Technical University of Denmark

DTU

Predictive Food Microbiology
new tools for risk assessment and dairy product development

Østergaard, Nina Bjerre; Dalgaard, Paw; Christiansen, Lasse Engbo

Publication date:
2014

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Østergaard, N. B., Dalgaard, P., & Christiansen, L. E. (2014). Predictive Food Microbiology: new tools for risk assessment and dairy product development. DTU Food.

DTU Library
Technical Information Center of Denmark

Predictive Food Microbiology
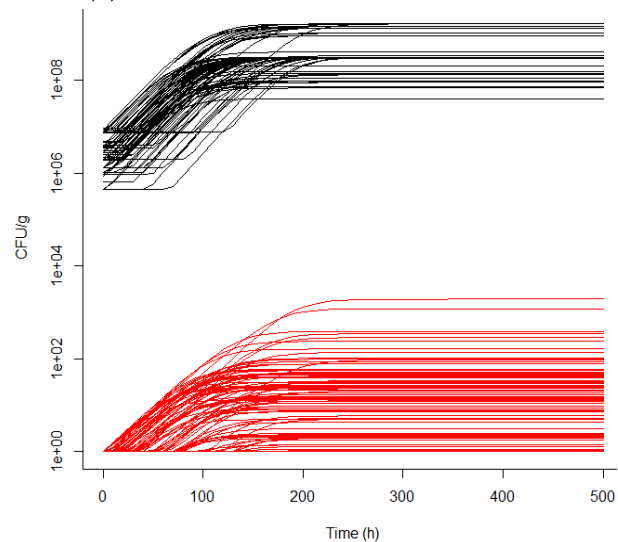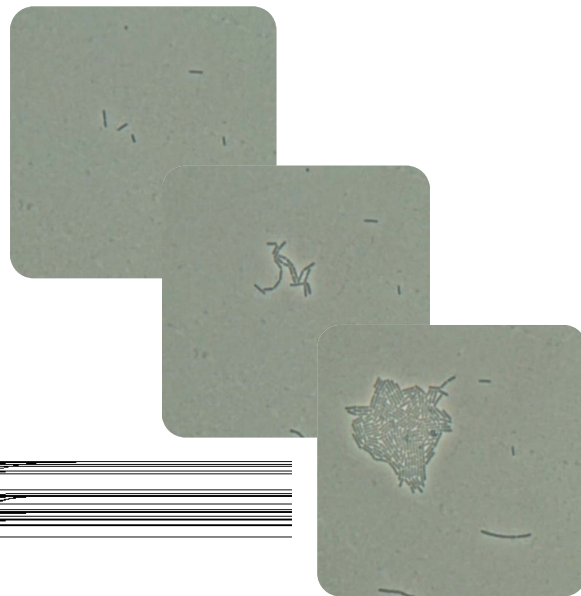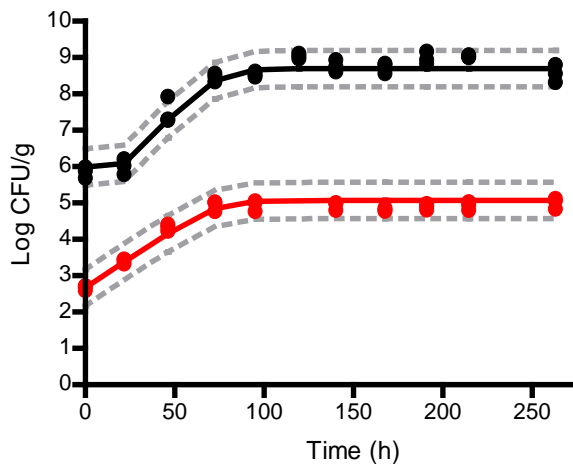– new tools for risk assessment and dairy product development

Nina Bjerre Østergaard
PhD Thesis
August 2014

DTU Food
National Food Institute

Predictive Food Microbiology

– new tools for risk assessment and dairy product development

Nina Bjerre Østergaard
PhD Thesis
August 2014

National Food Institute
Technical University of Denmark
Division of Industrial Food Research
Group of Predictive Food Microbiology
Søltofts Plads, DK-2800 Kgs. Lyngby
Denmark

DTU Food
National Food Institute

Principal supervisor
Professor Paw Dalgaard
National Food Institute
Technical University of Denmark

Co-supervisor
Associate Professor Lasse Engbo Christiansen
DTU Compute
Technical University of Denmark

Preface

This PhD study was carried out at the National Food Institute, Technical University of Denmark from August 2011 until August 2014 under the supervision of Professor Paw Dalgaard. The presented work was financed by Arla Foods amba and the Technical University of Denmark.

I wish to thank Paw Dalgaard for his support throughout the project and for the many valuable discussions we have had. I could not have found a better mentor within predictive microbiology and microbial food safety. I would also like to thank my co-supervisor Lasse Engbo Christiansen for introducing me to the world of stochastic models, I am indeed fascinated.

During my studies I spent two months at the School of Agriculture, Aristotle University of Thessaloniki, Greece and I would like to thank Konstantinos Koutsoumanis and his group for making my stay at the Laboratory of Food Microbiology and Hygiene both educational and enjoyable.

Special thanks go to Nadereh Samieian and Tina Dahl Devitt for their skilful and highly appreciated technical assistance in the laboratory. I wish to thank Arla Foods amba, and especially Research Scientist Annelie Eklöw, for being supportive throughout the project. PTU Assistant Kim Nielsen from Korsvej Dairy has also been most helpful – thank you very much.

Last but not least, I am deeply grateful for the patience and understanding I have met from Hans-Christian and the rest of my family – You mean the world to me.

Nina Bjerre Østergaard
Kgs. Lyngby, August 2014

Summary

Predictive Food Microbiology – new tools for risk assessment and dairy product development

Listeria monocytogenes is a well-known food borne pathogen that potentially causes listeriosis. No outbreaks or cases of listeriosis have been associated with cottage cheese, but several confirmed cases and outbreaks in the EU and the US have been related to dairy products made from raw or pasteurised milk. This, in combination with the fact that cottage cheese support growth of *Listeria monocytogenes,* induces a documentation requirement on the food producer. In the EU regulatory framework, mathematical models are recognised as a suitable supplement to traditional microbiological methods. The models can be used for documentation of compliance with microbiological criteria for *Listeria monocytogenes* under reasonably foreseeable conditions.

Cottage cheese is a fresh, fermented dairy product. It consists of a fermented cheese curd mixed with a fresh or cultured cream dressing. The product contains considerable concentrations of lactic acid bacteria from the added starter or aroma cultures. The presence of these microorganisms induces some complexity to the product, since the lactic acid bacteria metabolites and e.g. bacteriocins exhibit an inhibitory effect towards co-culture microorganisms such as *Listeria monocytogenes.* During storage at temperatures allowing the mesophilic lactic acid bacteria to grow (> 8-10°C), a pronounced inter-bacterial interaction and growth inhibition of co-culture *Listeria monocytogenes* was observed. These observations emphasised the need for inter-bacterial interaction models when predicting the growth response of *Listeria monocytogenes* in fermented dairy products.

The objective of the PhD-project was to develop new, or extend existing mathematical models to be used for risk assessment and product development. When the project was initiated, none of the existing predictive models were found to appropriately describe the simultaneous growth of lactic acid bacteria from the added starter or aroma culture and *Listeria monocytogenes* in cottage cheese.

New, deterministic growth models were developed for *Listeria monocytogenes*, starter lactic acid bacteria and aroma lactic acid bacteria. The new cardinal parameter type growth models included the effect of temperature, pH, NaCl, lactic and sorbic acid. The models were developed based on growth data obtained from absorbance measurements in liquid laboratory media and growth data obtained in cottage cheese with fresh or cultured cream dressing. An important step in the modelling procedure was the calibration of the reference growth rate (μ_{ref}, h^{-1} at 25°C) which was strongly affected by the dominating lactic acid bacteria culture. By combining the developed secondary growth models with the empirical Jameson approach, good predictions of the simultaneous growth of *Listeria monocytogenes* and lactic acid bacteria were obtained. Both growth rate and maximum population densities of *Listeria monocytogenes* was accurately described under constant and dynamic storage temperatures (between 5°C and 15°C).

The inter-bacterial interaction was clearly important to include when predicting growth response of *Listeria monocytogenes* in fermented dairy products. Alternative, semi-mechanistic,

modelling approaches were evaluated based on methods applied in the fermentation technology. The dynamics of lactic acid concentration and product pH was related to growth of lactic acid bacteria by the yield factor concept. The ability to predict the maximum population density of *Listeria monocytogenes* in cottage cheese based on dynamic lactic acid and pH was evaluated. For cottage cheese with fresh cream dressing, the semi-mechanistic interaction model successfully predicted the maximum population density. Lactic acid and pH was, however, insufficient to describe the growth inhibition of *Listeria monocytogenes* observed in cottage cheese with cultured cream dressing. Improved, mechanistic, prediction of *Listeria monocytogenes* in cottage cheese with cultured cream dressing would require that additional mechanisms were included in the model, such as other metabolites or bacteriocins. Finally, the semi-mechanistic and the empirical Jameson approach to inter-bacterial interaction modelling were compared. The empirical Jameson model consistently performed equally well or better than the more complex semi-mechanistic model.

In order to evaluate the growth response of more realistic concentrations of *Listeria monocytogenes* and to take variability into account, a stochastic approach was applied. The deterministic growth models were used in combination with stochastic input values for bacterial concentration; lag time duration and product characteristics. Good agreement between predicted and observed growth was obtained, when applying broth based lag time distributions for *Listeria monocytogenes* single cells in combination with the relative lag time concept. Furthermore, application of relative lag time distributions from *Listeria monocytogenes* population data provided good predictions of the growth response of only a few *Listeria monocytogenes* cells in cottage cheese at chilled temperatures.

From the results of the present PhD-project it was found that once solid, deterministic, secondary growth models have been developed and validated, they can be modified and/or extended to a range of other modelling procedures. Furthermore, inclusion of inter-bacterial interaction was considered to be an inevitable part when modelling and predicting growth of *L. monocytogenes* in fermented dairy products. In general, simple approaches to describe interaction and growth inhibition (empirical approach), lag time prediction of individual cells (variability in population *RLT*-values) and representation of e.g. variable product characteristics (bootstrapping from empirical distributions) were advocated. It is believed that it is necessary to define some applicable methodologies for the development of growth models for complex products such as fermented dairy products. Model development is a comprehensive process with an almost infinite data requirement and the findings of the present PhD-project is thought to be important in relation to the development of predictive models that are valuable for, and readily applicable in the food industry.

Sammendrag (summary in Danish)

Prædiktiv mikrobiologi – nye redskaber til risikovurdering og udvikling af mejeriprodukter

Listeria monocytogenes er en velkendt fødevarebåren patogen der kan føre til listeriose. Ingen udbrud eller tilfælde af listeriose er blevet sat i forbindelse med hytteost, men adskillige tilfælde og udbrud af listeriose i EU og USA er blevet knyttet til mejeriprodukter lavet med rå- eller pasteuriseret mælk. Disse tilfælde, i kombination med det faktum at *Listeria monocytogenes* er i stand til at vokse i hytteost, pålægger fødevareproducenten et dokumentationskrav. Indenfor europæisk fødevarelovgivning er matematiske anerkendt som et velegnet supplement til traditionelle mikrobiologiske analyse metoder. Modellerne kan anvendes til at dokumentere at produktet overholder de mikrobiologiske kriterier for *Listeria monocytogenes* under forventelige forhold.

Hytteost er et frisk, syrnet mejeriprodukt. Det består af en fermenteret ostemasse der er blandet med en flødedressing med eller uden aroma kultur. Produktet indeholder en betragtelig koncentration af mælkesyrebakterier fra den tilsatte starter- og/eller aromakultur. Tilstedeværelsen af disse mælkesyrebakterier tilfører produktet nogen kompleksitet, da deres metaboliske produkter og f.eks. bakteriociner kan virke hæmmende på andre mikroorganismer såsom *Listeria monocytogenes*. Når hytteoste opbevares ved temperaturer hvor de mesofile mælkesyrebakterier kan vokse (>8-10°C) da observeres en udtalt interaktion mellem mikroorganismerne og en hæmning af *Listeria monocytogenes*. Disse observationer understreger vigtigheden af at inkludere interaktion mellem mikroorganismer når vækst respons af *Listeria monocytogenes* skal forudsiges i fermenterede mejeri produkter.

Målsætningen for Ph.d.-projektet var at udvikle nye, eller udvide eksisterende, matematiske modeller der kan anvendes i forbindelse med risikovurdering og produktudvikling. Ved projektets start kunne ingen af de eksisterende modeller forudsige vækst af *Listeria monocytogenes* og mælkesyrebakterier fra starter- og aroma kultur i hytteost tilfredsstillende.

Nye, deterministiske vækstmodeller blev udviklet for *Listeria monocytogenes*, starter-mælkesyrebakterier og aroma-mælkesyrebakterier. Vækstmodellerne inkluderede effekten af opbevaringstemperatur, pH, salt, mælkesyre og sorbinsyre. Kardinalparameter-vækstmodellerne blev udviklet på basis af absorbans-data i modelsystemer samt vækstdata fra hytteost med eller uden aroma mælkesyrebakterier. Et vigtigt trin i modelleringsproceduren var kalibreringen af reference-væksthastigheden (μ_{ref}, t^{-1} ved 25°C). Den var stærkt afhængig af den dominerende mælkesyrebakteriekultur. Ved at kombinere de udviklede sekundære vækstmodeller med Jameson interaktionsmodellen, kunne der opnås gode forudsigelser for den sideløbende vækst af *Listeria monocytogenes* og mælkesyrebakterier. Både væksthastighed og den maksimale populationstæthed blev forudsagt præcist under konstant og dynamisk opbevaringstemperatur (mellem 5°C og 15°C).

Det er tydeligvis vigtigt at inkludere interaktionen mellem mikroorganismerne, når *Listeria monocytogenes'* vækstrespons skal forudsiges i fermenterede mejeriprodukter. Alternative, semi-

mekanistiske, metoder fra fermenteringsteknologi blev evalueret i forhold til at forudsige interaktion. Dynsmisk mælkesyrekoncentration og produkt pH var relateret til væksten af mælkesyrebakterier ved at anvende "yield-factor" konceptet. Det blev evalueret hvorvidt den maksimale populationstæthed for *Listeria monocytogenes* i hytteost kunne forudsiges baseret på dynamisk mælkesyrekoncentration og pH. I hytteost uden tilsat aroma-kultur, kunne den maksimale populationstæthed forudsiges præcist ved at bruge den semi-mekanistiske interaktionsmodel. Til gengæld var mælkesyre koncentration og pH ikke tilstrækkeligt til at forudsige den væksthæmning af *Listeria monocytogenes* der blev observeret i hytteost hvor der også var tilsat aroma-kultur. En forbedring af forudsigelsen vil sandsynligvis kræve at yderligere mekanismer inkluderes i modellen. Det kunne være andre metabolitter eller dannelsen af bakteriociner. Afslutningsvist blev den semi-mekanistiske interaktionsmodel sammenlignet med den mere simple empiriske Jameson model. Den empiriske Jameson model klarede sig konsekvent lige så godt eller bedre end den mere komplicerede, semi-mekanistiske interaktionsmodel.

For at kunne evaluere vækstresponset af mere realistiske koncentrationer af *Listeria monocytogenes* samt inkludere variabilitet i forudsigelserne blev der anvendt en stokastisk tilgang. De udviklede deterministiske vækstmodeller blev anvendt sammen med stokastiske input for startkoncentration af mikroorganismerne, varighed af nølefasen og produkt egenskaber. Ved at anvende bouillonbaseret nølefase data for *Listeria monocytogenes* enkeltceller, i kombination med konceptet for relativ nølefase, var det muligt at forudsige vækst af *Listeria monocytogenes* tilfredsstillende. Yderligere blev der opnået gode forudsigelser for lave koncentrationer af *Listeria monocytogenes* i hytteost ved at anvende den observerede variabilitet i relative nølefase værdier for *Listeria monocytogenes* populationer.

Baseret på de opnåede resultater i dette Ph.d.-projekt kan gode og validerede deterministiske vækstmodeller modificeres og/eller anvendes til en række andre modellerings-formål. Yderligere blev det konkluderet at inklusion af mikrobiel interaktion var uundgåeligt når vækst af *Listeria monocytogenes* skal forudsiges i fermenterede mejeriprodukter. Generelt blev simple tilgange til modellering promoveret, såsom en empirisk tilgang til modellering af interaktion, anvendelse af populationsdata for nølefase-variabilitet når vækst af få *Listeria monocytogenes* skal forudsiges samt anvendelsen af empiriske fordelinger (bootstrapping) til at repræsentere observeret variabilitet i f.eks. produktegenskaber. Det vurderes at det er nødvendigt at definere nogle simple og overkommelige metoder der kan følges når der skal udvikles vækstmodeller for forholdsvist komplekse produkter såsom fermenterede mejeriprodukter. Udviklingen af modeller er en meget omfattende proces og der kræves nærmest uendelige mængder af data. Det vurderes derfor at resultaterne fra dette projekt kan have vigtig indflydelse på udviklingen af værdifulde prædiktive modeller der kan finde anvendelse i fødevareindustrien.

Table of Contents

OUTLINE AND OBJECTIVES

1. Outline and objectives

This PhD thesis consists of the following structure; (i) an introductory part which serves to give an overview of the subjects under study, (ii) a presentation of the three scientific papers generated from the obtained results and (iii) a final, summarising discussion, conclusions and future perspectives. The introduction describes dairy products and the fermentation process, activity and metabolism of lactic acid bacteria and issues relevant for *Listeria monocytogenes* in food products such as organism- and growth characteristics, listeriosis and infection, legislative matters and *Listeria monocytogenes* prevalence and listeriosis outbreaks. Furthermore, predictive microbiology, bacterial growth and different modelling approaches are described as well, in order to provide a theoretical basis for the investigated subjects- and for the decisions made during the project work.

The introductory theory constitutes the basis of the scientific papers. Paper I [Modelling the effect of lactic acid bacteria from starter- and aroma culture on growth of *Listeria monocytogenes* in cottage cheese] presents deterministic models for the simultaneous growth of lactic acid bacteria and *Listeria monocytogenes* in cottage cheese with fresh- or cultured cream dressing. Paper II [Semi-mechanistic and empirical modelling of interaction between lactic acid bacteria and *Listeria monocytogenes* in cottage cheese] investigates the mechanisms of the observed inter-bacterial interactions further and considers different modelling approaches for their mathematical description. In Paper III [Stochastic modelling of *Listeria monocytogenes* single cell growth in cottage cheese with mesophilic lactic acid bacteria from starter cultures], focus moves from predicting population growth to the prediction of *Listeria monocytogenes* single cell growth in community with lactic acid bacteria and the inclusion of variability, hence applying a stochastic modelling approach.

The objective of the present PhD-project was to develop predictive models for *Listeria monocytogenes* growth in dairy products. Cottage cheese – a fresh fermented dairy product – was used as a case product. Supported by findings in previous studies, it soon became evident that evaluation of simultaneous growth of lactic acid bacteria and *Listeria monocytogenes* was highly relevant. Throughout the project, focus has been on maintaining an application-oriented approach without neglecting the ambition of every food modeller of progression and "taking a step forward" within the area of predictive food microbiology.

In each paper, the findings of the individual studies are discussed and related to existing knowledge. The final, summarising discussion facilitates extension of the discussions from the papers or supplementing discussion of new topics of relevance for the project.

Finally, the findings of the project have been consolidated in the conclusion and thoughts about areas deserving further investigation, interesting topics and potential future research are outlined in the future perspectives-section.

INTRODUCTION

2. Introduction

2.1 Dairy products

Dairy products are widely available in a range of forms varying from fresh milk and cultured milk products to cheeses that have ripened for several months. Since ancient times, milk and dairy products have been considered to be an important part of the diet, documented by rock drawings in the Sahara and by remains of cheese found in Egyptian tombs (Miller et al., 2006). Cheese making has a long history and it is believed that the first cheeses were made by an accident when milk was allowed to spontaneously ferment due to growth and acidification by lactic acid bacteria (LAB) naturally present in the milk (Porto de Souza Vandenberghe et al., 2013; Wouters et al., 2002). Cheese is considered to be one of the most ancient forms of manufactured foods and deliberate fermentation of milk was first performed in order to prolong the shelf life of the perishable raw material. Later on, technological advances were achieved and production of cheese became more widespread. The geographical distribution of cheese making became evident from the different types of products that developed. Cheeses produced in Europe required less salt for preservation than in the warmer Middle East. A lower salt concentration allowed growth of a variety of beneficial microbes and moulds producing the characteristic and interesting flavours of aged cheeses (Porto de Souza Vandenberghe et al., 2013; Walther et al., 2008).

According to the Danish Veterinary and Food Administration (DVFA) the current average daily intake for an adult Dane was recorded to be more than 300 ml of dairy products (milk and milk products) and more than 30 g of cheese. These reports were based on observations made from 2003-2008 (DVFA, 2010; Pedersen et al., 2010). Milk and milk products are nutrient-dense food products meaning that they supply a high concentration of a range of nutrients in relation to their energy value (Miller et al., 2006). Being a major source of calcium, milk and dairy products are main contributors to the dietary intake of calcium. In Denmark the recommended daily intake of calcium is covered by the average daily intake throughout the population (DVFA, 2010; Pedersen et al., 2010). Health promoting properties of cheese and dairy foods have been reported in relation to blood pressure, growth- and development of the human body, anti-carcinogenic properties and contribution to formation and maintenance of strong bones and teeth (Huth et al., 2006; Walther et al., 2008). Despite the positive nutritional value and the beneficial properties of milk and dairy products, they are also a source of unsaturated fat. Approximately 50% of the saturated fat in the Danish diet is estimated to come from dairy products. Therefore, fresh cheeses such as cottage cheese, low fat quark and smoked fresh cheese possess a high nutritional value but contribute with less saturated fat and they are a good alternative to more fat-dense dairy products (DVFA, 2010). Cottage cheese is, for instance, often recommended as a part of a low calorie diet (Piccinin and Shelef, 1995).

2.2 Fermented dairy products

A range of different food products, for instance ripened cheese, pickles, sauerkraut, wine, vinegar, bread, soy sauce, buttermilk, yoghurt and fermented sausages, owe their flavour characteristics and stable shelf life to the fermentation process and activity of LAB, yeasts or moulds (Hansen, 2002; Jay, 2000). The cluster of fermented dairy products are usually distinguished based on geographical origin, type of fermentation organism and/or type of starter culture (mesophilic, thermophilic and probiotic cultures) (Mehta and de Oliveira, 2012). Products such as acidophilus milk, kefir, yogurt, cultured buttermilk and cheeses all belong to the group of fermented dairy products. Furthermore, the group of cheeses is divided into subgroups according to e.g. structure and product characteristics (Table 1) (Jay, 2000; Mehta and de Oliveira, 2012; Walstra et al., 2005a).

Table 1 Examples of different types of cheese (Walstra et al., 2005a, 2005b)

Category	Characteristics and/or examples of products
Gouda type	Made from cows milk, 40-50% fat in the dry matter. Made with mesophilic starter cultures (L-, DL- and D-cultures, see Table 2) and have matured from 2-15 months.
Cheddar type	Similar to Gouda type cheeses but are more dry and acidic. Produced with non-aromatic, mesophilic O-cultures (see Table 2)
Fresh cheeses	Have a high water content and have matured for short time or not at all. These types are primarily produced with mesophilic L- and DL cultures (except cottage cheese which is produced with a mesophilic O-culture, Table 2)
Very hard cheeses	Parmesan and Pecorino Romano. These hard cheeses are produced using thermophilic starter cultures.
Cheese with propionic bacteria	Emmentaler and Jarlsberg. Produced with mesophilic/thermophilic propionic acid bacteria starter cultures.
Stretched curd/pasta-filata	Provolone, Kashkaval and Mozerella. Produced with thermophilic starter cultures.
Semisoft cheese	Saint Paulin, Monterey and Amsterdammer. Produced with mesophilic starter cultures.
White pickled cheese	Feta and Domiati. Produced with mesophilic starter cultures.
Soft cheese with white mould	Brie and Camembert. Produced with a mesophilic starter culture (pre-acidification) and a mould surface flora.
Blue veined cheese	Bleu d'Auvergne, Gorgonzola, Roquefort and Stilton. Pre-acidified with a mesophilic or thermophilic starter culture and subsequently mould spores are added.

2.2.1 Fermentation

Biochemically, fermentation is defined as a metabolic process where carbohydrates and related compounds are partially oxidised with release of energy in the absence of external electron acceptors. The final electron acceptors are organic compounds directly produced from the breakdown of carbohydrates (Jay, 2000; Josephsen and Jespersen, 2004). LAB are traditionally associated with fermentation of dairy products (Rodríguez et al., 2012; Von Wright and Axelsson, 2011) and the LAB used for dairy fermentation are generally considered safe for human

consumption and are therefore assigned the QPS-status (Qualified Presumption of Safety) (EFSA, 2007; Leuschner et al., 2010). The most commonly used mesophilic LAB strains are *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc lactis*. Especially *L. lactis* and *L. cremoris* are capable of rapid acidification of the milk and *L. diacetylactis*, *L. mesenteroides* subsp. *cremoris* and *L. lactis* can metabolise citrate into CO_2 (hole formation) and diacetyl (buttery flavour), hence often called aroma producers (Josephsen and Jespersen, 2004). The applied thermophilic strains include *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus acidophilus* (Josephsen and Jespersen, 2004; Walstra et al., 2005b). In addition to these LAB strains, different lactobacilli are found in cheeses and are occasionally used as adjunct cultures. Furthermore, some dairy propionibacteria are used in some cheeses where they catabolise lactate into propionate, acetate and CO_2 during ripening (Josephsen and Jespersen, 2004).

The Gram positive, non-sporulating, microaerophilic, non-motile LAB do not possess a respiratory system and therefore rely on obtaining their energy from substrate-level phosphorylation (Josephsen and Jespersen, 2004; Von Wright and Axelsson, 2011). The organisms are divided into two subgroups according to their metabolic pathway and the associated end-products from fermentation of the carbon source; (i) homofermentative bacteria, which utilise the glycolysis (Embden-Meyerhof-Parnas pathway) and produces only lactic acid and (ii) heterofermentative bacteria which utilise the pentose phosphoketolase pathway (also named hexose monophosphate shunt or 6-phospho-gluconate pathway) and produces lactic acid, ethanol and acetic acid (Mehta and de Oliveira, 2012; Von Wright and Axelsson, 2011). In addition to lactose, the LAB also catabolise proteins and to some extent lipids present in the milk (Fig. 1). LAB are fastidious and require a range of nutrients in order to grow (Josephsen and Jespersen, 2004). In milk, sufficient amounts of directly available nitrogenous compounds (low-molecular mass peptides and amino acids) are not readily available and a proteolytic system in the LAB cell membrane is important for growth. This system is a prerequisite for bacterial growth and acid production (Walstra et al., 2005b). Besides the impact on growth of LAB, the enzymatic hydrolysis of milk proteins (caseins) and the related release of peptides and amino acids affect e.g. gel formation of yogurt, the mouth-feel of cheese and they are essential precursors for a range of reactions yielding flavour compounds during ripening (Mehta and de Oliveira, 2012; Walstra et al., 2005c). The LAB display limited lipolytic activity and the main effect of these reactions is observed during ripening where free fatty acids, formed due to lipolysis, contribute to flavour characteristics of matured cheeses (Walstra et al., 2005b, 2005c).
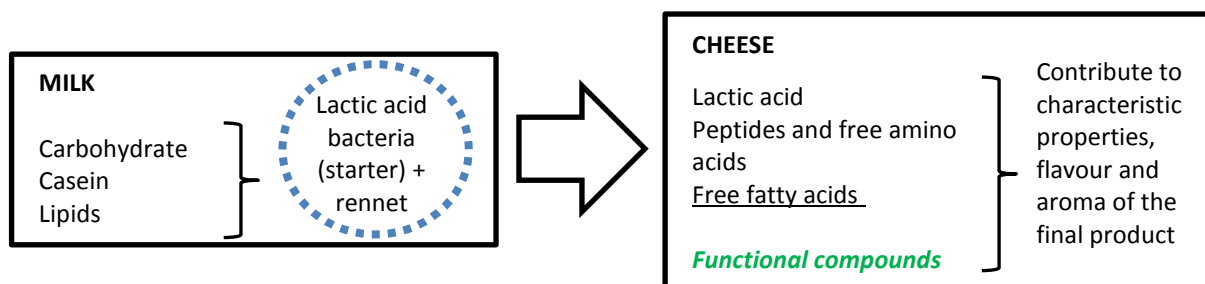
Figure 1 Simplified schematic presentation of the biochemical changes during fermentation of milk and subsequent ripening of cheese

2.2.2 Lactose metabolism and production of lactic acid

As described above, LAB are categorised, among others, based on their metabolism of carbohydrates and the associated metabolic products (homo- and heterofermentative organisms). In milk, the disaccharide lactose is the predominant carbohydrate and accounts for 50% of the milk solids (Mehta and de Oliveira, 2012). Lactose can enter the bacterial cell either by the means of a specific permease, where lactose is cleaved to glucose and galactose by β-galactosidase and subsequently these monosaccharides enter the fermentation pathways, or by a lactose specific PEP:PTS system (phosphoenolpyruvate dependent phosphotransferase system) as lactose phosphate (Fig. 2). Lactose phosphate is then cleaved by phosphor-β-D-galactosidase into glucose and galactose-6-phosphate. Subsequently, glucose enters the glycolytic pathway and galactose-6-phosphate enters the tagatose-6-phosphate pathway (Fig. 2) (Kandler, 1983; Von Wright and Axelsson, 2011). *L. lactis* typically possess the PEP:PTS system while in leuconostocs, *Streptococcus thermophiles*, thermophilic lactobacilli and other species, the permease system is typical. Pyruvate will most often act as electron acceptor to form lactic acid but alternative pathways utilising pyruvate exist in which formate, acetate, ethanol and acetoin may be formed (Von Wright and Axelsson, 2011; Walstra et al., 2005b). During homofermentative fermentation, where up to 95% of the lactose is converted into lactic acid, one mole of lactose yields four moles of lactic acid whereas heterofermentative fermentation only yields two mole lactic acid per mole lactose. The lactic acid formed from reduction of pyruvate to lactate can exist as D- or L-stereoisomers depending on specific lactate dehydrogenases found in the bacteria (Walstra et al., 2005b).

Homofermentative fermentation

$$\text{Lactose} + 4\ H_3PO_4 + 4\ ADP \rightarrow 4\ \text{lactic acid} + 4\ ATP + 3\ H_2O$$

Heterofermentative fermentation

$$\text{Lactose} + 2\ H_3PO_4 + 2\ ADP \rightarrow 2\ \text{lactic acid} + 2\ \text{ethanol} + 2\ CO_2 + 2\ ATP + H_2O$$
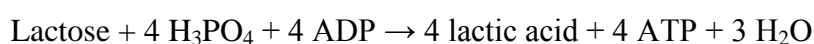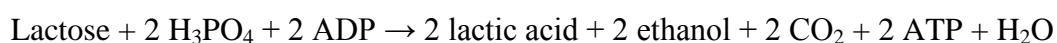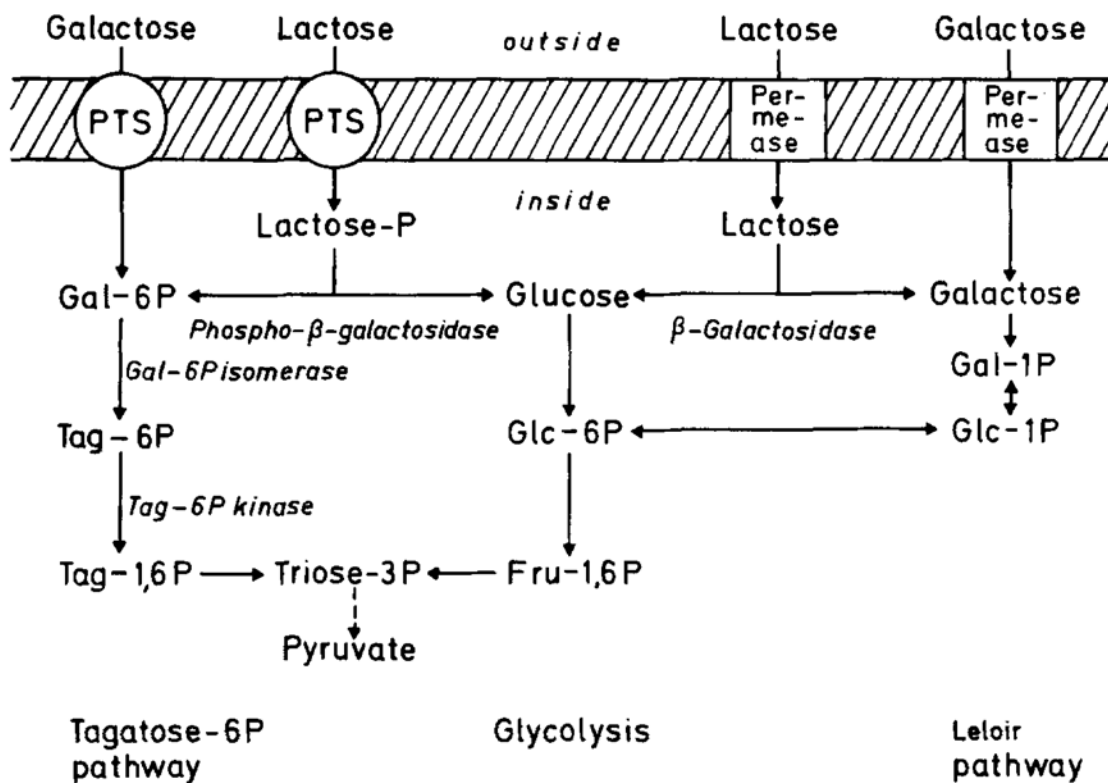
Figure 2 Lactose uptake and metabolism in LAB. From the exterior of the cell, lactose enters the cell either by specific permeases or by the PEP:PTS system. The end-product pyruvate usually acts as electron acceptor to form lactic acid but may also enter alternative pathways, e.g. the diacetyl/acetoin pathway (Kandler, 1983).

2.2.3 Lactic acid bacteria-starters and their activity

Dairy starter cultures are defined as a preparation of at least one type of microorganism, in high concentrations, to be added to raw or pasteurised milk in order to produce fermented dairy products. The main function of the starter culture is to produce lactic acid from the metabolism of lactose, resulting in a pH decrease which is important in relation to milk coagulation and product preservation (Leroy and De Vuyst, 2004; Rodríguez et al., 2012). In modern dairy production, commercially available LAB cultures are used to produce fermented products under highly controlled and standardised conditions (Leroy and De Vuyst, 2004). Based on their composition, these starters can be defined as (i) Single strain starters with a pure culture of one strain, (ii) Multiple strain starters with a defined mixture of pure cultures of more than one (2-6) strain and (iii) Mixed strain starters which are "natural" starters with an undefined mixture of multiple strains of different species of bacteria. The composition is based on a dynamic equilibrium between the different strains (Walstra et al., 2005b). Other measures used to classify starter cultures are the main function of the culture (primary (lactic acid production) or secondary (other metabolic compounds)) and their growth temperature (mesophilic- or thermophilic cultures) (Rodríguez et al., 2012). Different types of mixed cultures (D, L, DL, O, see Table 2) exist and are widely applied in the dairy industry. Examples of the composition and application of some mixed, mesophilic starter

cultures are provided in Table 2. The starter cultures highly affect properties such as structure, flavour, hole-formation and taste (Josephsen and Jespersen, 2004; Urbach, 1995).

Table 2 Examples of composition and application of some mixed mesophilic starter cultures (Josephsen and Jespersen, 2004; Walstra et al., 2005b)

Type	Organisms	Composition	Dairy product application
O[a]	*Lactococcus lactis* subsp. *lactis*	5-10%	Cheddar cheese
	Lactococcus lactis subsp. *cremoris*	90-95%	Cottage cheese
			Feta
			Quarg
L[b]	*Lactococcus lactis* subsp. *lactis*	5-10%	Lactic butter
	Lactococcus lactis subsp. *cremoris*	80-90%	Feta
	Leuconostoc mesenteroides subsp. *cremoris*	5-10%	Cheddar cheese
	Leuconostoc lactis		
D[c]	*Lactococcus lactis* subsp. *lactis*	5-10%	Lactic butter
	Lactococcus lactis subsp. *cremoris*	70-85%	
	Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*	10-20%	
DL[d]	*Lactococcus lactis* subsp. *lactis*	60-80%	Continental cheese (w. eyes)
	Lactococcus lactis subsp. *cremoris*	10-20%	Mould ripened cheese
	Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*	5-10%	Lactic butter
	Leuconostoc mesenteroides subsp. *cremoris*		Cultured buttermilk
	Leuconostoc lactis		Creme fraiche, ymer

[a] Contain neither leuconostocs or diacetylactis
[b] Contain leuconostocs
[c] Contain diacetylactis
[d] Contain both diacetylactis and leuconostocs

2.2.4 Citrate metabolising lactic acid bacteria

The so called aroma producers, capable of metabolising citrate into e.g. diacetyl, are responsible for desired flavour characteristics of dairy products such as cultured buttermilk and cottage cheese (Hugenholtz, 1993). Citrate is a normal constituent of milk where it is present in concentrations ~10 mmol/L. The concentration may vary with lactation stage and health status of the cow (Garnsworthy et al., 2006). An example of the importance of diacetyl in relation to consumer perception was presented by Antinone et al. (1994). They found that consumer aroma, flavour and overall liking attributes increased as a function of the concentration of diacetyl in the range of 0-4.0 ppm, peaking around 1.0 ppm. Similar results were reported by Drake et al. (2009) where the overall liking of cottage cheese with diacetyl scored higher than cottage cheese without diacetyl.

The pathways resulting in diacetyl and acetoin/2,3-butanediol requires a surplus of pyruvate which can be obtained from the breakdown of citrate present in the milk (Von Wright and Axelsson, 2011). Citrate is transported into the bacterial cell by citrate permease, where it is cleaved into acetate and oxaloacetate by citrate lyase. Subsequently, oxaloacetate is decarboxylated to yield pyruvate and CO_2. The most common route from pyruvate to diacetyl involves a spontaneous

reaction with the unstable molecule α-acetolactate as an intermediate. At low pH, α-acetolactate may be nonenzymatically decarboxylated to acetoin or, if oxygen is present, oxidatively to diacetyl (de Vos and Hugenholtz, 2004; Hugenholtz, 1993; Von Wright and Axelsson, 2011; Walstra et al., 2005b). In addition to the positive influence on flavour, diacetyl may also have an inhibitory effect on undesired microorganisms. Early studies on the inhibitory effect of diacetyl against Gram-positive lactic acid- and non-lactic acid bacteria, Gram-negative bacteria and yeasts revealed that the compound was effective at pH values < 7.0 and LAB were not affected by concentrations between 100 and 350 µg/ml. Inhibition of Gram-positive bacteria required concentrations of 300µg/ml whereas Gram-negative bacteria were inhibited by 200µg/ml. The overall conclusion was that diacetyl was considerably more effective as an antimicrobial against Gram-negative bacteria, yeasts and moulds than against Gram-positive bacteria (Jay, 1982). Similar results were obtained by Lanciotti et al. (2003) when they tested the inhibitory effect of diacetyl against *Escherischia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*. *L. monocytogenes* was the most resistant organism and inhibition of the organism required concentrations of diacetyl well above what would be expected to be present in e.g. fermented dairy products, which has been reported to be in the range of 1-2 ppm (Antinone et al., 1994).

2.2.5 Functional properties of lactic acid bacteria

Additional to the obvious impact on sensory properties caused by the LAB starter cultures, they may also contribute with functional properties. In relation to food spoilage and safety, the production of bacteriocins by LAB is of interest and will be described further in the following section.

Bacteriocins are small, ribosomally synthesised, low-molecular mass peptides or proteins displaying antimicrobial effects against related Gram positive bacteria (Cotter et al., 2005; Gálvez et al., 2007; Leroy and De Vuyst, 2004). Cotter et al. (2005) suggested to simply classify bacteriocins into two main categories; Class I are the lanthionine-containing lantibiotics which include single- and two-peptide lantibiotics containing the amino acids lanthionine, dehydroalanine and dehydrobutyrine. The peptides range from 18 to 38 amino acids in length. Class II are non-lanthionine-containing bacteriocins, which can be divided into four subclasses (IIa, IIb, IIc, IId) e.g. pediocin-like/*Listeria*-active, two-peptide and cyclic peptides. In addition to this, heat labile murerin hydrolases (non-bacteriocin lytic proteins) should be categorised in a third, non-bacteriocin category, as bacteriolysins (Fig. 3) (Cotter et al., 2005; Rodríguez et al., 2012).
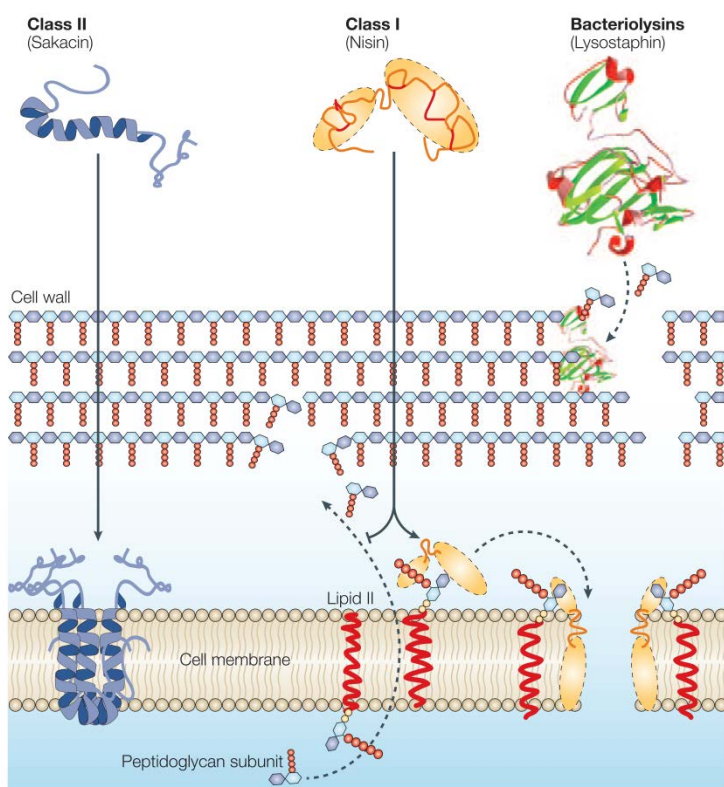
Figure 3 Mode of action of bacteriocins produced by LAB.
Class I bacteriocins, such as nisin, act by forming pores in the cell membrane and thereby disrupting the membrane potential *and* by binding to the docking molecule lipidII causing interruption of the cell wall synthesis which will kill the cell.
The general structure of Class II bacteriocins allow them to insert into the cell membrane which causes depolarisation and cell death.
The Bacteriolysins act directly on the cell wall of Gram-positive target organisms causing cell lysis and death (Cotter et al., 2005).

The Class I bacteriocins (e.g. the commercially applied nisin) are generally active through the formation of pores in the bacterial cell membrane of the target organism. This will lead to a loss of membrane potential and efflux of small metabolites. Another mechanism is enzyme inhibition and it has been established that nisin possess both mechanisms. Nisin binds to lipidII which act as a docking molecule and from this position both pore-formation and prevention of peptidoglycan synthesis is facilitated. The main proportion of Class II bacteriocins are active by inducing membrane permeabilisation and thereby leakage of molecules from the target organism. The third, non-bacteriocin group, catalyse the cell-wall hydrolysis causing cell lysis (Fig. 3) (Cotter et al., 2005).

From a food safety and quality point of view, bacteriocins are interesting due to a number of reasons: (i) they are generally regarded as safe compounds, (ii) they are not toxic to or active against eukaryotic cells, (iii) they have minimal influence on the gut microbiota as they are inactivated by digestive proteases, (iv) they are fairly heat- and pH tolerant, (v) they display a broad antimicrobial spectrum against food spoilage- and pathogenic organisms and (vi) no risk of cross resistance with antibiotics due to their mode of action (cytoplasmic membrane) (Gálvez et al., 2007). At present, only nisin, which is produced by strains of *Lactococcus lactic* subsp. *lactis*, is approved as a food preservative by the EU (E234) and the Food and Drug Administration in the US and its use as an additive must fulfil regulatory requirements (EC, 1995). Additionally, it must be labelled on the product (Sobrino-López and Martín-Belloso, 2008). Several studies have documented the antibacterial effect of nisin and other bacteriocins in a range of food/microorganism

combinations. However, far from exhaustive, a resume of some studies dealing with dairy application is provided in the succeeding section. Salih et al. (1990) investigated the effect of freeze dried and liquid Microgard™ on yeasts and Gram-negative bacteria in yogurt and cottage cheese, respectively. They found positive effects on shelf life of both products. 68% of the cottage cheese samples prepared with Microgard™ contained undetectable levels of spoilage organisms after 30 days of storage at 7°C and less than 1% displayed visible mould growth on the surface. For comparison, 33% of the samples prepared without Microgard™ displayed visible growth on the surface after 21 days of storage. In another study, low moisture (53%) pasteurised cheese spreads produced from cheddar cheese made with a nisin producing starter culture displayed a longer shelf life when stored at 22°C, compared to control samples without nisin. For instance, 6 of 8 batches prepared without nisin spoiled within 29-90 days of storage whereas 0 of eight batches of the cheese spread prepared with nisin spoiled within 90 days of storage at 22°C. (Roberts and Zottola, 1993). The effect of nisin and lacticin 3147 on *L. monocytogenes* in cottage cheese has also been investigated (Ferreira and Lund, 1996; McAuliffe et al., 1999). In both studies, pronounced inactivation of *L. monocytogenes* was observed, even when applying the most resistant strains found among a number of different *L. monocytogenes* strains. Davies et al. (1997) investigated the effect of commercially available nisin (Nisaplin®) on *L. monocytogenes* in ricotta-type cheese. They found that nisin efficiently inhibited growth of *L. monocytogenes* during storage at 6-8°C compared to control samples where critical concentrations of *L. monocytogenes* were reached within 1-2 weeks of storage.

However, as is the case for other antimicrobial compounds, resistance may also develop against bacteriocins. Davies and Adams (1994) studied the sensitivity to nisin of two strains of *L. monocytogenes*. They found, that mutants with increased resistance could be isolated from the one strain. Martínez et al. (2005) tested the sensitivity of wild *Listeria* isolates, from artisanal cheeses, to different bacteriocins (pediocin PA-1, enterocin AS-48, nisin and plantaricin C). They found some isolates to be highly resistant to pediocin PA-1. They concluded that nisin resistance should be considered when evaluating the risk of long term use of the compound as an anti-listerial agent.

Other antimicrobial compounds produced by LAB, besides organic acids (mainly lactic acid) and bacteriocins, include hydrogen peroxide, acetaldehyde and CO_2 (Gálvez et al., 2007; Rodríguez et al., 2012).

2.3 Listeria monocytogenes

The isolation- and thereby existence of *L. monocytogenes* was first reported in 1926 by Murray et al. and even though it may have been isolated prior to this, no isolates had been deposited and comparisons and identification was therefore not possible. In 1924 Murray and his colleagues observed sudden death of six young laboratory rabbits and the Gram positive rod was subsequently isolated from the blood of the dead animals (Hof, 2003; Rocourt and Buchrieser, 2007). The isolated bacterium was named *Bacterium monocytogenes* and in 1940 the genus was named *Listeria* (Hof, 2003). The genus *Listeria* contains six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*. Within the genus, two distinct lines of descent are recognised. The first contains *L. grayi* and the other contains *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. seeligeri*. Within the latter, the species can be divided into two different groups with *L. monocytogenes* and *L. innocua* in one and *L. ivanovii*, *L. seeligeri* and *L. welshimeri* in the other group. The six species can be grouped in five DNA relatedness groups. Group one contains the type strain of *L. monocytogenes* and include strains belonging to 13 different serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (Rocourt and Buchrieser, 2007). *L. monocytogenes* is the only species considered to be an important human pathogen even though *L. welshimeri*, *L. seeligeri* and *L. ivanovii* occasionally have been associated with illness in humans or animals (Adams and Moss, 2000).

2.3.1 Morphology

The *Listeria* cell is a regular, short rod of 0.4-0.5 by 1-2 µm. The cells occur as individual units, in short chains or they may be arranged in V or Y shapes. When cultured at 20-25°C all *Listeria* cells are motile with peritrichous flagella whereas they become

Figure 4
L. monocytogenes cells observed with phase contrast microscopy (25°C, BHI-agar, pH 5.3, 1% NaCl)

non-motile at 37°C. The organism is Gram-positive, catalase-positive, oxidase negative and non-spore forming. It display aerobic and facultative anaerobic metabolism (capable of growing with and without oxygen present). L(+)-lactic acid, acetic acid and some other end products are formed by homofermentative anaerobic catabolism of glucose (Adams and Moss, 2000; Rocourt and Buchrieser, 2007; Wagner and McLauchlin, 2008).

2.3.2 Listeria monocytogenes growth characteristics

L. monocytogenes occurs ubiquitously in the environment and it has been isolated from soil, decaying vegetation, river water, sewage sludge and animal feed (Stack et al., 2008). *Listeria* may also be the causative agent of mastitis in lactating cows and hence the raw milk can be contaminated (Bourry and Poutrel, 1996; Jensen et al., 1996). As a consequence of the widespread

occurrence, *L. monocytogenes* may contaminate raw materials to be used for food processing or they may settle in the production environment (Kornacki and Gurtler, 2007). Additionally, *L. monocytogenes* is highly suited as a foodborne pathogen due to a relative resistance to acid and salt and especially its ability to proliferate at refrigeration temperatures (Table 3) (Lado and Yousef, 2007), even though growth at refrigeration temperature is slow. The organism do not display increased heat resistance and is eliminated by pasteurisation (Wagner and McLauchlin, 2008).

Table 3 Growth and/or survival characteristics of *L. monocytogenes* in food products (Rocourt and Buchrieser, 2007; Schuchat et al., 1991; Wagner and McLauchlin, 2008)

Temperature	-0.15 – 45°C, optimum between 30-37°C
pH	4.3[a] – 9.4, optimum around pH 6 – 7
NaCl	Up to 10%[a]
a_w	> 0.92
MIC value, undissociated lactic acid	3.79 mM[b]
MIC value, undissociated sorbic acid	1.90 mM[c]

[a] Strongly dependent on temperature (Rocourt and Buchrieser, 2007)
[b] from UTAS model, see Gimenez and Dalgaard (2004)
[c] from Mejlholm and Dalgaard (2009)

As *Listeria* is frequently isolated from environmental sources and raw materials used for food production, strategies towards complete elimination of *Listeria* in the food industry are unrealistic (Gerner-Smidt et al., 2005; Stack et al., 2008). This realisation, combined with a demand for convenient and minimally processed food products (Gálvez et al., 2007), stresses the importance of *Listeria* management in the food industry.

2.3.3 Recovery and identification of Listeria monocytogenes from food samples

The traditional methods for recovery and identification of *L. monocytogenes* in food rely on selective enrichment broths, plating on selective media and subsequent biochemical tests (e.g. ISO 11290 method and the FDA bacteriological and analytical method (BAM)). This is considered to be the reference method but it is also time- and resource demanding. In recent years, attention has therefore been drawn to more rapid techniques based on antibodies or molecular methods such as ELISA, PCR or DNA hybridisation, real-time PCR and other methods (Gasanov et al., 2005). It is, however, beyond the scope of this thesis to elaborate on these different techniques. In the present project, *L. monocytogenes* from inoculated products was enumerated on selective media in order to quantify growth during storage. The underlying mechanisms of these commercially available selective media will therefore be described in the following section.

Listeria spp. usually require biotin, riboflavin, thiamine, thioctic acid and different amino acids (e.g. cysteine, glutamine, isoleucine, leucine and valine) in order to grow optimally. Additionally, proliferation require carbohydrates, such as glucose (Wagner and McLauchlin, 2008).

L. monocytogenes can be subcultured on most of the common bacteriological media (e.g. tryptone soy agar, brain heart infusion agar, nutrient agar, blood agar) but it is often challenging to isolate *L. monocytogenes* from food samples on these unselective media (Donnelly and Nyachuba, 2007). The psychrotolerance of *L. monocytogenes* has previously been exploited for cold enrichment procedures (first introduced by Gray et al. in 1948), but one of the main drawbacks of this method is the prolonged incubation period (several weeks) which is undesirable when testing food products for the presence of *L. monocytogenes* (Donnelly and Nyachuba, 2007; Gasanov et al., 2005). Contaminated food samples often contain low concentrations of *L. monocytogenes* and in order to be able to detect *L. monocytogenes* in 25 g of food sample, as required by most regulatory agencies, an enrichment step is necessary. However, high concentrations of other microorganisms are often present in the food and they may be able to outgrow *L. monocytogenes* during incubation (Gasanov et al., 2005; Jantzen et al., 2006). To overcome this problem, antimicrobial agents that suppress the competing microbiota are being employed to make the enrichment broths selective for *Listeria*. The most commonly used agents are (i) acriflavine, which inhibit growth of other Gram-positive bacteria, (ii) nalidixic acid which inhibit growth of Gram-negative bacteria and (iii) cycloheximide, which inhibit growth of fungi (Jantzen et al., 2006). Most of the selective agars for isolation and identification of *Listeriae* contain esculin and ferric iron (e.g. PALCAM agar, Oxford agar, modified Oxford agar (MOX)). All *Listeria* spp. hydrolyse esculin and complexation of the ferric iron with 6,7-dihidroxycoumarin, which is the product from esculin cleavage by β-D-glucosidase, produces a black precipitate. Colonies of *Listeria* spp. will therefore appear intensely black surrounded by a dense black halo (Donnelly and Nyachuba, 2007; Gasanov et al., 2005; Jantzen et al., 2006). Selective media capable of distinguishing between *L. monocytogenes* and other species of *Listeria* have also been developed, the so called ALOA-type media ("Agar *Listeria* according to Ottaviani and Agosti"). These chromogenic media primarily rely on two selective and differential substrates. 5-bromo-4-chloro-3-indolyl-β-D-glucopyanosid is an enzyme substrate utilised by β-D-glucosodase which is produced by all *Listeria* spp. The *Listeria* colonies become blue-turquoise with an average diameter of 1 mm. Differentiation is obtained by a second substrate, L-α-phosphatidylinositol, which is hydrolysed by the phospholipase C. This is a virulence factor only produced by the pathogenic *L. monocytogenes* and *L. ivanovii*. The colonies are blue-turquoise and surrounded by a white zone of precipitation. A range of commercially available media have been developed afterwards based on the original ALOA-composition (Jantzen et al., 2006; Wagner and McLauchlin, 2008).

During the experimental work of this project, *L. monocytogenes* in inoculated samples was quantified by surface plating directly onto selective PALCAM agar. For samples inoculated with low concentrations of *L. monocytogenes,* an enrichment step was included in order to estimate the most probable number (MPN) of *L. monocytogenes* in the analysed sample (Thomas, 1942).

2.4 Listeriosis

Despite the fact that *L. monocytogenes* is frequently isolated from environmental- and food samples, listeriosis remain an uncommon, yet highly serious disease with a high mortality rate (Gerner-Smidt et al., 2005). Fatality rates have been reported to range from 20-50% (Low and Donachie, 1997; Schuchat et al., 1991; Swaminathan and Gerner-Smidt, 2007; Vázquez-Boland et al., 2001) and between 22 and 45% for perinatal cases (Jackson et al., 2010). However, the European Food Safety Authorities (EFSA) reported a EU case-fatality rate of 17 % in 2010 (EFSA, 2012) and 17.8% in 2012 (EFSA, 2014) and a Danish survey on invasive listeriosis infections (1994-2003) reported an overall case fatality rate of 21 % (Gerner-Smidt et al., 2005). Certain groups of the population have been identified to be more susceptible to listeriosis. The YOPI's (Young – Old – Pregnant – Immunocompromised individuals) display increased risk of severe invasive listeriosis (Gerner-Smidt et al., 2005; Goulet et al., 2012; Schlech, 2000; Skogberg et al., 1992). Gerner-Smidt and co-workers analysed 299 cases of invasive listeriosis (excluding maternofetal cases) between 1994 and 2003 in Denmark. They found that for patients < 70 years, predisposing conditions had a major effect on mortality rate whereas that was not the case for patients > 70 years of age. For these patients, age was a predisposing factor in itself. Non-haematological malignancies was found to be the underlying factor most strongly increasing the risk of fatal outcome of an invasive listeriosis infection in patients < 70 years (Gerner-Smidt et al., 2005). Similar analysis of French cases between 2001 and 2008 (n = 1959) revealed that, compared to persons aged < 65 years and with no predisposing conditions, patients with chronic lymphocytic leukaemia had more than 1000 fold increased risk of acquiring listeriosis. Patients with liver cancer, myeloproliferative disorder, multiple myeloma, acute leukaemia, giant cell arthritis, dialysis, different forms of cancer (esophageal, stomach, pancreas, lung, brain), cirrhosis, organ transplants and pregnancy had 100-1000 fold increased risk of listeriosis compared to the reference group (Goulet et al., 2012). The outcome of a *Listeria* infection is also determined by the serovar of the infecting microorganism (Kuhn et al., 2008; Low and Donachie, 1997; Schuchat et al., 1991; Swaminathan and Gerner-Smidt, 2007). More than 95% of infections in humans are caused by serotypes 1/2a, 1/2b and 4b (Swaminathan and Gerner-Smidt, 2007) and most outbreaks are caused by strains of serotype 4b whereas sporadic cases are predominantly caused by serogroup 1/2 strains (Gerner-Smidt et al., 2005). Strains from serogroup 1/2 (a,b,c) are most often isolated from food or food-production environment (Swaminathan and Gerner-Smidt, 2007). Even for susceptible groups, the infective dose is estimated to be high ($>10^6$ cells), which is also evident based on the relative low frequency of infection compared to the presence of *Listeria* in food and environment (Kuhn et al., 2008). The density of *L. monocytogenes* in foods implicated in febrile gastroenteritis outbreaks has been found to vary between 3×10^1 CFU/g to 1.6×10^9 CFU/g (Painter and Slutsker, 2007). The incubation period for listeriosis has been reported to vary from one day up to 90 days, but usually it illness will occur within a few weeks (Adams and Moss, 2000).

2.4.1 Infection

Clinical signs of listeriosis are similar in all susceptible hosts. Generally, two basic forms of presentation are recognised; perinatal listeriosis and listeriosis in adult patients. The predominating, invasive forms are generalised infection with sepsis and bacteremia or local infections in different parts of the body (Kuhn et al., 2008; Vázquez-Boland et al., 2001). Non-invasive forms also occur and they are manifested by e.g. febrile gastroenteritis. It is assessed that the number of cases of non-invasive listeriosis is highly under reported due to the self-limiting nature of this presentation of infection (Swaminathan and Gerner-Smidt, 2007). An overview of clinical syndromes attributed to infection with *L. monocytogenes* is provided in Table 4.

Table 4 Clinical syndromes that have been attributed to *Listeria* infection (Schlech, 2000)

Neonatal sepsis and meningitis (early- and late-onset)	Hepatitis
Bacterial meningitis in adults	Liver abscess
Rhombencephalitis in adults	Febrile gastroenteritis
Sepsis syndrome in adults	Spontaneous bacterial peritonitis
Native or prosthetic valve endocarditis	Continuous ambulatory peritoneal dialysis peritonitis
Arterial infections	Osteomyelitis
Pneumonia	Septic arthritis

The primary source of *Listeria* infection is assessed to be food, with infections developing after translocation of *L. monocytogenes* from the gastrointestinal tract (Hof, 2003; Jackson et al., 2010; Kuhn et al., 2008; Schlech, 2000; Vázquez-Boland et al., 2001). *L. monocytogenes* can enter the host through the intestinal mucosa by two different mechanisms. (i) specific mechanism that rely on direct invasion of the enterocytes lining the absorptive epithelium of the microvilli causing infection of the intestinal cells and (ii) unspecific entry pathway involving phagocytosis by the M cells of the Peyer's patches. After entry, the bacteria localise within professional phagocytes and antigen presenting cells, where replication of the bacteria takes place. Dissemination occurs rapidly and the liver and spleen are the main target organs. Immunocompetent hosts are capable of confining infections so they remain subclinical. In immunocompromised hosts, bacteria can be released into the bloodstream (Fig. 5) from where the infection can progress towards clinical listeriosis presented as septicemic disease or localised infections in the brain or the fetoplacental unit. Thus listerial infection is a multistage process with several barriers prior to clinical infection of secondary target organs (Kuhn et al., 2008; Vázquez-Boland et al., 2001).
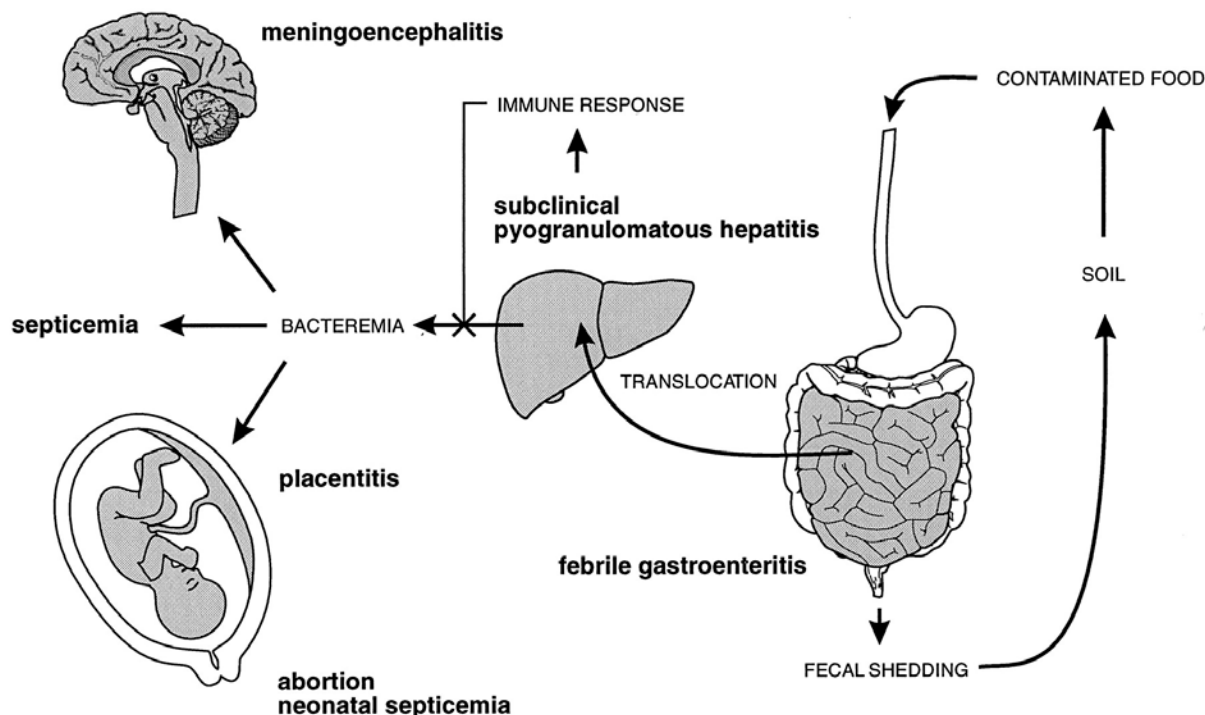
Figure 5 Schematic presentation of the pathophysiology of *Listeria* infection (Vázquez-Boland et al., 2001). After translocation from the gastrointestinal tract to the spleen and liver, *Listeria* cells may be released into the bloodstream in immunocompromised hosts. From here, the bacterial cells can spread to-, establish themselves and multiply in secondary target organs, e.g. the brain or the fetoplacental unit.

The severity of listeria infection combined with the relative high frequency of isolation from food- and food production environment, and the increased demand for convenient, ready-to-eat food products with no- or low concentrations of preservatives, makes management of *L. monocytogenes* throughout the food production chain highly important (Gálvez et al., 2007; Schlech, 2000). The pathogenic organism receives extensive attention both nationally-, at EU level and worldwide, and food production companies spend considerable resources on documentation and management. At EU level, all food production companies must comply with the General Food Law, hygiene regulations and specific criteria for hygiene and food safety (EC, 2005, 2004a, 2004b, 2002).

2.5 Listeria monocytogenes; Regulatory issues, Prevalence and Outbreaks
2.5.1 Legislation

Consumers are entitled to expect food products to be safe and suitable for consumption (CAC/RCP 1-1969). The General Food Law (EC, 2002) state that food business operators have the responsibility to produce safe food. Food safety must be ensured by a preventive approach which can be obtained by implementation of a food safety management system based on the Hazard Analysis and Critical Control Point (HACCP) system. In fact, the EU regulation on the hygiene of foodstuffs (EC, 2004a) clearly state that HACCP based food safety management systems must be implemented and that the management of food processing companies are responsible for implementation of, putting into practice- and compliance with these preventive systems.

Additionally, food business operators must implement, and follow, good hygiene practices (GHP) (EC, 2004a). For food products of animal origin, the EU regulation 853/2004 lay down more specific hygiene requirements regarding raw material quality, handling, treatment, storage of foodstuffs of animal origin etc. (EC, 2004b). Regulation 2073/2005 set up specific microbiological criteria that food processors must comply with. The criteria are categorised in two groups. (i) Food safety criteria, which define the acceptability of food in relation to the microbiological safety. These criteria apply throughout the shelf life of the food product and (ii) Process hygiene criteria, which serves as indicators of whether the production processes are operating in a hygienic manner or not. They apply at various stages during the production processes. For *Listeria*, food safety criteria are defined for ready-to-eat (RTE) foods (Table 5) (EC, 2005).

Table 5 Food safety criteria applicable to *Listeria monocytogenes* (EC, 2005)

Food category	Sampling plan		Limits[c]		Stage where the criterion applies
	n[a]	c[b]	m	M	
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
Ready-to-eat foods able to support the growth of *L. monocytogenes* 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
Ready-to-eat foods unable to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes[d,e]	5	0	100 CFU/g		Products placed on the market during their shelf-life

[a] n = number of units comprising the sample
[b] c = number of samples giving values above m=M
[c] m = M
[d] Under normal circumstances, regular testing against the criterion of certain ready-to-eat foods is not useful (EC 2073/2005)
[e] This criterion applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout the shelf-life. The operator may fix intermediate limits during the process that should be low enough to guarantee that the limit of 100 CFU/g is not exceeded at the end of the shelf-life.
[f] This criterion applies to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout the shelf-life.

It is evident that the legislation in place imposes a burden on the food manufacturers in relation to documentation of presence/absence, survival and growth of *L. monocytogenes* in ready-to-eat foods. Additionally, the food processors must ensure that the food safety criteria are complied with under "reasonably foreseeable conditions of distribution, storage and use" (EC, 2005). For documentation, the food producers must conduct studies and analyses in order to investigate compliance with the food safety criteria in place. In particular, this applies to RTE products supporting growth of *L. monocytogenes* and thereby induces a potential risk of *Listeria* infection. The wording of the Act specifies that the physico-chemical properties of the product, concentration of preservatives and type of packaging should be studied. In combination with this, scientific literature on growth and survival of the microorganism of interest should be consulted. If sufficient information is not available, additional studies must be conducted, including challenge testing and application of

predictive mathematical models developed for the product of interest (EC, 2005). The EU hereby expresses confidence in the use of predictive models, as a supplement to traditional microbiological testing, for the evaluation of growth in, and growth potential of specific food products.

2.5.2 Prevalence of- and outbreaks caused by Listeria monocytogenes in dairy products

Historically, food products were not considered to be a vehicle for *Listeria* transmission until the early eighties. However, in 1981 an outbreak occurred in the Maritime province in Canada, causing 34 perinatal- and seven adult cases. The mortality rate approached 30% for both groups. The outbreak provided evidence of a link to contaminated food products and commercially produced coleslaw, prepared with contaminated cabbage, was identified to be the source of infection. Subsequently, foods were recognised as vehicles of *Listeria* and numerous studies were published afterwards associating a range of food products with outbreaks of listeriosis (Conly and Johnston, 2008).

