Microbial changes and safety of lightly preserved seafood

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Microbial Changes and Safety of Lightly Preserved Seafood

By

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Preface

The present Ph.D.-project has been carried out at the Danish Institute for Fisheries Research (DIFRES) in Kgs. Lyngby, Denmark under the supervision of senior scientist Paw Dalgaard. The presented work was financed by the Danish Directorate for Food, Fisheries and Agri Business.

I wish to thank Paw Dalgaard for his support and many valuable discussions, and for introducing me to predictive microbiology.

I would like to thank our industrial partner Royal Greenland Seafood for being supportive throughout the project. Special thanks should go to Niels Bøknæs for his great enthusiasm and for many interesting discussions on “seafood” as well as other essential things in life.

Thanks to Prof. Johanna Björkroth and her group for making my stay at the University of Helsinki, Finland both educational and enjoyable.

I wish to thank Nadereh Samieian and Tina Dahl Devitt for their skilful and valuable technical assistance, and our librarian Søren Tørper Christensen for finding literature.

Last but not least, I would like to thank my beloved wife Lise for her patience and endless support, and Nikolai, Valdemar and Augusta – you are truly precious and unpredictable.

Ole Mejlholm
Kgs. Lyngby, July 2007
Summary

This thesis deals with microbial changes and safety aspects of lightly preserved seafood including cold-smoked and gravad fish, and shrimp products. Lightly preserved seafood is of significant economical importance to the Danish seafood industry. To maintain this position management of spoilage microorganisms and *Listeria monocytogenes* is important as they affect the shelf life and the safety of these products.

The objective of the present Ph.D.-project was to identify means to reduce and prevent growth of spoilage microorganisms and *L. monocytogenes*, respectively, in lightly preserved seafood. Furthermore, it was the aim to develop mathematical models to predict the effect of product characteristics and storage conditions on growth of spoilage microorganisms and *L. monocytogenes* in lightly preserved seafood.

The antimicrobial effect of modified atmosphere packaging (MAP) and organic acids against *L. monocytogenes* was examined in challenge tests with cold-smoked and gravad salmon and Greenland halibut. Growth of *L. monocytogenes* was prevented in MAP cold-smoked fish by using sensorially acceptable concentrations of diacetate and lactate. Thus, it is concluded that diacetate and lactate can be used as additional growth hurdles in lightly preserved seafood in order to prevent growth of *L. monocytogenes*.

A mathematical model was developed to predict the effect of diacetate, lactate, CO₂, smoke components (phenol), nitrite, pH, NaCl, temperature, and interactions between all these parameters on growth and the growth boundary of *L. monocytogenes* in lightly preserved seafood. Validation studies revealed that both growth and the growth boundary of *L. monocytogenes* in lightly preserved seafood were accurately predicted by the developed model. The growth and growth boundary model correctly predicted 73 of 76 growth and no growth responses of *L. monocytogenes* in lightly preserved seafood. The developed model can be used by the seafood industry in order to identify combinations of product characteristics and storage conditions that prevent growth of *L. monocytogenes*, and thereby facilitate production of lightly preserved seafood in compliance with the EU regulation on ready-to-eat foods (EC 2073/2005).

A review of the literature showed that lactic acid bacteria (LAB) are most frequently found as the dominating spoilage microflora of lightly preserved seafood and that they are able to cause spoilage of e.g. cold-smoked salmon. Thus, a predictive model
for growth of LAB in lightly preserved seafood was developed including the effect of diacetate, lactate, CO₂, smoke components (phenol), pH, NaCl, temperature, and interactions between all parameters. Growth of LAB in lightly preserved seafood was correctly predicted by the developed model. The LAB-model can be used by the seafood industry to predict the time for LAB to reach a specified concentration.

The availability of validated models for growth of *L. monocytogenes* and LAB made it possible to predict the effect of microbial interaction on growth (i.e. maximum population density, MPD) of the pathogen in lightly preserved seafood. The performance of the previously developed model for *L. monocytogenes* was improved when the effect of microbial interaction was taken into account. The observed and predicted MPD of *L. monocytogenes* in naturally contaminated vacuum-packed cold-smoked salmon were 0.7 and 0.6 log (CFU/g) when a relative lag time of 4.5 was used. The combined LAB-Lm model can be used to assure and document that the critical limit of 10² CFU/g of *L. monocytogenes* (EC 2073/2005) is not exceeded within the declared shelf life of e.g. cold-smoked salmon. Furthermore, it can be used to predict the concentration of *L. monocytogenes* in lightly preserved seafood e.g. at the time of consumption, and thereby be useful for exposure assessment studies.

The shelf life of cooked and peeled shrimp in MAP at chill temperatures is limited by the potential growth of *L. monocytogenes* as established in challenge tests. This pathogen reached critical concentrations prior to sensory spoilage and predicted time to toxin production by *Clostridium botulinum*. *Carnobacterium maltaromaticum* and *Brochothrix thermosphacta* were identified as the dominating spoilage microflora of cooked and peeled shrimp in MAP. Inoculation experiments with cooked and peeled MAP shrimp showed that a mixture *Cb. maltaromaticum* and *B. thermosphacta* produced a distinct off-flavour resembling the spoilage of naturally contaminated products.

The importance of brine composition on growth of *L. monocytogenes* in chilled brined shrimp was studied in challenge tests. Growth of *L. monocytogenes* was prevented in brined shrimp when benzoic, citric and sorbic acids were used as preservatives in concentrations resembling those of commercial products. However, it was shown that brined shrimp are sensitive to even small changes in the preserving profile, especially the concentration of benzoic acid. Thus, it would be relevant to expand the existing model for *L. monocytogenes* with the effect of benzoic, citric and sorbic acids, to be able to predict
how growth of the pathogen is affected by changes in brine composition. Storage trials were carried out to examine the shelf life and to identify the spoilage microflora of brined shrimp. The composition of the spoilage microflora of brined shrimp was affected by process hygiene, preserving parameters and storage conditions. Eighty-two isolates from the spoilage microflora of brined shrimp was identified and they included 53 LAB, 6 coagulase negative *Staphylococcus* spp., 18 *Pseudomonas fluorescens* and 5 yeast isolates. *Lactobacillus sakei* dominated the spoilage microflora of brined and drained shrimp in MAP whereas a more diverse microflora was found on shrimp in brine.
Sammendrag (summary in Danish)

Denne afhandling omhandler mikrobielle ændringer og sikkerheds aspekter for letkonservede fiskeprodukter herunder koldrøget og gravad fisk samt rejeprodukter. Letkonservede fiskeprodukter er af stor økonomisk betydning for fiskeindustrien. For at opretholde denne position er det vigtigt at kunne styre vækst af fordærvelsesbakterier og *Listeria monocytogenes* da disse påvirker holdbarheden og sikkerheden af produkterne.

Formålet med det foreliggende Ph.D.-projekt var, at identificere metoder til at reducere og forhindre vækst af henholdsvis fordærvelsesbakterier og *L. monocytogenes* i letkonservede fiskeprodukter. Derudover var det formålet at udvikle matematiske modeller til at forudsige effekten af produktkarakteristika og lagringsbetingelser på vækst af fordærvelsesbakterier og *L. monocytogenes* i letkonservede fiskeprodukter.


En gennemgang af litteraturen viste, at mælke-syrebakterier ofte udgør den dominerende fordærvelses-mikroflora for letkonservede fiskeprodukter og at disse

Udviklingen af validerede modeller for vækst af *L. monocytogenes* og mælkesyrebakterier gjorde det muligt at forudsige effekten af mikrobiel interaktion på vækst (maksimal populations densitet, MPD) af *L. monocytogenes* i letkonserverede fiskeprodukter. Vækst og vækstgrænse modellen for *L. monocytogenes* blev forbedret ved at inkludere effekten af mikrobiel interaktion. Den observerede og forudsagte MPD for *L. monocytogenes* i naturligt kontamineret vacuum pakket koldrøget laks var 0,7 og 0,6 log (CFU/g) når en relativ nølefase på 4,5 blev anvendt. Den kombinerede mælkesyrebakterie-*L. monocytogenes* model kan anvendes til at sikre og dokumentere, at den kritiske grænse på 10² CFU/g for *L. monocytogenes* ikke overskrides indenfor den deklarerede holdbarhed for letkonserverede fiskeprodukter heriblandt koldrøget laks. Derudover, kan den anvendes til at forudsige koncentrationen af *L. monocytogenes* i letkonserverede fiskeprodukter f.eks. på tidspunktet for konsum og derved være nyttig i forbindelse med eksponeringsvurderinger.

Holdbarheden af kølede, kogte og pillede rejer i MAP er begrænset af potentiel vækst af *L. monocytogenes* som bestemt i podningsforsøg. *L. monocytogenes* nåede kritiske koncentrationer i kogte og pillede MAP rejer forud for sensorisk fordærv og forudsagt tid til toksindannelse af *Clostridium botulinum*. *Carnobacterium maltaromaticum* og *Brochothrix thermosphacta* blev identificeret som den dominerende fordævelsesmikroflora i kogte og pillede MAP rejer. Podningsforsøg med kogte og pillede MAP rejer viste at en blanding af *Cb. maltaromaticum* and *B. thermosphacta* producerede en karakteristisk bilugt der mindede om fordærvet af naturligt kontaminerede produkter.

Betydningen af lagesammensætning for vækst af *L. monocytogenes* i kølede lagerejer blev undersøgt i podningsforsøg. Vækst af *L. monocytogenes* blev forhindret i lagerejer hvor benzoe-, citron- og sorbinsyre blev anvendt som konserveringsstoffer i koncentrationer der var tæt på dem der anvendes til kommercielle produkter. Det blev
imidlertid vist at lagerejer er følsomme overfor selv små ændringer i konserveringsprofilen, specielt indholdet af benzoesyre. Det vil derfor være relevant at udvide den eksisterende model for *L. monocytogenes* med effekten af benzoe-, citron- og sorbinsyre for at gøre det muligt, at forudsige hvordan vækst af *L. monocytogenes* påvirkes af ændringer i lagesammensætningen. Lagringsforsøg blev udført for at undersøge holdbarheden og for at identificere fordævelsesmikrofloraen for lagerejer. Sammensætningen af fordævelsesmikrofloraen var bestemt af produktionshygiejne, lagesammensætning og lagringsbetingelser. To og firs isolater fra lagerejers fordævelsesmikroflora blev identificeret og de inkluderede 53 mælkesyrebakterier, 6 koagulase negative *Staphylococcus* spp., 18 *Pseudomonas fluorescens* og 5 gær isolater. *Lactobacillus sakei* dominerede fordævelsesmikrofloraen fra drænede lagerejer i MAP, hvorimod en mere varieret mikroflora blev fundet på rejer i lage ("spanderejer").
Table of content

Preface .............................................................................................................................................................. II
Summary .......................................................................................................................................................... III
Sammendrag (summary in Danish).................................................................................................................. VI
Table of content ................................................................................................................................................ IX
1 Introduction ............................................................................................................................................... 1
2 Lightly preserved seafood ......................................................................................................................... 6
  2.1 Processing of lightly preserved seafood ............................................................................................... 7
      2.1.1 Shrimp products ............................................................................................................................... 7
      2.1.2 Cold-smoked and gravad fish .......................................................................................................... 8
  2.2 Preservation techniques/methods ...................................................................................................... 10
      2.2.1 Storage temperature ...................................................................................................................... 10
      2.2.2 Modified atmosphere packaging .................................................................................................... 11
      2.2.3 Brining ............................................................................................................................................ 12
      2.2.4 Smoking ......................................................................................................................................... 14
          2.2.4.1 Smoke generation and smoking procedure........................................................................... 14
          2.2.4.2 Chemical composition of smoke and smoked food................................................................... 16
          2.2.4.3 Analysis of smoke components............................................................................................. 17
  3 Microbial changes and shelf life of lightly preserved seafood ................................................................ 19
  3.1 Microbial changes and shelf life ......................................................................................................... 19
      3.2 Shelf life and spoilage microflora of shrimp and shrimp products...................................................... 20
          3.2.1 Raw shrimp .................................................................................................................................... 20
          3.2.2 Cooked shrimp ............................................................................................................................... 21
          3.2.3 Brined shrimp .................................................................................................................................... 25
      3.3 Shelf life and spoilage microflora of cold-smoked and gravad products............................................ 28
  4 Microbial safety of lightly preserved seafood .......................................................................................... 30
  4.1 Listeria monocytogenes ......................................................................................................................... 30
      4.1.1 Prevalence and level of L. monocytogenes in lightly preserved seafood ........................................... 32
      4.1.2 Listeriosis – cases associated with lightly preserved seafood ....................................................... 37
      4.1.3 Contamination sources ................................................................................................................... 39
          4.1.3.1 Shrimp ................................................................................................................................... 40
          4.1.3.2 Fish products ......................................................................................................................... 40
      4.1.4 Microbiological criteria for L. monocytogenes in RTE foods .......................................................... 42
      4.1.5 Preventing growth of L. monocytogenes in lightly preserved seafood ........................................... 43
          4.1.5.1 Brined shrimp......................................................................................................................... 44
          4.1.5.2 Cold-smoked and gravad fish................................................................................................ 46
  4.2 Clostridium botulinum .......................................................................................................................... 48
5 Predictive models ........................................................................................................................................ 51
  5.1 Modelling of growth ................................................................................................................................ 52
  5.2 Modelling the growth boundary .................................................................................................................. 57
  5.3 Predictive models for spoilage microorganisms ...................................................................................... 58
  5.4 Predictive models for Listeria monocytogenes .......................................................................................... 59
6 Application of validated predictive models ............................................................................................... 67
  6.1 Management of safety and shelf life ......................................................................................................... 68
    6.1.1 Cold-smoked salmon ............................................................................................................................ 68
    6.1.2 Gravad salmon .................................................................................................................................... 72
    6.1.3 Meat products .................................................................................................................................... 74
  6.2 Risk assessment ........................................................................................................................................ 75
7 Conclusions and perspectives ..................................................................................................................... 79
8 References ............................................................................................................................................... 82
The present thesis is based on the following papers:

**Paper 1:**

**Paper 2:**

**Paper 3:**

**Paper 4:**
1 Introduction

Lightly preserved seafood constitute a broad group of chilled stored ready-to-eat (RTE) foods characterised by having pH > 5.0 and < 6 % NaCl in the water phase of the product (DVFA, 2006). Apart from that, it is not easy to define this product category as different types of fish/shell fish, and processing (e.g. brining and smoking) and packaging methods (e.g. vacuum or modified atmosphere packaging) are used for production. The resulting differences in product characteristics and storage conditions makes it a challenge to evaluate and predict microbial changes and potential growth of relevant pathogens e.g. *Listeria monocytogenes* in lightly preserved seafood.

The present Ph.D project focuses on lightly preserved seafood of particular importance to the Danish seafood processing industry and how growth of spoilage microorganisms and *L. monocytogenes* can be reduced and prevented, respectively in these products, and ultimately predicted by mathematical models.

Brined cold-water shrimp comprise the largest and economically most important product to the Danish seafood processing industry with a yearly export value of 170-280 million US$ since 2000 followed by cold-smoked salmon (100-155 million US$ per year) (Figure 1a). In addition to that considerable amounts of gravad ("sugar-salted") salmon, cold-smoked trout and cold-smoked Greenland halibut are produced. The Danish export of brined cold-water shrimp has increased from 19,300 metric tons in 1997 to more than 37,800 metric tons in 2006 whereas the corresponding values of cold-smoked salmon has decreased from 16,700 to 7,700 metric tons during the same period (FAO, 2006). On a global scale, the Danish seafood processing industry contributes significantly with 20 % of brined cold-water shrimp and 30 % of cold-smoked salmon being sold on international export markets (Figure 1b). For both type of products a drop in the unit price by 15-30 % have been noticed since the early 1990’s due to increased international competition. However, it seems like this tendency has been reversed for cold-smoked salmon after a period characterised by closure of processing plants, and relocation of production to less cost expensive countries (e.g. Poland). In the case of brined cold-water shrimp this negative development in unit price is primarily caused by an extensive production and export of warm-water shrimp (*Penaeus* spp.) from e.g. Thailand, China and India (FAO, 2006).
Figure 1. World-wide (---) and Danish (—) export of brined cold-water shrimp (□) and cold-smoked salmon (●) (a). Danish share of the total international export of brined cold-water shrimp (□) and cold-smoked salmon (●) (b). Data from Fishstat Plus (FAO, 2006).

Brined cold-water shrimp are traditionally processed from cooked and peeled shrimp (*Pandalus borealis*) that are preserved in aqueous brine consisting of salt and different types of organic acids (e.g. benzoic, citric and sorbic acids) as preservatives. The majority of these products are produced and distributed as shrimp in brine; however, brined and drained shrimp in modified atmosphere packaging (MAP) are also processed. Recently, cooked and peeled shrimp in MAP have been introduced as a mildly preserved product on the European market, primarily in the UK (Bøknæs, 2007). Industrially processed cold-smoked salmon is typically salted by injection of brine and subsequently dried and smoked at 25-28 °C for 2-2½ hours (Bøknæs, 2007). As noticed for brined cold-water shrimp the application of MAP has also become more frequent and popular for production and distribution of e.g. cold-smoked salmon.

In addition to increased international competition producers of lightly preserved seafood also faces challenges and requirements from e.g. authorities and customers. The new EU regulation on RTE foods (EC, 2005) differentiates between products that prevent or do not prevent growth of *L. monocytogenes* and allows $10^2$ CFU/g in the former. Consequently, identification of product characteristics and storage conditions that prevents growth of this pathogen is an interesting perspective and possibility of improving the shelf life and safety of lightly preserved seafood. Relevantly, preventing growth of *L. monocytogenes* to high concentrations has been established as the strategy most likely to
reduce the cases of listeriosis (ILSI, 2005). The prospect of preventing growth of *L. monocytogenes* are also consistent with requirements for products with improved and extended shelf life as has been observed for some markets (e.g. cold-smoked salmon exported to Italy). However, the wish for longer shelf life is not necessarily in concordance with the increased demand for mildly preserved foods (Gould, 2000). To meet demands and regulations knowledge is needed about the spoilage microflora and potential growth of *L. monocytogenes* in lightly preserved seafood and how changes in preserving parameters influence microbial aspects.

The spoilage microflora and potential growth of *L. monocytogenes* in lightly preserved seafood is described to different extends in the literature. Very few studies have described these aspects in relation to brined cold-water shrimp (From and Huss, 1990; Dalgaard and Jørgensen, 2000; Dalgaard et al., 2003) and it has not been reported how changes in preserving parameters affect development and growth of microorganisms. Thus, research is needed within this area to obtain some basic knowledge about the microflora of brined cold-water shrimp and to be able to identify preserving parameters that reduce and prevent growth of spoilage microorganisms and *L. monocytogenes*, respectively. Unlike brined cold-water shrimp, several studies has described the spoilage microflora as well as the occurrence and growth of *L. monocytogenes* in cold-smoked and gravad fish (Cortesi et al., 1997; Leroy et al., 1998; Jørgensen and Huss, 1998; Paludan-Müller et al., 1998; Hansen and Huss, 1998; Lyhs et al., 1998; Lyhs et al., 1999; Jørgensen et al., 2000; Lyhs et al., 2001; Giménez and Dalgaard, 2004; Beaufort et al., 2007; Olofsson et al., 2007). The spoilage microflora has most often been determined to consist of lactic acid bacteria (LAB) but other spoilage associations have also been identified (Hansen et al., 1998; Olofsson et al., 2007). It is well-known that typical product characteristics (i.e. pH, salt, smoke components and naturally occurring lactate) of chilled cold-smoked and gravad fish are insufficient to prevent growth of *L. monocytogenes* (Huss et al., 2000b; FAO, 2004; ILSI, 2005). Thus, additional growth hurdles needs to be identified to make these products comply with the EU regulation (EC 2073/2005). To accomplish this task the use of diacetate and lactate seems interesting as these organic acids previously have been found to prevent growth of *L. monocytogenes* in meat products and vacuum-packed (VP) cold-smoked salmon (Seman et al., 2002; Mbandi and Shelef, 2002; Glass et al., 2002; Stekelenburg, 2003; Yoon et al., 2004). However, studies on
lightly preserved seafood in MAP with added diacetate and/or lactate have not been reported.

Storage trials and challenge tests are normally used to assess the effect of changes in product characteristics and storage conditions on growth of relevant microorganisms in food. However, this type of experiments is both expensive and time consuming. Mathematical models represent an interesting alternative to product experiments as fast answers are obtainable on how growth of microorganisms is affected by environmental parameters. Numerous mathematical models are available to predict the effect of different environmental parameters (e.g. temperature, water activity and pH) on growth of *L. monocytogenes*. Unfortunately, none of these models includes all of the parameters that most likely influence growth of *L. monocytogenes* in lightly preserved seafood (Ross et al., 2000; Giménez and Dalgaard, 2004). The same aspects and limitations apply for mathematical models intended to predict growth of spoilage microorganisms (e.g. LAB). Consequently, development of validated predictive models for *L. monocytogenes* and relevant spoilage microorganisms that includes environmental parameters of relevance to growth in lightly preserved seafood would be of great importance to e.g. the seafood industry. This type of models could be used actively by the industry to formulate products that prevent growth of *L. monocytogenes* to make them comply with the EU regulation on RTE foods (EC, 2005). Finally, growth of *L. monocytogenes* is normally observed to a lesser extend in naturally contaminated products as compared to inoculated products (i.e. challenge tests) (Dalgaard and Jørgensen, 1998). A better understanding of this phenomenon would improve the use of predictive models substantially and possibly make them useful for exposure assessments.

The main objectives of the present Ph.D project were to evaluate and predict microbial changes and growth of *L. monocytogenes* in different types of lightly preserved seafood. Storage trials and challenge tests with lightly preserved seafood were carried out to evaluate how microbial changes and growth of *L. monocytogenes* was affected by MAP and other preserving parameters (e.g. organic acids) (paper 1, 2, 3 and 4). Mathematical models was developed to predict the growth of *L. monocytogenes* and spoilage microorganisms in lightly preserved seafood as a function of relevant product characteristics and storage conditions (paper 3 and 4).
The first part of this thesis reviews lightly preserved seafood with the main emphasis on processing and preserving parameters. The next section reviews microbial changes and spoilage of lightly preserved seafood followed by a discussion of *L. monocytogenes* in relation to this product category. The fourth section reviews predictive models with special focus on available mathematical models for *L. monocytogenes* and spoilage microorganisms associated with lightly preserved seafood. Finally, the use of validated predictive models for management of safety and shelf life of lightly preserved seafood are discussed.
2 Lightly preserved seafood

Different processing and preservation methods are used for production of lightly preserved seafood, mainly to improve the shelf life and/or to obtain desirable sensory characteristics (e.g. smoke flavour) of the products. The use of different methods for production results in lightly preserved seafood with varied characteristics and storage conditions. Results of the present Ph.D.-project are summarised in Table 1 to demonstrate variations in product characteristics and storage conditions between different types of lightly preserved seafood and even within the same type (e.g. cold-smoked salmon). Knowledge about these variations is essential in order to understand and explain microbial changes and potential growth of pathogens in lightly preserved seafood. Furthermore, in the perspective of developing predictive models it is important to identify parameters that affect growth of microorganisms in lightly preserved seafood. In this section processing of selected lightly preserved seafoods are described followed by a discussion of the preservation techniques (i.e. storage temperature, MAP, brining, and smoking) used for these products. The antimicrobial effect of the above mentioned methods are only touched briefly in this section and will be discussed more thoroughly in subsequent chapters.

Table 1 Product characteristics and storage conditions of lightly preserved seafood

<table>
<thead>
<tr>
<th>Product</th>
<th>Measured product characteristics and storage conditions</th>
<th>Percentages of organic acids in the water phase</th>
<th>Phenol (ppm)</th>
<th>% CO₂ b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Water phase salt (%)</td>
<td>Benzoic</td>
<td>Citric</td>
</tr>
<tr>
<td>Cooked and peeled MAP shrimp 1</td>
<td>7.5</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shrimp in brine 2</td>
<td>5.6-5.8</td>
<td>2.9-3.5</td>
<td>0.11-0.15</td>
<td>0.61-0.79</td>
</tr>
<tr>
<td>Brined and drained MAP shrimp 2</td>
<td>5.6-5.7</td>
<td>2.6-3.6</td>
<td>0.12-0.13</td>
<td>0.54-0.66</td>
</tr>
<tr>
<td>VP cold-smoked salmon 1</td>
<td>6.0</td>
<td>5.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAP cold-smoked salmon 3</td>
<td>6.0-6.2</td>
<td>3.6-5.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAP gravad salmon 1</td>
<td>6.3</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VP cold-smoked Greenland halibut 1</td>
<td>6.4</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAP cold-smoked Greenland halibut 1</td>
<td>6.5</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are compiled from the present Ph.D.-project (Mejlholm et al., 2005; Mejlholm et al., 2007; Mejlholm and Dalgaard, 2007a)

Number of batches

Equilibrium concentration in headspace gas
2.1 *Processing of lightly preserved seafood*

2.1.1 *Shrimp products*

The Danish production of lightly preserved shrimp is based almost exclusively on cold-water shrimp (*Pandalus borealis*) from the North Atlantic Ocean. Following catch and maturing these shrimps are cooked, peeled and frozen. Different types of lightly preserved shrimp products are produced as convenient alternatives to frozen shrimp. Shrimp in brine (Figure 2a) and brined and drained MAP shrimp (Figure 2b) constitute the two main products processed from cooked and peeled cold-water shrimp (Figure 3).

![Figure 2a](image1.png) ![Figure 2b](image2.png)

**Figure 2.** Shrimp in brine (a) and brined and drained shrimp in modified atmosphere packaging (b). Pictures used with permission from Royal Greenland Seafood Ltd.

Shrimp in brine are produced by placing cooked and peeled shrimp in plastic cups followed by addition of brine and closure with plastic lids. The ratio between shrimp and brine is typically 1.0:0.8. Brined and drained shrimp in MAP are processed by marinating the shrimps in brine for 20-24 hours at 0-2 °C. Again, the ratio between shrimp and brine is often 1.0:0.8. Following the marinating step the brine is drained from the shrimps through the bottom-valve of a stainless-steel trolley used for production. Subsequently, brined and drained shrimp are packed in a CO₂ enriched atmosphere with N₂ as filler gas. Both of the above described products are stored at 0-2 °C prior to distribution (Figure 3). As a mildly preserved alternative to brined shrimp, cooked and peeled shrimp in MAP are also produced using a modified atmosphere (MA) composed of CO₂, N₂ and O₂. For some markets, cooked and peeled shrimp in MAP are distributed in their frozen state to improve the subsequent chilled shelf life of the product.
2.1.2 Cold-smoked and gravad fish

A wide range of lightly preserved fish products are processed by the Danish seafood industry including cold-smoked salmon (Figure 4a), gravad (“sugar-salted”) salmon (Figure 4b) and cold-smoked Greenland halibut (Figure 4c).
Farmed raised salmon (*Salmon salar*) from aquaculture in Norway or Chile are used for production of cold-smoked salmon and gravad salmon whereas Greenland halibut (*Reinhardtius hippoglossoides*) from the North Atlantic Ocean is processed into cold-smoked Greenland halibut. Salmon is supplied to the processors as fresh or frozen whole gutted fish or frozen fillets, while Greenland halibut merely is received as frozen fillets. Whole gutted fish of salmon is filleted and trimmed prior to processing of cold-smoked products. Initially, trimmed fillets of salmon or Greenland halibut are salted typically by dry-salting with crystalline salt and/or by injection of brine through needles. Next, salted fillets are dried at 25-27 °C for 2-5 hours followed by smoking at 25-28 °C for 2-2½ hours. Finally, salted and cold-smoked products are sliced and packed either in vacuum or MA with increased concentrations of CO₂ (Figure 5). The above described procedure do also apply to gravad salmon with the exceptions that this product is injected with brine composed of salt, sugar and “gravad extract”, and not smoked.
Figure 5. Production of cold-smoked salmon

2.2 Preservation techniques/methods

2.2.1 Storage temperature

Temperature is the single most important parameter to control growth of microorganisms in lightly preserved seafood. At the same time it is the parameter exposed to the greatest risk of fluctuation at different stages during production, retailing and/or home storage. The storage temperature has a huge impact on the types of microorganisms likely to grow in foods. Chill temperatures select for growth of psychrotolerant microorganisms including both spoilage and pathogen bacteria. The Danish legislation requires that lightly preserved seafood is stored at temperatures $\leq 5^\circ C$. 
Microbial changes and safety of lightly preserved seafood

(DVFA, 2006). In addition to being important as single parameter, the storage temperature also influence the effect of other preserving parameters (see section 2.2.2).

2.2.2 Modified atmosphere packaging

The use of MAP for foods has become increasingly popular since it was introduced for retail-packaging in the early 1980’s as a mean of providing fresh products with extended shelf life (Sivertsvik et al., 2002). Although, defined as any gas composition different from air, VP is not referred to as a MA in this thesis, which focuses on gas mixtures used for lightly preserved seafood. The preferred gasses for packaging of lightly preserved seafood are CO₂, N₂ and O₂ with the former gas possessing the main antimicrobial effect. The mode by which CO₂ exerts its antimicrobial effect is not fully understood but it is assumed that it affects different functional cell mechanisms resulting in extended lag phases and decreased growth rates of microorganisms (Farber, 1991b). The combined use of CO₂ and O₂ are also anticipated to inhibit the growth of certain microorganisms, especially anaerobes (e.g. Clostridium botulinum). In addition to the gas composition, several other parameters affect the efficiency of MAP including the storage temperature, gas/product (g/p) ratio and packaging material (i.e. gas permeability). The solubility of CO₂ in water (i.e. water phase of foods) is greatly enhanced at low temperatures (Carroll et al., 1991; Farber, 1991b; Sivertsvik et al., 2002). Consequently, to utilize the antimicrobial potential of CO₂ fully storage at chill temperatures is required. By increasing the g/p ratio it is possible to increase the concentration of dissolved CO₂ in the product (Sivertsvik and Birkeland, 2006) and thereby the antimicrobial effect of the MA. However, a higher g/p ratio also imply that larger packaging sizes and volumes of gasses are needed which is economically unfavourable to the producer. In general, when non-respiring foods (e.g. fish) are packed in MA it is important that only slight changes occur in the gas composition during storage, and consequently packaging materials with low gas permeability is used. Finally, the integrity of the packages is of outmost importance when MAP is used as even small leakages will result, more or less rapidly, in a gas composition resembling air.

Once packed, CO₂ is gradually dissolved in the product and an equilibrium gas composition within the package is obtained relatively fast. Nitrogen, used as inert gas, is not soluble in the product and it functions mainly to prevent the packages from collapsing.
Safety concerns on the use of VP and MAP in relation to growth and toxin production by *Cl. botulinum* are discussed in section 4.2.

2.2.3 Brining

Preservation of brined shrimp is based on the use of salt and organic acids. Benzoic, citric and sorbic acids constitute the most common organic acids for production of brined shrimp, but acetic and lactic acids are used as well. Salts of organic acids (e.g. benzoat, sorbat and acetate) are preferred for production of foods due to their higher solubility in water. The total allowable concentration of benzoic and/or sorbic acid in brined shrimp is 2000 mg/kg, whereas no upper limit has been established for addition of citric, acetic and lactic acids (EPC, 1995). The primary function of citric acid is to lower the pH of the product and to contribute to the sensory characteristics of brined shrimp. The equilibrium pH of brined shrimp is determined by the initial pH of the brine, and the ratio between brine and shrimp. The typical pH range of commercial brined shrimp is 5.6 to 5.9 (Dalgaard and Jørgensen, 2000; Mejlholm et al., 2007).

Added organic acids are not normally used as preservatives for cold-smoked and gravad fish; however, recently the use of modified brines containing e.g. diacetate in addition to salt has been introduced on an industrial scale for production of these types of products. Vogel et al. (2006) showed that the uptake of salt in cold-smoked salmon was affected negatively by addition of relatively high concentrations of lactate to the brines used for production. Thus, the use of organic acids as preservatives for cold-smoked and gravad fish is restricted by the requirement for > 3.5 % water phase salt (WPS) in lightly preserved seafood in order to prevent growth of *Cl. botulinum* (see section 4.2).

The mean by which organic acids exert their antimicrobial effect is not fully understood. However, some general mechanisms including depletion of energy and inhibition of metabolic processes have been suggested (Stratford, 2000). The antimicrobial effect of organic acids is mainly caused by their undissociated form:

\[
CH_3CH(OH)COOH \rightleftharpoons CH_3CH(OH)COO^- + H^+
\]

lactic acid (undissociated)    lactate (dissociated)    hydrogen ion
The concentration of undissociated acid is determined by the pKa-value of the organic acid and the pH of the food. At pH = pKa, 50 % of the organic acid will be present as undissociated acid. Within the normal pH-range of lightly preserved seafood, undissociated acid constitute only a small percentage of the added amount of organic acid (Table 2). Undissociated organic acids have a slightly lipophilic character and this makes them able to enter the bacterial cell membrane. Within the cell, the acid is dissociated due to a higher internal pH of approx. 7 and this result in accumulation of hydrogen ions (H⁺) (Ita and Hutkins, 1991; Houtsma et al., 1994). Thus, to transport H⁺ out of the cell, in an attempt to retain the internal pH, the microorganism needs to use energy and this result in inhibition of growth.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>pKa-value</th>
<th>pH 5.6</th>
<th>pH 5.8</th>
<th>pH 6.0</th>
<th>pH 6.2</th>
<th>pH 6.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid/diacetate</td>
<td>4.8</td>
<td>12.6</td>
<td>8.4</td>
<td>5.4</td>
<td>3.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>4.2</td>
<td>3.7</td>
<td>2.4</td>
<td>1.5</td>
<td>0.97</td>
<td>0.61</td>
</tr>
<tr>
<td>Citric acid</td>
<td>3.1</td>
<td>0.34</td>
<td>0.21</td>
<td>0.13</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>3.9</td>
<td>1.8</td>
<td>1.1</td>
<td>0.72</td>
<td>0.46</td>
<td>0.29</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>4.8</td>
<td>13.7</td>
<td>9.1</td>
<td>5.9</td>
<td>3.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Undissociated organic acids partition between the aqueous and the lipid phase of foods to different degrees (Brocklehurst, 2004). As microorganisms proliferate in the aqueous phase of foods it is desirable to minimize the fraction of undissociated acids in the lipid phase. The percentage of lipids in e.g. cold-smoked salmon (10-20 % of lipids) are relatively high and thus, organic acids with a low affection for lipids (i.e. low partition coefficient between oil and water) are most likely to be effective as preservatives. The content of lipids in shrimp is low (< 1 %) and this makes the selection of organic acids less critical. Partition coefficient of acetic, lactic and sorbic acids between sunflower oil and water has been estimated as 0.02, 0.033 and 2.15, respectively (Brocklehurst and Wilson, 2000). Thus, acetic acid is the one less likely to distribute in the lipid phase.

Salt is included as an important ingredient for production of brined shrimp, and cold-smoked and gravad fish. Addition of NaCl reduces the water activity of foods and this inhibits the growth of microorganisms to different extents.
2.2.4 Smoking

Smoking of foods has been carried out for thousands of years originally to extend the shelf life, but later also as a mean of adding flavour to the products. Nowadays, smoking is widely used for different types of fish including products of salmon, Greenland halibut, trout, tuna, mackerel, swordfish, cod and herring. Smoking of fish results in several changes of the products. The sensory characteristics are altered by the smoking process resulting in a desirable “smoky” flavour and colour. The intensity of smoke flavour and colour is determined by a wide range of process parameters e.g. smoking time and temperature, and the type of wood used for smoke generation. Furthermore, constituents of smoke having a preserving effect are also adsorbed or condensed on the products during production. The type and content of smoke compounds having a preserving effect is determined by the process used for smoke generation, and the actual smoking procedure (Tóth and Potthast, 1984; Sérot et al., 2004).

2.2.4.1 Smoke generation and smoking procedure

Smoke for food manufacturing is normally generated from pyrolysis of wood. Different types of wood categorized as either hardwood (e.g. oak, beech and hickory) or softwood (e.g. fir, pine and spruce) are used for generation of smoke. The main constituents of wood are cellulose (50 %), hemicellulose (25 %) and lignin (25 %). Cellulose and hemicellulose is composed of aggregated sugar molecules whereas the composition of lignin, being more complex, is mainly made up of interlinked phenolic molecules (Tóth and Potthast, 1984).

During generation of smoke both primary and secondary reactions takes place classified as pyrolysis of wood and changes in products of pyrolysis, respectively. Several types of methods are used for production of smoke with the most common being smouldering, friction and condensation (liquid smoke). Smouldering is normally carried out in a smoke chamber equipped with an external heat supply. Smoke is produced by placing sawdust or chips of wood on a heated metal surface inside the chamber. The generated smoke is then carried by air to a separate smoking chamber containing the product (e.g. fish). In the generation of smoke by friction, a bar of wood is pressed against a rapidly turning wheel. The resulting friction leads to glowing of the wood and thus the development of smoke. Smoke from smouldering is usually generated at higher temperatures (e.g. 450 °C) than smoke from friction (e.g. 350 °C) which influences the
Microbial changes and safety of lightly preserved seafood

chemical composition of the smoke (Tóth and Potthast, 1984). A higher degree of lignin pyrolysis occurs at 450 °C as compared to 350 °C which results in formation of more phenolic compounds. The chemical composition of smoke is also affected by the type of wood, the humidity of the wood, and the oxygen supply during generation of smoke. Oxygen is used to control the combustion of wood (i.e. to avoid the wood from catching fire) during smoke generation, and moreover it reacts with compounds of pyrolysis resulting in formation of smoke components (e.g. phenolics) with important flavour properties. Liquid smoke (artificial smoke) is produced by condensation of smoke in water (Holley and Patel, 2005). Unwanted constituents like ash, tar and soot are removed by this process but important volatile smoke components are not trapped by the water. In general, smoke produced by different methods is either washed or filtered as a mean of removing unwanted compounds like tar, soot and not least polyaromatic hydrocarbons (PAHs) being potentially carcinogenic (Tóth and Potthast, 1984).

In modern industries, the smoke generator and the smokehouse functions as separated chambers connected with pipes. By separating these processes, contamination of the products with ash, tar and soot, produced during generation of smoke, is avoided as such components are condensed within the pipes and/or trapped by installed filters.

Smoking of foods is typically classified as either cold-smoking (25-28 °C; 2-2½ hour) or hot-smoking (70-90 °C; 1½ hour) (Bøknæs, 2007). The textural properties of cold-smoked products are almost unaffected by the process contrary to hot-smoked products being more or less cooked. The microbial load of foods is not affected by the temperatures used for cold-smoking, however, a reduction in the concentration of L. monocytogenes has been observed as a result of the smoking procedure (Rørvik et al., 1995). During hot-smoking the main part of the microflora is killed by the elevated temperatures.

Smoke constituents are adsorbed or condensed on the surface of the products during smoking. The amount of adsorbed or condensed smoke components is affected by the smoke density, smokehouse humidity and temperature, duration of the smoking process and pre-treatment (e.g. drying) of the products. Condensation of smoke components is favoured by high humidity conditions in the smokehouse as well as large temperature differences between the smoke and the products. Sérot et al. (2004) established a linear relationship between the smoking time and the concentration of smoke components in the final product. Furthermore, it has been shown that higher
concentrations of phenolic compounds are condensed on products with a wet surface as compared to a dry product surface. The highest concentration of smoke constituents including phenols has been determined in the outer layer of smoked foods (Chan et al., 1975; Maga, 1987). This is important to consider when assessing the antimicrobial effect of the smoking procedure as samples from the outer layer of e.g. cold-smoked salmon are more likely to possess an antimicrobial effect than samples from the core of the fillets.

2.2.4.2 Chemical composition of smoke and smoked food

The chemical composition of smoke is very diverse including carbonyls, acids, alcohols, esters, aldehydes, phenols, furans, lactones and PAHs. Overall, more than 400 different chemical compounds have been identified from smoke (Maga, 1987; Clifford, 2000). The single most important group of chemical compounds in smoke is phenols (Tóth and Potthast, 1984). Phenolic compounds contribute significantly to the characteristic flavour of smoked foods. In addition, phenols have previously been shown to possess both antimicrobial and antioxidative effects (Suñen, 1998; Holley and Patel, 2005). Acids and carbonyls like acetic acid and formaldehyde respectively, are other constituents with a potential inhibiting effect against microorganisms (Niedziela et al., 1998).

The majority of phenolics in smoke are derived from pyrolysis of the lignin fraction of wood. Ferulic and sinapic acid are the precursors of phenolic compounds. Previous studies examining the chemical composition of smoked foods has established guaiacol, syringol, 4-methylguaiacol, 4-methylsyringol and phenol as being the predominant phenolic components (Lustre and Issenberg, 1970; Knowles et al., 1975; Hierro et al., 2004; Guillen et al., 2006). More than 75 different phenolic compounds have been identified from smoked foods (Maga, 1987). Guaiacol and other phenols with one methoxy group (e.g. 4-methylguaiacol) are predominant in smoke from softwood whereas phenols with two methoxy groups (e.g. syringol and 4-methylsyringol) prevail in hardwood smoke (Tóth and Potthast, 1984). This has been confirmed by Guillén et al. (2006) showing that syringol was the dominant phenolic compound occurring in both swordfish and cod treated with hardwood smoke. However, considerable amounts of guaiacol and phenol were also determined in these products. Sérot et al. (2004) established guaiacol, 4-methylguaiacol, syringol and phenol as the major phenolics in smoked fillets of herring when smoke from hardwood was used. This shows that no general rule exists on the type of wood used for smoke generation and the resulting chemical composition of the smoke. In this context it
should be mentioned that the chemical composition of smoke is not necessarily reflected in the smoked food, as not all constituents are likely to adsorb or condense on the product. It has moreover been hypothesised that new compounds might be formed when smoke and food items come in contact with each other during processing (Tóth and Potthast, 1984).

2.2.4.3 Analysis of smoke components

While well-known methods and apparatus are available to determine e.g. temperature, pH, NaCl/water activity, CO₂ and organic acids it is far more difficult to identify and quantify smoke components in foods. This is mainly caused by the wide range of compounds present in smoke having different chemical structures and physical properties. Consequently, to determine the entire range of smoke components it is not possible to use a single method. The most frequently used method for determination of smoke compounds in smoke fractions as well as in smoked foods is gas chromatography (GC) often combined with mass spectrometry (MS) (Knowles et al., 1975; Luten et al., 1979; Wittkowski et al., 1990; Jørgensen et al., 2001; Guillen and Errecalde, 2002; Serot and Lafficher, 2003; Hierro et al., 2004; Jira, 2004; Guillen et al., 2006; Varlet et al., 2007). This technique has primarily been used to identify smoke compounds whereas the quantifying ability still needs to be improved. Spectrophotometric methods have been used to determine the content of phenols (expressed as “total phenol”) in smoked foods (Chan et al., 1975; Cardinal et al., 2004). These methods are restricted as no information on identity and quantity of single phenolic compounds is given. Furthermore, it has been reported that para-substituted phenols (e.g. 4-methylguaiaiacol) is not measured by spectrophotometric methods (Lustre and Issenberg, 1970; Luten et al., 1979) This observation was confirmed by recent and unpublished results from our laboratory (results not shown). This limitation should of course be considered as para-substituted phenols have been determined to compromise a significant part of the phenolic fraction in smoked meat and fish products (Lustre and Issenberg, 1970; Knowles et al., 1975; Guillen and Errecalde, 2002; Guillen et al., 2006). Though, results obtained by spectrophotometric methods could still be used as a valuable and relatively simple measure of “smoke intensity” in smoked foods when the antimicrobial effect of smoking needs to be assessed. Values from below the detection limit to > 20 ppm of phenol have been reported for cold-smoked seafood (Leroi et al., 2001; Cardinal et al., 2001; Giménez and Dalgaard, 2004;
Lakshmanan and Dalgaard, 2004; Espe et al., 2004; Cardinal et al., 2004; Cornu et al., 2006; Mejilholm and Dalgaard, 2007a) whereas substantially higher concentrations of phenol have been detected in products treated with liquid smoke (Vitt et al., 2001; Montero et al., 2007). The antimicrobial effect of liquid smoke treatment seems far less evident as compared to the one observed for samples processed by the “true” smoking procedure (Vitt et al., 2001; Lebois et al., 2004; Montero et al., 2007). This phenomenon definitely needs further examination particularly if hazardous predictions by mathematical models, including the effect of smoke components (phenol) on growth of microorganisms, are to be avoided.
3 Microbial changes and shelf life of lightly preserved seafood

The spoilage microflora of lightly preserved seafood is described to different extents in the literature. For cold-smoked and gravad products (e.g. cold-smoked salmon) several studies has dealt with these aspects (see section 3.3 and Table 4). Contrary, very few studies has described the spoilage microflora of shrimp products (e.g. shrimp in brine). Identification of the spoilage microflora is important in an attempt to understand spoilage of lightly preserved seafood. Such knowledge would make it possible to direct preservation towards the spoilage microflora and facilitate development of predictive models to be used as decision tools when establishing the shelf life of lightly preserved seafood (Dalgaard, 2000). In the following sections the shelf life and spoilage microflora of lightly preserved seafood is reviewed with the main focus on shrimp products as these aspects previously has been discussed thoroughly for cold-smoked salmon in the literature as well as in Ph.D.-thesis from DIFRES (Hansen, 1995; Jørgensen, 2000).

3.1 Microbial changes and shelf life

The shelf life of lightly preserved seafood is most often limited by microbial activity resulting in production of off-flavours. The composition of the spoilage microflora is affected by several factors including (i) the inherent/natural microflora of the fish/shell fish; (ii) processing methods (e.g. cooking and packaging); (iii) microbial contamination during production; and (iv) product characteristics (e.g. preservation) and storage conditions (e.g. temperature). The spoilage microflora can be composed of a single microbial species or a mixture of which not all necessarily contributes to the formation of spoilage characteristics. For some products, including MAP cod fillets, it has been possible to identify the specific spoilage organism (SSO) from the spoilage microflora (Dalgaard, 1995). In general, SSOs are only represented as a minor part of the initial microflora but environmental parameters favours the growth of these microorganisms, ultimately resulting in spoilage of the product. The SSO, consisting of one or more microbial species, is identified by comparing spoilage characteristics of naturally contaminated products with those produced by single or mixtures of isolates from the spoilage microflora (Dalgaard, 2000). It is important to define the spoilage domain of SSOs as even small changes in product characteristics and storage conditions might alter the composition of the spoilage microflora. This is one of the
main reasons why it has been difficult to identify SSOs from lightly preserved seafood possessing a wide variation in e.g. preserving parameters (see section 2, Table 1).

3.2 Shelf life and spoilage microflora of shrimp and shrimp products

3.2.1 Raw shrimp

The shelf life of aerobically stored whole or peeled raw shrimp is very short, in the range 5-6 days, at chill temperatures (Matches, 1982). The neutral pH (~ 7.5) and high water activity of shrimp makes this product highly perishable as fast growth of microorganisms is allowed. The presence of relatively high concentrations of easily degradable free-amino acids (e.g. arginine and glycine) in shrimp (Laursen et al., 2006) is another factor contributing to the short shelf life. When not degraded free-amino acids contribute to the desirable sweet flavour of shrimp (Finne, 1992). High initial microbial concentrations (4-6 log, CFU/g) have been reported for different species of shrimp from both temperate and tropical waters (Lannelongue et al., 1982; Zuberi et al., 1983; Shamshad et al., 1990) with Gram-negative microorganisms representing the dominant part of the microflora. Pseudomonas, Acinetobacter-Moraxella, Aeromonas, Vibrio, Shewanella, Alcaligenes, Flavobacterium, Coryneforms and Enterobactericeae, typical to the aquatic environment, are the most frequently isolated microorganisms from raw shrimp (Harrison and Lee, 1968; Chinivasagam et al., 1996; Jeyasekaran et al., 2006). Chinivasagam et al., (1998) determined that different types of tropical prawns stored aerobically in ice or ice slurry were spoiled by Pseudomonas fragi and Shewanella putrefaciens, respectively. S. putrefaciens were assumed to dominate in ice slurry with low oxygen tension due to its ability to use trimethylamineoxide (TMAO), present in shrimp, as electron acceptor (Chinivasagam et al., 1998). Pseudomonas is not able to utilise TMAO and its growth is inhibited in environments with low concentrations of O₂ (Dainty and Mackey, 1992). The importance of temperature abuse on the shelf life of raw shrimp was more or less deliberately shown by Jeyasekaran et al., (2006). The shelf life of Indian white shrimps (Penaeus indicus) was established as being < 32 hours when stored in dry ice without reicing (Jeyasekaran et al., 2006). During this period the recorded storage temperature fluctuated between 32 °C and -4.8 °C, and remained > 10 °C for approx. 50 % of the time. This result shows the importance of immediate icing and reicing throughout storage if accelerated spoilage of raw shrimp is to be avoided. Pseudomonas and
Aeromonas was identified as the main spoilage microflora of white Indian shrimp stored in water ice whereas Flavobacterium prevailed when dry ice was used for storage. These differences in the composition of the spoilage microflora were attributed to the CO$_2$ environment created by dry ice (sublimation of CO$_2$: solid to gaseous phase), with Pseudomonas and Aeromonas being more sensitive towards this gas as compared to Flavobacterium (Jeyasekaran et al., 2006).

