



Predictive Food Microbiology - new models for safety and quality assessment of a broad range of dairy products

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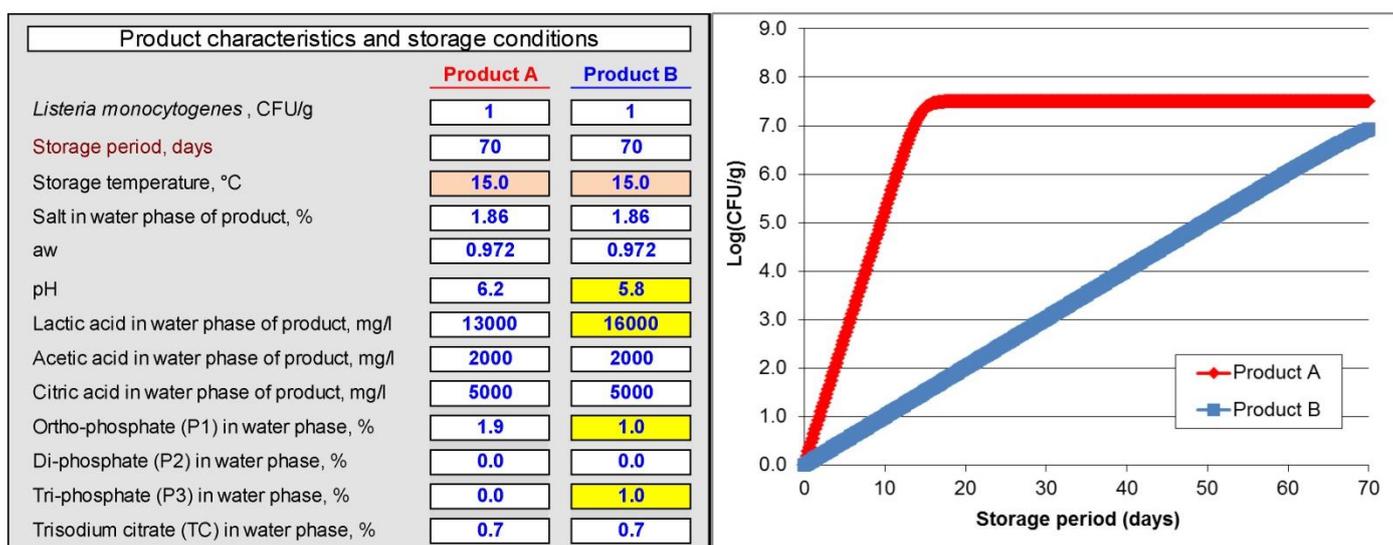
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Predictive Food Microbiology

- new models for safety and quality assessment of a broad range of dairy products



Veronica Martinez Rios
PhD Thesis
March 2019

Preface

The present PhD-project has been carried out at the National Food Institute (DTU Food) in Kgs. Lyngby, Denmark under the supervision of Professor Paw Dalgaard. The presented work was financed by the Danish Dairy Research Foundation and the Danish Veterinary and Food Administration.

I wish to thank Paw Dalgaard for his support, many valuable discussions, endless patience when correcting the papers included in this thesis and for providing me with the great opportunity to work in the field of predictive microbiology. I would like to thank Elissavet Gkogka for her enthusiasm throughout the project, support and immediate action when I asked for products, cultures or information regarding industrial processing of cheeses. Thanks to Chemist Mikael Pedersen and Professor Jørn Smedsgaard for support collaboration with the Analytical Food Chemistry group which was both educational and enjoyable. I wish to thank Tina Dahl Devitt for her skillful and valuable technical assistance. I would also like to thank Per Rosshaug and Kiki Ahlstøm for many conversations of non-scientific topics. Incommensurate thanks to my friend Annai for her contribution to this thesis translating the summary from English to Danish. And finally I want to thank Ioulia Koukou, Govand Babae and Jonas S. Sørensen for their support during the writing process of this thesis.

And lastly I would like to thank my partner, Salva. I will always be in debt with you. Only your unconditional support, patience and care of our very active and energetic boy as a single dad made the process of writing this thesis possible - you and Joel are the best of my life.

Summary

This PhD-thesis focused on development of mathematical models to predict growth of spoilage and pathogenic bacteria in a broad range of dairy products. The studied products included milk and smear-, veined-, ripened-, brined-, fresh-, cream-, processed- and chemically acidified cheeses. These products are of significant economic importance to the Danish dairy sector, therefore, management of psychrotolerant pseudomonads and *Listeria monocytogenes* in the studied products is important as their growth affects product shelf-life and safety. Furthermore, prevalence of *L. monocytogenes* in different types of cheeses was studied by a systematic review and meta-analysis.

The systematic review of the literature showed that *L. monocytogenes* primarily is involved in outbreaks related to smear- or fresh cheese. The data collected for prevalence of *L. monocytogenes* in different types of European cheeses revealed that the highest mean prevalence was observed for smear cheese. Meta-analysis of the results demonstrated that prevalence of *L. monocytogenes* in cheeses produced with un-pasteurized milk was similar to those produced with pasteurized milk highlighting the importance of post-pasteurization contamination.

Existing predictive models for *L. monocytogenes* were evaluated for their ability to predict growth in different types of cheeses including smear, veined, ripened, fresh and brined cheeses. Predictions were compared with growth responses of *L. monocytogenes* extracted from literature studies where constant storage temperature and constant product characteristics were assumed. Two models were identified as being able to accurately predict growth of *L. monocytogenes* in smear cheese and brined cheese, respectively. Challenge tests were performed to collect *L. monocytogenes* growth data and dynamic product characteristics for smear cheese during production, ripening and

storage. Growth of *L. monocytogenes* in smear cheese was correctly predicted from changes in storage temperature and changes in product characteristics by using an existing mathematical model including the effect of temperature, pH, lactic acid concentration in water phase and water activity.

The effect of temperature on the minimum pH that supports growth of *L. monocytogenes* (pH_{min}) was quantified in broth studies and by using literature data obtained with different pH-values and different constant temperatures. A model was developed to describe the effect of temperature on the minimum pH for *L. monocytogenes* growth. A growth and growth boundary model was developed by substituting the constant pH_{min} -value present in an existing model by the new pH_{min} -term. Challenge tests where *L. monocytogenes* was inoculated in chemically acidified cheese (glucono-delta-lactone; GDL) and cream cheese were performed to collect growth responses in low pH foods. In addition, literature data for growth of *L. monocytogenes* in products with or without GDL were collected. Growth rates of *L. monocytogenes* were accurately predicted by the new model in a broad range of foods. Growth and no-growth responses of *L. monocytogenes* in seafood, meat, non-fermented dairy products as well as fermented cream cheese were 90.3% correctly predicted with the incorrect predictions being 5.3% fail-safe and 4.4% fail-dangerous. The new model can support product development, reformulation or risk assessment of a wide variety of foods including meat, seafood and different dairy products (milk, cream, desserts, chemically acidified cheese and cream cheese) with pH-values as low as 4.6.

The antimicrobial effect of phosphate salts necessary to produce spreadable processed cheese was examined in broth studies and their inhibiting effect on *L. monocytogenes* growth was modelled. It was concluded that emulsifying salts can be used as additional growth hurdle in spreadable processed cheese in order to prevent growth if the pathogen e.g. is introduced by consumer handling after opening the hot-filled packaged. A mathematical model was developed to

predict the effect of phosphate salts, lactic-, acetic-, citric acid, pH, a_w , temperature and interaction amongst all these factors on growth and the growth boundary of *L. monocytogenes* in spreadable processed cheese. Challenge tests showed that both growth and the growth boundary were accurately predicted by the developed model. The growth and growth boundary model correctly predicted 54 of 60 growth and no growth responses of *L. monocytogenes* in spreadable processed cheese. The developed model can be used by the dairy sector to facilitate formulation of safe recipes and this approach seems faster and more cost effective than the traditional challenge testing.

The model described in the previous paragraph was further expanded to contain a term to account for the inhibiting effect of nisin A added as preservative to processed cheese. The antimicrobial activity of nisin A against *L. monocytogenes* was quantified in broth studies and additional antimicrobial data, obtained at different pH-values, were collected from the literature. A nisin-term was developed to describe the effect of pH on the antimicrobial activity of nisin A. Furthermore, a liquid chromatography/mass spectrometry (LC-MS/MS) method was developed and validated to quantify nisin A and Z present in cheese. Challenge tests were performed to generate data for model evaluation. When the quantified residual nisin A concentrations measured by LC-MS/MS in cheese were used as model input this resulted in accurate predictions of growth for *L. monocytogenes*. The model can support risk assessment and product development, but further studies with higher residual concentrations of nisin A in cheeses will be beneficial for model validation.

A growth boundary model for psychrotolerant pseudomonas in cottage cheese with cultured cream dressing, raw milk and heat treated milk was developed and validated. The model included terms for the effect of temperature, pH, NaCl/ a_w , lactic-, sorbic acid and interaction among all

factors can be used e.g. to optimized cottage cheese formulations to inhibit growth of psychrotolerant pseudomonads.

The present PhD-project has developed/validated five new predictive growth and growth boundary models that are ready to be applied by the dairy sector. This represents important progress for the use of predictive food microbiology models with dairy products. For further progress in this area there is a need for the dairy sector to increase attention on more detailed chemical product characterization of those dairy products where it is relevant to predict microbial responses.

Sammendrag (summary in Danish)

Denne ph.d-afhandling har fokus på udvikling af matematiske modeller til at forudsige vækst af fordærvelses- og patogene bakterier i en bred vifte af mejeriprodukter. De undersøgte produkter omfattede mælk samt smøre-, skimmel-, modnet-, frisk-, smelte-, fløde-, kemisk syrnede ost og ost i saltlage. Disse produkter har en væsentlig økonomisk betydning for den danske mejerisektor. Derfor er styrning af kuldolerante pseudomonader og *Listeria monocytogenes* vigtig da disse bakterier påvirker produkternes holdbarhed og sikkerhed. Desuden blev forekomst af *L. monocytogenes* i forskellige type oste undersøgt ved en systematisk litteraturgennemgang og meta-analyse.

Den systematiske gennemgang af litteraturen viste, at *L. monocytogenes* primært er involveret i udbrud af listeriose relateret til smøreost og frisk ost. Indsamlede data for forekomst af *L. monocytogenes* i forskellige typer af europæiske oste, viste, at den højeste gennemsnitlige forekomst blev observeret for smøreost. Meta-analysen af resultaterne viste, at forekomsten af *L. monocytogenes* i oste produceret med ikke-pasteuriseret mælk ikke var forskellig fra forekomsten i oste produceret med pasteuriseret mælk. Dette understreger betydningen af kontaminering med *L. monocytogenes* efter pasteurisering.

Eksisterende prædiktive modeller for *L. monocytogenes* blev vurderet for deres evne til at forudsige vækst af denne bakterie i forskellige typer af ost, herunder smøre-, skimmel-, modnet-, og frisk ost samt ost i saltlage. Forudsigelser blev sammenlignet med vækstrespons for *L. monocytogenes* fra den videnskabelige litteratur, hvor konstant opbevaringstemperatur og konstante produkttegenskaber var antaget. To modeller blev identificeret til, med acceptabel præcision, at kunne forudsige vækst af *L. monocytogenes* i, henholdsvis, smøreost og ost i saltlage. Belastningsundersøgelser, blev udført med det formål at indsamle *L. monocytogenes* vækstdata og

dynamiske produkttegenskaber for smøreost under produktion, modning og opbevaring. Vækst af *L. monocytogenes* blev korrekt forudsagt ud fra ændringer i opbevaringstemperatur og ændringer i produkttegenskaber ved anvendelse af en eksisterende matematiske model. Denne model indeholdt effekten af temperatur, pH, mælkesyrekoncentration i vandfasen samt vandaktivitet.

Effekten af temperatur på den minimale pH-værdi for vækst af *L. monocytogenes* (pH_{min}) blev kvantificeret i bouillonforøg samt ved anvendelse af litteratur-data opnået ved forskellige pH-værdier og ved forskellige konstante temperaturer. En model blev udviklet til at beskrive effekten af temperatur på pH_{min} for *L. monocytogenes*. En vækst og vækts-grænse model blev udviklet ved at erstatte den konstante pH_{min} -værdi i en eksisterende model med det nye pH_{min} -led. Belastningsundersøgelser med *L. monocytogenes* podet i ost, der var kemisk syrnede med gluconodelta-lactono (GDL) samt i flødeost blev udført til evaluering af den nye model i ost med lavt pH. Derudover blev litteraturdata for vækst af *L. monocytogenes* i produkter med eller uden GDL indsamlet. Væksthastigheden af *L. monocytogenes* blev nøjagtig forudsagt af den nye model for en bred vifte af fødevarer. Vækst og ikke-vækstrespons af *L. monocytogenes* i fisk og skaldyr, kød, ikke-fermenterede mejeriprodukter samt fermenteret flødeost var 90.3% korrekt forudsagt, med 5.3% 'fail-safe' forudsigelser og 4.4% 'fail-dangerous' forudsigelser. Den nye model kan understøtte produktudvikling, om-formulering samt risikovurdering af en bred vifte af fødevarer med pH over 4.6, herunder kød, fisk, skaldyr og forskellige mejeriprodukter (mælk, fløde, desserter, kemisk syrnede ost og flødeost).

Smeltesalte, primært fosfat- og citrat-salte, anvendes ved fremstilling smelteost. Den antimikrobielle effekt af smeltesalte blev undersøgt i forsøg med flydende bouillon og den væksthæmmende effekt på *L. monocytogenes* blev beskrevet med en matematisk model. Det blev konkluderet, at smeltesalte kan anvendes som supplerende væksthæmmere i smelteoste. Dette er relevant da disse varmfyldte produkter kan kontamineres med *L. monocytogenes* i forbindelse med

forbrugerhåndtering efter åbning af emballagen. En matematiske model blev udviklet for at forudsige effekten af fosfat-salte, mælkesyre, eddikesyre, citronsyre, pH, vandaktivitet, temperatur samt den hæmmende effekt af interaktionen mellem alle disse faktorer på vækst og vækstgrænse for *L. monocytogenes* i smelteost. Belastningsundersøgelser viste, at både vækst og vækstgrænse blev forudsagt med acceptabel præcision af den udviklede model. Specifikt forudsage den udviklede vækst og vækstgrænse model 54 ud af 60 vækstrespons for *L. monocytogenes* korrekt i smelteost. Den udviklede model kan bruges af mejerisektoren til at lette formulering af sikre opskrifter, og denne tilgang er hurtigere og billigere end traditionelle belastningsundersøgelser.

Modellen for smelteoste blev yderligt udvidet med et led for den inhiberende virkning af nisin A tilsat som konserveringsmidler til smelteost. Den antimikrobielle aktivitet af nisin A mod *L. monocytogenes* blev kvantificeret i bouillon og antimikrobielle data, blev indsamlet for forskellige pH-vardier fra den videnskabelige litteratur. Et nisin-led blev udviklet til at beskrive effekten af pH på den antimikrobielle aktivitet af nisin A. Endvidere blev en væskechromatografi/massespektrometri (LC-MS/MS) metode udviklet og valideret til at kvantificere nisin A og Z i oste. Belastningsundersøgelser blev udført for at generere data til modevaluering. Når den målte resterende koncentration af nisin A i ost var anvendt som model-input blev vækst vækstrespons for *L. monocytogenes* forudsagt med god præcision. Den udviklede vækstmodel kan understøtte risikovurdering og produktudvikling, men yderligere undersøgelser med højere restkoncentrationer af nisin A i oste vil være gavnelige til validering af modellen.

En vækst-grænsemiddel for psykotolerante pseudomonas i mælk og hytteost blev udviklet og valideret. Modellen indeholdt effekten af temperatur, pH, NaCl/vandaktivitet, mælkesyre, sorbinsyre samt effekten af interaktion mellem alle disse faktorer. Modellen kan anvendes f.eks. til at optimerede formulering af hytteost således at vækst af kuldetolerante pseudomonas hæmmes.

Dette ph.d.-projekt har udviklet og valideret fem nye prædiktive vækst og vækstgrænsemodeller, der er klar til at blive anvendt af mejerisektoren. Dette repræsenterer et vigtigt fremskridt med hensyn til anvendelse af prædiktive mikrobiologiske modeller for mejeriprodukter. I forhold til yderligere udvikling på dette område er der behov for at mejerisektoren fremover gør en større indsats med mere detaljeret kemisk produktkarakterisering af de mejeriprodukter, hvor det er relevant at forudsige mikrobiologisk vækst og overlevelse.

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The present thesis is based in the following papers:

Paper I:

Martinez-Rios, V., Dalgaard, P., (2018). Prevalence of *Listeria monocytogenes* in European cheeses: A systematic review and meta-analysis. *Food Control* 84, 205-214.

Paper II:

Martinez-Rios, V., Gkogka, E., Dalgaard, P., (2019). Evaluation of predictive models for growth of *Listeria monocytogenes* in cheeses. Manuscript in preparation.

Paper III:

Martinez-Rios, V., Gkogka, E., Dalgaard, P., (2019). New term to quantify the effect of temperature on pH_{min} -values used in cardinal parameter growth models for *Listeria monocytogenes*. *Frontiers in Microbiology*. Submitted.

Paper IV:

Martinez-Rios, V., Jørgensen, M.Ø., Koukou, I., Gkogka, E., Dalgaard, P., (2019). Growth and growth boundary model with terms for melting salts to predict growth responses of *Listeria monocytogenes* in spreadable processed cheese. *Food Microbiology*. Submitted.

Paper V:

Martinez-Rios, V., Pedersen, M., Pedrazzi, M., Gkogka, E., Smedsgaard, J., Dalgaard, P., (2019). Cardinal parameter model containing nisin term to predict growth of *Listeria monocytogenes* in processed cheese. *International Journal of Food Microbiology*. Submitted.

Paper VI:

Martinez-Rios, V., Østergaard, N.B., Gkogka, E., Rosshaug, P.S., Dalgaard, P., (2016). Modelling and predicting growth of psychrotolerant pseudomonads in milk and cottage cheese. *International Journal of Food Microbiology* 216, 110-120.

1. Introduction

Dairy products are a major component of the daily European diet and due to their abundant nutrient elements such as protein, fat, minerals and vitamins they are included as core elements of dietary recommendations worldwide (FAO, 2013; Nicklas et al., 2009; Park and Henlein, 2013). Dairy products constitute a broad range of chilled or ambient stored ready-to-eat (RTE) foods with few common characteristics other than being produced from milk. Dairy products can be produced with different (i) types of milk (e.g. cow, sheep and goat), (ii) heat treatments (e.g. none, low and high pasteurization, ultra-high temperature), (iii) acidification methods (e.g. none, lactic acid bacteria or chemical), (iv) maturation periods (e.g. none to 3 years) and (v) microbial complexity. Therefore, the broad range of available products in the market challenges the dairy industry to find the best ways to prevent spoilage and pathogenic microorganisms from entering a product or preventing growth of those that escaped good manufacturing practices. The present PhD thesis focused on fresh-, smear-, veined-, ripened-, brined-, cream-, processed-, chemically acidified cheeses and milk. These products are all of particular importance for the Danish dairy industry and models presented in this PhD-thesis can support product development by simulating optimum formulations that reduce or prevent growth for *Listeria monocytogenes* and psychrotolerant pseudomonads.

Since the removal of EU quota in 2015 large amounts of excess milk has become available in Denmark. A competitive solution for the excess milk is the production of cheese which has higher revenue than milk. Denmark is the fifth cheese exporter in the world (5.6% of the total world exports) with total revenue of 1.7 billion US\$. The Danish dairy industry is establishing in new markets where consumption exceeds production of dairy products (e.g. Asia, Africa and Middle East). For instance, Asian countries (such as Japan, China and South Korea) account for an 8.1% of

the total Danish cheese exports (OEC, 2017). However, some of the new markets may present challenges regarding control of the cold chain and the dairy products formulation might need to be optimized to achieve the desired shelf-life in the intended distribution chain.

Furthermore, in September 2015, the United Nations (UN) announced a goal of halving worldwide food waste and substantially reducing global food loss by 2030 as part of its Sustainable Development Goals (SDG) agenda. For instance, in Europe every year more than 29 million tons of dairy products go to waste with the majority of waste happening in private households (FAO, 2019). Not only this pose a significant economic and nutritional loss, it also comes at an environmental cost as dairy products have relatively high greenhouse gas emission (kg CO₂/ kg produce) (Clune et al., 2017). Therefore, extension of shelf-life has the potential for reducing food waste across the food supply chain. Increased shelf-life may help retailers sell more of a dairy product before it expires. While increasing the open shelf-life will get consumers more time to consume a product prior its expiration date and in turn reduce food waste. However, this poses a higher risk for potential growth of spoilage or pathogenic bacteria introduce during production or by consumers and thereby the need to reformulate dairy products.

Growth in dairy products of the pathogenic bacteria *L. monocytogenes* and the group of spoilage psychrotolerant pseudomonads have been extensively described in literature (Angelidis et al., 2010; Brocklehurst and Lund, 1985; Dalzini et al., 2017; Kapetanakou et al., 2017; Ryser et al., 1985; Ryser and Marth, 1989; Sørhaug and Stepaniak, 1997; Valero et al., 2018; Yousef and Marth, 1988). However, information about product characteristics (e.g. pH, NaCl/aw, organic acids, emulsifying salts, bacteriocins) that most likely affect growth is not reported in most of the studies. Therefore, it is necessary to obtain precise physico-chemical information of dairy products to be able to identified potential preserving parameters that will reduce or prevent growth of *L.*

monocytogenes or psychrotolerant pseudomonads. In relation to the safety of dairy products, identification of product characteristics that prevents growth of *L. monocytogenes* is an interesting perspective (EC, 2005) and possibility for extending the shelf-life or open shelf-life of dairy products. In fact, FAO/WHO and FDA/USDA risk assessment models predicted that the greatest impact for reducing listeriosis will be achieved by preventing growth to high numbers (FAO, 2004; FDA, 2003). Therefore, reformulating foods so they retard or do not support growth of *L. monocytogenes* is one of the recommended approaches to reduce listeriosis cases (ILSI, 2005).

The food industry can use durability studies or challenge tests to assess the effect of product recipes and storage conditions on growth of relevant spoilage or pathogenic microorganisms (EC, 2005; Membré and Lambert, 2008; Legan et al., 2009). However, this is a time consuming and expensive approach (Walker, 2000; Zwietering et al., 1996). Extensive and validated mathematical models containing the most important factors affecting growth of spoilage or pathogenic organisms potentially present in dairy products can be used to optimized product formulation. However, none of the available models includes those factors most likely to affect growth of *L. monocytogenes* or psychrotolerant pseudomonads in dairy products (see Tables 9 and 10).

The main objectives of the present PhD-project were to evaluate, develop and validate predictive models for safety and quality assessment of a broad range of dairy products. A systematic review and meta-analysis of *L. monocytogenes* data in European cheese was carried out to estimate mean prevalence of the pathogen and to compare prevalence among types of cheeses (Paper I). Secondly, the ability of existing *L. monocytogenes* models to predict growth in different types of cheese (smear-, veined, ripened, brined, fresh cheese) was evaluated with data collected from challenge tests and from literature (Paper II). Thirdly, challenge tests with chemically acidified cheese, cream cheese, processed cheese and cottage cheese were carried out to obtain precise

psysico-chemical information of cheeses and to evaluate how product characteristics affect growth of *L. monocytogenes* and psychrotolerant pseudomonads (Paper III, IV, V, VI). Lastly, mathematical models were developed to predict growth of *L. monocytogenes* and psychrotolerant pseudomonads in a broad range of dairy products as a function of the relevant product characteristics (Paper II, III, IV, V, VI).

The first section of this PhD thesis describes relevant issues in relation to the pathogenic bacteria *L. monocytogenes* and the spoilage psychrotolerant pseudomonads potentially present in dairy products. For *L. monocytogenes* the organism and its growth characteristics, disease, legislation, prevalence and outbreaks are discussed. For psychrotolerant pseudomonads a description of growth characteristics and spoilage of foods are provided. The next part describes the different dairy products studied in the present PhD thesis in relation to dairy specific ingredients and its relevance in connection with the studied bacteria. The fifth section reviews predictive food microbiological models available for the studied bacteria. Lastly, the application of validated predictive models for the quality and safety management of dairy products developed throughout the present PhD-project are presented and discussed.

2. Microbial safety of dairy products

Several bacterial pathogens such as *L. monocytogenes*, *Salmonella enterica*, *Escherichia coli* O157:H7, *Campylobacter*, *Staphylococcus aureus* and *Clostridium botulinum* are relevant regarding the safety of different dairy products. These microbial hazards have been reviewed extensively in literature (Cretenet et al., 2011; D'Amico and Donnelly, 2017; Farrokh et al., 2013; Verraes et al., 2015).

L. monocytogenes is the major concern for the dairy industry since it can be potentially present in the milk, in the dairy processing environment and consequently cross-contaminate pasteurized products. If this occurs, products characteristics and storage conditions of a wide range of dairy products are insufficient to prevent growth (Donnelly, 2018; Pérez-Rodríguez et al., 2017; FAO/WHO, 2004). Furthermore, this pathogen is considered by artisan cheese makers as the main biological hazard due to its severe impact in human health and consequences for food business (Le et al., 2014).

The safety of dairy products is discussed in the following section with focus in *L. monocytogenes*, its growth characteristics, prevalence, outbreaks, disease, and legislation.

2.1. *Listeria monocytogenes*

L. monocytogenes belongs to the genus *Listeria* along other 18 species subdivided in two major groups: (i) *Listeria sensu stricto*, constituted by the species *Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria marthii* and (ii) *Listeria sensu lacto*, constituted by the species *Listeria grayi* and *Listeria booriae* among others (Chiara et al., 2015; Leclercq et al., 2019; Núñez-Montero et al., 2018; Orsi and Wiedmann, 2016). Only two species are considered pathogens, *L. monocytogenes* which infects humans along with animals, and

L. ivanovii which infects other mammals. Nevertheless, there have been some reports of *L. seeligeri* and *L. ivanovii* causing illness in humans (Cummins et al., 1994; Guillet et al., 2010; Rocourt et al., 1986; Snapir et al., 2006).

L. monocytogenes is a Gram-positive facultative anaerobe, non-spore forming rod-shaped bacterium with a typical length between 0.5 and 2 μm (Holt et al., 1994). It is capable of growing or surviving in environments with high salt concentration (up to 13-16%), a wide pH range (pH 4.4 to 9.5) and temperatures -0.4 to 45°C (ICMSF, 1996). *L. monocytogenes* is relatively sensitive to heat, but heat resistance can vary depending on strains and food products (Aryani et al., 2015; van Lieverloo et al., 2013).

The psychrotolerant nature poses a challenge for ready-to-eat (RTE) foods with relative long shelf-life (such as cheese and other dairy products). However, the ability of *L. monocytogenes* to multiply at refrigeration temperature is strongly pH dependent (van der Veen et al., 2008; Tienungoon et al., 2000; Lado and Yousef, 2007; Paper III). *L. monocytogenes* is wide spread in nature (e.g. soil, plants and water). Importantly it has also been isolated within the dairy plant environment from floors, drains, freezers, equipment and ripening wooden shelf among other places where is able to persist (D'Amico and Donnelly, 2008; Parisi et al., 2013; R ckerl et al., 2014, Spanu et al., 2015). Biofilm formation is essential for survival and persistence of *L. monocytogenes* in the food processing environment, where cleaning and disinfection are daily routines (Tr moulet et al., 2002). Muhterem-Uyar et al. (2015) demonstrated that *L. monocytogenes* is a common colonizer of some European cheese processing facilities and that a consistent cross-contamination risk exists. Almeida et al. (2013) evaluated the presence of *L. monocytogenes* within three different size processing plants, an artisanal producer, a small-scale industrial producer and an industrial

cheese producer. They found that the percentage of positive samples (11%, 7% and 0.2%) for *L. monocytogenes* decreased with the increase in the size of the cheese plant.

2.2. Listeriosis

Listeriosis is a rare but serious disease caused by *L. monocytogenes*. The main route of *L. monocytogenes* transmission is through consumption of contaminated food. The disease has a well-defined risk group that includes the elderly, pregnant women and their neonates as well persons with chronic debilitating illnesses that impair their immune system including cancer, diabetes or alcoholism, HIV/AIDS patients and persons taking immunosuppressive medication (transplant patients) (FAO/WHO, 2004). Listeriosis can manifest depending on the barrier crossed by the pathogen as an (i) isolated bacteraemia (intestinal barrier) or (ii) invasive bacteraemia (placental and blood-brain barriers) (Maury et al., 2016).

The average number of confirmed cases per 100,000 persons in the USA in 2010 was 0.25 (Pohl et al., 2017) while for Europe in 2017 the number was 0.48 (EFSA, 2018a). Although the case incidence is low, invasive listeriosis has a significant impact on public health due to a high rate of hospitalization (>98%), high fatality rate and long-term morbidity due to the nature of the central nervous system syndromes it causes (Liu, 2008; EFSA, 2018a). Fatality rates have been reported between 14 to 54% (Farber and Peterkin, 1991; Filipello et al., 2017; Linnan et al., 1988; Mead et al., 1999; Siegman-Igra et al., 2002). Noordhout et al. (2014) assessed the global burden of listeriosis in 2010 by meta-analysis and estimated 23,150 illnesses, 5,463 deaths and 172,823 disability-adjusted life-years (DALYs). The proportion of perinatal cases was estimated in a 20.7%. Lately, the European Food Safety Authorities have reported fatality rates of 18% in 2015 (EFSA, 2016) and 14% in 2017 (EFSA, 2018a).

The outcome from a listeriosis infection is also related to the serotype of the infecting strain (Lamont et al., 2011; Maury et al., 2016). To this date, there have been described 12 serotypes of *L. monocytogenes* (1/2a, 1/2b, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7). Serotype 4b occurs more frequently among clinical isolates than serotypes 1/2b, 1/2a and 1/2c; however its relative frequency depends on the food categories studied (Jacquet et al., 2004; Maury et al., 2016). McLauchlin (1990) demonstrated that serotype 4b is predominant among clinical patients; however, serotypes were unequally distributed among three categories, (i) pregnancy associated cases, (ii) non-pregnant previously healthy and (iii) non-pregnant with severe underlying illness. Serotype 1/2b was mostly associated with non-pregnant persons having underlying illness and 1/2c was the least frequent in the pregnancy associated cases. Serotype 4b occurred more frequently in the pregnant associated cases followed by non-pregnant previously healthy and non-pregnant with severe underlying illness. Maury et al. (2016) studied the distribution of *L. monocytogenes* clonal complexes from clinical and food isolates. They demonstrated that clonal complexes belonging to serotypes 1/2b, 1/2a and 1/2c were more often isolated from highly immune-compromised patients, whereas serotype 4b was more prevalent among patients with few or no immunosuppressive comorbidities.

2.3. Prevalence and outbreaks caused by *L. monocytogenes* presence in cheeses

Prevalence of *L. monocytogenes* reported in cheese is very low. Specific information about the presence of *L. monocytogenes* in different ready-to-eat (RTE) products at retail was collected by the EU baseline survey performed during 2010 and 2011 (EFSA, 2013). Specifically, the EU prevalence for *L. monocytogenes* in cheese at retail was 0.47% (confidence interval (CI): 0.29-0.77%). However, other studies have reported higher prevalence of *L. monocytogenes* in cheese at the European retail level (Ianneti et al., 2016; O'Brien et al., 2009; Prencipe et al., 2010). Filiouis et al. (2009) reported an 8% prevalence of *L. monocytogenes* in cheeses obtained from open-air

Greek markets and Schoder et al. (2015) reported a 2% prevalence of *L. monocytogenes* in European cheese purchased online. Furthermore, from August 2015 to January 2019, 68 notifications on *L. monocytogenes* in cheese products were recorded in the EU Rapid Alert System for Food and Feed (RASFF), with 37 of them not been related to raw milk cheese (RASFF, 2019). In 2015, two of those notifications for presence of *L. monocytogenes* were in grated cheese and processed cheese. And lately, there have been notifications for presence in mozzarella di buffalo and grated mozzarella all indicating potential cross-contamination after pasteurization. Paper I summarized available data on prevalence of *L. monocytogenes* in different types of cheese produced in Europe. Meta-analysis models were used to estimate mean prevalence of the pathogen, prevalence among types of cheeses and prevalence for cheeses produced with pasteurized and non-pasteurized milk. Data for a total of 130,604 samples of cheese collected between 2005 and 2015 in Europe were analysed. Paper I found no significant difference in prevalence between pasteurized and non-pasteurized milk cheese, supporting the importance of cross-contamination after pasteurization for the presence of *L. monocytogenes* in cheese. Interestingly, a prevalence more than three times higher from scientific literature (2.3%; CI: 1.4-3.8%) than from EFSA reports (0.7%; CI: 0.5-1.1%) was estimated. Other studies about the prevalence of *L. monocytogenes* in non-European cheeses have found values between 0 and 12% (El Marnissi et al., 2013; Gebretsadik et al., 2011; Ismaiel et al., 2014; Reda et al., 2016; Torres-Vitela et al., 2012; Brito et al., 2008). Recently, Churchill et al. (2019) estimated a worldwide *L. monocytogenes* prevalence in soft cheese of 2.4% (95% CI: 1.6 to 3.6%); however they concluded that the substantial heterogeneity between studies indicates that the use of global summary prevalence estimates for risk assessments are not advisable.

The first reported outbreak of human listeriosis associated with consumption of fresh cheese produced with raw milk occurred during 1985 in the USA. A total of 142 cases were identified with 48 of them resulting in fatality (Linnan et al., 1988). However, a previous outbreak occurred in Switzerland between 1983 and 1987 due to consumption of smear cheese produced from pasteurized milk and caused 122 cases of listeriosis including 31 deaths (Büla et al., 1995; Bille et al., 2006). During 2009 and 2010, 34 cases of listeriosis in Austria, Germany and Czech Republic were associated with consumption of “quargel” which is a pasteurized milk cheese. The outbreak caused eight fatalities (Fretz et al., 2010; Rychli et al., 2014). In 2012, 22 cases of listeriosis (including 4 fatalities) occurred in the USA in relation with ricotta salatta manufacture in Italy. This cheese is produced by heating whey at 85°C for 30 min (Coroneo et al., 2016). An overview of cheese associated outbreaks reported in literature and databases is given in Table 1.

Although much attention is often paid to raw milk cheese, *L. monocytogenes* can be present in pasteurized milk cheese as shown above. Furthermore, Gould et al. (2014) demonstrated that eight out of the 12 confirmed outbreaks caused by consumption of *L. monocytogenes* contaminated cheese in the USA between 1998 and 2011 were caused by consumption of pasteurized milk cheese. Additionally, it is unclear if higher numbers of *L. monocytogenes* will be reached in pasteurized or raw milk cheese (D’Amico and Donnelly, 2008; Schwartzman et al., 2011), making raw milk cheeses and pasteurized cheeses equally important at risk products for listeriosis (Lahou and Uyttendaele, 2017).

New models for safety and quality assessment of a broad range of dairy products

Table 1. Overview of listeriosis outbreaks implicating cheese during the period from 1983 to 2019^a.

Country	Year	Serotype	No. ^b of cases (fatalities)	Implicated food	References
Switzerland	1983-1987	4b	122(31)	Smear cheese (Vacherin Mont d'Or)	Büla et al. (1995); Bille et al. (2006)
USA	1985	4b	142(48)	Fresh cheese (Queso Fresco)	Linnan et al. (1988)
Luxembourg	1989	NR ^c	2(0)	Smear cheese (Camembert)	Ries et al. (1990)
Denmark	1989-1990	4b	26(6)	Veined or ripened cheese	Jensen et al. (1994)
France	1995	4b	37(11)	Smear cheese (Brie de Meaux)	Goulet et al. (1995) Arnold and Coble (1995)
France	1997	4b	14(? ^e)	Smear cheese (Pont l'Évêque)	Ryser and Marth (2007); Goulet et al., 2013
USA	2000	4b	13(5)	Non-commercial fresh cheese (Queso Fresco)	MacDonald et al., 2005
Sweden	2001	1/2a	≥120(0)	Fresh cheese	Carrique-Mas et al., 2003; Danielsson-Tham et al., 2004
Japan	2001	1/2b	38(0)	Smear cheese	Makino et al., 2005
Canada	2002	4b	47(0)	Soft and semi-soft cheese	Gaulin et al., 2003
Canada	2002	4b	86(0)	Cheese made from pasteurized milk	Pagotto et al., 2006
Switzerland	2005	1/2a	10 (3+2 ^d)	Smear cheese (Soft "Tomme")	Bille et al., 2006
USA	2005	NR ^c	9(?)	Fresh cheese (Queso fresco)	FIOD, 2005
Czech Republic	2006		78(13)	Soft cheese	EFSA, 2007
Germany	2006-2007	4b	189(26)	Acid curd cheese	Koch et al., 2010
Norway	2007	NR ^c	17(3)	Smear cheese (Camembert)	Johnsen et al., 2010
Chile	2008	NR ^c	91(5)	Smear cheese (Brie)	Promed, 2008
Canada	2008	NR ^c	38(5)	Cheeses	Gaulin and Ramsay, 2010

New models for safety and quality assessment of a broad range of dairy products

USA	2008	1/2a	8(0)	Fresh cheese (Oaxaca cheese)	Jackson et al., 2011
Austria-Germany-	2009-2010	1/2a	34 (8)	Fresh cheese(Quargel)	Fretz et al., 2010; Rychli et al., 2014
Portugal	2009- 2012	4b	30 (11)	Fresh cheese (Cured cheese and queijo fresco)	Magalhães et al., 2015
USA	2010	NR ^c	5(0)	Fresh cheese (Panela, queso fresco, Requeson)	FIOD, 2010
USA	2010-2015	NR ^c	28(3)	Fresh cheeses	FIOD, 2015a
USA	2011	NR ^c	2(?^e)	Fresh cheese (Chives cheese)	FIOD, 2011
Austria-Germany	2011-2013	1/2b	7(? ^e)	Fresh cheese	Schmid et al., 2014
Spain	2012	1/2a	2(0)	Fresh cheese (Queso fresco)	De Castro et al., 2012
USA	2012	NR ^c	22(4)	Brined cheese (Ricotta salata)	CDC, 2012; Coroneo et al., 2016
USA	2013	NR ^c	5(1)	Smear cheese (Les Freres)	FIOD, 2013
Australia	2013	NR ^c	18(? ^e)	Smear cheese	NSW, 2013
USA	2013-2014	NR ^c	4 (1)	Fresh cheese	FIOD, 2014a
USA	2014	NR ^c	7(1)	Fresh cheese	FIOD, 2014b
USA	2015	NR ^c	3(1)	Fresh cheese (Panela, Queso Fresco, Requeson, Cotija)	FIOD, 2015b
USA	2017	NR ^c	8(2)	Smear cheese	CDC, 2017
Spain	2018	NR ^c	1(0)	Raw milk cheese	AECOSAN, 2018

^a Modified from Paper I

^b Number of listeriosis cases

^c Serotype not reported (NR)

^d Septic abortion i.e. fatality

^e Fatalities uncertain

Various authors have suggested that cross-contamination in homes and in food-service establishments could well be the major contributing factor to sporadic and epidemic food-borne illnesses (Beumer and Kusumaningrum, 2003; Bloomfield, 2003; Chen et al., 2001). In relation to cross-contamination in homes Beumer and te Giffel (1999) investigated presence of foodborne pathogens in 250 domestic kitchens and found *L. monocytogenes* in 10% of the investigated kitchens. Furthermore, Heiman et al. (2016) studied a listeriosis outbreak caused by ricotta salata in the USA (Table 1). They hypothesized that ricotta salata may have cross-contaminated multiple types and brands of other cheeses throughout cutting and repackaging at retail and distribution locations. This hypothesis was confirmed firstly when they isolated the outbreak strain exclusively from open samples of different brands and types of cheese other than ricotta salata and secondly most patients did not reported have eaten ricotta salata. Their study highlighted the importance of routine cleaning and disinfection of equipment after cutting each block or cheese wheel to minimize cross-contamination.

2.4. Legislation

The EU regulation 2073/2005 (EC, 2005) laid down specific microbiological criteria for *L. monocytogenes* that food business operator (FBO) must to comply with. The food safety criteria differentiate between foods that are able or unable to support growth of *L. monocytogenes* (Table 2). The FBO shall conduct microbiological studies to investigate compliance with the food safety criteria throughout the shelf-life of the products (EC, 2005). This applies primary for RTE food able to support growth of *L. monocytogenes* and therefore may pose a risk for public health.

For RTE foods intended for infants or for special medical purpose (category 1.1) the EU regulation lays a *L. monocytogenes* criterion of absence (in 10 samples of 25 g) during the declared

shelf-life on the market. If the product supports growth (category 1.2), the FBO must document that if contaminated, *L. monocytogenes* will not exceed the limit of 100 cfu/g throughout the declared shelf-life. Products with (i) $\text{pH} \leq 4.4$ or $a_w \leq 0.92$, (ii) products with $\text{pH} \leq 5.0$ and $a_w \leq 0.94$ and (iii) with a shelf-life of less than five days belong to category 1.3. Other products can also belong to category 1.3 with scientific justification that no-growth of *L. monocytogenes* is possible throughout the declared shelf-life. Such scientific justification can consist of predictive mathematical modelling, durability studies and/or challenge tests (EC, 2005).

Table 2. Microbiological criteria for foods concerning *L. monocytogenes*^a.

Food category	Sampling-plan		Limits ^b		Stage where the criterion applies
	n ^c	c ^d	m	M	
1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes ^d	10	0	Absence in 25 g		Products placed on the market during their shelf-life
1.2. Ready-to-eat foods able to support the growth of <i>L. monocytogenes</i> , other than those intended for infants and for special medical purposes	5	0	100 cfu/g ^e		Products placed on the market during their shelf-life
	5	0	Absence in 25 g ^f		Before the food has left the immediate control of the food business operator, who has produced it
1.3. Ready-to-eat foods unable to support the growth of <i>L. monocytogenes</i> , other than those intended for infants and for special medical purpose	5	0	100 cfu/g		Products placed on the market during their shelf-life

^a EC (2005)

^b m=M

^c n: number of units comprising the sample

^d c: number of sample units giving values over m and M

^d Regular testing against the criterion it is not useful for certain ready-to-eat foods (EC, 2005)

^e The criterion applies only if the manufacture can demonstrate to the satisfaction of the competent authority that the product will not exceed the limit throughout the shelf-life. The producer may fix intermediate limits during the production process that will ensure that the limit will not be exceeded at the end of shelf-life.

^f This criterion applies to products before they have left the control of the producer, when he is not able to demonstrate to the competent authority that the product will not exceed the limit throughout the shelf-life.

The fate of *L. monocytogenes* in RTE foods can be experimentally determined by performing durability studies or challenge tests (Figure 1). According to the technical guidelines for conducting shelf-life studies in relation to *L. monocytogenes* in RTE foods (Beaufort et al., 2014),

durability studies are considered more realistic than challenge tests, as the contamination is naturally occurring. A challenge test studies the growth potential or the maximum growth rate of *L. monocytogenes* in an artificially contaminated food by simulating as closely as possible the storage conditions of the product. In the present PhD-thesis multiple challenge tests were performed to quantify μ_{max} -values of *L. monocytogenes* in smear cheese, chemically acidified cheese, cream cheese, processed cheese and cottage cheese (Paper II - VI).

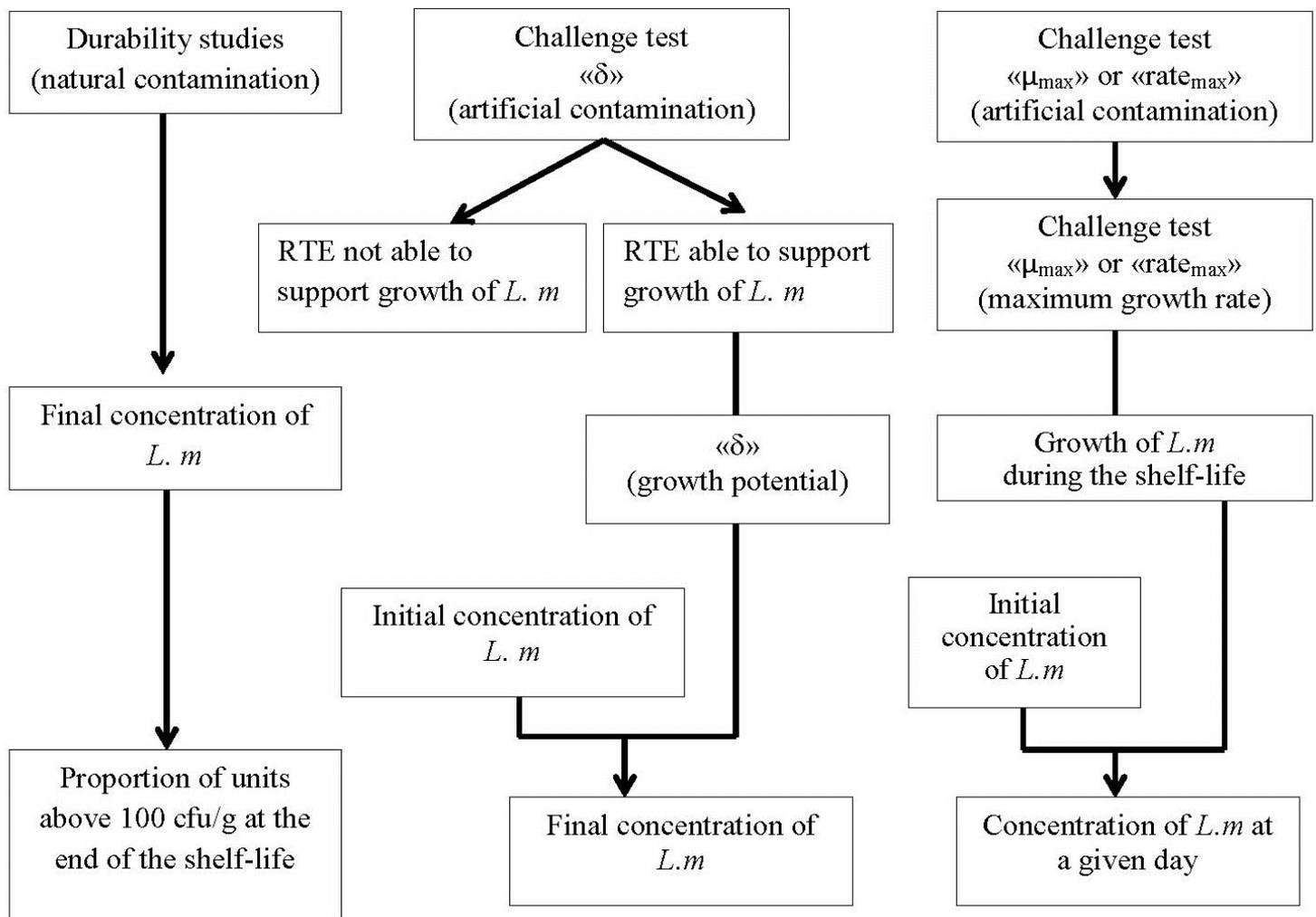


Figure 1. Microbiological procedures for determining the growth of *L. monocytogenes* (*L.m*) using durability studies or challenge tests (Beaufort et al., 2014).

Canada, Australia and New Zealand have similar regulations as the European establishing food safety criteria based on the ability of the food to support or not growth of *L. monocytogenes*. Predictive microbiology can be used following these countries regulations to document that *L. monocytogenes* will not grow in a RTE food (ANZ, 2018; CA, 2011). The *Codex Alimentarius* have proposed similar microbiological criteria for the verification and control of *L. monocytogenes* in RTE foods, keeping in mind the health of consumers while ensuring fair food trade practices (CAC, 2007). On the other hand, the USA applies a zero-tolerance since 1980s, stating that *L. monocytogenes* should not be detected in RTE foods (e.g. 25 g sample). Detection of *L. monocytogenes* in samples of e.g. cheese results in rejection or recall of the whole batch in question irrespective of whether the food is able or unable to support growth (CFR Title 21, 2018).

The relative risk of acquiring listeriosis in relation to the different food safety criteria around the world has been extensively discussed. It is well accepted that nearly all cases of listeriosis results from the consumption of foods with numbers of *L. monocytogenes* exceeding both a zero-tolerance limit and 100 cfu/g (FAO/WHO, 2004; ICMSF, 1996) and that foods containing low levels of *L. monocytogenes* (e.g. <100 cfu/g) pose very little risk (FAO/WHO, 2004; Chen et al., 2003).

3. Microbial spoilage of dairy products

Dairy spoilage microorganisms posing a challenge for the dairy industry included aerobic psychrotolerant gram-negative bacteria, yeasts, moulds, heterofermentative lactobacilli and spore-forming bacteria (Büchl and Seiler, 2011; Garnier et al., 2017; Ledenbach and Marshall, 2009; Sørhaug, 2011). Specifically, psychrotolerant pseudomonads and their enzymes are recognized as the predominant limiting factor for milk and fresh cheese shelf-life (Brocklehurst and Lund, 1985; Ternström et al., 1993; Martin et al., 2011; Dogan and Boor, 2003; Sørhaug & Stepaniak, 1997).

The spoilage of milk and fresh cheeses is discussed in the following section with focus on psychrotolerant pseudomonads including their growth characteristics and spoilage along with possibilities and perspectives for preventing growth of these spoilage organisms.

3.1. Psychrotolerant pseudomonads

Pseudomonas spp. are aerobic gram-negative, non-spore forming and rod-shaped bacteria. They are motile by one or more polar flagella and with a typical length of 1.5 to 5 µm, catalase positive and usually oxidase positive. Most *Pseudomonas* spp., if not all, fail to grow under acidic conditions ($\text{pH} \leq 4.5$) (Garrity et al., 2005). The genus *Pseudomonas* includes several psychrotolerant species with the ability to grow to high concentrations in various chilled foods. Some psychrotolerant pseudomonads have generation times as low as 4-5 h at 4°C in milk and therefore even with very low initial contamination level they can dominate the final spoilage microbiota (Cox and Rae, 1998). *P. fluorescens*, *P. fragi*, *P. lundensis* and *P. putida* are psychrotolerant species associated with spoilage of foods from animal and plant origin. These bacteria cause sensory spoilage by producing fruity and nauseous off-odours when they reach concentrations above 7 log cfu/g in fresh foods e.g. raw milk, fresh fish, poultry, meat and eggs

(Borch et al., 1996; Dainty and Mackey, 1992; Liao, 2006; Sørhaug and Stepaniak, 1997; Tryfinopoulou et al., 2002).

Psychrotolerant pseudomonads also play an important role as post-pasteurization contaminants of dairy products (D'Amico, 2014; Martin et al., 2018). *P. fluorescens* and *P. putida* can secrete pyoverdinin, a fluorescent yellow-green pigment (Meyer et al., 2002) and certain *P. fluorescens* can produce additional blue pigments, called pyocyanin. In fact, *P. fluorescens* biovar I and biovar IV were identified as the causing agents for green and blue discoloration defects in fresh cheeses due to the production of pyoverdinin and pyocyanin (Cantoni et al., 2001; Cantoni et al., 2003). Several episodes of fresh cheese spoilage by psychrotolerant pseudomonads due to the production of different pigments have been described in literature. The Italian authorities notified the RASFF system about two events of blue decolouration and high numbers of *P. fluorescens* in mozzarella cheese imported from Germany (RASFF, 2010). Martin et al. (2011) described a comparable spoilage event in Latin-style fresh cheese (Queso fresco) manufacture in the USA from pasteurized milk. They identified *P. fluorescens* biovar IV as the causing agent of the blue colouring defect of the cheese surface. The defect persisted for 9 months, suggesting a post-pasteurization contamination from the dairy environment. Carrascosa et al. (2015) described a similar incident in fresh cheese produced from raw milk in Spain. In this case, raw milk was replaced by pasteurized milk to produce the cheese, but the defect persisted. Finally, a new cleaning and disinfection program of the production site eliminated cross-contamination by psychrotolerant pseudomonads and the blue defect was managed.

Psychrotolerant pseudomonads can be responsible for spoilage of cottage cheese; a soft unripe, mildly acidic cheese consisting of fermented curd granules and cream dressing (Brocklehurst and Lund, 1985). Cottage cheese with cultured cream dressing is a popular product in

Scandinavia characterized by high water activity (>0.99), low concentration of salt in the water phase (ca. 1%), pH below 5.5 and lactic acid originating from fermentation (Østergaard et al., 2014; Paper VI). For this product, washing and handling of the curd granules after fermentation have been identified as the critical point to manage, as this processing step can introduce psychrotolerant pseudomonads (Ledenbach and Marshall, 2010). Spoilage of cottage cheese by *P. fragi* and *P. viscosa* produces a gelatinous or slimy curd defect (Parker et al., 1975). To reduce potential spoilage of cottage cheese by psychrotolerant pseudomonads the used of different approaches have been reported in literature. Cultured cream dressing with *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* has been studied as control step to reduce growth of psychrotolerant pseudomonads in cottage cheese (Kristoffersen and Chakraborty, 1964); however pseudomonas can grow in this product (Paper VI), reduce the diacetyl (Wang and Frank, 1981) and thereby lead to a “green” or yogurt-like flavour defect from the imbalance of the diacetyl to acetaldehyde ratio. Kristoffersen and Chakraborty (1964) studied the addition of sorbic acid and lactic acid to the cream dressing in order to extend shelf-life of cottage cheese. Shelf-life was extended by approximately 50% when 0.065% sorbic acid was added to the cream dressing. A higher shelf-life extension (68%) was achieved when lactic acid was added in order to adjust pH of the cream’s dressing to 6.0. The use of bifidobacteria to inhibit growth of psychrotolerant pseudomonads in cottage cheese was studied by O’Riordan and Fitzgerald (1998). They concluded that *Bifidobacterium infantis* NCFB 2255 and *Bifidobacterium breve* NCFN 2258 inhibit growth of pseudomonads; however the inhibitory effect was species-dependent. Salih and Sandine (1990) demonstrated that the use of 1% liquid Microgard™ added to cottage cheese cream dressing significantly reduced spoilage due to inhibition of psychrotolerant gram-negative bacteria. Results showed that 90% of cottage cheese samples contained less than 3 log cfu/g after 24 days and 68% of samples had undetectable levels of spoilage organisms after 30 days of storage at 7°C.

Pseudomonas spp. can produce proteases, lipases and pectinases. Enzymes production usually occurs in the mid to late exponential growth phase and in the stationary phase of bacterial growth (Nicodème et al., 2005; Rajmohan et al., 2002). Therefore, if raw milk is store on farms or dairy plants at refrigerate temperatures for a long time; they can produce thermotolerant lipolytic and proteolytic enzymes that will reduce quality and shelf-life of the processed milk and dairy products. (e.g. cheese, UHT milk, butter, yogurt) (Sørhaug and Stepaniak, 1997; Dogan and Boor, 2003; Wiedmann et al., 2000). *P. fragi* and *P. fluorescens* degrade the casein proteins in milk to obtain carbon for growth since they are unable to use lactose from milk as carbon source (Dodd, 2014). Many of these enzymes are stable after pasteurization and ultra-high (UHT) temperature treatments (Bhunja, 2008). Proteases are related to textural changes in milk, e.g. gelation and increased viscosity and to unclean and bitter flavours in cheese (Fairbairn and Law, 1986; Lemieux and Simard, 1991), whereas lipases have been associated to rancid and fruity aromas (Stead, 1986; Ledenbach and Marshall, 2009). White and Marshall (1973) studied the effect of high concentration of proteases present in milk used to produce Cheddar cheese. They observed higher proteolysis in Cheddar cheese made from milk containing 0.94 units of the enzyme per millilitre and lower flavour scores compared with the control.

The presence of psychrotolerant pseudomonads in the dairy environment might be due to improper cleaning or resistance to routine cleaning of surfaces due to their capability to form biofilms (Wirtanen et al., 2001). However, they might gain access to the dairy environment through the water used for cleaning and rinsing the equipment (D'Amico, 2014). Stellato et al. (2017) studied the occurrence of psychrotolerant pseudomonas in dairy processing plants and showed that the most frequently found species were *P. fluorescens* and *P. fragi*. They further identified the same strains occurring both in the dairy environment and food, highlighting the important role of the

processing environment as a source of food contamination. Similarly, Dogan and Boor (2003) examined the genetic diversity of *Pseudomonas* spp. isolated from dairy processing plants. The majority of the isolates were *P. fluorescens* and *P. putida* with high genetic diversity among the isolates suggesting multiple origins of contamination within the dairy processing plant.

4. Dairy products

Dairy products include a wide variety of food items all based on some kind of milk. Commonly consumed dairy products in addition to milk are cheese, yogurt, butter, cream, acidified milk or powder products. Results from this PhD-thesis are summarized in Table 3 to demonstrate variations in product characteristics among different types of dairy products. In order to develop predictive models it is essential to identify factors that can affect growth of microorganisms. In the following sections cheese processing steps will be described followed by a discussion of dairy specific ingredients present in some of these products.

Table 3. Products characteristics of a broad range of dairy products.

Product	pH	WPS ^a (%)	Concentrations in product water phase				Residual Nisin A (mg/kg)	Reference
			Lactic acid (%)	Acetic acid (%)	Citric acid (%)	Ortho- phosphate (%)		
Smear ^b cheese	4.7-7.3	0.1-4.5	0.00-1.76	- ^c	- ^c	ND ^d	ND ^d	Paper 2
Cream cheese	4.8-5.0	1.8-2.1	0.31-0.55	0.09-0.12	0.06-0.21	ND ^d	ND ^d	Paper 3
Chemically acidified cheese	4.5-4.6	3.9-4.4	- ^c	- ^c	- ^c	ND ^d	0.00-0.19	Paper 3 and 5
Processed cheese	5.9-6.2	0.3-2.1	0.64-1.31	0.06-0.24	0.05-0.77	1.47-2.14	0.16-0.19	Paper 4 and 5
Fresh cheese	5.3-5.4	0.9-1.1	0.04-0.10	- ^c	- ^c	ND ^d	ND ^d	Paper 6
Milk	6.5-6.7	- ^c	- ^c	- ^c	- ^c	ND ^d	ND ^d	Paper 6

^a WPS, water phase salt

^b Product characteristics represent the entire production process

^c Below the HPLC limit of detection

^d ND: not determined

4.1. Cheese

Cheese is the generic name for a group of fermented or acidified milk-based products, produced throughout the world in a great diversity of flavours, textures and forms (Fox et al., 2017). The *Codex Alimentarius* defines cheese as the ripened or unripened product obtained by coagulation of milk, cream, butter milk or a combination of the previous through the action of rennet or other agents and by partially draining the whey (CAC, 1978). Furthermore, whey cheese can be produced from the coagulation of whey by heat and with or without the addition of acids (CAC, 1971). Throughout this thesis the term “cheese” will refer to ripened or unripened products obtained by acidification of milk, cream, butter milk or a combination of the previous by the action of starter cultures or acids, coagulation by rennet or other agents and potential further heat or acid-heat treatment of the whey drained after coagulation. Several authors have further classified cheese into subgroups according to e.g. texture, coagulation method, maturation and product characteristics (Fig. 2) (Fox et al., 2017; Almena-Aliste and Mietton, 2014; McSweeney et al., 2017). Paper I classified different types of cheese based on maturation characteristics and definitions are presented as follows. Fresh cheeses are curd-style cheeses which do not undergo any ripening (CAC, 2013) e.g. queso fresco, cottage cheese, cream cheese, Mozzarella or Ricotta. These cheeses have pH of 4.8-6.7 and water phase salt content of 0.9-4.9%; however, information about lactic-, acetic- and citric acids are scarce (Table 4). Ripened cheeses are not ready for consumption shortly after manufacture and maturation is needed for development of specific product characteristics (CAC, 2013), e.g. Gouda, Edam, Cheddar or Parmesan.

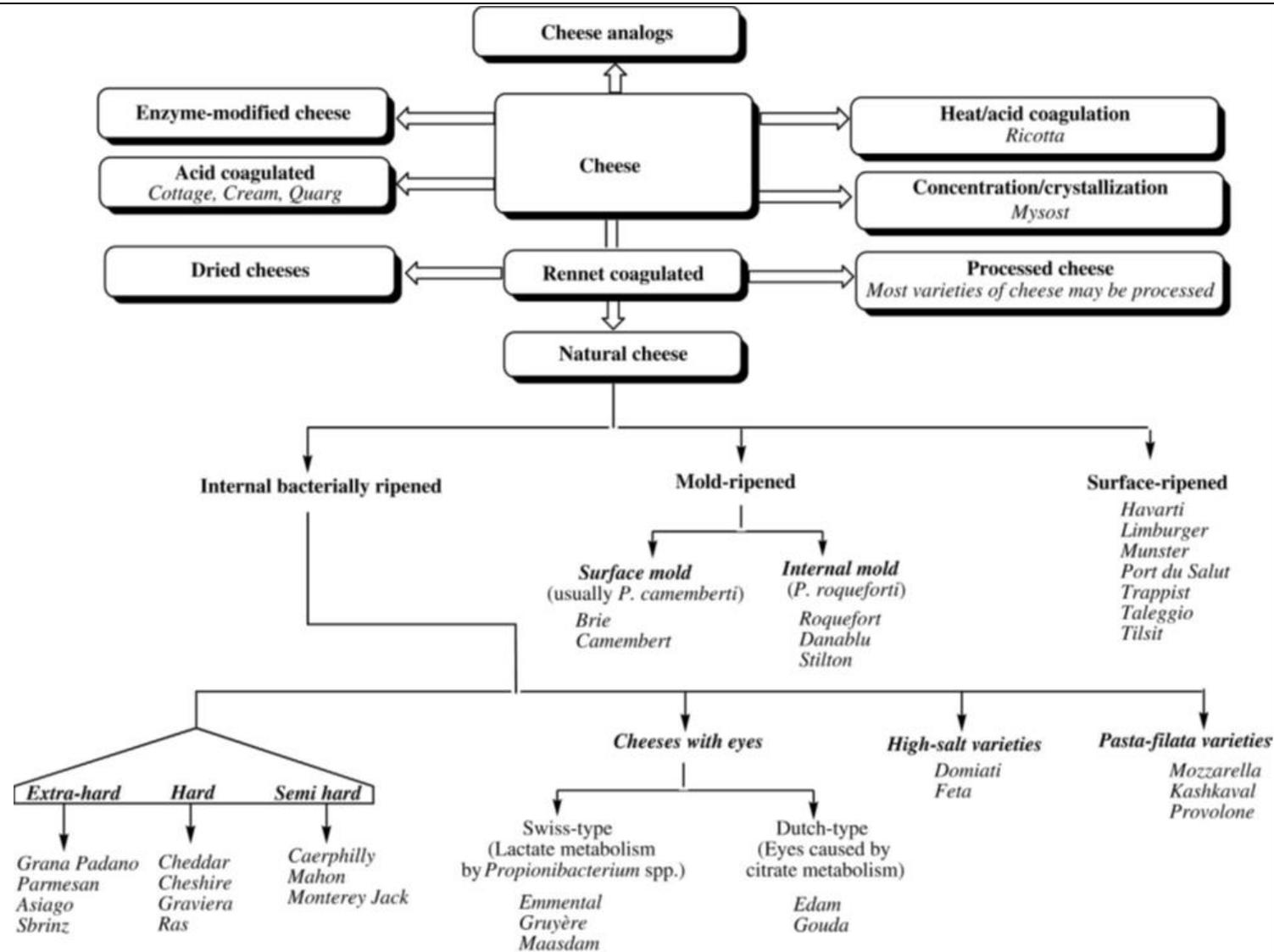


Figure 2. The diversity of cheeses. Cheeses classified into sub-groups based on the method of coagulation and further sub-divided based on the principal ripening agents and/or characteristic technology (Fox et al., 2017)

Table 4. Product characteristics of fresh cheese available in literature.

