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Published in:
Food Microbiology

Link to article, DOI:
[10.1016/j.fm.2020.103578](https://doi.org/10.1016/j.fm.2020.103578)

Publication date:
2020

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Martinez Rios, V., Gkogka, E., & Dalgaard, P. (2020). Predicting growth of *Listeria monocytogenes* at dynamic conditions during manufacturing, ripening and storage of cheeses – Evaluation and application of models. *Food Microbiology*, 92, [103578]. <https://doi.org/10.1016/j.fm.2020.103578>

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PII: S0740-0020(20)30167-2

DOI: <https://doi.org/10.1016/j.fm.2020.103578>

Reference: YFMIC 103578

To appear in: *Food Microbiology*

Received Date: 24 February 2020

Revised Date: 19 May 2020

Accepted Date: 20 June 2020

Please cite this article as: Martinez-Rios, V., Gkogka, E., Dalgaard, P., Predicting growth of *Listeria monocytogenes* at dynamic conditions during manufacturing, ripening and storage of cheeses – Evaluation and application of models, *Food Microbiology* (2020), doi: <https://doi.org/10.1016/j.fm.2020.103578>.

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Predicting growth of *Listeria monocytogenes* at dynamic conditions during manufacturing, ripening and storage of cheeses – evaluation and application of models

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Abstract

Mathematical models were evaluated to predict growth of *L. monocytogenes* in mould/smear-ripened cheeses with measured dynamic changes in product characteristics and storage conditions. To generate data for model evaluation three challenge tests were performed with mould-ripened cheeses produced by using milk inoculated with *L. monocytogenes*. Growth of *L. monocytogenes* and lactic acid bacteria (LAB) in the rind and in the core of cheeses were quantified together with changes in product characteristics over time (temperature, pH, NaCl/a_w, lactic- and acetic acid concentrations). The performance of nine available *L. monocytogenes* growth models was evaluated using growth responses from the present study and from literature together with the determined or reported dynamic product characteristics and storage conditions (46 kinetics). The acceptable simulation zone (ASZ) method was used to assess model performance. A reduced version of the Martinez-Rios et al. (2019) model (<https://doi.org/10.3389/fmicb.2019.01510>) and the model of Østergaard et al. (2014) (<https://doi.org/10.1016/j.ijfoodmicro.2014.07.012>) had acceptable performance with a ASZ-score of 71-70% for *L. monocytogenes* growth in mould/smear-ripened cheeses. Models from Coroller et al. (2012) (<https://doi.org/10.1016/j.ijfoodmicro.2011.09.023>) had close to acceptable performance with ASZ-scores of 67-69%. The validated models (Martinez-Rios et al., 2019; Østergaard et al., 2014) can be used to facilitate the evaluation of time to critical *L. monocytogenes* growth for mould/smear-ripened cheeses including modification of recipes with for example reduced salt/sodium or to support exposure assessment studies for these cheeses.

Keywords: Predictive microbiology, model validation, exposure assessment, food safety, smear cheese, white-mould cheese, blue/white cheese.

1. Introduction

Mould/smear-ripened cheeses have been involved in many listeriosis outbreaks worldwide (Amato et al., 2017; Martinez-Rios and Dalgaard, 2018; McIntyre et al., 2015) and in numerous recalls (FDA, 2018; RASFF, 2019) due to prevalence and high concentrations of *L. monocytogenes* in the products. Mould/smear-ripened cheeses have a complex microbiota that both evolves throughout production/maturation and affects product characteristics. During fermentation, lactic acid bacteria (LAB e.g. *Lactococcus lactis* and *Leuconostoc* species) transforms lactose into lactic acid (ca. 2%) resulting in lowering of the product pH to about 5 or below. Thereafter, moulds, yeast or bacteria (e.g. *Penicillium camemberti*, *Penicillium roqueforti*, *Geotrichum candidum*, *Debaryomyces hansenii*, *Brevibacterium linens*) can colonize the rind or core of cheeses throughout maturation and contribute to changes in product characteristics including consumption of lactic- and/or acetic acids resulting in increased product pH (Leclercq-Perlat et al., 2004; Suzuki-Iwashima et al., 2019).

Improved management of *L. monocytogenes* in these cheeses is highly relevant and validated predictive models for growth and growth boundary may help to establish process objectives, identify critical control points in HACCP plans (Membré, 2017) or support re-formulation of the products. However, documentation is limited on successfully validated *L. monocytogenes* growth models for mould/smear ripened cheeses.

Cardinal parameter models (CPMs) have been developed to predict the growth of *L. monocytogenes* in specific mould/smear-ripened cheeses. Schwartzman et al. (2014) developed a simple model to predict growth of *L. monocytogenes* in smear- and mould-ripened cheese. This model contained a single term to quantify the effect of pH and a calibrated optimal growth rate

parameter (μ_{opt}). The μ_{opt} -value was calibrated so that model predictions corresponded to the observed growth rate data. Rosshaug et al. (2012) developed a CPM with terms for the effect of temperature, pH, NaCl/ a_w and lactic acid concentrations to predict growth of *L. monocytogenes* in white/blue mould cheese. Schwartzman et al. (2011) used a similar approach to predict growth of *L. monocytogenes* in smear cheese. These studies used very few growth curves (≤ 4) to evaluate the performance of the developed models and product characteristics and their dynamics were not always reported. It has been recommended to use more than 20 kinetics from well-characterized products to evaluate predictive food microbiology growth models (Mejlholm and Dalgaard, 2013; Dalgaard and Mejlholm, 2019) and for instance the evaluation of *L. monocytogenes* models for seafood and meat relied on hundreds of growth responses (Mejlholm et al. 2010). Therefore, there is a need for further evaluation of *L. monocytogenes* models to predict growth in mould/smear-ripened cheese. Furthermore, dynamic product characteristics and temperature during manufacturing, ripening and storage of mould/smear-ripened cheeses represented a challenge for prediction of *L. monocytogenes* growth and for the evaluation of growth models. Models for diffusion of lactic acid/lactate and its consumption in smear soft cheese by a specific yeast has been suggested (Aldarf et al., 2006; Riahi et al., 2007). However, models or other tools to predict the dynamic product characteristics of mould/smear-ripened cheeses during manufacturing, ripening and storage are not available. The use of measured dynamic product characteristics as model inputs is another option to predict growth of *L. monocytogenes* in mould/smear-ripened cheeses.