All foods may potentially cause foodborne illnesses and being of animal origin, milk and milk products are no exception. Human pathogens can be transferred from the dairy animal to the milk or the milk can be contaminated during subsequent storage and processing (CAC/RCP 57-2004). From January 2010 to June 2014, 87 notifications on listeria in cheese products were recorded in the Rapid Alert System for Food and Feed (RASFF) in the EU. Of these, 61 notifications were not related to raw milk products, indicating cross contamination after pasteurisation (EC, 2014a). In 2012, a total of 1,642 cases of confirmed listeriosis were reported by the member states. The average number of confirmed cases per 100,000 citizens was 0.41. The rate in Denmark was 0.90 per 100,000 citizens (EFSA, 2014). Specific information and statistics on dairy products associated with outbreaks of listeriosis in the EU is scarce, however in 2010 the transmission route was stated for 8.26% (132) of reported outbreaks of which 13 outbreaks were associated with the consumption of cheese (EFSA, 2012). In 2009, 14 cases in Austria and Germany were associated with "quargel" (acid curd cheese). Of these, four cases had fatal outcome (Fretz et al., 2010). In August 2012, two cases of pregnancy related listeriosis were associated with the consumption of pasteurised Latin-style fresh cheese in Spain (de Castro et al., 2012). Additionally, 24 confirmed outbreaks of listeriosis linked to cheese and cheese products (raw and pasteurised) were reported from 1985 to 2014 in the US (Foodborne Illness Outbreak Database, 2014). An overview of cheese associated outbreaks reported in literature and American databases is given in Table 6.

Table 6 Listeriosis outbreaks implicating cheese products

Country	Year	No. of cases (fatalities)	Implicated food	Reference
Switzerland	1983-1987	57 (18)	Soft cheese	Bula et al., 1995
USA	1985	142 (48)	Mexican style soft cheese	Linnan et al., 1988
UK	1987	1	Soft cheese	Bannister, 1987
Luxembourg	1989	2	Camembert	Ries et al., 1990
Denmark	1989-1990	26 (6)	Blue- and hard cheese	Jensen et al., 1994; Norton and Braden, 2007
France	1995	37 (11)	Soft cheese	Goulet et al., 1995
USA	2000	13	Non-commercial Mexican style cheese	MacDonald et al., 2005
Sweden	2001	33	Soft cheese	Carrique-Mas et al., 2003
Japan	2001	?	Cheese	Papademas and Bintsis, 2010
Canada	2002	17	Soft or semi-hard cheese	Norton and Braden, 2007
USA	2003	12	Queso fresco	Foodborne Illness Outbreak database[a]
USA	2005	9	Queso fresco	Foodborne Illness Outbreak database[a]
USA	2006	3 (1)	Pasteurised cheese	Foodborne Illness Outbreak database[a]
Germany	2006-2007	189	Pasteurised cheese (acid curd)	Koch et al., 2010
Canada	2008	38	Pasteurised cheese	Foodborne Illness Outbreak database[a]
USA	2008	8	Asadero cheese	Foodborne Illness Outbreak database[a]
Austria and Germany	2009	14 (4)	"Quargel" (acid curd cheese)	Fretz et al., 2010
USA	2009	8	Mexican style cheese	Foodborne Illness Outbreak database[a]
USA	2009	2	Cheese	Foodborne Illness Outbreak database[a]
USA	2010	1	Queso fresco	Foodborne Illness Outbreak database[a]
USA	2010	5	Fresh cheese	Foodborne Illness Outbreak database[a]
USA	2010	2	Raw dairy (cheese?)	Foodborne Illness Outbreak database[a]
USA	2011	2	Chives cheese	Foodborne Illness Outbreak database[a]
Spain	2012	2	Latin-style fresh cheese	de Castro et al., 2012
USA	2012	22 (4)	Ricotta	Foodborne Illness Outbreak database[a]
USA	2012	1	Queso fresco	Foodborne Illness Outbreak database[a]
USA	2012	1	Cheese	Foodborne Illness Outbreak database[a]
USA	2013	5 (1)	Soft brie	Foodborne Illness Outbreak database[a]
USA	2013	8 (1)	Soft cheese	Foodborne Illness Outbreak database[a]

[a] (Foodborne Illness Outbreak Database, 2014)

The European Food Safety Authority (EFSA) conducted a baseline survey (2010-2011) of the prevalence of *Listeria* in soft- and semi-soft cheeses (excluding fresh cheeses) in the EU. It was found that the prevalence of *L. monocytogenes* contaminated cheese samples was 0.12% (EFSA, 2013).

These documentations of prevalence, notifications, withdrawals and outbreaks all underline the potential risk of *L. monocytogenes* in cheese and cheese products making it highly relevant to investigate the growth potential of the products and the control measures during processing and storage. For this, predictive microbiology can be a helpful and beneficial tool (DVFA, 2014; EC, 2005).

3. Case product: Cottage cheese

Cottage cheese was used as a case product throughout the project. This product presents some interesting challenges since it is a ready-to-eat product consisting of two distinct components and it contains considerable concentrations of LAB from the fermentation of the cheese curd. Additionally, some products are produced with a cultured cream dressing where a diacetyl producing LAB culture has been added prior to mixing (Examples, Fig. 6a and 6b).

Figure 6a Cottage cheese produced with a cultured cream dressing (Arla Karoline cottage cheese, 4.5% fat)

Figure 6b Cottage cheese with fresh cream dressing (Arla cottage cheese, 4.0% fat)

Cottage cheese belongs to the soft, high moisture, fresh cheeses, which contain 55-80% moisture (Fernandes, 2009). The nutritional value (Table 7) is characterised by a high protein and water content and a low content of fat and carbohydrates compared to other types of cheeses (Araújo et al., 2012).

Table 7 Nutritional value of cottage cheese, 20+ (Foodcomp, 2014)

Energy (kJ/100g)	Protein (g/100g)	Fat (g/100g)	Carbohydrate (g/100g)	Water (g/100g)
433	12.2	5.4	1.5	79.7

Cottage cheese is an unripened, particulate cheese made from skim milk. It exhibits a slightly acidic pH, low salt content and a moderate initial concentration of lactic acid. The cheese curd has a pH around 4.5-4.7 whereas the dressing has pH around 7.0 resulting in a final product pH around 5.1-5.4 (Table 8).

Table 8 Product characteristics of cottage cheese and its components (curd and dressing). Data obtained from the present PhD-project.

Product/component	pH (Avg ± SD)	NaCl (% in water phase ± SD)	Lactic acid (water phase ppm ± SD)
Cottage cheese with fresh cream dressing	5.18 ± 0.06	1.21 ± 0.07	718 ± 187
Fresh cream dressing (~10% fat)	7.00 ± 0.02	3.52 ± 0.02	-[a]
Curd of cottage cheese with fresh cream dressing	4.53 ± 0.05	0.04 ± 0.01	519 ± 112
Cottage cheese with cultured cream dressing	5.39 ± 0.07	1.09 ± 0.08	1029 ± 244
Cultured cream dressing (~10% fat)	6.89 ± 0.03	3.01 ± 0.03	547 ± 70
Curd of cottage cheese with cultured cream dressing	4.78 ± 0.00	0.19 ± 0.03	2838 ± 102

[a] Below the limit of detection

The grainy cheese curd is mixed with either fresh or cultured cream (Fig. 7, Walstra et al., 2005a). Acidification of the milk can be induced by addition of a starter culture or by addition of an organic or inorganic acid (direct-set method). Acidification induced by LAB can be done in three different ways; (i) long-set method, (ii) medium-set method and (iii) short-set method. The methods differ in setting time (time to cutting), temperature of set milk and in the concentration of the added starter (shorter time – higher concentration). Traditionally, the short-set method has been applied (Bylund, 1995; Walstra et al., 2005a).
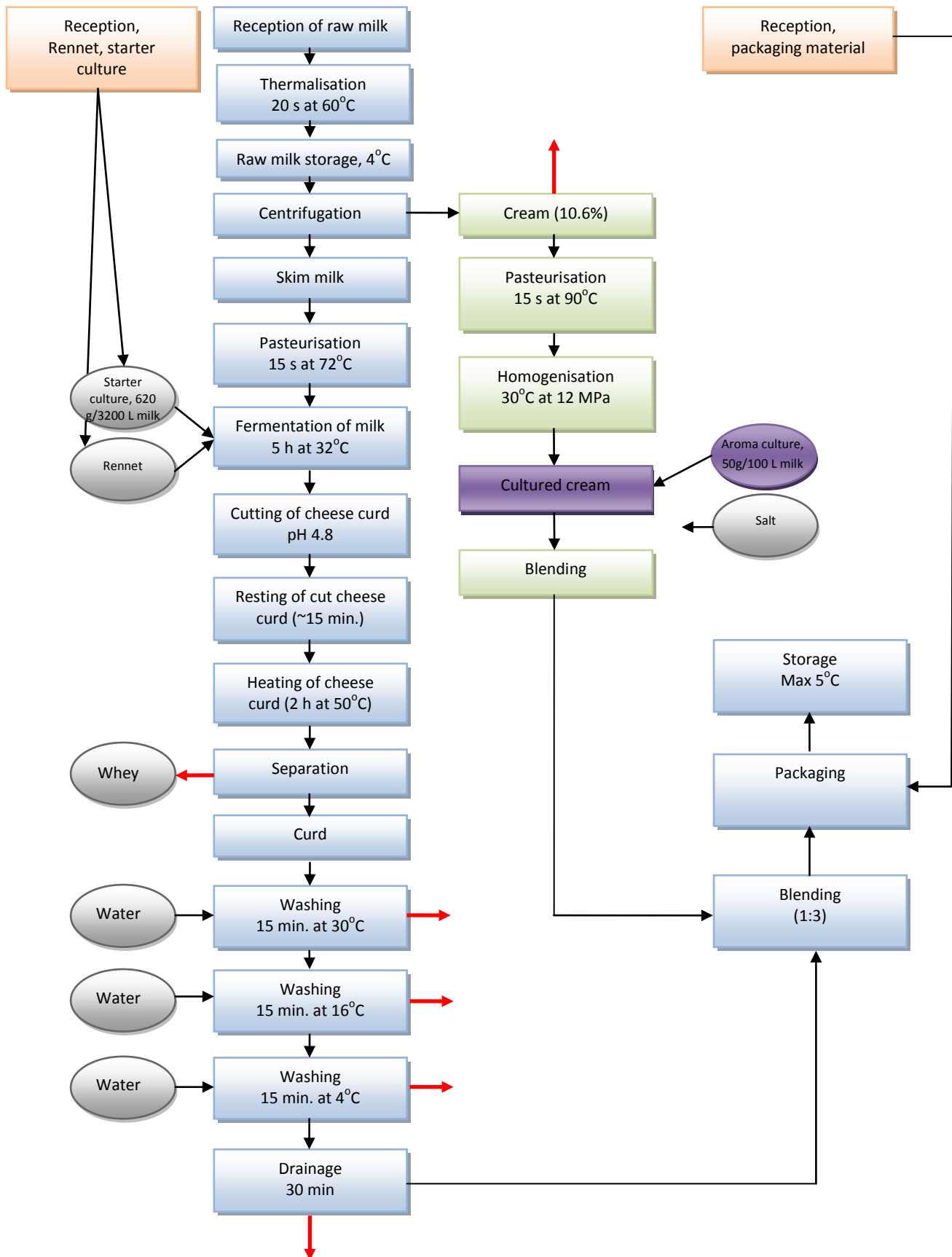
Figure 7 Flow sheet for the production of traditional short-set cottage cheese with fresh or cultured (purple) cream dressing. Red arrows indicate materials "leaving" the production flow and blue are the direct manufacture of cottage cheese. Modified from Bylund (1995) and Walstra et al. (2005a).

3.1 Production of cottage cheese

Raw milk is received and thermalised at 60°C for 20 seconds in order to prevent the formation of heat resistant lipases and proteinases. The thermalisation allows the milk to be stored at refrigeration temperatures before use, without affecting the quality adversely. Skim milk and cream is separated by centrifugal cream separation and part of the cream is kept for later addition to the cottage cheese. The separated skim milk is low-pasteurised (15 sec. at 72°C) in a plate heat exchanger and holding cell and subsequently cooled to 32°C, which is the clotting temperature applied in the manufacture of short-set cottage cheese. Rennet and starter culture (Mesophilic O-culture; *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *Lactis*, 620 g freeze dried culture to 3200 L of milk) is added. The milk is left to clot for 5 h at a constant temperature of 32°C. When pH has decreased to 4.8 the curd is cut and left to stand for approximately 15 minutes in order to expel some whey. The curd is not stirred. The cheese curd is heated for 2 h at 50°C and the rate of heating and heating temperature can be used to affect the properties of the cottage cheese. The expelled whey is separated from the curd which is then washed three times at different temperatures (30°C, 16°C and 4°C). The washing serves to dilute lactose and lactic acid and it also lower the temperature of the curd to approximately 4°C, which stops further lactic acid production and shrinkage of the curd. In between each washing step, the curd is drained. The cream which has been standardised to 10.6% fat, high-pasteurised (15 sec. at 90°C) and homogenised, is now added to the cheese curd in a ratio of 1:3. The dressing can be either fresh- or cultured. The cultured cream is prepared by adding 50 g of freeze dried LAB culture to 1000 L of cream at 33-37°C. The culture is distributed by agitation during cooling of the cream to 4°C. Salt is added to the cream dressing (1.75 % resulting in final salt concentration of approximately 1.0%). After blending, the creamy cottage cheese is packed and stored at 5°C until distribution to retail (Bylund, 1995; Nielsen, 2014; Walstra et al., 2005a).

3.2 Effect of processing on the product characteristics of cottage cheese

In the illustrated production of cottage cheese (Fig. 7), two heating steps are included (thermalisation and pasteurisation). The first heating step (thermalisation) is performed immediately after receiving the raw milk and serves to kill psychrotolerant bacteria which may produce heat resistant proteinases and lipases that can impair the yield and quality of the cheese (Lucey and Kelly, 1994). In cottage cheese, activity of these extracellular enzymes can cause flavour defects described as bitter, rancid, unclean, fruity and yeast-like. Furthermore, the breakdown of proteins reduces the yield when manufacturing cheese (Sørhaug and Stepaniak, 1997; Walker, 1988). If the milk is to be used immediately, this heating step can be omitted, since the pasteurisation will kill the undesired microorganisms.

When producing fresh, unripened cheese, the milk must be pasteurised. The intensity of the heat treatment affects the cheese processing and the quality of the final cheese (Bylund, 1995). The

rennetability and the rate of syneresis decreases as a consequence of the heat treatment, and especially more intense heat treatment than low pasteurisation lead to increased clotting time, a weaker curd and impaired syneresis. This is partly caused by a decrease in Ca^{2+} activity, but also because a part of the serum protein becomes covalently bound to κ-casein and to some proteins of the fat globule membrane (Walstra et al., 2005d, 2005e).

Syneresis is one of the most important steps in cheese production since it affect the moisture content, acidity and texture of the product. In a study by Castillo et al. (2006) on factors affecting cottage cheese-type gels they concluded the following. Increasing coagulation temperature had a significantly increasing effect on the rate of syneresis whereas increased inoculum concentration decreased the rate of syneresis. Syneresis parameters were correlated to acidification and formation of protein network (Castillo et al., 2006).

The pH of the curd at the time of cutting highly affects the properties of the product. The pH of the curd determines the firmness of the coagulum. The acid production is normally allowed until pH 4.6-4.8 is reached where after the curd is cut and left to rest before being heated. If the pH is lower at the point of cutting, the curd becomes too weak and if the pH is higher (> 4.9) then the curd becomes firm and tough (Walstra et al., 2005a).

Heating conditions are determining for the total solids in cottage cheese curd and hence indirectly also firmness and size distribution (Chua and Dunkley, 1979). The rate of heating during cooking affects consistency and firmness of the curd. The slower heating rate, the more even syneresis and in opposition to that, fast heating results in a curd with a more dry and firm rind. During heating the curd should be agitated gently to avoid fusing of the curd grains (Walstra et al., 2005a). Chua and Dunkley (1979) found a range of parameters that were affected by heating rate. The desired total solids was reached more rapidly with a higher heating rate, but higher rates of heating also results in a firmer curd which may not be desired.

Washing of the curd is normally performed three times at different temperatures (30°C, 16°C and 4°C; other temperatures may be applied but within the same temperature range). The washing serves to dilute lactose and lactic acid and to cool down the curd, which stops further production of lactic acid and prevents shrinkage of the curd, since syneresis is stopped by cooling. In between each washing step, whey-water is drained off (Bylund, 1995; Walstra et al., 2005e).

3.3 Behaviour of Listeria monocytogenes in cottage cheese during storage

When assessing the safety of food products in relation to *L. monocytogenes* the growth potential of the product must be clarified. As stated in the EU regulations (EC 2073/2005) existing scientific literature and growth studies should be used to assess the growth potential of a given product. For cottage cheese, different studies of *L. monocytogenes* growth exist in the literature. However, these studies do not provide unequivocal conclusions regarding the growth potential (Table 9).

Table 9 Information obtained from the scientific literature on growth/no growth of *L. monocytogenes* in cottage cheese during storage

Description of experiment	Growth (Yes/No)	Reference
Cottage cheese with fresh- and cultured cream dressing (pH 5.1-5.4) was inoculated with *L. monocytogenes* and growth was monitored during storage at constant temperatures between 5-15°C and during storage at dynamic temperatures between 5 and 12°C. *L. monocytogenes* grew at all investigated temperatures but was inhibited when LAB reached their maximum population densities at higher storage temperatures (see also Fig. 8)	Yes	Østergaard et al., 2014
A control cottage cheese was manufactured without enterocin A producing *Lactococcus lactis* and inoculated with different concentrations of *Listeria monocytogenes* EGD*elux* (~10^4, ~10^5, ~10^6, ~10^7 CFU/g). The pH of the cheese was not provided. *L. monocytogenes* was monitored during 15 days of storage. A slight decrease occured over time (~0.1-0.6 log CFU/g) in the samples without enterocin A producing *Lactococcus lactis*. The least decrease was found for the higher initial inoculation concentrations.	No	Liu et al., 2008
Cottage cheese was produced in lab scale facilities and the cottage cheese exhibited a pH of 5.2. In the control sample, manufactured without bacteriocin producing starter culture, the concentration of *L. monocytogenes* was hardly affected during 7 days of storage at 4 and 18°C, respectively. At 30°C *L. monocytogenes* was not detected in the control sample after 3 days of storage. By that time, the cheese had been excessively spoiled by moulds.	No	McAuliffe et al., 1999
The effect of acid adaption and growth/survival of a mutant (enhanced acid tolerance) was investigated using growth/survival of LO28 as reference. The cottage cheese applied was made in the laboratory and displayed a pH value of 4.71. The samples were stored at 4°C for 15 days. After 15 days ~0.1% of the initial concentration of LO28 (control sample) had survived, whereas ~5% of the mutant (ATM56) and the acid adapted LO28 had survived. The same cultures were inoculated into commercially purchased cottage cheese exhibiting a higher pH (5.15). These data were not included in the study, but it was reported that all strains (mutant, adapted/non-adapted LO28) survived (stored for 26 days) at the elevated pH and that there was no evident difference between strains.	No / Yes	Gahan et al., 1996
Growth curves for *L. monocytogenes* (Scott A) in commercial low-fat cottage cheese (pH 4.5) were obtained in this study (control samples). *L. monocytogenes* grew at 4°C when inoculated into cottage cheese. During the storage period of 11 weeks the concentration increased from ~10^3 CFU/g to ~10^5 CFU/g. Normally, the shelf life of cottage cheese is 2-3 weeks, and in this period a slight decrease/static concentration was observed.	Yes / No	Larson et al., 1996
L. monocytogenes in the control sample (- nisin, pH 4.6-4.7) did not grow when stored for 7 days at 20°C. A decrease of approximately 1 log unit was observed (log ~5.5 CFU/g → log ~4.3 CFU/g).	No	Ferreira and Lund, 1996
The fate of *L. monocytogenes* in cottage cheese containing sorbate and citric acid was investigated. It was found that *L. monocytogenes* was unable to increase in concentration, but the observed decrease was limited. No significant difference in the decline in cottage cheese containing sorbate and citric acid was observed, but sorbate containing cottage cheese remained sensory acceptable during the storage period (24 days) whereas samples containing citric acid spoiled due to off-odours (12-14 days) and visible surface moulds and yeast on some samples.	No	Piccinin and Shelef, 1995
Rapid growth of *L. monocytogenes* was observed in cottage cheese (pH 5.14) at 7°C, where an increase from 10^4 to >10^7 CFU/g (within the normal shelf-life of commercial cottage cheese) occurred after a lag phase of ~7 days. At 4°C *L. monocytogenes* also grew, but only after a lag-phase of 28 days. The concentration increased from 10^4 to >10^7 CFU/g (day 28 – day 60). In the study, the standard plate count (SPC) corresponded to the concentrations of the recovered *L. monocytogenes* during storage, indicating no interfering/parallel microbiota in the product.	Yes	Chen and Hotchkiss, 1993

The discrepancies in the reported growth responses of *L. monocytogenes* in cottage cheese (Table 9) should be considered in relation to the product characteristics of the monitored products. Generally, pH values in products displaying no growth of *L. monocytogenes* were close to the minimum pH allowing growth of *L. monocytogenes* (Table 3, Lado and Yousef, 2007).

Throughout the present PhD-project, a range of growth experiments were conducted in both cottage cheese and fresh- and cultured cream dressing, inoculated with different concentrations of *L. monocytogenes* (2-4 log CFU/g or 0.4-1 cell per gram). The cottage cheese was stored at constant temperatures between 5°C and 15°C or at dynamic temperatures in the range from 5°C to 12°C. During all experiments, *L. monocytogenes* grew (Fig. 8), except in one experiment where 1000 ppm sorbate had been added to the product. Simultaneously with *L. monocytogenes*, the behaviour of LAB from the added starter cultures was monitored during storage (Fig. 8). It was evident, that when LAB grew a pronounced growth inhibition of *L. monocytogenes* was observed when LAB reached their maximum population density. This phenomenon corresponded to the Jameson effect (Gimenez and Dalgaard, 2004; Jameson, 1962) and will be addressed in later sections.
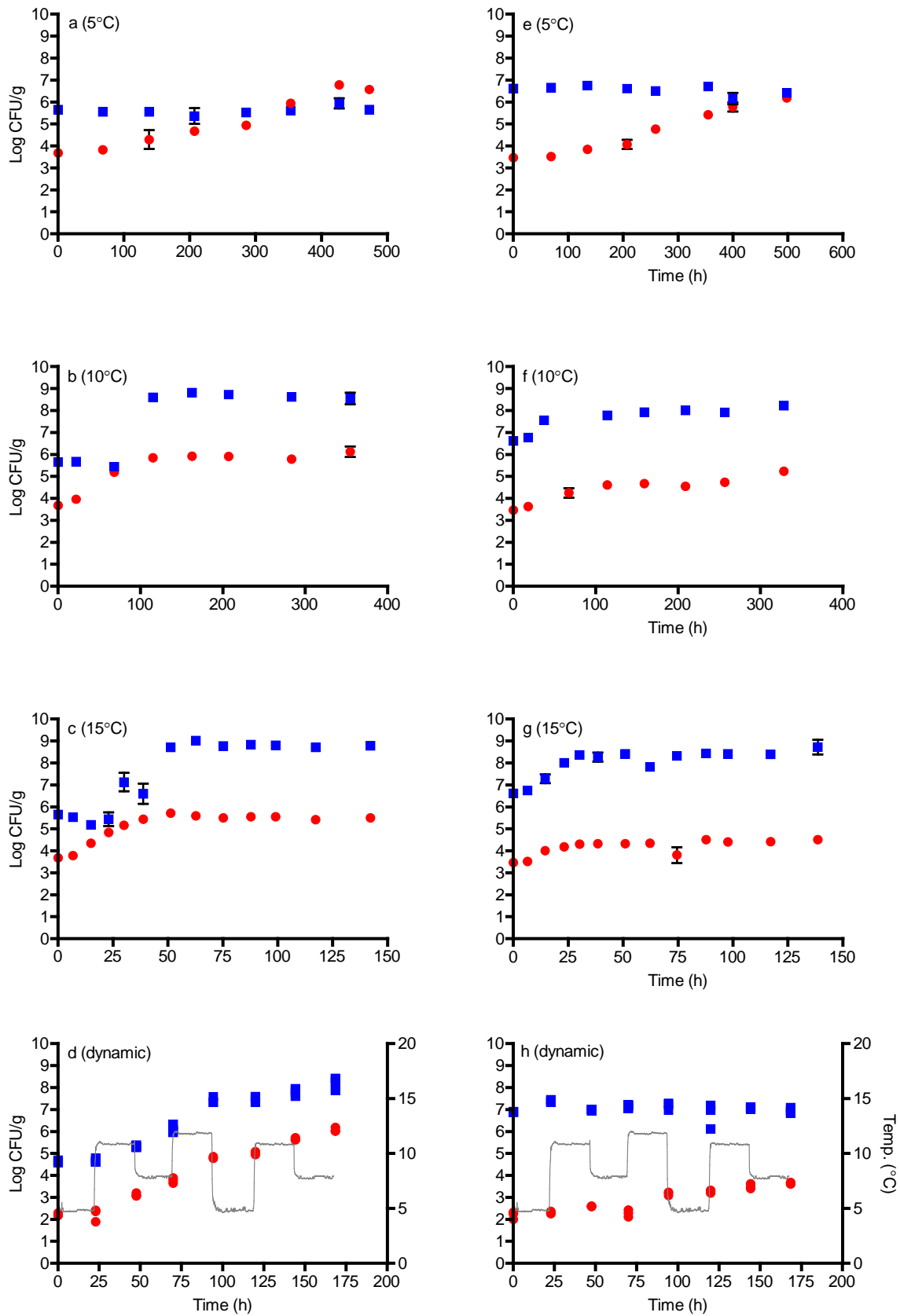
Figure 8 Growth of *L. monocytogenes* (●) and LAB (■) in cottage cheese under constant- and dynamic (—) storage temperatures. (a), (b), (c) and (d) illustrate growth in cottage cheese with fresh cream dressing and (d), (e), (f) and (g) represent growth in cottage cheese with cultured cream dressing.

27

3.4 Contamination levels in dairy products

When evaluating growth responses, and thereby also when developing predictive growth models, the initial bacterial concentration is not insignificant. Previous studies have investigated the effect of inoculum size on lag phase and the growth and growth-boundaries. Pascual and Robinson (2001) studied the ability of *L. monocytogenes* (concentrations between 10^6 cells/200 µl to 0.01 cells/200 µl) to initiate growth under suboptimal conditions (NaCl and pH at 37°C). They found that the growth/no-growth boundary for *L. monocytogenes* was represented by a region, rather than fixed values. Within this region, the growth probability rapidly decreased as the conditions became more severe. Under severe conditions, a critical inoculum size was observed and this phenomenon was attributed to cell-death in the inoculum rather than co-operative population effects. Similar results were obtained by Koutsoumanis and Sofos (2005) when they tested the combined temperature (4-30°C), pH (3.76-6.44), and a_w (0.888-0997) limits for the effect of inoculum size (0.90-6.81 log CFU/300 µl). They concluded that the inoculum size was important in relation to growth initiation of *L. monocytogenes* and that this knowledge could be applied when evaluating effects of the hurdle technology. Besse et al. (2006) found pronounced impact of inoculum size on lag phase duration and on maximum population density in model system mimicking smoked fishery products. Furthermore, the observed effect was dependent on a range of factors such as the pre-history of the bacterial cells, the background flora, the texture of the media and the packaging method. Specific studies on the effect of inoculum size on lag time were conducted by Augustin et al. (2000) and Robinson et al. (2001). In both studies, lag time duration under sub-optimal conditions, was extended with decreasing inoculum size. Under optimal conditions, Robinson et al. (2001), found the lag time duration to be little affected by inoculum size and the variability between replicates was little even for very low cell concentrations.

In addition to the above mentioned studies, a range of publications deal with quantification of individual cell variability, especially in relation to lag phase. Their findings also demonstrate that at low bacterial concentrations, the variability increases with increasing stress from the growth environment – represented by changed distribution parameters in lag time distributions. Francois et al. (2005) conducted a large study on the effect of temperature (2, 4, 7, 10 and 30°C) and pH (4.4, 4.7, 5.0, 5.5, 6.1, and 7.4) combinations on the individual cell lag time distributions. These authors found, that at low stress levels, the majority of the cells had short lag times resulting in compressed histograms close to zero. When increasing the applied stress, the lag time duration increased along with the variability between individual cells. Fig. 9 illustrates this very well for cells grown at 7°C. At pH 7.4, the fitted distribution was high and slim whereas the lag time duration and variability between individual cells (distribution width) increased markedly with decreasing pH (6.1 and 5.5).
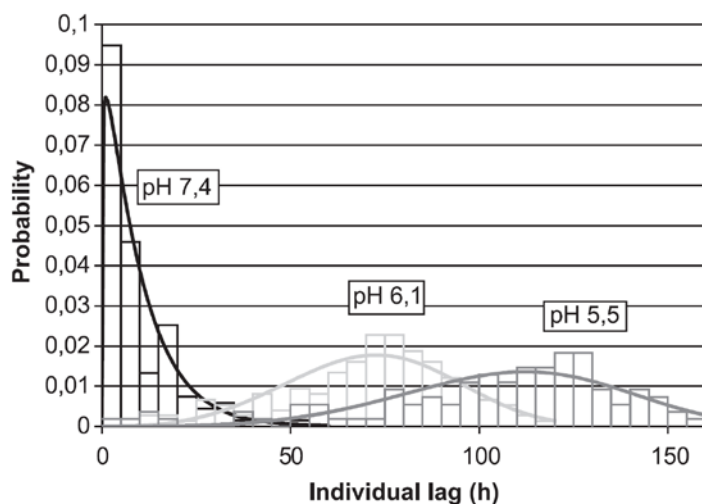
Figure 9 Lag times of individual *L. monocytogenes* cells at 7°C. Lag times at pH 7.4 were described best by a gamma distribution (α = 1.110 and β = 9.066), at pH 6.1 and 5.5 weibull distributions were used to represent lag time data with distribution parameters (α; β) of 3.678; 79.24 and 4.290; 119.4, respectively. It is evident that the applied stress influences not only the lag time duration but also the variability between cells demonstrated by increased distribution width with increasing stress. (Reprinted from Francois et al., 2005)

A search of the available scientific literature provides only a few studies on the prevalence- and realistic contamination levels in naturally contaminated dairy products. One of the most well-known, and often cited publications is the study of Kozak et al. from 1996. In that paper it was reported that only 3-4 % of raw milk samples could be expected to contain *Listeria* species and that concentrations in positive samples was low (<10 CFU/ml). Rudolf and Scherer (2001) investigated the prevalence of *L. monocytogenes* in European red smear cheese by qualitative detection. They found that 6.4% of 329 samples from six different European countries were contaminated with *L. monocytogenes*. A higher incidence of contamination with *L. monocytogenes* was observed in cheese made from pasteurised milk compared to raw milk cheeses indicating post-pasteurisation contamination. As described in a previous section (2.5.2) a recent report, prepared by EFSA, on the prevalence of *L. monocytogenes* in certain RTE food products analysed in 2010 and 2011 (fish, meat, cheese) showed that of 3452 analysed cheese samples, 3448 contained less than 10 CFU/g (likely to be zero). Only four samples were found positive for *L. monocytogenes* with concentrations ranging from 10 to 100000 CFU/g (EFSA, 2013).

Based on the knowledge of the effect of inoculum size on growth response and variability, and the general reporting of limited/sporadic prevalence of low concentrations of *L. monocytogenes* in dairy products, these aspects should undoubtedly be addressed when modelling and predicting growth of *L. monocytogenes* in dairy products.

Different approaches can be applied to address variability in e.g. product properties and lag time duration. When using deterministic models the input is represented by single values and the output is similarly represented by point estimates. One way to handle variability of the input values is to use mean- or median values and the output would thereby be an estimate of the most commonly occurring scenario. However, from a risk evaluation point of view it is more interesting to obtain knowledge of worst case scenarios (tail values) since they are most likely to cause illness. This can be obtained by using conservative input values and the output would hence represent a worst case estimate. The downside of this approach is that it may lead to unnecessary conservatism

and the predicted scenario may lose its value as useful information because it represents a very unlikely event (WHO/FAO, 2004). This problem can be approached by combining scenarios of worst case, average- and best case predictions from deterministic models. This will allow an evaluation of the potential span of the scenarios according to measured and observed product characteristics and growth responses. Of course, the approach does not take into account the potential skewness of distributions describing variability, but it still remain an informative alternative to more complex stochastic models. In some of the available software (e.g. Food Spoilage and Safety Predictor), it is possible to "tick off" whether e.g. *L. monocytogenes* lag time should be included in the prediction. Omitting the lag time return a worst case prediction assuming that *L. monocytogenes* will initiate growth immediately after contamination.

4. Risk analysis and predictive microbiology

Management of food borne threats is an ongoing challenge due to changes in primary- and secondary production, microbial adaption, increase in international trade, changes in consumer demands and behavioural- and demographic changes. As a mean to face these challenges and to evaluate- and control microbial risks, risk analysis has been introduced. Risk analysis include three components; (i) risk assessment, (ii) risk management and (iii) risk communication (CAC/GL 63-2007). Risk assessment is the scientific evaluation of known- or potential adverse health effects of a food product and comprises: hazard identification, hazard characterisation, exposure assessment and risk characterisation (Marvin et al., 2009). The outcome of the risk characterisation is an estimate of the likelihood of adverse health effects in the population due to exposure of the hazard in question (FAO/WHO, 1995). In a quantitative microbiological risk assessment, the exposure assessment describes the routes at which the microbiological hazard can be introduced, distributed and altered during the production, distribution and consumption of a given food product (WHO/FAO, 2004). Predictive microbiology is of particular interest in relation to evaluation of alterations (increase or decrease) of the hazard over time. For quantitative risk analyses, it is often stated that data is lacking and available data often originate from model experiments with e.g. high bacterial doses. In general, high quality, relevant and timely data is lacking (Gardner, 2004; Ross and Sumner, 2002; WHO/FAO, 2004). As a mean to provide information and to fill out data gaps, predictive models for growth- and inactivation can be helpful and efficient tools. Predictive models, successfully validated in growth environments comparable to the products of concern, can be used to predict the effect of intrinsic- and extrinsic factors on the response of the pathogen in question (WHO/FAO, 2004). This quantification is important since the effect of both spoilage- and pathogenic microorganisms is highly correlated to the numbers present in the food product at the point of consumption. For *L. monocytogenes,* and other foodborne pathogens, this relation is illustrated by the dose-response curve. Fig. 10 demonstrates that at low bacterial concentrations the population response (probability of illness, P_{ill}) is low. However, it should be noted that the risk of illness exist

even at these low concentration and many assume that one single infectious particle has the potential to cause illness (Ross and McMeekin, 2003). As the bacterial concentration increase, so does the population response and in the intermediate region P_{ill} is proportional to the bacterial concentration. At high bacterial concentrations (e.g. > 7.0 log CFU/g), it is assumed that the population response levels out and no differences in P_{ill} are observed at high bacterial concentrations (Fazil, 2005). Different models can be used to describe the relationship between ingested dose and P_{ill}.
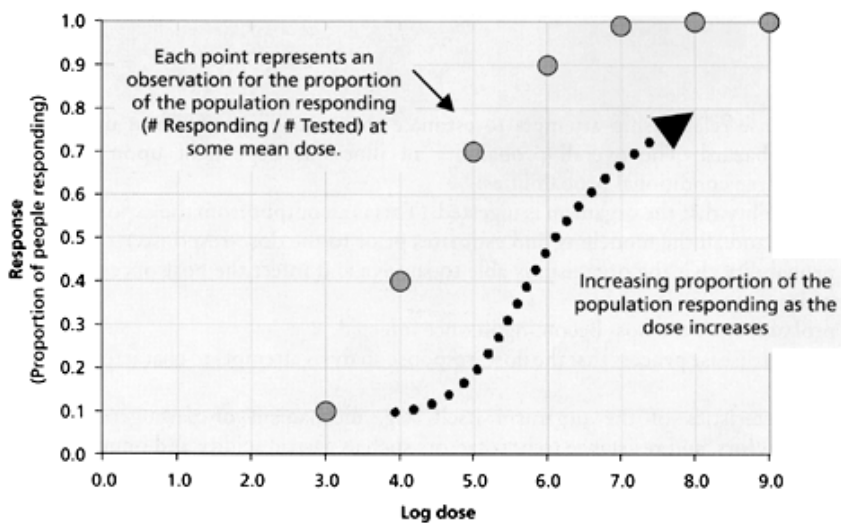
Figure 10 Example of a typical dose-response curve where the probability of illness (Y-axis) increases with increasing bacterial concentration (X-axis). At low concentrations very few people respond. At intermediate concentrations, the response increase proportionally with increasing bacterial concentration and at higher concentrations a plateau is reached meaning that the response at e.g. a concentration of 7.0 log CFU/g is the same as for concentrations of 9.0 log CFU/g (Reprinted from Fazil, 2005).

The simplest model is the exponential model which represents a direct relationship between dose and response; it assumes that there is no lower threshold for infection and that bacterial cells are randomly distributed throughout the product. Other models include the Beta-Poisson model which also predicts a direct relationship between dose and response but this type of model tend to be more flexible due to additional parameters compared to the exponential model. The Gompertz and the Weibull-Gamma model are also examples of alternative models. These models provide more complex predictions of the relationship between dose and response in the low dose region (Fazil, 2005; Ross and McMeekin, 2003). Due to the relationship between dose and response and the potential exponential growth- or inactivation of a microbial hazard over time, the food safety risk will – in many situations – also change exponentially over time (Ross and McMeekin, 2003). Thus, it is important to be able to estimate the bacterial concentration at any time-point during the lifespan of the food product in order to estimate the potential food safety risk.

PREDICTIVE MICROBIOLOGY

5. Predictive microbiology

5.1 Predictive microbiology – an introduction

Predictive microbiology – or the quantitative microbial ecology of foods – represents a proactive approach to food quality and safety by accumulating information on bacterial responses related to environmental factors and by summarising the responses in databases and mathematical models (McMeekin and Ross, 2002; McMeekin et al., 1997). The first mathematical models, or mathematical relations, within astronomy and architecture can be documented several thousand years back (Schichl, 2004). For food related processes the first model, documented in the scientific literature in 1922, describes the relation between heat treatment and inactivation of *Clostridium botulinum* spores (Esty and Meyer, 1922; McMeekin et al., 2002). It was, however, not until the 1980's that growth and survival of microorganisms in food started to receive more focused attention (Ross and McMeekin, 1994). Reasons for this (late) awakening of predictive food microbiology has been attributed to (i) a marked increase in food borne outbreaks during the 80's, (ii) that classical "rapid" microbiological analysis could only support a retrospective approach relying on end-product testing, contrary to the proactive approach of the Hazard Analysis and Critical Control Point (HACCP) system (CAC/RCP 1-1969, 1969) and (iii) increased availability of computing power (McMeekin et al., 2002; Perez-Rodriguez and Valero, 2013; Ross and McMeekin, 1994). During the last 30-40 years, predictive microbiology has achieved status as a scientific discipline within food microbiology. Furthermore, food professionals and food authorities are also showing interest and belief in the area (McMeekin et al., 2008, 2010b; McMeekin, 2007; Mejlholm et al., 2010). As described previously, the use of predictive models for documentation purposes is recognised by Danish- and EU food authorities (DVFA, 2014; EC, 2005). Moreover, the concept of *predictive* microbiology is consistent with the *proactive* approach of the HACCP methodology (McMeekin and Ross, 2002).

Traditionally, predictive microbiology is based on a two-step modelling approach including primary- and secondary models (Ross and McMeekin, 2003; van Boeckel, 2008). The primary models aim at accurately describing bacterial growth kinetics (initial concentration, lag-time, growth rate, maximum population density) with as few parameters as possible (McKellar and Lu, 2004) whereas the secondary growth models describe the effect of environmental factors on the bacterial growth kinetics from the primary models (Ross and Dalgaard, 2004; Ross and McMeekin, 2003).

5.1.1 Primary models

Different expressions have been used to mathematically describe the bacterial growth curve. The different applied models include the sigmoid functions (modified Logistic- and Gompertz model), models with an adjustment function (Baranyi model), compartmental models (Hills- and McKellar model), linear models (e.g. Buchanan three phase linear model) and the logistic with

delay model (Baty and Delignette-Muller, 2004; McKellar and Lu, 2004; Perez-Rodriguez and Valero, 2013). Describing the growth kinetics of bacterial populations has received extensive attention and especially during the 1990's, but also in recent years, a range of studies have been conducted comparing, evaluating and/or modifying primary growth models (Baty and Delignette-Muller, 2004; Buchanan et al., 1997; Dalgaard, 1995; Graham et al., 1996; McKellar, 1997; Membré et al., 2002; Pal et al., 2008; Zwietering et al., 1990). Despite the diversity of primary growth models it is not straightforward to select one model rather than another (McKellar and Lu, 2004) as also demonstrated by the above mentioned studies which presented different conclusions. However, it has been concluded in several studies that the Gompertz model return inaccurate estimates of lag-time and growth rate (Dalgaard, 1995; Fujikawa et al., 2004; Graham et al., 1996; McKellar and Knight, 2000; Membré et al., 2002; Whiting and Cygnarowicz-Provost, 1992) and systematic, 10-20%, over-estimation of growth rate has been reported (Dalgaard, 1995; Dalgaard et al., 1994; Whiting and Cygnarowicz-Provost, 1992). The over-estimation is explained by the fact that it fits a sigmoid function with a pronounced inflexion leading to an overestimation of growth rate (Graham et al., 1996). A few criteria may, after all, be designated as decisive for model selection and that is simplicity and biological interpretability (Dalgaard, 1995; McKellar and Lu, 2004). Throughout this present PhD-project, the logistic model with delay (Eq. (1)) has been used to fit to experimental data in order to obtain estimates of the kinetic parameters (lag time, growth rate, initial and final concentrations) of the microorganisms in question.

$$\log(N_t) = \log(N_0) \qquad\qquad\qquad \text{if } t < t_{lag} \qquad [1]$$

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

where t is the time of storage and t_{lag} the lag time, N_t, N_0 and N_{max} are the cell concentrations (CFU/g) at time t, zero and the maximum asymptotic cell concentration, respectively. μ_{max} is the maximum specific growth rate (h^{-1}). Additionally, the expanded logistic model (Eq. (2)) was used. This model include a growth dampening parameter, m which, despite poor statistical properties, has proven suitable when relating product formation (e.g. lactic acid and histamine formation) to bacterial growth (Dalgaard, 2002; Emborg and Dalgaard, 2008a) or when estimating μ_{max}-values from absorbance growth curves (Dalgaard and Koutsoumanis, 2001). The model is often designated the Richards model but it was introduced by Turner et al. (1969) as a modified version of the logistic law of growth.

$$\log(N_t) = \log(N_0) \qquad\qquad\qquad\qquad\qquad\qquad\text{if } t < t_{lag} \qquad [2]$$

$$\log(N_t) = \log\left(\frac{N_{max}}{\left(1+\left(\left(\frac{N_{max}}{N_0}\right)^m - 1\right)\cdot exp(-\mu_{max}\cdot m\cdot(t-t_{lag}))\right)^{1/m}}\right) \qquad \text{if } t \geq t_{lag}$$

where N_t, N_{max} and N_0 are the cell concentrations (CFU/g) at time t, zero and the maximum population density, respectively. t_{lag} is the lag time (h) and t is the time of storage, μ_{max} is the maximum specific growth rate (h^{-1}). The parameter m describes the dampening of growth when N_t approaches N_{max}.

5.1.2 Secondary growth models

The secondary models, relating environmental factors to bacterial kinetics, may roughly be divided into three groups; polynomial models, artificial neural networks and kinetic models (Dalgaard, 2002; Ross and Dalgaard, 2004). Polynomial models rely on multiple linear regressions and are fairly straightforward to use. There has, however, been pointed out some disadvantages of this model type. Primarily, the large number of parameters and their lacking biological interpretability has been criticised (Dalgaard, 2002; van Gerwen and Zwietering, 1998). Polynomial type models were used to develop the USDA Pathogen Modelling Program and the UK Food Micro-Model (Ross and McMeekin, 2003). Artificial Neural Networks can be defined as structures that are comprised of interconnected adaptive processing elements that can perform multiple parallel computations for data processing and knowledge representation. The neural networks are characterised by their ability to learn by mimicking information processing and knowledge acquisition in the human brain (Basheer and Hajmeer, 2000). An example of a predictive model based on an artificial neural network is the *Listeria* model developed by the Danish Meat Research Institute (freely accessible via http://dmripredict.dk; Mejlholm et al., 2010). The kinetic models include model-terms with biological interpretability and thereby meet the prerequisites for predictive microbiology valued by many authors (Augustin and Carlier, 2000a; Rosso et al., 1993; van Gerwen and Zwietering, 1998; Wijtzes et al., 1995). Ratkowsky et al. (1982) introduced the square-root type/Ratkowsky model relating bacterial growth rate to temperature. This modelling approach was suggested to be a suitable substitute for the Arrhenius Law since a non-linear relationship between the logarithm of the growth rate and the reciprocal absolute temperature was observed due to a temperature dependency of the activation energy (E_a). The square-root type models have subsequently been expanded to include the effect of various environmental factors (Ross and Dalgaard, 2004). In the early 1990's the Gamma concept was introduced by Zwietering and coworkers. This type of model describes the growth rate relative to its maximum value at optimum conditions. The concept rely on the assumption that factors affecting the growth rate act

independently and that the effects are multiplicative (Perez-Rodriguez and Valero, 2013; Ross and Dalgaard, 2004; Zwietering et al., 1993). Each environmental factor is represented by a dimensionless gamma-factor (γ) with a value between 0 and 1. The independent action of each individual factor enables (theoretically) existing models to be expanded to include other environmental factors (Ross and Dalgaard, 2004). The Cardinal Parameter Models (Rosso et al., 1995) resemble the Gamma concept models by their assumption of independency between the effects of environmental factors. One of the main advantages of this type of model is the potential for simplification related to specific cases where some factors may be of minor importance (Ross and McMeekin, 2003). In the present project, a simplified cardinal parameter type model structure was applied to describe the effect of environmental factors on growth of LAB and *L. monocytogenes* in cottage cheese and the Cardinal Parameter Models will be described in more detail in subsequent sections. Examples of application of the Cardinal Parameter modelling approach include the studies of Augustin and Carlier (2000a, 2000b) where data obtained from literature was used to estimate model parameter values to describe growth response of *L. monocytogenes*. The model was subsequently improved by including interaction between the parameters and thereby including the hurdle effect. This modification enabled more accurate predictions close to the growth boundary (Augustin and Carlier, 2000b). Also Le Marc et al. (2002) developed a *L. monocytogenes* growth model based on the multiplicative modelling approach. In that study, the interaction parameter Epsilon (ξ) was defined, providing an adjustment of the growth rate according to the combined effect of the environmental factors. Augustin et al. (2005) obtained good predictions of growth/no growth responses of *L. monocytogenes* in dairy, meat and seafood products, using a cardinal parameter model in combination with interaction between environmental factors (ξ). A simplified "square root type cardinal parameter model" including the interaction parameter ξ was used and extended by Mejlholm and Dalgaard (2013, 2009, 2007a, 2007b) and Mejlholm et al. (2014) for prediction of growth response of *L. monocytogenes* and LAB in lightly preserved seafoods. Examples of existing, published growth models for *L. monocytogenes* and LAB are provided in Table 10 and 11.

Table 10 Overview of existing predictive growth models for *L. monocytogenes/L. innocua*

Reference	Microorganism	Model parameters	Experimental conditions
Te Giffel and Zwietering, 1999	*L. monocytogenes*	T_{min}: -1.5 T_{opt}: 37 pH_{min}: 4.39 pH_{opt}: 7 pH_{max}: 9.6 $a_{w,min}$: 0.92 μ_{opt}: 2 h^{-1}	Application of a general gamma-model to predict growth of *L. monocytogenes*. Includes the effect of temperature, pH and water activity.
Alavi et al., 1999	*L. monocytogenes*	b: 0.0185 c: 0.1392 T_{min}: -1.73 T_{max}: 44.81	Modified Ratkowsky model to predict growth of *L. monocytogenes* in fluid whole milk. The effect of temperature was included in the model.
Augustin and Carlier, 2000a	*L. monocytogenes*	T_{min}: -2.7 T_{opt}: 37 T_{max}: 45.5 pH_{min}: 4.55 pH_{opt}: 7.10 pH_{max}: 9.61 $a_{w,opt}$: 0.997 $a_{w,min}$: 0.910 LAC_u: 5.4 mM SAC_u: 5.1 mM (potassium sorbate) μ_{opt}: 1.016 h^{-1}	Global predictive model for growth of *L. monocytogenes*. The model has been developed based on growth data collected from the literature. The effect of temperature, pH, water activity, lactic acid and sorbic acid is included. Furthermore, the effect of acetic and citric acid and sodium benzoate has been included together with sodium nitrite, glycerol monolaurate, butylated hydroxyanisole, tertiary butylhydroquinone, butylated hydroxytoluene, CO$_2$, caffeine and phenol. Only parameters relevant in relation to cottage cheese are reported here.
Devlieghere et al., 2001	*L. monocytogenes*	a: 7.1277*10^{-4} T_{min}: -3.5419 $a_{w,min}$: 0.9295 $CO_{2,max}$: 3140 diss. CO$_2$ (mg/L) NaL_{max}: 5.9547 % w/w	Square root type model developed to predict growth of *L. monocytogenes* in gas-packed cooked meat products. The pH in the model is fixed to simulate that of cooked ham (~6.2). The effect of water-activity, temperature, atmosphere (CO$_2$) and sodium-lactate is included in the model.
Le Marc et al., 2002	*L. innocua*	T_{min}: -4.5 T_1: 0.6 T_c: 10 T_{opt}: 37.4 T_{max}: 45.5 pH_{min}: 4.21 pH_{opt}: 7.21 pH_{max}: 10.07 LAC_u: 8 mM AAC_u: 20.3 mM $PropAC_u$: 8.8 mM μ_{opt}: 1.14 h^{-1}	A multiplicative predictive model including the effect of temperature, pH and three organic acids on growth of *L. innocua*. The interaction term, ξ, is introduced in the model to describe the effect of the interaction between environmental parameters. The temperature term has been extended (T$_1$ and T$_c$) to model the non-linearity between sqrt(μ_{max}) and temperature (reported by Bajard et al., 1996) in the sub-optimal temperature-range.
Gimenez and Dalgaard, 2004	*L. monocytogenes*	b: 0.14776 c: 0.536 T_{min}: 0.88 T_{max}: 41.4 $a_{w,min}$: 0.923 pH_{min}: 4.97 $MIC\ LAC_u$: 3.79 mM NIT: 350 ppm	Application of the UTAS-model (square root type model by Tom Ross) including the effect of temperature, water activity, pH, LAC and nitrite.
Augustin et al., 2005	*L. monocytogenes*	$\mu_{opt,\ liquid\ dairy}$: 0.74 h^{-1} $\mu_{opt,\ cheese}$: 0.21 h^{-1} T_{min}: -1.72 $pH_{min,\ HCl}$: 4.26 $pH_{min,\ LAC}$: 4.71 $a_{w,min}$: 0.913 MIC_{nit}: 25 µmol/l MIC_{phe}: 31.9 ppm MIC_{CO2}: 3.04	New cardinal parameters were suggested to the model of Le Marc et al. (2002) and the performance of the model was evaluated in different food commodities (meat, seafood, liquid dairy and cheese). For each commodity a specific μ_{opt} was suggested.

Table 10 continued

Reference	Microorganism	Model parameters	Experimental conditions
Panagou and Nychas, 2008	*L. monocytogenes*	*b*: 0.017 T_{min}: -6.30	Square root model to predict growth of *L. monocytogenes* in vanilla cream under dynamic temperatures. Only the effect of temperature was evaluated and the pH of the product was in the range of 6.3-6.7.
Gougouli et al., 2008	*L. monocytogenes*	*b*: 0.0203 T_{min}: -2.102	Square root model to predict growth of *L. monocytogenes* in ice cream under dynamic chilling and freezing conditions. The effect of temperature was included in the model. The pH of the product was in the range 6.50-6.67.
Mejlholm and Dalgaard, 2009, 2007a, 2007b	*L. monocytogenes*	Different *b* and T_{min} values. 2007a: *b*: 0.6802 T_{min}: -2.3 2007b and 2009: *b*: 0.419 T_{min}: -2.83 $a_{w,min}$:0.923 pH_{min}: 4.97 *MIC LAC_u*: 3.79 mM *MIC DAC_u*: 4.8 mM *MIC AAC_u*: 10.3 mM *MIC BAC_u*: 0.35 mM *MIC CAC_u*: 2.12 mM *MIC SAC_u*: 1.90 mM	Predictive model to describe growth of *L. monocytogenes* in lightly preserved seafood products. The effect of temperature, pH, water activity and six different organic acids was included. Furthermore, the model includes the effect of phenol, nitrite and CO_2 and also the interaction between the environmental parameters was included in the model by the ξ-factor. In SSSP (Seafood Spoilage and Safety Predictor software, now Food Safety and Spoilage Predictor, www.fssp.food.dtu.dk) it is possible to predict the inhibitory effect of LAB present in the product (the Jameson effect) which was also referred to in Mejlholm and Dalgaard (2007a).
Le Marc et al., 2010	*L. monocytogenes*	pH_{min}: 4.96 $a_{w,min}$: 0.915	Predictive model including the effect of abrupt acid- and osmotic shifts. Experiments were performed at 10˚C
Schvartzman et al., 2011	*L. monocytogenes*	Same model parameters as Augustin et al. (2005), but a range of different μ_{opt} were applied. An average μ_{opt} from the cheeses made from raw milk during ripening (13˚C and 8˚C and pH-values in the range og 6.7-5) was found to be most appropriate to use in the comparison with growth of *L. monocytogenes* in cottage cheese. μ_{opt} 0.175 h^{-1}	"Validation"/extension and application of the secondary cardinal parameter model of Augustin et al. (2005). The model should predict the fate of *L. monocytogenes* during ripening of smear cheese. Three different μ_{opt} values were used at different stages of the production (cheese-making + core and rind during ripening), to account for changes in the growth environment.
Pathogen Modeling Program Freely accessible software with user interface www.ars.usda.gov/services/software/software.htm	*L. monocytogenes* and other microorganisms		Polynomial model including the effect of temperature (4-37°C), pH (4.5-7.5), NaCl (0.5-10.5%) and nitrite. Predictions can be obtained with or without lag time estimates under aerobic or anaerobic conditions.
ComBase Freely accessible software with user interface www.modelling.combase.cc/	*L. monocytogenes* and other microorganisms		Polynomial model including the effect of temperature, pH, NaCl, CO_2, nitrite, lactic acid and acetic acid. Lag time predictions are related to the physiological state of the cells (between 0 and 1).

Table 11 Overview of existing predictive growth models for LAB

Reference	Microorganism	Model parameters	Experimental conditions
Zwietering *et al.*, 1994	*Lactobacillus plantarum*	b : 0.0385 c : 0.247 T_{min} : 3.29 T_{max} : 44.8	Modelling growth of *Lactobacillus plantarum* in chilled foods. Only the effect of temperature was included in the model (Ratkowsky model) Experiments were performed in MRS agar.
Gänzle *et al.*, 1998	*Lactobacillus sanfranciscensis*	μ_{opt}: 0.68 T_{min}: 3.0±0.6 and 4.1±0.5 T_{max}: 41.0±0.1 and 41.0±0.07 T_{opt}: 32.5 (avg. value) pH_{min}: 3.94 ±0.05 and 3.90 ±0.06 pH_{max}: 6.67 ± 0.05 and 6.64 ±0.05 pH_{opt}: 5.47 ± 0.05 and 5.53 ± 0.06	Modelling growth of two strains of *L. sanfranciscensis* in the process of sourdough fermentation. The effect of temperature, pH, ethanol, acetic acid, acetate and lactic acid was included in the model. Only parameters for μ_{opt}, temperature and pH are reported here.
Devlieghere *et al.*, 2000	*Lactobacillus sake* subsp. *carnosum*	b : 0.001187 T_{min}: -8,81 $a_{w, min}$: 0.9542 $CO_{2, max}$: 7799 ppm b : 0.000578 T_{min} : -9.03 $a_{w, min}$: 0.9544 $CO_{2, max}$: 6691 ppm NaL_{max} :5.87% w/w	Development of a growth model to predict growth of psychrotolerant LAB responsible for spoilage of chilled, cooked meat products. The model includes the effect temperature, CO_2, water activity and Na-lactate. (Extended Ratkowsky model). pH 6.2 (~cooked meat).
Wijtzes *et al.*, 2001	*Lactobacillus curvatus*	b_4 : -2.34 · 10^{-3} T_{min} : -3.63 pH_{min} : 4.24 pH_{max} : 9.53 $a_{w, min}$: 0.928	Modelling growth of *Lactobacillus curvatus* by application of a multiplicative model (gamma-concept) including the effect of temperature, pH and water activity. The model was developed in MRS broth.
Messens *et al.*, 2003	*Lactobacillus curvatus*	T_{min}: -3 T_{max}: 41 T_{opt}: 34.5 pH_{min}: 4.2 pH_{max}: 9.0 pH_{opt}: 6.0	Development of a model, based on the gamma-concept, to predict growth of *Lactobacillus curvatus* in sausages during fermentation. The model is validated for temperatures between 20 and 38°C and for pH values between 4.8 and 7.0.
Mejlholm and Dalgaard, 2007	LAB mix; *L. sakei, L. curvatus, C. maltaromaticum, E. maldoratus* and *Leuconostoc* spp.	b: 0.659 ± 0.002 T_{min}: -3.05 ± 0.66 pH_{min}: 4.24 (fromWijtzes et al., 2001) $a_{w,min}$: 0.928 (from Wijtzes et al., 2001) $MIC_{lactate}$: 12.0 ± 0.2 mM $MIC_{diacetate}$: 33.3 ± 1.3 mM	Development- and extension of a cardinal parameter model to predict growth of LAB in lightly preserved seafood. The effect of diacetate, lactate, CO_2, smoke components, pH, NaCl, temperature and the effect of interaction between the environmental parameters was included in the model. Only parameters relevant for cottage cheese and similar products are reported here. LAB model is included in SSSP/FSSP.
Manios *et al.*, 2009	LAB (spoilage microbiota)	b:0.019 T_{min}: -10.7	Development of a model to predict growth of LAB (spoilage) in acidic, cheese based Greek appetizers. Only temperature was included in the model. The products displayed pH values between 4.2 and 4.5.
Leroi *et al.*, 2012	*Lactococcus piscium*	T_{min}: -4.80 T_{max}: 27.19 T_{opt}: 23.39 pH_{min}: 4.79 pH_{opt}: 7.36 $NaCl_{opt}$: 4.69 g/L $NaCl_{max}$: 23.00 g/L	Application of the gamma concept in the modelling of growth of *Lactococcus piscium*. The effect of temperature, pH and NaCl is included in the model.

5.1.3 From deterministic- to stochastic models

Deterministic models can be said to be a reduced version of a model, based on the assumption that the stochastic elements of biology are small enough to be negligible (Baranyi and Pin, 2004). Traditionally, predictive food microbiology has relied on deterministic models either by applying the concept of "smoothing down" biological variability or by predicting worst case scenarios by using highest/lowest observed values as model input (Baranyi and Pin, 2004; WHO/FAO, 2004). In recent years, attention has been drawn towards inclusion of variability in the growth models in order to provide an estimate of all possible outcomes and their probability of occurring based on variability in input data (Couvert et al., 2010; Koutsoumanis et al., 2010; Mejlholm et al., 2014; Pouillot et al., 2007; WHO/FAO, 2004). Additionally, variability in lag time duration for individual cells has been investigated and quantified (Augustin et al., 2000; Besse et al., 2006; Elfwing et al., 2004; Francois et al., 2005; Guillier et al., 2005; Koutsoumanis and Sofos, 2005; Métris et al., 2005, 2003; Pascual and Robinson, 2001; Pin and Baranyi, 2006; Robinson et al., 2001; Smelt et al., 2002) and the collected lag time data has been applied to simulate growth responses of low bacterial concentrations in food products (Francois et al., 2006b; Manios et al., 2013). Variability in initial bacterial contamination level and the physiological state of contaminating cells, product characteristics and storage conditions all affect the growth response of e.g. *L. monocytogenes* and is relevant to consider when predicting growth responses in foods (Mejlholm et al., 2014). Specifically for cottage cheese, variability in e.g. product pH may have a pronounced effect on the growth response, since the pH value of cottage cheese approaches the lower growth limit (Table 3 and Table 8). The inclusion of stochasticity in predictive microbiology is described further in section 5.8.

5.1.4 Future trends in predictive microbiology

Besides a move towards stochastic modelling approaches, other subjects are also forecasted to be a part of the future of predictive microbiology. In 2004, Bernaerts and co-workers strongly advocated for the development of more mechanistically inspired predictive models in order to obtain a better understanding of the underlying mechanisms but also to develop more robust models (Bernaerts et al., 2004). McMeekin et al. (2010) suggested focusing on the ecophysiology of food-borne pathogens and to model growth responses from e.g. thermodynamics. The introduction of systems biology into predictive microbiology has been suggested by Brul et al. (2008) and Van Impe et al. (2013) in order to apply "bottom-up" approaches and to work at a microscopic level e.g. by developing metabolic network-based modelling approaches. Belief in systems-biology as an integrated part of predictive microbiology has also been expressed by McMeekin et al. (2013) in order to induce a shift from empirical predictive microbiology towards mechanistic predictive systems biology models. These new, emerging approaches within predictive microbiology should be considered and, if obtainable, tested when developing new models. For instance, in relation to

inter-bacterial interactions, application of mechanistic modelling approaches will not only predict the growth response of the microorganism under study, but will also (potentially) provide explanations of the underlying mechanisms (Bernaerts et al., 2004; Van Impe et al., 2005).

5.2 Bacterial growth

Food spoilage or food poisoning caused by food borne pathogens is most commonly due to bacterial growth in the food product. Bacterial growth patterns in food and food model systems have therefore been extensively studied and this accumulated knowledge constitutes a pivotal part in predictive microbiology (McMeekin et al., 2002; Peleg and Corradini, 2011). The bacterial growth curve (Fig. 11) often display a phase where the specific growth rate starts at zero and subsequently increases until the maximum specific growth rate is reached. This period is referred to as the lag time or lag phase (often described by λ). The period in which the bacteria grow at the maximum specific growth rate (μ_{max}) is the exponential phase which is followed by a period of decreasing specific growth rate until it reaches zero and the bacteria enter the stationary phase. In the fourth "mortality phase" the bacteria start to die and the concentration of live cells decreases. The kinetics (lag time and growth rate) of bacterial growth is dependent on a combination of the extracellular environment (e.g. pH, temperature, NaCl, preservatives, access to nutrients) and the intracellular environment (van Boeckel, 2008). In the following sections, the bacterial lag, exponential and stationary phase will be described more detailed.
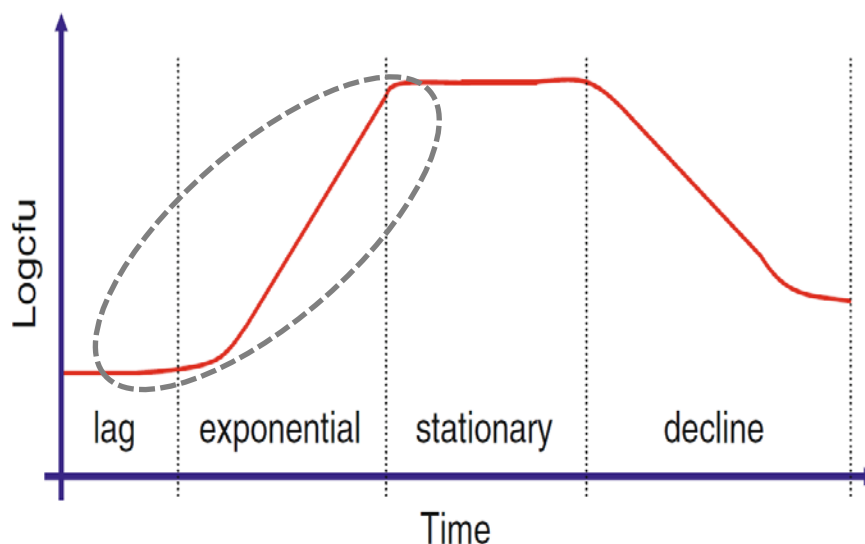
Figure 11 Theoretical presentation of the bacterial growth curve with four phases: (i) lag phase, (ii) exponential growth phase, (iii) stationary phase and (iv) decline (Perez-Rodriguez and Valero, 2013).

5.3 The bacterial growth curve

5.3.1 Lag time

The lag phase represents the time needed for the bacteria to adapt to a new growth environment in order to be able to resume cell multiplication (McMeekin et al., 2002; Robinson et al., 2001; Ross and Dalgaard, 2004). Depending on the new growth environment, the magnitude of the shift in environment and the physiological state of the cell, the duration of the lag time can range from zero to infinity (Ross and Dalgaard, 2004). Other factors influencing lag phase duration include the species and strain of the microorganism and for low inoculum levels also the bacterial concentration at the point of environmental change (Swinnen et al., 2004). From a predictive microbiology point of view, lag phase has caused challenges as it display high variability and low reproducibility and is thereby difficult to predict accurately (Baranyi, 2002; Baty and Delignette-Muller, 2004; McKellar, 1997; McMeekin et al., 2002). The lacking knowledge of the pre-history (physiological state) of contaminating cells has often been mentioned as one of the main reasons for the difficulties (Baty and Delignette-Muller, 2004). In 1994, Baranyi and Roberts introduced the dimensionless variable $q(t)$ which represent the physiological state of the cell. $q(t)$ is used to derive $\alpha(t)$ which is the product of the "adjustment function". The relationship between $q(t)$ and $\alpha(t)$ is transformed to yield $h(t)$ and $(h_0) = h(0)$ equals $-\ln(\alpha(0))$. Based on the assumption that lag time is inversely proportional to the maximum specific growth rate the physiological state of the microorganisms can be established (Eq. (3)).

$$\lambda\,(h) = \frac{h_0}{\mu_{max}(h^{-1})} \hspace{4cm} [3]$$

where λ is the lag time duration (h), h_0 is a measure of the physiological state and μ_{max} is the maximum specific growth rate (h^{-1}). Being the product of the maximum specific growth rate and the lag time, h_0 is a constant value for specific growth curves, provided that that the cells display identical physiological states (Baranyi and Roberts, 1994). The physiological state variable is also included in the ComBase Predictor as an input value in the predictive model. If this value is not entered, default values are used to describe the physiological state of the microorganisms.