Packaging of raw shrimp in MA with elevated concentrations of CO$_2$ has been shown to shift the microflora towards Gram-positive bacteria and the CO$_2$ resistant Gram-negative microorganism Photobacterium phosphoreum resulting in an extended shelf life (Lannelongue et al., 1982; Layrisse and Matches, 1984; Matches and Layrisse, 1985; López-Caballero et al., 2002).

3.2.2 Cooked shrimp

Cooking reduces the microbial load of shrimp substantially (Ridley and Slabyj, 1978). However, subsequent processing steps e.g. peeling and freezing implies that recontamination of shrimp is unavoidable. The concentration of microorganisms on cooked and peeled shrimp, reflecting the degree of recontamination and/or insufficient cooking, has been reported to vary between 2.0 and 4.8 log (CFU/g) (Ridley and Slabyj, 1978; Valdimarsson et al., 1998; Hatha et al., 1998; Mejlholm et al., 2007). Harrison and Lee (1968) established that cooked shrimp were recontaminated with a microflora being predominantly Gram-positive.

Sivertsvik et al. (1997) showed that the shelf life of whole unpeeled cooked shrimp (P. borealis) was extended from 7 to 21 days when MAP (60 % CO$_2$/40 % N$_2$) and storage at 3 °C was used instead of aerobic storage in ice. Shrimp used in that study was cooked in 12 % salt water resulting in 3.5-4.3 % WPS in the final product. Mejlholm et al. (2005) established the shelf life of cooked and peeled shrimp (P. borealis) in MAP (50 % CO$_2$, 30 % N$_2$ and 20 % O$_2$) based on both spoilage and safety aspects. This product had an initial pH of 7.7 and contained 1.9 % WPS. The sensory shelf life of cooked and peeled MAP shrimp was determined as 25-26 days, 15-16 days and 9-10 days at 2, 5 and 8 °C, respectively. This is in agreement with the findings of Sivertsvik et al. (1997) reporting a shelf life of 21 days at 3 °C for whole unpeeled cooked shrimp. The dominating spoilage
microflora of cooked and peeled MAP shrimp was identified as *Carnobacterium maltaromaticum* and *Brochothrix thermosphacta* at 2, 5 and 8 °C, producing distinct off-flavours characterised as sour, wet-dog and chlorine-like by the sensory panel (Mejlholm et al., 2005). *Psychrobacter* spp. was also identified as part of the spoilage microflora but their contribution to the spoilage pattern was not obvious as shown in inoculation tests with cooked and peeled MAP shrimp. When samples of cooked and peeled MAP shrimp were inoculated with a pure culture of *Cb. maltaromaticum* isolates, off-flavours characterised as chlorine-like were produced whereas isolates of *B. thermosphacta* produced off-flavours described as buttermilk-like and sour. Inoculation of cooked and peeled MAP shrimp with a mixed culture of *Cb. maltaromaticum* and *B. thermosphacta* resulted in off-flavours identical to those observed in storage trials (Mejlholm et al., 2005). Laursen et al. (2006) examined how metabolic activity of *Carnobacterium* spp. and their interaction with *B. thermosphacta* affected sensory characteristics of cooked and peeled MAP shrimp. It was confirmed that a co-culture of *Cb. maltaromaticum* and *B. thermosphacta* produced a very distinct wet-dog off-flavour in cooked and peeled MAP shrimp whereas this off-flavour were not produced by pure cultures of the above mentioned microorganisms. When identifying metabolites it was found that no new compounds were produced by the co-culture as compared to metabolites formed by pure cultures of *Cb. maltaromaticum* or *B. thermosphacta*. Thus, the wet-dog off-flavour is most likely a result of interaction between metabolites produced by *Cb. maltaromaticum* and *B. thermosphacta*, respectively, and not metabiosis where products formed by one microorganism are used as substrate by another microorganism to produce new metabolites. The characteristic wet-dog off-flavour was also observed in cooked and peeled MAP shrimp when *B. thermosphacta* were inoculated in co-culture with *Cb. divergens* or *Cb. mobile* (Laursen et al., 2006).
### Table 3 Shelf life and spoilage microflora of shrimp products

<table>
<thead>
<tr>
<th>Product</th>
<th>Storage conditions</th>
<th>% CO2</th>
<th>pH</th>
<th>Water phase salt (%)</th>
<th>Acetic (diacetate)</th>
<th>Benzoic</th>
<th>Citric</th>
<th>Lactic</th>
<th>Sorbic</th>
<th>TVC(^a) (log, CFU/g)</th>
<th>Shelf life (d)</th>
<th>Spoilage microflora</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brined shrimp</td>
<td>5 °C, aerobic</td>
<td>-</td>
<td>5.4</td>
<td>3.8</td>
<td>-</td>
<td>0.1</td>
<td>0.69</td>
<td>-</td>
<td>0.08</td>
<td>-</td>
<td>68-75</td>
<td>Streptococcus, Lactobacillus, yeast</td>
<td>From and Huss, 1990</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>10 °C, aerobic</td>
<td>-</td>
<td>5.4</td>
<td>3.8</td>
<td>-</td>
<td>0.1</td>
<td>0.69</td>
<td>-</td>
<td>0.08</td>
<td>&gt; 8</td>
<td>&gt; 40</td>
<td>Lactobacillus</td>
<td>From and Huss, 1990</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>0 °C, MAP</td>
<td>14</td>
<td>5.7</td>
<td>3.3</td>
<td>-</td>
<td>0.074</td>
<td>0.33</td>
<td>-</td>
<td>0.077</td>
<td>&lt; 4</td>
<td>&gt; 308</td>
<td>No isolates</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>5 °C, MAP</td>
<td>14</td>
<td>5.7</td>
<td>3.3</td>
<td>-</td>
<td>0.074</td>
<td>0.33</td>
<td>-</td>
<td>0.077</td>
<td>&gt; 8</td>
<td>&gt; 201</td>
<td>Carnobacterium spp.</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>8 °C, MAP</td>
<td>14</td>
<td>5.7</td>
<td>3.3</td>
<td>-</td>
<td>0.074</td>
<td>0.33</td>
<td>-</td>
<td>0.077</td>
<td>&gt; 8</td>
<td>63-84</td>
<td>Aerococcus viridans, Enterococcus spp.</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>15 °C, MAP</td>
<td>14</td>
<td>5.7</td>
<td>3.3</td>
<td>-</td>
<td>0.074</td>
<td>0.33</td>
<td>-</td>
<td>0.077</td>
<td>&gt; 8</td>
<td>21-28</td>
<td>E. faecalis, Enterococcus spp.</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>25 °C, MAP</td>
<td>14</td>
<td>5.7</td>
<td>3.3</td>
<td>-</td>
<td>0.074</td>
<td>0.33</td>
<td>-</td>
<td>0.077</td>
<td>&gt; 8</td>
<td>4-6</td>
<td>E. faecalis</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>0 °C, MAP</td>
<td>15</td>
<td>5.9</td>
<td>2.3</td>
<td>-</td>
<td>0.077</td>
<td>0.28</td>
<td>-</td>
<td>0.063</td>
<td>&gt; 7</td>
<td>217-246</td>
<td>Cb. divergens, Lb. curvatus</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>5 °C, MAP</td>
<td>15</td>
<td>5.9</td>
<td>2.3</td>
<td>-</td>
<td>0.077</td>
<td>0.28</td>
<td>-</td>
<td>0.063</td>
<td>&gt; 8</td>
<td>84-104</td>
<td>Cb. divergens, Enterococcus spp.</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>8 °C, MAP</td>
<td>15</td>
<td>5.9</td>
<td>2.3</td>
<td>-</td>
<td>0.077</td>
<td>0.28</td>
<td>-</td>
<td>0.063</td>
<td>&gt; 8</td>
<td>35-42</td>
<td>Cb. divergens, Enterococcus spp.</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>15 °C, MAP</td>
<td>15</td>
<td>5.9</td>
<td>2.3</td>
<td>-</td>
<td>0.077</td>
<td>0.28</td>
<td>-</td>
<td>0.063</td>
<td>&gt; 8</td>
<td>~ 21</td>
<td>Cb. divergens, Enterococcus spp.</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>25 °C, MAP</td>
<td>15</td>
<td>5.9</td>
<td>2.3</td>
<td>-</td>
<td>0.077</td>
<td>0.28</td>
<td>-</td>
<td>0.063</td>
<td>&gt; 8</td>
<td>4-6</td>
<td>E. faecalis, Lactococcus garvieae</td>
<td>Dalgaard and Jørgensen (2000); Dalgaard et al., (2003)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>1 °C, aerobic</td>
<td>-</td>
<td>4.8</td>
<td>1.9</td>
<td>-</td>
<td>ND</td>
<td>0.3</td>
<td>-</td>
<td>ND</td>
<td>2.0</td>
<td>40</td>
<td>No isolates</td>
<td>Cadun et al., (2005)</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>1 °C, aerobic</td>
<td>-</td>
<td>4.7</td>
<td>2.1</td>
<td>-</td>
<td>0.52</td>
<td>-</td>
<td>-</td>
<td>4.0</td>
<td>40</td>
<td>No isolates</td>
<td>Cadun et al., (2005)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Total viable count (TVC) at the end of shelf life
Table 3 Shelf life and spoilage microflora of shrimp products (continued)

<table>
<thead>
<tr>
<th>Product</th>
<th>Storage conditions</th>
<th>% CO2</th>
<th>pH</th>
<th>Water phase salt (%)</th>
<th>Organic acids (%)</th>
<th>TVC&lt;sup&gt;a&lt;/sup&gt; (log, CFU/g)</th>
<th>Shelf life (d)</th>
<th>Spoilage microflora</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&amp;P shrimp</td>
<td>2 °C, MAP</td>
<td>24.2</td>
<td>7.7</td>
<td>1.9</td>
<td></td>
<td>0.06</td>
<td>8.3</td>
<td>25-26</td>
<td>Cb. maltaromaticum, Brochothrix thermosphacta, Psychrobacter spp.</td>
</tr>
<tr>
<td>C&amp;P shrimp</td>
<td>5 °C, MAP</td>
<td>24.6</td>
<td>7.7</td>
<td>1.9</td>
<td></td>
<td>0.06</td>
<td>8.6</td>
<td>15-16</td>
<td>Cb. maltaromaticum, B. thermosphacta, Psychrobacter spp.</td>
</tr>
<tr>
<td>C&amp;P shrimp</td>
<td>8 °C, MAP</td>
<td>26</td>
<td>7.7</td>
<td>1.9</td>
<td></td>
<td>0.06</td>
<td>8.6</td>
<td>9-10</td>
<td>Cb. maltaromaticum, B. thermosphacta, Psychrobacter spp.</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.8</td>
<td>3.5</td>
<td>-</td>
<td>0.13</td>
<td>8.2</td>
<td>49-56</td>
<td>Pseudomonas fluorescens, E. faecalis-like, Cb. maltaromaticum, Staphylococcus spp.</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.8</td>
<td>3.5</td>
<td>-</td>
<td>0.15</td>
<td>7.0</td>
<td>53-60</td>
<td>Ps. fluorescens, E. malodoratus, Lb. sakei</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.6</td>
<td>3.6</td>
<td>0.14</td>
<td>0.13</td>
<td>5.7</td>
<td>56-63</td>
<td>Leuconostoc pseudomesenteroides, Cb. maltaromaticum, yeast</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.7</td>
<td>3.6</td>
<td>0.15</td>
<td>0.14</td>
<td>3.4</td>
<td>~ 61</td>
<td>Cb. maltaromaticum</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>12 °C, aerobic</td>
<td>-</td>
<td>5.6</td>
<td>3.6</td>
<td>0.14</td>
<td>0.13</td>
<td>8.6</td>
<td>28-35</td>
<td>E. faecalis-like, E. malodoratus, Staphylococcus spp.</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.6</td>
<td>3.5</td>
<td>0.15</td>
<td>-</td>
<td>8.2</td>
<td>35-42</td>
<td>E. faecalis-like, Staphylococcus spp.</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.8</td>
<td>3.3</td>
<td>0.86</td>
<td>-</td>
<td>6.6</td>
<td>42-49</td>
<td>yeast, Lb. sakei</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.8</td>
<td>3.3</td>
<td>1.07</td>
<td>-</td>
<td>3.7</td>
<td>69-84</td>
<td>No isolates</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, aerobic</td>
<td>-</td>
<td>5.7</td>
<td>3.2</td>
<td>1.23</td>
<td>-</td>
<td>2.2</td>
<td>69-84</td>
<td>No isolates</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>12 °C, aerobic</td>
<td>-</td>
<td>5.8</td>
<td>3.3</td>
<td>0.86</td>
<td>-</td>
<td>7.5</td>
<td>35-42</td>
<td>Lb. sakei, E. faecalis</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, MAP</td>
<td>26.9</td>
<td>5.7</td>
<td>3.3</td>
<td>-</td>
<td>0.13</td>
<td>8.1</td>
<td>28-35</td>
<td>Lb. sakei</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, MAP</td>
<td>25.8</td>
<td>5.6</td>
<td>3.8</td>
<td>-</td>
<td>0.13</td>
<td>1.8</td>
<td>&gt; 75</td>
<td>No isolates</td>
</tr>
<tr>
<td>Brined shrimp</td>
<td>7 °C, MAP</td>
<td>26.3</td>
<td>5.7</td>
<td>3.1</td>
<td>1.26</td>
<td>-</td>
<td>7.3</td>
<td>56-63</td>
<td>Lb. sakei</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total viable count (TVC) at the end of shelf life

<sup>b</sup> Cooked and peeled shrimp
3.2.3 Brined shrimp

Brining has been used for more than 30 years as a means of extending the shelf life of cooked and peeled shrimp (see section 2.1.1). In spite of this fact, the spoilage microflora of brined shrimp remains poorly described in the literature.

In one of the first reported studies on shrimp in brine, From and Huss (1990) described the spoilage microflora as being dominated by yeast and LAB (Streptococcus and Lactobacillus) at 5 °C whereas isolates of Lactobacillus prevailed at 10 °C. Later, Einarsson & Lauzon (1995) examined microbial changes for shrimp in brine (P. borealis) stored at 4.5 °C. Growth of microorganisms was prevented for the duration of the experiment (59 days) in samples with the following characteristics: 3 % NaCl, pH 5.7, 0.1 % Na-benzoate, 0.5 % citric acid, and 0.1 % K-sorbate. However, when benzoate and sorbate was omitted from the brine, total viable count increased from approx. 4 to 8 log (CFU/g) within 15 days at 4.5 °C. At the time of reaching > 6 log (CFU/g) the dominating microflora was composed of Coryneforms (67 %) and Moraxella (33 %) (Einarsson and Lauzon, 1995).

Dalgaard & Jørgensen (2000) determined the shelf life of brined and drained MAP shrimp at temperatures between 0 and 25 °C. Samples of both cold-water (P. borealis) and warm-water (Penaeus spp.) shrimp were examined. A pronounced effect of the storage temperature on microbial changes and consequently the shelf life was observed (Table 3). In general, a shorter shelf life was determined for samples of warm-water shrimp, though, this was not attributed to inter-species variations but rather differences in preserving parameters (Dalgaard and Jørgensen, 2000). At storage temperatures ≤ 8 °C Carnobacterium spp., Aerococcus viridans and Enterococcus spp. were identified as the spoilage microflora of brined and drained MAP cold-water shrimp whereas Cb. divergens, Lb. curvatus and Enterococcus spp. were found in samples of warm-water shrimp. Enterococcus faecalis/Enterococcus spp. were found to constitute the spoilage association at temperatures ≥ 15 °C, irrespective of shrimp species (Dalgaard et al., 2003).

Cadun et al. (2005) showed that microbial growth was prevented for more than 40 days at 1 °C when benzoic, citric and sorbic acids was used for production of cooked and peeled shrimp (Parapenaeus longirostris, Lucas, 1846) in brine. This product had an initial pH of 4.8, and contained 1.9 % WPS and 0.30 % citric acid. Unfortunately, concentrations
of benzoic and sorbic acids were not reported making a comparison with other studies difficult. For shrimp in brine without benzoic and sorbic acid (pH 4.7, 2.1 % WPS and 0.52 % citric acid), aerobic plate count increased by approx. 2 log (CFU/g) during 40 days of storage at 1 °C (Cadun et al., 2005). The main antimicrobial effect observed by Cadun et al. (2005) was most likely caused by the low storage temperature, the unusually low pH of the samples, and to some extend benzoic and sorbic acids. A low pH of 4.7-4.8 has a strong inhibiting effect in itself and it implies that markedly higher concentrations of undissociated organic acids will be present at least when compared to other studies on brined shrimp with pH values in the range 5.4-5.8 (Table 3). On the negative side, a pH of 4.7-4.8 would be expected to affect the sensory properties (i.e. texture) of shrimp negatively, however, this was not reported by Cadun et al. (2005). Although, growth of microorganisms was restricted in both of the above described products, samples were sensorially rejected after 40 days of storage at 1 °C due to formation of rancid off-flavours (Cadun et al., 2005). Thiobarbituric acid values of 6.5-7.3 mg/malonaldehyde/kg was measured at the end of storage indicating that lipid oxidation could be the cause to rancid off-flavours. Formation of rancid off-flavours was also observed for shrimp in brine when growth of microorganisms was prevented or strongly reduced during storage for > 60 days at 7 °C (Mejlholm et al., 2007). In addition, occurrence of yellow coloured shrimp was noticed for these samples, especially at the interface between air and brine. In the same way presence of yellow coloured shrimp was observed by Einarsson & Lauzon (1995) for shrimp in brine when microbial growth was prevented, though, the formation of rancid off-flavours was not reported. It should be mentioned that relatively low concentrations of lipids < 1 % have been reported for shrimp (Basavakumar et al., 1998; Cadun et al., 2005; Laursen et al., 2006) and consequently further studies are needed to elicit whether this phenomenon is caused by oxidation of lipids and/or proteins. Noteworthy, formation of rancid off-flavours or yellow coloured shrimp were not observed for brined and drained shrimp in MAP (40 % CO₂/60 % N₂) stored for > 75 days at 7 °C (Mejlholm et al., 2007).

In a total of 13 storage trials, Mejlholm et al. (2007) examined the importance of (i) process hygiene; (ii) preserving parameters (i.e. brine composition); and (iii) storage conditions (i.e. temperature and atmosphere) on microbial changes and shelf life of brined shrimp (P. borealis). A pronounced effect of process hygiene was observed by Mejlholm et al. (2007) as the initial concentration of microorganisms on brined shrimp from an
industrial scale processing line was 1.0-2.3 log (CFU/g) higher than comparable values for manually processed samples from the same batch of cooked and peeled shrimp. Isolates of *E. faecalis*-like, *Leuconostoc pseudomesenteroides*, coagulase negative *Staphylococcus* spp. and yeast were identified from the spoilage microflora of industrially processed samples but not from manually processed brined shrimp. Thus, contamination during processing of brined shrimp was not only quantitative but also qualitative as the spoilage microflora of industrially processed samples became more diverse. The effect of contamination was obvious as the shelf life of brined shrimp from an industrially scale processing line was substantially shorter than observed for samples being manually processed. This was particularly apparent for brined and drained MAP shrimp at 7 °C where a shelf life of more than 75 days were determined for manually processed samples whereas a shelf life of only 28-35 days were observed for samples from the industrial scale processing line (Mejlholm et al., 2007).

From a total of 82 isolates from the spoilage microflora of brined shrimp; 59 Gram-positive microorganisms (including 53 LAB isolates), 18 Gram-negative bacteria and 5 yeast isolates were found. *Ps. fluorescens*, *E. faecalis*-like, *E. malodoratus*, *Cb. maltaromaticum*, coagulase negative *Staphylococcus* spp. and *Lb. sakei* were determined to constitute the spoilage microflora of shrimp in brine at 7 °C when benzoic, citric and sorbic acids was used as preservatives. Addition of diacetate to shrimp in brine, corresponding to 0.14-0.15 % in the water phase, inhibited growth of *Ps. fluorescens*, *Enterococcus* spp. and coagulase negative *Staphylococcus* spp. at 7 °C. When acetic, citric and lactic acids were used as preservatives for production of brined shrimp the dominating spoilage microflora was exclusively composed of LAB and yeast with *Lb. sakei*. *Lb. sakei* were identified as the spoilage microflora of brined and drained shrimp in MAP (40 % CO₂/60 % N₂) at 7 °C, irrespective of brine composition. Increasing the storage temperature from 7 to 12 °C changed the composition of the spoilage microflora and shortened the shelf life of shrimp in brine (see Table 3).
### 3.3 Shelf life and spoilage microflora of cold-smoked and gravad products

#### Table 4 Spoilage microflora of cold-smoked and gravad fish

<table>
<thead>
<tr>
<th>Product</th>
<th>Storage conditions</th>
<th>Isolates (n)</th>
<th>Identified spoilage microflora</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold-smoked salmon</td>
<td>VP/MAP, 5 °C</td>
<td>271</td>
<td>Carnobacterium piscicola (82 %) Lactobacillus spp. (8 %) Cb. divergens (2 %) Lactococcus spp. (1 %) Brochothrix thermosphacta (2 %)</td>
<td>Paludan-Müller et al. (1998)</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>VP, 8 °C</td>
<td>155b</td>
<td>Cb. piscicola (63 %) Lb. farcinminis (24 %) Lb. sake (6 %) Lb. alimentarius (4 %) Brochothrix spp. b Photobacterium phosphoreum b</td>
<td>Leroi et al. (1998)</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>VP, 5 °C</td>
<td>179</td>
<td>Lb. curvatus (40 %) Lactobacillus spp. (13 %) Lb. plantarum (8 %) Carnobacterium spp. (6 %) Lb. saké (4 %) Leuconostoc mesenteroides (3 %) Enterobacteriaceae (26 %)</td>
<td>Hansen and Huss (1998)</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>VP, 5 °C</td>
<td>238</td>
<td>Lb. curvatus (34 %) Lb. saké (19 %) Carnobacterium spp. (6 %) Lc. carnosum (2 %) yeast (17 %) B. thermosphacta (12 %) Enterobacteriaceae (6 %)</td>
<td>Hansen and Huss (1998)</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>VP, 4 °C</td>
<td>309</td>
<td>Lactic acid bacteria (&gt; 70 %) Enterobacteriaceae (20 %) staphylococci/micrococci (7 %)</td>
<td>Lyhs et al. (1998)</td>
</tr>
<tr>
<td>Cold-smoked trout</td>
<td>VP, 8 °C</td>
<td>311</td>
<td>Lactic acid bacteria (~ 80 %) Enterobacteriaceae (11 %) staphylococci/micrococci (5 %) Pseudomonas aeruginosa (4 %)</td>
<td>Lyhs et al. (1998)</td>
</tr>
<tr>
<td>Cold-smoked trout</td>
<td>VP, 4 °C</td>
<td>212a</td>
<td>Lc. mesenteroides (52 %) Lb. sakei (35 %) Lc. citreum (4 %) Lb. curvatus (2 %)</td>
<td>Lyhs et al. (1999)</td>
</tr>
<tr>
<td>Cold-smoked trout</td>
<td>VP, 8 °C</td>
<td>193a</td>
<td>Lc. citreum (45 %) Lb. sakei (29 %) Lc. mesenteroides (12 %) Lb. curvatus (2 %)</td>
<td>Lyhs et al. (1999)</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>VP, 5 °C</td>
<td>180</td>
<td>Lb. curvatus (24 %) Cb. divergens (22 %) Lc. mesenteroides (20 %) Lb. sakei subsp. carnosus (9 %) P. phosphoreum (8 %) Aeromonas spp. (3 %)</td>
<td>Jørgensen et al. (2000)</td>
</tr>
<tr>
<td>Gravad trout</td>
<td>VP, 3 °C</td>
<td>128a</td>
<td>Lb. curvatus subsp. melibiosus (46 %) Cb. piscicola (29 %) Lb. curvatus subsp. curvatus (19 %) Lb. sakei subsp. sakei (3 %) Lb. sakei subsp. carnosum (2 %) Cb. divergens (1 %)</td>
<td>Lyhs et al. (2002)</td>
</tr>
<tr>
<td>Gravad trout</td>
<td>VP, 8 °C</td>
<td>168a</td>
<td>Lb. curvatus subsp. melibiosus (37 %) Lb. curvatus subsp. curvatus (34 %) Cb. piscicola (11 %) Lb. sakei subsp. sakei (10 %) Lb. sakei subsp. carnosum (10 %)</td>
<td>Lyhs et al. (2002)</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>VP, 7 °C</td>
<td>61</td>
<td>Lactobacillus spp. (60 %) Photobacterium spp. (30 %) B. thermosphacta (9 %)</td>
<td>Olofsson et al. (2007)</td>
</tr>
</tbody>
</table>

a Only lactic acid bacteria (LAB) isolates  
b Number of isolates not reported  
c Experiment 1 of Hansen and Huss (1998)  
d Experiment 2 of Hansen and Huss (1998)  
e 212 of 223 LAB isolates are further identified by Lyhs et al. (1999)  
f 193 of 246 LAB isolates are further identified by Lyhs et al. (1999)  
g Leuconostoc mesenteroides subsp. mesenteroides
The spoilage microflora of cold-smoked and gravad fish have been examined and described in several studies and some of these are listed in Table 4. Hansen (1995) described three scenarios for the spoilage microflora in cold-smoked salmon (i) dominance by LAB, (ii) dominance by a mixture of LAB and Enterobacteriaceae, and (iii) dominance by a mixture of LAB and Photobacterium phosphoreum. Studies summarised in Table 4 confirm these three scenarios for cold-smoked salmon as well as for other cold-smoked and gravad fish products. In addition, Brochothrix thermosphacta/Brochothrix spp. has also been identified as part of the spoilage microflora of cold-smoked salmon (Leroi et al., 1998; Paludan-Müller et al., 1998; Hansen and Huss, 1998; Olofsson et al., 2007). LAB is most frequently isolated from cold-smoked and gravad fish with Lactobacillus, Carnobacterium and Leuconostoc constituting the dominating species.

The spoilage potential of e.g. LAB, P. phosphoreum, B. thermosphacta and Enterobacteriacea has been studied in inoculation experiments with cold-smoked salmon (Paludan-Müller et al., 1998; Joffraud et al., 2001; Stohr et al., 2001; Joffraud et al., 2006). Paludan-Müller et al. (1998) established that even high concentrations of Cb. piscicola (> 10⁷ CFU/g) were unable to spoil samples of VP or MAP cold-smoked salmon at 5 °C. The low spoilage potential of Cb. piscicola has been confirmed by other studies on cold-smoked salmon (Leroi et al., 1996; Nilsson et al., 1999; Joffraud et al., 2001; Joffraud et al., 2006). Stohr et al. (2001) identified Lb. sake, Lb. farcininis and B. thermosphacta as the most offensive spoilage bacteria producing sulphurous, acidic and butter/rancid off-flavours, respectively, in inoculation trials with cold-smoked salmon. Joffraud et al. (2006) examined the spoilage potential of pure and mixed cultures of isolates from VP cold-smoked salmon. Lb. sakei and Serratia liquefaciens-like isolates possessed the strongest spoilage potential followed by P. phosphoreum and B. thermosphacta. Lb. sakei produced amine and sulphurous off-flavours. Importantly, it was shown that mixed cultures produced spoilage characteristics that were different from those of pure cultures (Joffraud et al., 2006). Considering the pronounced variation in the spoilage microflora of cold-smoked and gravad fish (Table 4), this explains why it is difficult to identify quality indices for this group of products (Hansen et al., 1998; Jørgensen et al., 2000; Leroi et al., 2001).
4 Microbial safety of lightly preserved seafood

A number of biological hazards are relevant and important to consider, when the safety of lightly preserved seafood needs to be evaluated. Most of these hazards have been described extensively in the literature and those of most concern have been identified (Huss et al., 2000b; Huss et al., 2004). *L. monocytogenes* represent the main risk in some chilled and lightly preserved seafood as typical product characteristics and storage conditions of cold-smoked and gravad fish are insufficient to prevent growth of this pathogen (Huss et al., 2000b; FAO, 2004; ILSI, 2005). The ability of *L. monocytogenes* to grow in brined shrimp, with a preserving profile different from cold-smoked and gravad fish, is described to a much lesser extent in the literature. Potential growth and toxin production by *Cl. botulinum* is another risk associated with seafood; however, it is possible to control this pathogen by environmental parameters e.g. chill storage and salt (Huss et al., 2004). Risks possessed by e.g. viruses, parasites, non-indigenous bacteria (e.g. *Salmonella*) and biogenic amines in relation to cold-smoked salmon has been described in previous Ph.D.-thesis from the Danish Institute of Fisheries Research (DIFRES), and consequently they are not discussed further in this thesis (Hansen, 1995; Jørgensen, 2000). Furthermore, the risk of biogenic amine (i.e. histamine) formation in seafood is evaluated in a parallel and ongoing Ph.D.-project at our institute as part of the BIOCOM project within SEAFOODplus.

The safety of lightly preserved seafood is discussed in the following section with the main focus on *L. monocytogenes*, and the perspectives and possibilities of preventing growth of this pathogen. A brief discussion of the risk constituted by *Cl. botulinum* is given at the end of this chapter.

4.1 *Listeria monocytogenes*

*Listeria monocytogenes* is a Gram-positive, foodborne pathogen causing the infection listeriosis (Farber and Peterkin, 2000). It is wide spread in nature (e.g. soil, plant material and water) and has often been isolated from e.g. seafood processing plants where it is able to persist (Vogel et al., 2001b; Thimothe et al., 2004; Lappi et al., 2004b; Wulff et al., 2006; Gudmundsdóttir et al., 2006a). *L. monocytogenes* is a psychro- and halotolerant bacteria, able to grow in the range of 1 °C to 45 °C, and between 0 and 10 % NaCl (Farber and Peterkin, 2000). This implies that growth of *L. monocytogenes* is often
Microbial changes and safety of lightly preserved seafood

not prevented in chilled lightly preserved seafood with typical concentrations of salt. A vast amount of lightly preserved seafood can be categorised as high-risk foods according to the definition proposed by ILSI (ILSI, 2005). A high-risk food is defined by the following characteristics (i) contamination with \textit{L. monocytogenes} is possible; (ii) growth of \textit{L. monocytogenes} is supported; (iii) RTE food; (iv) requires refrigeration; and (v) stored for an extended period of time. It is debatable whether the last characteristic needs to be fulfilled to classify a food item in the high-risk category. In fact, Jørgensen and Huss (1998) showed that both the frequency and level of \textit{L. monocytogenes} increased in naturally contaminated samples of cold-smoked salmon, cold-smoked trout and gravad fish stored for 14-28 days at 5 °C. Furthermore, in trials with inoculated products it has been shown that potentially infectious concentrations of \textit{L. monocytogenes} are reached within the period typically used for storage at retail level (FAO, 2004; Mejlholm et al., 2005; Mejlholm and Dalgaard, 2007a). Growth of \textit{L. monocytogenes} in naturally contaminated and in inoculated products is discussed further in section 5. Substantial efforts have been directed towards improving process hygiene and sanitation as well as identifying possible contamination routes and sources of \textit{L. monocytogenes} (section 4.1.3). However, these initiatives have not been able to eliminate \textit{L. monocytogenes} from the processing environment and/or many final products, and it is now generally accepted that contamination with this pathogen is very difficult to prevent (Huss et al., 2000a; Gram, 2001b). Thus, alternative strategies needs to be described to reduce the risk associated with \textit{L. monocytogenes}. Preventing growth of \textit{L. monocytogenes} is the strategy suggested to have the greatest impact on reducing the cases of listeriosis (ILSI, 2005). This strategy is in accordance with the latest EU regulation on RTE foods (EC, 2005) as well as the fact that most outbreaks of listeriosis is associated with foods in which \textit{L. monocytogenes} can grow to high numbers (FAO, 2004). FAO (2004) estimated that the risk of listeriosis on a per serving basis could be reduced by 100- to 1000-fold in RTE foods where growth of \textit{L. monocytogenes} is prevented as compared to products allowing growth. Finally, it has been proposed that development of validated mathematical models, facilitating identification of growth preventing parameters, would be most useful for accomplishing the above mentioned strategy (ILSI, 2005).
4.1.1 Prevalence and level of *L. monocytogenes* in lightly preserved seafood

From a total of nearly 15,000 samples of lightly preserved seafood including cold-smoked, hot-smoked, gravad, seafood salad, and shrimp products, the prevalence of *L. monocytogenes* was 9.4% (Table 5). The highest prevalence of *L. monocytogenes* was found in cold-smoked halibut with more than 45% of the samples (n = 58) being positive. Data on cold-smoked halibut were obtained from two independent studies (Jørgensen and Huss, 1998; Van Coillie et al., 2004). However, it is likely that samples from both studies originated from a Danish processor as Denmark accounts for almost 80% of the world production of cold-smoked halibut (FAO, 2006). The prevalence of *L. monocytogenes* in cold-smoked salmon (n = 2272) and cold-smoked trout (n = 104) was 18.6 and 16.4%, respectively. The frequency of *L. monocytogenes* was lower in hot-smoked fish than in cold-smoked products, although 10.7% of the former samples were tested positive (Figure 6). This clearly shows that contamination occurs after the hot-smoking step as *L. monocytogenes* is expected to be eliminated by the time-temperature combination (70-90 °C for 1½ hour) of this process. Concerns have been raised on this aspect as the natural microflora is eliminated as well during hot-smoking and this might result in better growth conditions for *L. monocytogenes* in the final products (Jørgensen and Huss, 1998; Rocourt et al., 2000). However, it seems unrealistic that hot-smoked products are contaminated solely with *L. monocytogenes* and not other microorganisms (e.g. LAB), which might act as a competitive microflora towards the pathogen.

![Figure 6. Percentages of samples being positive for *Listeria monocytogenes* within different sub-groups of lightly preserved seafood (generated from Table 5).](image-url)
## Table 5 Prevalence and concentration of *Listeria monocytogenes* in lightly preserved seafood

<table>
<thead>
<tr>
<th>Product</th>
<th>Country</th>
<th>Total number of samples (n)</th>
<th>Positive for <em>L. monocytogenes</em></th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Cooked and peeled shrimp</td>
<td>Canada</td>
<td>8</td>
<td>2</td>
<td>25.0</td>
</tr>
<tr>
<td>Shrimp</td>
<td>Canada</td>
<td>48</td>
<td>4</td>
<td>8.3</td>
</tr>
<tr>
<td>Shrimp</td>
<td>Iceland</td>
<td>11</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Shrimp</td>
<td>Iceland</td>
<td>13</td>
<td>3</td>
<td>23.1</td>
</tr>
<tr>
<td>Shrimp</td>
<td>Norway</td>
<td>16</td>
<td>3</td>
<td>18.8</td>
</tr>
<tr>
<td>Cooked and peeled shrimp</td>
<td>Iceland</td>
<td>3331</td>
<td>67</td>
<td>2.0</td>
</tr>
<tr>
<td>Cooked and peeled shrimp</td>
<td>Iceland</td>
<td>63</td>
<td>3</td>
<td>4.8</td>
</tr>
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<td>Shrimp salad</td>
<td>Belgium</td>
<td>5</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cooked and peeled shrimp</td>
<td>Iceland</td>
<td>82</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
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<td>Canada</td>
<td>32</td>
<td>10</td>
<td>31.3</td>
</tr>
<tr>
<td>Gravad salmon</td>
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<td>1</td>
<td>8.3</td>
</tr>
<tr>
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<td>Iceland</td>
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<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cold-smoked trout</td>
<td>Iceland</td>
<td>8</td>
<td>1</td>
<td>12.5</td>
</tr>
<tr>
<td>Gravad salmon salad</td>
<td>Iceland</td>
<td>5</td>
<td>2</td>
<td>40.0</td>
</tr>
<tr>
<td>Cold-smoked salmon salad</td>
<td>Iceland</td>
<td>11</td>
<td>2</td>
<td>18.2</td>
</tr>
<tr>
<td>Smoked salmon</td>
<td>Norway</td>
<td>33</td>
<td>3</td>
<td>9.1</td>
</tr>
<tr>
<td>Smoked salmon</td>
<td>New Zealand</td>
<td>12</td>
<td>9</td>
<td>75.0</td>
</tr>
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<td>Cold-smoked salmon</td>
<td>USA</td>
<td>61</td>
<td>49</td>
<td>80.3</td>
</tr>
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<td>Cold-smoked salmon</td>
<td>Norway</td>
<td>65</td>
<td>7</td>
<td>10.8</td>
</tr>
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<td>Cold-smoked salmon</td>
<td>Sweden</td>
<td>13</td>
<td>2</td>
<td>15.4</td>
</tr>
<tr>
<td>Cold-smoked trout</td>
<td>Sweden</td>
<td>13</td>
<td>1</td>
<td>7.7</td>
</tr>
<tr>
<td>Gravad salmon</td>
<td>Sweden</td>
<td>24</td>
<td>4</td>
<td>16.7</td>
</tr>
<tr>
<td>Gravad trout</td>
<td>Sweden</td>
<td>34</td>
<td>8</td>
<td>23.5</td>
</tr>
<tr>
<td>Hot-smoked salmon</td>
<td>Sweden</td>
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<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Hot-smoked trout</td>
<td>Sweden</td>
<td>6</td>
<td>1</td>
<td>16.7</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>Italy</td>
<td>165</td>
<td>31</td>
<td>18.8</td>
</tr>
</tbody>
</table>

* a Not reported
b Ready-to-eat food
c Import samples
d Both import and export samples
Table 5 Prevalence and concentration of *Listeria monocytogenes* in lightly preserved seafood (continued)

<table>
<thead>
<tr>
<th>Product</th>
<th>Country</th>
<th>Total number of samples (n)</th>
<th>Positive for <em>L. monocytogenes</em></th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive in 25 g or &lt; 1 log CFU/g</td>
<td>0-1 log CFU/g</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>Denmark</td>
<td>190</td>
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</tr>
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<td>Denmark</td>
<td>115</td>
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</tr>
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<td>Cold-smoked salmon</td>
<td>Denmark</td>
<td>75</td>
<td>32</td>
<td>42.7</td>
</tr>
<tr>
<td>Cold-smoked halibut</td>
<td>Denmark</td>
<td>20</td>
<td>9</td>
<td>45.0</td>
</tr>
<tr>
<td>Cold-smoked halibut</td>
<td>Denmark</td>
<td>20</td>
<td>12</td>
<td>60.0</td>
</tr>
<tr>
<td>Gravad fish</td>
<td>Denmark</td>
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<td>32.9</td>
</tr>
<tr>
<td>Gravad fish</td>
<td>Denmark</td>
<td>91</td>
<td>23</td>
<td>25.3</td>
</tr>
<tr>
<td>Cold-smoked fish</td>
<td>USA</td>
<td>291</td>
<td>52</td>
<td>17.9</td>
</tr>
<tr>
<td>Hot-smoked fish</td>
<td>USA</td>
<td>234</td>
<td>19</td>
<td>8.1</td>
</tr>
<tr>
<td>Fish and shrimp salad</td>
<td>Belgium</td>
<td>362</td>
<td>98</td>
<td>27.1</td>
</tr>
<tr>
<td>Preserved fish</td>
<td>Denmark</td>
<td>335</td>
<td>54</td>
<td>16.1</td>
</tr>
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<td>Cold-smoked trout</td>
<td>Finland</td>
<td>30</td>
<td>5</td>
<td>16.7</td>
</tr>
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<td>Finland</td>
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<td>16</td>
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</tr>
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<td>Finland</td>
<td>28</td>
<td>6</td>
<td>21.4</td>
</tr>
<tr>
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<td>5</td>
<td>5.4</td>
</tr>
<tr>
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<td>2</td>
<td>16.7</td>
</tr>
<tr>
<td>Smoked salmon</td>
<td>Spain</td>
<td>52</td>
<td>14</td>
<td>26.9</td>
</tr>
<tr>
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<td>21</td>
<td>2</td>
<td>9.5</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
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<td>42</td>
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<td>28.6</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>Spain</td>
<td>90</td>
<td>16</td>
<td>17.8</td>
</tr>
<tr>
<td>Cold-smoked trout</td>
<td>Spain</td>
<td>38</td>
<td>10</td>
<td>26.3</td>
</tr>
</tbody>
</table>

* Analysed at day 0
* b Analysed after 14-20 days at 5 °C
* c Analysed after 21-50 days at 5 °C
* d Analysed after 14-28 days at 5 °C
* e Smoked and cured, not heat-treated
### Table 5 Prevalence and concentration of *Listeria monocytogenes* in lightly preserved seafood (continued)

<table>
<thead>
<tr>
<th>Product</th>
<th>Country</th>
<th>Total number of samples (n)</th>
<th>Positive for <em>L. monocytogenes</em></th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive in 25 g or &lt; 1 log CFU/g</td>
<td>0-1 log CFU/g</td>
</tr>
<tr>
<td>Cold-smoked fish</td>
<td>Finland</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hot-smoked fish</td>
<td>Finland</td>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cold-smoked fish</td>
<td>Switzerland&lt;sup&gt;a&lt;/sup&gt;</td>
<td>814</td>
<td>114</td>
<td>14.0</td>
</tr>
<tr>
<td>Hot-smoked fish</td>
<td>Switzerland&lt;sup&gt;a&lt;/sup&gt;</td>
<td>471</td>
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<td>12.0</td>
</tr>
<tr>
<td>Marinated fish&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Switzerland&lt;sup&gt;a&lt;/sup&gt;</td>
<td>125</td>
<td>48</td>
<td>38.0</td>
</tr>
<tr>
<td>Smoked seafood</td>
<td>USA</td>
<td>2644</td>
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</tr>
<tr>
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<td>1</td>
<td>1.8</td>
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<tr>
<td>Hot-smoked salmon</td>
<td>USA</td>
<td>16</td>
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<td>0.0</td>
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<tr>
<td>Smoked salmon</td>
<td>Belgium</td>
<td>42</td>
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<td>19.0</td>
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<td>3</td>
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<tr>
<td>Smoked trout</td>
<td>Belgium</td>
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<td>0.0</td>
</tr>
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<td>Smoked halibut</td>
<td>Belgium</td>
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<td>6</td>
<td>33.3</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>Iceland</td>
<td>125</td>
<td>5</td>
<td>4.0</td>
</tr>
<tr>
<td>Smoked fish</td>
<td>USA</td>
<td>233</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>Cold-smoked salmon</td>
<td>Spain</td>
<td>100</td>
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<td>28.0</td>
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<td>France</td>
<td>626</td>
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<td>7.0</td>
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<td>Cold-smoked salmon</td>
<td>France</td>
<td>384</td>
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<td>Cold-smoked salmon</td>
<td>Italy</td>
<td>104</td>
<td>11</td>
<td>10.6</td>
</tr>
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<td>Smoked fish</td>
<td>Austria</td>
<td>88</td>
<td>18</td>
<td>20.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Both import and export samples

<sup>b</sup> e.g. gravad salmon
Microbial changes and safety of lightly preserved seafood

*L. monocytogenes* was found in 31.6 % of the samples of gravad fish (n = 403) and thus, this sub-group of lightly preserved seafood was the one with the highest prevalence of the pathogen (Figure 6). Represented as single products, samples of gravad salmon (n = 36) and gravad trout (n = 66) had a prevalence of *L. monocytogenes* of 14.0 % and 36.6 %, respectively. Unfortunately, for some of the studies compiled in Table 5 the type of gravad fish was not specified. This was also the case for cold-smoked products and a number of studies did not even distinguish between cold- and hot-smoked products. This type of information could have provided a more detailed picture on the occurrence of *L. monocytogenes* in lightly preserved seafood. A pronounced difference in the prevalence of *L. monocytogenes* was noticed between samples of gravad and cold-smoked products (Figure 6), and it can be speculated whether this is caused by the lack of potential listericidal steps (i.e. smoking) during production of the former product or if other aspects plays a part. In a study by Rørvik et al. (1995), *L. monocytogenes* was not isolated from samples of cold-smoked salmon taken immediately after smoking. The most likely sources of contamination are, however, situated after the smoking process and this explains why *L. monocytogenes* also are found frequently on smoked fish (see section 4.1.3.2).

Seafood salad, including mayonnaise-based products with e.g. smoked salmon and shrimp, constituted the largest group of samples (n = 5294), and had a prevalence of *L. monocytogenes* of 6.6 %. The presence of *L. monocytogenes* in these products varied between 0 and 50 %.

Only one study has described the occurrence of *L. monocytogenes* in brined shrimp and they found that 3 out of 16 samples (19 %) were positive for this pathogen. Samples tested positive for *L. monocytogenes* were obtained from three different shrimp processing plants (Rørvik and Yndestad, 1991). The fact that *L. monocytogenes* is sometimes present in cooked and peeled shrimp (2.2 % of the samples), used for production of brined shrimp, should encourage that more studies are conducted within this field including examination of potential contamination routes and sources during production.

Gombas et al. (2003) analysed more than 30.000 samples of RTE foods (e.g. meat, cheese and seafood) and found the highest prevalence of *L. monocytogenes* in smoked seafood (4.7 % of the samples) and seafood salad (4.3 % of the samples).
The concentration of *L. monocytogenes* was determined in 526 of the 14696 samples compiled in Table 5 (< 4 % of the samples). Eighty-two percent of the samples contained < 2 log (CFU/g) and 13 % had a level between 2 and 3 log (CFU/g). A concentration between 3 and 4 log (CFU/g), and > 4 log (CFU/g) was established in 4 % and 1 %, respectively of the samples. The latter category contained products of cold-smoked salmon (n = 2), cold-smoked trout (n = 1), hot-smoked salmon (n = 1) and smoked fish (n = 2). Uyttendaele et al. (1999) reported that concentrations of *L. monocytogenes* > 1 log (CFU/g) often were present in fish and shrimp salad from the Belgian retail market. In another study, all of the examined samples of gravad and smoked fish had concentrations of *L. monocytogenes* < 2 log (CFU/g), with the exception of one smoked salmon sample containing 3.04 log (CFU/g) (Jemmi et al., 2002).

4.1.2 *Listeriosis* – cases associated with lightly preserved seafood

Only a few outbreaks/cases of human listeriosis have been associated with consumption of lightly preserved seafood, although the prevalence of *L. monocytogenes* is relatively high in these products (Table 6). However, the concentration of *L. monocytogenes* in lightly preserved seafood is normally low (< 10^2 CFU/g) and at small risk of causing listeriosis (FAO, 2004). Furthermore, lightly preserved seafood is consumed to a smaller extent than, for example deli meats and cheese, and this is reflected in the number of outbreaks linked to the different types of food (FAO, 2004).

<table>
<thead>
<tr>
<th>Table 6 Seafood/lightly preserved seafood implicated in human listeriosis</th>
<th>Product</th>
<th>Year</th>
<th>Number of cases</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1988</td>
<td>3</td>
<td>Sweden</td>
<td>Loncarevic et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>Cooked shrimp</td>
<td>1989</td>
<td>10</td>
<td>USA</td>
<td>Riedo et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Smoked cod roe</td>
<td>1989</td>
<td>1</td>
<td>Denmark</td>
<td>Jensen et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Smoked mussels a</td>
<td>1991</td>
<td>4</td>
<td>Australia</td>
<td>Misrachi et al. (1991)</td>
<td></td>
</tr>
<tr>
<td>Smoked mussels</td>
<td>1992</td>
<td>3</td>
<td>New Zealand</td>
<td>Baker et al. (1993); Brett et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>Gravad/cold-smoked trout</td>
<td>1994-95</td>
<td>10</td>
<td>Sweden</td>
<td>Ericsson et al. (1997)</td>
<td></td>
</tr>
<tr>
<td>Cold-smoked trout</td>
<td>1998</td>
<td>5</td>
<td>Finland</td>
<td>Miettinen et al. (1999)</td>
<td></td>
</tr>
</tbody>
</table>

Modified from McLauchlin (1996); Elliot and Kvenberg (2000).

a Imported from New Zealand
An outbreak of listeriosis suspected to be caused by cooked shrimp was reported by Riedo et al. (1994). Ten out of 36 persons attending a party was affected by the illness with the majority experiencing symptoms like fever, and musculoskeletal and gastrointestinal pains. Two of the implicated patients were pregnant women and in one of these cases the illness resulted in fetal demise. No isolates of *L. monocytogenes* was obtained from foods eaten at the party as no left-overs remained. Based on the type and amount of food consumed by the individual persons at the party, shrimp was identified as the most likely source of listeriosis followed by non-alcoholic beverages, camembert cheese and cauliflower (Riedo et al., 1994).

