Promotors: Prof. dr. ir. Johan Debevere
Prof. dr. ir. Frank Devlieghere

Laboratory of Food Microbiology and Food Preservation
Department of Food Safety and Food Quality
Faculty of Bioscience Engineering
Ghent University

Dean: Prof. dr. ir. Herman Van Langenhove

Rector: Prof. dr. Paul Van Cauwenberghe

ir. Lieve Vermeiren

Biopreservation of anaerobically packaged sliced cooked meat products by non-bacteriocinogenic micro-organisms

Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Applied Biological Sciences

Bioconservering van anäeroob verpakte versneden gekookte vleesproducten met niet-bacteriocineproducerende micro-organismen

Illustratie: *Lactobacillus sakei* 10A © Lieve Vermeiren
Bacteriophage P200 © Steven Hagens (Swiss Federal Institute of Technology)

Vermeiren, L. (2006). Biopreservation of anaerobically packaged sliced cooked meat products by non-bacteriocinogenic micro-organisms. Doctoral dissertation. Universiteit Gent.

ISBN: 90-5989-132-5
The author and the promotor give the authorisation to consult and to copy parts of this work for personal use only. Every other use is subject to the copyright laws. Permission to reproduce any material contained in this work should be obtained from the author.

Woord vooraf

Proefbuizen, labojassen, cellen, microscopie, micro-organismen,… het fascineerde mij al heel vroeg. Het willen weten, onderzoeken, analyseren en trachten oplossen, dat heeft er altijd al wel ingezeten.

Na mijn thesis aan het Labo Levensmiddelenmicrobiologie en -conservering was ik zo gebeten door de labo-microbe, letterlijk en figuurlijk, dat ik dan ook met groot enthousiasme gevraagd heb aan Prof. dr. ir. J. Debevere om verder te blijven werken aan zijn labo. Daar is het uiteindelijk allemaal mee begonnen en ik ben Prof. dr. ir. J. Debevere zeer dankbaar voor die eerste kans maar uiteraard ook voor al de daaropvolgende kansen, die ik telkens kreeg, als assistente en ook nu weer in mijn toekomstige job. Professor, je interesse, je kritische opmerkingen en je bewonderenswaardige ervaring en kennis hebben sterk bijgedragen tot het tot een goed einde brengen van dit werk.

Prof. dr. ir. Frank Devlieghere of mag ik Frank zeggen, jij hebt mijn achtjarige carrière het meest van dichtbij gevolgd… en ik ben je extra dank verschuldigd. Al van in mijn thesistijd stond je deur altijd open. Ondanks je drukke agenda, wist je tijd vrij te maken voor het helpen oplossen van alledaagse onderzoeksprobleempjes, voor het grotere brainstormwerk op de kritieke momenten van dit werk en voor het kritisch beoordelen van het schrijfwerk. Je energie en enthousiasme werkten inspirerend.

Prof. Debevere en Prof. Devlieghere, bedankt voor het promotorschap op jullie te nemen maar vooral bedankt voor jullie vertrouwen.

Mijn werk was nooit alleen mijn werk. Mijn werk was en is ook mijn collega's… en die groep collega's werd door de jaren heen steeds groter. Jullie allemaal opnoemen is dan ook onmogelijk (ook al drongen sommigen hierop sterk aan), maar elk van jullie heeft wel hier of daar zijn steentje bijgedragen tot dit werk: wetenschappelijke steun, technische steun, administratieve steun, IT-steun, morele steun … BEDANKT daarvoor.

Ook wil ik een zeer groot woord van dank richten aan al de thesisstudenten die meegeholpen hebben aan dit werk: Ali, Veerle, Annelies, Isabelle, Ursula, Barbara. Jullie hebben uiteraard elk een belangrijk deel van het praktische werk voor jullie rekening genomen, maar veel belangrijker voor mij was dat jullie aanwezigheid mij het gevoel gaf er niet alleen voor te staan, het samen aan iets kunnen werken, het samen naar oplossingen zoeken, samen bloed, zweet en tranen vergieten,… was uiterst motiverend.

Dit doctoraat afleggen was een zware reis… en naast de wetenschappelijke kennis die ik opbouwde, leerde ik eigenlijk veel meer andere dingen… zoals geduld, volharding en relativeringsvermogen, maar niet zonder hulp van mijn gezin en mijn familie.

Aan mijn ouders en schoonouders kom ik woorden van dank tekort. Het is ongelooflijk hoe onvoorwaardelijk en nooit aflatend jullie steeds hulp aanbieden en geleidelijk aan, naarmate dit werk vorderde en de nood eraan toenam, leerde ik ook beter die hulp te aanvaarden.

Tom, jij hebt alle fasen van dit doctoraat mee beleefd… de perioden van mist, optrekkende mist en helder weer. Jij bent de enige die alle hoekjes van mijn ziel kent. Je laat me zijn zoals ik ben en dat is heel veel waard…. Bovendien schonk je mij twee schatten van zonen en dat 'mama' worden heeft ook op zijn manier bijgedragen tot dit werk… het plaatste dit doctoraat in de juiste context… en zo leerde ook ik stilaan relativeren.

Jorne en Brent, ook aan jullie een woordje. Nu zijn jullie nog veel te klein om dit te begrijpen maar ooit komt een tijd dat jullie dit wel zullen lezen. Het was niet altijd gemakkelijk om dit doctoraatswerk te combineren met mijn zorg voor jullie maar ik wil dat jullie weten dat jullie steeds op de eerste plaats stonden en dat ook altijd zo zal zijn.

Lieve

26 september 2006

Table of contents

Table of Contents

Table of Contents i

Introduction and objectives vii

Summary xi

Samenvatting xvi

Chapter 1: Antagonistic micro-organisms for biopreservation of food products 1

1. LACTIC ACID BACTERIA 1

1.1. INTRODUCTION 1
1.2. TAXONOMY 1
1.3. METABOLISM AND ENERGY GENERATION 2
1.4. LACTIC ACID BACTERIA OF IMPORTANCE IN FOOD PRODUCTS 4
1.4.1. The genus *Lactococcus* 4
1.4.2. The genus *Leuconostoc* 4
1.4.3. The genus *Pediococcus* 5
1.4.4. The genus *Lactobacillus* 5
1.5. ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA 8
1.5.1. Fermentation end-products 9
1.5.2. Bacteriocins 13
1.5.3. Other antagonistic systems 17

2. THE MICROBIAL ECOLOGY OF ANAEROBICALLY PACKAGED SLICED COOKED CURED MEAT PRODUCTS 18

2.1. PRODUCTION PROCESS 18
2.2. CONTAMINATION 18
2.3. MICROBIAL SPOILAGE 19
2.3.1. Factors determining the microbial growth in CMP 19
2.3.2. Specific spoilage organisms 19
2.3.3. Spoilage phenomena 21
2.4. SAFETY 24
2.4.1. Pathogens associated with cooked meat products 24
2.4.2. *Listeria monocytogenes* 24

3. BIOPRESERVATION 26

3.1. DEFINITION AND PRINCIPLE 26
3.2. BIOPRESERVATION BY MEANS OF MICROBIAL METABOLITES 27
3.2.1. Bacteriocins 27
3.2.2. Organic acids 29
3.2.3. Fermentates 30
3.3. BIOPRESERVATION BY MEANS OF MICRO-ORGANISMS 30
3.3.1. Protective LAB 30
3.3.2. Bacteriophages 41

3.4. EFFECT OF PROTECTIVE LAB ON THE SENSORY QUALITY OF ANAEROBICALLY PACKAGED COOKED MEAT PRODUCTS 46
3.5. MECHANISM OF INHIBITION OF PROTECTIVE LAB IN ANAEROBICALLY PACKAGED COOKED MEAT PRODUCTS 48
3.5.1. Production of inhibitory substances 49
3.5.2. Nutrient competition 50
3.5.3. Competition for space (attachment/adhesion sites) 53
3.5.4. Quorum sensing 54
3.6. PRACTICAL IMPLEMENTATION OF PROTECTIVE CULTURES 54
3.6.1. Safety aspects 54
3.6.2. Regulatory aspects 56
3.6.3. Technical aspects 58

Chapter 2: Evaluation of meat born lactic acid bacteria as protective cultures for biopreservation of cooked meat products 60

SUMMARY 60

1. INTRODUCTION 61

2. MATERIALS AND METHODS 62

2.1. COLLECTION OF RELEVANT STRAINS 62
2.2. ISOLATION AND IDENTIFICATION OF ISOLATES FROM MEAT PRODUCTS 62
2.3. PSYCHROTROPHIC CHARACTER AND SALT TOLERANCE 63
2.4. ANTIBACTERIAL ACTIVITY TOWARDS *L. MONOCYTOGENES*, *LEUC. MESENTEROIDES*, *LEUC. CARNOSUM* AND *B. THERMOSPHACTA* 63
2.5. GROWTH, ACIDIFICATION PROFILE AND LACTIC ACID PRODUCTION OF 12 SELECTED PUTATIVE PROTECTIVE CULTURES 64
2.6. BEHAVIOUR OF 12 SELECTED PUTATIVE PROTECTIVE CULTURES ON A MODEL COOKED HAM 66

3. RESULTS AND DISCUSSION 68

3.1. COLLECTION OF RELEVANT STRAINS 68
3.2. ISOLATION AND IDENTIFICATION OF ISOLATES FROM MEAT PRODUCTS 68
3.3. PSYCHROTROPHIC CHARACTER AND SALT TOLERANCE 71
3.4. ANTIBACTERIAL ACTIVITY TOWARDS *L. MONOCYTOGENES*, *LEUC. MESENTEROIDES*, *LEUC. CARNOSUM* AND *B. THERMOSPHACTA* 72
3.5. GROWTH, ACIDIFICATION PROFILE AND LACTIC ACID PRODUCTION OF 12 SELECTED PUTATIVE PROTECTIVE CULTURES 74
3.6. BEHAVIOUR OF 12 SELECTED PUTATIVE PROTECTIVE CULTURES ON A MODEL COOKED HAM 76

4. CONCLUSION 83

Chapter 3: In-vitro and in-situ growth characteristics and behaviour of spoilage organisms associated with anaerobically stored cooked meat products 84

SUMMARY 84

1. INTRODUCTION 85

2. MATERIALS AND METHODS 86

2.1. BACTERIAL STRAINS AND PREPARATION OF INOCULUM 86
2.2. GROWTH, ACIDIFICATION PROFILE AND METABOLITE PRODUCTION IN A LIQUID BROTH UNDER ANAEROBIC REFRIGERATED CONDITIONS 87
2.3. BEHAVIOUR ON A MODEL COOKED HAM PRODUCT 87

3. RESULTS AND DISCUSSION 89

3.1. GROWTH, ACIDIFICATION PROFILE AND METABOLITE PRODUCTION IN A LIQUID BROTH UNDER ANAEROBIC REFRIGERATED CONDITIONS 89
3.2. BEHAVIOUR ON A MODEL COOKED HAM PRODUCT 91

4. CONCLUSION 98

Chapter 4: Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham 99

SUMMARY 99

1. INTRODUCTION 100

2. MATERIALS AND METHODS 101

2.1. BACTERIAL STRAINS 101
2.2. MODEL COOKED HAM (MCH) 102
2.3. CO-CULTURE EXPERIMENTS 102
2.4. CHEMICAL ANALYSES 103
2.5. MICROBIOLOGICAL ANALYSES 103
2.6. DEVELOPMENT AND OPTIMISATION OF TC8-MRS-AGAR 104
2.7. STATISTICAL ANALYSES 105

3. RESULTS AND DISCUSSION 105

3.1. DEVELOPMENT OF TC8-MRS-AGAR 105
3.2. CO-CULTURE EXPERIMENTS 106
3.2.1. Co-culture studies between the potential protective cultures and *Brochothrix thermosphacta* 107
3.2.2. Co-culture studies between the potential protective cultures and *Leuconostoc mesenteroides* 113
3.2.3. Discussion 116

4. CONCLUSION 118

Chapter 5: The interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham 120

SUMMARY 120

1. INTRODUCTION 121

2. MATERIALS AND METHODS 122

2.1. BACTERIAL STRAINS 122
2.2. MODEL COOKED HAM 122
2.3. CO-CULTURE EXPERIMENTS 122
2.4. MICROBIOLOGICAL ANALYSES 124
2.5. PH-MEASUREMENTS 124
2.6. SENSORY ANALYSIS 124
2.7. STATISTICAL ANALYSES 124

3. RESULTS AND DISCUSSION 125

3.1. INFLUENCE OF INOCULUM RATIO 125
3.2. INFLUENCE OF STORAGE TEMPERATURE 128
3.3. INFLUENCE OF ATMOSPHERE IN THE HEADSPACE OF THE PACKAGING 129
3.4. INFLUENCE OF 10A ON THE ACIDITY OF THE MCH 130

4. CONCLUSION 132

Chapter 6: The sensory acceptability of cooked meat products treated with a protective culture depends on glucose content and buffering capacity: a case study with *Lactobacillus sakei* 10A 133

SUMMARY 133

1. INTRODUCTION 134

2. MATERIALS AND METHODS 135

2.1. BACTERIAL STRAINS 135
2.2. CHEMICAL ANALYSES 136
2.3. MICROBIOLOGICAL ANALYSES 137
2.4. SENSORY ANALYSES 137
2.5. SCREENING OF COOKED MEAT PRODUCTS 138
2.6. STORAGE EXPERIMENTS 139
2.7. STATISTICAL ANALYSES 140

3. RESULTS AND DISCUSSION 141

3.1. SCREENING OF COOKED MEAT PRODUCTS 141
3.2. STORAGE EXPERIMENTS 145
3.2.1. Antagonistic activity of *L. sakei* 10A 146
3.2.2. Glucose consumption and lactic acid production 149
3.2.3. Influence of 10A on pH and sensory properties 150
3.3. DISCUSSION 154
3.3.1. Antagonistic activity of *L. sakei* 10A 154
3.3.2. Role of glucose and buffering capacity in sensory acceptability of protective cultures 155

4. CONCLUSION 157

Chapter 7: The contribution of lactic acid production versus nutrient competition to the growth inhibition of *Listeria monocytogenes* by the non-bacteriocinogenic *Lactobacillus sakei* 10A 158

SUMMARY 158

1. INTRODUCTION 159

2. MATERIALS AND METHODS 160

2.1. BACTERIAL STRAINS 160
2.2. GROWTH MEDIA 160
2.3. EXPERIMENTAL SET-UP 162
2.4. CO-CULTURE EXPERIMENT IN B-BHI AND SUBSEQUENT CHALLENGE EXPERIMENT IN CFS OF 10A 163
2.5. ROLE OF LACTIC ACID PRODUCTION VERSUS NUTRIENT COMPETITION 164
2.5.1. Co-culture experiment 164
2.5.2. Challenge experiment in CFS 165
2.6. CO-CULTURE EXPERIMENTS – SUPPLEMENTATION WITH VITAMINS AND MINERALS 166

3. RESULTS AND DISCUSSION 167

3.1. CO-CULTURE EXPERIMENT IN B-BHI AND SUBSEQUENT CHALLENGE EXPERIMENT IN CFS OF 10A 167
3.2. ROLE OF LACTIC ACID PRODUCTION VERSUS NUTRIENT COMPETITION 170
3.2.1. Co-culture experiment 170
3.2.2. Challenge experiment in CFS 174
3.3. CO-CULTURE EXPERIMENTS - SUPPLEMENTATION WITH VITAMINS AND MINERALS 178
3.4. DISCUSSION 180

4. CONCLUSION 181

Chapter 8: Treatment with bacteriophage P100 as an effective biopreservation method to control *Listeria monocytogenes* on sliced and vacuum packaged cooked meat products 183

SUMMARY 183

1. INTRODUCTION 184

2. MATERIALS AND METHODS 185

2.1. BACTERIAL STRAINS AND BACTERIOPHAGE P100 185
2.2. EFFECT OF P100 ON *L. MONOCYTOGENES* IN BROTH 185
2.3. EFFECT OF P100 ON *L. MONOCYTOGENES* IN COOKED CHICKEN FILLET 186
2.4. EFFECT OF P100 ON *L. MONOCYTOGENES* IN COOKED HAM 187
2.5. CHEMICAL ANALYSES 188
2.6. MICROBIOLOGICAL ANALYSES 188
2.7. PHAGE TITER DETERMINATION 189

2.8. STATISTICAL ANALYSES 189
3. RESULTS AND DISCUSSION 190
3.1. EFFECT OF P100 ON *L. MONOCYTOGENES* IN BROTH 190
3.2. EFFECT OF P100 ON *L. MONOCYTOGENES* IN COOKED CHICKEN FILLET 192
3.3. EFFECT OF P100 ON *L. MONOCYTOGENES* IN COOKED HAM 194
4. CONCLUSION 198
General discussion, conclusions and perspectives 199
1. GENERAL DISCUSSION 199
1.1. RELEVANT SPOILAGE ORGANISMS FOR ANAEROBICALLY PACKAGED COOKED MEAT PRODUCTS 199
1.2. SHELF-LIFE EXTENDING CAPACITY OF PROTECTIVE LAB-CULTURES 200
1.3. CAPACITY OF PROTECTIVE LAB-CULTURES TO PREVENT PROLIFERATION OF *L. MONOCYTOGENES* 201
1.4. SENSORY ACCEPTABILITY OF CMP TREATED WITH *L. SAKEI* 10A 202
1.5. INHIBITION MECHANISM OF *L. SAKEI* 10A 203
1.6. BACTERIOPHAGE P100 TO CONTROL GROWTH OF *L. MONOCYTOGENES* 204
2. CONCLUSIONS 204
3. PERSPECTIVES 206
List of abbreviations 207
References 209
Curriculum vitae I

Introduction and objectives

Introduction and objectives

In recent years there has been an increase in consumer concern about the use of chemical additives to ensure product safety and to extend the shelf-life of foods. In response to these concerns, efforts have been made to introduce alternative biological preservatives (Guinane et al., 2005). In view of the consumer trends towards natural and healthy food products, preservation by the use of micro-organisms and/or their metabolites has been proposed. In biopreservation the applied micro-organisms are mostly bacteria, in particular lactic acid bacteria, and they are designated as protective cultures (Holzapfel et al., 1995; Lücke, 2000).
Considerable research has been done on the ability to inhibit growth of food born pathogens and in particular *Listeria monocytogenes* by means of protective cultures. The potential of these cultures to control growth of spoilage micro-organisms, including the endogenous lactic acid flora, has not been examined to the same extent (Leisner et al., 1996). The majority of the work in the field of biopreservation had the intention of demonstrating the effect of protective cultures being active through bacteriocin production. However, the sometimes limited effectiveness of these cultures in the meat substrate (Rodriguez et al., 2002) and the concern for resistance development (Ennahar et al., 2000) has driven the search for alternative non-bacteriocinogenic protective cultures. Furthermore, the concept of biopreservation has been well developed for applications in fermented products since it is common to use bacteria as starter cultures in these products. However, the addition of micro-organisms to non-fermented products is a rather new concept. Recently, there is also an increased interest in the use of bacteriophages, viruses that infect and kill bacteria, as a means of inactivating food born pathogens (Hudson et al., 2005).