The objectives of the present study were to evaluate mathematical models for their ability to predict growth of *L. monocytogenes* in mould/smear-ripened cheeses as influenced by measured dynamic changes in product characteristics and storage conditions. Firstly, mould-ripened cheese was studied by challenge testing to quantify growth of *L. monocytogenes* with dynamic changes in storage temperature and product characteristics (LAB, pH, NaCl/ a_w , lactic- and acetic acid

concentrations). Secondly, available data for growth of *L. monocytogenes* in mould/smear-ripened cheese was collected from literature. Finally, the performance of nine available *L. monocytogenes* growth models was evaluated using a total of 46 kinetics determined experimentally in the present study or obtained from literature together with the corresponding dynamic product characteristics and storage temperature.

2. Material and methods

2.1. Bacterial strains and pre-culture conditions

Four dairy related strains of *L. monocytogenes* were provided by Arla Foods amba and used as a cocktail (SLU92, 612, LM19, 6) for inoculation of challenge tests. Strains were transferred from storage at -80°C to Brain Heart Infusion (BHI) broth (CM1135, Oxoid, Hampshire, UK) and incubated 24h at 25°C. The individual stains were pre-cultured one day at a temperature of 8°C in BHI broth with pH 6.7 to simulate milk conditions. Pre-cultures were grown to a relative increase in absorbance (540 nm) of 0.05 to 0.2 (Novaspec II, Pharmacia Biotech, Allerød, Denmark). A *L. monocytogenes* cocktail of strains (*Lm*-mix) were produced by mixing equal volumes of the individual pre-cultures. The cell concentration of *Lm*-mix was determined by direct phase-contrast microscopy at 1000x magnification considering that one cell per field of view corresponded to a concentration of around 10^6 cfu/ml (Adams et al., 2016).

2.2. *L. monocytogenes* growth and measured dynamic product characteristics for mould-ripened cheese

Three challenge tests were performed to quantify growth of *L. monocytogenes* in three differently formulated white mould-ripened cheeses (see section 2.2.1). Growth in the rind and in the core of the cheeses were quantified separately and a total of 18 growth/no-growth responses were generated together with quantification of changes in storage conditions (temperature) and product characteristics (pH, NaCl/a_w, lactic- and acetic acids) (see section 2.2.2 and Figures 1, 2 and 3).

2.2.1. Production of white mould-ripened cheeses

Pasteurized milk with 3.5% fat was purchased from a local supermarket and used for cheese making in the laboratory. The milk was heated to 30-32°C, 0.4 ml/L of a 50% CaCl₂ w/v solution was added and then the milk was inoculated with different commercial LAB starter culture along with mould and/or yeast as ripening microbiota in order to simulate production of different white mould cheeses. For the first challenge test, milk (25.5 L) was inoculated with a freeze-dried probiotic strain (0.01 g/L of *Lactobacillus acidophilus*, LA-5, Chr. Hansen A/S, Hørsholm, Denmark), a freeze dried DL-culture (0.13 g/L of *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, *Leuconostoc* spp. and *L. lactis* subsp. *lactis* biovar diacetylactis, FLORA™ C501, Chr. Hansen A/S) and a ripening culture (4 mg/L of *P. camemberti*, PC HP 6 LYO and 2 mg/L of *G. candidum*, GEO 17, DuPont™ Danisco®, Brabrand, Denmark). For the second challenge test, milk (24.5 L) was inoculated with a freeze dried DL-culture (0.13 g/L of FLORA™ C503, Chr. Hansen A/S) and a ripening culture (2 mg/L of *G. candidum*, GEO 17, DuPont™ Danisco®). For the third challenge test, milk (53 L) was inoculated with a freeze dried DL-culture (0.02 g/L of *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, *Leuconostoc* spp. and *L. lactis* subsp. *lactis* biovar diacetylactis, CHN-11, Chr. Hansen A/S, Hørsholm, Denmark) and a ripening culture (6 mg/L of *P. camemberti*, SWING FD PCA-3 and 5 mg/L of *G. candidum*, SWING GEO CD-1, Chr. Hansen A/S). 30

minutes after the addition of cultures and in order to ensure an even distribution in the final product, milk was inoculated with 0.1% (v/w) of *Lm*-mix appropriately diluted in chilled saline water (0.85% NaCl) to obtain an initial concentration of 1 log cfu/ml of milk. Subsequently, 0.2 ml/L of rennet CHY-MAX[®] Plus (Chymosin, Chr. Hansen) for challenge test one and two or CHY-MAX[®] Special (Chymosin, Chr. Hansen A/S) for challenge test three was added to the milk. 60 minutes after rennet addition, the coagulum was cut into cubes with 1 cm edge and gently stirred for 5 sec. every 5 min during 30 min. The curd was transferred into round moulds (11.4 cm diameter and 8.5 cm height) and let to stand for draining of the whey at 20°C during 20-22 h. After six hours within the draining process the cheese moulds were flipped over. Cheeses were then removed from the moulds and cut vertically into halves. For the first and second challenge tests, cheeses were immersed 30 min. in brine (18-20% salt) at a temperature of 18°C. For the third challenge test, cheeses were dry-salted on the surface. After brining/salting by hand, cheeses were transferred to open maturation boxes placed in an incubator (TC 175 S, Lovibond[®], Amesbury, UK) and dried during 5 h at 18°C. Thereafter, maturation boxes were closed and cheeses were incubated at 10°C for 5 days. Temperature was increased to 12°C one week after the beginning of the fermentation in order to reach the optimum ripening temperature (Leclercq-Perlat et al., 2015). Cheeses were stored up to 30 days at a relative humidity of 90-95% as measured by data loggers (Tinytag view, Gemini Data Loggers Ltd, Chichester, UK). 60 cheeses (60±10 g) were produced for each of the challenge tests one and two, whereas 120 cheeses (60±10 g) were produced for challenge test three.