A similar, and less complicated, approach to predict lag phase based on the assumption that lag time and generation time are proportional, is the concept of the relative lag time (*RLT*) (Eq. (4)) or generation time equivalents (McMeekin et al., 2002; Mellefont and Ross, 2003; Ross and Dalgaard, 2004; Ross, 1999). From this expression, the lag time is related to the severity of the new growth conditions and the growth rate is used as a measure of the metabolic rate in a given growth environment (Ross, 1999).

$$\lambda \ (h) = \frac{RLT \cdot \ln(2)}{\mu_{max}(h^{-1})} \qquad\qquad\qquad\qquad [4]$$

where λ is the lag time duration (h) and μ_{max} is the maximum specific growth rate (h⁻¹).The *RLT*-value mirror the physiological state of the microorganisms when they are introduced to the new environment but also the difference between the new and the old environment and express the amount of work that the cell has to perform in order to change its physiology and resume growth at maximum specific growth rate (μ_{max}, h⁻¹) in the new growth environment (Ross and Dalgaard, 2004). Ross (1999) reviewed large amounts of bacterial growth data (*Escherischia coli, Listeria monocytogenes, Staphylococcus aureus, Salmonella, Clostridium Perfringens* and *Bacillus stearothermophilus*) obtained from literature and calculated *RLT*-values from the lag time and maximum specific growth rate estimates. Generally, the obtained *RLT*-values were in the range of 3-6 and more than 90% were between zero and 15 and in all cases the distributions of *RLT*-values were left shifted with a pronounced right tail (Fig. 12).
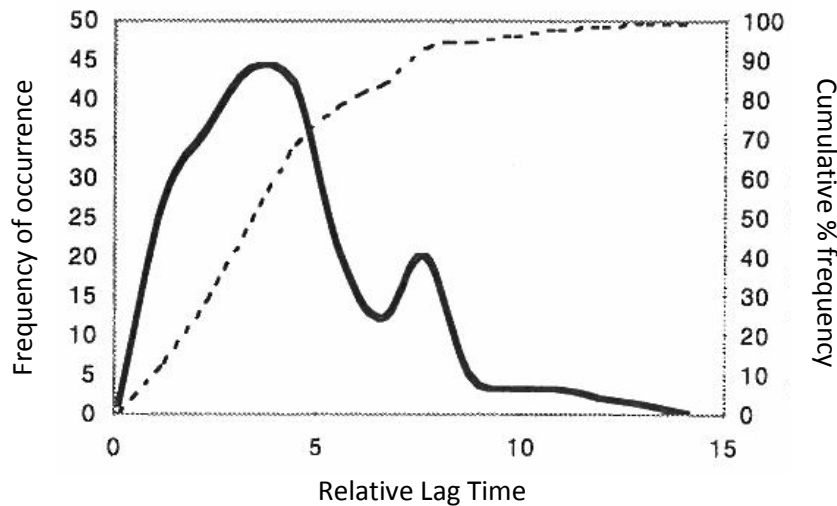
Figure 12 *RLT*-values for *L. monocytogenes* obtained from experiments performed in food products (Ross, 1999)

The concept of *RLT* has been evaluated to perform acceptably for shifts in temperature, however close to minimum temperatures allowing growth, lag time may increase more than the environment, represented by the growth rate, can account for (Zwietering et al., 1994b). These observations were further supported by the findings of Hereu et al. (2014). In that study a clear temperature effect on the *RLT*-value of *L. monocytogenes* was observed at 4°C, whereas it remained stable at 8°C and 12°C, respectively. Stecchini et al. (2004) found that *RLT*-values increased under more restrictive water activity conditions and Mellefont et al. (2003) found that especially osmotic downshifts (water activity reduction) induced larger *RLT*-values compared to corresponding upshifts (transfer to a new environment with higher water activity). However, secondary lag time

models based on the *RLT*-concept are manageable to use in combination with secondary growth models and the modelling approach has been applied in several studies (Augustin and Carlier, 2000a; Mejlholm and Dalgaard, 2007a; Mejlholm et al., 2014; Møller et al., 2013). Lag time estimates based on the *RLT*-concept are also obtained from predictions made with the Food Spoilage and Safety Predictor and for *L. monocytogenes* a default value of 4.5 has been found to be a suitable and qualified estimate of the relative lag time for *L. monocytogenes* (Mejlholm and Dalgaard, 2007a; Mejlholm et al., 2014; Ross, 1999). Application of a stochastic modelling approach has been suggested to be more appropriate when predicting lag time duration of bacteria in food since variability is then taken into account (Baty and Delignette-Muller, 2004; McMeekin et al., 2002; Ross, 1999) and this is even more relevant when considering individual bacterial cells or growth og low bacterial concentrations (see 5.7.1). Alternatives to models where proportionality between lag time and growth rate is assumed are secondary lag time models most often in the form of polynomial models or artificial neural networks (Ross and Dalgaard, 2004).

5.3.2 Exponential growth

As early as in 1949, Monod pointed out that bacterial growth obeyed relatively simple laws and that accuracy and reproducibility of growth constant determination was remarkable and unparalleled by other biological systems (Monod, 1949). There is a general consensus that bacterial growth rates are highly reproducible and the accuracy and reproducibility are some of the cornerstones of predictive microbiology (McMeekin et al., 2002).

The rate at which bacterial growth occurs in the exponential growth phase depends on intrinsic- and extrinsic factors. One of the most important factors is the storage temperature, but also product pH, salt concentration (a_w) and naturally occurring- or added antimicrobials affect bacterial growth (Montville and Matthews, 2007). From thermodynamics it is well known that temperature is determining for the rate of chemical reactions and for bacteria, temperature is also a cardinal factor controlling the rate at which multiplication occurs (Ratkowsky et al., 1982). However, for bacteria a constant increase in "reaction rate" only occurs within a limited temperature range, the normal physiological range (Mellefont and Ross, 2003; Nichols et al., 2000; Ross and Dalgaard, 2004), and below- and above this interval the temperature will have an inhibitory effect on bacterial growth. In Fig. 12a, which corresponds to a simplified Arrhenius plot, this principle is illustrated with minimum-, optimum- and maximum values. The shape of the "relationship-curve" and the location of the optimum value (Fig. 12a) depends on the nature of the microorganism in question (psychrotolerant, mesophilic or thermophilic bacteria) (Montville and Matthews, 2007). The stability and activity of macromolecules such as enzymes is highly affected by pH and the growth and metabolism of microorganisms is thus also affected by pH (Adams and Moss, 2000). The sensitivity towards pH is, however, affected by the nature of the acidulant (organic/inorganic) (Farber et al., 1989; Vasseur et al., 1999). Vasseur et al. (1999) investigated the

effect of acetic-, lactic- and hydrochloric acid (HCl) against five strains of *L. monocytogenes* in Meat Bacto Tryptone broth with 0.5% glucose. They concluded that acetic acid exhibited the most pronounced inhibitory effect followed by lactic- and hydrochloric acid. Similar results were reported by Augustin et al. (2005), who determined theoretical minimum values of pH (pH_{min}) allowing growth of *L. monocytogenes* in liquid microbiological media, based on growth data from literature. The average pH_{min}-values obtained with lactic acid were between 0.2 and 0.6 pH units higher than pH_{min}-values obtained with HCl. The inhibitory effect of lactic acid at low pH is recognised (Adams and Hall, 1988; Houtsma et al., 1994) and the inhibitory effect increase with lactic acid concentration (Fig. 12b). However, the inhibitory concentration decreases with declining pH. Houtsma et al. (1994) found that at pH 5.5 *L. innocua* was inhibited by 217 mM sodium lactate whereas at neutral pH 1071-1339 mM sodium lactate was required to induce inhibition of growth.
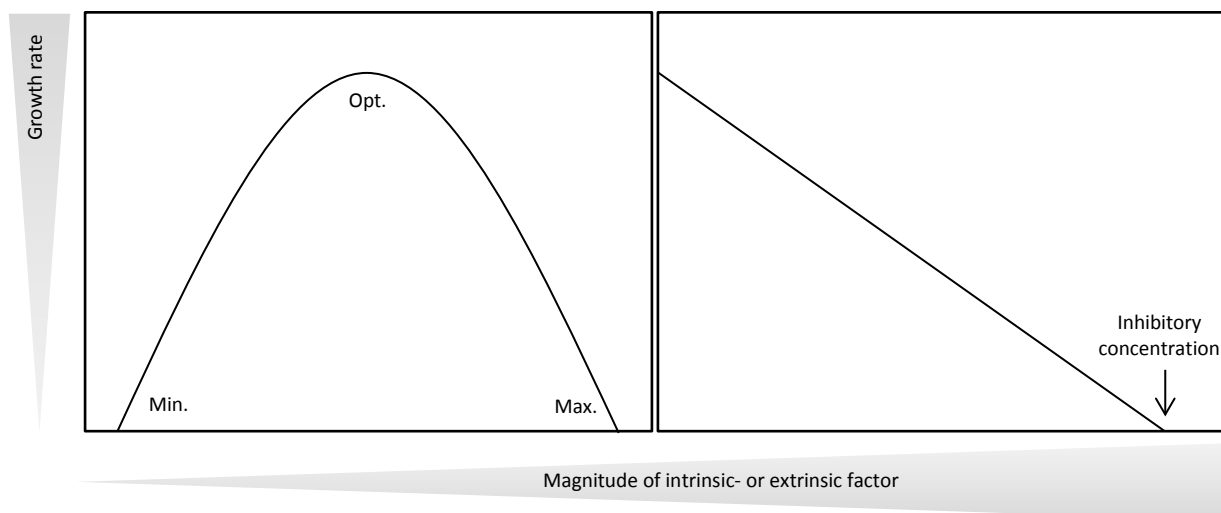
Figure 12a Relative effect of e.g. temperature, pH and NaCl on bacterial growth rate. Minimum-, optimum- and maximum values are often observed

Figure 12b Relative effect of e.g. organic acids on bacterial growth rate. Bacterial growth rate decreases with increasing concentration

Lactic acid ($C_3H_6O_3$, pK_a-value = 3.86) is naturally present in fermented dairy products due to the lactose metabolism exhibited by LAB from the added starter cultures (see 2.2.2; Ricke, 2003). The weak organic acid acts as an antimicrobial, pH control agent and flavouring compound in food products and it has been shown to inhibit *Clostridium botulinum, Clostridium sporogenes, L. monocytogenes, Salmonella, S. aureus, Y. enetrocolitica* and different spoilage bacteria (Davidson and Taylor, 2007). Lactic acid acts by lowering the product pH and by lowering the intracellular pH of the microorganism. The inhibitory effect is strongly dependent on pH as the degree of dissociation increases as the pH of the environment approach or become lower than the pK_a-value ($-\log(K_a)$) of the organic acid. The undissociated from of the acid can permeate the lipophilic membrane and after entering the more neutral cytoplasm, the acid dissociates and decrease the intracellular pH (Fig. 13). The bacterial cell tries to expel the protons generated from

the intracellular dissociation, using ATP to facilitate the pumps. If the cell manage to restore near-neutral pH it can resume delayed growth but if the effect of the acid is too strong the cell will terminate growth and eventually die (Davidson and Taylor, 2007; Wilson et al., 2000).
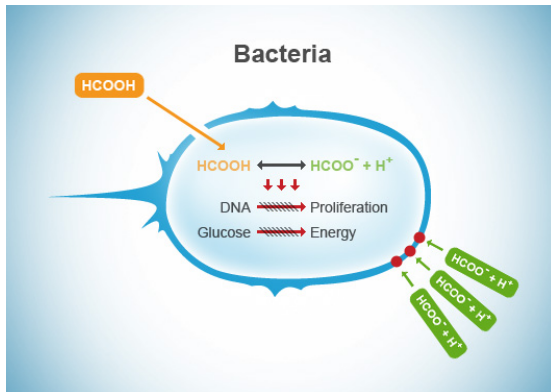
Figure 13 Illustration of the mechanisms fully- or partly responsible for growth inhibition caused by weak organic acids. Undissociated molecules penetrate the lipophilic membrane and dissociates in the cytoplasm resulting in a decrease in intracellular pH. The cell tries to restore near-neutral pH and uses energy to pump out protons generated from the dissociation of the acid (http://basfanimalnutrition.com).

In some studies it has, however, been suggested that it is not only the undissociated from of the organic acid that causes growth inhibition of microorganisms but it may involve other mechanisms as well (Houtsma et al., 1994). Ita and Hutkins (1991) proposed that the growth inhibition of *L. monocytogenes* was due to specific effects of the undissociated from of the acid on metabolic of physiological activities. In addition to this, Presser et al. (1997) obtained good results by including the effect of both the undissociated- and the dissociated form of lactic acid in a growth model for *E. coli*.

Sorbic acid ($C_6H_8O_2$, pK_a-value = 4.76) may be added to prolong the shelf-life of the food product and is inhibitory towards fungi and certain bacteria (Table 12) (Davidson and Taylor, 2007; González-Fandos and Dominguez, 2007; Thomas, 1999). Sorbic acid (E200) and its salts (potassium sorbate, E202 and calcium sorbate, E203) can be added in total concentrations up to 1000 ppm in unripened cheese (EC, 2014b). The compounds are generally considered to be safe (González-Fandos and Dominguez, 2007).

Table 12 Fungi, moulds and bacteria inhibited by sorbic acid (Davidson and Taylor, 2007; Thomas, 1999)

Organisms sensitive to sorbic acid			
Yeasts	*Brettanomyces*	*Hansenula*	*Sporobolomyces*
	Byssochlamys	*Kloeckera*	*Torulaspora*
	Candida	*Pichia*	*Torulopsis*
	Cryptococcus	*Rhdotorula*	*Zygosaccharomyces*
	Debaryomyces	*Saccharomyces*	
Moulds and mycotoxigenic moulds	*Alternaria*	*Helminthosporium*	*Aspergillus flavus*[a]
	Aspergillus	*Mucor*	*Aspergillus parasiticus*[a]
	Botrytis	*Penicillium*	*B. niveau*[a]
	Acremonium	*Pullularia*	*Penicillium expansum*[a]
	Cephalosporium	*Sporitrichum*	*Penicillium patulum*[a]
	Fusarium	*Trichoderma*	
	Geotrichum	*Cladosporium*	
Bacteria, Gram-positiv	*Arthrobacter*	*Lactobacillus*	*Mycobacterium*
	Bacillus	*L. monocytogenes*	*Pediococcus*
	Clostridium	*Micrococcus*	*Staphylococcus*
Bacteria, Gram-negative	*Acetobacter*	*Enterobacter*	*Pseudomonas*
	Acinetobacter	*Escherischia*	*Salmonella*
	Aeromonas	*Klebsielle*	*Serratia*
	Alcaligens	*Moraxella*	*Vibrio*
	Alteromonas	*Proteus*	*Y. enterocolitica*
	Campylobacter		

[a] Mycotoxigenic moulds

It has been theorised that the inhibitory mechanism of sorbic acid differs from other weak organic acid since the inhibitory effect display less pH dependency compared to e.g. lactic acid. It is therefore expected that the acid primarily acts through disruption of the cytoplasmic membrane and subsequent inhibition of the amino acid uptake, rather than by cytoplasmic acidification (Davidson and Taylor, 2007; Stratford and Anslow, 1998). Other observed or suggested mechanisms include uncoupling the nutrient transport system from the electron transport chain and damage on structure and fluidity of the membrane; cell wall disruption; prevention of endospore germination and disruption of nutrient transport, metabolism, cell growth and division by affecting the activity of different enzymes and reducing the active sites of certain enzymes (fumarase, aspartase, succinic dehydrogenase, ficin and yeast alcohol dehydrogenase) by binding to their sulphydryl groups (Thomas, 1999). Depending on organism and pH, *MIC* values (ppm) for yeasts have been reported between 30 and 400, for moulds between 10 and 1000, for Gram-negative bacteria between 50 and 1000 and for Gram-positive bacteria between 50 and 10000 (Thomas, 1999). Even though sorbic acid is mainly used as a preservative added to the food product its efficiency has also been shown against *L. monocytogenes* on the surface of chicken legs. González-Fandos and Dominguez (2007) found a 1.3 log reduction of *L. monocytogenes* on chicken legs after washing with a 5% sorbate solution compared to control samples that had been washed with distilled water.

Bacterial growth can also be affected by osmotic changes in the environment due to a decreased a_w from increasing concentrations of e.g. NaCl, KCl or sucrose (Mellefont et al., 2003). In brief, the reactions occurring in the cytoplasm are conducted in an aqueous environment and the

membrane surrounding the cytoplasm is permeable to water molecules allowing them to pass freely between the internal- and external environment. The cell becomes stressed if there is a net flow of water molecules out of the cytoplasm (plasmolysis) or if there is a net flow into the cytoplasm causing rupture. The latter is most often prevented in bacteria and fungi by the cell wall (Adams and Moss, 2000). For *L. monocytogenes* especially changes in osmolality, caused by NaCl, on growth rate, growth response, growth limits and lag time duration has been studied and modelled extensively in model systems and foods (Boziaris et al., 2007; Conner et al., 1986; Fernández et al., 1997; McClure et al., 1997; Mellefont and Ross, 2003; Thomas and Wimpenny, 1996; Tienungoon et al., 2000; Vasseur et al., 1999; Zaika and Fanelli, 2003). More recently, attention has been drawn towards reduction of the salt content due to public health concerns (He and MacGregor, 2010) and studies evaluating the effect of reduced NaCl and salt-replacers on *L. monocytogenes* growth have been conducted (Samapundo et al., 2013; Stollewerk et al., 2014). Other factors influencing bacterial growth include nutrients (limitations), relative humidity, gas composition of packaging atmosphere, other preservatives/antimicrobials, smoke components, redox potential etc. (Adams and Moss, 2000; Montville and Matthews, 2007; Ross and Dalgaard, 2004).

At the end of the exponential phase the bacterial culture enters the stationary phase (van Boeckel, 2008). Stationary phase cultures are cultures in which the bacterial cells no longer increase in number. This cessation can be due to nutrient depletion of one or more nutrients. The definition of the beginning of the stationary phase may be vague and is more appropriately described as a transition period in which e.g. DNA, protein and total cell mass stop to increase at equal rates and the transition phase continues until no further increase in cell number occur (Kolter et al., 1993). The stationary phase is of less interest when predicting growth of spoilage- and pathogenic bacteria since the food product most often will be unfit- or unsafe for human consumption when the stationary phase occurs (Peleg and Corradini, 2011). There are, however, exceptions. When bacteria grow in co-culture e.g. LAB and *L. monocytogenes*, the population that reach the maximum population density first will induce a stationary phase in the co-culture at cell concentrations below the expected maximum population density, corresponding to the Jameson effect (Gimenez and Dalgaard, 2004; Jameson, 1962). This early induction of stationary phase, caused by co-culture interactions, and its relevance in predictive microbiology and risk analysis will be addresses in later sections.

5.4 Deterministic modelling approach

The deterministic modelling approach used in the present project relies on a combination of elements from secondary square root type models, the Gamma concept models and Cardinal Parameter models. This type of model has previously been successfully applied for prediction of *L. monocytogenes* in, especially, meat and seafood and for prediction of LAB and *L. monocytogenes* growth in lightly preserved seafoods (Augustin et al., 2005; Le Marc et al., 2002; Mejlholm and Dalgaard, 2009, 2007a, 2007b). The model structure for both LAB- and *L. monocytogenes* growth models (Eq. (5)) allow model extension using newly determined- or existing model parameters due to the, at least theoretically, individual action of each environmental factor (Ross and Dalgaard, 2004; Ross and McMeekin, 2003). However, caution should be taken when expanding existing models due to the risk of extrapolation (Masana and Baranyi, 2000) and the potential of interaction between environmental factors (e.g. weak organic acids and pH) should also be kept in mind. It is therefore extremely important to evaluate and validate the extended models on independent growth data from challenge tests or naturally contaminated products (Ross and Dalgaard, 2004; Ross and McMeekin, 2003).

$$
\begin{aligned}
\mu_{max} = \mu_{ref} \ &\cdot \left[\left(\frac{T-T_{min}}{T_{ref}-T_{min}} \right)^2 \right] \\
&\cdot \left(1\text{-}10^{(pH_{min}-pH)} \right) \\
&\cdot \left(1\text{-}10^{(pH-pH_{max})} \right) \\
&\cdot \left(\frac{a_w - a_{w,min}}{1\text{-}a_{w,min}} \right) \\
&\cdot \left(1\text{-}\left(\frac{[LAC_U]}{MIC_{U\ Lactic\ acid}} \right)^{n1} \right)^{n2} \\
&\cdot \left(1\text{-}\left(\frac{[SAC_u]}{MIC_{U\ Sorbic\ acid}} \right)^{n1} \right)^{n2} \cdot \xi
\end{aligned}
\tag{5}
$$

μ_{ref} corresponds to μ_{max} at the reference temperature (T_{ref}) of e.g. 25°C when other studied environmental parameters are not inhibiting growth (Dalgaard, 2009). 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 (%WPS) and $a_{w,min}$ is the minimum theoretical water activity allowing growth. pH_{min} and pH_{max} are the theoretical minimum- and maximum pH values allowing growth of the microorganisms. $[LAC_U]$ and $[SAC_U]$ are the concentrations (mM) of undissociated lactic- and sorbic acid in the product and $MIC_{U\ Lactic\ acid}$ and $MIC_{U\ Sorbic\ acid}$ are fitted *MIC* (minimum inhibitory concentration) values (mM) of undissociated lactic- and sorbic acid that prevent growth of the modelled microorganisms. ξ is the interaction

parameter accounting for the combined inhibitory effect of the modelled environmental factors (Le Marc et al., 2002). n1 (values of 0.5 or 1.0) and n2 (values of 1.0 or 2.0) influence the model-term fitted to experimental data of the organic acids (Dalgaard, 2009) and it is important to use the estimated *MIC* value in combination with the model expression used to determine the specific *MIC* value (Augustin et al., 2005; Ross and Dalgaard, 2004). For instance, *MIC* values of undissociated lactic acid for the aroma LAB culture in cottage cheese ranged from 9.7 to 41.1 depending on the values of n1 and n2 (Eq. (5)) in the model term fitted to square root transformed μ_{max}-values against undissociated lactic acid

The apparent independency between the individual environmental factors has proven to be a reasonable assumption for a wide range of environmental conditions (Ross and Dalgaard, 2004) but at the same time it has been demonstrated that close to the growth boundary of the microorganisms, interaction between environmental factors must be taken into consideration since growth will be overestimated otherwise (Augustin and Carlier, 2000b; Augustin et al., 2005). This conclusion is supported by other growth response studies reporting changing cardinal values for growth with changing environmental conditions (Cole et al., 1990; Conner et al., 1986; Farber et al., 1989; McClure et al., 1991; Sorells et al., 1990). Different approaches to a mathematical description of the interaction between environmental factors have been applied within the area of growth boundary modelling, including empirical deterministic approaches, logistic regression techniques and artificial neural networks (Ross and Dalgaard, 2004). However, here focus will remain on the approach suggested by Le Marc et al. (2002), introducing the interaction parameter ξ.

$$\xi \; (\varphi(T, pH, a_w, \text{lactic acid, sorbic acid}) = \begin{cases} 1 & \psi \leq 0.5 \\ 2(1 - \psi) & 0.5 < \psi < 1 \\ 0 & \psi \geq 1 \end{cases} \qquad [6]$$

where ξ ($\varphi(T, pH, a_w$, lactic acid, sorbic acid), with a value between 0 and 1, describes the effect of interaction between the modelled environmental parameters on μ_{max}. The value of ξ is determined from Eq. (6) with contributions from the individual environmental parameters (Eq. (7)) used as input in equation (8), which summarise the environmental parameters, represented by e_i, in the parameter ψ. A ψ value above 1 indicates inhibition of growth by the combined effect of the environmental conditions. A ψ value between 0.5 and 1 indicate some reduction of μ_{max}, depending on the value of ψ, whereas ψ-values below 0.5 represent no reducing effect on μ_{max} (ξ=1) caused by the combined effect of the environmental conditions.

$$\varphi_T = \left(1 - \left[\frac{(T - T_{min})}{T_{ref} - T_{min}}\right]\right)^2$$

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

$$\varphi_{a_w} = \left(1 - \sqrt{\left[\frac{a_w - a_{w,min}}{1 - a_{w,min}}\right]}\right)^2$$

$$\varphi_{[LAC];[SAC]} = \left(1 - \left(\left[1 - \left(\frac{LAC_U}{MIC_{LAC,U}}\right)^{n1}\right]^{n2} \cdot \left[1 - \left(\frac{SAC_U}{MIC_{SAC,U}}\right)^{n1}\right]^{n2}\right)\right)^2 \qquad [7]$$

$$\psi = \sum_i \frac{\varphi_{e_i}}{2 \prod_{j \neq i}\left(1 - \varphi_{e_i}\right)} \qquad [8]$$

Contributions from lactic- and sorbic acid ($\varphi[LAC];[SAC]$) are modelled by multiplying their effects. This approach has been suggested by Coroller et al. (2005) as a mean to model the synergistic effects of acid mixtures. The assumption on which the interaction term (ξ) was developed is that the experimental range is divided into three areas: (i) the environmental factors act independently on bacterial growth, (ii) the environmental factors interact and the reduction of growth rate is greater than what would be expected from the effect of each factor individually and (iii) the no-growth zone where interactions between environmental factors prevent bacterial growth (Le Marc et al., 2002). The cardinal parameters are kept constant and the reduction of growth rate is based on a quantification of the relative effect of the interactions (Le Marc et al., 2002; Ross and Dalgaard, 2004). An example of the application of the interaction model of (Le Marc et al., 2002) is provided in the FSSP software (Fig. 14). In the illustrated example, the relationship between and the effect of water phase diacetate and water phase lactic acid at different temperatures, on growth of *L. monocytogenes,* is shown. The psi-value (Ψ) was set to 1 and the lines indicate the growth/no growth limits.
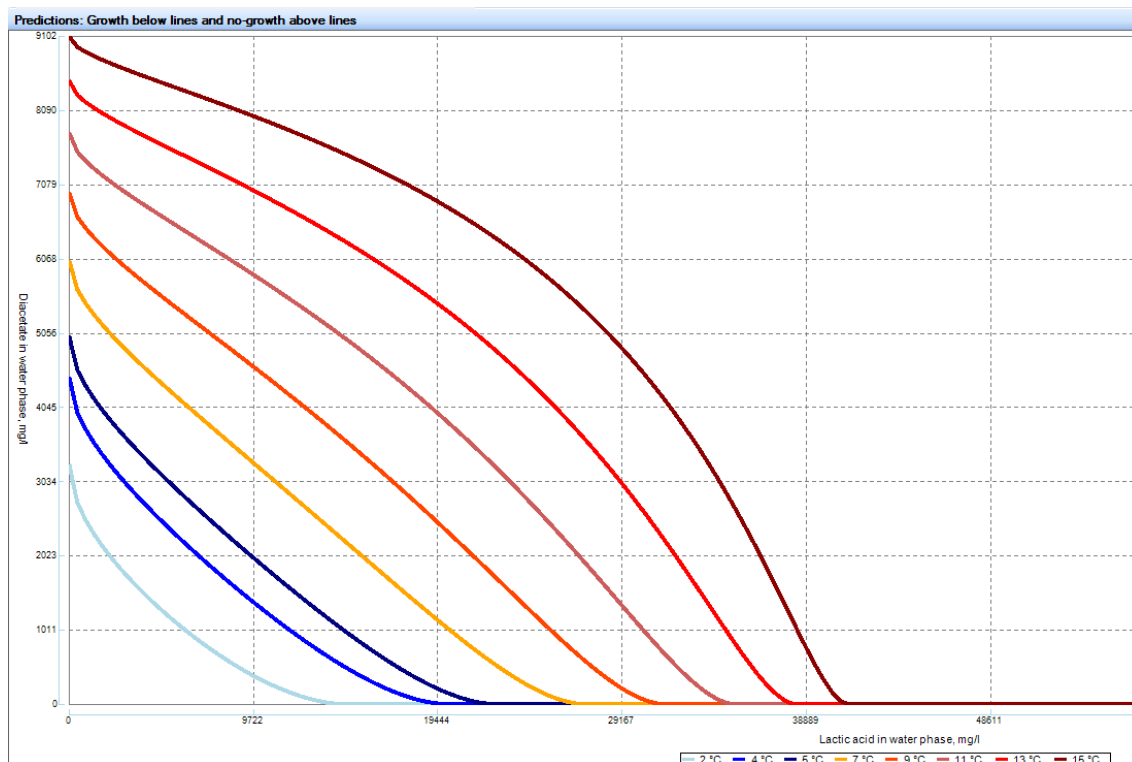
Figure 14 Example of growth boundary modelling of *L. monocytogenes*. The temperature dependent growth boundaries, as determined by the concentration of diacetic and lactic acid, respectively. The psi-value (Ψ) was set to 1, corresponding to the growth/no-growth boundary (from FSSP, www.fssp.dtu.dk).

The Gamma concept and the Cardinal Parameter models are based on the entire physiological range and rely on minimum, optimum, and maximum parameter values (Rosso et al., 1995; Zwietering et al., 1996). The modelling approach (Eq. (5)) applied in the present project has previously been designated as a "simplified cardinal parameter modelling approach" (Emborg and Dalgaard, 2008b). The simplification is implied by a reduction of the modelled biokinetic range compared to the cardinal parameter model originally suggested by Rosso et al. (1995, 1993) which include minimum, optimum and maximum cardinal parameters. The first example of a simplification was probably presented in a study by Rosso and Robinson (2001). They omitted an experimentally determined maximum value of $a_{w,max}$ and assumed that it was equal to 1. Arguments supporting the simple modelling approach include: (i) less data demanding since focus can be maintained on the region of interest and (ii) for e.g. chilled or low pH products, growth responses at high temperature and high pH are of less relevance. It should however be stressed that detailed specification of the range used for model development and validation should be provided and that the model should not be used outside this specific range (Ross and McMeekin, 2003). One of the factors contributing to the flexibility of the model (Eq. (5)) is the μ_{ref}-parameter which allows the model to be calibrated to specific products. Such calibration, refitting or correction has previously been performed (Mejlholm and Dalgaard, 2013, 2007a; Te Giffel and Zwietering, 1999) and it

51

allows complex factors (e.g. bacteriocins or other species specific properties), not addressed by the modelled environmental factors, in the food product to be accounted for in the growth model.

In complex food products, such as fermented dairy, where it is relevant to model growth of the pathogenic microorganism of interest *and* co-culture organisms such as LAB from starter culture, these simplifications and adjustment tools may facilitate a more manageable modelling procedure.

5.5 Inter-bacterial interactions

Jameson (1962) documented the phenomenon of co-culture inhibition of intestinal organisms. This phenomenon was subsequently termed the "Jameson effect" (Ross et al., 2000; Stephens et al., 1997). In brief, the study showed that two organisms, inoculated simultaneously, grow unaffected of each other's presence until one of the organisms reach concentrations close to the maximum population density. This will lead to a termination of multiplication of both organisms. Afterwards, this phenomenon – or versions of it – has been presented in several studies of *Listeria* and LAB or other spoilage- or pathogenic bacteria (Aguilar et al., 2010; Al-Zeyara et al., 2011; Mellefont et al., 2008; Nilsson et al., 2005). Also the effect of co-culture growth has been studied for *Staphylococcus aureus* and yeast in co-culture with LAB (Medveďová et al., 2008) and for interaction between spoilage organisms and yeast-yeast interactions (Malakar et al., 1999; Mounier et al., 2008). For growth experiments performed in this present project, a clear effect of LAB on *L. monocytogenes* growth, at storage temperatures allowing LAB growth, was observed (see Fig. 8).

There may be several factors involved in the inter-bacterial interactions observed for microorganisms in co-culture such as nutrient depletion, metabolite formation and quorum sensing (Irlinger and Mounier, 2009; Miller and Bassler, 2001; Nilsson et al., 2005; Vinderola et al., 2002). Dairy products often contain considerable concentrations of LAB from the added starter cultures, and thereby present contaminating pathogens for a co-culture environment. The inhibitory effect exercised by LAB has been attributed to the formation of lactic acid (see 5.3.2) and the associated decrease in product pH. Additional factors, inducing antimicrobial effects of LAB, are the production of bacteriocins (see 2.2.5) and low molecular mass compounds such as diacetyl, hydrogen peroxide, other organic acids, CO_2, cyclic dipeptides etc. (Dalié et al., 2010; Irlinger and Mounier, 2009; Šušković et al., 2010; Vandenbergh, 1993).

From a modelling perspective, the ultimate objective is to predict realistic growth kinetics of microorganisms in food, that is lag-phase duration, maximum specific growth rate and maximum population density. However, inter-bacterial interactions may induce growth termination and thereby a lower maximum population density than expected/predicted without inclusion of co-culture interaction (Gimenez and Dalgaard, 2004; Mejlholm and Dalgaard, 2007a; Powell et al., 2004). Malakar et al. (2003) considered the relevance of microbial interactions to predictive microbiology and concluded that microbial interactions can be ignored in predictive microbiology

under most conditions since high bacterial concentrations are required to induce an inhibitory effect. At that point, the product will be excessively spoiled. Exceptions include situations where high population densities occur, such as fermented products, without causing spoilage of the product in question. In contrast to these considerations, Ross and McMeekin (2003) pointed out that in vacuum packed foods, LAB can reach their maximum population density without causing detectable spoilage of the product and the microbial interactions are important when predicting the fate of pathogens in the product. Being a fermented product, microbial interactions are relevant for cottage cheese (Malakar et al., 2003). This conclusion is further supported by the growth curves displayed in Fig. 8, where a pronounced inhibition of *L. monocytogenes* was observed as the LAB from the starter culture reached the stationary phase.

5.5.1 Modelling inter-bacterial interaction

Modelling of microbial interactions has been performed in a range of studies using different modelling approaches e.g. the empirical Jameson term (Gimenez and Dalgaard, 2004; Le Marc et al., 2009; Mejlholm and Dalgaard, 2007a; Mejlholm et al., 2014; Møller et al., 2013), modelling the Jameson effect by predicting the evolution of the ratio between two populations (Cornu et al., 2002), descriptive Predator-Prey modelling with Lotka-Volterra equations (Dens et al., 1999; Mounier et al., 2008; Møller et al., 2013; Vereecken et al., 2000) and more mechanistic approaches used to model dynamic lactic acid, pH, a_w related to bacterial growth (Antwi et al., 2007; Breidt and Fleming, 1998; Janssen et al., 2006; Malakar et al., 1999; Vereecken et al., 2003). The most appropriate modelling approach has been discussed and as described in previous sections (see 5.1.4) there is a general trend towards more mechanistic- and "theoretical" modelling approaches among authors discussing the future of predictive microbiology (Bernaerts et al., 2004; McMeekin et al., 2008; Van Impe et al., 2013). In brief, three classes of models can be defined (Fig. 15a-c). (i) empirical black box models which are derived directly from experimental data and are generally considered curve-fitting models (Bernaerts et al., 2004; McMeekin et al., 2002), (ii) semi-mechanistic grey box models which encompass some mechanistic knowledge. Given the biological complexity of food and microorganisms, this type of hybrid modelling has been suggested to be the most appropriate in relation to predictive food microbiology (Bernaerts et al., 2004). And last, (iii) purely mechanistic white box models which provide a precise mathematical description of the mechanisms behind the observed response such as metabolic models (Bernaerts et al., 2004; McMeekin et al., 2002).
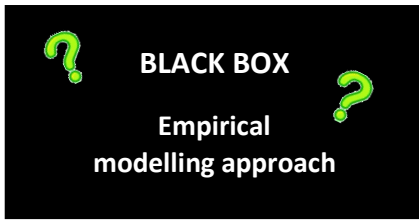
Figure 15a Black box models provides no explanation for the observed responses but fit data satisfactorily. The model may potentially be unique to the data used to generate the model.
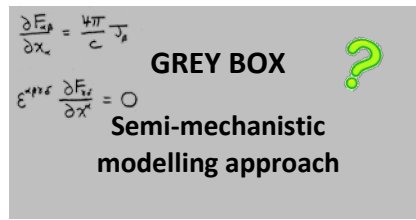
Figure 15b Hybrid Grey box models encompass some interpretation of the response but due to a high level of e.g. biological complexity, parts are not fully explained.
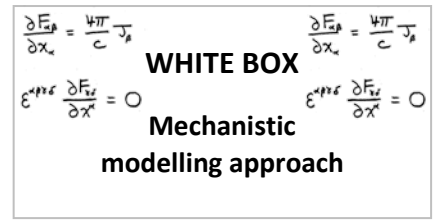
Figure 15c Purely mechanistic White box models provide mathematical descriptions of underlying mechanisms of the observed response.

Bernaerts et al. (2004) strongly advocated for the use of a mechanistic-, or at least semi-mechanistic, modelling approach to describe microbial dynamics and microbial interactions. They suggested that a generic model structure should be developed based on gradually increasing knowledge of microbial behaviour in food. In continuation hereof, Van Impe et al. (2005) presented a novel class of predictive models including the effect of nutrient exhaustion and/or metabolic end products. This novel class of models was analysed by Poschet et al. (2005) in relation to their performance of predicting mono- and co-culture growth termination and it was concluded that the new models performed equal to the Baranyi and Roberts model for prediction of the transition from exponential to stationary phase. As implied above, moving towards more mechanistic models prompt models with higher degree of complexity and Bernaerts et al. (2004) also stressed that in future work a satisfactory trade-off between "predictive power" and manageability should be determined.

In the subsequent section, the most widely used approaches for mathematical description of inter-microbial interaction are presented.

5.5.2 The Jameson term

The Jameson term (Eq. (9)) was introduced by Gimenez and Dalgaard (2004) in order to describe the simultaneous growth of *L. monocytogenes* and spoilage microbiota in cold-smoked salmon. The underlying basis for the term is the differential form of the Logistic growth model where the maximum specific growth rate is reduced as the microorganisms approach their maximum population density – hence describing the intra-species competition. The Jameson term is based on the assumption that high concentrations of LAB reduce the growth of *L. monocytogenes* in the same way that LAB reduce their own growth when their concentration approaches the maximum population density (LAB_{max}) and thereby describes inter-species competition (Gimenez and Dalgaard, 2004).

$$\frac{dLm/dt}{Lm_t} = 0, \qquad\qquad\qquad\qquad t < t_{lag,Lm}$$

$$\frac{dLm/dt}{Lm_t} = \mu_{max}^{Lm} \cdot \left(1 - \frac{Lm_t}{Lm_{max}}\right) \cdot \left(1 - \frac{LAB_t}{LAB_{max}}\right), \qquad t \geq t_{lag,Lm} \qquad [9]$$

where *Lm* and *LAB* represent concentrations (> 0 CFU/g) of *L. monocytogenes* and LAB at time t, respectively. Lm_{max} and LAB_{max} correspond to the maximum population density and μ_{max}^{Lm} is the maximum specific growth rate (h^{-1}) of *L. monocytogenes*. Despite the empirical nature of the expression it is not required to estimate new model parameters in order to implement the interaction model, provided that the growth models facilitate accurate prediction of Lm_t and LAB_t. The Jameson term has also been applied in modified versions using a critical concentration rather than LAB_{max} (Le Marc et al., 2009) or by introducing a γ-parameter allowing reduced growth of the co-culture after the dominating population reaches the maximum population density, N_{max}, instead og growth termination (Møller et al., 2013). Despite successful application of Jameson effect models over time (Cornu et al., 2002; Gimenez and Dalgaard, 2004; Le Marc et al., 2009; Mejlholm and Dalgaard, 2007a; Mejlholm et al., 2014; Møller et al., 2013; Vermeulen et al., 2011) this approach has been criticised for being too simplistic (Cornu et al., 2011). However, in the same paper Cornu and co-workers pointed out that further research was needed in relation to existing alternatives to the simple, empirical approach.

5.5.3 Lotka-Volterra inter-species competition models

The Predator-Prey theory laid the basis for the Lotka-Volterra interspecies competition model which was proposed independently by Alfred James Lotka in 1924 and Vito Volterra in 1931. The original Lotka-Volterra model (Eq. (10)) was based on the logistic theory of population growth and it assumes that intra- and inter-specific competitive interactions are linear (Ayala et al., 1973).

$$\frac{dN_i}{dt} = \left(\frac{r_i N_i}{K_i}\right)\left(K_i - N_i - \alpha_{ij} N_j\right), \qquad i \neq j \qquad [10]$$

where N_i is the number of individuals of species *i* (S_i) at time *t*, r_i is the innate capacity of increase per individual of the organisms of species *i*, α_{ij} is the coefficient of competition of an individual species *j* on an individual of species *i*, and K_i is the carrying capacity of the environment for S_i which is the number of S_i individuals that satisfies $\frac{dN_i}{dt} = 0$, r_i, $N_i > 0$ when no individuals of other competing species are present (Ayala et al., 1973). Within predictive microbiology a version unifying the Baranyi and Roberts model and the Lotka-Volterra model for two species competition has been suggested (Eq. (11)) (Dens et al., 1999; Vereecken et al., 2000).

$$\begin{cases} f_{Lm}(t) = \left(1 - \dfrac{Lm(t) + \alpha_{Lm-LAB}\, LAB(t)}{Lm_{max}}\right) \\ f_{LAB}(t) = \left(1 - \dfrac{LAB(t) + \alpha_{LAB-Lm}\, Lm(t)}{LAB_{max}}\right) \end{cases} \qquad [11]$$

where the underlying mechanism, competition for a common substrate, is described by the coefficients of interaction, $\alpha_{Lm\text{-}LAB}$ and $\alpha_{LAB\text{-}Lm}$, estimated from the microbial growth curves in mixed culture. This particular version of the two-species competition model has been applied for *E. coli* O157:H7 on ground beef (Powell et al., 2004), for *Aeromonas hydrophila* on fish surfaces during refrigerated storage (Giuffrida et al., 2007), for growth of *L. monocytogenes* in salami (Giuffrida et al., 2009) and for growth of *Salmonella* spp. in fresh pork (Møller et al., 2013). This type of descriptive model introduce new empirical parameter estimates in order to account for the inter-species competition (Le Marc et al., 2009).

5.5.4 The components of a mechanistic modelling approach

As described, the mechanistic modelling approach is based on mathematical descriptions of the underlying mechanisms of the observed responses. Thus, mechanistic models most often contain several components, each describing one underlying mechanism. In relation to fermented dairy products, especially the production of lactic acid and pH change has been pointed out as main factors affecting the growth environment (Vereecken et al., 2003; Wilson et al., 2000), but also production of other metabolic products and bacteriocins is of interest in relation to fermented dairy (De Vuyst and Leroy, 2007).

5.5.5 Dynamic lactic acid and pH

Lactic acid has wide applications in the food industry and other industries as well (Theron and Lues, 2011a) and biochemical engineering has, for many years, worked with optimisation and prediction of LAC production in batch- and continuous cultures (Bouguettoucha et al., 2011).

Studies dealing with the kinetics of lactic acid production focus on the prediction of bacterial growth rate and the rate of product formation. Different studies include different parameters as determinants of growth, e.g. nutrient depletion (nitrogen and carbon), inhibition by the product (lactic acid), inhibition by substrate (lactose), effect of pH and the associated degree of dissociation of the weak organic acid etc. Most studies have dealt with fermentation processes with controlled/stabilised pH, since the main objective of these studies has been to optimise fermentation processes applied in the industry. In the following, different approaches to the modelling of lactic acid formation are described. The main focus will be on the production kinetics rather than the growth kinetics since the methods applied in predictive microbiology are performing well in predicting growth of the microorganisms by inclusion of a range of environmental parameters affecting growth (Table 10 and Table 11).

5.5.6 Modelling metabolite formation

In fermentation technology, the aim is to optimise three things related to product formation. (i) the yield of product per gram of substrate, (ii) the concentration of product and (iii) the rate of product formation (Pirt, 1975). Formation of products can either be linked to bacterial growth or not.

The concept of the yield factor (*Y*) is, that the production of biomass of a microbial culture can be related to the substrate consumption by a constant growth yield factor as described by Monod (Eq. (12)) (Bailey and Ollis, 1986; Dalgaard, 2002; Monod, 1949).

$$Y_{X/S} = \frac{mass\ of\ cells\ produced\ (X)}{mass\ of\ substrate\ consumed\ (S)} \qquad [12]$$

Similarly, the absolute rate of the metabolite formation can be related to the absolute growth rate of the bacteria (Eq. (13)) describing strictly growth linked product formation (Dalgaard, 2002; Pirt, 1975).

$$Abs.\ rate\ of\ metabolite\ formation = Y_{P/X} \cdot \mu \qquad [13]$$

If product formation is partly growth linked and partly independent of growth, a maintenance factor can be included (Eq. (14)) as suggested by Luedeking and Piret (1959).

$$Abs.\ rate\ of\ metabolite\ formation = Y_{P/X} \cdot \mu + \beta \cdot X \qquad [14]$$

Equation (14) quantitatively relate the rate of product formation to the rate of bacterial growth and to the bacterial population in combination. It was concluded that during the logarithmic growth phase, the rate of the production of lactic acid was proportional to the bacterial growth rate or to the quantity of bacteria. Outside the logarithmic growth phase this was however not true and the rate of product formation should be based on the combined effect of growth rate and bacterial quantity (Luedeking and Piret, 1959). Over time, many expressions have been derived from the Luedeking and Piret expression and they combine the effect of growth associated and non-growth associated product formation. Additionally, the models include the effect of one or more parameters to the product formation kinetics. Substrate dependence, substrate limitation and substrate inhibition has been included (Bâati et al., 2004; Boonmee et al., 2003; Jørgensen and Nikolajsen, 1987; Rogers et al., 1978), the limitation and inhibition of lactic acid (the product) has been modelled (Bâati et al., 2004; Boonmee et al., 2003; Moldes et al., 1999; Monteagudo et al., 1997) and the effect of the undissociated form of the lactic acid has been included in some of the expressions (Balannec et al., 2007; Yeh et al., 1991). Amrane and Prigent (1994a, 1994b) included an additional term and a constant (*F*) to account for the decrease of the specific production rate at low specific growth rates.

When considering the formation of lactic acid due to growth of LAB in cottage cheese it is important to evaluate which parameters that is important. The "efficiency" of the lactic acid production cannot be compared to the efficiency of the product formation in a fermentation process and the differences should be considered. Dalgaard (2002) outlined that in many cases the simple, growth related, expression for product formation was sufficient to predict the formation of metabolites.

Emborg and Dalgaard (2008a, 2008b) used the yield factor concept to predict histamine formation caused by growth of *Morganella psychrotolerans* and *Morganella morganii* in tuna during storage. The rate of product formation was related to the growth rate of the microorganisms by a constant yield factor ($Y_{P/X}$), Eq. (15).

$$Absolute\ rate\ of\ product\ formation = \ Y_{P/X} \ \cdot \ \frac{dN}{dt} \cdot 1000 \qquad [15]$$

A primary model for the formation of product at time = t was obtained by combining the expanded Logistic growth model (N_t in Eq. (16) = Eq. (2), see XXX) with the yield factor concept (Dalgaard, 2002; Emborg and Dalgaard, 2008a, 2008b)

$$Product\ at\ time, t = \ Init. prod. conc. + Y_{P/X} \ \cdot \ (N_t - N_0) \ \cdot 1000 \qquad [16]$$

The yield factor constant was, as the only parameter, estimated based on fitted experimental data. The remaining (kinetic) parameters were known (initial product concentration, initial bacterial concentration, maximum specific growth rate, lag time and maximum bacterial population) and used when fitting the yield factor. The methodology was found successful to predict histamine formation in tuna during storage of the products (Emborg and Dalgaard, 2008a, 2008b). The yield factor concept has also been used to predict the formation of lactic acid related to growth of LAB and subsequent change in pH in microbial Time-Temperature Integrator media (Ellouze et al., 2008).

5.5.7 Modelling dynamic pH

Since lactic acid, produced by the LAB, has been shown to exhibit a pronounced inhibitory effect on co-culture organisms (Vereecken et al., 2003; Wilson et al., 2000) it could be essential to include the effect of dynamic pH in the prediction of co-culture inhibition. In literature, different equations have been proposed to describe the relation between lactic acid and pH. Poschet et al. (2005) and Vereecken and Van Impe (2002) used a pH model (based on Dabes-kinetics) relating pH to the concentration of undissociated lactic acid. Ellouze et al. (2008) used a model based on a modified version of the logistic model (Whiting, 1993) introducing two "acidification kinetics", each with individual acidification rates, and with a weighted relative importance of each

acidification process. A third model (Eq. (17)) was introduced by Martens et al. (1999) and applied by Malakar et al. (1999) and by Leroy and De Vuyst (2003). It is based on titration in media starting from a lactic acid concentration of zero (Fig. 16). In food, the initial concentration of lactic acid may not be equal to zero and it may be necessary to provide an estimate of the pH value, theoretically corresponding to no lactic acid in the product, based on knowledge of the raw materials etc. a_1 and a_2 in Eq. (17) are regression parameters of the model, LAC_t is the concentration of lactic acid at time t and pH_i is the initial pH value corresponding to a lactic acid concentration of zero.

$$pH = \frac{pH_i + a_1 [LAC_t]}{1 + a_2 [LAC_t]} \qquad [17]$$
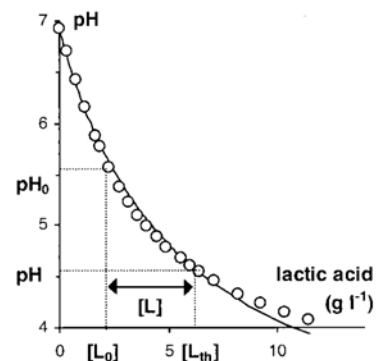
Figure 16 Illustration of the titration curve laying the basis for the pH model. The initial pH corresponds to no lactic acid whereafter pH decrease with increasing concentration of lactic acid (Leroy and De Vuyst, 2003).

5.5.8 Modelling the formation of other inhibitory compounds

The inhibitory effect of bacteriocins may also be of relevance when predicting contributors to growth inhibition of microorganisms in food products containing background microbiota. Production and activity of bacteriocins has been modelled under conditions similar to industrial fermentation processes (Guerra et al., 2007; Lejeune et al., 1998) and for conditions mimicking fermentation processes in food products (Leroy and De Vuyst, 2003, 1999; Messens et al., 2003, 2002). The formation and activity of nisin, sakacin K, amylovorin L471, curvacin A was modelled by relating a specific bacteriocin production rate to the biomass concentration. As the bacterial growth ceases, so does the bacteriocin production and degradation of the bacteriocins occurs instead, expressed by an apparent rate of bacteriocin inactivation (Leroy and De Vuyst, 1999). It may, however, not be straightforward to predict the formation of bacteriocins accurately in a complex food matrix. Lejeune et al. (1998) found that the specific bacteriocin production rate varied considerably with temperature and other authors observed effects of pH on the bioavailability of the bacteriocins (De Vuyst et al., 1996; Yang et al., 1992).

The aroma-contributing compound diacetyl has also proven as an antimicrobial especially against Gram negative bacteria (Jay, 1982; Vandenbergh, 1993) and it may be relevant to predict the production and effect of diacetyl in dairy products. Apparently, no studies presenting such

models for dairy products exist in literature but one model was found for diacetyl synthesis and degradation during beer production (García et al., 1994).

5.5.9 Dynamic modelling

To account for dynamic conditions during storage in the growth predictions (e.g. temperature, pH, lactic acid and LAB concentration) microbial growth must be integrated over time (Ross and McMeekin, 2003). Different approaches exist to solve ordinary differential equations (ODE) and here the Euler- and the Runge-Kutta methods are considered. The Euler method obtains the approximation through an interval, but it only use derivative information from the beginning of the interval (Fig. 17) which induce error in each step. Even though the Euler method is not recommended for practical use (Press et al., 1992) it has been applied successfully in a range of studies predicting microbial growth under dynamic conditions (Alavi et al., 1999; Leroy et al., 2002; Messens et al., 2003; Vaikousi et al., 2008; Vrancken et al., 2008). The reason for its unsuitability is that it is inaccurate compared to other methods performed at similar step size and it is found to be unstable (Press et al., 1992). The inaccuracy of the approximated values can, however, be minimised by minimising the step size.
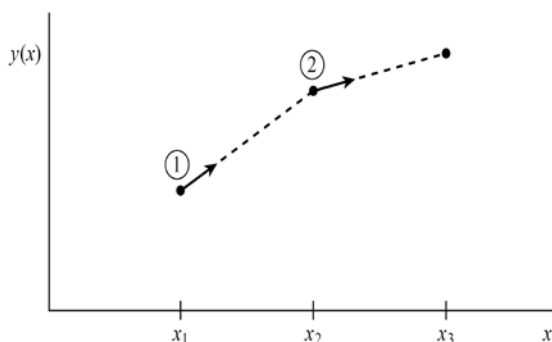
Figure 17 The simple Euler's method. The derivative at each starting point of the interval is extrapolated to find the next value. This method has first-order accuracy (Press et al., 1992).
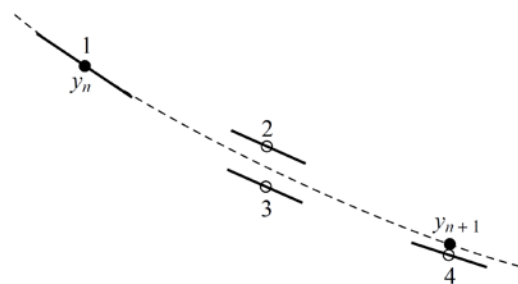
Figure 18 Fourth order Runge-Kutta method introduces four evaluations of the derivative in each step, once at the initial point, twice at the mid-point and once at the endpoint. From these evaluations, the final approximation is calculated (Press et al., 1992).

A more accurate and often used alternative is the Runge-Kutta method. A simple version is the second order, or mid-point method. In this method the start point *and* the mid-point of the derivative is evaluated and used to approximate the entire step. A higher-order solution is the fourth order Runge-Kutta method (Fig. 18) which may- or may not induce higher accuracy. This method relies on four evaluations of the derivatives of each step (first, 2 x mid-point, final). From these derivatives, the final approximation is calculated (Press et al., 1992). The fourth-order Runge-Kutta method has also been used for solving differential equations in predictive microbiology e.g. when predicting microbial growth at dynamic temperatures (Fujikawa et al., 2004; Huang, 2004).

When integrating microbial growth over time, lag time must also be dealt with. Zwietering et al. (1994) evaluated three hypotheses for the effect of temperature shifts within the lag phase of a bacterial culture. (i) a shift in temperature result in a new, full lag phase, unaffected by the previous lag phase (ii) a shift in temperature result in a new lag phase equal to the relative part of the lag phase that still needs to be completed and (iii) a temperature shift results in an additional lag phase added to the relative part of the lag phase that still needed to be completed. They obtained the best results for hypothesis (iii) but for practical reasons, it was suggested that the additional lag could be omitted, hence applying hypothesis (ii). Ross and McMeekin (2003) also considered the effect of fluctuating conditions on bacterial lag time and stated that new lag times are not induced each time the environmental conditions change. As a method to handle the effect of fluctuating conditions within the bacterial lag time, they suggested translating the predicted lag-time into lag-generations and until the total number of generations exceed the lag generations hidden in the *RLT*, no growth will occur. Thus it can be concluded that prediction of lag time under dynamic conditions do not induce new lag times at every shift but new environmental conditions must be related to the relative lag fraction that has not yet been resolved.

5.5.10 Interaction and dynamics – in summary

Microbial interactions are important to model, especially when evaluating microbial growth in fermented products such as cottage cheese. Different approaches to the modelling of interaction exist relying on empirical, semi-mechanistic and purely mechanistic methods. However, no purely mechanistic models exist for food products as they present a complex growth matrix and it may be difficult to explain all underlying mechanisms mathematically. The empirical Jameson and Predator-Prey based models are distinguished based on the need for new parameter estimates. The semi-mechanistic modelling approaches relevant for fermented products often rely on the modelling of dynamic lactic acid concentrations, changes in product pH and production of bacteriocins induced by the added LAB cultures. In relation to cottage cheese, application of semi-mechanistic approaches induces a need for further, extensive analyses and data collection.

When integrating growth over time it is important to consider the desired complexity of the approach. If using the simple Euler integration method, the integration step length must be considered. However, most applied mathematical programs and the available packages contain default algorithms optimising the integration procedures.

When modelling the effect of shifts in the environment on the lag phase duration, new full lag phases should not be included upon every shift in the environmental conditions. The approach to be used relies on an initial lag phase that must be resolved before growth can be initiated. The rate at which the lag time is resolved, depend on the (new) environmental factors.

5.6 Validation of deterministic models

An important step in the development of predictive microbial models is to evaluate the model performance against growth- or inactivation data obtained for microorganisms in food. An important mean to evaluate growth prediction is clearly to visually compare graphical presentations of observed and predicted growth. However, more "quantitative" and specific mean of evaluation include the bias- and accuracy factor (Ross, 1996) which has been used in numerous modelling studies as indices of performance (Cornu et al., 2006; Devlieghere et al., 2001, 2000; Mataragas et al., 2006; Mejlholm and Dalgaard, 2007a; Mejlholm et al., 2010; Te Giffel and Zwietering, 1999). The bias-factor (Eq. (18)) is a measure of whether the observed μ_{max}-values, on average, lie above or below predicted μ_{max}-values and it also serves to quantify contingent deviations. A bias factor >1 indicate that predicted growth is too fast whereas bias factor <1 indicate that predicted growth is too slow and hence the model is fail-dangerous (Ross, 1996).

$$\text{Bias factor} = 10^{\left(\sum \log\left(\frac{\mu_{observed}}{\mu_{predicted}}\right)/n\right)} \qquad [18]$$

Bias factors between 0.95 and 1.11 indicate good model performance and bias factors between 1.11-1.43 or 0.87-0.95 indicate acceptable model performance. Bias factors of <0.87 or >1.43 deems the model unacceptable (Baranyi et al., 1999; Mejlholm et al., 2010). An alternative range ($0.75 < B_f < 1.25$) of acceptable model performance for spoilage organisms, e.g. LAB, has previously been suggested by Dalgaard (2000). In continuation of this, Mejlholm and Dalgaard (2013) suggested an interval from 0.85 to 1.25 as appropriate for evaluation of models for spoilage *Lactobacillus* spp. in seafood and meat products. The accuracy factor (Eq. (19)) provides a measure of precision, which is the average distance between each point and the line of perfect match, to indicate how close predictions are to observations, on average (Ross, 1996; Te Giffel and Zwietering, 1999).

$$\text{Accuracy factor} = 10^{\left(\sum \left|\log\left(\frac{\mu_{observed}}{\mu_{predicted}}\right)\right|/n\right)} \qquad [19]$$

The bias- and accuracy factor should be used in combination in order to detect potential systematic deviations in the predictions of growth (Ross, 1996). Furthermore, Mejlholm and Dalgaard (2013) reported that A_f-values higher than 1.5 could be an indication of systematic deviation between observed and predicted μ_{max}-values. The Bias- and Accuracy factors are direct measures of the agreement between observed and predicted growth rates. Simulation of inter-bacterial interaction or growth under dynamic conditions induces a need for alternative evaluation measures, since the entire growth course needs to be evaluated. Oscar (2005) introduced the concept of the Acceptable Prediction Zone (APZ) based on relative error calculations for lag time and growth rate. In order to be acceptable, 70% of the prediction errors should fall inside the APZ. Velugoti et al. (2011)

considered their dynamic model for *Salmonella* growth acceptable as it predicted growth within 0.5 log from experimental values. These two evaluation procedures were combined (Møller et al., 2013) in an Acceptable Simulation Zone (ASZ) where the model was assessed acceptable if 70% of the observed values were within 0.5 log-units from the simulated growth.

5.7 Stochastic modelling approach

5.7.1 Stochasticity and variability

Point estimates obtained from deterministic models are in many cases sufficient to provide an estimate of the magnitude of growth potential and risk in a product (Nauta, 2007; WHO/FAO, 2004). However, variability is an inherent part of food and microorganisms and variability is observed (i) between contaminating strains, their initial physiological state and the initial concentration, (ii) within food processing, (iii) for the intrinsic factors of a food product, (iv) in the extrinsic factors e.g. storage temperature and (v) in relation to the microbial interaction between microorganisms during storage (Mejlholm et al., 2014). In order to obtain a prediction of the range and probability of possible outcomes, variability can be included in the predictive model (WHO/FAO, 2004). Variability can be observed and quantified and additional measurements will not lead to a reduction, contrary to uncertainty (Nauta, 2007; Vose, 2000). Variability can be incorporated in the predictive models by substituting point-estimate input values with probability distributions (Koutsoumanis et al., 2010; Nauta, 2007). It should be stressed that the possible range and probability of a simulated outcome can also be caused by the uncertainty associated with the input values. The uncertainty is representing a lack of knowledge, and it may be reduced by further measurements or by qualified assumptions and beliefs. The degree of uncertainty is a measure of how much we believe in e.g. a given parameter and uncertainty can, in some cases, be quantified by confidence intervals from a statistical analysis (Nauta, 2007; Vose, 2000). Even though variability and uncertainty are both described by probability distributions and in that sense are difficult to differentiate, it is important to be able to separate between the two and to be aware what the output of a simulation actually describes (Nauta, 2000). Mixing uncertainty and variability in one simulation makes it impossible to determine how much of the total uncertainty (combination of uncertainty and variability) that comes from either variability, which cannot be reduced by further data collection, or from uncertainty, which can be decreased by further data collection and hence improve future estimates (Vose, 2000). Important examples of variability and uncertainty, respectively, could be growth variability between strains and uncertainty of growth model parameters (Pouillot et al., 2003). Nauta (2000) illustrated the effect of second-order modelling where uncertainty and variability were separated. The prediction of the outbreak size depended on the way variability and uncertainty was separated and by omitting the separation of variability and uncertainty major outbreaks could be overlooked. These results were obtained based on a risk assessment of *Bacillus cereus* growth in pasteurised milk. Differences in the probability of large

outbreaks were observed depending on the separation of variability and uncertainty. The importance of the separation of variability and uncertainty is, however, dependent on the objective of the predicted range and probability estimates and on the questions to be answered (Nauta, 2007; Vicari, 2007). For a question such as "What is the expected concentration of X at time t?" a deterministic model may be sufficient. If estimates of relative frequency of a given situation are required, then sources of important variability should be included in the prediction to provide range and probability of certain outcomes. If, on the other hand, we need to know "how sure we are about a probability estimate" the uncertainty in the model must be quantified as well (Nauta, 2007).

In recent years, stochastic modelling approaches have been applied for a range of food/microorganism combinations and with different objectives. Examples include quantitative risk assessments (Cassin et al., 1998; Pouillot et al., 2007; WHO/FAO, 2004), evaluation of intervention strategies to ensure food safety (Koutsoumanis et al., 2010; Tenenhaus-Aziza et al., 2014), evaluation of growth of *L. monocytogenes* in refrigerated foods and evaluation of predicted growth of *L. monocytogenes* in naturally contaminated lightly preserved seafood samples (Couvert et al., 2010; Mejlholm et al., 2014) and evaluation of compliance with regulatory safety criteria (Koutsoumanis and Angelidis, 2007).

The above mentioned studies are mainly based on bacterial population growth and do not include the between-cell variability. However, one factor that is highly variable is the lag time duration of individual cells (Baranyi, 1998; Francois et al., 2006a, 2005; Nauta, 2007). Depending on the physiological state of the cell and the growth conditions, this variability may become even more evident and important (Guillier and Augustin, 2006; Guillier et al., 2005). Generally, population lag time is shorter than the average individual lag time due to the fact that fast growing cells contribute more to the population lag than does the slow growing cells (Baranyi, 1998). Several authors have investigated and quantified individual cell lag time variability using different approaches. The most commonly used methods are (i) absorbance measurements (D'Arrigo et al., 2006; Francois et al., 2006a, 2005; Guillier et al., 2005; Li et al., 2006; Métris et al., 2003; Smelt et al., 2002; Stephens et al., 1997) where cultures are diluted in microtiter plates to obtain individual cells e.g. by following the protocol of Francois et al. (2003). Bacterial growth is subsequently measured by absorbance measurements, either manually or by using automated instruments such as the Bioscreen C absorbance reader. Time-to-detection has been used as a measure of lag time under the assumption that the initial bacterial concentration and the detection limit is known (Pin and Baranyi, 2006). (ii) Flow chamber, where the cells proliferate while they are attached to a transparent solid surface. The daughter cells are removed by the shear force of the flow and single cells are thus monitored by microscopy and image capture. This method was described by Elfwing et al. (2004) and has been applied by Métris et al. (2005) and Pin and Baranyi (2006). Thirdly, time-lapse microscopy has been used to monitor single cell colonial growth of cells attached to an agar surface. This method differs from the flow chamber since the daughter cells are not removed and

colonial growth is monitored (Koutsoumanis and Lianou, 2013). Appropriate distributions are fitted to the quantified lag time data and these distributions are used as input data in simulation models evaluating growth of individual cells which is a realistic scenario within food risk assessment. It is obviously a prerequisite that accurate growth models are available.

5.7.2 Growth simulation

One approach to describe risks is to simulate growth. According to the theory of large numbers, accurate answers to a complex model can be obtained by solving the model repetitively while sampling from distribution input data (WHO/FAO, 2004). Simulation models are often based on a Monte Carlo sampling algorithm and the basic idea behind the Monte Carlo procedure is that the simulation is strengthened by performing a number of iterations. At each iteration, numbers are sampled from probability distributions and for a high number of iterations, real-world conditions are reasonably represented, provided that the distributions used to describe input data are realistic (Smid et al., 2010; WHO/FAO, 2004). Monte Carlo sampling relies on the generation of random numbers from input distributions. The cumulative distribution function ($F(x)$) is "reversed" and x-values are obtained from the inverse function ($G(F(x))$) (Fig. 19). To generate a random sample for a probability distribution, a random number (r) is generated between 0 and 1 and from this value, the corresponding x-value is determined. r is generated from a uniform distribution between 0 and 1 and that implies that the opportunity of a x-value being generated in any percentile is equal (Vose, 2000). Monte Carlo simulation tools are incorporated in most commercially available software (e.g. MS Excel add-in @Risk and Analytica) frequently used for stochastic simulations in predictive food microbiology.
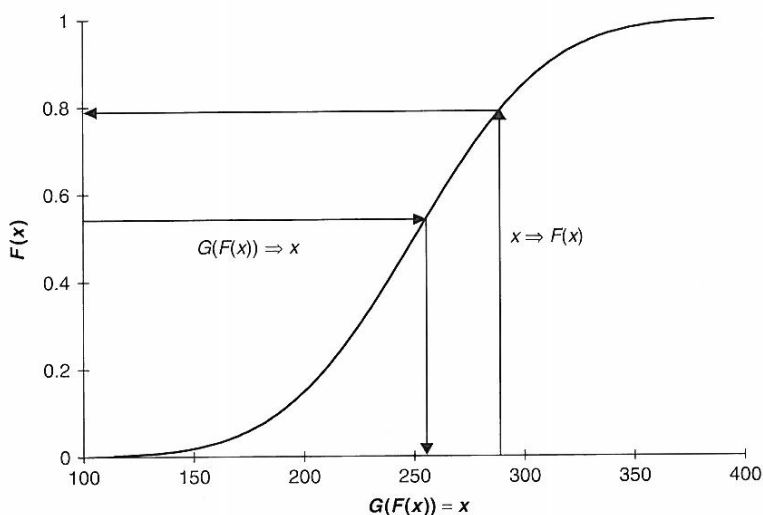
Figure 19 The relationship between x, $F(x)$ and $G(F(x))$ illustrating the principle of generating random numbers by sampling from input probability distributions (Vose, 2000).