The **primary objective** of this work was to investigate the possibility of preserving sliced, cooked meat products (CMP), which are packaged either under vacuum or under a modified atmosphere, by means of non-bacteriocinogenic micro-organisms and to study the mechanism of action behind this non-bacteriocinogenic type of biopreservation.
The main part (Chapters 2 to 7) of this work studied biopreservation by means of non-bacteriocinogenic lactic acid bacteria and this using an integrated approach since all different aspects concerning this preservation technology were investigated: effectiveness, sensory aspects and mechanism of inhibition. To realise the stipulated objectives, one particular protective LAB-culture had to be selected during the research to use this culture as a case

study. The results from the case-study could then be used to derive general conclusions on this biopreservation technology.
In the final chapter (Chapter 8) of this PhD-work, preservation of cooked meat products by means of bacteriophages was introduced as an additional subject of research.
The outline of this work is schematically presented in Figure 0.1. The following sub-objectives of this work can be stipulated:

- **Sub-objective 1**

  Before starting this PhD-work, it was of primary importance to study the existing knowledge in literature concerning antagonistic micro-organisms for biopreservation of food products (**Chapter 1**).

- **Sub-objective 2**

  In **chapter 2**, collected and isolated meat born lactic acid bacteria were tested for their suitability to be candidate protective cultures for cooked meat products. Besides testing whether the cultures had a 'protective' activity, they also needed to be adapted to the product/storage conditions (psychrotrophic character, salt tolerance). Furthermore, it was of primary concern to evaluate, already at this early stage of the work, the effect of the cultures on the sensory properties of a model cooked meat product.

- **Sub-objective 3**

  Chapter 3 aimed to better understand spoilage caused by different types of spoilage organisms, associated with vacuum packaged sliced CMP. The systematic study on the behaviour of several spoilage organisms on a model product, imitating cooked ham, was meant to establish the relationship between microbial growth, pH-evolution, metabolite formation and the occurrence of organoleptic deviations.

- **Sub-objective 4**

  Chapter 4 had the objective to examine the interaction between the candidate protective cultures, that had been selected in chapter 2, and the spoilage organisms, that were shown to be most relevant for anaerobically packaged sliced CMP in chapter 3. Chapter 4 aimed to answer the question: can homofermentative non-bacteriocinogenic lactic acid bacteria be used to prolong the shelf-life of CMP?

- **Sub-objective 5**

  Chapter 5 aimed at testing whether it was possible to extend the application field of the selected protective cultures. More specific, the usefulness of the cultures to control growth of *L. monocytogenes* and to improve in this way the food safety of CMP was investigated. The introduction of the new regulation on microbial criteria in food (the current Commission Regulation (EC) No 2073/2005) (European Commission, 2005) makes this study even more relevant. Furthermore, one of the objectives of chapter 5 was also to test the influence of inoculum level, storage temperature (4°C versus 7°C) and packaging type (vacuum versus modified atmosphere) on the biopreservative effect.

- **Sub-objective 6**

  The previous chapters had resulted in the selection of the promising protective culture *Lactobacillus sakei* 10A. However, up to that moment, the action of the culture was only tested on a model product. Therefore, it was the objective of **chapter 6** to validate the antagonistic effect of *L. sakei* 10A towards the spoilage organisms *Leuconostoc mesenteroides* and *Brochothrix thermosphacta* on the one hand and towards the food born pathogen *L. monocytogenes* on the other hand when this culture is applied to several industrially prepared cooked meat products. Furthermore, large efforts were made to obtain a better understanding of the impact of the protective culture *L. sakei* 10A on the sensory properties of these products. In this chapter, *L. sakei* 10A was in fact considered as a particular case study from which conclusions on protective cultures in general could be derived.

- **Sub-objective 7**

  The work presented in **chapter 7** tried to elucidate the mechanism by which the non-bacteriocinogenic *L. sakei* 10A inhibits the growth of *L. monocytogenes*.

- **Sub-objective 8**

  Finally, it was the objective of **chapter 8** to broaden our view on biopreservation by investigating biopreservation of vacuum packaged cooked meat products by means of bacteriophages.

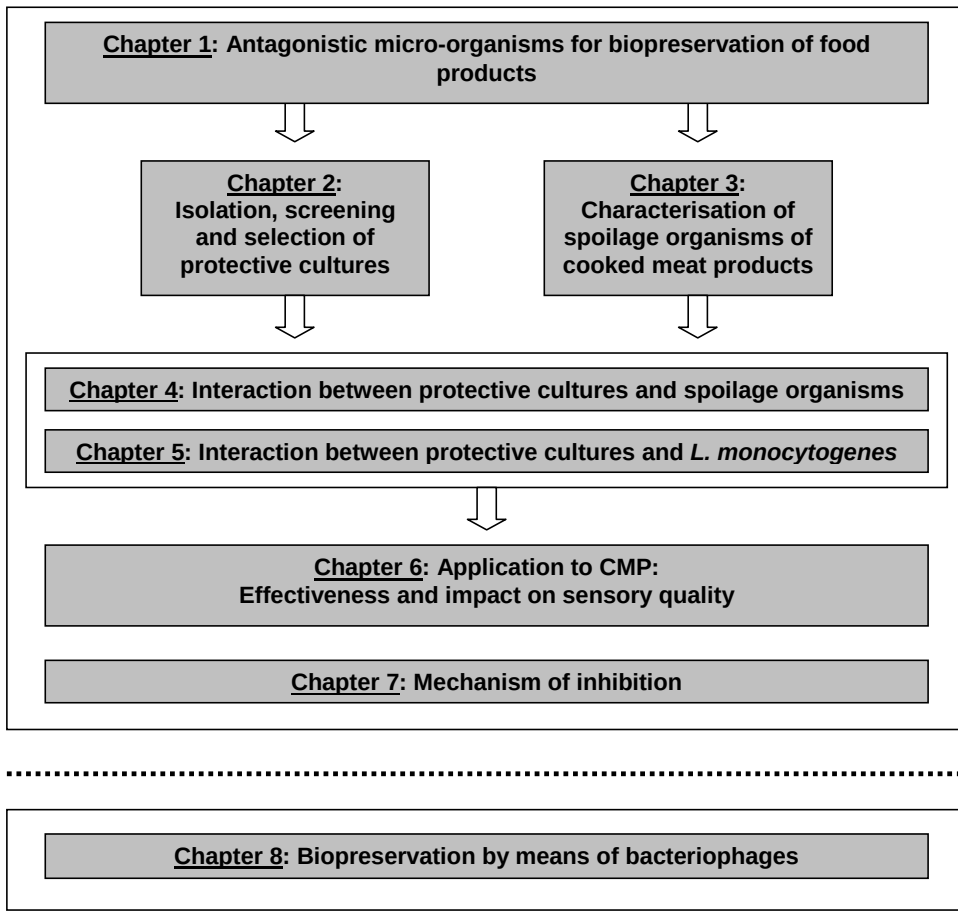

Figure 0.1. Outline of this PhD

Summary

Summary

In **chapter 1**, a literature review was given, discussing three major topics: (1) an introduction on lactic acid bacteria (LAB) and their antimicrobial activity, (2) a brief introduction on anaerobically packaged sliced cooked meat products (CMP) and their microbial ecology and (3) an in-depth overview on biopreservation.

In **chapter 2**, 91 bacterial isolates, originating from meat products, were subjected to a step-by-step screening and characterisation procedure to select potential protective cultures to be used in CMP. Strains were first tested on their homofermentative and psychrotrophic character and salt tolerance. Secondly, the antibacterial capacities towards *Listeria monocytogenes*, *Leuconostoc mesenteroides*, *Leuconostoc carnosum* and *Brochothrix thermosphacta* were determined in an agar spot test. Of the tested strains, 38% was inhibitory towards all indicator strains and 91%, 88% and 74% of the strains could inhibit *L. monocytogenes*, *B. thermosphacta* and *Leuc. mesenteroides*, respectively.

Further, 12 strains - those with the highest antibacterial capacities - were evaluated on their competitive nature by comparing their growth rate, acidifying character and lactic acid production at 7°C under anaerobic conditions in a liquid broth. All 12 strains, except a bacteriocin producing *Lactobacillus plantarum* strain and the lactocin S producing *Lactobacillus sakei* 148, combined a fast growth rate with a deep and rapid acidification due to the production of high levels of lactic acid.

The 12 selected strains were then further investigated for their growth capacity on a model cooked ham (MCH) product to establish whether the presence of these cultures on the ham did not negatively influence the sensory properties of the ham. All strains grew in 6 days at 7°C from a level of 10^5-10^6 cfu/g to 10^7-10^8 cfu/g and again the bacteriocin producing *L. plantarum* strain was the slowest growing strain. As the glucose level of the MCH was low (0.09 ± 0.03%), growth of the putative protective cultures resulted in glucose depletion and a limited lactic acid production and accompanying pH-decrease. Cooked ham, inoculated with isolates 13E, 10A, 14A (all three identified as *L. sakei* subsp. *carnosus*) and strains LS5 (*L. sakei* 148) and LS8 (*L. sakei* subsp. *carnosus* SAGA 777), was not rejected by the sensory panel at the 34th day of the vacuum packaged storage at 7°C. Therefore these strains were considered to have potential as protective culture in CMP.

Chapter 3 presented a systematic study on the behaviour of different types of spoilage organisms, relevant for vacuum packaged sliced CMP to better understand the spoilage they cause and to establish the relationship between microbial growth, pH-evolution, metabolite formation and organoleptic changes.

First, strains were characterised in a liquid growth medium to compare their growth rate, acidifying character and metabolite production under conditions imitating refrigerated vacuum packaged storage. *B. thermosphacta* grew faster than the lactic acid bacteria. All LAB-strains grew fast except *Leuc. mesenteroides* subsp. *dextranicum* and *Leuc. carnosum*. The acidification rate was related to the growth rate, while the acidification depth was more related to the fermentative nature of the strains (homofermentative or heterofermentative metabolism).

Secondly, the growth of the organisms was studied on the MCH. Strains spoiling the model product most rapidly belonged to the species *Leuc. mesenteroides* subsp. *mesenteroides* followed by the species *B. thermosphacta*, while *L. sakei* grew more slowly on the MCH. *Leuc. citreum*, *Leuc. carnosum* and *Weisella viridiscens* demonstrated an intermediate spoilage capacity, whereas *Leuc. mesenteroides* subsp. *dextranicum* and *Leuc. carnosum* grew very slowly compared to the other LAB. Growth of the strains on the MCH resulted in a limited pH-decrease which was a function of the growth rate of the strains. Also the glucose consumption was a function of this growth rate. For none of the strains a significant lactic acid production could be observed. Some small amounts of acetic acid, propionic acid and ethanol were detected for some strains near the end of the storage period. The time at which the MCH became unacceptable from a sensory point of view was linked to the growth rate of the strains, except for *Leuc. citreum* and *Leuc. mesenteroides* subsp. *dextranicum*, which were causing intensive spoilage despite their slow growth. Sensory rejection was mainly based on the attributes odour, taste and acid taste. No clear relationship could be observed between metabolite production and the occurrence of sensory changes.

In **chapter 4**, the usefulness of two LAB, a *L. sakei* subsp. *carnosus* strain (10A) and the lactocin S producing *L. sakei* 148 (LS5), to extend the shelf-life of CMP was investigated. The interactions between these potential protective cultures and the spoilage organisms, *Leuc. mesenteroides* (LM4) and *B. thermosphacta* (BT1), were examined in co-culture studies on a model cooked ham product (7°C, vacuum). The influence of the glucose content of the model cooked ham on the interaction phenomena was investigated by performing the co-culture studies on MCH with a low glucose content of about 0.2% (w/w) glucose and MCH

with a high glucose content of about 1.3% (w/w) glucose. The difficulty in quantifying such an interaction was to individually follow the growth of the homofermentative LAB-strain on the one hand and the growth of the heterofermentative LAB-strain on the other hand when growing in co-culture on the MCH. To resolve this issue, the medium TC8-MRS-agar, consisting of MRS-agar supplemented with tetracycline at 8 µg/ml, was developed. This agar medium allowed the differentiation of LM4-colonies from 10A-colonies or LS5-colonies after incubation for three days at 30°C under anaerobic conditions.

When artificially contaminating the MCH with BT1 at 10^2 cfu/g in combination with 10A at 10^5 cfu/g, the growth of BT1 was significantly slower compared to its simultaneous mono-culture growth. In a similar experiment with LM4, this strain reached a level of 10^7 cfu/g approximately 14 days later when LM4 grew together with *L. sakei* 10A compared to its growth in mono-culture. The lactocin S producing LS5 did not demonstrate an inhibitory action towards LM4 or BT1. On the MCH with low glucose content as well as on the MCH with high glucose content, antagonistic interactions of *L. sakei* 10A towards LM4 and BT1 occurred; the antagonistic effect of *L. sakei* 10A was not eliminated when glucose was abundant in the product.

The results of this chapter indicated that *L. sakei* 10A has potential as protective culture for the shelf-life prolongation of CMP, while *Lactobacillus sakei* LS5 had not.

Chapter 5 investigated the same potential protective cultures that had been studied in chapter 4, being the *Lactobacillus sakei* subsp. *carnosus* strain (10A) and the lactocin S producing *Lactobacillus sakei* 148 (LS5). Their capacity to increase the food safety and in particular to control the growth of *L. monocytogenes* on CMP was investigated. The interaction between these LAB and a cocktail of three *L. monocytogenes* strains was examined in co-culture studies on a MCH. Furthermore, the influence of the inoculum level (10^5 cfu/g versus 10^6 cfu/g), storage temperature (4°C versus 7°C) and packaging type (vacuum packaging versus modified atmosphere packaging) on the interaction phenomena was investigated. At 7°C, applying *L. sakei* 10A at 10^6 cfu/g limited the growth of *L. monocytogenes* to a level <1 $\log_{10}$(cfu/g) during 27 days, whilst an application level of 10^5 cfu/g failed to prevent growth to unacceptable levels. *L. sakei* LS5 did not demonstrate an antagonistic effect towards *L. monocytogenes* and is therefore not useful as protective culture on cooked meat products. Lowering the temperature to 4°C or switching from vacuum packaging to modified atmosphere packaging did not influence the ability of strain 10A to grow on the MCH, as its dominance did not change. A combination of strain 10A and 4°C or a combination of strain

10A and an atmosphere containing 50% of CO_2 completely inhibited the growth of *L. monocytogenes*. Sensory assessment and pH-measurements confirmed that *L. sakei* 10A, even when present at a high level (>7 $\log_{10}$(cfu/g)) for prolonged storage times (up to 42 days), did not acidify the cooked ham to a point of sensory rejection.

Biopreservation has been proven to be a promising natural preservation technique, but the impact of protective cultures on the sensory properties of CMP is not well documented. **Chapter 6** presented a case study on the protective culture *L. sakei* 10A to obtain a clear view on the real consequences of using protective cultures on the sensory quality of CMP. A preliminary screening study on 13 different CMP and more elaborate application trials at 7°C on vacuum packaged pâté, cooked ham, cooked sausage and two cooked poultry products demonstrated that *L. sakei* 10A inhibits the growth of the endogenous LAB-flora and of artificially inoculated *Leuc. mesenteroides*, *B. thermosphacta* and *L. monocytogenes* cells. Despite these promising antagonistic effects, the application of *L. sakei* 10A to CMP was in some cases limited by a significant acidification resulting in an acid taste of the product. This was most obvious in pâté and cooked sausage and less obvious in cooked turkey fillet. The hypothesis could be derived that high buffering capacity and low glucose content are essential properties to avoid sensory deviations when applying protective cultures on CMP.

The study presented in **chapter 7** investigated possible mechanisms by which the non-bacteriocinogenic *L. sakei* 10A inhibits *L. monocytogenes.*
First, the antagonistic character of *L. sakei* 10A was confirmed by the observation of growth inhibition of *L. monocytogenes* in the presence of 10A in buffered BHI-broth (b-BHI) (7°C, anaerobic). When assessing the growth of *L. monocytogenes* in the cell free supernatants (CFS), obtained after centrifugation of a 10A-culture at different time points during its growth in b-BHI, it became clear that the older the culture was, the more inhibitory properties it had, meaning that either production of (an) antimicrobial compound(s) or nutrient competition caused the inhibition.
The precise role of lactic acid production and nutrient competition was more obvious during co-culture experiments in two types of broth, differing from each other in their glucose level and in the presence/absence of yeast extract, Mn^{2+} and Mg^{2+}. The presence of more nutrients did not prevent the growth inhibition of *L. monocytogenes* by *L. sakei* 10A. In the nutrient-poor broth, inhibition coincided with the moment of glucose depletion. In the nutrient-rich broth, an increased lactic acid production was thought to cause the inhibition. Subsequent

challenge experiments with *L. monocytogenes* in the CFS, obtained from 10A's growth in the media with the two different nutrient levels, allowed distinction between the different antagonistic effects (pH-reduction, lactic acid production and nutrient competition). In the nutrient-poor broth, growth inhibition was exclusively caused by nutrient competition and competition for glucose was, at least partly, involved. In the nutrient-rich broth, growth inhibition was caused by a combination of several factors: the antimicrobial effect of the produced lactic acid/lactate, nutrient competition and pH-reduction as a consequence of lactic acid formation.

In a final experiment, no effect of supplementation with vitamins and minerals on the inhibition phenomena could be observed.