2.2.2. Product characteristics and microbiological analysis

For each of the three batches of white-mould cheese, product characteristics were determined separately and in triplicate for the rind and for the core, respectively. Product characteristics were determined during storage at every time of microbiological sampling. pH was

measured with a PHC10801 puncture combination probe (Hach, Brønshøj, Denmark) placed directly into the rind or core of each analysed cheese. NaCl was quantified by automated potentiometric titration (785 DMP Titrino, Metrohm, Hesisau, Switzerland) and a_w was measured by a water activity meter (Aqua Lab model CX-2, Decagon devices Inc., Pullman, US). The concentration of organic acids were determined by HPLC using external standard of lactic-, acetic- and citric acids for identification and quantification (Dalgaard and Jørgensen, 2000; Østergaard et al., 2014). To determine water phase concentrations of salt and organic acids the dry matter content was determined by oven drying at 105°C for 24±2h. Storage temperature was regularly recorded by data loggers placed inside the cheeses during maturation and storage (TinytagPlus, Gemini Data Loggers Ltd, Chichester, UK).

At each time of sampling in each challenge test, three cheeses (60±10 g) were analysed. Cheese rind samples were obtained from the 5 mm exterior layer of each cheese and core samples were taken from the centre of each cheese. 10 g of cheese rind or cheese core were independently diluted 10-fold with chilled physiological saline water (0.85% NaCl and 0.10% Bacto-peptone, BD Difco™, Wokingham, UK), homogenized for 30 s at normal speed in a Stomacher 400 (Seward Medical, London, UK) and then 10-fold serially dilutions were performed with chilled physiological saline water. Lactic acid bacteria (LAB) were enumerated by double layer pour plating in nitrite actidionine polymyxin (NAP) agar with pH 6.2 and incubated at 25°C for 72h (Davidson and Cronin, 1973). Viable counts of *L. monocytogenes* were determined by surface plating on PALCAM agar base (CM0877, Oxoid, Hampshire, UK) with PALCAM selective supplement (SR0150, Oxoid, Hampshire, UK) and incubation at 37°C for 48 h.

2.3. Collection of data from the scientific literature

The present study attempted to collect all accessible data for growth of *L. monocytogenes* in mould/smear-ripened cheeses with reported dynamic product characteristics as well as with dynamic or constant storage temperature. Schwartzman et al. (2011) inoculated pasteurized and raw milk used to produce a smear-ripened cheese with a single strain of the pathogen (C5, serotype 4b, isolated from cow faeces). The authors observed growth of *L. monocytogenes* during ripening of the smear cheese produced with raw milk and reported changes in pH, a_w and L-lactic acid concentrations in the rind and core. Rosshaug et al. (2012) inoculated a 13 days old blue/white mould cheese with the type strain of *L. monocytogenes* (ATCC 19115) and reported changes in storage temperature, pH, salt and lactic acid concentrations in the product over time. Schwartzman et al. (2014) inoculated the surface of a 25 days old mould cheese and 30 days old smear-ripened cheeses with a two strain cocktail of *L. monocytogenes* (6179, serotype 1/2a, isolated from cheese and C5, serotype 4b, isolated from cow faeces). Along with growth, changes in pH and a_w of the product were measured over time. Dalzini et al. (2017) inoculated three different batches of Gorgonzola cheese slices with the *L. monocytogenes* type strain (ATCC 19115) or with a cheese isolate (Lm273250). The authors reported average values for pH and a_w for three different batches of cheese that were analysed at different days (1, 14, 21, 49 and 100 days) of the challenge test at a constant storage temperature of 8°C. The PlotDigitizer v. 2.6.8 software (<https://sourceforge.net/projects/plotdigitizer/files/>) was used to extract data from graphs for *L. monocytogenes* growth in cheese, product characteristics and storage conditions for cheeses and results are shown in Supplementary material Table 1 and 2.

2.4. Predictive food microbiology models

The performance of nine predictive models for growth of *L. monocytogenes* in cheese was evaluated. These cardinal parameter models (CPM) were developed and evaluated to predict growth

of *L. monocytogenes* in cheeses (Table 1; Supplementary material). The number of environmental factors included in the studied models varied from one to five. The least complex model took into account exclusively the effect of pH, although it is well established that extensive models are necessary to accurately predict growth of *L. monocytogenes* in foods (Mejlholm et al., 2010). This simple model was included to determine the difference in performance when compared to more extensive models including the combined effect of organic acids, other product characteristics and interaction between environmental factors. A detailed description of the nine studied models including specific equations, parameter values used for simulations, assumptions and interaction terms has been provided as Supplementary material.