The precision of the simulation output is determined by the appropriateness of the input distributions describing the variability or uncertainty of a given parameter. A few basic distinguishing properties exist between the probability distributions. Whether to apply discrete or continuous distributions is determined by the nature of the variable described. Discrete distributions are used to describe variables that need to take specific values. Examples of discrete distributions include Binomial, Geometric, Hypergeometric and Poisson. Continuous distributions describe variables that can take any value within a defined range. Secondly, distributions can be bounded or unbounded. Bounded distributions are confined to lie within two defined values whereas unbounded distributions, theoretically, range from minus infinity to plus infinity. Distributions can also be truncated where an upper- or lower value has been defined in order to avoid tail-values – however, users should consider the appropriateness of distributions that need to be confined (Vose, 2000). The third property for distinguishing distributions is whether the distribution is model-based parametric or empirical non-parametric. In brief, the shape of the former is determined by the mathematics describing a theoretical problem whereas the mathematics of the latter is determined by the shape that is required to describe data most appropriately (Vose, 2000). In literature, a range of different distributions have been applied to describe variability- and/or uncertainty of input variables in stochastic simulation models. Couvert et al. (2010) used Normal distributions and truncated Normal distributions, applying the mean and standard deviation of model parameters, pH, water activity, bacterial concentrations, growth rate and physiological state. Gamma distributions were used to describe variability in model parameters and product properties by Mejlholm et al. (2014), and variability in temperature history of pasteurised milk during distribution, retail and domestic storage has been described by Normal-, Weibull- and Log-normal distributions (Koutsoumanis et al., 2010). Lag time variability for individual bacterial cells has also been described by different distributions over time. The Log-normal distribution was suggested to be suitable for both stressed and unstressed bacterial cells of *E. coli* (Li et al., 2006) and to describe lag time of individual *Salmonella* cells (Koutsoumanis and Lianou, 2013). Francois et al. (2005) suggested that the Gamma distribution was suitable to describe moderately stressed cells whereas the Weibull distribution was more appropriate to describe severely stressed cells. Extreme Value (type I and II), Exponential, Gamma, Weibull, Normal + Laplace and Normal distributions were used to describe the effect of different physiological states on the individual cell lag time (Guillier et al., 2005) and Smelt et al. (2002) also found the Extreme Value distributions appropriate to describe the effects of sub-lethal injuries on lag time of individual cells of *Lactobacillus plantarum*. It is thus evident that there is no direct consensus in literature on which distributions that describe specific variables most appropriately. One reason may be that only evaluated distributions can be chosen. Also the method used to assess evaluated distributions affect the choice of distribution. Often, goodness-of-fit statistics are used to evaluate distribution fitting (e.g. Chi-squared, Kolmogorov-Smirnoff and Anderson-Darling) but these measures present some shortages that

should be considered. For example, they do not penalise increased number of parameters in the best fitting distribution, which lead to increased flexibility on the expense of degrees of freedom. The concept of Information Criteria (Bayesian-, Akaike- and Hannan-Quinn Information Criterion) is a more modern approach to evaluate distribution fitting and this method do penalise increased number of parameters and thereby prioritise simplicity and conservation of degrees of freedom over model flexibility induced by increased number of model parameters (Vose, 2010). Accurate description of variability requires extensive amounts of data. Within food microbiology, data collection is time- and resource consuming even with efficient, automated methods. Even though there is no rule of thumb regarding appropriate sample size for distribution fitting, the confidence in the choice of probability distribution increases with increasing sample size (US EPA, 2001). If the distribution of a data set is not known or it is undesired to assume a theoretical distribution, the Bootstrap resampling simulation technique can be used to generate observations. This method was first introduced by Efron (1979) and basically relies on generating observations by sampling from the empirical distribution (original data) instead of a theoretical distribution. Individual observations from the original data set are randomly sampled with replacement. That means that at each step of the sampling process, individual observation from the original data has a probability of being sampled again and in each bootstrap population, some original individual observations may be represented more than once and some may not be represented at all (Grunkemeier and Wu, 2004). In predictive microbiology, bootstrap sampling has, for instance, been used by Schaffner (1994) to calculate variance in growth rates based on few observed data. In that study, it was concluded that at least 40 and ideally 1000 bootstrapped estimates were required to obtain consistent growth rate variance. Nowadays, with considerably increased computer power available, the number of samplings performed would probably be several thousand. Pouillot et al. (2007) used bootstrap sampling to determine uncertainty and variability of model parameters and consumption data and similarly, bootstrapping was used to obtain population statistics from limited dose-response data (Vicari, 2007).

5.7.3 The stochastic modelling approach – in summary

To round off the (limited) review of the stochastic modelling approach, found to be of particular relevance for the work presented in this present thesis, a summative outline of the "main-points to keep in mind" will be given here. First of all, the objective of the stochastic simulation should be clear, e.g. growth predictions according to variable product characteristics, authority risk assessment etc. It should be determined whether variability or uncertainty is of main concern and care should be taken to distinguish between the two. Secondly, when performing simulation modelling, distributions describing input data should be carefully selected by the use of proper evaluation tools and thirdly, population data can be generated from empirical data rather than theoretical probability distributions.

6. PAPER I

Modelling the effect of lactic acid bacteria from starter- and aroma culture on growth of *Listeria monocytogenes* in cottage cheese

Published in
International Journal of Food Microbiology

Modelling the effect of lactic acid bacteria from starter- and aroma culture on growth of *Listeria monocytogenes* in cottage cheese

Nina Bjerre Østergaard [a,*], Annelie Eklöw [b], Paw Dalgaard [a]

[a] National Food Institute (DTU Food), Technical University of Denmark, Kongens Lyngby, Denmark
[b] Arla Strategic Innovation Centre (ASIC), Stockholm, Sweden

ARTICLE INFO

ABSTRACT

Four mathematical models were developed and validated for simultaneous growth of mesophilic lactic acid bacteria from added cultures and *Listeria monocytogenes*, during chilled storage of cottage cheese with fresh- or cultured cream dressing. The mathematical models include the effect of temperature, pH, NaCl, lactic- and sorbic acid and the interaction between these environmental factors. Growth models were developed by combining new and existing cardinal parameter values. Subsequently, the reference growth rate parameters (μ_{ref} at 25 °C) were fitted to a total of 52 growth rates from cottage cheese to improve model performance. The inhibiting effect of mesophilic lactic acid bacteria from added cultures on growth of *L. monocytogenes* was efficiently modelled using the Jameson approach. The new models appropriately predicted the maximum population density of *L. monocytogenes* in cottage cheese. The developed models were successfully validated by using 25 growth rates for *L. monocytogenes*, 17 growth rates for lactic acid bacteria and a total of 26 growth curves for simultaneous growth of *L. monocytogenes* and lactic acid bacteria in cottage cheese. These data were used in combination with bias- and accuracy factors and with the concept of acceptable simulation zone. Evaluation of predicted growth rates of *L. monocytogenes* in cottage cheese with fresh- or cultured cream dressing resulted in bias-factors (B_f) of 1.07–1.10 with corresponding accuracy factor (A_f) values of 1.11 to 1.22. Lactic acid bacteria from added starter culture were on average predicted to grow 16% faster than observed (B_f of 1.16 and A_f of 1.32) and growth of the diacetyl producing aroma culture was on average predicted 9% slower than observed (B_f of 0.91 and A_f of 1.17). The acceptable simulation zone method showed the new models to successfully predict maximum population density of *L. monocytogenes* when growing together with lactic acid bacteria in cottage cheese. 11 of 13 simulations of *L. monocytogenes* growth were within the acceptable simulation zone, which demonstrated good performance of the empirical inter-bacterial interaction model. The new set of models can be used to predict simultaneous growth of mesophilic lactic acid bacteria and *L. monocytogenes* in cottage cheese during chilled storage at constant and dynamic temperatures. The applied methodology is likely to be applicable for safety prediction of other types of fermented and unripened dairy products where inhibition by lactic acid bacteria is important for growth of pathogenic microorganisms.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Cottage cheese is a fermented, slightly acidic, unripened milk product consisting of curd granules and a cream dressing. The cheese curd is made from low-pasteurised skim milk and acidified with a classical mesophilic starter culture containing *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (O-culture). The added cream dressing may be fresh or cultured using a diacetyl producing culture (*L. lactis* subsp. *lactis* biovar. *diacetylactis*). Both product types are widely available on the Scandinavian market. Cottage cheese has high moisture content (>80%), low salt content (1.0–1.2%) and pH below 5.5. As expected from these product characteristics, growth of the food-borne human pathogenic bacterium *Listeria monocytogenes* has been observed in this chilled food (Chen and Hotchkiss, 1993; Fernandes, 2009; Larson et al., 1996; Walstra et al., 2006). This growth potential is important as *L. monocytogenes* is ubiquitous in the environment, can be isolated from milk and may be introduced to the dairy production environment (Norton and Braden, 2007; Wiedmann and Sauders, 2007). However, some studies reported no growth or a reduction in the concentration of *L. monocytogenes* in cottage cheese in a temperature range between 4 °C and 30 °C (Ferreira and Lund, 1996; Gahan et al., 1996; Liu et al., 2008; McAuliffe et al., 1999). Further studies of cottage cheese are needed as EU regulations state that ready-to-eat products supporting growth of *L. monocytogenes* must not exceed 100 CFU/g throughout shelf life. Additionally, the food business operator must be able to document

compliance with this microbiological criterion depending on product characteristics and different, reasonably foreseeable storage conditions (EC, 2005). It is particularly relevant to investigate quantitative growth responses and predictive modelling of *L. monocytogenes* in cottage cheese. In fact, validated predictive mathematical models are indicated as a suitable supplement to traditional testing and analyses (EC, 2005). However, it must be expected that predictive models for fermented dairy products need to include inter-bacterial interaction since numerous studies have documented the inhibitory effect of lactic acid bacteria (LAB) or other microorganisms on growth of pathogenic bacteria (Antwi et al., 2007; Cornu et al., 2011; Gimenez and Dalgaard, 2004; Guillier et al., 2008; Jameson, 1962; Le Marc et al., 2009; Mellefont et al., 2008).

It is important to evaluate and manage *L. monocytogenes* growth in fermented dairy products. In fact, from January 2010 to October 2013, thirty six alerts related to cheese, not specified to have been produced with raw milk, were registered in the EU Rapid Alert System for Food and Feed (EC, 2013a). In 2012, two confirmed cases of listeriosis in Spain were associated with latin-style fresh cheese, made from pasteurised milk (de Castro et al., 2012). In the period 1998 to 2008 the number of listeriosis cases associated with dairy products in the US did not decrease and involved cases associated with pasteurised products (Cartwright et al., 2013). No cases of listeriosis have been associated with cottage cheese, but as indicated above, *L. monocytogenes* may be present in the dairy production environment and might contaminate products after pasteurisation.

The objective of the present study was to quantify the simultaneous growth of *L. monocytogenes* and LAB from starter- and aroma cultures in cottage cheese and to develop growth models including the effect of storage temperature, pH and water activity. Additionally, the inhibitory effect of lactate, originating from the fermentation process, and sorbate, potentially used as a food preservative in cottage cheese, was quantified and incorporated in the predictive models. Inter-bacterial interaction was investigated and modelled to predict growth of *L. monocytogenes* in cottage cheese. Cottage cheese with either fresh- or cultured cream dressing was characterised and growth of *L. monocytogenes* and LAB was quantified. Cardinal parameter values for growth of these bacteria were determined in liquid laboratory media and simplified cardinal parameter models were developed. To establish the range of applicability for the final models their predictions were compared with data for growth of *L. monocytogenes* and LAB in cottage cheese.

2. Materials and methods

2.1. Chemical characterisation of cottage cheese

Cottage cheese in containers with 0.45 kg to 3.0 kg was obtained from two different production sites producing cottage cheese with fresh cream dressing (cheese curd fermented with a mesophilic starter culture, R604, see Section 2.2.1) or cottage cheese with cultured cream dressing (cheese curd fermented with a mesophilic starter culture, R604, and cream dressing added a mesophilic, diacetyl producing culture, F-DVS SDMB-4, see Section 2.2.1). Product characteristics were studied over a period of 23 months. During transport to DTU Food, the products were packed with ice. When received, the products were stored at 2 °C until use (maximum 72 h). Prior to growth experiments the cottage cheese was analysed for initial chemical characteristics (pH, NaCl, and naturally occurring lactate). pH was measured with a PHM 250 Ion Analyzer (MetroLab™, Radiometer, Copenhagen, Denmark) in 5 g of product stirred with 25 ml water. NaCl was quantified by automated potentiometric titration (785 DMP Titrino, Metrohm, Herisau, Switzerland). The concentration of lactate was determined by HPLC using an external standard for identification and quantification (Dalgaard and Jørgensen, 2000). In order to improve the extraction of lactate, a centrifugation step, as also applied by Marsili et al. (1981), was included prior to two consecutive filtration steps.

2.2. Growth experiments in cottage cheese and liquid laboratory media

For model development, growth data for *L. monocytogenes* and LAB were obtained in four series of challenge tests (5–15 °C), using cottage cheese inoculated with the pathogen, and in one series of storage trials (10 °C and 15 °C) where kinetics of LAB added during production was followed. Additionally, one series of experiments in broth was performed to study the effect of LAB on growth of *L. monocytogenes* (see Section 2.3.3) and ten experiments with >1500 absorbance growth curves were performed in liquid laboratory media using Bioscreen C at temperatures between 5 °C and 21 °C (see Section 2.4.2). For model validation, four series of challenge tests at constant temperatures between 5 °C and 15 °C were performed to obtain growth data for *L. monocytogenes* (25 growth rates) and LAB (17 growth rates). Additionally, three series of challenge tests at dynamic temperature profiles (5–12 °C) yielded another 9 growth curves for both *L. monocytogenes* and LAB for validation purposes (calculation of B_f, A_f and ASZ values, see Section 2.5). During all growth experiments, temperature was regularly recorded by data loggers (TinyTag Plus, Gemini Data Loggers Ltd., Chichester, UK) or recorded automatically by the Bioscreen C software (Bioscreener, GrowthCurves USA, NJ, USA).

2.2.1. Bacterial strains and inoculation

Four strains of *L. monocytogenes* (6, LM19, SLU92, 612), provided by Arla Strategic Innovation Centre (ASIC), Stockholm, were used to inoculate products and laboratory media. The strains had been isolated from dairy production environments (LM19, SLU92, 612) or had been involved in a dairy related outbreak (6). The studied LAB cultures originated from the mesophilic O-culture used to ferment the cheese curd (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, R604, Chr. Hansen A/S, Hørsholm, Denmark) or from the cultured cream dressing (multiple strains of *L. lactis* subsp. *lactis* biovar. *diacetylactis*, F-DVS SDMB-4, Chr. Hansen A/S, Hørsholm, Denmark). All isolates were stored at −80 °C in a frozen storage medium with glycerol.

Before inoculation of products or laboratory media, isolates were pre-cultured to adapt cells to the subsequent environmental conditions. Frozen cultures (−80 °C) were transferred to either Brain Heart Infusion (BHI) broth (CM1135, Oxoid, Basingstoke, UK) (*L. monocytogenes*) or All Purpose Tween (APT) broth (Difco 265510, Becton Dickinson and Company, Le Pont de Claix, France) (LAB) and incubated during 24 h at 25 °C. Subsequently the cultures were re-inoculated in broth with pH adjusted to 5.3–5.5 using HCl, 1% NaCl and, when relevant, 800 ppm lactic acid. Inoculated broth was incubated at 10–12 °C for 24–48 h and the cultures were harvested in the late exponential phase, determined as a relative change in absorbance (540 nm) of 0.05–0.20 (Novaspec II, Pharmacia Biotech, Allerød, Denmark). The final pre-cultures were prepared either by mixing the *L. monocytogenes*-cultures (Lm-MIX) and diluting to the desired concentration in 0.85% saline solution or by using the individual LAB-cultures and likewise diluting to reach the desired bacterial concentration. Products were inoculated with 1.00% (vol/wt) of the diluted pre-cultures. When growth in liquid laboratory media was studied, media were inoculated with desired concentrations (10^2–10^6 CFU/ml). Cottage cheese used in challenge tests or storage trials was divided into portions of 75 to 100 g and stored in the same containers as used for commercial distribution of this product.

2.2.2. Microbiological analyses

At regular intervals during challenge tests and storage trials, microbiological analyses were performed in duplicate or triplicate. 10.00 g of cottage cheese was diluted 10-fold in chilled physiological saline (PS, 0.85% NaCl and 0.10% Bacto-peptone) and subsequently homogenised for 30 s at normal speed in a Stomacher 400 (Seward Medical, London, UK). Appropriate 10-fold dilutions were made in chilled PS. *L. monocytogenes* was enumerated by surface plating on Palcam agar (CM0877, Oxoid, Basingstoke, UK) with Palcam selective supplement (SR0150E, Oxoid Basingstoke, UK) and incubating at 37 °C for 48 h.

LAB were enumerated by pour plating in nitrite actidione polymyxin (NAP) agar (pH 6.2) and incubating with overlay at 25 °C for 72 h (Davidson and Cronin, 1973). Additionally, LAB in cottage cheese with cultured cream dressing was enumerated by surface plating on KMK-agar (Kempler and McKay, 1980) in order to confirm that the citrate fermenting-, diacetyl producing strains of *L. lactis*, from the aroma culture, dominated the LAB population during the entire storage period.

2.3. Modelling

2.3.1. Primary growth model

The integrated and log transformed logistic growth model with delay (four parameter model) or without delay (three parameter model), Eq. (1), was fitted to all individual growth curves of LAB and *L. monocytogenes*, obtained in challenge tests and storage trials. This primary modelling generated fitted parameter values and was performed using Prism (see Section 2.6). A comparison between the three- and four parameter logistic growth models was performed by an F-test to determine if the lag time was significant. The relative lag time ($RLT = t_{lag} \cdot \mu_{max}/\text{Ln}(2)$) was calculated from fitted parameter values for each growth curve.

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

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

$$(1)$$

where t is the time of storage and t_{lag} the lag time, N_t, N_0 and N_{max} are the cell concentrations (CFU/g) at time t, zero and the maximum asymptotic cell concentration, respectively. μ_{max} is the maximum specific growth rate (h^{-1}).

2.3.2. Inter-bacterial interaction model

Simultaneous growth of LAB and *L. monocytogenes* was described by the simple Jameson effect model (Eq. (2)). This equation relies on the assumption that high concentrations of LAB reduce the growth of *L. monocytogenes* in the same way that LAB reduce their own growth when their concentration approaches the maximum population density (LAB_{max}) (Gimenez and Dalgaard, 2004).

$$\frac{dLm/dt}{Lm_t} = 0, \qquad\qquad t < t_{lag,Lm}$$
$$\frac{dLm/dt}{Lm_t} = \mu_{max}^{Lm} \cdot \left(1 - \frac{Lm_t}{Lm_{max}} \right) \cdot \left(1 - \frac{LAB_t}{LAB_{max}} \right), \quad t \geq t_{lag,Lm}$$

$$(2)$$

where Lm and LAB represent concentrations (>0 CFU/g) of *L. monocytogenes* and LAB, respectively, and other parameters are as explained for Eq. (1). In cottage cheese, the initial concentration of LAB was >5.5 log CFU/g. It was therefore assumed that *L. monocytogenes* would not reach higher concentrations than LAB and the potential inhibiting effect of high concentrations of *L. monocytogenes* on growth of LAB was omitted in Eq. (2). In addition, it was tested if a modified version of Eq. (2), as suggested by Le Marc et al. (2009), with LAB_{max} replaced by a critical population density (LAB_{CPD}) lower than LAB_{max} more appropriately described the observed inhibitory effect of LAB from the starter- and aroma cultures on growth of *L. monocytogenes* in cottage cheese. This was studied in broth systems and subsequently tested on product growth data (see Section 2.3.3).

2.3.3. Growth of L. monocytogenes in cell free supernatant to determine the inhibitory concentration of LAB

Growth of *L. monocytogenes* in cell free supernatants from LAB cultures was studied to evaluate if a critical population density (LAB_{CPD}), lower than LAB_{max}, was required to describe the inhibitory effect of LAB on growth of the pathogen. A cocktail of four *L. monocytogenes* isolates (Lm-MIX, see Section 2.2.1) was grown in a series of cell free supernatants of both starter LAB- and aroma LAB-cultures. Supernatants were obtained by sterile filtration (0.20 μm, Minisart®, Sartorius Stedim Biotech, Sartorius, Goettingen, Germany) of APT broth in which LAB cultures (starter- or aroma culture) were grown at 15 °C. The APT broth had an initial pH of 5.3 (adjusted with HCl) and contained 1.0% NaCl and 800 ppm lactic acid. Samples of LAB cultures were taken every 12 h and the concentration of LAB was determined by plate counting using NAP-agar (see Section 2.2.2). Subsequently, seven cell free supernatants were prepared from these samples corresponding to seven specific concentrations of LAB from 0 log CFU/ml to 8.84 ± 0.02 log CFU/ml (starter culture) and 8.57 ± 0.07 log CFU/ml (aroma culture).

μ_{max} of *L. monocytogenes* (Lm-MIX) was determined in duplicate at 15 °C for each cell free LAB supernatant from absorbance detection times (Bioscreen C, Labsystems, Helsinki, Finland) of serially diluted inoculums of 10^2, 10^3, 10^4, 10^5 and 10^6 log CFU/ml. Detection times were determined as the time to a relative change in absorbance of 0.05 at 540 nm for each absorbance growth curve. μ_{max} (h^{-1}) of *L. monocytogenes* was estimated from detection times, as previously described (Dalgaard and Koutsoumanis, 2001). To describe the inhibitory effect of LAB on μ_{max} of Lm-MIX, the LAB concentrations in supernatants prior to filtration (N_{LAB}) were fitted to a modified Jameson effect model (Eq. (3)). Prism was used for curve fitting (see Section 2.6).

$$\frac{\mu_{max,\,Lm} \text{ with } N_{LAB}}{\mu_{max,\,Lm} \text{ without LAB}} = 1 - \frac{N_{LAB}}{LAB_{CPD}} \tag{3}$$

where LAB_{CPD} represents the critical population density of LAB corresponding to a μ_{max}-value of Lm-MIX equal to zero. The estimated LAB_{CPD}-values and LAB_{max}-values (see Section 2.2) for starter- and aroma cultures were used in Eq. (2) to predict simultaneous growth of LAB and *L. monocytogenes* in cottage cheese. For these simulations, Eq. (4) and the model parameters obtained in the present study (see Section 2.4), were used to predict simultaneous growth of LAB and *L. monocytogenes* in cottage cheese. Simulations were compared with observed growth from 11 challenge tests with cottage cheese. The root mean square error (RMSE) was calculated as a measure of model performance.

2.4. Modelling and predicting simultaneous growth of LAB and L. monocytogenes in cottage cheese

2.4.1. Evaluation of existing growth models for L. monocytogenes

Initially, three existing *L. monocytogenes* growth models were evaluated to assess their ability to predict the growth response of *L. monocytogenes* in cottage cheese, with either fresh- or cultured cream dressing, and thereby the need for development of new models. The studied growth models were: (i) the model of Mejlholm and Dalgaard (2009) which previously has been evaluated in a validation study using meat, seafood and some non-fermented dairy products (Mejlholm et al., 2010), (ii) the cardinal parameter model for liquid dairy and cheese products (model no. 5) presented by Augustin et al. (2005) and (iii) a refitted version of Augustin et al. (2005), model no. 5, introduced by Schvartzman et al. (2011). Bias- and accuracy factors (see Section 2.5) were calculated based on the 25 individual growth rates obtained for validation purposes (see Section 2.2).

2.4.2. Re-fitted LAB and L. monocytogenes growth models

A simplified cardinal parameter model was used to describe growth rates of LAB and *L. monocytogenes* in cottage cheese (Eq. (4), Mejlholm and Dalgaard, 2009, 2013). The model included the effect of temperature, pH, a_w, lactic- and sorbic acid. A pH_{max}-term was included in the LAB model where it was found to improve the fit of the parameters (Ross et al., 2003). The effect of interaction between these environmental parameters was taken into account by calculation of the dimensionless term, ξ (Le Marc et al., 2002).

$$\mu_{max} = \mu_{ref} \cdot \left[\left(\frac{T - T_{min}}{T_{ref} - T_{min}} \right)^2 \right] \cdot \left(1 - 10^{(pH_{min} - pH)} \right) \cdot \left(1 - 10^{(pH - pH_{max})} \right)$$
$$\cdot \frac{a_w - a_{w,min}}{1 - a_{w,min}} \cdot \left(1 - \left(\frac{[LAC_U]}{MIC_{U\ Lactic\ acid}} \right)^{n1} \right)^{n2}$$
$$\cdot \left(1 - \left(\frac{[SAC_u]}{MIC_{U\ Sorbic\ acid}} \right)^{n1} \right)^{n2} \cdot \xi$$

(4)

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). 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 (%WPS) according to the relation described by Resnik and Chirife (1988) ($a_w = 1 - 0.0052471 \cdot$ %WPS $- 0.00012206 \cdot$ %WPS^2) and $a_{w,min}$ is the minimum theoretical water activity allowing growth. pH_{min} and pH_{max} are the theoretical minimum- and maximum pH values allowing growth of the microorganisms. $[LAC_U]$ and $[SAC_U]$ are the concentrations (mM) of undissociated lactic- and sorbic acid in the product and $MIC_{U\ Lactic\ acid}$ and $MIC_{U\ Sorbic\ acid}$ are fitted MIC values (mM) of undissociated lactic- and sorbic acid that prevent growth of the modelled microorganisms. The effect on μ_{max} of interaction between the environmental factors was described by ξ. Contributions to the interactive effect from lactic- and sorbic acid ($\varphi[LAC];[SAC]$) were modelled by multiplying their effects as suggested by Coroller et al. (2005) whereas the interactive effects of the remaining factors were modelled as originally suggested (Le Marc et al., 2002). This modelling approach has been described and successfully applied in previous studies (Augustin et al., 2005; Le Marc et al., 2002; Mejlholm and Dalgaard, 2007a, 2007b, 2009).

Values of the cardinal parameters μ_{ref}, T_{min}, pH_{min} and pH_{max} were determined for each of the two mesophilic LAB cultures and for Lm-MIX. This was obtained by fitting Eq. (5) to square root transformed growth rate data using SigmaStat (see Section 2.6). μ_{max} data were obtained from Bioscreen C experiments at 7 °C, 11 °C, 16 °C and 21 °C using APT-broth with 1% NaCl and adjusted with HCl to pH 4.3, 4.7, 5.1, 5.6 and 6.3 for the LAB and at 5 °C, 10 °C and 15 °C in BHI-broth with 1% NaCl and adjusted to 8 different pH-values in the range from 4.4 to 6.9 in experiments with *L. monocytogenes*. μ_{max}-values were determined from absorbance detection times (Bioscreen C) of serially diluted inoculums as previously described.

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

(5)

Antimicrobial effects of lactic- and sorbic acid against the LAB cultures were investigated at 12 °C in APT-broth containing 1% NaCl and adjusted to pH 5.3 with HCl. The inhibitory effect of five to six concentrations of lactate (0 to 8.1 mM undissociated lactic acid; 60% Sodium DL lactate syrup, Sigma L1375, Sigma-Aldrich, St. Louis, MO, USA) and 12 concentrations of sorbate (0.0 to 2.6 mM undissociated sorbic acid; Potassium sorbate, SFK Food Inc., Viborg, Denmark) were examined

against both the mesophilic starter culture and the mesophilic aroma culture. Growth rates were determined from absorbance detection times as described above. Square root transformed μ_{max}-values were plotted against concentrations of undissociated organic acids. The minimum inhibitory concentrations (MIC, mM) of undissociated lactate and sorbate were estimated by fitting the organic acid terms from Eq. (4) using Prism. 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 evaluated from RMSE values. Concentrations of undissociated organic acid were calculated using Eq. (6) with pK_a values of 3.86 and 4.76 for lactic- and sorbic acid, respectively (Ross and Dalgaard, 2004).

Concentration of undissociated organic acid (mM)
$$= \frac{\text{Total concentration of organic acid}}{1 + 10^{pH - pK_a}}$$

(6)

2.4.3. Lag time model for LAB

The relative lag time (RLT) concept was used to include the influence of μ_{max} on lag time duration (t_{lag}) in the growth model for LAB. A one parameter model (Eq. (7)) (Mellefont and Ross, 2003; Ross and Dalgaard, 2004) was applied for the prediction of t_{lag}.

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

[7]

Data from 10 and 15 growth curves with starter- and aroma culture, respectively, were used to calculate RLT-values. The average, minimum and maximum RLT values were obtained from these individual growth curves.

2.5. Evaluation of model performance

Model performance was evaluated on independent growth data for LAB and *L. monocytogenes* obtained in cottage cheese with either fresh- or cultured cream dressing. Bias factor (B_f) and accuracy factor (A_f) values were calculated to evaluate the ability of the models to accurately predict μ_{max}. For pathogenic bacteria, $0.95 < B_f < 1.11$ indicate good model performance, with 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 (Ross, 1996; Mejlholm et al., 2010). For spoilage microorganisms, $0.85 < B_f < 1.25$ has been suggested for good model performance and in addition A_f-values > 1.5 was shown to indicate incomplete models or systematic deviation between observed and predicted μ_{max}-values (Mejlholm and Dalgaard, 2013). The acceptable simulation zone (ASZ) approach was used to evaluate the prediction of growth including lag phases and growth under dynamic temperature storage conditions. The acceptable interval was defined as $\pm 0.5 \log_{10}$-units from the simulated growth of LAB or *L. monocytogenes*. When at least 70% of the observed values were within the ASZ, the simulation was considered to be acceptable (Møller et al., 2013; Oscar, 2005; Velugoti et al., 2011).

Scenarios, demonstrating the applicability of the model in cottage cheese were performed to evaluate the effect of different pH and sorbic acid combinations near the growth boundary of *L. monocytogenes*.

2.6. Statistical analyses and curve fitting

Curve fitting was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego California USA). Data was reported as parameter estimate $\pm$ standard error. F-test, to determine significant lag time was performed in Prism. Model parameters were estimated by fitting secondary growth models (Eqs. (4) and (5)) to data using SigmaStat (version 3.5, Systat Software, Inc.). Data were

reported as parameter estimate $\pm$ standard error. Growth simulations were performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Product characteristics of cottage cheese

Cottage cheese with fresh- or cultured cream dressing showed little variation in product characteristics (pH, NaCl and lactic acid) during the 23 months where samples from different batches were analysed (Table 1). The initial concentrations of LAB were lower in the product with fresh cream dressing (5.6 $\pm$ 0.4 log CFU/g, mesophilic starter culture) compared to the product with cultured cream dressing (6.7 $\pm$ 0.2 log CFU/g, mesophilic aroma culture). The initial pH of the latter product (5.4 $\pm$ 0.1) was about 0.2 units higher than pH of cottage cheese with fresh cream dressing (Table 1).

3.2. Microbiological changes in cottage cheese

L. monocytogenes grew in cottage cheese with fresh cream dressing when stored at 5 °C, 10 °C and 15 °C whereas LAB exclusively grew at 10 °C and 15 °C (Fig. 1). At 5 °C *L. monocytogenes* grew from 3.7 $\pm$ 0.03 log CFU/g to 6.6 $\pm$ 0.11 log CFU/g during 470 h of storage whereas its maximum population densities (N_{max}) at 10 °C and 15 °C were 6.1 $\pm$ 0.24 log CFU/g and 5.5 $\pm$ 0.16 log CFU/g, respectively. In both cases, concentrations of *L. monocytogenes* reached a plateau when LAB approached their N_{max} of 8.6 $\pm$ 0.16 log CFU/g at 10 °C and 9.0 $\pm$ 0.03 log CFU/g at 15 °C. A similar growth pattern of *L. monocytogenes* and LAB was observed in cottage cheese with cultured cream dressing (Results not shown). For cottage cheese with cultured cream dressing and stored at 5, 10 or 15 °C, the concentrations of citrate fermenting LAB, as determined using KMK agar, were similar to LAB concentrations determined by NAP agar. The maximum difference was 0.3 log CFU/g.

3.3. Interaction between L. monocytogenes and LAB

Cell free supernatants from LAB cultures of different concentrations reduced the growth rate of *L. monocytogenes* (Fig. 2). The fitted minimum cell concentrations required for cell free supernatants to prevent growth of *L. monocytogenes* (LAB_{CPD}) were 8.41 log CFU/ml (R^2 = 0.999) and 7.87 log CFU/ml (R^2 = 0.886) for the starter- and aroma LAB cultures, respectively (Fig. 2). The corresponding LAB_{max}-values, determined in non-filtrated broth, were 8.82 $\pm$ 0.08 log CFU/ml and 8.43 $\pm$ 0.08 log CFU/ml (Results not shown). When using Eq. (2), the simultaneous growth of LAB and *L. monocytogenes* in cottage cheese was more appropriately described with LAB_{max}-values compared to LAB_{CPD}-values. This is shown in Fig. 3 for one challenge test and confirmed by the RMSE-values which, for seven of 11 challenge tests, were lower when using LAB_{max}-values compared to LAB_{CPD}-values. Therefore, subsequent simulations of the simultaneous LAB and *L. monocytogenes* growth were performed by using LAB_{max}-values.

Table 1
Product characteristics of cottage cheese with fresh- and cultured cream dressing.

	Cottage cheese with fresh cream dressing		Cottage cheese with cultured cream dressing	
	n[a]	Average $\pm$ SD	n[a]	Average $\pm$ SD
pH[b]	13	5.18 $\pm$ 0.06	15	5.39 $\pm$ 0.07
NaCl[b] (% in waterphase)	13	1.21 $\pm$ 0.07	21	1.09 $\pm$ 0.08
Lactic acid[b] (ppm in water phase)	12	718 $\pm$ 187	24	1029 $\pm$ 244
Lactic acid bacteria[b] (log CFU/g)	11	5.59 $\pm$ 0.39	13	6.65 $\pm$ 0.21

[a] Number of samples analysed. Different batches of cottage cheese were obtained during a period of 23 months.
[b] Initial values or concentrations.
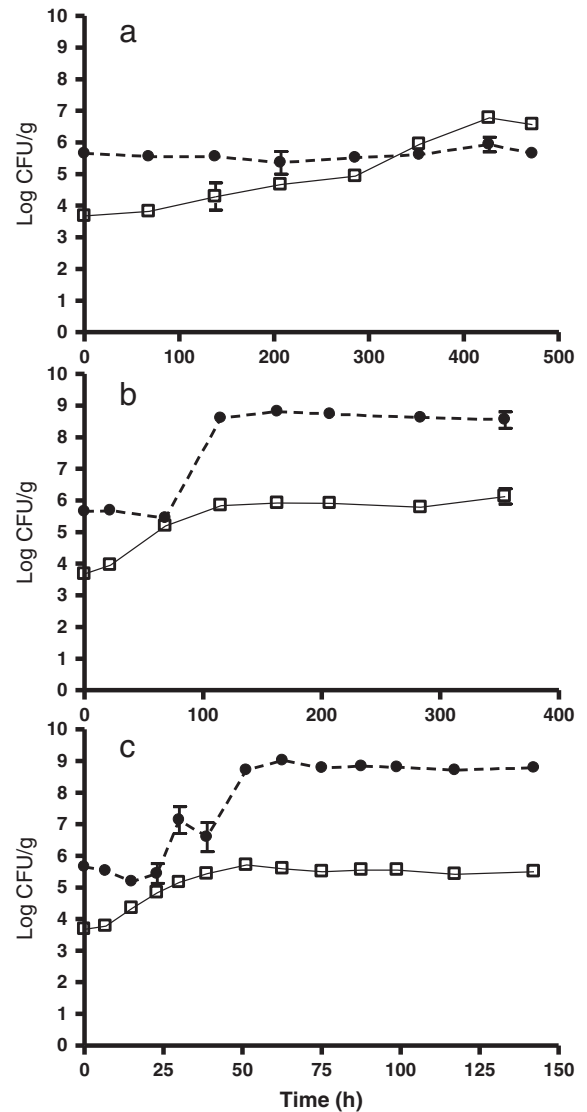
Fig. 1. Growth of lactic acid bacteria (mesophilic starter culture) (●) and *L. monocytogenes* (□) in cottage cheese (pH 5.18, 1.22% NaCl in water phase, ~800 ppm lactic acid in water phase) at 5 °C (a), 10 °C (b) and 15 °C (c) during storage of up to 470 h (20 days). Error bars indicate standard deviation of four samples.

3.4. Modelling the simultaneous growth of LAB and L. monocytogenes

3.4.1. Growth model for LAB in cottage cheese

The two studied LAB cultures displayed distinct growth responses and different T_{min}, pH_{min}, $MIC_{U\ Lactic\ acid}$ and $MIC_{U\ Sorbic\ acid}$ values were determined (Table 2).

μ_{ref}-values determined from growth in liquid laboratory medium were refitted to product growth data in order to calibrate the two secondary LAB-models to the specific products. This product calibration with cottage cheese had a pronounced effect and changed μ_{ref} for the LAB starter culture from 0.73 h^{-1} to 1.20 h^{-1}, whereas μ_{ref} of the LAB aroma culture was reduced from 0.78 h^{-1} to 0.57 h^{-1}. As determined from MIC values of the undissociated form of the organic acids, the LAB starter culture with a MIC of 4.09 $\pm$ 0.26 mM was more sensitive to lactic acid than the LAB aroma culture with a MIC value of 9.72 $\pm$ 0.47 mM. In contrast the two LAB-cultures had similar sensitivity to sorbic acid (Fig. 4). The fitted MIC values were 4.62 $\pm$ 0.35 mM and 5.50 $\pm$ 0.36 mM undissociated sorbic acid for the starter- and aroma culture, respectively. However, to appropriately describe the
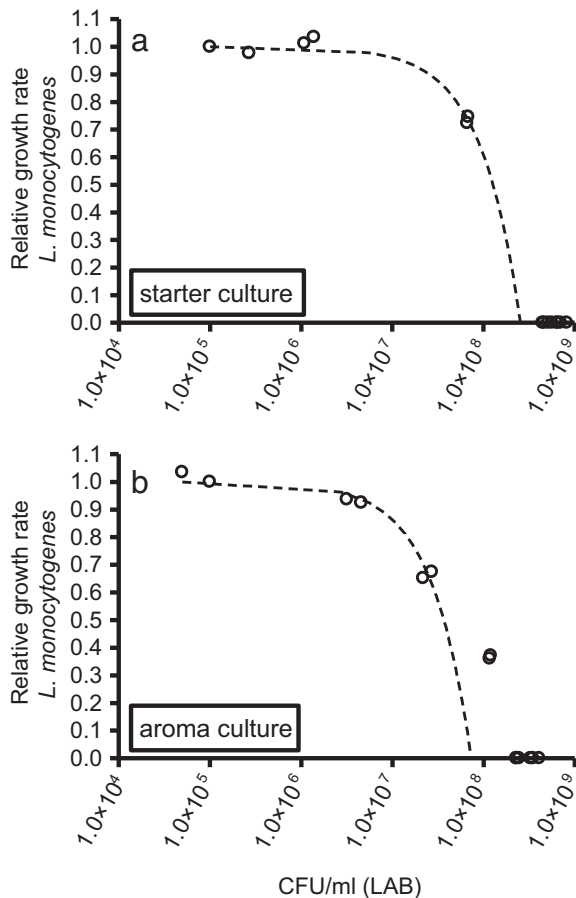
Fig. 2. Relative growth rate of *L. monocytogenes* (○) in cell free supernatants related to the concentration of lactic acid bacteria in growth media prior to filtration. Data was fitted (dashed line) to the Jameson term to estimate the inhibitory concentration of lactic acid bacteria on growth of *L. monocytogenes*.

experimental data, different exponents (values of n1 and n2) were used in the sorbic acid model terms for the two LAB cultures. Therefore, the MIC values for sorbic acid are not directly comparable (Eq. (4), Table 4).
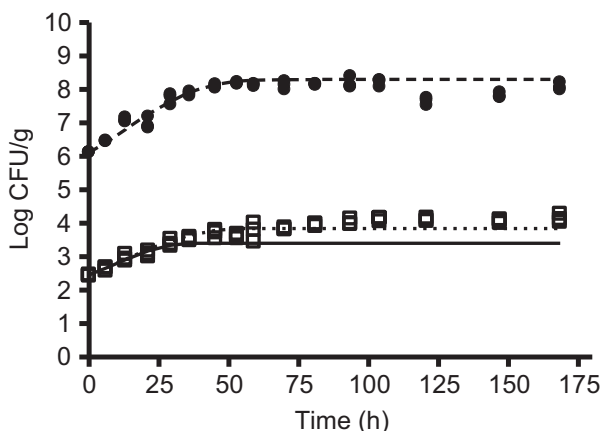

Fig. 3. Observed growth of lactic acid bacteria (●) and *L. monocytogenes* (□) in cottage cheese with cultured cream dressing (14.8 °C, pH 5.45, 1.1% NaCl in water phase and 800 ppm lactic acid in water phase). Growth of *L. monocytogenes* was predicted using Eq. (2) with the classical Jameson term including LAB_{max} (⋯) and by using the modified Jameson term with LAB_{CPD} (—).

3.4.2. Lag time model for LAB

11 of 17 generated LAB growth curves in cottage cheese at constant storage temperatures displayed a significant lag phase with P < 0.05 (Figs. 1 and 5). Therefore, the secondary LAB growth models were extended by using the *RLT* concept to include a lag phase. The *RLT* value of 4.04 ± 2.09 (AVG ± SD) for the LAB starter culture was longer than the corresponding value of 2.34 ± 1.85 for the LAB aroma culture (Table 2). *RLT* values showed considerable variability but no systematic effect of the environmental parameters on these values was observed (Results not shown).

3.4.3. L. monocytogenes growth model

Evaluation of the ability of existing models, to predict growth of *L. monocytogenes* in cottage cheese with fresh- or cultured cream dressing, resulted in B_f values between 0.05 and 1.31. The model of Augustin et al. (2005) for liquid dairy products on average resulted in good predictions for cottage cheese with cultured cream dressing (B_f 1.06) but the accuracy factor was above 1.5 (Table 3). Generally, the evaluated models predicted growth to be too slow ($B_f < 1.0$) and the accuracy factors were unacceptable. Based on these model evaluations, it was decided to develop cottage cheese specific *L. monocytogenes* growth models.

Fitted cardinal parameter values for μ_{ref}, T_{min} and pH_{min}, obtained in model system, were combined with terms from existing models that included $a_{w,min}$ and MIC values for undissociated lactic- and sorbic acid (Table 2). μ_{ref} estimates were additionally calibrated to data for the two specific types of cottage cheese in order to improve model performance. For cottage cheese with fresh cream dressing, μ_{ref} was adjusted from 0.67 h^{-1} to 0.72 h^{-1}. For cottage cheese with cultured cream dressing, predicted growth was too fast and μ_{ref} was adjusted from 0.67 h^{-1} to 0.34 h^{-1}. This improved the model performance, resulting in an A_f value of 1.5 compared to 2.2 before the calibration to product data.

3.5. Evaluation of the new growth models

The new and calibrated μ_{max}-models predicted growth rates of both LAB and *L. monocytogenes* with good or acceptable performance as determined from a total of 42 independent growth curves obtained with the two studied types of cottage cheese. Calculated B_f values were between 0.91 and 1.16 and A_f values between 1.11 and 1.32 (Table 4).

The simultaneous growth of LAB and *L. monocytogenes* in cottage cheese was well predicted by the new models with good agreement between simulated and observed growth (Table 5, Fig. 5). Of 13 *L. monocytogenes* growth curves at constant and dynamic storage temperatures 11 had more than 70% of the observations within the ASZ and the remaining two had 50% and 67% within the ASZ (Table 5). The overall ASZ score was 83% indicating acceptable model performance. Application of either the minimum- or the maximum observed LAB *RLT* value improved the ASZ score for *L. monocytogenes* predictions in six of twelve cases (Table 5, growth curve 3, 4, 7, 9, 10, 11). For LAB, nine of the 13 growth curves had more than 70% of the observations within the ASZ (Table 5) and the overall ASZ score was 75%. In two cases (Table 5, growth curve no. 8 and 12) the ASZ score was improved by applying the maximum *RLT* value and thereby prolonging the lag time. This change did not have any negative impact on the performance of the *L. monocytogenes* growth model (Table 5).

Simulation of scenarios at 7 °C showed pH to have a pronounced effect with no growth predicted at pH 5.0 and growth of 100-fold in 20 days at pH 5.4 (Fig. 6a). At pH 5.4, the highest pH observed in cottage cheese, growth of *L. monocytogenes* was predicted to be prevented by 700 ppm of sorbic acid, which is below the legal limit of 1000 ppm (Fig. 6b, EC, 2013b).

Table 2
Model parameter estimates for *L. monocytogenes* and the mesophilic starter- and aroma culture used in cottage cheese.

	Parameter values				
	Starter culture	Aroma culture	Reference	*L. monocytogenes*	Reference
μ_{ref} (h^{-1})	0.73[a] ± 0.02[b] (1.20[c])	0.78[a] ± 0.03[b] (0.57[c])	This study	0.67[h] ± 0.02[b] (0.72[i] and 0.34[j])	This study
T_{min} (°C)	2.31[a] ± 0.42[b]	3.69[a] ± 0.38[b]	This study	−2.01[h] ± 0.40[b]	This study
pH_{min}	4.15[a] ± 0.02[b]	3.87[a] ± 0.05[b]	This study	4.87[h] ± 0.01[b]	This study
pH_{max}	–	7.23[a] ± 0.17[b]	This study	–	–
$a_{w, min}$	0.928 ± 0.003[d]	0.928 ± 0.003[d]	Wijtzes et al. (2001)	0.923	UTAS model, see Giménez and Dalgaard (2004)
$MIC_{U\ Lactic\ acid}$ (mM)	4.09[e] ± 0.26[b]	9.72[e] ± 0.47[b]	This study	3.79	UTAS model, see
n1	1	1		1	Giménez and Dalgaard (2004)
n2	1	1		2	
$MIC_{U\ Sorbic\ acid}$ (mM)	4.62[e] ± 0.35[b]	5.50[e] ± 0.36[b]	This study	1.90	Mejlholm and
n1	1	0.5		1	Dalgaard (2009)
n2	2	2		1	
Relative lag time (RLT)					
Average value	4.04[f] ± 2.09	2.34[g] ± 1.85	This study	–	–
Min/Max value observed	0.06/7.28	0.05/5.20		–	–

[a] Estimated from model system data. Experiments performed in duplicate at four different temperatures (7, 11, 16, 21 °C) and five pH values (4.3–6.3).
[b] ± Standard error on fitted model parameter value.
[c] μ_{ref} Calibrated to cottage cheese.
[d] 95% Confidence interval on parameter value.
[e] Estimated from model system data. Experiments performed in duplicate at 12 °C with lactic acid (0–35,000 ppm) and sorbic acid (0–2000 ppm).
[f] RLT ± SD calculated from 10 growth curves.
[g] RLT ± SD calculated from 15 growth curves.
[h] Estimated from model system data. Experiments performed in duplicate at three different temperatures (5, 10, 15 °C) and eight pH values (4.4–6.9).
[i] μ_{ref} Calibrated to growth data obtained in cottage cheese with fresh cream dressing.
[j] μ_{ref} Calibrated to growth data obtained in cottage cheese with cultured cream dressing.

4. Discussion

Various mathematical models are available to predict growth and activity of mesophilic LAB during conditions of milk fermentation at temperatures of 25–55 °C (García-Parra et al., 2011; Kristo et al., 2003; Latrille et al., 1994; Oner et al., 1986; Poirazi et al., 2007). Other available models focus on industrial fermentation processes that include growth of LAB at temperatures between 26 °C and 45 °C (Boonmee et al., 2003; Cachon and Diviès, 1993; Guerra et al., 2007; Lejeune et al., 1998). These models concern kinetics of growth, consumption of substrates like lactose and glucose, product formation including lactic acid and synthesis of various bacteriocins. In some cases the inhibiting effect of LAB on other microorganisms has been quantified or modelled (Le Marc et al., 2009; Liptáková et al., 2006; Malakar et al., 1999; Martens et al., 1999;
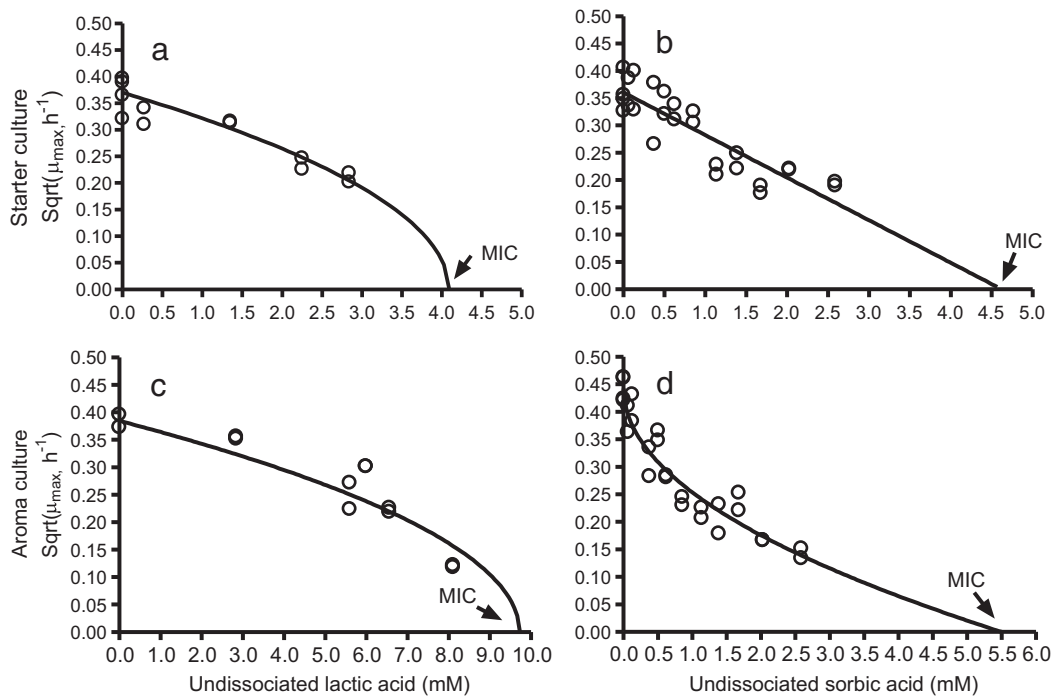
Fig. 4. Maximum specific growth rates (h^{-1}, ○) of mesophilic starter- (a, b) and aroma culture (c, d) related to the concentrations of undissociated sorbic- (b, d) and lactic acid (a, c). Experiments were performed at 12 °C in APT broth (pH 5.5, 1.0% NaCl). Minimum inhibitory concentrations (MIC) were estimated by fitting model terms from Eq. (4) to the experimental data. Solid lines represent the regression lines.
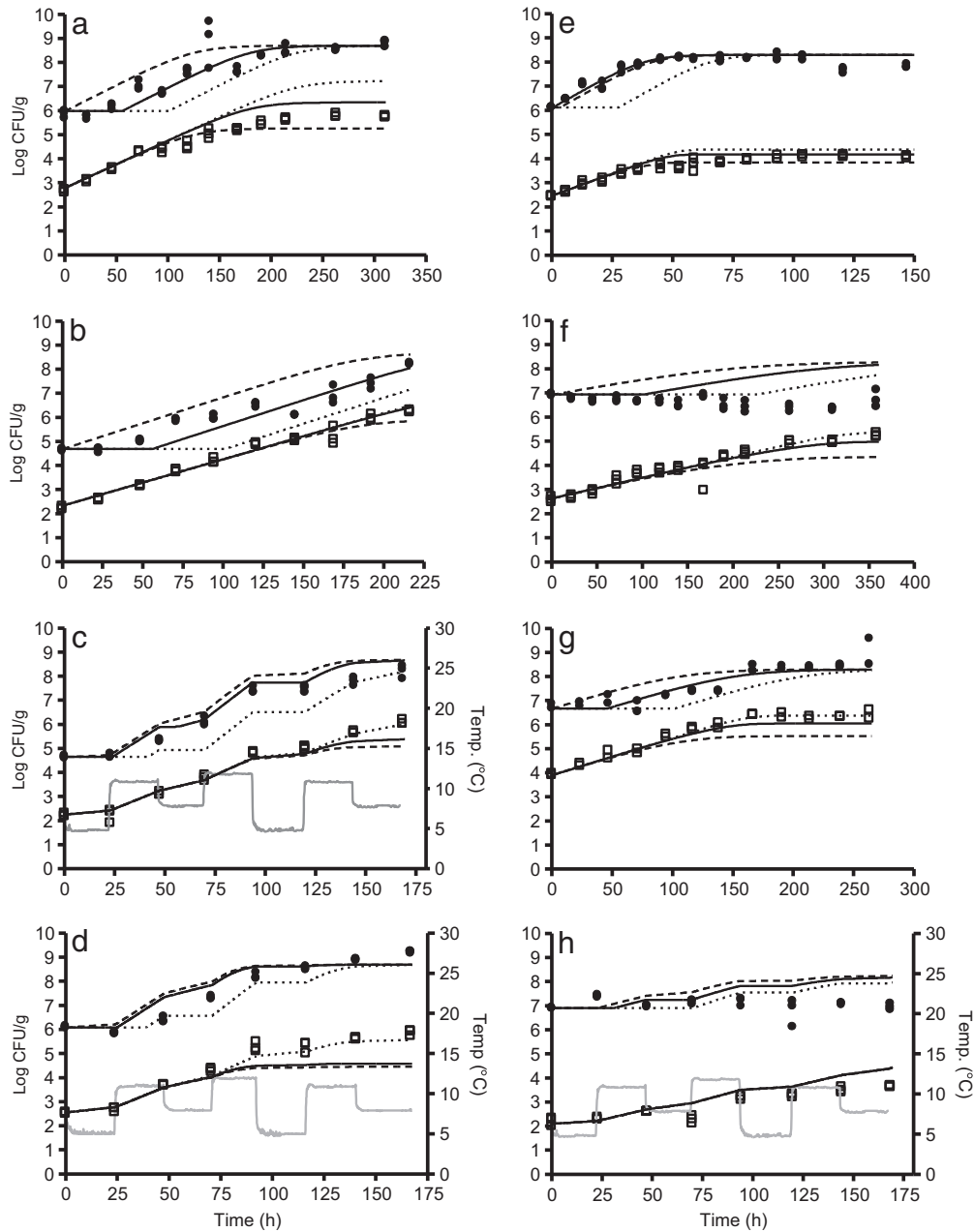
Fig. 5. Comparison of observed and predicted growth for *L. monocytogenes* (observed: □, predicted: —) and lactic acid bacteria (observed: ●, predicted:—) in cottage cheese. Predictions were also made with the minimum (—) and maximum (·····) *RLT* value observed during model development. Data was obtained in two different types of cottage cheese and at different constant and dynamic temperature storage conditions as shown in Table 5. Temperature profiles are illustrated with grey lines in (c), (d) and (h).

Table 3
Evaluation of the performance of existing *L. monocytogenes* growth models using data obtained in cottage cheese.

Model	Cottage cheese with fresh cream dressing			Cottage cheese with cultured cream dressing		
	n[a]	B_f[b]	A_f[b]	n[a]	B_f[b]	A_f[b]
Augustin et al. (2005), liquid dairy	6	0.26	3.90	19	1.06	1.64
Augustin et al. (2005), cheese	6	0.07	13.69	19	0.30	3.31
Mejlholm and Dalgaard (2009)	6	0.56	1.78	19	1.31	1.33
Schvartzman et al. (2011)	6	0.05	19.13	19	0.21	4.80

[a] Number of growth curves evaluated.
[b] Bias (B_f)- and accuracy (A_f) factor values were calculated from predicted and observed μ_{max} values (h^{-1}).

Medved'ová et al., 2008). However, during chilled storage and distribution of cottage cheese or other fermented dairy products, successfully validated mathematical models to predict the simultaneous growth of mesophilic LAB from starter cultures and human pathogens are not available although there is a need for such models for the assessment and management of microbial risks.

It is well known that growth and survival of *Listeria* in fermented dairy products is affected by various factors including the types and concentrations of starter cultures, product characteristics and processing (Ryser, 2007). The inhibiting effect of LAB can be due to substrate competition or product inhibition including bacteriocins, peptides, organic acids, fatty acids, volatile compounds, H_2O_2 and interaction between these factors. Consequently, it has been considered difficult to predict growth of *L. monocytogenes* in fermented dairy products (Irlinger and

Table 4
Evaluation of the performance of new models from the present study.

	Cottage cheese with fresh cream dressing			Cottage cheese with cultured cream dressing		
	n[a]	B_f[b]	A_f[b]	n[a]	B_f	A_f
L. monocytogenes	6	1.10	1.11	19	1.07	1.22
Lactic acid bacteria	6	1.16	1.32	11	0.91	1.17

[a] Number of growth curves evaluated.
[b] Bias (B_f)- and accuracy (A_f) factor values were calculated from predicted and observed μ_{max} values (h^{-1}).

Mounier, 2009). In fact, this has been observed for growth models calibrated to specific types of dairy products as they performed well for non-fermented dairy products but poorly for cheeses resulting in high A_f values above 3.5 (Augustin et al., 2005).

In the present study, models to predict the simultaneous growth of *L. monocytogenes* and mesophilic LAB in two different types of cottage cheese were developed and successfully validated. The mesophilic LAB cultures had an important inhibiting effect on the maximum population density (N_{max}) of *L. monocytogenes* and the new models were able to predict this microbial interaction for storage conditions and product characteristic of relevance for cottage cheese (Table 5, Fig. 5). The models' range of applicability, as determined from product validation studies, included cottage cheese with both fresh- and cultured cream dressing, pH from 5.0 to 5.5, initial concentrations of lactic acid below 2500 ppm and of sorbic acid below 1000 ppm, water phase salt of 1.0–1.25% and storage temperatures from 5 °C to 15 °C. The new models can be used to conveniently evaluate the growth potential of *L. monocytogenes* in cottage cheese depending on product characteristics and storage conditions.

The approach used in the present study to predict the potential growth of *L. monocytogenes* in cottage cheese relied on accurate models for the kinetics of added LAB cultures, *L. monocytogenes* and their interaction. Simplified cardinal parameter models including a reference growth rate (μ_{ref}) allowed secondary μ_{max}-models for both LAB and *L. monocytogenes* to be calibrated to product data to take into account the effect of the complex factors of the fermented cottage cheese as previously suggested for other foods (Mejlholm and Dalgaard, 2007a; Rosso, 1999). Interestingly, the calibrated μ_{ref} values seemed to depend more on the LAB cultures used for milk/cream fermentation than on product characteristics of the cottage cheese (Table 2).

As expected, available models for growth of psychrotolerant LAB were unable to accurately predict kinetics of the mesophilic starter- and aroma cultures in cottage cheese. A_f values above 1.70 were determined at 5–15 °C with four different models (Devlieghere et al., 2000; Leroi et al., 2012; Mejlholm and Dalgaard, 2007b; Wijtzes et al., 2001). In the same way, the model of Manios et al. (2014) for mesophilic spoilage LAB in low pH vegetable spreads resulted in B_f of 0.19 and A_f of 5.25 (n = 6) when evaluated for growth of LAB in cottage cheese. The studied dairy isolates of *L. monocytogenes* had T_{min} (−2.01 °C) and pH_{min} (4.87) values similar to isolates from other foods but the calibrated μ_{ref} values differed markedly (Augustin et al., 2005; Mejlholm et al., 2010). This supports that terms from different cardinal parameter models can be combined to facilitate development of complex models with a wider range of applicability. However, parameter estimates must be used in combination with the model terms they are consistent with (Augustin et al., 2005; Ross and Dalgaard, 2004) and the resulting models may need to be calibrated to specific types of food.

The measured variability in *RLT* values for LAB (Table 2) corresponds to previous observations for lag time of *Lactobacillus curvatus* (Wijtzes et al., 1995) and various other microorganisms (Ross, 1999). Mejlholm and Dalgaard (2013) mainly observed nonsignificant lag phases for psychrotolerant *Lactobacillus* spp. in seafood and meat products whereas observed lag phases of mesophilic LAB in cottage cheese were important. Predictions using average *RLT* values for mesophilic LAB in cottage

cheese were generally acceptable (Table 5, Fig. 5) but in some cases higher RLT values for LAB provided more appropriate simulations of *L. monocytogenes* growth (Fig. 5d and g). It therefore seems interesting, in future studies, to evaluate lag time distributions for both LAB and *L. monocytogenes* in combination with stochastic modelling. Stochastic lag time models have been studied for single species including *L. monocytogenes* (Couvert et al., 2010; Tenenhaus-Aziza et al., 2014) but remain little studied for more complex predictive models including microbial interaction (Delignette-Muller et al., 2006). No lag phase was included in the developed *L. monocytogenes* model (see Section 2.4) and the present study takes a worst case approach to growth prediction of the pathogen.

The empirical Jameson effect model (Eq. (2)) seemed appropriate to predict the inhibiting effect of the studied LAB cultures on growth of *L. monocytogenes*. The studied LAB cultures included different sub-species and various strains of *L. lactis* (see Section 2.2.1) but this had no clear effect on their ability to inhibit growth of *L. monocytogenes* (Figs. 3, 5). This simple inter-bacterial interaction model (Giménez and Dalgaard, 2004; Mejlholm and Dalgaard, 2007b) or its modifications (Le Marc et al., 2009; Møller et al., 2013) has previously been successful for various sets of microorganisms and foods and thereby represent an interesting alternative to more complex modelling approaches for the inhibitory effect of LAB on other microorganisms (Breidt and Fleming, 1998; Schvartzman et al., 2011). However, to explain this inter-bacterial interaction in cottage cheese it seems interesting in future studies to evaluate if changes in pH, lactic acid or concentrations of other compounds produced by the LAB cultures quantitatively can explain the growth inhibition of *L. monocytogenes*. Subsequently, this type of information may lead to more mechanistic inter-bacterial interaction models although formulation of such model for different sub-species and various strains of *L. lactis* seems challenging.

The developed secondary growth rate models for LAB and *L. monocytogenes* were successfully validated for cottage cheese at constant storage temperatures by using the classical B_f and A_f values as indices of model performance (Table 4; Ross, 1996; Mejlholm and Dalgaard, 2013; Mejlholm et al., 2010). In addition, the ASZ approach and simple graphs allowed the evaluation of the models including lag times, growth at dynamic storage temperatures and of the effect of microbial interaction as previously suggested (Møller et al., 2013; Oscar, 2005; Velugoti et al., 2011). At dynamic storage temperatures and for cottage cheese with fresh cream dressing (Table 5, growth curves 3 and 4), good model performance was observed with average and maximum observed RLT whereas less than 70% of observations in cottage

Table 5
Comparison of simulated and observed individual growth curves by using the acceptable simulation zone (ASZ) approach.

Growth curve no.	Storage temperature and growth data	% observations within ASZ[c]	
		L. monocytogenes	Lactic acid bacteria
1[a]	8 °C, Fig. 5a	86 (78/50)	81 (44/67)
2[a]	8 °C, Fig. 5b	100 (87/100)	70 (37/43)
3[a]	Dynamic (5–12 °C), Fig. 5c	71 (67/96)	71 (46/63)
4[a]	Dynamic (5–12 °C), Fig. 5d	50 (50/86)	67 (59/67)
5[b]	5 °C	82 (82/82)	100 (95/100)
6[b]	10 °C	91 (87/87)	51 (56/51)
7[b]	15 °C, Fig. 5e	94 (100/81)	73 (90/52)
8[b]	8 °C, Fig. 5f	97 (72/97)	49 (18/64)
9[b]	5 °C	71 (75/71)	96 (92/96)
10[b]	10 °C, Fig. 5g	96 (50/100)	83 (63/67)
11[b]	10 °C	71 (38/100)	92 (75/75)
12[b]	Dynamic (5–12 °C), Fig. 5h	67 (67/67)	46 (42/58)
13[b]	10 °C with 1000 ppm of sorbic acid	96 (96/96)	96 (96/96)
All data	Average ASZ score	83	75

[a] Data obtained in cottage cheese with fresh cream dressing.
[b] Data obtained in cottage cheese with cultured cream dressing.
[c] Calculation of ASZ score with average *RLT*-value and (minimum/maximum *RLT*-value).
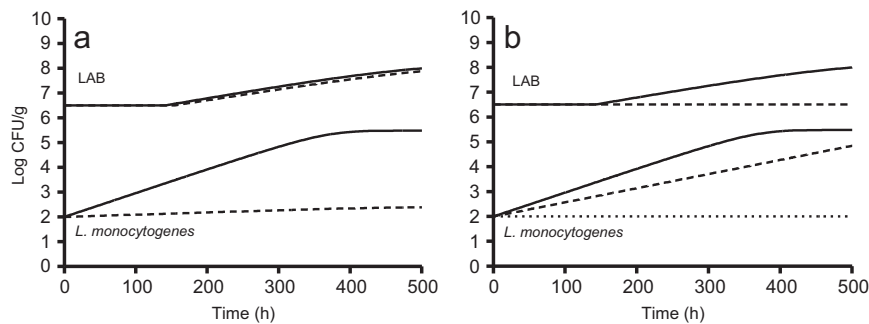
Fig. 6. Simulation of potential scenarios. a) Simulation of growth at 7 °C, 1.1% NaCl in the water phase and 1000 ppm lactic acid in the water phase. Dashed lines (−) indicate growth at pH 5.0 of *L. monocytogenes* and lactic acid bacteria in cottage cheese and solid lines (−) represent growth at pH 5.4. b) Simulated growth of lactic acid bacteria and *L. monocytogenes* with 0 ppm sorbate (▬), 350 ppm sorbate (−) and 700 ppm sorbate (···) in the water phase (7 °C, pH 5.4, 1.1% NaCl in the water phase and 1000 ppm lactic acid in the water phase).

cheese with cultured cream dressing (Table 5, growth curve 12) were within the ASZ. Application of maximum observed RLT-values did however improve model performance for LAB. Based on reported graphs, similar model performance at fluctuating temperatures has been obtained for *L. monocytogenes* in dairy and meat products (Gougouli et al., 2008; Lee et al., 2013; Panagou and Nychas, 2008) and for spoilage microbiota in meat (Koutsoumanis et al., 2006; Mataragas et al., 2006). We found the combined use of B_f, A_f and ASZ values useful for model evaluation although no previous studies used these indices of model performance in combination. Further studies are needed to compare limits of acceptable model validation based on these methods.

In summary, the present study developed mathematical models to predict growth of LAB and *L. monocytogenes* in cottage cheese. These models can be used for product re-formulation and to evaluate storage, distribution and handling by consumers as demonstrated by evaluation of potential scenarios (see Sections 2.5 and 3.5). Different LAB cultures had a pronounced effect on growth of *L. monocytogenes* and their individual kinetic characteristics were required in order to develop appropriate models. For cottage cheese, the effect of the added LAB culture must be regarded as an input parameter equal to e.g. pH and temperature when modelling growth response of *L. monocytogenes* in fermented dairy products. The application of reference growth rates (μ_{ref}) refitted to product data, and the empirical Jameson term to describe the inter-bacterial interaction, resulted in realistic predictions of *L. monocytogenes* growth and maximum population densities. The used methodology can, most likely, be successfully applied to other fermented dairy products in order to predict the simultaneous growth of LAB from added cultures and *L. monocytogenes* or other human pathogens. Since a range of different LAB cultures are applied in the dairy industry, it is essential to define a manageable methodology for the modelling of the important inter-bacterial interactions.

Acknowledgements

References

Antwi, M., Bernaerts, K., Van Impe, J.F., Geeraerd, A.H., 2007. Modelling the combined effects of structured food model system and lactic acid on *Listeria innocua* and *Lactococcus lactis* growth in mono-and coculture. Int. J. Food Microbiol. 120 (1), 71–84.

Augustin, J., Zuliani, V., Cornu, M., Guillier, L., 2005. Growth rate and growth probability of *Listeria monocytogenes* in dairy, meat and seafood products in suboptimal conditions. J. Appl. Microbiol. 99 (5), 1019–1042.

Boonmee, M., Leksawasdi, N., Bridge, W., Rogers, P.L., 2003. Batch and continuous culture of *Lactococcus lactis* NZ133: experimental data and model development. Biochem. Eng. J. 14 (2), 127–135.

Breidt, F., Fleming, H.P., 1998. Modeling of the competitive growth of *Listeria monocytogenes* and *Lactococcus lactis* in vegetable broth. Appl. Environ. Microbiol. 64 (9), 3159–3165.

Cachon, R., Diviès, C., 1993. Modeling of growth and lactate fermentation by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* in batch culture. Appl. Microbiol. Biotechnol. 40 (1), 28–33.

Cartwright, E.J., Jackson, K.A., Johnson, S.D., Graves, L.M., Silk, B.J., Mahon, B.E., 2013. Listeriosis outbreaks and associated food vehicles, United States, 1998–2008. Emerg. Infect. Dis. 19, 1–9.

Chen, J.H., Hotchkiss, J.H., 1993. Growth of *Listeria monocytogenes* and *Clostridium sporogenes* in cottage cheese in modified atmosphere packaging. J. Dairy Sci. 76 (4), 972–977.

Cornu, M., Billoir, E., Bergis, H., Beaufort, A., Zuliani, V., 2011. Modeling microbial competition in food: Application to the behavior of *Listeria monocytogenes* and lactic acid flora in pork meat products. Food Microbiol. 28 (4), 639–647.

Coroller, L., Guerrot, V., Huchet, V., Le Marc, Y., Mafart, P., Sohier, D., Thuault, D., 2005. Modelling the influence of single acid and mixture on bacterial growth. Int. J. Food Microbiol. 100 (1), 167–178.

Couvert, O., Pinon, A., Bergis, H., Bourdichon, F., Carlin, F., Cornu, M., Denis, C., Gnanou Besse, N., Guillier, L., Jamet, E., Mettler, E., Stahl, V., Thuault, D., Zuliani, V., Augustin, J.-C., 2010. Validation of a stochastic modelling approach for *Listeria monocytogenes* growth in refrigerated foods. Int. J. Food Microbiol. 144 (2), 236–242.

Dalgaard, P., 2009. Modelling of Microbial Growth. Chapter 10 Bulletin of the International Dairy Federation. A Revolution in Food Safety Management, vol. 433, pp. 45–57.