Chapter 8 reports on the use of bacteriophage P100 to prevent proliferation of post-processing contaminating *L. monocytogenes* cells on vacuum packaged sliced cooked meat products.

At first, broth experiments revealed that the three *L. monocytogenes* strains, used in chapters 5 and 6 of this PhD-work, were each susceptible to the action of bacteriophage P100 and this at 30°C as well as at 7°C. However, at 30°C the susceptibility towards P100 was strain-dependent since the time, at which the OD(600 nm) of the growth medium containing *L. monocytogenes* and P100 started to increase, differed among the three strains. Therefore subsequent application trials on cooked meat products were making use of a cocktail of the three *L. monocytogenes* strains.

In a preliminary application test on a sliced, cooked poultry product the presence of phage P100 resulted in a reduction of the *L. monocytogenes* count with 3.32 $\log_{10}$(cfu/g) compared to the untreated control after 21 days of storage (7°C, vacuum). A more elaborate application test on sliced cooked ham confirmed the antilisterial effect of P100 on CMP during storage at 7°C under vacuum packaged conditions. In the latter experiment, treatment with P100 at a level of 1×10^7 pfu/cm^2 or 5×10^6 pfu/cm^2 reduced the population of *L. monocytogenes* after 10 days of storage (7°C, vacuum) with 0.97 and 0.61 $\log_{10}$(cfu/g), respectively compared to the untreated control. However, the difference in antilisterial effect between the two different phage doses was shown to be not significant.

In conclusion, this chapter provided evidence on the usefulness of bacteriophage P100 to control the growth of *L. monocytogenes* on sliced, cooked meat products during anaerobic storage at 7°C.

Samenvatting

Samenvatting

De literatuurstudie (**Hoofdstuk 1)** omvat drie belangrijke onderwerpen: (1) een inleiding tot melkzuurbacteriën en hun antimicrobiële activiteit, (2) een beknopte inleiding tot anaëroob verpakte, versneden, gekookte vleesproducten en hun microbiële ecologie en (3) een grondig overzicht over bioconservering.

In **hoofdstuk 2** worden 91 bacteriële culturen, geïsoleerd uit vleesproducten, onderworpen aan een stapsgewijze screening en karakterisatie om potentiële protectieve culturen te selecteren die gebruikt kunnen worden voor de bioconservering van gekookte vleesproducten. De stammen worden eerst getest op hun homofermentatief en psychrotroof karakter en ook op zouttolerantie. Vervolgens worden hun antibacteriële eigenschappen tegenover *Listeria monocytogenes*, *Leuconostoc mesenteroides*, *Leuconostoc carnosum* en *Brochothrix thermosphacta* bepaald in een agar spot test. Van de geteste stammen was 38% groeiremmend tegenover alle indicator stammen; 91%, 88% en 74% van de stammen kon de groei remmen van *L. monocytogenes*, *B. thermosphacta* en *Leuc. mesenteroides*, respectievelijk.
Verder worden 12 stammen – deze met de meest uitgesproken antibacteriële capaciteit – geëvalueerd op basis van hun competitief karakter door een vergelijking te maken van hun groeisnelheid, verzurend karakter en melkzuurproductie bij 7°C onder anaërobe omstandigheden in een vloeibaar groeimedium. Alle 12 stammen, met uitzondering van een bacteriocineproducerende *Lactobacillus plantarum* stam en de lactocin S producerende *Lactobacillus sakei* 148, combineerden een snelle groei met een diepe en snelle verzuring die veroorzaakt werd door de productie van hoge concentraties aan melkzuur.
De 12 geselecteerde stammen worden vervolgens verder onderzocht op hun vermogen tot groei op een modelkookham om na te gaan of de aanwezigheid van deze culturen op de ham geen negatieve invloed had op de sensorische eigenschappen van het product. Alle stammen groeiden bij 7°C in 6 dagen van 10^5-10^6 kve/g tot 10^7-10^8 kve/g en het was opnieuw de bacteriocineproducerende *L. plantarum* die het traagst groeide. Aangezien de modelkookham weinig glucose bevatte (0.09 ± 0.03%), leidde de groei van de potentiële protectieve culturen tot uitputting van glucose en tot een beperkte melkzuurvorming en beperkte pH-daling. Kookham, beënt met isolaten 13E, 10A, 14A (alle drie geïdentificeerd als *L. sakei* subsp. *carnosus*) en stammen LS5 (*L. sakei* 148) en LS8 (*L. sakei* subsp. *carnosus* SAGA 777), werd na 34 dagen bewaring (vacuüm, 7°C) niet verworpen door het sensorisch panel. Daarom

werden deze stammen beschouwd als mogelijke protectieve culturen met potentieel voor de bioconservering van gekookte vleesproducten.

Hoofdstuk 3 beschrijft een systematische studie over het gedrag van verschillende soorten bederforganismen, die relevant zijn voor vacuümverpakte versneden gekookte vleesproducten. Deze studie heeft als doel inzicht te verwerven in het bederf dat deze stammen veroorzaken en in de relatie tussen hun groei, pH-evolutie, metabolietvorming en het optreden van organoleptische wijzigingen.
In een eerste fase worden negen bederforganismen met elkaar vergeleken wat betreft hun groeisnelheid, verzurend karakter en metabolietvorming in een vloeibaar groeimedium onder condities die gekoelde vacuümverpakte bewaaromstandigheden nabootsen. De *B. thermosphacta* stammen groeiden sneller dan de melkzuurbacteriën. Alle geteste melkzuurbacterie-stammen groeiden snel met uitzondering van *Leuc. mesenteroides* subsp. *dextranicum* en *Leuc. carnosum*. De snelheid van verzuring was gelinkt aan de groeisnelheid, terwijl de diepte van de verzuring gerelateerd was aan het fermentatief karakter van de stammen (homo- of heterofermentatief metabolisme).
Vervolgens wordt de groei van dezelfde negen bederforganismen bestudeerd op een modelkookham. De stammen, die het snelst tot bederf van het modelproduct leidden, behoorden tot het species *Leuc. mesenteroides* subsp. *mesenteroides* gevolgd door het species *B. thermosphacta*, terwijl *L. sakei* trager groeide op de modelkookham. *Leuc. citreum, Leuc. carnosum* en *Weissella viridiscens* vertoonden een meer gematigd bederfpatroon, terwijl *Leuc. mesenteroides* subsp. *dextranicum* en *Leuc. carnosum* zeer traag groeiden in vergelijking met de andere melkzuurbacteriën. De groei van de teststammen op de modelkookham resulteerde in een beperkte pH-daling die gelinkt was aan de groeisnelheid van de stammen. Ook het glucoseverbruik was gerelateerd aan deze groeisnelheid. Voor geen enkele stam kon een significante melkzuurvorming geobserveerd worden. Bij enkele stammen werd op het einde van de bewaartijd kleine hoeveelheden azijnzuur, propionzuur en ethanol gedetecteerd. Het moment waarop de modelkookham sensorisch gezien onaanvaardbaar werd, was gelinkt aan de groeisnelheid van de stammen, behalve voor *Leuc. citreum* en *Leuc. mesenteroides* subsp. *dextranicum,* die toch bederf veroorzaakten ondanks hun trage groei. Het sensorisch afkeuren van de modelkookham gebeurde hoofdzakelijk op basis van geur, smaak en zure smaak. Tussen metabolietvorming en het optreden van sensorische wijzigingen kon geen duidelijk verband gevonden worden.

In **hoofdstuk 4** wordt onderzocht in welke mate twee geselecteerde melkzuurbacteriën, *L. sakei* subsp. *carnosus* 10A en de lactocin S producerende *L. sakei* LS5, bruikbaar zijn voor het verlengen van de houdbaarheid van gekookte vleesproducten. De interacties tussen deze twee potentiële protectieve culturen enerzijds en de bederforganismen, *Leuc. mesenteroides* (LM4) en *B. thermosphacta* (BT1), anderzijds worden bestudeerd in co-cultuur experimenten op een modelkookham (vacuüm, 7°C). Bovendien wordt ook nagegaan wat de invloed is van het glucosegehalte van de modelkookham op de waargenomen interacties door de co-cultuurexperimenten uit te voeren op een modelkookham met een laag (0.2%) glucosegehalte en op een modelkookham met een hoog (1.3%) glucosegehalte. De moeilijkheid in het kwantificeren van een dergelijke interactie is het afzonderlijk opvolgen van de individuele groei van homofermentatieve en heterofermentatieve melkzuurbacteriën wanneer ze in co-cultuur met elkaar groeien. Daarom werd een nieuw medium, TC8-MRS-agar, ontwikkeld. Dit medium bestond uit MRS-agar gesupplementeerd met tetracycline (8 µg/ml) en liet toe om kolonies van *Leuc. mesenteroides* LM4 te onderscheiden van kolonies van *L. sakei* 10A of LS5 na anaërobe incubatie gedurende drie dagen bij 30°C. Wanneer de modelkookham beënt was met BT1 aan 10^2 kve/g in combinatie met 10A aan 10^5 kve/g, werd vastgesteld dat de groei van BT1 significant trager verliep in vergelijking met zijn gelijktijdige monocultuur groei. In een gelijkaardig experiment met LM4, bereikte deze stam in aanwezigheid van *L. sakei* 10A een celaantal van 10^7 kve/g ongeveer 14 dagen later dan wanneer LM4 groeide zonder *L. sakei* 10A. De lactocin S producerende stam LS5 vertoonde geen groeiremmend effect tegenover LM4 of BT1. Het antagonistische effect van *L. sakei* 10A tegenover LM4 en BT1 trad zowel op in de modelkookham met een laag glucosegehalte als in deze met een hoog glucosegehalte en werd dus niet geëlimineerd wanneer glucose in overmaat aanwezig was in het product.

De resultaten van dit hoofdstuk tonen aan dat *L. sakei* 10A mogelijkheden biedt als protectieve cultuur voor de houdbaarheidsverlenging van gekookte vleesproducten, terwijl *L. sakei* LS5 dit niet biedt.

In **hoofdstuk 5** wordt onderzocht of dezelfde twee melkzuurbacteriën, *L. sakei* 10A en *L. sakei* LS5, ook bruikbaar zijn om de voedselveiligheid van gekookte vleesproducten te verbeteren. De interactie tussen deze potentiële protectieve culturen en een cocktail van drie *L. monocytogenes* stammen wordt onderzocht door middel van co-cultuur experimenten op modelkookham. Daarnaast wordt ook onderzocht of de interactiefenomenen beïnvloed worden door het inoculumniveau van de protectieve cultuur (10^5 kve/g versus 10^6 kve/g), de

bewaartemperatuur (4°C versus 7°C) en de manier van verpakken (vacuüm versus gemodificeerde atmosfeer). De toepassing van *L. sakei* 10A aan 10^6 kve/g bij 7°C beperkte de uitgroei van *L. monocytogenes* tot minder dan 1 $\log_{10}$(kve/g) gedurende 27 dagen, terwijl een dosis van 10^5 kve/g onvoldoende was om uitgroei tot onaanvaardbare niveau's te voorkomen. *L. sakei* LS5 werkte niet antagonistisch tegenover *L. monocytogenes* en is daarom niet bruikbaar als protectieve cultuur op gekookte vleesproducten. Het verlagen van de temperatuur tot 4°C of overstappen van vacuümverpakking op MAP had geen invloed op het groeivermogen van *L. sakei* 10A op de modelkookham en dominantie van 10A bleef gegarandeerd. De groei van *L. monocytogenes* kon op de modelkookham volledig voorkomen worden door de toepassing van stam 10A te combineren met een bewaartemperatuur van 4°C of met een gemodificeerde atmosfeer die 50% CO_2 bevatte. De sensorische evaluatie en de pH-metingen bevestigden dat *L. sakei* 10A de modelkookham niet verzuurde tot een sensorisch onaanvaardbaar product zelfs wanneer de melkzuurbacterie gedurende lange tijd aanwezig was in hoge celaantallen.

Bioconservering is een veelbelovende natuurlijke vorm van conservering, maar de impact van protectieve culturen op de sensorische eigenschappen van gekookte vleesproducten is een aspect dat onvoldoende gedocumenteerd is. **Hoofdstuk 6** beschrijft de gevalstudie van de protectieve cultuur *L. sakei* 10A met de bedoeling een duidelijker beeld te krijgen over de werkelijke consequenties van het gebruik van protectieve culturen op de sensorische kwaliteit van gekookte vleesproducten. Een voorafgaande studie met 13 verschillende soorten gekookte vleesproducten en meer uitgebreide toepassingstesten bij 7°C met vacuümverpakte paté, kookham, kookworst, kippewit en kalkoenwit toonden aan dat *L. sakei* 10A in staat is de groei van de natuurlijke melkzuurflora te remmen alsook die van kunstmatig geënte *Leuc. mesenteroides*, *B. thermosphacta* en *L. monocytogenes* cellen. Ondanks deze veelbelovende antagonistische effecten was de toepassing van *L. sakei* 10A op gekookte vleesproducten in sommige gevallen beperkt door een significante verzuring die aanleiding gaf tot een zure smaak van het product. Dit fenomeen was het duidelijkst in paté en kookworst en minder duidelijk in kalkoenwit. Op basis van de resultaten werd de hypothese geformuleerd dat een hoge buffercapaciteit en een laag glucosegehalte cruciale eigenschappen zijn van gekookte vleesproducten in het voorkomen van sensorische afwijkingen ten gevolge van het toepassen van protectieve culturen.

Hoofdstuk 7 beschrijft onderzoek naar het werkingsmechanisme van de niet-bacteriocinogene cultuur *L. sakei* 10A tegenover *L. monocytogenes.*

Eerst en vooral werd het antagonistisch karakter van *L. sakei* 10A bevestigd, aangezien in aanwezigheid van 10A groeiremming van *L. monocytogenes* optrad in een gebufferde BHI-broth (b-BHI) (7°C, anaëroob). Bij het bepalen van de groei van *L. monocytogenes* in verschillende celvrije cultuurmedia, bekomen na centrifugatie van een 10A-cultuur op verschillende tijdstippen tijdens zijn groei in b-BHI, werd het duidelijk dat hoe ouder de cultuur was, hoe meer groeiremmende eigenschappen het celvrij medium vertoonde. Dit betekent dat de groeiremming ofwel veroorzaakt wordt door de productie van één of meerdere antimicrobiële component(en) ofwel door competitie voor nutriënten.

De exacte rol van melkzuurvorming en nutriëntcompetitie werd duidelijker tijdens co-cultuur experimenten in twee soorten vloeibaar groeimedium, die van elkaar verschilden in hun glucosegehalte en in de aanwezigheid/afwezigheid van gistextract, Mn^{2+} en Mg^{2+}. De aanwezigheid van meer nutriënten kon niet voorkomen dat er groeiremming optrad van *L. monocytogenes* door *L. sakei* 10A. In het nutriënt-arme medium viel het moment van groeiremming samen met het moment dat het medium geen glucose meer bevatte. In het nutriënt-rijke medium werd de uitgesproken melkzuurvorming ervan verdacht de groeiremming te veroorzaken. Daaropvolgende challenge testen met *L. monocytogenes* in het celvrij kweekmedium, afkomstig van de groei van 10A in de media met de verschillende gehalten aan nutriënten, liet toe om een onderscheid te maken tussen de verschillende antagonistische effecten (pH-daling, melkzuurvorming en nutriëntcompetitie). In het nutriënt-arme medium werd de groeiremming uitsluitend veroorzaakt door nutriëntcompetitie en competitie voor glucose was, tenminste gedeeltelijk, hierbij betrokken. In het nutriënt-rijke medium werd de groeiremming veroorzaakt door een combinatie van verschillende factoren: het antimicrobiële effect van het geproduceerde melkzuur/lactaat, nutriëntcompetitie en pH-daling als gevolg van melkzuurvorming.

In een laatste experiment werd vastgesteld dat extra toevoeging van vitaminen en mineralen aan het groeimedium geen effect had op de inhibitiefenomenen wat doet vermoeden dat competitie voor deze vitaminen en mineralen geen bijkomende rol speelt in het inhibitiemechanisme van *L. sakei* 10A.

Hoofdstuk 8 rapporteert over het gebruik van bacteriofaag P100 om te voorkomen dat nabesmetting met *L. monocytogenes* aanleiding geeft tot uitgroei van deze voedselpathogeen op vacuümverpakte gekookte vleesproducten.

In-vitro testen toonden aan dat de drie *L. monocytogenes* stammen, die gebruikt werden in hoofdstuk 5 en 6 van dit werk, elk op zich gevoelig waren voor de werking van bacteriofaag P100 en dit zowel bij 30°C als bij 7°C. Bij 30°C was de gevoeligheid voor P100 wel stamafhankelijk aangezien het tijdstip, waarop de OD(600 nm) van het groeimedium, dat *L. monocytogenes* en P100 bevatte, begon toe te nemen, verschilde tussen die drie stammen. Daarom werd bij de daaropvolgende toepassingstesten op gekookte vleesproducten gewerkt met een mengsel van deze drie *L. monocytogenes* stammen.

In een voorafgaande toepassingstest op versneden, gekookt kippewit resulteerde de aanwezigheid van faag P100 in een reductie van het celaantal van *L. monocytogenes* met 3.32 $\log_{10}$(kve/g) in vergelijking met een onbehandelde controle na 21 dagen bewaring (7°C, vacuüm).

Een meer uitgebreide toepassingstest op versneden kookham bevestigde de antilisteriale werking van P100 op gekookte vleesproducten tijdens bewaring bij 7°C onder vacuüm. In dit laatste experiment gaf een behandeling met een P100-dosis van 1×10^7 pve/cm^2 of 5×10^6 pve/cm^2 na 10 dagen bewaring (7°C, vacuüm) aanleiding tot een reductie van de *L. monocytogenes* populatie met 0.97 en 0.61 $\log_{10}$(kve/g), respectievelijk, in vergelijking met de onbehandelde controle. Het verschil in antilisteriaal effect tussen de twee P100-dosissen was echter statistisch niet significant.

Dit hoofdstuk levert dus het bewijs dat bacteriofaag P100 bruikbaar is om de uitgroei van *L. monocytogenes* tot onaanvaardbare niveau's tegen te gaan op versneden, gekookte vleesproducten die bewaard worden in vacuümverpakking bij 7°C.