2.5. Simulation and evaluation of models

Growth simulation with the different models and based on product characteristics and storage conditions for the different studies were performed using Microsoft Excel 2016 (Microsoft Corp. Redmond, WA, USA). The Euler-method for numerical integration (Press et al., 2007) was applied with a step-length of 0.5 h to predict growth under dynamic storage temperature and product characteristics and by using the differential form of the primary logistic model (Rosso et al., 1996) with delay ($dN/dt=0$ for $t < t_{lag}$ and $dN/dt=N \cdot \mu_{max} \cdot (1-(N/N_{max}))$ for $t \geq t_{lag}$). Parameter values were initial cell concentration (N , cfu/g), maximum specific growth rate (μ_{max} , h⁻¹), maximum population density (N_{max} , cfu/g), storage time (t , h) and lag time (t_{lag} , h). Growth simulations were performed using the initial cell concentrations observed experimentally (see section 2.2.2) or values reported in the literature (see section 2.3). The maximum cell concentration was assumed to be a constant value (7.4 log cfu/g) for all the simulations performed. It was calculated as an average value ($n = 19$) from maximum populations of *L. monocytogenes* observed in mould-ripened cheeses growth kinetics at different temperatures (Kapetanakou et al., 2017; Lobacz et al., 2013).

The acceptable simulation zone (ASZ) approach was used to compare predictions from the different models (see section 2.4) with experimentally determined growth (see section 2.2) and with observations extracted from the literature (see section 2.3). The acceptable interval was defined as ± 0.5 log cfu/g from the simulated growth of *L. monocytogenes* as suggested by Oscar (2005) and with 0.5 log cfu/g corresponding to the precision of microbiological testing in food (FAO/WHO, 2009). Fail-safe and fail-dangerous predictions had, respectively, differences between observed and predicted value below -0.5 log cfu/g and above +0.5 log cfu/g. When at least 70% of the predicted cell concentrations (log cfu/g) were within the ASZ, the simulation was considered acceptable (Oscar, 2005; Velugoti et al., 2011).

3. Results

3.1. *L. monocytogenes* growth and dynamic product characteristics for mould-ripened cheese

In challenge test one and two, *L. monocytogenes* grew during the first 22 h of cheese making. Concentrations increased from 1.0 ± 0.2 to 2.7 ± 0.3 log cfu/g while the temperature decreased from 32°C to 16.5°C, pH decreased from 6.5 ± 0.0 to 4.8 ± 0.1 and the concentration of lactic- and acetic acids in the water phase increased from non-detectable to $1.7 \pm 0.2\%$ and $0.2 \pm 0.0\%$, respectively. At the same time, the added LAB grew to their maximum cell concentration (8.6 ± 0.3 log cfu/g) and caused the observed increase in lactic- and acetic acid concentrations with the corresponding decrease of pH (Fig. 1 and 2). From 22 h until four days of storage, the concentration of *L. monocytogenes* remained constant or decreased slightly in the core but increased in the rind of the mould-ripened cheese (Fig. 1 and 2). After four days of storage in challenge tests one and two, the concentration of NaCl (%) in the water phase and a_w showed little

variability with values of 3.7 ± 0.2 (%) and 0.979 ± 0.001 , respectively as found in several previous studies (Guinee and Fox, 2017; Leclercq-Perlat et al., 2013).

In challenge test one, growth of *L. monocytogenes* was observed in the white-mould cheese rind from day four until day 15 and during this period where pH increased from 4.7 ± 0.0 to 5.8 ± 0.0 , the lactic- and the acetic acid concentrations in the water phase decreased from $1.8 \pm 0.1\%$ to $0.2 \pm 0.0\%$ and from $0.1 \pm 0.0\%$ to non-detectable, respectively (Fig. 1a and 1c). In the rind, *L. monocytogenes* reached a concentration of 6.8 ± 0.2 log cfu/g after 28 days of storage when pH was 7.3 ± 0.0 (Fig. 1a). In the core, pH-values were lower than in the rind after 15 and 28 days of storage (5.0 ± 0.1 and 6.3 ± 0.3) and a final cell concentration of 4.2 ± 0.0 log *L. monocytogenes*/g in the core was observed at day 28 (Fig. 1b and 1d).

In challenge test two, the concentration of *L. monocytogenes* decreased from 3.0 ± 0.1 log cfu/g on day four to 1.4 ± 0.4 log cfu/g on day 15 of storage (Fig. 2a). Growth was then observed in the rind of the white-mould cheese at day 21 following an increase of pH from 5.3 ± 0.1 to 6.8 ± 0.2 (Fig. 2c). *L. monocytogenes* reached a concentration in the rind of 4.0 ± 0.9 log cfu/g on day 28 when pH was 6.9 ± 0.1 (Fig. 2a and 2c). For the core, *L. monocytogenes* reached a concentration of 2.5 ± 0.9 log cfu/g with a pH of 5.5 ± 0.3 and a lactic acid concentration of 0.5 ± 0.0 % after 28 days of storage (Fig. 2b and 2d).

For challenge test three *L. monocytogenes* grew during the first 25 h of cheese making with concentrations increasing from 2.2 ± 0.0 to 3.9 ± 0.2 log cfu/g. The temperature decreased from 33°C to 16°C , pH decreased from 6.5 ± 0.0 to 4.7 ± 0.1 and the concentration of lactic- and acetic acids in the water phase increased from non-detectable to $1.6 \pm 0.5\%$ and $0.2 \pm 0.0\%$, respectively (Fig. 3). From 25 h and until the end of storage, inactivation of *L. monocytogenes* was observed both in the

rind and in the core of the studied cheese (Fig. 3a and 3b). This might be due to a low water activity of ~0.94 on day two that decreased to ~0.88 on day seven combined with a low pH (Fig. 3e and 3f).