Dalgaard, P., Jørgensen, L.V., 2000. Cooked and brined shrimps packed in a modified atmosphere have a shelf-life of >7 months at 0 °C, but spoil in 4–6 days at 25 °C. Int. J. Food Sci. Technol. 35 (4), 431–442.

Dalgaard, P., Koutsoumanis, K., 2001. Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. J. Microbiol. Methods 43 (3), 183–196.

Davidson, C.M., Cronin, F., 1973. Medium for the selective enumeration of lactic acid bacteria from foods. Appl. Microbiol. 26 (3), 439.

de Castro, V., Escudero, J., Rodriguez, J., Muniozguren, N., Uribarri, J., Saez, D., Vazquez, J., 2012. Listeriosis outbreak caused by latin-style fresh cheese, Bizkaia, Spain, August 2012. Eur. Surveill. 17 (42) (pii=20298. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20298).

Delignette-Muller, M.L., Cornu, M., Pouillot, R., Denis, J.-B., 2006. Use of Bayesian modelling in risk assessment: application to growth of *Listeria monocytogenes* and food flora in cold-smoked salmon. Int. J. Food Microbiol. 106, 195–208.

Devlieghere, F., Geeraerd, A.H., Versyck, K.J., Bernaert, H., Van Impe, J.F., Debevere, J., 2000. Shelf life of modified atmosphere packed cooked meat products: addition of Na-lactate as a fourth shelf life determinative factor in a model and product validation. Int. J. Food Microbiol. 58 (1), 93–106.

EC, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union 338 (1), 1–26.

EC, 2013a. RASFF Portal, Milk and Milk Products, Pathogenic Micro-organismsRetrieved October 25, 2013, from http://ec.europa.eu/food/food/rapidalert/index_en.htm.

EC, 2013b. Food Additives DatabaseRetrieved December 18, 2013, from https://webgate.ec.europa.eu/sanco_foods/main/?sector=FAD&auth=SANCAS.

Fernandes, R., 2009. Cheese. In Microbiology Handbook Dairy Products. Leatherhead Publishing and Royal Society of Chemistry, pp. 61–76.

Ferreira, M.A.S.S., Lund, B.M., 1996. The effect of nisin on *Listeria monocytogenes* in culture medium and long-life cottage cheese. Lett. Appl. Microbiol. 22 (6), 433–438.

Gahan, C.G., O'Driscoll, B., Hill, C., 1996. Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. Appl. Environ. Microbiol. 62 (9), 3128.

García-Parra, M.D., García-Almendárez, B.E., Guevara-Olvera, L., Guevara-González, R.G., Rodríguez, A., Martínez, B., Domínguez-Domínguez, J., Regalado, C., 2011. Effect of sub-inhibitory amounts of nisin and mineral salts on nisin production by *Lactococcus lactis* UQ2 in skim milk. Food Bioprocess Technol. 4 (4), 646–654.

Gimenez, B., Dalgaard, P., 2004. Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and spoilage microorganisms in cold smoked salmon. J. Appl. Microbiol. 96 (1), 96–109.

Gougouli, M., Angelidis, A.S., Koutsoumanis, K., 2008. A study on the kinetic behavior of *Listeria monocytogenes* in ice cream stored under static and dynamic chilling and freezing conditions. J. Dairy Sci. 91 (2), 523–530.

Guerra, N.P., Agrasar, A.T., Macías, C.L., Bernárdez, P.F., Castro, L.P., 2007. Dynamic mathe-matical models to describe the growth and nisin production by *Lactococcus lactis* subsp. *lactis* CECT 539 in both batch and re-alkalized fed-batch cultures. J. Food Eng. 82 (2), 103–113.

Guillier, L., Stahl, V., Hezard, B., Notz, E., Briandet, R., 2008. Modelling the competitive growth between *Listeria monocytogenes* and biofilm microflora of smear cheese wooden shelves. Int. J. Food Microbiol. 128 (1), 51–57.

Irlinger, F., Mounier, J., 2009. Microbial interactions in cheese: implications for cheese quality and safety. Curr. Opin. Biotechnol. 20 (2), 142–148.

Jameson, J.E., 1962. A discussion of the dynamics of *Salmonella* enrichment. J. Hyg. 60 (2), 193–207.

Kempler, G.M., McKay, L.L., 1980. Improved medium for detection of citrate-fermenting *Streptoccus lactis* subsp. *diacetylactis*. Appl. Environ. Microbiol. 39 (4), 926–927.

Koutsoumanis, K., Stamatiou, A., Skandamis, P., Nychas, G.-J., 2006. Development of a mi-crobial model for the combined effect of temperature and pH on spoilage of ground meat, and validation of the model under dynamic temperature conditions. Appl. En-viron. Microbiol. 72 (1), 124–134.

Kristo, E., Biliaderis, C.G., Tzanetakis, N., 2003. Modelling of rheological, microbiological and acidification properties of a fermented milk product containing a probiotic strain of *Lactobacillus paracasei*. Int. Dairy J. 13 (7), 517–528.

Larson, A.E., Yu, R.R.Y., Lee, O.A., Price, S., Haas, G.J., Johnson, E.A., 1996. Antimicrobial activ-ity of hop extracts against *Listeria monocytogenes* in media and in food. Int. J. Food Microbiol. 33 (2), 195–207.

Latrille, E., Corrieu, G., Thibault, J., 1994. Neural network models for final process time de-termination in fermented milk production. Comput. Chem. Eng. 18 (11), 1171–1181.

Lejeune, R., Callewert, R., Crabbé, K., De Vuyst, L., 1998. Modelling the growth and bacterocin production by *Lactobacillus amylovorus* DCE 471 in batch cultivation. J. Appl. Microbiol. 84 (2), 159–168.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P., Thuault, D., 2002. Model-ling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. Int. J. Food Microbiol. 73 (2), 219–237.

Le Marc, Y., Valík, L., Medved'ová, A., 2009. Modelling the effect of the starter culture on the growth of *Staphylococcus aureus* in milk. Int. J. Food Microbiol. 129 (3), 306–311.

Lee, S., Lee, H., Lee, J.-Y., Skandamis, P., Park, B.-Y., Oh, M.-H., Yoon, Y., 2013. Mathematical models to predict kinetic behavior and growth probabilities of *Listeria monocytogenes* on pork skin at constant and dynamic temperatures. J. Food Prot. 76 (11), 1868–1872.

Leroi, F., Fall, P.A., Pilet, M.F., Chevalier, F., Baron, R., 2012. Influence of temperature, pH and NaCl concentration on the maximal growth rate of *Brochothrix thermosphacta* and a bioprotective bacteria *Lactococcus piscium* CNCM I-4031. Food Microbiol. 31 (2), 222–228.

Liptáková, D., Lubomír, V., Bajúsová, B., 2006. Effect of protective culture on the growth of *Candida maltosa* YP1 in yoghurt. J. Food Nutr. Res. 45 (4), 147–151.

Liu, L., O'Conner, P., Cotter, P.D., Hill, C., Ross, R.P., 2008. Controlling *Listeria monocytogenes* in cottage cheese through heterologous production of enterocin A by *Lactococcus lactis*. J. Appl. Microbiol. 104 (4), 1059–1066.

Malakar, P.K., Martens, D.E., Zwietering, M.H., Beal, C., Van't Riet, K., 1999. Modelling the in-teractions between *Lactobacillus curvatus* and *Enterobacter cloacae*: II. Mixed cultures and shelf life predictions. Int. J. Food Microbiol. 51 (1), 67–79.

Manios, S.G., Lambert, R.J., Skandamis, P.N., 2014. A generic model for spoilage of acidic emulsified foods: combining physico-chemical data, diversity and levels of specific spoilage organisms. Int. J. Food Microbiol. 170, 1–11.

Marsili, R.T., Ostapenko, H., Simmons, R.E., Green, D.E., 1981. High performance liquid chro-matographic determination of organic acids in dairy products. J. Food Sci. 46 (1), 52–57.

Martens, D.E., Béal, C., Malakar, P., Zwietering, M.H., Van't Riet, K., 1999. Modelling the in-teractions between *Lactobacillus curvatus* and *Enterobacter cloacae*: I. Individual growth kinetics. Int. J. Food Microbiol. 51 (1), 53–65.

Mataragas, M., Drosinos, E.H., Vaidanis, A., Metaxopoulos, I., 2006. Development of a pre-dictive model for spoilage of cooked cured meat products and its validation under constant and dynamic temperature storage conditions. J. Food Sci. 71 (6), 157–167.

McAuliffe, O., Hill, C., Ross, R.P., 1999. Inhibition of *Listeria monocytogenes* in cottage cheese manufactured with a lacticin 3147-producing starter culture. J. Appl. Microbiol. 86 (2), 251–256.

Medved'ová, A., Liptáková, D., Hudecová, A., Valík, Ľ., 2008. Quantification of the growth competition of lactic acid bacteria: a case of co-culture with *Geotrichum candidum* and *Staphylococcus aureus*. Acta Chim. Slovaca 1 (1), 192–207.

Mejlholm, O., Dalgaard, P., 2007a. Modeling and predicting the growth boundary of *Listeria monocytogenes* in lightly preserved seafood. J. Food Prot. 70 (1), 70–84.

Mejlholm, O., Dalgaard, P., 2007b. Modeling and predicting the growth of lactic acid bac-teria in lightly preserved seafood and their inhibiting effect on *Listeria monocytogenes*. J. Food Prot. 70 (11), 2485–2497.

Mejlholm, O., Dalgaard, P., 2009. Development and validation of an extensive growth and growth boundary model for *Listeria monocytogenes* in lightly preserved and ready-to-eat shrimp. J. Food Prot. 72 (10), 2132–2143.

Mejlholm, O., Dalgaard, P., 2013. Development and validation of an extensive growth and growth boundary model for psychrotolerant *Lactobacillus* spp. in seafood and meat products. Int. J. Food Microbiol. 167, 244–260.

Mejlholm, O., Gunvig, A., Borggaard, C., Blom-Hanssen, J., Mellefont, L., Ross, T., Leroi, F., Else, T., Visser, D., Dalgaard, P., 2010. Predicting growth rates and growth boundary of *Listeria monocytogenes* — An international validation study with focus on processed and ready-to-eat meat and seafood. Int. J. Food Microbiol. 141 (3), 137–150.

Mellefont, L.A., Ross, T., 2003. The effect of abrupt shifts in temperature on the lag phase duration of *Escherichia coli* and *Klebsiella oxytoca*. Int. J. Food Microbiol. 83 (3), 295–305.

Mellefont, L.A., McMeekin, T.A., Ross, T., 2008. Effect of relative inoculum concentration on *Listeria monocytogenes* growth in co-culture. Int. J. Food Microbiol. 121 (2), 157–168.

Møller, C.O.A., Ilg, Y., Dalgaard, P., Christensen, B.B., Aabo, S., Hansen, T.B., 2013. Effect of natural microbiota on growth of *Salmonella* spp. in fresh pork — A predictive micro-biology approach. Food Microbiol. 34, 284–295.

Norton, D.M., Braden, C.R., 2007. Foodborne Listeriosis, In: Ryser, E.T., Marth, E.H. (Eds.), Listeria, Listeriosis, and Food Safety, 3rd ed.CRC Press, pp. 305–356.

Oner, M.D., Erickson, L.E., Yang, S.S., 1986. Analysis of exponential growth data for yoghurt cultures. Biotechnol. Bioeng. 28 (6), 895–901.

Oscar, T.E., 2005. Validation of lag time and growth rate models for *Salmonella* Typhimurium: acceptable prediction zone method. J. Food Sci. 70 (2), 129–137.

Panagou, E.Z., Nychas, G.-J.E., 2008. Dynamic modeling of *Listeria monocytogenes* growth in pasteurized vanilla cream after postprocessing contamination. J. Food Prot. 71 (9), 1828–1834.

Poirazi, P., Leroy, F., Georgalaki, M.D., Aktypis, A., De Vuyst, L., Tsakalidou, E., 2007. Use of artificial neural networks and a gamma-concept-based approach to model growth of and bacteriocin production by *Streptococcus macedonicus* ACA-DC 198 under sim-ulated conditions of Kasseri cheese production. Appl. Environ. Microbiol. 73 (3), 768–776.

Resnik, S.L., Chirife, J., 1988. Proposed theoretical water activity values at various temper-atures for selected solutions to be used as reference sources in the range of microbial growth. J. Food Prot. 51, 419–423.

Ross, T., 1996. Indices for performance evaluation of predictive models in food microbiol-ogy. J. Appl. Microbiol. 81 (5), 501–508.

Ross, T., 1999. Quantifying and utilising lag times in predictive microbiology. Predictive Microbiology for the Meat Industry. Meat and Livestock Australia, pp. 141–176.

Ross, T., Dalgaard, P., 2004. Secondary models. In: McKellar, R.C., Lu, X. (Eds.), Modeling Microbial Responses in Food. CRC Press, pp. 63–150.

Ross, T., Ratkowsky, D.A., Mellefont, L.A., McMeekin, T.A., 2003. Modelling the effects of temperature, water activity, pH and lactic acid concentration on the growth rate of *Escherichia coli*. Int. J. Food Microbiol. 82 (1), 33–43.

Rosso, L., 1999. Models using cardinal values. Predictive microbiology applied to chilled food preservation. Proceedings of Conference No 1997/2 of Commission C2, European Commission.

Ryser, E., 2007. Incidence and behavior of *Listeria monocytogenes* in cheese and other fermented dairy products, In: Ryser, E.T., Marth, E.H. (Eds.), Listeria, Listeriosis and Food Safety, 3rd ed.CRC Press, pp. 405–501.

Schvartzman, M.S., Maffre, A., Tenenhaus-Aziza, F., Sanaa, M., Butler, F., Jordan, K., 2011. Modelling the fate of *Listeria monocytogenes* during manufacture and ripening of smeared cheese made with pasteurised or raw milk. Int. J. Food Microbiol. 145, 31–38.

Tenenhaus-Aziza, F., Daudin, J.J., Maffre, A., Sanaa, M., 2014. Risk-based approach for mi-crobiological food safety management in the dairy industy: The case of Listeria monocytogenes in soft cheese made from pasteurized milk. Risk Anal. 34, 56–74.

Velugoti, P.R., Bohra, L.K., Juneja, V.K., Huang, L., Wesseling, A.L., Subbiah, J., Thippareddi, H., 2011. Dynamic model for predicting growth of *Salmonella* spp. in ground sterile pork. Food Microbiol. 28 (4), 796–803.

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2006. Cheese varieties, Dairy Science and Technology2nd ed. CRC Press, pp. 687–741.

Wiedmann, M., Sauders, B., 2007. Ecology of *Listeria* Species and *L. monocytogenes* in the natural environment, In: Ryser, E.T., Marth, E.H. (Eds.), Listeria, Listeriosis and Food Safety, 3rd ed.CRC Press, pp. 21–53.

Wijtzes, T., De Wit, J.C., Huis, I., Van't, R., Zwietering, M.H., 1995. Modelling bacterial growth of *Lactobacillus curvatus* as a function of acidity and temperature. Appl. Environ. Microbiol. 61 (7), 2533–2539.

Wijtzes, T., Rombouts, F.M., Kant-Muermans, M.L.T., Van't Riet, K., Zwietering, M.H., 2001. Development and validation of a combined temperature, water activity, pH model for bacterial growth rate of *Lactobacillus curvatus*. Int. J. Food Microbiol. 63 (1), 57–64.

7. PAPER II

Semi-mechanistic and empirical modelling of interaction between lactic acid bacteria and *Listeria monocytogenes* in cottage cheese

Submitted for publication in
International Journal of Food Microbiology

Semi-mechanistic and empirical modelling of interaction between lactic acid bacteria and *Listeria monocytogenes* in cottage cheese

Nina Bjerre Østergaard* and Paw Dalgaard
Division of Industrial Food Research, National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark

Running title: Modelling LAB-*L. monocytogenes* interaction in cottage cheese

Corresponding author: Division of Industrial Food Research, National Food Institute, Technical University of Denmark, Søltofts Plads, Building 221, DK-2800, Kgs. Lyngby, Denmark. Tel.: +45 45254918, Fax: +45 45884774. E-mail address: nbjos@food.dtu.dk (N.B. Østergaard)

ABSTRACT

The correlation between lactic acid bacteria growth, lactic acid formation and pH change in cottage cheese with either fresh- or cultured cream dressing was mathematically modelled. The aim was to predict the growth inhibition of *Listeria monocytogenes* in these products by using a semi-mechanistic model to simulate lactic acid formation and pH changes. A total of 14 kinetics for lactic acid bacteria growth, 460 analyses of lactic acid concentrations and 14 pH profiles were generated in cottage cheese. Primary modelling, relying on the yield factor concept, was used to describe lactic acid formation during storage. A two-parameter model was used to relate lactic acid concentrations and product pH. The developed primary models were used in combination with secondary cardinal parameter growth models for lactic acid bacteria and *Listeria monocytogenes* in cottage cheese [Østergaard, N.B., Eklöw, A. and Dalgaard, P. 2014. Modelling the effect of lactic acid bacteria from starter- and aroma culture on growth of *Listeria monocytogenes* in cottage cheese. International Journal of Food Microbiology, 188, 15-25]. Simulated growth of lactic acid bacteria, lactic acid formation and pH change were in agreement with observations in the product. In cottage cheese with fresh cream dressing, growth inhibition of *Listeria monocytogenes* was predicted accurately, indicating that lactic acid and pH were mainly responsible for growth inhibition. In contrast, lactic acid and pH could not fully explain the growth inhibition observed in cottage cheese with cultured cream dressing. For cottage cheese with fresh cream dressing the semi-mechanistic model performed as well as the empirical Jameson effect model whereas this empirical model more accurately predicted the observed inhibition of *Listeria monocytogenes* in cottage cheese with cultured cream dressing. To predict the inter-bacterial interaction in cottage cheese during chilled storage the empirical Jameson effect model was a performant alternative to a more complex semi-mechanistic model.

Keywords:
- Semi-mechanistic model
- Yield factor concept
- Growth inhibition
- Jameson effect
- Empirical model

Introduction

Predictive growth models for *Listeria monocytogenes* are beneficial tools to evaluate product safety and shelf life in relation to changes in product formulation, process deviations or risk assessments (EC, 2005; McMeekin et al., 2010; Papademas and Bintsis, 2010). For fermented dairy products, predictions should include the effect of interaction between starter lactic acid bacteria (LAB) and *L. monocytogenes*, since previous studies have demonstrated an inhibitory effect of LAB on growth of pathogenic bacteria in dairy products (Guillier et al., 2008; Le Marc et al., 2009; Østergaard et al., 2014). The inhibitory effect of LAB on *L. monocytogenes* in fermented dairy products and other foods has previously been modelled empirically by use of the Jameson term. With this approach it is assumed, and often demonstrated, that e.g. LAB inhibit co-culture microorganisms in the same way as they inhibit their own growth when approaching the maximum population density (Gimenez and Dalgaard, 2004; Le Marc et al., 2009; Mejlholm and Dalgaard, 2013, 2007; Østergaard et al., 2014). In fermented dairy products the empirical Jameson term represent the combined inhibitory effect of substrate competition or inhibitory products such as bacteriocins, peptides, organic acids, fatty acids, volatile compounds, H_2O_2 and the interaction between these factors (Irlinger and Mounier, 2009). Alternatively, the inhibitory effect of LAB on *L. monocytogenes* growth can be modelled more mechanistically by including individual factors, such as lactic acid formation and pH changes, in the model. It has been discussed, that the applicability and reliability of predictive models may be improved by unravelling the underlying mechanisms (Bernaerts et al., 2004; McMeekin et al., 2013; Van Impe et al., 2013). Such mechanistic models may be relevant for microbial interactions but substantial information and model development is lacking. Furthermore, it remains to be tested if mechanistic interaction models provide predictions that are superior to those of empirical models.

Previously, modelling of LAB product formation has been extensively studied within fermentation technology (Bouguettoucha et al., 2011). The Luedeking and Piret (1959) expression including yield and maintenance factors, or variations of this model, has often been used in that area whereas these approaches have not yet been widely used within predictive food microbiology. The yield factor concept (see Pirt, 1975) has been used to predict trimethylamine (TMA) and histamine formation in relation to shelf-life and safety of different seafoods (Dalgaard, 1995; Emborg and Dalgaard, 2008a, 2008b). The same approach was used to model lactic acid production by LAB in biological TTI indicator media (Ellouze et al., 2008; Vaikousi et al., 2008), to model growth of *Lb. curvatus* in a model system simulating sausage fermentation (Messens et al., 2003) and in stochastic modelling of *L. monocytogenes* behaviour during fermentation of Sicilian salami (Giuffrida et al., 2008). However, this type of semi-mechanistic models has not been evaluated for their ability to predict microbial interaction in fermented dairy products during chilled storage.

The objective of the present study was to mathematically describe the formation of lactic acid and the associated pH change in cottage cheese. These changes were described for cottage cheese

with fresh- or cultured cream dressing and related to LAB growth in these products. This allowed lactic acid formation, pH changes and their effect of on the growth response of *L. monocytogenes* to be predicted during chilled storage. Subsequently, predictions of these semi-mechanistic models were compared with predictions obtained using the empirical Jameson interaction model, to evaluate the performance of the two modelling approaches for simultaneous growth of LAB and *L. monocytogenes* in cottage cheese.

Materials and Methods

2.1 Storage trials, lactic acid analyses and pH change

For model development, LAB growth, lactic acid formation and pH change was determined in storage trials at 10-15°C using cottage cheese with either fresh- (two series of trials) or cultured (four series of trials) cream dressing. This resulted in a total of 14 growth curves for LAB with corresponding data for lactic acid formation and pH change. Cottage cheese (0.45 kg - 3.0 kg) was obtained from two different production sites, processing cottage cheese with either fresh- or cultured cream dressing. For cottage cheese with fresh cream dressing the dominating LAB population originated from the starter culture consisting of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (Mesophilic O-culture, R604, Chr. Hansen A/S, Hørsholm, Denmark). For cottage cheese with cultured cream dressing, the dominating LAB population originated from the aroma culture added to the cream dressing and included multiple strains of *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* (F-DVS SDMB-4, Chr. Hansen A/S, Hørsholm, Denmark). During transport to our laboratory, the samples were packed with ice and when received, the products were stored at 2°C and used within 72 h. Samples of 75-100 grams were prepared in containers used for commercial distribution of cottage cheese and they were stored at either 10°C or 15°C during the experiments. Storage temperature was recorded by data loggers (TinyTag Plus, Gemini Data Loggers Ltd., Chichester, UK). At regular intervals (every eight to 24 hour) microbiological analyses were performed in duplicate or triplicate. 10.0 g of cottage cheese was diluted 10-fold in chilled physiological saline solution (PS, 0.85% NaCl and 0.10% Bacto-peptone) and homogenised at normal speed for 30 s in a Stomacher 400 (Seward Medical, London, UK). Appropriate 10-fold dilutions were prepared in chilled PS and LAB were enumerated by pour plating with overlay in nitrite actidione polymyxin (NAP) agar (pH 6.2) incubated at 25°C for 72h (Davidson and Cronin, 1973). Additionally, LAB in cottage cheese with cultured cream dressing was enumerated by surface plating on KMK-agar (32°C for 48 h; Kempler and McKay, 1980) to confirm that the citrate fermenting *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* was the dominating LAB in these products. At each time of sampling pH was measured in 5.0 g of product, stirred with 25.0 ml distilled water, using a PHM 250 Ion Analyzer (MetroLab™, Radiometer, Copenhagen, Denmark). The remainder of the sample was stored at -20°C for subsequent High Performance Liquid Chromatography (HPLC) analyses to quantify the lactic acid concentrations. HPLC identification and quantification

of lactic acid was performed as previously described (Dalgaard and Jørgensen, 2000) and to improve the extraction, a centrifugation step (10 min at 5000 rpm, Sigma 4-16KS, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) was included prior to two consecutive filtration steps.

2.2 Modelling LAB growth, lactic acid formation and pH changes in cottage cheese

To estimate lag time, growth rate and maximum population density, the integrated and log transformed expanded logistic growth model with delay, Eq. (1), was fitted to individual growth curves of LAB (n=14, see 2.1). A fixed value of m = 1 was used for the fitting of growth curves.

$$\log(N_t) = \log(N_0) \qquad \text{if } t < t_{lag} \qquad [1]$$

$$\log(N_t) = \log\left(\frac{N_{max}}{\left(1+\left(\left(\frac{N_{max}}{N_0}\right)^m - 1\right)\cdot exp(-\mu_{max}\cdot m\cdot(t-t_{lag}))\right)^{1/m}}\right) \qquad \text{if } t \geq t_{lag}$$

where N_t, N_{max} and N_0 are the cell concentrations (CFU/g) at time t, zero and the maximum population density, respectively. t_{lag} is the lag time (h) and t is the time of storage. μ_{max} is the maximum specific growth rate (h^{-1}). The parameter m describes the dampening of growth when N_t approaches N_{max} (Dalgaard, 2002; Emborg and Dalgaard, 2008b; Turner et al., 1969).

A primary model for the formation of lactic acid was obtained by combining the expanded logistic growth model, Eq. (1), with the yield factor concept. In this way, lactic acid formation was related to LAB growth by a constant yield factor ($Y_{LAC/CFU}$, mg/CFU; Eq. (2)).

$$LAC_t = LAC_0 + Y_{LAC/CFU} \cdot (N_t - N_0) \cdot 1000 \qquad [2]$$

where LAC_t and LAC_0 is the concentration (mg/l) of lactic acid at time t and zero, respectively. $Y_{LAC/CFU}$ is the constant yield factor, N_t is the LAB concentration (CFU/g) at time t, described by Eq. (1) and N_0 is the initial LAB concentration (CFU/g) in the cottage cheese.

Eq. (2) was fitted to non-transformed data for lactic acid concentrations (mg/l in the water phase, 14 kinetics with a total of 460 data points) in cottage cheese during storage at 10°C or 15°C, to estimate the yield factor ($Y_{LAC/CFU}$) at specific environmental conditions. N_t in Eq. (2) was substituted with Eq. (1) using parameter values obtained from fitting of Eq. (1), with m = 1, to corresponding LAB growth curves. When fitting the kinetics for lactic acid formation with Eq. (2), different fixed values of m (0.10, 0.25, 0.35, 0.50 and 1.00) were used to determine the most appropriate value based on the residual sum of squares (RSS). Average $\log_{10}(Y_{LAC/CFU})$ values were calculated from $Y_{LAC/CFU}$-estimates obtained with the most appropriate value of m.

The relationship between lactic acid concentrations in the products and the associated pH values was determined by fitting Eq. (3) to observed data. This two-parameter model has previously been used to describe the relationship between lactic acid and pH in MRS broth during a fermentation process (Leroy and De Vuyst, 2003).

$$pH = \frac{pH_i + a_1[LAC_t]}{1 + a_2[LAC_t]}$$ [3]

where pH_i is the pH-value corresponding to no lactic acid in the product, a_1 and a_2 are parameters to be fitted and LAC_t is the measured concentration of lactic acid (mg/l in the water phase) in the product at time t. pH_i was fixed to 6.7 corresponding to the pH of milk before fermentation (Walstra et al., 2006). Average values of a_1 and a_2 in Eq. (3) resulted in a poor description of pH changes during storage at 10°C and 15°C of both types of cottage cheese. a_1 and a_2 were temperature dependent and this relationship was described by simple linear regression (Eq. (4)). The linear relationship for the effect of temperature on a_1 and a_2 improved the fit of the pH model with α and β being model constants and T the storage temperature (°C).

$$a_1 \, or \, a_2 = \alpha \cdot T + \beta$$ [4]

2.2.1 Secondary models to predict bacterial growth, lactic acid formation and pH change

The growth models developed by Østergaard et al. (2014) were used to predict the effect of temperature (°C), pH, NaCl (% water phase salt) and lactic acid (mg/l in the water phase) on the growth rates of *L. monocytogenes* and LAB in cottage cheese with added starter- or aroma cultures. Four individual cardinal parameter growth models were used. Growth of *L. monocytogenes* was modelled without a lag phase whereas a secondary lag-time model (Eq. (5)) was used for LAB growth (Østergaard et al., 2014).

$$\lambda = \frac{RLT \cdot \ln(2)}{\mu_{max}}$$ [5]

where λ is lag time (h), μ_{max} is the maximum specific growth rate and *RLT* is the relative lag time. Estimates of minimum-, average- and maximum *RLT*-values have previously been determined (Østergaard et al., 2014) and the *RLT*-value providing the best simulation of LAB growth was used.

Eqs. (1)-(5) were used in combination with the growth models from Østergaard et al. (2014) to predict growth of LAB and the associated lactic acid formation and pH change. Additionally, the effect of lactic acid and pH on growth of *L. monocytogenes* was simulated. The Euler method was used for numerical integration in combination with time steps of 0.083 h.

In addition, growth of *L. monocytogenes* was predicted by directly using the measured pH profiles in combination with the growth models from Østergaard et al. (2014).

2.3 Empirical modelling of microbial interaction

For comparative purposes, the models from Østergaard et al. (2014) were used to predict growth of LAB and *L. monocytogenes* in cottage cheese and their interaction as described by the Jameson term (Eq. (6); Gimenez and Dalgaard, 2004). LAB growth, including a lag phase, was predicted using the *RLT*-value (minimum-, average- or maximum) providing the best predictions of growth in cottage cheese (Østergaard et al., 2014).

$$\frac{dLm/dt}{Lm_t} = 0, \qquad\qquad\qquad\qquad t < t_{lag,Lm}$$

$$\frac{dLm/dt}{Lm_t} = \mu_{max}^{Lm} \cdot \left(1 - \frac{Lm_t}{Lm_{max}}\right) \cdot \left(1 - \frac{LAB_t}{LAB_{max}}\right), \qquad t \geq t_{lag,Lm} \qquad\qquad [6]$$

Lm_t and LAB_t are the concentrations (log CFU/g) of *L. monocytogenes* and lactic acid bacteria at time t. Lm_{max} and LAB_{max} are the maximum population densities of *L. monocytogenes* and LAB, respectively.

2.4 Evaluation of predicted microbial interaction

In order to evaluate the performance of the semi-mechanistic- and empirical modelling approaches for interaction, experimental data from a previous study was used (Østergaard et al., 2014). Growth data for the added LAB cultures and *L. monocytogenes* (a cocktail of four dairy related *L. monocytogenes* strains) was obtained in inoculated cottage cheese stored at temperatures between 5°C and 15°C. Microbiological analyses were performed at regular intervals following the same procedure as described above (see 2.1). LAB were enumerated on NAP-agar (pour plating, incubated for 72 h at 25°C) and *L. monocytogenes* was enumerated by surface plating on Palcam agar (CM0877, Oxoid, Basingstoke, UK) with Palcam selective supplement (SR0150E, Oxoid, Basingstoke, UK) incubated for 48 h at 37°C. Additionally, corresponding pH values were measured at each sampling time (see 2.1). A total of 15 individual growth curves for both LAB and *L. monocytogenes* and 15 pH profiles were used for model evaluation.

Performance of both the semi-mechanistic (2.2 and 2.3) and the empirical (2.4) modelling approaches were evaluated by the Acceptable Simulation Zone (ASZ) method. The acceptable interval was defined as ±0.5 $\log_{10}$ units from the simulated growth of LAB and *L. monocytogenes* in cottage cheese. When at least 70% of the observed values were within this ASZ, the simulation was considered acceptable (Møller et al., 2013; Oscar, 2005; Velugoti et al., 2011). Additionally, observed and predicted final concentrations (log CFU/g) were compared in order to evaluate if predictions were within ±0.5 log CFU/g from observed final concentrations.

2.5 Curve fitting and statistical analyses

Fitting of eqs. (1)-(3) was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, California, USA). Data was reported as parameter estimate ± standard error. Temperature dependency of a_1 and a_2 was determined by linear regression in Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA). Dynamic growth conditions were simulated by numerical integration using the Euler integration method in Microsoft Excel 2010.

Results

3.1 LAB growth, lactic acid formation and pH changes during storage

LAB from the added starter- and aroma cultures grew in cottage cheese during storage at 10°C and 15°C, produced lactic acid and decreased pH of the products (Fig. 1). On average, the LAB population in cottage cheese with fresh cream dressing increased from 5.42 ± 0.07 log CFU/g to 8.74 ± 0.04 log CFU/g at 10°C and from 5.42 ± 0.07 log CFU/g to 8.88 ± 0.00 log CFU/g at 15°C. The corresponding increase in lactic acid was from 381 ± 90 mg/l in the water phase to 2270 ± 53 mg/l in the water phase at 10°C and to 3823 ± 767 mg/l in the water phase at 15°C. The corresponding decrease in pH was from 5.07 ± 0.01 to 4.39± 0.00 and 4.16 ± 0.03. In cottage cheese with cultured cream dressing LAB grew from 6.58 ± 0.04 log CFU/g to 7.85 ± 0.02 log CFU/g at 10°C and to 8.48 ± 0.07 log CFU/g at 15°C. At 10°C the associated change in lactic acid concentration and product pH was from 1135 ± 365 to 3042 ± 342 mg/l in the water phase and from pH 5.34 ± 0.01 to 5.25 ± 0.01 at 10°C. At 15°C, lactic acid increased from 1135 ± 365 to 3676 ± 424 mg/l in the water phase resulting in a pH decrease from 5.34 ± 0.01 to 4.59 ± 0.02.

3.2 Modelling LAB growth, lactic acid formation and pH changes

For the 14 lactic acid formation curves generated in the present study, average m-values of 0.35 and 0.25 were determined for cottage cheese with fresh- and cultured cream dressing, respectively. The associated average $\log_{10}(Y_{LAC/CFU})$ estimates were -8.44 and -7.93 (Table 1). Predicted lactic acid formation and pH changes at 10°C and 15°C corresponded well to observed values for cottage cheese with cultured cream dressing (Fig. 1c, Fig. 1d, Table 2). Observed lactic acid formation showed some variability and the predicted final concentration of lactic acid in cottage cheese with fresh cream dressing at 15°C was lower than observed (Fig. 1a, Fig. 1b, Table 2). Nevertheless, pH was predicted in concordance with observed pH-profiles in both types of cottage cheese at 10°C and 15°C (Fig. 1; Table 2). This could be due to a low observed impact of high lactic acid concentrations on pH in cottage cheese (Fig. 2).

3.3 Evaluation of bacterial growth- and growth inhibition predicted with the semi-mechanistic modelling approach

Predicted growth of LAB in cottage cheese with fresh- and cultured cream dressing was generally within the ASZ and all values were above 60% (Table 3). On average, observed growth of *L. monocytogenes* in cottage cheese with fresh cream dressing was within the ASZ of growth predicted with the semi-mechanistic modelling approach. However, for cottage cheese with cultured cream dressing only 65% of the observations were, on average, within the ASZ. Additionally, final concentrations were predicted to be higher than observed final concentrations (Table 3). Calculated RMSE values for the predicted pH profiles indicated reasonable prediction of pH (Table 3). In agreement with this, application of measured dynamic pH from growth experiments, in the simulation of *L. monocytogenes* growth resulted in accurate prediction of growth inhibition for cottage cheese with fresh cream dressing (Fig. 3a, 3b). In contrast, *L. monocytogenes* was predicted to reach higher final concentrations than observed in cottage cheese with cultured cream dressing (Fig. 3c, 3d). This was especially evident at 10°C, where the pH decrease was less pronounced than at 15°C (Fig. 3; Table 3).

3.4 Evaluation of bacterial growth and microbial interaction predicted with the empirical Jameson approach

Growth simulations obtained using the same secondary growth models, resulted in similar average ASZ scores (84% and 78%) for the semi-mechanistic- and the empirical model for cottage cheese with fresh cream dressing. Both models displayed five of six predicted final concentrations of *L. monocytogenes* within ±0.5 log CFU/g distance from observed final concentrations. For cottage cheese with cultured cream dressing, 84% of the observed values were, on average, within the ASZ when predicting growth inhibition with the empirical Jameson approach. Only 65% of the observed values were within the ASZ of predictions obtained with the semi-mechanistic modelling approach. In continuation hereof, the number of predicted final concentrations of *L. monocytogenes* within ±0.5 log CFU/g distance from observed final concentrations increased when using the Jameson approach (Table 3).

Discussion

Several authors have advocated for development of mechanistic white box models rather than empirical black box models in predictive food microbiology (Bernaerts et al., 2004; Breidt and Fleming, 1998; Brul et al., 2008; McMeekin et al., 2013; Van Impe et al., 2013). Application of semi-mechanistic (grey-box) models has been proposed, in order to provide a better understanding of the process of growth and to improve the biological interpretability and extendability of the models (Bernaerts et al., 2004; Van Impe et al., 2005). It has also been stated that inclusion of micro- or mesoscopic information in the models will enable an understanding of cell dynamics,

which cannot be obtained with traditional macroscopic approaches (Van Impe et al., 2013). In the present study, a semi-mechanistic, grey-box approach has been used to quantify and, to some extent, explain the observed growth inhibition of *L. monocytogenes* in cottage cheese due to LAB from added starter- or aroma cultures.

Inter-bacterial interaction has previously been quantified for co-cultures and mathematically modelled by taking into account lactic acid formation and pH changes in laboratory media (Bernaerts et al., 2004; Breidt and Fleming, 1998; Janssen et al., 2006; Poschet et al., 2005; Van Impe et al., 2005; Vereecken et al., 2003). Besides the study of Giuffrida et al. (2008), where a stochastic competition model including dynamic lactic acid was evaluated using growth data from challenge tests, no studies have been found to apply predictions of lactic acid and pH to predict microbial interaction in food products. In the present study, well-founded modelling approaches from fermentation technology were used to quantitatively describe the dynamic change in lactic acid concentration and pH, related to growth of LAB, in cottage cheese during storage.

Our results indicate that, for cottage cheese with fresh cream dressing, the inhibitory effect of the starter culture can be attributed to the effect of pH with a minor additional effect of lactic acid (Fig. 3a, 3b, 4a, 4c). This has also been concluded for e.g. *Y. enterocolitica* in co-culture with *Lb. sakei* (Janssen et al., 2006; Vereecken et al., 2003), *L. monocytogenes* in co-culture with *Lc. lactis* (Breidt and Fleming, 1998) and *L. monocytogenes* in co-culture with *Lb. plantarum* (Wilson et al., 2005). On the contrary, growth inhibition of *L. monocytogenes* in cottage cheese with cultured cream dressing could not be explained exclusively by lactic acid formation and pH changes, indicating that "something else" contributed to the inhibitory effect of the aroma culture (Fig. 4e, 4g). Simulations made with the observed pH profile and excluding the effect of lactic acid (Fig. 3c, 3d) further supported this conclusion. *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* ferment citric acid and produce the well-known volatile flavour compound diacetyl, resulting in the characteristic buttery flavour detected in many cultured dairy products (Antinone et al., 1994). Previous studies investigated the antimicrobial effect of diacetyl, and it was concluded that concentrations above 100 ppm in the head space were required to induce bactericidal effects on *L. monocytogenes* in solid media. 100 ppm of diacetyl in laboratory media was observed to increase lag time slightly compared to control samples (Lanciotti et al., 2003). Concentrations of diacetyl in cottage cheese have been reported to be between 1 and 2 ppm (Antinone et al., 1994) and between 0.5 and 20 ppm in fermented dairy products generally (Lanciotti et al., 2003). It therefore seems unlikely that quantification and modelling of diacetyl production and the related inhibitory effect would have an important impact on the predicted growth response of *L. monocytogenes* in cottage cheese during storage. Another explanation for the observed inhibition, not accounted for by lactic acid formation and pH decrease, could be production of bacteriocins by the LAB-aroma culture. For many years, numerous studies have demonstrated the anti-listerial effect of these antimicrobial peptides or proteins and their effects and applications in the food industry has been thoroughly reviewed

(Cleveland et al., 2001; De Vuyst and Leroy, 2007). Bacteriocin production has been modelled for lactobacilli during fermentation of meat products (Messens et al., 2003), sourdough fermentation (model system) (Messens et al., 2002), olive fermentation (model system) (Delgado et al., 2005) and in batch fermentation processes (Lejeune et al., 1998). However, previous experiments performed in our lab (deferred inhibitory test, modified from Tagg et al., 1976) did not show systematically increased inhibitory effect of the aroma culture compared to the starter culture and further analyses are required to determine whether bacteriocins are produced or not. If they are produced, inclusion of their inhibitory effect in the semi-mechanistic model will most likely improve the performance of this interaction model but is may also markedly increase the complexity of the model. Lejeune et al. (1998) found that the specific bacteriocin production rate varied considerably with temperature and other authors observed effects of pH on the bioavailability of the bacteriocins (De Vuyst et al., 1996; Yang et al., 1992). It may therefore be a difficult task to predict formation- and activity of bacteriocins in a complex food matrix like cottage cheese with changing product characteristics during storage.

For cottage cheese with fresh cream dressing, the semi-mechanistic- and the simpler empirical models predicted growth of *L. monocytogenes* equally well (Table 3). The main purpose of predicting growth of *L. monocytogenes* in cottage cheese was to get realistic estimates of *L. monocytogenes* concentrations, in case of contamination and depending on storage conditions and product characteristics (Østergaard et al. 2014). For that purpose we find the simple empirical modelling approach for inter-bacterial interaction "good enough" and justified since precise predictions of growth inhibition and final concentrations of *L. monocytogenes* were obtained regardless of lactic acid, pH or potentially other factors were causing the growth inhibition (Table 3 and Fig. 4).

Increased acid tolerance of acid adapted *L. monocytogenes* cells due to the acid tolerance response (ATR) has been well established (O'Driscoll et al., 1996). It has also been shown, that acid adapted or mutated *L. monocytogenes* cells showed improved survival in acidified dairy products, e.g. in a model cottage cheese (Gahan et al., 1996). By the use of methods adapted from systems biology it could potentially be possible to investigate the ATR related to decreasing product pH. For low contamination levels it is relevant and interesting to quantify the potential inter-cellular variability of *L. monocytogenes* in relation to MIC values and pH$_{min}$ caused by the ATR. From our point of view, and as also mentioned by Van Impe et al. (2013), these new approaches present challenges when applied to bacterial communities in food. To develop and validate these new approaches for prediction of microbial responses in cottage cheese substantial work seems required.

The present study confirmed that modelling of microbial interaction in cottage cheese is important. Interestingly, we found the simple and empirical Jameson model to describe *L. monocytogenes* growth inhibition as well or better than a more complex, semi-mechanistic model including lactic acid formation and pH changes. The importance of a clear definition of the range of

applicability of the semi-mechanistic interaction models should also be emphasised. As demonstrated in this study, the effect of lactic acid and pH could not explain the inhibition of *L. monocytogenes* growth in one of the two studied products. It is therefore important to obtain detailed knowledge about the properties of the LAB cultures of interest.

These findings can be used to justify the use of more simple methodologies for interaction models which, in some cases, can be advantageous since massive amounts of data are already required for development and validation of predictive models to be used in the food industry.

References

Antinone, M., Lawless, H., Ledford, R., Johnston, M., 1994. Diacetyl as a flavor component in full fat cottage cheese. Journal of Food Science 59, 38–42.

Bernaerts, K., Dens, E., Vereecken, K., Geeraerd, A.H., Standaert, A.R., Devlieghere, F., Debevere, J., Van Impe, J.F., 2004. Concepts and tools for predictive modeling of microbial dynamics. Journal of Food Protection 67, 2041–2052.

Bouguettoucha, A., Balannec, B., Amrane, A., 2011. Unstructured models for lactic acid fermentation - A Review. Food Technology and Biotechnology 48, 3–12.

Breidt, F., Fleming, H.P., 1998. Modeling of the competitive growth of *Listeria monocytogenes* and *Lactococcus lactis* in vegetable broth. Applied and Environmental Microbiology 64, 3159–3165.

Brul, S., Mensonides, F.I.C., Hellingwerf, K.J., Teixeira de Mattos, M.J., 2008. Microbial systems biology: new frontiers open to predictive microbiology. International Journal of Food Microbiology 128, 16–21.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology 71, 1–20.

Dalgaard, P., 1995. Qualitative and quantitative characterization of spoilage bacteria from packed fish. International Journal of Food Microbiology 26, 319–333.

Dalgaard, P., 2002. Modelling and predicting the shelf-life of seafood. In: Bremner, H. (Ed.), Safety and quality issues in fish processing. Woodhead Publishing Ltd, Cambridge, pp. 191–219.

Dalgaard, P., Jørgensen, L. V, 2000. Cooked and brined shrimps packed in a modified atmosphere have a shelf-life of > 7 months at 0° C, but spoil in 4–6 days at 25° C. International Journal of Food Science & Technology 35, 431–442.

Davidson, C.M., Cronin, F., 1973. Medium for the selective enumeration of lactic acid bacteria from foods. Applied Microbiology 26, 439.

De Vuyst, L., Callewaert, R., Crabbe, K., 1996. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology 142, 817–827.

De Vuyst, L., Leroy, F., 2007. Bacteriocins from lactic acid bacteria: production, purification and food applications. Journal of Molecular Microbiology and Biotechnology 13, 194–199.

Delgado, A., Brito, D., Peres, C., Noé-Arroyo, F., Garrido-Fernández, A., 2005. Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. Food Microbiology 22, 521–528.

EC, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union 338, 1–26.

Ellouze, M., Pichaud, M., Bonaiti, C., Coroller, L., Couvert, O., Thuault, D., Vaillant, R., 2008. Modelling pH evolution and lactic acid production in the growth medium of a lactic acid bacterium: Application to set a biological TTI. International Journal of Food Microbiology 128, 101–107.

Emborg, J., Dalgaard, P., 2008a. Growth, inactivation and histamine formation of *Morganella psychrotolerans* and *Morganella morganii* - development and evaluation of predictive models. International Journal of Food Microbiology 128, 234–243.

Emborg, J., Dalgaard, P., 2008b. Modelling the effect of temperature, carbon dioxide, water activity and pH on growth and histamine formation by *Morganella psychrotolerans*. International Journal of Food Microbiology 128, 226–233.

Gahan, C.G., O'Driscoll, B., Hill, C., 1996. Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. Applied and Environmental Microbiology 62, 3128.

Gimenez, B., Dalgaard, P., 2004. Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and spoilage microorganisms in cold smoked salmon. Journal of Applied Microbiology 96, 96–109.

Giuffrida, A., Valenti, D., Ziino, G., Spagnolo, B., Panebianco, A., 2008. A stochastic interspecific competition model to predict the behaviour of *Listeria monocytogenes* in the fermentation process of a traditional Sicilian salami. European Food Research and Technology 228, 767–775.

Guillier, L., Stahl, V., Hezard, B., Notz, E., Briandet, R., 2008. Modelling the competitive growth between *Listeria monocytogenes* and biofilm microflora of smear cheese wooden shelves. International Journal of Food Microbiology 128, 51–57.

Irlinger, F., Mounier, J., 2009. Microbial interactions in cheese: implications for cheese quality and safety. Current Opinion in Biotechnology 20, 142–148.

Janssen, M., Geeraerd, A.H., Logist, F., De Visscher, Y., Vereecken, K.M., Debevere, J., Devlieghere, F., Van Impe, J.F., 2006. Modelling *Yersinia enterocolitica* inactivation in coculture experiments with *Lactobacillus sakei* as based on pH and lactic acid profiles. International Journal of Food Microbiology 111, 59–72.

Kempler, G., McKay, L., 1980. Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Applied and Environmental Microbiology 39, 926-927.

Lanciotti, R., Patrignani, F., Bagnolini, F., 2003. Evaluation of diacetyl antimicrobial activity against *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus*. Food Microbiology 20, 537–543.

Le Marc, Y., Valík, L., Medveďová, A., 2009. Modelling the effect of the starter culture on the growth of *Staphylococcus aureus* in milk. International Journal of Food Microbiology 129, 306–311.

Lejeune, R., Callewaert, R., Crabbé, K., De Vuyst, L., 1998. Modelling the growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in batch cultivation. Journal of Applied Microbiology 84, 159–168.

Leroy, F., De Vuyst, L., 2003. A combined model to predict the functionality of the bacteriocin-producing *Lactobacillus sakei* Strain CTC 494. Applied and Environmental Microbiology 69, 1093–1099.

Luedeking, R., Piret, E.L., 1959. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. Journal of Biochemical and Microbiological Technology and Engineering 1, 393–412.

McMeekin, T., Olley, J., Ratkowsky, D., Corkrey, R., Ross, T., 2013. Predictive microbiology theory and application: Is it all about rates? Food Control 29, 290–299.

McMeekin, T.A., Tamplin, M.L., Ross, T., Koseki, S., 2010. The way forward with predictive microbiology in the dairy industry. Australian Journal of Dairy Technology 65, 91–97.

Mejlholm, O., Dalgaard, P., 2007. Modeling and predicting the growth of lactic acid bacteria in lightly preserved seafood and their inhibiting effect on *Listeria monocytogenes*. Journal of Food Protection 70, 2485–2497.

Mejlholm, O., Dalgaard, P., 2013. Development and validation of an extensive growth and growth boundary model for psychrotolerant *Lactobacillus* spp. in seafood and meat products. International Journal of Food Microbiology 167, 244–260.

Messens, W., Neysens, P., Vansieleghem, W., Vanderhoeven, J., De Vuyst, L., 2002. Modeling growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in response to temperature and pH values used for sourdough fermentations. Applied and Environmental Microbiology 68, 1431–1435.

Messens, W., Verluyten, J., Leroy, F., De Vuyst, L., 2003. Modelling growth and bacteriocin production by *Lactobacillus curvatus* LTH 1174 in response to temperature and pH values used for European sausage fermentation processes. International Journal of Food Microbiology 81, 41–52.

Møller, C.O.A., Ilg, Y., Dalgaard, P., Christensen, B.B., Aabo, S., Hansen, T.B., 2013. Effect of natural microbiota on growth of *Salmonella* spp. in fresh pork - A predictive microbiology approach. Food Microbiology 34, 284-295.

O'Driscoll, B., Gahan, C., Hill, C., 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Applied and Environmental Microbiology 62, 1693–1698.

Oscar, T.E., 2005. Validation of lag time and growth rate models for *Salmonella* Typhimurium: acceptable prediction zone method. Journal of Food Science 70, 129–137.

Papademas, P., Bintsis, T., 2010. Food safety management systems (FSMS) in the dairy industry: A review. International Journal of Dairy Technology 63, 489–503.

Pirt, S.J., 1975. Product formation in microbial cultures, in: Principles of Microbe and Cell Cultivation. Blackwell Scientific Publications, pp. 156–169.

Poschet, F., Vereecken, K.M., Geeraerd, A.H., Nicolaï, B.M., Van Impe, J.F., 2005. Analysis of a novel class of predictive microbial growth models and application to coculture growth. International Journal of Food Microbiology 100, 107–24.

Tagg, J.R., Dajani, A.S., Wannamaker, L.W., 1976. Bacteriocins of gram-positive bacteria. Bacteriological Reviews 40, 722–56.

Turner, M., Blumenstein, B., Sebaugh, J., 1969. 265 Note: A generalization of the logistic law of growth. Biometrics 25, 577–580.

Vaikousi, H., Biliaderis, C.G., Koutsoumanis, K.P., 2008. Development of a microbial time/temperature indicator prototype for monitoring the microbiological quality of chilled foods. Applied and Environmental Microbiology 74, 3242–50.

Van Impe, J.F., Poschet, F., Geeraerd, A.H., Vereecken, K.M., 2005. Towards a novel class of predictive microbial growth models. International Journal of Food Microbiology 100, 97–105.

Van Impe, J.F., Vercammen, D., Van Derlinden, E., 2013. Toward a next generation of predictive models: A systems biology primer. Food Control 29, 336–342.

Velugoti, P.R., Bohra, L.K., Juneja, V.K., Huang, L., Wesseling, A.L., Subbiah, J., Thippareddi, H., 2011. Dynamic model for predicting growth of *Salmonella* spp. in ground sterile pork. Food Microbiology 28, 796–803.

Vereecken, K.M., Devlieghere, F., Bockstaele, A., Debevere, J., Van Impe, J.F., 2003. A model for lactic acid-induced inhibition of *Yersinia enterocolitica* in mono- and coculture with *Lactobacillus sakei*. Food Microbiology 20, 701–713.

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2006. Milk, in: Dairy Science and Technology, Second Edition, Food Science and Technology. CRC Press, pp. 3–16.

Wilson, A.R., Sigee, D., Epton, H.A.S., 2005. Anti-bacterial activity of *Lactobacillus plantarum* strain SK1 against *Listeria monocytogenes* is due to lactic acid production. Journal of Applied Microbiology 99, 1516–1522.

Yang, R., Johnson, M.C., Ray, B., 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 58, 3355–3359.

Østergaard, N.B., Eklöw, A., Dalgaard, P., 2014. Modelling the effect of lactic acid bacteria from starter- or aroma culture on growth of *Listeria monocytogenes* in cottage cheese. International Journal of Food Microbiology [FOOD-D-14-00067].

Tables and Figures

Table 1. Parameters obtained from primary lactic acid formation and pH modelling.

		Parameter values and statistics			
		Cottage cheese with fresh cream dressing		Cottage cheese with cultured cream dressing	
$\text{Log}(Y_{LAC/CFU})^a$ (mg/CFU)		-8.435 (n=4)	0.170^d	-7.931 (n=9)	0.409^d
m-value[b]		0.35	-	0.25	-
a_1[c]	α	-0.0037	0.992^e (n=3)	-0.0112	0.961^e (n=9)
	β	0.0747		0.1728	
a_2[c]	α	-0.0007	0.986^e (n=3)	-0.0021	0.959^e (n=9)
	β	0.0152		0.0326	

[a] Average value from observed data fitted to Eq. (2)
[b] m-value returning best fit evaluated by absolute sum of squares (ASS)
[c] Model-parameters determined from temperature (T, °C) dependent linear regression
[d] ± standard deviation
[e] r^2-value

Table 2. Evaluation of lactic acid- and pH predictions.

	Cottage cheese with fresh cream dressing		Cottage cheese with cultured cream dressing	
	10°C	15°C	10°C	15°C
RMSE (mg/l in water phase), lactic acid formation model[a]	307	1530	556	1544
RMSE, pH model[b]	0.129	0.164	0.036	0.107

[a] Corresponding lactic acid formation curves (observed- and predicted) are shown in Fig. 1
[b] Corresponding pH profiles (observed- and predicted) are shown in Fig. 1

Table 3. Comparison of mechanistic and empirical models to predict the effect of LAB on growth of *L. monocytogenes* in cottage cheese.

	Mechanistic modelling approach							Empirical Jameson approach					
Storage temperature (°C)	ASZ score (%) Dynamic lactic acid and pH		RMSE (pH)	LAB *RLT* value[c]	Log(Final LM concentration) observed[a]	Log(Final LM concentration) predicted	Log difference[b]	ASZ score (%) Jameson approach		LAB *RLT* value[c]	Log(Final LM concentration) observed[a]	Log(Final LM concentration) predicted	Log difference[b]
	L. monocytogenes	Lactic acid bacteria						*L. monocytogenes*	Lactic acid bacteria				
Cottage cheese with fresh cream dressing													
5	16	92	0.1229	7.28	6.57 ± 0.12	8.10	1.53	31	84	7.28	6.57 ± 0.12	7.58	1.00
10 (fig. 3a+b)	94	88	0.1482	4.04	6.13 ± 0.24	5.83	-0.30	53	84	4.04	6.13 ± 0.24	6.38	0.25
15	100	65	0.1195	7.28	5.50 ± 0.16	5.55	0.05	100	81	7.28	5.50 ± 0.16	5.65	0.15
8 (fig. 3c+d)	97	78	0.0776	4.04	5.75 ± 0.04	5.34	-0.41	86	81	4.04	5.75 ± 0.04	6.12	0.37
11	100	87	0.1964	4.04	5.01 ± 0.15	4.75	-0.26	100	97	4.04	5.01 ± 0.15	5.16	0.16
8	97	77	0.1451	4.04	6.25 ± 0.05	6.22	-0.03	100	70	4.04	6.25 ± 0.05	6.15	-0.10
Average	84	81					0.10 ± 0.72[d]	78	83				0.30 ± 0.38[d]
Cottage cheese with cultured cream dressing													
5	84	97	0.3357	2.34	6.20 ± 0.02	6.49	0.30	94	97	2.34	6.20 ± 0.02	6.29	0.09
10 (fig. 3e+f)	31	100	0.1830	0.05	5.24 ± 0.06	8.37	3.13	44	97	0.05	5.24 ± 0.06	5.61	0.37
15	42	71	0.0810	0.05	4.51 ± 0.10	5.42	0.91	94	90	0.05	4.51 ± 0.10	4.58	0.07
5	95	100	0.1498	2.34	6.24 ± 0.14	5.89	-0.35	82	100	2.34	6.24 ± 0.14	5.77	-0.47
10	67	71	0.0449	0.05	5.63 ± 0.18	7.68	2.05	87	56	0.05	5.63 ± 0.18	4.63	-1.00
15 (fig. 3g+h)	46	71	0.1005	0.05	4.15 ± 0.12	5.14	0.99	100	90	0.05	4.15 ± 0.12	3.85	-0.30
8	90	69	0.0347	5.20	5.25 ± 0.09	5.13	-0.12	97	67	5.20	5.25 ± 0.09	5.37	0.12
11	80	62	0.2657	5.20	5.02 ± 0.04	5.86	0.84	87	46	5.20	5.02 ± 0.04	4.49	-0.53
5	50	96	0.0918	2.34	4.17 ± 0.06	5.68	1.51	71	96	2.34	4.17 ± 0.06	4.95	0.78
Average	65	82					1.03 ± 1.09[d]	84	82				-0.10 ± 0.53[d]

[a] Average log CFU/g based on 3-4 replicates

[b] Difference between observed and predicted final concentration of *L. monocytogenes*. Positive values indicate higher final concentrations and negative values indicate lower final concentrations compared to observed values

[c] The applied *RLT*-values were either the minimum-, average- or maximum observed values.

[d] ± standard deviation
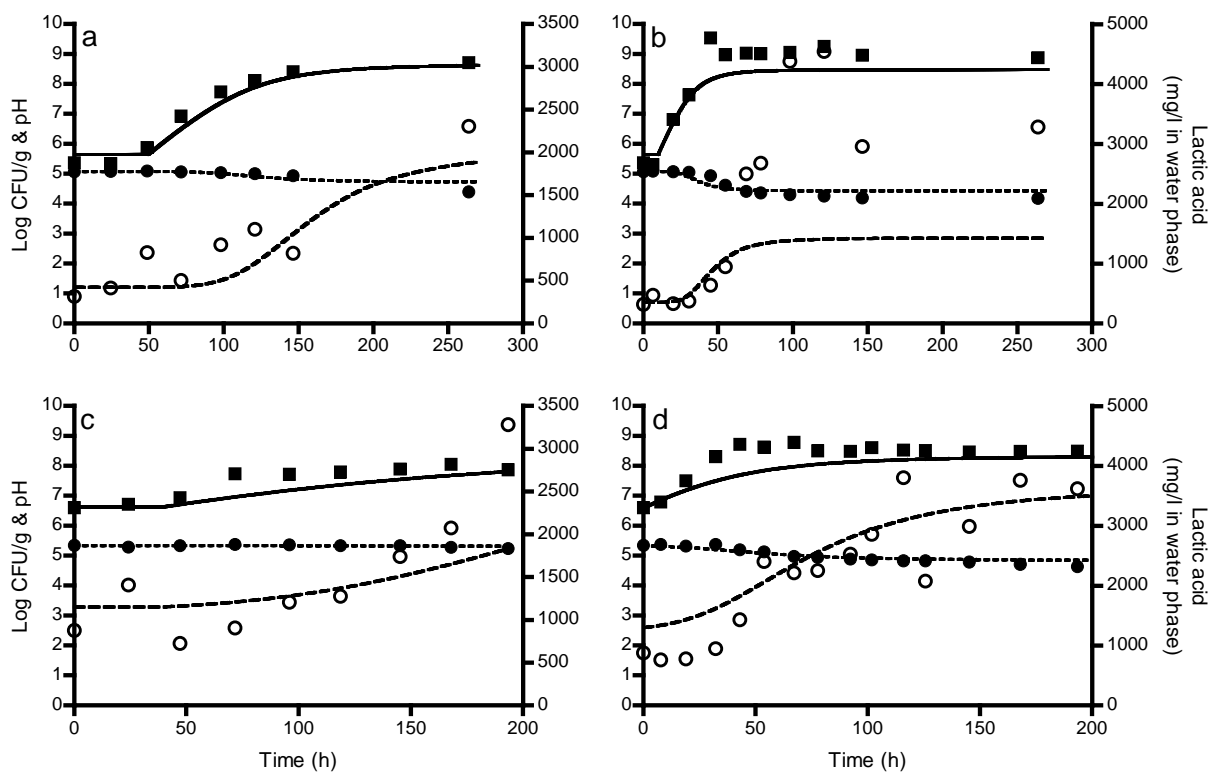
Figure 1. Measured growth of lactic acid bacteria (■), lactic acid formation (○) and pH (●) at (a) 10°C and (b) 15°C in cottage cheese with fresh cream dressing and similarly at (c) 10°C and (d) 15°C in cottage cheese with cultured cream dressing. Simulations of fitted models are displayed for lactic acid bacteria (—), lactic acid (---) and pH (⋯). RMSE values for lactic acid formation- and pH simulations are presented in Table 2.
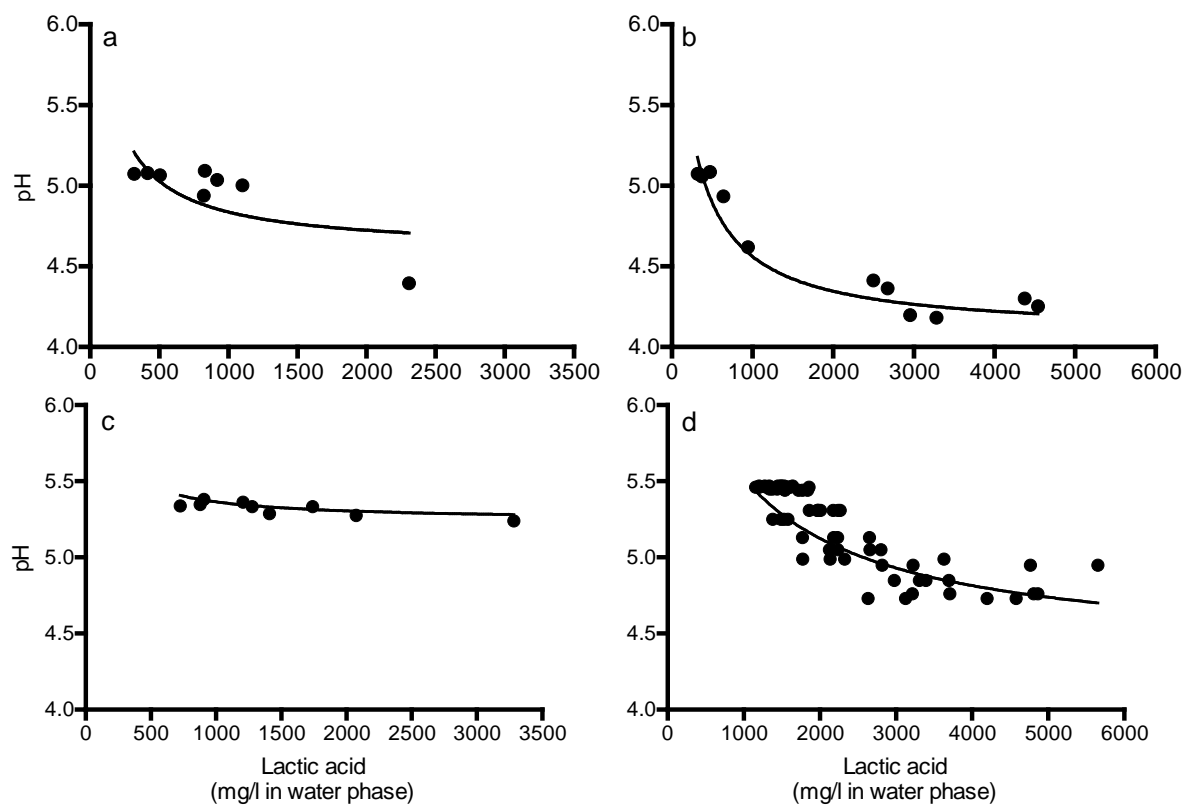
Figure 2. Measured pH and lactic acid concentrations (mg/l in water phase) for cottage cheese with fresh cream dressing at 10°C (a) and 15°C (b) and for cottage cheese with cultured cream dressing at 10°C (c) and 15°C (d).
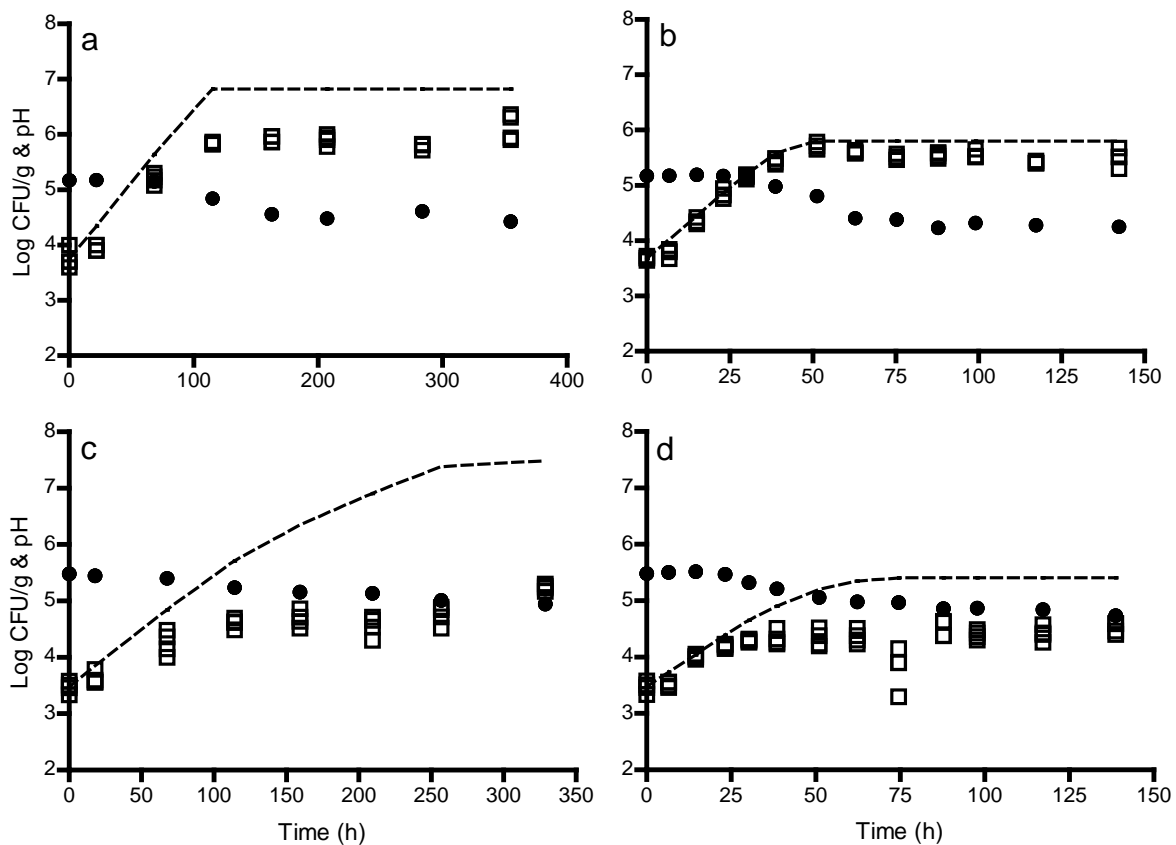
Figure 3. Measured (□) and simulated (---) growth of *L. monocytogenes* in cottage cheese. Observed pH-profiles (●) were used as input to predict growth. Simulations were performed for cottage cheese with fresh cream dressing at (a) 10°C and (b) 15°C and cottage cheese with cultured cream dressing at (c) 10°C and (d) 15°C.
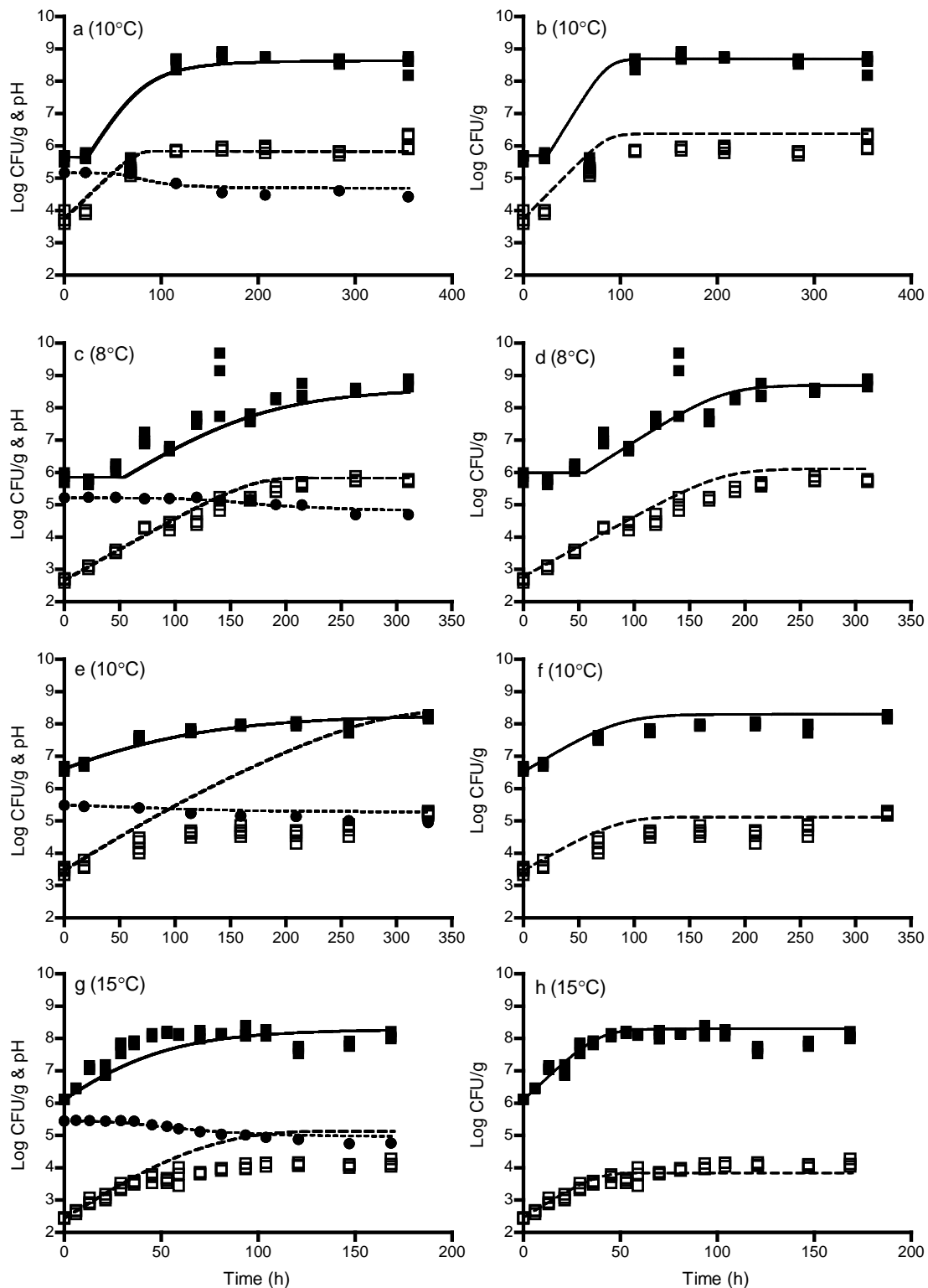
Figure 4 Simulated (lines) and observed (symbols) lactic acid bacteria growth (— and ■), *L. monocytogenes* growth (--- and □) and product pH (··· and ●) in cottage cheese with fresh- (a,b,c,d) and cultured cream dressing (e,f,g,h). Simulations were obtained using mechanistic (a, c, e, g) and empirical (b, d, f, h) interaction models.