Chapter 1

Antagonistic micro-organisms for biopreservation of food products

Chapter 1
Antagonistic micro-organisms for biopreservation of food products

1. Lactic acid bacteria

1.1. Introduction

Lactic acid bacteria (LAB) constitute a diverse group of micro-organisms widely distributed in nature and associated with dairy, vegetable and meat products. LAB are best known for their use as starter cultures in the manufacture of dairy products and are also commercially important in the processing of meat, alcoholic beverages and vegetables. Although LAB are often beneficial in the food industry, they also can be a nuisance as spoilage causing contaminants of food products (Carr et al., 2002).

Lactic acid bacteria are broadly defined as Gram-positive, non-spore-forming rods, cocci or coccobacilli with a DNA-base composition of less than 50 mol% G+C (Stiles & Holzapfel, 1997). They are oxidase negative and generally catalase negative; motility and nitrate reduction are highly unusual (Kandler & Weiss, 1986; Vandamme et al., 1996; Carr et al., 2002). Kandler & Weiss (1986) describe LAB as micro-aerophilic but other wordings for this feature are aerobic to facultatively anaerobic (Carr et al., 2002) or fermentative but aerotolerant (Liu, 2003).

1.2. Taxonomy

The concept of the LAB as a group of organisms developed at the beginning of the 20th century. LAB have a long and complex taxonomic history (Pot et al., 1994; Stiles & Holzapfel, 1997). The classical approach to bacterial taxonomy was based on morphological, physiological and biochemical features. This was expanded to chemical taxonomy including analysis of the cell wall composition, comparison of whole-cell protein patterns obtained by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and other techniques. The use of molecular characteristics, such as the mol% G+C of the DNA-content, and the application of genetic techniques, such as 16S rRNA sequence analysis, as taxonomic tools have resulted in significant changes in the taxonomy of LAB (Pot et al., 1994; Stiles & Holzapfel, 1997; Temmerman et al., 2004). Unfortunately, these new genetically based taxonomic relationships cut across the phenotypic lines that have been used for many years

(Stiles & Holzapfel, 1997). Another development of bacterial taxonomy, polyphasic taxonomy, aims at the integration of different kinds of data and information (phenotypic, genotypic and phylogenetic) on LAB (Vandamme et al., 1996).

The classification of LAB remains under investigation but this group is generally restricted to the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella*. Representatives of the genera *Aerococcus, Alloiococcus, Atopobium, Dolosigranulum, Gemella, Globicatella, Helcococcus, Melissococcus* and *Saccharococcus* are generally not considered to belong to the LAB, although they might meet the broad definition of this group (Vandamme et al., 1996).

Phylogenetically, LAB belong to the so-called *Clostridium* branch of the Gram-positive bacteria, a phylum consisting of bacteria with a DNA-base composition of less than 50 mol% G+C (Kandler & Weiss, 1986; Vandamme et al., 1996).

1.3. Metabolism and energy generation

Metabolically, LAB are at the threshold of anaerobic to aerobic life. They are told to have a fermentative metabolism and to have a need for a fermentable carbohydrate for growth because they possess efficient carbohydrate fermentation pathways coupled to substrate level phosphorylation (Kandler & Weiss, 1986; Vandamme et al., 1996). A second substrate level phosphorylation site is coupled to arginine fermentation, observed in most of the heterofermentative lactobacilli. In addition to substrate level phosphorylation, LAB may gain energy from the proton motive force by lactate efflux. Lactobacilli contain in general no isoprenoid quinines and no cytochrome systems to perform oxidative phosphorylation. However, they possess (per)oxidases to carry out the oxidation of $NADH_2$ and O_2 as the final electron acceptor. Furthermore, they are able to perform a Mn-catalysed scavenging of superoxide although they do not possess dismutase and catalase (Kandler & Weiss, 1986; Piard & Desmazeaud, 1991).

Lactic acid bacteria, although consisting of a number of diverse genera, are grouped as either homofermentatives or heterofermentatives based on the end-product(s) of their hexose fermentation (Figure 1.1). The homofermentatives produce lactic acid as the major end-product of glucose fermentation. The heterofermentatives produce a number of products

besides lactic acid, including carbon dioxide, acetic acid and ethanol from the fermentation of glucose (Carr et al., 2002).

Homofermentative LAB possess the enzyme aldolase and are able to convert one mol hexose through the Embden-Meyerhof-Parnas (EMP) pathway to two mol of lactic acid (homolactic fermentation). The heterofermentative LAB lack aldolase but possess the enzyme phosphoketolase and use the alternative 6-phosphogluconate pathway, resulting in one mol CO_2, one mol ethanol (or acetic acid) and one mol lactic acid (heterolactic fermentation) (Kandler & Weiss, 1986; Carr et al., 2002).

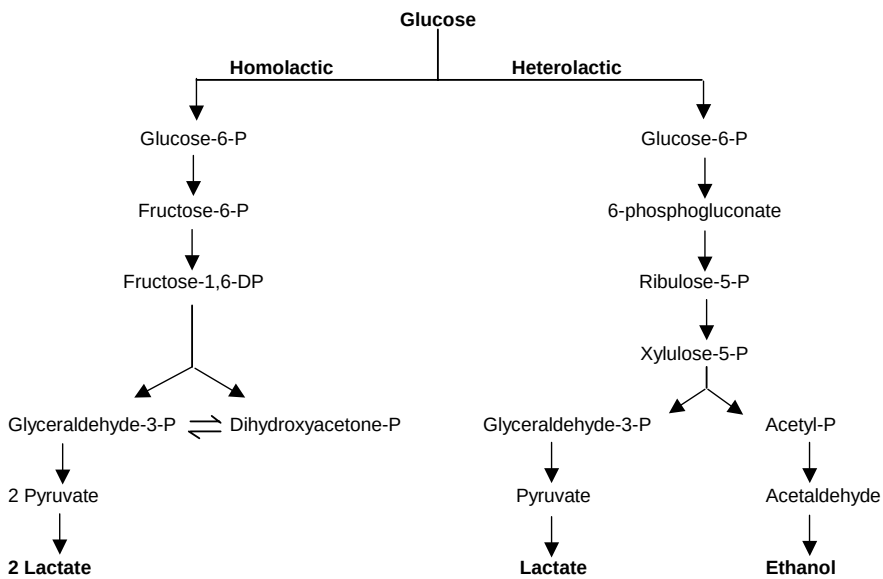

Figure 1.1. Glucose metabolism in homo- and heterofermentative LAB (Caplice & Fitzgerald, 1999)

Lactic acid bacteria are also able to metabolise a number of non-carbohydrates including organic acids such as citrate (Hugenholtz, 1993) and lactate (Liu, 2003), peptides, amino acids such as arginine (Kandler & Weiss, 1986; Urbach, 1995; Arena et al., 1999; Tavaria et al., 2002), etc.

Citrate can be degraded to unusual fermentation products with very distinct aroma properties such as diacetyl, acetoin, butanediol and acetaldehyde (Hugenholtz, 1993).

Lactic acid bacteria can hydrolyse peptides to free amino acids. Amino acid catabolism produces, in turn, a number of compounds, including ammonia, amines, aldehydes, phenols,

indole and alcohols, which play an important role in the flavour development of dairy products (Urbach, 1995; Tavaria et al., 2002). Arginine catabolism occurs in most of the heterofermentative lactobacilli and results in the production of citrulline, ornithine and ammonia (Kandler & Weiss, 1986; Arena et al. 1999).

1.4. Lactic acid bacteria of importance in food products

In food, LAB contribute to the taste and texture of fermented products and inhibit growth of bacteria by the production of growth-inhibiting substances. However, LAB are also known to be involved in spoilage of e.g. beer, wine, meat and meat products and some fish products. The genera of most importance in the microbial ecology of food products are *Lactobacillus, Pediococcus, Leuconostoc* and *Lactococcus* (Stiles & Holzapfel, 1997; Carr et al., 2002).

1.4.1. The genus *Lactococcus*

The genus *Lactococcus* includes several species but *Lactococcus lactis* is the most important species of the commercially used LAB. *Lactococcus lactis* is commonly isolated from plant material but the most recognised habitat is dairy products. They are non-motile, coccus-shaped, homofermentative bacteria that grow at 10°C and 40°C but not at 45°C, grow in 4% NaCl and produce L(+)-lactic acid from glucose (Stiles & Holzapfel, 1997; Carr et al., 2002). The use of lactococci, mainly in the dairy industry (production of cheese or yoghurt), is widespread and has the longest tradition in industrial starter culture technology. Some strains of *Lc. lactis* are also important because of nisin production (Stiles & Holzapfel, 1997).

1.4.2. The genus *Leuconostoc*

The leuconostocs are heterofermentative cocci that occur in pairs and chains and form D(-)-lactic acid and carbon dioxide from the fermentation of glucose. The leuconostocs require a less acidic environment (pH $\geq$ 4,5) than the lactobacilli and the pediococci, which are more acid tolerant (Carr et al., 2002). Plants are the natural habitat of this genus and *Leuc. mesenteroides* subsp. *mesenteroides* is the principal isolate. In fermented foods of plant origin, *Leuc. mesenteroides* is generally the first organism to grow and it is succeeded by the more acid-tolerant lactobacilli. *Leuconostoc mesenteroides*, *Leuconostoc gelidum* and *Leuconostoc carnosum* have been isolated from raw and processed meat and meat products packaged under vacuum or in a modified atmosphere (Stiles & Holzapfel, 1997). Spoilage of meat products through leuconostocs may be accompanied by the production of slime.

1.4.3. The genus *Pediococcus*

This genus is characterised by tetrad formation and a spherical shape, pseudocatalase production and salt tolerance. The pediococci have a homofermentative metabolism and produce DL- or L(+)-lactic acid from glucose according to the Embden-Meyerhof pathway. Pediococci occur on plant materials, in various food products and as spoilage agents in alcoholic beverages such as beer. *Pediococcus pentosaceus* and *Pediococcus acidilactici* are important starter cultures for fermented sausage production (Stiles & Holzapfel, 1997).

1.4.4. The genus *Lactobacillus*

1.4.4.1. Habitat

The lactobacilli grow in and are associated with many different habitats, e.g. plants, soil, water, sewage and manure, cereal products, silage, milk and dairy products, meat, fish and vegetable products. They are involved in spoilage of several food products and occur in the respiratory, intestinal and genital tracts of humans and animals (Stiles & Holzapfel, 1997).

1.4.4.2. Cell morphology and growth conditions

Lactobacilli meet the general definition for LAB. Their cells vary from long and slender, sometimes bent rods to short, often coryneform coccobacilli and chain formation is common. The lactobacilli are strictly fermentative and have complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates.

Their growth temperature ranges from 2 to 53-55°C. Most lactobacilli grow best at mesophilic temperatures with an upper limit of around 40°C. Some grow also below 15°C and some psychrophilic strains even below 5°C (as low as 2°C). The so-called thermophilic lactobacilli may have an upper limit of 55°C.

Lactobacilli are generally aciduric or acidophilic, they grow best in slightly acidic media with an initial pH of 6.4 to 4.5 and growth ceases when pH 4.0 to 3.6 is reached, depending on the species and strain.

Although most strains are fairly aerotolerant, optimum growth is achieved under micro-aerophilic or anaerobic conditions (Kandler & Weiss, 1986; Carr et al., 2002).

1.4.4.3. Classification

Although earlier subdivisions exist, the classical division of the lactobacilli is based on their fermentation characteristics: (1) obligately homofermentative; (2) facultatively heterofermentative and (3) obligately heterofermentative (Kandler & Weiss, 1986; Stiles & Holzapfel, 1997).

The obligately homofermentative lactobacilli (group 1) possess aldolase but no phophoketolase (Kandler & Weiss, 1986) and degrade hexoses almost exclusively to lactic acid by the Embden-Meyerhof pathway and do not ferment pentoses or gluconate (Vandamme et al., 1996). Some important food associated species of this group are *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Lactobacillus helveticus* (Stiles & Holzapfel, 1997).

The facultatively heterofermentative lactobacilli (group 2) ferment hexoses almost exclusively to DL-lactic acid by the Embden-Meyerhof pathway or to lactic acid, acetic acid, ethanol and formic acid under glucose limitation. Pentoses are fermented to lactic acid and acetic acid via an inducible pentose phosphoketolase (Kandler & Weiss, 1986; Vandamme et al., 1996). They may produce gas from gluconate but not from glucose. Important food-associated species in this group are *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus sakei* and *Lactobacillus curvatus* (Stiles & Holzapfel, 1997).

The obligately heterofermentative lactobacilli (group 3) lack aldolase and ferment hexoses to DL-lactic acid, acetic acid, ethanol and CO_2 via the 6-phosphogluconate pathway. Pentoses are fermented to lactic acid and acetic acid. In general, a pentose phosphoketolase is involved in both pathways (Kandler & Weiss, 1986; Vandamme et al., 1996). The production of gas from glucose is a characteristic feature of these bacteria. The most important food-associated species of this group are *Lactobacillus sanfranciscensis* involved in the production of sourdough bread, *Lactobacillus reuteri* of interest because of its antimicrobial metabolite reuterin and *Lactobacillus brevis* causing spoilage in citrus fruits, beer, wine and some meat products (Stiles & Holzapfel, 1997).

The past two decades, a whole range of taxonomic reassignments occurred within the genus *Lactobacillus*, based almost exclusively on the results of rRNA-sequencing or DNA-DNA hybridisations. The large number of nomenclature revisions is a striking indication of the discrepancies between the results obtained by former traditional phenotypic tests (classical

taxonomy) and the present phylogenetic insights obtained by rRNA-sequencing (molecular taxonomy) (Vandamme et al., 1996).

Based on 16S rRNA-homology, lactobacilli can be subdivided into three major groups: (1) the *Lactobacillus delbrueckii* group, (2) the *Lactobacillus casei-Pediococcus* group and (3) the *Leuconostoc* group (Vandamme et al., 1996). However, this redefinition of the *Lactobacillus* group of the LAB crosses the established metabolism-based classification.

1.4.4.4. The species Lactobacillus sakei

Lactobacillus sakei (Figure 1.2), formerly *L. sake*, was originally isolated from sake or rice wine. Its cells are rods with rounded ends, occurring singly and in short chains and frequently slightly curved (Kandler & Weiss, 1986).

Together with *L. curvatus, L. sakei* constitutes a subgroup of the facultatively heterofermentative lactobacilli (Stiles & Holzapfel, 1997) that are mannitol negative and ribose positive (Carr et al., 2002). Despite wide phenotypic heterogeneity, *L. sakei* strains are closely related to the genomic level (Champomier-Vergès et al., 2002). The species *L. sakei* was split into *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus* (Torriani et al., 1996). *Lactobacillus sakei* belongs to the main flora of fresh meat and becomes dominant flora when meat and meat products are stored under anaerobic conditions (Devlieghere et al., 1998; Samelis et al., 2000a). Furthermore, it is an important starter culture in fermented meat products (Stiles & Holzapfel, 1997; Carr et al., 2002).

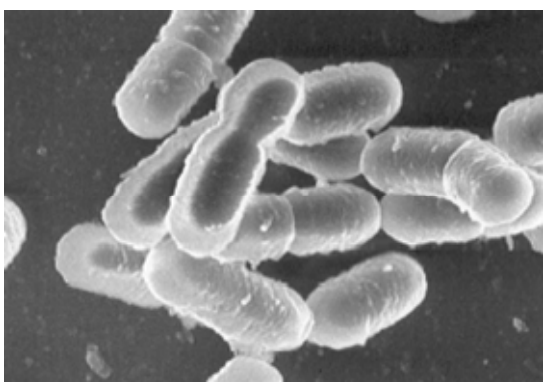

Figure 1.2. Scanning electron microscopy of a *L. sakei* strain (Champomier-Vergès et al., 2002)

Lactobacillus sakei is known to be one of the most psychrotrophic species of lactobacilli since some strains grow at 2-4°C (Zhang & Holley, 1999; Champomier-Vergès et al., 2002; Marceau et al., 2003). Although most LAB are catalase negative, *L. sakei* possesses a heme-dependent catalase responsible for the efficient decomposition of H_2O_2 (Champomier-Vergès et al., 2002). Growth of this species is possible up to pH 3.9 and 8% NaCl (Carr et al., 2002). Marceau et al. (2003) demonstrated that *L. sakei* is able to adapt to conditions of low

temperature (4°C) and high salt (9% NaCl). Although these conditions influence growth, survival was enhanced and differences in cell morphology were observed. In 2004, Marceau et al. found six proteins to be affected during growth of *L. sakei* at 4°C or in the presence of 4% NaCl. Two were proteins from general carbon metabolism, four were general stress proteins.

Lactobacillus sakei is known to have the most fastidious nutritional requirements of all lactobacilli (Lauret et al., 1996). Although *L. sakei* can generate energy by degrading arginine, leading to NH_3 and ATP production, via the arginine deiminase pathway, this amino acid alone does not allow growth of *L. sakei* but rather survival during the stationary phase (Champomier-Vergès et al., 1999; 2002). Instead, this species uses other energy sources to grow on meat. Among the few sugars present in meat, glucose and ribose are the only sugars that *L. sakei* can utilise for its growth (Stentz et al., 2001). Glucose is fermented via the homofermentative EMP-pathway while ribose is fermented via the heterofermentative phosphoketolase pathway. During sugar fermentation, D- and L-lactic acid are produced although only L-lactate dehydrogenase is present. The conversion of L- to D-lactic acid is catalysed by a lactate racemase (Carr et al., 2002; Champomier-Vergès et al., 2002).

Little is known about nucleotide and vitamin requirements. Thiamin is required for growth on pentoses such as ribose (Champomier-Vergès et al., 2002). In the study of Moretro et al. (1998), riboflavin was essential for growth, while biotin had no effect on the growth of two *L. sakei* strains. Furthermore, both strains needed purines and pyrimidines for growth.

1.5. Antimicrobial activity of lactic acid bacteria

Since ancient times, LAB are used in the production of a wide range of fermented foods. Lactic acid bacteria contribute to the stability and safety of these products mainly due to the acidic conditions they create during their development and this souring effect is primarily due to the fermentation of carbohydrates to organic acids. Although the preservative effect of LAB is known for a long time, only in the last twenty years it became clear that the antimicrobial activity of LAB is more than organic acid production alone. A whole range of antagonistic systems have been described for LAB (De Vuyst & Vandamme, 1994a).