Cheese	pH	WPS ^a (%)	Organic acids in product water phase			Reference
			Lactic acid (%)	Acetic acid (%)	Citric acid (%)	
Cream cheese	4.8-5.0	1.8-2.1	0.31-0.55	0.09-0.12	0.06-0.21	Paper 4
Cottage cheese	5.3-5.4	0.9-1.1	0.04-0.10	ND ^b	ND ^b	Paper 6
Cottage cheese	5.2-5.4	1.1-1.2	0.07-0.10	NR ^c	NR ^c	Østergaard et al. (2014)
Cottage cheese	4.5	NR ^c	NR ^c	NR ^c	NR ^c	Ryser et al. (1985)
Queso blanco	5.2	3.4	NR ^c	NR ^c	NR ^c	Glass et al. (1995)
Mozzarella	NR ^c	0.2-3.4	NR ^c	NR ^c	NR ^c	Stecchini et al. (1995)
Ricotta cheese	5.8-6.1	NR ^c	NR ^c	NR ^c	NR ^c	Davies et al. (1997)
Queso blanco	6.5	NR ^c	NR ^c	NR ^c	NR ^c	Mendoza-Yepes et al. (1999)
Mozzarella	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c	Menon & Garg (2001)
Queso blanco	6.8	4.9	NR ^c	NR ^c	NR ^c	Uhlich et al. (2006)
Katiki	4.6	2.9	NR ^c	NR ^c	NR ^c	Panagou (2008)
Queso fresco	6.6-6.7	1.7	NR ^c	NR ^c	NR ^c	Legget et al. (2012)
Fresh cheese	6.5	NR ^c	NR ^c	NR ^c	NR ^c	Coelho et al. (2014)
Queso fresco	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c	Gadotti et al. (2014)
Fresh cheese	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c	Hamedi et al. (2014)
Mozzarella	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c	Han et al. (2014)
Minas frescal	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c	Pimentel-Filho et al. (2014)
Mozzarella	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c	Han et al. (2015)
Fresh cheese	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c	Lobacz et al. (2016)
Minas frescal	5.6	NR ^c	NR ^c	NR ^c	NR ^c	Meira et al. (2016)
Fresh cheese ^d	6.2-6.4	1.1-2.1	NR ^c	NR ^c	NR ^c	Kapetanakou et al. (2017)
Queso fresco	6.0	NR ^b	NR ^c	NR ^c	NR ^c	Lourenço et al. (2017)

^a WPS, water phase salt

^b ND: not determined

^c Information not reported (NR)

^d Mascarpone, cottage cheese, Antonyous, Manouri, Mozzarella, Ricotta

Veined cheeses are ripened cheeses in which ripening has been accomplished primarily by the development of the mould *Penicillium roqueforti* in the interior e.g. Roquefort, Gorgonzola,

Cabrales, Stilton or Danablu. Smear cheeses are ripened cheeses where the surface is treated with e.g. *Penicillium camemberti*, *Geotrichum candidum*, *Debaryomyces hansenii* or *Brevibacterium linens*, e.g. Brie, Camembert, Limburger or Taleggio. Brined cheeses are ripened and stored in brine until they are sold or packaged, e.g. Feta, or Ricotta salata (Fox et al., 2000). Product characteristics extracted from the literature for ripened-, veined-, smear- and brined cheese shows a broad range of values regarding water phase salt content (1.7-10.6%) and pH values (4.4-7.5). Importantly, the performed literature search highlights the lack of published information about organic acid concentrations present in cheese (Table 5).

Cheese production involves a number of principal steps that are common to most types of cheese. The first step involves the standardization of milk to optimize the protein to fat ratio necessary to produce a good quality cheese followed by pasteurization, mild heat-treatment or a non-heat-treatment (raw) of the milk. The third step involves inoculation of the milk with starter cultures that will grow and acidify it. Optionally milk can be inoculated with a secondary microbiota that will support later during ripening/maturation. The fourth step is essential since all cheeses require coagulation of the milk protein. The majority of cheeses are produced by rennet coagulation but some are acid coagulated (e.g. cream cheese, mascarpone) or acid-heat coagulated. The acid-heat coagulated cheeses are produced from whey and represent a way to recover the nutritional valuable whey proteins (e.g. Ricotta) (Fox et al., 2000). After draining of whey by cutting the curd, heating and stirring, salting by hand or by placing the cheese in brine is the last cheese manufacturing step shared by all cheese. From this point, fresh cheese is ready to be consumed. However, a great number of cheeses (e.g. smear-, veined-, ripened- and brined-) will follow a ripening/maturation process.

Table 5. Product characteristics of ripened-, veined-, smear- and brined cheese available in literature.

Cheese-type	Cheese	pH	WPS ^a (%)	Lactic acid in the water phase (%)	Reference
Ripened	Cheddar	5.1	NR ^b	NR ^b	Ryser and Marth (1987)
	Colby	5.0-5.2	3.2-4.0	NR ^b	Yousef and Marth (1988)
	Brick	5.3	NR ^b	NR ^b	Ryser and Marth (1989)
	Graviera	5.6	8.3	NR ^b	Giannou et al. (2009)
	Ripened ^c	5.5	4.6	NR ^b	Valero et al. (2014)
	Cantal	5.3	6.3	NR ^b	Chateland-Chauvin et al. (2015)
	Ripened ^d	5.5-6.4	5.5-7.7	NR ^b	Kapetanakou et al. (2017)
	Gouda	5.1-5.6	1.7-13.0	1.2-1.8	Wemmenhove et al. (2018)
Veined	Stilton	NR ^b	9.4	NR ^b	Whitley et al. (2000)
	Blue/white	5.0-7.5	1.8	0.01-0.3	Rosshaug et al. (2012)
	Gorgonzola	5.9-6.8	5.0-10.6	NR ^b	Bernini et al. (2013)
	Blue	5.9	4	NR ^b	Lobacz et al. (2013)
	Gorgonzola	6.6	3.8	NR ^b	Dalzini et al. (2017)
Smear	Limburger	5.3	5.0	NR ^b	Ryser and Marth (1989)
	Munster	5.8-6.6	2.4-6.3	NR ^b	Ferrier et al. (2013)
	Camembert	NR ^b	1.7	NR ^b	Lobacz et al. (2013)
	Smear ^e	5.7	5.0-5.7	NR ^b	Tiwari et al. (2014)
	Camembert	6.3	4.6	NR ^b	Kapetanakou et al. (2017)
	Taleggio	6.0	10.6	NR ^b	Combase (2012)
	Smear	4.7-7.3	0.1-4.5	0.00-1.76	Paper 2
Brined	White pickled	6.4	5.2	NR ^b	Abdalla et al. (1993)
	Feta	4.4-6.6	3.9-4.4	NR ^b	Papageorgiou and Marth (1989)
	Ricotta salata	6.1	8.7	NR ^b	Spanu et al. (2012)
	Ricotta salata	6.3	8.7	NR ^b	Spanu et al. (2013)
	Ricotta salata	6.6-6.8	6.3-7.4	NR ^b	Coroneo et al. (2016)
	Haloumi	6.6	5.8	NR ^b	Kapetanakou et al. (2017)

^a WPS, water phase salt

^b Information not reported (NR)

^c Semi-hard cheese aged for three to six months

^d Mastelo, Edam, Gouda

^e Semi-soft rind washed

The primary biochemical changes that occur during ripening are glycolysis, lipolysis and proteolysis that will define the unique characteristics of the different cheeses regarding flavour, aroma and texture. These changes are caused by one or many of the following agents: (i) enzymes released by the death and lysis of the starter cultures, (ii) enzymes from the rennet or indigenous milk enzymes, particularly important in raw milk cheese, (iii) enzymes produced by the development of the nonstarter cultures or secondary microbiota (Collins et al., 2003; Upadhyay et al., 2004).

4.1.1. Starter cultures

The starter cultures are lactic acid bacteria (LAB) further divided in mesophilic with a maximum growth temperature ~39°C and thermophilic with an optimum growth temperature between 35°C and 45°C (Fox et al., 2000). The mesophilic starter cultures used in cheese making are *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc lactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. The latter three provide aroma and texture by metabolizing citrate into diacetyl, acetoin and CO₂, which are important for cheese aroma and eye formation. *Lactococcus lactis* subsp. *lactis* and *cremoris* are the major lactic acid producers from lactose (Hugenholtz, 1993). Commercial types of mesophilic starter cultures (O, L, D, DL, Table 2) are extensively applied by the dairy industry. The group of thermophilic starter cultures is constituted by *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus helveticus*, and *Streptococcus thermophiles* (Walstra et al., 2005). These are used in the production of Italian (e.g., Mozzarella, Grana Padano and Parmigiano Reggiano) and Swiss (e.g., Emmental, Gruyère) cheese varieties (Sheehan et al., 2007; Giraffa et al., 1998). An overview of the commercial mixtures of mesophilic starter cultures used in cheese making is provided in Table 6.

Table 6. Overview of mesophilic starter cultures and cheese applications.

Type	Organisms	Cheese	References
O ^a	<i>Lactococcus lactis</i> subsp. <i>lactis</i> <i>Lactococcus lactis</i> subsp. <i>cremoris</i>	Cheedar Cottage cheese Emmental ^b	Walstra et al., 2005; Düsterhöft et al., 2017; Belitz et al., 2004
L ^c	<i>Lactococcus lactis</i> subsp. <i>lactis</i> <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Leuconostoc lactis</i> <i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	Seldom used for cheese	
D ^c	<i>Lactococcus lactis</i> subsp. <i>lactis</i> <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i>	Seldom used for cheese	
DL ^c	<i>Lactococcus lactis</i> subsp. <i>lactis</i> <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> <i>Leuconostoc lactis</i> <i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	Camembert ^d Brie ^d Roquefort ^e Edam ^f Gouda ^f Danbo Havarti Samsø ^g	Walstra et al., 2005; Düsterhöft et al., 2017; Belitz et al., 2004

^a Citrate-negative cultures

^b Contains thermophilic cultures and propionibacterium

^c Citrate-negative and citrate-positive strains

^d Contains *Penicillium camemberti*

^e Contains *Penicillium roqueforti*

^f Contains *Brevibacterium linens*, *Micrococcus* spp. and yeasts

^g Contains propionicbacterium

4.1.2. Lactose and citrate fermentation

The transport of lactose into the cell by lactococci is performed by the phosphoenolpyruvate phosphotransferase system (PEP-PTS) in which lactose is phosphorylated as transported across the cell membrane. Whereas in other dairy bacteria such as *Streptococcus thermophilus*, *Leuconostoc* spp. and thermophilic lactobacilli, lactose is transported by a specific protein (Broome, 2007). Once inside the cell, the enzyme phospho-β-galactosidase hydrolysis lactose into glucose and galactose-6-phosphate. Subsequently, glucose enters the glycolytic pathway and galactose-6-phosphate enters the tagatose-6-phosphate pathway (Fig. 3) producing pyruvate that is metabolized in lactic acid by the action of the lactate dehydrogenase enzyme. Other substrates can be metabolized from pyruvate

by the action of the enzyme pyruvate formate lyase including formate, acetate, acetaldehyde and ethanol under anaerobic conditions and at high pH (> 7.0) (Broome et al., 2011). Under aerobic conditions and pH 5.5-6.5, pyruvate can be converted to acetate, acetaldehyde, ethanol and minor by-products (e.g., acetoin, diacetyl and 2,3-butanediol) via the multienzyme pyruvate dehydrogenase complex (Broome et al., 2011). In *Leuconostoc* spp. the glucose portion of the galactose is metabolized to CO₂, ethanol and lactic acid via the phosphoketolase pathway, while galactose is converted to lactic acid by the Leloir pathway (Broome, 2007).

Citrate is present in milk at concentrations of 8 to 9 mM and it can be fermented by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. In lactococci, citrate is converted to acetate, CO₂, and pyruvate, which is further metabolized into acetate, diacetyl, acetoin, 2,3-butanediol and CO₂ (García-Quintáns et al., 1998). In *Leuconostoc* spp. the pyruvate produced from citrate is metabolized to lactate, however, at low pH and in the absence of either glucose or lactose *Leuconostoc* spp. will produce diacetyl and acetoin (Vedamuthu, 1994).

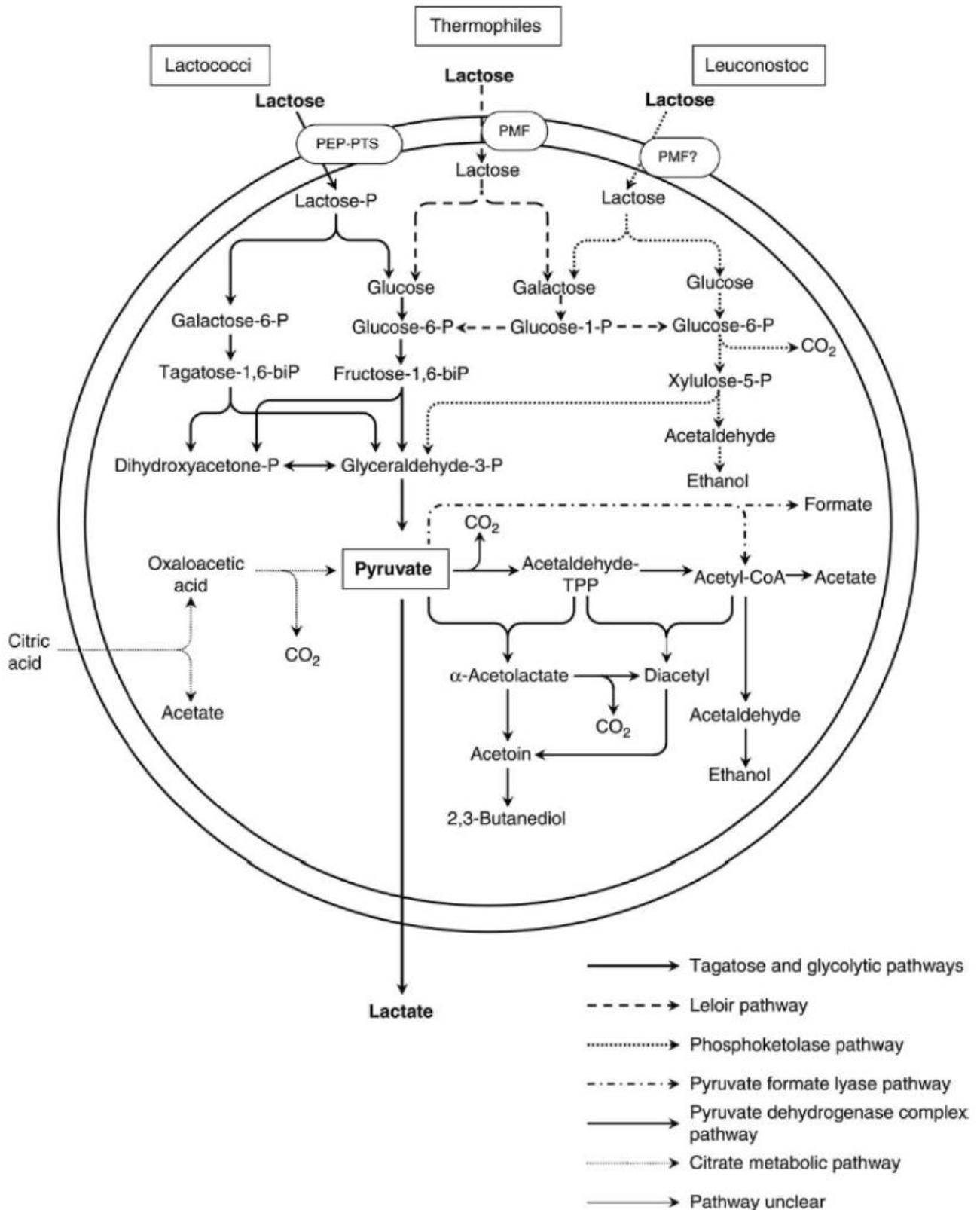


Figure 3. General pathway for lactose and citrate catabolism by lactic acid bacteria. PEP-PTS, phosphonolpyruvate phosphotransferase system; PMF, proton motive force or other transmembrane potential. (Broome et al., 2011).

4.1.3. Secondary microbiota

The secondary microbiota of cheese is formed by a broad group of microorganisms (Fox et al., 2017) and this section will focus in the microbiota present in smear cheese studied in Paper II. The “smear cheese” category used throughout this PhD-thesis and in Paper II includes surface ripened soft cheeses containing moulds, bacteria and yeast on the exterior (e.g. *Penicillium camemberti*, *Geotrichum candidum*, *Brevibacterium linens*, *Debaryomyces hanseii*). This category was created based on the ability of yeast and moulds to metabolize lactic acid into CO₂ and H₂O (Corsetti et al., 2001; Famelart et al., 1987; Fox et al., 2017; Larpin et al., 2006; McSeeney and Sousa, 2000; Riahi et al., 2007), production of NH₃ and keto acids causing the pH increase of the rind. For instance, Leclercq-Perlat et al. (2000) carried a detail study of microbial changes occurring on the surface of Camembert cheese produced in the laboratory with a secondary microbiota composed by *P. camemberti*, *G. candidum*, *Kluyveromyces lactis* and *B. linens*. They observed a rapid deacidification and consumption of lactate on the cheese surface paralleled to growth of *K. lactis* and *G. candidum*.

4.2. Processed cheese

The development of processed cheese was inspired by the possibility of increased cheese trade through the creation of more stable cheeses that will retain their original quality characteristics for a longer time and have extended shelf-life when compared to “traditional” cheese (Guinee, 2017). Processed cheese is produced by heating and blending together one or more types of cheeses mixed with emulsifying salts (phosphate and/or citrate salts) and maybe other ingredients such as dairy products, colourings, flavours or preservatives (e.g. nisin). Heat treatments necessary to manufacture processed cheese can be performed in batch cookers (e.g. 70-95°C for 4-15 min) or in

continuous cookers with direct steam injection (e.g. ~140°C for 10s). The final products are typically packaged hot-fill or aseptically (Fox et al., 2017).

4.2.1. Emulsifying salts

Emulsifying salts, such as sodium or potassium salts of phosphoric and citric acid are used to promote the formation of a homogeneous and stable processed cheese. Numerous broth studies (Table 7) and food applications have showed that phosphate and citrate salts can be used to control gram-positive and gram-negative bacteria.

Phosphate salts chelate essential metals from cation-binding sites located within the cell walls (Jen and Shelef, 1986; Knabel et al., 1991). Confirmation for the mechanism of action was provided when inhibition of bacteria growth was reversed by supplementation of the media by divalent cations, mainly magnesium (Wagner and Busta, 1985b; Maier et al., 1999; Lee et al., 1994). Post et al. (1963) suggested that sodium hexametaphosphate sequestering Mg^{2+} resulted in the inhibition of cell wall division and consequently compromised cell wall integrity. In the study by Elliott et al. (1964) a similar mechanism for inhibition of psychrotolerant pseudomonads was suggested, since addition of Mg^{2+} to the media reversed the inhibition. Jen and Shelef (1986) reported that addition to the broth of 0.25 to 1.0 mM Mg^{2+} was effective to overcome inhibition of *S. aureus* 196E produced by 0.5% sodium tripolyphosphate.

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Table 7. Antimicrobial activity of phosphate and citrate salts in broth media.

Compound	(% w/v)	Bacteria	Antimicrobial activity	Reference
Sodium hexametaphosphate	0.1	<i>S. aureus</i> and <i>B. subtilis</i>	Inhibition of growth for 24h at 30°C	Post et al. (1963)
	>10	<i>S. typhimurium</i> and <i>E. coli</i>	Growth for 24h at 30°C	
Commercial polyphosphate ^a	1	<i>P. fragi</i>	Inhibition of growth for 14 days at 4.4°C	Elliott et al. (1964)
Sodium hypophosphite	1	<i>C. botulinum</i> type E spores	Inhibition of spores germination for 72 h at 30°C	Seward et al. (1982)
Sodium pyrophosphate	0.5	<i>S. aureus</i> and LAB ^a	Inactivation of cells after 72 h at 30°C or 37°C	Molins et al. (1984)
Sodium polyphosphate	0.5	<i>S. aureus</i> and LAB ^a		
Tetrasodium pyrophosphate	0.5	<i>S. aureus</i> and LAB ^a		
Sodium tripolyphosphate	0.5	<i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> and LAB		
Sodium tripolyphosphate	0.5	<i>S. aureus</i> 196E	Inhibition in BHI broth for 72h at 35°C	Jen and Shelef (1986)
Sodium hexametaphosphate	0.5			
Sodium tripolyphosphate	0.3			
Sodium hexametaphosphate	0.3 to > 0.5	<i>B. cereus</i> , <i>S. aureus</i> , <i>P. fluorescens</i>	MIC from OD measurements after 24h at 25°C	Zessin and Shelef (1988)
Sodium pyrophosphate	0.4 to > 0.5			
	0.05	<i>S. aureus</i>		
Sodium polyphosphate ^a	0.1	<i>C. tyrobutyricum</i>	Inhibition of growth after 48h at 37°C	Loessner et al. (1997)
	0.3	<i>L. monocytogenes</i>		

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Sodium polyphosphate ^b	0.1	<i>B. cereus</i>	Cell elongation and lysis	Maier et al. (1999)
Sodium hexametaphosphate	0.5	<i>L. monocytogenes</i>	Increased of lag-times	Obritsch et al. (2008)
Sodium orthophosphate	14.9	<i>L. monocytogenes</i>		
Sodium pyrophosphate	9.4	<i>L. monocytogenes</i>		
Sodium tripolyphosphate	7.6	<i>L. monocytogenes</i>	MIC-values estimated from OD measurements at 25 °C	Paper IV
Trisodium citrate	11.0	<i>L. monocytogenes</i>		

^a Commercial polyphosphate salts (75% tri-phosphate, 25% di-phosphate and 1% orthophosphate)

^b Commercial polyphosphate salts (JOHA HBS; JOHA HBS-1; JOHA HBS-9)

^c Commercial polyphosphate salt (JOHA HBS)

Knabel et al. (1991) and Loessner et al. (1997) reported that *L. monocytogenes* grew better than other gram-positive bacteria on media containing different polyphosphate salts. Knabel et al. (1991) speculated that *L. monocytogenes* produced high levels of alkaline phosphatase which might hydrolysed the polyphosphates into less inhibitory orthophosphate. However, no further studies were performed to confirm the hypothesis. More recently, Obritsch et al. (2008) reported longer lag times of *L. monocytogenes* as results of adding 0.5% sodium hexametaphosphate to broth but they also observed shorter generation times. The chain phosphates (polyphosphates) chelate strongly than the ring phosphates or orthophosphates (van Wazer and Callis, 1958) and consequently they have a higher antimicrobial activity (Table 7; Paper IV).

Phosphates salts can be used to control production of toxin by *C. botulinum* or growth of *Clostridia* spp. and *L. monocytogenes* in processed cheese. Eckner et al. (1994) demonstrated that polyphosphates inhibited botulinum toxin production in spreadable processed cheese with low moisture concentration (52-56%) and delayed toxin production in cheese produced with 60% moisture. Growth of *C. tyrobutyricum* was studied in spreadable processed cheese with 55% moisture and produced with increasing concentration (0.1 to 1%) of commercial polyphosphate salts (Loessner et al., 1997). It was concluded that 1% polyphosphate salts totally inhibited growth of *C. tyrobutyricum* during seven weeks of storage at 35°C. Paper IV demonstrated that *L. monocytogenes* growth can be inhibited in spreadable processed cheese formulated with 1.5% of sodium tripolyphosphate and stored at 15°C when the other product characteristics are as it follows: pH 5.8, a_w 0.970, 2.2% (lactic acid), 0.35% (acetic acid), 0.2% (citric acid), 0.3% orthophosphate and 0.5% sodium pyrophosphate in the water phase.

Growth of *L. monocytogenes* can be inhibited by short chain organic acids passing through the bacterial cell membrane in their undissociated form. Once within the cytoplasm the acid

dissociates resulting in an increased of hydrogen ion concentration in the cell. To restore the intracellular pH, the hydrogen ions must be pumped out by an unfavourable process for the bacteria (Mitchell, 1961). Minimum inhibitory concentrations (MIC) of undissociated citric acid against *L. monocytogenes* have been reported by various authors (Augustin and Carlier, 2000; Mejlholm and Dalgaard, 2009; Wemmenhove et al., 2016). However, MIC-values for the emulsifying salt trisodium citrate were not available from literature and it was determined by the present study (Table 7, Paper IV).

4.2.2. Nisin

Nisin is an antimicrobial peptide belonging to the lantibiotic class of bacteriocins (Class I), composed of 34 aminoacids and with a molecular mass of 3.5 kDa. Five other naturally occurring variants of nisin A have been described. Nisin Z, F and Q like nisin A are produced by *Lactococcus lactis* whereas nisin U and U2 are produced by *Streptococcus* ssp. (Piper et al., 2010). Nisin Z differs from nisin A in aminoacid position 27 where histidine is replaced by asparagine (Figure 4). Nisin Z produced by *L. lastis* NIZO 22186 was identified as a natural nisin variant by Mulders et al. (1991).

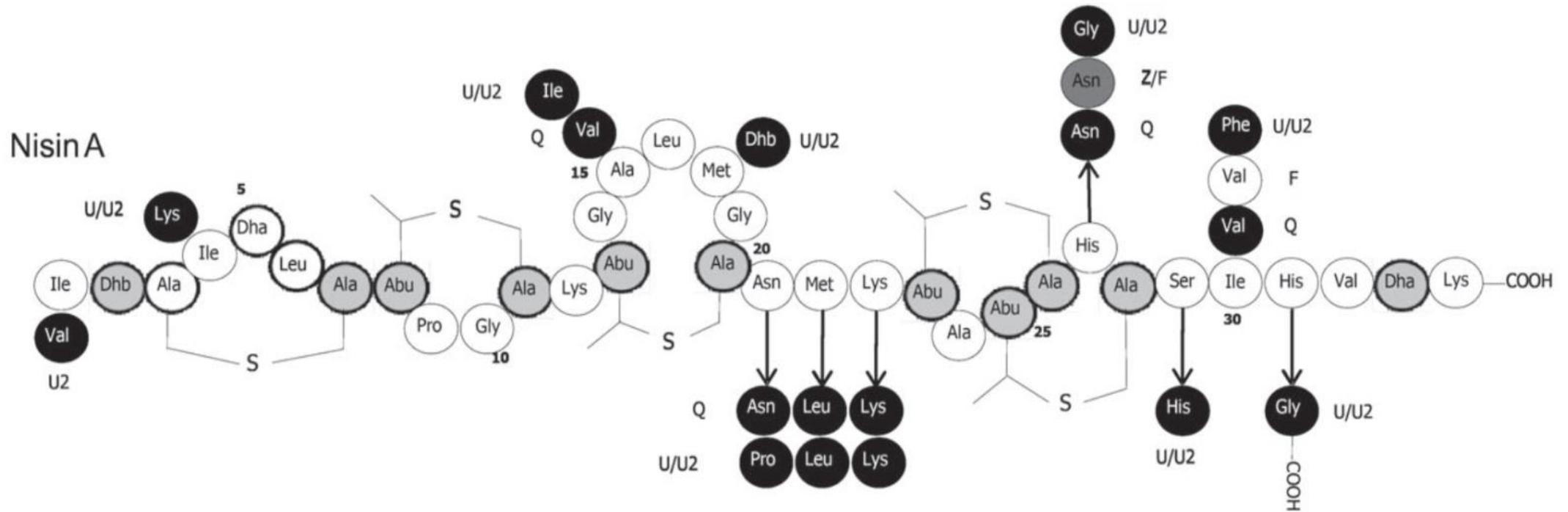


Figure 4. Comparison of natural nisin variants with respect to nisin A. Structure variants in nisin F, Q, Z, U and U2 that differ from nisin A are indicated by a white circle/grey text (nisin Z), dark grey circle/white text (nisin Q), a dark grey circle/black text (nisin Z) and black circles/white text (nisin U/U2). (Piper et al., 2010).

Nisin binding to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, results in prevention of cell wall synthesis that leads to cell death. Nisin can further use lipid II as a docking molecule to initiate membrane insertion and pore formation that results in rapid cell death (Cotter et al., 2005). Furthermore, it has been demonstrated that, due to its gene-encoded nature, the efficacy of nisin A as antimicrobial can be further improved through bioengineering-based approaches. For instance, nisin V was bioengineered with enhanced activity against *L. monocytogenes* (Field et al., 2010).

Nisin exhibits antimicrobial activity against a wide range of food-borne pathogens, including *B. cereus*, *C. botulinum*, *S. aureus* and *L. monocytogenes* (Thomas and Delves-Broughton, 2005). The antimicrobial activity of nisin against *L. monocytogenes* has been extensively studied by determination of the minimum inhibitory concentration (MIC) (Fig. 5; Table 8; Paper V).

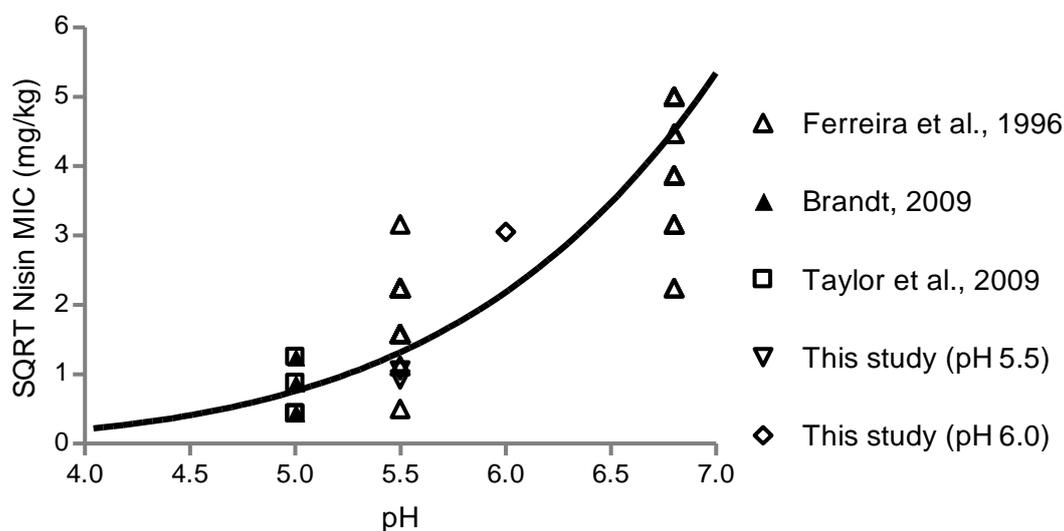


Figure 5. Effect of pH adjusted with HCL on nisin MIC-values for *L. monocytogenes* in broth media. Nisin stock solution (0.02N HCl and sterile filtration). Solid line represents the predict MIC-values of nisin A depending on pH obtained by the model developed in Paper V.

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Table 8. Minimum inhibitory concentration (MIC) of nisin as determined for *L. monocitogenes* in broth studies.

Nisin	Stock solution		Experimental design						MIC (µg/ml)	Reference
	A or Z	Stock solution	Heat treatment or filtration	Media	pH	Temp. (°C)	Time (h)	Inoculum (cfu/ml)		
Nisin ^a	A	Di H ₂ O ^b	0.22 µm	TSA/MRS	6.8	37	24	NR ^c	0.1-2950	Benkerroum and Sandine (1988)
Nisin and Nisaplin	A	0.02N HCl	0.22 µm	TSYGA	5.5	20	48	6	0.3-10.0	Ferreira and Lund (1996)
		0.02N HCl	0.22 µm	TSYGA	6.8	37	24	6	5.0-25.0	
No-commercial	A	NR ^c	NR ^c	MRS	6.5±0.2	30	12	NR ^c	2.4-38.4	Meghrouss et al. (1999)
No-commercial	Z	NR ^c	NR ^c	MRS	6.5±0.2	30	12	NR ^c	5.2-46.5	
Nisaplin	A	0.02N HCl	0.45 µm	BHI	7.4±0.2	30	24	NR ^c	3.1	Nilsson et al. (2000)
Nisaplin	A	HCl (pH 3)	NR ^c	TSB	7.3±0.3	37	48	NR ^c	3.9-7.8	Branen and Davison (2004)
Nisin	A	NR ^c	NR ^c	TSB	7.3±0.3	37	24	NR ^c	6.2-25.0	Martinez and Rodríguez (2005)
Sigma-Aldrich	A	Di H ₂ O ^b	NR ^c	NR ^c	NR ^c	35	20-24	5.7	9.0-11.0	Popowska et al. (2006)
Sigma-Aldrich	A	0.02M CH ₃ COOH	0.22 µm	TSBYE	5.5	35	24	NR ^c	4.8-19.0	Neetoo et al. (2008)
Sigma-Aldrich	A	0.02N HCl	100°C 4 min	2X TPB	5	35	24	5	0.2-1.6	Brandt (2009)
Nisaplin	A	Di H ₂ O ^b	NR ^c	MHA	7.2	37	24	6-8	20-40	Hara et al. (2009)
Sigma-Aldrich	A	0.02N HCl	100°C 4 min	2X TPB	5	35	24	NR ^c	0.2-0.1.6	Taylor et al. (2009)
					7.3	35	24	NR ^c	3.1-6.3	
Sigma-Aldrich	A	0.02N HCl	100°C 4 min	2X TPB	7.2±0.1	35	24	5	3.1-6.3	Brandt et al. (2010)
No-Commercial	A	NR ^c	NR ^c	BHI	7.4±0.2	37	16	5	12.6	Campion et al. (2013)
Nisaplin	A	0.02N HCl	Autoclave	TGE	NR ^c	37	18	NR ^c	0.3	Kaur et al. (2013)
No-Commercial and Nisaplin	A	TSB	NR ^c	TSB	7.3	37	-	5	9.8-12.5	Smith et al. (2016)
Commercial	Z	Di H ₂ O ^b	NR ^c	NR ^c	NR ^c	NR ^c	24	NR ^c	50	Niaz et al. (2018)
Nisaplin	A	0.02N HCl	0.2 µm	BHI	5.5	20 and 25	413	2-6	0.8-1.1	Paper V
Nisaplin	A	0.02N HCl	0.2 µm	BHI	6.0	25	264	2-6	9.3	Paper V

^a Nisin : Aplin & Barrett Ltd.

^b Di H₂O: distilled water

^c NR: not reported

Higher antimicrobial and more consistent activity of nisin have been reported when dissolved in 0.02N HCl compared to distilled water (Hall, 1966). This effect may also be one reason for the wide range of nisin MIC-values reported in literature (Table 8). In fact, studies using a nisin stock solution other than the standard (nisin in 0.02N HCl and without autoclaving) recommended by FAO/WHO (2009) have estimated markedly different nisin MIC-values. For instance, Neetoo et al. (2008) estimated nisin MIC-values at pH 5.5 between 4.8 and 19.0 mg/kg with a stock solution produced with acetic acid. Benkerroum and Sandine (1998) observed nisin MIC-values ranging from 0.1 to 2950 mg/kg when determined at pH 6.8 and using a nisin stock solution prepared in distilled water

The application of nisin to control growth of *L. monocytogenes* includes (i) inoculation of milk with a nisin-producer strain, (ii) addition of nisin as a food preservative or (iii) use of a product previously fermented with a nisin-producing strain as ingredient in dairy production (Chen and Hoover, 2003). Maisnier-Patin et al. (1992) studied *L. monocytogenes* growth/no-growth response when the milk used to manufacture Camembert cheese was inoculated with nisin-producer lactic acid bacteria. *L. monocytogenes* cell concentrations decreased during the initial two weeks, however, re-growth was observed thereafter as the cheese pH increased first on the rind followed by the core. Several studies that added commercial preparations of nisin (e.g. Nisaplin®) to various types of cheese observed a bactericidal effect on *L. monocytogenes* (Arqués et al., 2007; Davies et al., 1997; Gadotti et al., 2014). For instance, in the study by Lourenço et al. (2017) addition of 12.5 ppm of nisin (500 ppm Nisaplin®) to queso fresco caused an immediate 3 log cfu/g reduction of *L. monocytogenes* but as in the aforementioned studies the remaining cells were able to grow and reach high concentrations. Paper V studied *L. monocytogenes* growth/no-growth responses in processed cheese containing nisin as food additive. *L. monocytogenes* was able to grow in products with pH 5.9-6.1, water activity 0.98, 1% (lactic acid), 0.2% (acetic acid), 0.5% (citric acid), 0.8-

0.9% orthophosphate in the water phase of the product and containing 11.2 ppm of added nisin A. Zottola et al. (1994) studied growth of *L. monocytogenes* in Cheddar cheese spread manufacture with or without nisin produced during fermentation. Cheese spread pH was adjusted to 5.1 and nisin concentrations were estimated by the Tramer and Fowler (1964) method to be 0, 2.5 and 7.5 mg/kg. Products stored at 4°C or 23°C did not support growth of *L. monocytogenes* during 56 days regardless of nisin content.

4.3. Chemically acidified cheese

Acidification of the cheese curd is usually achieved via lactic acid production by the starter cultures (see section 4.1.). Alternatively, a direct-acid-set method with a food-grade acid such as glucono-delta-lactone (GDL) can be applied (Farkye, 2004). GDL facilitates the dairy industry to achieve standardization of the milk pH and ultra-filtrated retentates used to produce chemically acidified cottage cheese or white cheese (Serpelloni et al., 1990). Advantages of using GDL in cheese making are that hydrolyses to gluconic acid (GAC) occurs at a rate convenient for cheese making (Serpelloni et al., 1990) and eliminates problems associated with starter cultures performance (e.g. bacteriophages).

4.3.1. Glucono-delta-lactone

Limited information is available in literature studying the antimicrobial activity of GDL against *L. monocytogenes* in dairy products beyond lowering pH. For instance, El-Shewany and Marth (1990) suggested that using GDL at concentrations high enough to coagulate milk for cottage cheese production should contribute to control *L. monocytogenes* during the manufacturing process. However, information on concentration of GDL necessary to coagulate the milk and final pH of the product were not reported. In preliminary studies, they reported that addition of 1.5% GDL to milk inhibit growth of *L. monocytogenes* for 13 days with pH 4.7. Other authors have study the effect of

GDL on meat products. For example, Juncher et al. (2000) found a recipe for saveloys with 2.0% lactate and 0.25% GDL to prevent growth of *L. monocytogenes*. The addition of GDL reduced product pH from 6.37 to 6.08 resulting in an increase of undissociated lactic acid from 1.2 to 2.3 mM. Similarly, Qvist et al. (1994) found bologna-type sausage with 2% lactate and 0.5% GDL prevented growth of *L. monocytogenes* at 5°C and 10°C during 28 days of storage. Product pH was reduced from 6.6 to 6.0 by 0.5% GDL and this resulted in an increase of undissociated lactic acid from 0.7 to 2.8 mM. For the previous examples, *L. monocytogenes* growth inhibition can be explained by the combined effect of product pH and other product characteristics rather than by the antimicrobial effect suggested for GDL (Paper III).

5. Predictive microbiology

In recent years dairy products are increasingly being introduced in several new markets (e.g. Africa, China, and Middle East). This presents challenges in relation with refrigeration of food products since chilled distribution is often inadequate making ambient storage very important. Therefore, products that were developed for chilled storage need to be reformulated or re-design to inhibit growth of pathogens or spoilage organisms when stored at ambient temperatures. Predictive models can be used to adjust product formulations or processing parameters to maintain an acceptable level of microbial safety and quality comparable to products subjected to chilled storage. However, most of the existing models for *L. monocytogenes* or psychrotolerant pseudomonads includes the effect of a limited numbers of environmental factors e.g. temperature, a_w and pH. This is limiting their ability to support product reformulation/development. Furthermore, some processed dairy products contain specific ingredients such as organic acids, emulsifying/phosphate salts, GDL and nisin likely to affect the growth of *L. monocytogenes* and psychrotolerant pseudomonas. Therefore, the development of extensive mathematical models that predicts the effect of intrinsic/extrinsic factors on growth and growth boundary of *L. monocytogenes* and psychrotolerant pseudomonas in a broad range of dairy products would be interesting for the dairy sector.

The development of models is typically based on a two-step approach including primary- and secondary models (Ross and McMeekin, 2003; Swinnen et al., 2004). Primary models describe the evolution of microbial concentration with time (Fig. 6) whereas secondary models describe the relation between the primary model parameters and the influencing factors (e.g., environmental conditions) (McKellar and Lu, 2004; Ross and Dalgaard, 2004; Swinnen et al., 2004). Secondary models can be used proactively e.g. to design products with extended shelf-life or retrospectively e.g. to determine the remaining product shelf-life when storage temperature control has failed

(McMeekin et al., 1997; Membré and Lambert, 2008). Performance of developed models must be evaluated by product studies where observed growth is compared with predicted (Oscar, 2005; Ross, 1996). And lastly, the range of applicability of the model must be stated to minimize the misused of the models and invalid predictions (Ross and Dalgaard, 2004).

5.1. Primary growth models

Several primary growth models such as the modified Gompertz model, logistic model, exponential model, Baranyi model, Buchanan model and McKellar model have been developed (see reviews by Baranyi and Roberts, 1994; Skinner et al., 1994; McDonald and Sun, 1999; McKellar and Lu, 2004; Pérez-Rodríguez and Valero, 2013; Zwietering et al., 1990). The modified Gompertz model is known to overestimate maximum specific growth rate values by approximately 10-20% (Baranyi, 1992; Dalgaard et al., 1994; Membré et al., 1999) and may provide negative lag-time estimates (Dalgaard, 1995). The Baranyi and Roberts model provides practically identical maximum specific growth rate values and lag times compared to those obtained from the less complicated logistic model (Dalgaard et al., 1994; Dalgaard, 1995; Dalgaard and Koutsoumanis, 2001). Consequently, Augustin and Carlier (2000) estimated factors to correct published μ_{max} -values depending on the primary model used. If the logistic model with delay is used as the reference, μ_{max} -values estimated with the Gompertz, logistic, Baranyi and Roberts and log-linear models have to be multiplied by a factor of 0.84, 0.86, 0.97 and 1.00, respectively.

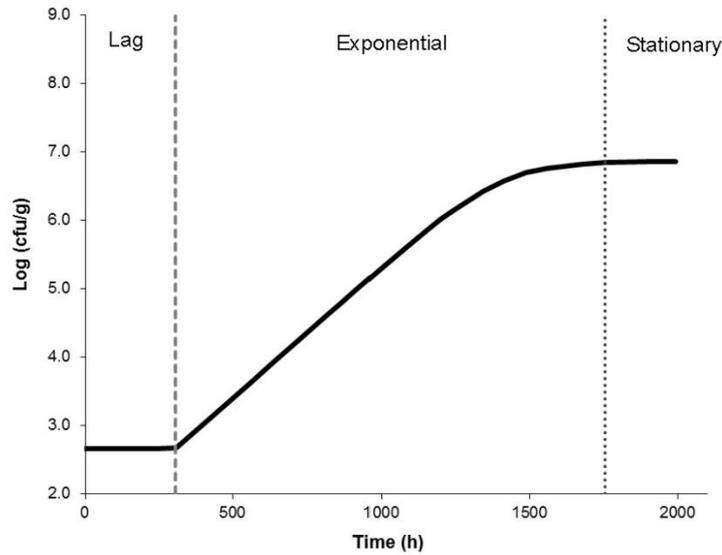


Figure 6. Bacterial growth curve

In the present thesis the integrated and log transformed logistic model with delay (four parameter model) or without delay (three parameter model) (eq. (1); Rosso et al., 1996) was fitted to all individual growth curves of *L. monocytogenes* (Fig. 7) and psychrotolerant pseudomonads obtained in challenge tests at constant temperatures.

$$\text{Log } (N_t) = \text{Log } (N_0) \quad \text{if } t < t_{lag}$$

$$\text{Log } (N_t) = \text{Log} \left(\frac{N_{max}}{1 + \left(\frac{N_{max}}{N_0} - 1 \right) \cdot \exp(-\mu_{max} \cdot (t - t_{lag}))} \right) \quad \text{if } t \geq t_{lag} \quad (1)$$

where $\text{Log } N_0$ and $\text{Log } N_{max}$ (log cfu/g) are the initial and maximum cell concentration, respectively. t_{lag} (h) is the lag-time, μ_{max} (1/h) is the maximum specific growth rate, t is the storage time (h) and N_t is the cell concentration (cfu/g) at time t .

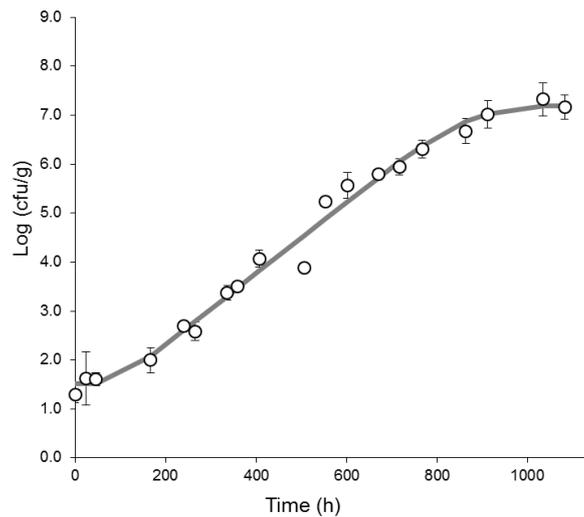


Figure 7. Observed growth of *L. monocytogenes* (log cfu/g) in spreadable processed cheese stored at 10.1 ± 0.2 °C (CT 13; Paper IV) (o) and fitted growth (solid grey line) by eq. (1).

5.2. Secondary growth models

Secondary growth models can be divided into (i) empirical or data based models and (ii) mechanistic models based on the underlying biology, chemistry or physics governing the process. These models have been extensively reviewed (Dalgaard and Mejlholm, 2019; Li et al., 2008; Ross and Dalgaard, 2004; Pérez-Rodríguez and Valero, 2013) and they can be summarized in probabilistic models, polynomial models, artificial neural networks (ANN), Arrhenius models, square root models, the gamma concept and cardinal parameter models.

The majority of the existing models for growth of *L. monocytogenes* and psychrotolerant pseudomonads includes a maximum of three or four environmental factors typically temperature, pH, NaCl/water activity and maybe lactic acid concentration (Table 9 and 10). Most processed foods contain additional compounds with potential antimicrobial effects that most of the available models do not take into account (Membré and Lambert, 2008). And as demonstrated by Mejlholm and Dalgaard (2007a, 2007b, 2009, 2013) and Mejlholm et al., (2010) extensive models containing several intrinsic and extrinsic environmental factors relevant for growth of spoilage and

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Table 9. Predictive models for *Pseudomonas* spp.

References	Type of model	Response variable	Factors and range	Developed in or evaluated for:	Model parameters
Davey (1989)	Polynomial	μ_{\max}	T (-1-30°C) a_w (0.960-0993)	Ox muscle	Temperature a_w
Fu et al. (1991)	Square root	μ_{\max}	T (2-22°C)	Reconstituted non-fat milk	$T_{\min} = -7.85^\circ\text{C}$
Membré and Burlot (1994)	Polynomial	μ_{\max} , lag-time	T (4-30°C) pH (6-8) NaCl (0-5%)	Broth	Temperature, pH, a_w and interactions
Garcia-Gimeno and Zarera-Cosano (1997)	Square root	μ_{\max}	T (4-15°C)	Salad	$T_{\min} = -250.85\text{ K}$
Neumeyer et al. (1997)	Square root	GT ^a	T(0-30°C) a_w (0.947-0966)	Broth	$T_{\min} = -7.6^\circ\text{C}$ $a_{w\min} = 0.947$
Giannuzzi et al. (1998)	Square root	μ_{\max}	T(0-10°C)	Beef	$T_{\min} = \text{not reported}$
Pin and Baranyi (1998)	Polynomial	μ_{\max}	T (2-11°C) pH (5.2-6.4)	Broth	Temperature and pH
Taoukis et al. (1999)	Square root	μ_{\max}	T (0-15°C)	<i>Boops boops</i> fish	$T_{\min} = -11.36^\circ\text{C}$
Koutsoumanis et al. (2000)	Square root	μ_{\max}	T (0-15°C) CO ₂ (0-100%)	<i>Mullus barbatus</i> fish	$T_{\min} = -11.4^\circ\text{C}$ CO _{2max} = 120.9 ppm
Koutsoumanis and Nychas (2000)	Square root	μ_{\max}	T (0-15°C)	<i>Sparus aurata</i> fish	$T_{\min} = -10.98^\circ\text{C}$
Lebert et al. (2000)	Polynomial	GT ^a	T (2-14°C) pH (5.2-6.4) NaCl (0-6.5%)	Broth	Temperature, pH, salt and interactions
Koutsoumanis (2001)	Square root	μ_{\max} , lag-time	T (0-15°C)	<i>Sparus aurata</i> fish	$T_{\min} = -11.80^\circ\text{C}$
Moore and Sheldon (2003)	Arrhenius	μ_{\max}	T (3-15°C)	Chicken drumsticks	$E_A^b = 59.0 \pm 15.1\text{ kJ/mol}$
Geysen et al. (2006)	Polynomial	μ_{\max} , lag-time	O ₂ (20-75%) CO ₂ (0-15%)	Lettuce	CO ₂ and O ₂

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Koutsoumanis et al. (2006)	Modified Arrhenius	μ_{\max} , lag-time	T (0-20°C) pH (5.3-6.1)	Beef and pork ground meat	$E_A^b = 68.8$ kJ/mol
Dominguez and Schaffner (2007)	Square root	μ_{\max}	T (0-25°C)	Raw poultry	$T_{\min} =$ not reported
Gospavic et al. (2008)	Square root	μ_{\max}	T (0-20°C)	Poultry	$T_{\min} = -5.88; -5.38; -5.82^\circ\text{C}$
Nuin et al. (2008)	Arrhenius	μ_{\max}	T (0-15°C)	<i>Psetta maxima</i> fish	$E_{\text{app}}^c = 86.1$ kJ/mol Apparent activation energy
Zhang et al. (2011)	Square root	μ_{\max}	T (0-20°C)	Beef <i>longissimus dorsi</i>	$T_{\min} = -0.085$ °C
Bruckner et al. (2013)	Arrhenius	μ_{\max}	T (2-15°C)	Pork and poultry	$E_A^b = 16.6$ kcal/mol (pork) $E_A^b = 24.6$ kcal/mol (poultry)
Paper VI	Simplified cardinal parameter model	μ_{\max} , lag-time	T (5-15°C) pH (5.0-7.0) NaCl (0.5-8%) Lactic acid (0.0-4.0%) Sorbic acid (0.0-0.45%)	Cottage cheese and milk	$T_{\min} = -7.01$ $\text{pH}_{\min} = 4.85$ $a_{w,\min} = 0.98$ $\text{MIC}_{\text{U lacti acid}} = 5.39$ mM $\text{MIC}_{\text{U sorbic acid}} = 4.74$ mM
Martinez-Rios and Dalgaard (2017)	Simplified cardinal parameter model	μ_{\max}	T (5-15°C) pH (5.0-7.0) NaCl (0.5-8%) Lactic acid (0.0-4.0%) Sorbic acid (0.0-0.45%) Acetic acid Benzoic acid Citric acid	Lightly preserved seafood	$T_{\min} = -7.01$ $\text{pH}_{\min} = 4.85$ $a_{w,\min} = 0.947$ $\text{MIC}_{\text{U lacti acid}} = 5.39$ mM $\text{MIC}_{\text{U sorbic acid}} = 4.74$ mM $\text{MIC}_{\text{U acetic acid}} = \text{NR}^d$ $\text{MIC}_{\text{U benzoic acid}} = \text{NR}^d$ $\text{MIC}_{\text{U citric acid}} = \text{NR}^d$
Gonçalves et al. (2017)	Square root	μ_{\max}	T (4-12°C) pH (5.5-6.3)	Meat broth	$T_{\min} = -14.66$ and -64.14 $\text{pH}_{\min} = -0.71$ and -0.63
Tsironi et al. (2017)	Arrhenius	μ_{\max}	T (2.5-15°C)	Salad	$E_A^b = 69.1$ kJ/mol

^a GT: generation time

^b E_A : activation energy

^c E_{aap} : apparent activation energy

^d NR: not reported

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Table 10. Examples of some predictive models for *L. monocytogenes*

References	Type of model	Response variable	Factors and range	Develop in or evaluated for:	Model parameters
Parente et al. (1998)	Probability	Probability of survival	T (30°C); pH (4.7-6.5) NaCl (0.7-4.5%) Nisin (1-2100 IU/ml) Leucocin F10 (1-2100 AU/ml) EDTA ^a (0-0.9 mmol/l)	Broth	Temperature, pH, NaCl, nisin, Leucocin, EDTA and interactions
Bouttefroy et al. (2000)	Polynomial	Log cfu/ml	T (37°C); pH (5.0-8.2) NaCl (0.0-6.0%) Nisin (0-100 IU/ml)	Broth	Temperature, pH, NaCl, nisin and interactions
Tienungoon et al. (2000)	Probability	Probability of growth	T (3.1-26.2°C) pH (3.7-7.78) a _w (0.928-0.995) Lactic acid (0-450 mM)	Broth	T _{min} = -1.62 a _{w,min} = 0.914 pH _{min} = 3.35 MIC _{ULactic acid} = 23.68 mM
Augustin et al. (2005)	Cardinal parameter	μ _{max}	T (-1.72-NR ^b °C) a _w (0.913-NR ^b); pH (4.26-NR ^b) nitrite (0.0-25.0 μM) CO ₂ (0.0-3.04 ^c) Phenol (0.0-31.9 ppm)	Cheese (Feta cheese, cottage cheese, Camembert cheese)	T _{min} = -1.72°C; T _{opt} = 37.0°C T _{max} = 45.5°C; pH _{min} = 4.71 pH _{opt} = 7.10; pH _{max} = 9.61 a _{w,min} = 0.913; a _{w,opt} = 0.997 a _{w,max} = 1.0
Boziaris and Nychas (2006)	Probability	Probability of growth	T (5-35°C) pH (4.05-6.70) a _w (0.937-0.998) Nisin (0-100 IU/ml)	Broth	Temperature, pH, a _w , nisin and interactions
Boziaris et al. (2007)	Probability	Probability of growth	T (5°C) a _w (0.951-0.999) pH (4.0-7.3) Nisin (0-100 IU/ml)	Broth	Temperature, a _w , pH, nisin and interactions
Skandamis et al. (2007)	Probability	Probability of growth	T (10-30°C); pH (3.82-7.42) NaCl (0.5-2.5%) Sodium lactate (0-10%) Sodium diacetate (0-0.5%)	Broth	Temperature, sodium lactate, sodium diacetate and interaction
Schwartzman et al. (2011)	Cardinal parameter	μ _{max}	T (-1.72-NR ^b °C) a _w (0.913-NR ^b) pH (4.26-NR ^b) Lactic acid (0-0.36 g/l)	Pasteurized and raw milk smeared cheese	T _{min} = -1.72°C; T _{opt} = 37.0°C T _{max} = 45.5°C; pH _{min} = 4.71 pH _{opt} = 7.10; pH _{max} = 9.61 a _{w,min} = 0.913; a _{w,opt} = 0.997 a _{w,max} = 1.0; MIC _{U lactic acid} = 0.4g/l

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Rosshaug et al. (2012)	Cardinal parameter	μ_{\max}	T (-1.72-NR ^b °C) pH (NR ^b) NaCl (NR ^b) Lactic acid (NR ^b)	Blue-white soft cheese	$T_{\min} = -1.72^{\circ}\text{C}$; $T_{\text{opt}} = 37.0^{\circ}\text{C}$ $T_{\max} = 45.5^{\circ}\text{C}$; $\text{pH}_{\min} = 4.41$ $\text{pH}_{\text{opt}} = 7.58$; $S_{\max} = 11.93$ $\text{MIC}_{\text{U lactic acid}} = 0.41 \text{ g/l}$
Østergaard et al. (2014)	Cardinal parameter	μ_{\max}	T (5-15°C) pH (4.4-6.9); % WPS ^d (2-6) Lactic acid (0-2%) Undissociated sorbic acid (0-0.82 mM)	Cottage cheese with cultured and non-cultured cream dressing	$T_{\min} = -2.01^{\circ}\text{C}$ $\text{pH}_{\min} = 4.87$ $a_{w,\min} = 0.923$ $\text{MIC}_{\text{U lactic acid}} = 3.79 \text{ mM}$ $\text{MIC}_{\text{U sorbic acid}} = 1.90 \text{ mM}$
Paper III	Cardinal parameter	μ_{\max}	T (2-35°C) pH (4.6-7.7) % WPS ^d (2-9) Phenol (0-20 ppm) Nitrite (0-150 ppm) % water phase diacetate (0-0.2) % water phase lactate (0-3.0)	Chemically acidified cheese and cream cheese	$T_{\min} = -2.83^{\circ}\text{C}$; $a_{w,\min} = 0.923$ $\text{pH}_{\min} = \text{term}^{\text{e}}$; Phenol = 32 ppm $a_{w,\min} = 0.923$; $\text{CO}_2 = 3140 \text{ ppm}$ Nitrite = 350 ppm $\text{MIC}_{\text{U acetic acid}} = 10.3 \text{ mM}$ $\text{MIC}_{\text{U benzoic acid}} = 0.35 \text{ mM}$ $\text{MIC}_{\text{U citric acid}} = 2.12 \text{ mM}$ $\text{MIC}_{\text{U diacetate}} = 4.80 \text{ mM}$ $\text{MIC}_{\text{U lactic acid}} = 3.79 \text{ mM}$ $\text{MIC}_{\text{U sorbic acid}} = 3.79 \text{ mM}$
Paper IV, V	Cardinal parameter	μ_{\max}	T (4-22°C) pH (6.1-6.6) aw (0.952-0.975) % water phase acetic (0.1-0.3) % water phase citric (0.1-3.8) % water phase lactic (0.6-1.5) % water phase ortho-phosphate (0.4-5.0) % water phase di-phosphate (0.0-5.0) % water phase tri-phosphate (0.0-5.2) % water phase trisodium citrate (0.2-5.0)	Spreadable processed cheese	$T_{\min} = -2.83^{\circ}\text{C}$; $a_{w,\min} = 0.923$ $\text{pH}_{\min} = \text{term}^{\text{e}}$; $\text{MIC}_{\text{U acetic acid}} = 10.3 \text{ mM}$ $\text{MIC}_{\text{U citric acid}} = 2.12 \text{ mM}$ $\text{MIC}_{\text{U lactic acid}} = 3.79 \text{ mM}$ $\text{MIC}_{\text{ortho-phosphate}} = 14.9\%$ $\text{MIC}_{\text{di-phosphate}} = 9.4\%$ $\text{MIC}_{\text{tri-phosphate}} = 7.6\%$ $\text{MIC}_{\text{trisodium citrate}} = 11.0\%$ $\text{MIC}_{\text{nisin}} = \text{model}^{\text{d}}$

^a EDTA: Ethylene-diamine-tetraacetic acid

^b NR: not reported

^c proportion of CO₂ in the modified atmosphere

^d WPS: water phase salt

^e pH_{min} is a new term presented in Paper III

^d MIC_{nisin} is a model presented in Paper V

pathogenic microorganisms are necessary not only to accurately predict growth but also to allow the use of the model for several food products (e.g. seafood, meat and non-fermented dairy products).

In relation to psychrotolerant pseudomonads (Table 9) numerous square root models have been developed with similar T_{min} -values (Fu et al., 1991; Koutsoumanis et al., 2000; Koutsoumanis & Nychas, 2000; Koutsoumanis, 2001; Neumeyer et al., 1997; Taoukis et al., 1999). Likewise, several Arrhenius models are available with comparable E_a -values (Bruckner et al., 2013; Koutsoumanis et al., 2006; Moore and Sheldon, 2003; Tsironi et al., 2017). Psychrotolerant pseudomonads models including the effect of temperature, pH, a_w , and the interaction between environmental factors have been developed (Lebert et al., 2000; Membré and Burlot, 1994); however the ability of these models to predict growth in foods was not evaluated. Paper VI represents the first attempt to develop extensive models for growth of psychrotolerant pseudomonas in foods. The mathematical models including the effect of temperature, pH, NaCl, lactic acid and sorbic acid can accurately predict growth in milk and cottage cheese with cultured cream dressing.