Ericsson et al., (1997) described an outbreak of listeriosis (invasive) suspected to be caused by gravad and cold-smoked trout. This outbreak involved ten cases and had a fatality rate of 20 %. All patients belonged to the risk group i.e. immunocompromised, elderly or pregnant persons. The same clonal type of *L. monocytogenes* was isolated from six of the patients, opened and unopened packages of cold-smoked and gravad trout from the same processor, and from the process environment of this plant. This type of *L. monocytogenes* (serovar 4b) was shown to persist in the process environment of the involved plant, and this might explain the length of the outbreak (11 months). No positive relations were established between three of the patients and the above mentioned processor; however, they all remembered to have been eating trout and/or salmon products recently. Gravad trout obtained from the refrigerator of one of the patients contained approx. 3.7 log (CFU/g) of *L. monocytogenes* whereas positive samples from retailers in general had concentrations < 2 log (CFU/g). However, one sample of gravad trout, left unrefrigerated at the post office over the weekend, contained > 6 log (CFU/g) of *L. monocytogenes*.

Another outbreak of listeriosis (non-invasive) associated with consumption of cold-smoked trout was reported by Miettinen et al. (1999). Four adults and a 3-year old child, with no underlying illnesses, became sick with febrile gastroenteritis less than 27 hours after having dined together. Based on patient interviews, cold-smoked trout was identified as the most likely source of *L. monocytogenes*. This was supported by analysis of stool samples from the patients, and samples of VP cold-smoked trout originating from the same batch and purchased at the same retail store as the implicated product. The same serovar (1/2a) and PFGE (Pulsed Field Gel Electrophoresis) type was isolated from both
types of samples. The concentration of *L. monocytogenes* in the fish sample was 5.3 log (CFU/g). The implicated product was packed 17 days prior to consumption and the storage temperature at retail level varied between 4.6 °C and 11.6 °C as measured at the bottom and top, respectively of the chill cabinet.

Gudmundsdóttir et al. (2006b) studied the virulence of *L. monocytogenes* isolates from humans and smoked salmon, peeled shrimp, and their processing environment. Sixty-eight (98.6 %) of the 69 isolates obtained from raw materials, final products and the process environment were established to be virulent. Similarly, Norton et al. (2001) examined the pathogenic potential of *L. monocytogenes* isolates from the raw material, semi-final and final products, and the environment of a plant processing smoked fish. Three ribotypes of *L. monocytogenes* previously implicated in outbreaks of listeriosis (e.g. coleslaw, Canada, 1981 and Mexican style soft cheese, USA, 1985) were detected among the isolates from the process plant. However, the virulence of these isolates were impaired as compared to human isolates (Norton et al., 2001).

4.1.3 Contamination sources

Numerous studies have been carried out in an attempt to identify possible contamination routes and sources of *L. monocytogenes* in seafood processing plants and seafood products (Eklund et al., 1995; Destro et al., 1996; Autio et al., 1999; Johansson et al., 1999; Dauphin et al., 2001; Miettinen et al., 2001; Aguado et al., 2001; Vogel et al., 2001a; Hoffman et al., 2003; Thimothe et al., 2004; Gudmundsdóttir et al., 2005; Hansen et al., 2006; Gudmundsdóttir et al., 2006a).

The initial contamination source of *L. monocytogenes*, introducing this pathogen to the process environment, has not been identified. However, considering the ubiquitous nature of *L. monocytogenes*, it seems most likely that multiple sources e.g. raw material, employees and transporting devices are involved (Gram, 2001b). Once introduced, *L. monocytogenes* is able to colonize and multiply, and persist in the process environment. This is critical as contamination of the final product with *L. monocytogenes* is a likely outcome. The availability of molecular typing techniques have made it possible to identify routes and sources of contamination as well as to establish if *L. monocytogenes* constitute a persistent “in-house flora” (Vogel et al., 2001a; Vogel et al., 2001b).
4.1.3.1 Shrimp

Possible contamination routes and sources of *L. monocytogenes* during production of brined shrimp has not been reported in the literature, however, a single study has described these aspects for cooked and peeled shrimp (Gudmundsdóttir et al., 2006a). They studied two Icelandic shrimp processing plants and *L. monocytogenes* was isolated from several sources throughout production but not from the final product. *L. monocytogenes* was frequently isolated from the raw material (20.9 % of the samples) and the low-risk area (15.4-40.6 % of the samples), in which the raw shrimp were handled before cooking. The results of Gudmundsdóttir et al. (2006a) clearly demonstrated the ability of *L. monocytogenes* to persist in the shrimp processing environment as this pathogen was isolated consecutively from the high-risk area, more precisely the peeling machine (1.7-5.6 % of the samples), of one of the plants. *L. monocytogenes* was not eliminated from the peeling machine although repeated and careful cleaning was applied. This problem was first solved when the peeling machine was dismantled and every part was cleaned separately. Isolates of *L. monocytogenes* with identical genetic patterns (PFGE) was obtained from different stages during production of cooked and peeled shrimp showing that cross contamination occurred. A general reduction in the prevalence of *L. monocytogenes* was observed following cleaning and disinfection of the process environment, but a total elimination of this pathogen was never seen (Gudmundsdóttir et al., 2006a). It is therefore relevant to study growth of *L. monocytogenes* in shrimp products.

4.1.3.2 Fish products

Eklund et al. (1995) described the raw fish as the primary source of contamination with *L. monocytogenes* in a cold-smoked fish processing plant. It was assumed that during processing (e.g. filleting and brining), *L. monocytogenes* was transferred from the fish to process equipment, employees and other surfaces which then served as secondary sites of contamination (Eklund et al., 1995). However, it was not determined if isolates of *L. monocytogenes* from the raw fish, process equipment and the finished product, respectively were of the same genetic type. In a study by Norton et al. (2000), examining two smokehouses, the same genetic type (clone) of *L. monocytogenes* was isolated from the raw fish, processing line and the final product of one of the plants. For the other plant, identical types of *L. monocytogenes* were found on the process line and the finished...
product, but not on the raw fish (Norton et al., 2000). These findings have been confirmed by other studies, isolating identical types (clones) of *L. monocytogenes* from the process environment and the final product, but not from the raw fish used for production (Rørvik et al., 1995; Autio et al., 1999; Dauphin et al., 2001; Vogel et al., 2001a; Thimothe et al., 2004). Thus, the process environment is assumed to be the most important source of contamination with *L. monocytogenes* (Autio et al., 1999; Johansson et al., 1999; Dauphin et al., 2001; Vogel et al., 2001a; Thimothe et al., 2004). The main sources of contamination during production of cold-smoked and gravad fish have been identified as the brining and slicing process (Autio et al., 1999; Johansson et al., 1999; Vogel et al., 2001a). Autio et al. (1999) paid special attention towards the practice of recirculating the brine as this increased the risk of introducing *L. monocytogenes* into the fish when needle-injection was used. Equipment used for slicing and brining are difficult to clean and thus colonisation of *L. monocytogenes* is a possibility (Autio et al., 1999). *L. monocytogenes* has been isolated from several other sources throughout the process environment including drains, floors, transporters, tubs, aprons and gloves. The ability of *L. monocytogenes* to persist in the processing environment has been shown in a number of studies (Autio et al., 1999; Vogel et al., 2001a; Thimothe et al., 2004; Gudmundsdóttir et al., 2005). Vogel et al. (2001a) showed that the same RAPD (Random Amplification of Polymorphic DNA) type of *L. monocytogenes* persisted over a period of 4 years in a cold-smoked salmon processing plant. It is generally accepted that it is possible to reduce the prevalence of *L. monocytogenes* in the process environment and on final products by implementing good hygiene practice (GHP) and HACCP systems pointing at critical control points, and by using improved and targeted cleaning/disinfection (Vogel et al., 2001a). However, the ability of *L. monocytogenes* to adhere to surfaces and its resistance towards cleaning and disinfection agents makes it a challenge to eliminate this pathogen from the process environment. Following thorough cleaning and heat treatment (i.e. hot steam, hot air and hot water) of slicing and brining equipment, *L. monocytogenes* was not isolated from the environment or the final products of a cold-smoked trout processing plant during a period of 5-months (Autio et al., 1999). Though, most studies have only demonstrated a reduction in the prevalence of *L. monocytogenes* following cleaning (Gudmundsdóttir et al., 2005), and even if this pathogen is eliminated it is likely to be reintroduced to the process environment from time to time (Huss et al., 2000a).
4.1.4 Microbiological criteria for *L. monocytogenes* in RTE foods

The most recent EU regulation on RTE foods (EC, 2005) differentiate between products that are able or unable to support growth of *L. monocytogenes* (Table 7). In RTE foods (e.g. cold-smoked salmon) supporting growth of *L. monocytogenes* it must be documented by the producer that the limit of 10^2 CFU/g is not exceeded within the declared shelf life. If not documentable, *L. monocytogenes* should not be detected in the product (i.e. samples of 25 g). A limit of 10^2 CFU/g is permitted in products where it can be documented that growth of *L. monocytogenes* is prevented. Products with (i) pH ≤ 4.4 or aw ≤ 0.92; (ii) pH ≤ 5.0 and aw ≤ 0.94; and (iii) shelf life < 5 days are automatically considered to belong to the latter category. However, the above mentioned characteristics are not consistent with the definition of lightly preserved seafood (pH > 5.0 and < 6 % WPS ~ aw > 0.96) or desirable sensory characteristics of these products. Moreover, a short shelf life of < 5 days is not in the interest of many within the seafood processing industry. Thus, alternative methods are needed to prevent growth of *L. monocytogenes* in lightly preserved seafood (see section 4.1.5). The EU regulation (EC, 2005) does not differentiate between RTE foods intended for infants and medicinal use, as *L. monocytogenes* should not be detected in these products, whether growth is prevented or not.

**Table 7 EU regulation (EC 2073/2005) on ready-to-eat foods and *Listeria monocytogenes***

<table>
<thead>
<tr>
<th>Ready-to-eat foods</th>
<th>Sampling plan</th>
<th>Limits</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Support growth</td>
<td>n = 5, c = 0</td>
<td>m = 10⁰ CFU/g</td>
<td></td>
</tr>
<tr>
<td>Support growth</td>
<td>n = 5, c = 0</td>
<td>M = Absence in 25 g</td>
<td>When the food leaves the producer</td>
</tr>
<tr>
<td>Unable to support growth</td>
<td>n = 5, c = 0</td>
<td>m = M = 10² CFU/g</td>
<td>It must be documented that growth of <em>L. monocytogenes</em> is prevented</td>
</tr>
</tbody>
</table>

A zero-tolerance, stating that *L. monocytogenes* should not be detected in RTE foods (i.e. samples of 25 g) is used in e.g. Italy, New Zealand, Australia and the United States (US). Detection of *L. monocytogenes* in samples of e.g. cold-smoked salmon results in rejection of the whole batch in question. Unlike the EU regulation, the zero-
tolerance policy does not distinguish between products that are able or unable to support growth of *L. monocytogenes*.

Much debate has been focused on whether microbiological criteria on *L. monocytogenes* in RTE foods should be based on qualitative (zero-tolerance) or quantitative aspects (e.g. a limit of $10^2$ CFU/g) (Tompkin, 2002; Chen et al., 2003; FAO, 2004; ILSI, 2005). Particularly, the relative risk of acquiring listeriosis in relation to the different criteria on *L. monocytogenes* has been discussed. By comparing the number of reported cases, no clear differences are noticed in the incidence of listeriosis between countries using either the zero-tolerance or a limit of $10^2$ CFU/g (Chen et al., 2003). It is well-known that most cases of listeriosis results from consumption of foods with high levels of *L. monocytogenes*, not in compliance with any of the above mentioned criteria (FAO, 2004). Studies examining the relationship between the level of *L. monocytogenes* and the occurrence of listeriosis (i.e. dose-response relationship) have estimated that foods with $\leq 10^2$ CFU/g account for less than 1 % of the cases (Buchanan et al., 1997; Chen et al., 2003). It has been proposed that a regulation less stringent than the zero-tolerance e.g. a limit of $10^2$ CFU/g would result in a reduction in the cases of listeriosis, if this new criterion resulted in improved control measures able to reduce the number of RTE foods with high levels of *L. monocytogenes* (Chen et al., 2003; FAO, 2004). A less stringent regulation would encourage the identification of measures/critical control points that are able to reduce or even prevent growth of *L. monocytogenes*. Chen et al. (2003) established that a decrease in the concentration of *L. monocytogenes* in RTE foods would be far more effective in reducing cases of listeriosis than a decrease in the prevalence of the pathogen. In fact, they estimated that a reduction in the level of *L. monocytogenes* to a maximum of $10^2$ CFU/g for all RTE foods would decrease the risk of listeriosis by more than 99 %.

4.1.5 Preventing growth of *L. monocytogenes* in lightly preserved seafood

Different methods have been studied in an attempt to identify parameters that are able to prevent growth of *L. monocytogenes*. In the following section some of these approaches are discussed with the main emphasis on the use of organic acids as preservatives.
4.1.5.1 Brined shrimp

Mejlholm et al. (2007) examined the potential growth of \textit{L. monocytogenes} for shrimp in brine, and brined and drained MAP shrimp with different preserving profiles. Brines used for the challenge tests contained (i) benzoic, citric and sorbic acids; (ii) citric and sorbic acids or (iii) benzoic and citric acids. In addition, it was studied if diacetate could replace benzoic acid as preservative in brined shrimp. Growth of \textit{L. monocytogenes} was prevented for shrimp in brine for more than 40 days at 6.8 °C when a preserving profile corresponding to the one of commercial products were used (Figure 7a; Table 8, line 1). In brined and drained MAP shrimp, with comparable preserving parameters (Table 8, line 2), a 1.5 log (CFU/g) increase in the concentration of \textit{L. monocytogenes} was observed during 70 days of storage at 8.1 °C (Figure 7b). However, it should be mentioned that the shelf life of brined shrimp typically is no longer than 6 weeks (42 days) and at that time no apparent increase in the level of \textit{L. monocytogenes} was observed. In a study by Dalgaard and Jørgensen (2000) growth of \textit{L. monocytogenes} was prevented in brined and drained MAP cold-water shrimp for more than 80 days at 8 °C. This is in agreement with the results of Mejlholm et al. (2007), although differences were observed between the preserving parameters of the two products (Table 8, line 2 and 8). In brined and drained MAP warm-water shrimp growth of \textit{L. monocytogenes} was observed at 5 °C (Dalgaard and Jørgensen, 2000). This product had a preserving profile slightly different from brined and drained MAP cold-water shrimp (Table 8, line 8 and 9). A pronounced effect on the growth of \textit{L. monocytogenes} was observed when benzoic acid was omitted from the brine used for production of shrimp products (Mejlholm et al., 2007). For shrimp in brine, the time to a 100-fold increase in the concentration of \textit{L. monocytogenes} was reduced from more than 40 days to 6 days at 6.8 °C when benzoic acid was omitted as preservative (Figure 7a; Table 8, line 3). Comparable results were obtained for brined and drained MAP shrimp without benzoic acid as the time to a 100-fold increase in cell numbers was reduced from more than 70 days to 7 days at 8.1 °C (Figure 7b; Table 8, line 4).
Table 8 Effect of product characteristics and storage conditions on growth of *Listeria monocytogenes* in challenge tests with brined shrimp

<table>
<thead>
<tr>
<th>pH</th>
<th>Water phase salt (%)</th>
<th>Benzoic %</th>
<th>Citric %</th>
<th>Diacetate %</th>
<th>Lactic %</th>
<th>Sorbic %</th>
<th>Time (days) to a 100-fold increase in <em>Listeria monocytogenes</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.6</td>
<td>2.9</td>
<td>0.11</td>
<td>0.61</td>
<td>-</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>2.6</td>
<td>0.12</td>
<td>0.54</td>
<td>-</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>5.6</td>
<td>2.9</td>
<td>-</td>
<td>0.63</td>
<td>-</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>4</td>
<td>5.6</td>
<td>2.6</td>
<td>-</td>
<td>0.65</td>
<td>-</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>5.6</td>
<td>2.5</td>
<td>0.12</td>
<td>0.60</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>5.4</td>
<td>2.9</td>
<td>-</td>
<td>0.64</td>
<td>0.23</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>7</td>
<td>5.4</td>
<td>2.5</td>
<td>-</td>
<td>0.57</td>
<td>0.14</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>8</td>
<td>5.7</td>
<td>3.3</td>
<td>0.07</td>
<td>0.33</td>
<td>-</td>
<td>-</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
<td>5.9</td>
<td>2.3</td>
<td>0.08</td>
<td>0.28</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
</tr>
</tbody>
</table>

1-7: From Mejholm et al. (2007)
8-9: From Dalgaard and Jørgensen (2000)

The effect of omitting sorbic acid was less evident as this change only reduced the time to a 100-fold increase from more than 70 days to 31 days in experiments with brined and drained MAP shrimp at 8.1 °C (Figure 7b; Table 8, line 5). The effect of reducing the concentration of benzoic acid was always more apparent on growth of *L. monocytogenes* than comparable changes in sorbic acid levels. The antimicrobial effect of diacetate against *L. monocytogenes* in brined shrimp was examined as an alternative to the use of benzoic acid. Although, growth of *L. monocytogenes* was reduced by addition of 0.14-0.23 % water phase diacetate this treatment was less efficient than 0.11-0.12 % water phase benzoic acid (Table 8, line 6 and 7) (Mejlholm et al., 2007).
Figure 7. Changes in concentrations of *Listeria monocytogenes* during storage of (a) shrimp in brine and (b) brined and drained shrimp in modified atmosphere packaging. Brined shrimp with benzoic, citric and sorbic acids (■); citric and sorbic acids (○); and benzoic and citric acids (▲). Error bars represent the standard deviation (n = 3). Modified from Mejlholm et al. (2007).

To conclude, preserving parameters currently used for brined shrimp are sufficient to prevent growth of *L. monocytogenes* at chill temperatures and within the desired shelf life of these products. However, the risk possessed by *L. monocytogenes* should not be neglected as brined shrimp are sensitive to changes in their preserving profile, particularly the concentration of benzoic acid. Unintended variations in the composition of brine and/or the ratio between shrimp and brine during production might result in conditions that allow growth of *L. monocytogenes* and these aspects definitely needs attention.

4.1.5.2 Cold-smoked and gravad fish

In a study by Yoon et al. (2004) growth of *L. monocytogenes* was prevented for 32 days at 4 °C in VP cold-smoked salmon added 0.06 % sodium diacetate and 0.84 % potassium lactate. Addition of 0.2 % sodium diacetate and 2.8 % potassium lactate was needed to obtain a similar effect at 10 °C (Yoon et al., 2004). In the same way Vogel et al. (2006) showed that a combination of 0.12 % sodium diacetate and 2.1 % potassium lactate prevented growth of *L. monocytogenes* in minced cold-smoked salmon for 27 days at 10 °C. When sodium-diacetate was substituted with 0.12 % sodium acetate, the concentration of *L. monocytogenes* increased by approx. 3 log (CFU/g) during 27 days at 10 °C (Vogel et al., 2006). Mejlholm and Dalgaard (2007a) studied the antimicrobial effect
of MAP, diacetate and lactate against *L. monocytogenes* in challenge tests with gravad and cold-smoked fish products, to identify parameters that were able to prevent its growth. Cold-smoked salmon and cold-smoked Greenland halibut, without any further treatment allowed growth of *L. monocytogenes* as observed in several other studies (Pelroy et al., 1994; Nilsson et al., 1997; Dalgaard and Jørgensen, 1998; Giménez and Dalgaard, 2004; Yoon et al., 2004; Lakshmanan and Dalgaard, 2004). Packaging of cold-smoked salmon and cold-smoked Greenland halibut in MAP reduced the growth of *L. monocytogenes* as compared to VP. Addition of 0.15 % (w/w) diacetate to MAP cold-smoked salmon prevented growth of *L. monocytogenes* for more than 40 days at 8 °C (Figure 8).

![Figure 8](image)

Figure 8. Changes in concentrations of *Listeria monocytogenes* during storage of cold-smoked salmon at 8 °C. ■ Vacuum packed; ○ Modified atmosphere packed (MAP); and △ MAP + 0.15 % (w/w) diacetate. Error bars represent the standard deviation (n = 3). Modified from Mejlholm and Dalgaard (2007a).

Importantly, MAP cold-smoked Greenland halibut added 0.15 % (w/w) diacetate allowed growth of *L. monocytogenes* at 8 °C. This difference between the two types of products was attributed to the higher content of naturally occurring lactate in cold-smoked salmon (1.2-1.5 % water phase lactate) as compared to cold-smoked Greenland halibut (0.18-0.21
% water phase lactate). In fact, when MAP cold-smoked Greenland halibut was added
0.15 % (w/w) diacetate and 0.75 % (w/w) lactate growth of *L. monocytogenes* was
prevented for more than 45 days at 8 °C. The final concentration of lactate in these
samples (1.1 % water phase lactate) corresponded well with the natural content of cold-
smoked salmon. Consequently, a synergistic effect of diacetate and lactate against *L.
monocytogenes* was observed. This synergistic effect also explains why addition of
diacetate to brined shrimp was not sufficient to prevent growth of *L. monocytogenes* as
this product contained as little as 0.04-0.06 % water phase lactate (Mejlholm et al., 2007).
In MAP marinated Greenland halibut, no clear differences in growth of *L. monocytogenes*
was observed between samples added 0.15 % (w/w) diacetate and 0.9 % (w/w) lactate,
and those without addition. This result was ascribed to a pH of 6.7 for marinated
Greenland halibut. At this high pH only a negligible fraction of the organic acids were in
their undissociated form. Cold-smoked salmon and cold-smoked halibut with added
diacetate and lactate, and no observed growth of *L. monocytogenes*, had pH in the range
5.8-6.2. Thus, pH is an important parameter to consider if organic acids should be used
successfully to reduce or prevent growth of *L. monocytogenes* (see section 2.2.3).

The antimicrobial effect of diacetate and lactate against *L. monocytogenes* has
been shown in a number of studies on different types of meat products (Stekelenburg and
Kant-Muermans, 2001; Mbandi and Shelef, 2002; Glass et al., 2002; Stekelenburg, 2003).

4.2 *Clostridium botulinum*

*Clostridium botulinum* is a Gram-positive, anaerobic, spore-forming pathogen
being the causative agent of foodborne botulism resulting from production of neurotoxin.
Based on the toxin produced, *Cl. botulinum* is divided into types designated by a letter
from A to G. Types pathogenic to humans are further divided into proteolytic *Cl. botulinum*
(type A, B and F) and non-proteolytic *Cl. botulinum* (type B, E and F) (Lund and Peck,
2000; Huss et al., 2004). *Cl. botulinum* type E is inherent to the aquatic environment and
the type most often associated with botulism from seafood. However, *Cl. botulinum* type A
and B, from the general environment, has also been the cause of botulism from seafood.
The level of *Cl. botulinum* type E on fish is generally low (0.1 spores/g), although, this
pathogen is widely distributed in temperate and artic waters (Huss et al., 2004).
Growth of Cl. botulinum can be controlled by a combination of chill storage and salt (Huss et al., 1995; Gram, 2001a; Huss et al., 2004). In products chilled to $\leq 5 ^\circ C$ and with $\geq 3$ % WPS, Cl. botulinum does not constitute a problem as growth is prevented (Huss et al., 1995). Gram (2001a) suggested a combination of 3.5 % WPS and storage at $< 4.4 ^\circ C$ for a maximum of 4 weeks as a means of controlling Cl. botulinum. Proteolytic Cl. botulinum type A and B is only considered a problem in temperature abused products as there is no risk of toxin production $< 10 ^\circ C$ (Huss et al., 2004). Brined shrimp and cold-smoked fish are stored at chill temperatures and the content of salt in these products is typically $> 3$ % WPS. This is manifested in the excellent safety record of brined shrimp and cold-smoked fish with no reported outbreaks of botulism (Gram, 2001a). However, safety concerns have been raised on the use VP and MAP with moderate to high levels of CO$_2$ and low concentrations of O$_2$ as growth of Cl. botulinum might be allowed during extended storage whereas growth of aerobic spoilage microorganisms, that normally keeps the consumers from eating the product due to formation of unpleasant odours, is at risk of being inhibited. The main worry has been the potential growth and toxin formation by Cl. botulinum in VP and MAP RTE products that normally are consumed without any preceding heat treatment able to destroy the toxin.

For brined and drained MAP shrimp with 2.3 % WPS, pH 5.9, and benzoic and sorbic acids as preservatives it was shown that a storage temperature of 25 $^\circ C$ ($> 15 ^\circ C$) were needed before toxin were produced by Cl. botulinum (mixture of type A, B and E spores) (Dalgaard and Jørgensen, 2000). However, in the same study toxin was not produced in a comparable product with 3.3 % WPS (Table 9). The risk possessed by Cl. botulinum type E is more present in a product like cooked and peeled MAP shrimp with approx. 2 % WPS and no additional preservatives. However, in challenge tests with cooked and peeled shrimp in MAP (50 % CO$_2$, 30 % N$_2$ and 20 % O$_2$) it was shown that the shelf life at 2, 5 and 8 $^\circ C$ was limited by the potential growth of L. monocytogenes and not the time to toxin production by Cl. botulinum as predicted by available mathematical models (Mejlholm et al., 2005). Furthermore, sensory spoilage of cooked and peeled MAP shrimp occurred prior to predicted toxin production by Cl. botulinum. However, at 8 $^\circ C$ the safety margin between observed sensory spoilage and predicted toxin formation was only 2 days (Mejlholm et al., 2005). The pH of cooked and peeled shrimp provides a safety barrier in itself as botulinum toxin is destroyed at pH-values $> 7.5$ (Huss et al., 2004). In a
study by Garren et al. (1994) it was shown that deheaded raw shrimp (*Penaeus* spp.) in VP spoiled in advance of toxin production by *Cl. botulinum* type E at 4 and 10 °C. For the majority of lightly preserved seafood, available mathematical models for *Cl. botulinum* are likely to provide a conservative (i.e. fail-safe) prediction of the growth potential or time to toxin production as the effect of important environmental parameters (e.g. organic acids and smoke components) are not yet included in the models. The importance of including relevant environmental parameters in predictive models is demonstrated when observed data on toxin production by *Cl. botulinum* in brined and drained MAP shrimp (Dalgaard and Jørgensen, 2000) are compared with predictions by the Pathogen Modeling Program (PMP) and the ComBase Predictor including the effect of temperature and competitive microflora, and temperature, pH and NaCl, respectively (Table 9).

### Table 9 Comparison of observed and predicted time to toxin formation by *Clostridium botulinum* in brined and drained shrimp in modified atmosphere packaging.

<table>
<thead>
<tr>
<th>Product</th>
<th>Temp (° C)</th>
<th>pH</th>
<th>Water phase salt (%)</th>
<th>Benzoic</th>
<th>Citric</th>
<th>Sorbic</th>
<th>% CO₂</th>
<th>Duration of experiment (days)</th>
<th>Toxin produced</th>
<th>Pathogen Modeling Program</th>
<th>ComBase Predictor&lt;sup&gt;c,d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>5.7</td>
<td>3.3</td>
<td>0.07</td>
<td>0.33</td>
<td>0.08</td>
<td>14</td>
<td>63-84</td>
<td>No</td>
<td>7.4</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.7</td>
<td>3.3</td>
<td>0.07</td>
<td>0.33</td>
<td>0.08</td>
<td>14</td>
<td>21-28</td>
<td>No</td>
<td>2.6</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5.7</td>
<td>3.3</td>
<td>0.07</td>
<td>0.33</td>
<td>0.08</td>
<td>14</td>
<td>4-6</td>
<td>No</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>5.9</td>
<td>2.3</td>
<td>0.08</td>
<td>0.28</td>
<td>0.06</td>
<td>15</td>
<td>35-42</td>
<td>No</td>
<td>7.4</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.9</td>
<td>2.3</td>
<td>0.08</td>
<td>0.28</td>
<td>0.06</td>
<td>15</td>
<td>~21</td>
<td>No</td>
<td>2.6</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5.9</td>
<td>2.3</td>
<td>0.08</td>
<td>0.28</td>
<td>0.06</td>
<td>15</td>
<td>4-6</td>
<td>Yes&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples were inoculated with a mixture of type A, B and E spores of *Cl. botulinum*

<sup>b</sup> Including the effect of temperature and the competitive microflora

<sup>c</sup> Including the effect of temperature, pH and NaCl

<sup>d</sup> Estimated as the time to reach 10⁶ CFU/g

<sup>e</sup> Toxin of type A and B were produced but not type E toxin

This comparison clearly shows the importance of including terms for the effect of e.g. organic acids if better predictions of time to toxin formation by *Cl. botulinum* are needed for brined shrimp, and other lightly preserved seafood.
5 Predictive models

Development of mathematical models that predicts the effect of environmental parameters on growth and the growth boundary of *L. monocytogenes* and spoilage microorganisms in lightly preserved seafood would be of considerable practical importance to the seafood sector and regulatory authorities. As one example a predictive model for *L. monocytogenes* could be used to identify combinations of product characteristics and storage conditions that prevent growth of the pathogen and thereby facilitate production of lightly preserved seafood in compliance with the EU regulation on RTE foods (EC, 2005). The practical application of validated predictive models is discussed further in section 6. Several environmental parameters, both intrinsic and extrinsic, are likely to affect growth of microorganisms in lightly preserved seafood, and this makes it a challenge to develop predictive models. The majority of the existing models for *L. monocytogenes* includes the effect of three to four environmental parameters typically temperature, pH and salt/water activity (Table 10). However, additional parameters e.g. organic acids, smoke components, packaging atmosphere and microbial interactions are likely to affect the growth of *L. monocytogenes* in lightly preserved seafood. Consequently, growth of *L. monocytogenes* is often overestimated, and sometimes severely overestimated, by existing models as pointed out by Dalgaard and Jørgensen (1998) and Giménez and Dalgaard (2004). This highlights the need for more complex models including the effect of all the relevant parameters. Then again, “more complex” models (Augustin and Carlier, 2000b; Augustin et al., 2005) including the effect of 5-6 environmental parameters as well as interactions between these factors have been shown to underestimate growth of *L. monocytogenes* in cold-smoked fish (Giménez and Dalgaard, 2004; Mejlholt and Dalgaard, 2007a). This is problematic as hazardous predictions might result.

Most predictive models are developed from well-defined experiments in model systems (i.e. broth media), with a composition far less complex than the one of foods. This could in part explain why growth of microorganisms is often overestimated in foods. Incorrect predictions by models not taking into account all relevant parameters have been termed “completeness error” (Ross et al., 2000). Although, not anticipated by everybody models that under- or overestimate the growth of microorganisms are often referred to as “fail-dangerous” and “fail-safe”, respectively, in the literature (Ratkowsky, 2004).
Ratkowsky (2004) addressed special concern towards the use of overtly “fail-safe” models, always predicting growth of pathogens irrespective of growth conditions, as this in theory would make it impossible to produce foods with a long shelf life. In agreement, Legan (2007) pointed at the challenge/problem of drawing a line between “fail-safe” and “too conservative” models, especially in the perspective of a more practical application of predictive models. A product-oriented modelling approach, using data from growth of microorganisms in well-characterized foods, could be one way to obtain a better agreement between observed and predicted growth in foods. This strategy is supported in the literature and described as a likely means of increasing the applicability of predictive models (Dalgaard, 2002; Dalgaard et al., 2002; ILSI, 2005; Legan, 2007).

Once developed and validated in product studies, predictive models should be incorporated into user-friendly software to increase their utility towards the industry and other interested parties (McMeekin et al., 2006). The type of product(s) for which the model is developed and the range of applicability should be stated clearly to minimize the risk of erroneous predictions (Dalgaard et al., 2002).

5.1 Modelling of growth

The growth curve of microorganisms is often composed of four different phases i.e. the lag phase, the exponential growth phase, the stationary phase and the death/decline phase (Figure 9). During the lag phase microorganisms adapt to the current environment and no increase in the population occurs. The length of the lag phase is determined by the growth environment and the physiological state of the microorganisms (Robinson et al., 1998; Ross and Dalgaard, 2004). During the exponential phase microorganisms multiply at a constant rate, often referred to as the exponential or the maximum specific growth rate ($\mu_{\text{max}}$). The growth rate is determined by the environmental conditions (e.g. temperature and pH). The stationary phase occurs when the microorganisms reach their maximum population density (MPD). Kinetic parameters (e.g. lag time and maximum specific growth rate) of the growth curve are estimated by primary models, whereas secondary models are used to predict how these parameters are affected by environmental conditions (Ross and McMeekin, 2003; Ross and Dalgaard, 2004).
The present thesis focuses on the use of secondary models with special emphasis on the gamma concept (Zwietering et al., 1992) and cardinal parameter models (CPMs) (Rosso et al., 1993). The gamma (γ) concept, based on the use of dimensionless growth parameters, was introduced by Zwietering et al. (1992). This concept assumes that environmental parameters act independently on growth of microorganisms, and that it is possible to predict the collective effect by multiplying the contribution of each parameter (γ-factor) (Eqn. 1).

\[ \mu_{\text{max}} = \mu_{\text{max,opt}} \cdot \gamma_{\text{temp}} \cdot \gamma_{\text{pH}} \cdot \gamma_{a_w} \cdot \gamma_{\text{other}_1} \cdot \gamma_{\text{other}_2} \cdots \gamma_{\text{other}_n} \]  

Eqn. 1
where $\mu_{\text{max opt}}$ is the maximum specific growth rate under optimal growth conditions, and $\gamma(i)$ is the relative effect of different environmental parameters with a value between 0 and 1 (see Eqn. 2). Under optimal growth conditions each $\gamma$-factor attains a value of 1 and the growth rate ($\mu_{\text{max opt}}$) is not affected whereas at suboptimal conditions growth is reduced in a predictable manner.

$$\gamma(a_w) = \frac{a_w - a_{w\text{min}}}{a_{w\text{opt}} - a_{w\text{min}}}$$

Eqn. 2

where $a_{w\text{min}}$ is the theoretical minimum water activity below which growth is not possible and $a_{w\text{opt}}$ is the optimal water activity for growth assumed to be 1.0.

Dimensionless factors can be used to expand different types of existing models due to the assumption of independent effects of environmental parameters on growth of microorganisms (Ross and Dalgaard, 2004). Thus, the gamma concept could be used to add the effect of e.g. organic acids and CO$_2$ to existing models for growth of *L. monocytogenes* in order to improve their performance for lightly preserved seafood. As an example, Giménez and Dalgaard (2004) used this approach to expand a square-root-type model with the effect of smoke components (phenol).

It can be argued whether all environmental parameters act independently on growth of microorganisms or if interactions occur. The pH of food has an antimicrobial effect in itself and at the same time it affects the concentration of undissociated organic acid. Similarly, the storage temperature is extremely important as single parameter, but, it also influences the solubility of CO$_2$ in the water phase of foods. Interactions between environmental can be modelled by using the concept of Le Marc et al. (2002) (see section 5.2).

Within predictive microbiology CPMs was introduced by Rosso et al. (1993). Like square-root-type models this approach uses cardinal parameters ($T_{\text{min}}$, $T_{\text{max}}$, pH$_{\text{min}}$, pH$_{\text{max}}$, $a_{w\text{min}}$, $a_{w\text{max}}$, etc.), with a biological significance, to describe the effect of environmental parameters on the growth rate of microorganisms. Cardinal parameters are estimated from experimental data that are fitted to the model by nonlinear regression. CPMs are closely linked to the gamma concept as it is assumed that the extent of growth inhibition can be determined by multiplying the relative effect of each environmental parameter. Ross and
Dalgaard (2004) demonstrated that different parameter values were obtained when CPMs and square-root-type models were fitted to identical sets of experimental data. Consequently, parameter values estimated for CPMs should not be used for square-root models and *visa versa*, although these two types of models resemble each other.

When comparing the different kinetic parameters of relevance to predictive microbiology, the maximum specific growth rate ($\mu_{\text{max}}$) is least complicated to determine. The growth rate of microorganisms is highly reproducible during the exponential phase, and this characteristic allows growth to be predicted from previous observations (McMeekin et al., 2002). However, the ability of kinetic models to predict growth accurately is impaired near the growth/no growth interface due to the greater variability in responses of microorganisms at severe conditions (McMeekin et al., 2002). The lag phase (lag time) of microorganisms is complicated to model and predict as this parameter is affected by several factors. In addition to the actual growth conditions, the duration of the lag phase is influenced by the physiological state of the microorganisms when these are transferred from one environment to another (Augustin and Carlier, 2000a; Robinson et al., 2001; Ross and Dalgaard, 2004). This could also be described as the readiness of bacteria to grow under new environmental conditions. If *L. monocytogenes* is transferred from low salt conditions (e.g. slicing equipment) to high salt environments (e.g. cold-smoked salmon), the pathogen needs to adapt to the increased concentration of salt before growth is initiated. The length of the lag phase may range from not present (i.e. growth is initiated/continues without delay) to infinite depending on the physiological state of the bacteria and the shift in environmental conditions (Ross and Dalgaard, 2004). Isolates of microorganisms used for challenge tests are sometimes adapted, as part of culturing, to the conditions (e.g. temperature and salt concentration) prevailing in the product of interest and thus growth is initiated more rapidly and a lag phase is rarely observed (Giménez and Dalgaard, 2004; Lakshmanan and Dalgaard, 2004; Mejlholm and Dalgaard, 2007a; Mejlholm and Dalgaard, 2007b). This procedure seems reasonable as it allows for the generation of data describing the effect of environmental conditions on the growth rate of microorganisms in foods. However, to predict growth of microorganisms accurately in naturally contaminated products the lag time can be relevant.

The influence of lag time on growth of microorganisms can be modelled by using the concept of relative lag times (RLTs) (Ross, 1999; Mellefont and Ross, 2003; Ross and
Dalgaard, 2004). This concept is based on the hypothesis that the lag time is determined by the amount of work that a microorganism has to perform to adapt to a new environment and the rate at which that work can be done (Robinson et al., 1998; Ross, 1999). The amount of work to be done, considered as the RLT, is described by the ratio of lag time to generation time under identical environmental conditions (Eqn. 3).

\[
\frac{\lambda}{T_g} = \frac{\lambda * \mu_{max}}{\ln(2)} = RLT, \quad \frac{RLT * \ln(2)}{\mu_{max}} = \lambda
\]

Eqn. 3

where \(\lambda\) is the lag time, and \(T_g\) is the generation time being reciprocal to the maximum specific growth rate (\(\mu_{max}\)). Ross (1999) showed that RLTs of \(L.\) monocytogenes had a sharp peak in the range 3-6 when estimated on data from broth experiments and foods covering a wide range of environmental conditions. In another study the median RLT of \(L.\) monocytogenes was determined as 3.09 on data (\(n = 1176\)) from culture broths and different types of food (Augustin and Carlier, 2000a). In this thesis, an average RLT of 3.5 ± 1.6 (\(n = 5\)) was determined for \(L.\) monocytogenes on data from cooked and peeled MAP shrimp, MAP cold-smoked Greenland halibut, and brined and drained shrimp (Mejlholm et al., 2005; Mejlholm et al., 2007; Mejlholm and Dalgaard, 2007a). These findings indicate that RLTs are more consistent and reproducible than lag times. However, shifts in environmental conditions (e.g. NaCl, pH and temperature) have been shown to influence the RLT of e.g. \(L.\) monocytogenes (Delignette-Muller, 1998; Mellefont et al., 2003) and thus this measure should be used with caution. When used appropriately RLTs allows for the prediction of growth rate and lag time by a single predictive model (Ross and Dalgaard, 2004).

Recently, Guillier and Augustin (2006) described how the mean and standard deviation of individual cell lag times is predictable from population lag times observed with high initial concentration experiments. The ability of the proposed model (Guillier and Augustin, 2006) to predict the lag time of low contamination levels (i.e. individual cell lag times) of \(L.\) monocytogenes in lightly preserved seafood from experiments with high inoculums is interesting and deserves further study. An estimate of the lag time distribution could be very useful as input for exposure assessment studies in order to predict the concentration of \(L.\) monocytogenes at the time of consumption.
5.2 Modelling the growth boundary

Different approaches have been suggested and used to model the interface between growth and no growth (i.e. the growth boundary) of microorganisms (Ratkowsky and Ross, 1995; Augustin and Carlier, 2000b; Le Marc et al., 2002). Ratkowsky and Ross (1995) developed a logistic regression model to predict the probability of growth or no growth of *Shigella flexneri*. This model was developed by transforming an existing kinetic model (square-root-type model) including the effect of temperature, pH, water activity and nitrite. Briefly, the kinetic model was converted to a growth/no growth model by taking the natural logarithm of both sides of the equation, and then substituting the left-hand side by \( \ln(P/(1-P)) \), with \( P \) being the predicted probability of growth expressed as a value between 0 and 1 (i.e. 0-100 % probability of growth). This approach has been used by others to develop probability models for *L. monocytogenes* (Bolton and Frank, 1999; Tienungoon et al., 2000; Koutsoumanis et al., 2004; Augustin et al., 2005; Le Marc et al., 2005; Koutsoumanis and Sofos, 2005; Mataragas et al., 2006; Valero et al., 2006). These probability models can be used to estimate the growth boundary of *L. monocytogenes* at different values of \( P \). Values corresponding to 10, 50 and 90 % probability of growth are frequently used in the literature to predict the growth/no growth interface as a function of environmental parameters. However, it has been shown by several studies that different values of \( P \) only results in very small displacements of the predicted growth boundary (Tienungoon et al., 2000; Koutsoumanis and Sofos, 2005; Mataragas et al., 2006). Thus, it seems that variability in product characteristics (e.g. pH and NaCl) is more important for the placement of the growth boundary than the value of \( P \).

Le Marc et al. (2002) modelled the growth boundary of *Listeria* by adding a term for the interactive effect between environmental parameters to a kinetic model (CPM) including the effect of temperature, pH and organic acid concentration. They assumed that the contribution of each environmental parameter to the interaction can be derived from their separated effect on the growth rate \( (\mu_{\text{max}}) \), and that the experimental range can be divided into three areas (Figure 10). In the first area, environmental parameters act independently on the growth rate as assumed by the gamma concept whereas in the second area the growth rate is reduced to an extend that is greater than the multiplicative effect of the environmental parameters (i.e. interactions occur). In the third area, growth is prevented due to the effect of interactions between environmental parameters (Le Marc et
Microbial changes and safety of lightly preserved seafood

al., 2002). A number of studies have pointed at the necessity of including interactions between environmental parameters in order to predict the growth boundary of microorganisms i.e. quantifying the hurdle concept described by Leistner (1992) (McMeekin et al., 2000; Augustin and Carlier, 2000b; Augustin et al., 2005).

![Graph showing growth boundary of Listeria monocytogenes and lower limit of interaction area](image)

Figure 10. Growth boundary of *Listeria monocytogenes* (solid line) and lower limit of interaction area (dotted line) determined by the model of Mejholm and Dalgaard (2007a) using the following parameters: \( T = 5 \, ^\circ\text{C} \), 4% water phase salt, pH 6.0, 8 ppm phenol and 25% CO\(_2\). (I) area in which the environmental parameters act independently; (II) interaction area; and (III) no growth area.

5.3 Predictive models for spoilage microorganisms

The spoilage microflora of lightly preserved seafood was reviewed in section 3. LAB is frequently found to constitute the dominating part of the spoilage microflora of
lightly preserved seafood. Consequently, growth models for LAB could be valuable to the seafood industry in order to predict the shelf life of lightly preserved seafood as a function of product characteristics and storage conditions. Models for growth of LAB including the effect of temperature, water activity, pH, CO₂ and lactic acid are available (Devlieghere et al., 2000; Wijtzes et al., 2001). However, it has been shown that smoke components (phenol), diacetate and interactions between environmental parameters can have an important effect on growth of LAB in lightly preserved seafood (Leroi et al., 2000; Mejlholm and Dalgaard, 2007a). Consequently, a model including the effect of diacetate, lactate, CO₂, smoke components (phenol), pH, NaCl/water activity, temperature and interactions between all parameters on growth of LAB in lightly preserved seafood was developed and validated as part of the present Ph.D.-project (Mejlholm and Dalgaard, 2007b). An existing model for *L. monocytogenes* (see section 5.4) (Mejlholm and Dalgaard, 2007a) was used as template in order to develop a model for growth of LAB in lightly preserved seafood. Initially, cardinal parameter values (i.e. *a*ₕ min, *pH* min and CO₂ max) of *L. monocytogenes* were substituted with corresponding values of LAB. Minimum inhibitory concentrations (MICs) of diacetate and lactate were determined in broth experiments for a mixture of LAB previously isolated from lightly preserved seafood. To determine values of the constant “b”, *T*ₘᵢₙ and *P*ₜₐₓ (i.e. the theoretical concentration of smoke components (phenol) preventing growth of LAB) the model was fitted to 48 *μ*ₘₐₓ-values of LAB obtained from lightly preserved seafood with well-characterised product characteristics and storage conditions. Validation of the fitted LAB-model resulted in average bias and accuracy factor-values of 1.2 and 1.5 for an independent set of data constituting of 33 *μ*ₘₐₓ-values of LAB from lightly preserved seafood. The developed LAB-model performed better than existing models for growth of LAB (Mejlholm and Dalgaard, 2007b).

5.4 Predictive models for *Listeria monocytogenes*

Some available models for growth of *L. monocytogenes* are summarized in Table 10.