3.2. Evaluation of *L. monocytogenes* growth models for mould/smear-ripened cheese under dynamic product characteristics and/or storage temperature

For challenge test one on day 15, a reduced version of the Martinez-Rios et al. (2019) model (Eq. (24)) predicted *L. monocytogenes* concentrations of 4.6 log cfu/g in the rind and 2.8 log cfu/g in the core when the corresponding observed values were 4.0 ± 0.4 and 2.6 ± 0.4 log cfu/g, respectively. On day 28, values of 7.4 log cfu/g in the rind and 7.3 log cfu/g in the core were predicted for *L. monocytogenes* while the observed values in the rind and the core were 6.8 ± 0.2 and 4.2 ± 0.0 log cfu/g, respectively (Fig. 4a and 4b). For challenge test two, predicted concentrations of *L. monocytogenes* in the rind and core after 15 days were 3.1 log cfu/g in the rind and 2.9 log cfu/g in the core whereas the observed values were lower within both the rind (1.4 ± 0.4 log cfu/g) and core (2.1 ± 0.2 log cfu/g). On day 28, the model predicted 7.4 log cfu/g in the rind and 3.4 log cfu/g in the core while the observed values were 4.9 ± 0.9 and 2.5 ± 0.9 log cfu/g, respectively (Fig. 4c and 4d). Eq. (24) overestimated growth of *L. monocytogenes* in the core for challenge test one (Fig 4c). For challenge tests two overestimated growth occurred both in the rind and core of cheese at day 28. However, predictions at day 15 were acceptable for cheese as for rind and the core of challenge test two cheese (Fig. 4b, 4d). For the 12 kinetics (4 growth curves in triplicate) from challenge test one and two with white mould cheese, 69% of the observed cell concentrations were within the ASZ for predictions with eq. (24) at dynamic storage temperatures and product characteristics (Fig. 4; Table 2). When predictions were performed without taking into account the measured concentrations of lactic- and acetic acids in the water phase or by excluding the effect of interaction between the environmental factors (Le Marc et al., 2002) then the obtained average ASZ-values were much

lower (57% and 39%) as predicted growth rates were markedly faster than observed (Table 3). For challenge test three at 25 h, eq. (24) predicted *L. monocytogenes* concentrations of 4.1 log cfu/g in the rind and in the core when the observed values were 3.8 ± 0.3 and 3.9 ± 0.1 log cfu/g, respectively. From 25 h and until the end of challenge test three the model predicted no-growth of *L. monocytogenes* in rind and core based on the measured product characteristics storage temperature, despite the observed inactivation and therefore challenge test three was excluded when calculating the ASZ. Eq. (24) was able to acceptably predict growth of *L. monocytogenes* in white/blue mould cheese and smear cheese (Rosshaugh et al., 2012; Schwartzman et al., 2011) with, respectively, 100% and 88% of the predictions within the ASZ (Table 2). 72% of the observations from a total of 18 kinetics in well characterized mould/smear-ripened cheese, were within the ASZ (Table 2). In contrast, eq. (24) was unable to accurately predict growth of *L. monocytogenes* as observed by Dalzini et al. (2017) and Schwartzman et al. (2014) (Table 2 and Fig. 5). Dalzini et al. (2017) and Schwartzman et al. (2014) did not quantify the concentrations of lactic or acetic acids and reported exclusively an average storage temperature, pH and a_w for the different batches of cheese. Furthermore, the used of different starter cultures might have had an effect on the growth of *L. monocytogenes* beyond the measured product characteristics. Specifically, Gorgonzola cheese studied by Dalzini et al. (2017) was produced using *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* as starter cultures rather than the DL-cultures (*L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, *Leuconostoc* spp. and *L. lactis* subsp. *lactis* biovar *diacetylactis*) used in the present study and both by Rosshaugh et al. (2012) and Schwartzman et al. (2011). Schwartzman et al., (2014) did not provide information about starter cultures used in the production of smear- and mould-ripened cheeses used in their study.

Eq. (24) acceptably predicted growth of *L. monocytogenes* in one of the smear-ripened cheeses and one of the mould-ripened cheese based on product characteristics reported by

Schvartman et al. (2014) (Fig. 5b and 5c). However, the model failed to predict the growth observed in a different batch of smear-ripened cheese (Fig. 5a). Similarly, eq. (24) was able to predict growth of *L. monocytogenes* ATCC 19115 and Lm273250 in Gorgonzola cheese based on the reported product characteristics for batch C (Fig. 5f and 5i) but the model overestimated growth of both strains in batch A and B of Gorgonzola cheese (Fig. 5d, 5e, 5g, 5h).

Based on the performed model evaluation, the range of applicability for eq. (24) included storage temperatures from 0°C to 33°C, pH between 4.5 and 7.3, water phase salt from 0% to 5.0%, water phase lactic acid from 0 to 2.0% (222 mM) and water phase acetic acid between 0 and 0.2% (36 mM). Variable model performance was observed, particularly, for insufficiently characterized cheeses (Fig. 5; Table 2) and further evaluation of models with well characterized mould/smear-ripened cheeses, including gorgonzola, is needed.

For these 18 kinetics the performance of the Augustin model in Coroller et al. (2012) and of the Østergaard et al. (2014) models were close to acceptable with, respectively, 69% and 67% of predictions within the ASZ (Table 2). With 26-63% of the predictions within the ASZ the remaining evaluated models failed to acceptably predict growth of *L. monocytogenes* in mould/smear-ripened cheeses with dynamic product characteristics and storage conditions (Table 2).

None of the evaluated models were able to accurately predict the growth observed by Dalzini et al. (2017) for Gorgonzola cheese slices and by Schvartzman et al. (2014) for smear-ripened cheese based on the reported average values of product characteristics and storage temperature (Table 2). This highlights the importance of knowing changes in relevant product characteristics to predict *L. monocytogenes* in mould/smear-ripened cheeses.