1.5.1. Fermentation end-products

1.5.1.1. Organic acids

Lactic acid bacteria are characterised primarily by their ability to form organic acids from the fermentation of carbohydrates. Depending on their homo- or heterofermentative nature, only lactic acid or lactic acid and other organic acids, e.g. acetic acid and propionic acid, are produced. The antimicrobial activity of these acidic end-products and the concomitant low pH constitute the most important antimicrobial system of LAB (De Vuyst & Vandamme, 1994a).

The organic acids produced by LAB are weak acids and follow the common equilibrium reaction and equation

$$HA \rightleftharpoons H^+ + A^- \qquad \frac{[H^+]\cdot[A^-]}{[HA]} = K_a$$

where $[H^+]$ is the concentration of protons, $[A^-]$ is the concentration of anions, [HA] is the concentration of undissociated acid and K_a is the dissociation constant (Eklund, 1989). Only in their undissociated and uncharged form, organic acids are able to penetrate the microbial cell to act antimicrobially (Russell, 1992; Stratford, 2000).
From the above equation it can be derived that the inhibitory activity of organic acids is determined by the pH, the dissociation constant (K_a) and the acid concentration. The antimicrobial activity of organic acids increases with decreasing pH-value since a greater proportion of undissociated molecules exists as the pH decreases. Because they are weak acids, organic acids have high pK_a values (acetic acid, pK_a = 4.73 and lactic acid, pK_a = 3.86) (Eklund, 1989; Bogaert & Naidu, 2000). At a given pH and acid concentration, the acid with the highest pK_a will have the most undissociated acid present, resulting in the strongest antimicrobial activity. This explains partly why, at a given pH, acetic acid is more inhibitory than lactic acid (Stratford, 2000).

Traditionally, the antimicrobial activity of organic acids was explained by reduction of the pH below the minimum pH for growth and inhibition by undissociated acid (Russell, 1992). The mechanism of action is complex and the uncoupling theory (Russell, 1992) or weak acid theory (Stratford, 2000) has been developed (Figure 1.3). This theory states that when

undissociated acid molecules enter a microbial cell, at the higher intracellular pH, they dissociate in charged anions and protons, both unable to diffuse out of the cell thus reducing the intracellular pH (Eklund, 1989; Russell, 1992). A low cytoplasmic pH causes structural changes in proteins, nucleic acids and phospholipids, influences rates of enzyme action and in this way interferes with essential metabolic functions and prevents active transport requiring a ΔH^+-gradient (Eklund, 1989; Stratford, 2000). To maintain or raise internal pH, a compensating transport of protons out of the cell takes place by membrane-bound H^+-ATPases but consumes excessive ATP (Stratford, 2000). The remaining anion inside the cell can be driven to the outside of the cell by the electrochemical gradient. The anion is protonated again to repeat the cycle until the proton motive force is dissipated and the cell is inhibited by energy depletion (Eklund, 1989; Russell, 1992; Stratford, 2000). The last decade, however, the organic acids are regarded as having not one but several mechanisms of action and inhibition of active nutrient transport by neutralisation of the proton motive force only is not believed to fully explain their antimicrobial activity (Eklund, 1989; Russell, 1992). Lactic acid is acting partly according to the weak acid theory, but other mechanisms of inhibition are also involved (Eklund, 1989; Bogaert & Naidu, 2000; Stratford, 2000). Furthermore, it has been shown that the effects of lactic acid on microbial growth at different pH can only be explained if both the undissociated and the dissociated form are taken into account (Gonçalves et al., 1997). Organic acids have a wide inhibitory spectrum including Gram-positive and Gram-negative bacteria, yeasts and moulds. Lactic acid mainly acts against bacteria and is ineffective against yeast and moulds (Eklund, 1989; Bogaert & Naidu, 2000). Lactic acid and its salts are reported to have an inhibitory effect on LAB (Houtsma et al., 1993), spore-forming *Clostridia* and *Bacillus* spp. (Bogaert & Naidu, 2000), *Listeria monocytogenes* (Houtsma et al., 1993) as well as on Gram-negative pathogens such as *E. coli* 0157:H7 and *Salmonella* spp. This property makes organic acids attractive food preservatives.

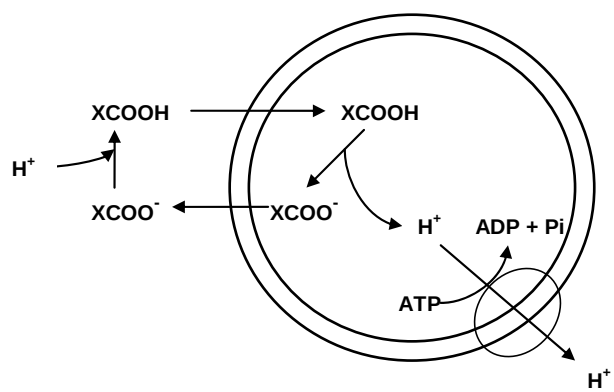

Figure 1.3. Weak acid theory and dissipation of the proton motive force (modified from Russell, 1992 and Stratford, 2000)

The ability of lactic acid to rotate under influence of polarised light results in the existence of two stereo-isomers being the L(+) (levorotary) and D(-) (dextrorotary) form. If there is a mixture of both, it is termed racemic (DL) (Bogaert & Naidu, 2000; Carr et al., 2002). The production of L(+) or D(-) isomers varies with the genus and species within a genus of LAB (Stiles & Holzapfel, 1997; Liu, 2003) and depends on the stereo-specificity of the present lactate dehydrogenase (Kandler & Weiss, 1986). From a nutritional point of view, D(-)-lactate in food is undesirable because it is not readily metabolised in humans compared with L(+)-lactate (Liu, 2003). It has been demonstrated that the antimicrobial activity of lactic acid is stereo-specific and it seems that micro-organisms are least sensitive to the isomer they intrinsically produce. *Listeria monocytogenes* is a L-lactic acid producer and is therefore more sensitive to D-lactic acid than to L-lactic acid. However, this difference in sensitivity is less than the strain variation in L-lactic acid sensitivity indicating that the stereo-specific activity of lactic acid has no practical importance regarding *L. monocytogenes*.

1.5.1.2. Hydrogen peroxide

In the presence of oxygen, LAB can produce hydrogen peroxide (H_2O_2). Its formation occurs through electron transport and is catalysed by the flavin enzyme NADH-oxidase. Furthermore, H_2O_2 can be synthesised from pyruvate, α-glycerophosphate and even from lactate. Accumulation of H_2O_2 can also result from dismutation of superoxide anions (O_2^-) by the action of a superoxide dismutase that is present in most LAB or by Mn-ions that are present in the cytoplasm of bacteria lacking superoxide dismutase (De Vuyst & Vandamme, 1994a). In general, LAB are catalase negative (Kandler & Weiss, 1986). Since the produced H_2O_2 cannot be hydrolysed, it accumulates in the growth medium where it can exert its action. However, a few species, including *L. sakei,* have been reported to possess a heme-dependent catalase (Hertel et al., 1998).

Ito et al. (2003) showed that H_2O_2, accumulated by LAB in a cell suspension, is very effective in reducing the counts of several food born pathogens. The bactericidal effect of H_2O_2 is mainly attributed to its peroxidising properties on the membrane lipids, causing increased membrane permeability, and on other basic molecular structures, e.g. nucleic acids and cell proteins (Piard & Desmazeaud, 1991). Furthermore, H_2O_2 may react further to produce additional inhibitory compounds, e.g. the generation of hypothiocyanite by the action of lactoperoxidase on H_2O_2 and thiocyanate (De Vuyst & Vandamme, 1994a).

1.5.1.3. Carbon dioxide

Carbon dioxide (CO_2) is mainly produced by heterofermentative LAB from hexoses (Kandler & Weiss, 1986). Furthermore, various LAB can produce CO_2 from malate and citrate (Hugenholtz, 1993) and also by metabolising arginine via the arginine deiminase pathway (Arena et al., 1999). Finally, decarboxylation of amino acids can also be a minor source of CO_2 (Urbach, 1995). Carbon dioxide contributes to the antagonistic activity of LAB (De Vuyst & Vandamme, 1994a) and its inhibitory properties are due to its role in creating an anaerobic environment, its extracellular and intracellular pH-decreasing effect and specific actions on enzymes and membranes (Devlieghere, 2000).

1.5.1.4. Diacetyl

Diacetyl, the characteristic flavour associated with butter and cheese, is synthesised from pyruvate aerobically as well as anaerobically by citrate fermenting LAB (Hugenholtz, 1993; Liu, 2003). In the presence of citrate and a metabolisable energy source (e.g. in milk), the production of excessive amounts of pyruvate can result in the production of diacetyl and acetoin. When hexoses are the only fermentable carbon source, no diacetyl and little if any acetoin is produced. Diacetyl has antimicrobial activities but Gram-negative bacteria, yeasts and moulds are more sensitive to diacetyl than Gram-positive bacteria and usually high levels are necessary for inhibition (De Vuyst & Vandamme, 1994a). Its mode of action is believed to be due to interference with the utilisation of arginine. Diacetyl is rarely produced at sufficient levels to make a major contribution to the antimicrobial activity of LAB (Piard & Desmazeaud, 1991; De Vuyst & Vandamme, 1994a).

1.5.1.5. Acetaldehyde and ethanol

Acetaldehyde, responsible for the typical aroma of yoghurt, is produced during the carbohydrate metabolism of heterofermentative LAB, principally *L. delbrueckii subsp. bulgaricus*, and further reduced to ethanol by an alcohol dehydrogenase. When the latter enzyme is absent or repressed, it may result in the excretion of acetaldehyde. The possible antagonistic effect of acetaldehyde is poorly documented and existing data suggest that this compound plays a minor antagonistic role (Piard & Desmazeaud, 1991; De Vuyst & Vandamme, 1994a). Similarly, although ethanol may be produced by LAB, the produced levels are so low that its contribution to the antimicrobial activity of LAB is minimal (Caplice & Fitzgerald, 1999).

1.5.2. Bacteriocins

Bacteriocins, produced by bacteria, can be defined as antimicrobial proteinaceous (peptides, proteins or protein complexes) substances displaying a bactericidal mode of action usually towards closely related species (Klaenhammer, 1988; De Vuyst & Vandamme, 1994a; Cleveland et al., 2001). They form a heterogeneous group with respect to producing bacterial species, molecular size, molecular, physical and chemical properties, stability, antimicrobial spectrum, mode of action, etc. Today, numerous bacteriocins have been isolated from a variety of LAB and a multitude of information is available and compiled in several reviews (Klaenhammer, 1993; De Vuyst & Vandamme, 1994a; Abee et al., 1995; Jack et al., 1995; Caplice & Fitzgerald, 1999; Cleveland et al., 2001; McAuliffe et al., 2001; Ross et al., 2002).

1.5.2.1. Classification

Bacteriocins are commonly classified into three classes (Table 1.1): (1) lantibiotics, (2) small heat-stable peptides and (3) large heat-labile proteins. A fourth class of complex proteins, whose activity requires the association of carbohydrate or lipid moieties, has been proposed but there is no consensus on the existence of this group (Klaenhammer, 1993; Jack et al., 1995; Caplice & Fitzgerald, 1999; Cleveland et al., 2001).

Table 1.1. Classification of bacteriocins from Gram-positive bacteria (modified from Cleveland et al., 2001)

Class	Subclass	Characteristics	Representative
I	Lantibiotics		
	Ia	Flexible molecules compared to Ib	Nisin
	Ib	Globular peptides; no net charge or net negative charge	Mersacidin
II	Small heat-stable peptides (non-lantibiotics)		
	IIa	Antilisterial single peptides	Pediocin PA-1; Sakacin A
	IIb	Two-peptide bacteriocins	Lactacin F; Plantaricin JK
III	Large heat-labile proteins (non-lantibiotics)		Helveticin J; Lactacin A
IV	Complex bacteriocins carrying lipid or carbohydrate moieties		none

Class I, termed lantibiotics, is further subdivided into subclasses Ia and Ib based on their structural and functional features. In general, the small peptides of class I typically have from 19 to more than 50 amino acids and they are characterised by unusual amino acids e.g.

lanthionine and beta-methyllanthionine. Subclass Ia bacteriocins, which include nisin, consist of cationic and hydrophobic peptides that form pores in target membranes and have an elongated flexible structure compared to the more rigid subclass Ib. Subclass Ib bacteriocins are globular peptides with no net charge or a net negative charge and they interfere with cellular enzymatic reactions (Cleveland et al., 2001; McAuliffe et al., 2001; Ross et al., 2002). Class II contains small heat-stable, non-modified peptides and can be further subdivided in subclasses IIa and IIb (Cleveland et al., 2001). Subclass IIa includes pediocin-like *Listeria*-active peptides with a conserved N-terminal sequence Tyr-Gly-Asn-Gly-Val and two cysteines forming a S-S bridge in the N-terminal half of the peptide (Ennahar et al., 1999). Bacteriocins composed of two different peptides comprise subclass IIb (Garneau et al., 2002). Class III is the least well characterised group and consists of large and heat-labile bacteriocins, e.g. helveticin (Cleveland et al., 2001).

1.5.2.2. Mode of action

All bacteriocins are synthesised via the common ribosomal protein biosynthesis mechanism involving transcription and translation. They appear to be formed initially as prepeptides, which are subsequently cleaved enzymatically to form the biologically active molecule. In the case of the lantibiotics, post-translational modifications are introduced into the precursor molecule before cleavage (De Vuyst & Vandamme, 1994a; Jack et al., 1995; Cleveland et al., 2001). Once produced, most bacteriocins are translocated to the outside of the cell (Cleveland et al., 2001).

The mechanism, by which bacteriocins act, is not fully understood but it is generally accepted that the primary mode of action of bacteriocins is attributed to pore formation in the phospholipid bilayer of the cytoplasmic membrane. In particular, lantibiotics of class Ia act by forming pores in the membrane, depleting the transmembrane potential and/or pH-gradient, resulting in leakage of cellular materials and dissipation of the proton motive force and as a main secondary effect cessation of energy-requiring reactions such as biosynthetic reactions (Montville & Bruno, 1994; Jack et al., 1995; Cleveland et al., 2001). Other reported modes of action such as inhibition of peptidoglycan biosynthesis or cell lysis are rather believed to be secondary effects of bacteriocins' primary action although not fully agreed on (Montville & Bruno, 1994; McAuliffe et al., 2001; Ross et al., 2002).

Electrostatic interactions of the positively charged bacteriocins with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding with the target cell. Further, it is likely that the hydrophobic patches of the bacteriocin

molecule inserts into the membrane forming a pore (Cleveland et al., 2001). In the case of nisin, pore formation is studied in detail and two models are suggested. In the 'barrel-stave' model (Abee et al., 1995; Garneau et al., 2002), during insertion, each nisin molecule adopts a transmembrane orientation, forming a pore by subsequent aggregation of the different inserted monomers (Figure 1.4). Whether aggregation occurs prior to insertion or in the membrane after insertion is unknown but insertion followed by aggregation is the favoured model. In the 'wedge' model, after a critical number of nisin molecules associate with the membrane, they insert concurrently, forming a wedge-like pore (Cleveland et al., 2001; McAuliffe et al., 2001).

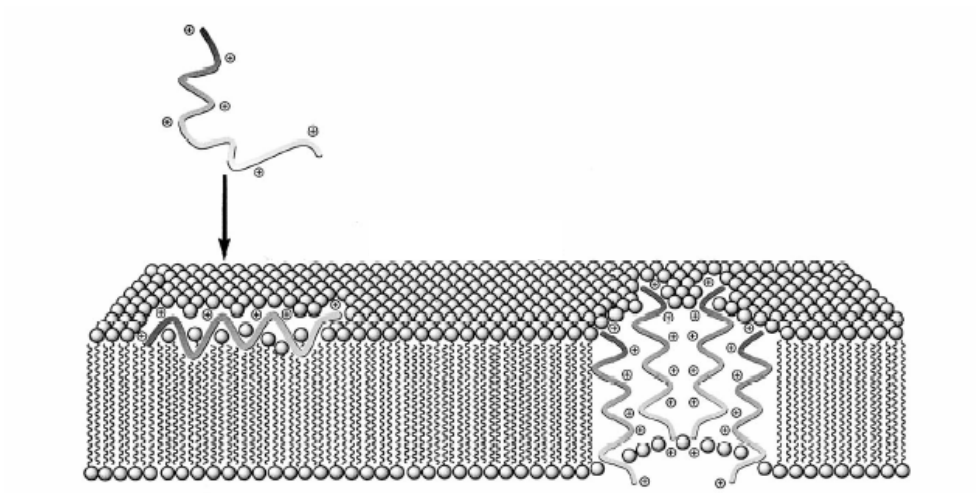

Figure 1.4. Barrel-stave model for pore formation by cationic bacteriocins (Garneau et al., 2002)

Recent studies demonstrate that bacteriocin activity is more complex and it appears that 'docking' molecules on the target cell membrane facilitate the interaction between bacteriocin and target cell. In the case of nisin, the peptidoglycan precursor lipid II acts as a docking molecule; initial binding to lipid II is followed by pore formation resulting in rapid killing of the target cell (Cleveland et al., 2001; Wiedemann et al., 2001; Ross et al., 2002). Other bacteriocins, e.g. those from Class IIa, interact with specific proteins on target cell membranes. However, the role of these 'receptor' molecules remains unclear (Montville & Bruno, 1994; Ennahar et al., 1999; Cleveland et al., 2001).

1.5.2.3. Antimicrobial spectrum

Klaenhammer (1988) distinguishes two groups of bacteriocins from LAB with respect to their inhibitory spectrum. One group includes bacteriocins with a narrow inhibitory spectrum, either only against closely related bacteria of the same genus either against other bacterial genera (other LAB, *Clostridium*, *Listeria*, etc.). Bacteriocins from lactobacilli belong to this group. A second group of bacteriocins, including nisin, has a relatively broad spectrum of activity but only against Gram-positive bacteria. In general, bacteriocins are not active against Gram-negative bacteria and this may be explained by their mode of action and the difference in cell wall composition between Gram-positive and Gram-negative bacteria. Gram-negatives become sensitive to e.g. nisin after exposure to treatments that change the permeability properties of their outer membrane (Abee et al., 1995).