A growth and growth boundary model for *L. monocytogenes* in lightly preserved seafood was developed and validated as part of the present Ph.D.-project (Figure 11). This model included the effect of diacetate, lactate, CO₂, smoke components (phenol), nitrite, pH, NaCl/water activity, temperature and interactions between all parameters (Eqn. 4). The model of Mejlholm and Dalgaard (2007a) was developed by expanding an existing
Microbial changes and safety of lightly preserved seafood

square-root-type model (Giménez and Dalgaard, 2004) with the effect of diacetate, CO₂ as well as interactions between environmental parameters. The approach of Le Marc et al. (2002) was used successfully to model the interactive effect between environmental parameters. Terms for the effect of temperature, water activity and CO₂ was transformed to dimensionless parameters in resemblance with the other model components (Figure 11, Eqn. 4).

\[
\mu_{\text{max}} = b \\
\cdot (T - T_{\text{min}})/(25 - T_{\text{min}})^2 \\
\cdot (a_w - 0.923)/(0.077) \\
\cdot 1 - 10^{(4.97 - pH)} \\
\cdot 1 - [\text{LAC}_U]/3.79 \\
\cdot ((350 - \text{NIT})/350)^2 \\
\cdot (32.0 - P)/(32.0) \\
\cdot (3140 - \text{CO}_2_{\text{equilibrium}})/(3140) \\
\cdot 1 - \sqrt{[\text{DAC}_U]/4.8} \\
\cdot \xi
\]

Eqn. 4

where [LACₜₜ] and [DACₜₜ] is the concentration (mM) of undissociated lactic acid and diacetate, respectively. NIT is the concentration of nitrite (ppm), P is the concentration of phenols (ppm), CO₂ equilibrium is the concentration of dissolved carbon dioxide (ppm) at equilibrium, and \( \xi \) is the interaction between environmental parameters. The model (Eqn. 4) was fitted to growth data (\( \mu_{\text{max}} \) values) of \textit{L. monocytogenes} from lightly preserved seafood (n = 39) with carefully determined product characteristics and storage conditions to determine the values of “b” and \( T_{\text{min}} \). Thus, a product-oriented approach was used to develop the model as an alternative to the more common practise of using data from broth experiments. Dalgaard et al. (2002) and Pinon et al. (2004) pointed at the perspectives of building models from product data in order to obtain better agreement between observed and predicted growth in foods.
<table>
<thead>
<tr>
<th>Type of model</th>
<th>Response variable</th>
<th>Variables and ranges</th>
<th>Interactions between environmental parameters</th>
<th>Microbial interactions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardinal parameter</td>
<td>$\mu_{\text{max}}, \text{lag}$</td>
<td>$T$ (-2.7-45 °C); $a_w$ (0.910-0.997); pH (4.55-9.61); acetic acid (0-20.1 mM); lactic acid (0-5.4 mM); citric acid (0-1.6 mM); Na-benzoate (0-0.7 mM); K-sorbate (0-5.1 mM); Na-nitrite (0-11.4 µM); $\text{CO}_2$ (0-1.64 proportion); phenol (0-12.5 ppm)</td>
<td>+</td>
<td>-</td>
<td>Augustin and Carlier (2000a, b)</td>
</tr>
<tr>
<td>Probability</td>
<td>Probability of growth</td>
<td>$T$ (3.1-30 °C); pH (3.7-7.8); $a_w$ (0.928-0.995)</td>
<td>-</td>
<td>-</td>
<td>Tienungoon et al. (2000)</td>
</tr>
<tr>
<td>Square-root, polynomial</td>
<td>$\mu_{\text{max}}, \text{lag}$</td>
<td>$T$ (4-12 °C); $a_w$ (0.9622-0.0882); Na-lactate (0-3.0 %); Na-nitrite (20 ppm); pH (6.2)</td>
<td>-</td>
<td>-</td>
<td>Devlieghere et al. (2001)</td>
</tr>
<tr>
<td>Cardinal parameter</td>
<td>$\mu_{\text{max}}, \text{lag}$</td>
<td>$T$ (0.5-43 °C); pH (4.5-9.4); acetic acid (16-64 mM); lactic acid (40-138 mM); propionic acid (18-55 mM)</td>
<td>+</td>
<td>-</td>
<td>Le Marc et al. (2002)</td>
</tr>
<tr>
<td>Square-root</td>
<td>$\mu_{\text{max}}$</td>
<td>$T$ (4-10 °C); % WPS (2-6 %); phenol (3-10 ppm); pH (5.9-6.3); lactic acid (0-20000 ppm)</td>
<td>- With lactic acid bacteria</td>
<td>-</td>
<td>Giménez and Dalgaard (2004)</td>
</tr>
<tr>
<td>Probability</td>
<td>Probability of growth</td>
<td>$T$ (4-30 °C); pH (4.24-6.58); $a_w$ (0.900-0.993)</td>
<td>+</td>
<td>-</td>
<td>Koutsoumanis et al. (2004)</td>
</tr>
<tr>
<td>Cardinal parameter</td>
<td>$\mu_{\text{max}}$</td>
<td>$T$ (-1.72-$X_a$ °C); $a_w$ (0.913-$X_a$); pH (4.26-$X_a$); nitrite (0-25.0 µM); $\text{CO}_2$ (0-3.04 proportion); phenol (0-31.9 ppm)</td>
<td>+</td>
<td>-</td>
<td>Augustin et al. (2005)</td>
</tr>
<tr>
<td>Probability</td>
<td>Probability of growth</td>
<td>$T$ (4-30 °C); pH (3.76-6.44); $a_w$ (0.888-0.997)</td>
<td>+</td>
<td>-</td>
<td>Koutsoumanis and Sofos (2005)</td>
</tr>
<tr>
<td>Square-root</td>
<td>$\mu_{\text{max}}$</td>
<td>$T$ (4-25 °C)</td>
<td>- With &quot;food flora&quot;</td>
<td>-</td>
<td>Delignette-Muller et al. (2006)</td>
</tr>
<tr>
<td>Probability</td>
<td>Probability of growth</td>
<td>$T$ (4-30 °C); pH (4.5-6); citric acid (0-0.4 %); ascorbic acid (0-0.4 %)</td>
<td>+</td>
<td>-</td>
<td>Valero et al. (2006)</td>
</tr>
<tr>
<td>Cardinal parameter</td>
<td>$\mu_{\text{max}}$</td>
<td>$T$ (2-15 °C); % WPS (2-9); pH (5.8-7.5); % water phase lactate (0-3.0); phenol (0-20 ppm); % $\text{CO}_2$ equilibrium (0-100); % water phase diacetate (0-0.2)</td>
<td>+ With lactic acid bacteria</td>
<td>-</td>
<td>Mejlholm and Dalgaard (2007a, b)</td>
</tr>
</tbody>
</table>

* Values not reported
Microbial changes and safety of lightly preserved seafood

Model of Giménez and Dalgaard (2004) including the effect of temperature, $a_w$, pH, lactate, smoke components (phenol) and nitrite

Transformed into dimensionless terms (gamma concept):
- Temperature
- $a_w$
- $CO_2$

+ Diacetate
+ $CO_2$ (Devlieghere et al., 2001)
+ Interactions between environmental parameters (Le Marc et al., 2002)

Fitted to 39 $\mu_{max}$-values for *Listeria monocytogenes* from lightly preserved seafood

$\rightarrow$ estimation of $b$ (constant) and $T_{min}$

Growth and growth boundary model of Mejlholm and Dalgaard (2007a) including the effect of diacetate, lactate, $CO_2$, smoke components (phenol), nitrite, pH, $a_w$, temperature and interactions between all parameters

Validated on 21 $\mu_{max}$-values for *L. monocytogenes* from lightly preserved seafood (bias-/accuracy factor values = 1.1/1.6) and 76 growth and no growth responses of *L. monocytogenes* in lightly preserved seafood (73 out of 76 responses correctly predicted = 96 %)

LAB-model of Mejlholm and Dalgaard (2007b) including the effect of diacetate, lactate, $CO_2$, smoke components (phenol), pH, $a_w$, temperature and interactions between all parameters

+ Interaction between *L. monocytogenes* and lactic acid bacteria (Giménez and Dalgaard (2004)
+ Relative lag time = 4.5 (Ross, 1999)

LAB-Lm model of Mejlholm and Dalgaard (2007b)

Validated on 13 maximum population density (MPD) values of *L. monocytogenes* from naturally contaminated vacuum-packed cold-smoked salmon (Jørgensen and Huss, 1998)

- Average MPD observed = 0.7 log CFU/g
- Average MPD predicted = 0.6 log CFU/g

Figure 11. Modelling approach of Mejlholm and Dalgaard (2007a, b)
When validated on an independent set of data for growth of *L. monocytogenes* in lightly preserved seafood (n = 21), average bias and accuracy factor values (Ross, 1996) of 1.1 and 1.6, respectively, were obtained for the model of Mejlholm and Dalgaard (2007a). The CPM of Augustin et al. (2005) underestimated the growth of *L. monocytogenes* in lightly preserved seafood, particularly smoked products, when evaluated on growth data (n = 60) collected in the present Ph.D.-project. This is in agreement with the findings of Augustin et al. (2005) who found their own model to underestimate the growth of *L. monocytogenes* in cold-smoked salmon. To improve predictions they suggested that the effect of phenol should be ignored. However, in the present thesis as well as in other studies (Giménez and Dalgaard, 2004) the importance of smoke components as antilisterial parameter has been demonstrated. In fact, smoke components could be important in an attempt to identify control measures for *L. monocytogenes* in RTE foods and in my opinion their effect should not be ignored from predictive models.

The growth and growth boundary model of Mejlholm and Dalgaard (2007a, b) correctly predicted 73 out of 76 growth and no growth responses of *L. monocytogenes* in lightly preserved seafood, corresponding to a correct prediction percentage of 96. The ability of this model to predict the growth/no growth interface of *L. monocytogenes* accurately was clearly demonstrated in challenge tests with MAP cold-smoked Greenland halibut added the same concentration of diacetate and increasing levels of lactate (Figure 12). The model (Eqn. 4) performed substantially better than other growth boundary models for *L. monocytogenes* (Tienungoon et al., 2000; Koutsoumanis et al., 2004; Legan et al., 2004; Augustin et al., 2005; Le Marc et al., 2005; Koutsoumanis and Sofos, 2005) when evaluated on samples of cold-smoked and gravad fish (Mejlholm and Dalgaard, 2007a, b).
A term modelling interactions between LAB and \textit{L. monocytogenes} (i.e. the Jameson effect) was added to the model of Mejholm and Dalgaard (2007a) to improve its performance, especially the ability to predict the MPD of the pathogen (Mejholm and Dalgaard, 2007b). Microbial interactions between LAB and \textit{L. monocytogenes} was modelled and predicted by combining the developed LAB-model (see section 5.3) and the \textit{L. monocytogenes} model (Mejholm and Dalgaard, 2007a) using the approach of Giménez and Dalgaard (2004). This model is referred to as the LAB-Lm model (Figure 11). From a total of 40 experiments with lightly preserved seafood, all inoculated with \textit{L. monocytogenes}, the average MPD of the pathogen were observed and predicted as 4.7 and 4.1 log (CFU/g), respectively. An average MPD of 6.3 log (CFU/g) was predicted when
interactions between microbial species were omitted. In thirteen batches of naturally contaminated VP cold-smoked salmon (Dalgaard and Jørgensen, 1998) the observed and predicted average MPD of *L. monocytogenes* was 0.7 and 0.6 log (CFU/g), respectively, when a RLT of 4.5 was used. Thus, by including the effect of microbial interaction it is possible to predict the MPD of *L. monocytogenes* in naturally contaminated samples when a lag phase of the pathogen is taken into account.

![Figure 13. Comparison of observed and predicted maximum population density (MPD) of *Listeria monocytogenes* in thirteen batches of naturally contaminated vacuum-packed cold-smoked salmon (■△) (Dalgaard and Jørgensen, 1998) and naturally contaminated cooked and peeled shrimp (■△) (Farber, 1991a). Predictions by the LAB-Lm model developed as part of the present Ph.D.-project and using a relative lag time of 4.5 (■■) and by the ComBase Predictor including the effect of temperature, pH, NaCl and lactate, and a “physiological state value” corresponding to a RLT of 4.5 (△△). The solid line represents the perfect adequacy between observed and predicted values. Modified from (Mejlholm and Dalgaard, 2007b).](image-url)
The ability of the LAB-Lm model to predict the MPD of *L. monocytogenes* in lightly preserved seafood is superior as compared to other available models (Figure 13). The ComBase Predictor, including the effect of temperature, pH, NaCl and lactate, predicted MPD-values of *L. monocytogenes* in the range of 1 to more than 8 log (CFU/g) when evaluated on thirteen sub-batches of naturally contaminated VP cold-smoked salmon (Figure 13). However, the highest observed MPD-value for these sub-batches were 2.1 log (CFU/g) (Jørgensen and Huss, 1998). MPD-values in the range of < 0 (i.e. no growth) to 1.6 log (CFU/g) was predicted by the LAB-Lm model of Mejlholm and Dalgaard (2007b). The LAB-Lm model and the ComBase Predictor performed equally well on samples of naturally contaminated cooked and peeled shrimp with respect to predicting the MPD of *L. monocytogenes* (Figure 13). Growth of *L. monocytogenes* in cooked and peeled shrimp is primarily determined by the parameters (i.e. temperature, pH, NaCl and lactate) included in the ComBase Predictor and thus a better prediction of the MPD was obtained. Additional experiments/data on growth of *L. monocytogenes* in naturally contaminated lightly preserved seafood are needed to establish the distribution of RLTs in this type of products. In this thesis a RLT of 4.5 was used, however, a lower value would provide a more conservative prediction of growth of *L. monocytogenes*. 
6 Application of validated predictive models

Validated models for growth of *L. monocytogenes* and spoilage microorganisms can be used to assist management of safety (risk) and shelf life (“quality”) of lightly preserved seafood. Possible applications of validated predictive models are shown in Figure 14.

![Figure 14. Application of validated predictive models](image)

To summarize, the most recent EU regulation on RTE foods (EC, 2005) distinguish between products that are able or unable to support growth of *L. monocytogenes* (see Table 7). For lightly preserved seafood supporting growth of *L. monocytogenes* it must be documented by the producer that the critical limit of $10^2$ CFU/g is not exceeded within the declared shelf life. A limit of $10^2$ CFU/g is permitted in products where it can be documented that growth of *L. monocytogenes* is prevented. Documentation should be based on physico-chemical characteristics of the products and consultation of available scientific literature, and when necessary on predictive models and/or challenge tests (EC, 2005). In this section the potential use of predictive models are discussed and demonstrated with special emphasis on the LAB-Lm model developed within the present Ph.D.-project (see section 5.3 and 5.4). At the end of this chapter the
perspectives of using the LAB-Lm model for future risk assessment studies are considered.

6.1 Management of safety and shelf life

To demonstrate the potential use of the LAB-Lm model for management of safety and shelf life, two examples are provided below using realistic product characteristics and storage conditions of cold-smoked salmon and gravad salmon, respectively. In these examples variability in product characteristics and storage conditions are not taken into account, however, such aspects are important to consider when using the model in “real life” (see Figure 22 for an example). A preliminary software version of the LAB-Lm model was made in Excel to demonstrate and illustrate its use (see e.g. Figure 15).

6.1.1 Cold-smoked salmon

<table>
<thead>
<tr>
<th>PRODUCT CHARACTERISTICS AND STORAGE CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Listeria monocytogenes, CFU/g</strong></td>
</tr>
<tr>
<td><strong>Lactic acid bacteria, CFU/g</strong></td>
</tr>
<tr>
<td>Storage period, days</td>
</tr>
<tr>
<td>Storage temperature, °C</td>
</tr>
<tr>
<td>Salt in water phase of product, %</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Lactic acid in water phase of product, mg/l</td>
</tr>
<tr>
<td>Smoke components (phenol, mg/kg)</td>
</tr>
<tr>
<td>% CO2 in headspace gas at equilibrium</td>
</tr>
<tr>
<td>Disaccharate in water phase of product, mg/l</td>
</tr>
<tr>
<td>Nitrile, mg/kg (only for <em>L. monocytogenes</em>)</td>
</tr>
</tbody>
</table>

Figure 15. Predicted growth of *Listeria monocytogenes* and lactic acid bacteria in MAP cold-smoked salmon with typical product characteristics (see Table 1). Predictions were obtained by the LAB-Lm model of (Mejlholm and Dalgaard, 2007b) using a relative lag time of 4.5 for *L. monocytogenes*.
Predictions by the LAB-Lm model using a RLT of 4.5 showed that *L. monocytogenes* is able to grow in MAP cold-smoked salmon with typical product characteristics and storage conditions (Figure 15). According to the prediction a MPD of approx. 2 log (CFU/g) for *L. monocytogenes* is reached after 35 days at 5 °C (Figure 15). This is in agreement with the findings of Jørgensen and Huss (1998) who found *L. monocytogenes* to multiply in naturally contaminated samples of VP cold-smoked salmon at 5 °C. Eighty percent of their samples contained less than 2 log (CFU/g) of *L. monocytogenes* when analysed after 14-50 days at 5°C, and 5 % had a concentration > 3 log (CFU/g) (Table 5). When the effect of CO₂ (i.e. MAP) is omitted from the prediction in Figure 15, corresponding to VP, *L. monocytogenes* reach a MPD of 2 log (CFU/g) after 30 days at 5 °C (result not shown) Beaufort et al. (2007) examined naturally contaminated samples of VP cold-smoked salmon and found that approx. 70 % of the samples contained < 2 log (CFU/g) of *L. monocytogenes* after 7-15 days at 4 °C succeeded by 7 days at 8 °C. The highest reported concentration in that study was 3.4 log (CFU/g).

By using the LAB-Lm model it is possible to identify combinations of parameters that prevent growth of *L. monocytogenes* in cold-smoked salmon. Thus, the LAB-Lm model can be used by the seafood industry for product development and reformulation of existing products to make them comply with EU regulation on RTE foods (Figure 14). Once identified, growth controlling parameters can be used as critical control points (CCPs) in connection with quality control. By using diacetate for production of cold-smoked salmon, corresponding to 1000 mg/l in the water phase of the product, growth of *L. monocytogenes* is prevented according to the prediction by the LAB-Lm model (Figure 16).
Microbial changes and safety of lightly preserved seafood

Figure 16. Predicted growth of *Listeria monocytogenes* and lactic acid bacteria in MAP cold-smoked salmon with added diacetate. Predictions were obtained by the LAB-Lm model of (Mejlholm and Dalgaard, 2007b) using a relative lag time of 4.5 for *L. monocytogenes*. Yellow shaded parameters are changed as compared to Figure 15. Addition of diacetate results in a slight decrease in the pH of the product.

The LAB-Lm model cannot predict the time to sensory spoilage. However, it can be used to establish the time it will take to reach a certain level of LAB, defined by e.g. customers and/or regulatory authorities. This can e.g. be used to document that the defined maximum level of LAB is not exceeded within the declared shelf life of a given product or to regulate the shelf life if required. If a maximum concentration of 7 log (CFU/g) is used as the limit for LAB this value is reached after 24-25 days in MAP cold-smoked salmon, with characteristics as described in Figure 15. The time to reach 7 log (CFU/g) is increased from 24-25 days to approx. 28 days when diacetate corresponding to 1000 mg/l in the water phase of the product is added to cold-smoked salmon Figure 16.
Microbial changes and safety of lightly preserved seafood

**PRODUCT CHARACTERISTICS AND STORAGE CONDITIONS**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes, CFU/g</em></td>
<td>10</td>
</tr>
<tr>
<td>Lactic acid bacteria, CFU/g</td>
<td>1</td>
</tr>
<tr>
<td>Storage period, days</td>
<td>40</td>
</tr>
<tr>
<td>Storage temperature, °C</td>
<td>5.00</td>
</tr>
<tr>
<td>Salt in water phase of product, %</td>
<td>4.50</td>
</tr>
<tr>
<td>pH</td>
<td>6.10</td>
</tr>
<tr>
<td>Lactic acid in water phase of product, mg/l</td>
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</tr>
<tr>
<td>Smoke components (phenol, mg/kg)</td>
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</tr>
<tr>
<td>% CO2 in headspace gas at equilibrium</td>
<td>25.0</td>
</tr>
<tr>
<td>Diacetate in water phase of product, mg/l</td>
<td>0</td>
</tr>
<tr>
<td>Nitrite, mg/kg (only for <em>L. monocytogenes</em>)</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 17. Predicted growth of *Listeria monocytogenes* and lactic acid bacteria in MAP cold-smoked salmon with typical product characteristics (see Table 1). Predictions were obtained by the LAB-Lm model of (Mejlholm and Dalgaard, 2007b) using a relative lag time of 4.5 for *L. monocytogenes*.

The antimicrobial effect of e.g. diacetate and lactate against LAB is not as apparent as observed for *L. monocytogenes* (Mejlholm and Dalgaard, 2007b), and thus only a small delay in growth of LAB results from addition of these organic acids to lightly preserved seafood. The higher tolerance of LAB towards environmental parameters implies that these microorganisms are predicted to overgrow *L. monocytogenes*, even if the initial balance between LAB and *L. monocytogenes* is in favour of the pathogen (Figure 17). Giménez and Dalgaard (2004) showed that equal MPD of LAB and *L. monocytogenes* were reached in challenge tests with VP cold-smoked salmon at 5 °C when the initial level of the pathogen (~ 2 log, CFU/g) was approx. ten times as high as the inherent concentration of LAB (~ 1 log, CFU/g). Comparable results were shown by Mejlholm and Dalgaard (2007a) in challenge tests with MAP cold-smoked salmon and Greenland halibut. However, in both of the above mentioned studies isolates of *L. monocytogenes* were
adapted to the growth conditions of the challenge tests, and thus a lag phase was not observed. By ignoring the lag phase of \textit{L. monocytogenes}, MPD values of 8.5 and 4.5 log (CFU/g) is predicted for LAB and the pathogen, respectively, when product characteristics and storage conditions of Figure 17 is used (result not shown). This prediction is not in agreement with the observations of Giménez and Dalgaard (2007) and Mejlholm and Dalgaard (2007a).

<table>
<thead>
<tr>
<th>PRODUCT CHARACTERISTICS AND STORAGE CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Listeria monocytogenes}, CFU/g</td>
</tr>
<tr>
<td>Lactic acid bacteria, CFU/g</td>
</tr>
<tr>
<td>Storage period, days</td>
</tr>
<tr>
<td>Storage temperature, °C</td>
</tr>
<tr>
<td>Salt in water phase of product, %</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Lactic acid in water phase of product, mg/l</td>
</tr>
<tr>
<td>Smoke components (phenol, mg/kg)</td>
</tr>
<tr>
<td>% CO2 in headspace gas at equilibrium</td>
</tr>
<tr>
<td>Diacete in water phase of product, mg/l</td>
</tr>
<tr>
<td>Nitrite, mg/kg (only for \textit{L. monocytogenes})</td>
</tr>
</tbody>
</table>

Figure 18. Predicted growth of \textit{Listeria monocytogenes} and lactic acid bacteria in MAP gravad salmon with typical product characteristics (see Table 1). Predictions were obtained by the LAB-Lm model of (Mejlholm and Dalgaard, 2007b) using a relative lag time of 4.5 for \textit{L. monocytogenes}.

6.1.2 Gravad salmon

As described for cold-smoked salmon (section 6.1.1) the LAB-Lm model can be used to identify combinations of products characteristics and storage conditions that prevent growth of \textit{L. monocytogenes} in gravad salmon. \textit{L. monocytogenes} is predicted to grow to a higher level in gravad salmon than established for cold-smoked salmon (Figure 15; Figure 18). This is attributed to the fact that gravad salmon is not smoked, and that this
product has a higher pH and often also a lower content of WPS. Addition of diacetate to gravad salmon, corresponding to 1500 mg/l in the water phase of the product is predicted to prevent growth of *L. monocytogenes* (Figure 19). Thus, this formulation can be used to document that gravad salmon complies with the EU regulation on RTE foods. At 8 °C, corresponding to a slight but not unrealistic temperature abuse, *L. monocytogenes* is predicted to reach a MPD of 1 log (CFU/g) after 13-14 days if product characteristics and storage conditions of Figure 19 are used (result not shown). In naturally contaminated VP gravad salmon, 15 of 23 samples (65 %) were reported to contain < 2 log (CFU/g) of *L. monocytogenes* after 14-28 days at 5 °C, and 13 % of the samples had a concentration > 3 log (CFU/g) (Jørgensen and Huss, 1998).

![PRODUCT CHARACTERISTICS AND STORAGE CONDITIONS](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em>, CFU/g</td>
<td>1</td>
</tr>
<tr>
<td>Lactic acid bacteria, CFU/g</td>
<td>10</td>
</tr>
<tr>
<td>Storage period, days</td>
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</tr>
<tr>
<td>Storage temperature, °C</td>
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</tr>
<tr>
<td>Salt in water phase of product, %</td>
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<tr>
<td>pH</td>
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</tr>
<tr>
<td>Lactic acid in water phase of product, mg/l</td>
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</tr>
<tr>
<td>Smoke components (phenol, mg/kg)</td>
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</tr>
<tr>
<td>% CO2 in headspace gas at equilibrium</td>
<td>25.0</td>
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<tr>
<td>Diacetate in water phase of product, mg/l</td>
<td>1500</td>
</tr>
<tr>
<td>Nitrite, mg/kg (only for <em>L. monocytogenes</em>)</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 19. Predicted growth of *Listeria monocytogenes* and lactic acid bacteria in MAP gravad salmon with added diacetate. Predictions were obtained by the LAB-Lm model of (Mejlholm and Dalgaard, 2007b) using a relative lag time of 4.5 for *L. monocytogenes*. **Yellow shaded** parameters are changed as compared to Figure 18. Addition of diacetate results in a decrease in the pH of the product.
6.1.3 Meat products

In a recent study by Carrasco et al. (2007) it was described how mathematical models can be used for risk management of *L. monocytogenes* in RTE meat products in order to comply with the EU regulation (EC, 2005). To predict the lag time and the growth rate ($\mu_{\text{max}}$) of *L. monocytogenes* they used the model of Devlieghere et al. (2001), including the effect of temperature, CO$_2$, water activity and sodium-lactate concentration. Using experimental data, obtained from ComBase, a linear regression was performed to correlate temperature and MPD of *L. monocytogenes*. This correlation was used to determine the MPD of *L. monocytogenes* in cooked ham as a function of storage temperature (Carrasco et al., 2007). Three commercial brands of cooked ham were collected at retail stores and analysed for CO$_2$, water activity and sodium-lactate (Carrasco et al., 2007).

![Figure 20. Predicted growth of *Listeria monocytogenes* and lactic acid bacteria in MAP cooked ham. Predictions were obtained by the LAB-Lm model of (Mejlholm and Dalgaard, 2007b) using product characteristics and storage conditions as reported by Carrasco et al. (2007) and Casiraghi et al. (2007), and a relative lag time of 4.5 for *L. monocytogenes*. The initial concentration of LAB was assumed to be ten times as high as the initial level of *L. monocytogenes*.](image-url)
Based on an initial concentration of 32 *L. monocytogenes*/g, Carrasco et al. (2007) predicted a MPD of *L. monocytogenes* in the range of 5.1 to 6.9 log (CFU/g) for the three brands of cooked ham. To comply with the EU regulation on RTE foods and the critical limit of $10^2$ CFU/g, they suggested that the shelf life of cooked ham was reduced from 30-50 days to 10 days at 5°C. However, levels of 5 to 7 log (CFU/g) of *L. monocytogenes* is hardly ever reported in RTE meat products (Nørrung et al., 1999; Gombas et al., 2003; Koutsoumanis et al., 2005; Angelidis and Koutsoumanis, 2006; Sagoo et al., 2007). Sagoo et al. (2007) examined 2981 samples of RTE meat products at the end of shelf life. *L. monocytogenes* was isolated from 6.4% of the samples and 86% of these contained less than 2 log (CFU/g) of the pathogen. In one sample, more than 6 log (CFU/g) of *L. monocytogenes* were detected (Sagoo et al., 2007). Similarly, Gombas et al. (2003) analysed more than 9000 samples of RTE meat products and never found the concentration of *L. monocytogenes* to be above 3 to 4 log (CFU/g). Using the LAB-Lm model of Mejlholm and Dalgaard (2007b) and a RLT of 4.5, a MPD of 3 log (CFU/g) was predicted for *L. monocytogenes* in cooked ham, when product characteristics and storage conditions reported by Carrasco et al. (2007) were used (Figure 20). It should be noticed that the predicted MPD of *L. monocytogenes* would have been lower than the limit of $10^2$ CFU/g, if a more realistic initial level of the pathogen (e.g. 1 CFU/g) had been used. Thus, based on the prediction by the model of Mejlholm and Dalgaard (2007b), cooked ham seems to comply with the EU regulation on RTE foods. Particularly as additional parameters e.g. smoke components and nitrite are likely to reduce growth of *L. monocytogenes* in cooked ham. Growth of *L. monocytogenes* is predicted to be prevented in cooked ham with a content of 70 mg/kg nitrite and 8 mg/kg phenol (i.e. smoke components), and additional characteristics as described in Figure 20 (results not shown).

### 6.2 Risk assessment

To assess the risk of *L. monocytogenes* in foods knowledge is required about the concentration of the pathogen at the time of consumption (Ross and McMeekin, 2003). Validated predictive models can be used to estimate the number of *L. monocytogenes* at the time of consumption and thus be valuable as part of exposure assessment studies. From the predicted concentration of *L. monocytogenes* and the serving size of the food it is possible to determine the exposure to the pathogen.
Recently, Zwietering and Nauta (2007) described and demonstrated how predictive models can be useful for risk assessment studies. In one example they predicted the development in the number of *L. monocytogenes* on cold-smoked salmon during production and subsequent storage at 5 °C. Cardinal parameter values for temperature, pH and water activity were used to predict growth of *L. monocytogenes* (the specific model used was not reported).

The concentration of *L. monocytogenes* was predicted to increase from approx. 1 log (CFU/g) to 8 log (CFU/g) during storage at 5 °C for 35 days (Zwietering and Nauta, 2007). This increase is in agreement with the prediction of the LAB-Lm model when the effect of lactate, phenol, microbial interactions and lag phase was ignored (Figure 21a). However, including realistic values for these parameters, a concentration of 3 log (CFU/g) is predicted for *L. monocytogenes* by the LAB-Lm model (Figure 21b). If a serving size of 100 g cold-smoked salmon is assumed, intake of 10 and 5 log *L. monocytogenes* cells is estimated from concentrations of 8 and 3 log (CFU/g). Using the dose-relationship curve of
Buchanan et al. (1997), the probability of acquiring listeriosis is 80 % and 0 % from ingestion of 10 and 5 log \( L. \text{monocytogenes} \) cells, respectively. Considering the safety record of cold-smoked salmon it is most likely that growth of \( L. \text{monocytogenes} \) is severely overestimated by Zwietering and Nauta (2007). However, the risk of listeriosis from consumption of cold-smoked salmon should not be neglected as \( L. \text{monocytogenes} \) can be present and are able to grow particularly > 5 °C.

Beaufort et al. (2007) used the model of Delignette-Muller et al. (2006) to predict growth of \( L. \text{monocytogenes} \) in naturally contaminated samples of VP cold-smoked salmon from three French processing plants. Concentrations of \( L. \text{monocytogenes} \) from below the detection limit to 8 log (CFU/g) were predicted with approx. 20-40 % of the predictions being above 4 log (CFU/g) (Beaufort et al., 2007). Using the LAB-Lm model concentrations from below the detection threshold to 3.4 log (CFU/g) were predicted when a RLT of 4.5 was used (Mejlholm and Dalgaard, 2007b). This is in accordance with the observed concentrations reported by (Beaufort et al., 2007) in the range of <0.2 to 3.5 log (CFU/g). The high concentrations of \( L. \text{monocytogenes} \) predicted by the model of Delignette-Muller et al. (2006) is probably explained by the lack of relevant environmental parameters as this model only includes the effect temperature and microbial interactions between \( L. \text{monocytogenes} \) and the “food flora”.

Variability in product characteristics, storage conditions, and the initial level of \( L. \text{monocytogenes} \) and LAB are important aspects to consider when growth is predicted. Stochastic modelling using distributions for each of the relevant parameters instead of point-estimates could be used to describe the impact of variability on growth of \( L. \text{monocytogenes} \). In the present Ph.D.-project the focus has been on developing predictive models, however, a logical next step will be to integrate these models and the approach of stochastic modelling. To do this more data on product characteristics and storage conditions of lightly preserved seafood, and initial numbers of \( L. \text{monocytogenes} \) are desirable in order to reduce the degree of uncertainty. One example is provided in Figure 22 to illustrate the importance of variability in product characteristics and storage conditions on growth of \( L. \text{monocytogenes} \) in cold-smoked salmon. In this example, based on actual product characteristics and storage conditions of MAP cold-smoked salmon, a difference of 0.7-0.8 log (CFU/g) in concentrations of \( L. \text{monocytogenes} \) is predicted between worst-case and best-case scenarios.
Figure 22. Predicted growth of *L. monocytogenes* in MAP cold-smoked salmon (pH 5.8 ± 0.01; 5.3 ± 0.2 % water phase salt; 8.9 ± 0.9 ppm phenol; 0.70 ± 0.1 % water phase lactate and 30.0 ± 1.7 % CO<sub>2</sub>) stored at 7.8 ± 0.5 °C. Dotted lines represents growth of *L. monocytogenes* predicted by taking variability (± standard deviation) in product characteristics (pH, NaCl, phenol and lactate) and storage conditions (CO<sub>2</sub> and temperature) into account. Modified from Mejholm and Dalgaard (2007b).
7 Conclusions and perspectives

Addition of diacetate and lactate to cold-smoked fish were shown to prevent growth of *L. monocytogenes* in a predictable manner. A synergistic effect was observed against *L. monocytogenes*, when a combination of diacetate and lactate was used for cold-smoked fish, and when the former organic acid was added to products with a relatively high content of naturally occurring lactate (e.g. cold-smoked salmon). Thus, diacetate and lactate, approved for food manufacturing, can be used as extra growth hurdles in lightly preserved seafood.

By using a product-oriented modelling approach, a growth and growth boundary model for *L. monocytogenes* in lightly preserved seafood was successfully developed and validated in the present Ph.D.-project. This model, including the effect of diacetate, lactate, CO₂, smoke components, nitrite, pH, NaCl/water activity, temperature and interactions between all parameters, can be used to identify combinations of product characteristics and storage conditions that prevent growth of *L. monocytogenes*. Risk assessments of cold-smoke and gravad fish has established *L. monocytogenes* as the one risk for which no effective control measures has been identified. However, the growth and growth boundary model could facilitate identification of growth controlling parameters (critical control points) for *L. monocytogenes* in order to improve the safety of lightly preserved seafood, and to assure and document that these products comply with the EU regulation on RTE foods.

A product-oriented modelling approach was used as well to develop a predictive model for growth of LAB in lightly preserved seafood. This model included the effect diacetate, lactate, CO₂, smoke components, pH, NaCl/water activity, temperature and interactions between all parameters. A model for growth of LAB can be used by the seafood industry to predict the time for LAB to reach high concentrations in lightly preserved seafood.

The development of validated models for *L. monocytogenes* and LAB made it possible to predict the effect of microbial interaction on the growth (i.e. maximum population density, MPD) of the pathogen. The observed MPD of *L. monocytogenes* in naturally contaminated VP cold-smoked salmon corresponded well with the predicted MPD-values, when the effect of microbial interactions was included, and a RLT of 4.5 was
used. Thus, the combined LAB-Lm model can be used to predict the concentration of *L. monocytogenes* in lightly preserved seafood at the time of consumption, and be valuable as part of exposure assessment studies.

Although, the LAB-Lm model was developed and validated for lightly preserved seafood it would be interesting and relevant to examine its application for meat products.

Both the *L. monocytogenes* model and the LAB model were developed from relatively small sets of data on growth of *L. monocytogenes* and/or LAB in well-characterised lightly preserved seafood. Nevertheless, both models were validated successfully on independent growth data from lightly preserved seafood, and they performed substantially better than existing models for growth of *L. monocytogenes* and LAB, respectively. The applied product-oriented modelling approach therefore seems a very promising means of developing predictive models with a considerable number of environmental parameters. Once incorporated in user-friendly application software the developed model can be used as an important decision tool by the seafood industry as well as regulatory authorities.

Uncertainty and variability in product characteristics, storage conditions and the initial level of *L. monocytogenes* and LAB are important aspects to consider when growth is predicted. In the present Ph.D.-project the focus has been on developing predictive models, however, a logical next step will be to integrate the LAB-Lm model and the approach of stochastic modelling taking into account the importance of variability. To reduce the degree of uncertainty more data are desirable, especially on growth of *L. monocytogenes* in naturally contaminated products with carefully described characteristics and storage conditions.

Potential growth of *L. monocytogenes* limited the shelf life of cooked and peeled MAP shrimp at chill temperatures as this pathogen reached critical levels prior to sensory spoilage and predicted time to toxin formation by *Cl. botulinum*. A shelf life of no more than 20-21 days at 2 °C was recommended. Storage and inoculation trials with cooked and peeled MAP shrimp, established *Cb. maltaromaticum* and *B. thermosphacta* as the spoilage microflora, producing a distinct off-flavour described as sour, wet-dog and chlorine-like.

Growth of *L. monocytogenes* was prevented in brined shrimp with benzoic, citric and sorbic acids, when a preserving profile comparable to the one of commercial products
was used. However, brined shrimp are sensitive to changes in preserving parameters, especially the concentration of benzoic acid. Thus, to identify alternative and efficient combinations of preservatives an expanded version of the LAB-Lm model including the additional effect of benzoic, citric and sorbic acids could be valuable to the seafood sector. Such a model could be valuable not only for brined shrimp but possibly also for brined products of crayfish, lobster and caviar as well as for seafood salads.

In the future it seems relevant to examine how organic acids distribute in mayonnaise-based seafood salads, and to evaluate the potential growth of *L. monocytogenes* in these products. A review of the literature revealed that the prevalence of *L. monocytogenes* in seafood salads, including products with smoked salmon or shrimp, was 6.6 %. However, a better understanding of how organic acids partition between different phases of foods (i.e. lipid and water) is needed to develop models for seafood salads. Based on distribution coefficients of organic acids and knowledge about the lipid content of foods, it might be possible to incorporate a term into predictive models that estimate the concentration of these preservatives in the water phase of the products.

No clear patterns were observed in the spoilage microflora of brined shrimp. The composition of the spoilage microflora depended on process hygiene (i.e. degree of contamination), preserving parameters and storage conditions. This situation is comparable to the one of cold-smoked and gravad fish where it has not been possible to identify a specific spoilage organism (SSO).
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Paper 1
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Shelf life and safety aspects of chilled cooked and peeled shrimps (Pandalus borealis) in modified atmosphere packaging

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Shelf life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging

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ABSTRACT


Aims: To evaluate the growth of *Listeria monocytogenes* and shelf life of cooked and peeled shrimps in modified atmosphere packaging (MAP).

Methods and Results: Storage trials with naturally contaminated cooked and peeled MAP shrimps (*Pandalus borealis*) were carried out at 2, 5 and 8°C. Challenge tests at the same conditions were performed after inoculation with *Listeria monocytogenes*. Both storage trials and challenge tests were repeated after 4 months of frozen storage (−22°C). *Brochothrix thermosphacta* and *Carnobacterium maltaromaticum* were responsible for sensory spoilage of cooked and peeled MAP shrimps. In challenge tests, growth of *L. monocytogenes* was observed at all of the storage temperatures studied. At 5 and 8°C the concentration of *L. monocytogenes* increased more than a 1000-fold before the product became sensory spoiled whereas this was not observed at 2°C. Frozen storage had only a minor inhibiting effect on growth of *L. monocytogenes* in the thawed product.

Conclusions: To prevent *L. monocytogenes* becoming a safety problem, cooked and peeled MAP shrimps should be distributed at 2°C and with a maximum shelf life of 20–21 d. At higher temperatures shelf life is significantly reduced.

Significance and Impact of the Study: Information is provided to establish shelf life of cooked and peeled MAP shrimps.

Keywords: *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, challenge test, frozen storage, *Listeria monocytogenes*, ready-to-eat seafood.

INTRODUCTION

In recent years, interest in mildly preserved convenience foods has increased, and establishment of storage conditions to assure product safety and sufficient shelf life for distribution is an important issue (Gould 2000). Raw shrimps have a very short shelf life and freezing or the combined use of brining and chilling are common preservation methods for shelf life extension of shrimp products (Dalgaard and Jørgensen 2000). Modified atmosphere packaging (MAP) facilitates distribution of seafood from chill cabinets in supermarkets and it has been studied for raw and lightly preserved shrimp products (Dalgaard and Jørgensen 2000; López-Caballero et al. 2002). However, for cooked and peeled MAP shrimps we found no previous studies on the chilled shelf life, growth of spoilage micro-organisms or the important food-borne pathogen *Listeria monocytogenes*. This ready-to-eat (RTE) product is mildly preserved and frozen; retail distribution prior to chilled distribution in supermarkets could be appropriate to overcome potential...
shelf life problems. In addition to reducing the time of chilled distribution, frozen storage has previously been reported to inhibit or inactivate both spoilage and pathogen micro-organisms (Guldager et al. 1998; Lund 2000).

Shelf life of chilled raw shrimps is short (6 d at 5–6°C) as their high pH (c. 7-5) allows Gram-negative micro-organisms, including Pseudomonas fragi and Shewanella putrefaciens, to grow rapidly under aerobic storage conditions (Lannelongue et al. 1982; Matches 1982; Chinivasagam et al. 1996). Packaging of raw shrimps in modified atmospheres with elevated concentrations of CO2 extends shelf life and changes the spoilage microflora to become dominated by Gram-positive bacteria or by the CO2-resistant Gram-negative bacterium Photobacterium phosphoreum (Lannelongue et al. 1982; López-Caballero et al. 2002). When cooked, the concentration of micro-organisms on shrimps is markedly reduced but during chilling and peeling, the product can be recontaminated by a predominantly Gram-positive microflora (Harrison and Lee 1968). The effect of MAP on the chilled shelf life and growth of spoilage and potential pathogenic micro-organisms in cooked and peeled shrimps is of practical importance but as indicated above remains to be documented.

Listeria monocytogenes and Clostridium botulinum type E are pathogenic and psychrotolerant bacteria of potential importance in a chilled RTE product such as cooked and peeled MAP shrimps. Listeria monocytogenes can grow close to 0°C and Cl. botulinum type E produce toxin above 3°C (Huss et al. 2004). Listeria monocytogenes was detected in 2% of 3331 samples of cooked and peeled shrimps from Icelandic factories (Valdimarsson et al. 1998). Clearly, it is important to limit growth of L. monocytogenes in this product as high concentrations in foods may cause serious illness with fatality rates of 20–40% for susceptible consumers (Farber and Peterkin 2000). Clostridium botulinum type E is indigenous to the aquatic environment where coldwater shrimps are caught and the boiling procedure used for shrimps is insufficient to inactivate the spores of Cl. botulinum type E (Huss et al. 2004). Consequently, an evaluation of the growth of L. monocytogenes and toxin formation by Cl. botulinum type E in chilled cooked and peeled MAP shrimps is important for safety of this product.

The objectives of the present study were to evaluate shelf life and safety of cooked and peeled MAP shrimps. The product was studied by storage trials and challenge tests carried out just after packing in a modified atmosphere and again after 4 months frozen storage of the MAP product. First, shelf life and microbiological and chemical changes were determined in storage trials at 2, 5 and 8°C. Secondly, growth of L. monocytogenes was evaluated in challenge tests at 2, 5 and 8°C. Finally, growth of L. monocytogenes observed in the present study was compared with available predictive models, and toxin formation by Cl. botulinum was predicted for the product characteristics and storage conditions of the cooked and peeled MAP shrimps.

MATERIALS AND METHODS

Storage trials

Two series of storage trials with cooked and peeled shrimps packed in a modified atmosphere were carried out during April–May 2003, without frozen storage, and during August–September 2003 after approx. 4 months frozen storage of the MAP product. For both series of experiments individually quick frozen shrimps (Pandalus borealis) from the North Atlantic Ocean were used. The shrimps were caught, peeled, frozen and glazed in Greenland, and supplied in the frozen state to Danish Institute for Fisheries Research (DIFRES) by a Danish shrimp processor. At DIFRES the shrimps were kept at −22°C until the start of the experiments. Shrimps were randomly divided into six sub-batches and approx. 115 g of frozen shrimps were packed in a modified atmosphere initially containing 50% CO2, 30% N2 and 20% O2 (AGA Ltd, Copenhagen, Denmark). A Multivac C500 packaging machine (Multivac, Vejle, Denmark) and packaging film (NEN 40 HOB/LLPDE 75, Amcore Flexibles, Horsens, Denmark) with low gas permeability (0.45 ± 0.15 cm3 m−2 for O2 and 1.8 ± 0.6 cm3 m−2 for CO2) were used for packaging. The gas/shrimp ratio was 4/1 in the final packed product. During handling and packaging the shrimps remained in the frozen state. Immediately after packaging three sub-batches were stored at 2, 5 and 8°C, respectively, whereas the remaining three sub-batches were kept frozen for 4 months at approx. −22°C before chilled storage at 2, 5 and 8°C. For all sub-batches the temperature of the shrimps was recorded continuously throughout frozen and/or chilled storage by data loggers (Tinytag, Gemini Data Loggers Ltd, Chichester, UK). At regularly intervals during the chilled storage three packs from each sub-batch were analysed by microbiological and chemical methods whereas two packs were analysed by sensory evaluation.

Microbiological analyses. Twenty grams of shrimp were diluted 10-fold in chilled (5°C) physiological saline (0.85% NaCl) with 0.1% peptone (PS) and homogenized 60 s in a Stomacher 400 (Seward Medical, London, UK). Further appropriate 10-fold dilutions of the homogenates were made in chilled PS. Aerobic Plate Counts (APC) were determined by spread plating (15°C, 7 d) on Long and Hammer agar (LH) with 1% NaCl (van Sprekens 1974) and lactic acid bacteria (LAB) were enumerated by pour plating (25°C, 3 d) in nitrite actidione polymyxin agar (NAP) with pH 6.2 (Davidson and Cronin 1973). At the time of sensory rejection Lactobacillus spp. were determined by spread...
plating (25°C, 5 d) on Rogosa agar (Oxoid CM627) incubated anaerobically (5–10% CO₂/90–95% N₂); Enterococcus were determined by spread plating (44–45°C, 2 d) on Slanetz and Bartley agar (CM0377); Enterobacteriaceae were enumerated by spread plating (25°C, 2 d) on tryptone soya agar (Oxoid CM0131) covered with a double layer of violet red bile glucose (VRBG) agar (Oxoid CM0485); H₂S-producing micro-organisms were determined by pour plating (25°C, 3 d) in iron agar Lyngby (IA) (Oxoid CM964); Brochothrix thermosphacta were enumerated by spread plating (25°C, 2–3 d) on streptomycin-sulphate, thallous-acetate and actidione (STAA) agar (Oxoid CM881) with STAA Selective Supplement (Oxoid SR0151) and L. monocytogenes were determined qualitatively in 25 g of sample as described previously (Jørgensen and Huss 1998).

To characterize the spoilage microflora, c. 20 colonies from each sub-batch were isolated from LH at the time of sensory rejection of the shrimps. All types of colonies growing on the plates were represented among the isolates, in numbers reflecting their relative proportion of the total microflora. The isolates were initially characterized by: colony morphology, cellular morphology and motility by the use of phase-contrast microscopy, Gram reaction (KOH method), catalase (H₂O₂ method), cytochrome oxidase (Dryslide™ Oxidase strips, Difco Laboratories), metabolism of glucose (Hugh and Leifson 1953) and growth on STAA agar. Based on these data the isolates were divided into: (i) LAB being Gram-positive, fermentative, catalase and cytochrome oxidase-negative rods and cocci; (ii) Brochothrix-like being Gram-positive, fermentative, positive for growth on STAA agar, catalase-positive and cytochrome oxidase-negative rods; and (iii) Gram-negative isolates being catalase and cytochrome oxidase positive. LAB isolates were further characterized by growth on acetate agar, final pH in La-broth, production of NH₃ from arginine (aerobically and anaerobically with 1% and 2% glucose), production of gas from glucose and gluconate, production of acetoin (Voges-Proskauer) and fermentation of inulin, lactose, mannitol, methyl-z-D-glucoside, methyl-ß-D-mannoside and xylose. Tests were carried out as previously described (Wilkinson and Jones 1977; Dalgaard et al. 2003) and isolates identified according to Collins et al. (1987) and Mora et al. (2003). Reference and type strains of Carnobacterium piscicola (DSM 20730T), C. divergens (DSM 20623T), Lactobacillus curvatus subsp. curvatus (DSM 20019T) and Lact. sakei subsp. sakei (DSM 20017T) were included for comparison. Brochothrix-like isolates were further characterized by growth with 1%, 6.5% and 8.0% NaCl, growth with 0.5% potassium telluride and fermentation of rhamnose. Brochothrix-like isolates were identified according to Wilkinson and Jones (1977) and Holley (2000). The type strain of B. thermosphacta (ATCC 11509T) was included. Gram-negative isolates were further identified for vibriostatic sensitivity (150 µg), production of indole, reduction of trimethylamine-oxide (TMAO) and production of H₂S. In addition, for four selected isolates a partial 16S rDNA sequence consisting of 869–883 nucleotides was determined by BCCM™/LMG, University of Ghent, Belgium, as previously described (Vancanneyt et al. 2001).

**Chemical analyses.** Initially and at the time of sensory rejection salt content and percentage dry matter was determined (Jørgensen et al. 2000). Drip loss, pH, total volatile nitrogen (TVN), TMAO and trimethylamine (TMA) were determined as previously described (Dalgaard et al. 1993). Formation of biogenic amines and organic acids were measured at regular intervals using previously described HPLC methods (Dalgaard and Jørgensen 2000; Jørgensen et al. 2000). External standards were used for identification and quantification of compounds. Gas composition within packs was measured using a Combi Check 9800-1 gas analyser (PBI, Dansensor, Ringsted, Denmark).

**Sensory analyses.** Five to six trained panellists carried out sensory evaluations. Samples were coded with 3-digit numbers and served in randomized order to the panellists. Prior to serving, the samples were placed at 10°C for 30 min to allow the temperature of the samples to be the same. Each panellist evaluated two portions of shrimps from each sub-batch at each time of analysis. At each sensory session, samples of freshly thawed shrimps were included to reduce the risk of panellists guessing the development in sensory scores. Changes in overall acceptance (appearance, texture, smell and taste) of the samples were evaluated by using a simple three-class scale (I, II or III) with class III corresponding to sensory rejection (Dalgaard 2000). Time of sensory rejection was defined as the time when 50% of the panellists evaluated samples from a sub-batch to be in class III. Furthermore the panellists were asked to describe the sensory characteristics of the samples using a predefined vocabulary and/or their own words.

**Spoilage activity of microbial isolates.** Shrimps were inoculated with mixtures of strains from each of the dominating groups of micro-organisms found on the naturally contaminated shrimps at the time of sensory rejection. Each mixture consisted of four strains precultured individually (5°C, 1–2 d) in APT broth (Difco 265510) or tryptone soya broth (Oxoid CM129), respectively, for Gram-positive and Gram-negative isolates. Precultures were harvested late in their exponential growth phase, defined as an increase in absorbance of 0.05–0.5 at 540 nm (Novaspec II, Pharmacia LKB Biochrom, Cambridge, UK). Inoculation mixtures were prepared by mixing the four precultures followed by a dilution in PS to a cell density of 10⁷ CFU ml⁻¹. Shrimps were added 1% (v/w) of the
relevant mixture(s) to achieve an initial concentration of $10^5$ CFU g$^{-1}$. Inoculation mixtures were added as four portions ($4 \times 0.25\%$, v/v) and after each addition the shrimps were manually tumbling to ensure an even distribution of micro-organisms on the samples. The samples were packed as previously described for the storage trials and stored at 5°C. Three packs from each sub-batch were analysed at the day of packaging and after 10 d using the following analyses previously described for the storage trials: LH, NAP, STAA and sensory evaluation.