With respect to *L. monocytogenes* (Table 10) several models containing a term to account for the antimicrobial effect of nisin have been developed (Bouttefroy et al., 2000; Boziaris et al., 2007; Boziaris and Nychas, 2006; Parente et al., 1998). However, none of the studies compared probability of growth predicted by the models with results obtained from e.g. challenge tests with foods. This limits the applicability of the aforementioned models since as discuss in Paper V the activity and stability of nisin in foods depends on many factors including heat treatment (temperature and time), storage temperature, pH, fat and protein content (Delves-Broughton et al., 1996; Oshima et al., 2014). On the other hand, numerous models have been developed and evaluated/validated to predict growth of *L. monocytogenes* in different types of cheeses (Augustin et al., 2015; Østergaard et al., 2014; Rosshaug et al., 2012; Schwartzman et al., 2011). These models as

demonstrated by Paper II can over- or underestimated growth of *L. monocytogenes* depending on the type of cheese. These models were calibrated to growth rates observed in different cheeses, meaning that the effect of any antimicrobials not included in the model was lumped into the reference or optimal growth rate parameter (μ_{ref} or μ_{opt}). This approach was successfully used by Østergaard et al., (2014) where the μ_{ref} -value determined in broth (0.67 1/h) was calibrated for growth of *L. monocytogenes* in cottage cheese with cultured cream dressing (0.34 1/h) or cottage cheese with fresh cream dressing (0.72 1/h). Paper II demonstrated that the model of Østergaard et al., (2014) with μ_{ref} -value of 0.34 1/h can accurately predict growth of *L. monocytogenes* from changes in storage temperature and changes in product characteristics observed during production, ripening and storage of smear cheese. Whereas the same model but with μ_{ref} -value of 0.72 1/h was able to predict growth in brined cheese from constant storage temperature and constant product characteristics collected from the literature. Augustin et al. (2005) used the same approach where μ_{opt} was calibrated to *L. monocytogenes* growth rates observed in different types of cheeses (Camembert, Brick, Brie, cottage cheese, queso fresco, queso panella, queso ranchero, Ricotta, Teleme, white pickle and Emmental). However, model evaluation resulted in acceptable bias factor but predictions were little accurate with accuracy factor of 3.42.

Secondary models developed in the present PhD-thesis are cardinal parameter models (CPM). This approach has previously been successfully applied for predictions of *L. monocytogenes* growth in meat, seafood, non-fermented dairy products and cheeses (Augustin et al., 2005; Le Marc et al., 2002; Mejlholm and Dalgaard, 2009; Østergaard et al., 2014).

CPMs can cover the entire physiological range and rely on minimum, optimal and maximum parameter values (Rosso et al., 1993; Rosso et al., 1995). However, simplified CPM

focus on describing how the maximum specific growth rate (μ_{max} , h⁻¹) at the reference temperature (e.g. 25°C) is reduced when conditions become less favourable for growth (Eq. (3)).

$$\mu_{max} = \mu_{ref} \cdot \left[\frac{(T - T_{min})}{(T_{ref} - T_{min})} \right]^2 \cdot \frac{(a_w - a_{w,min})}{(a_{w,opt} - a_{w,min})} \cdot [1 - 10^{(pH_{min} - pH)}] \quad (3)$$

where μ_{ref} is equal to μ_{max} at the reference temperature (T_{ref}) of e.g. 25°C, T is the temperature (°C), T_{min} is the theoretical minimum temperature allowing growth. a_w is the water activity calculated from the concentration of NaCl in the water phase (%) or measured and $a_{w,min}$ is the minimum theoretical water activity allowing growth. pH_{min} is the theoretical minimum pH value allowing growth of microorganisms. The basic structure presented in eq. (3) can be modified and/or extended with newly determined or existing model terms (Ross and Dalgaard, 2004). The effect of environmental factors like undissociated concentrations of organic acids (OA) has been included in CPMs for growth of different spoilage and foodborne bacteria (Le Marc et al., 2002; Mejlholm and Dalgaard, 2009; Østergaard et al., 2014; Paper VI). The undissociated concentrations of OA are calculated by eq. (4) with the correspondent pKa value (Ross and Dalgaard, 2004). The effect of increasing concentrations of undissociated organic acids (OA) on μ_{max} -values can be modelled by eq. (5).

$$\text{Undissociated OA (mM)} = \frac{OA \text{ (mM)}}{1 + 10^{pH - pK_a}} \quad (4)$$

$$\sqrt{\mu_{max}} = \sqrt{\mu_{refexp} \cdot \left(1 - \left(\frac{[OA_U]}{MIC_{U OA}} \right)^{n1} \right)^{n2}} \quad (5)$$

where is the reference maximum specific growth rate obtained when performing the experiment at a specific temperature; $[OA_U]$ are the concentrations (mM) of undissociated organic acid and MIC_U

μ_{OA} is the fitted minimum inhibitory concentration (MIC) value (mM) of organic acid that prevents growth of the microorganism.

When fitting eq. (5) to experimental data, n_1 can be set to 0.5 or 1 and n_2 can be set to 1 or 2 (Dalgaard, 2009) in order to describe data most appropriately (Fig. 8) and this is determined from root mean square error (RMSE) values. By using n_1 and n_2 the effect of any antimicrobial on μ_{max} -values whether linear or not can be described. For instance, Le Marc et al., (2002) observed that increasing concentrations of undissociated lactic acid had a linear effect on *Listeria* μ_{max} -values. However, it was the square root of the undissociated acetic and propionic acid concentrations that had a linear effect on μ_{max} -values of *Listeria*. The effect of increasing concentrations of other antimicrobials (AM) e.g. phosphate salts and nisin can be modelled by eq. (6) (Paper IV-V).

$$\sqrt{\mu_{max}} = \sqrt{\mu_{refexp} \cdot \left(1 - \left(\frac{[AM]}{MIC_{AM}}\right)^{n_1}\right)^{n_2}} \quad (6)$$

where [AM] are the concentrations (%) of individual antimicrobial and MIC_{AM} is the fitted minimum inhibitory concentration (MIC) value (%) of individual antimicrobials that prevents growth of microorganisms.

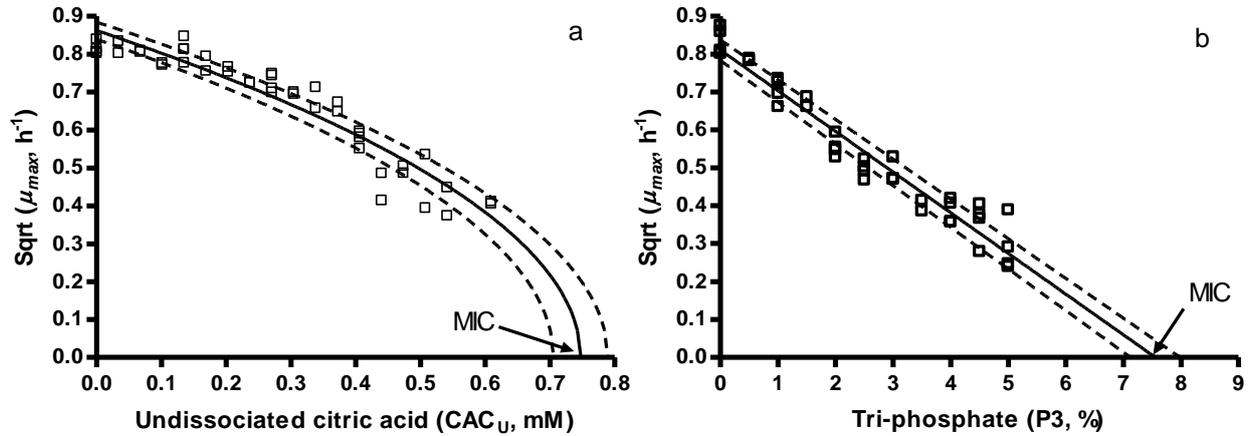


Figure 8. Effect of undissociated citric acid (CAC_U) (a) and tri-phosphate (P3) (b) on maximum specific growth rates (μ_{max} , 1/h) of *L. monocytogenes* in BHI broth at 25°C and pH 6.2. MIC-values of undissociated citric acid and tri-phosphate were determined by fitting eq. (5 and 6) to observed data (□). Solid and dashed lines represent the fitted (eq. (5 and 6)) and confidence intervals (95%), respectively (Paper IV).

Paper III, IV and V represent the first attempt to develop extensive models to predict growth of *L. monocytogenes* in different types of cheeses including chemically acidified cheese, cream cheese and processed cheese.

It has been demonstrated that growth of microorganisms for one environmental condition is affected by other environmental factor (Augustin and Carlier, 2000; Davey, 1989; Salter et al. 2000; Koutsoumanis et al., 2004; Le Marc et al., 2002). This interaction is more important close to the growth boundary of microorganisms where interaction between environmental factors has to be taken into consideration to avoid overestimation of growth (Augustin et al., 2005). The interaction between environmental parameters (ξ) has been modelled in this PhD-thesis by using the Le Marc approach (Le Marc et al., 2002; Mejlholm and Dalgaard, 2009; Paper III-IV) where the effect of interaction between environmental factors is divided into three regions: (i) if ψ is less than 0.5, then no interactive effect between environmental factors occurs ($\xi = 1$); (ii) if ψ is greater than 1, then no

growth occurs ($\xi = 0$); and (iii) if ψ is less than 1 and greater than 0.5, then the growth rate (μ_{max} , 1/h) is reduced depending on the value of ψ . A ψ value greater than 1 (e.g., 1.5 or 2.0) provides a measure of how far the properties of a specific food product is from the predicted growth boundary of *L. monocytogenes* (Mejlholm and Dalgaard, 2009). For chilled products with shelf-life of more than 5 weeks products formulations resulting in a ψ -value >2 has been recommended (Dalgaard and Mejlholm, 2019).

The concept of relative lag time (*RLT*, eq. (8), Mellefont and Ross, 2003) was used to describe lag times of *L. monocytogenes* growing in spreadable processed cheese (Paper IV) and psychrotolerant pseudomonads growing in cottage cheese and milk (Paper VI). The concept can be interpreted as the amount of work a cell has to do to change its physiology when introduced in a new environment. *RLT* are calculated as the ratio of lag time (t_{lag}) and generation time (t_g) at identical conditions (eq. (8)).

$$RLT = \frac{t_{lag}}{t_g} = \frac{t_{lag} \cdot \mu_{max}}{\ln(2)} ; t_{lag} = \frac{RLT \cdot \ln(2)}{\mu_{max}} \quad (8)$$

5.3. Predictive microbiological software for dairy products

To use a risk based management approach to evaluate and to improve the quality and safety of food products as recommended by *Codex Alimentarius*, FBOs need to use mathematical models to predict microbial behaviour throughout the food chain (CAC, 1999). Several researchers have developed predictive microbiology software (e.g. FSSP, Sym'Previus, ComBase, GroPIN) (see Tenenhaus-Aziza and Ellouze, 2015). These software also known as tertiary models allows experts and non-experts to obtain predictions of microbial behaviour by using products characteristics and storage conditions of their products (e.g. pH, a_w , temperature, lactic acid, etc.) as inputs for predictions. The Dairy Product Safety Predictor is commercial software that performs simulations

of an exposure and risk assessment for specific microorganisms and specific dairy products. The microorganisms include *L. monocytogenes*, *Salmonella* spp, *S. aureus* and *E. coli* (VTEC) and the dairy products are blue cheese, cooked pressed cheese and soft cheese. The software includes factors to account for the effect of temperature, pH, a_w and lactic acid on growth of the aforementioned pathogens. The predictive models presented by this PhD-thesis will markedly increase the ability of the dairy sector to use predictive models in product formulation and risk assessment when included in Food Spoilage and Safety Predictor (FSSP) software.

6. Application of predictive models

Validated models for growth of *L. monocytogenes* and psychrotolerant pseudomonads in different types of dairy products can be used to support (i) innovation and development of new products (ii) risk assessment (iii) or to identify the maximum achievable shelf-life in the intended distribution conditions (Legan et al., 2009; Membré and Lambert, 2008). To demonstrate the potential use of models developed and validated throughout this PhD-project examples are provided in the following sections to exemplify how product developers and food safety management can benefit from their use. These examples contain growth predictions for *L. monocytogenes* and psychrotolerant pseudomonads in products available in the market and the effect on growth caused by a reformulated recipe.

6.1. Processed cheese

Presence of *L. monocytogenes* in processed cheese is highly unlikely before the opening of packages by consumers. For instance, Kim et al. (2018) analysed 800 processed cheese samples for presence of *L. monocytogenes* and none was detected positive for the pathogen. This is the result of good hygiene practices, high temperature processing (>70°C) and hot-filling of the end product (see section 4.2). Therefore, scenarios presented in here are related to consumer cross-contamination of the product. Perditions for growth of *L. monocytogenes* in spreadable processed cheese are performed with the model developed in Paper IV.

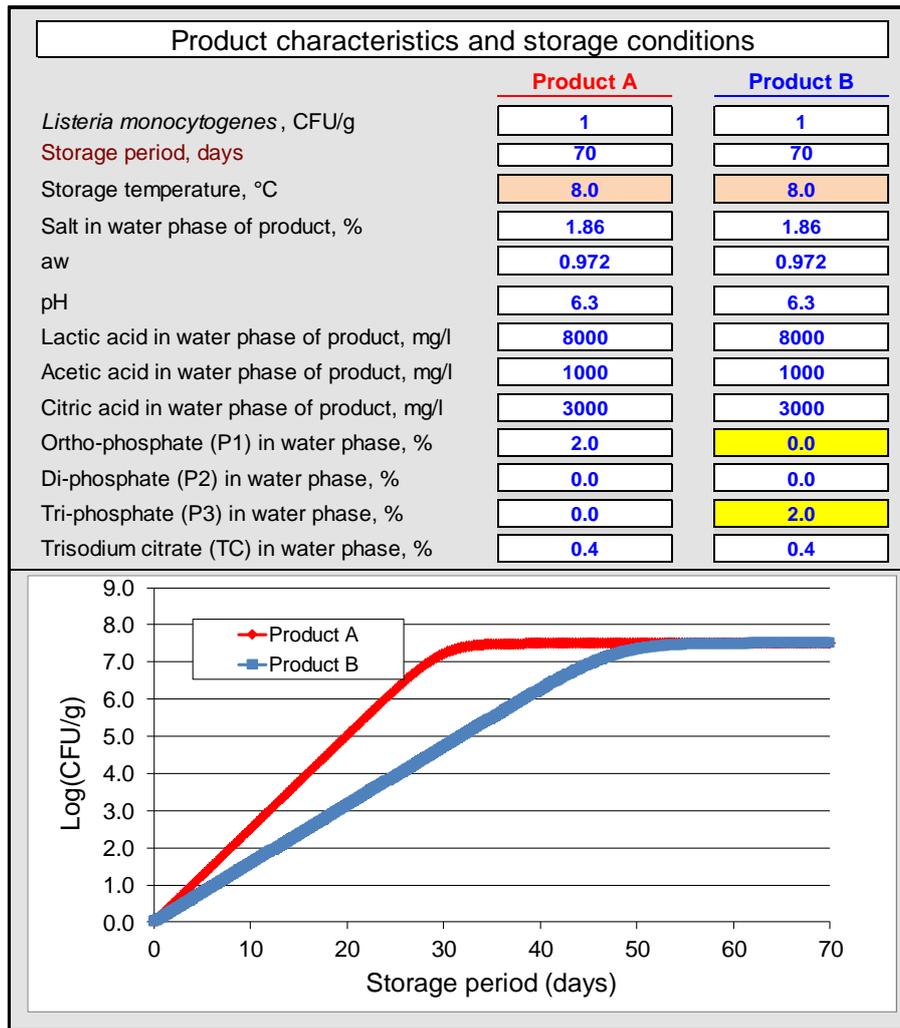


Figure 9. Predicted growth of *L. monocytogenes* in spreadable processed cheese. Predictions were obtained by the model present in Paper IV. Highlighted in yellow are changed parameters in product B compared to product A.

Spreadable processed cheese with pH 6.3, a_w 0.972 and water phase organic acid concentrations of 0.8% (lactic acid), 0.1% (acetic acid), 0.3% (citric acid) and 2.0% (orthophosphate) is studied (Fig.9-Product A). If product A is contaminated with 1 cfu/g and stored at 8°C, *L. monocytogenes* will reach the critical concentration of 2 log cfu/g after eight days of storage. A larger open-shelf life and safety margin may be desirable and the new model predicts that by substituting the 2.0% orthophosphate with 2.0% tri-phosphate, *L. monocytogenes* will require 13 days to reach the critical concentration in the reformulated product B (Fig.9-Product B).

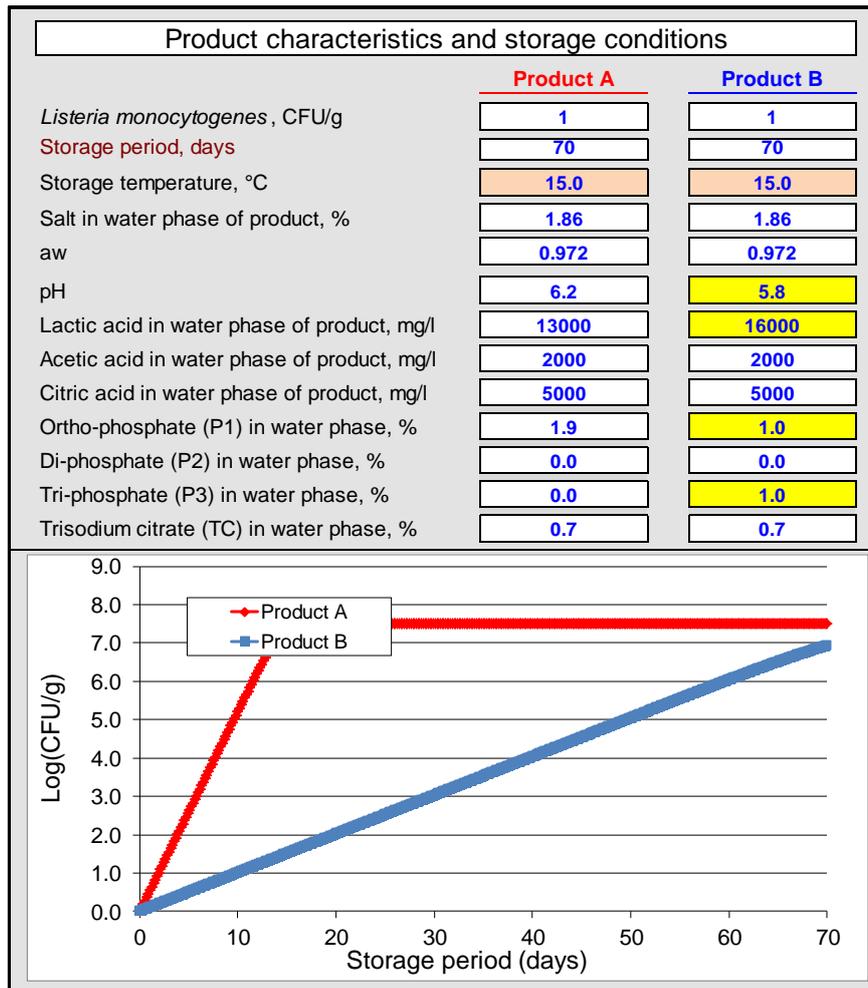


Figure 10. Predicted growth of *L. monocytogenes* in spreadable processed cheese. Predictions were obtained by the model present in Paper IV. Product characteristics for product A represent parameters measured in commercial spreadable processed cheese used in challenge tests 15, 16 and 17 from Paper IV. Highlighted in yellow are changed parameters in product B compared to product A.

As another example, the model can be used to modify several product characteristics of spreadable process cheese and simulate growth at ambient temperature of a product with pH 6.2, a_w 0.972, 1.3% (lactic acid), 0.2% (acetic acid), 0.5% (citric acid) and 1.9% P1 in the water phase of a product and stored at 15°C (Fig.10-product A). If product A is contaminated with 1 cfu/g and stored at 15°C it will take four days for *L. monocytogenes* to reach the critical concentration of 2 log cfu/g. However, if the product is reformulated with pH 5.8, a_w 0.972, 1.6% (lactic acid), 0.2% (acetic acid), 0.5% (citric acid), 1.0% orthophosphate and 1% trip-phosphate in the water phase of the

product B, *L. monocytogenes* will be prevented from reaching the critical concentration for 20 days of storage at 15°C (Fig.10-product B). In the present PhD-project the focus has been on developing predictive models for *L. monocytogenes* growth on various types of cheese; however, for processed cheese the major pathogen of concern before opening a product will be *C. botulinum*. Therefore, the logic next step will be to assess growth potential and toxin production of *C. botulinum* in the reformulated product (Fig.10-product B). Several publications recommend using lactic acid to delay toxin production. Tanaka et al. (1986) recommended the addition of 0.25% of lactic acid to processed cheese to inhibit toxin production; however, no information was reported on lactic acid concentration present previously to the addition in processed cheese. Glass and Johnson (2004) observed that toxin production was delayed in processed cheese containing 1.5% sodium lactate. Therefore, the extended model of ter Steeg and Cuppers (1995) containing terms for temperature, pH, % of citrate in total salts, % of salts in moisture and % of lactate in moisture was used to estimate time necessary to a 100-fold increase of *C. botulinum* cells. The model predicted the 100-fold increase to occur after 4.3 days of storage at 15°C; however, food developers and food safety management from the dairy industry will benefit from a more flexible and comprehensive model to be used in combination with the model present in Paper IV.

Nisin is a preservative with a well-documented use for the control of spore-forming bacteria in processed cheese (Delves-Broughton, 1990; Delves-Broughton, 2008; Glass and Doyle, 2013; Somers and Taylor, 1987). However, little information is available with regards to its protective effect against *L. monocytogenes*, when this pathogen is introduced by cross-contamination of processed cheese at the consumer phase. Prediction of *L. monocytogenes* growth in processed cheese containing nisin is possible by using model A present in Paper V. For instance, a processed cheese with pH 5.9, a_w 0.981, water phase concentrations of 0.94% (lactic acid), 0.25% (acetic acid), 0.5% (citric acid), water phase orthophosphate of 1.6% and residual nisin A of 0.2 mg/g is

used (Fig. 11-product A). If product A is contaminated with 1 cfu/g and stored at 15°C it will take five days for *L. monocytogenes* to reach the critical concentration of 2 log cfu/g. However, if the product is reformulated to contain 2.0 mg/g of residual nisin A then *L. monocytogenes* will be prevented from reaching the critical concentration for 12 days of storage at 15°C (Fig.11-product B).

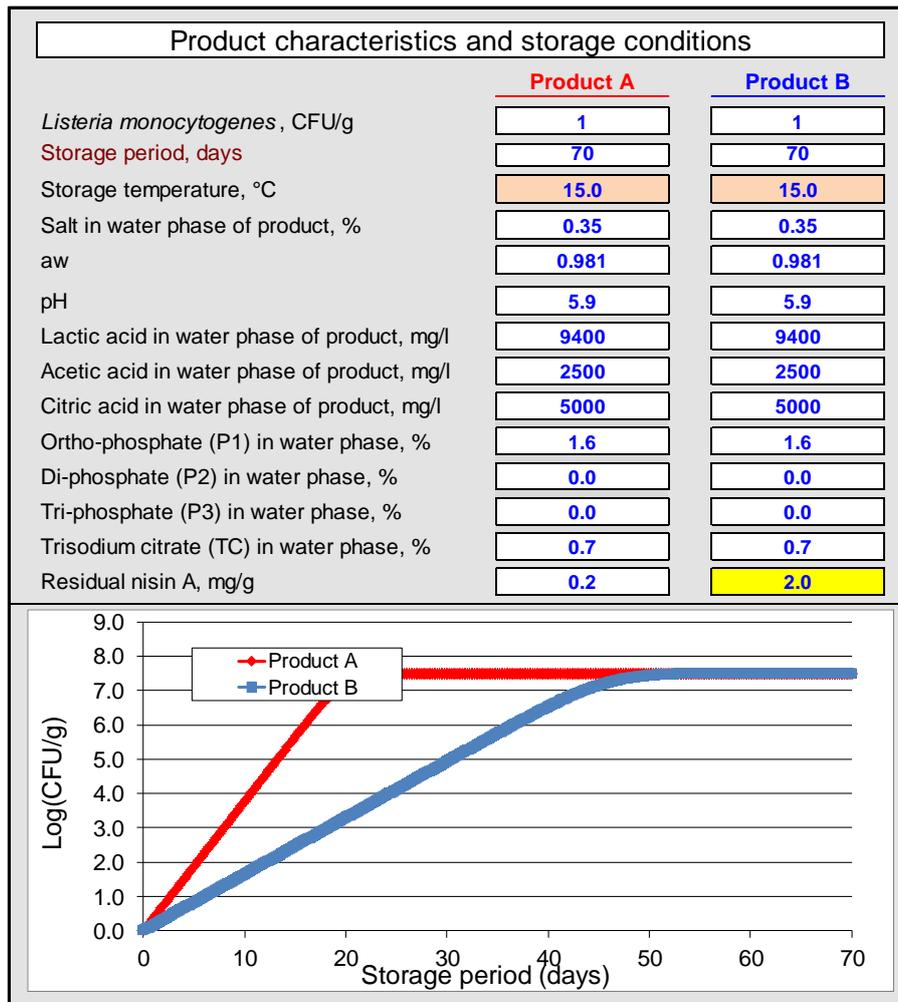


Figure 11. Predicted growth of *L. monocytogenes* in processed cheese containing nisin. Predictions were obtained by the model present in Paper V. Product characteristics of product A represent parameters measured in commercial processed cheese used in challenge test 10 from Paper V. Highlighted in yellow are changed parameters in product B compared to product A.

6.2. Chemically acidified cheese

Chemically acidified cheese might become contaminated with *L. monocytogenes* after pasteurization when adding GDL to acidify the milk (Tamine and Kirkegaard, 1996). The model present in Paper III can be used to assess *L. monocytogenes* growth in chemically acidified cheeses depending on storage conditions and product characteristics. As an example, if a chemically acidified cheese with pH 4.6 and 4.4% NaCl in the water phase (Fig.12-product A) is contaminated with 1 *L. monocytogenes*/g after pasteurization and subsequently chill stored at 5 °C then the product will not support growth. However, if the product is stored at 25°C (Fig.12-product B) the critical concentration of 100 cfu/g will be exceeded in less than two days. If the product B is reformulated by including 0.17% lactic acid in the water phase the model predicts that *L. monocytogenes* will exceed the critical concentration after 237 days of storage at 25°C (Fig.12-product C). Nevertheless, if lactic acid concentration is increased up to 0.21%, then the model calculates a ψ -value of 2.5, meaning that growth of *L. monocytogenes* is unlikely to occur as consequence of intrinsic variability of product characteristics or strain.

6.3. Cream cheese

The model present in Paper III can be used to predict growth/no-growth of *L. monocytogenes* in cream cheese. As example, a cream cheese with pH 5.2, 1.9% water phase NaCl, and water phase organic acids concentrations of 0.20% (lactic), 0.10% (acetic) and 0.10% (citric) was used. If the product is contaminated with 1 cfu/g growth of *L. monocytogenes* will not be supported (ψ of 2.1); however if the same contaminated product is stored at 25°C then the critical cell concentration will be exceeded in 2.5 days (ψ of 0.4). The model can be used to design a reformulated product with lower pH (5.0) and increased concentrations in the water phase of lactic

acid (0.45%) and acetic acid (0.15%) where growth of *L. monocytogenes* will be prevented if stored at 25°C (ψ of 2.4).

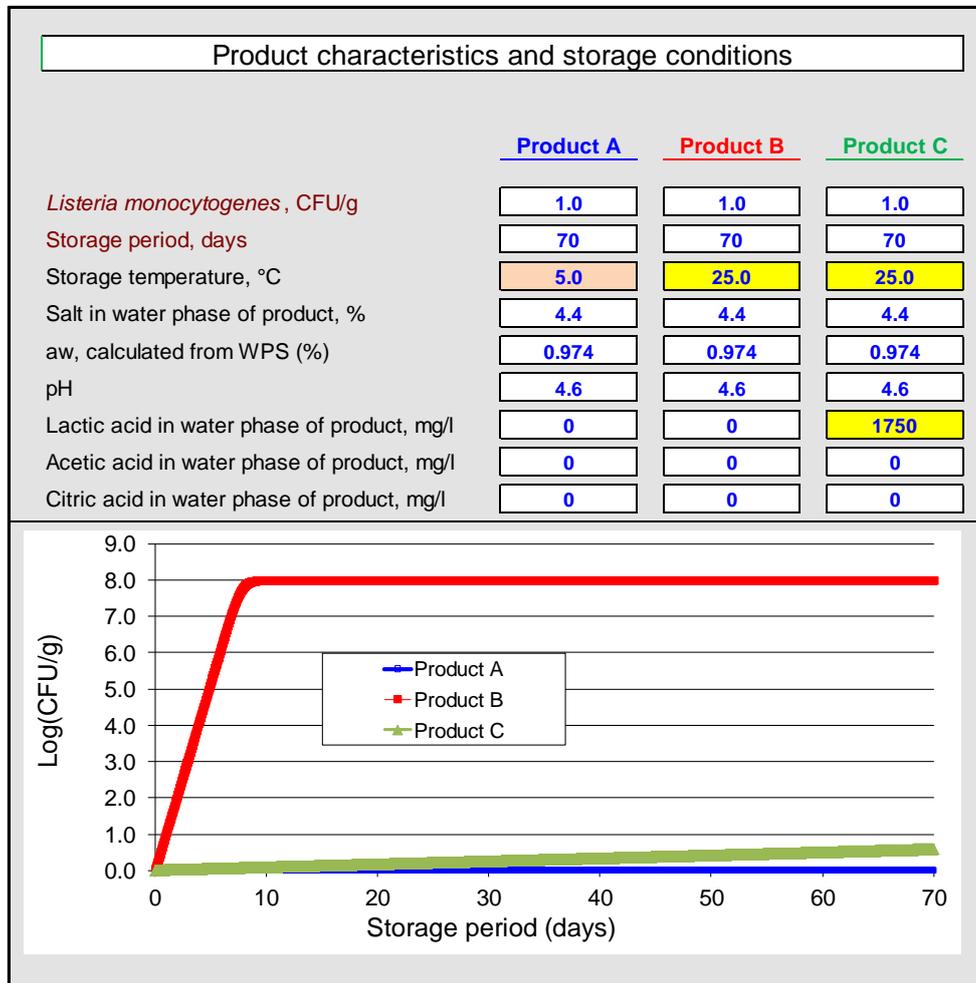


Figure 12. Predicted growth of *L. monocytogenes* in chemically acidified cheese. Predictions were obtained by the model present in Paper III. Product characteristics for product A represent parameters measured in commercial chemically acidified cheese used in challenge test 5 from Paper III. Highlighted in yellow are changed parameters in product B and C compared to product A.

6.4. Smear cheese

Predictive microbiology models are essential tools to model bacterial growth in quantitative microbial risk assessments (Pouillot and Lubran, 2011). Validated predictive models can be used to

estimate the number of *L. monocytogenes* at the time of consumption and therefore are valuable tools as part of exposure assessment studies (Zwietering and Nauta, 2007). However, the growth models used in published quantitative microbiological risk assessments (QMRA) remain simple compared to those developed in predictive microbiology (Campagnollo et al., 2018; FAO/WHO, 2004; FDA/FSIS, 2003; Pouillot and Lubran, 2011; Sanaa et al., 2004; Tiwari et al., 2015). For instance, the EFSA (2018b) QMRA of the risk from *L. monocytogenes* in ready-to-eat products used a secondary growth model containing a term for temperature and an exponential growth rate log-normal distribution for cheese data extracted from seven studies (Perez-Rodriguez et al., 2017). At least for cheese, this might be due to the lack of information available in the literature about product characteristics that will preclude the use of complex models (see section 4.1).

Food producer might know more about their products physico-chemical characteristics and therefore be able to use extensive models when performing risk assessment. Paper II demonstrated that the model of Østergaard et al. (2014) developed to predict growth of *L. monocytogenes* in cottage cheese can be used to estimate growth of *L. monocytogenes* in smear cheese (Fig. 13) at the time of consumption. Thereby, the model can be used to quantify contribution of every individual process step (e.g. acidification, ripening, retail, consumer storage) to the total number of *L. monocytogenes*. Furthermore, the model can be used to simulate different scenarios that can affect growth of *L. monocytogenes* or to evaluate critical control points suggested by other studies for similar cheeses (e.g. soft cheese). For instance, Lamboni et al. (2014) proposed that temperature during transportation and conservation of soft cheese should be maintained between 4.3°C and 5.5°C; and between 11.4°C and 13.5°C during ripening. Their suggestion is based on a sensitivity analysis for determination of critical control points using a growth model for *L. monocytogenes* containing terms for temperature, pH and a_w . It will be interesting to use a similar approach but substituting their growth model by the model presented in Østergaard et al. (2014).

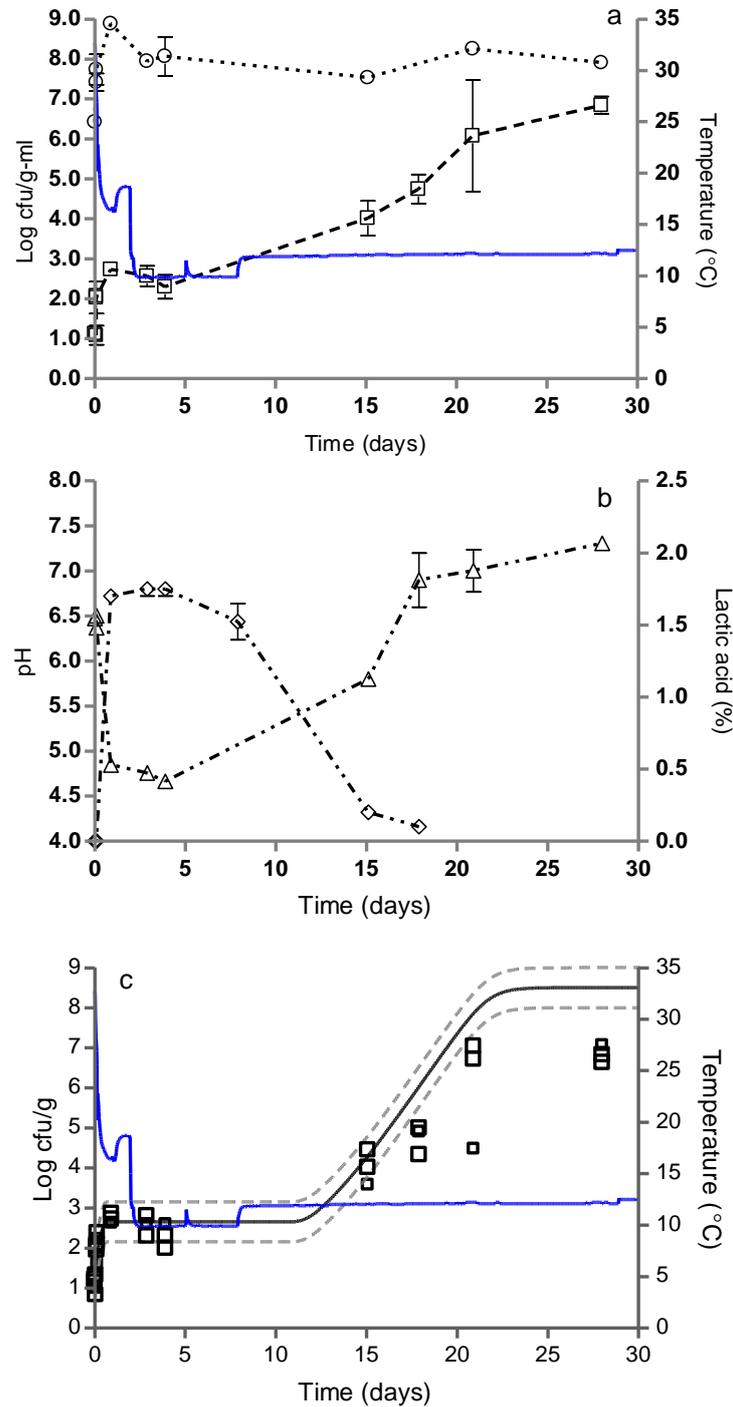


Figure 13. Data collected in the rind of smear cheese. *L. monocytogenes* was inoculated in the milk used to produce smear cheese. (a) *L. monocytogenes* (□) and lactic acid bacteria (○) behaviour in smear cheese. (b) Evolution of pH (△) and lactic acid concentration in % (◇). Symbols represent average values and error bars of the standard deviation for three samples. (c) Comparison of observed (□) and predicted (-) growth of *L. monocytogenes* in the rind of smear cheese. Solid lines represent the predicted growth by the model presented in Paper II when using pH and lactic acid concentrations shown in (b) as model inputs. Graphs include the acceptable simulation zone (± 0.5 Log cfu/g, dashed lines). The temperature profiles are shown as blue lines in the top figures.

6.5. Cottage cheese

Time to reach the minimal spoilage level of cottage cheese by psychrotolerant pseudomonads can be predicted by the model present in Paper VI. Psychrotolerant pseudomonas can become part of the cottage cheese microbiota by contamination during production (see section 3.1). If psychrotolerant pseudomonads grow during storage of the product, metabolites responsible for off-odours will be produced and will result in a sensory rejection. During the model development process, changes in overall appearance and odour of the samples were evaluated and scored by panellists. Analysis of the panellists scores indicated that the minimal spoilage level for psychrotolerant pseudomonads in cottage cheese is 8.4 ± 0.4 log cfu/g. Therefore, the growth model in combination with the minimal spoilage level estimated by sensory analysis can be used for example to document that the defined shelf-life is adequate for the intended storage conditions or to reformulate the product to extend shelf-life if necessary (Fig. 14). To demonstrate how variability in product characteristics can affect growth of psychrotolerant pseudomonas, simulations with the least and the most preserved products measured by Østergaard et al. (2014) are shown in Fig. 14. If the most preserved cottage cheese with culture cream dressing and pH 5.3, 1.1% NaCl and 0.14% lactic acid in the water phase (Fig.14-product A) is contaminated with 1 cfu/g and subsequently chill stored at 5 °C then the minimal spoilage level will not be exceeded within the 21 days of declare shelf-life. However, if the least preserved product with pH 5.5, 0.9% NaCl and 0.04% lactic acid in the water phase is stored at 7°C (Fig.14-product B) the minimal spoilage level will be reach on the 16th day of storage. If then product B is reformulated by including 0.05% sorbic acid in the water phase of the product, this will result in a difference in shelf-life of eight extra days when store at 7°C (Fig.14-product C).

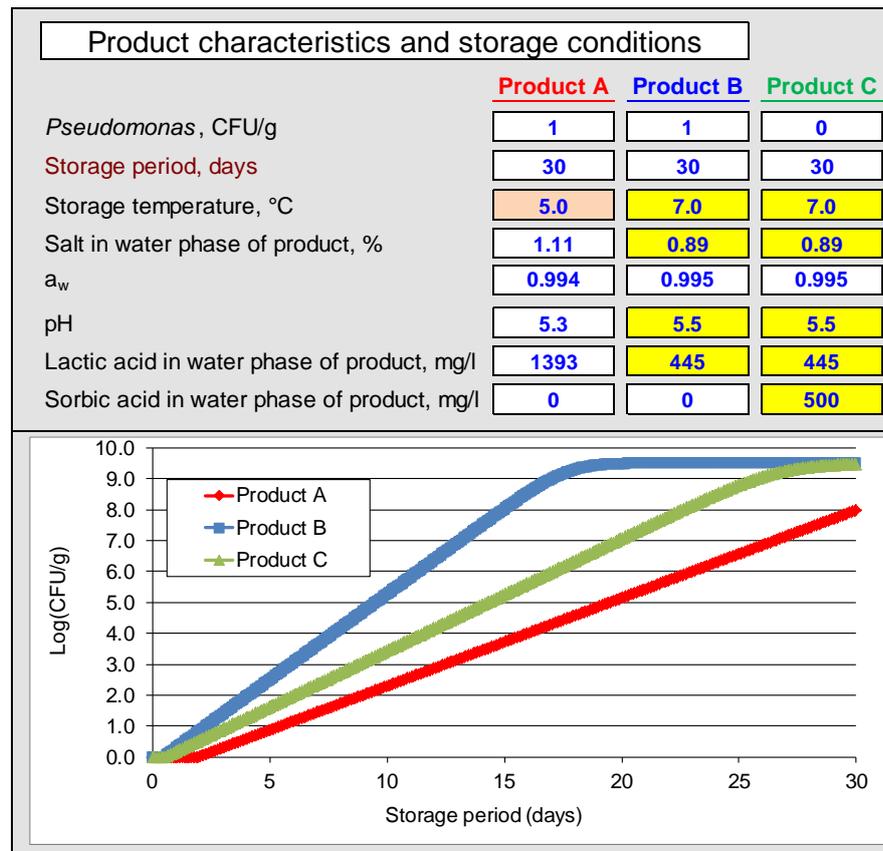


Figure 14. Predicted growth of psychrotolerant pseudomonas in cottage cheese with cultured cream dressing. Predictions were obtained by the model present in Paper VI. Product characteristics for product A and B represent parameters measured in commercial cottage cheese with cultured cream dressing measured by Østergaard et al., 2014. Highlighted in yellow are difference in parameter for product B and C compared to product A.

7. Conclusions and perspectives

This PhD-thesis has demonstrated that accurate prediction of *L. monocytogenes* growth in different types of cheeses is possible when using extensive models containing all relevant intrinsic/extrinsic factors and products are fully physico-chemically characterized.

Systematic review of the literature showed that *L. monocytogenes* primarily is involved in outbreaks related to smear- or fresh cheese. Meta-analysis of *L. monocytogenes* prevalence in different types of European cheeses revealed (i) that the highest mean prevalence is observed in smear cheese and (ii) mean prevalence in cheeses produced with un-pasteurized milk is similar to those produced with pasteurized milk highlighting the importance of post-pasteurization contamination (Paper I).

The model of Østergaard et al., (2014) developed to predict growth of *L. monocytogenes* in cottage cheese with cultured and fresh cream dressing was validated for smear cheese by using data collected from challenge tests and from literature. As well, the model for fresh cream dressing was validated to predict growth of *L. monocytogenes* in brined cheese with literature data (Paper II).

A growth and growth boundary model for *L. monocytogenes* in chemically acidified cheese and cream cheese was successfully developed by substituting the constant pH_{min} -value present in the Mejlholm and Dalgaard (2009) model by a new pH_{min} -term. The model including 12 intrinsic/extrinsic parameters and interactions was validated with an extensive data set containing a total of 10 parameters. The model can be used to optimize product characteristics that prevent growth of *L. monocytogenes* in a broad range of foods including meat, seafood and different dairy products (milk, cream, desserts, chemically acidified cheese and cream cheese) with pH-values as low as 4.6 (Paper III).

A second growth and growth boundary model for *L. monocytogenes* in spreadable processed cheese and processed cheese containing nisin was successfully developed and validated. This model including the effect of temperature, pH, a_w , lactic-, acetic-, citric-, orthophosphate, di-phosphate, tri-phosphate, residual nisin A and interactions can be used to optimized processed cheese formulations that will inhibit or prevent growth of *L. monocytogenes* if contaminated by consumers and stored at chilled or ambient temperatures. Furthermore, a LC-MS/MS method to quantified concentrations of residual nisin A and Z in processed cheese was developed and validated. This method can be used to support product development by quantifying and optimizing concentrations of residual nisin A and Z present in processed cheese that will inhibit or prevent growth of *L. monocytogenes* (Paper IV and V).

Lastly, a growth and growth boundary model for psychrotolerant pseudomonads growth in cottage cheese with cultured cream dressing and milk was developed and validated. This model including the effect of temperature, pH, NaCl/ a_w , lactic-, sorbic acid and interactions can be used to optimized cottage cheese formulations that will prevent psychrotolerant pseudomonads from reaching the minimal spoilage level during the intended shelf-life (Paper VI).

Once all these models are incorporated in Food Spoilage and Safety Predictor (FSSP) they will become valuable support tools for the dairy industry and food safety authorities.

In relation to spreadable processed cheese, it seems relevant in future studies to examine if fat and moisture concentrations have any effect on the ability of the developed model to predict growth of *L. monocytogenes* (Paper IV and V). Furthermore, it is necessary to evaluate model performance with a wider product pH range in order to study if the new MIC-value for undissociated concentration of citric acid provides with more accurate predictions than using the trisodium citrate MIC-value.

Regarding nisin, it appears interesting to evaluate the effect of the heat treatment (temperature and time) used to produce processed cheese on the residual concentrations of nisin A quantify by LC-MS/MS. Secondly, it is relevant to evaluate the developed model containing a nisin term with higher concentrations of residual nisin A than used in Paper V and to evaluate if a nisin Z term will be necessary to be included in the model. Lastly, LC-MS/MS quantification of residual nisin A should be compared with results from the agar diffusion methods extensively used by the food industry to quantify nisin activity.

The model of Østergaard et al., (2014) is a promising candidate to predict growth of *L. monocytogenes* in a broad range of fermented cheeses with different maturation processes. It seems necessary to further evaluate the model with other smear cheese than those employed in Paper II. Challenge test with other soft-smear cheese such as Taleggio or soft Limburger inoculated with *L. monocytogenes* will provide valuable data since this cheeses have a different microbiota than those study in challenge tests. Lastly, it is interesting to evaluate different scenarios of *L. monocytogenes* contamination. In Paper II the milk to produced cheese was inoculated with *L. monocytogenes* while future studies should simulate different scenarios, such as contamination during the demolding process, ripening period, portioning of products and contamination by the consumer.

Last but not least, it seems very interesting to evaluate the ability of the Østergaard et al. (2014) model to predict growth of *L. monocytogenes* in a well characterized veined cheese, were growth information and product characteristics are collected from specific locations of the cheese (e.g. from the mold area and from the none-mold area).

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Paper I

Martinez-Rios, V., Dalgaard, P.

Prevalence of *Listeria monocytogenes* in European cheeses: A systematic review and
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Prevalence of *Listeria monocytogenes* in European cheeses: A systematic review and meta-analysis

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ABSTRACT

Both in Europe and worldwide cheese has caused important outbreaks of listeriosis and can be a vehicle for transmission of *Listeria monocytogenes* to consumers. A systematic review and meta-analysis were conducted using scientific literature and European Food Safety Authority (EFSA) reports to summarize available data on the prevalence of *L. monocytogenes* in different types of cheeses produced in Europe. Meta-analysis models were used to estimate mean prevalence of the pathogen and to compare prevalence among types of cheeses (fresh, ripened, veined, smear and brined) and cheeses produced using, respectively, pasteurized or un-pasteurized milk. Data from a total of 130,604 samples were analysed. Mean prevalence for presence during 2005-2015 estimated from scientific literature (2.3% with confidence interval (CI): 1.4-3.8%) was more than three times higher than results from EFSA reports (0.7%; CI: 0.5-1.1%). The prevalence differed among types of cheeses including fresh (0.8%; CI: 0.3-1.9%), ripened (2.0%; CI: 0.8-4.9%), veined (2.4%; CI: 0.9-6.3%), smear (5.1%; CI: 1.9-13.1%) and brined (11.8%; CI: 3.5-33.3%). Mean prevalence of *L. monocytogenes* in soft/semi-soft cheeses were not significantly different ($P > 0.05$) for cheeses produced from pasteurized (0.9%; CI: 0.4-1.9%) or un-pasteurized (1.0%; CI: 0.4-2.2%) milk. For cheese samples reported by EFSA 0.2% CI: 0.1-0.4% had concentration of *L. monocytogenes* above the critical European limits of 100 cfu/g. In addition, this systematic review focused on groups/species of microorganisms suitable as indicator organisms for *L. monocytogenes* in cheeses to reflect the level of production hygiene or as index organisms to assess the prevalence of *L. monocytogenes* in cheeses. However, no suitable indicator or index organisms were identified. The performed meta-analyses improved our understanding of *L. monocytogenes* prevalence in different types of cheeses and provided results that can be useful as input for quantitative microbiological risk assessment modelling.

Keywords: Occurrence, fresh cheese, soft and semi-soft cheeses, risk assessment

1. Introduction

The genus *Listeria* includes more than 20 species that can be divided into three clades (Weller et al. 2015). Two *Listeria* species belonging to the same clade are generally considered to be pathogenic, *L. monocytogenes* in humans and *L. ivanovii* in other mammals. Nevertheless, there have been some reports of *L. seeligeri* and *L. ivanovii* causing illness in humans (Cummins et al., 1994; Rocourt et al., 1986). The likelihood of *L. monocytogenes* infection leading to listeriosis is greatest among certain groups; including pregnant woman, neonates, immunocompromised adults and the elderly (Ryser & Marth, 2007). Within the European Union (EU) there has been a statistically significant increasing trend of listeriosis over the period 2009-2015. Specifically, the numbers of confirmed human cases of listeriosis were 1,331 and 2,206 in 2009 and 2015, respectively (EFSA, 2016). A total of 270 deaths due to listeriosis were reported within nineteen EU member states. The overall EU notification rate of listeriosis was 0.46 cases per 100.000 population with a case-fatality rate of 17.7% (EFSA, 2016). Seven EU Member States and Norway provided information from conventional serotyping of *L. monocytogenes* (accounting for 23.3 % of all confirmed cases). The most common serotypes in 2013 were 1/2a (57.5 %) and 4b (34.3 %), followed by 1/2b (6.4 %), 1/2c (1.4 %), 3a and 3b (both 0.2 %) (EFSA, 2015).

In 2010-2011 an EU baseline survey (EFSA, 2013a) collected data about presence of *L. monocytogenes* and the non-compliance for different ready-to-eat (RTE) food categories at retail. The proportion of *L. monocytogenes* positive samples at retail was highest in fish products (mainly smoked fish), followed by soft and semi-soft cheeses and RTE meat products. Specifically, the EU prevalence of *L. monocytogenes* in cheeses at retail was 0.47 % (CI: 0.29-0.77%) determined as 16 positive samples out of 3393 at the end of shelf-life. For these 2010-2011 samples 0.06% (CI: 0.02-0.24 %) determined as two samples out of 3393 exceeded the critical concentration of 100 cfu/g

(EFSA, 2013a). In 2015 fifteen samples out of 3039 exceeded the critical concentration of 100 cfu/g (EFSA, 2016).

The first reported outbreak of human listeriosis associated with consumption of cheese occurred in the USA during 1985 (Linnan *et al.*, 1988) and was caused by a fresh cheese. Since then, several outbreaks associated with consumption of cheese have occurred worldwide and fatalities continue to be reported (Table 1). Clearly, it is important to collect information and to analyse data in an attempt to improve our understanding and options to better manage this risk.

Meta-analysis is a statistical approach that can be used to analyse, for example, prevalence data (effect size) originating from various sources (primary studies) and in this way provide an overview of effects and variability (Glass, 1976; Sutton, et al., 2001). Lately, meta-analysis has been used to study several food safety issues and the quantitative results obtained can be used as inputs in risk assessment models (Baron et al., 2009).

Fortunately, prevalence and concentrations of *L. monocytogenes* in cheeses and cheese processing environments are low. Therefore, to evaluate its potential presence other index or indicator microorganisms that are easier to determine or quantify can be relevant to analyse. Index organisms can be used to assess likelihood of the presence of a pathogen whereas indicator organisms demonstrate a failure in Good Hygiene Practices (GHP) (Brodsky, 1995; Mossel, 1978). EU Regulation (EC) No 2073/2005 use coagulase-positive staphylococci as index organisms to assess the likelihood of staphylococcal enterotoxins in cheese made from raw or pasteurized milk and *E. coli* is used as an indicator for the level of production hygiene in cheese made from milk that has undergone heat treatment. Furthermore, *Listeria* spp. has been used as index organisms for the likely presence of *L. monocytogenes* in food (FSIS, 2014; Gilbert et al., 2000).

The objective of the present study was to perform a systematic review and a meta-analysis of the prevalence of *L. monocytogenes* in different types of European cheeses and study potential indicator organisms for assessment of production hygiene or index organisms for implementation in the assessment of product safety.

Table 1

Overview of listeriosis outbreaks caused by cheese during the period from 1983 to 2016.

Country	Year	Serotype	No. ^a of cases (fatalities)	Implicated food	References
Switzerland	1983-1987	4b	122(31)	Smear cheese (Vacherin Mont d'Or)	Billa et al., 1995; Bille et al., 2006
USA	1985	4b	142(48)	Fresh cheese (Queso Fresco)	Linnan et al., 1988
Luxembourg	1989	NR ^b	2(0)	Smear cheese (Camembert)	Ries et al., 1990
Denmark	1989-1990	4b	26(6)	Veined or ripened cheese	Jensen et al., 1994
France	1995	4b	37(11)	Smear cheese (Brie de Meaux)	Goulet et al., 1995; Arnold & Coble, 1995
France	1997	4b	14(? ^c)	Smear cheese (Pont l'Evêque)	Ryser & Marth, 2007; Goulet et al., 2013
USA	2000	4b	13(5)	Non-commercial fresh cheese (Queso Fresco)	MacDonald et al., 2005
Sweden	2001	1/2a	≥120(0)	Fresh cheese	Carrique-Mas et al., 2003; Danielsson-Tham et al., 2004
Japan	2001	1/2b	38(0)	Smear cheese	Makino et al., 2005
Canada	2002	4b	47(0)	Soft and semi-soft cheese	Gaulin et al., 2003
Canada	2002	4b	86(0)	Cheese made from pasteurized milk	Pagotto et al., 2006
Switzerland	2005	1/2a	10 (3+2 ^d)	Smear cheese (Soft "Tomme")	Bille et al., 2006
USA	2005	NR ^b	9(?)	Fresh cheese (Queso fresco)	FIOD, 2005
Czech Republic	2006		78(13)	Soft cheese	EFSA, 2007
Germany	2006-2007	4b	189(26)	Acid curd cheese	Koch et al., 2010
Norway	2007	NR ^b	17(3)	Smear cheese (Camembert)	Johnsen et al., 2010
Chile	2008	NR ^b	91(5)	Smear cheese (Brie)	Promed, 2008
Canada	2008	NR ^b	38(5)	Cheeses	Gaulin & Ramsay, 2010
USA	2008	1/2a	8(0)	Fresh cheese (Oaxaca cheese)	Jackson et al., 2011

Austria-Germany- Czech Republic	2009-2010	1/2a	34 (8)	Fresh cheese (Quargel)	Fretz et al., 2010; Rychli et al., 2014
Portugal	2009- 2012	4b	30 (11)	Fresh cheese (Cured cheese and queijo fresco)	Magalhães et al., 2015
USA	2010	NR ^b	5(0)	Fresh cheese (Panela, queso fresco, Requeson)	FIOD, 2010
USA	2010-2015	NR ^b	28(3)	Fresh cheeses	FIOD, 2015b
USA	2011	NR ^b	2(? ^c)	Fresh cheese (Chives cheese)	FIOD, 2011
Austria-Germany	2011-2013	1/2b	7(? ^c)	Fresh cheese	Schmid et al., 2014
Spain	2012	1/2a	2(0)	Fresh cheese (Queso fresco)	De Castro et al., 2012
USA	2012	NR ^b	22(4)	Brined cheese (Ricotta salatta)	CDC, 2012; Coroneo et al., 2016
USA	2013	NR ^b	5(1)	Smear cheese (Les Freres)	FIOD, 2013
Australia	2013	NR ^b	18(? ^c)	Smear cheese	NSW, 2013
USA	2013-2014	NR ^b	4 (1)	Fresh cheese	FIOD, 2014a
USA	2014	NR ^b	7(1)	Fresh cheese	FIOD, 2014b
USA	2015	NR ^b	3(1)	Fresh cheese (Panela, Queso Fresco, Requeson, Cotija)	FIOD, 2015b

^a Number of listeriosis cases

^b Serotype not reported (NR)

^c Fatalities uncertain

^d Septic abortion i.e. fatality

2. Materials and methods

2.1. Literature search and inclusion criteria

A systematic review was performed following the protocol presented by Sargeant et al., 2005. Literature searches were carried out to identify suitable scientific literature using Web of Science (2017) or DTU Findit (2017) databases for papers indexed since 1985 as well as Google searches using English, French, Italian, and Spanish terms for combinations of *Listeria* spp., *L. monocytogenes*, cheese, dairy, prevalence, incidence and occurrence. Electronic searches were carried out to identify reports of the prevalence for *Listeria* spp. in cheese. This included reports by

national and international organizations such as World Health Organization (WHO), EFSA and the International Commission for Microbiological Specification in Foods (ICMSF).

For inclusion in the meta-analysis results had to meet three requirements: (i) to come from original studies, (ii) to be obtained by using approved (FDA/FIL-IDF or ISO) microbiological methods for detection of *Listeria* spp. and (iii) originate from cheeses produced in Europe during the period of 2005 to 2015.

2.2. Data and definitions

Cheese-type definitions were necessary in order to categorize studies from scientific literature. Available information allowed for a classification based in maturation characteristics. For the purpose of this paper, the following definitions apply. Fresh cheeses are curd-style cheeses which do not undergo any ripening (CAC, 2013), for example, queso fresco, cottage cheese, Mozzarella or Ricotta. Ripened cheeses are not ready for consumption shortly after manufacture and maturation is needed for development of specific cheese characteristics (CAC, 2013), for example, Gouda, Edam, Cheddar or Parmesan. Veined cheeses are ripened cheeses in which ripening has been accomplished primarily by the development of the mould *Penicillium roqueforti* throughout the interior and/or on the surface, for example, Roquefort, Gorgonzola, Cabrales, Stilton or Danablu. Smear cheeses are ripened cheeses where the surface is treated with *Penicillium candidum*, *Penicillium camemberti* or *Brevibacterium linens*, for example, Brie, Camembert, Limburger or Taleggio. Brined cheeses are ripened and stored in brine until they are sold or packed, for example, Feta or Ricotta salata (Fox et al., 2000).

Classification of cheese in EFSA reports are based on cheese moisture content. Soft-cheeses have a percentage of moisture, on a fat-free basis, higher than 67 %. Semi-soft cheeses have 62 to

67 % fat-free moisture and are characterized by their firm but elastic feel. Hard cheeses have 49 to 56 % fat-free moisture (CAC, 2013; EFSA, 2013b).

2.3. Problem statement

To estimate prevalence of *L. monocytogenes* in cheese during the period 2005-2015 (i) from scientific literature data, (ii) from data in EFSA reports, (iii) from scientific literature and data in EFSA reports when combined and (iv) to study groups/species of microorganisms suitable as indicator or index organisms to assess prevalence of *L. monocytogenes* in cheeses.

2.4. Description of data sets for meta-analysis and regression modelling

From each primary study the number of samples positive for *L. monocytogenes* (s) and the total number of samples (n) were extracted. Information about year of survey, country, sample weight and information on sampling at production site or at retail were also collected from each primary study. Meta-analysis for prevalence of *L. monocytogenes* in cheese as reported in the scientific literature was based on 17 primary studies including a total of 7,221 samples (Table 2), while data from seven EFSA reports with a total of 123,383 samples were included (Table 3 and Table 4). The regression model used to evaluate indicator/index organisms for *L. monocytogenes* in European cheeses was based in 16 primary studies all from the scientific literature and including a total of 3,852 samples (Table 5).

Table 2

Prevalence data (s/n) from the scientific literature.

References	Survey year	Number of <i>L. monocytogenes</i> positive (s) /total number of cheese samples (n)				
		Fresh	Ripened	Veined	Smear	Brined
Filioussis et al., 2009	2005-2006		4/20			0/10
Little et al., 2009	2006-2007		2/1240			
O'Brien et al., 2009	2007	0/29	1/104	1/33	14/79	
Di Pinto et al., 2010	2007-2009	2/294				
Pesavento et al., 2010	2008	2/258				
Prencipe et al., 2010	2005-2006	1/437	1/449	21/444	24/802	
Angelidis et al., 2012	2010	0/83		0/38	0/16	
Lambertz et al., 2012	2006-2012			2/465	0/62	
Dambrosio et al., 2013	2009-2010	0/404				
Doménech et al., 2013	2005-2009	0/77				
Parisi et al., 2013	2008-2010	3/70				
Gyurova et al., 2014	2011-2012		0/17	0/7		0/34
Doménech et al., 2015	2006-2012	9/507	3/100			
Schoder et al., 2015	NS ^a		1/15	0/50	1/22	
Spanu et al., 2015	2011-2013		3/50			7/33
Iannetti et al., 2016	2011-2012	0/421	0/106	8/190	11/177	
Coroneo et al., 2016	NS ^a					15/87
Total		17/2,580	15/2,101	32/1,218	50/1,158	24/164

^a Not specified; but assumed within the period 2005-2015.**Table 3**

Prevalence data (s/n) from EFSA reports.

Type of cheese	Number of <i>L. monocytogenes</i> positive (s) /total number of cheese samples (n)							
	EFSA, 2006 ^a	EFSA, 2007 ^a	EFSA, 2009 ^a	EFSA, 2010 ^a	EFSA, 2011 ^a	EFSA, 2015 ^a	EFSA, 2016 ^a	
	2005 ^b	2006 ^b	2007 ^b	2008 ^b	2009 ^b	2013 ^b	2015 ^b	
Hard	Un-pasteurized	0/969	38/718	16/3,242	2/1,606	2/1,001	15/1,618	11/858
	Pasteurized	0/1,367	5/3,284	68/9,449	85/10,877	15/7,246	77/8,288	19/2,384
Soft/ Semi-soft	Un-pasteurized	29/1,505	13/1,959	16/5,943	5/4,203	6/774	155/2,880	10/707
	Pasteurized	25/5,973	22/4,736	853/16,333	70/5,585	41/4,087	49/10,668	67/5,123

^a References^b Survey year

Table 4Cheese samples in non-compliance with EU food safety limits for *L. monocytogenes* in RTE foods.

Type of cheeses	Cheese samples (n) with > 100 <i>L. monocytogenes</i> /g /Total number of samples (n)							
	EFSA, 2006 ^a	EFSA, 2007 ^a	EFSA, 2009 ^a	EFSA, 2010 ^a	EFSA, 2011 ^a	EFSA, 2015 ^a	EFSA, 2016 ^a	
	2005 ^b	2006 ^b	2007 ^b	2008 ^b	2009 ^b	2013 ^b	2015 ^b	
Hard	Un-pasteurized	? ^c	? ^c	2/1,569	0/133	2/940	1/2,854	0/880
	Pasteurized	0/672	7/1,701	14/2,292	3/4,005	1/9,894	10/3,041	0/141
Soft/ Semi-soft	Un-pasteurized	1/1,174	0/64	2/1,008	17/484	0/775	3/2,718	10/809
	Pasteurized	0/3,231	3/1,093	1/2,727	10/3,230	12/4,702	9/1,351	5/1,209

^a References^b Survey year^c Not reported**Table 5**European studies reporting the prevalence of *Listeria* species in cheeses.