4. Discussion

It is evident from the present and several previous studies that mould/smear-ripened cheeses support growth of *L. monocytogenes* due to activity of the mould/smear microbiota resulting in organic acid consumption and increasing product pH during ripening and storage (Fig. 4; Back et al., 1993; D'Amico et al., 2008; Leclercq-Perlat et al., 2006; Liu et al., 2007; Picque et al., 2006; Ryser and Marth, 1987). Depending on storage temperature and time of potential contamination with *L. monocytogenes* critical concentrations of the pathogen above 100 cfu/g may be attained (ANZ, 2018; CA, 2011; EC, 2005) and focus on evaluation and management of this hazard is important.

A single model was identified to predict growth of *L. monocytogenes* in different mould/smear-ripened cheeses from measured changes in storage temperature and product characteristics (Eq. (24); Fig. 4; Table 2). This reduced version of the Martinez-Rios et al. (2019) model allowed *L. monocytogenes* growth in both rind and core of cheeses to be predicted with near to acceptable accuracy. This is the first study where growth of *L. monocytogenes* in different mould/smear-ripened cheeses have been acceptably predicted for all accessible data (n=18) from fully characterized cheeses by a single model, an important aspect For practical application of predictive microbiology with this group of cheeses. However, further evaluation of the model with additional dynamic data from well characterized cheese is desirable. The growth and growth boundary model of Martinez-Rios et al. (2019) included the effect of ten environmental factors and eq. (24) might easily be expanded to include the effect of for example CO₂ to predict growth of *L. monocytogenes* in modified atmosphere packed cheeses and with the effect of citric acid that might be relevant for other types of cheeses (Martinez-Rios et al., 2019; Leclercq-Perlat et al., 2006).

Importantly, the acceptable model performance for *L. monocytogenes* growth predictions in mould/smear-ripened cheeses required the effect of organic acids and interaction between all environmental factors to be taken into account (Table 3). As an example, *L. monocytogenes* grew 100-fold during 17 days from day one to 18 in the rind of cheese in challenge test one (Fig. 1). The predicted time for this growth was, respectively, two days and 14-15 days without and with including the effect of lactic acid and using other product characteristics and storage temperature as measured for challenge test one. It has previously been shown that growth prediction for well characterized food products by extensive models, including the effect of relevant growth inhibiting factors, can differ markedly from prediction by simpler models and less characterized foods (Mejlholm et al., 2010; Østergaard et al., 2014). However, most previous studies did not quantify profiles of lactic and other organic acids during fermentation, ripening and storage mould/smear-ripened cheeses (Back et al., 1993; D'Amico et al., 2008; Ryser and Marth, 1987) and as a consequence the possibility for evaluation and predicting the growth potential of *L. monocytogenes* was limited.

Classical predictive food microbiology models allow growth or survival of pathogens and spoilage bacteria to be predicted for different constant product characteristics, constant or dynamic storage temperatures and in some cases including the inhibiting effect of the food microbiota on growth of pathogens. Predictions can be used to document food safety, in exposure assessment studies or for safe product reformulations where one set of preserving parameters is changed into another set to reduce for example the concentration of NaCl/sodium or a specific food preservative (Dalgaard and Mejlholm, 2019; Tenenhaus-Aziza and Ellouze, 2015). In the same way eq. (24) can be used to document food safety and in exposure assessment studies for mould/smear-ripened cheeses but importantly with measured profiles, rather than constant values, for temperature, pH, lactic and acetic acid concentrations and NaCl/ a_w must be used as model input. However, to

evaluate potential *L. monocytogenes* growth in mould/smear-ripened cheeses after ripening for example due to contamination by consumers or retailers (Evans and Redmond, 2018; Heiman et al., 2016) and subsequent storage at different temperatures, eq. (24) can be applied as classical predictive food microbiology models because changes in product characteristics will be limited and they might be assumed as constant. Therefore, at this point the major factor to reduce growth of *L. monocytogenes* is temperature. As an example, for a mould-ripened cheese with pH 6.3, a_w 0.973 and water phase lactic acid concentration of 0.15%, the predicted time for *L. monocytogenes* to reach the critical cell concentration was 11-22 days at 4.5°C if this product was contaminated with 1 cfu/g (high) or 1 cfu/125g (low). This contamination may result from consumer or retailer handling of cheeses e.g. by cross-contamination from a different food item. On the other hand, if the cheese was stored at a higher temperature e.g. 8°C, then the predicted time to reach the critical cell concentration will be 5-10 days for a product contaminated with a high or a low concentration. Storage of refrigerated foods is recommended to be performed by consumers at temperatures below 5°C. However, several studies have shown that domestic refrigerators work above a mean value of 6°C and with some extreme mean values as high as 12°C (Dumitraşcu et al. 2020; Roccato et al., 2017). Eq. (24) can be used to educate/advise consumers and retailers on safe storage profiles (time-temperature) of these cheeses after opening of the packaging and cutting of the product.

There is increasing demand for cheeses with reduced salt/sodium content and it is interesting to predict the effect of product reformulation on *L. monocytogenes* growth. If the rind of mould-ripened cheese with product characteristics as in challenge test one was contaminated with *L. monocytogenes* (1 cfu/125g) on day 15 and stored at 5°C then the predicted critical cell concentration (100 cfu/g) would be reached, respectively, 18 days and 15 days after contamination when using the studied NaCl profile or a profile with NaCl concentrations reduced by 25%. In agreement with these predictions other studies found pH and lactic acid concentrations to have more

impact than NaCl on *L. monocytogenes* growth/no-growth/inactivation responses (Shrestha et al., 2011; Wemmenhove et al., 2013).

Several models have been developed to describe the relationship between temperature, lactic acid production in foods and the associated pH value (Leroy and De Vuyst, 2003; Østergaard, 2014). Other models have been developed for growth of moulds and their lactic acid consumption in broth or during ripening of smear soft cheese (Riahi et al., 2007; Aldarf et al., 2006; Adour et al., 2002). However, models are not yet available to predict changes in product characteristics for mould/smear-ripened cheese during fermentation, ripening and storage. The present study therefore used measured product characteristics to predict growth of *L. monocytogenes*.