**Challenge test**

Shrimps were inoculated with a mixture of four strains to an initial level of approx. $10^2 L. monocytogenes$ per gram. The four strains, originally isolated from seafood (Jørgensen and Huss 1998) were precultured, mixed and inoculated on shrimps as described above under the section Spoilage activity of microbial isolates. Two experiments (April–May 2003 and August–September 2003) each consisting of three sub-batches were carried out as previously described for the storage trials. In the first experiment the three sub-batches were stored at 2, 5 and 8°C just after packaging whereas in the second experiment all three sub-batches of the inoculated MAP shrimps were stored frozen at approx. –22°C during 120 d (April–July 2003) and then stored at 2, 5 and 8°C respectively. The temperature of all sub-batches of shrimps was recorded throughout the challenge tests as described for storage trials above.

**Sampling and analyses.** At regular intervals, three packs from each sub-batch were analysed. Samples were homogenized and diluted as described above for the storage trials. *Listeria monocytogenes* was enumerated by spread plating (37°C, 48 h) on Palcam agar (CM877, Oxoid) supplemented with Palcam Selective Supplement (SR0150). LAB was enumerated using NAP agar to examine the importance of LAB on growth of *L. monocytogenes*. To study the effect of frozen storage on inactivation of *L. monocytogenes* during storage at –22°C three samples were, at regular intervals, removed during the frozen storage period and analysed as described above.

**Evaluation of models to predict microbial growth in MAP shrimps**

To determine lag time (h), maximum specific growth rate ($\mu_{max}$, h$^{-1}$) and maximum population density (MPD, log CFU g$^{-1}$), the four-parameter logistic model (Dalgaard 1995) were fitted to growth data determined on NAP in storage trials and on NAP and Palcam agar in challenge tests. The software package Fig.P (v. 2.98, Biosoft, Cambridge, UK) was used for curve fitting. An F-test to compare fits of the three- and four-parameter logistic models was used to evaluate whether lag phases of the microbial growth curves were significant (Dalgaard 1995). One-way ANOVA and multifactor ANOVA were used to determine whether differences between mean values of repeated measurements were statistically significant. Calculations were carried out using Statgraphics Plus (Anon. 1998). A simple model for the inhibitory effect of LAB on growth of *L. monocytogenes* (Giménez and Dalgaard 2004) was evaluated with data from the cooked and peeled MAP shrimps. In addition, $\mu_{max}$-values observed for *L. monocytogenes* in challenge tests were compared with available predictive models (Augustin and Carlier 2000; Devlieghere et al. 2001; Growth Predictor (IFR 2003)). Observed and predicted $\mu_{max}$-values were compared by calculation of bias- and accuracy-factors (Ross 1996).

Time to toxin production by *Clostridium botulinum* type E was predicted by using both the Growth Predictor (IFR 2003) and the Pathogen Modelling Program (USDA 2004).

**RESULTS**

**Storage trial**

**Product characteristics and storage conditions.** The shrimps studied had an initial pH of 7.7 ± 0.0 and contained 1.91 ± 0.02% water phase salt, 20.0 ± 1.2% dry matters, 10.3 ± 0.2 mg TVN 100 g$^{-1}$ and 599 ± 52 ppm of water phase lactic acid. After frozen storage at –22°C the thawed MAP shrimps reached their chill storage temperatures of 2, 5 and 8°C, after c. 4, 6, 2 and 0 d respectively. Chill storage temperatures were 1.8 ± 0.3, 5.0 ± 0.4 and 8.0 ± 0.2°C in the experiment without previous frozen storage and 1.7 ± 0.4, 5.0 ± 0.3 and 7.9 ± 0.3°C for the thawed MAP shrimps.

Average temperature during the frozen storage period (120 d) was –21.7 ± 0.5°C. The CO$_2$ and O$_2$ concentration in the modified atmosphere measured immediately after packaging was 44.7 ± 1.8 and 22.5 ± 0.7%, respectively. The CO$_2$ concentration decreased rapidly after packaging due to absorption in the shrimp flesh and equilibrium levels of 24.2 ± 1.2, 24.6 ± 1.1 and 26.0 ± 1.4% CO$_2$ were reached at 2, 5 and 8°C respectively. After frozen storage the equilibrium CO$_2$ concentrations were 25.1 ± 1.0, 24.8 ± 2.1 and 26.4 ± 1.5% at 2, 5 and 8°C, respectively, for the thawed MAP shrimps. The gas volume in each pack decreased when CO$_2$ dissolved in the shrimps and this caused the concentration of O$_2$ in the modified atmosphere to increase to c. 30%. At the end of the storage period the concentration of CO$_2$ and O$_2$ increased and decreased respectively.

**Microbiological changes.** Four months frozen storage at –21.7 ± 0.5°C significantly reduced ($P = 0.013$) the initial concentration of micro-organisms (APC) in MAP shrimps from 2·70 ± 0·0 log (CFU g$^{-1}$) to 2·43 ± 0·11
log (CFU g\(^{-1}\)). In the MAP shrimps without frozen storage
the initial concentration of LAB was 1·30 ± 0·0 log
(CFU g\(^{-1}\)) corresponding to c. 5% of APC but in the
frozen and thawed MAP shrimps LAB was not detectable,
by the method used, at the first time of sampling after
thawing (Fig. 1). Nevertheless, LAB always dominated the
spoilage microflora of the MAP shrimps and frozen storage
had no significant effect on their maximum specific growth
rates (\(\mu_{\text{max}}\)) and maximum cell densities (\(P = 0.09-0.57\)
(Table 1, Fig. 1). In addition to LAB, presumptive
\textit{B. thermosphacta} growing on STAA agar constituted a
considerable part of the spoilage microflora (Table 1) and
\textit{B. thermosphacta} was enumerated at regular intervals during
storage of the frozen and thawed MAP shrimps (Fig. 1).

Of 116 isolates from the dominating spoilage microflora of
MAP shrimps 60% were identified as \textit{C. maltaromaticum},
27% as \textit{B. thermosphacta} and 13% as \textit{Psychrobacter} spp.
(Table 2). \textit{Listeria monocytogenes} was not detected in
shrimps that had not been inoculated (results not shown).

**Chemical and sensory changes.** At the time of sensory
spoilage of the MAP shrimps, drip losses of 17·1 to 19·9% were observed but no significant effect of previous frozen
storage or chill storage temperature was observed
\((P = 0.32-0.76)\) (Table 1). Compared with the initial con-
centration of TVN (10·3 ± 0·2 mg-N 100 g\(^{-1}\)) relatively
small amounts were formed during storage of the MAP
shrimps (Table 1). At 2°C, previous frozen storage, signi-
ficantly lowered \((P < 0.0001)\) the formation of TVN but this
was not observed at 5 and 8°C \((P = 0.25-0.87)\). TMA was
not produced in any of the sub-batches at the time of
sensory spoilage (results not shown). The concentration of
lactic acid decreased during chilled storage of MAP shrimps
\((P = 0.017-0.047)\) but this was not observed for the frozen
and thawed MAP shrimps \((P = 0.25-0.94)\) an observation
that needs to be further examined (Table 1). Irrespective of
the treatment, no organic acids or biogenic amines were
formed in the MAP shrimps at the time of spoilage.

**Spoilage activity of microbial isolates.** A mixture of
\textit{C. maltaromaticum} and \textit{B. thermosphacta} isolates produced

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Evolution in aerobic plate count (APC), lactic acid bacteria (LAB) and \textit{Brochothrix thermosphacta} of cooked and peeled shrimps in modified atmosphere packaging (50% CO\(_2\)/30% N\(_2\)/20% O\(_2\)) stored at (a, b) 2°C, (c, d) 5°C and (e, f) 8°C respectively. (■, □) APC; (●, ○) LAB and
(▲, △) \textit{B. thermosphacta}. Solid and open symbols correspond to samples from storage trials without and with frozen storage respectively. Downward arrows indicate levels of micro-organisms below detection level. Error bars represent SD \((n = 3)\).}
\end{figure}
Sensory analysis

*Oxidase and catalase negative, fermentative, unable to grow on acetate agar and yielded a final pH in La-broth of 4

sequences have been deposited in the EMBL, GenBank and DDBJ nucleotide databases (AJ871596, AJ871597, AJ871598 and AJ871599).

but only

P. psychrophila

/C160

/C224

Gram-negative 2 (11) 2 (10) 4 (21) 3 (16) 2 (11) 2 (10)

Gram-positive 17 (89) 18 (90) 15 (79) 16 (84) 17 (89) 18 (90)

Total number of isolates 19 (100) 20 (100) 19 (100) 19 (100) 19 (100) 20 (100)

Table 2 Identification of isolates from the spoilage microflora of cooked and peeled shrimps in modified atmosphere packaging at 2, 5 and 8 °C

<table>
<thead>
<tr>
<th>Number of isolates (%)</th>
<th>Without frozen storage</th>
<th>With frozen storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2°C</td>
<td>5°C</td>
</tr>
<tr>
<td>Total number of isolates</td>
<td>19 (100)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Gram-positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Carnobacterium malataromaticum</em></td>
<td>11 (58)</td>
<td>12 (60)</td>
</tr>
<tr>
<td><em>Brochothrix thermosphaeta</em></td>
<td>6 (31)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Gram-negative</td>
<td>2 (11)</td>
<td>2 (10)</td>
</tr>
<tr>
<td><em>Psychrobacter</em> spp.‡</td>
<td>2 (11)</td>
<td>2 (10)</td>
</tr>
</tbody>
</table>

*Oxidase and catalase negative, fermentative, unable to grow on acetate agar and yielded a final pH in La-broth of 4–5.5, produced NH₃ from arginine and acetoin from glucose (Voges-Proskauer), unable to produce gas from glucose or gluconate, fermented inulin, mannitol, methyl-α-D-glucoside, methyl-α-D-mannoside and lactose but not xylose.

‡Oxidase and catalase positive, nonfermentative, unable to produce indole and H₂S, did not reduce trimethylamine-oxide and were sensitive towards vibriostaticum (150 μg). Partial 16S rDNA sequences for three of the four isolates studied by this technique showed ≥98.8% sequence similarity with each other and with *Psychrobacter immobilis* and *P. fozii*. The fourth isolate showed 99.5% sequence similarity with *P. maritimus* and *P. psychrophila* but only c. 97% sequence similarity with the other three *Psychrobacter* isolates from the present study. The four partial 16S rDNA sequences have been deposited in the EMBL, GenBank and DDBJ nucleotide databases (AJ871596, AJ871597, AJ871598 and AJ871599).

the same off-flavours in cooked and peeled MAP shrimps as those observed in the naturally contaminated spoiled product (Tables 1 and 3). *Brochothrix thermosphaeta* alone produced strong buttermilk-like and sour off-odours but these clearly differed from the sensory spoilage characteristics of the naturally contaminated product. Alone *C. malataromaticum* and *Psychrobacter* spp. produced only weak off-flavours (Table 3).

Table 1 Spoilage characteristics and sensory shelf life of cooked and peeled shrimps in modified atmosphere packaging at 2, 5 and 8°C.

<table>
<thead>
<tr>
<th>Spoilage characteristics</th>
<th>Without frozen storage</th>
<th>With frozen storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2°C</td>
<td>5°C</td>
</tr>
<tr>
<td>LH (log CFU g⁻¹)</td>
<td>8.3 ± 0.1</td>
<td>8.6 ± 0.1</td>
</tr>
<tr>
<td>NAP (log CFU g⁻¹)</td>
<td>8.1 ± 0.2</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>STAA (log CFU g⁻¹)</td>
<td>7.8 ± 0.1</td>
<td>8.3 ± 0.0</td>
</tr>
<tr>
<td>IA-H₂S (log CFU g⁻¹)</td>
<td>6.2 ± 0.2</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>Rogosa (log CFU g⁻¹)</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Slanetz &amp; Bartley (log CFU g⁻¹)</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>VRBG (log CFU g⁻¹)</td>
<td>&lt;2</td>
<td>NT</td>
</tr>
<tr>
<td>Drip loss (%)*</td>
<td>18.1 ± 1.9</td>
<td>17.1 ± 2.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.5 ± 0.0</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>TVN (mg·N/100 g)</td>
<td>196 ± 3.6</td>
<td>179 ± 9.5</td>
</tr>
<tr>
<td>Water phase lactic acid (ppm)</td>
<td>488 ± 15</td>
<td>511 ± 18</td>
</tr>
</tbody>
</table>

Sensory analysis

Shelf life (d) 25–26 15–16 9–10 25–26 14–15 9–10

Sensory spoilage characteristics Sour, wet-dog and chlorine-like Sour, wet-dog and chlorine-like

*Includes c. 11% glazing. LH, Long and Hammer agar; NAP, nitrite actidione polymyxin agar; STAA, streptomycin-sulphate, thallous-acetate, actidione agar; IA, iron agar; TVN, total volatile nitrogen; VRBG, violant red bile glucose agar.

Storage and product characteristics. Shrimps used for the challenge test had product characteristics as described previously for the storage trial. The three sub-batches without preceding frozen storage were stored at 1–2, 4.6 ± 0.5 and 7.8 ± 0.2°C respectively. The average temperature during the frozen storage period was

**Challenge test**

Table 3 Spoilage activity of bacteria isolated from cooked and peeled modified atmosphere packaging (MAP) shrimps

<table>
<thead>
<tr>
<th>Micro-organisms</th>
<th>Microbial and sensory characteristics of inoculated cooked and peeled MAP shrimps stored at 5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 d</td>
</tr>
<tr>
<td></td>
<td>LH (log CFU g⁻¹)</td>
</tr>
<tr>
<td>Control</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Carnobacterium maltaromaticum</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Brochothrix thermosphacta</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>C. maltaromaticum in co-culture</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>with B. thermosphacta</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Psychrobacter spp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 d</td>
</tr>
<tr>
<td></td>
<td>LH (log CFU g⁻¹)</td>
</tr>
<tr>
<td>Control</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>Carnobacterium maltaromaticum</td>
<td>9.0 ± 0.2</td>
</tr>
<tr>
<td>Brochothrix thermosphacta</td>
<td>8.6 ± 0.1</td>
</tr>
<tr>
<td>C. maltaromaticum in co-culture</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>with B. thermosphacta</td>
<td></td>
</tr>
<tr>
<td>Psychrobacter spp.</td>
<td>8.8 ± 0.1</td>
</tr>
</tbody>
</table>

I–II, acceptable; III, unacceptable.

Microbiological changes. The concentration of L. monocytogenes after inoculation of the MAP shrimps was 1.5 ± 0.3 log (CFU g⁻¹) and this level was not influenced by 120 d of frozen storage at −22°C (Fig. 2). At all the conditions tested L. monocytogenes grew without significant lag phases (Table 4) and the frozen storage period had no significant effect on its maximum specific growth rate (P = 0.010–0.29). However, the time for a 100-fold increase in the cell concentration of L. monocytogenes was increased by previous frozen storage (Table 4). The MPD of L. monocytogenes increased with the storage temperature and previous frozen storage significantly lowered the MPD values at 2°C (P = 0.00064) and 5°C (P = 0.0026) but not at 8°C (P = 0.27) (Table 4). In the challenge test, LAB that occurred naturally in the MAP shrimps grew 3–3 to 4–3 times faster than L. monocytogenes at 2°C and LAB always reached MPD of 8.6–9.0 log (CFU g⁻¹) (Table 4).

Evaluation of models to predict microbial growth in MAP shrimps

The interaction model for the inhibitory effect of LAB on L. monocytogenes predicted MPD appropriately at 5°C but at 2 and 8°C MPD of L. monocytogenes were underestimated (Table 4). Maximum specific growth rates (μₘₐₓ) predicted with available models for L. monocytogenes were on average 28–32% higher than the μₘₐₓ-values observed in the present study with cooked MAP shrimps at 2, 5 and 8°C (Table 5). Time to toxin production by nonproteolytic Cl. botulinum at 5°C and 8°C was predicted to be 25 and 12 d, respectively, using both the Growth Predictor software (IFR 2003) and the Pathogen Modelling Program (USDA 2004). At 2°C toxin was predicted not to be formed.

DISCUSSION

Natural growth of L. monocytogenes limited shelf life of cooked and peeled MAP shrimps more than growth of spoilage bacteria and nonproteolytic Cl. botulinum (Tables 1 and 4). To prevent growth of L. monocytogenes to critical concentrations our data suggest that cooked and peeled MAP shrimps should be kept at 2°C or colder. In addition, maximum declared shelf life, corresponding to the time required for L. monocytogenes to multiply 100-fold with no lag phase, should be limited to 20–21 d at 2°C, 8–9 d at 5°C and 4–5 d at 8°C (Table 4, Fig. 2). Listeria monocytogenes is inactivated by cooking of shrimps but the pathogen can be present in the cooked and peeled product (Destro et al. 1996; Valdimarsson et al. 1998) and limits to storage temperature and shelf life, as just indicated, are therefore important for this RTE product.

Frozen storage (120 d at −22°C) extended the time for a 100-fold increase in cell concentration of L. monocytogenes by 10–3, 3–2 and 1–4 d at 2, 5 and 8°C respectively (Table 4). This delay in growth was longer than the c. 4–6, 2–7 and 0–4 d required for shrimps to reach 2, 5 and 8°C, respectively, after frozen storage and this indicated an inhibitory effect of frozen storage on the subsequent growth of L. monocytogenes in thawed and chilled MAP shrimps, especially when stored at 2°C. Listeria monocytogenes can be inactivated by frozen storage of food with pH below 5 but the limited effect observed in the present study with shrimps (pH = 7–7) corresponds to results for other foods with pH above 5.5 and kept at −18°C (Palumbo and Williams 1991). Consequently, frozen storage cannot be used to inactivate L. monocytogenes or substantially delay growth in thawed MAP shrimps.

The storage temperature had a pronounced effect on both shelf life, determined by sensory evaluation, and growth of L. monocytogenes in the cooked and peeled MAP shrimps (Tables 1 and 4). In fact, the effect of temperature on shelf life was more pronounced than predicted by the classical...
The square-root spoilage model for fresh fish from temperate waters as also previously observed for cooked and brined MAP shrimps (Dalgaard and Jørgensen 2000). The shelf life of the cooked and peeled shrimps was appropriately described by the exponential spoilage model when using a slope parameter of $0.16 \, {\text{C}}^{-1}$ (http://www.dfu.min.dk/micro/SSSP). This high temperature sensitivity most likely results from inactivation by cooking of the natural psychrotolerant microflora on shrimps. For *L. monocytogenes* growth at 2°C was markedly slower than at both 5 and 8°C (Table 4). This may be related to a high antilisterial effect of CO$_2$ at 2°C. The equilibrium concentrations in the modified atmosphere indicated only a slightly increased solubility of CO$_2$ at 2°C as compared with 5 and 8°C and this was expected based on the temperature, initial CO$_2$ concentration and the gas/product ratio (Ross and Dalgaard 2004, pp. 129–131). Claire et al. (2004) observed a marked inhibiting effect of MAP (40% CO$_2$/60 N$_2$ and 80% CO$_2$/20% N$_2$) on growth of *L. monocytogenes* in hard-boiled eggs (pH = 7.7) at 4°C but not at 8 and 12°C. However, the present study cannot determine whether a combined effect of temperature and CO$_2$ or simply the low temperature of 2°C which is close to the growth limit of *L. monocytogenes* accounted for its slow growth in cooked MAP shrimps at 2°C.

The growth of *L. monocytogenes* were inhibited in cooked and peeled MAP shrimps by high concentrations of LAB and this effect was most pronounced at 2°C where the difference in growth rates between LAB and *L. monocytogenes* was largest (Fig. 1, Table 4). The simple interaction model to predict the growth-inhibiting effect of LAB on *L. monocytogenes* on average overestimated the inhibiting effect of LAB (Table 4). The model was developed and validated for cold-smoked salmon (Giménez and Dalgaard 2004) and this could explain our data as the inhibitory effect of bacteriocins, produced by LAB, against *L. monocytogenes* can be stimulated by NaCl in concentrations found in cold-smoked salmon (Lebois et al. 2004). In cooked MAP shrimps the interaction of LAB and *L. monocytogenes*, however, seems of little practical importance as the product becomes sensory spoiled by the same concentrations of LAB required to inhibit the pathogen (Table 1, Fig. 1).

Growth of *L. monocytogenes* in cooked and peeled MAP shrimps was slightly overestimated by the three evaluated predictive models (Table 5). All models included the effect of temperature, CO$_2$, NaCl and pH on growth of *L. monocytogenes* in ranges reflecting the conditions of the present study with cooked and peeled MAP shrimps. However, none of the evaluated models included the effect of O$_2$ previously described as inhibiting the growth of *L. monocytogenes* in gaseous atmospheres containing CO$_2$ (Martin and Fischer 1999). Thus, expanded *L. monocytogenes* growth models including the effect of atmospheres with both CO$_2$ and O$_2$ seem likely to improve their usefulness for the cooked and peeled MAP shrimps studied here.

Irrespective of previous frozen storage, *C. maltaromaticum*, *B. thermosphacta* and Psychrobacter spp. dominated the spoilage microflora of cooked MAP shrimps (Table 2) and this was not surprising. In fact, *Carnobacterium* has been

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Fig. 2 Evolution in *Listeria monocytogenes* and lactic acid bacteria (LAB) of cooked and peeled shrimps in modified atmosphere packaging (50% CO$_2$/30% N$_2$/20% O$_2$) stored at (a) 2°C, (b) 5°C and (c) 8°C respectively. (■, □) *L. monocytogenes* and (●, ○) LAB. Solid and open symbols correspond to samples from challenge tests without and with frozen storage respectively. Error bars represent SD ($n = 3$)
found frequently in high concentrations in thawed and chilled MAP seafood. For *B. thermosphacta* growth is relatively unaffected by CO₂ when O₂ is present and this bacteria was previously shown to dominate the microflora of vacuum packed (VP) or MAP meats and a few seafood products with O₂ concentrations above 0.2% (Gardner 1981; López-Gálves et al. 1995; Koutsoumanis et al. 2000). We found no previous studies with high concentrations of *Psychrobacter* spp. in MAP seafoods but tentatively identified *Moraxella*-like bacteria have been reported and these may have been *Psychrobacter* spp. (van Spreekens 1977; Gennari et al. 1999).

Spoilage characteristics of the cooked and peeled MAP shrimps (sour, wet-dog and chlorine-like) were due to the combined activity of *B. thermosphacta* and *C. maltaromaticum* rather than activity of a single spoilage organism (Table 3). Similar concentrations of *B. thermosphacta* and *C. maltaromaticum* produced the sensory spoilage characteristics in naturally contaminated and in inoculated cooked MAP shrimps and this supports their importance for sensory spoilage (Tables 1 and 3). How metabolites of *B. thermosphacta* and *C. maltaromaticum* influence each other and causes the spoilage of shrimps remain to be determined but in a few situations it has been shown that metabolism of one micro-organism can be stimulated by the metabolism of another (metabiosis) and that *B. thermosphacta* and *C. maltaromaticum* together can contribute to spoilage of seafood (Jorgensen et al. 2000; Joffraud et al. 2001).

*Brochothrix thermosphacta* is a well-known meat spoilage bacteria but in seafood it is less common and most often reported in high concentrations in fresh MAP fish from warmer waters (López-Gálves et al. 1995; Drosinos and Nychas 1996; Koutsoumanis et al. 2000). In fact, we found no previous studies with high concentrations of *B. thermosphacta* in shrimp products. *Brochothrix thermosphacta* is known to produce acetoin, diacetyl, 2,3-butanediol, acetic acid, isobutyric acid, isovaleric acids, 2-heptanone and 2-hexanone resulting in unpleasant sour, butter, cheesy, blue-cheese, pungent and sweaty feet off-odours. Off-odours are particularly offensive when oxygen is present, and this

Table 4 Kinetic parameters describing the growth of *Listeria monocytogenes* and lactic acid bacteria in cooked and peeled MAP shrimps at 2, 5 and 8°C.

<table>
<thead>
<tr>
<th>Models</th>
<th>Temp. (°C)</th>
<th>% CO₂</th>
<th>% NaCl*</th>
<th>% Lactate</th>
<th>pH</th>
<th>Bias factors: 2, 5 and 8°C (n = 6)</th>
<th>Accuracy factors: 2, 5 and 8°C (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Augustin and Carlier (2000)</td>
<td>-2.7–45.5</td>
<td>0–164</td>
<td>0–126</td>
<td>0–3–2</td>
<td>4.5–9.6</td>
<td>1.32</td>
<td>1.32</td>
</tr>
<tr>
<td>Devlieghere et al. (2001)</td>
<td>4–12</td>
<td>0–80</td>
<td>20–62</td>
<td>0–3</td>
<td>6–7</td>
<td>1.28</td>
<td>1.28</td>
</tr>
<tr>
<td>Growth Predictor v. 1.01‡</td>
<td>1–35</td>
<td>0–100</td>
<td>0–114</td>
<td>–</td>
<td>4–4.76</td>
<td>1.31</td>
<td>1.31</td>
</tr>
</tbody>
</table>

*Table 5* Observed and predicted maximum specific growth rates for *Listeria monocytogenes* in cooked and peeled MAP shrimps as evaluated by bias and accuracy factors.

*Converted from water activity (a_w).*

*Corresponds to a pressure of 1.64 atm.

‡Available free of charge at: http://www.ifr.ac.uk/Safety/GrowthPredictor.

corresponds to the results of the present study (Table 3) (Dainty and Hibbard 1980; Blickstad and Molin 1984; Leroi et al. 1998; Joffraud et al. 2001; Pin et al. 2002).

To our knowledge chlorine-like off-odours production by *C. maltaromaticum* was not previously reported (Table 3) and the spoilage activity of this bacterium seems poorly understood. *Carnobacterium maltaromaticum* produced off-odours and caused spoilage of beef slices stored under vacuum at 2°C and then transferred to aerobic storage at 7°C (Leisner et al. 1995). However, in cold-smoked salmon *C. maltaromaticum* produced either no off-flavours (Paludan-Müller et al. 1998) or only weak butter-like off-odours possibly resulting from its formation of 2,3-butanedione and 2,3-pentanedione (Joffraud et al. 2001).

*Psychrobacter* spp. produced weak fishy off-odours in cooked MAP shrimps (Table 3) but cell concentrations much higher than those observed in the naturally contaminated product were required and these bacteria seem of no importance for spoilage of cooked MAP shrimps. This concur with van Spreckens (1977) and Gennari et al. (1999) describing the spoilage potential of *Psychrobacter* spp. and *Moraxella*-like bacteria as low. Although, some isolates produced musty or mushroom-like odours in sterile sardine juice and rehydrated salted cod (Gennari et al. 1999; Bjørkevoll et al. 2003).

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**REFERENCES**


Paper 2

Mejlholm, O., Kjeldgaard, J., Modberg, A., Vest, M.B., Bøknæs, N., Koort, J., Björkroth, J. and Dalgaard, P.

Microbial spoilage and growth of *Listeria monocytogenes* during chilled storage of brined shrimp (*Pandalus borealis*)

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Microbial changes and growth of *Listeria monocytogenes* during chilled storage of brined shrimp (*Pandalus borealis*)

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Abstract

Thirteen storage trials and ten challenge tests were carried out to examine microbial changes, spoilage and the potential growth of *Listeria monocytogenes* in brined shrimp (*Pandalus borealis*). Shrimp in brine as well as brined and drained shrimp in modified atmosphere packaging (MAP) were produced. Different recipes were used to study the effect of variations in the preserving parameters (organic acids, pH and NaCl) on growth of microorganisms and shelf life at 7-8 °C or 12 °C. Brines with different concentrations of (i) benzoic, citric and sorbic acids or (ii) acetic, citric and lactic acids were studied. Furthermore, the effect of adding diacetate to brined shrimp was evaluated. A single batch of cooked and peeled shrimp was used to study both industrially and manually processed brined shrimp with respect to the effect of process hygiene on microbial changes and the shelf life of products. Concentrations of microorganisms on newly produced brined shrimp from an industrial scale processing line
was 1.0-2.3 log (CFU g⁻¹) higher than comparable concentrations in manually processed samples. This resulted in a substantially shorter shelf life and a more diverse spoilage microflora of the industrially processed brined shrimp. In addition, shelf life of brined shrimp was affected markedly by variations in the types and concentrations of organic acids in brines and as expected by the storage temperature. The effect of MAP was less pronounced. Eighty-two isolates from the spoilage microflora of brined shrimp was identified and they included 53 lactic acid bacteria, 6 coagulase negative Staphylococcus spp., 18 Pseudomonas fluorescens and 5 yeast isolates. After storage at 7 °C, Pseudomonas fluorescens, Enterococcus faecalis-like, E. malodoratus, Carnobacterium maltaromaticum, coagulase negative Staphylococcus spp. and Lactobacillus sakei constituted the dominating microflora of shrimp in brines that contained benzoic, citric and sorbic acids as preservatives. Lb. sakei dominated the spoilage microflora of brined and drained MAP shrimp, and of brined shrimp preserved using acetic, citric and lactic acids, irrespective of packaging conditions. Shrimp in brine with benzoic, citric and sorbic acids prevented growth of L. monocytogenes during more than 40 days at 7 °C when the preserving parameters resembled those of commercial products. However, small changes in the preserving parameters and particularly reduced concentrations of benzoic acid lead to growth of L. monocytogenes in brined shrimp. The present study provides significant new information on microbial changes, shelf life and growth of L. monocytogenes in brined shrimp. This information can facilitate development of new and safe brined shrimp products.

1. Introduction

Brined shrimp (Pandalus borealis) are economically a most important product to the Danish seafood processing industry with an annual export value of 170-200 million Euros during 2001 to 2005. This corresponds to about 30 percent of brined shrimp exported from European countries (FAO, 2006). Brined shrimp are produced from cooked and peeled shrimp, and for some processes the cooked and peeled raw material is frozen, transported and subsequently thawed prior to brining and packaging (Fig. 1). The aqueous brine normally contains salt and organic acids of which combinations of benzoic, citric and sorbic acids are used most commonly. However, products containing acetic, citric and lactic acids are also available on some markets in Europe. Brined shrimp are usually distributed at chill temperatures using plastic cups with shrimp and brine. However, brined and drained shrimp
in modified atmosphere packaging (MAP) are becoming increasingly popular. Despite the significant production and distribution of brined shrimp in Europe very few published scientific studies have described shelf-life, spoilage reactions, spoilage microflora and potential growth of relevant pathogens e.g. *Listeria monocytogenes* in these products (From and Huss, 1990; Einarsson and Lauzon, 1995; Dalgaard and Jørgensen, 2000; Dalgaard, Vancanneyt, Vilalta, Swings, Fruekilde and Leisner, 2003). The microbial status of cooked, peeled and frozen shrimp, used for production of brined shrimp, has been described but information on the impact of further processing is limited (Harrison and Lee, 1968; Zapatka and Bartolomeo, 1973; Ridley and Slabyj, 1978; Valdimarsson, Einarsson, Gudbjörnsdottir and Magnusson, 1998; Gudmundsdottir, Gudbjörnsdottir, Einarsson, Kristinsson and Kristjansson, 2006). *L. monocytogenes* was isolated from 2% of 3331 samples of cooked, peeled and frozen cold-water shrimp (Valdimarsson et al., 1998). It is also known that this pathogen can grow to high concentrations in inoculated samples of brined and drained MAP shrimp, with benzoic, citric and sorbic acids, at 8-25 °C but not at 0 °C and 5 °C (Dalgaard and Jørgensen, 2000). The occurrence of *L. monocytogenes* in commercial products of brined shrimp has only been reported in a single study (Rørvik and Yndestad, 1991). Thus, the effect of processing and variations in product characteristics of brined shrimp with respect to microbial changes and shelf life during chilled storage deserves further study.

For brined shrimp as for many other foods there is demand for less preserved products with reduced concentrations of salt and other food preservatives (Gould, 2000). Consequently, it is interesting to obtain information about the effect of processing and variations in product characteristics (organic acids, pH and NaCl) and storage conditions (atmosphere and temperature) on microbial changes and shelf life of brined shrimp. Based on recent risk assessments and existing EU-regulations, it seems particularly important to evaluate if reduced concentrations of food preservatives will allow *L. monocytogenes* to grow to high concentrations in this ready-to-eat product. In fact, EU-regulations on ready-to-eat foods e.g. differentiates between products that are able or unable to support growth of *L. monocytogenes* and allows 100 CFU g⁻¹ in the latter (Walls, 2005; EC, 2005).

Diacetate in combination with naturally occurring or added lactate can be used to control the growth of *L. monocytogenes* in different smoked and marinated seafoods (Yoon, Burnette, Abou-Zeid and Whiting, 2004; Vogel, Ng, Hyldig, Mohr and Gram, 2006; Mejlholm and Dalgaard, 2007a). Thus, diacetate may also be useful to control growth of *L. monocytogenes*
in brined shrimp where concentrations of other food preservatives are reduced. The antimicrobial effect of diacetate in combination with other organic acids, however, to our knowledge has not previously been studied in brined shrimp.

The objectives of the present study were to evaluate microbial spoilage and growth of *L. monocytogenes* during chilled storage of brined cold-water shrimp. Firstly, storage trials with brined shrimp were carried out to evaluate the importance of (i) process hygiene (ii) brine composition and (iii) storage conditions (atmosphere and temperature) on microbial changes and shelf life determined by sensory evaluation. Secondly, challenge tests using brined shrimp inoculated with *L. monocytogenes* were carried out to evaluate the effect of brine composition and storage atmosphere on growth of this pathogen.

![Flow diagram for production of shrimp in brine (dashed lines), and brined and drained shrimp in modified atmosphere packaging (MAP) (dotted lines). Arrows indicate transportation of shrimp by e.g. conveyor belts.](image_url)
2. **Materials and methods**

2.1 **Storage trials**

Two series of storage trials were carried out to evaluate the effect of (i) processing hygiene; (ii) brine composition; and (iii) storage atmosphere and temperature on microbial changes during chilled storage of brined shrimp (Table 1). Cold-water shrimp (*Pandalus borealis*) from the North Atlantic Ocean were used for all storage trials. The shrimps were caught, stored on ice, cooked, peeled and frozen in blocks of 6.2 kg in Greenland and supplied to a Danish shrimp processor where they were kept at -24 °C until used in the storage trials. The frozen shrimps were thawed overnight to reach a temperature of 1-2 °C and then they were divided into two sub-batches corresponding to the two series of storage trials carried out. One sub-batch of thawed shrimp was processed using the production lines of a commercial shrimp processing factory (Fig. 1). In contrast, the other sub-batch of shrimp did not pass through the commercial processing line. Instead this sub-batch of shrimp was processed manually. The two series of storage trials consisted of eight and five treatments, respectively, as shown in Table 1. Three types of brines were used for the storage trials and they contained (i) sodium-benzoate (SFK Food Inc., Viborg, Denmark), citric acid (Jungbunzlauer AG, Basel, Switzerland), potassium-sorbate (SFK Food Inc., Viborg, Denmark) and salt (Akzo Nobel Salt Inc., Mariager, Denmark); (ii) citric acid, potassium-sorbate and salt; and (iii) acetic acid (80 % [wt/wt] syrup, Brenntag Nordic Inc., Hellerup, Denmark), citric acid, lactic acid (80 % [wt/wt] syrup, Purac) and salt. These brines were further modified by addition of 0.26-0.27 % (wt/vol) sodium-diace
tate (Macco Organiques Inc., Vallyfield, Quebec, Canada) (Table 1).

For shrimp in brine 105 ± 1 g of thawed shrimp were placed into 200 ml plastic cups (Superfoss Inc, Vipperød, Denmark) and added 75 ml brine resulting in a 1.0:0.7 ratio between shrimp and brine. Shrimp from sub-batch one were portioned using a multihead weigher as part of a commercial processing line for packaging of shrimp in brine (Treatments A, C, E, H, I and L, Table 1). Shrimp from sub-batch two were portioned manually using sterile rubber gloves (Treatments B, D, G, J and K, Table 1). For all treatments of sub-batch one and sub-batch two, brines were prepared in carefully cleaned plastic buckets and added manually to the plastic cups.
For brined and drained MAP shrimp the thawed shrimp was marinated in brine during 24 h at 0-1 °C using a shrimp to brine ratio of 1.0:0.8 and industrial scale stainless steel trolleys (sub-batch one; treatment F and M, Table 1) or carefully cleaned plastic containers (sub-batch two; treatment G, Table 1). Following this marinating step the brine was drained off. 100 ± 1 g portions of the brined and drained shrimp were then packed in a modified atmosphere initially containing 40 % CO₂ and 60 % N₂ (AGA Ltd, Copenhagen, Denmark). A Multivac R7000 packaging machine (Multivac, Vejle, Denmark) and packaging material APET 400 (Wipak Food) with low gas permeability were used for packaging. The gas/shrimp ratio was 1.5/1 for the packed product. Brined and drained shrimp from both sub-batches were packed on an industrial scale packaging line but for sub-batch two shrimp were manually placed directly into the plastic trays used for packaging thereby preventing contact with conveyor belts and the weighing machinery.

2.1.1 Product characteristics and storage conditions

Shrimp in brine and the brined and drained MAP shrimp were chilled to 0-1 °C immediately after packaging and transported to the Danish Institute for Fisheries Research (DIFRES) where they were stored at 7 or 12 °C (Table 1). Storage temperatures of the brined shrimp were recorded continuously throughout the storage trials by data loggers (Tinytag Gemini Data Loggers Ltd., Chichester, UK). Three packs from each of the 13 treatments were analyzed by chemical methods prior to chill storage. The concentration of dry matter and pH were measured as previously described (Dalgaard, Gram and Huss, 1993). Salt content was determined using an automated potentiometric titration method. Organic acids were determined by using a previously described HPLC method (Dalgaard and Jørgensen, 2000). External standards were used for identification and quantification of acetic acid/diacetate, benzoic acid, citric acid, lactic acid and sorbic acid. Finally, composition of the headspace gas in brined and drained MAP shrimp was measured at regular intervals during storage using a Combi Check 9800-1 gas analyzer (PBI, Dansensor, Ringsted, Denmark).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shrimp from processing line</th>
<th>Organic acids in brine</th>
<th>Added Na-diacetate</th>
<th>MAP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Water phase salt (%)</th>
<th>Acetic (Diacetate)</th>
<th>Benzoic</th>
<th>Citric</th>
<th>Lactic</th>
<th>Sorbic</th>
<th>% CO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Yes</td>
<td>Benzoic, citric, sorbic</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>5.8 ± 0.0</td>
<td>3.49 ± 0.08</td>
<td>-</td>
<td>0.13 ± 0.01</td>
<td>0.67 ± 0.03</td>
<td>0.073 ± 0.007</td>
<td>-</td>
<td>0.048 ± 0.003</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>No</td>
<td>Benzoic, citric, sorbic</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>5.8 ± 0.0</td>
<td>3.52 ± 0.03</td>
<td>-</td>
<td>0.15 ± 0.01</td>
<td>0.79 ± 0.00</td>
<td>0.073 ± 0.007</td>
<td>-</td>
<td>0.051 ± 0.010</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>Yes</td>
<td>Benzoic, citric, sorbic</td>
<td>+</td>
<td>-</td>
<td>7</td>
<td>5.6 ± 0.1</td>
<td>3.59 ± 0.13</td>
<td>0.14 ± 0.07</td>
<td>0.13 ± 0.01</td>
<td>0.68 ± 0.04</td>
<td>0.073 ± 0.007</td>
<td>-</td>
<td>0.051 ± 0.004</td>
<td>-</td>
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<tr>
<td>D</td>
<td>No</td>
<td>Benzoic, citric, sorbic</td>
<td>+</td>
<td>-</td>
<td>7</td>
<td>5.7 ± 0.0</td>
<td>3.55 ± 0.05</td>
<td>0.15 ± 0.04</td>
<td>0.14 ± 0.03</td>
<td>0.80 ± 0.02</td>
<td>0.073 ± 0.007</td>
<td>-</td>
<td>0.051 ± 0.010</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>Yes</td>
<td>Benzoic, citric, sorbic</td>
<td>+</td>
<td>-</td>
<td>12</td>
<td>5.6 ± 0.0</td>
<td>3.59 ± 0.13</td>
<td>0.14 ± 0.07</td>
<td>0.13 ± 0.01</td>
<td>0.68 ± 0.04</td>
<td>0.073 ± 0.007</td>
<td>-</td>
<td>0.051 ± 0.004</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>Benzoic, citric, sorbic</td>
<td>-</td>
<td>+</td>
<td>7</td>
<td>5.7 ± 0.1</td>
<td>3.32 ± 0.08</td>
<td>-</td>
<td>0.13 ± 0.01</td>
<td>0.65 ± 0.02</td>
<td>0.073 ± 0.007</td>
<td>0.047 ± 0.004</td>
<td>26.9 ± 0.7</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>G</td>
<td>No</td>
<td>Benzoic, citric, sorbic</td>
<td>-</td>
<td>+</td>
<td>7</td>
<td>5.6 ± 0.0</td>
<td>3.76 ± 0.01</td>
<td>-</td>
<td>0.13 ± 0.00</td>
<td>0.66 ± 0.07</td>
<td>0.073 ± 0.007</td>
<td>0.047 ± 0.001</td>
<td>25.8 ± 0.2</td>
<td>6.9 ± 0.3</td>
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<tr>
<td>H</td>
<td>Yes</td>
<td>Citric, sorbic</td>
<td>+</td>
<td>-</td>
<td>7</td>
<td>5.6 ± 0.0</td>
<td>3.53 ± 0.01</td>
<td>0.15 ± 0.05</td>
<td>-</td>
<td>0.66 ± 0.03</td>
<td>0.073 ± 0.007</td>
<td>0.078 ± 0.003</td>
<td>-</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>I</td>
<td>Yes</td>
<td>Acetic, citric, lactic</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>5.8 ± 0.0</td>
<td>3.28 ± 0.07</td>
<td>0.86 ± 0.04</td>
<td>-</td>
<td>0.19 ± 0.01</td>
<td>0.85 ± 0.06</td>
<td>-</td>
<td>-</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>J</td>
<td>No</td>
<td>Acetic, citric, lactic</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>5.8 ± 0.0</td>
<td>3.33 ± 0.05</td>
<td>1.07 ± 0.03</td>
<td>-</td>
<td>0.21 ± 0.03</td>
<td>1.10 ± 0.00</td>
<td>-</td>
<td>-</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>K</td>
<td>No</td>
<td>Acetic, citric, lactic</td>
<td>+</td>
<td>-</td>
<td>7</td>
<td>5.7 ± 0.0</td>
<td>3.24 ± 0.04</td>
<td>1.23 ± 0.04</td>
<td>-</td>
<td>0.19 ± 0.03</td>
<td>1.08 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>L</td>
<td>Yes</td>
<td>Acetic, citric, lactic</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>5.8 ± 0.0</td>
<td>3.28 ± 0.07</td>
<td>0.86 ± 0.04</td>
<td>-</td>
<td>0.19 ± 0.01</td>
<td>0.85 ± 0.06</td>
<td>-</td>
<td>-</td>
<td>11.9 ± 0.2</td>
</tr>
<tr>
<td>M</td>
<td>Yes</td>
<td>Acetic, citric, lactic</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td>5.7 ± 0.0</td>
<td>3.14 ± 0.04</td>
<td>1.26 ± 0.00</td>
<td>-</td>
<td>0.17 ± 0.00</td>
<td>0.97 ± 0.03</td>
<td>-</td>
<td>26.3 ± 0.8</td>
<td>7.1 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Modified atmosphere packaging (MAP) with an initial gas composition of 40 % CO<sub>2</sub> and 60 % N<sub>2</sub>. Brined and drained shrimp were stored in MAP whereas shrimp in brine was stored without MAP.

<sup>b</sup> Equilibrium concentrations in headspace gas

<sup>c</sup> Natural content of lactic acid in cold-water shrimp
### Table 2 Microbiological characteristics and shelf life of brined shrimp depending on the treatments studied

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shrimp from processing line</th>
<th>APC (log CFU g⁻¹)</th>
<th>Lactic acid bacteria (LAB)</th>
<th>Spoilage characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shrimp from</td>
<td>Lag phase (d)</td>
<td>µₘ₅₅ (d⁻¹)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>processing line</td>
<td>(d)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Yes</td>
<td>3.2</td>
<td>8.2</td>
<td>NS</td>
</tr>
<tr>
<td>B</td>
<td>No</td>
<td>1.8</td>
<td>7.0</td>
<td>22.3</td>
</tr>
<tr>
<td>C</td>
<td>Yes</td>
<td>3.2</td>
<td>5.7</td>
<td>NS</td>
</tr>
<tr>
<td>D</td>
<td>No</td>
<td>1.6</td>
<td>3.4</td>
<td>30.4</td>
</tr>
<tr>
<td>E</td>
<td>Yes</td>
<td>3.2</td>
<td>8.6</td>
<td>NS</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>3.5</td>
<td>8.1</td>
<td>NS</td>
</tr>
<tr>
<td>G</td>
<td>No</td>
<td>1.8</td>
<td>1.8</td>
<td>&gt; 75</td>
</tr>
<tr>
<td>H</td>
<td>Yes</td>
<td>3.2</td>
<td>8.2</td>
<td>NS</td>
</tr>
<tr>
<td>I</td>
<td>Yes</td>
<td>3.9</td>
<td>6.6</td>
<td>NS</td>
</tr>
<tr>
<td>J</td>
<td>No</td>
<td>2.2</td>
<td>3.7</td>
<td>&gt; 75</td>
</tr>
<tr>
<td>K</td>
<td>No</td>
<td>2.2</td>
<td>2.2</td>
<td>&gt; 75</td>
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<td>L</td>
<td>Yes</td>
<td>3.9</td>
<td>7.5</td>
<td>NS</td>
</tr>
<tr>
<td>M</td>
<td>Yes</td>
<td>3.5</td>
<td>7.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

ᵃ See Table 1
ᵇ Initial population density (IPD)
ᶜ Maximum population density (MPD)
2.1.2 Microbiological and sensory analyses

At regular intervals during storage three samples from each treatment were analyzed by microbiological methods. Twenty grams of shrimp were diluted 10-fold in chilled (5 °C) physiological saline (0.85 % NaCl) with 0.1 % peptone (PS) and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Further appropriate 10-fold dilutions of the homogenates were made in chilled PS. Aerobic Plate Counts (APC) were determined by spread plating (15 °C, 7 d) on Long and Hammer agar (LH) with 1 % NaCl (NMKL, 2006). Lactic acid bacteria (LAB) were enumerated by pour plating (25 °C, 3 d) in nitrite acididione polymyxin agar (NAP) with pH 6.2 (Davidson and Cronin, 1973). To characterize the dominating spoilage microflora of shrimp, colonies were isolated from LH at the time of sensory rejection of the shrimps or at the end of the storage period for treatments where the brined shrimp were not sensory spoiled (Table 2). Types of colonies growing on LH were isolated in numbers reflecting their relative proportion of the total microflora. These isolates were pure-cultured and characterized initially by: colony morphology, cellular morphology and motility by the use of phase-contrast microscopy, Gram reaction (KOH method), catalase (H2O2 method), cytochrome oxidase (Dryslide™ Oxidase strips, Difco laboratories), metabolism of glucose, and growth on acetate agar and oxytetracycline gentamycin yeast extract agar (OGYE). Tests were carried out as previously described (Dalgaard et al., 2003; Mejlholm, Bøknæs and Dalgaard, 2005). Based on these data the isolates were divided into (i) LAB being Gram-positive, fermentative, catalase and cytochrome oxidase-negative rods and cocci; (ii) Staphylococcus-like isolates being Gram-positive, fermentative, catalase-positive and cytochrome oxidase-negative; (iii) Gram-negative, catalase and cytochrome oxidase-positive isolates; and (iv) yeast isolates able to grow on OGYE agar. LAB isolates were further characterized by final pH after growth in La-broth, production of NH3 from arginine (aerobically and anaerobically with 0.1 and 2.0 % glucose), production of gas from glucose and gluconate, production of acetoin (Voges-Proskauer) and fermentation of inulin, lactose, mannitol, methyl-α-D-glucoside, methyl-α-D-mannoside and xylose. Tests were carried out as previously described (Wilkinson and Jones, 1977; Dalgaard et al., 2003) and isolates were identified according to Collins et al. (1987); Stiles and Holzapfel (1997); Manero and Blanch (1999); and Mora (2003) (Table 3). Reference and type strains of LAB previously used by (Mejlholm et al., 2005) were included for comparison. In addition, LAB isolates were identified using a 16 and 23 S rDNA HindIII RFLP (ribotyping) database. The HindIII
ribopatterns obtained from shrimp isolates were compared with the corresponding patterns in the LAB database of the Department of Food and Environmental Hygiene, University of Helsinki, Finland. This database comprises HindIII ribopatterns of over 6000 LAB including patterns of over 300 type and reference strains and for example strains belonging to the species Carnobacterium, Enterococcus, Lactobacillus and Leuconostoc (Björkroth and Korkeala, 1996; Björkroth and Korkeala, 1997; Björkroth, Vandamme and Korkeala, 1998; Lyhs, Björkroth and Korkeala, 1999; Vihavainen, Lundström, Susiluoto, Koort, Paulin, Auvinen et al., 2007). The HindIII ribopatterns of these strains are utilized as operational taxonomic units in numerical analyses. Identification of unknown isolates is based on their locations in the clusters containing the type and reference strains. Reliability of the clusters to distinguish between different species has been validated in several polyphasic taxonomy studies of LAB (Björkroth, Geisen, Schillinger, Weiss, De Vos, Holzapfel et al., 2000; Björkroth, Schillinger, Geisen, Weiss, Hoste, Holzapfel et al., 2002; Koort, Vandamme, Schillinger, Holzapfel and Björkroth, 2004; Koort, Coenye, Vandamme, Sukura and Björkroth, 2004; Koort, Murros, Coenye, Eerola, Vandamme, Sukura et al., 2005).