References	Country	Sample size	Number of samples positive for different <i>Listeria</i> species					
			<i>L. monocytogenes</i>	<i>L. innocua</i>	<i>L. grayi</i>	<i>L. welshimer</i>	<i>L. ivanovii</i>	<i>L. seeligeri</i>
Comi et al., 1990	Italy	1740	65	145	15	185	18	0
Massa et al., 1990	Italy	121	2	2	0	0	0	0
Quagilo et al., 1992	Italy	246	29	42	0	0	0	2
Rota et al., 1992	Spain	58	1	2	0	0	0	0
Pinto & Reali, 1996	Italy	132	7	30	0	2	0	1
Theodoridis et al., 1998	Greece	334	26	8	0	0	0	10
Bottarelli et al., 1999	Italy	100	2	2	0	0	0	0
Rudolf & Scherer, 2000	Germany	50	2	13	0	0	0	0
Rudolf & Scherer, 2001	Austria	274	19	33	0	0	0	4
Vitas et al., 2004	Spain	99	1	6	1	0	0	0
Pintado et al., 2005	Portugal	63	32	23	0	0	0	2
Pesavento et al., 2010	Italy	258	2	6	1	1	0	0

Angelidis et al., 2012	Greece	137	0	1	2	0	0	1
Parisi et al., 2013	Italy	70	3	3	0	0	0	0
Schoder et al., 2015	Europe	87	2	8	0	0	0	0
Spanu et al., 2015	Italy	83	10	3	0	0	0	0
Total		3,852	203	327	19	188	18	20

2.5. Meta-analysis

Prevalence ($p_i = s_i/n_i$) data was studied as observed effect size (θ_i) and they were logit transformed in order to restrict values to a range between 0-1 and to stabilize variance (Eq. 1; Viechtbauer, 2010). The parameter measuring effect size (θ_i) is a common metric that permits direct comparison and summation of primary studies (Borenstein et al., 2009).

$$\theta_i = \text{logit } p_i = \ln\left(\frac{p_i}{1-p_i}\right) = \ln\left(\frac{s_i}{n_i-s_i}\right) \quad (1)$$

Models with random-effects were used to calculate prevalence values (mean and 95% CI) of *L. monocytogenes* across primary studies (Eq. 2; Borenstein et al., 2009):

$$T_i = \theta_i + \varepsilon_i = \mu + u_i + \varepsilon_i \quad (2)$$

where T_i is the true effect size for each primary study ($i = 1, 2, \dots$), ε_i is the sampling error and μ is the mean true effect size. u_i represents the true variation in effect sizes being composed of within-study (σ^2) and between-study variance (τ^2).

The between-study variance (τ^2) is estimated from the Q-statistic (DerSimonian & Laird 1986),

$$\hat{t}^2 = \begin{cases} \frac{Q-(k-1)}{\sum w_i \frac{\sum w_i^2}{\sum w_i}}, & \text{for } Q > (k-1) \\ 0 & , \text{for } Q \leq (k-1) \end{cases} \quad (3)$$

where Q is calculated by Eq. 4 and 5, k is the number of studies and w_i the weight assigned to each study (Eq.5).

$$Q = \sum w_i (T_i - \mu)^2 \quad (4)$$

$$\mu = \frac{\sum_i w_i T_i}{\sum_i w_i} \quad (5)$$

$$w_i = \frac{1}{\sigma_i^2 + \tau_i^2} \quad (6)$$

A significant value of the Q -statistic indicates a real effect difference between primary studies and suggests the use of a multilevel model (Xabier et al., 2014). The I^2 index was used to measure the extent of between-study variance dividing the difference between the result of the Q -statistic and its degrees of freedom ($k - 1$) by the Q value itself, and then multiply by 100. Higgins & Thompson (2002) proposed a classification of I^2 values with percentages of around 25% ($I^2 = 25$), 50% ($I^2 = 50$) and 75% ($I^2 = 75$) corresponding to low, medium and high between-study variance, respectively. The τ^2 and I^2 indices are related and higher τ^2 values corresponds to higher I^2 index values.

Multilevel meta-analysis including type of cheese and pasteurized or unpasteurized milk were used to account for some of the observed between-study variance in prevalence data.

The multilevel models used were formulated as:

$$T_i = \beta_0 + \beta_1 X_{1i} + \dots + \beta_k X_{ki} + u_i + \varepsilon_i \quad (7)$$

with (X_1 to X_k) being study characteristics and β_k the moderator effects.

Meta-analysis modelling was performed by using R version 3.1.3 (R Development Core Team) and the “metafor” package (Viechtbauer, 2010), which provides functions for fitting of random-effects and multilevel models as well as meta-analytical graphs including forest plots.

2.6. Regression modelling

A linear regression model ($y = a + bx$) was used to evaluate the relation between prevalence of *Listeria* spp. (x) and prevalence of *L. monocytogenes* (y). Regression modelling was performed with R and an F-test was used to evaluate if the linear model could be reduced to $y = bx$.

3. Results

3.1. Meta-analysis of prevalence data from scientific literature

The overall prevalence for presence of *L. monocytogenes* in cheese was 2.3% (CI: 1.4-3.8%). Variability in reported prevalence among studies was high (Table 6 and Fig.1) and the between-study variance slightly decrease from $\tau^2 = 1.72$ to 1.12 when cheeses were grouped in categories by the multilevel model. Nevertheless, unexplained variability remained high ($I^2 = 75\%$; p-value < 0.001 in Table 6).

Fresh cheese had the lowest mean prevalence of 0.8% (CI: 0.3-1.9%), followed by ripened cheese 2.0% (CI: 0.8- 4.9%), veined cheese 2.4% (CI: 0.9- 6.3%) and smear cheese 5.1% (CI: 1.9-13.1%). Brined cheese had the highest *L. monocytogenes* prevalence of 11.8% (CI: 3.5-33.3%) (Table 6 and Fig. 1).

Table 6Meta-analysis results for prevalence of *L. monocytogenes* from scientific literature

Meta-analysis type	Prevalence (CI) ^a	τ^2 ^b	I ² (%) ^c	Q ^d
Random-effects	0.023 (0.014-0.038)	1.72	86	197*** ^e (df = 35)
Multilevel		1.12	75	108*** ^e (df = 31)
Fresh cheese	0.008 (0.003-0.019) ^{Af}			
Ripened cheese	0.020 (0.008-0.049) ^{ABf}			
Veined cheese	0.024 (0.009-0.063) ^{Bf}			
Smear cheese	0.051 (0.019-0.131) ^{Bf}			
Brined cheese	0.118 (0.035-0.333) ^{Bf}			

^a 95% confidence interval.^b Between-study variance.^c Between-study variance index proposed by Higgins & Thompson (2002).^d Q-statistic proposed by DerSimonian & Laird (1986).^e P-value < 0.001.^f Mean values for classes with the same capital letter do not differ significantly ($p > 0.05$).

3.2. Meta-analysis of prevalence data from EFSA reports

The overall prevalence for presence of *L. monocytogenes* in cheese was 0.7% (CI: 0.5 – 1.1%) with high between-studies variance (Table 7). A multilevel model determined the prevalence of *L. monocytogenes* in hard and soft/semi-soft cheeses produced from un-pasteurized or pasteurized milk. No significant effect of pasteurization ($p > 0.05$) was observed within hard or soft/semi-soft cheeses (Table 7).

A second random-effects meta-analysis was performed to assess non-compliance with the criterion of 100 cfu/g for *L. monocytogenes* in ready-to-eat (RTE) foods. 0.2% (CI: 0.1-0.4) of the cheese samples had more than 100 *L. monocytogenes*/g and high between-study variance was observed (Table 8). Prevalence of *L. monocytogenes* in hard and soft/semi-soft cheese produced with un-pasteurized or pasteurized milk was estimated. Pasteurization of milk had no significant effect ($p > 0.05$) within hard or soft/semi-soft cheeses (Table 8).

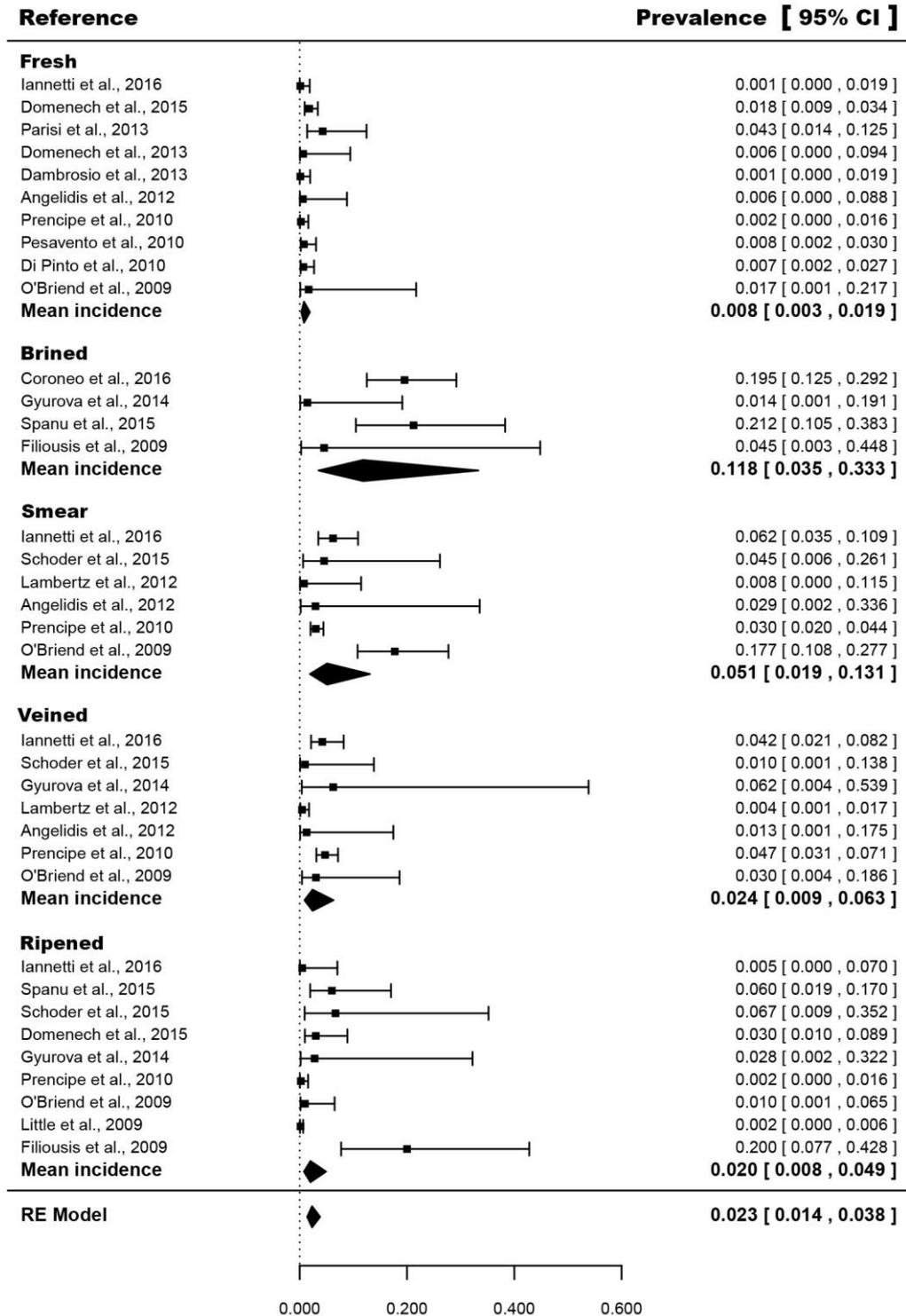


Fig. 1. Forest plot of the multilevel model based on scientific literature reporting prevalence of *L. monocytogenes* in different types of cheeses

Table 7Meta-analysis results for prevalence of *L. monocytogenes* from EFSA reports

Meta-analysis type	Prevalence (CI) ^a	τ^2 ^b	I ² (%) ^c	Q ^d
Random-effects	0.007 (0.005-0.011)	1.09	98	1712*** ^e (df = 27)
Multilevel		1.17	88	1174*** ^e (df = 24)
Hard and un-pasteurized	0.006 (0.003-0.015) ^f			
Hard and pasteurized	0.012 (0.002-0.010) ^f			
Soft/semi-soft and un-pasteurized	0.009 (0.004-0.019) ^g			
Soft/semi-soft and pasteurized	0.010 (0.004-0.022) ^g			

^a 95% confidence interval.^b Between-study variance.^c Between-study variance index proposed by Higgins & Thompson (2002).^d Q-statistic proposed by DerSimonian & Laird (1986).^e P-value < 0.001.^f Mean values within hard cheeses do not differ significantly ($p > 0.05$).^g Mean values within soft/semi-soft cheeses do not differ significantly ($p > 0.05$).**Table 8**Meta-analysis results assessing non-compliance with the criterion of “> 100 cfu/g” for *L. monocytogenes* in cheeses as reported by EFSA.

Meta-analysis type	Prevalence (CI) ^a	τ^2 ^b	I ² (%) ^c	Q ^d
Random-effects	0.002 (0.001-0.004)	1.22	84	154*** ^e (df = 25)
Multilevel		1.18	82	95*** ^e (df = 22)
Hard and un-pasteurized	0.001(0.000-0.004) ^f			
Hard and pasteurized	0.002 (0.001-0.005) ^f			
Soft/semi-soft and un-pasteurized	0.004 (0.002-0.012) ^g			
Soft/semi-soft and pasteurized	0.002 (0.001-0.006) ^g			

^a 95% confidence interval.^b Between-study variance.^c Between-study variance index proposed by Higgins & Thompson (2002).^d Q-statistic proposed by DerSimonian & Laird (1986).^e P-value < 0.001.^f Mean values within hard cheeses do not differ significantly ($p > 0.05$).^g Mean values within soft/semi-soft cheeses do not differ significantly ($p > 0.05$).

3.3. Meta-analysis of combined prevalence data from scientific literature and EFSA reports

The overall prevalence of *L. monocytogenes* in European cheeses was 1.2% (CI: 0.8-1.8%).

High between-study variance was observed and a significant difference ($p < 0.001$) was determined

between data from the scientific literature and from EFSA reports data (Table 9).

Table 9Meta-analysis results for prevalence of *L. monocytogenes* from combined data

Meta-analysis type	Prevalence (CI) ^a	τ^2 ^b	I ² (%) ^c	Q ^d
Random-effects	0.012 (0.008-0.018)	1.78	97	1961*** ^e (df = 63)
Multilevel		1.38	97	1909*** ^e (df= 62)
Scientific literature	0.007 (0.004-0.011) ^{Af}			
EFSA reports	0.024 (0.015-0.038) ^{Bf}			

^a 95% confidence interval.^b Between-study variance.^c Between-study variance index proposed by Higgins & Thompson (2002).^d Q-statistic proposed by DerSimonian & Laird (1986).^e P-value < 0.001.^f Mean values for classes with different capital letters differed significantly ($p < 0.001$).

3.4. Evaluation of index organisms for prevalence of *L. monocytogenes* in European cheeses

Of 3852 samples reporting presence of *Listeria* spp., 203 (5.3%) were positive for *L. monocytogenes*, 327 (8.5%) *L. innocua*, 19 (0.5%) *L. grayi*, 188 (4.9%) *L. welshimer*, 18 (0.5%) *L. ivanovii* and 20 (0.5%) *L. seeligeri*. The correlation factor was sufficient to describe the relation between prevalence of *Listeria* spp. (x) and prevalence of *L. monocytogenes* (y) in cheeses ($y = 0.52x$, $r^2 = 0.86$, Fig. 2).

4. Discussion

It is critical to understand and quantified prevalence of *L. monocytogenes* in cheeses since they are an important vehicle for transmission of the pathogen and infection causes the highest fatality case rate among zoonotic diseases (EFSA, 2016).

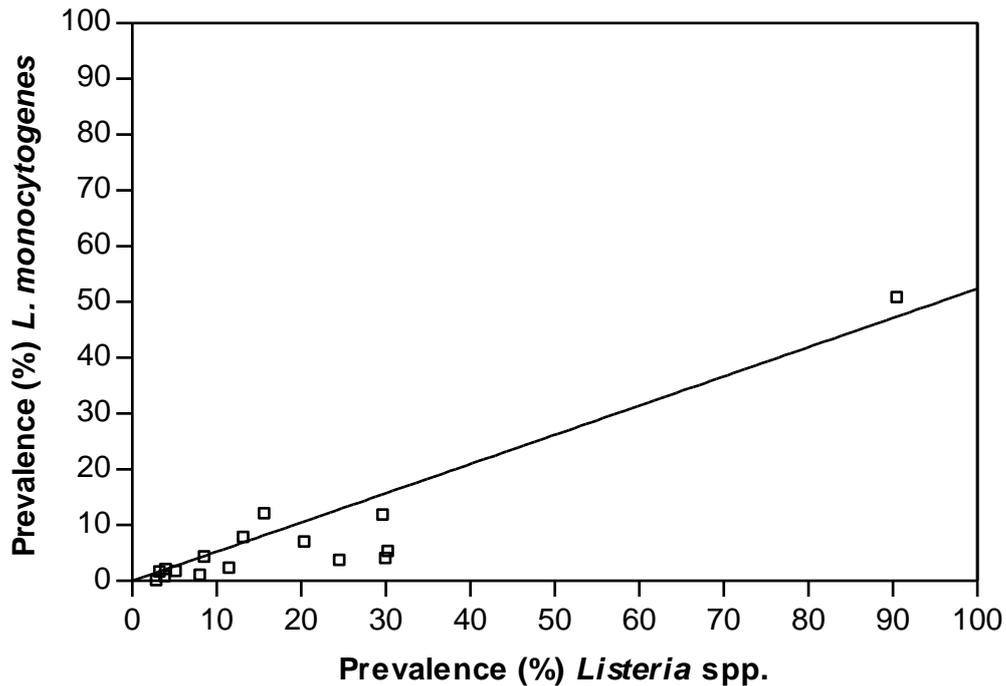


Fig. 2. Comparison of observed prevalence (%) for *Listeria* spp. and *L. monocytogenes* in European cheeses.

EU mean prevalence of *L. monocytogenes* in cheese from scientific literature exceeded what was reported by EFSA for the same period. This may result from a focus on problematic cheese products in scientific studies whereas EFSA reports include a larger number of samples from hard cheeses where *L. monocytogenes* can be inactivated and prevalence therefore is lower. The data from scientific studies corresponded to previous studies reporting prevalence between 0 and 4.8% (Esho et al., 2013; Manfreda et al., 2005; Rosengren et al., 2010), but some other studies reported more than 40% prevalence (Loncarevic et al., 1995; Pintado et al., 2005).

Mean prevalence of *L. monocytogenes* in fresh cheese was similar to the overall prevalence obtained from EFSA data. In 1985 consumption of contaminated fresh cheese (queso blanco) was directly linked to more than 142 cases of listeriosis, including 48 deaths (Linnan *et al.*, 1988). From 2009 to 2012 there was an outbreak in Portugal linked to 30 cases of listeriosis, including 11 deaths

and related to consumption of fresh cheeses (curded cheese and queijo fresco) (Magalhães *et al.*, 2015). Furthermore, Greco *et al.*, (2014) for example demonstrated how prevalence of *L. monocytogenes* can be high (24.4%) in mozzarella cheese as result of cross-contamination.

Fresh cheeses were excluded from the EFSA baseline survey on prevalence of *L. monocytogenes* in certain RTE foods within EU during 2010-2011 (EFSA, 2013a). Interestingly, EFSA (2015) started to differentiate between fresh and soft/semi-soft cheeses but included only 2.1% fresh cheese samples compared to 80.1% hard cheese samples from a total of 13,718 cheese samples. Hard cheese have never been linked to a listeriosis outbreak (Table 1) and as it does not support growth of *L. monocytogenes* (Dalmaso & Jordan, 2014; Wemmenhove *et al.*, 2013; Yousef & Marth, 1990) the large number of these samples does not correspond to a risk-based sampling approach.

It is important to note that mean prevalence for brined cheese was estimated from only four studies with smaller sample sizes compare with other types of cheese. Consequently, there is a high level of uncertainty and results may be biased by results from a single study (Fig. 1; Table 6). In 2012, Ricotta salata imported from Italy and contaminated with *L. monocytogenes* was involved in a listeriosis outbreak in the USA with 22 hospitalizations and 4 deaths (CDC, 2012). Furthermore, ricotta salata supports growth of *L. monocytogenes* (Coroneo *et al.*, 2016; Spanu *et al.*, 2012) and production of this cheese includes manual processing of the curd and exposure to processing environments that increase the risk of *L. monocytogenes* contamination (Spanu *et al.*, 2013). Our findings suggest that prevalence of *L. monocytogenes* in fresh and brined cheese are not negligible; therefore we encourage EFSA to increase and independently report sampling of fresh and brined cheeses since they have been related with listeriosis outbreaks recurrently (Table 1).

As shown by EFSA reports, contamination of cheese by *L. monocytogenes* is not specific to un-pasteurized milk cheeses since cheeses made from pasteurized milk can be contaminated due to

inadequate pasteurization or post-pasteurization contamination (De Buyser et al., 2001; Donnelly, 2001). Our report is the first of our knowledge to analysed EFSA prevalence data of cheeses made from un-pasteurized and pasteurized milk. There was no significant difference in prevalence between cheeses produced with un-pasteurized or pasteurized milk; either for hard or soft/semi-soft cheeses (Table 7 and 8). This may be due to requirements leading to the use of milk of high microbiological quality for the production of un-pasteurized milk cheese and to post-pasteurization contamination of pasteurized milk cheese. Tiwari et al., (2015) compared the risk of soft/semi-soft cheese made from un-pasteurized or pasteurized milk and estimated a higher risk for un-pasteurized milk cheese as a consequence of the higher contamination rate of milk due to the lack of pasteurization and growth of *L. monocytogenes* in un-pasteurized milk cheese but inactivation in the same pasteurized milk cheese. But this study observed no significant effect of pasteurization in prevalence of *L. monocytogenes* in soft/semi-soft cheese. We provide mean prevalence and distributions for *L. monocytogenes* in soft/semi-soft cheese that can be combined with concentration data of *L. monocytogenes* (cfu/g) for the same period in un-pasteurized and pasteurized milk cheese to perform a quantitative risk assessment of the end product (Crépet et al., 2007) and results from both studies could be compared.

Prevalence and concentration of *L. monocytogenes* in cheeses are low, hence evaluation of potential presence of other index or indicator microorganisms easier to determine or quantify was considered. *Listeria* spp. has been proposed as index organisms for presence of *L. monocytogenes* in RTE foods and as indicator of inadequate hygiene conditions in food production practices and environment (FSAI, 2011; Gilbert et al., 2000; McLauchlin, 1997). These findings were confirmed by the present study and we found prevalence of *L. monocytogenes* corresponded to prevalence of *Listeria* spp. when multiplied by a factor of 0.52. This was further supported by Trmčić et al., (2016) where 273 cheese samples had 12 positive for *Listeria* spp. and five of these positive for *L.*

monocytogenes. Silva et al., (2003) also found 33% of *Listeria* spp. positive samples from cheese and dairy processing plants to be *L. monocytogenes* positive. However, Arrese & Arroyo-Izaga (2012) found no *L. monocytogenes* positive amongst 51 cheese samples with five samples positive for other *Listeria* spp. Microbiological methods for detection and quantification of *Listeria* spp. are not more performant than available methods for *L. monocytogenes* (Gasnov et al., 2005). Therefore, we do not consider *Listeria* spp. a useful index- or indicator-organism *L. monocytogenes* despite the relation reported in the present study (Fig. 2).

5. Conclusions

Meta-analysis provided pooled prevalence estimates for *L. monocytogenes* in specific types of cheeses, however, significant between-study variance was observed. Overall prevalence of *L. monocytogenes* in cheese as estimated from scientific literature data was higher than reported by data from EFSA during the same period 2005-2015. Considering prevalence of *L. monocytogenes* in cheeses produced with un-pasteurized or pasteurized milk no significant difference in prevalence was observed. The results obtained provided a broad picture of *L. monocytogenes* prevalence in cheeses and can be used as an important input in quantitative microbial risk assessments. *Listeria* spp. was not a useful index- or indicator-organism for *L. monocytogenes* in cheeses although prevalence of *Listeria* spp. was related to prevalence of *L. monocytogenes*.

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Paper II

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Evaluation of predictive models for growth of *Listeria monocytogenes* in cheeses

Manuscript in preparation

Evaluation of predictive models for growth of *Listeria monocytogenes* in cheeses

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Abstract

The present study evaluated mathematical models for their ability to predict growth of *L. monocytogenes* in cheeses with (i) known dynamic changes in product characteristics and (ii) assumed constant product characteristics. To generate growth data for model evaluation three challenge tests were performed with inoculated milk to produce smear cheese. Growth data for *L. monocytogenes* and lactic acid bacteria (LAB) present in the rind and core of cheese were determined along with quantification of product characteristics over time (temperature, pH, NaCl/aw and lactic acid concentration). The model of Østergaard et al. (2014) acceptably predicted growth of *L. monocytogenes* for dynamic storage conditions and product characteristics. The performance of ten available *L. monocytogenes* growth models was evaluated using growth responses from literature (n = 319) and the corresponding reported constant storage temperature and product characteristics for smear, veined, ripened, brined and fresh cheeses. The model of Østergaard et al. (2014) appropriately predicted growth in smear and brined cheeses. The validated model can be used to support risk assessment of products.

Keywords: Predictive microbiology, mathematical modelling, model validation, risk assessment, food safety, smear, white-mould.

1. Introduction

Fresh and smear cheese has been involved in the majority of listeriosis outbreaks caused by consumption of cheeses and the European prevalence of *L. monocytogenes* in smear cheese is higher than for other categories of cheeses (Martinez-Rios and Dalgaard, 2018; Rudolf and Scherer, 2001). Improved management of *L. monocytogenes* in smear cheese seems relevant and validated predictive models for this pathogen/product combination may help identify changes in storage conditions or product characteristics resulting in an increased safety margin. However, documentation of successfully validated *L. monocytogenes* growth models for smear cheese is limited.

Cheese can have a complex microbiota that evolves during milk fermentation, curd formation and ripening. Lactic acid bacteria (LAB e.g. *Lactococcus lactis* and *Leuconostoc* species) transforms lactose into lactic acid resulting in lowering of the product pH. Thereafter moulds, yeast or bacteria (e.g. *Penicillium camemberti*, *Penicillium roqueforti*, *Geotrichum candidum*, *Debaryomyces hansenii*, *Brevibacterium linens*, *Propionibacterium freudenreichii*) can colonize the rind or core of cheeses and contribute to changes in product characteristics including consumption of lactic acid and increased product pH (Fox et al., 2017).

Several mathematical models have been developed to predict the potential growth of *L. monocytogenes* in cheeses. A few of these models take into account the dynamic changes in temperature and product characteristics (water activity, pH and lactic acid concentration) during fermentation, ripening and storage of the cheeses (Ferrier et al., 2013, Østergaard, 2014; Rosshaug et al., 2012; Schwartzman et al., 2011). Other models predict the potential growth of *L. monocytogenes* in cheeses based on an assumption of constant product characteristics and for those

models values of the optimal (μ_{opt}) or reference (μ_{ref}) growth rate parameters have been determined by calibration of the models to data for growth in specific cheeses (Augustin et al. 2005; Østergaard et al. 2014). For both types of models a limited number of growth responses have been used to evaluate their performance with respect to prediction of *L. monocytogenes* growth in cheeses. There is a need for further model evaluation particularly when taking into account that 1014 growth/no-growth responses have been used to evaluate *L. monocytogenes* growth models for seafood, meat products and non-fermented dairy products (Mejlholm et al. 2010). Models have also been developed for growth of *Debaryomyces hansenii* and lactic acid consumption during ripening of a smear soft cheese (Riahi et al., 2007) or for the growth of *Geotrichum candidum* and *Penicillium camembert*, lactic acid consumption and production of ammonia in broth (Adour et al., 2002). These models, however, were not related to *L. monocytogenes* growth.

The model of Østergaard et al. (2014) was developed and validated for growth of *L. monocytogenes* in cottage cheese with fresh- or aroma cultured cream dressing. The model includes the effect of temperature, pH, NaCl/ a_w , lactic acid concentration and the effect of interaction between these environmental factors. Furthermore, the inhibiting effect on *L. monocytogenes* growth by simultaneous growth of LAB is quantified by the empirical Jameson effect (Gimenez and Dalgaard, 2004). The reference growth rate parameter (μ_{ref}) obtained in broth was calibrated for growth of *L. monocytogenes* in cottage cheese with fresh cream dressing (O-culture; μ_{ref} of 0.72 h⁻¹) and for cottage cheese with cultured cream dressing (DL-culture; μ_{ref} of 0.34 h⁻¹).

The objectives of the present study were to evaluate mathematical models for their ability to predict growth of *L. monocytogenes* in cheeses with (i) known dynamic changes in product characteristics and (ii) assumed constant product characteristics. Firstly, smear cheese was studied to quantify growth of *L. monocytogenes* with dynamic changes in storage temperature and

product characteristics (LAB, pH, lactic acid concentration, NaCl/a_w). Secondly, the model of Østergaard et al. (2014), with and without simultaneous LAB growth, was evaluated for the generated smear cheese data. Finally, the performance of ten available *L. monocytogenes* growth models was evaluated using growth responses from literature (n = 319) and the corresponding reported constant storage temperature and product characteristics for smear, veined, ripened, brined and fresh cheeses.

2. Material and methods

2.1. *L. monocytogenes* growth and dynamic product characteristics for smear cheese

Three challenge tests were performed to quantify growth of *L. monocytogenes* in smear cheese with well described physico-chemical product characteristics. Growth in rind and in the core of the smear cheese was quantified separately and a total 18 growth/no-growth responses were generated together with quantification of changes in storage conditions (temperature) and product characteristics (pH, lactic acid, NaCl/a_w). These data was used to evaluate the performance of the *L. monocytogenes* growth and growth boundary model of Østergaard et al. (2014).

2.1.1. Smear cheese production

Pasteurized milk with 3.5% fat was purchased from a local supermarket and used for cheese making. The milk was heated to 30-32°C, added 0.4 ml/L of a 50% CaCl w/v solution and then inoculated with starter culture and with moulds as ripening microbiota. For the first challenge test, milk was inoculated with a probiotic strain, a DL-culture (0.01 g/L of *Lactobacillus acidophilus*, LA-5 and 0.13 g/L of FLORA™ C501, Chr. Hansen A/S, Hørsholm, Denmark) and a ripening culture (4 mg/L of *Penicillium camemberti*, PC HP 6 LYO and 2 mg/L of *Geotrichum candidum*, GEO 17,

DuPont™ Danisco®, Brabrand, Denmark). For the second challenge test, milk was inoculated with a DL-culture (0.13 g/L of FLORA™ C503, Chr. Hansen A/S) and a ripening culture (2 mg/L of *Geotrichum candidum*, GEO 17, DuPont™ Danisco®). For the third challenge test, milk was inoculated with a DL-culture (0.02 g/L of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc* spp. and *Lactococcus lactis* subsp. *lactis* biovar diacetylactis, CHN-11, Chr. Hansen A/S, Hørsholm, Denmark) and a ripening culture (6 mg/L of *Penicillium camemberti*, SWING FD PCA-3 and 5 mg/L of *Geotrichum candidum*, SWING GEO CD-1, Chr. Hansen A/S). Thirty minutes after the addition of cultures, milk was inoculated with *L. monocytogenes* to reach an initial concentration of 1 log cfu/ml. 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 min. after rennet addition, the coagulum was cut into cubes with 1 cm edge and gently stirred 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 h the cheese moulds were flipped over. Cheeses were then removed from the moulds and cut in half. 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 5h 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 production. Cheeses were stored up to 30 days at a relative humidity of 90-95% measured by data loggers (Tinytag view, Gemini Data Loggers Ltd, Chichester, UK). Sixty 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.

2.1.2. Product characteristics

For each of the three batches of smear cheeses, product characteristics were determined separately and in triplicate for, respectively, the rind and the core. Product characteristics were determined during storage at the time of sampling for microbiological analysis. 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 for 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.

2.1.3. Bacterial strains, pre-culture conditions, inoculation and microbiological analysis

Four dairy related strains of *L. monocytogenes* were provided by Arla Foods 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). The *L. monocytogenes* cocktail of strains (*Lm*-mix) were produced by mixing equal volumes of individual pre-cultures of isolates. The cell concentration of *Lm*-mix was determined by direct phase-contrast microscopy prior to inoculation.

Milk for cheese making 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. For the three challenge tests a total of 18 growth/no-growth responses of *L. monocytogenes* were determined in the rind (n = 9) and in the core (n = 9) of the studied smear cheeses. These growth/no-growth responses were determined together with quantification of dynamic storage temperature and dynamic product characteristics (Fig.1, 2, 3). Storage temperature was regularly recorded by data loggers placed inside the cheeses (TinytagPlus, Gemini Data Loggers Ltd, Chichester, UK). At each time of sampling a 60 ± 10 g cheese was 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), 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.

2.2. Performance evaluation of growth and growth boundary model

The model of Østergaard et al. (2014) (Eq. (1)) was used to predict growth in the studied smear cheeses. This model was originally developed to predict growth and growth boundary of *L. monocytogenes* in cottage cheese including a cream dressing with or without aroma culture. The model included terms for the effect of temperature, NaCl/ a_w , pH, lactic acid and the effect of interaction between these environmental factors. The simultaneous growth of LAB and *L. monocytogenes* was predicted by the simple Jameson effect model (Gimenez and Dalgaard, 2004).

The model relies on the assumption that high concentrations of LAB reduce the growth of *L. monocytogenes* in the same way as LAB reduces their own growth when their concentration approaches the maximum population density (Le Marc et al., 2009; Mejlholm and Dalgaard, 2007; Møller et al., 2013).

$$\mu_{max} = \mu_{ref} \cdot \left[\left(\frac{T - T_{min}}{T_{ref} - T_{min}} \right)^2 \right] \cdot (1 - 10^{(pH_{min} - pH)}) \cdot \left(\frac{a_w - a_{w,min}}{1 - a_{w,min}} \right) \cdot \left(1 - \left(\frac{[LAC_U]}{MIC_{U \text{ Lactic acid}}} \right) \right) \cdot \xi \quad (1)$$

where μ_{ref} is a fitted parameter that corresponds to μ_{max} at the reference temperature (T_{ref}) of 25°C when other studied environmental parameters are not inhibiting growth (Dalgaard, 2009). This model included separate μ_{ref} -values for cottage cheese with aroma culture (0.34 1/h; DL-culture) or without aroma culture (0.72 1/h; O-culture). T (°C) is the storage temperature, T_{min} is the theoretical minimum temperature allowing growth, a_w is the water activity calculated from the concentration of NaCl in the water phase according to Resnik and Chirife (1998) and $a_{w,min}$ is the minimum theoretical water activity allowing growth. pH_{min} is the theoretical minimum pH-value allowing growth. $[LAC_U]$ is the concentration (mM) of undissociated lactic acid in the product and $MIC_{U \text{ Lactic acid}}$ is the fitted MIC value (mM) of undissociated lactic acid that prevents growth. The interaction between environmental parameters (ξ) was modelled as previously described using the Le Marc approach (Le Marc et al., 2002; Mejlholm and Dalgaard, 2009; Martinez-Rios et al., 2019). The model of Østergaard et al. (2014) included the following cardinal parameter values: $T_{min} = -2.01^\circ\text{C}$; $a_{w,min} = 0.923$; $pH_{min} = 4.87$; $MIC_{U \text{ lactic acid}} = 3.79$ mM undissociated lactic acid.

The acceptable simulation zone (ASZ) approach was used to compare the prediction of *L. monocytogenes* growth by the Østergaard et al., (2014) model (μ_{ref} -value of 0.34 1/h) with growth (log cfu/g) determined during the three challenge tests with smear cheese (see section 2.1). The acceptable interval was defined as ± 0.5 log cfu/g from the simulated growth of *L. monocytogenes*.

When at least 70% of the observed values (log cfu/g) were within the ASZ, the simulation was considered acceptable (Oscar, 2005; Velugoti et al., 2011).

2.3. Evaluation of available growth models using constant product characteristics

A total of 319 growth responses of *L. monocytogenes* in cheeses were extracted from 40 literature studies and from ComBase (Table 1). In contrast to the experiments described above, these studies reported growth responses of *L. monocytogenes* for assumed constant storage temperature and product characteristics. The collected responses described growth, no-growth or inactivation of *L. monocytogenes* in cheeses divided into five types as defined by Martinez-Rios and Dalgaard (2018). In accordance with FAO/WHO (2009) and Skandamis et al. (2007) an increase of more than 0.5 log cfu/g was considered as growth and a decrease of more than 1.0 log cfu/g was defined as inactivation. A total of 46 responses distributed as 38 growth responses, six no-growth and two inactivation responses of *L. monocytogenes* in smear cheese were collected from six literature studies and ComBase. For veined cheese 37 growth responses were obtained from five studies and a total of 64 responses of *L. monocytogenes* in ripened cheese distributed as 11 growth responses, 13 no-growth and 40 inactivation responses were extracted from eight literature studies. Six literature studies contained 24 responses of *L. monocytogenes* in brined cheese which included 18 growth and 6 inactivation responses. For fresh cheese 19 studies were identified containing a total of 148 responses of *L. monocytogenes* distributed as 109 growth, 11 no-growth and 28 inactivation responses (Table 1).

Table 1. Product characteristics and storage conditions in experiments (n = 319) used for evaluation of *Listeria monocytogenes* growth models.

Type of cheese	Reference	Cheese	n ^a	No. of strains inoculated	T (°C)	Water phase salt (%)	aw	pH	Lactic acid reported (%)
Smear	Ryser and Marth (1988)	Limburger	12	4	15	5.04 ^b	0.970^c	5.8	NR ^d
	Combase (2012)	Taleggio	2	-	6	10.64 ^b	0.930	6.0	NR ^d
	Ferrier et al. (2013)	Smear-soft	3	1	15	6.26 ^b	0.962	5.8	NR ^d
	Ferrier et al. (2013)	Smear-soft	3	1	15	2.43 ^b	0.986	6.6	NR ^d
	Lobacz et al. (2013)	Camembert	15	1	3-15	1.70	0.990 ^e	6.3^c	NR ^d
	Tiwari et al. (2014)	Semi-soft smear	6	2	4-20	5.04-5.66 ^b	0.996-0.970	5.7	NR ^d
	Kapetanakou et al. (2017)	Camembert	5	2	7	4.56 ^b	0.973	6.3	NR ^d
Veined	Whitley et al. (2000)	Stilton	5	1	5	9.36 ^b	0.940	6.2^c	NR ^d
	Rosshaug et al. (2012)	Soft blue/white	7	1	4-13	1.83	0.990 ^e	5.0-7.5	0.01-0.35
	Bernini et al. (2013)	Gorgonzola	4	5	4-8	5.04-10.64 ^b	0.930-.0970	5.90-6.8	NR ^d
	Lobacz et al. (2013)	Blue cheese	15	1	3-15	4	0.977 ^d	5.9	NR ^d
	Dalzini et al. (2017)	Gorgonzola	6	2	8	3.76 ^b	0.978	6.6	NR ^d
Ripened	Ryser and Marth (1987)	Cheddar	18	3	6-13	8.00 ^b	0.950^c	5.1	NR ^d
	Yousef and Marth (1988)	Ripened	6	2	4	3.18-3.98 ^b	0.977-0.982	5.0-5.2	NR ^d
	Ryser and Marth (1989)	Brick-aged	24	4	10	5.04 ^b	0.970^c	5.3	NR ^d
	Giannou et al. (2009)	Ripened	6	5	4-25	8.28 ^b	0.948	5.6	NR ^d
	Wemmenhove et al. (2013)	Gouda	2	3	5	3.43 ^b	0.980	5.5-5.4	1.4
	Valero et al. (2014)	Ripened	3	3	4-22	4.25 ^b	0.975	5.5	NR ^d
	Chatelard-Chauvin et al. (2015)	Ripened	2	5	9	6.26 ^b	0.962	5.3	NR ^d
	Kapetanakou et al. (2017)	Mastelo, Edam, Gouda	3	2	7	5.50-7.72	0.952-0.967 ^e	5.5-6.4	NR ^d
Brined	Abdalla et al. (1993)	White pickled	2	1	4	5.15	0.964 ^e	6.4	NR ^d
	Papageorgiou and Marth (1989)	Feta	12	2	4-22	3.92-4.40 ^b	0.974-0.977	4.4-6.6	NR ^d
	Spanu et al. (2012)	Ricotta salata	1	3	4	8.69 ^b	0.945	6.1	NR ^d
	Spanu et al. (2013)	Ricotta salata	1	3	4	8.69 ^b	0.945	6.3	NR ^d
	Coroneo et al. (2016)	Ricotta salata	2	3	4-8	6.26-7.43 ^b	0.954-0.962	6.6-6.8	NR ^d
	Kapetanakou et al. (2017)	Haloumi	6	3	7	5.81 ^b	0.965	6.6	NR ^d
Fresh	Ryser et al. (1985)	Cottage	8	2	3	2.10^c	0.988 ^e	4.5	NR ^d

Glass et al. (1995)	Queso Blanco	42	5	4-20	3.43 ^b	0.980	5.2	NR ^d
Stecchini et al. (1995)	Mozzarella	2	1	5	0.16-3.43 ^b	0.980-0.999^c	5.4	NR ^d
Davies et al. (1997)	Ricotta	2	5	7	0.52 ^b	0.997^c	5.8-6.1	NR ^d
Mendoza-Yepes et al. (1999)	Queso fresco	4	3	7	3.43 ^b	0.980^c	6.5	NR ^d
Menon & Garg (2001)	Mozzarella	4	2	7-30	3.43 ^b	0.980^c	5.4	NR ^d
Uhlich et al. (2006)	Queso blanco	12	5	5-25	4.88 ^b	0.971	6.8	NR ^d
Panagou (2008)	Katiki	4	5	5-20	2.94 ^b	0.983^c	4.6	NR ^d
Leggett et al. (2012)	Queso fresco	4	5	4-10	1.67 ^b	0.990	6.6-6.7	NR ^d
Coelho et al. (2014)	Fresh	16	1	4	1.75 ^b	0.990^c	6.5	NR ^d
Gadotti et al. (2014)	Queso fresco	7	5-6	4	0.16 ^b	0.999^c	6.4	NR ^d
Hamed et al. (2014)	White cheese	1	1	10	0.16 ^b	0.999^c	6.4	NR ^d
Han et al. (2014)	Mozzarella	1	5	10	0.16 ^b	0.999^c	5.8	NR ^d
Pimentel-Filho et al. (2014)	Minas Frescal	1	1	4	3.43 ^b	0.980^c	5.1	NR ^d
Han et al. (2015)	Mozzarella	6	4	4-10	0.16 ^b	0.999^c	5.8	NR ^d
Lobacz et al. (2016)	Fresh	15	1	3-15	1.75 ^b	0.990^c	6.5	NR ^d
Meira et al. (2016)	Minas Frescal	1	1	4	1.75 ^b	0.990^c	5.6	NR ^d
	Mascarpone, cottage,							
Kapetanakou et al. (2017)	Antonyous, Mozzarella, ricotta	13	2	7	1.05-2.09 ^b	0.988-0.994	6.23-6.45	NR ^d
Lourenço et al. (2017)	Queso fresco	5	6	4	1.75 ^b	0.990^c	6.0	NR ^d

^a n, number of experiments

^b Calculated from a_w using Resnik and Cherife (1988).

^c Bold type: assumed values. See explanation in section 2.3)

^d NR: not reported

^e Calculated from water phase salt (%) using Resnik and Cherife (1988).

Growth of *L. monocytogenes* was described by fitting the integrated and log transformed logistic model with delay (Eq. (1); Rosso et al., 1996). Fitted parameter values for initial cell concentration (Log N_0 , log cfu/g), lag time (t_{lag} , h), maximum specific growth rate (μ_{max} , 1/h) and maximum population density (Log N_{max} , log cfu/g) were determined for each growth curve collected from literature.

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

where t is the storage time (h) and N_t is the cell concentration (cfu/g) at time t . Other parameters were indicated above.

When a_w was not reported for growth responses from literature, it was estimated from concentration of NaCl in the water phase of the cheese according to eq. (2) obtained from data by Resnik and Cherife (1988).

$$a_w = 1 - 0.0052471 \cdot WPS - 0.00012206 \cdot WPS^2 \quad (2)$$

$$\% WPS = \frac{100 \cdot \%NaCl}{\%moisture + \%NaCl} \quad (3)$$

where %WPS (eq.(3)) is the water phase salt calculated from NaCl concentration in percentage (% NaCl, w/v) and the water in the product (%moisture, w/v). Eq.(4) was used to estimate %WPS when a_w was reported (Jørgensen et al. 2000).

$$\% WPS = 8 - 140.07 \cdot (a_w - 0.95) - 405.12 \cdot (a_w - 0.95)^2 \quad (4)$$

When no information was provided regarding NaCl/ a_w and pH, then values were assumed based on information for similar type of cheeses (Table 1).

2.4. Evaluation of available growth model and indices of model performance

Predicted and observed μ_{max} -values (n= 319) were evaluated by calculation of B_f - and A_f -values. B_f -values from 0.85 to 1.11 indicate a good model performance, whereas B_f -values of 1.11-1.43 and 0.87-0.95 correspond to acceptable model performance. B_f -values <0.87 or > 1.43 corresponds to unacceptable model performance (Ross, 1996). A_f is a measure of the average difference between observed and predicted μ_{max} -values. $A_f > 1.5$ indicates an incomplete model or systematic deviation between observed and predicted μ_{max} -values (Mejlholm and Dalgaard, 2013).

Predicted and observed growth and no-growth responses were further assess by calculating the percentage of all samples that were correctly predicted. Incorrect predictions were described as fail-safe (growth predicted when no growth was observed) or fail-dangerous (no growth predicted but growth was observed) (Mejlholm and Dalgaard, 2009).

The ability of the model of Østergaard et al. (2014), 4 other secondary growth models and two software were evaluated for their ability to predict growth of *L. monocytogenes* in smear-, veined-, ripened-, brined- and fresh cheeses. This evaluation relied on calculation of B_f - and A_f -values from a total of 213 growth responses for specific types of cheeses (Table 2).

The studied existing secondary growth models were: (i) the model of Augustin et al. (2005) including the effect of temperature, pH, a_w , interactions among environmental factors and μ_{opt} -values of 0.742 or 0.212 1/h for liquid dairy products and cheeses, respectively. (ii) The model of Schwartzman et al. (2011) included the effect of temperature, a_w , pH and interactions among environmental factors with μ_{opt} -values of 1.19 1/h or 0.17 1/h for pasteurized or raw milk cheese, respectively.

Table 2. Comparison of observed (literature data) and predicted maximum specific growth rates (μ_{\max} -values) of *L. monocytogenes* in different types of cheese

Type of cheese	G ^a	Østergaard et al. (2014) ($\mu_{ref}=0.34$) ^b	Østergaard et al. (2014) ($\mu_{ref}=0.72$) ^c	Augustin et al. (2005) ($\mu_{opt}=0.742$) ^d	Martinez-Rios et al. (2019)	Sym'Previus	Augustin et al. (2005) ($\mu_{opt}=0.212$) ^e	Schwartzman et al. (2011) ($\mu_{opt}=1.19$) ^f	Schwartzman et al. (2011) ($\mu_{opt}=0.17$) ^g	Rosshaug et al. (2012)	ComBase
Smear	38	1.0/1.5	2.1/2.2	1.4/1.7	1.4/1.6	1.8/2.0	0.4/2.6	2.2/2.4	0.3/3.3	2.1/2.2	2.2/2.2
Veined	37	1.3/2.4	2.7/3.3	2.3/2.9	1.7/2.6	3.5/3.7	0.6/2.0	3.0/3.2	0.6/1.9	3.0/3.0	2.9/3.2
Ripened	11	0.6/2.5	1.2/1.8	0.5/2.8	0.7/2.3	1.0/1.9	0.1/7.3	0.8/2.2	0.1/9.3	1.2/1.8	0.9/2.0
Brined	18	0.6/2.1	1.2/1.6	0.8/1.8	0.7/1.9	1.1/1.6	0.2/4.4	1.3/1.9	0.2/5.5	1.3/1.8	1.3/1.7
Fresh	109	0.7/2.4	1.4/2.2	1.0/2.3	0.9/2.2	0.9/2.9	0.3/4.2	1.6/2.5	0.2/5.0	0.8/3.9	1.4/2.2
Total	213	0.8/2.2	1.6/2.2	1.1/2.3	1.1/2.1	1.3/2.6	0.3/3.7	1.7/2.6	0.2/4.4	1.1/3.2	1.7/2.3

^a G, number of experiments where growth was observed

^b Østergaard et al., 2014, aroma culture (DL-culture)

^c Østergaard et al., 2014, no-aroma culture (O-culture)

^d Augustin et al., 2005, liquid dairy products

^e Augustin et al., 2005, cheese

^f Schwartzman et al., 2011, pasteurized milk cheese

^g Schwartzman et al., 2011, raw milk cheese

(iii) The model of Rosshaug et al. (2012) to predict growth and the growth boundary of *L. monocytogenes* in soft blue-white cheese and including the effect of temperature, NaCl, pH, lactic acid concentration and interaction among the environmental factors with a μ_{opt} -value of 0.43 1/h obtained by fitting model predictions with measurements of *L. monocytogenes* strain ATCC 19115 growing in a 9 weeks aged soft blue-white cheese. (iv) The model of Martinez-Rios et al. (2019) developed and validated for growth of *L. monocytogenes* in chemically acidified cheese and cream cheese. (v) Models from ComBase and (vi) the Sym'Previous software including the effect of temperature, pH, a_w and lactic acid.

3. Results

3.1. *L. monocytogenes* growth and dynamic product characteristics for smear cheese

In all three challenge tests, *L. monocytogenes* grew during the first 22 h of cheese making where 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 acid in the water phase increased from non-detectable to $1.7 \pm 0.2\%$. 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 acid concentrations and the correspondent decrease of pH (Fig. 1 and 2). From 22 h and up to four days of storage, *L. monocytogenes* cell concentrations remained constant or decreased slightly both in the rind and in the core of the smear 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 (Fox et al., 2017).

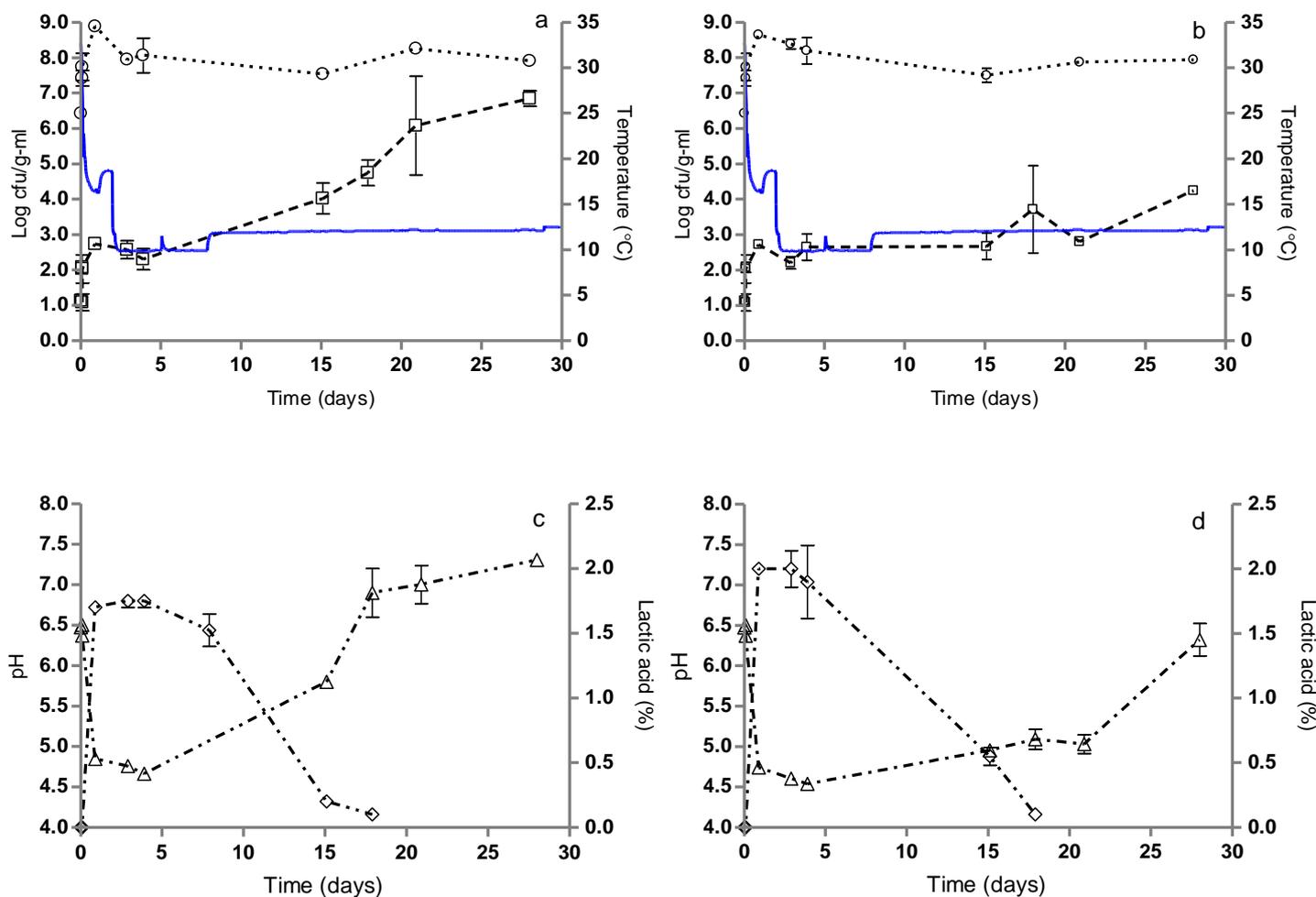


Figure 1. Data for the rind (a, c) and the core (b, d) of challenge test one smear cheese. *L. monocytogenes* (□) and lactic acid bacteria (○) behaviour in smear cheese (*Lactobacillus acidophilus*, LA-5 and FLORA™ C501, Chr. Hansen A/S, *Penicillium camemberti*, PC HP 6 LYO and *Geotrichum candidum*, GEO 17, DuPont™ Danisco®). Evolution of pH (△) and lactic acid concentration in % (◇). The temperature profiles are shown as blue lines in the top figures. Symbols represent average values and error bars of the standard deviation for three samples.

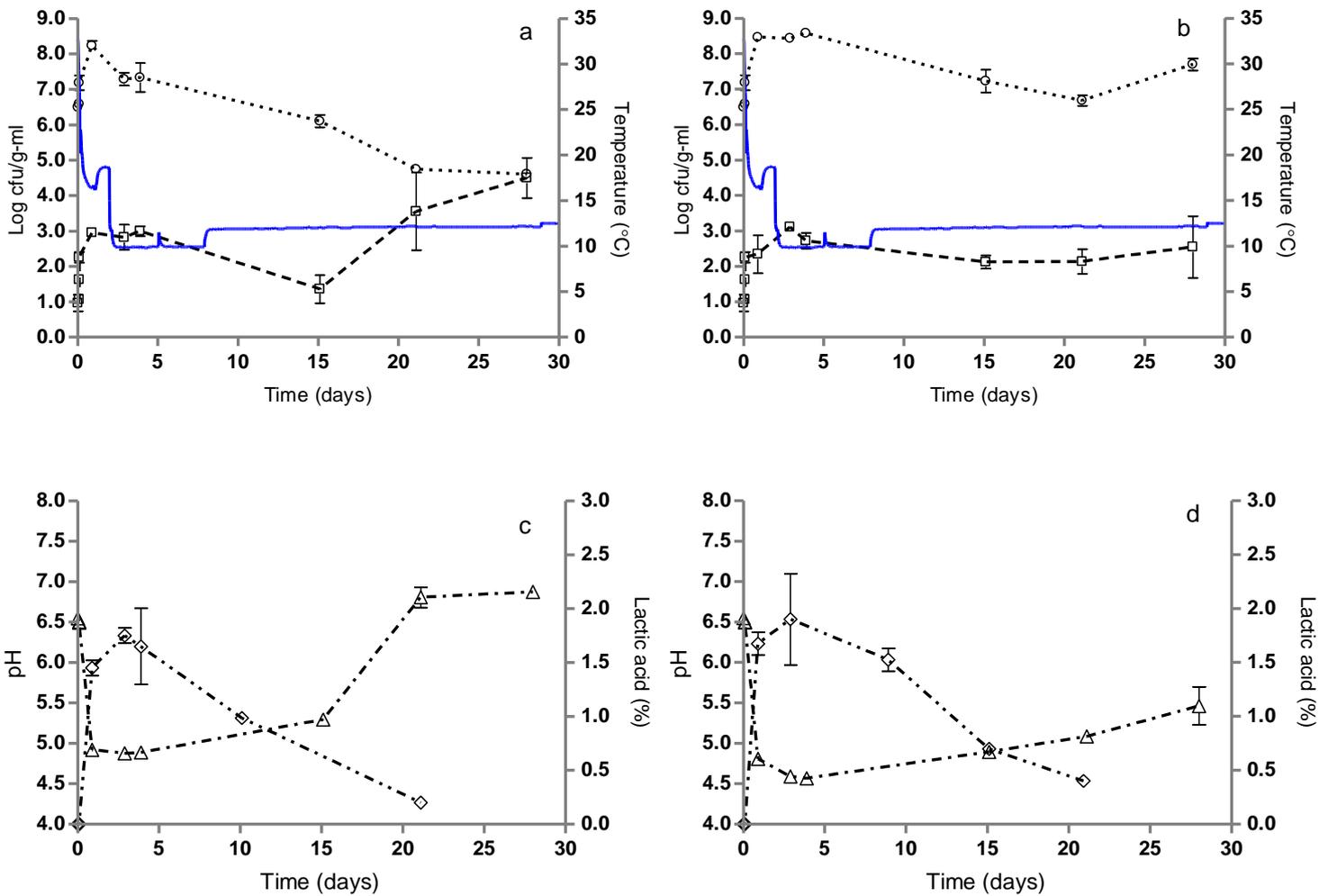


Figure 2. Data for the rind (a, c) and the core (b, d) of challenge test two smear cheese. *L. monocytogenes* (□) and lactic acid bacteria (○) in smear cheese (FLORA™ C503, Chr. Hansen A/S and *Geotrichum candidum*, GEO 17, DuPont™ Danisco®). Evolution of pH (△) and lactic acid concentration in % (◇). The temperature profiles are shown as blue lines in the top figures. Symbols represent average values and error bars of the standard deviation for three samples.

In challenge test one, growth of *L. monocytogenes* was observed in the rind of smear cheese from day four to day 15 where pH increased from 4.7 ± 0.0 to 5.8 ± 0.0 and the lactic acid concentration in the water phase decreased from $1.8 \pm 0.1\%$ to $0.2 \pm 0.0\%$ (Fig. 1a). 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 was observed at day 28 (Fig.1b).

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. Growth was then observed in the rind of the smear 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 and with a pH of 6.9±0.1 (Fig. 2c). *L. monocytogenes* reached a concentration in the core of 2.5±0.9 log cfu/g when pH was 5.5±0.3 after 28 days of storage (Fig. 2d).

For challenge test three, *L. monocytogenes* was inactivated both in the rind and in the core of studied cheese. This was due to a water activity of ~0.94 on day two and this value decreased to ~0.88 on day seven and stayed below 0.92 for the rest of challenge test.

3.2. Evaluation of *L. monocytogenes* model for smear cheese with dynamic product characteristics

For challenge test one on day 15, the model of Østergaard et al. (2014) with simultaneous growth of LAB and *L. monocytogenes*, predicted *L. monocytogenes* concentrations of 2.8 log cfu/g in the rind and 1.4 log cfu/g in the core. The corresponding LAB concentration being of 7.7 log cfu/g. On day 28, 3.8 log cfu/g in the rind and 1.4 log cfu/g in the core were predicted for *L. monocytogenes* with 9 log cfu/g for LAB. For challenge test two, predicted concentrations of *L. monocytogenes* in the rind and core after 28 days were 2.0 log cfu/g and 1.5 log cfu/g, respectively, with LAB at a concentration of 9 log cfu/g. The model markedly underestimated growth of *L. monocytogenes* in smear cheese for both challenge tests one and two (Fig. 1 and 2).

Without the inhibiting effect of LAB (Jameson effect), the model of Østergaard et al., (2014), for challenge tests one and two, appropriately predicted the time when concentrations of *L. monocytogenes* in rind and core started to increase (Fig. 3). Predicted growth was slightly faster and reached higher concentration than the observed growth (Fig. 3). On average 67% of the predicted cell concentrations were within the ASZ for smear cheese when calculated for challenge test one and two including a total of 12 growth curves at dynamic temperatures (Fig. 3; Table 3). When predictions were performed without taking into account the measured concentrations of lactic acid in the water phase then the obtained ASZ score was 41% as predicted growth rates were markedly faster than observed (Table 3).

3.3. Evaluation of available growth models using constant product characteristics

For smear cheese (n=38), evaluation of the model of Østergaard et al. (2014) (μ_{ref} of 0.34 1/h for DL-culture) resulted in acceptable B_f - and A_f -values of 1.0/1.5 (Table 2). 83% of the growth/no-growth responses were correctly predicted with incorrect predictions being fail-safe (17%) (Table 4). When using an assumed average lactic acid concentration in the water phase of 0.55% (average value observed at day 15) the B_f - and A_f -values became 0.9/1.5. Due to high A_f -values validation of the Østergaard et al. (2014) model was not successful for the other types of cheeses studied (Table 3).

B_f - and A_f -values of 1.4/1.7 were obtained for smear cheese with the model of Augustin et al., (2005) including a μ_{opr} -value of 0.742 1/h, as obtained for liquid dairy products. 87% of the growth/no-growth responses were correctly predicted with the rest being fail-safe (13%) (Table 4). Evaluation of the model of Martinez-Rios et al. (2019) with smear cheese data resulted in B_f - and A_f - values of 1.4/1.6. 83% of predictions were correct with the incorrect ones being fail-safe (17%).

