaminated with higher levels, the $a_w$ value needed to achieve a 0.1 probability of growth would be 0.94. The addition of salt to cheese or milk is a common practice, and there is a wide range of cheese varieties (for example, Domiati-type cheese) on the market with salt concentrations ranging up to 10%. Salt is added in most of the cheese technologies once the curd is separated from the whey. In these cases, the $a_w$ throughout the whole cheesemaking process is high (>0.99) before the addition of salt, and the probabilities of getting initiated growth of $L$. monocytogenes during the cheesemaking are therefore very high ($P > 0.9$).

The narrow range of pH used for these experiments (5.6 to 6.5) could explain the limited effect of pH on the log increase of $L$. monocytogenes during the cheesemaking.

All pH and $a_w$ combinations used in these experiments are known to support growth of $L$. monocytogenes in laboratory media over a longer period of time (4, 24, 25, 47). However, only 63% of the cases resulted in growth initiation of $L$. monocytogenes during cheesemaking in this study; the ComBase Modelling Toolbox predicted growth initiation in 57% of the cases, but the combinations of pH, $a_w$, and contamination level leading to growth according to the ComBase tool were different from those experimentally observed. Some of the combinations tested resulted in complete inactivation of $L$. monocytogenes during the 8 h of cheesemaking; however, the ComBase Modelling Toolbox is limited to predicting strictly thermal inactivation or growth; the nonthermal inactivation cases observed in this work were therefore not predicted by ComBase.

When comparing the ComBase-predicted log increase against the observed, large deviations were encountered (adjusted $R^2 = 0.3564$). ComBase underestimated growth in 28% of the cases and overestimated the growth in 56% of the cases. The remaining 16% were more accurate ($\pm 0.5$) predictions, corresponding to cases with 8% NaCl ($a_w$, 0.938 to 0.955). This suggests that conditions encountered in foods differ considerably from those conditions present in laboratory media and that caution must be taken when applying laboratory media-developed models to simulate food systems, as they do not reflect the intrinsic conditions of the food that cause higher variability in the microbial behavior. Nonetheless, the authors of this application state on the Web page that “ComBase Predictor models are based on extensive experimental data obtained in liquid culture media under well-controlled laboratory conditions. This is why model predictions are usually ‘fail-safe’ compared with observations in food, and there can be no guarantee that predicted values will match those that would occur in any specific food system” (5). This reflects the need to generate more data on specific food systems, to feed models used in ComBase with food data and to further validate existing models with these data.

Variability can be confined not only to the type of microorganism but also to the strain type (genotype and phenotype); in order to determine if one strain would grow better than another, a two-strain mix of natural isolates of $L$. monocytogenes was used in this work. Analysis of the isolates from all the cheesemaking conditions used by PFGE indicated that strain C5 dominated over strain 6179 and therefore had a better ability to grow and survive during the cheesemaking process. This could be related to its improved ability to grow in milk or its ability to withstand the stressful conditions of cheesemaking. Further experiments are required to clarify the strain difference observed. Using a six-strain mix of $L$. monocytogenes, Hwang (19) studied the probability of growth in cooked salmon. Similar to the results presented here, salt was a factor that influenced the growth probability, but no effort was made to determine strain dominance in the six-strain mixture.

In this study, the level of contamination used was found to affect the growth limits of $L$. monocytogenes. This effect of inoculum size affecting growth boundaries has been previously reported (32). Koutsoumanis and Sofos (25) studied the growth/no growth responses of $L$. monocytogenes at four contamination levels and different temperature, pH, and $a_w$ values. They found that the growth limits of the pathogen were affected by the size of the inoculum, and they stressed the importance of the effect of the inoculum size on microbial growth initiation.
In the present study, pasteurized milk at constant pH and $a_w$ values were used, as microbial interactions (as with raw milk) with dynamic conditions (as with starter culture) would have been difficult to interpret. Therefore, the predictions of our model refer to initial pH and $a_w$ conditions. Due to the limitations of the rennet action, it was not possible to perform the present experiments at a wider range of pH values. In preliminary experiments, it was observed that milk containing 8% sodium chloride did not coagulate at or below pH 5.5, so a lower limit of pH 5.6 was predetermined for the experiments. The milk coagulation is carried out with the addition of an enzyme called chymosin (rennet), which is a protease. The chymosin acts on the caseins (insoluble milk proteins) in a two-step reaction consisting in a destabilization of the casein micelles followed by an aggregation step. The activity of chymosin is pH-, temperature-, and photodependent. Under acidic conditions (pH 3 to 4), it loses its activity, probably by autodegradation. Chymosin is also more stable at 2°C than at room temperature, and its solubility depends on pH, temperature, and ionic strength; thus, in solutions with $\geq 137$ g of NaCl per liter at pH 5.5, chymosin appears to be insoluble (19, 45). The unexpected lack of coagulation of the milk at two different pH and NaCl combinations (pH 5.6 and 8 or 4.5% NaCl) cannot be explained by the chymosin solubility, as the rennet was diluted prior to its addition in all of the cases. This phenomenon could therefore be explained by a loss of chymosin activity, probably due to the pH and NaCl combination. It is not well understood why the rennet could act at an NaCl concentration of 6% but not at 4.5 and 8%.

The model was finally validated with an independent set of data, and cases leading to growth initiation were well predicted by the model, although further validation at pH values around 6.0 is required.

Although the numbers of reported cases of listeriosis are relatively low, the severity of the disease and the high mortality rate have resulted in stringent criteria on the presence of $L. monocytogenes$ in foods, particularly those that are ready-to-eat products (11). Growth boundaries in food systems are industrially important, as current European Union regulations (12) refer to the ability of products to support the growth of $L. monocytogenes$. This study shows that in order to meet the microbiological criteria, the conditions of cheesemaking that would ensure no growth initiation include the addition of high concentrations of salt, which is contrary to the current general trend of low-salt food products.

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REFERENCES