For ribotyping, DNA from pure cultures was extracted by the guanidinium thiocyanate method of Pitcher (1989) as modified by Björkroth and Korkeala (1996) by combined lysozyme and mutanolysin (Sigma-Aldrich, St. Louis, MO, USA) treatment. After cleaving 8 µg of DNA with HindIII (New England Biolabs, Beverly, MA, USA), the DNA fragments were separated by agarose gel electrophoresis. The resulting fingerprint patterns were transferred onto a nylon membrane using a Vacugene apparatus (Pharmacia, Uppsala, Sweden). A mixture of five cDNA oligonucleotide probes specific for 16 and 23 S rRNA genes and double-labeled with digoxigenin was used in hybridizations. Membranes were hybridised at 53 °C overnight and detection of the digoxigenin-labeled fragments (ribopatterns) was performed as recommended by Roche Molecular Biochemicals. The ribopatterns were scanned (ScanJet 4c/T scanner, Hewlet Packard, Boise, ID, USA) and then transferred to the BioNumerics software (version 4.5, Applied Maths, Sint-Martens-Latem, Belgium) as tiff files. The patterns were normalised based on the mobility of the standards (digoxigenin-labeled DNA Molecular Weight Marker II, Roche), and a similarity matrix was created. The similarity between all pairs was expressed by using the Dice coefficient correlation and UPGMA (unweighed pair group method using arithmetic averages) clustering was then applied for the construction of a dendrogram. Based on the use of internal controls
in the database, pattern optimisation and band position tolerance of 0.5 and 1.5, respectively, were allowed.

*Staphylococcus*-like isolates were further characterized for production of coagulase (Cowan, 1938). Gram-negative isolates were further characterized by growth on Kings B agar and by using API-20NE (bioMérieux, Marcy l’Etoile, France). Type strains of *Pseudomonas fragi* (ATCC 4973), *Ps. fluorescens* (ATCC 13525) and *Alcaligenes aquamarinus* (ATCC 14400) were included for comparison. Yeast isolates was not characterized further.

At regular intervals during storage three samples from each treatment were analyzed by 3-4 trained and experienced sensory panellists. Changes in overall acceptance (appearance, odour, texture and taste) of the samples were evaluated by using a simple three-class scale (I, II and III) with class III corresponding to sensory rejection (Dalgaard, 2000). Time of sensory rejection (shelf life) was defined as the time when 50 % of the panellists evaluated samples from a treatment in the storage trials to be in class III. In addition, the panellists were asked to describe the sensory characteristics of the samples and particularly off-flavours using a predefined vocabulary and/or their own words.

### 2.2 Challenge tests with *Listeria monocytogenes*

A series of challenge tests with brined shrimp inoculated with *L. monocytogenes* and consisting of 11 treatments were carried out to examine the effect of brine composition and storage atmosphere on growth of this pathogen (Table 4). Three types of brines were used and they contained (i) sodium-benzoate, citric acid, potassium-sorbate and salt; (ii) sodium-benzoate, citric acid and salt or (iii) citric acid, potassium-sorbate and salt (Table 4). For selected treatments sodium-diacetate (Macco Organiques Inc., Vallyfield, Quebec, Canada) was added to cups with brined shrimp corresponding to final concentrations of diacetate of 0.13-0.23 % (wt/wt) in the water phase of the shrimps (Table 4).

For all challenge tests cooked and peeled cold-water shrimp (*Pandalus borealis*) were supplied by a Danish shrimp processor and transported to DIFRES in their frozen state. Frozen shrimp were thawed overnight at 5 °C and then randomly divided into sub-batches prior to addition of brines. Shrimp in brine were produced manually by weighing 100 ± 1 g of shrimp into plastic cups followed by addition of 80 ml brine (1.0:0.8 ratio between shrimp and brine). Cups were closed with plastic lids. For brined and drained MAP shrimp the
thawed shrimps was marinated in brine during one day at 0-1 °C with a shrimp to brine ratio of 1.0:0.7 and subsequently the brine was drained off, prior to inoculation and packaging.

2.2.1 Inoculation and packaging of samples

For all the treatments studied (Table 4), shrimp were inoculated with a mixture of four *L. monocytogenes* isolates previously obtained from seafood (Jørgensen and Huss, 1998; Giménez and Dalgaard, 2004). The isolates were precultured in Brain Heart Infusion (BHI, Oxoid CM225) broth with pH 6.0 and 4 % NaCl as previously described (Mejlholm and Dalgaard, 2007a). Precultures were harvested in late exponential growth phase, defined as a relative change in absorbans of 0.05-0.2 at 540 nm (Novaspec II, Pharmacia Biotech, Allerød, Denmark). Inoculums of *L. monocytogenes* (Lm-mix) with concentrations of $10^5$ or $10^4$ CFU ml$^{-1}$ were prepared by mixing the four precultures and diluting in 0.85 % NaCl. Shrimp in brine were added 0.1 % (vol/wt) of Lm-mix ($10^5$ CFU ml$^{-1}$) and cups were carefully shaken to ensure an even distribution of *L. monocytogenes*. Brined and drained shrimp were added 1 % (vol/wt) of Lm-mix ($10^4$ CFU ml$^{-1}$). The inoculum was added to drained shrimp as four portions of 0.25 % (vol/wt) and after each addition the products were manually tumbled to distribute *L. monocytogenes* on the shrimps. After inoculation, 100 ± 1 g of brined and drained shrimp were packaged in a modified atmosphere initially containing 40 % CO$_2$ and 60 % N$_2$ as previously described (Mejlholm and Dalgaard, 2007a).

2.2.2 Product characteristics and storage conditions

Prior to chilled storage three samples from each treatment were analyzed by chemical methods (See Section 2.1.1). Both shrimp in brine, and brined and drained MAP shrimp were then stored at 7-8 °C and storage temperature was recorded by data loggers as described above for the storage trials.

2.2.3 Microbiological analyses

At regular intervals during storage, three samples from each treatment were analyzed. Samples were homogenized and diluted as previously described (Section 2.1.2). *L. monocytogenes* was enumerated by spread plating (37 °C, 2 d) on Palcam agar (Oxoid CM0877) with Palcam Selective Supplement (Oxoid SR0150).
2.3 Statistical analyses and curve fitting

A classical t-test was used to evaluate differences between the mean values of repeated measurements. Calculations were carried out using Statgraphics Plus version 5.1 (Manugistics Inc., Rockville, Md., USA). To determine the lag phase \( (d) \) and maximum specific growth rate \( (\mu_{\text{max}}, \text{d}^{-1}) \) of bacteria in the differently treated samples of brined shrimp the four parameter logistic model (Dalgaard, 1995) was fitted to growth data obtained on NAP and Palcam agar. The software package Fig.P (version 2.98, Biosoft, Cambridge, UK) was used for curve fitting. An \( F \)-test to compare fits of the three- and four parameter logistic models was used to evaluate whether lag phases of the microbial growth curves were significant (Dalgaard, 1995).

3. Results and discussion

3.1 Storage trials and microbial spoilage

3.1.1 Product characteristics and storage conditions

Product characteristics and storage conditions of brined shrimp used in the storage trials are shown in Table 1. No significant differences \( (P > 0.05) \) in concentrations of benzoic and sorbic acid, respectively, were observed between products added equal amounts of the two organic acids. However, small differences \( (P < 0.05) \) in lactic acid concentrations were observed for treatment J and K as compared to treatment I and L.

Similarly, small differences \( (P < 0.05) \) in acetic acid concentrations were found for treatment J as compared to treatment I and L. Finally, higher \( (P < 0.05) \) concentrations of citric acid were determined in treatment B and D as compared to treatment A, C, E, F and G (Table 1). The studied concentrations of organic acids were within the legal limits in EU regulations (EC, 1995).
Fig. 2 Microbial changes (APC) during storage of brined shrimp at 7 °C. (a) Shrimp in brine with benzoic, citric and sorbic acids (■, □) (treatment A and B) as well as diacetate (●, ○) (treatment C and D). (b) Brined and drained MAP shrimp with benzoic, sorbic and citric acids (■, □) (treatment F and G). (c) Shrimp in brine with acetic, citric and lactic acids (■, □) (treatment I and J). Solid and open symbols represent industrial and manually processed samples, respectively. Error bars represent SD (n = 3).
3.1.2 Microbiological and sensory changes

The cooked, peeled and frozen cold-water shrimp, used in the present study for production of shrimp in brine and brined and drained MAP shrimp, contained slightly lower concentrations of bacteria than previously reported in other studies (Ridley and Slabyj, 1978; Valdimarsson et al., 1998; Hatha, Paul and Rao, 1998). However, we found the concentration of microorganisms to increase substantially when an industrial scale processing line was used for production of brined shrimp (Table 2). The initial APC of manually brined shrimp varied between 1.6 and 2.2 log (CFU g\(^{-1}\)) whereas 3.2 to 3.9 log (CFU g\(^{-1}\)) were found on brined shrimp from the industrial scale processing line (Fig. 2, Table 2). These differences, however, were not reflected in the initial concentrations of LAB (Table 2). The spoilage microflora of chilled brined shrimp seemed more diverse when an industrial scale processing line was used for production. *E. faecalis*-like, *E. faecalis*, *Lc. pseudomesenteroides*, coagulase negative *Staphylococcus* spp. and yeast were isolated from these products but not from manually processed brined shrimp (Table 3). Isolates of *Ps. fluorescens*, *E. malodoratus*, *Cb. maltaromaticum* and *Lb. sakei* were identified from both industrial and manually processed samples (Table 3). The microbial contamination seemed to have a pronounced effect on the subsequent shelf life of chilled brined shrimp (Fig. 2, Table 2). Particularly, for brined and drained MAP shrimp where a shelf life of more than 75 days at 7 °C were determined for manually brined and packed samples whereas a shelf life of only 28-35 days at 7 °C were observed for samples from the industrial scale processing line (Table 2, treatment F and G). The latter product spoiled due to formation of sour off-flavours and gas was formed in the packages. These spoilage characteristics were most likely caused by *Lb. sakei* as it was present in high concentrations of 7.9 log CFU g\(^{-1}\) and dominated the spoilage microflora (Table 2 and 3, treatment F). *Lb. sakei* has previously been identified as the dominating microflora from different types of lightly preserved seafoods e.g. cold-smoked salmon where this bacteria produced off-flavours associated with spoilage (Hansen and Huss, 1998; Lyhs et al., 1999; Jørgensen, Huss and Dalgaard, 2000; Joffraud, Leroi, Roy and Berdagüé, 2001; Stohr, Joffraud, Cardinal and Leroi, 2001; Lyhs, Korkeala and Björkroth, 2002). Corresponding to treatment G of the present study Dalgaard and Jørgensen (2000) reported a shelf life of 63-84 days at 8 °C for brined and drained MAP cold-water shrimp. However, in that study the spoilage microflora was dominated by *Aerococcus viridans*, *E. saccharolyticus* and other Gram-positive bacteria (Dalgaard et al., 2003) and the initial APC was approx. 3.5
which is much higher than for treatment G in the present study (Table 2). Brined and drained MAP shrimp of the present study were packed with more CO₂, and contained more benzoic and citric acid but less sorbic acid as compared to the data reported by Dalgaard and Jørgensen (2000) and (Dalgaard et al., 2003). Clearly, these differences in preserving parameters may influence shelf-life and microbial changes during chilled storage. Nevertheless, data from treatment F and G (Table 2) together with those reported by Dalgaard and Jørgensen (2000) and Dalgaard et al. (2003) suggest that the initial microbial contamination can have a major effect on both shelf life and the dominating spoilage microflora of brined and drained MAP shrimp. For this product the use of acetic acid/diacetat and lactic acid (Treatment M) instead of benzoic and sorbic acid (Treatment F) had a positive effect on the shelf life (Table 2) although *Lb. sakei* remained as a dominating part of the spoilage microflora (Table 3).

The effect of initial microbial contamination on shelf life and on microbial changes makes is difficult to evaluate the effect of MAP as shrimp in brine, and brined and drained MAP shrimp were produced by different processes and thereby possibly with different degrees of microbial contamination. However, data for manually processed brined shrimp with low initial microbial contamination and similar preserving parameters suggest that MAP with an equilibrium concentration of approx. 26 % CO₂ extended the shelf-life from 53-60 days for shrimp in brine to more than 75 days for brined and drained MAP shrimp (Table 2, treatment B and G). The opposite effect was observed by comparison of treatment A and F but this could be due to differences in their higher initial microbial contamination.

The impact of contamination during production was also shown for shrimp in brine, especially for samples containing acetic, citric and lactic acids. The shelf life of processed and manually packed shrimp in brine was determined as 42-49 days and 69-84 days, respectively, at 7 °C (Fig. 2, Table 2, treatment I and J). The spoilage microflora of the former product was dominated by yeast and *Lb. sakei* resulting in formation of sour and buttermilk-like off-flavours (Table 2 and 3). When yeast constituted the dominating microflora of shrimp in brine, cell concentrations of approx. 10⁶ CFU g⁻¹ were sufficient to spoil the products (Table 3, treatment C and I). Sensory rejection of manually processed shrimp in brine (treatment J), with a maximum APC of 3.7 log (CFU g⁻¹), was not caused by microbial activity but rather chemical changes possibly lipid and/or protein oxidation resulting in rancid off-flavours and yellow coloured shrimp. This phenomenon was also observed for treatment D and K (Table
The occurrence of yellow coloured shrimp and rancid off-flavours has previously been reported for brined shrimp where growth of microorganisms were prevented or strongly inhibited (Einarsson and Lauzon, 1995; Cadun, Cakli and Kisla, 2005).

The dominating microflora of shrimp in brine stored at 7 °C consisted of *Ps. fluorescens*, *E. faecalis*-like, *E. malodoratus*, *Cb. maltaromaticum*, *Staphylococcus* spp. and *Lb. sakei* when benzoic, citric and sorbic acids were used as preservatives (Table 1 and 3, treatment A and B). The importance of processing conditions (i.e. industrial or manually processed samples) on the sensory shelf life of these samples were not as apparent as observed for shrimp in brine with acetic, citric and lactic acids, and brined and drained MAP shrimp (Table 2). Spoilage characteristics were not very distinct although high concentrations of e.g. *Ps. fluorescens* were present. *Pseudomonas* spp. frequently dominates the spoilage microflora of fresh chilled shrimp/prawn where they form fruity and sulphurous off-flavours (Matches, 1982; Zuberi, Qadri and Siddiqui, 1983; Chinivasagam, Bremner, Thrower and Nottingham, 1996; Chinivasagam, Bremner, Wood and Nottingham, 1998; Lalitha and Surendran, 2006). At DIFRES *Ps. fluorescens* was previously determined as part of the spoilage microflora of brined shrimp at 5 °C with 3.1 % WPS, 0.11 % kalium-sorbat, 0.35 % citric acid and a pH of 6.0 (Jessen, Schæffer, Priess and Jakobsen, 1980). In another study at DIFRES, *Ps. fluorescens* was not isolated from the spoilage microflora of brined shrimp at 5 or 10 °C, although this microorganism was found on the cooked and peeled shrimp used for production. The examined samples of brined shrimp contained 3.0 % WPS, 0.10 % sodium-benzoat, 0.08 % kalium-sorbat, 0.69 % citric acid and had a pH of 5.4 (From, 1986). Thus, the products examined by Jessen et al. (1980) and From (1986) had a preservation profile being, respectively, less and more stringent, than treatment A and B of the present study (Table 1).
**Table 3 Dominating spoilage microflora of chilled brined shrimp**

<table>
<thead>
<tr>
<th>Experimental design&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Shrimp from processing line</th>
<th>Total</th>
<th>Gram-negative</th>
<th>Gram-positive</th>
<th>Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudomonas fluorescens</td>
<td>Enterococcus spp.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Carnobacterium maltaromaticum&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A Yes</td>
<td>12 (100)</td>
<td>4 (33)</td>
<td>4 (33)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1 (9)</td>
<td>-</td>
</tr>
<tr>
<td>B No</td>
<td>30 (100)</td>
<td>14 (47)</td>
<td>12 (40)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>4 (13)</td>
</tr>
<tr>
<td>C Yes</td>
<td>4 (100)</td>
<td>-</td>
<td>-</td>
<td>1 (25)</td>
<td>-</td>
</tr>
<tr>
<td>D No</td>
<td>3 (100)</td>
<td>-</td>
<td>3 (100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E Yes</td>
<td>3 (100)</td>
<td>-</td>
<td>2 (66)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F Yes</td>
<td>6 (100)</td>
<td>-</td>
<td>-</td>
<td>6 (100)</td>
<td>-</td>
</tr>
<tr>
<td>G No</td>
<td>Ni&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H Yes</td>
<td>6 (100)</td>
<td>-</td>
<td>4 (67)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I Yes</td>
<td>7 (100)</td>
<td>-</td>
<td>-</td>
<td>3 (43)</td>
<td>-</td>
</tr>
<tr>
<td>J No</td>
<td>Ni&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K No</td>
<td>Ni&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L Yes</td>
<td>6 (100)</td>
<td>-</td>
<td>1 (17)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
<td>5 (83)</td>
</tr>
<tr>
<td>M Yes</td>
<td>5 (100)</td>
<td>-</td>
<td>-</td>
<td>5 (100)</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Table 1

<sup>b</sup> Identification based on both phenotypic characteristics and ribotyping

<sup>c</sup> All isolates are shown in Fig. 3

<sup>d</sup> Eleven of the 23 isolates are shown in Fig. 3

<sup>e</sup> Both isolates are shown in Fig. 3

<sup>f</sup> *E. faecalis*-like (5 of the 9 isolates are shown in Fig. 3)

<sup>g</sup> *E. malodoratus* (2 of the 13 isolates are shown in Fig. 3)

<sup>h</sup> No isolates (NI)

<sup>i</sup> *E. faecalis* (isolate are shown in Fig. 3)
Addition of diacetate to shrimp in brine stored at 7 °C inhibited the growth of *Ps. fluorescens* and Gram-positives identified as *E. faecalis*-like, *E. malodoratus* and coagulase negative *Staphylococcus* spp. (Table 3, treatment C and D). The inhibiting effect of acetic acid/diacetate against *Ps. fluorescens* has previously been demonstrated in broth experiments (Shelef and Addala, 1994; Ouattara, Simard, Holley, Piette and Begin, 1997). The present
study confirmed this effect in shrimp at both 7°C and 12°C. The antimicrobial effect of diacetate against *E. faecalis*-like, *E. malodoratus* and coagulase negative *Staphylococcus* spp. was not sufficient to prevent growth to high concentrations when samples were stored at 12 °C or when brine without benzoic acid was studied (Table 3, treatment E and H). As expected, an increase of the storage temperature from 7 to 12 °C changed the dominating spoilage microflora and reduced shelf life of shrimp in brine (Table 2 and 3, treatment C vs. E and I vs. L) (Dalgaard and Jørgensen, 2000; Mejlhølm et al., 2005).

*Cb. maltaromaticum* was identified as part of the dominating spoilage microflora in brined shrimp containing 0.14-0.15 % water phase diacetate (Table 3, treatment C and D). This is surprising as carnobacteria are known for their sensitivity to acetate and have a minimum inhibitory concentration 0.10 % acetic acid (wt/vol) (Ouattara et al., 1997). However, in brined shrimp with higher concentrations of acetic acid/diacetate (0.15-1.23 %) *Carnobacterium* spp. was not isolated (Table 1 and 3, treatment H-M).

Microbial contamination during production of brined shrimp as observed in the present study may be attributed to the presence of a persistent microflora on the industrial scale processing line of the shrimp plant. In fact, following cleaning and disinfection, *Pseudomonas* spp. and yeast were previously detected as dominating microorganisms on equipment for processing of cold-smoked salmon, semi-preserved herring and brined caviar (Bagge-Ravn, Ng, Hjelm, Christiansen, Johansen and Gram, 2003). Lower proportions of *Staphylococcus* spp. and LAB were also identified in that study. Similarly, *Pseudomonas* spp. and yeast constituted 66 and 10 percent, respectively of the microflora attached to production surfaces of a shrimp processing plant (Gudbjornsdottr, Suihko, Gustavsson, Thorkelsson, Salo, Sjoberg et al., 2004). The high frequencies of *Pseudomonas* spp. seems related to its ability to form biofilm and to adapt to disinfectants (Langsrud and Sundheim, 1997; Bagge-Ravn et al., 2003). The present study has illustrated the potential for optimization of hygiene procedures in relation to shelf life of brined shrimp. However, identification of the routes of contamination needs further study.
Table 4 Effect of product characteristics and storage atmosphere on growth of *Listeria monocytogenes* in challenge tests with brined shrimp at 7-8°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Organic acids in brine</th>
<th>Added Na-diacetate</th>
<th>MAP</th>
<th>pH</th>
<th>Water phase salt (%)</th>
<th>Acetic (Diacetate)</th>
<th>Benzoic</th>
<th>Citric</th>
<th>Lactic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sorbic</th>
<th>% CO₂&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Temp. (°C)</th>
<th>Lag phase (d)</th>
<th>µmax (d⁻¹)</th>
<th>Time to 100-fold increase (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Benzoic, sorbic, citric</td>
<td>-</td>
<td>-</td>
<td>5.6 ± 0.0</td>
<td>2.85 ± 0.05</td>
<td>-</td>
<td>0.11 ± 0.02</td>
<td>0.61 ± 0.02</td>
<td>0.060 ± 0.017</td>
<td>0.043 ± 0.011</td>
<td>-</td>
<td>6.8 ± 0.1</td>
<td>&gt; 40</td>
<td>&lt; 0.02</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>O</td>
<td>Sorbic, citric</td>
<td>-</td>
<td>-</td>
<td>5.6 ± 0.1</td>
<td>2.85 ± 0.05</td>
<td>-</td>
<td>-</td>
<td>0.63 ± 0.02</td>
<td>0.047 ± 0.011</td>
<td>0.038 ± 0.005</td>
<td>-</td>
<td>6.8 ± 0.1</td>
<td>NS</td>
<td>0.65</td>
<td>6</td>
</tr>
<tr>
<td>P</td>
<td>Sorbic, citric</td>
<td>+</td>
<td>-</td>
<td>5.4 ± 0.0</td>
<td>2.85 ± 0.05</td>
<td>0.23 ± 0.01</td>
<td>-</td>
<td>0.64 ± 0.03</td>
<td>0.041 ± 0.011</td>
<td>0.032 ± 0.008</td>
<td>-</td>
<td>6.8 ± 0.1</td>
<td>NS</td>
<td>0.12</td>
<td>27</td>
</tr>
<tr>
<td>Q</td>
<td>Benzoic, sorbic, citric</td>
<td>-</td>
<td>+</td>
<td>5.7 ± 0.0</td>
<td>2.55 ± 0.07</td>
<td>-</td>
<td>0.12 ± 0.01</td>
<td>0.54 ± 0.05</td>
<td>0.050 ± 0.002</td>
<td>0.046 ± 0.003</td>
<td>34 ± 1.5</td>
<td>8.1 ± 0.1</td>
<td>&gt; 40</td>
<td>0.05</td>
<td>&gt; 70</td>
</tr>
<tr>
<td>R</td>
<td>Benzoic, sorbic, citric</td>
<td>-</td>
<td>+</td>
<td>5.7 ± 0.1</td>
<td>2.50 ± 0.06</td>
<td>-</td>
<td>0.056 ± 0.004</td>
<td>0.56 ± 0.04</td>
<td>0.056 ± 0.000</td>
<td>0.045 ± 0.002</td>
<td>34 ± 1.5</td>
<td>8.1 ± 0.2</td>
<td>NS</td>
<td>0.24</td>
<td>23</td>
</tr>
<tr>
<td>S</td>
<td>Benzoic, sorbic, citric</td>
<td>-</td>
<td>+</td>
<td>5.6 ± 0.0</td>
<td>2.54 ± 0.07</td>
<td>-</td>
<td>0.063 ± 0.003</td>
<td>0.60 ± 0.08</td>
<td>0.058 ± 0.001</td>
<td>0.023 ± 0.001</td>
<td>34 ± 1.5</td>
<td>8.1 ± 0.3</td>
<td>NS</td>
<td>0.31</td>
<td>15</td>
</tr>
<tr>
<td>T</td>
<td>Sorbic, citric</td>
<td>-</td>
<td>+</td>
<td>5.6 ± 0.1</td>
<td>2.55 ± 0.07</td>
<td>-</td>
<td>-</td>
<td>0.65 ± 0.09</td>
<td>0.062 ± 0.002</td>
<td>0.052 ± 0.004</td>
<td>34 ± 1.5</td>
<td>8.1 ± 0.4</td>
<td>NS</td>
<td>0.70</td>
<td>7</td>
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<tr>
<td>U</td>
<td>Sorbic, citric</td>
<td>+</td>
<td>+</td>
<td>5.4 ± 0.0</td>
<td>2.52 ± 0.05</td>
<td>0.14 ± 0.00</td>
<td>-</td>
<td>0.57 ± 0.05</td>
<td>0.057 ± 0.003</td>
<td>0.051 ± 0.002</td>
<td>34 ± 1.5</td>
<td>8.1 ± 0.5</td>
<td>NS</td>
<td>0.26</td>
<td>20</td>
</tr>
<tr>
<td>V</td>
<td>Benzoic, sorbic, citric</td>
<td>-</td>
<td>+</td>
<td>5.6 ± 0.0</td>
<td>2.50 ± 0.06</td>
<td>-</td>
<td>0.12 ± 0.05</td>
<td>0.60 ± 0.08</td>
<td>0.046 ± 0.017</td>
<td>-</td>
<td>34 ± 1.5</td>
<td>8.1 ± 0.6</td>
<td>NS</td>
<td>0.22</td>
<td>31</td>
</tr>
<tr>
<td>W</td>
<td>Citric</td>
<td>+</td>
<td>+</td>
<td>5.5 ± 0.1</td>
<td>2.55 ± 0.03</td>
<td>0.14 ± 0.00</td>
<td>-</td>
<td>0.58 ± 0.05</td>
<td>0.060 ± 0.002</td>
<td>-</td>
<td>34 ± 1.5</td>
<td>8.1 ± 0.7</td>
<td>NS</td>
<td>0.36</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Natural content of lactic acid in cold-water shrimp

<sup>b</sup> Equilibrium concentrations in headspace gas
3.2 Challenge tests with Listeria monocytogenes

Product characteristics and storage conditions of the challenge tests are shown in Table 4. No differences (P > 0.05) in concentrations of acetic acid/diacetate, benzoic acid and sorbic acids were observed between products added equal amounts of the different organic acids. However, slightly higher (P < 0.05) concentrations of citric acid were determined in treatment O, P and T as compared to treatment Q and for treatment T as compared to treatment R (Table 4).

Fig. 4 Growth of *Listeria monocytogenes* during storage of brined and drained shrimp in modified atmosphere packaging (MAP) at 7-8 °C. Brined and drained MAP shrimp with benzoic, citric and sorbic acids (■) (treatment Q); citric and sorbic acids (○) (treatment T); and benzoic and citric acids (▲) (treatment V). Error bars represent SD (n = 3).

For brined shrimp growth of *L. monocytogenes* was prevented during more than 40 days at 6.8 °C whereas only a 1.5 log (CFU g⁻¹) increase in the concentration of the pathogen was observed in brined and drained MAP shrimp during 70 days of storage at 8.1 °C (Table 4, treatment N and Q). This is important as the preserving parameters of treatment N and Q corresponded to the ones used for commercial products. Dalgaard and Jørgensen (2000) observed no growth of *L. monocytogenes* in brined and drained MAP cold-water shrimp for
more than 80 days at 8 °C. That product contained lower concentrations of CO₂, water phase salt, and benzoic and citric acids but a higher concentration of sorbic acid than samples from treatment N and Q in the present study. However, in brined and drained MAP warm-water shrimp with moderately changed preserving parameters (higher pH and lower concentration of WPS) growth of L. monocytogenes was determined at 5 °C (Dalgaard and Jørgensen, 2000).

Omitting benzoic acid from brined shrimp resulted in a markedly increase in the growth of L. monocytogenes with the time to a 100-fold increase in cell concentration being reduced from more than 40-70 d to 6-7 d (Fig. 4, Table 4, treatment N vs. O and Q vs. T). The effect of sorbic acid on growth of L. monocytogenes in brined and drained MAP shrimp was less pronounced (Fig. 4, Table 4, treatment T and V) and this is in agreement with results from broth experiments and meat products (Elshenawy and Marth, 1988; Samelis, Sofos, Kain, Scanga, Belk and Smith, 2001; Islam, Chen, Doyle and Chinnan, 2002). In addition, the present study documented how growth of L. monocytogenes can be sensitive to rather small changes of the preserving parameters in brined shrimp. This corresponds to the effect of pH and salt reported by Dalgaard and Jørgensen (2000) and mentioned above.

Addition of diacetate reduced growth of L. monocytogenes in brined shrimp but 0.14-0.23 % water phase diacetate was less efficient than 0.11-0.12 % water phase benzoic acid (Table 4, treatment N vs. P and Q vs. U). Comparable concentrations of diacetate has previously been shown to prevent growth of L. monocytogenes in other lightly preserved seafoods e.g. cold-smoked salmon (Yoon et al., 2004; Mejilholm and Dalgaard, 2007a; Mejilholm and Dalgaard, 2007b). The concentration of naturally occurring lactate in shrimp is low of < 0.1 % water phase lactate (Mejlholm et al., 2005; Laursen, Leisner and Dalgaard, 2006) as compared to salmon with approx. 0.7 % water phase lactate (Mejlholm and Dalgaard, 2007a). This explains why the effect of diacetate on growth of L. monocytogenes is less pronounced in shrimp as compared to salmon. Importantly, mathematical models are now available to predict the effect of diacetate and lactate on growth of L. monocytogenes in lightly preserved seafood. As an example no growth of L. monocytogenes is predicted in brined shrimp with product characteristics and storage conditions corresponding to treatment I to M (Table 1) (Mejlholm and Dalgaard, 2007a). Currently, the model of Mejlholm and Dalgaard (2007a) includes the effect of diacetate, lactate, CO₂, smoke components, nitrite, pH, NaCl, temperature and interactions between all these parameters, and this makes it applicable for a
range of lightly preserved seafood. However, for brined shrimp it is relevant to expand the model to include the effect of benzoic, citric and sorbic acids, and this is the aim of ongoing research at our institute.

No clear effect of MAP against growth of *L. monocytogenes* was observed in the present study.

4. **Conclusion**

The present study demonstrated the importance of processing hygiene on shelf life and microbial changes of brined shrimp. Brined shrimp from an industrial scale process line had a shorter shelf life and a more diverse spoilage microflora as compared to manually brined and packed samples. It was shown how changes in preserving parameters and storage conditions affected the shelf life and spoilage microflora of brined shrimp. This information can be used actively to improve the shelf life of these products.

Challenge tests with *L. monocytogenes* showed how unacceptable growth of this pathogen is prevented by typical product characteristics and storage conditions of brined shrimp. However, growth of *L. monocytogenes* in brined shrimp is sensitive to changes in the preserving parameters. Thus, relatively small changes in product characteristics can be important for growth to the critical concentration of 100 *L. monocytogenes*/g.

5. **Acknowledgments**

We thank Tina Julien and Nadereh Samieian from DIFRES for skilful technical assistance with storage trials and challenge tests. The research was financed by Royal Greenland Seafood Ltd and the Directorate for Food, Fisheries and Agri Business (Project nr. 3401-66-04-869).
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Paper 3

Mejlholm, O. and Dalgaard, P.

Modeling and predicting the growth boundary of *Listeria monocytogenes* in lightly preserved seafood

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Modeling and Predicting the Growth Boundary of Listeria monocytogenes in Lightly Preserved Seafood

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ABSTRACT

The antimicrobial effect of diacetate and lactate against Listeria monocytogenes was evaluated in challenge tests with vacuum-packaged or modified atmosphere packaged (MAP) cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon. MAP cold-smoked salmon with the addition of 0.15% (wt/wt) diacetate prevented the growth of L. monocytogenes for more than 40 days at 8°C, whereas the addition of 0.15% (wt/wt) diacetate reduced the growth rate of the pathogen in MAP cold-smoked Greenland halibut. This difference between the two types of products was explained by a higher content of naturally occurring lactate in cold-smoked salmon (0.77 to 0.98%, wt/wt) than in cold-smoked Greenland halibut (0.10 to 0.15%, wt/wt). In fact, the addition of 0.15% (wt/wt) diacetate and 0.75% (wt/wt) lactate to MAP cold-smoked Greenland halibut prevented the growth of L. monocytogenes for more than 45 days at 8°C. A mathematical model that included the effect of diacetate, lactate, CO2, smoke components, nitrite, pH, NaCl, temperature, and interactions between all these parameters was developed to predict the growth boundary of L. monocytogenes in lightly preserved seafood. The developed growth boundary model accurately predicted growth and no-growth responses in 68 of 71 examined experiments from the present study as well as from literature data. Growth was predicted for three batches of naturally contaminated cold-smoked salmon when a no-growth response was actually observed, indicating that the model is fail-safe. The developed model predicts both the growth boundary and growth rate of L. monocytogenes and seems useful for the risk management of lightly preserved seafood. Particularly, the model facilitates the identification of product characteristics required to prevent the growth of L. monocytogenes, thereby making it possible to identify critical control points, and is useful for compliance with the new European Union regulation on ready-to-eat foods (EC 2073/2005).

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Despite considerable efforts to improve process hygiene and sanitation procedures, it has not been possible to successfully prevent the contamination of several types of ready-to-eat (RTE) seafood with Listeria monocytogenes. This is critical, as typical product characteristics, including pH, salt, and smoke components, are insufficient to prevent the growth of L. monocytogenes, e.g., in chilled and vacuum-packaged (VP) smoked and gravad products. Especially products with a long, chilled shelf life constitute a risk with respect to the growth of L. monocytogenes to critical concentrations (17, 46, 47, 49).

Cold-smoked and gravad seafoods are lightly preserved RTE products of considerable economic importance. Control of L. monocytogenes in these products represents a substantial challenge to reduce the risk of listeriosis and to prevent recalls due to a lack of compliance with national and international regulations. For RTE seafood, European Union (EU) regulations differentiate between products that are able or unable to support the growth of L. monocytogenes and allow 100 CFU g⁻¹ in the latter (EC 2073/2005). In the United States, the U.S. Food and Drug Administration demands the absence of L. monocytogenes in 25-g samples of lightly preserved seafood (47). Preventing the growth of L. monocytogenes to high concentrations is the strategy expected to have the greatest impact on reducing cases of listeriosis (49). Consequently, preservation procedures that prevent the growth of L. monocytogenes in lightly preserved seafood are of major interest, particularly procedures that can be used without altering the sensory characteristics of classical lightly preserved seafood.

Traditionally, cold-smoked and gravad seafoods have been distributed as VP products, but recently, the use of modified atmosphere packaging (MAP) with CO2 has been increasing (1, 33, 43). Carbon dioxide in MAP delays the growth of L. monocytogenes in several lightly preserved foods (15, 33), and 100% CO2 prevents growth in cold-smoked salmon at 4°C (43). More frequently, organic acids and especially combinations of diacetate and lactate have been reported to prevent the growth of L. monocytogenes in various meat products (6, 18, 40, 41) as well as in chilled VP cold-smoked salmon (50). Depending on concentrations, the addition of diacetate and lactate may alter the sensory characteristics of seafood, and further studies are needed to identify the combinations of these compounds that prevent the growth of L. monocytogenes without having a negative effect on product properties. Storage conditions (temperature and atmosphere) and product characteristics (NaCl, pH, smoke components, and naturally occurring lactate) in lightly preserved seafood most likely influence the antimicrobial effect of added diacetate and lactate.
Thus, a mathematical model to predict the combined effect of all these parameters on the growth boundary of *L. monocytogenes* would be of considerable practical importance to assist in the formulation of safe products in compliance with the EU regulation on RTE seafood (EC 2073/2005).

Mathematical models for the growth limits of *L. monocytogenes* as a function of temperature, pH, NaCl, CO₂, smoke components (phenol), and organic acids are available (3–5, 23, 26, 27, 45), and the combined effect of diacetate, lactate, salt, and moisture in cooked meat at 4°C has also been modeled (25, 40). Clearly, it is interesting to use the combined effect of product characteristics and storage conditions as a means of identifying critical control points for *L. monocytogenes* in lightly preserved seafood. However, to our knowledge, mathematical models that include the effect of diacetate and lactate in combination with the environmental parameters that most likely influence the growth of *L. monocytogenes* in lightly preserved seafood are not available.

The objectives of the present study were to evaluate and mathematically model the antimicrobial effect of diacetate and lactate against *L. monocytogenes* in chilled, lightly preserved seafood. Cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon were studied to evaluate the importance of different product characteristics on the growth of *L. monocytogenes*. A new growth boundary model was developed to predict the effect of diacetate, lactate, CO₂, smoke components, nitrite, pH, NaCl, temperature, and interactions between the parameters on the growth boundary of *L. monocytogenes* in lightly preserved seafood.

**MATERIALS AND METHODS**

**Challenge tests.** A series of challenge tests were carried out with cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon. Two different batches of cold-smoked salmon and cold-smoked Greenland halibut as well as one batch of marinated salmon, marinated Greenland halibut, and gravad salmon were studied in a total of 24 challenge tests. Products were VP or MAP and stored at 8 or 15°C, as shown in Table 1. Salmon (*Salmo salar*) from aquaculture in Norway and Greenland halibut (*Reinhardtius hippoglossoides*) caught in the North Atlantic Ocean were used as raw materials and processed into sliced and packed fillets by a company in Denmark. After processing, the products were frozen and transported to the Danish Institute for Fisheries Research in their frozen state. The frozen, processed fillets were thawed overnight at 5°C and divided into slices. For each batch of each product, slices were randomly divided into subbatches prior to the addition of organic acids. Selected subbatches of the five products were added from 0.0 to 0.15% (wt/wt) diacetate and from 0.0 to 1.5% (wt/wt) lactate as shown in Table 1. A total of 0.183% (wt/wt) sodium diacetate (98%; Spectrum S1266, Spectrum, New Brunswick, N.J.) and 2.36% (vol/wt) sodium lactate (60% [wt/wt] syrup, Sigma L-1375, Sigma, St. Louis, Mo.) were added to the samples, corresponding to concentrations of 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate, respectively. Proportional concentrations of sodium diacetate and sodium lactate were added for other levels of diacetate and lactate. Solutions containing sodium diacetate and sodium lactate or only sodium diacetate were prepared by dissolving sodium diacetate in deionized water corresponding to 1% (vol/wt) of the weight of the product in question, and subsequently, when required, sodium lactate was added. To all subbatches was added the same amount of liquid, with sterile water being added to products with less than 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate (Table 1). For each subbatch, the liquid was added as four portions, and after each addition, the product was manually tumbled to ensure an even distribution of liquid and preservatives. Following this treatment, all subbatches were stored at 1 to 2°C for 24 h to allow distribution of preservatives in the samples.

**Inoculation and packaging of samples.** All subbatches were inoculated with a mixture of four *L. monocytogenes* isolates (94-203D, 95-54A, 95-442A, and 94-167B) previously obtained from seafood (17, 21). Initially, each isolate was grown (25°C, 1 day) in brain heart infusion broth (Oxoid CM225, Oxoid, Basingstoke, UK) and subsequently precultured (5°C, 4 to 5 days) in brain heart infusion broth with 4% NaCl. Preculturing at 5°C in brain heart infusion broth with 4% NaCl was chosen in order to adapt the isolates to the experimental conditions of the challenge tests resembling the actual salt concentrations and storage conditions of lightly preserved seafood. Precultures were harvested in late exponential growth phase, defined as a relative change in absorbance of 0.05 to 0.2 at 540 nm (Novaspec II, Pharmacia Biotech, Allerød, Denmark). The inoculum was prepared by mixing the four precultures and diluting them in 0.85% NaCl to a cell density of approximately 10⁶ *L. monocytogenes* ml⁻¹ (MIX-Lm). To all subbatches was added 1% (vol/wt) MIX-Lm as four portions of 0.25% (vol/wt), and after each addition, the products were manually tumbled to distribute *L. monocytogenes* on the samples.

After inoculation, 100-g portions of the differently treated products (Table 1) were either VP or packaged in a modified atmosphere initially containing 40% CO₂ and 60% N₂ (AGA Ltd., Copenhagen, Denmark). A Multivac A 300/16 packaging machine (Multivac Ltd., Vejle, Denmark) and a packaging film (NEN 40 HOB/LLPDE 75, Amcor Flexible, Horsens, Denmark) with low gas permeability (0.45 ± 0.15 cm³ m⁻² atm⁻¹ for O₂ and 1.8 ± 0.6 cm³ m⁻² atm⁻¹ for CO₂) were used. For MAP samples, the gas/fish ratio was approximately 2:1. Following packaging, subbatches were stored at 8 or 15°C, and data loggers (TinytagPlus, Gemini Data Loggers Ltd., Chichester, UK) recorded the storage temperatures continuously throughout the storage period.

**Microbiological and chemical analyses.** At regular intervals during the storage of each subbatch, three samples were analyzed by microbiological methods. Product samples of 20 g were diluted 10-fold in chilled (5°C) physiological saline (0.85% NaCl) with 0.1% peptone and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Further appropriate 10-fold dilutions of the homogenates were made in chilled physiological saline (0.85% NaCl) with 0.1% peptone. *L. monocytogenes* was determined by spread plating (37°C, 2 days) on PALCAM agar (Oxoid CM0877) with PALCAM Selective Supplement (Oxoid SR0150). Lactic acid bacteria (LAB) were enumerated by pour plating (25°C, 3 days) in nitrite actidion polymyxin agar with pH 6.2 (14), and aerobic plate counts were determined by spread plating (15°C, 7 days) on Long and Hammer agar with 1% NaCl (48).

Characteristics of the seven different batches of the five types of products (cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon) were determined by an analysis of three samples from each batch at the start of the challenge tests (day 0). Dry matter and pH were measured as previously described (10). Salt content was determined by an automated potentiometric titration method (2). Diacetate and lactate were determined by a previously described
<table>
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<tr>
<th>Products and sub-batches (code)</th>
<th>Atmosphere</th>
<th>Temp (°C)</th>
<th>Added (% wt/wt)</th>
<th>pH</th>
<th>Water phase lactate (%)</th>
<th>Water phase diacetate (%)</th>
<th>Water phase salt (%)</th>
<th>Phenol (ppm)</th>
<th>Temp (°C)</th>
<th>% CO₂</th>
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<td>—</td>
<td>6.0</td>
<td>0.98 ± 0.36</td>
<td>—</td>
<td>6.0</td>
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<td>6.1</td>
<td>0.90 ± 0.08</td>
<td>—</td>
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<td>7.8 ± 0.5</td>
<td>30.0 ± 1.7</td>
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<td>0.35 ± 0.07</td>
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<td>6.5</td>
<td>0.13 ± 0.00</td>
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<td>0.14 ± 0.01</td>
<td>6.2</td>
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<td>—</td>
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<td>0.12 ± 0.01</td>
<td>6.1</td>
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</table>

*a Equilibrium concentrations in headspace gas.

*b Measured but not detected (ND).
high-pressure liquid chromatography method (12). External standards were used for the identification and quantification of the compounds. Smoke components were estimated as phenols by a spectrophotometric method (7). Finally, the equilibrium composition of gas in MAP samples was measured with a Combicheck 9800-1 gas analyzer (PBI Dansenor, Ringsted, Denmark). The sensorial effect of adding diacetate and lactic acid to the products was examined prior to the challenge test. Different concentrations of diacetate (0.0 to 2.0%, wt/wt), lactic acid (0.0 to 2.0%, wt/wt), or both were added to samples of fish as previously described. Subsequently, the sensorial properties (appearance, taste and smell, and texture) of the products were evaluated by an internal panel (five to six panelists), which judged the samples as either acceptable or not acceptable.

**Statistical analyses and curve fitting.** A one-way analysis of variance was used to determine whether differences between the mean values of repeated measurements were statistically significant. Calculations were carried out by Statgraphics Plus version 5.1 (Manugistics Inc., Rockville, Md.). To determine the lag time (expressed as hours) and maximum specific growth rate (μmax per hour), the four-parameter logistic model (9) was fitted to growth data determined on PALCAM and nitrite actidione polynixin agar in a challenge test. The software package Fig.P (version 2.98, Biosoft, Cambridge, UK) was used for curve fitting. An F-test to compare fits of the three- and four-parameter logistic models was used to evaluate whether lag phases of the microbial growth curves were significant (9).

**Modeling and predicting the growth of L. monocytogenes in lightly preserved seafood.** Sixty μmax values were obtained from the growth of *L. monocytogenes* in lightly preserved seafood as a function of storage conditions (temperature and CO2) and product characteristics (water phase salt concentration or water activity [aw], pH, lactic acid, nitrite, CO2, smoke components [phenol], and diacetate). Eighteen growth curves were generated in the present study, and 42 μmax values were obtained from the literature, together with relevant storage conditions and product characteristics (17, 24, 35, 36, 43, 44, 50). A relatively limited number of μmax values were obtained from the literature, as many previous studies unfortunately did not report or measure important antimicrobial parameters, such as the concentration of smoke components (phenol) or even, in some cases, the aw, pH, or lactic acid concentrations. Growth data were compared with predictions from existing secondary models and expanded versions of these models by calculation of bias and accuracy factor values from observed and predicted μmax values (11, 38). The effect of temperature, water phase salt or aw, and pH as well as concentrations of lactic acid, nitrite, and smoke components (phenol) was predicted by the secondary model previously studied by Giménez and Dalgaard (17). This model was then expanded by the addition of a term for the growth-inhibiting effect of CO2 and a term for the effect of diacetate as indicated in equation 1. The CO2 term was adapted from the model of Devlieghere et al. (15) and transformed into a model component (CO2 max − CO2 equilibrium)/(CO2 max − CO2 opt) with a value between 0 and 1. The optimal concentration of carbon dioxide (CO2 opt) for the growth of *L. monocytogenes* was assumed to be zero. A CO2 max value of 3,140 ppm was used with a 95% confidence interval of 2,917 to 3,365 ppm of CO2 (15). The diacetate term (1 − \sqrt[3]{\text{DAC}_{\text{ci}}}/\text{MIC}_{U\text{DAC}}) was obtained from equations previously used to express the antimicrobial effect of organic acids (3, 26). In addition, the temperature and aw terms of Giménez and Dalgaard (17) were reformulated to obtain terms with values between 0 and 1 in resemblance with the other model components. For the temperature term ((T − T_min)/

\[(T_{\text{ref}} − T_{\min})^2\], this was achieved by introducing a reference temperature (T_ref) of 25°C. The original aw term (aw − aw_min) was transformed to (aw − aw_min)/(aw opt − aw_min), where the optimal aw (aw opt) for the growth was assumed to be 1.0.

\[μ_{\text{max}} = \frac{[\text{LAC}]^2}{[\text{DAC}]^2} \left(\frac{\text{CO2 opt} - \text{CO2 equilibrium}}{\text{CO2 max} - \text{CO2 opt}}\right) \left(\frac{\text{aw} - 0.923}{0.077}\right) \left(1 - 10^{(4.97 - \text{pH})}\right) \left(1 - \frac{\text{DAC}_{\text{ci}}}{\text{MIC}_{U\text{DAC}}}\right) \left(1 - \frac{\text{DAC}_{\text{ci}}}{\text{MIC}_{U\text{DAC}}}\right)^{-1}\]

(1)

where \[\text{DAC}_{\text{ci}}\] is the concentration (expressed as millimoles) of undissociated lactic acid, NIT is the concentration of nitrite (expressed as parts per million), P is the concentration of phenols (expressed as parts per million), and CO2 equilibrium is the concentration of dissolved CO2 (expressed as parts per million) at equilibrium. The concentration of dissolved CO2 was calculated by the measured percentage of CO2 in the headspace gas at equilibrium and Henry’s constant at the appropriate storage temperature (39). \[\text{DAC}_{\text{ci}}\] is the concentration (expressed as millimoles) of undissociated diacetate, and \[\text{MIC}_{U\text{DAC}}\] is the theoretical concentration of undissociated diacetate preventing the growth of *L. monocytogenes*. The MIC of diacetate \[\text{MIC}_{U\text{DAC}}\] was established in brain heart infusion broth (Oxoid CM225) (pH 6.0) at 8°C. The antimicrobial effect of 10 different concentrations of diacetate (0.0, 0.025, 0.05, 0.075, 0.10, 0.125, 0.15, 0.20, 0.35, and 0.50%, wt/vol) was examined against each of the four *L. monocytogenes* isolates (94-203D, 95-54A, 95-442A, and 94-167B) previously described. Isolates of *L. monocytogenes* were cultured as previously described, and an inoculum of 104 *L. monocytogenes* ml−1 was used. For each treatment, the growth of the four *L. monocytogenes* isolates was determined in triplicate by automated absorbance measurements at 540 nm (Bioscreen C, Labsystems, Helsinki, Finland). For each absorbance growth curve, the maximum specific growth rate (μmax per hour) was determined by the logistic model (13). Square root-transformed μmax values were plotted against concentrations of undissociated diacetate, and the MIC was estimated by a simple square root model (3). The concentration (expressed as millimoles) of undissociated organic acid was calculated as \[\text{HA}/(1 + 10^{(pK_a - \text{pH})})\]. [HA] was the total concentration (expressed as millimoles) of organic acid, and the pK_a values of 3.86 and 4.76 were used for lactic acid and diacetate, respectively. A Pmax value of 28.1 ppm of phenol with a standard error of 2.8 ppm was used (17).