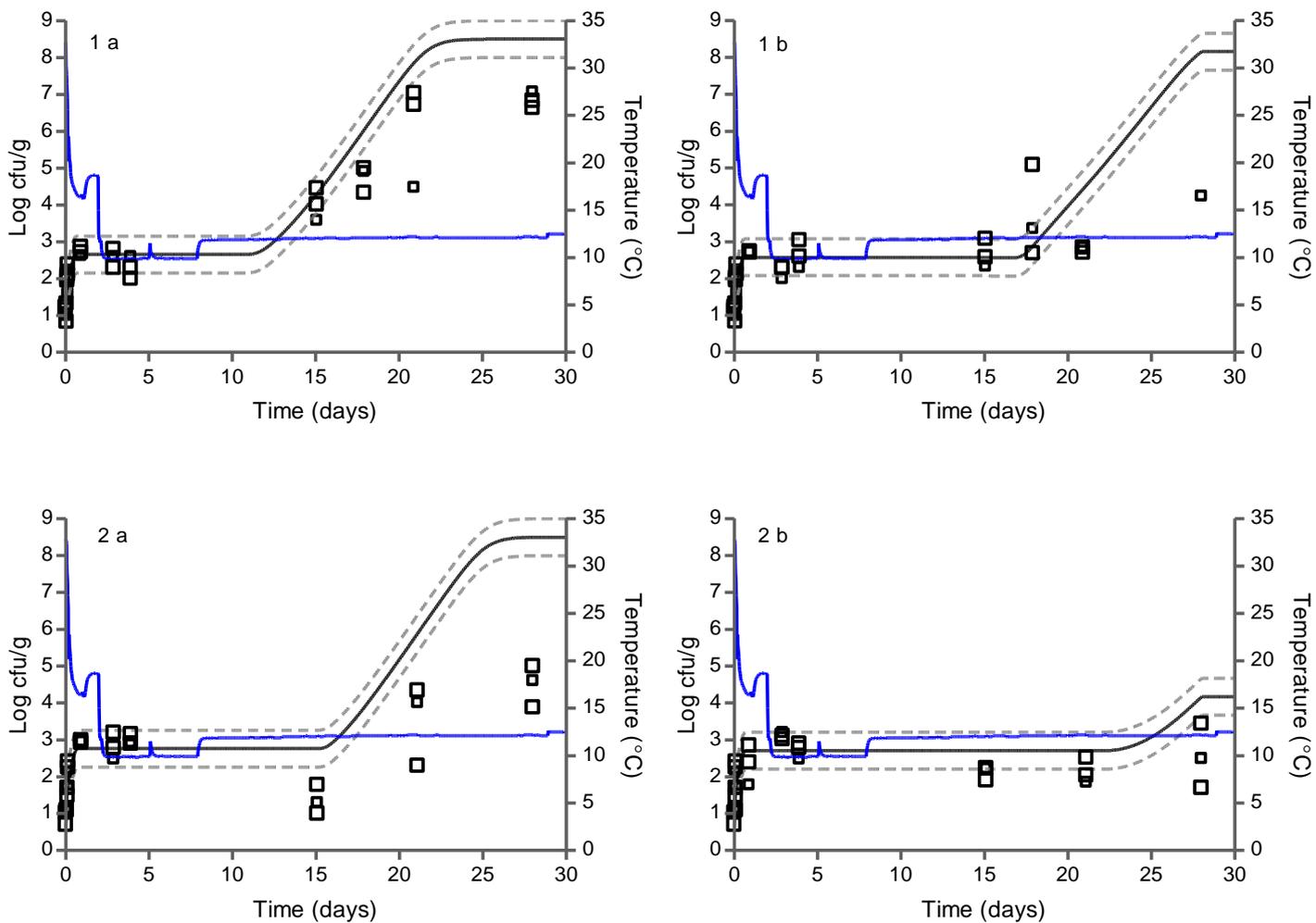


Figure 3. Comparison of observed (\square) and predicted (-) growth of *L. monocytogenes* in the rind (a) and core (b) of challenge test one (1) and two (2) with smear cheese. Solid lines represent the predicted growth by eq. (24). Graphs include the ASZ (± 0.5 Log cfu/g, dashed lines).

Table 3. Comparison of predicted and observed growth data using the acceptable simulation zone (ASZ) method.

CT ^a	Storage conditions and growth data	Predictions performed including lactic acid concentrations in the water phase % observations within AZS ^b	Predictions performed without including lactic acid concentrations in the water phase % observations within AZS ^b
1	Rind	58	30
1	Core	70	33
2	Rind	69	48
2	Core	73	53
Average AZS score			
All data		67	41

^a CT: challenge tests

Table 4. Comparison of observed and predicted growth/no-growth responses of *Listeria monocytogenes* in different types of cheese.

Type of cheese	n ^a	Observed		Correct/fail-safe/fail-dangerous (%)										
		G ^b	NG (I) ^c	Østergaard et al. (2014) ($\mu_{ref}=0.34$) ^d	Østergaard et al. (2014) ($\mu_{ref}=0.72$) ^e	Augustin et al. (2005) ($\mu_{opt}=0.742$) ^f	Martinez-Rios et al. (2019)	Sym'Previus	Augustin et al. (2005) ($\mu_{opt}=0.212$) ^g	Schwartzman et al. (2011) ($\mu_{opt}=1.19$) ^h	Schwartzman et al. (2011) ($\mu_{opt}=0.17$) ⁱ	Rosshaug et al. (2012)	ComBase	
Smear	46	38	8 (2)	83/17/0	83/17/0	87/13/0	83/17/0	87/13/0	87/13/0	87/13/0	87/13/0	87/13/0	87/13/0	83/17/0
Veined	37	37	0	97/0/3	97/0/3	97/0/3	97/0/3	84/0/16	97/0/3	97/0/3	97/0/3	97/0/3	97/0/3	100/0/0
Ripened	64	11	53 (40)	20/80/0	20/80/0	58/42/0	28/72/0	42/58/0	58/42/0	58/42/0	58/42/0	58/42/0	47/53/0	17/83/0
Brined	24	18	6 (6)	100/0/0	100/0/0	100/0/0	100/0/0	92/0/8	100/0/0	100/0/0	100/0/0	100/0/0	100/0/0	75/25/0
Fresh	148	109	39 (28)	80/19/1	80/19/1	84/6/9	80/19/1	79/20/1	84/6/9	84/6/9	84/6/9	84/6/9	83/7/9	74/26/0
Total	319	213	106(76)	72/27/1	72/27/1	82/13/5	74/25/1	74/23/3	82/13/5	82/13/5	82/13/5	82/13/5	79/16/4	67/33/0

^a n, number of experiments

^b G, number of experiments where growth was observed

^c NG, no-growth observed; I, inactivation observed.

^d Østergaard et al., 2014, aroma culture (DL-culture)

^e Østergaard et al., 2014, no-aroma culture (O-culture)

^f Augustin et al., 2005, liquid dairy products

^g Augustin et al., 2005, cheese

^h Schwartzman et al., 2011, pasteurized milk cheese

ⁱ Schwartzman et al., 2011, raw milk cheese

Further evaluation of the model of Martinez-Rios et al. (2019) including an average lactic acid concentration of 0.45% in the water phase resulted in B_f - and A_f -values of 1.2/1.5.

Both Sym'Previus and the model of Østergaard et al. (2014), with μ_{ref} of 0.72 1/h for an O-culture, resulted in acceptable B_f -values of 1.2 and 1.1, respectively, for *L. monocytogenes* growth in brined cheese (Table 2). A_f -values, however, were slightly above the acceptable level of 1.5 (Table 2). 92% of the growth/no-growth responses predicted by SymPrevius were correct with the incorrect ones being fail-dangerous (8%) predictions. On the other hand, the model of Østergaard et al. (2014) predicted growth/no-growth responses 100% correctly (Table 4).

None of the evaluated models resulted in acceptable B_f - and A_f -values for veined-, ripened- or fresh cheese. For *L. monocytogenes* growth in veined cheese the model of Østergaard et al. (2014) with a μ_{ref} of 0.34 1/h resulted in an acceptable B_f -value of 1.3 but with an unacceptable A_f -value of 2.4 (Table 2). 97% of the growth/no-growth responses were correctly predicted but the incorrect were fail-dangerous (3%) (Table 4).

None of the evaluated models and software was able to predict growth of *L. monocytogenes* with acceptable B_f - and A_f -values for cheese in general (n=213). Evaluation of the model of Augustin et al., (2005) with a μ_{opt} -value of 0.742 1/h and the model of Martinez-Rios et al. (2019) resulted in acceptable B_f -values for cheese in general and with A_f -values lower than for other models. However, A_f -values were above the suggested limit of 1.5 (Table 2).

4. Discussion

Growth of *L. monocytogenes* in smear cheese can be predicted from changes in storage temperature and product characteristics by using the model of Østergaard et al. (2014) (Fig. 3). Importantly, when the measured lactic acid concentrations in the smear cheese was used as model

input this resulted in 26% more accurate predictions of growth than predictions based exclusively in temperature, pH and water activity (Table 3). As an example, if the initial concentration at 22 h is 2.6 ± 0.0 log cfu/g and the model is used to predict the time required for a two log increase without including lactic acid concentration, the model will predict 6 days. If then the lactic acid concentrations are included as model inputs, the model predicts a two log increase by day 15 whereas the observed two log increase occurred at day 17. Therefore, predictions including lactic acid measurements as model inputs are fail-safe by 2 days rather than 11 days.

In the present study, *L. monocytogenes* growth observed during the first 22 h was predicted correctly by the model based on measurement of temperature, pH, a_w and lactic acid concentration. However, several studies have suggested that the increase during curd formation is the result of cell entrapment rather than growth (Schvartzman et al., 2011; Wemmenhove et al., 2013). Furthermore, the absence of growth observed in challenge tests from 22 h to day four can be explained by the model as the combined effect of low pH and high lactic acid concentration resulting in an undissociated lactic acid concentration above MIC-value of 3.79 mM and pH-value below 4.97. Some previous studies suggested that *L. monocytogenes* growth inhibition in similar cheeses might be the result of low temperature, low pH and high concentration of organic acids; however no quantitative data for organic acid concentrations was provided (Morgan et al., 2001; Papageorgiou and Marth, 1989).

The decrease in *L. monocytogenes* concentrations as observed in challenge test two between day four and day 15 (Fig. 2a) has previously been observed for smear cheese during the initial stages of ripening (Back et al., 1993; D'Amico et al., 2008; Liu et al., 2007). Specifically, Maisnier-Patin et al. (1992) observed a 1.7 log-reduction of *L. monocytogenes* in a similar product after one week of ripening. Thereafter, regrowth was observed first in the rind followed by the core as a

result of pH increase. For veined cheese *L. monocytogenes* inactivation has also been observed during the initial weeks of ripening where pH was as high as 5.5 to 6.5 (Dalzini et al., 2017; Ryser and Marth, 1989). The mechanisms underlying inactivation responses in these cheeses remain uncertain. Production of bacteriocins, peptides, organic acids, fatty acids, volatile compounds by LAB and secondary microbiota can be responsible but quantitative data to support this hypothesis are lacking (Cantor et al., 2017; De Vuyst and Vandamme, 1994; Dieuleveux et al., 1998; Spinnler and Gripon, 2017).

In the present study re-growth of *L. monocytogenes* was initiated at pH values between 5.1 and 5.4, and increased with increasing pH, as found in other studies (D'Amico et al., 2008; Millet et al., 2006; Ryser and Marth, 1987).

The complete model of Østergaard et al. (2014) including the Jameson effect for the inhibitory effect of high concentrations of LAB on *L. monocytogenes* underestimated growth of the pathogen in smear cheese (See 3.2). The Østergaard et al. (2014) model was developed and validated for growth of LAB and *L. monocytogenes* in cottage cheese at 5-15°C and with assumed constant pH and lactic acid concentration. The present study used this model at temperatures up to 32°C and thus out of its range of applicability where it was successfully validated. This may explain the underestimation of *L. monocytogenes* growth. However, smear cheese contains a more complex microbiota than cottage cheese and this is likely to influence the measured difference between observed and predicted growth. Smear cheese includes a white-mould exterior (e.g. *Penicillium camemberti*) and soft-smear cheeses are ripened by bacteria and yeasts present in the exterior that were not washed during production (e.g. *Geotrichum candidum*, *Brevibacterium linens*, *Debaryomyces hansenii*) (Corsetti et al., 2001; Larpin et al., 2006; Famelart et al., 1987; McSweeney and Sousa, 2000; Riahi et al., 2007). In the present study smear cheese included *Penicillium*

camemberti and/or *Geotrichum candidum* and these ripening cultures may reduce the inhibiting effect of high concentration of LAB on *L. monocytogenes* growth. Further studies are needed to test this hypotheses where the effect must be due to factors other than temperature, pH, lactic acid concentration and NaCl/a_w which are already taken into account by the model of Østergaard et al. (2014).

Data summarized in Table 1 highlights the lack of literature reported information about organic acid concentrations present in cheese when the growth of *L. monocytogenes* was studied. As demonstrated by the present study and by other authors (Martinez-Rios et al., 2019; Mejlholm and Dalgaard, 2007; Wemmenhove et al., 2018) lactic acid concentration has a marked effect on growth of *L. monocytogenes* depending on the products pH and organic acids quantification will improve the understanding of *L. monocytogenes* growth in cheese.

Evaluation of existing models demonstrated that there is not a single model that will accurately predict growth of *L. monocytogenes* in different types of cheeses. Depending on the type of cheese the same model can over- or underestimate growth. For instance, Sym'Previus predicted growth of *L. monocytogenes* in brined cheese correctly but growth in veined cheese was markedly overestimated (Table 2). Surprisingly, Augustin et al. (2005) with a μ_{opt} -value of 0.212 1/h obtained from different cheeses underestimated growth in all the studied types of cheeses as shown by B_f-values between 0.1 and 0.6 (Table 2). However, if a quick assessment of *L. monocytogenes* growth in cheese is required, the model of Augustin et al. (2005) with a μ_{opt} -value of 0.742 1/h obtained in liquid dairy products and Martinez-Rios et al. (2019) are the recommended models; nonetheless both models A_f-values (n= 213) were above the recommended acceptable limit of 1.5 and predictions should be used with caution. Lastly, the aforementioned models predictions were of 82

and 74% correct, 13 and 25% fail-safe and 5 and 1% fail-dangerous predictions for Augustin et al. (2005) and Martinez-Rios et al. (2019), respectively (Table 4)

Some models available in literature might be able to predict growth of *L. monocytogenes* in specific types of cheese (Augustin et al., 2005; Martinez-Rios et al., 2019; Rosshaug et al., 2012; Schwartzman et al., 2011). However, the model of Østergaard et al., (2014) previously validated for cottage cheese can additionally predict growth in smear- and brined cheese simply by using different μ_{ref} -values calibrated for growth of *L. monocytogenes* in presence of aroma or non-aroma cultures. Furthermore, Dalzini et al., (2017) suggested that the aforementioned model can be used as an alternative to challenge tests as the model predicted similar μ_{max} -values of *L. monocytogenes* in one batch out of three of Gorgonzola cheese.

In conclusion, the model of Østergaard et al., (2014) is a promising candidate to accurately predict growth of *L. monocytogenes* in different types of cheese and it might be necessary to be expanded in the future with terms to account for the effect of other compounds produced during fermentation or ripening of various types of cheese (e.g. acetic acid, propionic acid).

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Paper III

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New term to quantify the effect of temperature on pH_{min} -values used in cardinal
parameter growth models for *Listeria monocytogenes*

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New term to quantify the effect of temperature on pH_{min} -values used in cardinal parameter growth models for *Listeria monocytogenes*

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ABSTRACT

The aim of this study was to quantify the influence of temperature on pH_{min} -values of *L. monocytogenes* as used in cardinal parameter growth models and thereby improve the prediction of growth for this pathogen in food with low pH. Experimental data for *L. monocytogenes* growth in broth at different pH-values and at different constant temperatures were generated and used to determine pH_{min} -values. Additionally, pH_{min} -values for *L. monocytogenes* available from literature were collected. A new pH_{min} -term was developed to describe the effect of temperatures on pH_{min} -values obtained experimentally and from literature data. A growth and growth boundary model was developed by substituting the constant pH_{min} -value present in the Mejlholm and Dalgaard (2009) model (J. Food. Prot. 72, 2132–2143) by the new pH_{min} -term. To obtain data for low pH food, challenge tests were performed with *L. monocytogenes* in commercial and laboratory-produced chemically acidified cheese including glucono-delta-lactone (GDL) and in commercial cream cheese. Furthermore, literature data for growth of *L. monocytogenes* in products with or without GDL were collected. Evaluation of the new model by comparison of observed and predicted μ_{max} -values resulted in a bias factor of 1.01 and an accuracy factor of 1.48 for a total of 1129 growth responses from challenge tests and literature data. Growth and no-growth responses of *L. monocytogenes* in seafood, meat, non-fermented dairy products and fermented cream cheese were 90.3% correctly predicted with incorrect predictions being 5.3% fail-safe and 4.4% fail-dangerous. The new pH_{min} -term markedly extended the range of applicability for the Mejlholm and Dalgaard (2009) model from pH 5.4 to pH 4.6 and therefore the model can now support product development, reformulation or risk assessment of low pH chemically acidified cheese and cream cheese.

1. Introduction

Cardinal parameter models (CPMs) contain parameters with biological or graphical interpretation (Rosso, 1995). CPMs to predict growth and growth boundary of *Listeria monocytogenes* (CPM-*Lm*) are popular, extensively validated and widely used in the assessment and risk management of processed and ready-to-eat foods (Augustin and Carlier, 2000; Augustin et al., 2005; Mejlholm and Dalgaard, 2009; Østergaard et al., 2014; Corbion, 2017; te Giffel & Zwietering, 1999; Zuliani et al., 2007). These models include terms to quantitatively describe the growth inhibiting effect of different environmental factors and each term include at least one cardinal parameter related to growth limiting conditions e.g. for temperature (T_{min}), pH (pH_{min}) and water activity ($a_{w_{min}}$). For several microorganisms, including *L. monocytogenes*, different studies using similar model terms have found similar T_{min} - and $a_{w_{min}}$ -values (Le Marc et al., 2010; Østergaard et al., 2014). Remarkably, available CPM-*Lm* include very different pH_{min} -values ranging from 4.3 to 5.0. This can be due to differences in the mathematical terms used to estimate pH_{min} -values along with different acidulants and strain variability as often suggested (Augustin et al., 2005; Arayi et al., 2015). However, the experimental conditions used to estimate pH_{min} -values have been little studied quantitatively, although the minimal pH-value supporting growth is known to depend on environmental conditions including temperature (Rocourt and Buchrieser, 2007).

As for other predictive food microbiology models the performance of CPM-*Lm* can be evaluated by comparison of predicted growth responses with observed growth in foods. Often, indices of model performance, including bias (B_f) and accuracy (A_f) factors, are used to facilitate model evaluation and to determine the range of environmental conditions where a model can be successfully validated (Augustin et al., 2005; Østergaard et al., 2014; Ross, 1996). Mejlholm et al.,

(2010) evaluated the performance of four of the more extensive CPM-*Lm*, including the effect of several environmental factors, by using 1014 growth responses in meat, seafood, poultry and non-fermented dairy products. The model of Mejlholm and Dalgaard (2009) performed better than the other models, with B_r - and A_r - values for growth rate predictions of 1.0 and 1.5, respectively. However, the range of applicability for this model has been limited to pH-values above 5.4 as predicted growth rates at lower pH-values were too low due to a constant pH_{min} -value of 4.97 used in the pH-term (Mejlholm et al., 2010; Mejlholm and Dalgaard, 2015). *L. monocytogenes* can grow at pH values as low as 4.3-4.4 (Farber, 1989; ICMSF 1996; Tienungoon et al. 2000), which is important for several types of food including products acidified with glucono-delta-lactone (GDL) and gluconic acid (GAC). El-Shenawy and Marth (1990) found growth of *L. monocytogenes* in milk containing GDL or GAC at pH lower than 5.0 when products were stored at 13°C and 35°C. Genigeorgis et al. (1991) showed that *L. monocytogenes* has the potential to grow in cottage cheese with pH 4.9 to 5.1 when stored at 4, 8 or 30°C. More recently, Nyhan et al. (2018) showed that béarnaise sauce and zucchini purée with pH of 4.7 can support growth of *L. monocytogenes* at 30°C. To assess and manage *L. monocytogenes* growth in food with pH below 4.3-4.4 it is interesting to study the performance of predictive models. Furthermore, it remains unclear if GDL or GAC have any antimicrobial effect beyond that of lowering product pH.

The objective of the present study was to quantify the influence of temperature on pH_{min} -values of *L. monocytogenes* as used in cardinal parameter models. Firstly, the growth inhibiting effect of pH and GAC was studied at different temperatures to determine values for pH_{min} and the minimum inhibitory concentration (MIC) of undissociated GAC (MIC_{GACu}). Secondly, a new pH-term was developed, including the effect of temperature on pH_{min} -values, and this new pH-term was included in the growth and growth boundary model of Mejlholm and Dalgaard (2009) along with a GAC-term containing the MIC-value for undissociated GAC. Finally, the performance of the

expanded model was evaluated by comparison of predicted and observed growth for *L. monocytogenes*. Data included new challenge test with chemically acidified cheese, cream cheese and available growth responses from literature.

2. Material and methods

2.1. Bacterial strains, pre-culture conditions and inoculation

Eight strains of *L. monocytogenes* from milk, cheese, butter or the dairy environment were provided by Arla Foods and used as a cocktail (SLU 92, 612, LM 19, 6) or individually (ISO 570, 99714, SLU 2493, SLU 2265) to determine μ_{max} -values in broth and/or for inoculation of challenge tests. Each strain was transferred from storage at -80°C to Brain Heart Infusion (BHI) broth (CM1135, Oxoid, Hampshire, UK) and incubated for 24h at 25°C . Subsequently, for broth studies all strains were pre-cultured one or two days at 8°C to 20°C in BHI broth with 0.5 % NaCl and pH 5.5. For challenge tests the individual strains, later used as a cocktail, were pre-cultured one or two days at a temperature ranging from 8°C to 20°C in BHI broth with pH 5.5 and 3% NaCl or pH 5.2, 1% NaCl and 500 ppm lactic acid to simulate conditions encountered in chemically acidified and cream cheese as used in the present study. Pre-cultures were grown to a relative increase in absorbance (540 nm) of 0.05 to 0.2 (Novaspec II, Pharmacia Biotech, Allerød, Denmark) equivalent to late exponential phase-beginning stationary phase. The *L. monocytogenes* cocktail of strains (*Lm-mix*) were obtained by mixing equal volumes of individual pre-cultured strains. For *Lm-mix* and pre-cultures of individual strains the cell concentration was determined by direct phase contrast microscopy prior to dilution and subsequent inoculation of experiments.

2.2. Cardinal parameter values for pH and gluconic acid

The effect of pH and gluconic acid concentrations on μ_{max} -values of *L. monocytogenes* were determined at different temperatures. For each condition, growth of *Lm-mix* or individual strains

was determined in duplicate by automated absorbance measurements at 540 nm (BioScreen C, Labsystems, Helsinki, Finland). Detection times defined as incubation time necessary to observe an increase in absorbance of 0.05 from the lowest absorbance measured in the beginning of incubation; was determined for each absorbance growth curve. μ_{max} -values of *Lm-mix* and individual strains were determined from absorbance detection times for serially diluted inoculation levels of 10^2 , 10^3 , 10^4 , 10^5 and 10^6 CFU/ml as previously described (Dalgaard and Koutsoumanis, 2001).

The effect of 17 pH-values from 4.4-6.8 on μ_{max} -values were determined separately at different temperatures (5°C, 8°C, 10°C, 15°C, 20°C, 25°C, 35°C and 37°C) by using BHI broth adjusted to the desired pH values with HCl, autoclaved (121°C, 15 min.) and pH readjusted if necessary. A total of 221 μ_{max} -values were determined experimentally in BHI-broth. Seventeen pH_{min} -values were estimated by fitting eq. (1) to square root transformed μ_{max} -values from broth experiments obtained for the studied pH range at different constant temperatures.

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref-1}(*^{\circ}C) \cdot (1 - 10^{(pH_{min}-pH)})} \quad (1)$$

where μ_{max} is the maximum specific growth rate (h^{-1}) and $\mu_{ref-1}(*^{\circ}C)$ is the fitted reference maximum specific growth rate for each studied temperature. Additionally, 44 pH_{min} -values (obtained in broth adjusted to different pH-values with HCl) were extracted from literature and used to model the effect of storage temperature on pH_{min} -values of *L. monocytogenes* (Aryani et al., 2015; Brocklehurst et al., 1995; Duffy et al., 1994; Farber et al., 1989; George et al., 1988; Koutsoumanis et al., 2004; Petran et al., 1989; Ryser and Marth, 1988).

The effect of 54 gluconic acid (D-gluconic acid sodium salt, G9005 Sigma-Aldrich, St. Louis, USA) concentrations (0 – 26.7% (w/v)) on μ_{max} -values were determined separately at different temperatures (8°C, 20°C and 25°C) in BHI broth adjusted with HCl to pH 5.5 after addition of the organic acid and again after autoclaving (121°C, 15 min.) the broth if necessary. In

total 144 μ_{max} -values were determined experimentally in BHI-broth. Cardinal parameter value for undissociated gluconic acid (MIC_{GAC_U}) was determined from concentrations of undissociated GAC calculated by using eq. (2) with a pK_a -value of 3.7 (Quitmann et al., 2014). The cardinal parameter values (MIC_{GAC_U} and T_{min}) were estimated by fitting eq. (3) to square root transformed μ_{max} -values.

$$\text{Undissociated organic acid (mM)} = \frac{\text{Organic acid (mM)}}{1 + 10^{pH-pka}} \quad (2)$$

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref-3} \cdot \left(\frac{T-T_{min}}{T_{ref}-T_{min}}\right)^2 \cdot \left(1 - \left(\frac{[GAC_U]}{MIC_{U\ GAC}}\right)^{n1}\right)^{n2}}$$

(3)

where T is the temperature ($^{\circ}C$) and T_{min} the theoretical minimum temperature ($^{\circ}C$) that prevents growth; $[GAC_U]$ is the concentrations (mM) of undissociated gluconic acid and $MIC_{U\ GAC}$ is the fitted MIC value (mM) of undissociated gluconic acid that prevent growth of *L. monocytogenes*. In eq. (3), $n1$ was set to 1 or 0.5 and $n2$ was set to 1 or 2 (Dalgaard, 2009) in order to describe data most appropriately and this was determined from root mean square error (RMSE) values.

2.4. Challenge tests with chemically acidified cheese and cream cheese

A total of 20 challenge tests were performed to generate *L. monocytogenes* growth data in GDL chemically acidified cheese ($n = 12$) and cream cheese ($n = 8$) for model evaluation (see section 2.6).

2.4.1. Chemically acidified cheese and cream cheese

Chemically acidified cheese was prepared from five different batches of ultra-filtrated milk concentrate (UF-conc.) provided by Arla Foods and containing 40% dry matter. Cheese was prepared in batches of 2000 g of UF-conc. by adding different volumes of a glucono-delta-lactone

solution (GDL 54%, Roquette®, Lestrem, France) and 36 ml of rennet solution (3.3% Hannilase® XP 200 NB, Chr. Hansen, Hørsholm, Denmark). For the four batches of UF-conc. the salt concentration was adjusted by adding 3.5% or 5% NaCl (Merck, Kenilworth, US). In total, 11 laboratory-produced and one commercial chemically acidified cheese, with variation in salt, pH and added amount of GDL solution were studied in challenge tests (Table 1). Additionally, four batches of two types of cream cheese were purchased from a supermarket and were used in eight challenge tests (Table 2).

2.4.2. Inoculation and microbiological analysis

Growth of *L. monocytogenes* in chemically acidified cheese and cream cheese was determined in 20 challenge tests including a total of 60 curves with growth or no-growth responses at constant and dynamic storage temperature (Table 1 and 2). Chemically acidified cheese and cream cheese were inoculated with 0.1% (v/w) of *Lm-mix* appropriately diluted in chilled saline water (0.85% NaCl) to obtain an initial concentration in the range of 1 log (cfu/g) to 3.5 log (cfu/g). Inoculation of chemically acidified cheese was performed in each batch following addition of GDL solution. After the chemically acidified cheese was set it was packaged into 50 ± 1 g cheese containers and stored at 4.4-24°C during 10-30 days depending on the storage temperature. Thirty three individual packages of cream cheese (150 g) were combined to form a 5000 g sample which was then inoculated, re-packaged into 50 ± 1 g cheese containers and stored at 4.5-22°C during 30 days. Storage temperature during challenge tests was regularly recorded by data loggers (TinytagPlus, Gemini Data Loggers Ltd, Chichester, UK). Six to 12 times during storage samplings were performed to quantify growth responses. At each sampling a container with 50 ± 1 g of cheese was analysed and then discarded.

Table 1. Data obtained from challenge tests performed with chemically acidified cheese inoculated with *L. monocytogenes*.

CT ^b	Batch	n ^c	Storage temp. (°C)	Product characteristics (Avg.±SD) ^a				Growth parameter values (Avg. ±SD)			
				pH	Water phase salt (%)	Gluconic acid in water phase (ppm)	t _{lag} (h)	RLT (h)	Log N ₀ (Log cfu/g)	Log N _{max} (Log cfu/g)	μ _{max} (h ⁻¹)
1	1	3	14.0±0.4	4.8±0.2	7.24±0.06	43,871±5,715	0.0±0.0	0.0±0.0	2.8±0.1	5.6±0.4	0.030±0.01
2	1	3	14.2±0.4	5.5±0.1	7.58±0.74	24,428±10,675	0.0±0.0	0.0±0.0	3.0±0.2	7.3±1.0	0.107±0.03
3	1	3	14.1±0.4	5.2±0.1	7.44±0.08	32,492± 2,835	0.0±0.0	0.0±0.0	3.0±0.1	7.7±1.7	0.040±0.00
4	1	3	14.0±0.3	4.9±0.0	11.70±0.01	41,129± 5,146	0.0±0.0	0.0±0.0	3.1±0.2	3.0±0.0	0.000±0.00
5 ^d	2	3	21.0±1.4	4.6±0.1	4.43±0.28	45,162± 10,935	0.0±0.0	0.0±0.0	3.5±0.1	6.5±0.2	0.055±0.00
6	3	3	20.2±0.2	4.7±0.1	3.82±0.39	39,944±2,390	142±22	15.9±4.3	1.1±0.1	6.3±0.1	0.126±0.01
7	3	3	14.6±0.2	4.8±0.1	4.05±0.05	41,841±2,334	142±33	17.7±10.9	1.0±0.1	6.0±0.4	0.080±0.03
8	4	3	24.1±0.0	4.7±0.1	4.60±0.31	39,638±1,916	46.9±0.0	9.4±0.0	2.6±0.1	6.8±0.0	0.139±0.00
9	4	3	14.1±0.1	4.8±0.1	4.38±0.19	44,622±4,615	50.9±8.3	4.5±2.1	2.6±0.1	4.6±0.1	0.059±0.02
10	4	3	10.3±0.1	4.8±0.1	4.58±0.08	32,329±10,992	0.0±0.0	0.0±0.0	2.5±0.1	4.1±0.3	0.013±0.00
11	5	3	4.4-25.4	4.8±0.0	4.48±0.14	29,286±15,672	- ^e	- ^e	2.9±0.1	5.9±0.1	- ^e
12	5	3	5.2-25.3	4.8±0.1	4.65±0.19	27,742±3,290	- ^e	- ^e	2.7±0.2	6.7±0.1	- ^e

^a Avg.: average; SD: standard deviation

^b Challenge test.

^c Number of growth curves per challenge test (CT).

^d Commercial chemically acidified cheese

^e Not determined

Table 2. Storage conditions and product characteristics for challenge tests with cream cheese.

CT ^a	Batch	n ^b	Storage temp. (°C)	Product characteristics (Avg.±SD) ^c						Growth parameter values (Avg. ±SD) ^c			
				LAB (Log cfu/g) ^d	pH	Water phase salt (%)	Lactic acid in water phase (ppm)	Acetic acid in water phase (ppm)	Citric acid in water phase (ppm)	<i>t</i> _{lag} (h)	Log N ₀ (Log cfu/g)	Log N _{max} (Log cfu/g)	μ _{max} (h ⁻¹)
A	1	3	22.0±0.2	3.9±0.2	4.9±0.2	2.07±0.10	3,539±376	980±151	618±3	0.0±0.0	2.1±0.0	<1.0±0.0	0.000±0.00 ^e
B	2	3	4.5±0.1	4.6±0.3	5.1±0.1	1.79±0.04	3,102± 1,220	1,188±594	2,136±1433	0.0±0.0	1.9±0.8	2.0±0.1	0.000±0.00 ^e
C	2	3	10.1±0.2	4.3±0.2	5.1±0.1	1.79±0.04	3,102± 1,220	1,188±594	2,136±1433	0.0±0.0	2.1±0.1	2.0±0.1	0.000±0.00 ^e
D	2	3	14.6±0.2	4.8±0.1	5.1±0.0	1.79±0.04	3,102± 1,220	1,188±594	2,136±1433	0.0±0.0	2.1±0.2	2.3±0.0	0.000±0.00 ^e
E	3	3	4.5±0.1	4.4±0.1	4.9±0.1	1.93±0.04	5,452±1,941	911±443	1,954±836	0.0±0.0	3.0±0.1	3.1±0.2	0.000±0.00 ^e
F	3	3	10.1±0.2	4.4±0.1	4.8±0.1	1.93±0.04	5,452±1,941	911±443	1,954±836	0.0±0.0	3.0±0.1	3.0±0.3	0.000±0.00 ^e
G	3	3	14.6±0.2	3.2±0.1	4.7±0.2	1.93±0.04	5,452±1,941	911±443	1,954±836	0.0±0.0	3.0±0.1	2.5±1.2	0.000±0.00 ^e
H	4	3	4.7-14.6 ^f	ND ^g	4.7±0.0	1.84±0.03	10,930±1,815	1,808±485	5,121±569	- ^f	1.4±0.4	1.0±0.0	- ^f

^a Challenge test.

^b Number of growth curves per experiment.

^c Avg.: average; SD: standard deviation

^d LAB: lactic acid bacteria

^e No growth observed for the 30 days duration of experiment.

^f Not determined due to dynamic storage temperatures.

^g ND: not determined

10 g of cheese were diluted 10-fold with chilled physiological saline (PS, 0.85% NaCl and 0.10% Bacto-peptone) and subsequently homogenized for 30 s at normal speed in a Stomacher 400 (Seward Medical, London, UK). 10-fold dilutions were performed with chilled PS. Aerobic viable counts (AVC) for chemically acidified cheese were enumerated by surface plating on standard plate count agar (CM0463, Oxoid, Hampshire, UK) and incubation at 25°C for 24 h. For cream cheese viable counts of lactic acid bacteria (LAB) were determined by double layer pour plating in nitrite actidione polymyxin (NAP) agar (pH 6.2) with incubation at 25°C for 72h (Davidson and Cronin, 1973). Viable counts of *L. monocytogenes* were determined for both cheeses 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.

2.4.3. Product characteristics

Product characteristics of cheeses were determined by analysis of three packages (50 ± 1 g) for each treatment at the start of the challenge test. pH was measured directly in the cheese with a PHC10801 puncture combination probe (Hach, Brønshøj, Denmark) at all times of sampling for microbiological analysis. 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 lactic-, acetic, citric and gluconic acid was determined by HPLC using external standards for identification and quantification (Dalgaard and Jørgensen, 2000; Østergaard et al., 2014).

2.4.4. Primary growth model

The integrated and log transformed logistic model with lag-time (four parameter model) or without lag-time (three parameter model) (eq. 4; Rosso et al., 1996) was fitted to all individual growth curves of *L. monocytogenes* obtained in challenge tests at constant temperature. Fitted parameter values for lag time (t_{lag} , h), maximum specific growth rate (μ_{max} , h^{-1}) initial cell concentration (N_0 , cfu/g), and maximum population density (N_{max} , cfu/g) were determined for each growth curve and data was reported as average \pm standard deviation for each treatment (Table 1). An F-test was used to determine if the lag time was significant.

$$\begin{aligned} \log (N_t) &= \log (N_0) && \text{if } t < t_{lag} \\ \log (N_t) &= \log \left(\frac{N_{max}}{1 + \left(\frac{N_{max}}{N_0} \right) - 1 \cdot \exp(-\mu_{max} \cdot (t - t_{lag}))} \right) && \text{if } t \geq t_{lag} \quad (4) \end{aligned}$$

where t is the storage time (h) and N_t is the cell concentration (cfu/g) at time t . Other parameters were described above.

2.5. Growth data of *L. monocytogenes* from literature

A total of 170 growth responses of *L. monocytogenes* in milk, meat products and other products at different temperatures were collected from literature. Growth of *L. monocytogenes* was described using the growth parameters t_{lag} (h), μ_{max} (h^{-1}), N_0 (log cfu/g) and N_{max} (log cfu/g) obtained by fitting growth data from graphs with eq. (4). Published growth rates available in tables were adjusted by multiplying them with a correction factor. The logistic model with delay was used as the reference model; therefore, the maximum specific growth rates estimated with the Baranyi model (Baranyi and Roberts, 1994) were multiplied by 0.97 (Augustin et al., 2005). For 60 of the 170 growth responses collected from literature one or more of the relevant product characteristics

were not reported (Table 3). In 21 experiments the pH of milkshake and fresh pork were assumed to be 6.7 and 6.2, respectively. For 33 and 27 experiments with meat products 0.7% water phase lactic acid and 50 ppm nitrite were assumed to be present, respectively.

2.6. Evaluation of new terms and models

The new terms for pH and GAC were evaluated by comparison of predicted and observed growth responses. We used this approach to establish if the expanded model of Mejlholm and Dalgaard (2009) including new pH- and GAC-terms (see section 3.3) could predict growth of *L. monocytogenes* as determined in the present study for chemically acidified cheese and cream cheese with pH from 4.6 to 5.5 (n = 20; Table 1 and 2) as well as for a broad range of data from literature (n = 1129; Table 6).

For predicted and observed μ_{max} -values the calculated B_f - and A_f -values were evaluated as previously suggested with $0.95 < B_f < 1.11$ indicating good model performance, B_f of 1.11-1.43 or 0.87-0.95 corresponding to acceptable model performance and $B_f < 0.87$ or > 1.43 reflecting unacceptable model performance (Mejlholm et al., 2010; Ross, 1996; Ross et al., 2000). With $A_f > 1.5$ indicating an incomplete model or systematic deviation between observed and predicted μ_{max} -values (Mejlholm and Dalgaard, 2013).

Predicted and observed growth and no-growth responses were assessed by calculating the percentage of all samples that were correctly predicted. Incorrect predictions were described as fail-safe (growth predicted when no growth was observed) or fail-dangerous (no growth predicted when growth was observed).

Table 3. Storage conditions and product characteristics in experiments (n= 170) used for evaluation of the model.

Product	Food	Reference	n ^a	No. of strains ^b	Temp. (°C)	Water phase salt (%)	a _w ^c	pH	Acetic acid (%)	Diacetate (%)	Lactic acid (%)	GDL (%)	GAC (%)	Nitrite (ppm)
Dairy	Milk	El-Shenawy and Marth (1990)	15	1	13	0 ^e	0.999	3.7-6.4	- ^d	- ^d	- ^d	0-1	0-1.5	- ^d
	Milkshake	Salazar et al. (2018)	14	6	5-25	0	0.999	6.7	- ^d	- ^d	- ^d	- ^d	- ^d	- ^d
	Pudding	Lianou et al. (2018)	8	5	4-16	0	0.999	6.5	- ^d	- ^d	- ^d	- ^d	- ^d	- ^d
	UHT milk	Lobacz et al. (2015)	15	2	3-15	0	0.999	6.7	- ^d	- ^d	- ^d	- ^d	- ^d	- ^d
Meat	Bologna	Barmpalia et al. (2005)	15	10	4-10	3.6	0.979	6.3	- ^d	0-0.2	0.7-2.6	0.12-0.25	- ^d	50
	Saveloy	Juncher et al. (2000)	12	5	5-10	1.9	0.989	6.1-6.4	- ^d	0-0.9	0.7-3.5	0-0.25	- ^d	60-150
	Fresh pork	Luo et al. (2014)	7	3	5-35	0	0.999	6.2	- ^d	- ^d	- ^d	- ^d	- ^d	d
	Mortadella	Daminelli et al. (2014)	6	2	8	5.3 ^f	0.968	6.2	- ^d	- ^d	0.7	- ^d	d	50
	Bacon	Taormina and Dorsa (2010)	6	5	4-22	10.1-19.0 ^f	0.620-0.910	5.1-5.6	- ^d	- ^d	- ^d	- ^d	- ^d	50
Purée	Zucchini	Nyhan et al. (2018 ^g)	36	5 ^h	30	3.4-10.6 ^f	0.930-0.980	4.7-5.3	0-0.1	- ^d	- ^d	- ^d	- ^d	- ^d
Sauce	Béarnaise	Nyhan et al. (2018 ^g)	36	5 ^h	30	3.4-10.6 ^f	0.930-0.980	4.7-5.3	0-0.1	- ^d	- ^d	- ^d	- ^d	- ^d

^a n, number of experiments/growth curves.

^b Number of strains inoculated as a cocktail in experiments.

^c Measured or calculated from the concentration of water phase salt.

^d Information not reported.

^e Bold type: assumed values. See explanation in Section 2.5.

^f Calculated from a_w using Resnik and Cherife (1988).

^g Some experiments contain propionic acid (1,2 mM).

^h One *Listeria innocua* strain was included in the inoculated cocktail of strains.

The ψ -value was calculated for all predicted growth responses to indicate if they were close to the growth boundary of *L. monocytogenes* ($\psi = 1.0$) or well into the growth ($\psi < 1$) or no-growth ($\psi > 1$) regions. For chilled products with shelf-life of more than 5 weeks products formulations resulting in a ψ -value >2 has been recommended (Dalgaard and Mejlholm, 2019). Predicted and observed growth in challenge tests performed with chemically acidified cheese at dynamic storage temperature were visually assess.

2.7. Statistical analysis and curve fitting

Model parameters and standard errors were estimated by using GraphPad PRISM (version 8, GraphPad Software, San Diego, CA, USA). F-tests to determine significant lag times were performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Cardinal parameter values for pH and gluconic acid

Temperature had a marked effect on pH_{min} -values determined by fitting eq. 1 to μ_{max} -values of *Lm-mix* or of individual strains grown in BHI broth (Fig. 1). pH_{min} -values on average decreased from 4.9 at 5°C to 4.3 at 15-20°C and then increased to 4.7 at 37°C (Fig.1). The cardinal parameter value for GAC i.e. the MIC-value of undissociated GAC ($MIC_{U\ GAC}$) was 26.4 ± 1.1 mM as determined at 8, 20 and 25°C by using eq. 3 with n1 and n2 equal to 1.

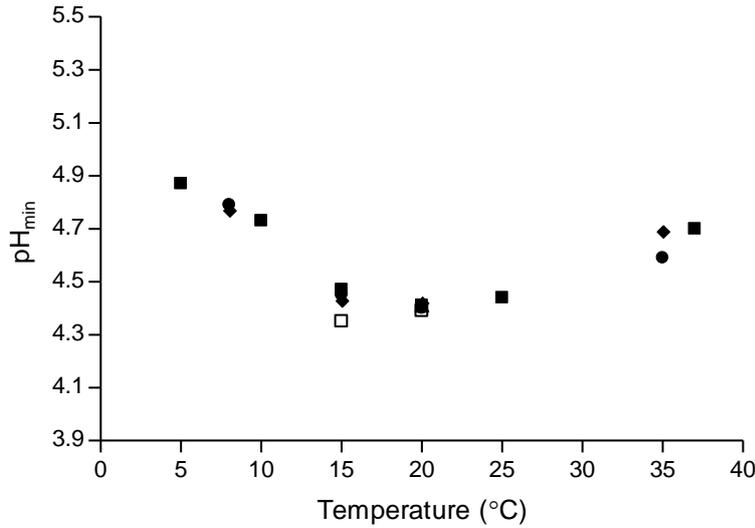


Figure 1. Cardinal parameter values (pH_{min}) at different temperatures for a cocktail of *L. monocytogenes* strains (SLU 92, 612, LM19, 6, (■)) and individual strains (ISO570 (◆), 99714 (●), SLU 2493 (□), SLU 2265 (△))

3.2. New cardinal parameter pH_{min} -term for *L. monocytogenes*

Eq. (5) was used to describe the observed effect of storage temperature on pH_{min} -values of *L. monocytogenes*.

$$pH_{minT} = pH_{min0} - T * \left(\frac{(pH_{min0} - pH_{minR})}{T_R} \right) \quad 0 \leq T < T_R$$

$$pH_{minT} = pH_{minR} + (T - T_R) * \left(\frac{(pH_{min37} - pH_{minR})}{(37 - T_R)} \right) \quad T_R < T < 37^\circ\text{C} \quad (5)$$

where T_R is the temperature (°C) corresponding to the lowest pH_{min} -value; T is the storage temperature (°C); pH_{minT} is the estimated pH_{min} -value at T (°C); pH_{min0} and pH_{min37} are, respectively, the estimated pH_{min} -value at 0°C and 37°C; pH_{minR} is the pH_{min} -value at T_R (°C) (Fig.2). The parameter values (Table 4) were estimated by fitting eq. (5) to pH_{min} -values for *Lm*-mix, individual stains and literature data (Fig. 2).

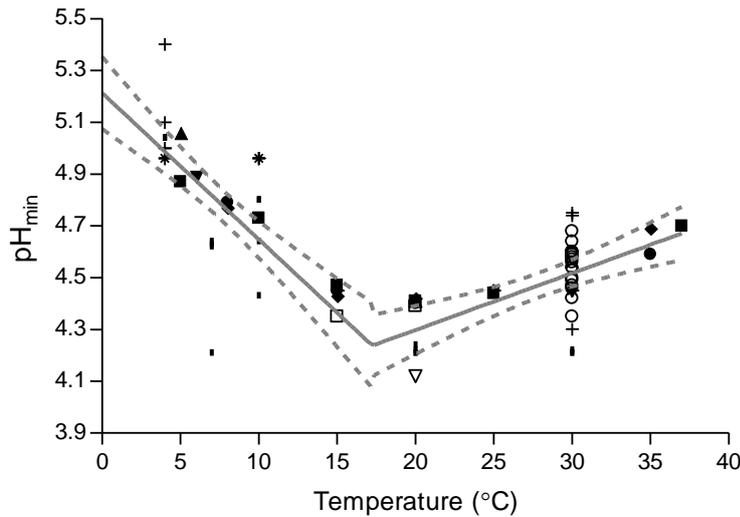


Figure 2. Observed and fitted pH_{min} -values from the present study and from literature. *L. monocytogenes* cocktail of the strains (SLU92, 612, LM19, 6, (■)) and individual strains (ISO570 (◆), 99714 (●), SLU 2493 (□), SLU 2265 (△) are from the present study. Data are from Aryani et al., 2015 (○), Brocklehurst et al., 1995 (▽), Duffy et al., 1994 (▲), Farber et al., 1989 (+), George et al., 1988 (l), Koutsoumanis et al., 2004 (*), Petran et al., 1989 (x) and Ryser and Marth 1988 (▼). Solid line (—) and dashed line (---) represent the fitted and confidence interval (95%).

Table 4. Fitted parameter values for new pH_{min} -term.

Parameters	Values (Avg. \pm SE) ^a
pH_{min_0}	5.2 ± 0.1
pH_{min_R}	4.2 ± 0.0
T_R	17.3 ± 1.3
$pH_{min_{37}}$	4.7 ± 0.1

^a Avg: average; SE, standard error

3.3. Expanded model for growth of *L. monocytogenes* in foods

The model of Mejlholm and Dalgaard (2009) was expanded by substituting the constant pH_{min} -value of 4.97 in the existing CPM-*Lm* by the new pH_{min} -term (Eq. (5)) (Model 1). Model 1 was further expanded by adding a GAC-term including the $MIC_{U\ GAC}$ obtained in the present study (Eq. (3)) (Model 2). As for the model of Mejlholm and Dalgaard (2009) the effect of interaction between environmental parameters (ξ) in model 1 and model 2 was taken into account by using the Le Marc approach (Le Marc et al., 2002; Mejlholm and Dalgaard, 2009) (Table S1 and S2).

3.4. Challenge tests with chemically acidified cheese and cream cheese

The chemically acidified cheese produced in the laboratory (Batch 1, 3, 4 and 5; Table 1) had pH of 4.6-5.5, water phase salt content of 4.05-11.7% and gluconic acid in the water phase of 2,443-4,516 % (w/v). Commercially available chemical acidified cheese had pH 4.6 ± 0.1 , water phase salt of $4.43\pm 0.28\%$ and gluconic acid in the water phase of $45,162\pm 10,935$ ppm (Table 1). Commercial cream cheese had pH of 4.7-5.1, water phase salt content of 1.79-2.07 %, lactic-, acetic and citric acid in the water phase of 3,102-10,930 ppm, 980-1808 ppm and 618-5121 ppm, respectively.

L. monocytogenes grew in the studied chemically acidified cheese with pH-values of 4.6-5.5 (Table 1). However, *L. monocytogenes* did not grow in challenge test 4 with chemically acidified cheese performed at 14.0°C due to a high water phase salt concentration ($11.7\pm 0.0\%$) in that product. Nevertheless, growth of *L. monocytogenes* was observed in challenge test 1 with chemically acidified cheese where the product had low pH (4.8 ± 0.2) and relatively high water phase salt ($7.24\pm 0.06\%$) (Table 1). *L. monocytogenes* did not grow in any challenge test performed with cream cheese (Table 2).

3.5. Evaluation of predictive models for *L. monocytogenes*

For chemically acidified cheese and cream cheeses the original model of Mejlholm and Dalgaard (2009) predicted no-growth in 15 out of the 17 challenge tests at constant temperatures resulting in a high percentage (35%) of fail-dangerous predictions (Table 5). For the two challenge tests with pH 5.2 and 5.5 where growth was both predicted (ψ of 0.2 and 0.3) and observed the model significantly underestimated growth rates of *L. monocytogenes* as shown by a B_f -value of 0.51 (Table 6).

Table 5. Comparison of observed and predicted maximum specific growth rate (μ_{max} -values) of *L. monocytogenes* in experimental data^a.

	Observed			Predicted		
	n ^c	Growth	No growth	Predicted growth/no-growth responses		
				Mejlholm & Dalgaard, 2009 ^d	Model 1 ^e	Model 2 ^f
Table 1	10	9	1	2/8	9/1	8/2
Table 2	7	0	7	0/7	0/7	0/7
			Correct (%)	65	100	90
			Fail-safe(%)	0	0	0
			Fail-dangerous (%)	35	0	10

^a See Table 1 and 2 for information on product characteristics and storage conditions of chemically acidified and cream cheese inoculated with *L. monocytogenes*.

^b B_f, bias factor; A_f, accuracy factor.

^c n, number of experiments.

^d Mejlholm and Dalgaard, 2009 model.

^e Model^d including only new pH_{min} -term.

^f Model^d including new pH_{min} -term and MIC_{GAC_U} (mM).

Table 6. Comparison of observed and predicted growth of *L. monocytogenes* obtained from experimental and literature data (n= 1129).

	n ^d	Observed		Predicted		
		Growth	No growth	Mejlholm & Dalgaard, 2009 ^a	Model 1 ^b	Model 2 ^c
				B _f / A _f ^e	B _f / A _f	B _f / A _f
Table 1-Chemically acidified cheese	10	9	1	0.51/1.97	1.03/1.26	0.26/3.85
Table 2- Cream cheese	7	0	7	- ^f	- ^f	- ^f
Table 3-Dairy	52	48	4	0.79/10.7	0.94/1.33	0.92/1.31
Table 3-Meat	46	30	16	0.85/1.39	0.91/1.39	0.91/1.39
Meat, seafood, poultry and non-fermented dairy products ^g	1014	707	307	1.00/1.49	1.02/1.50	1.02/1.50
All data	1129	794	335	0.98/1.50	1.01/1.48	1.00/1.50
			Correct (%)	89.2	90.3	89.8
			Fail-safe (%)	5.5	5.3	5.3
			Fail-dangerous (%)	5.3	4.4	4.9

^a Mejlholm and Dalgaard (2009) model.

^b Model^a including only new pH_{min} -term.

^c Model^a including new pH_{min} -term and MIC_{GAC_U} (mM).

^d n, number of experiments.

^e B_f, bias factor; A_f, accuracy factor.

^f B_f/A_f cannot be calculated from no-growth data

^g Data set available in Mejlholm et al. (2010).

However, growth rates of *L. monocytogenes* in chemically acidified cheese were accurately predicted by model 1, including the new pH_{min} -term (Eq. (5)), as shown by B_f - and A_f -values of 1.03 and 1.26 (n = 9; Table 6). Model 1 predicted growth in 9 out of the 17 challenge tests resulting in 100% correct predictions of growth and no-growth (Table 5). For challenge test with cream cheese, model 1 correctly predicted no-growth and ψ -values of 1.5 to >10 were determined showing that most of the studied products were far from the growth boundary (ψ -values of 1).

Model 2, developed in the present study and including the new pH_{min} -term (Eq. (5)) as well as a GAC-term, significantly underestimated growth rates of *L. monocytogenes* in chemically acidified cheese as shown by a B_f -value of 0.26 (n = 8, Table 6). The model predicted growth in 8 out of the 17 experiments resulting in 90% correct and 10% fail-dangerous predictions (Table 5). These results for evaluation of model 1 and model 2 suggest GAC, beyond lowering the pH, has no inhibiting effect on growth of *L. monocytogenes*. Inclusion of the gluconic acid MIC-term in model 2 decreased model performance and consequently this term is unnecessary to correctly predict growth of *L. monocytogenes* in the studied chemically acidified cheese. Further evaluation of model 1 and model 2 was performed with μ_{max} -data obtained from the literature. The Mejlholm and Dalgaard (2009) model slightly underestimated growth rates of *L. monocytogenes* in dairy and meat products as shown by B_f -values of 0.79 and 0.85, respectively (Table 6). For these products without GAC, similar B_f -values were obtained with model 1 and 2 indicating that growth can be accurately predicted with both models (Table 6). However, exclusively model 1 was able to accurately predict growth in chemically acidified cheese with low pH as shown above (Table 5). Importantly, model 1 predicted growth of *L. monocytogenes* in meat, seafood, poultry and non-fermented dairy products (n=707) with good precision and resulted in B_f -/ A_f -values of 1.02/1.50 (Table 6). Model 1 and the Mejlholm and Dalgaard (2009) model were further evaluated with a data set composed by experimental and literature data (n=1129, Table 6). B_f and A_f -values for model 1 were of 1.01 and

1.48, whereas values of 0.98 and 1.50 were obtained with the Mejlholm and Dalgaard, (2009) model. Model 1 predicted growth/no-growth responses correctly for 90.3% of the growth responses with the incorrect predictions distributed as 5.3% fail-safe and 4.4% fail-dangerous, resulting in a better performance compared with either of the other two models (Table 5). Model predictions were fail-safe or correct for the two challenge tests with chemically acidified cheese stored at dynamic temperature. An N_{\max} -value of 6.8 log cfu/g was used for these predictions as this value was observed in products with similar characteristics (Table 1, Fig. 3). For zucchini purée and béarnaise sauce, with low pH and storage at 30°C, model 1 had an acceptable B_f -value of 1.26 but the A_f -values of 1.56 and 38% fail-safe prediction indicated unacceptable precision of the model (Table 7).

4. Discussion

The present study quantified the effect of temperature on pH_{\min} -values for *L. monocytogenes* and included this effect (Eq. (5)) in an extensive growth and growth boundary model that was subsequently successfully validated for pH values as low as 4.6 (Table S1 and S2). This expanded model (Model 1, section 3.3) including the effect of both general product characteristics (temperature, NaCl/aw, pH) and product specific ingredients (organic acids and other preserving factors) provides new options to predict *L. monocytogenes* growth responses. These predictions are useful in the assessment and management of *L. monocytogenes* growth for processed and ready-to-eat foods including non-fermented dairy products and cream cheese with pH of 4.6 or above. Based in the performed model evaluation, the range of applicability for model 1 in foods includes storage temperatures from 2°C to 35°C, pH between 4.6 and 7.7 and water phase salt concentrations as low as 0% with the range of the other environmental factors as reported previously (Mejlholm et al., 2010; Mejlholm and Dalgaard, 2015).

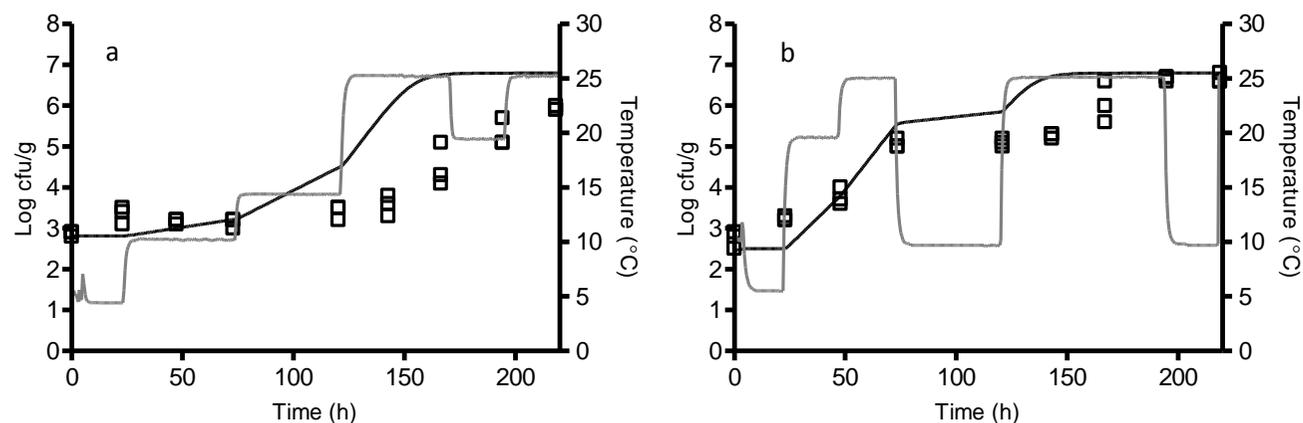


Figure 3. Comparison of observed (\square) and predicted (-) growth of *L. monocytogenes* under dynamic storage temperature. Chemically acidified cheese was studied at 4.4-25.4°C (a; CT 11) and 5.2-25.3°C (b; CT 12). Temperature profiles are shown as grey lines. Solid lines represent the predicted growth by model 1 with N_{\max} of 6.8 log cfu/g.

Table 7. Observed and predicted growth of *L. monocytogenes* with data from Nyhan et al., (2018).

	Observed growth		Predicted growth (Model 1 ^a)	Predicted growth (Model 1 ^a with propionic acid ^b)				
	n ^c	Growth	No growth	B_f / A_f^d	B_f / A_f^d	Correct (%)	Fail-safe (%)	Fail-dangerous
Total (Zucchini purée and Béarnaise sauce)	72	36	36	1.26/1.56	1.14/1.49	63	38	0

^a Mejlholm and Dalgaard (2009) model including new pH_{\min} -term

^b Propionic acid MIC value available in Le Marc et al., 2002

^c n, number of experiments

^d B_f , bias factor; A_f , accuracy factor

The successfully validated model 1 can be used to assess *L. monocytogenes* growth in chemically acidified cheeses and cream cheeses depending on storage conditions and product characteristics. As an example, if a chemically acidified cheese (pH 4.6 and 4.4% water phase NaCl) is contaminated with 1 *L. monocytogenes*/g after pasteurization (e.g. while adding GDL) and subsequently chill stored at 5 °C then the product will not support growth. However, if the product is stored at 25°C a critical concentration of 100 cfu/g (ANZ, 2018; CA, 2011; EC, 2005) will be exceeded after less than two days. Model 1 predicts that a formulation with 0.21% lactic acid in the water phase will prevent growth of *L. monocytogenes* for that product also at 25°C (ψ of 2.5). As another example the model can be used to predict growth/no-growth for cream cheese at 5°C with pH 5.2, 1.9% water phase NaCl, water phase organic acids concentrations of 0.20% (lactic), 0.10% (acetic) and 0.10% (citric). If the product is contaminated with 1 cfu/g growth of *L. monocytogenes* will not be supported (ψ of 2.1); however if the same contaminated product is stored at 25°C then the critical cell concentration will be exceeded in 2.5 days (ψ of 0.4). Model 1 predicts that a cream cheese reformulated with lower pH (5.0) and increased concentrations in the water phase of lactic acid (0.45%) and acetic acid (0.15%) will prevent growth of *L. monocytogenes* at 25°C (ψ of 2.4).

The observed effect of temperature on pH-growth-limits of *L. monocytogenes* (Fig. 1) are in agreement with previous studies based on broth media acidified with hydrochloric acid. Koutsoumanis et al. (2004a) found that the minimum pH supporting growth of *L. monocytogenes* at 4°C and 10°C was 4.96, while at 15°C and 30°C it was 4.45. Farber et al. (1989) determined pH of 5.0 to 5.4 needed to prevent *L. monocytogenes* growth at 4°C whereas as 30°C lower pH-values of 4.3 to 4.7 were required. For a_w of 0.990, 0% lactic acid and temperatures of 4°C, 15°C and 30°C the model of Tienungoon et al., (2000) predicted pH-growth-limits of *L. monocytogenes* to be 5.38, 4.40 and 4.38. These data are in agreement with the present study, where the effect of temperature on pH_{min} -values was quantified with markedly more data. Furthermore, the new model 1 includes

more environmental factors than the model of Tienungoon et al. (2000) and therefore has wider application e.g. for product formulation or documentation of food safety.

The effect of temperature on pH_{min} -values for *L. monocytogenes* as quantified in the present study (Fig. 1) has been important to accurately predict growth of this pathogen in food with low pH (Table 6). Temperature may have a similar effect on other microorganisms than *L. monocytogenes* as indicated by growth data for e.g. *Escherichia coli* (McKellar and Lu, 2001; Slater et al. 2000), *Salmonella* (Koutsoumanis et al. 2004b) and *Staphylococcus aureus* (Valero et al. 2009). It seems interesting in future studies to evaluate if CPMs with temperature dependent pH_{min} -terms could be valuable to predict growth and growth boundary responses of other microorganisms as well as to obtain more information on why a minimum pH_{min} -value is observed at a temperature markedly below the optimum temperature for growth.