The ability of several bacteriocins, mainly from Class IIa, to inhibit various food born pathogens, including *L. monocytogenes* (Harris et al., 1989; Motlagh et al., 1992; Hugas et al., 1995; Muriana, 1996; Katla et al., 2002; Aasen et al., 2003; Teixeira de Carvalho et al., 2006), makes them attractive as potential food preservation agents and this aspect is further discussed in section 3.2.1. of this literature review.

1.5.2.4. Bacteriocins of lactobacilli

Lactobacilli, producing bacteriocins, have been isolated from fermented dairy products but also from non-dairy fermentations. In general, they have a narrow range of inhibitory activity that affects only closely related species within the lactobacilli (Klaenhammer, 1988). The plantaricins (*L. plantarum*) and the sakacins (*L. sakei*) are probably the best known but other examples are lactocins, helveticins (*L. helveticus*) and lactacins (*L. acidophilus*) (Nettles & Barefoot, 1993).

Sakacin M, produced by *L. sakei* 148, was isolated from a Spanish dry fermented sausage and exhibits a bacteriostatic mode of action towards various lactobacilli, *Leuc. mesenteroides* and several Gram-positive bacteria including also *L. monocytogenes* (Sobrino et al., 1991; Sobrino et al., 1992; Rodriguez et al., 1994). According to Nes et al. (1994), a thorough characterisation of the bacteriocin indicated that it is identical to lactocin S, a Class I bacteriocin produced by *L. sakei* 45 (Abee et al., 1995).

Three bacteriocins, sakacin A, sakacin P and lactocin S have been isolated from *L. sakei* strains (Champomier-Vergès et al., 2002).

1.5.3. Other antagonistic systems

1.5.3.1. Reuterin

Reuterin is a low-molecular weight, non-proteinaceous, antimicrobial agent produced by *Lactobacillus reuteri*. Reuterin has been identified as an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde formed during anaerobic metabolism of glycerol. It is a broad-spectrum antimicrobial substance effective against Gram-negative (e.g. *Salmonella, Shigella*) and Gram-positive bacteria (e.g. *Clostridium*, *Staphylococcus* and *Listeria*), yeasts, moulds and protozoa (De Vuyst & Vandamme, 1994a; El-Ziney & Debevere, 1998). It acts by the inhibition of sulfhydryl enzymes such as ribonucleotide reductase, an enzyme involved in DNA-biosynthesis (De Vuyst & Vandamme, 1994a).

1.5.3.2. Reutericyclin

Reutericylin is an inhibitory compound produced by sourdough isolates of *Lactobacillus reuteri*. The tetramic acid reutericylin is the first low-molecular weight antibiotic from LAB and it is bacterostatic or bactericidal to Gram-positive bacteria only, based on its activity as a proton-ionophore resulting in the dissipation of the transmembrane ΔpH. A broad range of food-related spoilage and pathogenic organisms are inhibited by reutericylin and therefore this compound might be of interest for the food industry (Gänzle, 2004).

1.5.3.3. Competition for nutrients

Lactic acid bacteria may have a negative influence on the growth of other micro-organisms by competition for nutrients. Because of their high nutritional requirements, multiplying LAB may rapidly lead to nutrient depletion causing growth arrest of other bacteria growing in the same environment (De Vuyst & Vandamme, 1994a; Buchanan & Bagi, 1997; Nilsson et al., 2005).

1.5.3.4. Antifungal compounds

Recently, a number of antifungal metabolites, e.g. cyclic dipeptides, proteinaceous compounds ('fungicins'), special organic acids (3-phenyl-L-lactic acid and caproic acid) and 3-hydroxylated fatty acids have been isolated from LAB (De Muynck et al., 2004; Schnürer & Magnusson, 2005).

2. The microbial ecology of anaerobically packaged sliced cooked cured meat products

2.1. Production process

Cooked cured meat products are meat products that include a curing as well as a pasteurisation step in their production process. Typical examples are pâté, cooked ham, emulsion-style sausages (e.g. frankfurters, luncheon meat) and cooked poultry products. They are distributed chilled and are normally not heated before consumption.

Cooked cured meat products are made from intact muscles and cuts of meat (e.g. ham), from meat pieces that have been massaged and tumbled and then formed into casings or moulds (e.g. pressed shoulder) or from fully comminuted meats that are extruded into casings or moulds (e.g. emulsion-style sausage). The cure is added to the meat by injection of brine, soaking in brine or blending during emulsion preparation and contains sodium nitrite and salt. Frequently, other compounds may be added such as sugar, ascorbate, phosphate, spices, antioxidants, preservatives (e.g. sodium lactate), smoke compounds, etc. The product is then pasteurised to a temperature of 65-75°C, cooled and removed from the casing or mould prior to further slicing and repackaging. Vacuum packaging (VP) and modified atmosphere packaging (MAP) (50-70% CO_2/ 30-50% N_2) are the most widely used packaging techniques for industrial cooked meat products (CMP). When sold as whole products, pasteurisation of CMP can occur in the packaging in which they are marketed. For retail purposes, whole CMP may also be stored unpackaged under aerobic atmospheres (Korkeala & Björkroth, 1997; Devlieghere, 2000; Bell & Kyriakides, 2005; Roberts et al., 2005).

2.2. Contamination

The pasteurisation process will normally inactivate most vegetative cells. Some of the more heat-resistant LAB, streptococci and *Weissella viridiscens* (Borch et al., 1988) and also spores of bacilli and clostridia may survive the mild heat treatment (Roberts et al., 2005). Immediately after cooking, the contamination level on the surface of CMP is generally very low (Mäkelä & Korkeala, 1987). During further handling (removal from cooking form), slicing and packaging, the CMP are subjected to post-heat treatment recontamination with micro-organisms such as LAB, *Enterobacteriaceae*, *Brochothrix thermosphacta* and *Listeria* spp. (Korkeala & Björkroth, 1997; Samelis et al., 1998; 2000b; Roberts et al., 2005). This

explains why CMP, immediately after production, have an initial contamination level of 0.5-3 $\log_{10}$(cfu/g) of total bacteria (Samelis et al., 1998; 2000a; b).

2.3. Microbial spoilage

2.3.1. Factors determining the microbial growth in CMP

The combination of intrinsic and extrinsic factors determines the microbiology of CMP (McDonald & Sun, 1999). Main intrinsic parameters are pH, water activity (a_w) and the presence of antimicrobials. The pH is affected by the type and level of carbohydrate addition and the use of acidulants and phosphates. The water activity depends on the presence of salt, sugars, phosphates, lactates and fat. Antimicrobials in CMP are mainly salt, nitrite, lactates and smoke flavours. Extrinsic parameters include processing parameters, mainly time/temperature during cooking/cooling, storage, distribution and display conditions (packaging type, time/temperature) and the type and level of natural microbial flora remaining in the product after processing or due to post-process contamination. The high water activity (0.96-0.99), the low salt content (3-5%, Samelis et al. (2002)), the moderate pH (5.5-6.5) and the presence of readily available sources of energy and nutrients makes CMP highly perishable products.

2.3.2. Specific spoilage organisms

Lowering the redoxpotential of CMP by packaging under vacuum or in atmospheres enriched with CO_2, inhibits the respiratory Gram-negative flora, mainly consisting of *Pseudomonas* spp. The combination of the micro-aerophilic conditions under VP/MAP, the presence of curing salt and nitrite and a reduced a_w favours the growth of a Gram-positive facultative anaerobic microflora, mainly consisting of psychrotrophic LAB. *Brochothrix thermosphacta* may also be a dominant part of the bacterial flora, particularly when the film permeability is high (Borch et al., 1996; Nychas et al., 1998; Samelis et al., 2000a; Aymerich et al., 2002).

2.3.2.1. Lactic acid bacteria

Lactic acid bacteria are the major group of spoilage bacteria developing on refrigerated CMP under VP/MAP. The main LAB-strains associated with spoilage of CMP belong to the genera *Lactobacillus* and *Leuconostoc.* Furthermore, species from the genera *Weissella* and *Carnobacterium* are also reported to cause spoilage (Borch et al., 1996; Korkeala &

Björkroth, 1997; Holzapfel, 1998; Samelis et al., 2000a; Aymerich et al., 2002). The genus/species of LAB responsible for spoilage depends on the manufacturing site, the processing method and the product composition (Mäkelä & Korkeala, 1987; Borch et al., 1996; Samelis et al., 2000a). Lowering pH, lowering temperature, reducing a_w, increasing salt and/or applying smoke results in dominance of the LAB-flora of CMP by *Lactobacillus (L. sakei* and *L. curvatus), Leuconostoc, Weissella* and *Carnobacterium*, in the order listed (Zhang & Holley, 1999; Samelis & Georgiadou, 2000).

Lactobacilli

Lactobacillus sakei and *L. curvatus* are the main spoilage causing lactobacilli in VP/MAP, sliced CMP (Korkeala & Björkroth, 1997; Devlieghere et al., 1998; Samelis & Georgiadou, 2000; Samelis et al., 2000a; b). Generally, these homofermentative meat lactobacilli cause slow and mild sour spoilage, with the exception of some ropy-slime producing *L. sakei* strains. *Lactobacillus sakei* is found to predominate in emulsion-style sausages (Korkeala & Björkroth, 1997) and in smoked CMP (smoked pork loin, bacon, pariza and mortadella (Samelis et al., 2000a), smoked turkey breast (Samelis et al., 2000b)) immediately after slicing and packaging. However, at the end of the shelf-life *L. sakei* is present in a much wider range of products, including also non-smoked CMP such as cooked ham and cooked poultry products (Björkroth et al., 1998; Samelis et al., 2000a).

Leuconostocs

Several studies have reported on isolation of *Leuconostoc* spp., *Leuc. mesenteroides* subsp. *mesenteroides, Leuc. mesenteroides* subsp. *dextranicum, Leuc. gelidum, Leuc. citreum* and *Leuc. carnosum* from CMP (Björkroth et al., 1998; Samelis et al., 2000a; b; Samelis & Georgiadou, 2000; Hamasaki et al., 2003). *Leuconostoc* and other heterofermentative LAB are commonly found to predominate in non-smoked CMP such as cooked ham or cooked poultry, immediately after production (Björkroth et al., 1998; Samelis et al., 2000a; b). However, during subsequent storage up to the end of shelf-life, *L. sakei* can outgrow these leuconostocs (Samelis et al., 2000a), *Leuc. mesenteroides* can outgrow *L. sakei* (Samelis et al., 1998) or they compete equally (Samelis et al., 2000b) depending on their initial ratio and product characteristics (Zhang & Holley, 1999).

Leuconostoc spp. causes more severe spoilage (slime, gas, off-odours) due to its heterofermentative metabolism (Samelis et al., 2000a; b).

Weissella

Weissella viridiscens has been isolated less frequently and mainly from smoked CMP such as smoked pork loin (Samelis & Georgiadou, 2000; Samelis et al., 2000a). Its occurrence is often due to heat-surviving cells since this species has an increased thermotolerance (Borch et al., 1988).

2.3.2.2. Brochothrix thermosphacta

Brochothrix thermosphacta can form a significant part of the spoilage flora of vacuum packaged CMP and spoil them more readily than lactobacilli (Kotzekidou & Bloukas, 1996; Cayré et al., 2005). The amount of growth of *B. thermosphacta* relative to that of LAB is reduced by higher nitrite concentration, lower pH and lower film permeability (Roberts et al., 2005). Indeed, most associated growth studies involving *B. thermosphacta* and LAB in meat, stored under vacuum, found that LAB restrict the growth of *B. thermosphacta*, when using films with low oxygen transmission rate (Holzapfel et al., 1998; Samelis et al., 2000a). In contrast, Cayré et al. (2005) observed no inhibition of *B. thermosphacta* growth when the meat product was packaged in films with low oxygen permeability since under these conditions its growth paralleled LAB-growth. In films with a higher oxygen transmission rate, growth of *B. thermosphacta* was inhibited. The authors hypothesised that incoming O_2 may have resulted in H_2O_2-production by the accompanying LAB, inhibiting in this way *B. thermosphacta* in the presence of O_2 and not in its absence.

The spoilage of *B. thermosphacta*, associated with anaerobic metabolism, is slower developing and less offensive than that associated with aerobic growth (Pin et al., 2002). *B. thermosphacta* is homofermentative and produces L(+)-lactic acid from glucose, but under glucose limitation, small amounts of other metabolites are detected (Stiles & Holzapfel, 1997). The main metabolites resulting from glucose consumption under anaerobic conditions are lactic acid and ethanol, but no acetoin-diacetyl and only small or no amounts of short-chain fatty acids which may cause off-odours (Pin et al., 2002).

2.3.3. Spoilage phenomena

Spoilage of anaerobically packaged CMP is quite common but varies greatly with product types and brands (Yang & Ray, 1994). In a German study from 2001 on the quality of pre-packaged meat products at the stage of retail, 30% of cooked sausages and 40% of other cured CMP were rejected due to signs of sensory spoilage prior to the expiry date (Stolle et al.,

2001). The predominant spoilage characteristics are accumulation of gas, ropy slime, milky exudate, souring and off-odours (Yang & Ray, 1994).

2.3.3.1. Souring due to acid production

Spoilage causing LAB produce lactic acid, acetic acid and smaller amounts of other organic acids, especially at the stationary growth phase (Korkeala & Björkroth, 1997; Samelis & Georgiadou, 2000). An appreciable increase in lactic acid occurs when LAB reach 1 to 5×10^7 cfu/g on the surface of VP cooked sausages (Korkeala et al., 1990). Obligate heterofermentative leuconostocs only produce D-lactic acid, heterofermentative lactobacilli produce a racemix mixture of D- and L-lactic acid and *B. thermosphacta* produces only L-lactic acid (Nychas et al., 1998). D-lactate has been suggested to be an indicator for bacterial contamination of anaerobically packaged CMP (Dainty, 1996). Korkeala et al. (1990) found most of cooked sausages to be unacceptable above a level of 0.3-0.4% (w/w) lactic acid, corresponding to pH-values below 5.8-5.9.

The production of lactic acid is often accompanied by that of other (partly volatile) end-products such as short-chain fatty acids (e.g. acetic acid and butyric acids). The increase of acetate in certain CMP stored under VP/MAP could be attributed to (1) the presence of heterefermentative leuconostocs, (2) a shift from homo- to heterofermentative metabolism of the facultatively heterofermentative lactobacilli under conditions of substrate limitation or (3) the predominance of another organism e.g. *B. thermosphacta* (Nychas et al., 1998; Samelis & Georgiadou, 2000).

2.3.3.2. Off-odours

Spoilage of anaerobically stored CMP due to the development of objectionable off-odours is mainly caused by microbial production of volatile compounds such as ethanol, diacetyl, etc.

Ethanol and other alcohols such as propanol are fermentation products of heterofermentative leuconostocs or products coming from the switch of homofermentative LAB to a heterofermentative metabolism under influence of e.g. oxygen or glucose limitation. Ethanol is also one of the main metabolic end-products of *B. thermosphacta* at low O_2-tension (Nychas et al., 1998).

A sour-sweet offensive odour, mainly associated with acetoin, is the main sign of spoilage by *B. thermosphacta* in meat products packaged in films with a high oxygen permeability (Pin et al., 2002).

2.3.3.3. Discolouration

Green discolouration, often evident as green spots, is caused when H_2O_2, produced by LAB in the presence of oxygen, oxidises nitrosohaemochrome to choleomyoglobine (Roberts et al., 2005). Greening in the centre of CMP is caused by bacteria (e.g. *W. viridiscens*), surviving the heating process, which after exposure to air start to produce H_2O_2. Surface greening is caused by bacteria which contaminate the product after cooking such as *Lactobacillus* spp., *Leuconostoc* spp. and *C. divergens* (Borch et al., 1996).

2.3.3.4. Gas production

Accumulation of gas (CO_2), leading to swelling of packages, is typically associated with the growth of heterofermentative lactobacilli and leuconostocs such as *Leuc. mesenteroides, Leuc. carnosum* and *Leuc. gelidum* (Yang & Ray, 1994; Borch et al., 1996; Korkeala & Björkroth, 1997; Samelis et al., 2000b).

2.3.3.5. Slime formation

The formation of ropy slime on vacuum packaged CMP (Samelis et al., 2000b) is a common spoilage problem. The slime is often formed before the sell-by-date and consumers find the appearance of slimy products very offensive. Slime formation is due to the secretion by LAB, mainly homofermentative *Lactobacillus* and *Leuconostoc,* of long-chain, high-molecular mass, viscosifying or gelling, extracellular polysaccharides (EPS), e.g. dextran, into the environment (Aymerich et al., 2002). *L. sakei, Leuc. gelidum, Leuc. carnosum, Leuc. mesenteroides* subsp. *mesenteroides* and *Leuc. mesenteroides* subsp. *dextranicum* have been associated with slime formation (Borch et al., 1996; Korkeala & Björkroth, 1997; Carr et al., 2002). It is not advisable to use sucrose in the formulation of cooked meat products since this is the most suitable carbon source for EPS-production by slime-producing LAB (Aymerich et al., 2002).

2.3.3.6. Milky exudate

Accumulation of a milky fluid in the packages of cooked sausages during storage under vacuum has been observed. Souring of the product due to prolific growth of LAB is often associated with excessive separation of exudate or drip. The presence of lactic acid, formed by the rich LAB-population, may change the appearance of drip from transparent to white or gray (Korkeala & Björkroth, 1997).

2.4. Safety

2.4.1. Pathogens associated with cooked meat products

Though pasteurisation destroys vegetative pathogens, they can gain access to CMP during slicing and packaging. However, provided storage temperatures are kept below 7°C, only psychrotrophic pathogens are likely to grow depending on the a_w, pH, nitrite and lactate content, atmosphere, temperature and spoilage flora (Roberts et al., 2005). One of the most important psychrotrophic food born pathogens occurring in CMP is *Listeria monocytogenes* (Devlieghere, 2000).