By using the approach of Le Marc et al. (26), a term was added to the expanded model of Giménez and Dalgaard (17) for the interaction between the environmental parameters and in this way was used to predict the effect of temperature, water phase salt or aw, lactic acid, nitrite, CO2, smoke components (phenol), and diacetate on both the μmax and the growth boundary of *L. monocytogenes* (equation 1). In brief, this approach expands the growth rate models by adding a term for the interactive inhibiting effect of environmental parameters. The hypothesis is that the contribution of each environmental factor to the interaction can be derived from its separated effect on the μmax values (26). A term for the effect of interaction between environmental parameters (ξ) was included in equation 1, as indicated in equation 2, with contributions from the different environmental parameters calculated as indicated in equations 3 and 4. In equation 4, e_i represents environmental factors. It is assumed that (i) if η is lower than a predefined threshold value (θ), then no interactive effect between
environmental parameters occurs \((\xi = 1)\); (ii) if \(\psi\) is higher than 1, then a no-growth response occurs \((\xi = 0)\); and (iii) if \(\psi\) is lower than 1 and higher than 0, then the growth rate \(\mu_{\text{max}}\) (per hour) is reduced, depending on the value of \(\psi\). A threshold value \((\theta)\) of \(\frac{1}{2}\) was used, as suggested by Le Marc et al. (26).

The expanded model with interactions between environmental parameters (equation 1) was fitted to 39 of the 60 obtained \(\mu_{\text{max}}\) values (previously described), and the values of the parameters \(b\) and \(T_{\text{max}}\) were estimated. This expanded model (equation 1) was fitted using nonlinear regression and the software SigmaStat (Systat Software GmbH, Erkrath, Germany). The remaining 21 \(\mu_{\text{max}}\) values were used to evaluate the performance of the fitted model. An F-test was used to evaluate if terms for the different environmental parameters in the model had a significant effect on the \(\mu_{\text{max}}\) values of \(L.\) monocytogenes.

\[
\xi(\psi(T, a_w, \text{pH}, \text{[LAC]}, \text{NIT}, P, \text{CO}_2, \text{[DAC]})) = \begin{cases} 
1, & \psi \leq 0 \\
2(1 - \psi), & 0 < \psi < 1 \\
0, & \psi \geq 1 
\end{cases} 
\tag{2}
\]

where \(\xi(\psi(T, a_w, \text{pH}, \text{[LAC]}, \text{NIT}, P, \text{CO}_2, \text{[DAC]}))\) is the term describing the effect of interactions between environmental parameters on \(\mu_{\text{max}}\):

\[
\phi_{\psi} = \left[1 - \left(\frac{T - T_{\text{min}}}{T_{\text{ref}} - T_{\text{min}}}\right)^2\right]^2
\]

\[
\phi_{a_w} = (1 - \sqrt{(a_w - 0.923)/0.077})^2
\]

\[
\phi_{\text{pH}} = (1 - \sqrt{1 - 10^{(0.497 - \text{pH})}})^2
\]

\[
\phi_{\text{NIT}} = (1 - (350 - \text{NIT}/350))^2
\]

\[
\phi_{[\text{LAC}]/[\text{DAC}]} = \left[1 - \left(1 - \sqrt{[\text{LAC}]/[\text{DAC}]}\right)/3.79\right]
\times \left(1 - \sqrt{[\text{MIC}_{[\text{LAC}]/[\text{DAC}]}]/[\text{MIC}_{[\text{LAC}]/[\text{DAC}]}]}\right)^2
\]

\[
\phi_P = (1 - \sqrt{(28.1 - P)/28.1})^2
\]

\[
\phi_{\text{CO}_2} = (1 - \sqrt{(3,140 - \text{CO}_2)/3,140})^2
\]

\[
\psi = \sum_{j=1}^{2} \prod_{i=1}^{2} (1 - \phi_{\psi})
\tag{4}
\]

The contribution of lactate and diacetate to the interaction term \((\phi_{[\text{LAC}]/[\text{DAC}]})\) in equation 3 was modeled by the multiplication of their effects as previously described (8).

The model recently suggested by Augustin et al. (5) was used to predict the effect of temperature, water phase salt or \(a_w\), pH, nitrite, smoke components (phenol), CO2, and interactions between these environmental parameters on the growth rate \((\mu_{\text{max}})\) value of \(L.\) monocytogenes. In addition, this model was expanded by lactate and diacetate terms from equation 1 and the contribution of these organic acids to the effect of interaction between environmental parameters (equations 3 and 4).

Growth and no-growth responses of \(L.\) monocytogenes in lightly preserved seafood were obtained, together with product characteristics from the present study \((n = 24)\), from challenge tests reported in the literature \((n = 34)\) and from 13 lots of naturally contaminated cold-smoked salmon \((11, 35, 43, 50)\). A no-growth response was defined as an increase in \(L.\) monocytogenes concentration that was less than 0.5 log CFU g\(^{-1}\) for the duration of the experiment. These growth and no-growth data were compared with predictions from two growth boundary models: (i) the expanded and calibrated model from the present study (equation 1) and (ii) model \#5 from Augustin et al. (5). In addition, the growth and no-growth data \((n = 71)\) were compared with the growth probability models, including the effect of (i) temperature, \(a_w\), and pH \((22, 23, 27)\); (ii) temperature, \(a_w\), pH, and lactic acid \((45)\); and (iii) salt, moisture, lactate, and diacetate \((25)\). Growth and no-growth responses were defined as probability values above 0.5 and below 0.5, respectively. The model of Legan et al. (25), developed for the prediction of the growth and no-growth response at 4°C and expressed as the time to a 1-log (CFU per gram) increase of \(L.\) monocytogenes, was compared only with experiments carried out at this specific temperature \((n = 6)\). Predicted and observed growth and no-growth responses were compared by calculating the correct prediction percentage that corresponded to the percentage of all samples that were correctly predicted (19). Furthermore, the positive predictive value, representing the probability that growth is observed when growth is predicted, and the negative predictive value, representing the probability that the no-growth response is observed when a no-growth response is predicted, were estimated (5).

RESULTS

Product characteristics and storage conditions. Table 1 shows the characteristics and storage conditions of the products and subbatches studied. The concentration of naturally occurring water phase lactate was substantially higher in cold-smoked salmon, marinated salmon, and gravid salmon than in cold-smoked Greenland halibut and marinated Greenland halibut \((P < 0.01)\), and the content of smoke components (phenol) was significantly lower \((P < 0.01)\) in marinated salmon, marinated Greenland halibut, and gravid salmon than in cold-smoked salmon and cold-smoked Greenland halibut (Table 1). The addition of 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate had no adverse effects on the sensory characteristics of the samples, whereas the addition of 0.2% (wt/wt) diacetate and 2.0% (wt/wt) lactate seemed to affect the texture of the products negatively (data not shown).

Microbiological changes. No lag phases of \(L.\) monocytogenes were observed in VP and MAP products without added diacetate (Fig. 1 and Table 2). MAP, when compared with VP, significantly reduced both \(\mu_{\text{max}}\) and the maximum population density (expressed as log CFU per gram) of \(L.\) monocytogenes in cold-smoked salmon \((P < 0.01)\), whereas this was not observed for cold-smoked Greenland halibut \((P = 0.15\) to 0.69). \(L.\) monocytogenes grew significantly \((P < 0.01)\) faster in cold-smoked Greenland halibut than in cold-smoked salmon, and MAP extended the time to a 100-fold increase in the concentration of \(L.\) monocytogenes by approximately 70 to 75% in cold-smoked salmon but by only 23% in cold-smoked Greenland halibut (Table 2).

Importantly, the addition of 0.15% (wt/wt) diacetate prevented the growth of \(L.\) monocytogenes for more than 40 days at 8°C in MAP cold-smoked salmon with 0.77 to 0.98% water phase lactate (Fig. 1 and Table 2). The pathogen grew without a significant lag phase in MAP cold-smoked Greenland halibut with added 0.15% (wt/wt) diacetate (Table 2), but the naturally occurring water phase lactate concentration of this product was only 0.13 to 0.15% (Table 1). In cold-smoked Greenland halibut with 0.79 to 1.03% water phase lactate, the addition of 0.15% (wt/wt) diacetate was sufficient to prevent the growth of \(L.\) mono-
cytogenes for more than 45 days at 8°C (Table 2). The addition of 0.15% (wt/wt) diacetate strongly inhibited the growth of L. monocytogenes in MAP cold-smoked salmon (0.60% water phase lactate) stored at 15°C, and its concentration increased only by 1.3 log (CFU per gram) during 14 days of storage. In MAP marinated Greenland halibut, the growth of L. monocytogenes was not affected by the addition of 0.15% (wt/wt) diacetate and 0.9% (wt/wt) lactate (Table 2). In MAP gravad salmon with 0.62% water phase lactate, the addition of 0.15% (wt/wt) diacetate had only a limited inhibitory effect on the growth of L. monocytogenes at 15°C (Table 2).

The initial concentrations of LAB varied between 1.5 ± 0.1 and 2.6 ± 1.4 log (CFU per gram) in cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, and marinated Greenland halibut. In gravad salmon, the initial concentration of LAB was 3.4 ± 0.1 log (CFU per gram). MAP, when compared with VP, had no significant effect (P = 0.06 to 0.29) on the μmax values of LAB, but it significantly reduced (P < 0.01) the maximum population density (expressed as log CFU per gram) in cold-smoked salmon and cold-smoked Greenland halibut. The addition of 0.15% (wt/wt) diacetate to MAP cold-smoked salmon prevented the growth of LAB for more than 25 days at 8°C, and only a small increase in the concentration of LAB and no increase in aerobic plate counts were observed after 40 days (Table 2). The inhibitory effect of 0.15% (wt/wt) diacetate against LAB was reduced in MAP cold-smoked salmon at 15°C when compared with storage at 8°C (Table 2). In MAP cold-smoked Greenland halibut, the growth of LAB and aerobic plate counts was observed in subbatches added 0.15% (wt/wt) diacetate and as much as 1.5% (wt/wt) lactate (Table 2).

Modeling and predicting the growth of L. monocytogenes in lightly preserved seafood. The average MIC of diacetate (MIC_{diacetate}) against L. monocytogenes in liquid medium was established in the present study as 4.8 ± 0.3 mM undissociated diacetate at 8°C. No significant differences (P > 0.05) in the MICs of diacetate were found between the four examined isolates of L. monocytogenes.

The addition of terms for the antilisterial effect of CO2 and diacetate to the model of Giménez and Dalgaard (17) slightly improved the model and changed the bias and accuracy factor values from 1.4 and 1.8 to 1.3 and 1.7 (Table 3). However, the bias factor values of both the original (17) and the expanded model increased as a function of the storage temperature (Table 3). This effect of storage temperature on the bias factor was eliminated by the fitting of equation 1 to 39 of the 60 obtained μmax values and a constant (b value) of 0.6802, and a T_{min} value of −2.3°C was estimated with a standard error of 1.0°C (Table 3). The fitting of equation 1 showed a significant effect (P < 0.01) of temperature, smoke components (phenol), diacetate, and water phase salt or a_w on the growth rate (μ_{max}) of L. monocytogenes. Average bias and accuracy factor values for the fitted model (equation 1) were 1.0 and 1.5 (Table 3) for the data used to determine T_{min} and b and 1.1 and 1.6 for the 21 μ_{max} values used to validate the growth rate model (Table 3). The model of Augustin et al. (5), including the effect of temperature, pH, a_w, nitrite, phenol, CO2, and interactions between the parameters, resulted in bias and accuracy factor values of 0.7 and 1.9. However, bias and accuracy factor values of 0.4 and 2.6 were obtained when the model of Augustin et al. (5) was expanded by terms that modeled the effect of lactate and diacetate, as in equation 1 (data not shown).

The growth and no-growth model (equation 1) developed in the present study, including the effect of temperature, water phase salt or a_w, pH, lactate, nitrite, CO2, smoke components (phenol), diacetate, and the interactions between all these parameters (ξ), clearly performed better than previously developed models that predicted the growth boundary of L. monocytogenes (Table 4). The proposed model accurately predicted whether the growth of L. monocytogenes was observed or not in 96% of the tested experiments (n = 71), which represented different types of lightly preserved seafood (Fig. 2 and Table 4). In comparison, the models of Tienungoon et al. (45), Koutsoumanis and Sofos (22, 23), Le Marc et al. (27), and Augustin et al. (5) resulted in correct prediction percentages of 68, 68, 68, 51, and 70, respectively. Predictions by the model of Legan et al. (25) resulted in a correct prediction percentage of 83 when applied to experiments carried out at 4°C. Importantly, when no growth was predicted by the model of the present study, no growth was actually observed, resulting in a negative predictive value of 100%. In contrast, the models of Legan et al. (25), Le Marc et al. (27), and Augustin et al. (5) in some cases predicted a no-growth response when growth was actually observed in cold-smoked salmon and cold-smoked Greenland halibut. The developed model (equation 1) predicted the growth of L. monocytogenes in the 13 naturally contaminated batches of cold-smoked salmon, although a no-growth response was actually observed in three of the lots (Table 4).
TABLE 2. Kinetic parameters describing the growth of Listeria monocytogenes and lactic acid bacteria (LAB) in cold-smoked salmon (CSS), marinated salmon (MS), cold-smoked Greenland halibut (CSGH), marinated Greenland halibut (MGH), and gravad salmon (GS) at 8°C, 15°C, or both

<table>
<thead>
<tr>
<th>Products and subbatches (code)</th>
<th>Listeria monocytogenes</th>
<th>Lactic acid bacteria (LAB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lag phase (days)</td>
<td>μ&lt;sub&gt;max&lt;/sub&gt; (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>CSS-1 (a)</td>
<td>NS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.021 ± 0.002</td>
</tr>
<tr>
<td>CSS-1 (b)</td>
<td>NS</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>CSS-1 (c)</td>
<td>&gt;40</td>
<td>0</td>
</tr>
<tr>
<td>CSS-1 (d)</td>
<td>&gt;40</td>
<td>0</td>
</tr>
<tr>
<td>CSS-2 (a)</td>
<td>NS</td>
<td>0.038 ± 0.003</td>
</tr>
<tr>
<td>CSS-2 (b)</td>
<td>NS</td>
<td>0.012 ± 0.000</td>
</tr>
<tr>
<td>MS-1 (a)</td>
<td>NS</td>
<td>0.032 ± 0.002</td>
</tr>
<tr>
<td>MS-1 (b)</td>
<td>NS</td>
<td>0.027 ± 0.002</td>
</tr>
<tr>
<td>MS-1 (c)</td>
<td>NS</td>
<td>0.021 ± 0.006</td>
</tr>
<tr>
<td>MS-1 (d)</td>
<td>NS</td>
<td>0.018 ± 0.002</td>
</tr>
<tr>
<td>MS-1 (e)</td>
<td>NS</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>MS-1 (f)</td>
<td>NS</td>
<td>0.005 ± 0.002</td>
</tr>
<tr>
<td>CSGH-1 (a)</td>
<td>NS</td>
<td>0.029 ± 0.003</td>
</tr>
<tr>
<td>CSGH-1 (b)</td>
<td>NS</td>
<td>0.026 ± 0.001</td>
</tr>
<tr>
<td>CSGH-1 (c)</td>
<td>&gt;40</td>
<td>0</td>
</tr>
<tr>
<td>CSGH-2 (a)</td>
<td>NS</td>
<td>0.006 ± 0.001</td>
</tr>
<tr>
<td>CSGH-2 (b)</td>
<td>12</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>CSGH-2 (c)</td>
<td>28</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>CSGH-2 (d)</td>
<td>&gt;45</td>
<td>0</td>
</tr>
<tr>
<td>CSGH-2 (e)</td>
<td>&gt;45</td>
<td>0</td>
</tr>
<tr>
<td>MGH-1 (a)</td>
<td>NS</td>
<td>0.044 ± 0.006</td>
</tr>
<tr>
<td>MGH-1 (b)</td>
<td>NS</td>
<td>0.039 ± 0.004</td>
</tr>
<tr>
<td>GS-1 (a)</td>
<td>NS</td>
<td>0.105 ± 0.005</td>
</tr>
<tr>
<td>GS-1 (b)</td>
<td>NS</td>
<td>0.034 ± 0.006</td>
</tr>
</tbody>
</table>

<sup>a</sup> MPD, maximum population density within the storage period.

<sup>b</sup> Lag phase was not significant (NS) (<i>P</i> > 0.05).

<sup>c</sup> —, not significantly different from the inoculation level.

<sup>d</sup> ND, not determined as no growth was observed.
TABLE 3. Observed and predicted maximum specific growth rates for *Listeria monocytogenes* in VP and MAP cold-smoked salmon (CSS), marinated salmon (MS), cold-smoked Greenland halibut, marinated Greenland halibut and gravad salmon as evaluated by bias and accuracy factors

<table>
<thead>
<tr>
<th>Reference</th>
<th>n</th>
<th>Temp (°C)</th>
<th>Water phase salt (% WPS)</th>
<th>pH</th>
<th>aw&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phenol (ppm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Nitrite (ppm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% CO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Water phase lactate (%)</th>
<th>Water phase diacetate (%)</th>
<th>Gimez&lt;sup&gt;17&lt;/sup&gt; and Dalgaard model&lt;sup&gt;d&lt;/sup&gt; (b = 0.1478, T&lt;sub&gt;min&lt;/sub&gt; = 0.88°C)</th>
<th>Expanded Gimez&lt;sup&gt;17&lt;/sup&gt; and Dalgaard model&lt;sup&gt;d&lt;/sup&gt; (b = 0.1478, T&lt;sub&gt;min&lt;/sub&gt; = 0.88°C)</th>
<th>Equation 1&lt;sup&gt;e&lt;/sup&gt; (b = 0.6802, T&lt;sub&gt;min&lt;/sub&gt; = −2.3°C)</th>
<th>Augustin et al.&lt;sup&gt;f&lt;/sup&gt; (5) model #5</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>6</td>
<td>7.6–7.8</td>
<td>3.74–5.26</td>
<td>6.0–6.5</td>
<td>0.969–0.978</td>
<td>8.9–20.1</td>
<td>ND</td>
<td>0–30</td>
<td>0.10–0.98</td>
<td>0.0–0.14</td>
<td>1.4/1.6</td>
<td>1.1/1.3</td>
<td>0.6/1.7</td>
<td>0.1/9.3</td>
</tr>
<tr>
<td>This study</td>
<td>4</td>
<td>14.3</td>
<td>2.95–5.26</td>
<td>5.9–6.5</td>
<td>0.969–0.983</td>
<td>4.8–20.1</td>
<td>ND</td>
<td>0–30</td>
<td>0.10–0.98</td>
<td>0.0–0.17</td>
<td>3.4/3.4</td>
<td>2.2/2.2</td>
<td>1.2/1.4</td>
<td>1.2/1.7</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>5</td>
<td>3–6</td>
<td>6.2</td>
<td>0.964–0.983</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0.70&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
<td>1.0/1.6</td>
<td>1.0/1.6</td>
<td>1.1/1.6</td>
<td>1.0/1.6</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>10</td>
<td>3–6</td>
<td>6.2</td>
<td>0.964–0.984</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0.70&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
<td>1.5/1.5</td>
<td>1.5/1.5</td>
<td>1.0/1.3</td>
<td>0.9/1.3</td>
</tr>
<tr>
<td>44</td>
<td>6</td>
<td>4</td>
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</tr>
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<td>0.6/1.6</td>
</tr>
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<td>0.70&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>1.9/2.7</td>
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<td>0.6/1.6</td>
<td>0.5/1.9</td>
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<tr>
<td>24</td>
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<td>4.92</td>
<td>6.1</td>
<td>0.971</td>
<td>14.6</td>
<td>ND</td>
<td>0</td>
<td>0.59</td>
<td>—</td>
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<td>6.03</td>
<td>0.971</td>
<td>12.6</td>
<td>ND</td>
<td>0</td>
<td>0.37</td>
<td>—</td>
<td>2.3/2.3</td>
<td>2.3/2.3</td>
<td>2.4/2.4</td>
<td>—/—</td>
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<tr>
<td>17</td>
<td>1</td>
<td>10</td>
<td>4.9</td>
<td>6.03</td>
<td>0.971</td>
<td>12.6</td>
<td>ND</td>
<td>0</td>
<td>0.37</td>
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<td>1.5/1.5</td>
<td>0.6/1.8</td>
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<td>17.5</td>
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<td>6.03</td>
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<td>12.6</td>
<td>ND</td>
<td>0</td>
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<td>—</td>
<td>1.9/1.9</td>
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<td>1.0/1.1</td>
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<td>Average bias/accuracy factor values</td>
<td>1.4/1.8</td>
<td>1.3/1.7</td>
<td>1.0/1.5</td>
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</table>

Data for model validation

<table>
<thead>
<tr>
<th>Reference</th>
<th>n</th>
<th>Temp (°C)</th>
<th>Water phase salt (% WPS)</th>
<th>pH</th>
<th>aw&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phenol (ppm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Nitrite (ppm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% CO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Water phase lactate (%)</th>
<th>Water phase diacetate (%)</th>
<th>Gimez&lt;sup&gt;17&lt;/sup&gt; and Dalgaard model&lt;sup&gt;d&lt;/sup&gt; (b = 0.1478, T&lt;sub&gt;min&lt;/sub&gt; = 0.88°C)</th>
<th>Expanded Gimez&lt;sup&gt;17&lt;/sup&gt; and Dalgaard model&lt;sup&gt;d&lt;/sup&gt; (b = 0.1478, T&lt;sub&gt;min&lt;/sub&gt; = 0.88°C)</th>
<th>Equation 1&lt;sup&gt;e&lt;/sup&gt; (b = 0.6802, T&lt;sub&gt;min&lt;/sub&gt; = −2.3°C)</th>
<th>Augustin et al.&lt;sup&gt;f&lt;/sup&gt; (5) model #5</th>
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<td>This study</td>
<td>8</td>
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<td>3.40–3.67</td>
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<td>0</td>
<td>ND</td>
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<td>0.17–0.62</td>
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<td>2.4/2.4</td>
<td>1.7/1.7</td>
<td>1.3/1.4</td>
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<tr>
<td>35</td>
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<td>0–145</td>
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<td>0–2.01&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>0.7/1.9</td>
<td>1.9/1.9</td>
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<td>6.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
<td>0–100&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>1.0/1.7</td>
<td>0.9/1.3</td>
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<td>4</td>
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<td>3.5&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>0.980&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
<td>0</td>
<td>0–0.84&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0–0.06&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.3/3.4</td>
<td>1.2/2.4</td>
<td>1.4/1.7</td>
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<td>Average bias/accuracy factor values</td>
<td>2.0/2.5</td>
<td>1.6/2.0</td>
<td>1.1/1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Relation between percentage of water phase salt (% WPS) and water activity (aw<sub>∞</sub>) calculated as aw<sub>∞</sub> = 1 − 0.0052471 × % WPS − 0.00012206 × % WPS² (37).

<sup>b</sup>Measured but not detected (ND).

<sup>c</sup>Equilibrium concentrations in headspace gas.

<sup>d</sup>Expanded with the CO₂ term of Devlieghere et al. (15) and the acetate term of Le Marc et al. (26). See Equation 1.

<sup>e</sup>If not reported in the literature, the following product characteristics were used for prediction of μ<sub>max</sub> values in CSS: 3.5% water phase salt (aw<sub>∞</sub> = 0.980), pH 6.1, 6 ppm of phenol, and 0.70% water phase lactate (natural content of lactate in CSS).

<sup>f</sup>No growth predicted by the model.

<sup>g</sup>Added concentrations (% wt/wt) of diacetate, lactate, or both to the products.

<sup>h</sup>Initial concentrations of CO₂ (equilibrium concentrations not reported in the study).
## TABLE 4. Growth and no growth responses of *Listeria monocytogenes* in lightly preserved seafood as predicted by growth boundary models and probability models

<table>
<thead>
<tr>
<th>Reference</th>
<th>Products Packaging</th>
<th>Temp (°C)</th>
<th>Water phase salt (%)</th>
<th>pH</th>
<th>Phenol (ppm)</th>
<th>Sodium nitrite (ppm)</th>
<th>Added (% wt/wt)</th>
<th>Observed (growth or no growth)</th>
<th>Growth boundary models</th>
<th>No. of expts correctly predicted</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Inoculated</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Growth CSS, MS, CSGH, MGH, GS</td>
<td>8–15</td>
<td>3.03–5.26</td>
<td>59–6.8</td>
<td>0.0–20.1</td>
<td>0–15</td>
<td>0–0.9</td>
<td>19</td>
<td>Equation 1 Augustin et al. (5) model #5b</td>
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<tr>
<td></td>
<td>No growth CSS, CSGH</td>
<td>8</td>
<td>3.74–5.25</td>
<td>5.8–6.2</td>
<td>8.9–20.1</td>
<td>ND</td>
<td>0.15</td>
<td>0–1.5</td>
<td>5</td>
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<tr>
<td></td>
<td>Growth CPS</td>
<td>VP</td>
<td>5–10</td>
<td>2–3</td>
<td>6.1</td>
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<td>0–145</td>
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<td>0–2.01</td>
<td>11</td>
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<tr>
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<td>0–145</td>
<td>—</td>
<td>1.61–2.41</td>
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<tr>
<td></td>
<td>Growth CSS VP</td>
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<td>—h</td>
<td>6.3</td>
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<td>—h</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>No growth CSS MAP</td>
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<td>6.3</td>
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<td>Growth CSS VP</td>
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<td>—h</td>
<td>0–0.06</td>
<td>0–0.84</td>
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<tr>
<td></td>
<td>No growth CSS VP</td>
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<td>—h</td>
<td>—h</td>
<td>0.05–0.20</td>
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<tr>
<td></td>
<td>Growth CSS VP</td>
<td>5</td>
<td>3.5–5.8</td>
<td>6.1–6.3</td>
<td>—h</td>
<td>ND</td>
<td>—</td>
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<tr>
<td></td>
<td>No growth CSS VP</td>
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<td>6.2–6.3</td>
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<td>0–60</td>
<td>—</td>
<td>0.88–1.11</td>
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</tr>
</tbody>
</table>

| Correct prediction percentage (CPP) | 96 | 70 | 68 | 68 | 51 |
| Positive predictive value (PPV) | 94 | 72 | 67 | 67 | 64 |
| Negative predictive values (NPV) | 100 | 64 | 100 | 100 | 31 |

*a* If not reported, measured samples were assumed to contain 0.70% water phase lactate (natural content of lactate in CSS) in addition to the added amount of lactate.

*b* Includes the effect of temperature, pH, aw, nitrite, phenol, CO2, and interactions between parameters.

*c* Predicts the probability of growth expressed as values between 0 and 1 (0–100% probability of growth). Growth or no-growth were predicted when the growth probabilities were above 0.5 or below 0.5, respectively.

*d* Includes the effect of temperature, pH, aw, and lactic acid.

*e* Includes the effect of temperature, pH, aw, and interactions between the parameters.

*f* Not detected in the product.

*g* Cold-processed salmon (CPS). Produced as cold-smoked salmon without the smoking procedure.

*h* If not reported, measured products were assumed to contain 3.5% water phase salt (aw = 0.980), 6 ppm of phenol, and 0 ppm of sodium nitrite.

*i* Recalculated from the added concentrations of PURASAL P OptiForm 4 containing 4% sodium diacetate and 56% potassium lactate (60% [vol/vol] lactate syrup).

*j* CPP, percentage of all samples that were correctly predicted; PPV, representing the probability that growth is observed when growth is predicted; NPV, representing the probability that no growth is observed when no growth is predicted.
FIGURE 2. Comparison of predicted growth boundaries and observed growth and no-growth responses of *Listeria monocytogenes* in MAP cold-smoked Greenland halibut (a), MAP cold-smoked salmon (b), and vacuum-packaged (VP) cold-smoked salmon stored at 4 and 10°C (c and d). Growth data of VP cold-smoked salmon stored at 4 and 10°C were reported by Yoon et al. (50). Open and solid symbols represent no-growth and growth of *L. monocytogenes*, respectively.

**DISCUSSION**

The growth boundary model developed in the present study correctly predicted 68 of 71 growth and no-growth responses in lightly preserved seafood, but growth was predicted for three lots of naturally contaminated cold-smoked salmon where no-growth was observed (Table 4). The model performed substantially better than the existing *L. monocytogenes* growth boundary models (5, 25, 27, 45) (Table 4). The model of Legan et al. (25) also seemed promising, but its use is restricted to a storage temperature of 4°C only. The model developed in the present study included the effect of temperature, water phase salt or aw, pH, lactate, nitrite, phenol, CO₂, and diacetate as well as interactions between these parameters (equation 1), and its superior performance when compared with the existing and less complex models is most likely due to an important inhibiting effect of all these parameters with respect to the growth boundary of *L. monocytogenes* in lightly preserved seafood.

In contrast to the present study, Augustin et al. (5) found the growth of *L. monocytogenes* in lightly preserved seafood to be better predicted when the effect of phenol was excluded from their model. We observed a pronounced effect of smoke components on the growth boundary of *L. monocytogenes*, particularly in products with more than 10 ppm of phenol (Fig. 3d). Phenol concentrations above 10 ppm are common in smoked seafood (16, 17, 24, 29). The phenol term in equation 1 therefore is important to predict both the growth (*P < 0.01*) and the growth boundary of *L. monocytogenes* in smoked seafood, and in the present study, this was shown specifically for cold-smoked Greenland halibut with 16 to 20 ppm of phenol (Tables 1 and 4). In fact, smoke components (phenol) can be used together with sensorially acceptable concentrations of diacetate in an attempt to control the growth of *L. monocytogenes* in lightly preserved seafood (Table 5, line 5).

Within predictive food microbiology, growth boundary models have most often been developed from large sets of growth and no-growth data typically obtained from broth cultures in microwell plates and by polynomial models, logistic regression, or artificial neural networks (39). The present study used an alternative approach suggested by Le Marc et al. (26). The idea behind this approach is to divide the space of environmental parameters into three regions where the effect of interaction between the environmental...
FIGURE 3. Effect of temperature (a), water phase salt (WPS) (b), pH (c), and phenol (d) on the growth and no-growth boundary of Listeria monocytogenes at different concentrations of diacetate and lactate. Default parameters used were 8°C, pH 6.0, 3.5% water phase salt, 10 ppm of phenol, 25% CO₂ (equilibrium), and 0 ppm of nitrite.

parameters is expressed by the parameter \( \xi \); this interaction term can prevent growth (\( \xi = 0 \)), reduce growth rate (\( 0 < \xi < 1 \)), or have no effect on growth rate (\( \xi = 1 \)) (equation 2). The Le Marc approach to the development of growth boundary models thus relies on growth rate data. For L. monocytogenes, very large amounts of such data and existing growth rate models are available, and this facilitated the development of a growth boundary model for L. monocytogenes in the present study. We used \( \mu_{\text{max}} \) values for L. monocytogenes from challenge tests with lightly preserved seafood and in this way developed a product-specific growth boundary model: (i) an existing model for the growth of L. monocytogenes was expanded to include the effect of diacetate, CO₂, phenol, nitrite, lactate, pH, water phase salt or \( a_w \), and temperature; (ii) a term to take into account the inhibitory effect of the interaction between the environmental parameters was added (equation 1); and (iii) the model was calibrated to growth data (\( \mu_{\text{max}} \) values) ob-

### TABLE 5. Combinations of environmental parameters preventing growth of Listeria monocytogenes as predicted by the growth boundary model

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Water phase salt (%)</th>
<th>pH</th>
<th>Phenol (ppm)</th>
<th>Nitrite (ppm)</th>
<th>CO₂ (%)</th>
<th>Water phase lactate (%)</th>
<th>Water phase diacetate (%)</th>
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</thead>
<tbody>
<tr>
<td>5.0</td>
<td>4.5</td>
<td>6.0</td>
<td>13</td>
<td>0</td>
<td>25</td>
<td>0.70</td>
<td>0.07</td>
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<td>6.0</td>
<td>13</td>
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<td>0.70</td>
<td>0.22</td>
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<td>6.0</td>
<td>6.0</td>
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<td>25</td>
<td>0.70</td>
<td>0.16</td>
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<tr>
<td>8.0</td>
<td>4.5</td>
<td>5.9</td>
<td>13</td>
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<td>0.70</td>
<td>0.14</td>
</tr>
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<td>98</td>
<td>0.70</td>
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</table>

* Equilibrium concentrations in headspace gas.
tained for *L. monocytogenes* in challenge tests with lightly preserved seafood with well-characterized product characteristics and storage conditions (Table 3). Subsequently, the developed model was used to predict the growth and no-growth of *L. monocytogenes* in lightly preserved seafood in order to validate the growth boundary model.

For RTE foods, EU regulations (EC 2073/2005) include a critical limit (m = M) of 100 CFU g⁻¹ for *L. monocytogenes* if a product is unable to support the growth of this pathogen. To determine if lightly preserved seafood, depending on product characteristics and storage conditions, is able or unable to support the growth of *L. monocytogenes*, the model developed in the present study seems most useful, particularly because critical control points for *L. monocytogenes* have not been identified during the processing and chilled distribution of VP cold-smoked salmon and similar lightly preserved seafood (49). The present study has documented that the combined effect of several product characteristics is predictable and that it can be used to prevent the growth of *L. monocytogenes* in lightly preserved seafood. Thus, the developed growth boundary model facilitates a selection of relevant product characteristics and storage conditions useful as critical control points for different seafood (Table 5). The developed growth boundary model can be used actively by the seafood industry in connection with product development and quality control of existing products as a means of ensuring that products comply with the EU regulation on RTE foods. Furthermore, if documentation of growth and no-growth responses is needed by the authorities or retailers, the model can be used to predict if *L. monocytogenes* is able or unable to grow in a given product. The more general use of the developed model was shown when previously estimated growth rates (n = 6) of *L. monocytogenes* in cooked and peeled MAP shrimps (32) were compared with predictions of equation 1, resulting in bias and accuracy factor values of 0.9 and 1.1. Product characteristics of cooked and peeled MAP shrimps (<2% water phase salt, pH 7.5, and 0.05% water phase lactate) differ substantially from the characteristics of the products examined in the present study (Table 1). On the basis of our product validation studies, the range of applicability for equation 1 seems to include temperature (2 to 15°C), water phase salt (2 to 9%), pH (5.8 to 7.5), water phase lactate (<3.0%), smoke components (phenol) (<20 ppm), CO₂ equilibrium (0 to 100%), and water phase diacetate (<0.2%). The number of studies that examine the effect of nitrite was not sufficient to establish a reliable range of applicability for this preservative.

The suggested model is complex, and to improve its usefulness and value to the seafood sector, it should be incorporated in application software such as the Seafood Spoilage and Safety Predictor (available at: http://www.difres.dk/micro/ssp/).

The developed growth boundary model is fail-safe and has a relatively wide range of applicability, as validation studies have included seafood with varied product characteristics and inoculations with different cocktails of *L. monocytogenes* with a total of 13 strains (Table 4). The growth boundary of *L. monocytogenes* as a function of environmental parameters depends on the initial concentration of the pathogen (23). The low initial concentrations of *L. monocytogenes* in naturally contaminated cold-smoked salmon (11) may therefore explain why growth was predicted in three batches of the product when a no-growth response was actually observed (Table 4). Furthermore, differences in the physiological state between strains of *L. monocytogenes* on naturally contaminated and inoculated products might explain these results. Additional product validation of a predictive model is always desirable, and evaluation of different *L. monocytogenes* isolates, particularly evaluation of naturally contaminated seafood with new combinations of environmental parameters, will benefit the growth boundary model developed in the present study. To improve the suggested model, the calibration carried out in the present study to obtain a T<sub>min</sub> value of −2.3°C can be performed for other environmental parameters. This, however, requires a significant amount of new growth data with corresponding and carefully determined product characteristics and storage conditions. Furthermore, the inhibiting effect of the dominating spoilage microbiota, i.e., the Jameson effect, should be taken into account when the growth of *L. monocytogenes* in lightly preserved seafood is predicted (17). To do so, models for the growth of LAB and possibly other spoilage microorganisms must be expanded with terms for relevant environmental parameters, including diacetate and smoke components (phenol), and validated in well-characterized, lightly preserved seafood.

Treatment of MAP cold-smoked salmon with diacetate provided an effective method to prevent the growth of *L. monocytogenes* at 8°C (Fig. 1 and Table 2). The growth of *L. monocytogenes* was reduced only in MAP cold-smoked Greenland halibut treated with diacetate, whereas a combination of diacetate and lactate prevented growth (Table 2). The higher content of naturally occurring lactate in cold-smoked salmon (0.77 to 0.98% water phase lactate) when compared with cold-smoked Greenland halibut (0.13 to 0.15% water phase lactate) explained this difference between the products. The capability of equation 1 to predict the combined effect of diacetate and lactate on the growth boundary of *L. monocytogenes* in lightly preserved seafood and the importance of a thorough product characterization were obvious when the results of cold-smoked Greenland halibut were compared (Fig. 2a). In cold-smoked Greenland halibut with 0.15% (wt/wt) diacetate and 0.5% (wt/wt) lactate, growth of *L. monocytogenes* was observed, whereas a no-growth response was observed in samples with 0.15% (wt/wt) diacetate and 0.75% (wt/wt) lactate. In spite of these small differences in product characteristics, the model of the present study correctly predicted whether growth was observed or not (Fig. 2a).

The antimicrobial effect of diacetate and lactate against *L. monocytogenes* has previously been documented for different types of meat products (18, 31, 40, 41). Glass et al. (18) showed that 0.1% sodium diacetate and 1.0% sodium lactate prevented the growth of *L. monocytogenes* in smoked wieners for 60 days at 4.5°C. In beef bologna, the growth of *L. monocytogenes* was prevented for 45 days at 4°C by the addition of 0.2% sodium diacetate and 2.5%
sodium lactate (31), and in wiener and smoked-cooked ham treated with 0.15% sodium diacetate and 1.5% potassium lactate, comparable antimicrobial effects were observed for 18 weeks at 4°C (40). Stekeelenburg (41) showed that the growth of L. monocytogenes was inhibited at 4°C in frankfurter sausage treated with 0.1% sodium diacetate. The natural content of lactate in frankfurter sausage was 0.68% potassium lactate corresponding to 0.47% lactate. However, treatment of frankfurter sausage with 0.1% sodium diacetate and 1.4% potassium lactate prevented the growth of L. monocytogenes for >28 days at 4°C, once again confirming that a certain amount of lactate has to be present to prevent the growth of L. monocytogenes in products treated with sensorially acceptable concentrations of diacetate. The model of the present study was evaluated on data from meat products (n = 23), including the growth and no-growth responses of L. monocytogenes and product characteristics (18, 41). The correct prediction percentage of the newly developed model was 83 (data not shown). In comparison, the model of Augustin et al. (5) had a correct prediction percentage of 53.

Only recently have studies been conducted that examine the antimicrobial effect of diacetate and lactate in lightly preserved seafood. Yoon et al. (50) showed that treatment of VP cold-smoked salmon with 0.06% sodium diacetate and 0.84% potassium lactate prevented the growth of L. monocytogenes for 32 days at 4°C, whereas 0.2% sodium diacetate and 2.8% potassium lactate were needed to prevent the growth of L. monocytogenes at 10°C. Previous studies have established a similar relationship between the antimicrobial effect of diacetate-lactate and the applied storage temperature (6, 30, 31). Results of the present study showed a pronounced antimicrobial effect of diacetate in MAP cold-smoked salmon stored at 15°C. In MAP cold-smoked salmon treated with 0.15% (wt/wt) diacetate, the growth of L. monocytogenes was prevented for 7 days at 15°C, and after 14 days, the concentration of L. monocytogenes had increased by only 1.3 log (CFU per gram) (Table 2). The effect of storage temperature on the antimicrobial effect of diacetate and lactate was obvious when the growth boundaries of L. monocytogenes were predicted (Fig. 3a).

Although MAP did not prevent the growth of L. monocytogenes, a substantial extension of the time to a 100-fold increase in the concentration of L. monocytogenes was observed in MAP cold-smoked salmon when compared with VP samples. The growth boundary model developed in the present study predicted that equilibrium concentrations of approximately 98% CO₂ were needed to prevent the growth of L. monocytogenes in MAP cold-smoked salmon with typical storage and product characteristics (Table 5, line 7). Szabo and Cahill (43) showed that the packaging of cold-smoked salmon in 100% CO₂ prevented the growth of L. monocytogenes at 4°C and reduced the growth at 10°C. The model of the present study (Table 4) accurately predicted the results obtained by Szabo and Cahill (43).

Model simulation showed a pronounced effect of pH on the growth boundary of L. monocytogenes. Smaller concentrations of diacetate, lactate, or both were needed to prevent the growth of L. monocytogenes at pH 5.8 when compared with pH values of 6.0 and 6.2 (Fig. 3c). This effect of pH is most likely explained by the higher concentration of undissociated lactic acid and diacetate molecules at pH 5.8. This hypothesis was confirmed by the results of the present study, showing no antimicrobial effect of 0.15% (wt/wt) diacetate and 0.9% (wt/wt) lactate against L. monocytogenes in MAP marinated Greenland halibut with pH 6.7 to 6.8 (Table 2). The impact of water phase salt or aw on the growth limit of L. monocytogenes was not as pronounced as noticed for the temperature, pH, and phenol (Fig. 3b).

In subbatches of cold-smoked salmon, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon supporting the growth of LAB, these bacteria constituted the dominating natural microflora. This is in agreement with previous studies of both VP and MAP cold-smoked salmon (20, 28, 34). The growth of LAB was strongly inhibited at 8°C in MAP cold-smoked salmon treated with 0.15% (wt/wt) diacetate, resulting in only a negligible increase in the concentration of LAB throughout the challenge test (Table 2). In contrast, treatment of MAP cold-smoked Greenland halibut with 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate inhibited the growth of LAB to a much lesser extent (Table 2). Stekeelenburg and Kant-Muermans (42) showed that treatment of VP, cooked, cured ham with 0.1% sodium diacetate had no antimicrobial effects against Lactobacillus curvatus at 4°C, whereas 0.2% sodium diacetate or 1.98% sodium lactate inhibited the growth of this microorganism. In VP frankfurter sausage, no antimicrobial effect of 0.1% sodium diacetate against Lactobacillus sake was observed, whereas 1.68% potassium lactate and 0.12% sodium diacetate or 1.8% potassium lactate inhibited growth at 4°C (41).

In addition to a pronounced antimicrobial effect against L. monocytogenes, the results of the present study indicate that diacetate and lactate can be used to prolong the sensory shelf life, especially of MAP cold-smoked salmon.

ACKNOWLEDGMENTS

We thank Carina Vigsø Christensen, Nadered Samieian, and Tina Dahl Devitt for skilful technical assistance. An early version of equation 1, including the effect of temperature, aw, pH, lactic acid, and nitrite, was posted on the Internet in 2000, and we thank Tom Ross from the University of Tasmania for this contribution. The research was financed by Royal Greenland Seafood Ltd. and the Directorate for Food, Fisheries, and Agriculture Business as part of the project “Lightly preserved seafood in modified atmosphere packaging—management of quality and safety,” 3401-FF-03-535.

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Paper 4

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Modeling and predicting the growth of lactic acid bacteria in lightly preserved seafood and their inhibiting effect on *Listeria monocytogenes*

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Modeling and Predicting the Growth of Lactic Acid Bacteria in Lightly Preserved Seafood and Their Inhibiting Effect on \textit{Listeria monocytogenes}

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ABSTRACT

A cardinal parameter model was developed to predict the effect of diacetate, lactate, \(\text{CO}_2\), smoke components (phenol), \(pH\), NaCl, temperature, and the interactions between all parameters on the growth of lactic acid bacteria (LAB) in lightly preserved seafood. A product-oriented approach based on careful chemical characterization and growth of bacteria in ready-to-eat seafood was used to develop this new LAB growth model. Initially, cardinal parameter values for the inhibiting effect of diacetate, lactate, \(\text{CO}_2\), \(pH\), and NaCl–water activity were determined experimentally for a mixture of LAB isolates or were obtained from the literature. Next, these values and a cardinal parameter model were used to model the effect of temperature \(T_{\text{max}}\) and smoke components \(P_{\text{max}}\). The cardinal parameter model was fitted to data for growth of LAB \(\mu_{\text{max}}\) values in lightly preserved seafood including cold-smoked and marinated products with different concentrations of naturally occurring and added organic acids. Separate product validation studies of the LAB model resulted in average bias and accuracy factor values of 1.2 and 1.5, respectively, for growth of LAB \(\mu_{\text{max}}\) values in lightly preserved seafood. Interaction between LAB and \textit{L. monocytogenes} was predicted by combining the developed LAB model and an existing growth and growth boundary model for the pathogen (O. Mejlholm and P. Dalgaard, \textit{J. Food Prot.} \textbf{70}:70–84). The performance of the existing \textit{L. monocytogenes} model was improved by taking into account the effect of microbial interaction with LAB. The observed and predicted maximum population densities of \textit{L. monocytogenes} in inoculated lightly preserved seafoods were 4.7 and 4.1 log CFU g\(^{-1}\), respectively, whereas for naturally contaminated vacuum-packed cold-smoked salmon the corresponding values were 0.7 and 0.6 log CFU g\(^{-1}\) when a relative lag time of 4.5 was used for the pathogen.

The shelf life of lightly preserved seafoods such as cold-smoked, sugar-salted (gravad), and marinated products with added organic acids must often is determined by growth of microorganisms that affect the sensory quality or safety of these foods. Specific spoilage organisms that actually cause sensory spoilage of products have been identified for a few lightly preserved seafoods only (10). Nevertheless, lactic acid bacteria (LAB) often are isolated and described as the dominating microflora of lightly preserved seafoods, and these bacteria have the potential to spoil some products (20, 22, 25, 30, 40, 52). Consequently, mathematical models for growth of LAB in lightly preserved seafood could be valuable for predicting concentrations of the dominating microflora during storage as a function of product characteristics and storage conditions. Mathematical models for growth of LAB as a function of temperature, water activity \(a_w\), \(pH\), \(\text{CO}_2\), and lactic acid concentration are available (17, 56). However, lightly preserved seafoods include smoked and marinated products, and smoke components (phenol), diacetate, and interactions between environmental parameters can have important effects on growth of LAB in these products (35, 40). An LAB growth model including all these parameters has not previously been developed.

High concentrations of LAB in lightly preserved seafood can inhibit growth of \textit{L. monocytogenes}, through a phenomenon called the Jameson effect (19, 48). Initial concentrations and the growth rates of LAB in lightly preserved seafood are generally higher than those of \textit{L. monocytogenes} (29, 40), which means that the maximum population density (MPD) of \textit{L. monocytogenes} is restricted by growth of LAB. Results of recent risk assessments suggest that preventing growth of \textit{L. monocytogenes} to high concentrations in foods would have a greater impact on the number of listeriosis cases than would lowering the prevalence of this pathogen (55). New European Union regulations for ready-to-eat seafood differentiate between products that are able and unable to support growth of \textit{L. monocytogenes} and allow 100 CFU g\(^{-1}\) in those unable to support growth (6). Predicting the growth of LAB, therefore, will contribute to more realistic evaluations of the potential for \textit{L. monocytogenes} to reach high concentrations in lightly preserved seafood. Recently, an \textit{L. monocytogenes} growth model including the effects of diacetate, lactate, \(\text{CO}_2\), smoke components (phenol), nitrite, \(pH\), NaCl–\(a_w\), temperature, and the interactions between all these parameters was developed and validated for lightly preserved seafoods (40). Expansion of this model to include microbial interaction...
with LAB would improve its performance and particularly improve its use for predicting the MPD of \textit{L. monocytogenes} in different lightly preserved seafoods, depending on product characteristics and storage conditions.