The performed experiments with chemically acidified cheese highlighted an important limitation of the Mejlholm and Dalgaard (2009) model to accurately predict growth of *L. monocytogenes* in foods with low pH (Table 5; Table 6). This limitation is due to a constant pH_{min} -value for *L. monocytogenes* of 4.97 and consequently, no-growth is predicted below that pH-value, irrespective of the storage temperature. Model 1, with a new pH_{min} -term (Eq. (5)), did not have this limitation and showed good model performance for non-fermented dairy products with pH as low 4.6 (Table 6).

The unacceptable performance of model 1 for zucchini purée and béarnaise sauce (B_f - and A_f -values of 1.26 and 1.56; Table 7) could be due to inhibiting compounds in these products that were not included in model 1. In fact, some of the treatments studied by Nyhan et al. (2018) included propionic acid. It was therefore investigated if including a propionic acid term and MIC value from Le Marc et al. (2002) could improve the performance of model 1. Addition of the Le Marc et al. (2002) propionic acid term and MIC value improved performance of the expanded

model 1 (B_f - and A_f -values of 1.14 and 1.49; Table 7), however, further evaluation of the expanded model containing a propionic acid term is necessary for vegetable products and sauces due to a high percentage of fail-safe predictions (38%; Table 7).

Despite the inhibitory effect of GAC observed in broth, with $MIC_{U\ GAC}$ of 26.4 ± 1.1 mM (Section 3.1), comparison of predicted and observed *L. monocytogenes* growth in foods (Table 6) showed no need to include a GAC-term in the developed growth and growth boundary model (Model 1, section 3.3). This result is not in contradiction with available data although an antimicrobial effect of GDL and GAC against *L. monocytogenes* has been reported by several studies. For instance, Juncher et al. (2000) found a recipe for saveloys with 2.0% lactate and 0.25% GDL to prevent growth of *L. monocytogenes*. The addition of GDL reduced product pH from 6.37 to 6.08 resulting in an increase of undissociated lactic acid from 1.2 to 2.3 mM. Similarly, Qvist et al. (1994) found bologna-type sausage with 2% lactate and 0.5% GDL prevented growth of *L. monocytogenes* at 5°C and 10°C during 28 days of storage. Product pH was reduced from 6.6 to 6.0 by 0.5% GDL and this resulted in an increase of undissociated lactic acid from 0.7 to 2.8 mM. El-Shewany and Marth (1990) suggested that using GAC or GDL at concentrations high enough to coagulate milk for cottage cheese production should contribute to control *L. monocytogenes* during the manufacturing process. For these examples, the *L. monocytogenes* growth inhibition can be explained by the combined effect of product pH and other product characteristics rather than by the suggested effect of GAC or GDL as shown in the present study for different foods by using model 1.

In conclusion, the present study quantified and modelled the effect of temperature used to estimate pH_{min} -values of *L. monocytogenes* and showed the importance of this effect for accurate prediction of growth in low pH foods. The new model can support product development, reformulation or risk assessment of a wide range of foods including meat, seafood and different

dairy products (milk, cream, desserts, chemically acidified cheese and cream cheese). The new model can be included in predictive microbiology application software such as the Food Spoilage and Safety Predictor (FSSP <http://fssp.food.dtu.dk/>) to facilitate its use by the industry and food safety authorities.

Acknowledgements

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Supplementary Table 1. Cardinal parameter models for *L. monocytogenes* (Eq. 1, 3 and 4) and new pH_{min} -term (Eq. 2)

Reference	Model	Equation
Mejlholm and Dalggaard (2009)	$\mu_{max} = \mu_{ref} \cdot \left[\frac{(T-T_{min})}{(T_{ref}-T_{min})} \right]^2 \cdot \frac{(a_w-a_{w\ min})}{(1-a_{w\ min})} \cdot [1 - 10^{(pH_{min}-pH)}] \cdot \left(\frac{P_{max}-P}{P_{max}} \right) \cdot \left(\frac{CO_2\ max-CO_2\ equilibrium}{CO_2\ max} \right) \cdot$ $\left(\frac{MIC_{NIT}-NIT}{MIC_{NIT}} \right)^2 \cdot \left(1 - \left(\frac{[AAC_U]}{[MIC_U\ AAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[BAC_U]}{[MIC_U\ BAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[CAC_U]}{[MIC_U\ CAC]} \right)^{n1} \right)^{n2} \cdot$ $\left(1 - \left(\frac{[DAC_U]}{[MIC_U\ DAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[LAC_U]}{[MIC_U\ LAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[SAC_U]}{[MIC_U\ SAC]} \right)^{n1} \right)^{n2} \cdot \xi$	1
The present study eq. (5)	$pH_{minT} = pH_{min0} - T * \left(\frac{(pH_{min0}-pH_{minR})}{T_R} \right) \quad 0 \leq T < T_R$ $pH_{minT} = pH_{minR} + (T - T_R) * \left(\frac{(pH_{min37}-pH_{minR})}{(37-T_R)} \right) \quad T_R < T < 37^\circ C$	2
The present study Model 1	$\mu_{max} = \mu_{ref} \cdot \left[\frac{(T-T_{min})}{(T_{ref}-T_{min})} \right]^2 \cdot \frac{(a_w-a_{w\ min})}{(1-a_{w\ min})} \cdot [1 - 10^{(Eq.(2)-pH)}] \cdot \left(\frac{P_{max}-P}{P_{max}} \right) \cdot \left(\frac{CO_2\ max-CO_2\ equilibrium}{CO_2\ max} \right) \cdot$ $\left(\frac{MIC_{NIT}-NIT}{MIC_{NIT}} \right)^2 \cdot \left(1 - \left(\frac{[AAC_U]}{[MIC_U\ AAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[BAC_U]}{[MIC_U\ BAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[CAC_U]}{[MIC_U\ CAC]} \right)^{n1} \right)^{n2} \cdot$ $\left(1 - \left(\frac{[DAC_U]}{[MIC_U\ DAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[LAC_U]}{[MIC_U\ LAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[SAC_U]}{[MIC_U\ SAC]} \right)^{n1} \right)^{n2} \cdot \xi$	3
The present study Model 2	$\mu_{max} = \mu_{ref} \cdot \left[\frac{(T-T_{min})}{(T_{ref}-T_{min})} \right]^2 \cdot \frac{(a_w-a_{w\ min})}{(1-a_{w\ min})} \cdot [1 - 10^{(Eq.(2)-pH)}] \cdot \left(\frac{P_{max}-P}{P_{max}} \right) \cdot \left(\frac{CO_2\ max-CO_2\ equilibrium}{CO_2\ max} \right) \cdot$ $\left(\frac{MIC_{NIT}-NIT}{MIC_{NIT}} \right)^2 \cdot \left(1 - \left(\frac{[AAC_U]}{[MIC_U\ AAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[BAC_U]}{[MIC_U\ BAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[CAC_U]}{[MIC_U\ CAC]} \right)^{n1} \right)^{n2} \cdot$ $\left(1 - \left(\frac{[DAC_U]}{[MIC_U\ DAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[LAC_U]}{[MIC_U\ LAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[SAC_U]}{[MIC_U\ SAC]} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[GAC_U]}{[MIC_U\ GAC]} \right)^{n1} \right)^{n2} \cdot \xi$	4

Supplementary Table 2. Parameter values for *L. monocytogenes* cardinal parameter models.

Model parameter	<i>Listeria monocytogenes</i>		
	Value	n1	n2
μ_{ref} (1/h)	0.419	-	-
T_{min} (°C)	-2.83	-	-
$a_{w\ min}$	0.923	-	-
pH_{min}	4.97	-	-
Phenol (P_{max} , ppm)	32	-	-
$CO_2\ max$ (ppm)	3140	-	-
Nitrite (MIC _{NIT} , ppm)	350	-	-
Minimum inhibitory concentration (MIC) of undissociated organic acids (mM)			
Acetic acid (AAC)	10.3	0.5	1
Benzoic acid (BAC)	0.35	1	1
Citric acid (CAC)	2.12	1	1
Diacetate (DAC)	4.80	0.5	1
Lactic acid (LAC)	3.79	1	1
Sorbic acid (SAC)	1.9	1	1
Gluconic acid (GAC)	26.4	1	1
pH_{min_0}	5.2	-	-
pH_{min_R}	4.2	-	-
T_R	17.3	-	-
$pH_{min_{37}}$	4.7	-	-

Paper IV

Martinez-Rios, V., Jørgensen, M.Ø., Koukou, I., Gkogka, E., Dalgaard, P.

Growth and growth boundary model with terms for melting salts to predict growth

responses of *Listeria monocytogenes* in spreadable processed cheese

Food Microbiology. Submitted

Growth and growth boundary model with terms for melting salts to predict growth responses of *Listeria monocytogenes* in spreadable processed cheese

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ABSTRACT

The aim of this study was to develop and validate a growth and growth boundary model with terms for melting salts to predict growth of *L. monocytogenes* in spreadable processed cheese. Cardinal parameter terms for phosphate salts and citric acid were developed in broth studies and used to expand an available growth and growth boundary model. The expanded model includes the effect of nine environmental factors (temperature, pH, a_w , lactic acid, acetic acid, citric acid, orthophosphate, di-phosphate and tri-phosphate). To generate growth data for model evaluation challenge tests with inoculated commercial (n = 10) and customized (n = 10) spreadable processed cheeses were performed. Evaluation of the new model by comparison of observed and predicted μ_{max} -values resulted in a bias factor of 1.12 and an accuracy factor of 1.33 (n = 42). Prediction of growth and no-growth responses in processed cheese (n = 60) were 89% correct with 11 % fail-safe and 0 % fail-dangerous predictions. The developed model can be used to support product development, reformulation or risk assessment for spreadable processed cheese.

Keywords: Phosphate salts, product development, risk assessment, predictive microbiology

1. Introduction

Spreadable processed cheese is a ready-to-eat product manufactured by blending cheese, melting salts (e.g. sodium or potassium salts of phosphoric or citric acid) and other dairy and non-dairy ingredients, followed by heating and mixing to obtain a uniform molten mass which is typically hot-filled into the final packaging (Fox et al., 2017). Formulation parameters for spreadable processed cheese may vary considerably in terms of pH (4.7-6.3), moisture (ca. 50-70%) and salt content (Maurer, 2012; Kim et al., 2018). Food-grade hydrocolloids (e.g. carob bean gum, guar gum, xanthan gum, gelatine and/or carrageenan) can be used to influence product texture and to reduce the water activity of spreadable processed cheese (Guinee et al., 2004). Melting salts are ingredients known to contribute to the microbiological safety and stability of spreadable processed cheese, besides their main function as emulsifying agents. Among melting salts, phosphates are well known to inhibit the growth of spore-forming bacteria which are key microorganisms to control in processed cheeses (Tanaka et al., 1986; Tompkin, 1984). However, little information is available about their inhibitory effect against pathogens that may potentially contaminate the product during open shelf-life and especially under conditions of temperature abuse by the consumer.

Unsafe food handling by consumers, including cross-contamination and storage conditions, is believed to contribute significantly to foodborne illness (De Jong et al., 2008; Evans and Redmond, 2018; Redmond and Griffith, 2003). Based on data for several countries, more than one third of domestic refrigerators operate at temperatures above 5°C which is the maximum recommended chilled temperature for most ready-to-eat products (James et al., 2008; Roccato et al., 2017; WHO, 2006). Hygiene and temperature control can be critical in relation to food safety with EFSA estimates showing that prevention of growth of *L. monocytogenes* in ready-to-eat products at the consumer phase can reduce annual listeriosis cases in the Member States by 37% (EFSA, 2018).

Within the EU, it is mandatory for food business operators to evaluate and manage potential *L. monocytogenes* growth depending on product characteristics and different reasonably foreseeable storage conditions of ready-to-eat foods (EC, 2005). Melting salts are known to inhibit growth of foodborne pathogens such as *Bacillus cereus*, *Clostridium botulinum* and *Staphylococcus aureus* (ter Steeg et al., 1995; Maier et al., 1999; Loessner et al., 1997). In the same way, melting salts may be important in controlling *L. monocytogenes* growth in spreadable processed cheeses but their anti-listerial effect remains little studied. The potential growth of *L. monocytogenes* e.g. after opening a hot-filled packaged food product can be evaluated by challenge tests or predictive mathematical modelling (EC, 2005). Application of validated predictive models is typically a faster and more cost effective approach but to accurately predict growth responses of *L. monocytogenes* mathematical models must include the effect of all important preserving factors (Mejlholm et al., 2010; Ross and Dalgaard, 2004). Many *L. monocytogenes* growth models are available, some including the inhibitory effect of several intrinsic along with extrinsic factors and a few models have been successfully validated for different types of dairy products (Augustin et al., 2005; Martinez-Rios et al. 2019; Mejlholm et al., 2010). However, none of the available *L. monocytogenes* growth models include the inhibitory effect of melting salts or have been successfully validated for spreadable processed cheeses.

The objective of the present study was to expand and validate a mathematical model to predict growth and growth boundary of *L. monocytogenes* in spreadable processed cheese including phosphate salts and/or organic acids. Firstly, the growth inhibiting effects of phosphate and citrate salts on *L. monocytogenes* were studied in broth and their minimum inhibitory concentrations (MIC) were determined. Secondly, new mathematical terms for the inhibiting effect of these compounds were included in the growth and growth boundary model of Mejlholm and Dalgaard

(2009). Finally, the performance of the expanded model was evaluated by comparison of predicted and observed growth for *L. monocytogenes* in spreadable processed cheese.

2. Materials 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 (SLU 92, 612, LM19, 6) to determine μ_{max} -values in broth and for inoculation of challenge test. Prior to use, each strain was transferred from storage at -80°C to Brain Heart Infusion (BHI) broth (CM1135, Oxoid, Hampshire, UK) and incubated for 24h at 25°C . Subsequently, for broth studies the individual strains were pre-cultured one day at 25°C in BHI broth with pH 6.2 and 0.5% NaCl. For challenge testing the individual stains were pre-cultured one or two days at 8°C to 20°C in BHI broth with pH 6.2 and 1% NaCl to simulate temperature abuse conditions encountered in spreadable processed cheese during open-shelf life. Pre-cultures were grown to a relative increase in absorbance (540 nm) of 0.05 to 0.2 (Novaspec II, Pharmacia Biotech, Allerød, Denmark). The *L. monocytogenes* cocktail of strains (*Lm*-mix) used in broth and challenge test studies were produced by mixing equal volumes of individual pre-cultures. The cell concentration of *Lm*-mix was determined by direct phase-contrast microscopy.

2.2. Phosphate and citrate salts

Minimum inhibitory concentrations (MICs) were determined for three different phosphate salts and trisodium citrate. Furthermore, the anti-listerial effect of eight commercially available melting salts preparations were determined (Table 1).

Table 1. Product information for phosphate, citrate and commercial melting salts.

Abbreviation ^a	Name	Chemical compound name	Product number	Producer	E-number
P1	Orthophosphate	Sodium phosphate monobasic 2-hydrate	04269	Sigma-Aldrich	NA ^b
P2	Di-phosphate	Sodium pyrophosphate 10-hydrate	221368	Sigma-Aldrich	NA
P3	Tri-phosphate	Pentasodium tripolyphosphate	238503	Sigma-Aldrich	NA
TC	Trisodium citrate	Trisodium citrate	NA	BK Giulini GmbH	E331
DP	Disodium phosphate	Disodiumhydrogenphosphate-2 hydrate	NA	BK Giulini GmbH	E339ii
BUDAL	Budal [®] Na 322	Trisodium phosphate 12-hydrate	NA	Budenheim KG	E339iii
PZ35	JOHA [®] PZ35	Sodium orthophosphate, trisodium citrate	NA	BK Giulini GmbH	E339, E331
PZ6	JOHA [®] PZ6	Sodium orthophosphate, sodium diphosphate, sodium polyphosphates	NA	BK Giulini GmbH	E339, E450, E452
S9	JOHA [®] S9	Orthophosphates, polyphosphates	NA	BK Giulini GmbH	E339, E452
PZ189	JOHA [®] 189	Orthophosphates, polyphosphates, trisodium citrate	NA	BK Giulini GmbH	E339, E452, E331
S9K	JOHA [®] S9K	Potassium orthophosphates, potassium triphosphates	NA	BK Giulini GmbH	E340, E451

^a Product name abbreviation used in the text

^b NA: information not available

2.3. Cardinal parameter values for phosphate and citrate salts

The inhibitory effect of eight to 17 different concentrations of P1 (0 to 6.5%), P2 (0 to 6%), P3 (0 to 5%) and TC (0 to 9%; corresponding to 0-137,000 ppm of citric acid) on *Lm*-mix were determined at 25°C in BHI-broth with pH 6.2. A total of 154 μ_{max} -values were determined. For each condition, growth of *Lm*-mix was determined in duplicate by using automated absorbance measurements at 540 nm (BioScreen C, Labsystems, Helsinki, Finland). Detection times, defined as incubation time necessary to observe an increase in absorbance of 0.05 from the lowest absorbance measured in the beginning of incubation, were determined for each absorbance growth curve. μ_{max} -values of *Lm*-mix were determined from absorbance detection times for serially diluted inoculation levels of 10^2 , 10^3 , 10^4 , 10^5 and 10^6 cfu/ml as previously described (Dalgaard and Koutsoumanis, 2001). The cardinal parameter values for the different phosphate and citrate salts (P1, P2, P3 and TC) were estimated by fitting eq. (1) to square root transformed μ_{max} -values of *L. monocytogenes*.

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref\ 25^\circ C} \cdot \left(1 - \left(\frac{[P\ or\ TC]}{MIC_{P\ or\ TC}}\right)^{n1}\right)^{n2}} \quad (1)$$

where [P or TC] are the concentrations (%) of individual phosphates (P1, P2, P3) or citrate salt (TC) and $MIC_{P\ or\ TC}$ is the fitted minimum inhibitory concentration (MIC) value (%) of individual phosphates (P1, P2, P3) or citrate salt (TC) that prevents growth of *L. monocytogenes*. The cardinal parameter value for citric acid ($MIC_{U\ CAC}$) was determined from concentrations of undissociated citric acid calculated by eq. (2) with a pKa value of 3.13 (Ross and Dalgaard, 2004) from concentrations of TC. The cardinal parameter value was estimated by fitting eq. (3) to square root transformed μ_{max} -values of *Lm*-mix.

$$Undissociated\ organic\ acid\ (mM) = \frac{Citric\ acid\ (mM)}{1 + 10^{pH - pKa}} \quad (2)$$

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref\ 25^{\circ}C} \cdot \left(1 - \left(\frac{[CAC_U]}{MIC_{U\ CAC}}\right)^{n1}\right)^{n2}} \quad (3)$$

where $[CAC_U]$ is the concentration (mM) of undissociated citric acid and $MIC_{U\ CAC}$ is the fitted MIC-value of undissociated citric acid that prevents growth of *L. monocytogenes*. When fitting eq. (1 and 3), $n1$ was set to 0.5 or 1 and $n2$ was set to 1 or 2 (Dalgaard, 2009) in order to describe data most appropriately and this was determined from root mean square error (RMSE) values.

2.3.1. Growth inhibiting effect of interaction between phosphate and citrate salts

The effect of interaction for different combinations of phosphate and citrate salts concentrations (P1: 0-6%; P2: 0-5.5%; P3: 0-5%; TC: 0-8%) on *Lm*-mix were determined in BHI-broth with pH 6.2 at 25°C. A total of 66 μ_{max} -values were generated experimentally as explained above (Section 2.3). Experiments were designed to include combinations of concentrations that were close to the growth boundary of *L. monocytogenes*.

2.3.2. Anti-listerial effect of commercial melting salt preparations

The inhibitory effect for different concentrations of DP (0 to 8.6%), BUDAL (0 to 15.5%), PZ6, PZ35, S9, PZ189 (0 to 4.5%) and S9K (0 to 8%) on μ_{max} -values of *Lm*-mix were determined. A total of 94 μ_{max} -values were generated in BHI-broth with pH 6.2 at 25°C (See 2.3).

2.4. Development of a new *L. monocytogenes* growth and growth boundary model

New mathematical terms including MIC values for P1, P2, P3 and either TC or CAC_U were added to an existing cardinal parameter growth and growth boundary model previously validated for growth of *L. monocytogenes* in some non-fermented dairy products (Mejlholm and Dalgaard,

2009; Mejlholm et al., 2010). Of the 12 environmental factors in that model, exclusively terms for the effect of temperature, pH, NaCl/a_w, lactic acid and acetic acid were used in the present study and included in a new *L. monocytogenes* growth and growth boundary model with terms for the inhibitory effect of phosphate salts and either citrate salt or undissociated citric acid (eq. 4). A recently developed cardinal parameter pH_{min} -term was used to estimate pH_{min} -values for *L. monocytogenes* depending on the storage temperature (Martinez-Rios et al., 2019).

$$\begin{aligned} \mu_{max} = & \mu_{ref} \cdot \left[\frac{(T+2.83)}{(T_{ref}+2.83)} \right]^2 \cdot \frac{(a_w-0.923)}{(1-0.923)} \cdot [1 - 10^{(pH_{min}-pH)}] \cdot \left(1 - \frac{[LAC_U]}{3.79} \right) \cdot \left(1 - \sqrt{\frac{[AAC_U]}{10.3}} \right) \cdot \\ & \left[\left(1 - \left(\frac{[CAC_U]}{MIC_{UCAC}} \right) \right) \text{or} \left(1 - \left(\frac{[TC]}{MIC_{TC}} \right)^{n1} \right)^{n2} \right] \cdot \left(1 - \left(\frac{[P1]}{MIC_{P1}} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{[P2]}{MIC_{P2}} \right)^{n1} \right)^{n2} \cdot \\ & \left(1 - \left(\frac{[P3]}{MIC_{P3}} \right)^{n1} \right)^{n2} \cdot \xi \quad (4) \end{aligned}$$

where μ_{ref} is equal to μ_{max} at the reference temperature (T_{ref}) of 25°C; T is the temperature (°C) and a_w is the water activity measured in the product (Supplementary Table S1). [LAC_U], [AAC_U], [CAC_U] are the concentrations (mM) of undissociated lactic acid, acetic acid and citric acid in the water phase, respectively. [P1], [P2], [P3] and [TC] are the concentrations (%) in water phase of orthophosphate, di-phosphate, tri-phosphate and trisodium citrate respectively. [MIC_{P1}], [MIC_{P2}], [MIC_{P3}], and [MIC_{TC}] are the fitted MIC-values (% in the water phase) of orthophosphate, di-phosphate, tri-phosphate and trisodium citrate, respectively, that prevents growth of *L. monocytogenes*. The interaction between environmental parameters (ξ) was modelled as previously described using the Le Marc approach (Le Marc et al., 2002; Mejlholm and Dalgaard, 2009). The effect of interaction between environmental factors in eq. (4) was expressed by the parameter ξ , which has a value of between 0 and 1. The value of ξ , was calculated according to eq. (5), with contributions from different environmental factors as shown in eq. (6) and (7). In eq. (7), e_i

represents the environmental factors. Eq. (5) divides the space of environmental factors into three regions: (i) if ψ is less than 0.5, then no interactive effect between environmental factors occurs ($\xi = 1$); (ii) if ψ is greater than 1, then no growth occurs ($\xi = 0$); and (iii) if ψ is less than 1 and greater than 0.5, then the growth rate (μ_{max} , 1/h) is reduced depending on the value of ψ . A ψ value greater than 1 (e.g., 1.5 or 2.0) provides a measure of how far the properties of a specific food product is from the predicted growth boundary of *L. monocytogenes* (Mejlholm and Dalgaard, 2009).

$$\xi(\varphi(T, a_w, pH, [LAC_U], [AAC_U], [CAC_U], [P1], [P2], [P3], [TC]))$$

$$= \begin{cases} 1, & \psi \leq 0.5 \\ 2(1 - \psi), & 0.5 < \psi < 1 \\ 0, & \psi \geq 1 \end{cases} \quad (5)$$

where $\xi(\varphi(T, a_w, pH, [LAC_U], [AAC_U], [CAC_U], [P1], [P2], [P3], [TC]))$ is the term describing the effects of interactions between environmental factors on μ_{max} :

$$\varphi_T = \left[1 - \sqrt{(T + 2.83)/(T_{ref} + 2.83)} \right]^2$$

$$\varphi_{a_w} = \left[1 - \sqrt{(a_w - 0.923)/(1 - 0.923)} \right]^2$$

$$\varphi_{pH} = \left[1 - \sqrt{1 - 10^{(4.97 - pH)}} \right]^2$$

$$\varphi_{[LAC]; [AAC]; [CAC]}$$

$$= \left[1 - \left((1 - \sqrt{[LAC_U]/3.79}) \cdot (1 - \sqrt{[AAC_U]/10.3}) \cdot (1 - [CAC_U]/MIC_{CAC_U}) \right) \right]^2$$

$$\varphi_{[P1]; \varphi [P2]; \varphi [P3]; \varphi [TC]}$$

$$= \left[1 - \left(\left(1 - \left(\frac{P \text{ or } TC}{MIC_{P \text{ or } TC}} \right)^{n1} \right)^{n2} \right) \right]^2 \quad (6)$$

$$\psi = \sum_i \frac{\varphi_{e_i}}{2 \prod_{j \neq i} (1 - \varphi_{e_j})} \quad (7)$$

The inhibiting effect of organic acids in eq. (4) was modelled by multiplication of their effects as previously suggested (Coroller et al., 2005).

2.5. Challenge test with spreadable processed cheese

To generate data for model evaluation, growth of *L. monocytogenes* in spreadable processed cheese was determined in 20 inoculated challenge tests including 60 growth/no-growth responses at constant and dynamic storage temperatures (see section 2.6.). These included ten batches/formulations of customized spreadable processed cheese and 4 batches of commercially available spreadable processed cheese (Table 2).

2.5.1. Product characteristics

Ten customized spreadable processed cheese recipes were designed to evaluate the effect of phosphate salts, citrate salt and undissociated citric acid. The customized recipes were produced in the pilot plant at Arla Innovation Centre in Aarhus and transported on ice to DTU Food where they were stored upon arrival at 2°C for a maximum of 48h until further studied. Individual batches of customized spreadable processed cheese were produced with 3% or 6% orthophosphate (P1), di-phosphate (P2) or trisodium citrate (TC) and 2% or 5% tri-phosphate (P3). A commercially available emulsifying salt preparation (S9K) was used to produce spreadable processed cheese with two different concentrations (3% or 6%). Two commercial spreadable processed cheeses were obtained from a local supermarket.

Table 2. Storage conditions and product characteristics for challenge tests with processed spreadable cheese.

CT ^b	Batch	Type of cheese	n ^c	Storage temp. (°C)	Product characteristics (Avg.±SD) ^a								
					pH	a _w	Organic acids in water phase (ppm)			Melting salts in water phase (%)			
							Lactic acid	Acetic acid	Citric acid	P1 ^d	P2 ^e	P3 ^f	TC ^g
1	1	Customized	3	14.9±0.2	6.1±0.1	0.972±0.000	9,970±2,013	1,158±53	1,337±293	2.61±NA ^h	<0.01	<0.01	0.23±NA
2	2	Customized	3	14.9±0.2	6.2±0.3	0.970±0.004	11,605±588	1,272±57	1,493±91	4.98±NA	<0.01	<0.01	0.25±NA
3	3	Customized	3	15.0±0.3	6.4±0.2	0.967±0.001	11,969±1,611	3,483±934	1,709±384	0.52±NA	1.62±NA	<0.01	0.25±NA
4	4	Customized	3	15.0±0.3	6.2±0.1	0.971±0.001	14,768±523	3,231±922	2,134±125	0.48±NA	5.09±NA	<0.01	0.26±NA
5	5	Customized	3	15.0±0.3	6.6±0.1	0.970±0.002	9,559±1,630	1,451±1,135	1,642±281	0.68±NA	0.49±NA	0.63±NA	0.23±NA
6	6	Customized	3	15.0±0.3	6.1±0.1	0.967±0.000	11,859±598	1,701±13	2,368±212	0.74±NA	1.45±NA	5.17±NA	0.28±NA
7	7	Customized	3	14.9±0.2	6.4±0.1	0.970±0.000	7,051±1,030	1,116±18	11,510±290	0.42±NA	<0.01	<0.01	2.78±NA
8	8	Customized	3	14.9±0.2	6.3±0.1	0.963±0.001	12,339±1,620	2,162±1,116	38,282±5,319	0.44±NA	<0.01	<0.01	5.01±NA
9	9	Customized	3	15.0±0.3	6.4±0.2	0.964±0.001	9,514±2,760	1,116±90	1,289±479	2.04±NA	0.34±NA	0.29±NA	0.27±NA
10	10	Customized	3	15.0±0.3	6.3±0.1	0.952±0.001	15,328±1,768	1,630±227	2,490±1,390	3.90±NA	0.54±NA	3.67±NA	0.28±NA
11	1	Commercial	3	22.0±0.2	6.2±0.0	0.969±0.001	6,371±22	958±4	518±12	1.90±NA	<0.01	<0.01	0.49±NA
12	2	Commercial	3	4.8±0.4	6.2±0.0	0.969±0.001	7,641±865	568±178	2,558±290	1.94±NA	<0.01	<0.01	0.50±NA
13	2	Commercial	3	10.1±0.2	6.2±0.0	0.969±0.001	7,641±865	568±178	2,558±290	1.94±NA	<0.01	<0.01	0.50±NA
14	2	Commercial	3	14.5±0.2	6.2±0.0	0.969±0.001	7,641±865	568±178	2,558±290	1.94±NA	<0.01	<0.01	0.50±NA
15	3	Commercial	3	4.8±0.4	6.2±0.0	0.972±0.001	13,105±4,612	1,559±345	5,392±1,826	1.91±NA	<0.01	<0.01	0.49±NA
16	3	Commercial	3	10.1±0.2	6.2±0.0	0.972±0.001	13,105±4,612	1,559±345	5,392±1,826	1.91±NA	<0.01	<0.01	0.49±NA
17	3	Commercial	3	14.5±0.2	6.2±0.0	0.972±0.001	13,105±4,612	1,559±345	5,392±1,826	1.91±NA	<0.01	<0.01	0.49±NA
18	4	Commercial	3	7.2±0.2	6.1±0.0	0.969±0.001	12,624±1,468	1,436±159	7,612±1,062	2.14±NA	<0.01	<0.01	0.57±NA
19	4	Commercial	3	11.1±0.2	6.1±0.0	0.969±0.001	12,633±763	1,594±140	7,708±402	2.13±NA	<0.01	<0.01	0.58±NA
20	4	Commercial	3	3.8-19.4 ⁱ	6.3±0.0	0.975±0.000	8,368±717	1,042±226	5,416±284	1.92±NA	<0.01	<0.01	0.53±NA

^a Avg.: average; SD: standard deviation

^b Challenge test.

^c Number of growth curves per challenge test.

^d P1: orthophosphate

^e P2: di-phosphate

^f P3: tri-phosphate

^g TC: trisodium citrate

^h NA, not available as pooled sample was analysed by Eurofins.

ⁱ Dynamic storage temperature.

Product pH was measured with a PHM 250 Ion Analyzer (MetroLab™, Radiometer, Copenhagen, Denmark) after 1h stirring of a 5 g sample in 25 ml of distilled water. NaCl was quantified by automated potentiometric titration (785 DMP Titrino, Metrohm, Hesisau, Switzerland) and a_w was calculated from the concentration of NaCl in the water phase (%WPS) according to the relation derived from Resnik and Chirife (1988) ($a_w = 1 - 0.0052471 \cdot \%WPS - 0.0002206 \cdot \%WPS^2$). In addition a_w was measured by a water activity meter (Aqua Lab model CX-2, Decagon devices Inc., Pullman, US) (Supplementary Table 1). The concentrations of lactic, acetic and citric acid were determined by HPLC using external standards for identification and quantification. In order to improve extraction, a centrifugation step was applied (Dalgaard and Jørgensen, 2000; Martinez-Rios et al., 2016). Phosphate and citrate salt concentrations were determined by Eurofins, New Orleans, USA (test method QA02S).

2.5.2. Inoculation, storage conditions and microbiological analysis

Cheese was inoculated with 0.1% (v/w) of *Lm*-mix appropriately diluted in chilled saline water (0.85% NaCl) to obtain an initial concentration in the range of 1-3 Log cfu/g. Following inoculation, 50 ± 5 g of cheese was placed in containers similar to those used for commercial distribution of the product. Samples were stored at 5, 10, 15 and 22°C or under dynamic temperatures (Table 2). Storage temperature was recorded every 30 minutes by data loggers (TinytagPlus, Gemini Data Loggers Ltd., Chichester, UK). Storage time was from 8 to 83 days for different treatments with 7 to 27 sampling times per treatment.

At each time of sampling a container with 50 ± 5 g of cheese was analysed and then discarded. 10 g of cheese was diluted 10-fold in chilled physiological saline water with peptone (0.85% NaCl, 0.1% Bacto™ Peptone, 211677, BD Bioscience, San Jose, USA) and subsequently homogenized for 30s at normal speed in a stomacher (Stomacher 400 Circulator, Seward Medical,

London, UK). Viable counts of *L. monocytogenes* were determined by surface plating in Palcam agar base (CM0877, Oxoid, Basingstoke, UK) with selective supplement (SR0150, Oxoid) and incubated at 37°C for 48h.

2.5.3. Primary growth model

The integrated and log transformed logistic model with delay (four parameter model) or without delay (three parameter model) (eq. (8); Rosso et al., 1996) was fitted to all individual growth curves of *L. monocytogenes* obtained in challenge tests at constant temperatures. Fitted parameter values for initial cell concentration (Log N_0 , Log cfu/g), lag time (t_{lag} , h), maximum specific growth rate (μ_{max} , 1/h) and maximum population density (Log N_{max} , Log cfu/g) were determined for each growth curve and data was reported as average \pm standard deviation for challenge tests (Table 2). An F-test was used to determine if the lag time was significant.

$$\begin{aligned} \text{Log } (N_t) &= \text{Log } (N_0) && \text{if } t < t_{lag} \\ \text{Log } (N_t) &= \text{Log} \left(\frac{N_{max}}{1 + \left(\frac{N_{max}}{N_0} - 1 \right) \cdot \exp(-\mu_{max} \cdot (t - t_{lag}))} \right) && \text{if } t \geq t_{lag} \quad (8) \end{aligned}$$

where t is the storage time (h) and N_t is the cell concentration (cfu/g) at time t . Other parameters were indicated above.

The relative lag time ($RLT = t_{lag} \cdot \mu_{max} / \ln(2)$) (Mellefont and Ross, 2003) was calculated for all growth curves of *L. monocytogenes* in challenge tests (Table 3). It was evaluated if RLT-values were constant ($RLT = K_1$) or dependent on storage temperature ($RLT = K_1 + K_2/T^2$) as reported by Hereu et al. (2014).

Table 3. Growth parameters of *L. monocytogenes* in challenge tests with processed spreadable cheese.

CT ^b	Type of cheese	Growth parameter values (Avg. ±SD) ^a					Duration of exp. (days)	ψ^c	Predicted growth/no-growth responses
		Lag-time (h)	RLT (h)	Log N_0 (cfu/g)	Log N_{max} (cfu/g)	μ_{max} (1/h)			
1	Customized	222±11	13.9±2.1	2.3±0.2	4.9±0.1	0.043±0.01	19.1	0.3	Correct
2	Customized	114±18	7.2±1.8	2.5±0.1	7.0±0.6	0.043±0.00	20.1	0.3	Correct
3	Customized	0.0±0.0 ^d	0.0±0.0	2.4±0.1	2.2±0.1	0.000±0.00 ^e	51.0	0.4	Fail-safe
4	Customized	0.0±0.0 ^d	0.0±0.0	2.4±0.1	2.1±0.1	0.000±0.00 ^e	65.1	1.5	Correct
5	Customized	0.0±0.0 ^d	0.0±0.0	2.0±0.1	6.1±0.2	0.034±0.00	17.9	0.2	Correct
6	Customized	0.0±0.0 ^d	0.0±0.0	2.4±0.1	1.3±0.3	0.000±0.00 ^e	65.0	2.4	Correct
7	Customized	0.0±0.0 ^d	0.0±0.0	2.4±0.2	7.7±0.2	0.102±0.00	8.0	0.1	Correct
8	Customized	0.0±0.0 ^d	0.0±0.0	2.6±0.1	7.8±0.1	0.037±0.00	20.0	0.3	Correct
9	Customized	0.0±0.0 ^d	0.0±0.0	2.2±0.2	2.4±0.2	0.000±0.00 ^e	51.0	0.3	Fail-safe
10	Customized	0.0±0.0 ^d	0.0±0.0	2.4±0.2	1.7±0.4	0.000±0.00 ^e	65.0	1.2	Correct
11	Commercial	0.0±0.0 ^d	0.0±0.0	2.0±0.1	7.8±0.0	0.106±0.00	17.9	0.2	Correct
12	Commercial	0.0±0.0 ^d	0.0±0.0	1.4±0.1	3.4±0.3	0.006±0.00	32.0	0.3	Correct
13	Commercial	0.0±0.0 ^d	0.0±0.0	1.3±0.2	7.2±0.2	0.016±0.00	45.1	0.2	Correct
14	Commercial	0.0±0.0 ^d	0.0±0.0	1.3±0.0	7.0±0.1	0.051±0.00	16.2	0.2	Correct
15	Commercial	306±22	3.9±0.4	2.6±0.1	7.2±0.2	0.009±0.00	83.0	0.5	Correct
16	Commercial	43±16	1.6±0.6	2.9±0.0	7.8±0.1	0.027±0.00	29.8	0.4	Correct
17	Commercial	7±10 ^f	0.6±0.9	2.7±0.1	7.9±0.2	0.056±0.00	17.0	0.3	Correct
18	Commercial	157±56	4.3±1.9	1.0±0.0	4.5±0.1	0.015±0.00	24.0	0.5	Correct
19	Commercial	0.0±0.0 ^d	0.0±0.0	1.2±0.3	7.6±0.0	0.035±0.00	24.0	0.4	Correct
20	Commercial	-	-	1.9±0.2	7.6±0.3	-	24.0	-	

^a Avg: average; SD: standard deviation

^b Challenge test.

^c ψ -value indicate how far the properties of a specific food product is from the predicted growth boundary of *L. monocytogenes* with $\psi= 1.0$.

^d No significant lag-time

^e No growth observed for duration of experiment.

^f One growth curve had a significant lag time out of three growth curves.

2.6. Evaluation of the new *L. monocytogenes* growth and growth boundary model

Comparison of observed and predicted μ_{max} -values was carried out by calculation of bias (B_f) and accuracy (A_f) factor values (Ross, 1996). For pathogenic bacteria, $0.95 < B_f < 1.11$ indicate a good model performance, with B_f 1.11-1.43 or 0.87-0.95 corresponding to acceptable model performance and $B_f < 0.87$ or > 1.43 reflecting unacceptable model performance (Mejlholm et al., 2010). $A_f > 1.5$ has been suggested to indicate an incomplete model or systematic deviation between observed and predicted μ_{max} -values (Mejlholm and Dalgaard, 2013). Firstly, we used this approach to evaluate the effect of interaction among environmental factors (eq. (4)). Secondly, the approach was applied to evaluate if the new model could appropriately predict the inhibitory effect of commercial melting salt preparations on the growth of *L. monocytogenes*. For these predictions the concentrations of individual phosphates in the commercial melting salt preparations were analysed and concentrations of P1, P2 and P3 were used as model input to obtain predictions. Finally, the performance of the new model was evaluated by comparing predicted and observed growth responses in 20 challenge tests with spreadable processed cheese (see section 2.5). Predicted and observed growth and no-growth responses were assessed by calculating the percentage of all samples that were correctly predicted with or without inclusion in eq. 4 of the term for interaction between environmental factors (ξ). Incorrect predictions were considered as fail-safe (growth predicted with no growth observed) or fail-dangerous (no growth predicted with growth observed). ψ -values (eq. (5)) was used to describe how far the predicted response (growth or no-growth) was from the growth boundary ($\psi = 1$).

The acceptable simulation zone (ASZ) approach was used to compare observed and predicted growth in challenge tests where growth was observed under constant or dynamic temperature storage. The acceptable interval was defined as +0.5 and -1.0 Log cfu/g from the simulated growth of *L. monocytogenes*. When at least 70% of the observed values were within the ASZ, the simulation was considered acceptable (Oscar, 2007; Velugoti et al., 2011).

2.7. Evaluation of existing models

Three existing *L. monocytogenes* growth models were evaluated to assess their ability to predict growth responses in spreadable processed cheese. The studied models were: (a) the model of Mejlholm and Dalgaard (2009) previously evaluated for different non-fermented dairy products (Mejlholm et al., 2010), (b) the model of Augustin et al. (2005) developed for cheese and including terms for temperature, pH, NaCl/a_w, phenol, nitrite and CO₂ and (c) the ComBase model including the effect of temperature, pH, NaCl/a_w and lactic acid (Combase, 2012).

2.8. Statistical analysis and curve fitting

Model parameters and standard errors were estimated by using GraphPad PRISM (version 8, GraphPad Software, San Diego, CA, USA). F-tests to determine significant lag times were performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Development of a new *L. monocytogenes* growth and growth boundary model

The fitted MIC-values for phosphates were 14.9±1.1 %, 9.4±0.4 %, 7.6±0.2 % in the water phase for orthophosphate (P1), di-phosphate (P2), tri-phosphate (P3), respectively (Fig. 1; Table 4).

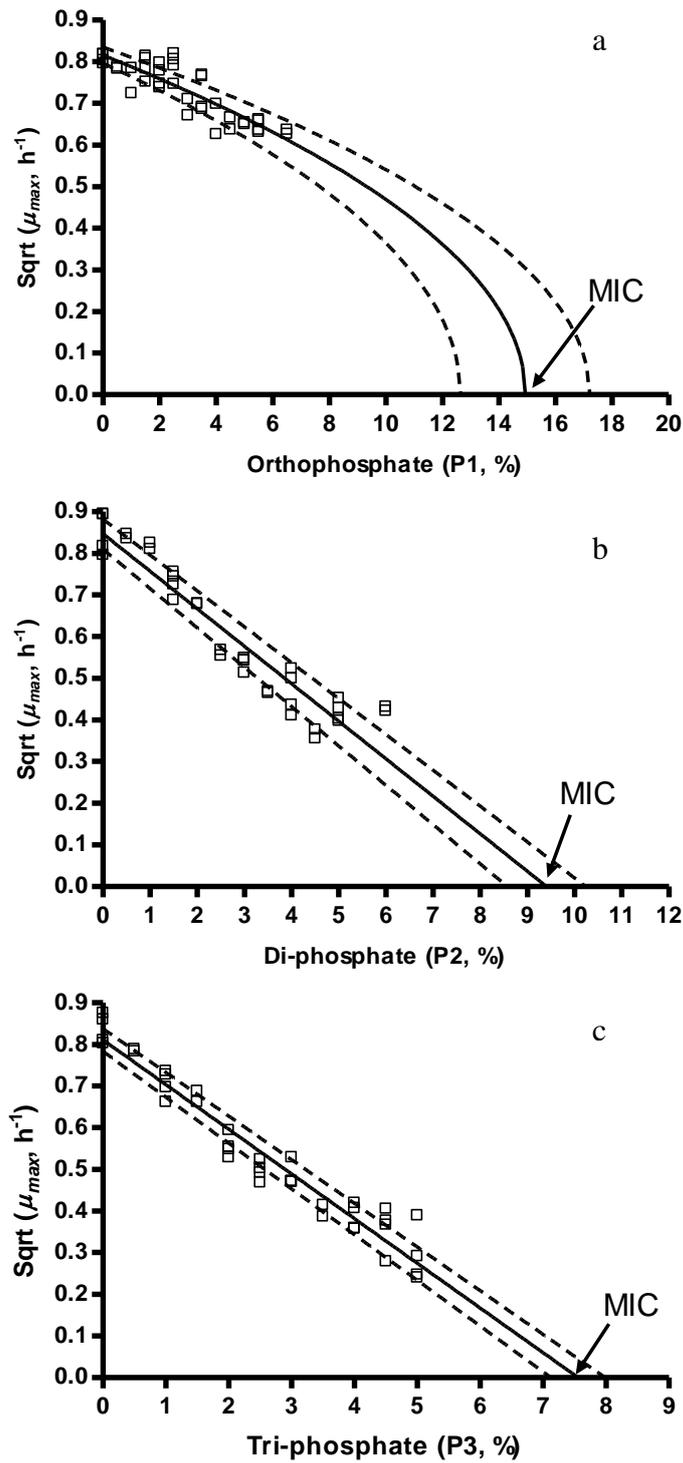


Fig.1. Maximum specific growth rates (μ_{max}, h^{-1}) of *L. monocytogenes* in BHI broth at 25°C and pH 6.2 as influenced by increasing concentrations of orthophosphate (a; P1), di-phosphate (b; P2) and tri-phosphate (c; P3). MIC-values for phosphate salts were determined by fitting eq. (3) to observed data (\square). Solid and dashed lines represent the fitted (eq. (3)) and confidence intervals (95%), respectively.

Table 4. Cardinal parameter values for the effect of melting salts on *L. monocytogenes* growth.

	Parameter values (value \pm SE) ^a	n1	n2
MIC _{P1} (%)	14.9 \pm 1.1	1	1
MIC _{P2} (%)	9.4 \pm 0.4	1	2
MIC _{P3} (%)	7.6 \pm 0.2	1	2
MIC _{TC} (%) or MIC _{CAC_U} (mM)	11.0 \pm 0.3 or 0.75 \pm 0.02	1	1

^a SE: standard error

The fitted MIC-values for trisodium citrate (TC) salts and undissociated citric acid (CAC_U) were 11.0 \pm 0.3 % and 0.75 \pm 0.02 mM, respectively (Fig. 2; Table 3). These MIC-values were used in eq. (4) together with a μ_{ref} -value of 0.419 1/h as determined at 25°C by Mejlholm and Dalgaard, (2009). When predictions by the new model (Eq. (4)) were performed either the MIC-value for TC or CAC_U was used.

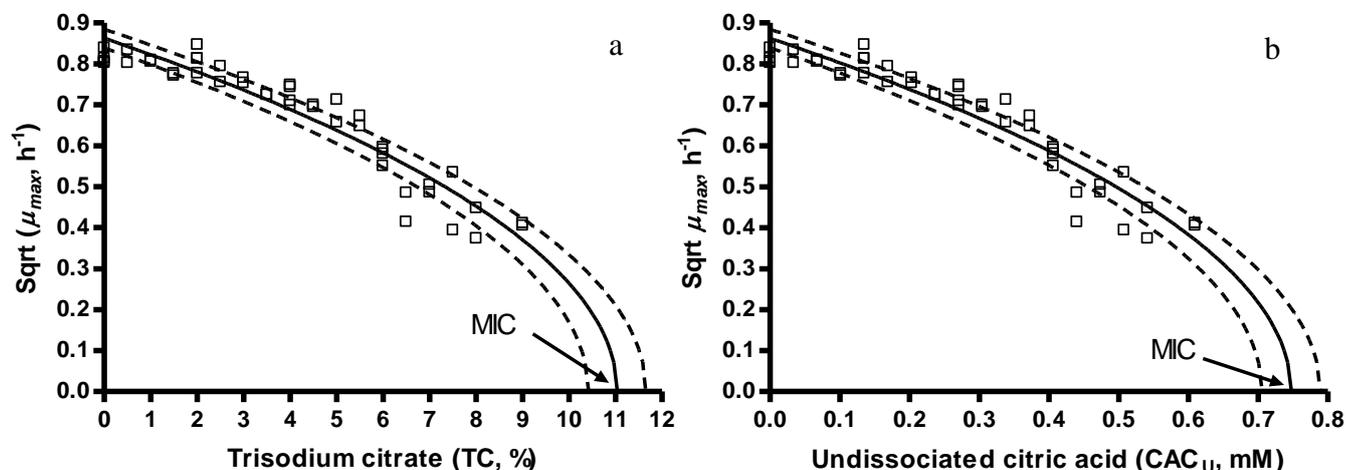


Fig.2. Effect of trisodium citrate (TC) (a) or undissociated citric acid (b) on maximum specific growth rates (μ_{max} , 1/h) of *L. monocytogenes* in BHI broth at 25°C and pH 6.2. MIC-values of citrate salts and undissociated citric acid were determined by fitting eq. (3) to observed data (\square). Solid and dashed lines represent the fitted (eq. (3)) and confidence intervals (95%), respectively.

3.2. Challenge test with spreadable processed cheese

Commercial spreadable processed cheese showed little variation in initial pH (6.1-6.3), a_w (0.969-0.975) or concentrations of P1 (1.90-2.14%) and TC (0.5%) (Table 2). More variability was observed for water phase concentrations of lactic acid (4120-12,624 ppm), acetic acid (619-1,594 ppm) and citric acid (518-7,708 ppm) (Table 2).

L. monocytogenes grew in commercial spreadable processed cheese at 5, 10, 15 and 22°C and μ_{max} -values were influenced by storage conditions and product characteristics (Table 2 and 3). As expected storage temperature had a pronounced effect on *L. monocytogenes* growth rate as seen for challenge tests 15, 16 and 17 which were performed with batch 3 of a commercial spreadable processed cheese and therefore had the same product characteristics (Table 2; Table 3). Triphosphate (P3) concentration had a major effect on *L. monocytogenes* μ_{max} -values, as suggested by their fitted MIC-values and confirmed by challenge tests 5 and 6 (Table 2, 3 and 4).

3.3. Evaluation of the new *L. monocytogenes* growth and growth boundary model

Broth studies with combinations of phosphates and citrate salt or undissociated citric acid suggested the need to include the growth inhibiting effect of interaction between these factors in the new growth and growth boundary model. By including φ [P1]; φ [P2]; φ [P3]; and either φ [TC] or φ [CAC_U] in eq. (7) the B_f and A_f values changed from 1.55/1.67 to 1.00/1.61. The seven studied commercial melting salt preparations all reduced growth rates of *L. monocytogenes* at 25 °C in BHI broth with pH 6.2 and this growth inhibiting effect was on average acceptably predicted by eq. 4 when using concentrations of P1, P2 and P3 as model input. On average for the 94 μ_{max} -values determined in broth the B_f and A_f were 1.42 and 1.46, respectively (Table 5). Specifically, the model predicted growth with acceptable model performance for DP, PZ35, PZ189 and S9K but overestimates growth for BUDAL and to a lesser extend for PZ6 and S9 (Table 5).

Table 5. Observed and predicted effect of commercial melting salt preparations for growth of *L. monocytogenes*.

Commercial melting salts	Conc. studied (g/ml)	Percentage composition				n ^a	B _f ^b	A _f ^c
		P1	P2	P3	TC			
DP	0.0-9.1	55	0	0	0	12	1.32	1.47
BUDAL	0.0-15.7	26	0	0	0	8	2.09	2.26
PZ35	0.0-8.2	15	0	51	0	18	1.24	1.24
PZ6	0.0-3.9	3	15	6	11	24	1.52	1.53
S9	0.0-3.4	4	5	15	0	14	1.53	1.53
PZ189	0.0-4.5	25	0	0	32	8	1.34	1.35
S9K	0.0-3.5	26	2	28	0	10	1.16	1.18
All data						94	1.42	1.46

^a n, number of experiments

^b B_f, bias factor

^c A_f, accuracy factor

For challenge test with spreadable processed cheese the new model predicted growth rates of *L. monocytogenes* at constant temperature with a good performance as determined from independent growth curves (n= 42) belonging to a total of 14 challenge test where growth was observed (Table 3). Comparison of observed and predicted μ_{max} -values, using either MIC_{TC} (%) or $MIC_{U\ CAC}$ (mM) (Fig. 2; eg. (1); eg. (3)), resulted in B_f/A_f –values of 1.06/1.35 or 1.12/1.29, respectively (Table 6). For commercial (n= 27) or customized (n= 15) spreadable processed cheese, B_f/A_f –values were 1.15/1.33 and 0.91/1.39, respectively, when using MIC_{TC} (%). When using $MIC_{U\ CAC}$ (mM) similar B_f/A_f–values of 1.17/1.36 and 1.05/1.29 were obtained.

RLT-values for growth in spreadable processed cheese showed considerable variability and they were not dependent on storage temperature (Table 3). The minimum, average and maximum *RLT*-values were 0.0, 1.2 and 13.9 .

Table 6. Comparison of observed and predicted growth of *L. monocytogenes* in spreadable processed cheese by bias and accuracy factors.

	B_f^a	A_f^b
New model ^c using MIC_{TC} (%)	1.06	1.35
New model ^c using MIC_{CACU} (mM)	1.12	1.29
Modified Mejlholm & Dalgaard (2009) model ^d	1.15	1.34
Original Mejlholm and Dalgaard (2009) model ^e	1.70	1.82
Augustin et al. (2005), cheese ^e	0.93	1.30
ComBase ^e	2.65	2.65

^a B_f , bias factor

^b A_f , accuracy factor

^c Predicted by the new model (eq. 2 through 5)

^d Predicted by the Mejlholm and Dalgaard (2009) model using its MIC_{UAC} -value (2.21 mM) but expanded with MIC-terms for phosphate salts (P1, P2, P3) as determined in the present study

^e Growth and no-growth prediction responses were 74% correct with 26% fail-safe and 0% fail-dangerous

For challenge tests, eq. (4) with interaction between environmental factors (ξ) resulted in 89 % correct prediction of growth and no-growth responses with 11% being fail-safe (Table 3). Without interaction between environmental factors (ξ) 74 % of the growth and no-growth responses were correctly predicted with 26% being fail-safe. Clearly, inclusion of the interaction term (ξ) in eq. (4) was important to accurately predict growth responses of *L. monocytogenes*. The two fail-safe predictions (11%) had ψ -values of 0.3 and 0.4 and these were not close to the growth boundary with ($\psi = 1$). Three correctly predicted no-growth responses had ψ -values of 1.2, 1.5 and 2.4 (Table 3).

On average 58% of the predicted cell concentrations were within the ASZ for spreadable processed cheese when calculated for 15 challenges where growth was observed resulting in 45 growth curves at constant and dynamic temperatures (Table 7, Fig. 3). Predictions were obtained using the minimum observed RLT -value for *L. monocytogenes*, N_{max} of 7.9 log cfu/g and the fitted MIC_{UAC} -value of 0.75 mM (Table 7, Fig. 3).

Table 7. Comparison of predicted and observed growth data using acceptable simulation zone (ASZ) method.

CT ^a	Storage conditions and growth data	Predictions performed with new model including $MIC_{U\ CAC}$ (mM) % observations within ASZ ^d
1 ^c	14.9±0.2, Fig 3a	17 (22/56)
2 ^c	14.9±0.2	32 (45/41)
5 ^c	15.0±0.3	41 (46/26)
7 ^c	14.9±0.2	67 (48/15)
8 ^c	14.9±0.2, Fig.3b	95 (79/13)
11 ^b	22.0±0.2, Fig. 3c	97 (93/43)
12 ^b	4.8±0.4	62 (64/44)
13 ^b	10.1±0.2	32 (32/49)
14 ^b	14.5±0.2	40 (30/30)
15 ^b	4.8±0.4, Fig. 3d	31 (70/33)
16 ^b	10.1±0.2, Fig. 3e	98 (100/21)
17 ^b	14.5±0.2, Fig. 3f	100 (70/18)
18 ^b	7.2±0.2, Fig. 3g	88 (88/54)
19 ^b	11.1±0.2	67 (45/27)
20 ^b	Dynamic (3.8-19.4°C), Fig. 3h	91 (21/18)
Average ASZ score		
All data		58 (57/33)
Commercial		63 (61/34)
Customized		49 (49/31)

^a Challenge test

^b Customized spreadable processed cheese

^c Commercial spreadable processed cheese

^d Calculation of ASZ score with minimum RLT-value (average/maximum RLT-value).

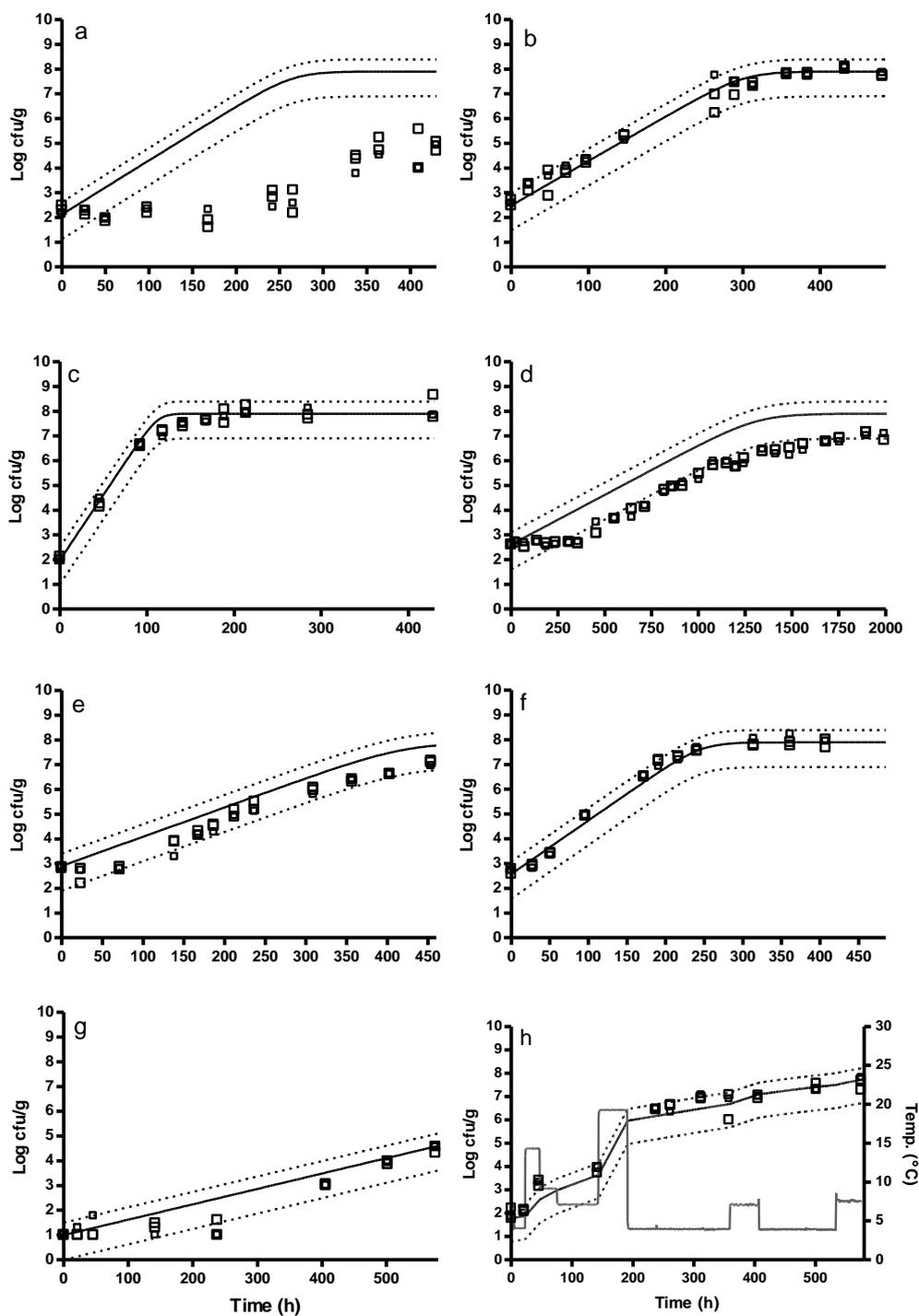


Fig.3. Comparison of observed (\square) and predicted (-) growth of *L. monocytogenes*. Spreadable processed spread cheese was studied at $22.0 \pm 0.2^\circ\text{C}$ (a), $4.8 \pm 0.4^\circ\text{C}$ (b), $10.1 \pm 0.2^\circ\text{C}$ (c), $14.5 \pm 0.2^\circ\text{C}$ (d), $14.9 \pm 0.2^\circ\text{C}$ (e), $14.9 \pm 0.2^\circ\text{C}$ (f), $7.2 \pm 0.2^\circ\text{C}$ (g) and dynamic storage temperature $3.8\text{--}19.4^\circ\text{C}$ (h, temperature profile is shown as grey lines). Solid lines represent the predicted growth by eq. (4) when using $\text{MIC}_{\text{CAC}_U}$ (mM). Graphs include the ASZ (+0.5 and -1.0 Log cfu/g, dashed lines).

Lag times had a major effect on the ASZ scores. As examples, challenge test 15 with a significant lag time (306 h, Table 3) resulting in a very low ASZ value (31%), however, when no significant lag time was observed at the same storage temperature (challenge test 12) a ASZ value of 62% was found (Table 7). For challenge test 1 and 15 growth rates were accurately predicted by the model but the presence of lag times resulted in low ASZ scores (Fig.3. a, d). To overcome this limitation of the model, we evaluated the use of average and maximum RLT-values but results were inferior to those obtained by using the minimum RLT-values (Table 7).

3.4 Evaluation of existing models

As expected, for spreadable processed cheese with melting salts, unacceptable model performance with B_f -values well above 1.43 were observed for both the model of Mejlholm and Dalgaard (2009) and the ComBase model. Acceptable performance with B_f and A_f of 0.93/1.30 were determined for the model of Augustin et al. (2005) developed for cheese (Table 6).

4. Discussion

A new mathematical model to predict growth and growth boundary of *L. monocytogenes* in spreadable processed cheese was developed by expanding an existing cardinal parameter model with terms to account for the effect of orthophosphate, di-phosphate, tri-phosphate and a new MIC-value for undissociated citric acid of 0.75 mM (eq. 4). The new model predicted acceptably the growth at constant and dynamic storage temperatures as well as the growth boundary of *L. monocytogenes* in spreadable processed cheese (Table 6, Table 7, Fig. 3h). The low average ASZ score of 58% was due to significant lag times in some challenge tests and predictions being fail-safe (Fig. 3 a, d). Similar effects of lag times on ASZ scores were previously observed for both *L. monocytogenes* and *Salmonella* spp. (Hereu et al., 2014; Velugoti et al. 2011).