8. PAPER III

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

[To be submitted]

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

Nina Bjerre Østergaard[a], Lasse Engbo Christiansen[b] and Paw Dalgaard[a]

[a]National Food Institute (DTU Food), Technical University of Denmark, Kgs. Lyngby, Denmark

[b]Department of Applied Mathematics and Computer Science (DTU Compute), Technical University of Denmark, Kgs. Lyngby, Denmark

Running title: Stochastic modelling of *L. monocytogenes* growth in cottage cheese

Corresponding author: Division of Industrial Food Research, National Food Institute, Technical University of Denmark, Søltofts Plads, Building 221, DK-2800, Kgs. Lyngby, Denmark. Tel.: +45 45254918, Fax: +45 45884774. E-mail address: nbjos@food.dtu.dk (N.B. Østergaard)

ABSTRACT

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

Keywords
- Stochastic modelling
- Bacterial interaction
- Low contamination levels
- Fresh fermented dairy products
- *L. monocytogenes*

Introduction

Listeria monocytogenes is a well-known food borne pathogenic bacterium. The organism has received extensive attention in relation to (i) growth response in different food products (Beumer et al., 1996; Bolton and Frank, 1999; Jørgensen and Huss, 1998), (ii) development of deterministic- and stochastic predictive growth- and growth boundary models (Augustin et al., 2005; Mejlholm et al., 2014), and (iii) in studies dealing with risk assessment and risk management (McLauchlin et al., 2004; Tenenhaus-Aziza et al., 2014). A range of deterministic- and stochastic growth models are available and their use, as a supplement to traditional testing in risk assessment and product evaluation, has been recognised by the European Union (EC, 2005). These well founded predictive models are based on population data which represent an average growth response of a bacterial population (Pin and Baranyi, 2006). However, contamination of food products with *L. monocytogenes* often occur with low cell numbers (EFSA, 2013; Kozak et al., 1996) and individual cell behaviour seems relevant to consider when predicting growth of *L. monocytogenes* in naturally contaminated food products. The time until growth is initiated by each individual cell is determined by the physiological state of the cell in combination with the environment it is transferred to (Standaert et al., 2007). This lag time has been shown to vary considerably between cells (Francois et al., 2006a) and with increasing variability observed for injured cells (Guillier et al., 2005). Modelling growth of low cell numbers aims at imitating growth of *L. monocytogenes* in naturally contaminated products. Luckily, naturally contaminated batches of cottage cheese are rarely available but that also makes model validation more complicated. As an alternative to naturally contaminated products, samples inoculated with low cell numbers have been used in previous studies to evaluate growth of individual cells in food. For these data sets the inoculum has been prepared under controlled conditions in the laboratory and less variability in the physiological state is likely to be observed (Augustin et al., 2014; Ferrier et al., 2013; Francois et al., 2006b; Manios et al., 2013). A stochastic modelling approach facilitate inclusion of e.g. variable product characteristics, lag time variability, and variability between strains and enables simulation of all possible outcomes related to the observed or estimated variability (Couvert et al., 2010; Koutsoumanis et al., 2010). As previously demonstrated, accurate predictions of *L. monocytogenes* growth in fermented dairy products should also include the inter-bacterial interaction and associated inhibition caused by growth of lactic acid bacteria (LAB) from added LAB cultures (Guillier et al., 2008; Le Marc et al., 2009; Østergaard et al., 2014a, 2014b). Few *L. monocytogenes* single cell or low inoculum stochastic models include inter-bacterial interactions (Mejlholm et al., 2014; Pouillot et al., 2007) and this type of models has not been evaluated and validated for cottage cheese.

The aim of the present study was to quantify growth of low *L. monocytogenes* cell numbers in cottage cheese with a natural high concentration of LAB from starter cultures. The effect of the pre-history of the *L. monocytogenes* inoculum was investigated, using three different pre-cultures to reflect conditions of potential routes of contamination during cottage cheese processing. In order to

include the effect of variability of (i) product characteristics, (ii) initial LAB concentration, and (iii) lag time duration in the predictions of growth, a stochastic modelling approach was applied for both LAB populations and individual cells of *L. monocytogenes*. Validated secondary growth models for LAB and *L. monocytogenes* in cottage cheese was used in combination with input data represented by theoretical- or empirical distributions. The variability in product characteristics (pH, and lactic acid) and initial- and final concentrations of LAB was included in the model by using observed data (100 datasets) to bootstrap from. Variability in lag time was included in the model by fitting distributions to observed lag time data for LAB after these were transformed into relative lag times (*RLT*). For *L. monocytogenes* lag time and *RLT* distributions extracted from literature were used. The observed growth of low concentrations of *L. monocytogenes* as determined in the present study was then compared with simulated growth. Finally, the developed stochastic model was applied for three different scenarios to evaluate *L. monocytogenes* growth and safety of cottage cheese.

Materials and Methods

2.1 Collection of variable input data

During a period of more than two years, 23 growth kinetics of LAB populations were determined in four independent batches of cottage cheese with added aroma culture (Østergaard et al., 2014a, 2014b). Microbiological analyses were performed as described by Østergaard et al. (2014). In brief, the logistic growth model with delay (Eq. 1) was fitted to the 23 LAB growth curves to obtain estimates of initial concentrations (log CFU/g), lag time duration, and maximum population densities (log CFU/g) at different storage temperatures.

$$\log(N_t) = \log(N_0) \qquad\qquad \text{if } t < t_{lag} \qquad [1]$$

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

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

$$t_{lag} = \frac{RLT \cdot \ln(2)}{\mu_{max}} \qquad \Rightarrow \qquad RLT = \frac{t_{lag} \cdot \mu_{max}}{\ln(2)} \qquad\qquad [2]$$

2.1.1 Variability of lag time for lactic acid bacteria

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

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

During previous studies (Østergaard et al., 2014a, 2014b) initial product pH and lactic acid concentration (ppm in the water phase) of six individual batches of cottage cheese were determined. Corresponding initial- and final concentrations of LAB were also recorded and a total of 100 coupled observations of pH, lactic acid, LAB N_0 and LAB N_{max} were compiled (Table 1). This dataset was used for bootstrap sampling during simulation in order to maintain the potential relationship between product characteristics (pH and lactic acid) and initial and final LAB concentrations.

2.1.3 Lag time data for individual L. monocytogenes *cells*

Data from four studies (Francois et al., 2006a, 2005; Guillier and Augustin, 2006; Guillier et al., 2005) was assessed to represent potential responses of *L. monocytogenes* in cottage cheese inoculated with the pre-cultures used in the present study (see Section 2.2.1). Variability in *L. monocytogenes* lag times (Table 2) was represented by Weibull, Extreme Value type I and II, and shifted Gamma distributions in the literature. The distributions represented either lag times (Francois et al., 2005; Guillier and Augustin, 2006; Standaert et al., 2007) or standardised detection times (Guillier et al., 2005) which were related to lag times by Eq. (3) (Baranyi and Pin, 1999).

$$lag = T_d - \frac{(\ln(N_d) - \ln(N_0))}{\mu} \tag{3}$$

where T_d is the detection time from absorbance measurements, N_d is the bacterial concentration at T_d (estimated to 1.8×10^7 *L. monocytogenes* cells/well (Guillier et al., 2005)), N_0 is the initial number of cells, assumed to be one and μ is the constant specific growth rate at exponential growth as reported in the studies (Francois et al., 2006a, 2005; Guillier and Augustin, 2006; Guillier et al., 2005). Sampled lag times and lag times calculated from recorded detection times were transformed into *RLT*-values by applying Eq. (2). It was assumed that *RLT*-values were temperature independent within the applied temperature range although these values may increase near the lower temperature limit of growth (Hereu et al., 2014). Subsequently, the *RLT*-values were used in combination with

predicted growth rates (see Section 2.3) to estimate the lag time (Eq. (2)) associated with the simulated growth conditions. In addition, growth was simulated using the 283 *RLT*-values for *L. monocytogenes* populations in food collected and reported by Ross (1999). These *RLT*-values were represented by fitting a suitable distribution using *fitdistrplus* in R (Table 2). Simulated growth was compared to pooled growth data of pre-culture 1, 2 and 3 (see Section 2.2.1), and the initial *L. monocytogenes* concentration was represented by a Poisson distribution fitted to all (n = 24) estimated initial concentrations in inoculated cottage cheese. The pooled data was intended to mimic unknown pre-history of contaminating cells.

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

2.2.1 Preparation of inoculum and inoculation of products

L. monocytogenes isolate SLU92 from a dairy production environment was provided by Arla Strategic Innovation Centre (ASIC). This isolate was stored at -80°C in freezing media with glycerol. The isolate was cultured in brain heart infusion (BHI) broth (CM1135, Oxoid, Basingstoke, UK) for 24 h at 25°C. Subsequently, three different inoculums were prepared. Pre-culture 1 represented a reference/worst case scenario with cells in the late exponential phase and adapted to the environment encountered in cottage cheese. Pre-culture 2 represented contamination with starved and hence stressed cells and pre-culture 3 represented transfer from high temperature and high pH environment to a chilled food product with lower pH. Pre-culture 1 was prepared by transferring the isolate (BHI broth, pH 7.4; 0.5% NaCl; 24 h at 25°C) to BHI broth (pH 5.3, 1% NaCl) followed by incubation at 10°C for 24 h. For pre-culture 2, the isolate (BHI broth, pH 7.4; 0.5% NaCl; 24 h at 25°C) was diluted tenfold in 0.85% saline H_2O and incubated at 10°C for 24 h. Preparation of pre-culture 3 was performed by transferring the isolate (BHI broth, pH 7.4; 0.5% NaCl; 24 h at 25°C) to BHI broth (pH 6.2, 3.0% NaCl) and incubating at 25°C for 20 h resulting in stationary phase cells. Growth of pre-culture 1 and 3 was followed by absorbance measurements (540 nm, Novaspec II, Pharmacia Biotech, Allerød, Denmark). Preparation of the pre-cultures was performed according to a modified version of the protocol presented by Francois et al. (2003). The concentration of the pre-cultures was determined by microscopy (100x magnification, Olympus BH-2, Olympus, Tokyo, Japan), assuming that one cell per field of view corresponded to a concentration of 10^6 CFU/ml (Adams and Moss, 2000). The pre-cultures were adjusted to a concentration of 10^7 CFU/ml in 0.85% saline H_2O and subsequently tenfold dilution series were made in 0.85 % saline H_2O to reach a concentration of 10^2 CFU/ml for SLU-92 from each pre-culture. Microtiter plates were prepared with 150 µl 0.85% saline H_2O in each well and 150 µl of the inoculum was transferred to each of the first wells of the microtiter plate (8x12 wells). Half-dilutions were made throughout the eight rows of the plate to reach the desired concentrations. Based on pre-experiments, performed to standardise the procedure, the final inoculum was obtained

after five half-dilutions for pre-culture 1 and 2 and after four half-dilutions for pre-culture 3. During preparation, the pre-cultures were kept on ice.

Prior to the experiment, the cottage cheese was distributed in portions of 10 g into small plastic containers. Each individual container was inoculated with 150 µl inoculum (1.50% vol/wt) resulting in an initial concentration between 1 and 10 bacterial cells per portion (10 g). Control of the inoculum size was made by surface plating the content of 3×10 wells, identical with those used for inoculation (150 µl inoculum), onto BHI agar and incubating for 72 h at 25°C. Inoculated cottage cheese was stored at 8°C during the experiment and the storage temperature was recorded by data loggers (TinyTag Plus, Gemini Data Loggers Ltd., Chichester, UK).

2.2.2 Microbiological analysis; growth of L. monocytogenes *and lactic acid bacteria in cottage cheese*

At regular intervals (approximately every 45 h) inoculated samples were analysed by transferring the content of one container (10 g) into a filter stomacher bag and diluting 5x by adding 40 ml of chilled physiological saline (PS, 0.85% NaCl and 0.10% Bacto-peptone) solution. The diluted sample was homogenised for 30 s at normal speed in a Stomacher 400 (Seward Medical, London, UK). Appropriate tenfold dilutions were prepared in chilled PS and LAB was enumerated by pour plating in nitrite actidione polymyxin (NAP) agar (pH 6.2) and incubating with overlay at 25°C for 72 h (Davidson and Cronin, 1973). The initial concentration of *L. monocytogenes* in the product was below the detection limit using general plate counts (0.7 log CFU/g) and the enumeration of *L. monocytogenes* by surface plating on Palcam agar (CM 0877, Oxoid, Basingstoke, UK) with Palcam selective supplement (SR0150E, Oxoid, Basingstoke, UK) (incubated for 48 h at 37°C) was supplemented with estimates of most probable number (MPN, Thomas, 1942) inducing a lower detection limit of -0.48 log CFU/g. For this, one or two ml (depending on the expected bacterial concentration) of the diluted sample was transferred to 10 ml of ONE-broth Listeria (CM1066, Oxoid, Basingstoke, UK) added ONE-broth Listeria selective supplement (SR0234E, Oxoid, Basingstoke, UK). This was performed using eight replicates for each sample. The inoculated tubes with ONE-broth Listeria were incubated at 30°C for 24 h and the presence or absence of *L. monocytogenes* was evaluated by streaking 10 µl of the inoculated ONE-broth Listeria onto Brilliance™ Listeria agar (CM1080, Oxoid, Basingstoke, UK) supplemented with Brilliance™ Listeria selective supplement (SR0227E, Oxoid, Basingstoke, UK) and Brilliance™ Listeria differential supplement (SR0228E, Oxoid, Basingstoke, UK). The maximum likelihood estimate (MLE) of the initial concentration of *L. monocytogenes* in the samples was calculated using Eq. (4) and the 95% likelihood intervals (Table 3) of the estimates were determined numerically.

$$MLE = \frac{-ln\left(\frac{[number\ of\ negative\ tubes]}{[total\ number\ of\ tubes]}\right) \cdot [dilution\ factor]}{[ml\ of\ sample\ per\ tube]} \qquad [4]$$

A Poisson distribution was fitted to rounded MLE estimates of the initial *L. monocytogenes* concentrations in the product and in the control samples of each pre-culture (1-3). Distributions were fitted using the *fitdistrplus* package in R.

2.2.3 Analysis of product properties

The cottage cheese, used in the challenge test, was analysed for initial chemical characteristics (pH, NaCl, dry matter and naturally occurring lactic acid). pH was measured with a PHM 250 Ion Analyzer (MetroLab™, Radiometer, Copenhagen, Denmark) in 5 g of product stirred with 25 ml of deionised water. NaCl was quantified by automated potentiometric titration (785 DMP Titrino, Metrohm, Herisau, Switzerland). The dry-matter content was determined by keeping 2.0 g of sample at 105.0°C for 24 h. Subsequently the weight of the dehydrated sample was measured and related to the total weight of the sample (AOAC, 1990). The concentration of lactic acid was determined by HPLC using an external standard for identification and quantification (Dalgaard and Jørgensen, 2000). In order to improve the extraction of lactic acid, a centrifugation step, as also applied by Marsili et al. (1981), was included prior to two consecutive filtration steps.

2.3 Stochastic modelling of simultaneous growth of L. monocytogenes *and lactic acid bacteria in cottage cheese*

The secondary, cardinal parameter models of Østergaard et al. (2014) (Eq. (5), Table 4) were used to predict growth rates (h⁻¹) for LAB and *L. monocytogenes* in cottage cheese with added aroma culture. The models included the effect of temperature, pH, water activity (NaCl), lactic- and sorbic acid. For the simulations in the present study, the water activity was kept constant (0.994, corresponding to 1.1 % NaCl in the water phase) and sorbic acid was set to zero ppm.

$$\mu_{max}= \mu_{ref} \cdot \left[\left(\frac{T-T_{min}}{T_{ref}\text{-}T_{min}}\right)^2\right] \cdot \left(1\text{-}10^{(pH_{min}-pH)}\right) \cdot \left(1\text{-}10^{(pH-pH_{max})}\right)$$

$$\cdot \left(\frac{a_w - a_{w,min}}{1\text{-}a_{w,min}}\right) \cdot \left(1\text{-}\left(\frac{[LAC_U]}{MIC_{U\ Lactic\ acid}}\right)^{n1}\right)^{n2} \cdot \left(1\text{-}\left(\frac{[SAC_u]}{MIC_{U\ Sorbic\ acid}}\right)^{n1}\right)^{n2} \cdot \xi \qquad [5]$$

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). T (°C) is the storage temperature, T_{min} is the theoretical minimum temperature allowing growth, a_w is the water activity and $a_{w,min}$ is the minimum theoretical water activity allowing growth. pH_{min} and pH_{max} are the theoretical minimum- and maximum pH values allowing growth of the microorganisms. [LAC_U]

and [SAC_U] are the concentrations (mM) of undissociated lactic- and sorbic acid in the product. $MIC_{U\ Lactic\ acid}$ and $MIC_{U\ Sorbic\ acid}$ are fitted MIC values (mM) of undissociated lactic- and sorbic acid that prevent growth of the modelled microorganisms. The effect on μ_{max} of interaction between the environmental factors was represented by ξ, and becomes particularly important as the growth boundaries are approached (Le Marc et al., 2002).

Growth simulations were performed with 10000 iterations using bootstrapped input values of observed data for product characteristics and corresponding initial and final LAB concentrations. The *boot* function with replacement in R was used. *RLT*-estimates for LAB and *L. monocytogenes* single cells were sampled from distributions fitted to observed data or distributions extracted from literature (see 2.1.1 and 2.1.3). The storage temperature, which was assumed to be normally distributed, was obtained from the data-logger. The growth over time was simulated by numerical integration using the *ode* function of the *deSolve* package in R. The effect of inter-bacterial interaction was included in this step by combining the primary growth model including delay (Eq. (1)) and the empirical Jameson term (Eq. (6)).

$$\frac{dLm/dt}{Lm_t} = 0, \qquad\qquad\qquad t < t_{lag,Lm}$$

$$\frac{dLm/dt}{Lm_t} = \mu_{max}^{Lm} \cdot \left(1 - \frac{Lm_t}{Lm_{max}}\right) \cdot \left(1 - \frac{LAB_t}{LAB_{max}}\right), \qquad t \geq t_{lag,Lm} \qquad\qquad [6]$$

where *Lm* and *LAB* represent concentrations (> 0 CFU/g) of *L. monocytogenes* and LAB, respectively. Other parameters are as explained for Eq. (1). The interactive effect of the LAB culture on *L. monocytogenes* was evaluated for each time-step resulting in a $\mu_t^{Lm} = \left(\frac{dLm}{dt}\right)/Lm_t$ value (Eq. (6)) corresponding to a given concentration of LAB (LAB_t). Application of this μ_t^{Lm} value instead of μ_{max} in Eq. (1) returned a *L. monocytogenes* concentration at time *t*, related to LAB_t and as LAB_t approach LAB_{max}, $\mu_t^{Lm} = \left(\frac{dLm}{dt}\right)/Lm_t$ will approach zero and the *L. monocytogenes* concentration cease to increase.

2.4 Scenarios related to production and distribution of cottage cheese

Three different scenarios (A, B, and C) were defined in order to evaluate the effect of different interventions on growth of low concentrations of *L. monocytogenes* in cottage cheese. It is well known that temperature and pH are important controlling factors in relation to bacterial growth and the effect of a somewhat strict temperature regime (5°C ± 0.25) was evaluated in Scenario A. The effect of a decreased initial product pH was evaluated by shifting the observed pH distribution 0.2 pH units downwards in Scenario B. That adjustment conserved the observed variability in product pH but at the same time evaluated the effect of a systematic decrease of initial product pH.

In the third scenario (Scenario C) an estimated concentration of one *L. monocytogenes* cell per container (450.0 g) was evaluated at 5°C ±0.25 using the *L. monocytogenes* population *RLT-*distribution from Ross (1999) in combination with the observed product- and LAB variability. This concentration (0.002 CFU/g) corresponded to the lowest possible contamination level in a commercially available container of cottage cheese (450.0 g). Similarly, an initial concentration of one *L. monocytogenes* cell per 125.0 g of product was evaluated under identical conditions. This scenario corresponded to the lowest level of non-compliance with the food safety criteria of ready-to-eat foods supporting growth of *L. monocytogenes* (absence in 5 x 25.0 g during processing) (EC, 2005).

Results

3.1 Variable input data

3.1.1 Lactic acid bacteria lag time data obtained from cottage cheese

The exponential distribution with a rate-parameter (± standard error) of 0.48 ± 0.10 described data well and returned the lowest BIC value of the five distributions tested (Results not shown).

3.1.2 Variability in product characteristics, initial and final concentrations of lactic acid bacteria

Mean values were 5.35 for pH, 1286 for lactic acid (ppm in water phase), 6.4 log CFU/g for the initial concentration of LAB and 8.5 log CFU/g for the maximum population density of LAB in cottage cheese. The average storage temperature (± standard deviation) during the experiment was 7.47°C ± 0.24 and it was assumed to be normally distributed (N(7.47,0.24)).

3.1.3 Lag time data for individual L. monocytogenes *cells*

Transformation of sampled lag times (10000 iterations) into *RLT*-values returned *RLT*-values in the range from -5.1 (assumed to correspond to no lag phase (*RLT*=0) for cells in the exponential phase (Guillier et al., 2005)) to 112.7 (Table 5). A mean value of 3.70 was obtained from the *L. monocytogenes* population data reported by Ross (1999) (Table 5).

3.2 Growth data for lactic acid bacteria and for low concentrations of L. monocytogenes *in cottage cheese*

All three pre-cultures grew in cottage cheese (Fig. 1) displaying an initial product pH of 5.32 ± 0.02 and initial concentration of lactic acid of 1099 ± 185 ppm in the water phase. The variability of the estimated initial concentration (CFU/g) of *L. monocytogenes* in products inoculated with pre-culture 1, 2, and 3 was represented by Poisson distributions fitted to the initial, rounded MPN estimates, returning λ-values (± standard error) of 0.63 ± 0.28, 1.12 ± 0.37 and 1.00 ± 0.35 for pre-culture 1, 2, and 3. For the pooled data on the estimated initial *L. monocytogenes* concentrations in cottage cheese a λ-value of 0.92 ± 0.20 was obtained. The average concentrations (CFU/g ±

standard deviation), based on eight replicates, were 0.40 ± 0.19, 0.91 ± 0.34 and 1.04 ± 0.25 for control samples of pre-culture 1, 2 and 3, respectively. Poisson distributions fitted to the concentrations of the control-wells displayed λ-values (± standard error) of 0.60 ± 0.17, 0.85 ± 0.21 and 0.70 ± 0.19 for pre-culture 1, 2 and 3, respectively.

3.3 Simulation of simultaneous growth of L. monocytogenes *and lactic acid bacteria in cottage cheese*

All growth simulations of LAB populations and low initial concentrations of *L. monocytogenes* are represented by the 50, 90, 99 and 100% confidence intervals (CI). Simulated growth of adapted *L. monocytogenes* cells (pre-culture 1) in cottage cheese (Fig. 2a) illustrated growth during storage and for the most extreme case the time to reach 100 CFU/g was 110 h and the mean time to reach 100 CFU/g was 195 h. Of 10000 iterations, 4026 growth curves did not reach 100 CFU/g or contained zero cells initially. The observed growth data was located within the 50% CI (dark red in Fig. 2a). Growth data for starved cells (pre-culture 2) was compared to simulated growth using two different distributions for *L. monocytogenes* lag time data (Fig 2b and 2c). For *L. monocytogenes* growth simulations with Gamma distributed lag times (Table 2), the minimum time to reach 100 CFU/g was 160 h and the mean was 351 h. Of 10000 iterations, 8256 growth curves did not reach concentrations of 100 CFU/g or did not contain any *L. monocytogenes* cells initially. The observed growth data for *L. monocytogenes* was located within the 90% CI (Fig. 2b). A similar simulation was made using the *L. monocytogenes* lag time data represented by the Extreme Value type II distribution (Table 2, Guillier and Augustin, 2006), returning a minimum time to reach 100 CFU/g of 330 h and a mean value of 441 (Fig. 2c). In this situation, of the 10000 iterations, 9432 growth curves were predicted not to reach concentrations of 100 CFU/g or to contain zero cells initially. Observed growth data was distributed within the 99% CI of the simulated growth curves (Fig. 2c). For pre-culture 3, representing *L. monocytogenes* cells transferred from high pH and temperature to a low pH chilled product, growth was simulated using two differently parameterised Weibull distributions (Francois et al., 2006a, 2005) (Fig. 2d and 2e). The minimum time to reach 100 CFU/g was 270 h (Fig. 2d) and 240 h (Fig. 2e), respectively, and mean values of 415 h and 421 h were obtained. In both cases more than 9800 simulated growth curves did not reach 100 CFU/g or contained zero cells initially. The observed growth of *L. monocytogenes* was distributed in the upper 5% of the simulated growth curves and in some cases concentrations of 100 CFU/g were reached before (after approximately 187 h) the minimum simulated time. Growth simulations of LAB were almost identical for all evaluated pre-cultures (Fig. 2a-2e, blue) and the observed growth was distributed within or close to the 50% CI of the simulated growth curves.

Growth simulations of LAB and *L. monocytogenes* in cottage cheese at 7.47°C ± 0.24, using the lag time variability for populations as reported by Ross (1999) displayed minimum time to reach

100 CFU/g of 120 h and a mean time of 270 h. Of 10000 iterations, 5336 growth curves did not reach concentrations of 100 CFU/g or did not contain any *L. monocytogenes* cells initially. The observed, pooled, growth data were distributed primarily within the 50% CI of the simulated growth (Fig. 3).

3.4 Simulation of scenarios related to production and distribution of cottage cheese

The effect of a storage temperature of 5°C with little variability (SD of ± 0.25) (Scenario A) was evaluated in relation to growth of LAB and *L. monocytogenes* in cottage cheese (Fig. 4). Of 10000 repetitions, more than 70% did not reach a concentration of 100 CFU/g or contained zero cells initially. The mean time until 100 cells for the growth curves reaching that concentration was 397 h and in the most extreme case, 100 CFU/g was reached after 220 h. Similarly, the effect of decreased product pH was evaluated (Scenario B, Fig. 5). A systematic, downwards shift of 0.2 pH units resulted in a minimum time to reach 100 *L. monocytogenes* cells/g of 270 h and the mean time was 441 h during storage at 5°C ± 0.25. Approximately 95% of the simulated growth curves did not reach the critical limit of 100 CFU/g within 500 h or the product contained zero cells initially.

The third evaluated scenario focused on the growth response of very low numbers of *L. monocytogenes* cells in cottage cheese (Fig. 6a and 6b). At 5°C ± 0.25, none of the simulated growth curves, starting from one *L. monocytogenes* cell per 450 g of cottage cheese (Fig. 6a), reached 100 CFU/g within the storage period of 500 h. Identical results were obtained for concentrations of one *L. monocytogenes* cell per 125.0 g cottage cheese during 500 h at 5°C ± 0.25.

Discussion

As described by several authors, accurate prediction of lag time duration has been challenging for food modellers for many years (Baranyi, 2002; McKellar, 1997; McMeekin et al., 2002; Robinson et al., 1998). The poor predictability of the lag time has primarily been attributed to the unknown physiological state of the bacterial cells at the point of contamination (Baty and Delignette-Muller, 2004; Robinson et al., 1998). One approach to handle lag time has been to omit it from the predictive models (McMeekin et al., 2002) and thereby apply a conservative modelling approach assuming that cells initiate growth immediately after contamination. However, exorbitant conservatism is inappropriate since the predictive models should provide a realistic estimate of the bacterial behaviour in a food product (Couvert et al., 2010; McMeekin, 2007). A range of studies have been conducted in order to quantify lag time variability between individual cells in laboratory media-based systems by absorbance measurements (Francois et al., 2006a, 2005; Guillier et al., 2005; Métris et al., 2003; Smelt et al., 2002; Stephens et al., 1997), in flow chamber (Métris et al., 2005; Pin and Baranyi, 2006) and by microscopy (Koutsoumanis and Lianou, 2013). Application of variable lag time data from broth based systems to predict growth responses in food products has previously been attempted with inconclusive findings. Francois et al. (2006b) modelled growth of

individual *L. monocytogenes* cells on liver pâté and cooked ham based on lag time data from Francois et al. (2006a). Their results confirmed that broth based lag time variability data could be used to predict growth responses on food and especially the predicted growth response on liver pâté, despite being slightly too fast, was in agreement with observed growth. For cooked ham, the over-prediction was more pronounced. Manios et al. (2013) also conducted growth simulations of individual *L. monocytogenes* cells using the lag time data from Francois et al. (2006a). Their conclusion was, however, that broth-based data exhibited poor transferability to food products and they obtained either too fast- or too slow growth when simulating growth of *L. monocytogenes* on lettuce and in cabbage at 7°C. The predicted growth of *L. monocytogenes* in cottage cheese corresponded satisfactorily with the observed growth of pre-culture 1 (Fig. 2a, adapted cells) and pre-culture 2 (Fig. 2b and 2c, starved cells) whereas predicted lag times of pre-culture 3 (Fig. 2d and 2e, cells from high to low pH) were too long leading to fail-dangerous predictions. The preparation of pre-culture 1 and 2 was highly similar to the preparation of the pre-cultures used to collect the lag time data (Guillier et al., 2005) whereas larger differences existed between pre-culture 3 and the pre-culture from Francois et al. (2006a) and Francois et al. (2005). Generally, the observed growth was located within the 50% CI (Fig. 2a and 2b) which would be expected since the particular product used in the challenge test displayed product characteristics (pH and lactic acid) close to the mean values of the input data (see Sections 3.1.2 and 3.2). For pre-culture 1 and 3 an apparent systematic difference between the enumeration methods (MLE and plate counts) existed, which may be explained by the size of the likelihood intervals of the MLE's (Table 3). In order to decrease the uncertainty of the MLE, a large increase in the number of replicates of each sample is required. However, the agreement between simulated growth of *L. monocytogenes* in cottage cheese, based on broth-based lag time data used in combination with the *RLT*-concept, and observed growth of *L. monocytogenes* suggest that the applied method may be suitable to describe growth responses in food products. Despite the fact that we obtained good agreement between observed growth of *L. monocytogenes* cells with a known prehistory and simulations using data obtained from cells of identical physiological state, the unknown physiological state of contaminating cells is a perennial problem in relation to lag time prediction. With the currently available data for variability of individual cell lag time, only predictions of worst case (e.g. adapted cells), best case (e.g. very stressed cells) or "something in between" is possible. It has been advocated to use variable input data for lag time predictions for population growth (McMeekin, 2007; McMeekin et al., 2002; Ross, 1999) in order to account for the unknown physiological state of the cells. Lag time duration in a bacterial population is determined by the fastest growing fraction (Baranyi, 1998) and the variability between cells is therefore of less relevance. Using the population *RLT*-values (Ross, 1999) to simulate growth of individual *L. monocytogenes* cells in cottage cheese provided a reasonable estimate of the observed growth when combining all observations regardless of pre-culture method (Fig. 3). Similarly, Mejlholm et al. (2014) obtained good estimates of the lag time

duration of *L. monocytogenes* in naturally contaminated cold smoked salmon and cold smoked Greenland halibut (initial *L. monocytogenes* concentrations of -0.12 ±0.44 and -0.49 ± 0.11 log CFU/g) when applying a fixed *RLT*-value of 3.0 in an otherwise stochastic model. Applying observed population lag time variability to predict individual cell lag time duration is, however, a somewhat empirical approach since Baranyi (1998) showed mathematically that the population lag would always be shorter than the average individual lag and Kutalik et al. (2005) demonstrated that the shape of an individual cell lag time distribution could not be inferred from population growth curves. We do, however, suggest that population *RLT* distributions can be used as a qualified default assumption if no lag time data is available for individual cells of *L. monocytogenes*.

In future, lag time data from naturally contaminated products or from challenge tests with low inoculum and different preparations of inoculum could be collected in a similar way as done by Ross (1999) for population lag time. In that way, lag time data used in combination with the *RLT*-concept, could be applied to predict representative variability in lag time duration for cells with unknown physiological state.

The nature and the representation of input data in stochastic models should also be considered. Distributions describing input data may have infinitely long tails leading to inadvertent extrapolation of the simulation model (Ross and McMeekin, 2003). To avoid extreme and unrealistic input variables, Ross and McMeekin (2003) suggested that distributions should be truncated with upper- or lower bounds in order to match the interpolation range. On the other hand, Vose (2000) recommended to refrain from adding constraints to the distributions and rather consider alternative distributions to represent data. There is no rule of thumb on when the quantity of data is sufficient to provide a reliable distribution fit, except that the confidence in the choice of probability distribution increases with increasing sample size (US EPA, 2001). An alternative to distribution fitting may be to bootstrap sample from observed data, where individual observations from the original dataset are randomly sampled with replacement (Grunkemeier and Wu, 2004). For the simulation of *L. monocytogenes* growth in cottage cheese, bootstrapping was used to generate input data for pH, lactic acid, and initial and final concentration of LAB. This approach allowed potential correlations between e.g. initial LAB concentration, lactic acid, and pH to be accounted for based on observed data. Furthermore, only observed values were used and no non-observed extreme values were used in the simulation process. From the simulation of scenario A (Fig. 4a) it was evident that lag time duration and input data for product characteristics affected the final concentration of *L. monocytogenes* whereas the initial number of *L. monocytogenes* cells had less impact on the concentration at the end of storage time (500 h). Food processors, routinely, analyse their final products for e.g. pH, organic acids, LAB concentration etc. in order to document compliance with product specifications and requirements (Vasconcellos, 2003). As demonstrated, such collected data is well suited to be used in a stochastic modelling process by sampling from the empirical distributions for product variability. This approach provides realistic information on the

impact of product variability over time, on the growth response of, for instance, *L. monocytogenes* in a given food product.

The deterministic growth model with stochastic input values was used to evaluate scenarios (A, B and C; Fig. 4, 5, and 6) assessed to be of relevance in the production of cottage cheese. A systematic pH decrease of 0.2 pH units was assessed to be an efficient tool to control *L. monocytogenes* growth in cottage cheese (Fig. 5). A similar conclusion was reached using a purely deterministic population growth model (Østergaard et al., 2014a). It may, however, not be feasible to decrease product pH due to adverse effects on product characteristics (Walstra et al., 2005) and alternative preservation methods may be required such as addition of sorbic acid. Low temperature was not, in itself, sufficient to control *L. monocytogenes* in cottage cheese (Fig. 4). Furthermore, it is not recommended to solely rely microbial safety of a food product on storage temperature alone, since several studies have reported a risk of temperature abuse during distribution, retail, and domestic storage (Jol et al., 2006; Kennedy et al., 2005; Likar and Jevšnik, 2006; Marklinder et al., 2004; Sergelidis et al., 1997).

Evaluation of very low contamination levels demonstrated that during refrigerated storage and provided that the food safety criteria during processing is met (absence in 5 x 25.0 g), cottage cheese complies with the food safety criteria of <100 CFU/g for *L. monocytogenes*, throughout the shelf-life of approximately 360 h (15 days) and up to >500 h of storage (Fig. 6b).

The performed simulations exclusively focused on variability in product characteristics, initial concentrations of *L. monocytogenes* and LAB and variability in the lag time duration, expressed by *RLT*-values, for both LAB populations and individual cells of *L. monocytogenes*. The simulated growth of cells with a known pre-history provided good agreement with observed growth. Our results suggest that the *RLT*-concept used in combination with lag time data for individual cells may be an appropriate method to predict lag time duration of individual cells. Furthermore, if single cell lag time is unavailable, the use of variable population *RLT*-data provided acceptable simulations and, if an empirical approach can be accepted, these data can be used for simulation purposes. The stochastic model presented in this study has the potential to provide growth estimates of low concentrations of *L. monocytogenes* in cottage cheese, taking variability into consideration. We do, however, suggest that the model is evaluated further at different intrinsic and extrinsic conditions to confirm its accuracy.

References

Adams, M.R., Moss, M.O., 2000. Food Microbiology, Second. ed. Royal Society of Chemistry.

AOAC, 1990. AOAC Official methods of analysis, method 950.46.

Augustin, J.-C., Ferrier, R., Hezard, B., Lintz, A., Stahl, V., 2014. Comparison of individual-based modeling and population approaches for prediction of foodborne pathogens growth. Food Microbiology. DOI: 10.1016/j.fm.2014.04.006

Augustin, J.-C., Zuliani, V., Cornu, M., Guillier, L., 2005. Growth rate and growth probability of *Listeria monocytogenes* in dairy, meat and seafood products in suboptimal conditions. Journal of Applied Microbiology 99, 1019–1042.

Baranyi, J., 1998. Comparison of Stochastic and Deterministic Concepts of Bacterial Lag. Journal of Theoretical Biology 192, 403–408.

Baranyi, J., 2002. Stochastic modelling of bacterial lag phase. International Journal of Food Microbiology 73, 203-206.

Baranyi, J., Pin, C., 1999. Estimating bacterial growth parameters by means of detection times. Applied and Environmental Microbiology 65, 732–736.

Baty, F., Delignette-Muller, M., 2004. Estimating the bacterial lag time: which model, which precision? International Journal of Food Microbiology 91, 261-277.

Beumer, R., Te Giffel, M.C., de Boer, E., Rombouts, F.M., 1996. Growth of *Listeria monocytogenes* on sliced cooked meat products. Food Microbiology 13, 333–340.

Bolton, L.F., Frank, J.F., 1999. Defining the growth/no-growth interface for *Listeria monocytogenes* in Mexican-style cheese based on salt, pH, and moisture content. Journal of Food Protection 62, 601–609.

Couvert, O., Pinon, A., Bergis, H., Bourdichon, F., Carlin, F., Cornu, M., Denis, C., Gnanou Besse, N., Guillier, L., Jamet, E., 2010. Validation of a stochastic modelling approach for *Listeria monocytogenes* growth in refrigerated foods. International Journal of Food Microbiology 144, 236–242.

Dalgaard, P., 2009. Chapter 10: Modelling of Microbial Growth, in: Bulletin of the International Dairy Federation A Revolution in Food Safety Management. pp. 45–57.

Dalgaard, P., Jørgensen, L. V, 2000. Cooked and brined shrimps packed in a modified atmosphere have a shelf-life of > 7 months at 0° C, but spoil in 4–6 days at 25° C. International Journal of Food Science & Technology 35, 431–442.

Davidson, C.M., Cronin, F., 1973. Medium for the selective enumeration of lactic acid bacteria from foods. Applied Microbiology 26, 439.

EC, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, Official Journal of the European Union.

EFSA, 2013. Analysis of the baseline survey on the prevalence of *Listeria monocytogenes* in certain ready-to-eat foods in the EU, 2010-2011 Part A: *Listeria monocytogenes* prevalence estimates. EFSA Journal 11(6):3241, 1-75.

Ferrier, R., Hezard, B., Lintz, A., Stahl, V., Augustin, J.-C., 2013. Combining individual-based modeling and Food microenvironment descriptions to predict the growth of *Listeria monocytogenes* on smear soft cheese. Applied and Environmental Microbiology 79, 5870–5881.

Francois, K., Devlieghere, F., Smet, K., Standaert, A.R., Geeraerd, A.H., Van Impe, J.F., Debevere, J., 2005. Modelling the individual cell lag phase: effect of temperature and pH on the individual cell lag distribution of *Listeria monocytogenes*. International Journal of Food Microbiology 100, 41–53.

Francois, K., Devlieghere, F., Standaert, A.R., Geeraerd, A.H., Van Impe, J.F., Debevere, J., 2003. Modelling the individual cell lag phase. Isolating single cells: protocol development. Letters in Applied Microbiology 37, 26–30.

Francois, K., Devlieghere, F., Standaert, A.R., Geeraerd, A.H., Van Impe, J.F., Debevere, J., 2006a. Effect of environmental parameters (temperature, pH and a w) on the individual cell lag phase and generation time of *Listeria monocytogenes*. International Journal of Food Microbiology 108, 326–335.

Francois, K., Devlieghere, F., Uyttendaele, M., Standaert, A.R., Geeraerd, A.H., Nadal, P., Van Impe, J.F., Debevere, J., 2006b. Single cell variability of *L. monocytogenes* grown on liver pâté and cooked ham at 7°C: comparing challenge test data to predictive simulations. Journal of Applied Microbiology 100, 800–12.

Grunkemeier, G., Wu, Y., 2004. Bootstrap Resampling Methods: Something for Nothing? The Annals of Thoracic Surgery 77, 1142–1144.

Guillier, L., Augustin, J., 2006. Modelling the individual cell lag time distributions of *Listeria monocytogenes* as a function of the physiological state and the growth conditions. International Journal of Food Microbiology 111, 241-251.

Guillier, L., Pardon, P., Augustin, J.-C., 2005. Influence of stress on individual lag time distributions of Listeria monocytogenes. Applied and environmental microbiology 71, 2940–8.

Guillier, L., Stahl, V., Hezard, B., Notz, E., Briandet, R., 2008. Modelling the competitive growth between *Listeria monocytogenes* and biofilm microflora of smear cheese wooden shelves. International Journal of Food Microbiology 128, 51–57.

Hereu, A., Dalgaard, P., Garriga, M., Aymerich, T., Bover-Cid, S., 2014. Analysing and modelling the growth behaviour of *Listeria monocytogenes* on RTE cooked meat products after a high pressure treatment at 400MPa. International Journal of Food Microbiology 186C, 84–94.

Jol, S., Kassianenko, A., Wszol, K., Oggel, J., 2006. Issues in time and temperature abuse of refrigerated foods. Food Safety 11, 30–32.

Jørgensen, L.V., Huss, H.H., 1998. Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. International Journal of Food Microbiology 42, 127–131.

Kennedy, J., Jackson, V., Blair, I.S., McDowell, D.A., Cowan, C., Bolton, D.J., 2005. Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators. Journal of Food Protection 68, 1424–1430.

Koutsoumanis, K., Pavlis, A., Nychas, G.-J.E., Xanthiakos, K., 2010. Probabilistic model for *Listeria monocytogenes* growth during distribution, retail storage, and domestic storage of pasteurized milk. Applied and Environmental Microbiology 76, 2181–91.

Koutsoumanis, K.P., Lianou, A., 2013. Stochasticity in colonial growth dynamics of individual bacterial cells. Applied and Environmental Microbiology 79, 2294–301.

Kozak, J., Balmer, T., Byrne, R., Fisher, K., 1996. Prevalence of *Listeria monocytogenes* in foods: Incidence in dairy products. Food Control 7, 215–221.

Kutalik, Z., Razaz, M., Elfwing, A., 2005. Stochastic modelling of individual cell growth using flow chamber microscopy images. International Journal of Food Microbiology 105, 177-190.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P., Thuault, D., 2002. Modelling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. International Journal of Food Microbiology 73, 219–237.

Le Marc, Y., Valík, L., Medveďová, A., 2009. Modelling the effect of the starter culture on the growth of *Staphylococcus aureus* in milk. International Journal of Food Microbiology 129, 306–311.

Likar, K., Jevšnik, M., 2006. Cold chain maintaining in food trade. Food Control 17, 108–113.

Manios, S., Konstantinidis, N., Gounadaki, A.S., Skandamis, P., 2013. Dynamics of low (1–4 cells) vs high populations of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut salads and their sterile liquid or solidified extracts. Food Control 29, 318–327.

Marklinder, I.M., Lindblad, M., Eriksson, L.M., Finnson, A.M., Lindqvist, R., 2004. Home storage temperatures and consumer handling of refrigerated foods in Sweden. Journal of Food Protection 67, 2570–2577.

Marsili, R.T., Ostapenko, H., Simmons, R.E., Green, D.E., 1981. High performance liquid chromatographic determination of organic acids in dairy products. Journal of Food Science 46, 52–57.

McKellar, R.C., 1997. A heterogeneous population model for the analysis of bacterial growth kinetics. International Journal of Food Microbiology 36, 179–186.

McLauchlin, J., Mitchell, R.T., Smerdon, W.J., Jewell, K., 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. International Journal of Food Microbiology 92, 15–33.

McMeekin, T.A., 2007. Predictive microbiology: quantitative science delivering quantifiable benefits to the meat industry and other food industries. Meat Science 77, 17–27.

McMeekin, T.A., Olley, J., Ratkowsky, D.A., Ross, T., 2002. Predictive microbiology: towards the interface and beyond. International Journal of Food Microbiology 73, 395–407.

Mejlholm, O., Bøknæs, N., Dalgaard, P., 2014. Development and validation of a stochastic model for potential growth of *Listeria monocytogenes* in naturally contaminated lightly preserved seafood. Food Microbiology. DOI: 10.1016/j.fm.2014.06.006

Mejlholm, O., Dalgaard, P., 2009. Development and validation of an extensive growth and growth boundary model for *Listeria monocytogenes* in lightly preserved and ready-to-eat shrimp. Journal of Food Protection 72, 2132–2143.

Métris, A., George, S.M., Peck, M.W., Baranyi, J., 2003. Distribution of turbidity detection times produced by single cell-generated bacterial populations. Journal of Microbiological Methods 55, 821–827.

Métris, A., Le Marc, Y., Elfwing, A., Ballagi, A., Baranyi, J., 2005. Modelling the variability of lag times and the first generation times of single cells of *E. coli*. International Journal of Food Microbiology 100, 13–9.

Pin, C., Baranyi, J., 2006. Kinetics of single cells: observation and modeling of a stochastic process. Applied and Environmental Microbiology 72, 2163–9.

Pouillot, R., Miconnet, N., Afchain, A.-L., Delignette-Muller, M.L., Beaufort, A., Rosso, L., Denis, J.-B., Cornu, M., 2007. Quantitative risk assessment of *Listeria monocytogenes* in French cold-smoked salmon: I. Quantitative exposure assessment. Risk Analysis 27, 683–700.

R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org.

Robinson, T.P., Ocio, M.J., Kaloti, A., Mackey, B.M., 1998. The effect of the growth environment on the lag phase of *Listeria monocytogenes*. International Journal of Food Microbiology 44, 83–92.

Ross, T., 1999. Chapter 5: Quantifying and utilising lag times in predictive microbiology, in: Predictive Microbiology for the Meat Industry. Meat and Livestock Australia, pp. 141–176.

Ross, T., McMeekin, T.A., 2003. Modeling microbial growth within food safety risk assessments. Risk Analysis 23, 179–197.

Sergelidis, D., Abrahim, A., Sarimvei, A., Panoulis, C., Karaioannoglou, P., Genigeorgis, C., 1997. Temperature distribution and prevalence of *Listeria* spp. in domestic, retail and industrial refrigerators in Greece. International Journal of Food Microbiology 34, 171–177.

Smelt, J., Otten, G., Bos, A., 2002. Modelling the effect of sublethal injury on the distribution of the lag times of individual cells of *Lactobacillus plantarum*. International Journal of Food Microbiology 73, 207-212.

Standaert, A.R., Francois, K., Devlieghere, F., Debevere, J., Van Impe, J.F., Geeraerd, A.H., 2007. Modeling individual cell lag time distributions for *Listeria monocytogenes*. Risk Analysis 27, 241–254.

Stephens, P.J., Joynson, J.A., Davies, K.W., Holbrook, R., Lappin-Scott, H.M., Humphrey, T.J., 1997. The use of an automated growth analyser to measure recovery times of single heat-injured *Salmonella* cells. Journal of Applied Microbiology 83, 445–455.

Tenenhaus-Aziza, F., Daudin, J.-J., Maffre, A., Sanaa, M., 2014. Risk-based approach for microbiological food safety management in the dairy Industry: The case of *Listeria monocytogenes* in soft cheese made from pasteurized milk. Risk Analysis 34, 56–74.

Thomas, H.A., 1942. Bacterial densities from fermentation tube tests. Journal of American Water Works Association 34, 572–576.

US EPA, 2001. Risk Assessment Guidance for Superfund (RAGS) Volume III - Part A: Process for Conducting Probabilistic Risk Assessment, Appendix B.

Vasconcellos, A., 2003. Introduction, in: Quality Assurance for the Food Industry: A Practical Approach. CRC Press, pp. 1–18.

Vose, D., 2000. Risk analysis A quantitative guide, 2nd ed. John Wiley & Sons, Ltd.

Vose, D., 2010. Fitting distributions to data, www.vosesoftware.com (accessed 7.12.14).

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2005. Cheese Varieties, in: Dairy Science and Technology, Second Edition, Food Science and Technology. CRC Press, pp. 687–741.

Wijtzes, T., Rombouts, F.M., Kant-Muermans, M.L.T., Van't Riet, K., Zwietering, M.H., 2001. Development and validation of a combined temperature, water activity, pH model for bacterial growth rate of *Lactobacillus curvatus*. International Journal of Food Microbiology 63, 57–64.

Østergaard, N.B., Dalgaard, P., Eklöw, A., 2014a. Modelling the effect of lactic acid bacteria from starter- or aroma culture on growth of *Listeria monocytogenes* in cottage cheese. International Journal of Food Microbiology 188, 15-25.

Østergaard, N.B., Dalgaard, P., Eklöw, A., 2014b. Semi-mechanistic and empirical modelling of interaction between lactic acid bacteria and *Listeria monocytogenes* in cottage cheese. International Journal of Food Microbiology [FOOD-D-14-00412]

Tables and Figures

Table 1 Datasets of related variable input data obtained from analysed cottage cheese.

No	Sample	Replicate	Lactic acid (WP ppm)	pH	$LAB\ N_0$ (log CFU/g)	$LAB\ N_{max}$[a] (log CFU/g)	$LM\ N_{max}$[b] (log CFU/g)
1	1	a	1156	5.44	6.54	8.48	8.50
2	1	b	1297	5.45	6.69	8.48	8.50
3	1	c	1334	5.44	6.66	8.48	8.50
4	1	d	1206	5.43	6.59	8.48	8.50
5	2	a	1180	5.44	6.54	8.29	8.50
…	…	…	…	…	…	…	…
…	…	…	…	…	…	…	…
99	13	ax	975	5.32	5.81	8.51	8.50
100	13	ay	1293	5.32	5.81	8.51	8.50

[a] In experiments where LAB N_{max} was not reached, average N_{max} values were used
[b] Theoretical maximum population density of *L. monocytogenes*

Table 2 Lag time distributions for *L. monocytogenes* as reported in the literature.

Conditions	Distribution type	Distribution parameters	Reference
Late exponential phase from high temperature 7°C pH = 5.47 BHI-broth Generation time = 7.0 h [6.79-7.20] = 0.099 h^{-1} [0.102-0.096] Cell stress level comparable to pre-culture 3	Weibull Lag times	α = 4.290 β = 119.4 Shift = 0	Francois et al., 2005
Starved cells 30°C pH = 7.1 a_w = 0.997 Observed growth rate = 0.90 h^{-1} Cell stress level comparable to pre-culture 2	Extreme value type II (c = 5) Lag times	a = -12.06 b = 17.74	Guillier and Augustin, 2006
Exponential growth phase cells 30°C in TSB-ye broth Observed GR = 0.90 h^{-1}	Extreme value type I Standardised detection times	a = 18.678 [18.674-18.682] b = 0.372 [0.367-0.376]	Guillier et al., 2005
Starved cells 30°C in TSB-ye broth Observed GR = 0.90 h^{-1} Cell stress levels comparable to pre-culture 1 and pre-culture 2	Gamma Standardised detection times	a = 19.49 [18.96-19.66] b = 3.98 [2.65-5.60] c = 1.93 [1.21-3.33]	
Late exponential phase from high temperature 7°C pH 5.54 a_w = 0.995 Mean generation time 7.35 h ± 1.23 = 0.094 h^{-1} Cell stress level comparable to pre-culture 3	Weibull Lag times	α = 4.258 β = 146.44	Francois et al., 2006a
Data from 25 different sources on *L. monocytogenes* population growth in food products. A total of 283 observations were used to fit distribution.	Exponential *RLT*-values	Rate = 0.27 Std. error = 0.02	Ross, 1999

Table 3 95% likelihood intervals (LI) for the MLE estimates of the initial *L. monocytogenes* concentration in inoculated cottage cheese.

Sample vol. (ml)	Number of negative tubes	Total number of tubes	MLE estimate[a]	Lower bound of 95% LI	Upper bound of 95% LI
1	1	8	10.40	4.04	24.41
1	2	8	6.93	2.63	15.33
1	3	8	4.90	1.71	11.05
1	4	8	3.47	1.06	8.27
1	5	8	2.35	0.58	6.18
1	6	8	1.44	0.24	4.47
1	7	8	0.67	0.04	2.95
2	1	8	5.20	2.02	12.20
2	2	8	3.47	1.31	7.67
2	3	8	2.45	0.86	5.53
2	4	8	1.73	0.53	4.14
2	5	8	1.18	0.29	3.09
2	6	8	0.72	0.12	2.24
2	7	8	0.33	0.02	1.47

[a] CFU/ml, Calculated from Eq. (3)

Table 4 Cardinal parameter values of secondary growth models for lactic acid bacteria and *L. monocytogenes* in cottage cheese from Østergaard et al. (2014a). Parameters were used in combination with Eq. (5).

	Cardinal parameter values	
	Lactic acid bacteria	*L. monocytogenes*
μ_{ref} (h^{-1})	0.57	0.34
T_{min} (°C)	3.69 ± 0.38[a]	-2.01 ± 0.40[a]
pH_{min}	3.87 ± 0.05[a]	4.87 ± 0.01[a]
pH_{max}	7.23 ± 0.17[a]	-
$a_{w, min}$	0.928 ± 0.003[b,c]	0.923[d]
$MIC_{U\,Lactic\,acid}$ (mM)	9.72 ± 0.47[a]	3.79[d]
n1	1	1
n2	1	2
$MIC_{U\,Sorbic\,acid}$ (mM)	5.50 ± 0.36[a]	1.90[d]
n1	0.5	1
n2	2	1

[a] standard error on fitted model parameter value
[b] 95% confidence interval on parameter value
[c] Model parameter estimate from Wijtzes et al. (2001)
[d] Model parameter estimate from Mejlholm and Dalgaard (2009)

Table 5 *RLT*-values calculated from literature data for lag time duration of individual cells.

Distribution/Reference	Minimum *RLT*-value[a]	Mean *RLT*-value[a]	Maximum *RLT*value[a]
Weibull, Francois et al., 2005	1.9	15.5	28.2
Weibull, Francois et al., 2006a	1.0	18.1	37.5
Extreme Value II, Guillier and Augustin, 2006	7.4	13.1	112.7
Extreme Value I, Guillier et al., 2005[b]	-5.1	-0.1	1.2
Shifted Gamma, Guillier et al., 2005	1.2	11.2	61.5
Exponential, Ross, 1999	0.0	3.7	42.9

[a] Based on 10000 iterations
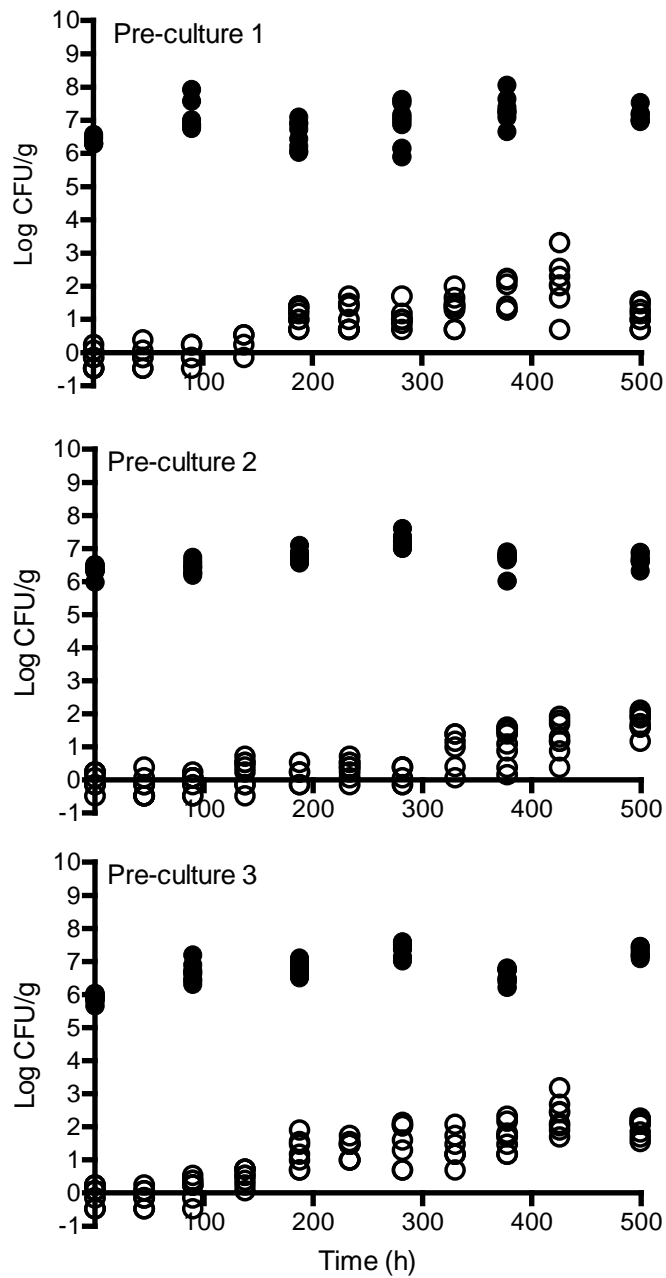[b] Data serve as reference and negative *RLT*-values were set to zero in the simulation model
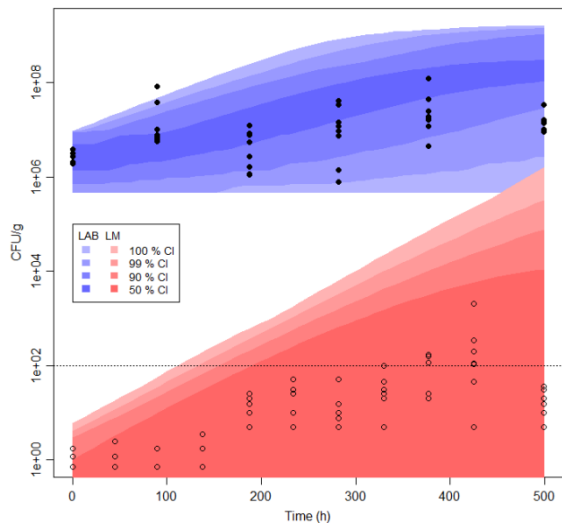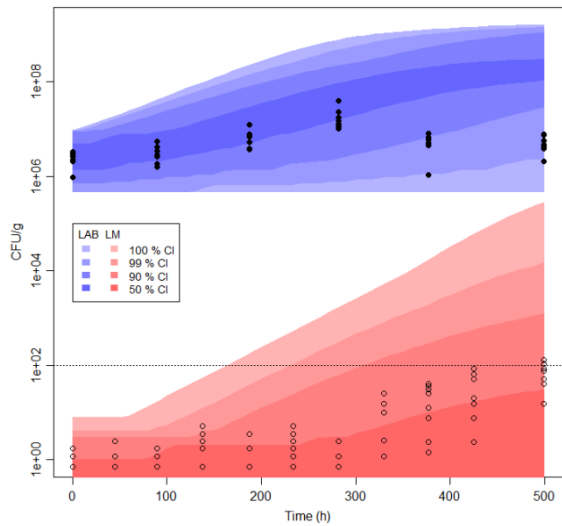
Figure 1 Observed growth of *L. monocytogenes* (○) (Pre-culture 1, 2 or 3) and LAB (●) in cottage cheese stored at 7.47°C ± 0.24. Eight replicates were analysed at each sampling point for both *L. monocytogenes* and LAB.

a

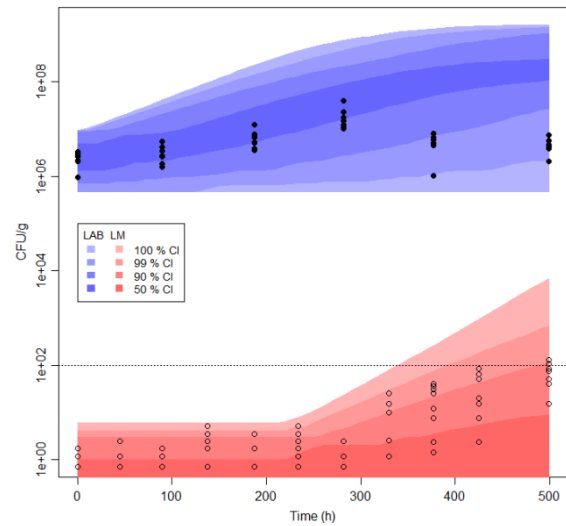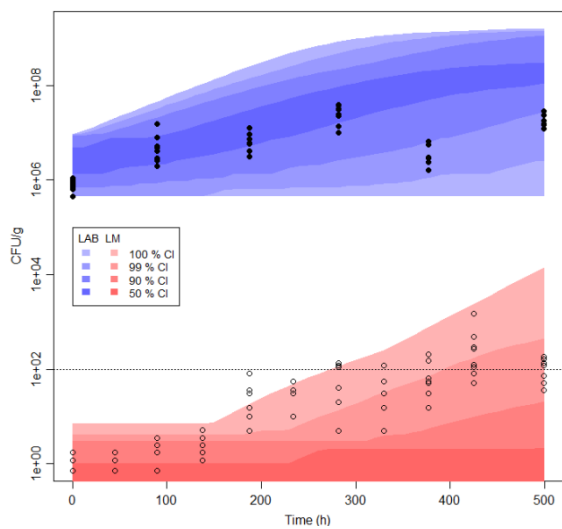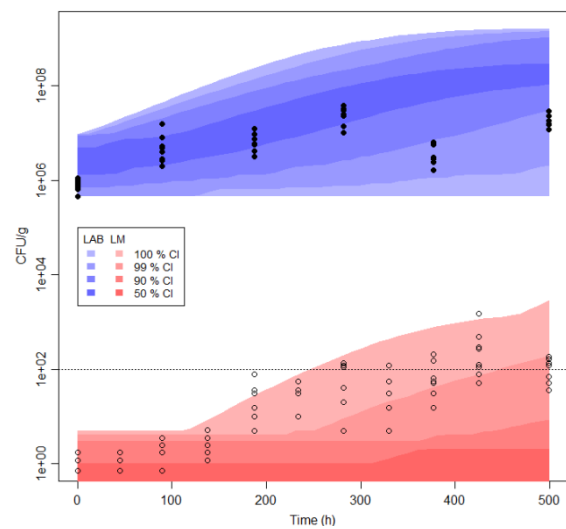Figure 2 Evaluation of simulated growth (n=10000) of *L. monocytogenes* (red and ○) and LAB (blue and ●) in cottage cheese stored at 8°C. Three differently prepared inoculums were evaluated. (a) adapted and exponentially growing cells, (b) and (c) starved cells and (d) and (e) cells transferred from 25°C to 8°C upon inoculation. *L. monocytogenes* single cell lag time distributions from literature (see Table 4) were used in combination with observed data for product variability, initial- and final concentration of LAB, initial concentration of *L. monocytogenes* and LAB *RLT*-values. Simulated growth is illustrated by the confidence intervals (50, 90, 99 and 100%). The dashed line indicates a concentration of 100 CFU/g.

b

c

d

e
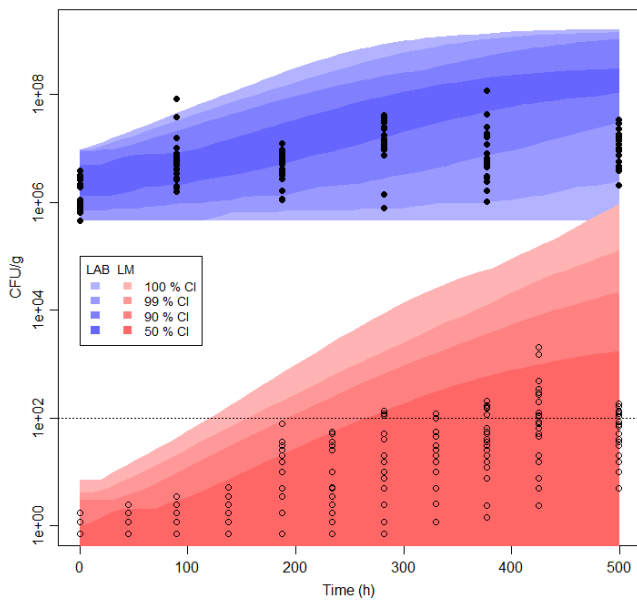
Figure 3 Simulated growth (n=10000) of *L. monocytogenes* (red and ○, Pre-culture 1,2 and 3 combined) and LAB (blue and ●) in cottage cheese stored at 8°C. *L. monocytogenes* lag time duration was predicted using (population) *RLT*-values as reported by Ross (1999) represented by an exponential distribution. The dashed line indicates a concentration of 100 CFU/g.