2.4.2. *Listeria monocytogenes*

Listeria monocytogenes is a widely distributed opportunistic food born pathogen causing listeriosis. This is a rare disease but of great concern due to its high human case fatality risk (Bell & Kyriakides, 2005). Major concerns with *L. monocytogenes* are its high mortality rate, wide distribution on raw products, growth at low temperatures and its ability to establish itself in various food processing environments (Muriana, 1996). *L. monocytogenes* is well-adapted to survival on equipment and in production facilities and its occurrence in CMP is connected with (1) cross contamination after heat treatment, e.g. during slicing or packaging (Bredholt et al., 1999), and (2) insufficient thermal processing and heat survivors (Samelis & Metaxopoulos, 1999). Uyttendaele et al. (1999) found that incidence rates for CMP were higher after slicing (6.65%) than before slicing (1.65%).

The incidence of *L. monocytogenes* on anaerobically packaged sliced CMP has been reported by several authors. In a survey of CMP on the Belgian retail market (Uyttendaele et al., 1999), the overall incidence of *L. monocytogenes* in 25 g was 4.90% (167/3405). However, only a small proportion (0.53%) of samples contained high contamination levels (>10 cfu/g). The incidence rate was higher for minced CMP (e.g. pâté) than for whole CMP (e.g. cooked ham, cooked poultry), 6.14% and 3.96%, respectively. According to De Boer (1990), luncheon meat, ham and cooked chicken breast are the most frequently contaminated CMP in The Netherlands. De Boer & van Netten (1990) found 11% (9/83) of pâté samples to be contaminated with *L. monocytogenes*. Rijpens et al. (1997) reported an incidence of *L. monocytogenes* of 2.6% in 3065 samples of pâté, which was in good agreement with the contamination level of 2.76% (217 samples) in the survey of Uyttendaele et al. (1999). A more recent study on the occurrence of *Listeria* on sliced cooked meat products in Spain

reported an incidence of *L. monocytogenes* in 25 g of 8.8% (35/396) (Vitas & Garcia-Jalon, 2004).

Further proliferation of the pathogen on CMP has been demonstrated although the extent of its growth depends on several factors including temperature, pH, water activity, level of lactate, composition of the headspace atmosphere and the presence of a competitive flora on a product (Beumer et al., 1996; Blom et al., 1997; Barakat & Harris, 1999; Bredholt et al., 2001; Devlieghere et al., 2001; Uyttendaele et al., 2004). Growth of *L. monocytogenes* in CMP has resulted in outbreaks of listeriosis. Belgian pâté was the vehicle of infection in listeriosis in the UK between 1987-1989 with more than 350 cases and more than 90 deaths. In 2000, an outbreak of listeriosis in New Zealand following the consumption of cooked meats (ham and corned beef) and in the US following the consumption of delicatessen turkey meat was responsible for 60 cases in total. In 2001, precooked sliced turkey was implicated in 10 cases of listeriosis (US) (Bell & Kyriakides, 2005).

Public and regulatory concern related to *L. monocytogenes* has led to the implementation of microbiological standards, aiming at regulating the levels of *L. monocytogenes* in food products (Bell & Kyriakides, 2005). Since 1st of January 2006, an EU regulation on microbiological criteria for foodstuffs has come into force (Commission Regulation (EC) No 2073/2005) (European Commission, 2005). This regulation sets a maximum level of 100 cfu/g for *L. monocytogenes* at the end of the shelf-life of ready-to-eat foods (including CMP) when absence of *L. monocytogenes* in 25 g of the product can not be guaranteed.

3. Biopreservation

Since the role of micro-organisms in spontaneous food fermentation processes became clear, man has tried to apply 'controlled' fermentations in order to preserve food products. Today, food safety is more than ever an important issue and the search for new preservation methods goes on. An increasing number of consumers prefer minimally processed food products, prepared with less or without chemical preservatives. The consumer wants food products to be 'fresh', 'natural', 'healthy' and 'convenient'. Many of the new ready-to-eat and novel food types bring along new health hazards and new spoilage associations. Against this background and relying on improved understanding and knowledge of microbial interactions, milder preservation approaches such as biopreservation have been developed (Holzapfel et al., 1995; Hugas, 1998).

3.1. Definition and principle

Biopreservation or biological preservation can be defined as a preservation method to improve safety and stability of food products in a natural way by using 'desired' micro-organisms (cultures) and/or their metabolites without changing the sensory quality (Holzapfel et al., 1995; Lücke, 2000).

Protective cultures (PC) can be defined as antagonistic micro-organisms (cultures) that are added to a food product only to inhibit pathogens and/or to extend the shelf-life, while changing the sensory properties of the product as little as possible (Lücke, 2000). Protective cultures differ from starter cultures in their functional objectives. Starter cultures are, by definition, used in food fermentations in order to modify the raw material to give it new sensory properties and this relying on the metabolic activity (acid production) of the culture, while the preservation effect (antimicrobial action) is of secondary importance. For a protective culture, the functional objectives are the inverse. Although distinguished by their definition, in reality a starter culture and a protective culture may be the same culture applied for different purposes under different conditions (Holzapfel et al., 1995).

Biopreservation can be applied in food products by two basic methods:

- adding crude, semi-purified or purified microbial metabolites;
- adding pure and viable micro-organisms (Hugas, 1998).

The use of micro-organisms or their metabolites as food preservatives is not meant as a primary means of preservation but as a way to contribute to the hurdle approach in food preservation (Muriana, 1996).

3.2. Biopreservation by means of microbial metabolites

Some microbial metabolites may be added directly to a food product to inhibit the growth of spoilage or pathogenic organisms.

3.2.1. Bacteriocins

The bacteriocins most studied for their biopreservative effect in food products, and more specific in meat and meat products, include nisin, pediocins and sakacins (De Vuyst & Vandamme, 1994b; Hugas, 1998; Cleveland et al., 2001).

Nisin, produced by *Lc. lactis* subsp. *lactis*, is the only bacteriocin that has found practical application in food products. It is mainly applied in the prevention of late-blowing of cheese by inhibiting the outgrowth of *Clostridium* spores (O'Sullivan et al., 2002) and in selected pasteurised cheese spreads to inhibit *Clostridium* and *Listeria* (Holzapfel et al., 1995; Ross et al., 2002). Its use has been approved throughout the world in various food products (Cleveland et al., 2001). In Europe, it was added to the positive list of EU food additives in 1983 as additive number E234 (Guinane et al., 2005) and some EU-countries have approved the use of nisin for preservation of some food products, e.g. processed and fresh cheese (Holzapfel et al., 1995). Typical levels that are used in food products range from 2.5 to 100 ppm (Caplice & Fitzgerald, 1999). The effectiveness of nisin has not only been demonstrated in dairy products (Davies et al., 1997) but also in brined shrimps (Einarsson & Lauzon, 1995) and during beer, wine and vegetable fermentations to inhibit spoilage (De Vuyst & Vandamme, 1994b; Ross et al., 2002). In meat products, nisin has not been very successful because of its low solubility at the pH of meat, uneven distribution, binding to meat proteins and lack of stability (Chung et al., 1989). Furthermore the required dose is uneconomical and exceeds the acceptable daily intake (Chung et al., 1989; Holzapfel et al., 1995; McMullen & Stiles, 1996; Hugas, 1998). The potential applications of lactococcal bacteriocins, other than nisin, are reviewed by Guinane et al. (2005).

Pediocins, in particular pediocin PA-1 (also AcH) from *P. acidilactici*, have been used successfully to control growth of *L. monocytogenes* in cottage cheese, half-and-half cream and cheese sauce (Pucci et al., 1988), raw or fresh meat (Nielsen et al., 1990; Skyttä et al.,

1991; Motlagh et al., 1992), cooked meat products (Metaxopoulos et al., 2002; Mataragas et al., 2003a; Mattila et al., 2003) and fermented meat products (Lauková et al., 1999). Pediocin PA-1 was also found to be active towards *L. curvatus* in a meat product model but when incorporated in a commercially manufactured luncheon corned beef product, just as nisin, no preservative effect was observed (Coventry et al., 1995). In general, the pediocins seem to be more effective in meat products than nisin (Montville & Winkowski, 1997; Hugas, 1998; Ennahar et al., 1999) but they are not approved for use (Cleveland et al., 2001). However, the use of pediocin PA-1 in different food products is patented and this bacteriocin is believed to be the next in line if more bacteriocins are to be approved in the future (Ennahar et al., 1999).
Also using sakacins, some successful results have been obtained. Sakacin K inhibited growth of *Listeria innocua* to different extents in anaerobically packaged raw minced pork, chicken breasts and cooked pork at 7°C (Hugas et al., 1998). Growth of *L. monocytogenes* was completely inhibited for at least three weeks in chicken cold cuts and cold-smoked salmon by addition of sakacin P (Aasen et al., 2003), while sakacin K was not able to prevent slime production by a *L. sakei* strain on a cooked ham product (Aymerich et al., 2002). The use of sakacins as additives is not officially approved.

Existing studies show large variations in the degree of inhibition of pathogenic and spoilage organisms by added bacteriocins (Aasen et al., 2003), depending on the culture, the bacteriocin and the food system (McMullen & Stiles, 1996). The production of a certain bacteriocin in a laboratory medium does not imply its effectiveness in a food system (Hugas, 1998). The effectiveness of bacteriocins in food can be limited by a range of factors such as a narrow activity spectrum (not active towards Gram-negatives), uneven distribution due to limited diffusion in solid food matrices, inactivation through proteases or binding to food ingredients such as lipids (Aasen et al., 2003) or proteins and finally the emergence of bacteriocin-resistant bacteria (Holzapfel et al., 1995; Rodriguez et al., 2002). Furthermore, also the processing conditions (pH, T, a_w and Eh) and the product formula (salt/nitrite content, presence of spices) may influence the effectiveness of bacteriocins (Gänzle, 2004; Leroy & De Vuyst, 1999a; b; Hugas et al., 2002). Finally, the use of bacteriocins, other than nisin, as biopreservatives in food products is hampered by the fact that they are not legally accepted as food additives.

3.2.2. Organic acids

In general, reviews on biopreservation do not discuss the use of organic acids although these compounds might be from microbial origin and thus meet the definition of biopreservation as given in section 3.1. A detailed description of the use of organic acids in foods is however beyond the scope of this study.

Lactic acid can be produced by controlled fermentation of refined sucrose or other carbohydrate sources by numerous micro-organisms, mainly LAB and fungi (*Rhyzopus Oryzae*), and this has led to the commercial production of large quantities of lactic acid. This method of producing lactic acid is considered superior to the chemical synthesis of lactic acid by hydrolysis of lactonitrile (Shelef, 1994; Bogaert & Naidu, 2000). Lactic acid and its salts are widely used for decontamination purposes of e.g. meat carcasses and for shelf-life enhancement in several food products (Bogaert & Naidu, 2000). The shelf-life extending effect of lactic acid and its salts in meat and meat products, by their inhibitory effect on LAB (Houtsma et al., 1993), has been reported (Debevere, 1989; Devlieghere et al., 2000; Stekelenburg & Kant-Muermans, 2001; Peirson et al., 2003; Stekelenburg, 2003). Furthermore, several authors described the antibacterial action of lactic acid or lactate on the food born pathogen *L. monocytogenes* (Houtsma et al., 1993), also in meat and meat products (Greer & Dilts, 1995; Blom et al., 1997; Devlieghere et al., 2001; Stekelenburg & Kant-Muermans, 2001; Samelis et al., 2002; Stekelenburg, 2003). In anaerobically packaged sliced CMP, levels of 1.8% (Samelis et al., 2002), 2% (Debevere, 1989; Qvist et al., 1994), 2.5% (Stekelenburg & Kant-Muermans, 2001), 3% (Peirson et al., 2003) up to 3.3% (Stekelenburg & Kant-Muermans, 2001) are used to prolong shelf-life and/or prevent growth of *L. monocytogenes* without sensory deviations. From 3% and more, lactate can result in organoleptic deviations (Qvist et al., 1994; Stekelenburg, 2003).

Acetic acid is formed in a four-step reaction involving conversion of starch to sugar by amylases, anaerobic conversion of sugars to ethanol by yeast fermentation, conversion of ethanol to hydrated acetaldehyde, and dehydrogenation to acetic acid by aldehyde dehydrogenase. The last two steps are performed aerobically with the aid of acetic acid forming bacteria (Marshall et al., 2000). Apart from its traditional use in pickled foods, acetic acid can also be used as a decontaminating agent of meat products, poultry, seafood, fresh produce,… and as a natural food preservative. Sodium acetate at 0.5% and 0.25% was, however, less effective in controlling growth of *L. monocytogenes* on vacuum packaged frankfurters at 4°C compared to 3% sodium lactate (Bedie et al., 2001).

Combinations of lactate and acetate have also proven to be effective. Addition of 2.5% lactate and 0.25% acetate controlled growth of *L. monocytogenes* in vacuum packaged servelat sausage and cooked ham, stored at 4°C and this without affecting the sensory quality (Blom et al., 1997).

3.2.3. Fermentates

The Microgard™ products marketed by Danisco are fermentates of *Propionibacterium freundenreichii* subsp. *shermanii* that are commonly used commercially as biopreservatives in cottage cheese. Their inhibitory activity is attributed to propionic acid, acetic acid and a heat-stable peptide (Lemay et al., 2002b; Guinane et al., 2005). Alta™ and Perlac™ are fermented whey-based products used as shelf-life extenders but no significant inhibitory effect of these antimicrobials could be found in a chicken meat model (Lemay et al., 2002b).

3.3. Biopreservation by means of micro-organisms

Many successful biopreservation applications add the micro-organism itself rather than the microbial metabolite. In general, the applied micro-organisms are bacteria and in particular LAB, although some reports on the biopreservative properties of bacteria other than LAB, e.g. propionibacteria (Lind et al., 2005), do exist. The use of viruses and in particular bacteriophages for preservation of food products is not described in the literature of biopreservation although this application meets the definition of biopreservation.

3.3.1. Protective LAB

3.3.1.1. General characteristics of protective LAB

Lactic acid bacteria show special promise for implementation as protective cultures because of their long history of use and their reputation as safe and food-grade bacteria (Holzapfel et al., 1995). The desired properties of a protective LAB-culture are summarised in Table 1.2.
Psychrotrophic protective LAB are used to actively preserve refrigerated food products at their low storage temperature while mesophilic protective LAB are inactive at refrigeration temperatures but start to grow and to exert their protective effect when temperature abuse occurs (Lücke, 2000).

Furthermore, protective LAB can be divided in two groups (bacteriocinogenic and non-bacteriocinogenic) based on the property to produce bacteriocins or not (Devlieghere et al., 2004).

In general, high inoculua (10^6-10^9 cfu/g) are needed to create an antagonistic effect (Skyttä et al., 1991; Buncic et al., 1997; Rodgers, 2001; Nilsson et al., 2005).

Table 1.2. Desired properties of a protective LAB-culture (modified from Holzapfel et al., 1995)

1. No health risks
    - No toxin production
    - No production of biogenic amines or other metabolites detrimental to health
    - Non pathogenic
2. Bring about beneficial effects in product
    - Adaptation to product/storage conditions (e.g. psychrotrophic, salt tolerant)
    - Reliable/consistent protective activity
    - Predictability of metabolic activity under given conditions (e.g. lactic acid/gas production)
    - Competitiveness against endogeneous microbial flora
3. No negative sensory effects on product
    - no slime/gas/acid formation
    - no discolouration
    - no proteolytic or lipolytic activity
4. Function as indicator under abuse conditions

3.3.1.2. Applications of protective LAB in different food products

The effectiveness of PC has been studied in different food products but mainly in meat and meat products. The majority of these inoculation experiments were performed with the intention of demonstrating the effectiveness of bacteriocinogenic strains in controlling *L. monocytogenes.*

Milk and dairy products

Cheese suffers from spoilage through *Clostridium* spp. (late blowing) and is, furthermore, susceptible to contamination with *L. monocytogenes.* This latter problem arises mainly in cheeses in which the pH increases during ripening, such as the Italian cheeses Taleggio, Gorgonzola and Mozarella (Schillinger et al., 1996). Several studies tried to prevent in these

products late blowing and/or growth of *L. monocytogenes* through the addition of PC (Schillinger et al., 1996; O'Sullivan et al., 2002).

Much of the early work to prevent late blowing concentrated on the use of nisin-producing lactococci. Although effective, nisin interfered with the cheese fermentation process. More recent work tested multiple strain starters composed of nisin-producing strains and nisin-resistant fast-acid starters (O'Sullivan et al., 2002). The addition of this paired nisin-producing starter system to make cheddar cheese provided enough nisin to increase the shelf-life of pasteurised processed cheese, made from this cheddar, from 14 to 87 days at 22°C and to control *L. monocytogenes, Cl. sporogenes* and *St. aureus* (Zottola et al., 1994). Antilisterial effects were also observed for a bacteriocinogenic *Enterococcus faecium* strain during Taleggio production (Giraffa et al., 1994) and for a *Lc. lactis* strain, genetically transformed for pediocin AcH production in Cheddar cheese (Buyong et al., 1998). An overview of studies on bacteriocinogenic PC in dairy products is given by O'Sullivan et al. (2002).

Another interesting dairy application is the inhibition of *Bacillus cereus* in milk by acid production of added *Lactobacillus* and *Lactococcus* strains (10^6-10^7 cfu/ml) (Rossland et al., 2003).

Vegetable products

Bacteriocinogenic LAB are reported to have potential for the biopreservation of foods of plant origin, especially minimally processed vegetables and fermented vegetables (Schillinger et al., 1996; Bennik et al., 2000; O'Sullivan et al., 2002).

In minimally processed vegetables such as pre-packaged mixed salads and different types of sprouts, bacteriocinogenic LAB have been found to act on coliforms and enterococci (Vescovo et al., 1995) and on *L. monocytogenes* (Cai et al., 1997). Moreover, bacteriocinogenic starter cultures may be useful for the fermentation of sauerkraut (Breidt et al., 1995) or olives (Ruiz-Barba et al., 1994) to prevent spoilage.

Furthermore, biological strategies have been developed to control growth and mycotoxin production of fungi within or on plants or plant food products, e.g. patulin production in fruit. Biocompetitive control or the use of biocompetitive micro-organisms to inhibit mycotoxin forming moulds can be obtained by (1) the use of biocompetitive non-aflatoxinogenic moulds or (2) the use of antagonistic yeasts or bacteria (Schillinger et al., 1996). The antifungal effects of LAB are well-known and their potential application in the preservation of food and feed is reviewed by Schnürer & Magnusson (2005).