Based on the performed evaluation of the model, its range of applicability included orthophosphate (0.14 to 4.98 %), di-phosphate (<0.01 to 5.09 %), tri-phosphate (<0.01 to 5.17 %), lactic acid (6,371 to 15,328 ppm), acetic acid (568 to 3,483 ppm), citric acid (518 to 38,282 ppm) in the product water phase, pH (6.1 to 6.6), a_w (0.952 to 0.975) and temperature (3.8 to 22.0°C). The inhibitory effect of several dairy specific ingredients is included in the new model (Eq. (4)) and this makes the model of practical importance for product development, reformulation or risk assessment of spreadable processed cheese. As an example, for a spreadable processed cheese with pH 6.3, a_w 0.972 and water phase organic acid concentrations of 0.8% (lactic acid), 0.1% (acetic acid), 0.3% (citric acid) and 2.0 % (orthophosphate), the predicted time for *L. monocytogenes* to reach the critical concentration of 2 log cfu/g is 4-8 days if this product is contaminated with 1-10 cfu/g by consumer handling, e.g. when opening a package, and then stored at 8°C. A longer open shelf-life or larger safety margin may be desirable and the new model predicts that by substituting the orthophosphate with 2.0 % tri-phosphate the reformulated product requires 13-17 days at 8°C to reach the same critical concentration for *L. monocytogenes*. It seems interesting to apply the new model in combination with available models to predict growth or toxin formation by *Clostridium botulinum* in spreadable process cheese containing melting salts (Glass et al., 2017; Schaffner et al., 1998; ter Steeg and Cuppers, 1995) to formulate recipes that will inhibit growth of the relevant pathogens. For these applications the new model has the advantages of including the inhibitory effect of ingredients specific to spreadable processed cheese and being validated for these products. When food products are reformulated, product characteristics must be selected at a sufficient distance from the growth boundary so that *L. monocytogenes* does not grow as a consequence of intrinsic variability of product characteristics, storage conditions or strain variability. In this respect, the new model (Eq. (4)) includes the parameter ψ as a quantitative measurement for the distance between specific environmental conditions and the growth boundary of *L. monocytogenes* ($\psi = 1$).

As an example, a ψ value of 0.20 was determined by the model for spreadable processed cheese with the following characteristics: pH 6.6, a_w 0.970, 1.0% (lactic acid), 0.1% (acetic acid), 0.2% (citric acid), 0.7% P1, 0.5% P2, 0.6% P3 in the water phase of product and stored at 15°C (Table 2, CT 5). These product characteristics are placed on the growth side of the growth boundary ($\psi < 1$). The new model can be used to optimize product characteristics to prevent growth of *L. monocytogenes*. The formulation studied in CT 5 can be changed to prevent growth and to obtain a product with a desired ψ -value of e.g. 2. With pH reduced from 6.6 to 5.8, water phase concentrations of lactic acid increased from 1% to 2.2%, acetic acid increased from 0.1% to 0.35% and P1 reduced from 0.7 to 0.3% and P3 changed from 0.6% to 1.5% the predicted ψ -value becomes 2.1.

The model of Augustin et al. (2005) also provided acceptable prediction for growth rates of *L. monocytogenes* in spreadable processed cheese (Table 6) and included the effect of temperature, pH, NaCl/ a_w , phenol, nitrite and CO₂. Without terms for organic acids and melting salts the potential of the Augustin et al. (2005) model to contribute to development and reformulation of spreadable processed cheese, however, is limited and in this respect the new model developed in the present study is more performant.

The present study estimated a lower undissociated citric acid MIC-value ($MIC_{U\ CAC}$) for *L. monocytogenes* dairy strains (0.75 mM) than Mejlholm and Dalgaard (2009) observed for seafood isolates (2.21 mM). Future studies should compare model performance when using either $MIC_{U\ CAC}$ -value for spreadable processed cheese with lower pH-values than the products evaluated in the present study.

The approach used in the present study to develop an extensive model including the inhibiting effect of both organic acids and phosphate salts could also be interesting for *Clostridium*

botulinum as available predictive models with relevance for spreadable processed cheese include few environmental parameters (Glass et al., 2017; Schaffner et al., 1998; ter Steeg and Cuppers, 1995).

5. Conclusion

The present study developed and validated a new model to predict growth and growth boundary of *L. monocytogenes* in spreadable processed cheese. The obtained results demonstrate that interaction among environmental factors improved the performance of the model. This study confirmed that increasing concentrations of phosphate salts reduces the growth of *L. monocytogenes* and therefore, these salts can be used as growth inhibiting compounds. The model can be used to support spreadable processed cheese product development, reformulation or risk assessment. It seems interesting to include the new model in predictive microbiology application software such as the Food Spoilage and Safety Predictor (FSSP <http://fssp.food.dtu.dk/>) to facilitate prediction of the effect of product characteristics at constant and dynamic temperature storage conditions on growth of *L. monocytogenes* in spreadable processed cheese.

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Supplementary Table 1. Differences in water activity (a_w) calculated by Resnik and Chirife (1988) or measured by water activity meter.

CT ^a	Type of cheese	n ^c	% WPS	a_w	
				Resnik and Chirife, (1988)	measured
1	Customized	3	1.71±0.00	0.990±0.000	0.972±0.000
2	Customized	3	1.71±0.01	0.990±0.000	0.970±0.004
3	Customized	3	3.16±0.05	0.982±0.000	0.967±0.001
4	Customized	3	1.79±0.03	0.990±0.000	0.971±0.001
5	Customized	3	2.60±0.03	0.985±0.000	0.970±0.002
6	Customized	3	1.90±0.02	0.989±0.000	0.967±0.000
7	Customized	3	2.25±0.00	0.987±0.000	0.970±0.000
8	Customized	3	1.93±0.01	0.989±0.000	0.963±0.001
9	Customized	3	3.28±0.08	0.981±0.001	0.964±0.001
10	Customized	3	1.97±0.02	0.989±0.000	0.952±0.001
11	Commercial	3	2.08±0.10	0.988±0.000	0.969±0.001
12	Commercial	3	1.86±0.08	0.989±0.000	0.969±0.002
13	Commercial	3	1.86±0.08	0.989±0.000	0.969±0.002
14	Commercial	3	1.86±0.08	0.989±0.000	0.969±0.002
15	Commercial	3	1.84±0.02	0.990±0.000	0.972±0.001
16	Commercial	3	1.84±0.02	0.990±0.000	0.972±0.001
17	Commercial	3	1.84±0.02	0.990±0.000	0.972±0.001
18	Commercial	3	1.42±0.05	0.992±0.000	0.969±0.001
19	Commercial	3	1.41±0.08	0.992±0.000	0.969±0.001
20	Commercial	3	1.70±0.13	0.990±0.001	0.975±0.000

Paper V

Martinez-Rios, V., Pedersen, M., Pedrazzi, M., Gkogka, E., Smedsgaard, J.,

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Cardinal parameter model containing nisin term to predict growth of *Listeria monocytogenes* in processed cheese

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Cardinal parameter model containing nisin term to predict growth of *Listeria monocytogenes* in processed cheese

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ABSTRACT

A mathematical model was developed to predict growth of *L. monocytogenes* in cheese containing added nisin. Minimum inhibitory concentration (MIC) values for nisin at pH 5.5 and 6.0 were determined experimentally in broth and literature MIC-values at different pH-values were collected. A MIC-term was developed to describe the effect of pH on nisin MIC-values. Two growth and growth boundary models were expanded with the new MIC-term for nisin to predict growth of *L. monocytogenes* in chemically acidified cheese (Model A expanded from Martinez-Rios et al., 20019a) and in processed cheese (Model B expanded from Martinez-Rios et al., 2019b). To generate growth data for model evaluation challenge tests were performed with *L. monocytogenes* inoculated in chemically acidified cheese and processed cheese containing added nisin (n = 45). A LC-MS/MS method was developed and validated to quantify nisin A and Z present in cheese. The nisin recoveries ranged from 83 to 110 % for nisin A and from 95 to 113 % for nisin Z. The limits of detection and quantification for both nisin A and nisin Z were 0.04 mg/kg and 0.12 mg/kg, respectively. Applicability of the LC-MS/MS method was evaluated by analysing 13 different cheeses containing nisin. Five cheese samples were found to contain nisin A at concentrations in the range from 0.16 to 0.19 mg/kg. Evaluation of model B by comparison of observed and predicted μ_{max} -values resulted in a bias factor of 1.05 and an accuracy factor of 1.12 for a total of 18 growth responses in processed cheese. Further studies with higher concentrations of nisin will be beneficial to validate the new nisin-terms including the effect of pH on nisin MIC-values.

Keywords: Predictive microbiology, LC-MS/MS, product development, risk assessment

1. Introduction

Nisin is an antimicrobial peptide belonging to the lantibiotic class of bacteriocins (Class I), composed of 34 aminoacids and with a molecular mass of 3.5 kDa. Nisin Z differs from nisin A in aminoacid position 27, where histidine is replaced by asparagine (Mulders et al., 1991). Nisin has antimicrobial activity against a wide range of gram-positive food-borne pathogens and it influences both cell membrane pore formation and inhibition of cell wall synthesis caused by prevention of lipid II transport of peptidoglycan subunits from the cytoplasm to the cell wall (Cotter et al., 2005). Commercial nisin preparations have been used for over 30 years and remain the only bacteriocin allowed in food as an added preservative (FDA, 1988; Thomas and Delves-Broughton, 2005). Activity and stability of nisin in foods depends on many factors including heat treatment (temperature and time), storage temperature, pH, fat and protein content (Delves-Broughton et al., 1996; Oshima et al., 2014). For instance, nisin is used in processed cheese to control potential growth of spore-forming bacteria. The anaerobic environment, high moisture and pH ranging from 5.4 to 6.0 usually found in processed cheese may favour germination of spores and growth of *Clostridium* spp. or facultative anaerobic *Bacillus* spp. if present in the product and not controlled by means of formulation (Delves-Broughton, 2008; Glass et al., 2017). Though nisin has a well-documented use for the control of these spore-forming bacteria in processed cheese formulations, little information is available with regards to its protective effect against vegetative pathogens such as *L. monocytogenes*, when potentially introduced by cross-contamination at the consumer phase.

The antimicrobial activity of nisin against *L. monocytogenes* has been extensively studied by determination of the minimum inhibitory concentration (MIC) (Benkerroum and Sandine, 1988; Ferreira and Lund 1996; Martínez and Rodríguez, 2005). However, the stock solution and the experimental design used to estimate nisin MIC-values (e.g. pH) needs to be taken into

consideration when using those results, since both solubility and stability of nisin in solution are affected by pH (Thomas and Delves-Broughton, 2005).

Several nisin bioassays have been developed over the years to measure nisin antimicrobial activity in foods. The most widely used is the horizontal agar diffusion method relying on the test organism *Micrococcus luteus* (Fowler et al., 1975; Papagianni et al., 2006; Tramer and Fowler, 1964). Other more sensitive assays for pure nisin analysis, such as ELISA assays have been developed (Falahee et al., 1990; Suárez et al., 1996). However, studies of nisin retention in foods including processed cheese indicate that correlation between bioactivity measured by the agar diffusion method and ELISA is poor (Thomas and Delves-Broughton, 2005). Heat treatment results in quantitative losses of nisin antimicrobial activity but no quantitative loss was observed when measured by the ELISA method; most probably because the ELISA method quantifies a partially degraded nisin molecule without biological activity (Abee and Delves-Broughton, 2012). In the last years, more advance analytical methods have been developed to quantify nisin present in foods. An ISO standard was published in 2009 for the determination of nisin A content in cheese by liquid chromatography/mass spectrometry (LC-MS) and LC-MS/MS (ISO, 2009). This LC-MS/MS method has been modified and extended to also quantify nisin Z (Fuselli et al., 2012; Ko et al., 2016; Schneider et al., 2011). However, the relation between concentrations of nisin measured by LC-MS/MS and its antimicrobial activity has not been studied.

The objectives of the present study were to develop and validate a LC-MS/MS method for quantification of residual nisin in different cheeses and to determine the inhibitory effect of nisin concentrations on growth responses of *L. monocytogenes* in cheese. The anti-listerial effect of nisin was studied by using a predictive food microbiology approach for comparison of observed and predicted growth. Firstly, a nisin MIC-term was developed including the effect of pH on nisin MIC-values and the term was added to two cardinal parameter growth and growth boundary models.

Secondly, the performance of the expanded models was evaluated by comparison of observed and predicted growth for *L. monocytogenes* in cheeses when assuming (a) no presence of nisin, (b) measured residual nisin concentrations and (c) concentrations of nisin added to the cheeses.

2. Material and methods

2.1. Nisin quantification by LC-MS/MS and method validation

The analytical technique described in this section combined a simple nisin extraction method with quantification by LC-MS/MS. LC-MS/MS combines the chromatographic separation of multiple compounds with the capability of mass spectrometry to separate compounds according to their m/z -ratios and furthermore achieving results with high specificity and detection sensitivity of bacteriocins (Zendo et al., 2007).

2.1.1 Standards and reagents

Nisin A (2.5%) was from Sigma-Aldrich (N5764, St. Louis, MO, USA) and Chrisin C (2.5% nisin Z) from Chr. Hansen (Hørsholm, Denmark). Methanol (HPLC grade, VWR chemicals, Søborg, Denmark) was used as solvent to extract nisin from cheese where pH was adjusted with formic acid (695076, Sigma-Aldrich, St. Louis, MO, USA).

2.1.2. Preparation of extraction solvent and nisin stock solution

As extraction solvent, a buffer consisting of 0.1% formic acid in water (pH 2) and methanol (1:1 v/v) was prepared. 50 ppm stock solution of nisin A and nisin Z was prepared in 0.02N HCl. The stock solution was filtered using a 0.2 μm filter (Minisart[®], Sartorius Stedim Biotech GmbH) and stored at 4°C for a maximum of 7 days.

2.1.3. Extraction method

Blended cheese (2.0 g) was homogenized in 30 mL extraction solvent with a T25 Ultra Turrax (IKA, Staufen, Germany) The suspension was sonicated for 10 min. at room temperature and then centrifuged (Eppendorf centrifuge 5810 R, Hørsholm, Denmark) at 5000 rpm for 10 min at 4°C to extract nisin from cheese. The supernatant was transferred to volumetric flask and the precipitate was extracted twice with 10 mL extraction solvent. Supernatants were combined and supplemented as necessary with extraction solvent to 50 ml and filtered (0.2 µm Ministart[®], Sartorius Stedim Biotech GmbH) prior to LC-MS/MS analysis.

2.1.4. LC-MS/MS analysis

For the LC-MS/MS analysis, an EVOQ Elite[™] triple quadrupole mass spectrometer (Bruker, Frederikssund, Denmark), equipped with an electrospray ionization (ESI) source was coupled to an UltiMate 3000 ultra-high-performance liquid chromatography system (UHPLC) from Dionex Thermo scientific (Waltham, MA, USA). The UHPLC consisted of a pump, autosampler and column compartment. Chromeleon Xpress (Thermo Fisher Scientific, Waltham, USA) and MS Workstation 8 (Agilent, Santa Clara, USA) were used to perform system control and data acquisition on the UHPLC and mass spectrometer, respectively. Chromatographic separation was performed using a Zorbax 300 SB-C18 (2.1 x 50 mm, 1.8 micron particle size, 300 Å) from Agilent Technologies, Inc. (Santa Clara, CA, USA).

The mobile phase consisted of an aqueous solution with 0.1% formic acid (mobile phase A) and 100% acetonitrile (mobile phase B; Chromasolv[™] LC-MS, Honeywell, Seelze, Germany). A linear gradient was used as follows: 0-2 min 80% A; 2-6 min 20% A; 6-7 min 80% A, and held for 3 min to equilibrate the column. The column was maintained at 40°C. The flow rate was 0.3

mL/min and the injection volume was 10 μ l. The diverter valve was used to eliminate NaCl interferences, directing the flow into the discharge for the first 3 minutes of each run, avoiding contamination of the ion source. Parent ions $[M+4H]^{4+}$ were identified as 839.0 and 833.6 m/z for nisin A (average mass, 3354) and nizin Z (average mass, 3331), respectively. The fragment ions of nisin A were also selected as 1081 (quantitative ion) and 811 (qualitative ion), and those selected for nizin Z were 1073.5 (quantitative ion) and 985.1, 805.7 and 801.5 (qualitative ions). Diagnostics ions were found using an infusion experiment. Separate standard solutions of nisin A and nisin Z (1 μ g/mL) were infused directly into the ion source without collision gas and collision energy applied and parent ions were identified. Fragment ions were likewise identified with infusion of standard solution and collision gas and collision energy applied.

2.1.6. Validation of the nisin quantitative method

The quantification method was validated for linearity, specificity, recovery and precision (NMKL, 2009). Validation was performed using four cheese matrixes: processed cheese (PC), spreadable processed cheese (SPC), white cheese (WC) and mascarpone (MP). Each calibration curve was built with four concentration levels (including zero) using a linear function of concentration (x-axis) versus peak area (y-axis). Linearity was evaluated using two replicates per level, in three different days. Acceptance level criterion was an average regression coefficient (r^2) > 0.99. Linearity was further studied by plotting residuals against concentrations of standard curves performed for the four types of cheese. Specificity was checked by analysing samples of each matrix, to evaluate the possibility for interference of some endogenous compound. The results were assessed by checking the presence of interfering substances with the same retention time as for nisin A and Z, in comparison to the cheese samples fortified with nisin A and Z. The recovery and precision (repeatability and reproducibility) were determined by spiking experiments where samples

were fortified with 0.1, 0.5 and 1 mg/kg of nisin A and Z before the extraction procedure. Precision (CV%) and recovery (%) were based on duplicate analysis on three different days for each concentration level. Data analyses were performed using Microsoft Excel software. The limit of detection (LOD) was calculated as three times the within sample standard deviation for reproducibility (S_R) and limit of quantification (LOQ) was calculated as 10 times S_R . Matrix effect (suppression or enhancement of signal due to other substances in the sample) were calculated by comparing the slope of two types of calibration curves. Curve type I, was prepared by spiking extraction solvent with nisin A and nisin Z to reach 0.1, 0.5 and 1 mg/kg. Curve type II, was prepared by spiking cheese samples with nisin A and Z to reach 0.1, 0.5 and 1 mg/kg. Matrix effect was calculated by division of the slope of curve type II with slope of curve type I.

2.1.7. Quantification of nisin A and Z in cheese samples

The LC-MS/MS method was used to quantify nisin A and Z in cheeses produced in the laboratory, purchased from a supermarket or supplied by Arla Foods (non-commercial and commercial samples with and without nisin A). Four batches of cheese were produced in the laboratory with pH adjusted to 5.7-5.8 by adding glucono-delta-lactone (GDL 54%, Roquette®, Lestrem, France), rennet (3.3% Hannilase® XP 200 NB, Chr. Hansen, Hoersholm, Denmark) and 2.5% of NaCl (Merck, Kenilworth, US) to milk (3.5% fat). Two out of the four batches were supplemented with a 0.02N HCl stock solution of Nisaplin® (DuPont, UK) to reach 2.5 ppm of nisin in cheese. Mascarpone cheese purchased in a supermarket was supplemented with the aforementioned Nisaplin® (DuPont, UK) stock solution to reach 2.5 or 5 ppm in the cheese. Nine batches of cheeses containing different concentration of nisin A (0-25 mg/kg) were supplied by Arla Foods. Uncertainty of measurements was reported by multiplying the analytical result by two times the CV (%).

2.2. Bacterial strains and pre-culture conditions

Four dairy related strains of *L. monocytogenes* were provided by Arla Foods and used as a cocktail (SLU92, 612, LM19, 6) to determine μ_{max} -values in broth and for inoculation of challenge tests. Prior to studies each strain was transferred from storage at -80°C to Brain Heart Infusion (BHI) broth (CM1135, Oxoid, Hampshire, UK) and incubated for 24h at 25°C . For broth studies the strains were pre-cultured at 20°C in BHI broth adjusted with HCl to pH 5.5 or 6.0. For challenge tests the strains were pre-cultured at a temperature ranging from 10°C to 20°C in BHI broth with 2.5% NaCl and pH 5.5 (HCl) to simulate conditions in chemically acidified cheese and processed cheese. Pre-cultures were grown to a relative increase in absorbance (540 nm) of 0.05 to 0.2 (Novaspec II, Pharmacia Biotech, Allerød, Denmark). The *L. monocytogenes* cocktail of strains (*Lm-mix*) used in broth and challenge tests was obtained by mixing equal volumes of individually pre-cultured strains. The *Lm-mix* concentration was determined by direct phase contrast microscopy at 1000x magnification considering that one cell per field of view corresponded to a concentration around 10^6 cfu/ml (Adams and Moss, 2016).

2.3. Cardinal parameter term for nisin

The effect of 16 nisin A concentrations (0-22 mg nisin/L, Nisaplin[®], DuPont Nutrition Biosciences ApS, Marlborough, UK in 0.02N HCl) on μ_{max} -values of *Lm-mix* were determined in duplicate by automated absorbance measurements at 540 nm (BioScreen C, Labsystems, Helsinki, Finland) at 20°C and 25°C using BHI broth adjusted to pH 5.5 or 6.0 with HCl and sterilized by filtration (0.2 μm , Minisart[®], Sartorius Stedim Biotech GmbH, Goettingen, Germany). Detection times, defined as incubation time necessary to observe an increase in absorbance of 0.05 from the lowest absorbance measured in the beginning of incubation were determined for each absorbance growth curve. μ_{max} -values of *Lm-mix* were determined from absorbance detection times for serial diluted inoculation levels of 10^2 , 10^3 , 10^4 , 10^5 and 10^6 cfu/ml as previously described (Dalgaard and

Koutsoumanis, 2001). A total of 41 μ_{max} -values of *Lm*-mix were determined experimentally in BHI-broth. Nisin MIC-values were estimated by fitting eq. (1) to square root transformed μ_{max} -values from experimental data obtained for the studied nisin concentrations.

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref-1} \cdot \left(1 - \left(\frac{nisin}{MIC_{nisin}}\right)^{n_1}\right)^{n_2}} \quad (1)$$

where μ_{max} is the maximum specific growth rate (h^{-1}) and μ_{ref-1} is the reference maximum specific growth rate for the studied temperature. When fitting eq. (1), n_1 was set to 0.5 or 1 and n_2 was set to 1 or 2 (Dalgaard, 2009) in order to describe data most appropriately and this was determined from root mean square error (RMSE) values.

A total of 71 nisin MIC-values of different *L. monocytogenes* strains determined in broth or agar with different pH values (relevant for cheese) were extracted from literature. The studied nisin stock solutions were produced in 0.02N HCl and without autoclaving. Eight MIC-values were obtained at pH 5.0, 39 MIC-values at pH 5.5 and 24 MIC-values at pH 6.8 (Brandt, 2009; Ferreira and Lund 1996; Taylor, 2009). A simple equation was used to model and predict the effect of pH on nisin MIC-values.

2.4. Models for growth of *L. monocytogenes* in cheese with added nisin

The model of Martinez-Rios et al. (2019a) previously validated for chemically acidified cheese was expanded with the new MIC-term for nisin (see section 2.3) by using the gamma approach with interaction between environmental factors (Le Marc et al. 2002). The new expanded model (Model A) was used to predict growth of *L. monocytogenes* in chemically acidified cheese. In the same way, the model of Martinez-Rios et al. (2019b) previously validated for spreadable processed cheese and containing terms for the inhibitory effect of phosphate salts was expanded with the nisin-term to predict growth of *L. monocytogenes* in processed cheese containing nisin (Model B).

2.5. Challenge tests

To generate data for model evaluation (see section 2.6), growth of *L. monocytogenes* in chemically acidified cheese and processed cheese containing nisin was determined in 15 challenge tests including 45 growth/no-growth responses at constant temperatures.

2.5.1. Inoculation of cheese and microbiological analysis

Cheeses were inoculated with 0.1% (v/w) of *Lm-mix* appropriately diluted in chilled saline water (0.85% NaCl) to obtain an initial concentration between 1 and 3 log cfu/g. After inoculation cheeses were packaged and stored in containers with 50 ± 1 g cheese. Storage temperature was recorded by data loggers (TinytagPlus, Gemini Data Loggers Ltd, Chichester, UK). At each time of sampling, 10 g of cheese from a container were diluted 10-fold with chilled physiological saline (0.85% NaCl and 0.10% Bacto-peptone), homogenized for 30 s at normal speed in a Stomacher 400 (Seward Medical, London, UK) and then 10-fold serial dilutions were performed. Microbial population was enumerated by surface plating on BHI agar (CM1136, Oxoid, Hampshire, UK) with incubation at 25°C for 24 h. 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.

2.5.2. Product characteristics

Product characteristics of cheeses were determined by analysis of three packages for each batch at the start of the challenge test. In addition, pH was measured every day of microbiological analysis by using a PHC10801 puncture combination probe (Hach, Brønshøj, Denmark) placed directly into the chemically acidified cheese. For processed cheese, pH was measure by a PHC725

SN (Hach, Brønshøj, Denmark) after 1h stirring of 5 g sample in 25 ml of distilled water. 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). Lactic-, acetic- and citric acid concentrations were determined by HPLC using external standards for identification and quantification (Østergaard et al., 2014). Phosphate salts concentrations were determined by Eurofins (test method QA02S). Nisin concentration was measured by LC-MS/MS (see section 2.1).

2.6. Models evaluation

Comparison of observed and predicted μ_{max} -values was carried out by calculation of bias (B_f) and accuracy (A_f) factor values (Ross, 1996). For pathogenic bacteria, $0.95 < B_f < 1.11$ indicate a good model performance, with B_f 1.11-1.43 or 0.87-0.95 corresponding to acceptable model performance and $B_f < 0.87$ or > 1.43 reflecting unacceptable model performance (Mejlholm et al., 2010). $A_f > 1.5$ has been suggested to indicate an incomplete model or systematic deviation between observed and predicted μ_{max} -values (Mejlholm and Dalgaard, 2013). Predicted and observed growth and no-growth responses were assessed by calculating the percentage of all samples that were correctly predicted. Incorrect predictions were described as fail-safe (growth predicted when no growth was observed) or as fail-dangerous (no growth predicted when growth was observed). The ψ -value was used to expressed the distance between combination of environmental factors and the growth boundary ($\psi = 1$). For products with more than 5 weeks shelf-life, a ψ -value > 2 has been suggested to ensure no-growth responses despite intrinsic variability of product characteristics, storage conditions or strain variability (Dalgaard and Mejlholm, 2019).

3. Results

3.1. LC-MS/MS method for quantification of nisin in cheese

Clearly distinguishable peaks with retention times of 4.48 min for nisin A and 4.59 min for nisin Z were observed (Fig. 1). The LC-MS/MS method showed linear responses for nisin A and Z concentrations ranging from 0.1 to 1 mg/kg with correlation coefficients $r^2 > 0.99$. Residuals were randomly distributed, indicating variance homogeneity (Results not shown). The LOD for both nisin A and nisin Z in cheese was 0.04 mg/kg, and the LOQ was 0.12 mg/kg. Recovery of nisin A and Z in cheese tended to increase with nisin concentrations (Table 1). No matrix interference was observed for nisin A and Z detection in the tested cheeses. Precision was $< 16\%$ and nisin recoveries from the four cheeses ranged from 83 to 110 % and from 95 to 113 % for nisin A and Z, respectively (Table 1).

3.2. Quantification of nisin A and Z in cheese samples

Five out of the 13 analysed cheese samples were found to contain nisin A with concentrations from 0.16 to 0.19 mg/kg (Table 2). The product description of sample E specified a nisin A concentration of 12.5 mg/kg and the amount quantified in the present study was 98% lower. Samples H and J were produced with 11.2 and 18.8 mg/kg nisin A but concentrations quantified by LC-MS/MS were below the LOD and LOQ, respectively (Table 2).

3.3. Cardinal parameter term for nisin

The experimentally determined nisin MIC-values for pH 5.5 at 20°C or 25°C were 1.1 ± 0.1 ppm and 0.8 ± 0.1 ppm, respectively. At pH 6 and 25°C the nisin MIC-value was 9.3 ± 1.5 ppm (Fig. 2).

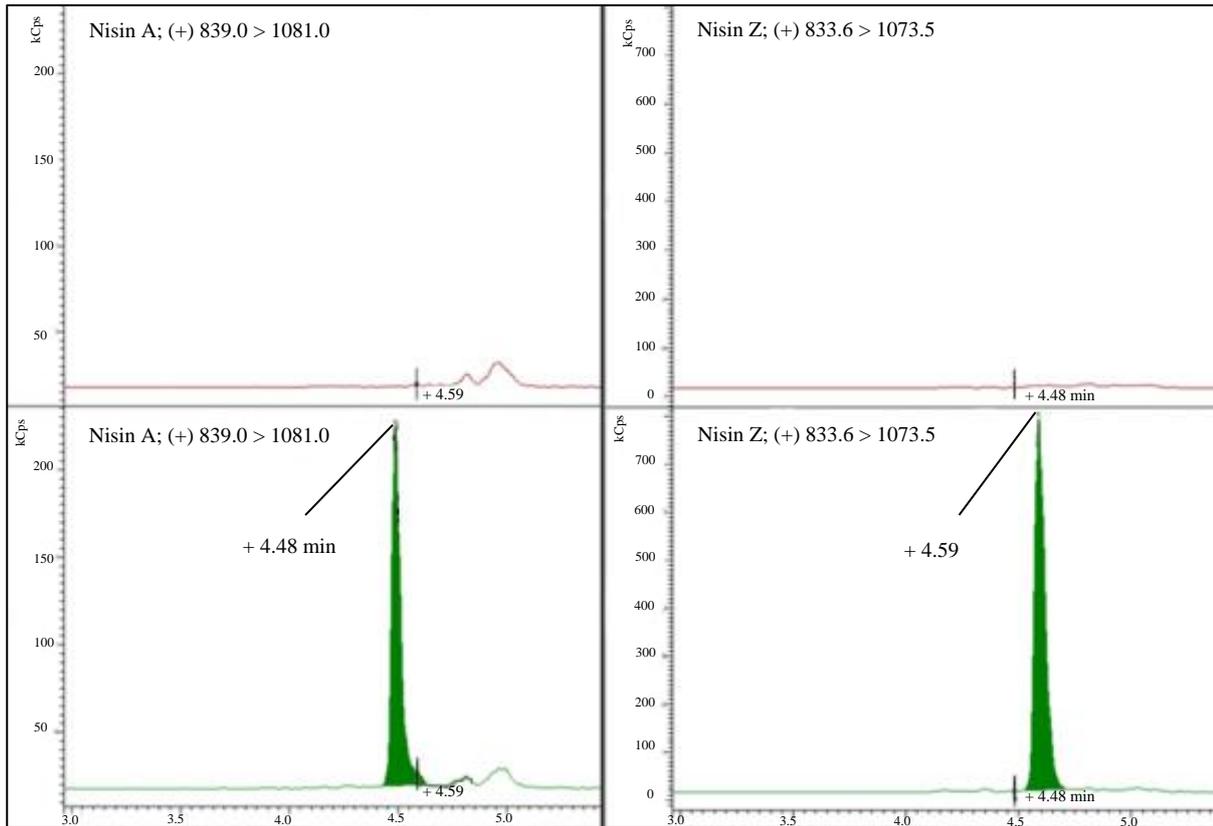


Figure 1. Chromatogram obtained after LC-MS/MS analysis of mascarpone cheese matrix non-spiked (top) and spiked (bottom) with nisin A and nisin Z (0.5 mg/kg).

Table 1. Recovery and precision for determination of nisin A and Z by LC-MS/MS

Nisin	Matrix	Conc. (mg/kg)	Recovery (%)	CV (%)
A	Processed cheese	0.1	99	13
		0.5	108	2
		1	110	4
	Spreadable processed cheese	0.1	83	14
		0.5	95	11
		1	100	9
	White cheese	0.1	90	15
		0.5	92	9
		1	101	7
	Mascarpone	0.1	88	14
		0.5	89	11
		1	95	6
Z	Processed cheese	0.1	113 ^a	8
		0.5	109	2
		1	107	5
	Spreadable processed cheese	0.1	97	13
		0.5	98	16
		1	98	12
	White cheese	0.1	99	12
		0.5	99	2
		1	102	8
	Mascarpone	0.1	105	15
		0.5	106	7
		1	95	6

^a Result outside acceptability range being: CV < 16 % and recovery within 80%-110%

Table 2. Quantification of nisin A and Z in cheeses by LC-MS/MS

Cheese sample	Matrix	ID ^a	Nisin	Concentration (mg/kg ± U) ^b	Added (mg/kg)
Laboratory ^c	White cheese	A	A	<LOD	2.5
			Z	<LOD	0
Laboratory ^c	White cheese	B	A	<LOD	2.5
			Z	<LOD	0
Commercial ^d	Mascarpone	C	A	<LOQ	2.5
			Z	<LOD	0
Commercial ^d	Mascarpone	D	A	0.16 ± 0.04	5
			Z	<LOD	0
Commercial ^e	White cheese	E	A	0.19 ± 0.05	12.5
			Z	<LOD	0
Commercial ^e	Unknown ^f	F	A	<LOQ	25
			Z	<LOD	0
Non-commercial ^e	Spreadable processed cheese	G	A	<LOD	0
			Z	<LOD	0
Non-commercial ^e	Spreadable processed cheese	H	A	<LOD	11.2
			Z	<LOD	0
Non-commercial ^e	Spreadable processed cheese	I	A	<LOD	11.2
			Z	<LOD	0
Non-commercial ^e	Spreadable processed cheese	J	A	<LOQ	18.8
			Z	<LOQ	0
Commercial ^e	Processed cheese	K	A	0.18 ± 0.05	11.2
			Z	<LOD	0
Commercial ^e	Processed cheese	L	A	0.19 ± 0.05	11.2
			Z	<LOD	0
Commercial ^e	Processed cheese	M	A	0.17 ± 0.04	11.2
			Z	<LOD	0

^a ID: Identification^b average ± uncertainty^c Produced in the laboratory^d Purchased in supermarket^e Supplied by Arla Foods^f Unknown matrix

In order to describe μ_{max} -values the best, n1 and n2 were found to be 1. Experimental and literature nisin MIC-values for *L. monocytogenes* increased with pH (Fig. 2). To predict MIC-values of nisin depending on pH a nisin MIC-term based on a regression model was developed (Eq. 2)

$$\sqrt{MIC_{nisin}(ppm)} = \sqrt{6.7 \cdot 10^{-5} \cdot pH^{5.8}} \quad R^2 = 0.94 \quad (2)$$

3.4. Challenge tests for chemically acidified and processed cheeses with added nisin

Nisin concentrations of < LOQ - 0.19 mg/kg as quantified by LC-MS/MS were found for chemically acidified and processed cheeses with 0-25 mg/kg of added nisin. *L. monocytogenes* grew in these cheeses with pH of 5.7 to 6.7 (Table 3 and 4). However, *L. monocytogenes* did not grow during 21 days at 15°C in challenge test 8 for chemically acidified cheese with pH 4.4, 12.5 mg/kg of added nisin (0.19±0.05 mg/kg quantified by LC-MS/MS) and 3.9% of water phase salt.

In challenge tests 5, 6 and 7 with mascarpone cheese a minor decrease in μ_{max} -values were observed for added nisin concentrations increasing from 0 to 5 mg/kg, but lower N_{max} (log cfu/g) was observed in products containing nisin (Table 3).

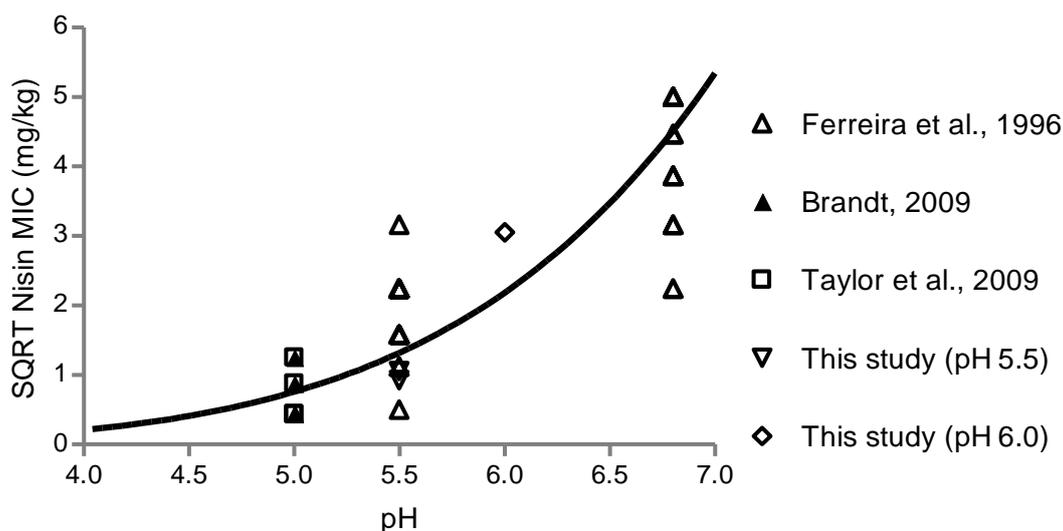


Figure 2. Effect of pH on nisin A MIC-values for *L. monocytogenes* in broth media.

3.5. Evaluation of model performance

For chemically acidified cheese (Table 3) model A used with no nisin or with residual concentrations of nisin measured by LC-MS/MS as model input predicted growth in all nine challenge tests resulting in 11% of fail-safe predictions (Table 5). Model A slightly underestimated growth rates of *L. monocytogenes* as shown by a B_f -value of 0.73-0.74 (Table 5).

However, when used with added concentrations of nisin as input to model A predicted growth rates were markedly underestimated as shown by a B_f -value of 0.57 (Table 5). Furthermore, with added nisin concentrations as model input as little as 56% of the growth/no-growth responses were correctly predicted and the 44% incorrect predictions were fail-dangerous (Table 5).

For processed cheese (Table 4), the performance of Model B was good with no nisin or with residual concentrations of nisin measured by LC-MS/MS as model input. B_f/A_f -values were 1.09/1.13 and 1.00/1.11, respectively (Table 5). However, when added concentrations of nisin were used as input, model B predicted no-growth in all challenge tests resulting in 100% of the predictions being fail-dangerous (Table 5).

4. Discussion

The present study included a first and very positive attempt to incorporate the antimicrobial effect of nisin in cardinal parameter models. Predictive models for chemically acidified cheese and processed cheese were developed and combined with quantification of residual nisin concentrations in these products by LC-MS/MS. In this way, both growth rates and growth/no-growth responses of *L. monocytogenes* were predicted with a precision that suggest the approach to be promising for further model development (Table 5). Importantly, when the quantified residual nisin concentrations in cheese were used as model input this resulted in markedly more accurate predictions for both growth rates and growth/no-growth responses than predictions based on the concentrations of nisin added to cheeses during processing (Table 5). The effect of nisin on growth of *L. monocytogenes* in combination with other environmental factors (temperature, pH, NaCl/ a_w) have previously been studied in broth and predictive models were suggested (Bouttefroy et al., 2000; Boziaris and Nychas, 2006; Parente et al., 1998).

Table 3. Storage conditions and product characteristics for challenge tests with chemically acidified cheese.

CT ^b	ID	n ^c	Nisin A ^d (mg/kg)	Storage temp. (°C)	Product characteristics measured (Avg.±SD) ^a				Observed growth parameter values (Avg. ±SD)			
					pH	Water phase salt (%)	Lactic acid in water phase (mg/kg)	Nisin A (mg/kg)	Lag-time (h)	Log N ₀ (Log cfu/g)	Log N _{max} (Log cfu/g)	μ _{max} (1/h)
1	1	3	0.0	9.1±0.2	5.7±0.0	2.8±0.0	ND ^e	- ^f	0.0±0.0	2.2±0.0	7.3±0.1	0.072±0.00
2	A	3	2.5	9.1±0.2	5.7±0.0	2.8±0.0	ND ^e	< LOD ^g	0.0±0.0	2.2±0.0	7.2±0.0	0.068±0.00
3	3	3	0.0	9.7±0.3	5.8±0.1	2.8±0.0	ND ^e	- ^f	0.0±0.0	2.2±0.1	7.4±0.1	0.094±0.01
4	B	3	2.5	9.7±0.3	5.8±0.1	2.8±0.0	ND ^e	< LOD ^g	0.0±0.0	2.0±0.4	8.1±0.1	0.080±0.01
5	5	3	0.0	6.1±0.1	6.7±0.2	0.5±0.0	1,222± 256	- ^f	0.0±0.0	2.4±0.1	8.0±0.1	0.060±0.00
6	C	3	2.5	6.1±0.1	6.7±0.0	0.4±0.1	1,458±157	< LOQ ^h	0.0±0.0	2.1±0.0	6.8±0.1	0.054±0.00
7	D	3	5.0	6.1±0.1	6.7±0.2	0.5±0.0	1,157±125	0.16±0.04	0.0±0.0	2.1±0.0	6.8±0.1	0.056±0.00
8	E	3	12.5	15.0±0.1	4.4±0.1	3.9±0.0	ND ^e	0.19±0.05	0.0±0.0	1.0±0.0	<1.0±0.0	0.000±0.00
9	F	3	25	15.1±0.1	6.6±0.1	0.2±0.0	ND ^e	< LOQ ^h	0.0±0.0	3.1±0.1	8.2±0.1	0.187±0.01

^a Avg: average; SD: standard deviation^b Challenge test.^c Number of growth curves per experiment.^d Added nisin or nisin concentration specified by the producer.^e ND: not detected by the HPLC^f Not analysed for nisin A^g LOD: limit of detection^h LOQ: limit of quantification

Table 4. Storage conditions and product characteristics for challenge tests with processed cheese (11.2 mg/kg nisin)^a.

CT ^c	ID	n ^d	Storage temp. (°C)	Product characteristics (Avg.±SD) ^b							Observed growth parameter values (Avg. ±SD)			
				pH	a _w	Lactic acid in water phase (ppm)	Acetic acid in water phase (ppm)	Citric acid in water phase (ppm)	P1 in water phase (%) ^e	Nisin A (mg/kg)	Lag-time (h)	Log N ₀ (Log cfu/g)	Log N _{max} (Log cfu/g)	μ _{max} (1/h)
10	L	3	10.0±0.3	5.9±0.1	0.981±0.001	9,415±293	2,471±60	5,030±26	0.9±NA ^f	0.19±0.05	0.0±0.0	0.8±0.0	4.2±0.3	0.022±0.00
11	L	3	15.0±0.1	6.0±0.1	0.983±0.003	9,132±147	1,908±685	3,766±1,731	0.9±NA	0.19±0.05	0.0±0.0	0.9±0.1	7.1±0.1	0.050±0.00
12	L	3	19.5±0.1	5.9±0.1	0.982±0.001	9,345± 275	2,384±2	4,947±47	0.9±NA	0.19±0.05	0.0±0.0	1.0±0.0	6.5±0.1	0.074±0.00
13	M	3	10.0±0.3	6.0±0.1	0.983±0.001	9,869± 46	2,305±81	5,070± 282	0.8±NA	0.17±0.04	0.0±0.0	0.9±0.1	4.6±0.2	0.023±0.00
14	M	3	15.0±0.1	6.1±0.0	0.981±0.001	9,364± 291	2,317±110	4,710± 119	0.8±NA	0.17±0.04	0.0±0.0	1.0±0.1	5.4±0.1	0.045±0.00
15	M	3	19.5±0.1	6.1±0.0	0.982±0.000	9,679±401	2,397±105	5,234±447	0.8±NA	0.17±0.04	0.0±0.0	1.1±0.1	5.6±0.1	0.063±0.01

^a Nisin concentration specified by producer^b Avg: average; SD: standard deviation^c Challenge test.^d Number of growth curves per experiment.^e P1: orthophosphate salt^f NA, not available. Information not provided by Eurofins.

Table 5. Observed and predicted growth of *L. monocytogenes*.

	Observed			Predicted		
	n ^a	Growth	No growth	Without nisin	Nisin by LC-MS/MS	Added nisin
	27	24	3	0 mg/kg	0-0.2 mg/kg	0-25 mg/kg
Model A-chemically acidified cheese			B _f /A _f ^b	0.74/1.35	0.73/1.36	0.57/1.74
			Correct (%)	89	89	56
			Fail-safe (%)	11	11	0
			Fail-dangerous (%)	0	0	44
	n ^a	Growth	No growth	Without nisin	Nisin by LC-MS/MS	Added nisin
	18	18	0	0 mg/kg	0-0.2 mg/kg	11.2 mg/kg
Model B-processed cheese			B _f /A _f ^b	1.09/1.13	1.00/1.11	- ^c
			Correct (%)	100	100	0
			Fail-safe (%)	0	0	0
			Fail-dangerous (%)	0	0	100

^a Number of growth curves

^b B_f: bias factor A_f: accuracy factor

^c Model predicted no-growth and therefore B_f/A_f –values could not be calculated.

These models, however, did not consider the distinction between residual and added nisin and they were not evaluated for their ability to predict growth of *L. monocytogenes* in foods containing nisin. The present study, represents substantial progress in this area although further studies are desirable to determine the precision and range of applicability for the two developed growth and growth boundary models developed in the present study to predict the inhibitory effect of nisin in cheeses.

Several previous studies reported no-growth or inactivation of *L. monocytogenes* in different types of processed cheese (Angelidis et al., 2010; Valero et al., 2018; Zottola et al., 1994). If these data concur with the present study cannot be determined precisely as residual concentrations of nisin and other product characteristic with influence on *L. monocytogenes* growth responses were not always quantified. Zottola et al. (1994) found inactivation of *L. monocytogenes* at 4°C or 23°C in spreadable Cheddar cheese with pH 5.1 and containing 0, 2.5 or 7.5 mg/kg of nisin as determined by the bioassay of Tramer and Fowler (1964). For this product the model developed in the present study (Model B) correctly predicted no-growth when assuming a realistic water activity of 0.95. Without nisin, model B predicted growth in the cheddar cheese but this incorrect prediction could be due to organic acid concentrations that were not reported by Zottola et al. (1994) and therefore not taken into account when growth was predicted.

The suggested model for the effect of pH on nisin A MIC-values of *L. monocytogenes* (Eq. 2) is in agreement with previous studies where nisin was more effective in acidic environments (Khan et al., 2015; Thomas and Wimpenny, 1996). Furthermore, higher antimicrobial and more consistent activity of nisin have been reported when dissolved in 0.02N HCl compared to distilled water (Hall, 1966). This effect may also be one reason for the wide range of nisin MIC-values reported in literature (Fig. 2). In fact, studies using a nisin stock solution other than the standard (nisin in 0.02N HCl and without autoclaving) recommended by FAO (2009) have estimated markedly different nisin MIC-values. For instance, Neetoo et al. (2008) estimated nisin MIC-values

at pH 5.5 between 4.8 and 19.0 mg/kg with a stock solution produced with acetic acid. Benkerroum and Sandine (1998) observed nisin MIC-values ranging from 0.1 to 2950 mg/kg when determined at pH 6.8 and using a nisin stock solution prepared in distilled water. Limited data on nisin Z MIC-values for *L. monocytogenes* is available (Meghroun et al., 1999; Niaz et al., 2018). To quantify nisin Z MIC-values and the effect of pH on these values for *L. monocytogenes* the approach used in the present study seems interesting particularly as the developed LC-MS/MS methods can quantify nisin Z in cheese.

The LC-MS/MS method developed for quantification of nisin A and Z in cheese provided recovery values similar to those previously obtained by Ko et al. (2016); however the present study used an extraction solvent without NaCl as it can interfere with ionization, cause ion suppression and broader peaks in the LC-MS/MS (Sterling et al., 2010).

Residual concentrations of nisin A quantified by the present study in processed cheese using LC-MS/MS were 98% lower than added concentrations stated by the producers. This difference, however, is in agreement with other studies. As example, Schneider et al. (2011) quantified 92% less nisin A by LC-MS/MS than initially measured in processed cheese after a 30 min. heat treatment at 120°C. Ko et al. (2016) also quantified low nisin A concentrations in processed cheese by LC-MS/MS (0.34-0.64 mg/kg) but no information was supplied about nisin concentrations described by producers. The degree of nisin losses depends, amongst others factors, on the heat treatment (temperature-time combination) involved in the manufacture of processed cheese. Therefore, it seems interesting in future studies to evaluate by LC-MS/MS the degree of nisin degradation depending on different heat treatments and to compared these results with both other methods for nisin quantification such as bio-assays and to the anti-listerial effect of the determined residual nisin concentrations.

For chemically acidified cheese it was surprising to quantify a low level of residual nisin A in mascarpone cheese as this cheese was not heat processed (Table 3, D). However, this may be the result of nisin degradation by binding of water to the aminoacid dehydroalanine and dehydrobutyrine units (Chan et al., 1989; Rolleman et al., 1996). Future studies should consider including quantification of nisin degradation compounds to attempt to fully describe nisin concentrations quantified by LC-MS/MS in cheese.

In conclusion, the present study quantified and modelled the effect of pH on nisin A MIC-values for *L. monocytogenes* and included the effect in cardinal parameter models that can predict the effect of residual nisin concentrations on growth in cheese. The model can support risk assessment and product development, but further studies with higher residual concentrations of nisin in cheeses will be beneficial for their validation.

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Paper VI

Martinez-Rios, V., Østergaard, N.B., Gkogka, E., Rosshaug, P.S., Dalgaard, P.

Modelling and predicting growth of psychrotolerant pseudomonads in milk and
cottage cheese

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Modelling and predicting growth of psychrotolerant pseudomonads in milk and cottage cheese

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Running title: Predicting pseudomonads growth in fresh dairy products

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ABSTRACT

Mathematical models were developed and evaluated for growth of psychrotolerant pseudomonads in chilled milk and in cottage cheese with cultured cream dressing. The mathematical models include the effect of temperature, pH, NaCl, lactic acid and sorbic acid. A simplified cardinal parameter growth rate model was developed based on growth in broth. Subsequently, the reference growth rate parameter $\mu_{ref25^{\circ}C}$ -broth of 1.03 1/h was calibrated by fitting the model to a total of 35 growth rates from cottage cheese with cultured cream dressing. This resulted in a $\mu_{ref25^{\circ}C}$ -cottage cheese value of 0.62 1/h. Prediction from both growth rate models were evaluated by comparison with literature and experimental data. Growth of psychrotolerant pseudomonads in heat-treated milk (n = 33) resulted in a bias factor (B_f) of 1.08 and an accuracy factor (A_f) of 1.32 ($\mu_{ref25^{\circ}C}$ -broth), whereas growth in cottage cheese with cultured cream dressing and in non-heated milk (n = 26) resulted in B_f of 1.08 and A_f of 1.43 ($\mu_{ref25^{\circ}C}$ -cottage cheese). Lag phase models were developed by using relative lag times and data from both the present study and from literature. The acceptable simulation zone method showed the developed models to successfully predict growth of psychrotolerant pseudomonads in milk and cottage cheese at both constant and dynamic temperature storage conditions. The developed models can be used to predict growth of psychrotolerant pseudomonads and shelf life of chilled cottage cheese and milk at constant and dynamic storage temperatures. The applied methodology and the developed models seem likely to be applicable for shelf life assessment of other types of products where psychrotolerant pseudomonads are important for spoilage.

Keywords. Simplified cardinal parameter model, dairy products, model validation, spoilage, shelf life

1.0 Introduction

The genus *Pseudomonas* includes several psychrotolerant species with the ability to grow to high concentrations in various chilled foods. Different psychrotolerant *Pseudomonas* species including *P. fluorescens*, *P. fragi*, *P. lundensis* and *P. putida* have been associated with spoilage of foods of both plant- and animal origin. Often these bacteria cause sensory spoilage, with fruity and nauseous off-odours, when they reach concentrations above 7 log CFU/g in for example, raw milk, pasteurized milk, fresh fish, poultry, lamb, pork and beef products (Borch et al., 1996; Dainty and Mackey, 1992; Liao, 2006; Sørhaug and Stepaniak, 1997; Tryfinopoulou et al., 2002). Specifically, *P. fluorescens* were determined as causative agent of a blue pigment on the surface of fresh and low-acid cheese (Martin et al., 2011). Furthermore, psychrotolerant pseudomonads have been responsible for spoilage of cottage cheese; a soft unripe, mildly acidic cheese consisting of fermented curd granules and a cream dressing. The fermented curd is produced from skim milk by using a classical mesophilic starter culture containing *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The cream dressing can be fresh or cultured using *L. lactis* subsp. *lactis* biovar. *diacetylactis*. Cottage cheese with cultured cream dressing is a popular product in Scandinavian and is characterized by high water activity (> 0.99), a low concentration of salt in the water phase (ca. 1%), pH below 5.5 and a content of lactic acid originating from the fermentation (Mather and Babel, 1959; Østergaard et al., 2014, Østergaard et al., 2015).

Growth of psychrotolerant pseudomonads can be substantially reduced by packaging using vacuum (Liao, 2006) or modified atmospheres with high concentrations of CO₂ or both CO₂ and O₂ (Geysen et al., 2005). However, for aerobic storage of cottage cheese it is most important to manage the washing and handling of curd granules after fermentation, as these processing steps can introduce psychrotolerant pseudomonads (Ledenbach and Marshall, 2010). Cream dressing with sorbic acid, bifidobacteria or cultured with *L. lactis* subsp. *lactis* biovar. *diacetylactis* has also been

used to reduce growth of psychrotolerant pseudomonads in cottage cheese (Brocklehurst and Lund, 1985; Kosikowski and Brown, 1973; Kristoffersen and Chakraborty, 1964; O’Riordan and Fitzgerald, 1998).

Predictive food microbiology models have the potential to evaluate the effect of temperature conditions on microbial growth during food distribution and extensive models, including the effect several environmental factors, can be used to determine how product characteristics can be modified to reduce growth to an acceptable level. This has been demonstrated for different spoilage and human pathogenic microorganisms including lactic acid bacteria and *Listeria monocytogenes* (Augustin et al., 2005; Mejlholm and Dalgaard, 2013; Østergaard et al., 2014). Despite their importance for spoilage and shelf life of various fresh and some lightly preserved foods, no extensive growth model has been developed for psychrotolerant pseudomonads. In contrast, numerous simpler predictive models have been developed. Various models include the effect of temperature (See e.g. Bruckner et al., 2013; Chandler and McMeekin, 1989), modified atmosphere (Geysen et al., 2005), temperature and modified atmosphere (Koutsoumanis et al., 2000), temperature and water activity (Davey, 1989; Neumeyer et al., 1997), temperature and pH (Koutsoumanis et al., 2006; Pin et al., 1999) or temperature, pH and salt/water activity (Braun and Sutherland, 2003; Lebert et al., 2000; Membre and Burlot, 1994).

It can be challenging to model and predict growth of spoilage or pathogenic microorganisms in fermented food during chilled storage (De Vuyst and Leroy, 2007; Irlinger and Mounier, 2009). For cottage cheese inhibiting substances produced by lactic acid bacteria during fermentation or later during storage and distribution may inhibit growth of spoilage or pathogenic microorganisms. However, Østergaard et al. (2014) recently modelled and predicted growth of *L. monocytogenes* in cottage cheese by using simplified cardinal parameter models and by taking into account the inhibiting effect of lactic acid bacteria. A similar approach seems interesting for growth of

psychrotolerant pseudomonads in both milk and cottage cheese where they can be responsible for spoilage. The objective of the present study was therefore to develop and evaluate mathematical models for growth of psychrotolerant pseudomonads in chilled milk and in cottage cheese with cultured cream dressing.

2. Materials and methods

2.1. Overview of experimental work

A simplified cardinal parameter growth rate model for dairy isolates of psychrotolerant pseudomonads was developed from maximum specific growth rate values (μ_{max} , 1/h) determined in broth using serially diluted cultures and absorbance detection times obtained with a BioScreen C instruments. More than 1800 absorbance detection times were generated in broth to quantify the effect of temperature, pH, water activity/salt, lactic acid and sorbic acid. Secondly, growth curves of psychrotolerant pseudomonas were generated in challenge tests using milk and cottage cheese with cultured cream dressing (Table 1). These product data were used to (i) evaluate if there was an inhibiting effect of lactic acid bacteria on growth of psychrotolerant pseudomonads in cottage cheese, (ii) determine growth parameters of psychrotolerant pseudomonads, (iii) calibrate the μ_{max} -model developed using broth data to growth of psychrotolerant pseudomonas in cottage cheese and (iv) to develop lag time models based on relative lag times (*RLT*). Finally, the developed models were evaluated by comparison of predicted growth and growth of psychrotolerant pseudomonads determined in products not used for calibration or development of the models. Bias- and accuracy factors as well as acceptable simulation zone (*ASZ*) method were used as indices of model performance in validation studies. For the broth model, 30 μ_{max} -values from literature and three

μ_{max} -values determined in this study were used to calculate bias- and accuracy factor values for heated milk (Table 2, Table 3). For the cottage cheese model, 20 μ_{max} -values from literature and six μ_{max} -values determined in this study were used to calculate bias- and accuracy factor values for non-heated milk and cottage cheese (Table 2, Table 3). The ASZ was calculated for three growth curves of psychrotolerant pseudomonads in pasteurized milk at dynamic temperature storage conditions, six growth curves in cottage cheese, three growth curves in non-heated milk at constant temperatures and three growth curves in cottage cheese at dynamic temperature storage conditions (Table 1).

2.2. Bacterial strains, pre-culture conditions and inoculation

Two strains of psychrotolerant *Pseudomonas* species from milk and cottage cheese (strain 11A6 and 11A65) were provided by Arla Strategic Innovation Center (ASIC). The two strains were used as a cocktail (Ps-mix) to determine μ_{max} -values in broth and for inoculation of challenge tests. Prior to studies of growth each strain was transferred from storage at -80 °C to Brain Heart Infusion (BHI) broth (84626, Merck, Darmstadt, Germany) and incubated 24 h at 25 °C. Subsequently, for broth studies the strains were pre-cultured two days at 8 °C in BHI broth with 1% NaCl, 0.2% glucose, 0.3 % yeast extract (BHI-G-Y) at pH 5.2. For challenge tests the strains were pre-cultured two days at 8 °C in BHI-G-Y at pH 5.2 and with 500 ppm of lactic acid (LAC) to simulate conditions of cottage cheese. Pre-cultures were grown to a relative increase in absorbance (540 nm) of 0.05 to 0.2 (Novaspec II, Pharmacia Biotech, Allerød, Denmark). Ps-mix was prepared from the two pre-cultures and then diluted to the desired concentration.

2.3. Development of growth rate model in broth

2.3.1. Effect of environmental parameters on growth rates

The effect of temperature, pH, a_w , lactic acid and sorbic acid on μ_{max} -values of Ps-mix were determined. For each condition studied, growth of Ps-mix was determined in duplicate or triplicate by automated absorbance measurements at 540 nm (BioScreen C, LabSystems, Helsinki, Finland). Detection time, defined as incubation time until an increase in absorbance of 0.05 from the lowest absorbance value after inoculation, was determined for each absorbance growth curve. μ_{max} -values of Ps-mix were determined from absorbance detection times for serially diluted inoculation levels of ca. 10^2 , 10^3 , 10^4 , 10^5 and 10^6 CFU/ml as previously described (Dalgaard and Koutsoumanis, 2001). The effect of temperature and pH on μ_{max} -values was determined at 5 °C, 10 °C and 15 °C using BHI-G-Y adjusted with HCl to pH 5.00, 5.28, 5.59, 5.87, 6.33 and 6.93. The effect of water activity (a_w) on μ_{max} -values was studied at 8 °C in BHI-G-Y with pH 5.2 and for a range of NaCl concentrations from 0.5 % to 8% (w/v). a_w -values were calculated from the concentration of water phase salt (%WPS) as $a_w = 1 - 0.0052471 \cdot \%WPS - 0.00012206 \cdot \%WPS^2$ (Chirife and Resnik, 1984). Effects of lactic acid (0 – 40,000 ppm) and sorbic acid (0 – 4,500 ppm) on μ_{max} -values were determined separately at 8 °C in BHI-G-Y adjusted to pH 5.2 after addition of the organic acid and again after autoclaving the broth if relevant.

2.3.2. Determination of cardinal parameter values and development of growth rate model from broth data

To determine the cardinal parameter values for temperature (T_{min}) and pH (pH_{min}) eq. (1) was fitted to the determined and square root transformed μ_{max} -values.

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref\ 25^\circ C} \cdot \left(\frac{T - T_{min}}{T_{ref} - T_{min}}\right)^2 \cdot (1 - 10^{(pH_{min} - pH)})} \quad (1)$$

where $\mu_{ref_{25^\circ C}}$ is a fitted parameter that corresponds to μ_{max} at the reference temperature (T_{ref}) of 25°C when other studied environmental parameters are not inhibiting growth (Dalgaard, 2009, Delignette-Muller et al., 2006). T (°C) is the storage temperature, T_{min} is the theoretical minimum temperature for growth and pH_{min} is the theoretical minimum pH for growth.

The cardinal parameter value for water activity ($a_{w_{min}}$) was determined by fitting eq. (2) to square root transformed μ_{max} -values.

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref_{8^\circ C}} \cdot \left(\frac{a_w - a_{w_{min}}}{1 - a_{w_{min}}} \right)^2} \quad (2)$$

where $a_{w_{min}}$ is the theoretical minimum water activity for growth.

Cardinal parameter values for lactic acid ($MIC_{U \text{ Lactic acid}}$) and sorbic acid ($MIC_{U \text{ Sorbic acid}}$) were determined from concentrations of the undissociated acids as calculated by eq. (3) with pK_a values of 3.86 and 4.76 for lactic- and sorbic acid, respectively (Ross and Dalgaard, 2004). The cardinal parameter values were estimated by fitting eq. (4) and eq. (5) to square root transformed μ_{max} -values.

$$\text{Undissociated organic acid (mM)} = \frac{\text{Organic acid (mM)}}{1 + 10^{pH - pK_a}} \quad (3)$$

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref_{8^\circ C}} \cdot \left(1 - \left(\frac{[LAC_U]}{MIC_{U \text{ Lactic acid}}} \right)^{n1} \right)^{n2}} \quad (4)$$

$$\sqrt{\mu_{max}} = \sqrt{\mu_{ref_{8^\circ C}} \cdot \left(1 - \left(\frac{[SAC_U]}{MIC_{U \text{ Sorbic acid}}} \right)^{n1} \right)^{n2}} \quad (5)$$

where $[LAC_U]$ and $[SAC_U]$ are the concentrations (mM) of undissociated lactic- and sorbic acid and $MIC_{U \text{ Lactic acid}}$ and $MIC_{U \text{ Sorbic acid}}$ are, respectively, fitted minimum inhibitory concentration (MIC) values (mM) of undissociated lactic- and sorbic acid that prevent growth of Ps-mix. For each

organic acid term, n1 was set to 1 or 0.5 and n2 was set to 1 or 2 (Dalgaard, 2009) in order to describe data most appropriately and this was determined from root mean square error (RMSE) values.