The inhibitory effect of high concentrations of lactic acid bacteria on growth of *L. monocytogenes* in cottage cheese, meat and seafood has previously been described empirically by using the Jameson effect model where growth of a pathogen stops when LAB reach their maximum cell concentration (Østergaard et al., 2014; Dalgaard and Mejlholm, 2019). When used in combination with measured dynamic changes in product characteristics the Jameson effect model (Østergaard et al., 2014) markedly underestimated growth of *L. monocytogenes* in mould-ripened cheese (Results not shown). This was expected as the Jameson effect empirically describe the effect of lactose consumption, lactic acid formation and related decrease of pH whereas this simple model cannot describe the effect of the subsequent lactic acid consumption and pH increase in mould-ripened cheese. It seems interesting in future studies to model growth and activity of the complex microbiota including LAB and moulds in mould/smear-ripened cheeses and their microbial interaction with *L. monocytogenes*. If these models will be able to predict *L. monocytogenes* growth in mould/smear-ripened cheeses more accurately than the CPM suggested in the present study (Eq. (24)) remain to be evaluated. To improve management of potential *L. monocytogenes* growth in

mould/smear-ripened cheeses microbial interaction may be important as yeast and moulds with antilisterial activity may be identified and used as bioprotective cultures (Goerges et al. 2006; Goerges et al., 2011).

The models of Augustin in Coroller et al. (2012) and Østergaard et al. (2014) had a “close to acceptable” performance when predicting growth of *L. monocytogenes* in mould/smear-ripened cheese (Table 2). However, we selected eq. (24) as it performed better and had a much more realistic T_{min} -value of -2.83°C (Delignette-Muller et al., 2006; Tienungoon et al., 2000; Ross, 1993) compared to the value of -5.82°C estimated by Coroller et al. (2012). This unusually low T_{min} -value of -5.82°C will result in marked overestimation of *L. monocytogenes* growth rates at low storage temperatures of for example $2\text{--}5^{\circ}\text{C}$. For mould/smear-ripened cheeses the model of Østergaard et al. (2014) had the limitation of a pH_{min} -value for *L. monocytogenes* of 4.87. Consequently, no-growth will be predicted below that pH-value, irrespective of other product characteristics. This is not optimal as pH of about 4.5 is common for mould/smear-ripened cheeses and because *L. monocytogenes* can grow at pH-values below 4.87 especially at temperatures of $10\text{--}20^{\circ}\text{C}$ (Martinez-Rios et al., 2019; Nyhan et al., 2018).

In conclusion, a model (Eq. (24)) was identified to acceptably predict growth of *L. monocytogenes* during manufacturing, ripening and storage of mould/smear-ripened cheese. This model can be used to support exposure assessment studies, documentation of food safety and safe reformulations of mould/smear-ripened cheeses. It seems interesting in the future to evaluate if prediction of *L. monocytogenes* growth in cheeses can be further improved by expanding this model to include terms for the effect of for example butyric acid, propionic acid or other compounds produced during fermentation or ripening of various types of cheeses.

Acknowledgements

The Danish Dairy Research Foundation funded the present study as part of the project ‘Predictive food microbiology tool for risk assessment and documentation of food safety’.

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Table 1. Overview of the *L. monocytogenes* growth models evaluated in the present study.

Model	Developed/validated for:	Number of environmental factors in model	Specific environmental factors in models					
			Temp.	NaCl/a _w	pH	Lactic acid	Acetic acid	Interactions between environmental factors
Augustin et al. (2005)	Liquid dairy products/cheeses	3	+	+	+	-	-	+ ^a
Schvartzman et al. (2011)	Smear cheese	4	+	+	+	+	-	+ ^a
Rosshaug et al. (2012)	White/blue cheese	4	+	+	+	+	-	+ ^a
Weak acid model ^b	Meat, seafood, cheese and vegetables/fruit	4	+	+	+	+	-	+ ^a
Augustin model ^b	Meat, seafood, cheese and vegetables/fruit	4	+	+	+	+	-	+ ^a
Dalgaard model ^b	Meat, seafood, cheese and vegetables/fruit	4	+	+	+	+	-	+ ^a
Schvartzman et al. (2014)	Smear/mould cheese	1	-	-	+	-	-	-
Østergaard et al. (2014)	Cottage cheese	4	+	+	+	+	-	+ ^a
Martinez-Rios et al. (2019)	Meat, seafood, dairy products and cheeses ^c	5	+	+	+	+	+	+ ^a

^a Interactions between environmental factors were modelled by using the Le Marc approach (Le Marc et al., 2002).

^b Models re-fitted by Coroller et al. (2012) using equations from Zuliani et al. (2007); Augustin et al. (2005) and Mejlholm and Dalgaard (2009).

^c Chemically acidified cheese and cream cheese.

Table 2. Comparison of predicted and observed growth using the acceptable simulation zone (ASZ) method.