Figure 4 Simulated growth (n=10000) of *L. monocytogenes* (red) and LAB (blue) in cottage cheese stored at 5°C (N(5,0.25)). *L. monocytogenes* lag time duration was predicted using (population) *RLT*-values as reported by Ross (1999) represented by an exponential distribution. Full lines are random samples of the simulated growth curves showing that the highest initial concentrations do not necessarily lead to the highest concentration at the end of storage. The minimum time until a critical concentration of 100 CFU/g was 220 h, with a mean of 397 h and maximum of 490 h. Of the 10000 simulated growth curves, 7143 did not reach a concentration of 100 cells/g within the shelf-life of the product or contained zero cells initially.
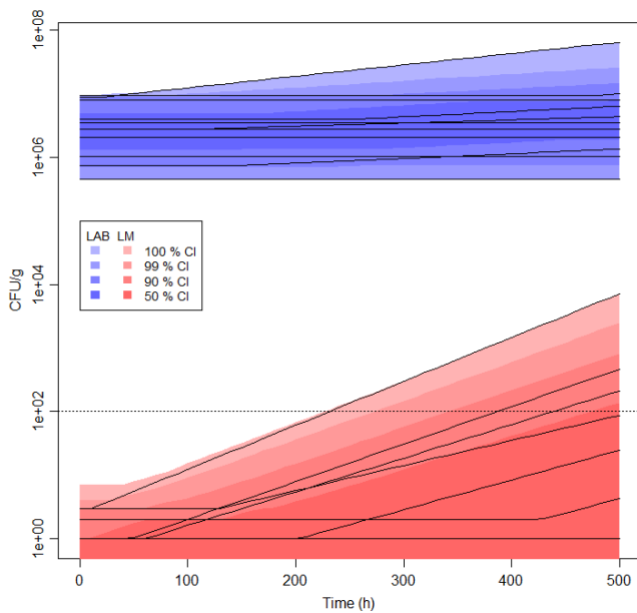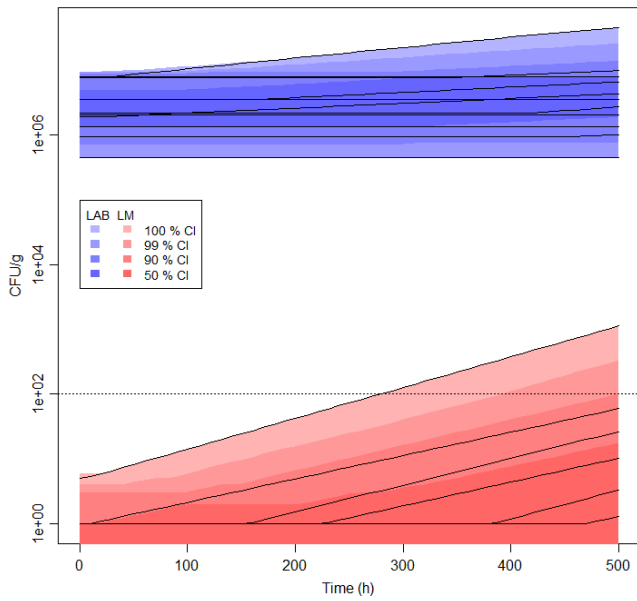
Figure 5 Simulated growth (n=10000) of *L. monocytogenes* (red) and LAB (blue) in cottage cheese stored at 5°C (N(5,0.25)) when initial pH is shifted 0.2 units left. *L. monocytogenes* lag time duration was predicted using (population) *RLT*-values as reported by Ross (1999) represented by an exponential distribution. Full lines are random samples of the simulated growth curves and the dashed line indicates a critical concentration of 100 CFU/g. The minimum time to reach 100 CFU/g was 270 h, and approximately 95% of the simulated growth curves did not reach the critical concentration or started with zero cells initially.

a   b
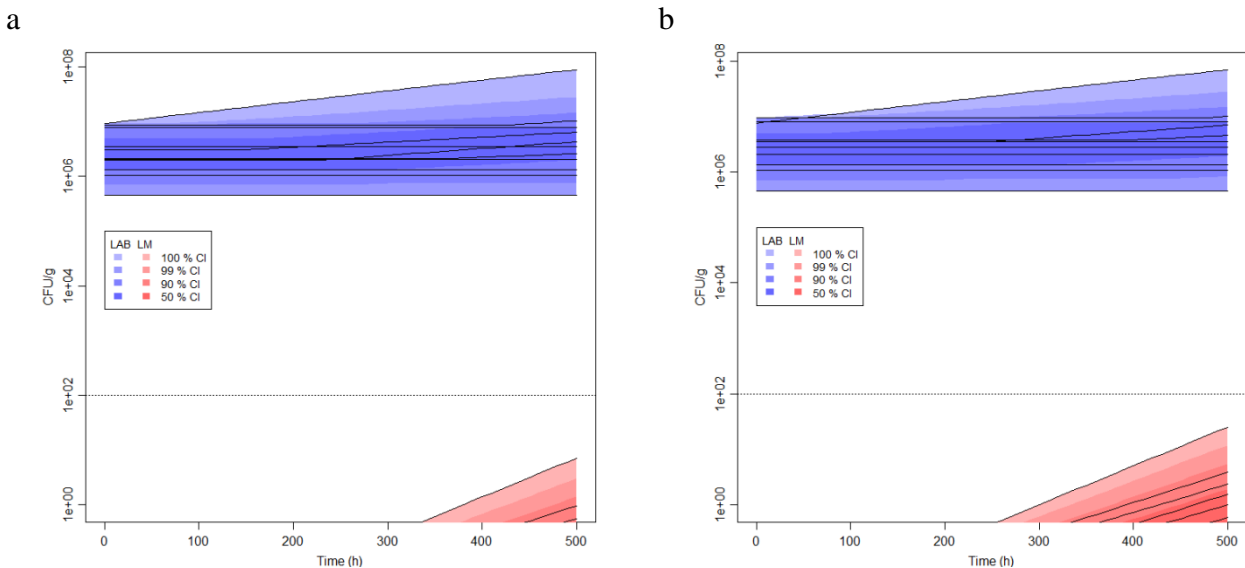
Figure 6 Simulated growth (n=10000) of (a) one *L. monocytogenes* (red) cell per 450 g (equals one large container) and (b) 0.008 CFU/g (red), corresponding to one *L. monocytogenes* cell per 125.0 g of product. Simulations were performed at 5°C (N(5.0,0.25)). For both concentrations (a and b) none of the simulated growth curves reached the critical concentration of 100 CFU/g during storage for 500 h, regardless of product- and lag time variability.

DISCUSSION

9. Discussion

9.1 A short outline

Throughout the present PhD-project, focus has been on cottage cheese with fresh- or cultured cream dressing and how to accurately predict growth of *L. monocytogenes* in this product during storage. As described in Paper I, no existing *L. monocytogenes* growth models were found to predict growth of *L. monocytogenes* in cottage cheese satisfactorily at the time of initiation of the present PhD-project. Based on this conclusion and the fact that *L. monocytogenes* was able to grow in the product (see Fig. 8 and Paper I and III), it was assessed that growth models should be developed or extended using new- or existing model parameters. Furthermore, it was evident that the interaction between LAB from the starter cultures and *L. monocytogenes* was important in this type of product and that it should be taken into account in the modelling procedure. Inclusion of the inhibitory effect of LAB has been suggested for fermented products (Augustin et al., 2005; Malakar et al., 2003), and performed for inoculated and naturally contaminated food products (Gimenez and Dalgaard, 2004; Mejlholm and Dalgaard, 2007a; Mejlholm et al., 2014). From authority incident and scientific reports it has been documented that contamination of e.g. cheeses with *L. monocytogenes* may occur sporadic and with low bacterial concentrations (EFSA, 2013) – Therefore it was highly relevant to evaluate the growth response of low bacterial concentrations in cottage cheese. That induced a need for a stochastic modelling approach (Francois et al., 2005; Guillier and Augustin, 2006; Pin and Baranyi, 2006) in order to account for lag time variability between bacterial cells. The numerous physico-chemical analyses performed on the product during the project, served as a suitable representation of product variability over time.
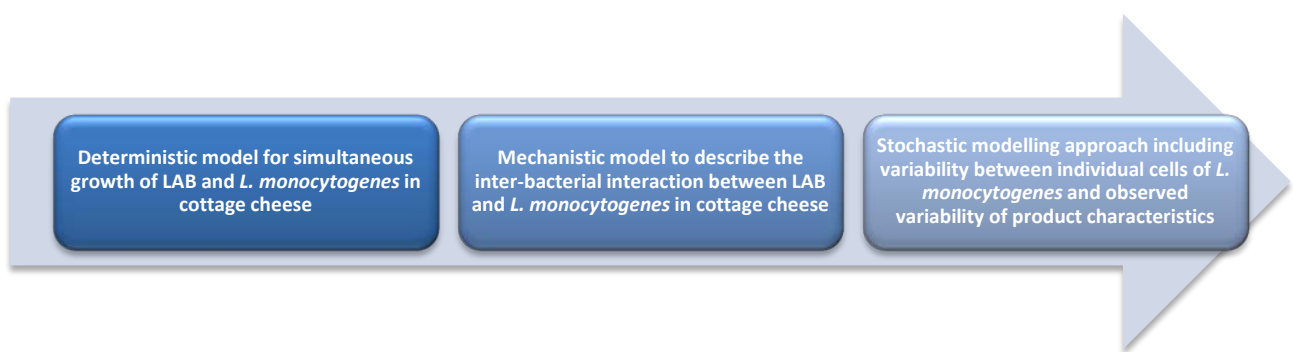
Figure 20 Modelling work-flow throughout the PhD-project. From deterministic model with inter-bacterial interaction via a mechanistic modelling approach to a stochastic model for low concentrations of *L. monocytogenes* in cottage cheese with variable product characteristics.

In brief, the work performed in the present project was done as an underpinning and supplementary process (Fig. 20) starting from the development of solid and validated deterministic growth models describing the simultaneous growth response of LAB and *L. monocytogenes* in cottage cheese with either fresh- or cultured dressing (Paper I). As the inter-bacterial interaction proved to be important in cottage cheese and more mechanistic modelling approaches had been

promoted by several authors (Bernaerts et al., 2004; Van Impe et al., 2005) it was a natural next step to analyse and model the formation of lactic acid and the related change in product pH. The procedure was rather laborious and required extensive amounts of data and analyses. It was therefore assessed valuable to compare the more simple and less resource demanding, albeit empirical, Jameson approach (Gimenez and Dalgaard, 2004) with the results obtained from the (semi)-mechanistic modelling approach (Paper II). The move from predicting *L. monocytogenes* population growth to prediction of growth of a few *L. monocytogenes* cells was performed by combining the solid and successfully validated secondary growth models with variable input data, hence making the model stochastic (Paper III). Focus was especially on the prediction of *L. monocytogenes* lag time duration from broth-based lag time data in combination with the *RLT*-concept. Including the stochastic elements in the deterministic model allowed an evaluation of the expected growth response of individual cells or very few bacterial cells in the product, taking observed product variability into account. The summarising discussion below will follow the papers more or less chronologically and discuss the findings of the studies and the applicability of the applied approaches in relation to other published studies. Finally, potential problems, challenges and possibilities, prompted by the present project, will be discussed.

9.2 Deterministic model for simultaneous growth of LAB and L. monocytogenes in cottage cheese

New, deterministic growth models for *L. monocytogenes* and LAB in cottage cheese were developed in order to facilitate prediction of simultaneous growth in cottage cheese during chilled storage (5-15°C). The model development and evaluation induced discussion of different topics including modelling of microbial interactions and the importance of such modelling approaches. During the model development, calibration of μ_{ref} became an essential step, introducing product or starter culture specificity in the *L. monocytogenes* growth models. The obtained results in relation to LAB lag-time variability supported the findings of other authors (Ross, 1999; Swinnen et al., 2004). Predicted lag times of the LAB cultures varied and could not exclusively be described by the use of a single *RLT*-value. As inter-bacterial interaction played an important role in the deterministic model, it was discussed whether alternative modelling approaches could be appropriate and this discussion gave rise to a second publication (Paper II) investigating the performance of a semi-mechanistic modelling approach vs. an empirical modelling approach. Growth predictions under constant temperatures were evaluated by the bias- and accuracy factors (Ross, 1996) and by the ASZ (Møller et al., 2013; Oscar, 2005; Velugoti et al., 2011) in order to evaluate the accuracy of the predicted growth rate but also the performance of the interaction model causing a growth inhibition of *L. monocytogenes* related to the growth of LAB. The combination of these methods was found suitable and provided essential information of the model performance. The general conclusion of the study was that inter-bacterial interactions should be included when predicting growth of *L.*

monocytogenes in products where LAB are present and able to grow. The applied methodology, including the combined use of simplified cardinal parameter models and the empirical Jameson term, was manageable and provided good and stable predictions of growth rate (μ_{max}, h^{-1}) and microbial interactions. The methodology was assessed to be applicable to other, similar product types requiring inclusion of microbial interactions.

As described, the calibration of μ_{ref} (h^{-1}) was decisive for the performance of the growth models. Calibration or refitting of μ_{ref} (h^{-1}) or μ_{opt} (optimal specific growth rate, h^{-1}) to specific food products has previously been applied in studies where modelling of growth in dairy products was performed. Te Giffel and Zwietering (1999) introduced an additional γ-factor in order to correct the predicted growth rates to obtain a bias-factor of 1.0. The corrective γ-factor was product dependent, and products such as egg (0.35), vegetables (0.35), milk (0.57) and dairy products (0.54) required this additional γ-factor. A similar approach was applied by Augustin et al. (2005) who evaluated the performance of a new cardinal parameter model. They estimated product specific μ_{opt}-values (h^{-1}) and for liquid dairy (non-fermented) the estimate was 0.74 h^{-1} ± 0.13 whereas the estimate for fermented cheese was 0.21 h^{-1} ± 0.19. For comparison, microbiological media and meat products displayed μ_{opt}-values of 1.05 h^{-1} ± 0.17 and 1.17 h^{-1} ± 0.35. Despite the calibration, they obtained highly variable predictions in cheese (A$_f$ > 3.5). Product dependent differences in μ_{opt}-values were also reported by Augustin and Carlier (2000). Compared to culture broth, seafood displayed identical μ_{opt}-estimates; meat was slightly higher whereas estimates in dairy products and liquid eggs were lower than the broth estimates. The results of Paper I in combination with the above mentioned examples suggest that calibration is an often required part of model development for fermented dairy products since the μ_{opt} or μ_{ref}-values are markedly different from those obtained in broth systems. In the case of cottage cheese, different types of the product (fresh or fermented cream dressing) required different μ_{ref}-values in the *L. monocytogenes* growth models. In this case it can therefore be argued that the μ_{ref}-value is starter-culture dependent rather than product dependent. This is further supported by the fact that cottage cheese with fresh cream dressing displayed systematically lower pH than cottage cheese with cultured cream dressing (Table 1, Paper I). Normally, and as demonstrated in the simulation of scenarios in Paper I and III, pH is regarded as an important factor in relation to bacterial growth (Montville and Matthews, 2007). Despite this, the calibrated μ_{ref}-value in cottage cheese with fresh cream dressing was higher than in cottage cheese with cultured cream dressing (Table 2, Paper I). This observation indicated that the LAB culture strongly influenced the growth potential for *L. monocytogenes* in this type of product and may be due to the effect of metabolic compounds, bacteriocins etc. as described by Irlinger and Mounier (2009). The development of the deterministic growth models demonstrated the importance of including the effect of LAB in order to obtain realistic predictions of the maximum population density of *L. monocytogenes* in community with LAB. But the effect of LAB was also evident

before inhibitory concentrations of LAB were reached, as demonstrated by the calibrated μ_{ref}-values in the *L. monocytogenes* growth models.

In Paper I, the evaluation of the deterministic LAB growth models was performed using average and maximum *RLT*-values due to the variability in the observed *RLT*-values (Table 2, Paper I). It has previously been suggested to use distributions to represent *RLT*-values (McMeekin et al., 2002; Ross, 1999) but in deterministic models, alternative approaches must be considered in order to address the poor predictability of bacterial lag time. The deterministic models from Paper I have been included in the Food Spoilage and Seafood Predictor (FSSP) software (http:/fssp.food.dtu.dk). In this user-interface (Fig. 21) growth in cottage cheese, with fresh- or cultured cream dressing, can be predicted by applying worst case *RLT*-values. That implies zero lag time for *L. monocytogenes,* and longer than average lag times for LAB. The inhibitory effect of LAB will thus be delayed and *L. monocytogenes* initiates growth immediately.

Figure 21 Worst-/"average" case approach to predicting LAB lag phase duration.
(From Food Spoilage and Safety Predictor, FSSP)

Similar approaches have been applied in ComBase (http://modelling.combase.cc) where the lag time duration can be adjusted by the physiological state parameter (with values between 0 and 1 where 0 result in infinite lag and 1 result in zero lag). In the Pathogen Modeling Program (PMP, http://pmp.errc.ars.usda.gov) growth predictions are presented with- and without lag. The results of the present PhD-project suggest that the application of worst case/"average" case approach to the prediction of lag time of LAB and *L. monocytogenes* provide appropriate estimates of growth during chilled storage. This procedure allows the user to decide whether a very conservative approach should be applied. Furthermore, worst case and "average" case scenarios can be compared, serving as a simplified approximation to the inclusion of lag-time variability in the predictions.

From the results of Paper I it was evident that *L. monocytogenes* grew in cottage cheese with fresh or cultured cream dressing (5-15°C) even though equivocal results has been presented in the literature (Chen and Hotchkiss, 1993; Ferreira and Lund, 1996; Gahan et al., 1996; Larson et al., 1996; Liu et al., 2008; McAuliffe et al., 1999). One reason may be the differences in product pH reported in the different studies. Inter-bacterial interaction was assessed to be important and

pronounced growth inhibition of *L. monocytogenes* was observed when LAB reached their maximum population density. However, during storage at low temperatures, the mesophilic LAB cultures either do not grow or grow slowly and growth of *L. monocytogenes* will not be inhibited by LAB growth. As demonstrated in Paper I and III, the cottage cheese growth models can be useful tools to evaluate the effect of different scenarios in order to assess the impact on food safety of different temperature scenarios, changed product pH, addition of preservatives (sorbic acid) and different initial concentrations of contaminating *L. monocytogenes*. Despite the fact that cottage cheese, with product characteristics as measured during the present PhD-project, support growth of *L. monocytogenes*, no dairy related outbreaks have been associated with cottage cheese in Denmark, the EU or the US (see Table 6) from the early 80's until present. Reasons for this are likely to be (i) low frequency of contamination due to extensive food safety management systems applied in the food/dairy industry (Papademas and Bintsis, 2010), (ii) bactericidal steps (pasteurisation) in the production. It has however been reported, that the incidence of *L. monocytogenes* was higher in European soft and semi-soft red smear cheese made from pasteurised milk compared to cheeses made from raw milk (Rudolf and Scherer, 2001). The potential risk of contamination *after* heat treatment should be addressed in the food safety management systems. (iii) *if* food products are contaminated with *L. monocytogenes* it is most likely to be only a few bacterial cells (EFSA, 2013). As illustrated in the evaluated scenario C in Paper III, contamination levels between one cell/container (450.0 g) and one cell/125.0 g of product will not lead to critical concentrations during refrigerated storage (shelf life of 15 days), not even for the worst case combination of product characteristics, storage temperature and lag-times (Fig. 6, Paper III).

As described in Paper I, cottage cheese can be produced from cheese curd and a fresh cream dressing or by adding a cultured cream dressing to the fermented cheese curd. Two independent, but known, LAB populations (from starter and aroma culture) are hence present in the latter product. Experiments were performed using KMK-agar (Kempler and McKay, 1980) to differentiate between citrate metabolising and non-citrate metabolising organisms in cottage cheese with cultured cream dressing. These results confirmed that the citrate metabolising aroma culture was dominating throughout the experiments within the temperature range of 5°C to 15°C. It could, however, be interesting to quantify the population dynamics during storage. Primarily a destinction between the "classic" starter microorganisms (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) and the citrate metabolising and diacetyl producing aroma culture (*Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*). With the available, culture based microbiological methods, identification of sub-populations is not possible. Even though the applied KMK agar facilitated separate identification of citrate metabolising and non-citrate metabolising organisms the sensitivity threshold was the limiting factor. Theoretically, the method should cover a span of 2 log units, meaning that a concentration of 6 log CFU/g of one culture and 4 log CFU/g of the other culture would be detectable. In reality, this may not be the case, and reliable identification

and quantification require that the concentrations are more identical. PCR-based methods do, however, facilitate identification of sub-populations also in the presence of other, dominating, populations (Postollec et al., 2011). Population dynamics of LAB has been studied during spontaneous sourdough fermentations (Van der Meulen et al., 2007) where dominating LAB strains were identified by PCR-DGGE (Denaturing Gradient Gel Electrophoresis). Camu et al. (2007) used an identical methodology to identify the dominating clusters of LAB and acetic acid bacteria during spontaneous fermentation of cocoa beans. A dairy related example was presented by Dolci et al. (2008) who investigated the LAB dynamics throughout the manufacture and ripening of the traditional Italian Castelmagno PDO (Protected Designation of Origin, EC, 2006) cheese where acidification was caused by indigenous LAB. Population dynamics was characterised by the frequency of isolation and molecular identification from the raw milk sample until 90 days of ripening. None of the above mentioned studies provided actual quantitative measures of the dominating population, but only focused on qualitative determination of the dominating populations over time. A more quantitative approach was applied by Grattepanche et al. (2005). They monitored the population of *Lactococcus lactic* supsp. *cremoris* in mixed culture with *Lactobacillus rhamnosus* and *Lactococcus lactic* subsp. *lactis* biovar. *diacetylactis* by the use of specific primers for *Lc. lactis* subsp. *cremoris*. Good agreement was obtained between real time qPCR results and plate counts in pure culture. In cottage cheese, the LAB originates from defined starter- and aroma cultures. Since different studies have presented qPCR primers specific for *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (Friedrich and Lenke, 2006; Ladero et al., 2012; Stevenson et al., 2006) it is assessed to be less complex to quantitatively determine the population dynamics in cottage cheese, compared to products with unknown microbiota, by molecular methods.

Due to the limited knowledge of the population dynamics in cottage cheese with cultured cream dressing, all predictions have been made using the LAB-aroma model (dominating population) in combination with the *L. monocytogenes* growth model. However, since the cheese curd has been fermented using a classic mesophilic O-culture, it is expected that residuals of the culture will be present in the product. Evaluation of a theoretical scenario, where the LAB model developed for the starter- and for the aroma culture are combined (Fig. 22), shows that at low temperatures (prediction performed at 8°C) the starter culture may be able to outgrow the aroma culture during storage (Fig. 22). However, care should be taken to draw any conclusions since the applied LAB-starter model was developed based on products without aroma culture. As demonstrated for *L. monocytogenes*, cottage cheese with aroma culture provided a poorer growth environment than cottage cheese with fresh cream dressing, illustrated by markedly different μ_{ref}-values after calibration (aroma: 0.34 and starter: 0.72). Reasons for this could, as described previously, be caused by the presence of metabolic compounds, bacteriocins, diacetyl etc. produced by the aroma culture (Irlinger and Mounier, 2009; Nilsson et al., 2005; Vinderola et al., 2002). The same could be in evidence for the LAB cultures as bacteriocins often inhibit closely related bacteria

(Cleveland et al., 2001; Morgan et al., 1997). It could be interesting and informative, from a microbiologist's point of view, to survey the population dynamics of LAB in cottage cheese with cultured cream dressing during storage. It is, however, important to focus on the actual value of this information in relation to the modelling process and the accuracy and usability of the developed model. When the aim is to accurately predict the growth response and maximum population density of *L. monocytogenes* in cottage cheese it may be sufficient to obtain a simple empirical description of the inter-bacterial interaction and related growth inhibition. As demonstrated in Paper I, II and III for cottage cheese with cultured cream dressing, growth predictions including inter-bacterial interactions between *L. monocytogenes* and aroma-LAB (dominating culture) were in good agreement with observed growth.
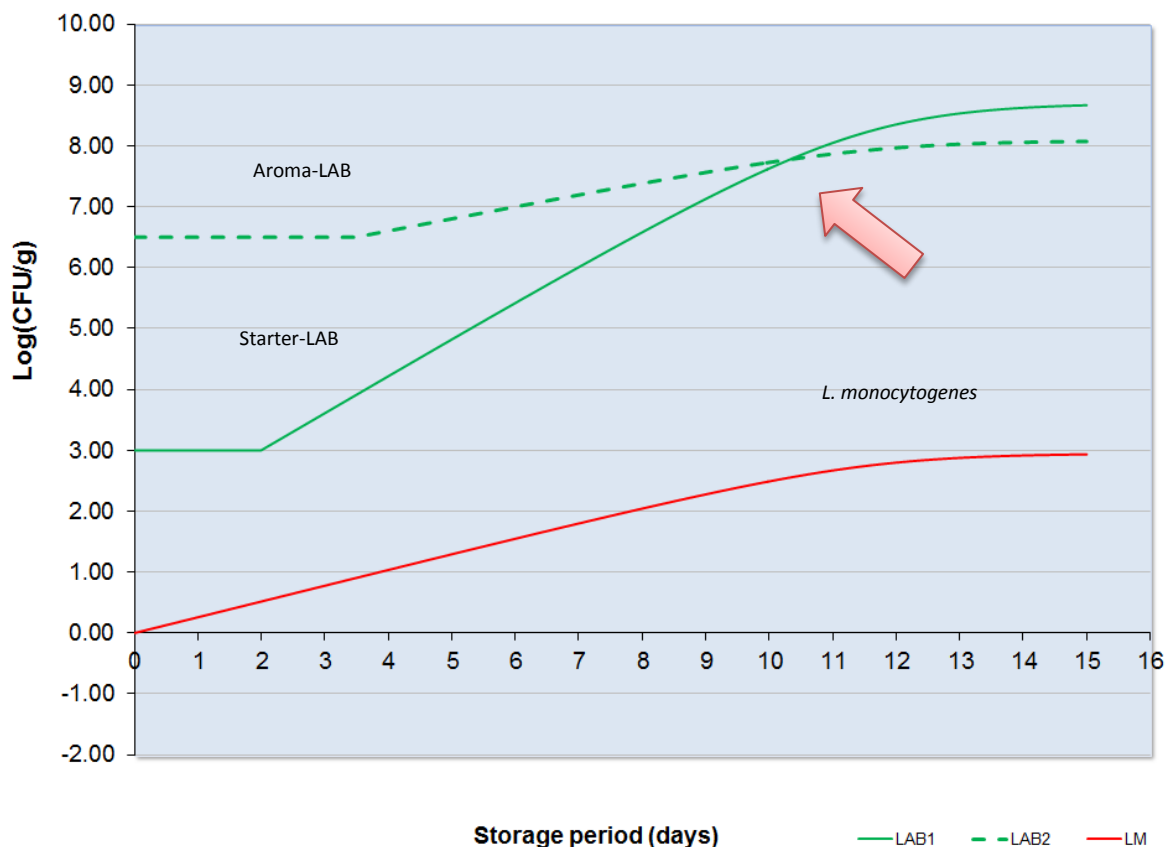
Figure 22 Theoretical scenario where growth of *L. monocytogenes* (—) and the combined growth of LAB from starter culture (—) and LAB from aroma culture (- - -) was predicted at 8°C, pH 5.3, 1.10 % water phase NaCl and 800 ppm lactic acid in the water phase. LAB lag phase was calculated using average *RLT*-values determined for each culture (Paper I).
Predictions were based on the *L. monocytogenes* growth model developed for cottage cheese with added aroma culture in combination with the growth models for starter-LAB and aroma-LAB.

9.3 Application of a (semi)-mechanistic modelling approach to simulate growth inhibition of L. monocytogenes in cottage cheese

The main objective was to model inter-bacterial interaction between LAB and *L. monocytogenes* in cottage cheese, focusing on the formation of lactic acid and the associated change in product pH. Subsequently, the performance of the semi-mechanistic modelling approach was evaluated against the performance of the empirical Jameson approach. The results confirmed that by using modelling approaches from fermentation technology (Bouguettoucha et al., 2011; Luedeking and Piret, 1959; Martens et al., 1999; Pirt, 1975) the observed growth inhibition of *L. monocytogenes,* in cottage cheese with fresh cream dressing, could be explained by dynamic lactic acid concentration and product pH. For cottage cheese with cultured cream dressing, dynamic lactic acid and product pH could not fully explain the observed growth inhibition (Figure 4g and 4e, Paper II). Additional causes of the growth inhibition were considered and factors as diacetyl and bacteriocin activity were discussed in relation to their relevance. Based on the existing knowledge, it is most likely that bacteriocin activity causes the additional growth inhibition. As demonstrated (Lanciotti et al., 2003) diacetyl concentrations well above the expected concentrations in fermented dairy products (Antinone et al., 1994) are required to inhibit growth of *L. monocytogenes*. In general, it was concluded that the simpler, empirical Jameson approach performed equally well - or better - than the more complex semi-mechanistic modelling approach. This conclusion facilitated a discussion of the importance of the purpose of modelling in relation to the type of modelling approach applied. The general opinion was that the obtained results could promote choosing the more simple empirical approaches when developing growth models for *L. monocytogenes* in fermented products, since this approach facilitated accurate and reliable predictions.

In continuation of the topics discussed in Paper II, another important issue is the data requirement related to the two modelling approaches. Both approaches require solid, validated growth models for the LAB culture in question. For the Jameson approach (Eq. (9)) no additional data is required and no new parameter estimates are added to the model (Gimenez and Dalgaard, 2004; Le Marc et al., 2009). For the semi-mechanistic approach, data on lactic acid concentration related to LAB concentration and corresponding product pH values are needed for model evaluation. Since pH is an interesting factor in relation to microbial growth (Montville and Matthews, 2007), it may often be routinely measured during such growth experiments. The lactic acid concentration should be determined at each sampling point in order to relate the concentration of lactic acid at time, t, to the corresponding concentration of lactic acid bacteria and product pH. Analyses of lactic acid can be performed by spectrophotometric- and chromatographic methods or by the use of enzymatic methods (Theron and Lues, 2011b). The modelling of lactic acid formation in Paper II was based on 14 lactic acid formation curves (an example of one curve is provided in Fig. 23b with the corresponding LAB growth curve (Fig. 23a)) generated from a total of 460 individual analyses of lactic acid in cottage cheese at a given sampling time. It was evident from the

obtained data that the applied HPLC method resulted in some variability between the analysed samples and even between replicates of one extraction (Fig. 23b). The illustrated data was not transformed, but e.g. log or square root transformation could be applied to stabilise the observed variance (Zwietering et al., 1994a). However, reasonable fits and parameter estimates (Table 1, Paper II) were obtained when fitting the Yield-factor concept to data, despite the observed variance.
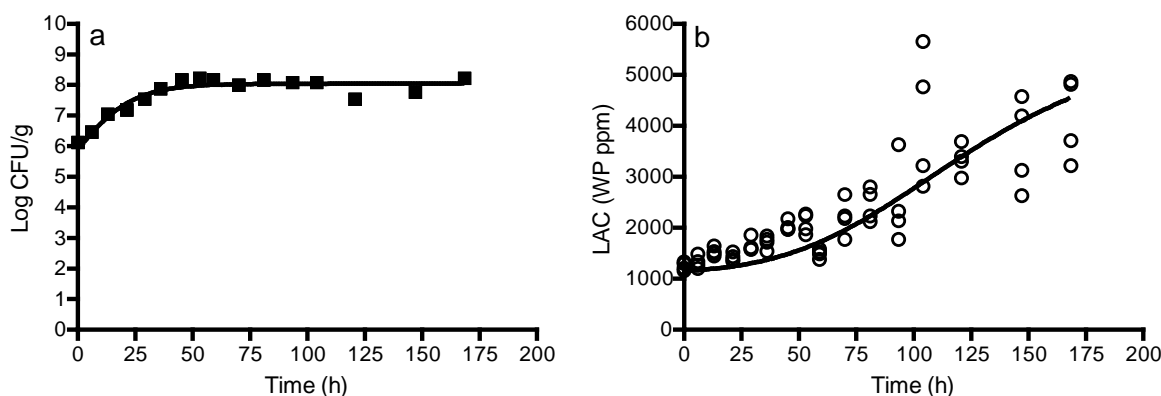
Figure 23 (a) Observed (■) and fitted (—) growth of LAB aroma culture at 15°C and (b) corresponding (untransformed) lactic acid concentrations (○). The Yield-factor concept (Eq. (19)), in combination with the expanded logistic growth model, was fitted to observed data. In both cases (a, b), $m = 0.25$ was applied.

Another important issue to consider is the impact of pH on the microbial growth response. Due to the logarithmic nature of the pH scale, even small changes in pH may have pronounced impact on the growth response of the co-culture organism(s) – especially when the growth boundary is approached (cf. Fig. 12a). Small inaccuracies in the pH model may therefore have considerable impact on the predicted growth and growth inhibition. Most pH models in the literature have been reported to perform satisfactorily (Poschet et al., 2005; Venkatesh et al., 1993; Vereecken and Van Impe, 2002; Vereecken et al., 2003) but, for instance, the pH model of Malakar et al. (1999) predicted the pH decrease to be more rapid than observed. Such predictions, in models aiming at describing microbial interaction and associated inhibition of pathogenic microorganisms, may lead to fail-dangerous predictions. The performance of the pH model presented in Paper II was strongly influenced by the observed temperature dependency described by simple linear regression between model parameters and temperature. Comparably, Ellouze et al. (2008) used simple linear regression to introduce a pH_0-dependent constant (F) in their pH model. The above mentioned models were all developed in well-defined laboratory media under controlled conditions. Salts, organic aids and milk proteins affect the buffering capacity of dairy products and hence the product's ability to acidify. Variability in buffering capacity of milk may be observed due to breed, stage of lactation, health status of the cow but also production processes such as heat treatment has either in- or decreasing effect (Salaün et al., 2005). Even though the fat- and protein content of raw materials (milk and cream) used for cottage cheese production are standardised prior to fermentation and

mixing (Nielsen, 2014; Walstra et al., 2005a) small variability is likely to be expected. From the numerous growth experiments performed during the present PhD-project, it was evident that for independent experiments, performed under identical conditions, variability in the obtained pH profiles was observed (Fig. 24).
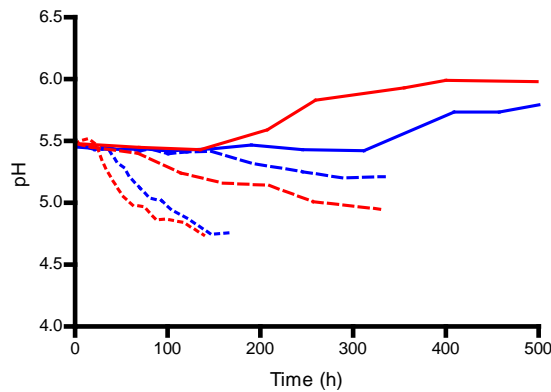
Figure 24 Observed pH profiles in cottage cheese with cultured cream dressing during storage at 5°C (— and —), 10°C (--- and ---) and 15°C (⋯ and ⋯). Despite similar experimental conditions, product and initial pH, variations in the pH profiles were observed.

Some of this variation may be explained by differences in LAB growth and associated formation of lactic acid but probably not all and differing buffer capacity could be one of the explanations. Similarly, Salaün et al. (2005) concluded in their review, that an improved understanding of the variations in buffering capacity of dairy products, due to variations in minerals and proteins, could provide a better interpretation of pH variations in dairy products. Due to this variability, relying solely on the estimated pH profile when predicting growth inhibition of *L. monocytogenes* in cottage cheese seems to be a fragile approach, where model inaccuracies may induce fail-dangerous predictions.

There is, however, no doubt that increased level of mechanistic knowledge is desirable in order to obtain better understanding of the underlying mechanisms of the observed growth responses (Bernaerts et al., 2004; McMeekin et al., 2013, 2008; Van Impe et al., 2013). However, as also emphasised in Paper II, it is important to establish the objective of the predictive model in question. For prediction of simultaneous growth of LAB and *L. monocytogenes* in cottage cheese during chilled storage, it seems sufficient to apply the empirical Jameson approach in order to obtain accurate predictions. The aim was to develop growth models applicable to risk assessment and product development of dairy products, more specifically cottage cheese. In relation to that, it is relevant to consider practices applied in the industry as well, when assessing the most appropriate model type. Within industrial fermentation and cheese production in particular, bacteriophage infection of the starter culture is of concern (Hansen, 2002). The virus infection caused by bacteriophages can kill the bacterial cell by lysis of the host cells. Bacteriophages are capable at proliferating when high numbers of bacteria are present, which is the case for starter cultures and during such infections, the bacteriophages will rapidly outnumber the bacterial cells (Walstra et al., 2005b). Complete elimination of bacteriophages is unrealistic since they occur ubiquitously and are

greatly diverse. Focus has therefore been on different control measures, and one widely applied strategy is starter culture rotation in order to avoid recurrent amplification of the same bacteriophage during consecutive fermentation processes (Garneau, 2011). In the production of cottage cheese, the rotation strategy is also applied (Eklöw, 2012; Nielsen, 2014) and that could argue against the semi-mechanistic modelling approach in this particular case. For the alternative starter cultures, it may be necessary to develop independent models for growth, lactic acid formation and pH change or at least thoroughly validate the existing LAB-, lactic acid- and pH models before they can be used in relation to risk assessment and product development of cottage cheese (Pinon et al., 2004). Furthermore, as demonstrated for the aroma culture in Paper II, other factors than lactic acid formation and the associated pH change cause growth inhibition and that should be investigated as well in order to obtain accurate predictions of *L. monocytogenes* growth inhibition in cottage cheese. If the empirical Jameson term is used to predict inter-bacterial interaction between the starter-LAB and *L. monocytogenes*, growth model development may also be necessary and validation is required as well. However, numerous resource demanding analyses can be omitted, making the process slightly more manageable.

Despite being ardent proponents of (semi)-mechanistic modelling approaches, Bernaerts et al. (2004) did recognise the potential need for trade-offs between "predictive power" (model complexity) and manageability of the developed predictive models. Mathematical models, that should be implemented in the industry need to be rapid and convenient (McMeekin et al., 2006) and they will often be applied by non-experts. Hence, the complexity of the models must not overshadow the usability (Bernaerts et al., 2004; Membré and Lambert, 2008). These opinions and realisations lead to a final question, inspired by Buchanan et al. (1997); *When is Simple Good Enough?* From the results of Paper II, the answer may very well be: In this case! Accurate and stable predictions were obtained by combining solid and successfully validated secondary growth models for LAB and *L. monocytogenes* in cottage cheese with the empirical Jameson term, whatever the reason for the observed growth inhibition (pH, lactic acid, bacteriocins?, diacetyl?).

9.4 Moving from a deterministic- to a stochastic modelling approach

In Paper III, the deterministic growth models for *L. monocytogenes* and LAB in cottage cheese with added aroma culture were used as a basis for the modelling procedure. A stochastic approach was applied by including observed, measured and reported variability in product characteristics, initial- and final bacterial concentrations and lag time durations for populations and individual bacterial cells. Reasonably good predictions were obtained when combining broth-based lag time data for *L. monocytogenes* with the *RLT*-concept. Previous studies had reported equivocal results for similar approaches, where lag time variability from broth based systems was used to predict growth in food products (Francois et al., 2006b; Manios et al., 2013). An alternative approach to predict lag time duration for *L. monocytogenes* cells with an "unknown" pre-history

(Paper III, pre-culture 1, 2 and 3 together) was evaluated. Variability in population lag time, represented by *RLT*-values (Ross, 1999), was used to predict growth of low bacterial numbers. Noteworthy good agreement between observed and predicted growth was obtained from this approach. It was thus considered whether application of observed population lag time variability (as *RLT*-values) could be used as an efficient and satisfactorily precise method to predict lag time duration for low bacterial concentrations as well. Besides lag time variability- and prediction, the representation of variability in input data was considered. Most existing studies on stochastic growth models apply theoretical distributions (Couvert et al., 2010; Koutsoumanis et al., 2010; Mejlholm et al., 2014; Pouillot et al., 2007), fitted to observed data or estimated, for the individual input parameters. In Paper III, the empirical distributions of product variability and initial- and final LAB concentrations were used to bootstrap from and in this way only observed data was used in the simulation process. The stochastic growth model was used to evaluate three different scenarios relevant for cottage cheese production and distribution. During the modelling procedure, focus was on observed and measured variability of input data. It was concluded that the developed model, used in combination with the *RLT*-concept, provided reasonable predictions taking product and lag time variability into consideration, and the model has the potential to be used to evaluate the safety of cottage cheese.

The main topics of Paper III were lag time predictions and representation of input data in order to obtain good simulations for simultaneous growth of LAB and *L. monocytogenes* in cottage cheese during storage. In the following section, these topics will be subject for further considerations.

As described, most available lag time data for individual bacterial cells (mainly *L. monocytogenes*) has been obtained for cells where the pre-history was known (laboratory conditions) and under specific conditions (defined temperature, pH, a_w etc.). Similarly, evaluation of lag time data to predict growth in food products has been performed for temperatures, pH values and a_w's identical to those used to collect the lag time data, and the lag time duration was sampled directly from the applied distribution (Francois et al., 2006b; Manios et al., 2013). However, this approach allows no flexibility and remains theoretical. If the predictive models are to be used by industry or authorities, some (limited) flexibility is needed in order to make the models valuable and useful (Membré and Lambert, 2008). The *RLT*-concept has previously been described and demonstrated to be a suitable measure of the amount of work to be done before growth could be resumed for cells in populations (Abou-Zeid et al., 2007; Mejlholm and Dalgaard, 2007a; Mellefont and Ross, 2003; Mellefont et al., 2005, 2004, 2003; Møller et al., 2013; Ross, 1999) and, when related to the μ_{max}-value obtained from the secondary growth model, the effects of environmental factors are included in the predicted lag time duration of bacterial populations (Mellefont et al., 2003; Ross and McMeekin, 2003). Based on the results of Paper III, it cannot be dismissed that the *RLT*-concept is also an appropriate method to induce flexibility into single cell lag time modelling

approaches. From the applied lag time data for *L. monocytogenes* the *RLT*-values were in the range from 0.0 (-5.1) to 112.7 for the most extreme case. The mean values were between 0.0 (-0.1) and 18.1 and for populations (Ross, 1999) the mean *RLT*-value was 3.7 (derived from *L. monocytogenes* lag time data obtained in food) (Table 5, Paper III). Mellefont and colleagues have studied the impact of temperature- and osmotic shifts on bacterial lag time duration and the *RLT*-concept extensively (Mellefont and Ross, 2003; Mellefont et al., 2005, 2004, 2003). In one study it was sought to define upper- and lower limits of the *RLT*-values. Related to abrupt osmotic shifts for *Salmonella* Typhimurium, no *RLT*-values above ~8 were observed within the applied experimental setup (Mellefont et al., 2004). However, for *Klebsiella oxytoga* the maximum *RLT*-values, obtained from viable counts, were larger than 12 (Mellefont et al., 2003). Population data for *L. monocytogenes*, *Escherischia coli*, *Clostridium perfringens*, *Salmonella* and *Staphylococcus aureus* indicated that the main proportion of the *RLT*-values were in the range from 3-6 (Ross, 1999). Based on the *L. monocytogenes* data presented by Ross (1999), Mejlholm and Dalgaard (2007) applied a *RLT*-value of 4.5 to predict *L. monocytogenes* lag time duration in lightly preserved seafoods. Møller et al. (2013) found average *RLT*-values of 3.10 (2.50-3.70) and 4.12 (3.35-4.89) to appropriately describe lag time of *Salmonella* and the natural microbiota, respectively, in fresh pork. The magnitude of the *RLT*-values for individual *L. monocytogenes* cells presented in Paper III was somewhat larger than reported in previous studies even though Hereu et al. (2014) obtained *RLT*-values > 50 for freeze stressed *L. monocytogenes* populations in ham and mortadella at low temperatures (4°C). One reason for the higher *RLT*-values may simply be the systematically increased stress level since all individual cells in each treatment have been subjected to the same pre-treatment (e.g. starvation). This is supported by the findings of Hereu et al. (2014), where the effect of freeze treatment, leading to stressed cells, had a significant impact on the *RLT*-value at low temperature. Another reason may be the fact that data was obtained for individual cells and therefore longer (average) lag times, hence larger *RLT*-values, are to be expected (Baranyi, 1998). The fitted maximum *RLT*-values of the literature data reported in Paper III was strongly affected by the distribution fitted to data. A clear example of this was the lag-time data for starved cells presented by Guillier and Augustin (2006) and Guillier et al. (2005) (Table 2 and Table 5, Paper III). The shifted Gamma distribution (Guillier et al., 2005) resulted in a mean *RLT*-value of 11.2 and a maximum *RLT*-value of 61.5 whereas the Extreme Value Type II distribution (Guillier and Augustin, 2006) returned a mean which was close to the mean obtained from the Gamma distribution (13.1) and a maximum value of 112.7, which was remarkably higher than the maximum obtained with the shifted Gamma distribution. The maximum *RLT*-values obtained from the Weibull distributions from Francois et al. (2006a and 2005) were similar to the maximum *RLT*-value obtained from the population data (Ross, 1999). The above reflections induce further confidence in the application of the *RLT*-concept to predict lag time duration of individual cells. The method provides a quick and flexible approach to obtaining lag time estimates. A similar

example of application was provided by Guillier and Augustin (2006) who used the constant k-value ($\mu_{max} \times lag$ corresponding to $RLT \times ln(2)$) $\pm$ standard deviation to predict the lag time duration $\pm$ standard deviation for *L. monocytogenes* at other temperatures than what was used to obtain lag time data experimentally (15°C vs. 30°C). For the tested scenario, they obtained estimated values close to the observed ones. However, the problem with unknown pre-history still remains, presenting a challenge on how to use the collected lag time data for individual cells in a "random manner", hence simulating the unknown pre-history of contaminating cells. Ross and McMeekin (2003) suggested that frequency distributions for relative lag times could be used in risk assessment as a plausible default assumption if no lag time data was available. As demonstrated in Paper III, application of population *RLT*-distributions provided reasonably good predictions for pooled experimental data (Fig. 3, Paper III) and it is suggested to use these variability data to obtain estimates of lag time duration when predicting growth of low bacterial concentrations. At worst, the estimated lag time duration will, theoretically, be too short (Baranyi, 1998), and more conservative predictions will be obtained. But, compared to worst case approaches where the lag phase is omitted (Mellefont et al., 2004) application of observed population lag time variability is suggested as a qualified alternative or "plausible default assumption" (c.f. Ross and McMeekin, 2003), until further research on quantification, analysis and application of single cell lag time has been performed. The importance of including a lag phase when predicting growth of *L. monocytogenes* in naturally contaminated cold smoked fish was also emphasised by Mejlholm et al. (2014).

Another, potential challenge, is the application and implementation of such stochastic growth model in industry for product evaluation and risk assessment purposes (Membré and Lambert, 2008). A basic rule of stochastic modelling is that no scenario should be modelled that cannot actually occur (Ross and McMeekin, 2003; Vose, 2000). Based on this statement and the approach used to represent product variability data in Paper III, it could be suggested to use empirical distributions to bootstrap from when evaluating microbial product safety. In that way, only observed data is used in the simulations. An alternative approach is to define distributions for each input factor (e.g. pH, NaCl, preservatives, co-culture microorganisms etc.). This approach is applied in the Sym'previus software (Couvert et al., 2010, www.symprevius.net, requires subscription). Couvert et al. (2010) validated that model for growth of *L. monocytogenes* at refrigeration temperatures and in that study input parameters were described by Normal distributions. However, other studies have reported that alternative distributions were more appropriate to describe input data (Mejlholm et al., 2014) and it may be difficult for food producers to assess which distributions that fit data best, potentially leading to simulation of unrealistic and/or erroneous simulations. It is therefore suggested that systematically collected data for e.g. product variability is used "directly" as input data for stochastic simulations and ongoing product evaluations.

Mejlholm et al. (2014) compared predictions obtained from a stochastic growth model with predictions obtained from a deterministic growth model. Input values from most- and least

preserved samples of cold smoked Greenland halibut and cold smoked salmon were used to deterministically predict growth of *L. monocytogenes* during chilled storage. They concluded that the deterministic model performed equally well as the stochastic model in relation to the predicted maximum population densities of *L. monocytogenes*. It was further concluded that the stochastic model provided additional features such as the possibility to evaluate considerably higher number of combinations of input values and to obtain a probability distribution of a given outcome. A similar comparison was made for *L. monocytogenes* growth in cottage cheese during storage at 8°C (Fig. 25 and Fig 26).
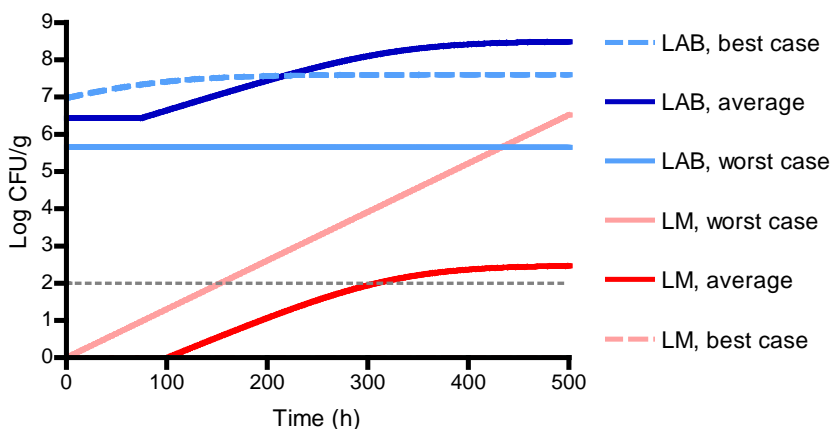
Figure 25 Predicted growth of one *L. monocytogenes* cell/g (red colours) and LAB (blue colours) in cottage cheese stored at 8°C for 500 h. Growth predictions were performed using worst case (light red and light blue full lines), best-case (light red (not visible) and light blue dashed lines) and average- (red and blue full lines) input values for initial- and final LAB concentration, LAB and *L. monocytogenes* lag time estimates, product pH and lactic acid concentration in the product.
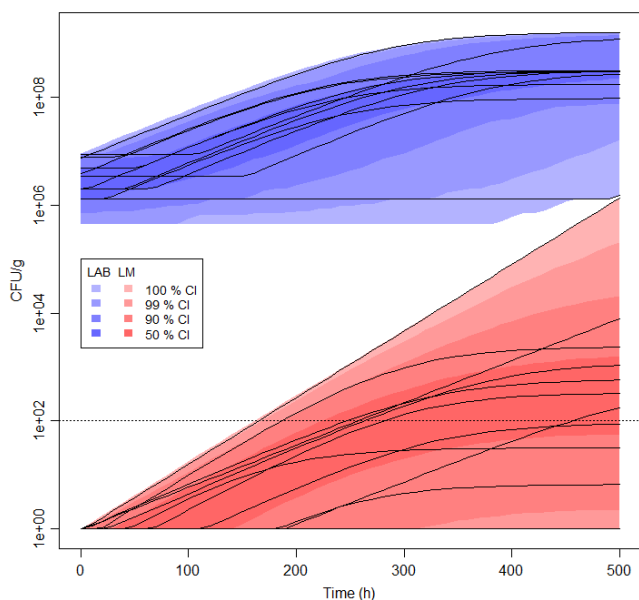
Figure 26 Simulated growth of one *L. monocytogenes* cell/g (red colours) and LAB (blue colours) in cottage cheese stored at 8°C for 500 h. The simulation is based on 10000 iterations and the 50, 90, 99 and 100% confidence intervals are provided (shaded). Input values was obtained from theoretical distributions fitted to observed- (LAB *RLT*) or reported (*L. monocytogenes RLT*) data or from empirical distributions (Initial- and final concentrations of LAB and product characteristics) used for bootstrap sampling. Full lines (—) represent randomly selected simulated growth curves of *L. monocytogenes* and LAB, respectively.

Simultaneous growth of LAB and *L. monocytogenes* was predicted in cottage cheese with cultured cream dressing using the deterministic models developed in Paper I including interaction between environmental parameters (ξ) and inter-bacterial interaction represented by the Jameson term (Fig. 26). Worst case, best case and average case values (Table 13) were used as input in the growth model. The worst case scenario focus on the optimal conditions for *L. monocytogenes* including low

initial concentrations of LAB, extended LAB lag time and the maximum observed maximum population density. Since the lag time was very long, no LAB growth occurred at the worst case scenario (light blue, full line).

Table 13 Input values for deterministic predictions of growth at worst case, best case and average case conditions.

Input	Worst Case	Best Case	Average Case
Temperature (°C)	8.0	8.0	8.0
Product pH	5.47	5.20	5.35
Lactic acid concentration (water phase ppm)	877	1924	1285
NaCl (% in water phase)	1.1	1.1	1.1
Sorbic acid (water phase ppm)	0.0	0.0	0.0
Initial *L. monocytogenes* concentration (CFU/g)	1	1	1
Initial LAB concentration (log CFU/g)	5.7	7.6	6.4
Final LAB concentration (log CFU/g)	9.2	7.6	8.5
LAB *RLT*	19.4	0.0	2.1
L. monocytogenes RLT	0.0	42.9	3.7

Similarly, stochastic simulation was performed with 10000 iterations at 8°C and starting from one *L. monocytogenes* cell/g (Fig. 26). Generally, good agreement between the deterministic- and the stochastic model was obtained in relation to the prediction of shortest- and average time to reach 2.0 log CFU/g during storage at 8°C. This result is not surprising since both approaches are based on the same model. From a risk-evaluation point of view, the results obtained from the stochastic growth model may, however, be more informative in relation to evaluation of the actual risk. Risk is defined as a function of the probability of an adverse effect and the magnitude of that effect, related to a specific hazard in food (FAO/WHO, 1995). It is therefore important to include the expected variability that determine the *range of results* that can be expected (CAC/GL 63-2007). The obtained results and subsequent considerations are fully in agreement with the conclusions of Mejlholm et al. (2014).

9.5 Value and applicability of the models presented in Paper I, II and III

To synthesise the outcome of the present PhD-project, extensive and not previously available, information regarding simultaneous growth- and growth responses of *L. monocytogenes* and LAB in cottage cheese was obtained. From these data, solid and successfully validated deterministic secondary growth models for *L. monocytogenes* and LAB in cottage cheese with fresh- and cultured cream dressing were developed. Using the developed, secondary models as starting point, the inter-bacterial interaction and associated inhibition of *L. monocytogenes* was investigated further using a semi-mechanistic modelling approach. As the empirical inter-bacterial interaction model (the Jameson approach) performed equally well, or better than the corresponding semi-mechanistic interaction model, the Jameson term was used in the subsequent modelling procedures. The relevant move from *L. monocytogenes* population growth predictions to growth predictions of low bacterial concentrations required a stochastic modelling approach. Again, the developed secondary growth models were used as the point of origin, in combination with the stable and well performing

empirical inter-bacterial interaction model. Based on accumulated data on product variability and LAB lag time and reported data for *L. monocytogenes* single cell lag times, simultaneous growth of *L. monocytogenes* and LAB was simulated. Compared to (limited) observed growth data, the model and the variable input resulted in predictions in correspondence with the observed growth. All things considered, the activities performed during the project underpinned each other and promoted progress and better understanding in relation to the prediction of *L. monocytogenes* growth in fresh, fermented dairy products (cottage cheese) in community with LAB from starter cultures. Furthermore, it is assessed that the developed deterministic- and stochastic models can be used as efficient tools for assessment of product safety and in relation to e.g. recipe alterations in the production of cottage cheese. In the Papers and in this thesis, the applicability has been demonstrated by the evaluation of relevant scenarios where quick and precise answers were obtained.

CONCLUSION AND
FUTURE PERSPECTIVES

10. Conclusion

The objective of the present PhD-project was to develop mathematical models in order to predict growth responses of *L. monocytogenes* in cottage cheese. The developed models should be applicable for risk assessment and product development in the production of cottage cheese.

During numerous growth experiments it was shown that *L. monocytogenes* was able to grow during storage of cottage cheese with either fresh or cultured cream dressing. According to the EU legislation in place, such growth potential of a ready-to-eat product induces a documentation requirement on the food processor. In relation to that, mathematical models can be beneficial and efficient tools for documentation of compliance. The residual LAB from the starter cultures induced a pronounced growth inhibition of *L. monocytogenes* at storage temperatures allowing LAB to grow to their maximum population density. This phenomenon corresponded to the well-known Jameson effect. In order to ensure accurate predictions of the bacterial kinetics, inclusion of bacterial interactions is highly relevant.

A theoretical introduction to dairy- and fermented dairy products, fermentation and starter cultures, *Listeria* and listeriosis, cottage cheese and cottage cheese production, legislative requirements, deterministic, empirical, mechanistic and stochastic modelling approaches was provided in the beginning of the thesis. Subsequently, three independent – yet supplementing – studies were presented in three manuscripts.

The first study (Paper I) was based on a combination of broth based *L. monocytogenes* and LAB growth data and growth data obtained in cottage cheese with fresh- and cultured cream dressing, respectively. These data were used to develop and calibrate deterministic, cardinal parameter growth models for *L. monocytogenes* in community with starter-LAB or aroma-LAB, respectively. The developed models, including the effect of temperature, pH, NaCl (water activity), lactic- and sorbic acid, were successfully validated at constant and dynamic storage temperatures using independently obtained growth data. The findings of Paper I showed that the *L. monocytogenes* growth response in cottage cheese with fresh- and cultured cream dressing was highly product dependent and affected by the dominating LAB population. Furthermore, the mesophilic starter-LAB and the mesophilic aroma-LAB could not be described by the same growth model and individual growth models were required. The calibration of the reference growth rate (μ_{ref}, h^{-1} at 25°C) was found to be an efficient method to improve the model performance. The simultaneous growth of *L. monocytogenes* and LAB was accurately described by combining the empirical Jameson term with the secondary growth model.

The overall conclusion of the study was that the developed models were applicable for product re-formulation and for evaluation of storage, distribution and handling of cottage cheese by consumers. An important realisation was that for cottage cheese, the effect of the added LAB culture should be regarded as an input parameter equal to e.g. pH and temperature when modelling growth response of *L. monocytogenes.* The applied methodology was found manageable and the

modelling procedure was considered to be efficient and likely to be applicable to other, similar, product types where inter-bacterial interactions are important.

In the second study (Paper II) the mathematical description if the observed inter-bacterial interaction was investigated further. Based on the deterministic growth models presented in Paper I, simultaneous growth of *L. monocytogenes* and LAB from starter- or aroma culture was predicted. The observed growth inhibition of *L. monocytogenes* was described either by applying a semi-mechanistic modelling approach or by the empirical Jameson term. The semi-mechanistic modelling approach was based on methodologies from fermentation technology and in Paper II lactic acid formation and pH changes, related to growth of either starter-LAB or aroma-LAB, was modelled. A comparison of the semi-mechanistic and the empirical modelling approaches revealed that lactic acid formation and pH change was sufficient to describe the *L. monocytogenes* growth inhibition induced by the starter-LAB. On the contrary, lactic acid formation and pH change was not sufficient to describe the *L. monocytogenes* growth inhibition induced by the aroma-LAB. The empirical modelling approach, relying on the Jameson term, accurately described the *L. monocytogenes* growth inhibition induced by both starter- and aroma-LAB in cottage cheese. Even though it was believed that the semi-mechanistic model for aroma-LAB could be improved by including additional mechanisms (e.g. bacteriocin formation and activity) the result of the comparison between the semi-mechanistic and the empirical modelling approach was found to be important. With no requirement of additional data and no introduction of additional parameter estimates, the empirical modelling approach provided accurate predictions regardless of the inhibitory cause. For fermented dairy products, where inter-bacterial interactions must be considered, such simplified and still accurate modelling approaches were found highly beneficial.

The overall conclusion was that the findings of Paper II can be used to justify the use of more simple methodologies for interaction models which, in some cases, can be advantageous since massive amounts of data are already required for development and validation of predictive models to be used in the food industry.

The third study (Paper III) was based on the deterministic growth models for simultaneous growth of *L. monocytogenes* and LAB in cottage cheese with cultured cream dressing (Paper I). The prediction of the inter-bacterial interaction relied on the empirical Jameson interaction model. In this study, growth responses of individual *L. monocytogenes* cells were evaluated taking the effect of variability in bacterial concentration, lag time durations and product characteristics into consideration. *L. monocytogenes* single cell lag time data obtained from previously published studies was used to represent expected lag time duration, depending on the prehistory of the *L. monocytogenes* cells. The data was used in combination with the *RLT*-concept. Prediction of growth response of cells with an unknown pre-history was done by using collected population data for variability in *RLT*-values for *L. monocytogenes*. The simulated growth of cells with a known pre-history provided good agreement with observed growth. Based on the obtained results it was

suggested that the *RLT*-concept used in combination with lag time data for individual cells may be an appropriate method to predict lag time duration of individual cells. Another essential result was the satisfactorily performance of the stochastic growth model in combination with *L. monocytogenes* population *RLT* data. If single cell lag time data is unavailable, these data can be used as a qualified default assumption when performing growth simulations for cells with unknown history.

The overall conclusions of the present PhD-project are that once solid, deterministic, secondary growth models have been developed and validated, they can be modified and/or extended to a range of other modelling procedures. Furthermore, inclusion of inter-bacterial interaction is an inevitable part when modelling and predicting growth of *L. monocytogenes* in fermented dairy products. In general, simple approaches to describe interaction and growth inhibition (empirical approach), lag time prediction of individual cells (variability in population *RLT*-values) and representation of e.g. variable product characteristics (bootstrapping from empirical distributions) were spoken in favour of. It is believed that it is necessary to define some applicable methodologies for the development of growth models for complex products such as fermented dairy products. Model development is a comprehensive process with an almost infinite data requirement and the findings of the present PhD-project is thought to be important in relation to the development of predictive models that are valuable for, and readily applicable in the food industry.

11. Future perspectives

Prospectively, it would be interesting and valuable to test the developed models and the applied modelling approach on other products similar to cottage cheese. The objective would be to see how much additional work that is required to obtain precise models within a portfolio of comparable products. In relation to cottage cheese, the impact of the different components could be interesting to investigate further. Studies have already been published, dealing with macro- and microscopic environments. Furthermore, since "omics" and mechanistic modelling have been strongly advocated for, it seems relevant to look into the application of such approaches and to evaluate where they could actually provide essential information. If combining food safety evaluation and process/product evaluation, predictive models including different mechanisms (dynamics in organic acids and pH, bacteriocin formation, production of diacetyl etc.) can most likely be beneficial and valuable.

Secondly, more work need to be performed in relation to the quantification and application of individual cell lag time data. *If* e.g. a dairy product becomes contaminated, the most likely scenario is a single or a few cells of *L. monocytogenes*. It is therefore important to develop fast and reliable methods to collect data in order to be able to describe the expected variability. It could be interesting to collect and combine available data from inoculated and naturally contaminated products. However, it still remains challenging to evaluate growth of individual cells in an efficient manner.

Keeping the link between academia and industry in mind, a third area of interest is the representation of variability. As discussed previously it is not straightforward to define and evaluate the most appropriate distribution to represent data. As demonstrated in the present PhD-project, bootstrapping from empirical distributions of initial LAB concentration, product pH and lactic acid concentrations provided good representation of variability. It could be interesting to work on an implementation of continuous collection and accumulation of such data in the industry. The could then be used for ongoing evaluation of the products of interest, based on observed product characteristics.

REFERENCES

12. References

Abou-Zeid, K.A., Yoon, K.S., Oscar, T.P., Schwarz, J.G., Hashem, F.M., Whiting, R.C., 2007. Survival and growth of *Listeria monocytogenes* in broth as a function of temperature, pH, and potassium lactate and sodium diacetate concentrations. Journal of Food Protection 11, 2460–2694.

Adams, M.R., Hall, C.J., 1988. Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. International Journal of Food Science & Technology 23, 287–292.

Adams, M.R., Moss, M.O., 2000. Food Microbiology, Second. ed. Royal Society of Chemistry.

Aguilar, C., Vanegas, C., Klotz, B., 2010. Antagonistic effect of Lactobacillus strains against *Escherichia coli* and *Listeria monocytogenes* in milk. The Journal of Dairy Research 78, 136–143.

Alavi, S.H., Puri, V.M., Knabel, S.J., Mohtar, R.H., Whiting, R.C., 1999. Development and validation of a dynamic growth model for *Listeria monocytogenes* in fluid whole milk. Journal of Food Protection 62, 170–176.

Al-Zeyara, S., Jarvis, B., Mackey, B.M., 2011. The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broths. International Journal of Food Microbiology 145, 98–105.

Amrane, A., Prigent, Y., 1994a. Lactic acid production from lactose in batch culture: analysis of the data with the help of a mathematical model; relevance for nitrogen source and preculture assessment. Applied Microbiology and Biotechnology 40, 644–649.

Amrane, A., Prigent, Y., 1994b. Mathematical model for lactic acid production from lactose in batch culture: Model development and simulation. Journal of Chemical Technology and Biotechnology 60, 241–246.

Antinone, M., Lawless, H., Ledford, R., Johnston, M., 1994. Diacetyl as a flavor component in full fat cottage cheese. Journal of Food Science 59, 38–42.

Antwi, M., Bernaerts, K., Van Impe, J.F., Geeraerd, A.H., 2007. Modelling the combined effects of structured food model system and lactic acid on *Listeria innocua* and *Lactococcus lactis* growth in mono-and coculture. International Journal of Food Microbiology 120, 71–84.

Araújo, E., Pires, Ac., Pinto, M., de Carvalho, A., 2012. Cottage cheese: Fundamentals and technology, in: Hui, Y. (Ed.), Handbook of Animal-Based Fermented Food and Beverage Technology. CRC Press, pp. 257–267.

Augustin, J.-C., Carlier, V., 2000a. Mathematical modelling of the growth rate and lag time for *Listeria monocytogenes*. International Journal of Food Microbiology 56, 29–51.

Augustin, J.-C., Carlier, V., 2000b. Modelling the growth rate of *Listeria monocytogenes* with a multiplicative type model including interactions between environmental factors. International Journal of Food Microbiology 56, 53–70.

Augustin, J.-C., Zuliani, V., Cornu, M., Guillier, L., 2005. Growth rate and growth probability of *Listeria monocytogenes* in dairy, meat and seafood products in suboptimal conditions. Journal of Applied Microbiology 99, 1019–1042.

Augustin, J.-C.J., Brouillaud-Delattre, A., Rosso, L., Carlier, V., 2000. Significance of inoculum size in the lag time of *Listeria monocytogenes*. Applied and Environmental Microbiology 66, 1706–1710.

Ayala, F.J., Gilpin, M.E., Ehrenfeld, J.G., 1973. Competition between species: Theoretical models and experimental tests. Theoretical Population Biology 4, 331–356.

Bâati, L., Roux, G., Dahhou, B., Uribelarrea, J.-L., 2004. Unstructured modelling growth of *Lactobacillus acidophilus* as a function of the temperature. Mathematics and Computers in Simulation 65, 137–145.

Bailey, J., Ollis, D., 1986. Kinetics of substrate utilization, product formation and biomass production in cell cultures, in: Biochemical Engineering Fundamentals. McGraw Hill, pp. 373–456.

Bajard, S., Rosso, L., Fardel, G., Flandrois, J.P., 1996. The particular behaviour of *Listeria monocytogenes* under sub-optimal conditions. International Journal of Food Microbiology 29, 201–211.

Balannec, B., Bouguettoucha, A., Amrane, A., 2007. Unstructured model for batch cultures without pH control of *Lactobacillus helveticus*—Inhibitory effect of the undissociated lactic acid. Biochemical Engineering Journal 35, 289–294.

Bannister, B.A., 1987. *Listeria monocytogenes* meningitis associated with eating soft cheese. Journal of Infection 15, 165–168.

Baranyi, J., 1998. Comparison of stochastic and deterministic concepts of bacterial lag. Journal of Theoretical Biology 192, 403–408.