The objective of the present study was to model and predict the growth of LAB in lightly preserved seafood and the inhibiting effect of LAB on \textit{L. monocytogenes}. A new cardinal parameter model was developed to predict the effect of diacetate, lactate, CO$_2$, smoke components (phenol), pH, NaCl, temperature, and the interactions between all these parameters on the growth of LAB in lightly preserved seafood. This new model was developed by using a product-oriented approach. In addition, interaction between LAB and \textit{L. monocytogenes} was evaluated, and the effect of preculture conditions and inoculum size on the growth of \textit{L. monocytogenes} in cold-smoked salmon was studied.

**MATERIALS AND METHODS**

**Characterization and selection of LAB isolates.** Fifteen LAB isolates from the dominating microflora of lightly preserved seafood (Table 1) were identified and characterized. The objective was to find the fastest growing isolates for determination of MICs and for inoculation in challenge tests. The dominating microflora were isolated and initially characterized as previously described \cite{39}. Isolates were categorized as LAB if they were rods and cocci that were gram positive, fermentative, and catalase and cytochrome oxidase negative \cite{39}. Tests previously used and described were carried out for further characterization of LAB isolates \cite{14,39,57}, and isolates were identified (Table 1) according to Collins et al. \cite{5}, Mora et al. \cite{42}, and Stiles and Holzapfel \cite{51}. Reference and type strains of LAB previously used by Mejlholm et al. \cite{39} were included for comparison. In addition, partial 16S rDNA sequences (801 to 806 nucleotides) were determined for four selected isolates by Belgium Coordinated Collections of Microorganisms/Laboratorium Microbiologie Ghent (University of Ghent, Ghent, Belgium) as previously described \cite{53}. The obtained partial 16S rDNA sequences have been deposited in the EMBL, GenBank, and DDBJ nucleotide databases.

The maximum specific growth rate ($\mu_{\text{max}}$, h$^{-1}$) of each LAB isolate was determined at 8°C in APT broth (265510, Difco, Becton Dickinson, Sparks, Md.) with pH 6.0 and 3% NaCl. The $\mu_{\text{max}}$ values were determined from absorbance detection times of serially diluted cultures \cite{13}.

A mixture consisting of the fastest growing strain for each of the five identified LAB species (LAB mix; Table 1) was used for determination of MICs and inoculation in challenge tests. Individual isolates of LAB were grown at 25°C for 1 day in APT broth and subsequently precultured at 8°C for 2 to 3 days in APT broth at pH 6.0 and 4% NaCl. Isolates were precultured under these conditions to simulate the conditions of lightly preserved seafood. Precultures were harvested in the late exponential growth phase, defined as a relative change in absorbance of 0.05 to 0.2 at 540 nm (Novaspec II, Pharmacia Biotech, Allerød, Denmark). The inoculum was prepared by diluting each of the precultures in 0.85% NaCl to a cell density of 10$^7$ CFU ml$^{-1}$.

**Cardinal parameter values.** MICs of lactate and diacetate were determined for the LAB mix at 8°C in APT broth at pH 6.0. The effect of seven different concentrations of lactate (0.0, 0.48, 1.45, 2.42, 3.39, 4.35, and 5.32%, wt/vol) and diacetate (0.0, 0.42, 0.84, 1.26, 1.68, 2.10, and 2.52%, wt/vol) on absorbance detection times of the LAB mix was determined. Absorbance detection times were defined as the time (hours) needed to reach a relative change in absorbance of 0.05 at 540 nm (Bioscreen C, Labsystems, Helsinki, Finland). Sodium lactate (60%, wt/wt) syrup (L-1375, Sigma, St. Louis, Mo.) and sodium diacetate (98%, S1266, Spectrum, New Brunswick, N.J.) were used for the experiments. LAB inoculation levels of 10$^2$, 10$^3$, 10$^4$, and 10$^5$ CFU ml$^{-1}$ were used for each of the examined concentrations of lactate and diacetate. For each treatment, the growth of the LAB mix was

### Table 1. Identification and characterization of LAB isolates from lightly preserved seafood

<table>
<thead>
<tr>
<th>Isolate</th>
<th>$n$</th>
<th>Origin</th>
<th>$\mu_{\text{max}}$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Lactobacillus sakei}$^b$</td>
<td>4</td>
<td>Brined shrimp or cold-smoked Greenland halibut</td>
<td>0.081–0.091</td>
</tr>
<tr>
<td>\textit{L. curvatus}$^c$</td>
<td>5</td>
<td>Cold-smoked Greenland halibut</td>
<td>0.063–0.092</td>
</tr>
<tr>
<td>\textit{Carnobacterium maltaromaticum}$^d$</td>
<td>1</td>
<td>Brined shrimp</td>
<td>0.098</td>
</tr>
<tr>
<td>\textit{Enterococcus malodoratus}$^e$</td>
<td>2</td>
<td>Brined shrimp</td>
<td>0.044–0.059</td>
</tr>
<tr>
<td>\textit{Leuconostoc spp.}$^f$</td>
<td>3</td>
<td>Cold-smoked Greenland halibut</td>
<td>0.109–0.140</td>
</tr>
</tbody>
</table>

$^a$ Maximum specific growth rate as determined in APT broth at 8°C.

$^b$ Growth on acetate agar. The culture yielded a final pH in La broth of 3.83 to 4.10, produced NH$_3$ from arginine, and was unable to produce acetoin from glucose (Voges-Proskauer), unable to produce gas from glucose, able to produce gas from gluconate, and unable to ferment D-xylene, D-mannitol, and inulin. The partial 16S rDNA sequence (EF555204) showed ≥99.6% sequence similarity with \textit{L. curvatus} subsp. melibiosus, \textit{L. sakei} subsp. carnosus, and \textit{L. sakei} subsp. \textit{sakei}.

$^c$ Growth on acetate agar. The culture yielded a final pH in La broth of 3.90 to 4.03, was unable to produce NH$_3$ from arginine, produced acetoin from glucose (Voges-Proskauer), and was unable to produce gas from glucose and gluconate (60%) and unable to ferment D-xylene, D-mannitol, and inulin.

$^d$ No growth on acetate agar. The culture yielded a final pH in La broth of 4.77, produced NH$_3$ from arginine and acetoin from glucose (Voges-Proskauer), was unable to produce gas and gluconate, and was able to ferment D-mannitol and inulin but not D-xylene. The partial 16S rDNA sequence (EF555206) showed 99.9% sequence similarity with \textit{C. maltaromaticum}.

$^e$ No growth on acetate agar. The culture yielded a final pH in La broth of 4.28 to 4.34 and was unable to produce NH$_3$ from arginine and acetoin from glucose (Voges-Proskauer), unable to produce gas from glucose and gluconate, and able to ferment D-mannitol but not D-xylene and inulin. The partial 16S rDNA sequence (EF555205 and EF555207) showed 99.8% sequence similarity with \textit{E. malodoratus}.

$^f$ No growth on acetate agar. The culture yielded a final pH in La broth of 4.35 to 4.50, was unable to produce NH$_3$ and acetoin from glucose (Voges-Proskauer), produced gas from glucose and gluconate, and was able to ferment D-xylene and D-mannitol but not inulin.
determined in duplicate by automated absorbance measurements at 540 nm (Bioscreen C). The $p_{\text{max}}$ values were determined from detection times of the serially diluted cultures as previously described (13). Square root–transformed $p_{\text{max}}$ values were plotted against concentrations of undissociated organic acids, and MICs of lactate and diacetate were estimated by a simple square root model (1). The concentration of undissociated organic acids was calculated from the pH as previously described (40).

The $a_{w_{\text{min}}}$ and pH$_{\text{min}}$ values determined for *Lactobacillus curvatus* as 0.928 ± 0.003 and 4.24 ± 0.13, respectively, were obtained from Witjzes et al. (56), and a CO$_{2\text{max}}$ value determined for *Lactobacillus sakei* subsp. *carnosum* as 6.691 ± 1.200 ppm was obtained from Devlieghere et al. (17). The $a_{w_{\text{min}}}$ and pH$_{\text{min}}$ values are the theoretical $a_{w}$ and pH values, respectively, above which growth of LAB is possible, and CO$_{2\text{max}}$ is the theoretical concentration of dissolved CO$_2$ below which growth of LAB is possible.

**Challenge tests.** Two series of challenge tests consisting of six and five experiments were carried out with cold-smoked salmon. In the first series, the antimicrobial effects of diacetate, lactate, and modified atmosphere packaging (MAP) on LAB and *L. monocytogenes* were examined to generate data for model validation. The effect of preculturing conditions (before product inoculation) and inoculum concentration on growth of *L. monocytogenes* in cold-smoked salmon was studied in the second series of challenge tests. For both series, products were packed in a modified atmosphere and stored at 6 or 8°C (Table 2). Salmon (*Salmo salar*) from aquaculture in Chile was used as raw material and processed into sliced and vacuum-packed fillets by a company in Denmark. After processing, the product was frozen and transported to the Danish Institute for Fisheries Research in its frozen state. Frozen cold-smoked salmon fillets were thawed overnight at 5°C, and then slices were randomly divided into subbatches prior to addition of organic acids. For the first series of challenge tests, selected subbatches were treated with 0.0 to 0.20% (wt/wt) diacetate and 0.0 to 2.0% (wt/wt) lactate (Table 2) as previously described (40). Diacetate (0.22%, wt/wt) was added to the subbatches of the second series of challenge tests to ensure that *L. monocytogenes* would grow very slowly at 8°C and be unable to grow at 6°C. The required concentration of diacetate was determined by using a recently developed growth and growth boundary model for *L. monocytogenes* (40) with carefully determined product characteristics of the cold-smoked salmon (Table 2).

**Inoculation and packaging of samples.** Five of the six subbatches in the first series of challenge tests were inoculated with LAB mix. A total of 10 subbatches from the two series of challenge tests were inoculated with a mixture of four *L. monocytogenes* isolates (Lm mix). These isolates originated from seafood, the integrated and log-transformed form of the four-parameter logistic model with carefully determined product characteristics of the cold-smoked salmon (Table 2).

After inoculation, 100-g portions of the different subbatches of the two series of challenge tests (Table 2) were packaged in a modified atmosphere initially containing 40% CO$_2$ and 60% N$_2$ or 80% CO$_2$ and 20% N$_2$, as previously described (40). The gas ratio was approximately 2:1. Following packaging, subbatches were stored at 6 or 8°C, and data loggers (TinytagPlus, Gemini Data Loggers Ltd., Chichester, UK) were used to record the storage temperatures continuously throughout the storage period.

**Microbiological and chemical analyses.** At regular intervals during the storage of each subbatch, three samples were analyzed by microbiological methods. Product samples of 20 g were diluted 10-fold in chilled (5°C) physiological saline (0.85% NaCl) with 0.1% peptone and homogenized for 60 s in a stomacher bag (Stomacher 400, Seward Medical, London, UK). Further appropriate 10-fold dilutions of the homogenates were made in chilled physiological saline (0.85% NaCl) with 0.1% peptone. LAB were enumerated by pour plating in nitrite acidified polynymyxin (NAP) agar at pH 6.2 and incubating at 25°C for 3 to 4 days (15). *L. monocytogenes* were enumerated by spread plating on Palcam agar (CM0877, Oxoid) with Palcam Selective Supplement (SR0150, Oxoid) and incubating at 37°C for 2 days. Aerobic viable counts were determined by spread plating on Long and Hammer agar with 1% NaCl and incubating at 15°C for 7 days (19).

Product characteristics of the cold-smoked salmon were determined by analysis of three samples from selected subbatches at the start of the challenge tests. The concentration of dry matter and the pH were measured as previously described (11). Salt content was determined by an automated potentiometric titration method, and organic acid concentrations (lactic acid and diacetate or acetic acid) were determined by a previously described high-performance liquid chromatography method (19). Smoke components estimated as the concentration of phenols were determined by a spectrophotometric method (4). The equilibrium concentration of gasses in the headspace of MAP samples was measured with a Combi Check 9800–1 gas analyzer (PBI, Dansensor, Ringsted, Denmark).

**Statistical analyses and curve fitting.** A classical $t$ test was used to determine whether differences between the mean values of repeated measurements were significantly different. A two-sample comparison analysis was carried out to determine whether different preculturing conditions or inoculum concentrations of *L. monocytogenes* had a significant effect on the subsequent growth in MAP cold-smoked salmon during storage. Calculations were carried out using Statgraphics Plus version 5.1 (Manugistics Inc., Rockville, Md.). To determine the lag phase (hour) and maximum specific growth rate ($\mu_{\text{max}}$, h$^{-1}$) of bacteria in lightly preserved seafood, the integrated and log-transformed form of the four-parameter logistic model (9) was fitted to growth data obtained from cultures on NAP and Palcam agar. The software package Fig.P (version 2.98, Biosoft, Cambridge, UK) was used for curve fitting. An $F$ test to compare fits of the three- and four-parameter logistic models was used to evaluate whether lag phases of the microbial growth curves were significant (9).

**Development and evaluation of an LAB growth model.** The structure of an existing cardinal parameter growth and growth boundary model for *L. monocytogenes* including the effect of diacetate, lactate, CO$_2$, smoke components, nitrite, pH, NaCl-$a_{w}$,
<table>
<thead>
<tr>
<th>Subbatch</th>
<th>Inoculum:</th>
<th>Atmosphere</th>
<th>Temp (°C)</th>
<th>Additive (%, w/w):</th>
<th>Measured mean ± SD values for product characteristics and storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LAB mix</td>
<td>Lm mix</td>
<td></td>
<td></td>
<td>pH</td>
</tr>
<tr>
<td><strong>First series of challenge tests</strong></td>
<td></td>
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<tr>
<td>CSS (a)</td>
<td>NC&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>40% CO₂, 60% N₂</td>
<td>8</td>
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<tr>
<td>CSS (b)</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>40% CO₂, 60% N₂</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>CSS (c)</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>80% CO₂, 20% N₂</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>CSS (d)</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>40% CO₂, 60% N₂</td>
<td>8</td>
<td>0.20</td>
</tr>
<tr>
<td>CSS (e)</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>40% CO₂, 60% N₂</td>
<td>8</td>
<td>2.0</td>
</tr>
<tr>
<td>CSS (f)</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>80% CO₂, 20% N₂</td>
<td>8</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Second series of challenge tests</strong></td>
<td></td>
<td></td>
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<tr>
<td>CSS (g)</td>
<td>NC&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>40% CO₂, 60% N₂</td>
<td>6</td>
<td>0.22</td>
</tr>
<tr>
<td>CSS (h)</td>
<td>NC&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>40% CO₂, 60% N₂</td>
<td>6</td>
<td>0.22</td>
</tr>
<tr>
<td>CSS (i)</td>
<td>NC&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>40% CO₂, 60% N₂</td>
<td>8</td>
<td>0.22</td>
</tr>
</tbody>
</table>

<sup>a</sup> Equilibrium concentrations in headspace gas.  
<sup>b</sup> NC, naturally contaminated.  
<sup>c</sup> ND, measured but not detected.  
<sup>d</sup> As predicted by the growth and growth boundary models of Mejholm and Dalgaard (40).  
<sup>e</sup> Precultured in BHI broth without additional NaCl.  
<sup>f</sup> Precultured in BHI broth at 37°C.
temperature, and interactions between all these parameters (40) was used as a template to develop a model for growth of LAB in lightly preserved seafood (equation 1). Parameter values for L. monocytogenes were substituted for corresponding values of LAB:

\[
\mu_{\text{max}} = b \left( \frac{T - T_{\text{min}}}{25 - T_{\text{min}}} \right)^2 \left( \frac{a_w - 0.928}{1 - 0.928} \right) \left( 1 - 10^{(4.24 - \text{pH})} \right) \left( 1 - \frac{[\text{LAC}_t]}{[\text{MIC}_{\text{lactate}}]} \right) \left( \frac{P_{\text{max}} - P}{P_{\text{max}}} \right) \left( 6.691 - \frac{\text{CO}_2_{\text{equilibrium}}}{(6.691)} \right) \left( 1 - \frac{[\text{DAC}_t]}{[\text{MIC}_{\text{diacetate}}]} \right) \xi \tag{1}
\]

where \([\text{LAC}_t]\) and \([\text{DAC}_t]\) are the concentrations (mM) of undissociated lactic acid and diacetate, respectively; \([\text{MIC}_{\text{lactate}}]\) and \([\text{MIC}_{\text{diacetate}}]\) are the theoretical minimum concentrations (mM) of undissociated lactic acid and diacetate, respectively, preventing growth of LAB; \(P_{\text{max}}\) is the theoretical concentration (ppm) of smoke components (phenol) preventing growth of LAB; \(P\) is the concentration (ppm) of phenols, and \(\text{CO}_2_{\text{equilibrium}}\) is the concentration (ppm) of dissolved CO\(_2\) at equilibrium. The concentration of dissolved CO\(_2\) was calculated using the measured percentage of CO\(_2\) in the headspace gas at equilibrium and Henry’s constant at the appropriate storage temperature (47). The interaction between environmental parameters was modeled as previously described (34, 40). The effect of the interaction between environmental parameters in equation 1 was expressed by a parameter \(\xi\) with a value between 0 and 1. The value of \(\xi\) was calculated according to equation 2, with contributions from the different environmental parameters as shown in equations 3 and 4. In equation 4, \(e_i\) represents the environmental factors. Equation 2 divides the space of environmental parameters into three regions: (i) if \(\psi\) is less than a predefined threshold value \(\theta\), then no interactive effect between environmental parameters occurs (\(\xi = 1\)); (ii) if \(\psi\) is greater than 1, then no growth occurs (\(\xi = 0\)); and (iii) if \(\psi\) is less than 1 and greater than 0, then the growth rate \((\mu_{\text{max}} - \text{b}^{-1})\) is reduced depending on the value of \(\psi\). A threshold value \(\theta\) of 0.5 was used as suggested by Le Marc et al. (34):

\[
\xi[\varphi(T, a_w, \text{pH}, [\text{LAC}], P, \text{CO}_2, [\text{DAC}])] = \begin{cases} 
\psi \leq \theta, & \text{if } \psi \leq \theta \\
2(1 - \varphi), & \text{if } 0 < \psi < 1 \\
0, & \text{if } \psi \geq 1 
\end{cases} \tag{2}
\]

where \(\xi[\varphi(T, a_w, \text{pH}, [\text{LAC}], P, \text{CO}_2, [\text{DAC}])]\) is the term describing the effect of interactions between environmental parameters on \(\mu_{\text{max}}\):

\[
\varphi_T = \left( 1 - \frac{(T - T_{\text{min}})}{(25 - T_{\text{min}})} \right)^2 \\
\varphi_{a_w} = \left( 1 - \sqrt{(a_w - 0.928)/0.072} \right)^2 \\
\varphi_{\text{pH}} = \left( 1 - \sqrt{10^{(4.24 - \text{pH})}} \right)^2 \\
\varphi_{[\text{LAC}]/[\text{LAC}]} = \left( 1 - \sqrt{[\text{LAC}_t]/[\text{MIC}_{\text{lactate}}]} \right) \left( 1 - \sqrt{[\text{DAC}_t]/[\text{MIC}_{\text{diacetate}}]} \right)^2 \\
\varphi_P = \left( 1 - \sqrt{(P_{\text{max}} - P)/P_{\text{max}}} \right)^2 \\
\varphi_{\text{CO}_2} = \left( 1 - \sqrt{(6.691 - \text{CO}_2_{\text{equilibrium}})/6.691} \right)^2 \\
\psi = \sum_i 2 \prod_j (1 - \varphi_{e_j}) \tag{3}
\]

To estimate values for the constant \(b\), \(T_{\text{min}}\), and \(P_{\text{max}}\), 81 \(\mu_{\text{max}}\) values were obtained or calculated from the growth of LAB in lightly preserved seafood as a function of storage conditions and product characteristics. Six growth curves were generated in the present study, and 75 \(\mu_{\text{max}}\) values were obtained from the literature, with relevant storage conditions and product characteristics (Table 3). Equation 1 was fitted to 48 of the 81 obtained \(\mu_{\text{max}}\) values, and 33 \(\mu_{\text{max}}\) values were used for evaluation of the model. The model was fitted to square-root-transformed \(\mu_{\text{max}}\) values using nonlinear regression (SigmaStat v. 3, Systat Software GmbH, Erkrath, Germany).

The performance of the fitted model was evaluated by comparison of maximum specific growth rates observed in lightly preserved seafood with \(\mu_{\text{max}}\) values predicted by the model under corresponding product characteristics and storage conditions (Table 3). The comparison was carried out by calculation of bias and accuracy factors (40, 45). The performance of the developed model also was compared with that of existing models for growth of L. sakei subsp. carnosum, including the effect of temperature, \(a_w\), \(\text{CO}_2\), and lactate (17) and for L. curvatus including the effect temperature, \(a_w\), and \(\text{pH}\) (56).

Predicting growth of L. monocytogenes. As described for LAB, the growth and growth boundary model of Mejillohm and Dalgaard (40) for L. monocytogenes were refitted to 41 \(\mu_{\text{max}}\) values of the pathogen, including 39 \(\mu_{\text{max}}\) values previously described (40) and 2 values from the present study. This refitting was done to determine the constant \(b\), \(T_{\text{min}}\), and \(P_{\text{max}}\) and thereby evaluate the \(P_{\text{max}}\) value previously determined by Giménez and Dalgaard (19) with a smaller data set (\(n = 16\)). The performance of the refitted model was evaluated with 76 growth–no growth responses of L. monocytogenes in lightly preserved seafood, consisting of 71 responses from experiments previously reported (40) and 5 growth–no growth responses from the present study.

The differential form of the simple logistic model (equation 5) describes how the specific growth rate \((\mu_s)\) of a species is reduced when the cell concentration \((N, \text{CFU g}^{-1})\) approaches its maximum value \((N_{\text{max}}, \text{CFU g}^{-1})\). An expanded version of equation 5 was used to model microbial interaction between LAB and L. monocytogenes (equation 6) as previously described by Giménez and Dalgaard (19). This model is based on the assumption that LAB and L. monocytogenes inhibit each other to the same extent as they inhibit their own growth. However, because of higher concentrations of LAB, the inhibiting effect of L. monocytogenes on LAB was not evaluated in the present study.

\[
\frac{dN}{dt} = \mu_s = \mu_{\text{max}} \left( 1 - \frac{N_t}{N_{\text{max}}} \right) \tag{5}
\]

\[
t < t_{\text{lag} - \text{LAB}}, \quad \frac{dLm_{\text{dt}}}{Lm} = 0 \\
t \geq t_{\text{lag} - \text{LAB}}, \quad \frac{dLm_{\text{dt}}}{Lm} = \mu_{\text{LAB}} max \left( 1 - \frac{Lm_t}{Lm_{\text{max}}} \right) \left( 1 - \frac{LAB}{LAB_{\text{max}}} \right) \tag{6}
\]

\[
t < t_{\text{lag} - \text{LAB}}, \quad \frac{dLAB_{\text{dt}}}{LAB} = 0 \\
t \geq t_{\text{lag} - \text{LAB}}, \quad \frac{dLAB_{\text{dt}}}{LAB} = \mu_{\text{LAB}} max \left( 1 - \frac{LAB_t}{LAB_{\text{max}}} \right) \left( 1 - \frac{Lm_t}{Lm_{\text{max}}} \right) \tag{6}
\]
TABLE 3. Comparison of observed and predicted maximum specific growth rates of lactic acid bacteria in lightly preserved seafoods as evaluated by bias and accuracy factors

| Reference | n   | Temp (°C) | Water phase salt (%) | pH | $a_w^a$ | Phenol (ppm) | CO$_2$ (%)$^b$ | Water phase lactate (%) | Water phase diacate (%) | Observed $\mu_{max}$ (h$^{-1}$) | Devlieghere et al. (17) | Wijtze et al. (56) | Equation 1$^c$ | Bias/accuracy factors based on comparison of observed and predicted $\mu_{max}$ |
|-----------|-----|-----------|----------------------|----|---------|--------------|--------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|-------------------|------------------|----------------------------------------------------------------------------------|
| Data used for development of model | | | | | | | | | | | | | | | |
| Mejhlom and Dalgard (40) | 15 | 7.6–14.3 | 2.95–5.26 | 6.0–6.8 | 0.969–0.983 | 0–20.1 | 0–26.0 | 0.18–1.49 | 0.0–0.23 | 0.017–0.138 | 1.9/1.9 | 2.7/2.7 | 1.1/1.2 | |
| Hansen et al. (22) | 1 | 5.0 | 4.6 | 6.1$^d$ | 0.973 | 6.0$^d$ | 0 | 0.70$^d$ | 0.041 | 1.0/1.0 | 1.2/1.2 | 0.7/1.5 | |
| Hansen and Huss (24) | 2 | 5.0 | 4.2–4.8 | 6.1$^d$ | 0.972–0.975 | 6.0$^d$ | 0 | 0.70$^d$ | 0.033–0.043 | 1.1/1.1 | 1.3/1.3 | 0.8/1.3 | |
| Leroi et al. (36) | 1 | 8.0 | 4.6 | 6.0 | 0.973 | 6.6 | 0 | 0.70$^d$ | 0.043 | 0.9/1.1 | 1.3/1.3 | 0.8/1.3 | |
| Leroi et al. (35) | 1 | 5.0 | 2.6 | 6.1$^d$ | 0.985 | 4.6 | 0 | 0.70$^d$ | 0.067 | 1.6/1.6 | 1.5/1.5 | 0.9/1.2 | |
| Jørgensen et al. (30) | 12 | 5.0 | 3.9–7.9 | 6.1 | 0.951–0.977 | 6.0$^d$ | 0 | 0.70$^d$ | 0.018–0.029 | 1.4/1.5 | 1.7/1.7 | 0.9/1.2 | |
| Stohr et al. (52) | 2 | 6.0 | 3.5$^d$ | 6.2 | 0.980 | 6.0$^d$ | 0 | 0.70$^d$ | 0.022–0.043 | 2.1/2.1 | 2.4/2.4 | 1.3/1.4 | |
| Lakshmanan and Dalgard (32) | 2 | 5.5–9.9 | 4.93 | 6.12 | 0.971 | 14.6 | 0 | 0.60 | 0.012–0.027 | 2.9/2.9 | 4.4/4.4 | 1.9/1.9 | |
| Giménez and Dalgard (19) | 5 | 2.0–25.0 | 4.9 | 6.0 | 0.971 | 12.6 | 0 | 0.37 | 0.018–0.270 | 1.2/1.2 | 1.8/1.8 | 0.7/1.5 | |
| Dalgard et al. (12) | 2 | 5.0–15.0 | 4.4 | 6.1 | 0.974 | 6.0$^d$ | 0 | 0.70$^d$ | 0.021–0.123 | 1.5/1.5 | 2.2/2.2 | 1.2/1.2 | |
| Joffraud et al. (27) | 5 | 8.0 | 4.4–4.8 | 6.1 | 0.972–0.974 | 8.2–8.8 | 0 | 0.70$^d$ | 0.038–0.046 | 1.4/1.4 | 2.1/2.1 | 1.1/1.1 | |
| Avg bias/accuracy factors | | | | | | | | | | | 1.5/1.6 | 2.1/2.1 | 1.0/1.3 | |
| Data used for model validation | | | | | | | | | | | | | | |
| Present study | 6 | 7.9 | 5.22 | 6.0–6.2 | 0.969 | 3.8 | 25.1–61.0 | 0.66–2.93 | 0.0–0.30 | 0.026–0.056 | 0.7/1.5 | 1.6/1.6 | 0.8/1.3 | |
| Mejhlom and Dalgard (40) | 6 | 7.6–14.3 | 3.12–5.26 | 5.9–6.7 | 0.969–0.982 | 0–20.1 | 16.2–30.0 | 0.14–1.38 | 0.0–0.26 | 0.020–0.100 | 1.7/1.8 | 2.8/2.8 | 1.2/1.3 | |
| Hansen et al. (23) | 1 | 5.0 | 3.9 | 6.1$^d$ | 0.977 | 6.0$^d$ | 0 | 0.70$^d$ | 0.027 | 1.9/1.9 | 2.0/2.0 | 1.1/1.1 | |
| Hansen et al. (25) | 11 | 5.0 | 4.1–6.1 | 6.1$^d$ | 0.963–0.976 | 6.0$^d$ | 0 | 0.70$^d$ | 0.010–0.026 | 2.0/2.1 | 2.7/2.7 | 1.5/1.5 | |
| Leroi et al. (36) | 3 | 5.0 | 4.8–5.5 | 6.2–6.3 | 0.967–0.972 | 2.7–10.8 | 0 | 0.70$^d$ | 0.032–0.038 | 1.0/1.1 | 1.4/1.4 | 0.7/1.4 | |
| Lyhs et al. (38) | 2 | 3.0–8.0 | 5.0 | 6.49 | 0.970 | 0 | 0 | 0.70$^d$ | 0.017–0.092 | 0.9/1.6 | 1.3/1.3 | 0.8/1.3 | |
| Giménez and Dalgard (19) | 4 | 5.0–25.0 | 4.9 | 6.0 | 0.971 | 12.6 | 0 | 0.37 | 0.005–0.190 | 2.8/2.8 | 4.7/4.7 | 2.3/2.3 | |
| Avg bias/accuracy factors | | | | | | | | | | | 1.4/1.7 | 2.2/2.2 | 1.2/1.5 | |

$^a$ Relation between percentage of water phase salt (% WPS) and water activity ($a_w$) calculated as $a_w = 1 - 0.0052471 \times$ WPS $- 0.00012206 \times$ WPS$^2$ (43, 47).

$^b$ Equilibrium concentrations in headspace gas.

$^c$ Where $b = 0.659$, $T_{min} = -3.05^\circ C$, and $P_{max} = 40.3$ ppm phenol.

$^d$ If not reported in the literature, the following product characteristics were used for prediction of $\mu_{max}$: 3.5% WPS ($a_w = 0.980$), pH 6.1, 6.0 ppm phenol, and 0.70% water phase lactate.
where \( Lm \) and LAB, both >0 CFU g\(^{-1}\), signify \( L. monocytogenes \) and LAB, respectively. The \( \mu_{\text{max}} \) values for LAB were obtained from equation 1, and those for \( L. monocytogenes \) were determined with the refitted model of Mejlholt and Dalgaard (40). Data from 53 experiments on the growth of LAB and \( L. monocytogenes \) in lightly preserved seafoods were used to compare the observed and predicted MPD (log CFU per gram) of \( L. monocytogenes \) (Table 4). A relative lag time (RLT; lag time/generation time) of 4.5 was used to predict growth and the MPD of \( L. monocytogenes \) in 13 batches of naturally contaminated vacuum-packed cold-smoked salmon (19). For comparison, the MPD of \( L. monocytogenes \) in naturally contaminated vacuum-packed cold-smoked salmon was predicted by the ComBase Predictor (www.combase.cc) including the effect of temperature, pH, NaCl, and lactate. The physiological state (0 to 1) of \( L. monocytogenes \) was set to 0.04 for the latter model corresponding to an RLT of 4.5.

**RESULTS**

**Characterization and selection of LAB isolates.** The 15 studied LAB isolates were identified as \( L. curvatus \) (33% of the isolates), \( L. sakei \) (27%), Leuconostoc spp. (20%), Enterococcus malodoratus (13%), and Carnobacterium maltaromaticum (7%) (Table 1). LAB mix was prepared as a mixture of the fastest growing isolate from each of these five species of LAB.

**Challenge tests.** Characteristics and storage conditions of the studied product and subbatches are shown in Table 2. The concentration of naturally occurring water phase lactate in cold-smoked salmon varied between 0.62 and 0.72% (wt/wt). Addition of 0.20 to 0.22% (wt/wt) diacetate lowered the pH of the cold-smoked salmon from 6.2 to 6.0 (Table 2).

In naturally contaminated cold-smoked salmon, the concentration of LAB as determined on NAP agar increased from below the detection limit (<1 log CFU g\(^{-1}\)) to 5.5 ± 1.3 log CFU g\(^{-1}\) during 19 days of storage at 8°C. The initial concentration of LAB after inoculation with LAB mix was 3.7 ± 0.1 log CFU g\(^{-1}\). No lag phases of LAB were observed in MAP cold-smoked salmon (results not shown). Packaging of cold-smoked salmon in a CO2-enriched atmosphere (80% CO2 and 20% N2) had no significant effect on the growth rate \((P = 0.67)\) and MPD \((P = 0.24)\) of LAB as compared with an atmosphere initially containing 40% CO2 and 60% N2. In contrast, addition of 0.20% diacetate (wt/wt) and 2.0% (wt/wt) lactate significantly reduced the growth rate \((P = 0.003)\) and MPD \((P = 0.001)\) of LAB in MAP cold-smoked salmon (results not shown).

**Development and validation of a LAB growth model.** MIC\(_{\text{lactate}}\) and MIC\(_{\text{diacetate}}\) as determined in broth by automated absorbance measurements for LAB mix at 8°C were 12.0 ± 0.2 mM for undissociated lactate and 33.3 ± 1.3 mM for undissociated diacetate. With these MICs and the values for \( a_w, \) \( p_H, \) and \( CO_2_{\text{max}} \) obtained from the literature, fitting of equation 1 to \( \mu_{\text{max}} \) values of LAB in lightly preserved seafoods resulted in an estimated \( b \) value of 0.659 ± 0.002 h\(^{-1}\), a \( T_{\text{min}} \) of −3.05 ± 0.66°C, and a \( P_{\text{max}} \) of 40.3 ± 7.0 ppm phenol. The model described
88.3% of the variability in the square-root–transformed \( \mu_{\text{max}} \) data.

Evaluation of the fitted LAB model (equation 1) resulted in average bias and accuracy factors of 1.2 and 1.5 for the 33 \( \mu_{\text{max}} \) values used to validate the growth model, whereas the 48 \( \mu_{\text{max}} \) values used to estimate \( b, T_{\text{min}}, \) and \( P_{\text{max}} \) resulted in a bias factor of 1.0, as expected (Table 3). Lower bias and accuracy factors indicated that the new LAB model performed better than did the model of Devlieghere et al. (17) including the effect of temperature, \( a_w, \) \( C_O_2, \) and lactate and better than the model of Wijtzes et al. (56) including the effect of temperature, \( a_w, \) and pH (Table 3).

**Predicting growth of *L. monocytogenes***. Refitting of the growth and growth boundary model of Mejlholm and Dalgaard (40) resulted in a \( b \) value of 0.419 ± 0.002 h\(^{-1}\), a \( T_{\text{min}} \) of \(-2.83 \pm 1.06^\circ C\), and a \( P_{\text{max}} \) of 32.0 ± 5.4 ppm phenol. The model described 79.6% of the variability in the square-root–transformed \( \mu_{\text{max}} \) data. In contrast, 46% of the variability was explained by taking into account the effect of temperature only. The refitted model correctly predicted whether growth of *L. monocytogenes* was observed or not observed in 96% of the evaluated experiments (\( n = 76 \)) representing different types of lightly preserved seafoods (results not shown). The refitted model predicted growth of *L. monocytogenes* in three batches of naturally contaminated vacuum-packed cold-smoked salmon where no growth was actually observed (fail-safe prediction), as previously described (40).

In the present study, the MPD of *L. monocytogenes* in both inoculated and naturally contaminated products were predicted by taking into account the microbial interaction between LAB and the pathogen. From a total of 40 experiments with lightly preserved seafoods, all inoculated with *L. monocytogenes*, the average MPD of the pathogen was observed and predicted as 4.7 and 4.1 log CFU g\(^{-1}\), respectively (Table 4). In the thirteen batches of naturally contaminated vacuum-packed cold-smoked salmon the average MPD of *L. monocytogenes* was 0.7 log CFU g\(^{-1}\) and the predicted MPD was 0.6 log CFU g\(^{-1}\) when an RLT of 4.5 was used (Table 4). Without the inclusion of a lag phase, an average MPD of 1.8 log CFU g\(^{-1}\) was predicted (Table 4). In comparison, the ComBase Predictor estimated an average MPD of 4.4 log CFU g\(^{-1}\) when a “physiological state value” of *L. monocytogenes* corresponding to an RLT of 4.5 was used (results not shown).

In the second series of challenge tests, growth and no growth of *L. monocytogenes* were correctly predicted for MAP cold-smoked salmon with 0.22% (wt/wt) diacetate added and stored at 6 and 8°C (Fig. 1a). The concentration of *L. monocytogenes* increased by approximately 1 log CFU g\(^{-1}\) during 58 days of storage at 8°C, corresponding well with the 1.5 log CFU g\(^{-1}\) increase predicted by the refitted growth and growth boundary model of Mejlholm and Dalgaard (40). These results confirmed the usefulness of the model when designing experiments very close to the growth boundary of *L. monocytogenes*.

Preculturing of *L. monocytogenes* in BHI broth with 0.5% NaCl (no added salt) significantly affected the subsequent growth of *L. monocytogenes* \(( P < 0.001)\) in MAP cold-smoked salmon at 8°C as compared with preculturing in BHI broth with 4% NaCl (Fig. 1b). In fact, no increase in the concentration of *L. monocytogenes* was observed in the former subbatch after 58 days of storage at 8°C. Thus, a marked lag phase for growth of *L. monocytogenes*, close to its growth boundary, in MAP cold-smoked salmon was caused by the reduced concentration of NaCl in the preculturing medium. In contrast, preculturing of *L. monocytogenes* at 37°C instead of 8°C had no significant effect \(( P = 0.28)\) on subsequent growth in MAP cold-smoked salmon at 8°C (Fig. 1c). Increasing the *L. monocytogenes* inoculation level from \(10^2\) to \(10^5\) CFU g\(^{-1}\) in MAP cold-smoked salmon at 6°C influenced the growth boundary of the pathogen less than did an increase in the storage temperature from 6 to 8°C (Fig. 1a and 1d).

**DISCUSSION**

In the present study, we developed and successfully validated an LAB growth model (equation 1) including the effects of diacetate, lactate, \( C_O_2, \) smoke components (phenol), \( pH, \) NaCl-\( a_w, \) temperature, and interactions between all these parameters. The model was developed on the basis of data for growth of LAB in naturally contaminated \(( n = 63 \) and inoculated \(( n = 18 \) lightly preserved seafoods. Based on characteristics and storage conditions of the products used to develop and validate the model (Table 3), its range of applicability includes temperature (3 to 25°C), water phase salt (2.6 to 7.9%), \( pH, \) \( 5.9 \) to 6.8), water phase lactate (<3.0%), smoke components (phenol) (<20 ppm), equilibrium concentrations of \( C_O_2, \) (0 to 60%), and water phase diacetate (<0.3%). The new LAB model (equation 1) includes the effects of a larger number of environmental parameters than do the previously suggested LAB growth models, and it predicted the growth of LAB in lightly preserved seafood more accurately than did other less complex models (Table 3).

Complex growth models are needed for at least some lightly preserved foods, and development of these models is a challenge in the field of predictive microbiology. Factorial experiments designed to quantify the effect of more than four or five environmental parameters are extremely labor intensive, particularly when data to develop polynomial or artificial neural network models are needed. In the present study, an alternative product-oriented approach was used and a cardinal parameter type model was developed to overcome these problems. Model terms and cardinal parameter values from previous studies were merged into a model with a relevant degree of complexity (see equation 1). Then, values of key parameters in the model (in this case \( b, T_{\text{min}}, \) and \( P_{\text{max}} \)) were fitted using growth data from relevant and well-characterized foods. This approach has the advantage that previously obtained information in the form of cardinal parameter values and data for growth in specific foods can be used in a simple way to develop new and more accurate predictive models. This improvement was confirmed by product validation studies using our own
and previously published data for growth of LAB in lightly preserved seafood (Table 3). The product validation studies also revealed that product characteristics and storage conditions frequently were poorly described in the literature, even in studies where growth kinetics were carefully determined. This lack of information is unfortunate because the understanding of the microbial ecology in such products remains qualitative.

Growth of *L. monocytogenes* in lightly preserved seafood can be controlled to a large extent by addition of diacetate and lactate (40, 54, 58), but LAB are much more resistant to the effects of these organic acids (40, 49, 50). Predicting the growth boundaries of LAB and *L. monocytogenes* with the studied models clearly demonstrated this difference in sensitivity to diacetate and lactate (Eq. 2b). Few data are available to evaluate the predicted growth boundary for LAB in lightly preserved seafood. However, in MAP cold-smoked salmon addition of 0.15% (wt/wt) diacetate and 1.5% (wt/wt) lactate restricted growth of LAB to 1.3 log CFU g⁻¹ during 40 days of storage at 8°C (40), and these environmental parameters were close to the growth boundary predicted by equation 1.

Components from wood smoke are important for the control of *L. monocytogenes* growth in seafood (19, 40). To predict this inhibiting effect as accurately as possible, we determined $P_{\text{max}}$ (the theoretical concentration of smoke components measured as phenol that prevents growth) from data for growth of *L. monocytogenes* in inoculated seafood ($n = 41$). The fitted value of 32.0 ± 5.4 ppm phenol was similar to the $P_{\text{max}}$ of 28.1 ± 2.8 ppm phenol previously determined for a smaller set of data based on growth in broth (19). The performance of the refitted *L. monocytogenes* model, however, was improved as shown by the bias and accuracy factors that changed from 1.0 and 1.5 to 1.0 and 1.3 (results not shown). The ability of the refitted model to predict growth or no growth of *L. monocytogenes* was unchanged and the growth and growth boundary model of Mejlholm and Dalgaard (40) should therefore be used with
the following parameter values: $b = 0.419$, $T_{\text{min}} = -2.83^\circ C$, and $P_{\text{max}} = 32.0$ ppm phenol.

Successfully validated mathematical models for growth of LAB (equation 1) and *L. monocytogenes* (40) in different lightly preserved seafoods are now available. These models make it possible to predict the inhibiting effect of LAB on growth of *L. monocytogenes* (Fig. 3) as previously shown specifically for vacuum-packed cold-smoked salmon (19) and more generally in ready-to-eat foods (18). By using the simple interaction model suggested by Giménez and Dalgaard (19), we found that the effect of LAB on the MPD of *L. monocytogenes* (the Jameson effect) was appropriately predicted for naturally contaminated samples when a lag phase of the pathogen was taken into account (Tables 4 and 5). In naturally contaminated vacuum-packed cold-smoked salmon, maximum concentrations of *L. monocytogenes* from below the detection limit to 3.4 log CFU g$^{-1}$ have been observed for more than 1,300 samples obtained from 20 different processing plants (2, 8, 29, 33). These MPDs correspond well with predictions from the combined LAB–*L. monocytogenes* model (LAB–Lm model) developed in the present study that includes the effects of seven environmental parameters (Tables 4 and 5). For comparison, the model of Delignette-Muller et al. (16), which was without a lag phase and included only the effect of temperature and microbial interaction, predicted MPDs of *L. monocytogenes* in cold-smoked salmon from below the detection limit to 8 log CFU g$^{-1}$ (2) with approximately 20 to 40% of the predictions above 4 log CFU g$^{-1}$. Predictions by the ComBase Predictor based on the effects of temperature, pH, NaCl, and lactate are even more extreme, with MPDs in the range of 1 to more than 8 log CFU g$^{-1}$ (Fig. 4). These predictions for MPDs of *L. monocytogenes* in cold-smoked salmon suggest that several environmental parameters, the lag phase of *L. monocytogenes*, and the microbial interaction (the Jameson effect) must be taken into account when growth of this pathogen is predicted in lightly preserved seafood (Tables 4 and 5).
TABLE 5. Predicted maximum population density (MPD) of Listeria monocytogenes in naturally contaminated vacuum-packed cold-smoked salmon

<table>
<thead>
<tr>
<th>Initial conc (log CFU g⁻¹)ᵃ</th>
<th>Storage conditionsᵃ</th>
<th>Product characteristicsᵇ</th>
<th>Predicted MPD of L. monocytogenes (log CFU g⁻¹)ᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. monocytogenes</td>
<td>LABᵈ</td>
<td>Water phase salt (%)</td>
<td>Without lag time</td>
</tr>
<tr>
<td>Below detection limit</td>
<td>1.3</td>
<td>7 days at 4°C and 7 days at 8°C</td>
<td>7.1</td>
</tr>
<tr>
<td>0.9</td>
<td>1.3</td>
<td>15 days at 4°C and 7 days at 8°C</td>
<td>2.7</td>
</tr>
</tbody>
</table>

ᵃ As reported by Beaufort et al. (2).
ᵇ As reported by Cornu et al. (7) and Mejlholm and Dalgaard (40).
ᶜ Predicted by the LAB-Lm model of the present study.
ᵈ Specified as “food flora” by Beaufort et al. (2).
ᵉ A relative lag time of 4.5 was used as proposed by Ross (46). NG, no growth.

**Figure 4.** Comparison of predicted and observed maximum population density (MPD) of Listeria monocytogenes in 13 batches of naturally contaminated vacuum-packed cold-smoked salmon (29). Predictions were made from the LAB-Lm model developed in the present study and using a relative lag time of 4.5 (●) and from the ComBase Predictor including the effects of temperature, pH, NaCl, and lactate (▲). The solid line represents the perfect match between observed and predicted values.

Likely, LAB growth rates were overestimated (Table 3), resulting in the prediction of lower MPDs for *L. monocytogenes* than those obtained in the present study.

*L. monocytogenes* most likely goes through a lag phase in naturally contaminated products before growth is initiated, but documentation of this pattern remains limited because sufficiently sensitive detection methods are lacking. Close to the growth boundary of *L. monocytogenes*, an NaCl shift up from 0.5 to 5.2% salt resulted in a pronounced lag phase (Fig. 1b). This finding and the inhibiting effect of LAB may explain the limited growth of the pathogen actually observed in several studies with naturally contaminated cold-smoked salmon (2, 8, 29, 33). In agreement with these findings, in previous studies using liquid media and meat models an NaCl shift up increased the lag phase of *L. monocytogenes* (26, 41). These results together suggest that it is reasonable to use a lag phase when growth of *L. monocytogenes* is predicted in naturally contaminated lightly preserved seafood. For *L. monocytogenes*, average RLTs from 3.1 to 5.7 have been reported (1, 21, 46), and an RLT of 4.5 was used in the present study (Tables 4 and 5).

In some previous studies, pronounced effects were associated with the inoculum concentration of *L. monocytogenes* on growth in liquid media with different inhibiting conditions (31, 44). However, the effect of the inoculum concentration seems less pronounced for combinations of inhibiting conditions than for single growth-reducing factors (31). This lack of effect was observed previously for aerobically stored and vacuum-packed cold-smoked salmon (3) and might explain why no marked effect of the inoculum concentration was observed in MAP cold-smoked salmon with several growth hurdles, including NaCl, smoke components, pH, lactate, and CO₂ (Fig. 1d and Table 2).

The developed model for growth of LAB can be used to predict concentrations of the dominating microflora in different lightly preserved seafoods during storage and the antimicrobial effect of such additives as diacetate and lactate on growth of LAB. For example, 0.20% (wt/wt) diacetate and 2.0% (wt/wt) lactate added to MAP cold-smoked salmon doubled the time for LAB to reach 10⁷ CFU g⁻¹ at 8°C (results not shown). In agreement with this observation, the LAB model predicted that the growth rate (μmax, h⁻¹) of LAB would be reduced by 94% when these same concentrations of diacetate and lactate were used. This type of information can be used to predict and extend the shelf life of lightly preserved seafood.

The LAB-Lm model developed in the present study can be used by the seafood industry when developing new or reformulating existing products to make them comply with *L. monocytogenes* requirements of the EU regulation on ready-to-eat foods (EC 2073/2005) (6). The developed LAB-Lm model also can be used to evaluate how growth and the growth boundary of *L. monocytogenes* are influenced by variation in product characteristics and storage conditions (Fig. 2a and 2b) and to provide documentation.
as required by authorities and customers. Clearly, variability in product characteristics and storage conditions is an important factor influencing the growth of *L. monocytogenes*. Consequently, this variability should be taken into account when predicting growth and growth boundaries (Fig. 2a and 2b). Variability in product characteristics and storage conditions could be described by various methods, such as the Bayesian modeling approach (16). However, the current amount of product data including all relevant parameters is not sufficient to determine the variation in such factors as the concentration of smoke components and water phase lactate. The LAB- *Lm* model could be valuable for determining the concentration of *L. monocytogenes* in products at the time of consumption in exposure assessment studies (18, 55). To facilitate and enhance the usefulness of the combined LAB-*Lm* model, it should be incorporated into user-friendly software such as the Seafood Spoilage and Safety Predictor (www.difres.dk/micro/sssp).

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