Finally, the estimated cardinal parameter values $\mu_{ref_{25^{\circ}C}}$, T_{min} , pH_{min} , $a_{w_{min}}$, $MIC_{U \text{ Lactic acid}}$ and $MIC_{U \text{ Sorbic acid}}$ were combined with terms for the effect of temperature, pH, a_w , lactic acid and sorbic acid on μ_{max} -values of Ps-mix and included in eq. (6).

$$\begin{aligned} \mu_{max} = & \mu_{ref_{25^{\circ}C}} \cdot \left(\frac{T - T_{min}}{T_{ref} - T_{min}} \right)^2 \cdot (1 - 10^{(pH_{min} - pH)}) \cdot \left(\frac{a_w - a_{w_{min}}}{1 - a_{w_{min}}} \right)^2 \\ & \cdot \left(1 - \left(\frac{[LAC_U]}{MIC_{U \text{ Lactic acid}}} \right)^{n1} \right)^{n2} \cdot \left(1 - \left(\frac{SAC_U}{MIC_{U \text{ Sorbic acid}}} \right)^{n1} \right)^{n2} \cdot \xi \end{aligned} \quad (6)$$

The effect on μ_{max} -values of interaction between environmental parameters (ξ) was modelled as previously described using the Le Marc approach (Le Marc et al., 2002; Mejlholm and Dalgaard, 2013). This growth rate model (Eq. (6)) based on μ_{max} -values in broth had a $\mu_{ref_{25^{\circ}C}}$ -value of 1.03 1/h and it was used directly to predict μ_{max} -values of psychrotolerant pseudomonads in heated milk. Furthermore, the model's $\mu_{ref_{25^{\circ}C}}$ -value was calibrated by fitting eq. (6) to a total of 35 μ_{max} -values for psychrotolerant pseudomonads growing in cottage cheese with cultured cream dressing while keeping all other cardinal parameter values constant. This was performed as previously described (See e.g. Østergaard et al. 2014) and resulted in a $\mu_{ref_{25^{\circ}C}}$ -value of 0.62 1/h.

2.4. Challenge tests and storage trials

2.4.1. Product characteristics

Product characteristics for five batches of cottage cheese with cultured cream dressing (multiple strains of *L. lactis* subsp. *lactis* biovar. *diacetylactis*, F-DVS SDMB-4, Chr. Hansen A/S, Hørsholm, Denmark) and one batch of pasteurized milk were studied over a period of 36 months (Table 1). Cottage cheese and pasteurized milk were produced by Arla Foods and packed with ice during transport to DTU Food. Once received, the products were stored at 2°C for a maximum of 48h until further studied. pH was measured with a PHM 250 Ion Analyzer (MetroLab™, Radiometer, Copenhagen, Denmark) in 10 g of product homogenised with 90 g of physiological saline (PS, 0.85% NaCl and 0.10% Bacto-peptone). NaCl was quantified by automated potentiometric titration (785 DMP Titrino, Metrohm, Hesisau, Switzerland). The concentration of lactic- and sorbic acids was determined by HPLC using external standards for identification and quantification. In order to improve extraction of acids a centrifugation step was applied (Dalgaard and Jørgensen, 2000; Østergaard et al., 2014). Lactic acid bacteria (LAB) were enumerated by pour plating in nitrite actidione polymyxin (NAP) agar with pH 6.2 (see Section 2.4.2).

[Table 1 close to here]

2.4.2. Inoculation, storage conditions, microbiological analysis and sensory evaluation

Growth of psychrotolerant pseudomonads were determined for cottage cheese in 12 inoculated challenge tests including 38 growth curves and in two storage trial with naturally contaminated samples and including six growth curves. In addition, two challenge test including six growth curves were performed with milk (Table 1).

1 **Table 1** Storage conditions and product characteristics for challenge tests and storage trials with cottage cheese and milk.

Use of data	Exp.	Product	Reference/data source	Inoculum	n ^f	Storage Temp. (°C)	Product characteristics prior to storage			
							LAB (Log CFU/g)	pH	Water phase salt (%)	Lactic acid in water phase (ppm)
Calibration of growth rate model and development of lag time model ^a	A	Cottage cheese	This study	I ^d	4	4.6 ± 0.9	5.6 ± 0.1	5.4 ± 0.0	1.11 ± 0.00	996 ± 246
	B	Cottage cheese	This study	I ^d	4	10.4 ± 0.3	5.6 ± 0.1	5.4 ± 0.0	1.11 ± 0.00	996 ± 246
	C	Cottage cheese	This study	I ^d	3	4.7 ± 0.1	8.5 ± 0.3	5.3 ± 0.0	0.89 ± 0.00	971 ± 202
	D	Cottage cheese	This study	I ^d	3	9.6 ± 0.2	8.5 ± 0.3	5.4 ± 0.0	0.89 ± 0.00	971 ± 202
	E	Cottage cheese	This study	I ^d	3	14.9 ± 0.3	8.5 ± 0.3	5.4 ± 0.0	0.89 ± 0.00	971 ± 202
	F	Cottage cheese	This study	I ^d	3	4.8 ± 0.3	7.3 ± 0.3	5.4 ± 0.0	1.09 ± 0.00	445 ± 37
	G	Cottage cheese	This study	I ^d	3	9.9 ± 1.1	7.3 ± 0.3	5.4 ± 0.0	1.09 ± 0.00	445 ± 37
	H	Cottage cheese	This study	I ^d	3	14.7 ± 0.2	7.3 ± 0.3	5.4 ± 0.0	1.09 ± 0.00	445 ± 37
	I	Cottage cheese	This study	I ^d	3	9.7 ± 0.3	ND ^h	5.3 ± 0.0	0.93 ± 0.00	556 ± 107
	J	Cottage cheese	This study	I ^d	3	19.4 ± 0.1	7.4 ± 0.2	5.4 ± 0.0	0.99 ± 0.00	541 ± 114
	K	Cottage cheese	This study	I ^d	3	19.4 ± 0.1	7.2 ± 0.2	5.3 ± 0.0	0.99 ± 0.00	549 ± 97
Development of lag time model ^b	L	Pasteurized milk	This study	I ^d	3	6.8 ± 0.1	ND ^h	6.7 ± 0.0	ND ^h	ND ^h
	M	Heated milk	Matis et al. (1994)	I ^d	2	7 ± 0.0	- ⁱ	6.7 ± 0.0	- ⁱ	- ⁱ
	N	Heated milk	Shelley et al. (1986)	I ^d	4	5 ± 0.0	- ⁱ	6.7 ± 0.0	- ⁱ	- ⁱ
Evaluation of models ^c	O	Pasteurized milk	This study	I ^d	3	4.6 - 9.8 ^g	ND ^h	6.7 ± 0.0	0.00 ± 0.00	ND ^h
	P	Cottage cheese	This study	NC ^e	3	9.6 ± 0.2	8.5 ± 0.3	5.4 ± 0.0	0.89 ± 0.00	971 ± 202
	Q	Cottage cheese	This study	NC ^e	3	14.9 ± 0.3	8.5 ± 0.3	5.4 ± 0.0	0.89 ± 0.00	971 ± 202
	R	Cottage cheese	This study	I ^d	3	4.1 - 19.3 ^g	7.3 ± 0.3	5.4 ± 0.0	1.09 ± 0.00	445 ± 37
	S	Non-heated milk	Sierra et al. (1996)	NC ^e	1	7 ± 0.0	- ⁱ	6.8 ± 0.0	- ⁱ	- ⁱ
	T	Non-heated milk	Zapico et al. (1995)	I ^d	1	4 ± 0.0	- ⁱ	6.5 ± 0.0	- ⁱ	- ⁱ
	U	Non-heated milk	Zapico et al. (1995)	I ^d	1	8 ± 0.0	- ⁱ	6.5 ± 0.0	- ⁱ	- ⁱ

- 2 ^a Data used for calibration of growth rate model from broth and for development of lag time model for cottage cheese.
3 ^b Data used for development of lag time model for heated milk.
4 ^c Data used for evaluation of heated milk and cottage cheese/non-heated milk models at constant and dynamic temperatures by using the ASZ approach.
5 ^d I, inoculated with psychrotolerant pseudomonads (Ps-mix).
6 ^e NC, naturally contaminated.
7 ^f Number of growth curves.
8 ^g Challenge test at dynamic temperatures.
9 ^h ND, not determined.
10 ⁱ Information not available in the specific study.

11 Cottage cheese or milk used in challenge tests were inoculated with 0.1% (v/w) of Ps-mix
12 appropriately diluted in PS to obtain an initial concentration in the range of 2-5 log (CFU/g).
13 Following inoculation, 100 ± 5 g of cottage cheese was placed in the same type of containers as
14 used for commercial distribution of the product. The same procedure was followed for storage
15 trials, but without inoculation. Samples were then stored at 5, 10, 15, 20°C or under dynamic
16 temperatures (Table 1). Storage temperature was regularly recorded by data loggers (TinytagPlus,
17 Gemini Data Loggers Ltd, Chichester, UK). At each time of sampling a container with 100 ± 5 g of
18 cottage cheese was analysed and then discarded. 10 g of cottage cheese were diluted 10-fold in
19 chilled PS and subsequently homogenized for 30 s at normal speed in a Stomacher 400 (Seward
20 Medical, London, UK). For milk, at each time of sampling, 10 g were aseptically sampled from a
21 1L container, 10-fold diluted in chilled PS and subsequently homogenized for 30 s at normal speed
22 in a Stomacher 400. 10-fold dilutions were then performed with chilled PS. Viable counts of
23 psychrotolerant pseudomonads and LAB were, respectively, determined by surface plating on
24 Pseudomonads agar (CM0559, Oxoid, Basingstoke, UK) with CFC selective supplement (SR0103,
25 Oxoid) and incubation at 25 °C for 48 h and by pour plating in nitrite actidione polymyxin (NAP)
26 agar (pH 6.2) with incubation in 90 % N₂ and 10 % CO₂ at 25 °C for 72 h (Davidson and Cronin,
27 1973).

28 Growth of psychrotolerant pseudomonads and LAB were determined for cottage cheese by
29 performing 12 challenge tests including 35 growth curves at constant temperature (Table 2, Exp. A-
30 K), three growth curves under dynamic temperature storage conditions (Table 2, Exp. R) and two
31 storage trials including six growth curves at constant temperature (Table 2, Exp. P and Q). Two
32 challenge tests exclusively with enumeration of psychrotolerant pseudomonads and including six
33 growth curves were performed with milk under constant and dynamic temperature storage
34 conditions (Table 2, Exp. L and O).

Table 2 Growth parameters of psychrotolerant pseudomonads and sensory spoilage in challenge tests and storage trials with cottage cheese and milk.

Use of data	Exp.	Product	Growth parameter values (Avg \pm SD) ^a					Shelf life (days)	Sensory spoilage characteristics
			t_{lag} (h)	RLT (h)	$Log N_0$ (Log CFU/g)	$Log N_{max}$ (Log CFU/g)	μ_{max} (1/h)		
Calibration of growth rate model and development of lag time model	A	Cottage cheese	37.3 \pm 1.2	2.0 \pm 0.2	5.4 \pm 0.2	9.9 \pm 0.2	0.037 \pm 0.00	ND ^c	ND ^c
	B	Cottage cheese	66.0 \pm 33.1	6.1 \pm 3.8	5.2 \pm 0.2	9.8 \pm 0.0	0.047 \pm 0.01	ND ^c	ND ^c
	C	Cottage cheese	112 \pm 3.4	8.2 \pm 0.2	3.8 \pm 0.1	9.9 \pm 0.1	0.051 \pm 0.00	12 - 16	Fruity-melon odour
	D	Cottage cheese	26.4 \pm 4.7	2.9 \pm 0.5	3.6 \pm 0.1	9.7 \pm 0.0	0.075 \pm 0.00	8 - 10	Fruity, matured cheese
	E	Cottage cheese	6.1 \pm 1.4	0.96 \pm 0.2	3.4 \pm 0.1	9.5 \pm 0.1	0.96 \pm 0.00	6 - 7	Sweet off-odour
	F	Cottage cheese	46.4 \pm 9.0	4.4 \pm 1.0	3.9 \pm 0.1	9.6 \pm 0.1	0.066 \pm 0.00	9 - 12	Sweet off-odour, yellow
	G	Cottage cheese	4.6 \pm 3.1	0.68 \pm 0.5	3.6 \pm 0.1	9.4 \pm 0.1	0.10 \pm 0.00	4 - 5	Sweet off-odour, yellow
	H	Cottage cheese	9.4 \pm 1.0	2.8 \pm 0.3	3.6 \pm 0.1	9.0 \pm 0.0	0.21 \pm 0.00	4 - 5	Sweet off-odour, yellow
	I	Cottage cheese	1.3 \pm 1.9 ^b	0.2 \pm 0.3	1.95 \pm 0.04	9.0 \pm 0.0	0.11 \pm 0.01	5 - 6	Fruity, matured cheese
	J	Cottage cheese	10.5 \pm 1.3	5.4 \pm 0.9	3.6 \pm 0.2	9.1 \pm 0.0	0.35 \pm 0.02	3 - 4	Sweet off-odour, yellow
	K	Cottage cheese	9.3 \pm 1.5	2.0 \pm 0.3	3.9 \pm 0.1	9.1 \pm 0.0	0.15 \pm 0.01	4 - 6	Sweet off-odour, yellow
Development of lag time model	L	Pasteurized milk	3.8 \pm 5.4 ^b	0.6 \pm 0.9	3.9 \pm 0.4	8.2 \pm 0.2	0.13 \pm 0.01	ND ^c	ND ^c
	M	Heated milk	15.7 \pm 4.1	4.4 \pm 1.7	3.6 \pm 0.6	8.1 \pm 0.7	0.20 \pm 0.01	-	-
	N	Heated milk	20.9 \pm 8.0	3.5 \pm 0.7	3.6 \pm 0.3	7.5 \pm 0.5	0.12 \pm 0.02	-	-
Evaluation of models	O	Pasteurized milk	-	-	-	-	-	ND ^c	ND ^c
	P	Cottage cheese	25.7 \pm 0.0	3.1 \pm 0.2	3.2 \pm 0.1	10.2 \pm 0.1	0.083 \pm 0.01	8 - 10	Fruity-melon odour
	Q	Cottage cheese	9.9 \pm 14.0 ^b	2.2 \pm 3.1	3.2 \pm 0.1	9.5 \pm 0.1	0.11 \pm 0.00	6 - 7	Fruity, matured cheese
	R	Cottage cheese	-	-	-	-	-	ND ^c	ND ^c
	S	Non-heated milk	0.81 \pm 0.0	0.09 \pm 0.0	5.1 \pm 0.0	8.2 \pm 0.0	0.081 \pm 0.00	-	-
	T	Non-heated milk	4.11 \pm 0.0	0.49 \pm 0.0	4.15 \pm 0.0	8.3 \pm 0.0	0.083 \pm 0.00	-	-
	U	Non-heated milk	5.43 \pm 0.0	1.01 \pm 0.0	4.15 \pm 0.0	8.5 \pm 0.0	0.129 \pm 0.00	-	-

^a t_{lag} : lag time; ; RLT : relative lag time; $log N_{max}$: maximum population density; μ_{max} : maximum specific growth rate.

^b One growth curve had a significant lag time out of three growth curves.

^c ND, not determined.

40 Sensory evaluation was performed for cottage cheese at regular intervals. At each time of
 41 sampling three containers with product from each treatment were analysed by 3-4 trained and
 42 experienced sensory panellists. Changes in overall appearance and odour of the samples were
 43 evaluated by using a simple three-class scale (I, II and III) with class III corresponding to sensory
 44 rejection (Dalgaard, 2000). Shelf life was determined as the time range corresponding the times of
 45 sampling within which 50% of the panellists evaluated samples from a treatment in the challenge
 46 test or storage trials to be in class III. In addition, the panellists were asked to describe the sensory
 47 characteristics of the samples and particularly off-odours using a predefined vocabulary and/or their
 48 own words.

49

50 2.4.3. Primary growth model

51 The integrated and log transformed logistic growth model with delay (four parameter model)
 52 or without delay (three parameter model) (Eq. (7); Rosso et al., 1996) was fitted to all individual
 53 growth curves of psychrotolerant pseudomonads obtained in challenge test and storage trials at
 54 constant temperatures. Fitted parameter values for initial cell concentration ($\log N_0$, \log CFU/g), lag
 55 time (t_{lag} , h), maximum specific growth rate (μ_{max} , 1/h) and maximum population density ($\log N_{max}$,
 56 \log CFU/g) were determined for each growth curve and data was reported as average \pm standard
 57 deviation for each treatment (Table 2). An F-test was used to determine if the lag time was
 58 significant.

$$59 \quad \log(N_t) = \log(N_0) \quad \text{if } t < t_{lag}$$

$$60 \quad \log(N_t) = \log\left(\frac{N_{max}}{1 + \left(\frac{N_{max}}{N_0} - 1\right) \cdot \exp(-\mu_{max} \cdot (t - t_{lag}))}\right) \quad \text{if } t \geq t_{lag} \quad (7)$$

61

62 where t is the storage time (h) and N_t the cell concentration (CFU/g) at time t . Other parameters are
63 as indicated above.

64

65 2.4.4. Lag time model for psychrotolerant pseudomonads

66 The relative lag time ($RLT = t_{lag} \cdot \mu_{max}/\ln(2)$) (Mellefont and Ross, 2003) was calculated for
67 all growth curves of psychrotolerant pseudomonads in challenge tests and storage trials (Table 2).
68 The applied lag time model is shown below (Eq. (8)). Three RLT -values from this study (Table 2,
69 Exp. L) and six RLT -values from literature (Table 2, Exp. M and N) were used to develop a lag time
70 model for heated milk. For cottage cheese, 35 RLT -values from this study (Table 2, Exp. A to K)
71 were used to develop a lag time model. It was evaluated if RLT -values were constant ($RLT = K_1$) or
72 dependent on storage temperature ($RLT = K_1 + K_2/T^2$) as reported by Hereu et al. (2014).

$$73 \quad t_{lag} = \frac{RLT \cdot \ln(2)}{\mu_{max}} = \frac{(K_1 + K_2/T^2) \cdot \ln(2)}{\mu_{max}} \quad (8)$$

74

75 2.5. Evaluation of growth models for psychrotolerant pseudomonads

76 This evaluation was carried out (i) to estimate the performance of growth rate models at
77 constant storage temperatures and (ii) to assess the performance of the combined primary (Eq. (7))
78 and secondary growth models (Eq. (6) and Eq. (8)), that include lag time and growth rate models,
79 under constant and dynamic storage temperatures.

80

81 2.5.1. Evaluation of growth rate models

82 Performance of growth rate models was evaluated by calculation of bias- (B_f) and accuracy
83 (A_f) factors (Ross, 1996) from observed and predicted μ_{max} -values. For spoilage microorganisms,
84 $0.85 < B_f < 1.25$ has been suggested as a criterion for good model performance and in addition A_f -

85 values > 1.5 was shown to indicate incomplete models or systematic deviation between observed
86 and predicted μ_{max} -values (Mejlholm and Dalgaard, 2013).

87 Three growth curves of psychrotolerant pseudomonads generated in this study in a challenge
88 test (Table 2, Exp. L) and 30 growth rates in heated milk obtained from literature at constant storage
89 temperatures (Table 3) were used to evaluate the performance of the developed growth rate model
90 from broth data.

91 Six growth curves of psychrotolerant pseudomonads generated in this study in storage trials
92 with cottage cheese (Table 2, Exp. P and Q) and 23 growth rates from literature in non-heated milk
93 (Table 3) were used to evaluate the performance of the cottage cheese growth rate model at constant
94 temperatures.

95 For literature data, if not reported, it was assumed that milk had a_w of 0.997 and pH of 6.7.
96 Maximum specific growth rates, μ_{max} (1/h) and lag times (h), estimated from literature data were
97 obtained by fitting the logistic growth model with delay (Eq. (7)).

98 [Table 3 close to here]

99

100 2.5.2. Evaluation of combined growth rate and lag time models

101 The combined growth rate (Eq. 6 with $\mu_{ref25^\circ C}$ of 1.03 1/h) and heated milk lag time model
102 were evaluated using three growth curves of psychrotolerant pseudomonads at dynamic temperature
103 storage conditions (Table 2, Exp. O). To evaluate the performance of the combined growth rate (Eq.
104 6 with $\mu_{ref25^\circ C}$ of 0.62 1/h) and cottage cheese lag time model we used six growth curves of
105 psychrotolerant pseudomonads in cottage cheese at constant temperatures (Table 2, Exp. P and Q),
106 three growth curves in non-heated milk at constant temperature (Table 2, Exp. S to U) and three
107 growth curves in cottage cheese at dynamic temperature storage conditions (Table 2, Exp. R).

108 **Table 3** Literature data on psychrotolerant pseudomonads growing in milk and used for model evaluation at constant storage temperatures.

Exp.	Products	Reference	Data source ^a	n ^b	Inoculum	Range of temperature storage	<i>a_w</i>	pH	Initial log CFU/ml	<i>t_{lag}</i> (h)	μ_{max} (1/h)
Heated milk											
a	UHT-milk	Cox and McRae (1988)	A	8	<i>P. fragi</i> <i>P. fluorescens</i>	4°C	0.997^d	6.7^d	2	- ^e	0.13 ± 0.01
b	Skim milk	Greene and Jezeski (1954)	A	6	<i>Pseudomonas spp.</i>	0, 5, 10, 15, 20 and 30°C	0.997^d	6.7^d	- ^e	- ^e	0.28 ± 0.26
c	Milk	Matias et al. (1994)	C	2	<i>P. fluorescens</i>	7°C	0.997^d	6.7^d	4.3	15.7 ± 4.1	0.20 ± 0.01
d	UHT-milk	Shelley et al. (1986)	C	4	<i>P. fragi</i> <i>P. fluorescens</i>	5°C	0.997^d	6.7^d	3.3 – 4.1	20.9 ± 8.0	0.12 ± 0.02
e	Milk	Tatini et al. (1991)	A	9	<i>Pseudomonas spp.</i> <i>P. fluorescens</i>	4 and 7°C	0.997^d	6.7^d	- ^e	- ^e	0.14 ± 0.03
f	Milk	Langeveld and Cuperus (1980)	C	1	<i>Pseudomonas spp.</i>	27°C	0.997^d	6.7^d	2.3	- ^e	0.73 ± 0.00
Non-heated milk											
g	Raw milk	Cox and McRae (1988)	A	8	<i>P. fragi</i> <i>P. fluorescens</i>	4°C	0.997^d	6.7^d	2	- ^e	0.072 ± 0.03
h	Raw milk	Griffiths et al. (1987)	C	2	<i>Pseudomonas spp.</i>	2 and 6°C	0.997^d	6.7^d	3.7	- ^e	0.070 ± 0.01
i	Raw milk	Maxcy and Liewen (1989)	A	10	<i>P. fluorescens</i>	10, 15, 20, 25 and 30°C	0.997^d	6.7^d	3 - 4	- ^e	0.44 ± 0.28
j	Raw milk	Sierra et al. (1996)	B	1	NC ^c	7°C	0.997^d	6.83	5.1	0.81 ± 0.0	0.081 ± 0.00
k	Raw goat milk	Zapico et al. (1995)	B	2	<i>P. fluorescens</i>	4 and 8°C	0.997^d	6.5	4.1	4.77 ± 0.7	0.11 ± 0.02

109 ^aLag times and μ_{max} -values reported directly (A), calculated from tabulated values (B) and calculated from graphs (C).

110 ^bNumber of growth curves.

111 ^cNC, naturally contaminated.

112 ^dBold type: Assumed value not reported in original study. See explanation in section 2.5.1.

113 ^eInformation not available in the specific study.

114 The ASZ method was used to compare observed and predicted growth (log CFU/g). The
115 acceptable interval was defined as ± 0.5 log CFU/g from the predicted growth. When at least 70%
116 of the observed values were within the ASZ, the prediction was considered acceptable (Møller et
117 al., 2013; Oscar, 2005; Østergaard et al., 2014).

118

119 2.6. Statistical analyses and curve fitting

120 Model parameters and standard errors were estimated by fitting eq. (1), (2), (4) and (5)
121 using SigmaStat (version 3.5, Systat Software, Inc., Point Richmond, CA, USA) or GraphPad
122 PRISM (version 4.03, GraphPad Software, San Diego, CA, USA). F-tests, to determine significant
123 lag time, to determine if the maximum population density was influenced by storage conditions and
124 growth simulations at dynamic temperature conditions were performed using Microsoft Excel 2010
125 (Microsoft Corp., Redmond, WA, USA). The Runge-Kutta 4th order method (Press et al., 2007) was
126 applied to predict growth under constant and dynamic temperature storage conditions by using the
127 differential form of eq. (7) i.e. the primary logistic growth model with delay ($dN/dt = 0$ for $t < t_{lag}$
128 and $dN/dt = N \cdot \mu_{max} \cdot (1 - (N/N_{max}))$ for $t \geq t_{lag}$).

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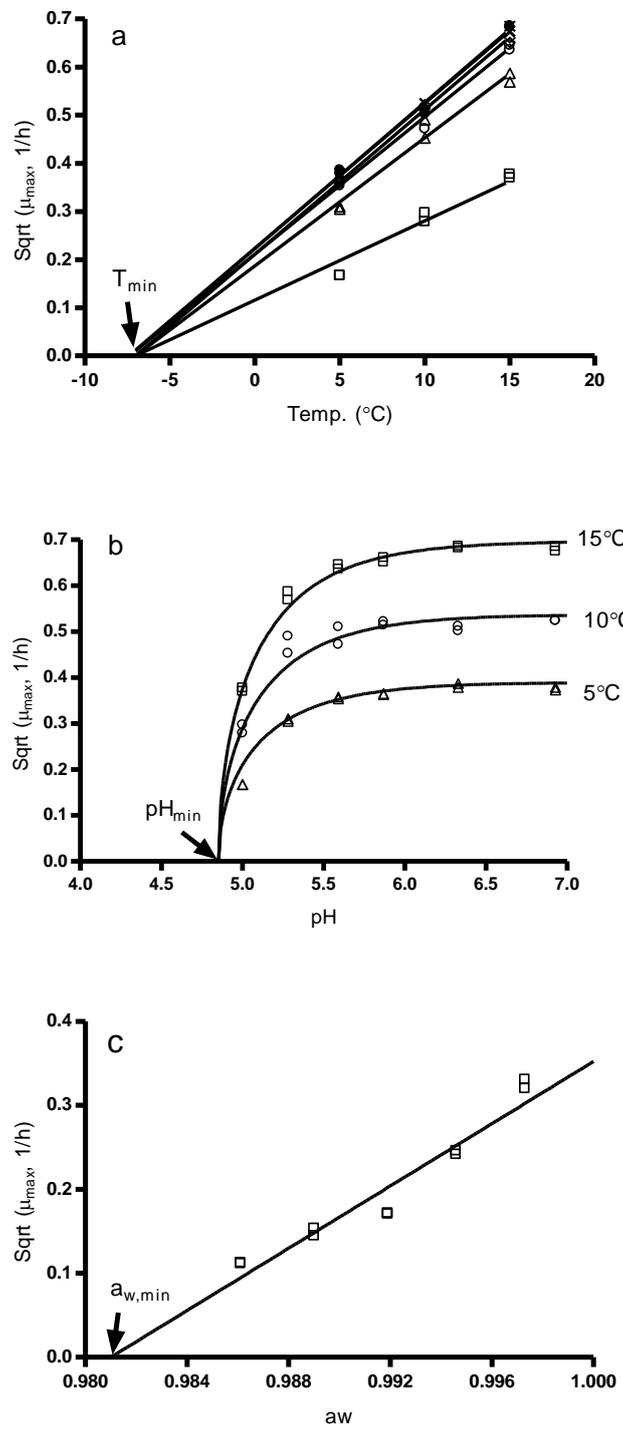
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131 3. Results

132

133 3.1. Growth rate models for psychrotolerant pseudomonads

134 Eq. (1) appropriately described the effect of temperature and pH on the obtained μ_{max} -values
135 for Ps-mix growing in BHI-G-Y broth (Fig. 1a, Fig. 1b). This resulted in fitted cardinal parameter
136 values ($\mu_{ref25^{\circ}C}$, T_{min} , pH_{min}) with small standard errors (Table 4).



137

138 **Fig. 1.** Maximum specific growth rates (μ_{max} , 1/h) of psychrotolerant pseudomonads as influenced by temperature (5, 10
 139 and 15 $^{\circ}\text{C}$), pH (5.00 (\square), 5.28 (Δ), 5.59 (\circ), 5.87 (\bullet), 6.33 (\diamond), 6.93 (\times)) and a_w in BHI-G-Y broth. T_{min} , pH_{min} and $a_{w,min}$
 140 were estimated by fitting of Eq. (1) and Eq. (2) (solid lines).
 141

142

143 **Table 4** Cardinal parameter values for psychrotolerant pseudomonads as determined in the present study.

	Parameter values
$\mu_{ref25^{\circ}C}$ (1/h); broth and heated milk	1.03 ± 0.03^a
$\mu_{ref25^{\circ}C}$ (1/h); cottage cheese and non-heated milk	0.62 ± 0.03^a
T_{min} ($^{\circ}C$)	-7.01 ± 0.51^a
pH_{min}	4.85 ± 0.01^a
$a_{w_{min}}$	0.98 ± 0.001^a
$MIC_{U \text{ Lactic acid}}$ (mM)	5.39 ± 0.17^a
n1	1
n2	1
$MIC_{U \text{ Sorbic acid}}$ (mM)	4.74 ± 0.23^a
n1	1
n2	1

144 ^a Standard error of fitted model parameter values.

145

146 The cardinal parameter values $a_{w_{min}}$, $MIC_{U \text{ Lactic acid}}$ and $MIC_{U \text{ Sorbic acid}}$ were determined separately
 147 from experiments where the effect of a single factor was studied. Eq. (2), eq. (4) and eq. (5)
 148 appropriately described the effect of a_w , lactic acid and sorbic acid on the obtained μ_{max} -values (Fig.
 149 1c, Fig. 2, Table 4). The developed model for μ_{max} -values of Ps-mix growing in BHI-G-Y broth
 150 had a $\mu_{ref25^{\circ}C}$ -value of 1.03 ± 0.03 1/h (Table 4). When eq. (6) was calibrated to 35 μ_{max} -values
 151 (Table 2, Exp. A-K) for psychrotolerant pseudomonads growing in cottage cheese with cultured
 152 cream dressing the $\mu_{ref25^{\circ}C}$ -value was reduced to 0.62 ± 0.03 1/h (Table 4). This result shows that
 153 cottage cheese with cultured cream dressing includes factors that on average reduce μ_{max} -values for
 154 psychrotolerant pseudomonads by 42% compared to growth in broth.

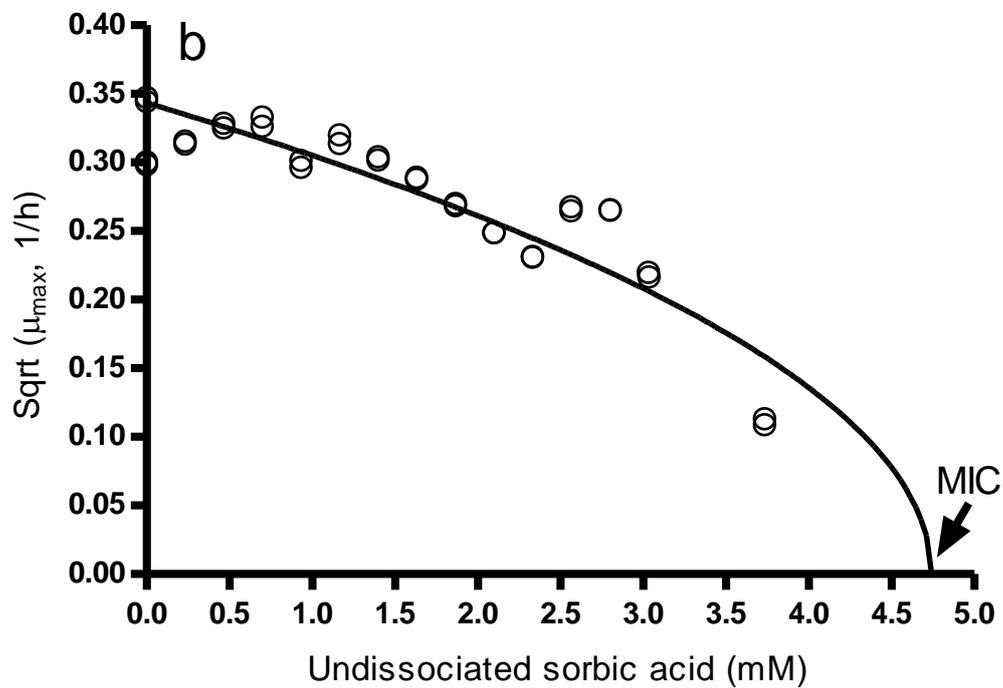
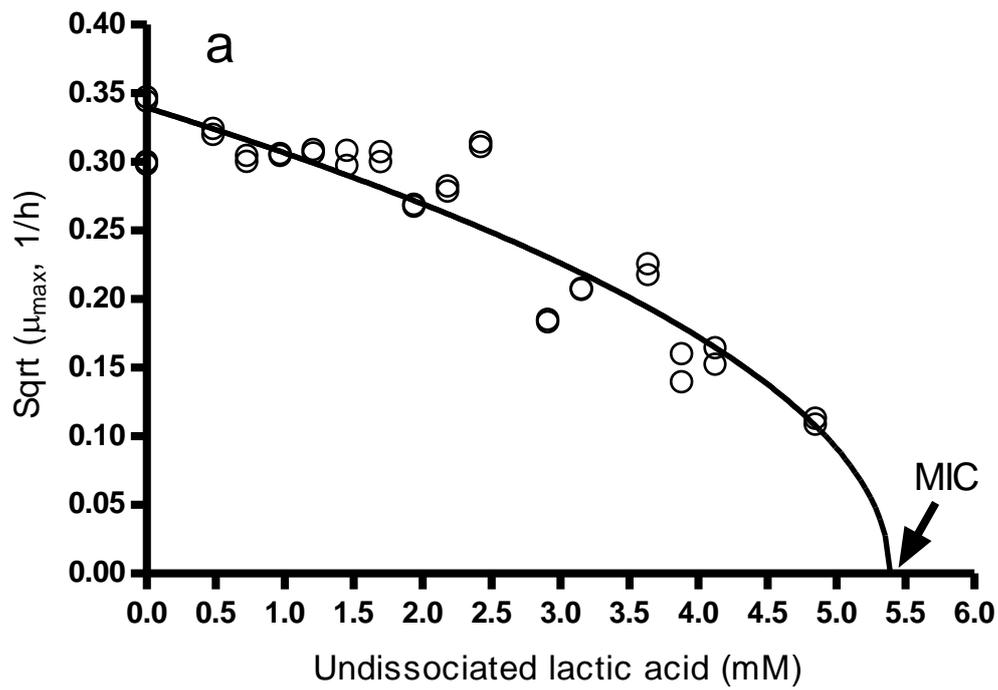
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161 **Fig. 2.** Effect of lactic- and sorbic acids on maximum specific growth rates (μ_{max} , 1/h) of psychrotolerant pseudomonads.
 162 Experiments were performed at 8 °C in BHI-G-Y broth at pH 5.2. Minimum inhibitory concentrations (MIC) were
 163 estimated by fitting Eq. (4) and Eq. (5) to the experimental data (Solid lines).

164

165 3.2. Challenge tests and storage trials

166 Cottage cheese with cultured cream dressing showed little variation in their initial pH
167 prior to chilled storage (5.3-5.4) and in their concentration of NaCl in the water phase (0.89-1.11%).
168 More variability was observed for the initial concentration of LAB (5.6-8.5 log CFU/g) and lactic
169 acid in the water phase of the product (445-996 ppm) (Table 1).

170 Psychrotolerant pseudomonads grew in cottage cheese at 5, 10, 15 and 20°C (Table 2, Exp.
171 A to K) while LAB exclusively grew at 10, 15 (Fig. 3) and 20 °C (Results not shown). As expected
172 μ_{max} -values and lag times (t_{lag} , h) of psychrotolerant pseudomonads were influenced by storage
173 conditions and product characteristics (Table 2). In contrast, the maximum population density of 9.5
174 ± 0.4 log CFU/g was not significantly influenced by storage conditions and product characteristics
175 ($P > 0.05$). Interestingly, high concentrations of LAB present in some batches prior to chilled
176 storage did not seem to influence the maximum population density of the psychrotolerant
177 pseudomonads (Fig. 3). The growth pattern for psychrotolerant pseudomonads and LAB was
178 similar for all batches of cottage cheese with cultured cream dressing (Results not shown).

179 As expected shelf life of cottage cheese decreased with increasing storage temperature.
180 Panellist described sensory spoilage characteristics as fruity-melon odour, sweet off-odour, matured
181 cheese odour and a yellow colour (Table 2). At the time of sensory spoilage concentrations of
182 psychrotolerant pseudomonads was 8.4 ± 0.5 log CFU/g.

183

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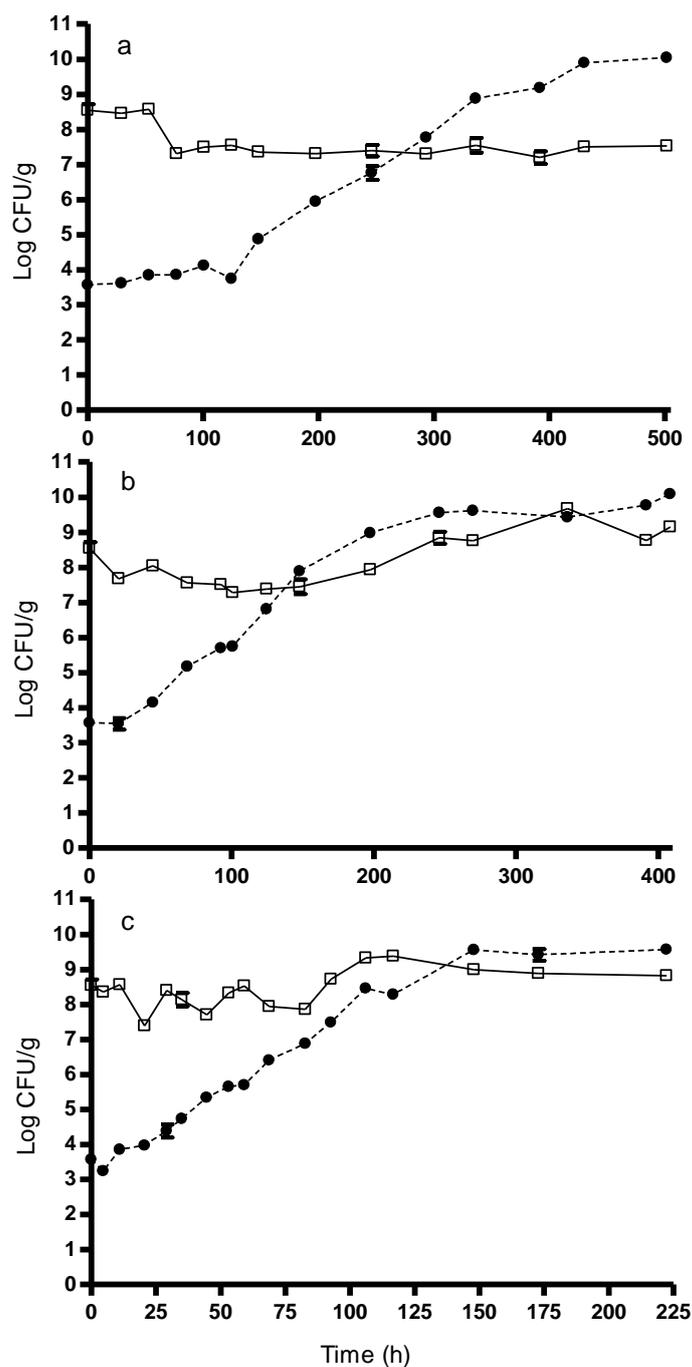
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191 **Fig. 3.** Growth of psychrotolerant pseudomonads (•) and lactic acid bacteria (□) in cottage cheese with cultured cream
 192 dressing at 4.7 °C (a), 9.6 °C (b) and 14.9 °C (c). The product had pH 5.4, 0.89% NaCl in the water phase and 971 ppm
 193 lactic acid in water phase. Symbols represent average values and errors bars the standard deviation for three samples
 194 (Table 1, Exp. C, D and E).

195

196

197

198 3.2.1. Lag time models for psychrotolerant pseudomonads

199 *RLT*-values for growth in heated milk and cottage cheese showed considerable variability
200 (Table 2). For heated milk the average *RLT*-value of 2.8 ± 1.9 (h) ($n = 9$; Table 2, Exp. L, M, N)
201 was significantly influenced by temperature ($P < 0.05$) and described as $RLT = 0.7 + 72.0/T^2$. For
202 cottage cheese and non-heated milk the average *RLT*-value was 3.1 ± 2.8 (h) ($n = 35$; Table 2, Exp.
203 A to K) was also influenced by temperature ($P < 0.05$) and described as $RLT = 2.0 + 56.0/T^2$.

204

205 3.3. Evaluation of the developed growth models

206

207 3.3.1. Evaluation of growth rate models

208 The developed growth rate model for broth (Eq. (6) with $\mu_{ref25^\circ C} = 1.03$ 1/h; Table 4)
209 acceptably predicted growth rates in heated milk. The average bias- and accuracy factor values were
210 1.08 and 1.32 for the evaluated 33 growth curves (Table 5, Fig. 4a). As expected this model
211 overestimated growth rates in cottage cheese with cultured cream dressing ($B_f = 2.21$) and growth
212 rates in non-heated milk were also markedly overestimated ($B_f = 1.69$) (Table 5). The developed
213 growth rate model for cottage cheese with culture cream dressing (Eq. (6) with $\mu_{ref25^\circ C} = 0.62$ 1/h;
214 Table 4) slightly overestimated the six growth rates for naturally contaminated cottage cheese with
215 psychrotolerant pseudomonads determined in independent experiments not used for development of
216 this model ($B_f = 1.31$). Interestingly, this model acceptably predicted growth rates in non-heated
217 milk ($B_f = 1.02$) (Table 5, Fig. 4b).

218

219

Table 5 Evaluation of growth rate models for psychrotolerant pseudomonads.

Products	Data source and experiments	n ^a	Model for growth rates in broth ($\mu_{ref25^{\circ}C} = 1.03$ 1/h)		Model calibrated for cottage cheese with cultured cream dressing ($\mu_{ref25^{\circ}C} = 0.62$ 1/h)	
			B _f ^b	A _f ^b	B _f ^b	A _f ^b
Pasteurized milk	Table 2, Exp. L	3	1.22	1.22	0.74	1.36
Heated milk	Table 3	30	1.07	1.33	0.64	1.59
Total		33	1.08	1.32	0.65	1.57
Cottage cheese	Table 2, Exp. P-Q	6	2.21	2.21	1.31	1.31
Non-heated milk	Table 3	20	1.69	1.72	1.02	1.45
Total		26	1.80	1.85	1.08	1.43

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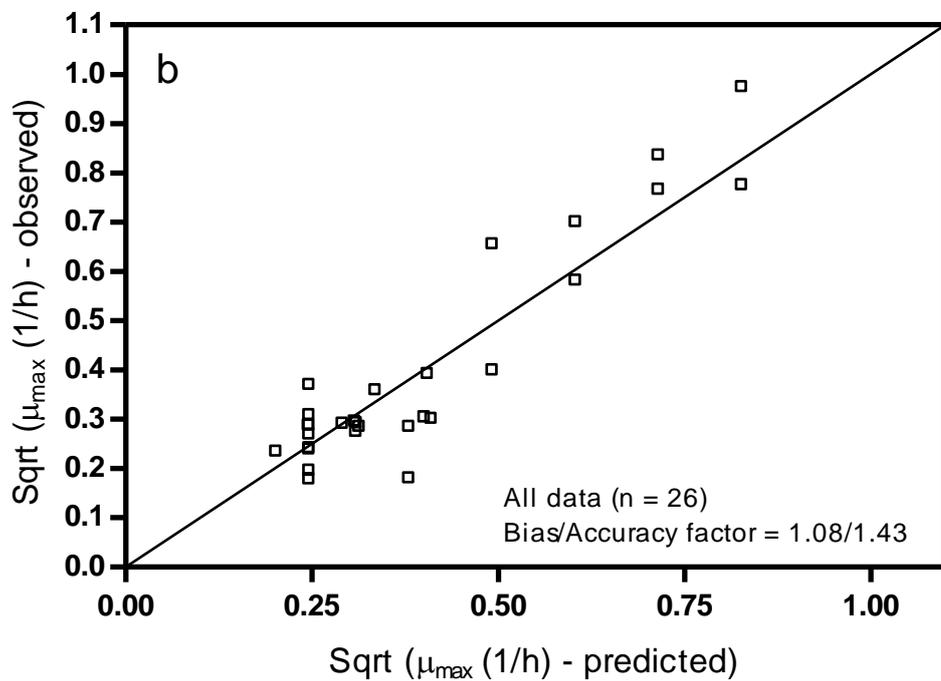
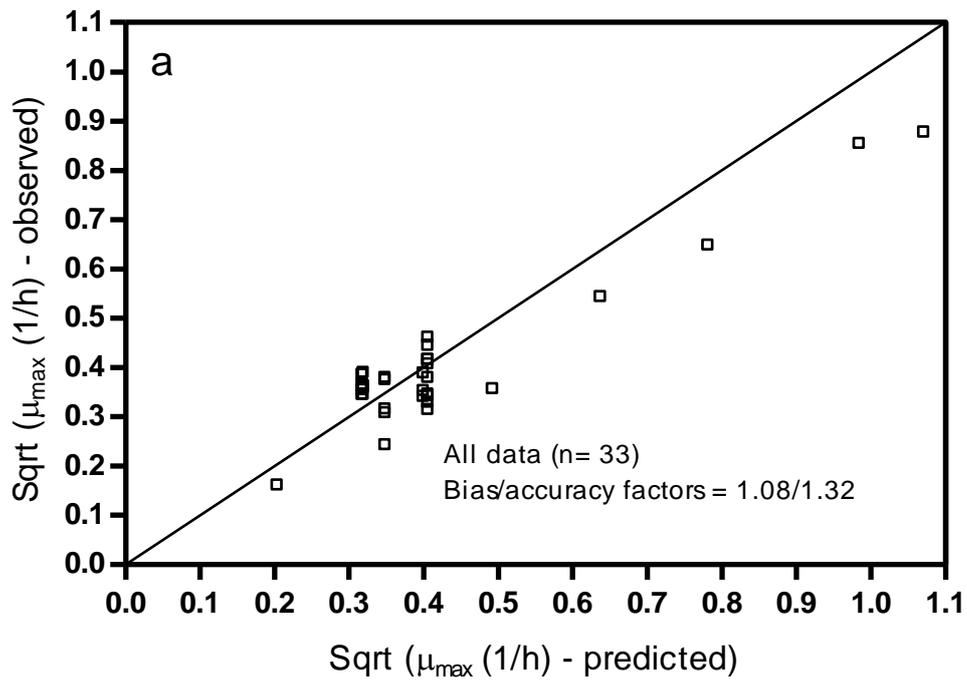
^a Number of growth curves evaluated.

221

^b Bias (B_f) and accuracy (A_f) factor values calculated from observed and predicted μ_{max} -values.

222

223



224

225 **Fig. 4.** Comparison of observed and predicted growth rates (μ_{\max} , 1/h) of psychrotolerant pseudomonads in heat treated
 226 milk (a) and in non-heated milk or cottage cheese with cultured cream dressing (b).

227

228

229 3.3.2. Evaluation of combined growth rate and lag time models

230 To predict growth as the increase in concentrations of psychrotolerant pseudomonads (log
231 CFU/g) during storage the growth rate (Eq. (6)) and lag time (Eq. (8)) models were combined. Eq.
232 (6) was used with parameter values as shown in Table 4 and eq. (8) was used with *RLT*-values as
233 indicated in section 3.2.1. The acceptable simulation zone (ASZ) method can be used to compare
234 observed and predicted growth including growth rates and lag phases under constant and dynamic
235 temperature storage conditions. In agreement with the model evaluation based on growth rates
236 (3.3.1.) the ASZ method showed growth of psychrotolerant pseudomonads to be well predicted by
237 the new models. ASZ were evaluated for twelve growth curves and on average 78 % of the
238 predicted cell concentrations were within the ASZ for cottage cheese and non-heated milk (Table
239 6; Fig. 5). Specifically, for naturally contaminated cottage cheese with cultured cream dressing at
240 constant temperatures (n = 6) 85 % of the cell concentrations were within the ASZ (Table 6; Fig. 5
241 e and f). At dynamic temperatures this value was 67 % (Table 6) but as shown by Fig. 5a and Fig.
242 5b there was small differences between observed and predicted growth. For non-heated milk the
243 three growth curves evaluated had 63% of the observations within the ASZ (Table 6; Fig. 5 d). For
244 heat-treated milk the three growth curves at dynamic storage temperature had 96 % of the cell
245 concentrations within ASZ (Table 6; Fig. 5 c).

246

247

248

249

250

251

252 **Table 6** Comparison of predicted and observed growth data using the acceptable simulation zone (ASZ) method.

Product	Data source	Inoculum	Storage temperature	Observations within ASZ (%)
Pasteurized milk	Table 2 Exp. O	I ^a	Dynamic (4.6 – 9.8°C), Fig 5c	88
Pasteurized milk	Table 2 Exp. O	I ^a	Dynamic (4.6 – 9.8°C)	100
Pasteurized milk	Table 2 Exp. O	I ^a	Dynamic (4.6 – 9.8°C)	100
Average ASZ score				96
Cottage cheese	Table 2 Exp. P	NC ^b	9.6 ± 0.2°C, Fig. 5e	93
Cottage cheese	Table 2 Exp. P	NC ^b	9.6 ± 0.2 °C	93
Cottage cheese	Table 2 Exp. P	NC ^b	9.6 ± 0.2 °C	100
Cottage cheese	Table 2 Exp. Q	NC ^b	14.9 ± 0.3°C, Fig 5f	65
Cottage cheese	Table 2 Exp. Q	NC ^b	14.9 ± 0.3°C	77
Cottage cheese	Table 2 Exp. Q	NC ^b	14.9 ± 0.3°C	82
Cottage cheese	Table 2 Exp. R	I ^a	Dynamic (4.1 – 19.3 °C), Fig 5a	50
Cottage cheese	Table 2 Exp. R	I ^a	Dynamic (4.1 – 19.3 °C)	63
Cottage cheese	Table 2 Exp. R	I ^a	Dynamic (4.1 – 19.3°C), Fig 5b	88
Non-heated milk	Table 2 Exp. S	NC ^b	7°C	86
Non-heated milk	Table 2 Exp. T	I ^a	4°C	25
Non-heated milk	Table 2 Exp. U	I ^a	8°C, Fig 5d	75
Average ASZ score				78

253 ^aI, inoculated with psychrotolerant pseudomonads (Ps-mix).

254 ^bNC, naturally contaminated with psychrotolerant pseudomonads.

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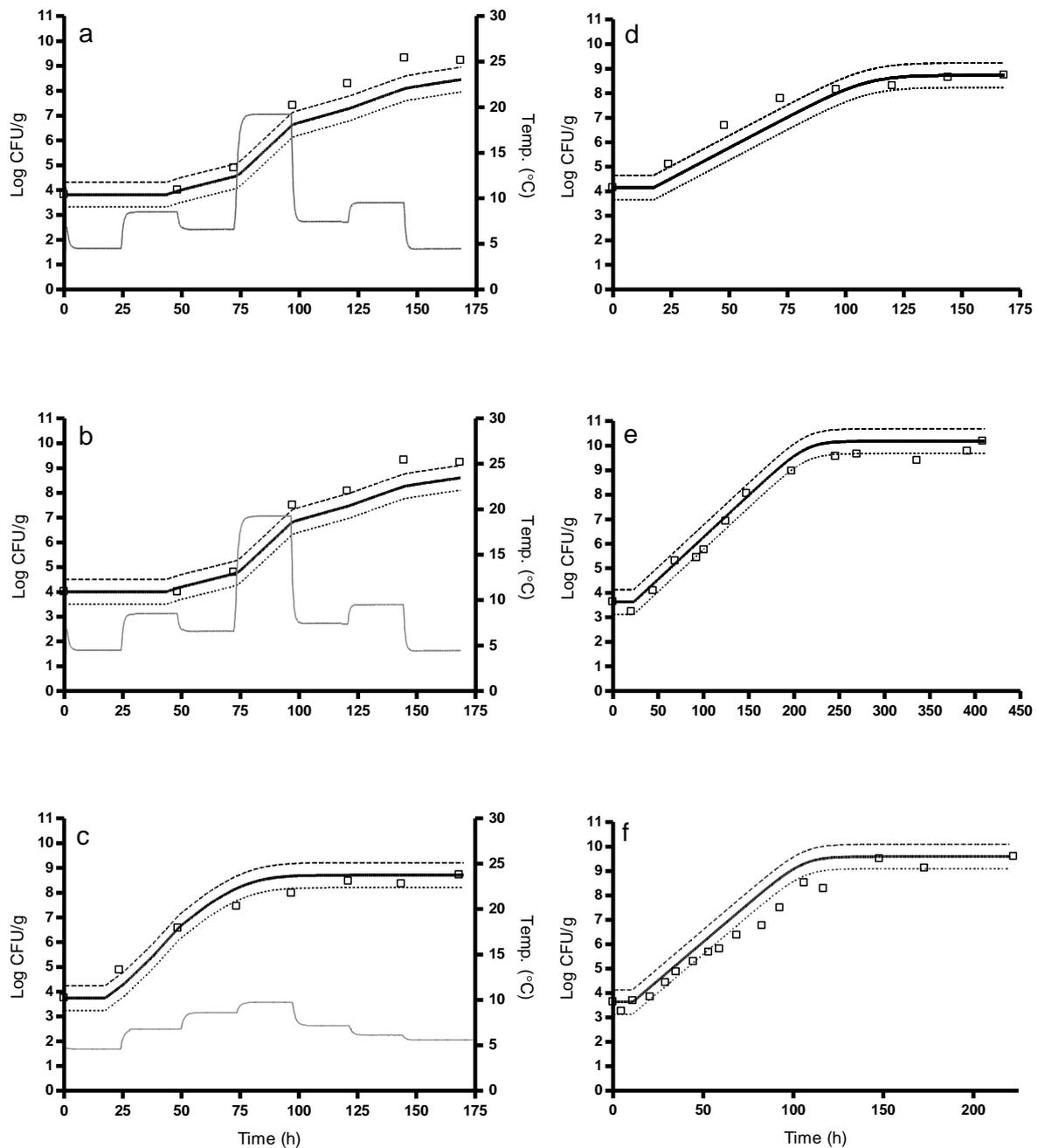


Fig. 5. Comparison of observed (\square) and predicted ($—$) growth of psychrotolerant pseudomonads. Graphs include ± 0.5 log CFU/g (dashed lines) defining the acceptable simulation zone (ASZ). Data was obtained for growth in cottage cheese with cultured cream dressing at 4.1 – 19.3 °C (a and b); heat-treated milk under dynamic storage temperature conditions at 4.6 – 9.8°C (c) ; non-heated milk at 8°C (d) and cottage cheese with cultured cream dressing at 9.6°C (e) and at 14.9°C (f). Temperatures profiles are shown as grey lines in (a), (b) and (c).

4. Discussion

Mathematical models to predict growth of psychrotolerant pseudomonads in milk and cottage cheese and including the effect of temperature, pH, a_w , lactic acid and sorbic acid were developed and evaluated in the present study. As determined from product evaluation studies the models' range of applicability included temperatures from 2 °C to 20 °C, pH from 5.3 to 6.8, water-phase salt content up to 1% and an initial concentrations of lactic acid up 996 ppm. Further studies are needed to evaluate the ability of the developed models to predict the effect a wider range of storage temperatures, product characteristics including the effect of sorbic acid and the effect of variation in products from different producers. The developed models can be used to predict growth of psychrotolerant pseudomonads and shelf life of products. As one example a reduction of storage temperature from 7°C to 5 °C was predicted to increase shelf life of (i) cottage cheese with cultured cream dressing by seven days, (ii) non-heated milk by three days and (iii) heated milk by a day and a half when products initially were contaminated with one psychrotolerant pseudomonads per 10 g of product (0.1 log CFU/g). As another example the observed variability in product characteristics of cottage cheese including pH from 5.30 to 5.47 and initial water phase concentrations of lactic acid from 445 ppm to 1393 ppm (Table 1; Østergaard et al., 2015) was predicted to result in a difference in shelf life of more than six days at 5°C for a product 0.1 log CFU/g of psychrotolerant pseudomonads.

The developed models had a T_{min} -value of -7.0 ± 0.5 °C (Table 4) and this value was similar to the T_{min} -values of -7.9 °C and -7.6 °C reported by Fu et al. (1991) and Neumeyer et al. (1997) but higher than the T_{min} -value of -11.4 °C reported by Koutsoumanis et al. (2000) for growth of *Pseudomonas* spp. in fresh MAP fish. The estimated a_{wmin} -value of 0.98 (Table 4) was higher than the value of 0.95 reported by Neumeyer et al. (1997). For pH_{min} , MIC_U Lactic acid and MIC_U Sorbic acid we found no values in the available literature to compare our results with although the effect of pH

has been included in polynomial models in combination with temperature and water activity (Braun and Sutherland, 2003; Lebert et al., 2000; Membré and Burlot, 1994; Pin et al., 1999).

The present study confirmed that an extensive growth model can be developed by the simplified cardinal parameter modelling approach as previously shown for other food spoilage or human pathogenic microorganisms including LAB and *L. monocytogenes* (Augustin et al., 2005; Mejlholm and Dalgaard, 2013; Østergaard et al., 2014; Rosso, 1999). This is important because extensive growth model are needed to obtain accurate prediction of growth responses in lightly preserved foods and because cardinal parameter models can be conveniently calibrated to predict growth in various foods. For psychrotolerant pseudomonads it seems interesting to further evaluate the developed growth models for queso fresco, queso blanco, ricotta or mozzarella where growth of psychrotolerant pseudomonads has been problematic (Andreani et al., 2015; Caputo et al., 2015; Martin et al., 2011; Mendoza-Yepes et al., 1999) but where available data are insufficient for model evaluation.

The present study did not further evaluate factors in cottage with cultured cream dressing and in raw milk that resulted in the observed 42% reduction of growth rates for psychrotolerant pseudomonads compared to growth in broth and heated milk (Table 4). It is interesting, however, that Østergaard et al. (2014) in the same way found a 49% reduction of growth rate for *L. monocytogenes* in cottage cheese with cultured cream dressing compared to growth in broth. The cultured cream dressing included *L. lactis* subsp. *lactis* biovar. *diacetylactis* and it is known to reduce μ_{max} -values of *P. fragi* (Mather and Babel, 1959). The inhibition may be caused by diacetyl and/or bacteriocins (Juffs, 1975; Price and Lee, 1970; Yap, 2000). Reduced growth rates in raw milk compared to heated milk can be due to the lactoperoxidase system or diacetyl (De Leonardis et al., 2013; Zapico, 1995). The effect of these factors is not easily modelled and the calibration of $\mu_{ref25^{\circ}C}$ in the present study was important to develop predictive models with sufficient accuracy for

cottage cheese and raw milk (Table 5). Although, cottage cheese with cultured cream dressing reduced μ_{max} -values for both psychrotolerant pseudomonads (42 %) and *L. monocytogenes* (49 %) the effect on their maximum population density (N_{max}) was fundamentally different with no effect on psychrotolerant pseudomonads (Fig. 3) and with $\log N_{max}$ of *L. monocytogenes* being reduced by several log-units due to inhibition by high concentration of LAB (Østergaard et al., 2014).

The observed variability in *RLT*-values for psychrotolerant pseudomonads (3.2.1) corresponded to data for several previous studies of other bacteria (Mellefont and Ross, 2003; Østergaard et al., 2015). Temperature influenced *RLT*-values as observed by Hereu et al. (2014) and it seems interesting, in future studies, to include lag time distributions of psychrotolerant pseudomonads in stochastic models together with variability in product characteristics to predict growth as recently reported for *L. monocytogenes* (Østergaard et al., 2015). As a simple alternative the worst case approach may be used where lag times are omitted when growth is predicted.

The growth rate model developed for cottage cheese with cultured cream dressing resulted in a B_f -value of 1.31 ($n = 6$). This value is slightly above the upper limit for good performance ($0.85 < B_f < 1.25$) of a model for spoilage microorganisms (Mejlholm and Dalgaard, 2013). However, when the combined lag time and growth rate model was evaluated for constant and dynamic temperature storage conditions ($n = 9$) the AZS method and graphical comparison of observed and predicted values showed the model to perform well (Table 5, Fig. 5). These results support the use of the classical B_f and A_f values in combination with the AZS method and graphs for evaluation of the performance of growth models as previously suggested (Møller et al., 2013; Oscar, 2005; Østergaard et al., 2014).

In summary, the properties of cottage cheese with cultured cream dressing and non-heated milk reduced growth rates of psychrotolerant pseudomonads compared with heated milk. If equally contaminated with psychrotolerant pseudomonads the reduced growth rates will result in longer

shelf life as similar storage conditions. The present study developed and validated mathematical models to predict growth of psychrotolerant pseudomonads in cottage cheese with cultured cream dressing and milk. These models can be used to evaluate the effect of storage conditions on shelf life of actual products or the effect of product reformulations on growth of psychrotolerant pseudomonads. The new models can for example be entered as generic growth models and used in combination with the Food Spoilage and Safety Predictor (FSSP) software to predict the effect of product characteristics and constant or dynamic temperature storage conditions (<http://fssp.food.dtu.dk>).

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