Baranyi, J., 2002. Stochastic modelling of bacterial lag phase. International Journal of Food Microbiology 73, 203–206.

Baranyi, J., Pin, C., 2004. Modeling the history effect on microbial growth and survival, in: McKellar, R., Lu, X. (Eds.), Modeling Microbial Responses in Food, Contemporary Food Science. CRC Press, pp. 285–301.

Baranyi, J., Pin, C., Ross, T., 1999. Validating and comparing predictive models. International Journal of Food Microbiology 48, 159–166.

Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. International Journal of Food Microbiology 23, 277–294.

Basheer, I.., Hajmeer, M., 2000. Artificial neural networks: fundamentals, computing, design, and application. Journal of Microbiological Methods 43, 3–31.

Baty, F., Delignette-Muller, M., 2004. Estimating the bacterial lag time: which model, which precision? International Journal of Food Microbiology 91, 261–277.

Bernaerts, K., Dens, E., Vereecken, K., Geeraerd, A.H., Standaert, A.R., Devlieghere, F., Debevere, J., Van Impe, J.F., 2004. Concepts and tools for predictive modeling of microbial dynamics. Journal of Food Protection 67, 2041–2052.

Besse, N.G., Audinet, N., Barre, L., Cauquil, A., Cornu, M., Colin, P., 2006. Effect of the inoculum size on *Listeria monocytogenes* growth in structured media. International Journal of Food Microbiology 110, 43–51.

Boonmee, M., Leksawasdi, N., Bridge, W., Rogers, P.L., 2003. Batch and continuous culture of *Lactococcus lactis* NZ133: experimental data and model development. Biochemical Engineering Journal 14, 127–135.

Bouguettoucha, A., Balannec, B., Amrane, A., 2011. Unstructured models for lactic acid fermentation - A review. Food Technology and Biotechnology 49, 3–12.

Bourry, A., Poutrel, B., 1996. Bovine mastitis caused by *Listeria monocytogenes*: kinetics of antibody responses in serum and milk after experimental infection. Journal of Dairy Science 79, 2189–2195.

Boziaris, I., Skandamis, P., Anastasiadi, M., Nychas, G.-J., 2007. Effect of NaCl and KCl on fate and growth/no growth interfaces of *Listeria monocytogenes* Scott A at different pH and nisin concentrations. Journal of Applied Microbiology 102, 796–805.

Breidt, F., Fleming, H.P., 1998. Modeling of the competitive growth of *Listeria monocytogenes* and *Lactococcus lactis* in vegetable broth. Applied and Environmental Microbiology 64, 3159–3165.

Brul, S., Mensonides, F.I.C., Hellingwerf, K.J., Teixeira de Mattos, M.J., 2008. Microbial systems biology: new frontiers open to predictive microbiology. International Journal of Food Microbiology 128, 16–21.

Buchanan, R., Whiting, R., Damert, W., 1997. When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. Food Microbiology 14, 313–326.

Bula, C.J., Bille, J., Glauser, M.P., 1995. An epidemic of food-borne listeriosis in Western Switzerland: Description of 57 cases involving adults. Clinical Infectious Diseases 20, 66–72.

Bylund, G., 1995. Dairy Processing Handbook. Tetra Pak Processing Systems AB, Lund.

CAC/GL 63-2007, 2007. Principles and guidelines for the conduct of microbial risk management (MRM). Codex Alimentarius 1–19.

CAC/RCP 1-1969, 1969. General Principles of Food Hygiene. Codex Alimentarius 1–31.

CAC/RCP 57-2004, 2004. Code of hygienic practice for milk and milk products 1–32.

Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J.S., Vancanneyt, M., De Vuyst, L., 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Applied and Environmental Microbiology 73, 1809–1824.

Carrique-Mas, J.J., Hökerberg, I., Andersson, Y., Arneborn, M., Tham, W., Danielsson-Tham, M.-L., Osterman, B., Leffler, M., Steen, M., Eriksson, E., Hedin, G., Gieseke, J., 2003. Febrile gastroenteritis after eating on-farm manufactured fresh cheese – an outbreak of listeriosis? Epidemiology and Infection 130, 79–86.

Cassin, M.H., Lammerding, A.M., Todd, E.C.D., Ross, W., McColl, R.S., 1998. Quantitative risk assessment for *Escherichia coli* O157:H7 in ground beef hamburgers. International Journal of Food Microbiology 41, 21–44.

Castillo, M., Lucey, J.A., Wang, T., Payne, F.A., 2006. Effect of temperature and inoculum concentration on gel microstructure, permeability and syneresis kinetics. Cottage cheese-type gels. International Dairy Journal 16, 153–163.

Chen, J.H., Hotchkiss, J.H., 1993. Growth of *Listeria monocytogenes* and *Clostridium sporogenes* in cottage cheese in modified atmosphere packaging. Journal of Dairy Science 76, 972–977.

Chua, T.E.H., Dunkley, W.L., 1979. Influence of cooking procedures on properties of cottage cheese curd. Journal of Dairy Science 62, 1216–1226.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology 71, 1–20.

Cole, M.B., Jones, M. V., Holyoak, C., 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. Journal of Applied Bacteriology 69, 63–72.

Conly, J., Johnston, B., 2008. *Listeria*: A persistent food-borne pathogen. The Canadian Journal of Infectious Diseases & Medical Microbiology 19, 327–328.

Conner, D., Brackett, R., Beuchat, L., 1986. Effect of temperature, sodium chloride, and pH on growth of *Listeria monocytogenes* in cabbage juice. Applied and Environmental Microbiology 52, 59–63.

Cornu, M., Beaufort, A., Rudelle, S., Laloux, L., Bergis, H., Miconnet, N., Serot, T., Delignette-Muller, M.L., 2006. Effect of temperature, water-phase salt and phenolic contents on *Listeria monocytogenes* growth rates on cold-smoked salmon and evaluation of secondary models. International Journal of Food Microbiology 106, 159–168.

Cornu, M., Billoir, E., Bergis, H., Beaufort, A., Zuliani, V., 2011. Modeling microbial competition in food: Application to the behavior of *Listeria monocytogenes* and lactic acid flora in pork meat products. Food Microbiology 28, 639–647.

Cornu, M., Kalmokoff, M., Flandrois, J.-P., 2002. Modelling the competitive growth of *Listeria monocytogenes* and *Listeria innocua* in enrichment broths. International Journal of Food Microbiology 73, 261–274.

Coroller, L., Guerrot, V., Huchet, V., Le Marc, Y., Mafart, P., Sohier, D., Thuault, D., 2005. Modelling the influence of single acid and mixture on bacterial growth. International Journal of Food Microbiology 100, 167–178.

Cotter, P., Hill, C., Ross, R., 2005. Bacteriocins: developing innate immunity for food. Nature Reviews Microbiology 3, 777–788.

Couvert, O., Pinon, A., Bergis, H., Bourdichon, F., Carlin, F., Cornu, M., Denis, C., Gnanou Besse, N., Guillier, L., Jamet, E., 2010. Validation of a stochastic modelling approach for *Listeria monocytogenes* growth in refrigerated foods. International Journal of Food Microbiology 144, 236–242.

D'Arrigo, M., García de Fernando, G.D., Velasco de Diego, R., Ordóñez, J.A., George, S.M., Pin, C., 2006. Indirect measurement of the lag time distribution of single cells of *Listeria innocua* in food. Applied and Environmental Microbiology 72, 2533–2538.

Dalgaard, P., 1995. Modelling of microbial activity and prediction of shelf life for packed fresh fish. International Journal of Food Microbiology 26, 305–317.

Dalgaard, P., 2000. Fresh and lightly preserved seafood, in: Man, C., Jones, A. (Eds.), Shelf Life Evaluation of Foods. Aspen Publishing Inc Maryland, USA, pp. 110–139.

Dalgaard, P., 2002. Modelling and predicting the shelf-life of seafood., in: Bremner, H. (Ed.), Safety and Quality Issues in Fish Processing. Woodhead Publishing Ltd, Cambridge, pp. 191–219.

Dalgaard, P., 2009. Chapter 10: Modelling of Microbial Growth, in: Bulletin of the International Dairy Federation A Revolution in Food Safety Management. pp. 45–57.

Dalgaard, P., Koutsoumanis, K., 2001. Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. Journal of Microbiological Methods 43, 183–196.

Dalgaard, P., Ross, T., Kamperman, L., Neumeyer, K., McMeekin, T.A., 1994. Estimation of bacterial growth rates from turbidimetric and viable count data. International Journal of Food Microbiology 23, 391–404.

Dalié, D.K.D., Deschamps, A.M., Richard-Forget, F., 2010. Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. Food Control 21, 370–380.

Davidson, P.M., Taylor, T.M., 2007. Chemical peservatives and natural antimicrobial compounds., in: Doyle, M.P., Beuchat, L.R. (Eds.), Food Microbiology, Fundamentals and Frontiers. ASM Press, pp. 713–745.

Davies, E.A., Adams, M.R., 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. International Journal of Food Microbiology 21, 341–347.

Davies, E.A., Bevis, H.E., Delves-Broughton, J., 1997. The use of the bacteriocin, nisin, as a preservative in ricotta-type cheeses to control the food-borne pathogen *Listeria monocytogenes*. Letters in Applied Microbiology 24, 343–346.

De Castro, V., Escudero, J., Rodriguez, J., Muniozguren, N., Uribarri, J., Saez, D., Vazquez, J., 2012. Listeriosis outbreak caused by Latin-style fresh cheese, Bizkaia, Spain, August 2012. Euro surveillance: European communicable disease bulletin 17.

De Vos, W.M., Hugenholtz, J., 2004. Engineering metabolic highways in Lactococci and other lactic acid bacteria. Trends in Biotechnology 22, 72–79.

De Vuyst, L., Callewaert, R., Crabbe, K., 1996. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology 142, 817–827.

De Vuyst, L., Leroy, F., 2007. Bacteriocins from lactic acid bacteria: production, purification, and food applications. Journal of Molecular Microbiology and Biotechnology 13, 194–199.

Dens, E., Vereecken, K., Impe, J. Van, 1999. A prototype model structure for mixed microbial populations in homogeneous food products. Journal of Theoretical Biology 201, 159–170.

Devlieghere, F., Geeraerd, A.H., Versyck, K.J., Bernaert, H., Van Impe, J.F., Debevere, J., 2000. Shelf life of modified atmosphere packed cooked meat products: addition of Na-lactate as a fourth shelf life determinative factor in a model and product validation. International Journal of Food Microbiology 58, 93–106.

Devlieghere, F., Geeraerd, A.H., Versyck, K.J., Vandewaetere, B., Van Impe, J., Debevere, J., 2001. Growth of *Listeria monocytogenes* in modified atmosphere packed cooked meat products: a predictive model. Food Microbiology 18, 53–66.

Dolci, P., Alessandria, V., Rantsiou, K., Rolle, L., Zeppa, G., Cocolin, L., 2008. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. International Journal of Food Microbiology 122, 302–311.

Donnelly, C., Nyachuba, D., 2007. Conventional methods to detect and isolate *Listeria monocytogenes*, in: Ryser, E., Marth, E. (Eds.), *Listeria*, Listeriosis, and Food Safety, Third Edition, Food Science and Technology. CRC Press, pp. 215–256.

Drake, S.L., Lopetcharat, K., Drake, M.A., 2009. Comparison of two methods to explore consumer preferences for cottage cheese. Journal of Dairy Science 92, 5883–5897.

DVFA, 2010. Danish Veterinary and Food Administration. Milk and Cheese. http://www.altomkost.dk/Fakta/Foedevarer/maelk_og_ost/forside.htm (accessed 6.4.14).

DVFA, 2014. Danish Veterinary and Food Administration. *Listeria monocytogenes*. http://www.altomkost.dk/Fakta/Foedevarer/maelk_og_ost/forside.htm (accessed 6.4.14).

EC, 1995. European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners 1–56.

EC, 2002. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matter of food safe. Official Journal of the European Communities 1–24.

EC, 2004a. Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. Official Journal of the European Union 1–26.

EC, 2004b. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for the hygiene of foodstuffs. Official Journal of the European Union 1–55.

EC, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union 338, 1–26.

EC, 2006. Council Regulation (EC) No 510/2006 of 20 March 2006on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. Official Journal of the European Communities L93/12 1–6.

EC, 2014a. RASFF Portal, Milk and milk products, Pathogenic micro-organisms. Rapid Alert System for Food and Feed. http://ec.europa.eu/food/food/rapidalert/index_en.htm (accessed 10.25.13).

EC, 2014b. Food Additives Database. https://webgate.ec.europa.eu/sanco_foods/main/?sector=FAD&auth=SANCAS (accessed 12.18.13).

Efron, B., 1979. Bootstrap methods: another look at the jackknife. The Annals of Statistics 7, 1–26.

EFSA, 2007. Opinion of the Scientific Commitee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. The EFSA Journal 587, 1–16.

EFSA, 2012. Scientific Report of EFSA and ECDC. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic agents and Food-borne Outbreaks in 2010. EFSA Journal 10, 1–312.

EFSA, 2013. Analysis of the baseline survey on the prevalence of *Listeria monocytogenes* in certain ready-to-eat foods in the EU, 2010-2011 Part A: *Listeria monocytogenes* prevalence estimates. EFSA Journal 11(6):3241, 1-75.

EFSA, 2014. Scientific Report of EFSA and ECDC. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic agents and Food-borne Outbreaks in 2010. EFSA Journal 12, 1–312.

Eklöw, A., 2012. Personal communication. Research Scientist, Arla Strategic Innovation Centre.

Elfwing, A., LeMarc, Y., Baranyi, J., Ballagi, A., 2004. Observing growth and division of large numbers of individual bacteria by image analysis. Applied and Environmental Microbiology 70, 675–678.

Ellouze, M., Pichaud, M., Bonaiti, C., Coroller, L., Couvert, O., Thuault, D., Vaillant, R., 2008. Modelling pH evolution and lactic acid production in the growth medium of a lactic acid bacterium: Application to set a biological TTI. International Journal of Food Microbiology 128, 101–107.

Emborg, J., Dalgaard, P., 2008a. Growth, inactivation and histamine formation of *Morganella psychrotolerans* and *Morganella morganii*—development and evaluation of predictive models. International Journal of Food Microbiology 128, 234–243.

Emborg, J., Dalgaard, P., 2008b. Modelling the effect of temperature, carbon dioxide, water activity and pH on growth and histamine formation by *Morganella psychrotolerans*. International Journal of Food Microbiology 128, 226–33.

Esty, J., Meyer, K., 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes. XI. Journal of Infectious Diseases 31, 650–663.

FAO/WHO, 1995. Application of risk analysis to food standards issues. Report of the Joint FAO/WHO Expert Consultation, WHO/FNU/FOS. World Health Organization, Geneva.

Farber, J.M.J., Sanders, G.G.W., Dunfield, S., Prescott, R., 1989. The effect of various acidulants on the growth of *Listeria monocytogenes*. Letters in Applied Microbiology 9, 181–183.

Fazil, A., 2005. FAO Fisheries Technical Paper 462: A primer on risk assessment modelling: focus on seafood products.

Fernandes, R., 2009. Cheese, in: Microbiology Handbook: Dairy Products. Leatherhead Publishing. Royal Society of Chemistry, pp. 61–76.

Fernández, P., George, S., Sills, C.C., Peck, M.W., 1997. Predictive model of the effect of CO 2, pH, temperature and NaCl on the growth of *Listeria monocytogenes*. International Journal of Food Microbiology 37, 37–45.

Ferreira, M.A.S.S., Lund, B.M., 1996. The effect of nisin of *Listeria monocytogenes* in culture medium and long-life cottage cheese. Letters in Applied Microbiology 22, 433–438.

Foodborne Illness Outbreak Database, 2014. Foodborne Illness Outbreak Database. http://outbreakdatabase.com/search/?organism=Listeria+monocytogenes (accessed 6.4.14).

Foodcomp, 2014. Foodcomp.dk. National Food Institute, Technical University of Denmark. http://www.foodcomp.dk/v7/fvdb_details.asp?FoodId=0260 (accessed 6.24.14).

Francois, K., Devlieghere, F., Smet, K., Standaert, A.R., Geeraerd, A.H., Van Impe, J.F., Debevere, J., 2005. Modelling the individual cell lag phase: effect of temperature and pH on the individual cell lag distribution of *Listeria monocytogenes*. International Journal of Food Microbiology 100, 41–53.

Francois, K., Devlieghere, F., Standaert, A.R., Geeraerd, A.H., Van Impe, J.F., Debevere, J., 2003. Modelling the individual cell lag phase. Isolating single cells: protocol development. Letters in Applied Microbiology 37, 26–30.

Francois, K., Devlieghere, F., Standaert, A.R., Geeraerd, A.H., Van Impe, J.F., Debevere, J., 2006a. Effect of environmental parameters (temperature, pH and a w) on the individual cell lag phase and generation time of *Listeria monocytogenes*. International Journal of Food Microbiology 108, 326–335.

Francois, K., Devlieghere, F., Uyttendaele, M., Standaert, A.R., Geeraerd, A.H., Nadal, P., Van Impe, J.F., Debevere, J., 2006b. Single cell variability of *L. monocytogenes* grown on liver pâté and cooked ham at 7 C: comparing challenge test data to predictive simulations. Journal of Applied Microbiology 100, 800–812.

Fretz, R., Sagel, U., Ruppitsch, W., Pietzka, A., Stöger, A., Huhulescu, S., Heuberger, S., Pichler, J., Much, P., Pfaff, G., Stark, K., Prager, R., Flieger, A., Feenstra, O., Allerberger, F., 2010. Listeriosis outbreak caused by acid curd cheese "Quargel", Austria and Germany 2009. EuroSurveillance 15, 1–2.

Friedrich, U., Lenke, J., 2006. Improved enumeration of lactic acid bacteria in mesophilic dairy starter cultures by using multiplex quantitative real-time PCR and flow cytometry-fluorescence in situ hybridization. Applied and Environmental Microbiology 72, 4163–4171.

Fujikawa, H., Kai, A., Morozumi, S., 2004. A new logistic model for *Escherichia coli* growth at constant and dynamic temperatures. Food Microbiology 21, 501–509.

Gahan, C.G., O'Driscoll, B., Hill, C., 1996. Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. Applied and Environmental Microbiology 62, 3128–3132.

Gálvez, A., Abriouel, H., López, R., Omar, N., 2007. Bacteriocin-based strategies for food biopreservation. International Journal of Food Microbiology 120, 51–70.

García, A.A.I., García, L.A. LA, Díaz, M., 1994. Modelling of diacetyl production during beer fermentation. Journal of the Institute of Brewing 100, 179–183.

Gardner, I.A., 2004. An epidemiologic critique of current microbial risk assessment practices: The importance of prevalence and test accuracy data. Journal of Food Protection 67, 2000–2007.

Garneau, J.E., 2011. Bacteriophages of lactic acid bacteria and their impact on milk fermentations. Microbial Cell Factories 10, 1–10.

Garnsworthy, P.C., Masson, L.L., Lock, A.L., Mottram, T.T., 2006. Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. Journal of Dairy Science 89, 1604–1612.

Gasanov, U., Hughes, D., Hansbro, P.M., 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. FEMS Microbiology Reviews 29, 851–875.

Gerner-Smidt, P., Ethelberg, S., Schiellerup, P., Christensen, J.J., Engberg, J., Fussing, V., Jensen, A., Jensen, C., Petersen, A.M., Bruun, B.G., 2005. Invasive listeriosis in Denmark 1994-2003: a review of 299 cases with special emphasis on risk factors for mortality. Clinical Microbiology and Infection 11, 618–624.

Gimenez, B., Dalgaard, P., 2004. Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and spoilage microorganisms in cold smoked salmon. Journal of Applied Microbiology 96, 96–109.

Giuffrida, A., Valenti, D., Ziino, G., Spagnolo, B., Panebianco, A., 2009. A stochastic interspecific competition model to predict the behaviour of *Listeria monocytogenes* in the fermentation process of a traditional Sicilian salami. European Food Research and Technology 228, 767–775.

Giuffrida, A., Ziino, G., Valenti, D., Donato, G., Panebianco, A., 2007. Application of an interspecific competition model to predict the growth of *Aeromonas hydrophila* on fish surfaces during the refrigerated storage. Archiv für Lebensmittelhygiene 58, 136–141.

González-Fandos, E., Dominguez, J.L., 2007. Effect of potassium sorbate washing on the growth of *Listeria monocytogenes* on fresh poultry. Food Control 18, 842–846.

Gougouli, M., Angelidis, A.S., Koutsoumanis, K., 2008. A study on the kinetic behavior of *Listeria monocytogenes* in ice cream stored under static and dynamic chilling and freezing conditions. Journal of Dairy Science 91, 523–530.

Goulet, V., Hebert, M., Hedberg, C., Laurent, E., Vaillant, V., De Valk, H., Desenclos, J.-C., 2012. Incidence of listeriosis and related mortality among groups at risk of acquiring listeriosis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 54, 652–60.

Goulet, V., Jacquet, C., Vaillant, V., Rebiere, I., 1995. Listeriosis from consumption of raw-milk cheese. The Lancet 345, 1581–1582.

Graham, A.F., Mason, D.R., Peck, M.W., 1996. Predictive model of the effect of temperature, pH and sodium chloride on growth from spores of non-proteolytic *Clostridium botulinum*. International Journal of Food Microbiology 31, 69–85.

Grattepanche, F., Lacroix, C., Audet, P., Lapointe, G., 2005. Quantification by real-time PCR of *Lactococcus lactis* subsp. *cremoris* in milk fermented by a mixed culture. Applied Microbiology and Biotechnology 66, 414–421.

Gray, M.L., Stafseth, H.J., Thorp, F., Sholl, L.B., Riley, W.F., 1948. A New Technique for Isolating *Listerellae* from the Bovine Brain. Journal of Bacteriology 55, 471–476.

Grunkemeier, G., Wu, Y., 2004. Bootstrap Resampling Methods: Something for Nothing? The Annals of Thoracic Surgery 77, 1142–1144.

Guerra, N.P., Agrasar, A.T., Macías, C.L., Bernárdez, P.F., Castro, L.P., 2007. Dynamic mathematical models to describe the growth and nisin production by *Lactococcus lactis* subsp. *lactis* CECT 539 in both batch and re-alkalized fed-batch cultures. Journal of Food Engineering 82, 103–113.

Guillier, L., Augustin, J., 2006. Modelling the individual cell lag time distributions of *Listeria monocytogenes* as a function of the physiological state and the growth conditions. International Journal of Food Microbiology 111, 241–251.

Guillier, L., Pardon, P., Augustin, J.-C., 2005. Influence of stress on individual lag time distributions of *Listeria monocytogenes*. Applied and Environmental Microbiology 71, 2940–2948.

Hansen, E.B., 2002. Commercial bacterial starter cultures for fermented foods of the future. International Journal of Food Microbiology 78, 119–131.

He, F.J., MacGregor, G.A., 2010. Reducing population salt intake worldwide: from evidence to implementation. Progress in Cardiovascular Diseases 52, 363–382.

Hereu, A., Dalgaard, P., Garriga, M., Aymerich, T., Bover-Cid, S., 2014. Analysing and modelling the growth behaviour of *Listeria monocytogenes* on RTE cooked meat products after a high pressure treatment at 400MPa. International Journal of Food Microbiology 186, 84–94.

Hof, H., 2003. History and epidemiology of listeriosis. FEMS Immunology & Medical Microbiology 35, 199–202.

Houtsma, P.C., Kusters, B.J.M., de Wit, J.C., Rombouts, F.M., Zwietering, M.H., 1994. Modelling growth rates of *Listeria innocua* as a function of lactate concentration. International Journal of Food Microbiology 24, 113–123.

Huang, L., 2004. Numerical analysis of the growth of *Clostridium perfringens* in cooked beef under isothermal and dynamic conditions. Journal of Food Safety 24, 53–70.

Hugenholtz, J., 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiology Reviews 12, 165–178.

Huth, P., DiRienzo, D., Miller, G., 2006. Major scientific advances with dairy foods in nutrition and health. Journal of Dairy Science 89, 1207–1221.

Irlinger, F., Mounier, J., 2009. Microbial interactions in cheese: implications for cheese quality and safety. Current Opinion in Biotechnology 20, 142–148.

Ita, P., Hutkins, R., 1991. Intracellular pH and survival of *Listeria monocytogenes* Scott A in tryptic soy broh containing acetic, lactic, citric, and hydrochloric acids. Journal of Food Protection 54, 15–19.

Jackson, K., Iwamoto, M., Swerdlow, D., 2010. Pregnancy-associated listeriosis. Epidemiology and Infection 138, 1503–1509.

Jameson, J.E., 1962. A discussion of the dynamics of *Salmonella* enrichment. Journal of Hygiene 60, 193–207.

Janssen, M., Geeraerd, A.H., Logist, F., De Visscher, Y., Vereecken, K.M., Debevere, J., Devlieghere, F., Van Impe, J.F., 2006. Modelling *Yersinia enterocolitica* inactivation in coculture experiments with *Lactobacillus sakei* as based on pH and lactic acid profiles. International Journal of Food Microbiology 111, 59–72.

Jantzen, M.M., Navas, J., Corujo, A., Moreno, R., López, V., Martínez-Suárez, J.V., 2006. Review. Specific detection of *Listeria monocytogenes* in foods using commercial methods: from chromogenic media to real-time PCR. Spanish Journal of Agricultural Research 4, 235.

Jay, J., 1982. Antimicrobial properties of diacetyl. Applied and Environmental Microbiology 44, 525–532.

Jay, J., 2000. Fermentation and fermented dairy products, in: Jay, J. (Ed.), Modern Food Microbiology. Springer, pp. 113–130.

Jensen, A., Frederiksen, W., Gerner-Smidt, P., 1994. Risk factors for listeriosis in Denmark, 1989-1990. Scandinavian Journal of Infectious Disease 26, 171–178.

Jensen, N.E., Aarestrup, F.M., Jensen, J., Wegener, H.C., 1996. *Listeria monocytogenes* in bovine mastitis. Possible implication for human health. International Journal of Food Microbiology 32, 209–216.

Josephsen, J., Jespersen, L., 2004. Starter cultures and fermented products., in: Hui, Y.H., Meunier-Goddik, L., Hansen, Å.S., Josephsen, J., Nip, W.-K., Stanfeld, P.S., Toldra, F. (Eds.), Handbook of Food and Beverage Fermentation Technology. Marcel Dekker, pp. 23–49.

Jørgensen, M.H., Nikolajsen, K., 1987. Mathematic model for lactic acid formation with *Streptococcus cremoris* from glucose. Applied Microbiology and Biotechnology 25, 313–316.

Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie van Leeuwenhoek 49, 209–224.

Kempler, G., McKay, L., 1980. Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Applied and Environmental Microbiology 39, 926–927.

Koch, J., Dworak, R., Prager, R., 2010. Large listeriosis outbreak linked to cheese made from pasteurized milk, Germany, 2006–2007. Foodborne Pathogens and Disease 7, 1581–1584.

Kolter, R., Siegele, D., Tormo, A., 1993. The stationary phase of the bacterial life cycle. Annual Reviews in Microbiology 47, 855–874.

Kornacki, J., Gurtler, J., 2007. Incidence and control of *Listeria* in food processing facilities, in: Ryser, E., Marth, E. (Eds.), *Listeria*, Listeriosis, and Food Safety, Third Edition, Food Science and Technology. CRC Press, pp. 681–766.

Koutsoumanis, K., Angelidis, A.S., 2007. Probabilistic modeling approach for evaluating the compliance of ready-to-eat foods with new European Union safety criteria for *Listeria monocytogenes*. Applied and Environmental Microbiology 73, 4996–5004.

Koutsoumanis, K., Pavlis, A., Nychas, G.-J.E., Xanthiakos, K., 2010. Probabilistic model for *Listeria monocytogenes* growth during distribution, retail storage, and domestic storage of pasteurized milk. Applied and Environmental Microbiology 76, 2181–2191.

Koutsoumanis, K.P., Sofos, J.N., 2005. Effect of inoculum size on the combined temperature, pH and a w limits for growth of *Listeria monocytogenes*. International Journal of Food Microbiology 104, 83–91.

Koutsoumanis, K.P., Lianou, A., 2013. Stochasticity in colonial growth dynamics of individual bacterial cells. Applied and Environmental Microbiology 79, 2294–2301.

Kozak, J., Balmer, T., Byrne, R., Fisher, K., 1996. Prevalence of *Listeria monocytogenes* in foods: Incidence in dairy products. Food Control 7, 215–221.

Kuhn, M., Scortti, M., Vázquez-Boland, J., 2008. Pathogenesis, in: Liu, D. (Ed.), Handbook of *Listeria Monocytogenes*. CRC Press, pp. 97–136.

Ladero, V., Cañedo, E., Pérez, M., Martín, M.C., Fernández, M., Alvarez, M.A., 2012. Multiplex qPCR for the detection and quantification of putrescine-producing lactic acid bacteria in dairy products. Food Control 27, 307–313.

Lado, B., Yousef, A., 2007. Characteristics of *Listeria monocytogenes* important to food processors, in: Ryser, E., Marth, E. (Eds.), *Listeria*, Listeriosis, and Food Safety, Third Edition, Food Science and Technology. CRC Press, pp. 157–213.

Lanciotti, R., Patrignani, F., Bagnolini, F., 2003. Evaluation of diacetyl antimicrobial activity against *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus*. Food Microbiology 20, 537–543.

Larson, A.E., Yu, R., Lee, O.A., Price, S., Haas, G.J., Johnson, E.A., 1996. Antimicrobial activity of hop extracts against *Listeria monocytogenes* in media and in food. International Journal of Food Microbiology 33, 195–207.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P., Thuault, D., 2002. Modelling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. International Journal of Food Microbiology 73, 219–237.

Le Marc, Y., Skandamis, P.N., Belessi, C.I.A., Merkouri, S.I., George, S.M., Gounadaki, A.S., Schvartzman, S., Jordan, K., Drosinos, E.H., Baranyi, J., 2010. Modeling the effect of abrupt acid and osmotic shifts within the growth region and across growth boundaries on adaptation and growth of *Listeria monocytogenes*. Applied and Environmental Microbiology 76, 6555–6563.

Le Marc, Y., Valík, L., Medveďová, A., 2009. Modelling the effect of the starter culture on the growth of *Staphylococcus aureus* in milk. International Journal of Food Microbiology 129, 306–311.

Lejeune, R., Callewaert, R., Crabbé, K., De Vuyst, L., 1998. Modelling the growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in batch cultivation. Journal of Applied Microbiology 84, 159–168.

Leroy, F., De Vuyst, L., 2003. A combined model to predict the functionality of the bacteriocin-producing *Lactobacillus sakei* strain CTC 494. Applied and Environmental Microbiology 69, 1093–1099.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science & Technology 15, 67–78.

Leroy, F., Degeest, B., De Vuyst, L., 2002. A novel area of predictive modelling: describing the functionality of beneficial microorganisms in foods. International Journal of Food Microbiology 73, 251–259.

Leroy, F.F., De Vuyst, L., 1999. Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin Sakacin K. Applied and Environmental Microbiology 65, 974–981.

Leuschner, R.G.K., Robinson, T.P., Hugas, M., Cocconcelli, P.S., Richard-Forget, F., Klein, G., Licht, T.R., Nguyen-The, C., Querol, A., Richardson, M., 2010. Qualified presumption of safety (QPS): a generic risk assessment approach for biological agents notified to the European Food Safety Authority (EFSA). Trends in Food Science & Technology 21, 425–435.

Li, Y., Odumeru, J., Griffiths, M., McKellar, R., 2006. Effect of environmental stresses on the mean and distribution of individual cell lag times of *Escherichia coli* O157: H7. International Journal of Food Microbiology 110, 278–285.

Linnan, M., Mascola, L., Lou, X., Goulet, V., May, S., Salminen, C., Hird, D., Yonekura, M., Hayes, P., Weaver, R., Audurier, A., Plikaytis, B., Fannin, S., Kleks, A., Broome, C., 1988. Epidemic listeriosis associated with Mexican-style cheese. New England Journal of Medicine 319, 823–828.

Liu, L., O'Conner, P., Cotter, P.D., Hill, C., Ross, R.P., 2008. Controlling *Listeria monocytogenes* in cottage cheese through heterologous production of enterocin A by *Lactococcus lactis*. Journal of Applied Microbiology 104, 1059–1066.

Low, J.C., Donachie, W., 1997. A review of *Listeria monocytogenes* and listeriosis. The Veterinary Journal 153, 9–29.

Lucey, J., Kelly, J., 1994. Cheese yield. International Journal of Dairy Technology 47, 1–14.

Luedeking, R., Piret, E.L., 1959. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. Journal of Biochemical and Microbiological Technology and Engineering 1, 393–412.

MacDonald, P.D.M., Whitwam, R.E., Boggs, J.D., MacCormack, J.N., Anderson, K.L., Reardon, J.W., Saah, J.R., Graves, L.M., Hunter, S.B., Sobel, J., 2005. Outbreak of listeriosis among Mexican immigrants as a result of consumption of illicitly produced Mexican-style cheese. Clinical Infectious Diseases 40, 677–82.

Malakar, P., Barker, G., Zwietering, M., van't Riet, K., 2003. Relevance of microbial interactions to predictive microbiology. International Journal of Food Microbiology 84, 263–272.

Malakar, P.K., Martens, D.E., Zwietering, M.H., Beal, C., Van't Riet, K., 1999. Modelling the interactions between *Lactobacillus curvatus* and *Enterobacter cloacae*: II. Mixed cultures and shelf life predictions. International Journal of Food Microbiology 51, 67–79.

Manios, S., Konstantinidis, N., Gounadaki, A.S., Skandamis, P., 2013. Dynamics of low (1–4 cells) vs high populations of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut salads and their sterile liquid or solidified extracts. Food Control 29, 318–327.

Martens, D.E., Béal, C., Malakar, P., Zwietering, M.H., van 't Riet, K., 1999. Modelling the interactions between *Lactobacillus curvatus* and *Enterobacter cloacae*. International Journal of Food Microbiology 51, 53–65.

Martínez, B., Bravo, D., Rodríguez, A., 2005. Consequences of the development of Nisin-resistant *Listeria monocytogenes* in fermented dairy products. Journal of Food Protection 68, 2383–2388.

Marvin, H.J.P., Kleter, G.A., Frewer, L.J., Cope, S., Wentholt, M.T.A., Rowe, G., 2009. A working procedure for identifying emerging food safety issues at an early stage: Implications for European and international risk management practices. Food Control 20, 345–356.

Masana, M.O., Baranyi, J., 2000. Adding new factors to predictive models: the effect on the risk of extrapolation. Food Microbiology 17, 367–374.

Mataragas, M., Drosinos, E.H., Vaidanis, A., Metaxopoulos, I., 2006. Development of a predictive model for spoilage of cooked cured meat products and its validation under constant and dynamic temperature storage conditions. Journal of Food Science 71, M157–M167.

McAuliffe, O., Hill, C., Ross, R.P., 1999. Inhibition of *Listeria monocytogenes* in cottage cheese manufactured with a lacticin 3147-producing starter culture. Journal of Applied Microbiology 86, 251–256.

McClure, P., Beaumont, A., Sutherland, J., Roberts, T., 1997. Predictive modelling of growth of *Listeria monocytogenes* The effects on growth of NaCl, pH, storage temperature and NaNO 2. International Journal of Food Microbiology 34, 221–232.

McClure, P., Kelly, T., Roberts, T., 1991. The effects of temperature, pH, sodium chloride and sodium nitrite on the growth of *Listeria monocytogenes*. International Journal of Food Microbiology 14, 77–91.

McKellar, R., 1997. A heterogeneous population model for the analysis of bacterial growth kinetics. International Journal of Food Microbiology 36, 179–186.

McKellar, R., Knight, K., 2000. A combined discrete–continuous model describing the lag phase of *Listeria monocytogenes*. International Journal of Food Microbiology 54, 171–180.

McKellar, R., Lu, X., 2004. Primary models, in: McKellar, R., Lu, X. (Eds.), Modeling Microbial Responses in Food. CRC Press, pp. 21–62.

McMeekin, T., Baranyi, J., Bowman, J., Dalgaard, P., Kirk, M., Ross, T., Schmid, S., Zwietering, M.H., 2006. Information systems in food safety management. International Journal of Food Microbiology 112, 181–194.

McMeekin, T., Bowman, J., McQuestin, O., Mellefont, L., Ross, T., Tamplin, M., 2008. The future of predictive microbiology: Strategic research, innovative applications and great expectations. International Journal of Food Microbiology 128, 2–9.

McMeekin, T., Brown, J., Krist, K., 1997. Quantitative microbiology: a basis for food safety. Emerging Infectious Diseases 3, 541–549.

McMeekin, T., Olley, J., Ratkowsky, D., Corkrey, R., Ross, T., 2013. Predictive microbiology theory and application: Is it all about rates? Food Control 29, 290–299.

McMeekin, T., Ross, T., 2002. Predictive microbiology: providing a knowledge-based framework for change management. International Journal of Food Microbiology 78, 133–153.

McMeekin, T.A., 2007. Predictive microbiology: quantitative science delivering quantifiable benefits to the meat industry and other food industries. Meat Science 77, 17–27.

McMeekin, T.A., Hill, C., Wagner, M., Dahl, A., Ross, T., 2010a. Ecophysiology of food-borne pathogens: essential knowledge to improve food safety. International Journal of Food Microbiology 139, S64–S78.

McMeekin, T.A., Olley, J., Ratkowsky, D.A., Ross, T., 2002. Predictive microbiology: towards the interface and beyond. International Journal of Food Microbiology 73, 395–407.

McMeekin, T.A., Tamplin, M.L., Ross, T., Koseki, S., 2010b. The way forward with predictive microbiology in the dairy industry. Australian Journal of Dairy Technology 65, 91–97.

Medveďová, A., Liptáková, D., Hudecová, A., Valík, Ľ., 2008. Quantification of the growth competition of lactic acid bacteria: a case of co-culture with *Geotrichum candidum* and *Staphylococcus aureus*. Acta Chimica Slovaca 1, 192–207.

Mehta, B.M., de Oliveira, M., 2012. Fermented Dairy Products, in: Fermentation, Chemical & Functional Properties of Food Components. CRC Press, pp. 259–283.

Mejlholm, O., Bøknæs, N., Dalgaard, P., 2014. Development and validation of a stochastic model for potential growth of *Listeria monocytogenes* in naturally contaminated lightly preserved seafood. Food Microbiology. DOI: 10.1016/j.fm.2014.06.006

Mejlholm, O., Dalgaard, P., 2007a. Modeling and predicting the growth of lactic acid bacteria in lightly preserved seafood and their inhibiting effect on *Listeria monocytogenes*. Journal of Food Protection 70, 2485–2497.

Mejlholm, O., Dalgaard, P., 2007b. Modeling and predicting the growth boundary of *Listeria monocytogenes* in lightly preserved seafood. Journal of Food Protection 70, 70–84.

Mejlholm, O., Dalgaard, P., 2009. Development and validation of an extensive growth and growth boundary model for *Listeria monocytogenes* in lightly preserved and ready-to-eat shrimp. Journal of Food Protection 72, 2132–2143.

Mejlholm, O., Dalgaard, P., 2013. Development and validation of an extensive growth and growth boundary model for psychrotolerant *Lactobacillus* spp. in seafood and meat products. International Journal of Food Microbiology 167, 244–260.

Mejlholm, O., Gunvig, A., Borggaard, C., Blom-Hanssen, J., Mellefont, L., Ross, T., Leroi, F., Else, T., Visser, D., Dalgaard, P., 2010. Predicting growth rates and growth boundary of *Listeria monocytogenes* -An international validation study with focus on processed and ready-to-eat meat and seafood. International Journal of Food Microbiology 141, 137–150.

Mellefont, L.A., McMeekin, T.A., Ross, T., 2003. The effect of abrupt osmotic shifts on the lag phase duration of foodborne bacteria. International Journal of Food Microbiology 83, 281–293.

Mellefont, L.A., McMeekin, T.A., Ross, T., 2004. The effect of abrupt osmotic shifts on the lag phase duration of physiologically distinct populations of *Salmonella* Typhimurium. International Journal of Food Microbiology 92, 111–20.

Mellefont, L.A., McMeekin, T.A., Ross, T., 2005. Viable count estimates of lag time responses for *Salmonella* Typhimurium M48 subjected to abrupt osmotic shifts. International Journal of Food Microbiology 105, 399–410.

Mellefont, L.A., McMeekin, T.A., Ross, T., 2008. Effect of relative inoculum concentration on *Listeria monocytogenes* growth in co-culture. International Journal of Food Microbiology 121, 157–168.

Mellefont, L.A., Ross, T., 2003. The effect of abrupt shifts in temperature on the lag phase duration of *Escherichia coli* and *Klebsiella oxytoca*. International Journal of Food Microbiology 83, 295–305.

Membré, J.-M., Lambert, R.J.W., 2008. Application of predictive modelling techniques in industry: From food design up to risk assessment. International Journal of Food Microbiology 128, 10–15.

Membré, J.-M., Ross, T., McMeekin, T., 2002. Behaviour of *Listeria monocytogenes* under combined chilling processes. Letters in Applied Microbiology 28, 216–220.

Messens, W., Neysens, P., Vansieleghem, W., Vanderhoeven, J., De Vuyst, L., 2002. Modeling growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in response to temperature and pH values used for sourdough fermentations. Applied and Environmental Microbiology 68, 1431–1435.

Messens, W., Verluyten, J., Leroy, F., De Vuyst, L., 2003. Modelling growth and bacteriocin production by *Lactobacillus curvatus* LTH 1174 in response to temperature and pH values used for European sausage fermentation processes. International Journal of Food Microbiology 81, 41–52.

Métris, A., George, S.M., Peck, M.W., Baranyi, J., 2003. Distribution of turbidity detection times produced by single cell-generated bacterial populations. Journal of Microbiological Methods 55, 821–827.

Métris, A., Le Marc, Y., Elfwing, A., Ballagi, A., Baranyi, J., 2005. Modelling the variability of lag times and the first generation times of single cells of *E. coli*. International Journal of Food Microbiology 100, 13–9.

Miller, G.D., Jarvis, J.K., McBean, L.D., 2006. The Importance of Milk and Milk Products in the Diet, in: Handbook of Dairy Foods and Nutrition. CRC Press, pp. 1–53.

Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annual Reviews in Microbiology 55, 165–199.

Moldes, A.B., Alonso, J.L., Parajó, J.C., 1999. Cogeneration of cellobiose and glucose from pretreated wood and bioconversion to lactic acid: A kinetic study. Journal of Bioscience and Bioengineering 87, 787–792.

Monod, J., 1949. The growth of bacterial cultures. Annual Reviews in Microbiology 3, 371–394.

Monteagudo, J.M., Rodríguez, L., Rincón, J., Fuertes, J., 1997. Kinetics of lactic acid fermentation by *Lactobacillus delbrueckii* grown on beet molasses. Journal of Chemical Technology & Biotechnology 68, 271–276.

Montville, T., Matthews, K., 2007. Growth, Survival and Death of Microbes in Foods, in: Doyle, M.P., Beuchat, L. (Eds.), Food Microbiology, Fundamentals and Frontiers. ASM Press, pp. 3–22.

Morgan, S., Ross, R.P., Hill, C., 1997. Increasing starter cell lysis in cheddar cheese using a bacteriocin-producing adjunct. Journal of Dairy Science 80, 1–10.

Mounier, J., Monnet, C., Vallaeys, T., Arditi, R., Sarthou, A.-S., Hélias, A., Irlinger, F., 2008. Microbial interactions within a cheese microbial community. Applied and Environmental Microbiology 74, 172–181.

Murray, E.G.D., Webb, R.A., Swann, M.B.R., 1926. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). Journal of Pathology and Bacteriology 29, 407 – 439.

Møller, C.O.A., Ilg, Y., Dalgaard, P., Christensen, B.B., Aabo, S., Hansen, T.B., 2013. Effect of natural microbiota on growth of *Salmonella* spp. in fresh pork - A predictive microbiology approach. Food Microbiology 34, 284–295.

Nauta, M., 2000. Separation of uncertainty and variability in quantitative microbial risk assessment models. International Journal of Food Microbiology 57, 9–18.

Nauta, M., 2007. Uncertainty and variability in predictive models of microorganisms in food, in: Brul, S., van Gerwen, S.J.C., Zwietering, M.H. (Eds.), Modelling Microorganisms in Food. Woodhead Publishing in Food Science, Technology and Nutrition, pp. 44–66.

Nichols, D.S., Olley, J., Garda, H., Brenner, R.R., McMeekin, T.A., 2000. Effect of temperature and salinity stress on growth and lipid composition of *Shewanella gelidimarina*. Applied and Environmental Microbiology 66, 2422–2429.

Nielsen, K., 2014. Personal communication. PTU Assistant, Korsvej Dairy, Fredericia, Denmark.

Nilsson, L., Hansen, T.B., Garrido, P., Buchrieser, C., Glaser, P., Knøchel, S., Gram, L., Gravesen, A., 2005. Growth inhibition of *Listeria monocytogenes* by a nonbacteriocinogenic *Carnobacterium piscicola*. Journal of Applied Microbiology 98, 172–183.

Norton, D.M., Braden, C.R., 2007. Foodborne Listeriosis, in: Ryser, E.T., Marth, E.H. (Eds.), *Listeria*, Listeriosis, and Food Safety, Food Science and Technology. CRC Press, pp. 305–356.

Oscar, T.E., 2005. Validation of lag time and growth rate models for *Salmonella* Typhimurium: acceptable prediction zone method. Journal of Food Science 70, 129–137.

Painter, J., Slutsker, L., 2007. Listeriosis in Humans, in: Ryser, E., Marth, E. (Eds.), *Listeria*, Listeriosis, and Food Safety, Third Edition, Food Science and Technology. CRC Press, pp. 85–109.

Pal, A., Labuza, T.P., Diez-Gonzalez, F., 2008. Comparison of primary predictive models to study the growth of *Listeria monocytogenes* at low temperatures in liquid cultures and selection of fastest growing ribotypes in meat and turkey product slurries. Food Microbiology 25, 460–70.

Panagou, E.Z., Nychas, G.-J.E., 2008. Dynamic modeling of *Listeria monocytogenes* growth in pasteurized vanilla cream after postprocessing contamination. Journal of Food Protection 71, 1828–1834.

Papademas, P., Bintsis, T., 2010. Food safety management systems (FSMS) in the dairy industry: A review. International Journal of Dairy Technology 63, 489–503.

Pascual, C., Robinson, T., 2001. The effect of inoculum size and sublethal injury on the ability of *Listeria monocytogenes* to initiate growth under suboptimal conditions. Letters in Applied Microbiology 33, 357–361.

Pedersen, A.N., Fragt, S., Groth, M. V, Christensen, T., Biltoft-Jensen, A., Matthiesen, J., Andersen, N.., Kørup, K., Hartkopp, H., Ygil, K.H., Hinsch, H.-J., Saxholt, E., Trolle, E., 2010. Dietary habits in Denmark 2003-2008. DTU Food.

Peleg, M., Corradini, M.G., 2011. Microbial growth curves: what the models tell us and what they cannot. Critical Reviews in Food Science and Nutrition 51, 917–945.

Perez-Rodriguez, F., Valero, A., 2013. Predictive Microbiology in Foods. Springer New York, New York, NY.

Piccinin, D.M., Shelef, L.A., 1995. Survival of *Listeria monocytogenes* in cottage cheese. Journal of Food Protection 174 58, 128–131.

Pin, C., Baranyi, J., 2006. Kinetics of single cells: observation and modeling of a stochastic process. Applied and Environmental Microbiology 72, 2163–2169.

Pinon, A., Zwietering, M., Perrier, L., Membré, J.-M., Leporq, B., Mettler, E., Thuault, D., Coroller, L., Stahl, V., Vialette, M., Membre, J.-M., 2004. Development and validation of experimental protocols for use of cardinal models for prediction of microorganism growth in food products. Applied and Environmental Microbiology 70, 1081–1087.

Pirt, S.J., 1975. Product formation in microbial cultures, in: Principles of Microbe and Cell Cultivation. Blackwell Scientific Publications., pp. 156–169.

Porto de Souza Vandenberghe, L., Yamaguishi, C.T., Rodrigues, C., Prado, M.R.M., Spier, M.R., Medeiros, A.B.P., de Carvalho, J.C., 2013. Production of Dairy Products, in: Soccol, C.R., Pandey, A., Larroche, C. (Eds.), Fermentation Processes Engineering in the Food Industry. CRC Press, pp. 323–354.

Poschet, F., Vereecken, K.M., Geeraerd, A.H., Nicolaï, B.M., Van Impe, J.F., 2005. Analysis of a novel class of predictive microbial growth models and application to coculture growth. International Journal of Food Microbiology 100, 107–24.

Postollec, F., Falentin, H., Pavan, S., Combrisson, J., Sohier, D., 2011. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiology 28, 848–861.

Pouillot, R., Albert, I., Cornu, M., Denis, J.-B., 2003. Estimation of uncertainty and variability in bacterial growth using Bayesian inference. Application to *Listeria monocytogenes*. International Journal of Food Microbiology 81, 87–104.

Pouillot, R., Miconnet, N., Afchain, A.-L., Delignette-Muller, M.L., Beaufort, A., Rosso, L., Denis, J.-B., Cornu, M., 2007. Quantitative risk assessment of *Listeria monocytogenes* in French cold-smoked salmon: I. Quantitative exposure assessment. Risk Analysis 27, 683–700.

Powell, M., Schlosser, W., Ebel, E., 2004. Considering the complexity of microbial community dynamics in food safety risk assessment. International Journal of Food Microbiology 90, 171–179.

Press, W., Teukolsky, S., Vetterling, W., Flannery, B., 1992. Integration of ordinary differential equations, in: Numerical Recipes in C The Art of Scientific Computing. Cambridge University Press, pp. 707–752.

Presser, K., Ratkowsky, D., Ross, T., 1997. Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. Applied and Environmental Microbiology 63, 2355–2360.

Ratkowsky, D.A., Olley, J., McMeekin, T.A., Ball, A., 1982. Relationship between temperature and growth rate of bacterial cultures. Journal of Bacteriology 149, 1–5.

Ricke, S., 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry Science 82, 632–639.

Ries, F., Dicato, M., Hemmer, R., Arendt, F., 1990. Camembert, *Listeria* and the immunocompromised patient. Bulletin de la Société des sciences médicales du Grand-Duché de Luxembourg 127, 41–43.

Roberts, R., Zottola, E., 1993. Shelf-life of pasteurized process cheese spreads made from cheddar cheese manufactured with a nisin-producing starter culture. Journal of Dairy Science 76, 1829 – 1836.

Robinson, T., Aboaba, O., Kaloti, A., 2001. The effect of inoculum size on the lag phase of *Listeria monocytogenes*. International Journal of Food Microbiology 70, 163–173.

Rocourt, J., Buchrieser, C., 2007. The Genus *Listeria* and *Listeria monocytogenes*, in: *Listeria*, Listeriosis, and Food Safety, Third Edition, Food Science and Technology. CRC Press, pp. 1–20.

Rodríguez, A., Martínez, B., Suárez, J., 2012. Dairy Starter Cultures, in: Handbook of Animal-Based Fermented Food and Beverage Technology, Second Edition. CRC Press, pp. 31–48.

Rogers, P.L., Bramall, L., McDonald, I.J., 1978. Kinetic analysis of batch and continuous culture of *Streptococcus cremoris* HP. Canadian Journal of Microbiology 24, 372–380.

Ross, T., 1996. Indices for performance evaluation of predictive models in food microbiology. Journal of Applied Microbiology 81, 501–508.

Ross, T., 1999. Chapter 5: Quantifying and utilising lag times in predictive microbiology, in: Predictive Microbiology for the Meat Industry. Meat and Livestock Australia, pp. 141–176.

Ross, T., Dalgaard, P., 2004. Secondary Models, in: McKellar, R., Lu, X. (Eds.), Modeling Microbial Responses in Food, Contemporary Food Science. CRC Press, pp. 63–150.

Ross, T., Dalgaard, P., Tienungoon, S., 2000. Predictive modelling of the growth and survival of *Listeria* in fishery products. International Journal of Food Microbiology 62, 231–245.

Ross, T., McMeekin, T.A., 1994. Predictive microbiology. International Journal of Food Microbiology 23, 241–264.

Ross, T., McMeekin, T.A., 2003. Modeling microbial growth within food safety risk assessments. Risk Analysis 23, 179–197.

Ross, T., Sumner, J., 2002. A simple, spreadsheet-based, food safety risk assessment tool. International Journal of Food Microbiology 77, 39–53.

Rosso, L., Lobry, J.R., Bajard, S., Flandrois, J.P., 1995. Convenient model to describe the combined effects of temperature and pH on microbial growth. Applied and Environmental Microbiology 61, 610–616.

Rosso, L., Lobry, J.R., Flandrois, J.P., 1993. An unexpected correlation between cardinal temperatures of microbial growth highlighted by a new model. Journal of Theoretical Biology 162, 447–463.

Rosso, L., Robinson, T.P., 2001. A cardinal model to describe the effect of water activity on the growth of moulds. International Journal of Food Microbiology 63, 265–273.

Rudolf, M., Scherer, S., 2001. High incidence of *Listeria monocytogenes* in European red smear cheese. International Journal of Food Microbiology 63, 91–98.

Salaün, F., Mietton, B., Gaucheron, F., 2005. Buffering capacity of dairy products. International Dairy Journal 15, 95–109.

Salih, M., Sandine, W., Ayres, J., 1990. Inhibitory effects of Microgard™ on yogurt and cottage cheese spoilage organisms. Journal of Dairy Science 73, 887–893.

Samapundo, S., Anthierens, T., Ampofo-Asiama, J., Xhaferi, R., Van Bree, I., Szczepaniak, S., Goemaere, O., Steen, L., Dhooge, M., Paelinck, H., Devlieghere, F., 2013. The effect of NaCl reduction and replacement on the growth of *Listeria Monocytogenes* in broth, cooked ham and white sauce. Journal of Food Safety 33, 59–70.

Schaffner, D.W., 1994. Application of a statistical bootstrapping technique to calculate growth rate variance for modelling psychrotrophic pathogen growth. International Journal of Food Microbiology 24, 309–14.

Schichl, H., 2004. Models and history of modeling, in: Kallrath, J. (Ed.), Modeling Languages in Mathematical Optimization, Applied Optimization. Springer US, Boston, MA, pp. 25–36.

Schlech, W.F., 2000. Foodborne listeriosis. Clinical Infectious Diseases 31, 770–775.

Schuchat, A., Swaminathan, B., Broome, C., 1991. Epidemiology of human listeriosis. Clinical Microbiology Reviews 4, 169–183.

Schvartzman, M.S., Maffre, A., Tenenhaus-Aziza, F., Sanaa, M., Butler, F., Jordan, K., 2011. Modelling the fate of *Listeria monocytogenes* during manufacture and ripening of smeared cheese made with pasteurised or raw milk. International Journal of Food Microbiology 145, S31–S38.

Skogberg, K., Syrjanen, J., Jahkola, M., Renkonen, O.-V., Paavonen, J., Ahonen, J., Kontiainen, S., Ruutu, P., Valtonen, V., 1992. Clinical presentation and outcome of listeriosis in patients with and without immunosuppressive therapy. Clinical Infectious Diseases 14, 815–821.

Smelt, J., Otten, G., Bos, A., 2002. Modelling the effect of sublethal injury on the distribution of the lag times of individual cells of *Lactobacillus plantarum*. International Journal of Food Microbiology 73, 207–212.

Smid, J.H., Verloo, D., Barker, G.C., Havelaar, A.H., 2010. Strengths and weaknesses of Monte Carlo simulation models and Bayesian belief networks in microbial risk assessment. International Journal of Food Microbiology 139, S57–S63.

Sobrino-López, A., Martín-Belloso, O., 2008. Use of nisin and other bacteriocins for preservation of dairy products. International Dairy Journal 18, 329–343.

Sorells, K., Enigl, D., Hatfield, J., 1990. Effect of pH, acidulant, time and temperature on the growth and survival of *Listeria monocytogenes*. Journal of Food Protection 52, 571–573.

Stack, H., Hill, C., Gahan, C.M., 2008. Stress Responses, in: Liu, D. (Ed.), Handbook of *Listeria Monocytogenes*. CRC Press, pp. 61–96.

Stecchini, M.L., Del Torre, M., Venir, E., 2004. Growth of *Listeria monocytogenes* as influenced by viscosity and water activity. International Journal of Food Microbiology 96, 181–187.

Stephens, P.J., Joynson, J.A., Davies, K.W., Holbrook, R., Lappin-Scott, H.M., Humphrey, T.J., 1997. The use of an automated growth analyser to measure recovery times of single heat-injured *Salmonella* cells. Journal of Applied Microbiology 83, 445–455.

Stevenson, D.M., Muck, R.E., Shinners, K.J., Weimer, P.J., 2006. Use of real time PCR to determine population profiles of individual species of lactic acid bacteria in alfalfa silage and stored corn stover. Applied Microbiology and Biotechnology 71, 329–338.

Stollewerk, K., Jofré, A., Comaposada, J., Arnau, J., Garriga, M., 2014. NaCl-free processing, acidification, smoking and high pressure: Effects on growth of *Listeria monocytogenes* and *Salmonella enterica* in QDS processed® dry-cured ham. Food Control 35, 56–64.

Stratford, M., Anslow, P.A., 1998. Evidence that sorbic acid does not inhibit yeast as a classic "weak acid preservative". Letters in Applied Microbiology 27, 203–206.

Šušković, J., Kos, B., Beganović, J., Leboš Pavunc, A., Habjanič, K., Matošić, S., 2010. Antimicrobial activity – The most important property of probiotic and starter lactic acid bacteria. Food Technology and Biotechnology 48, 296–307.

Swaminathan, B., Gerner-Smidt, P., 2007. The epidemiology of human listeriosis. Microbes and Infection 9, 1236–1243.

Swinnen, I.A.M., Bernaerts, K., Dens, E.J.J., Geeraerd, A.H., Van Impe, J.F., 2004. Predictive modelling of the microbial lag phase: a review. International Journal of Food Microbiology 94, 137–159.

Sørhaug, T., Stepaniak, L., 1997. Psychrotrophs and their enzymes in milk and dairy products: quality aspects. Trends in Food Science & Technology 8, 35–41.

Te Giffel, M.C., Zwietering, M.H., 1999. Validation of predictive models describing the growth of *Listeria monocytogenes*. International Journal of Food Microbiology 46, 135–149.

Tenenhaus-Aziza, F., Daudin, J.-J., Maffre, A., Sanaa, M., 2014. Risk-based approach for microbiological food safety management in the dairy industry: The case of *Listeria monocytogenes* in soft cheese made from pasteurized milk. Risk Analysis 34, 56–74.

Theron, M., Lues, J., 2011a. Large-scale organic acid production, in: Organic Acids and Food Preservation. CRC Press, pp. 165–184.

Theron, M., Lues, J., 2011b. Detection of Organic Acids, in: Organic Acids and Food Preservation. CRC Press, pp. 293–306.

Thomas, H.A., 1942. Bacterial densities from fermentation tube tests. Journal of American Water Works Association 34, 572–576.

Thomas, L., Wimpenny, J., 1996. Investigation of the effect of combined variations in temperature, pH, and NaCl concentration on nisin inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*. Applied and Environmental Microbiology 62, 2006–2012.

Thomas, L. V., 1999. Permitted preservatives - Sorbic acid, in: Encyclopedia of Food Microbiology. Elsevier, pp. 1769–1776.

Tienungoon, S., Ratkowsky, D.A., McMeekin, T.A., Ross, T., 2000. Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. Applied and Environmental Microbiology 66, 4979–4987.

Turner, M., Blumenstein, B., Sebaugh, J., 1969. 265 Note: A Generalization of the Logistic Law of Growth. Biometrics 25, 577–580.

Urbach, G., 1995. Contribution of lactic acid bacteria to flavour compound formation in dairy products. International Dairy Journal 5, 877–903.

US EPA, 2001. Risk Assessment Guidance for Superfund (RAGS) Volume III - Part A: Process for Conducting Probabilistic Risk Assessment, Appendix B.

Vaikousi, H., Biliaderis, C.G., Koutsoumanis, K.P., 2008. Development of a microbial time/temperature indicator prototype for monitoring the microbiological quality of chilled foods. Applied and Environmental Microbiology 74, 3242–3250.

Van Boeckel, M., 2008. Kinetics of Microbial Growth, in: Kinetic Modelling of Reactions in Foods. CRC Press, pp. 12–1 – 12–40.

Van der Meulen, R., Scheirlinck, I., Van Schoor, A., Huys, G., Vancanneyt, M., Vandamme, P., De Vuyst, L., 2007. Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. Applied and Environmental Microbiology 73, 4741–4750.

Van Gerwen, S.J.C., Zwietering, M.H., 1998. Growth and inactivation models to be used in quantitative risk assessments. Journal of Food Protection 61, 1541–1549.

Van Impe, J.F., Poschet, F., Geeraerd, A.H., Vereecken, K.M., 2005. Towards a novel class of predictive microbial growth models. International Journal of Food Microbiology 100, 97–105.

Van Impe, J.F., Vercammen, D., Van Derlinden, E., 2013. Toward a next generation of predictive models: A systems biology primer. Food Control 29, 336–342.

Vandenbergh, P.A., 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiology Reviews 12, 221–237.

Vasseur, C., Baverel, L., Hebraud, M., Labadie, J., 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. Journal of Applied Microbiology 86, 469–476.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* Pathogenesis and Molecular Virulence Determinants. Clinical Microbiology Reviews 14, 581–640.

Velugoti, P.R., Bohra, L.K., Juneja, V.K., Huang, L., Wesseling, A.L., Subbiah, J., Thippareddi, H., 2011. Dynamic model for predicting growth of *Salmonella* spp. in ground sterile pork. Food Microbiology 28, 796–803.

Venkatesh, K.V., Okos, M.R., Wankat, P.C., 1993. Kinetic model of growth and lactic acid production from lactose by *Lactobacillus bulgaricus*. Process Biochemistry 28, 231–241.

Vereecken, K.M., Dens, E., Van Impe, J.F., 2000. Predictive modeling of mixed microbial populations in food products: evaluation of two-species models. Journal of Theoretical Biology 205, 53–72.

Vereecken, K.M., Devlieghere, F., Bockstaele, A., Debevere, J., Van Impe, J.F., 2003. A model for lactic acid-induced inhibition of *Yersinia enterocolitica* in mono- and coculture with *Lactobacillus sakei*. Food Microbiology 20, 701–713.

Vereecken, K.M., Van Impe, J.F., 2002. Analysis and practical implementation of a model for combined growth and metabolite production of lactic acid bacteria. International Journal of Food Microbiology 73, 239–250.

Vermeulen, A., Devlieghere, F., De Loy-Hendrickx, A., Uyttendaele, M., 2011. Critical evaluation of the EU-technical guidance on shelf-life studies for *L. monocytogenes* on RTE-foods: a case study for smoked salmon. International Journal of Food Microbiology 145, 176–185.

Vicari, A.S., 2007. Second-order modeling of variability and uncertainty in microbial hazard characterization. Journal of Food Protection 70, 363 – 372.

Vinderola, C.G., Costa, G.A., Regenhardt, S., Reinheimer, J.A., 2002. Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. International Dairy Journal 12, 579–589.

Von Wright, A., Axelsson, L., 2011. Lactic Acid Bacteria: An Introduction, in: Lactic Acid Bacteria. CRC Press, pp. 1–16.

Vose, D., 2000. Risk analysis A quantitative guide, 2nd ed. John Wiley & Sons, Ltd.

Vose, D., 2010. Fitting distributions to data. www.vosesoftware.com (accessed 7.12.14).

Vrancken, G., Rimaux, T., De Vuyst, L., Leroy, F., 2008. Kinetic analysis of growth and sugar consumption by *Lactobacillus fermentum* IMDO 130101 reveals adaptation to the acidic sourdough ecosystem. International Journal of Food Microbiology 128, 58–66.

Wagner, M., McLauchlin, J., 2008. Biology, in: Liu, D. (Ed.), Handbook of *Listeria Monocytogenes*. CRC Press, pp. 3–25.

Walker, S.J., 1988. Major spoilage micro-organisms in milk and dairy products. International Journal of Dairy Technology 41, 91–92.

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2005a. Cheese Varieties, in: Dairy Science and Technology, Second Edition, Food Science and Technology. CRC Press, pp. 687–741.

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2005b. Lactic Fermentations, in: Dairy Science and Technology, Second Edition, Food Science and Technology. CRC Press, pp. 357–398.

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2005c. Cheese Ripening and Properties, in: Dairy Science and Technology, Second Edition, Food Science and Technology. CRC Press, pp. 641–676.

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2005d. Heat Treatment, in: Dairy Science and Technology, Second Edition, Food Science and Technology. CRC Press, pp. 225–272.

Walstra, P., Wouters, J.T.M., Geurts, T.J., 2005e. Cheese Manufacture, in: Dairy Science and Technology, Second Edition, Food Science and Technology. CRC Press, pp. 583–639.

Walther, B., Schmid, A., Sieber, R., Wehrmüller, K., 2008. Cheese in nutrition and health. Dairy Science and Technology 88, 389–405.

Whiting, R.C., 1993. Modeling bacterial survival in unfavorable environments. Journal of Industrial Microbiology 12, 240–246.

Whiting, R.C., Cygnarowicz-Provost, M., 1992. A quantitative model for bacterial growth and decline. Food Microbiology 9, 269–277.

WHO/FAO, 2004. Risk assessment of *Listeria monocytogenes* in ready-to-eat foods Technical report. World Health Organization.

Wijtzes, T., De Wit, J.C., Huis, I., Van't Riet, K., Zwietering, M.H., 1995. Modelling bacterial growth of *Lactobacillus curvatus* as a function of acidity and temperature. Applied and Environmental Microbiology 61, 2533–2539.

Wijtzes, T., Rombouts, F.M., Kant-Muermans, M.L.T., Van't Riet, K., Zwietering, M.H., 2001. Development and validation of a combined temperature, water activity, pH model for bacterial growth rate of *Lactobacillus curvatus*. International Journal of Food Microbiology 63, 57–64.

Wilson, P.D., Wilson, D.R., Waspe, C.R., 2000. Weak acids: dissociation in complex buffering systems and partitioning into oils. Journal of the Science of Food and Agriculture 80, 471–476.

Wouters, J.T.., Ayad, E.H.., Hugenholtz, J., Smit, G., 2002. Microbes from raw milk for fermented dairy products. International Dairy Journal 12, 91–109.

Yang, R., Johnson, M.C., Ray, B., 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 58, 3355–3359.

Yeh, P.L.-H., Bajpai, R.K., Iannotti, E.L., 1991. An improved kinetic model for lactic acid fermentation. Journal of Fermentation and Bioengineering 71, 75–77.

Zaika, L., Fanelli, J., 2003. Growth kinetics and cell morphology of *Listeria monocytogenes* Scott A as affected by temperature, NaCl, and EDTA. Journal of Food Protection 7, 1208–1215.

Zwietering, M., Cuppers, H., De Wit, J., van't Riet, K., 1994a. Evaluation of data transformations and validation of a model for the effect of temperature on bacterial growth. Applied and Environmental Microbiology 60, 195–203.

Zwietering, M., De Wit, J., Cuppers, H., Van't Riet, K., 1994b. Modeling of bacterial growth with shifts in temperature. Applied and Environmental Microbiology 60, 195–203.

Zwietering, M.H., de Wit, J.C., Notermans, S., 1996. Application of predictive microbiology to estimate the number of *Bacillus cereus* in pasteurised milk at the point of consumption. International Journal of Food Microbiology 30, 55–70.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van't Riet, K., 1990. Modeling of the bacterial growth curve. Applied and Environmental Microbiology 56, 1875–1881.

Zwietering, M.H., Wijtzes, T., Rombouts, F.M., Van't Riet, K., 1993. A decision support system for prediction of microbial spoilage in foods. Journal of Industrial Microbiology 12, 324–329.

Østergaard, N.B., Dalgaard, P., Eklöw, A., 2014. Modelling the effect of lactic acid bacteria from starter- or aroma culture on growth of *Listeria monocytogenes* in cottage cheese. International Journal of Food Microbiology 188, 15–25.