% of observations within the ASZ (% fail-safe, % fail-dangerous)								
Well characterized cheeses					Insufficiently characterized cheeses			
	The present study	Rosshaug et al. (2012)	Schvartzman et al. (2011)	ASZ (%) ^a	Dalzini et al. (2017)	Schvartzman et al. (2014)	ASZ (%) ^a	
Measured environmental factors	Temp., pH, NaCl/a _w , lactic- and acetic acids	Temp., pH, NaCl/a _w and lactic acid	Temp., pH, NaCl/a _w and lactic acid		Temp., pH, NaCl/a _w	Temp., pH, NaCl/a _w		
Kinetics	n=4x3 ^b	n=1x2 ^b	n=2x2 ^b	n=18 ^b	n=6x2 ^b	n=8x2 ^b	n=28 ^b	
Observations	N= 122 ^c	N=6 ^c	N=16 ^c	N=144 ^c	N=70 ^c	N=14 ^c	N=84 ^c	
General models	Eq. (24)	69 (23, 8)	80 (20, 0)	88 (12, 0)	71 (22, 7)	21 (76, 3)	43 (43, 14)	25 (70, 5)
	Weak acid in Coroller et al. (2012)	66 (25, 9)	55 (36, 9)	81 (13, 6)	67 (24, 9)	21 (78, 1)	50 (43, 7)	26 (72, 2)
	Augustin in Coroller et al. (2012)	68 (26, 6)	60 (30, 10)	81 (19, 0)	69 (25, 6)	19 (77, 4)	50 (43, 7)	24 (71, 5)
	Dalgaard in Coroller et al. (2012)	48 (52, 0)	55 (45, 0)	81 (19, 0)	53 (47, 0)	20 (79, 1)	43 (50, 7)	24 (74, 2)
	Augustin et al. (2005) ^d	49 (49, 2)	55 (45, 0)	81 (19, 0)	53 (45, 2)	33 (64, 3)	50 (43, 7)	36 (61, 4)
	Augustin et al. (2005) ^e	37 (6, 57)	63 (38, 0)	38 (25, 38)	38 (10, 52)	40 (36, 24)	36 (29, 36)	39 (35, 26)
Product-specific models	Østergaard et al. (2014)	70 (21, 9)	71 (0, 29)	69 (25, 6)	70 (21, 9)	29 (65, 6)	43 (50, 7)	31 (63, 6)
	Schvartzman et al. (2014)	25 (0, 75)	50 (0, 50)	19 (0, 81)	24 (0, 76)	40 (21, 39)	- ^f	-
	Rosshaugh et al. (2012)	44 (55, 1)	- ^f	81 (19, 0)	51 (47, 2)	20 (79, 1)	43 (50, 7)	24 (74, 2)
	Schvartzman et al. (2011)	35 (0, 65)	75 (0, 25)	- ^f	42 (2, 55)	23 (16, 59)	43 (50, 7)	29 (21, 50)

^a ASZ (%): Percentage of observed cell concentrations (log cfu/g) within the ASZ (fail-safe, fail-dangerous).^b n: number of growth curves at dynamic product characteristics and/or storage temperatures.^c N: number of observation (log cfu/g)^d Predictions performed with μ_{opt} for liquid dairy products (0.742 h⁻¹).^e Predictions performed with μ_{opt} for cheese (0.212 h⁻¹).^f Data used in model development and therefore excluded when evaluating the performance of the model.

Table 3. Effect of product characteristics and interaction between environmental factors on the acceptable simulation zone (ASZ) scores when using Eq. (24) to performed predictions.

Challenge tests in present study	Storage conditions and growth data	% observations within ASZ			
		Including lactic- and acetic acid concentrations in the water phase		Excluding lactic- and acetic acid concentrations in the water phase	
		With interaction	Without interaction	With interaction	Without interaction
One	Rind	55	55	39	39
One	Core	77	47	37	37
Two	Rind	72	72	41	41
Two	Core	73	63	40	40
Average ASZ score		69	57	39	39

Figure 1. Challenge test one: Growth of *L. monocytogenes* (□), lactic acid bacteria (○) and changes in pH (Δ), lactic acid (◇), acetic acid (×) (% in water phase) as well as a_w (▽) for the rind (a, c, e) and for the core (b, d, f) of white-mould cheese. The temperature profiles are shown as blue lines (a, b). Symbols represent average values and error bars the standard deviation for three samples.

Figure 2. Challenge test two: Growth of *L. monocytogenes* (□), lactic acid bacteria (○) and changes in pH (Δ), lactic acid (◇), acetic acid (×) (% in water phase) as well as a_w (▽) for the rind (a, c, e) and for the core (b, d, f) of white-mould cheese. The temperature profiles are shown as blue lines (a, b). Symbols represent average values and error bars the standard deviation for three samples.

Figure 3. Challenge test three: Growth of *L. monocytogenes* ($\square$), lactic acid bacteria ($\circ$) and changes in pH (Δ), lactic acid ($\diamond$), acetic acid ($\times$) (% in water phase) as well as a_w (∇) for the rind (a, c, e) and for the core (b, d, f) of white-mould cheese. The temperature profiles are shown as blue lines (a, b). Symbols represent average values and error bars the standard deviation for three samples.

Figure 4. Comparison of observed (□) and predicted (-) growth of *L. monocytogenes* in the rind (a, c) and in the core (b, d) of challenge test one (a, c) and two (b, d) with white mould-ripened cheese. Solid lines represent the predicted growth by Eq. (24). Graphs include the ASZ (±0.5 Log cfu/g, grey dashed lines). The temperature profiles are shown as blue lines.

Figure 5. Comparison of observed ($\square$) and predicted (-) growth of *L. monocytogenes* in the rind of two different batches of smear-ripened cheese (a, b), mould-ripened cheese (c) and gorgonzola slices (d-i). Solid lines represent the predicted growth by Eq. (24). Graphs include the ASZ (± 0.5 Log cfu/g, grey dashed lines). Data from Schwartzman et al. (2014) (a, b, c) and from Dalzini et al. (2017) (d-i). Growth of *L. monocytogenes* ATCC19115 (d-f) and *L. monocytogenes* Lm273250 (g-i) in batch A (d, g), batch B (e, h) and batch C (f, i) of Gorgonzola cheese.

Highlights

- Extensive evaluation of models to predict growth of *L. monocytogenes* in mould/smear-ripened cheeses
- Model including the effect of temperature, pH, NaCl/a_w, lactic and acetic acid concentrations
- Predictions can facilitate estimation of safe shelf-life for mould/smear-ripened cheeses

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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