Fish, fish products and seafood

Spoilage of fresh fish is generally caused by Gram-negative bacteria (Roberts et al., 2005) and because of this, few attempts have focused on biopreservation of fresh fish (O'Sullivan et al., 2002). However, when vacuum packaged the spoilage of fresh fish, smoked fish and seafood is dominated by mainly Gram-positive bacteria, in particular LAB, and also *L. monocytogenes* can cause problems (Roberts et al., 2005). Most biopreservation studies in these products focused on the addition of bacteriocins rather than bacteriocinogenic PC (O'Sullivan et al., 2002). However, in the last number of years, different research groups have confirmed the potential of *Carnobacterium* spp. to control *L. monocytogenes* in cold-smoked salmon. Promising results were obtained with the divercin V41 producing *Carnobacterium divergens* V41 (Brillet et al., 2005), the bacteriocinogenic *Carnobacterium maltaromaticum* (previous *piscicola*) A9b and the non-bacteriocinogenic *C. maltaromaticum* A10a (Nilsson et al., 1999).

Meat and meat products

Since long, LAB adapted to meat have improved the safety of fermented sausages. However, using selected strains, the safety and stability of non-fermented, perishable meat and meat products can be improved (Lücke, 2000). Since the first study on biopreservation of chill-stored vacuum-packaged raw meat (Schillinger & Lücke, 1987), many different studies have been performed either in raw, cooked or fermented meat products (Hugas, 1998). Most of these studies are directed towards control of *L. monocytogenes* while only a minor part investigated the option of shelf-life prolongation.

Raw meat

Raw meat, stored aerobically under chilled conditions, is spoiled by Gram-negative bacteria, predominantly pseudomonads, and LAB compete poorly under these conditions. Hence, very high inocula of LAB are required to observe an effect on the shelf-life of aerobically stored raw meat. Pathogens of most importance in raw meat, *Salmonella, Campylobacter*, *Escherichia coli* and *Yersinia enterocolitica* are Gram-negative and thus insensitive towards bacteriocinogenic LAB. Therefore, the sole benefit of a protective culture on aerobically stored refrigerated raw meat is in the control of *L. monocytogenes* (Lücke, 2000).

The microflora of anaerobically packaged chilled raw meat is dominated by mainly LAB and inoculation with selected psychrotrophic LAB can be used to extend the shelf-life and to

protect against *L. monocytogenes* (Lücke, 2000). An overview of studies on the effectiveness of bacteriocinogenic and non-bacteriocinogenic LAB in raw meat is given in Table 1.3.
Two very specific types of biopreservation of raw meat should be mentioned here. First of all, controlled lactic acid fermentation on the meat surface has been reported as an efficient method to stabilise fresh meat in tropical areas by Minor-Pérez et al. (2004) in vacuum packaged pork at 20°C and by Kalalou et al. (2004) in aerobically stored minced camel meat at 22°C. Inhibition is fully explained by the desired pH-reduction and acid formation.
Secondly, Brashears et al. (1998) and Senne & Gilliland (2003) described the antagonistic action of *Lactobacillus* cells against Gram-negative spoilage and pathogenic micro-organisms in aerobically stored fresh meat by hydrogen peroxide production.

Fermented meat products

Besides the action of traditional 'starter cultures' in fermented sausage production, these products might benefit from the use of an additional protective culture to inhibit the food born pathogen *L. monocytogenes* (Työppönen et al., 2003b). Especially LAB producing antilisterial bacteriocins are useful as they can reduce the level of *L. monocytogenes* in fermented meat products further by about one or two log cycles compared with a non-bacteriocinogenic control (Table 1.4) (Hugas et al., 1995; Lücke, 2000). Other food born pathogens associated with fermented meat products such as *Salmonella* and *E. coli* are Gram-negative and thus insensitive towards bacteriocinogenic LAB. Today, several of these antilisterial cultures for sausage fermentation are available on the market and sometimes they function simultaneously as 'starter' and as 'protective' culture.

Cured cooked meat products (CMP)

The shelf-life of CMP is mainly limited by growth of LAB, recontaminating the product after heating during further handling and slicing. In anaerobically packaged CMP, recontamination with LAB occurs at low numbers and growth of *L. monocytogenes* is probable (Schmidt & Leistner, 1993; Lücke, 2000). Therefore, controlled growth of protective LAB in these meat products is a potential preservation strategy.

Table 1.3. Effect of (non-)bacteriocinogenic LAB on the shelf-life and/or safety of anaerobically packaged raw meat under refrigerated storage

Culture (inoculation level, bacteriocin)	Product	Target	Effect (log_{10} cfu/g)	Reference
Bacteriocinogenic				
Three *Pediococcus* strains (10^8 cfu/g, pediocin-like inhibitors)	Minced meat	*Y. enterocolitica*, *L. monocytogenes* and *Pseudomonas* spp. (10^2 cfu/g)	7[a]	Skyttä et al. (1991)
L. bavaricus MN (10^5 cfu/g, bavaricin-MN)	Beef cubes	*Listeria monocytogenes*	± 5[a]	Winkowski et al. (1993)
Leuconostoc gelidum UAL187-22 (10^4/cm^2, leucocin A)	Beef	No inoculation	No negative effect on odour/appearance compared to a non-inoculated control	Leisner et al. (1995)
Leuconostoc gelidum UAL187 (10^4/cm^2, leucocin A)	Beef	Sulphide-producing *L. sakei* strain (10^2/cm^2)	4[a] or 3.5[b]	Leisner et al. (1996)
L. sakei CTC494 (10^6 cfu/g, sakacin K)	Chicken breast Minced raw meat	*L. innocua*	2.5-3[b] 1-2[b]	Hugas et al. (1998)
L. sakei CTC494 & *E. faecium* CTC 492 (10^5 cfu/g, sakacin and enterocin)	Model cooked pork	Slime producing *L. sakei* & *L. carnosum*	Partial prevention of ropiness	Aymerich et al. (2002)
Lactobacillus casei CRL705 (10^6 cfu/ml spraying solution, 2 lactocins)	Beef	*L. monocytogenes* & *B. thermosphacta*	1.25[a] for *B. thermosphacta* and complete prevention of growth of *L. monocytogenes*	Castellano & Vignolo (2004)
L. sakei CETC[a] 4808 (10^7 cfu/g, BLIS[c])	Sliced beef	*Enterobacteriaceae*, *Pseudomonas* spp., *B. thermosphacta*	1-2[a]	Katikou et al. (2005)
Non-bacteriocinogenic				
L. sakei BJ-33 (10^6/cm^2)	Beef	Endogenous LAB-flora	Inhibition of heterofermentative LAB	Jelle (1987)
L. sakei BJ-33 (10^{6-7}/g)	Ground beef	*L. monocytogenes*	3[a]	Juven et al. (1998)

[a], difference in cell count at the final storage day between the product containing the PC and a non-inoculated control product; [b], difference in cell count at the final storage day between the product containing the bacteriocinogenic LAB-strain and a control product containing a non-bacteriocinogenic LAB-strain; [c], BLIS = bacteriocin-like inhibitory substances

Table 1.4. Effect of various bacteriocinogenic LAB on *L. monocytogenes* in fermented sausages (modified from Lücke, 2000)

Culture	Bacteriocin	Effect (log_{10}(cfu/g))	Reference
Pediococcus JD1-23	Pediocin PA-1/AcH	1.4[a]	Berry et al. (1990)
L. sakei Lb706	Sakacin A	±1[a] or 0-0.5[b]	Schillinger et al. (1991)
P. acidilactici PAC 1.0	Pediocin PA-1/AcH	0-1.5[a]	Foegeding et al. (1992)
P. acidilactici	Pediocin PA-1/AcH	± 2.5[a]	Luchansky et al. (1992)
L. plantarum MCS	Not specified	0-0.5[b] or 0.5-1[c]	Campanini et al. (1993)
L. sakei CTC 494	Sakacin K	1.25[b]	Hugas et al. (1995)
L. sakei CTC 494	Sakacin K	± 1.5[b]	Hugas et al. (1996)
L. sakei Lb706	Sakacin A	0-0.5[b]	Hugas et al. (1996)
L. curvatus LHT 1174	Curvacin A	± 2[b]	Hugas et al. (1996)
E. faecium CCM 4231 & RZS C13	Not specified	3.3[e]	Callewaert et al. (2000)
L. sakei CTC 494	Sakacin K	1.5[e]	Callewaert et al. (2000)
L. plantarum ALC01	Pediocin AcH	ND[d]	Työppönen et al. (2003a)
Lc. lactis M	Class IIa bacteriocin	1.5[a]	Benkerrroum et al. (2003)
L. curvatus LBPE	Class IIa bacteriocin	2[a]	Benkerrroum et al. (2005)
Lc. lactis LMG 21206	Class IIa bacteriocin	< 0.5[a]	Benkerrroum et al. (2005)

[a], Reduction of *Listeria* count in the presence of the bacteriocinogenic strain (Bac^+) compared to in the presence of a bacteriocin negative mutant (Bac^-) at the end of the fermentation process; [b], Reduction of *Listeria* count in the presence of the Bac^+-strain compared to in the presence of the Bac^-- mutant at the end of the maturation process; [c], Difference in *Listeria* count between the product containing the Bac^+-strain and a product not containing inoculated LAB at the end of the maturation period; [d], no quantitative determination of *L. monocytogenes* but a *Listeria*-negative product was obtained 1 week earlier (after 21 days) during ripening compared to a non-bacteriocinogenic control (after 28 days); [e], Reduction of *Listeria innocua* count after 28 days of ripening compared to its initial level.

According to Lücke (2000) there are three approaches to develop protective cultures for CMP, involving the selection of

- psychrotrophic bacteriocinogenic LAB
- psychrotrophic non-bacteriocinogenic LAB
- mesophilic LAB rapidly becoming active if the product is temperature abused.

Nisin-producing *Lc. lactis* strains would be prime candidates for in-situ bacteriocin production in cooked meat products because of the broad antimicrobial spectrum of nisin. However, *Lc. lactis* is mesophilic (Stiles & Holzapfel, 1997) and does not grow in chill-stored meat products (McMullen & Stiles, 1996). Furthermore, nisin itself has also a poor

performance as a biopreservative in meat products (Chung et al., 1989; Holzapfel et al., 1995; McMullen & Stiles, 1996). This has resulted in the search for psychrotrophic bacteriocinogenic LAB that are suited to grow in the chilled meat environment. An overview of studies on the effectiveness of bacteriocinogenic LAB in CMP is given in Table 1.5. Recently, Budde et al. (2003) demonstrated that *Leuc. carnosum* 4010, producing leucocin A-4010 and leucocin B-4010, is suitable as a PC for anaerobically CMP. This strain immediately reduced the number of *L. monocytogenes* cells to a level below the detection limit, when applied at 10^7 cfu/g in a vacuum packaged meat sausage, and this without causing adverse effects on the sensory quality. Meanwhile, this bioprotective culture has been commercialised by Chr. Hansen under the name B-SF-43 SafePro™.

Despite the successful results of some studies, the effectiveness of bacteriocinogenic cultures in food products and more specific in meat products can be limited by a range of factors. First of all, some factors affect the efficacy of the bacteriocin itself, as mentioned before in section 3.2.1., such as a narrow activity spectrum, poor solubility, limited diffusion and uneven distribution in solid matrices, inactivation through proteolytic enzymes, binding to food ingredients such as lipids or proteins, the emergence of the bacteriocin-resistant bacteria, pH effects on bacteriocin stability and activity. A second series of factors are related to the bacteriocin-producing LAB and include spontaneous loss of bacteriocin-producing property through genetic instability, poor adaptation of the culture to the food environment (pH, temperature, nutrients), a low production level (Buncic et al., 1997) and phage infection (Holzapfel et al., 1995; Schillinger et al., 1996; Rodriguez et al., 2002). An alternative to overcome these disadvantages is the use of psychrotrophic non-bacteriocinogenic but nevertheless very competitive cultures, e.g. *L. sakei* BJ-33 (Andersen, 1995a), *L. sakei* TH1 (Bredholt et al., 2001), etc. (Devlieghere et al., 2004). An overview of studies on the effectiveness of non-bacteriocinogenic LAB in CMP is given in Table 1.6. *Lactobacillus sakei* BJ-33 (earlier *L. alimentarius*) is probably the first and most studied PC of this type. This psychrotrophic, homofermentative and non-bacteriocinogenic strain has been commercialised by Chr. Hansen (Denmark) as B-2 SafePro™ (earlier FloraCarn and Bactoferm) for bioprotection of anaerobically packaged meat products. The culture has been shown to inhibit spoilage causing LAB (Jelle, 1987; Andersen, 1995b) and *B. thermosphacta* (Bactoferm™ technical information, 2003) and its application has resulted in the extension of the sensory shelf-life of cooked ham (Kotzekidou & Bloukas, 1996) and frankfurters (Kotzekidou & Bloukas, 1998). Furthermore, *L. sakei* BJ-33 could control the growth of

Table 1.5. Studies on the effect of psychrotrophic bacteriocinogenic protective LAB on anaerobically packaged cooked meat products stored under refrigeration

Protective culture	Bacteriocin	Inoculum (cfu/g)	Target organism	Meat product	Effect (log_{10}(cfu/g))	Reference
Pediococcus acidilactici JD1-23	Pediocin AcH	10^7	*L. monocytogenes*	Frankfurters	0-0.5[a]	Berry et al. (1991)
Carnobacterium maltaromaticum LK5	ND[c]	10^{6-7}	*L. monocytogenes*	Frankfurters	2[b]	Buchanan & Klawitter (1992)
L. sakei Lb706	Sakacin A	10^3	*L. monocytogenes*	Emulsion-type sausage	No effect[a]	Buncic et al. (1997)
L. sakei CTC494	Sakacin K	10^6	*L. innocua*	Cooked pork product	0.5-2[a]	Hugas et al. (1998)
L. sakei Lb790	Sakacin P	10^4	*L. monocytogenes*	Chicken cold cuts	0-0.5[a], ± 2[b]	Katla et al. (2002)
Leuc. mesenteroides L124 or *L. curvatus* L442	ND	10^4	*B. thermosphacta* & enterococci	Frankfurter-type sausages and sliced cooked cured pork shoulder	1[b] (*B. thermosphacta)* and 3[b] (enterococci)	Metaxopoulos et al. (2002)
Leuc. mesenteroides L124 or *L. curvatus* L442	ND	10^5-10^6	*L. innocua*	Sliced cooked cured pork shoulder	± 1.5[a]/± 4.5[b]	Mataragas et al. (2003b)
Leuc. carnosum 4010	Leucocins	1×10^5	*L. monocytogenes*	Cooked pork meat sausage	2.5[b] after 2 weeks / 7[b] after 4 weeks	Budde et al. (2003)
		6×10^6			5[b] after 2 weeks / 7[b] after 4 weeks	
Leuc. carnosum 4010	Leucocins	10^7	*L. monocytogenes*	Pork saveloys	4[b]	Jacobsen et al. (2003)

[a], difference in cell count at the final storage day between a product containing the bacteriocinogenic LAB-strain and a control product containing a non-bacteriocinogenic LAB-strain; [b], difference in cell count between a product containing the bacteriocinogenic LAB-strain and a non-inoculated control product; [c], ND= the nature of the bacteriocin-like substance was not determined

Table 1.6. Studies on the effect of psychrotrophic non-bacteriocinogenic protective LAB on anaerobically packaged cooked meat products stored under refrigeration

Protective culture	Inoculum	Target organism	Food product	Effect	Reference
L. sakei BJ-33	10^7 cfu/cm^2	*L. monocytogenes*	Frankfurters	2[a]	Andersen (1995a)
L. sakei BJ-33	10^{10} cfu/g [b]	Spoilage flora	Cooked ham	Shelf-life extension (7 days)[c]	Kotzekidou & Bloukas (1996)
L. sakei BJ-33	10^7 cfu/cm^2	Ropy slime producing *Lactobacillus sakei*	Frankfurters	No inhibition	Björkroth & Korkeala (1997)
L. sakei BJ-33	10^7 cfu/g[d]	Spoilage flora	Frankfurters	Shelf-life extension (7 days)[c]	Bloukas et al. (1997)
L. sakei BJ-33	10^5 cfu/cm^2	*Pseudomonas* spp. & *B. thermosphacta*	Frankfurter-type sausage	Shelf-life extension (19 days) 3[a] (*B. thermosphacta)* 1.5-2[a] (*Pseudomonas* spp.)	Kotzekidou & Bloukas (1998)
L. sakei BJ-33	10^5 cfu/cm^2	*Salmonella enteritidis*	Frankfurter-type sausage	0-0.5[a]	Kotzekidou & Bloukas (1998)
L. sakei BJ-33	10^7 cfu/g	*L. monocytogenes*	Cooked ham	5[a]	Andersen (2000)
			Emulsion sausages	3[a]	
			Rolled cooked pork belly	5[a]	
L. sakei TH1	10^4/10^6 cfu/g	*L. monocytogenes*	Cooked ham	5[a]	Bredholt et al. (1999)
		E. coli O157:H7		2-3[a]	
		Y. enterocolitica O:3		0[a]	
L. sakei TH1	10^5-10^6 cfu/g	*L. monocytogenes*	Servelat sausage	1[a] (4°C) and 2.5[a] (8°C)	Bredholt et al. (2001)
P. acidilactici D3, *L. casei*	10^7 cfu/ml	*L. monocytogenes*	Frankfurters	4.2-4.7[a]	Amezquita & Brashears (2002)
D6 & *L. paracasei* I5	rinsate		Cooked ham	2-2.6[a]	

[a], reduction (log_{10}(cfu/g)) compared to non-inoculated control product; [b], added via the curing solution before pasteurisation; [c], shelf-life is based on a sensory evaluation; [d], added in the batter before heating

L. monocytogenes in frankfurters (Andersen, 1995a), in cooked ham, in an emulsion sausage and in rolled cooked pork belly (Andersen, 2000). This commercial biopreservative failed, however, to inhibit *Salmonella enteritidis* (Kotzekidou & Bloukas, 1998) and four ropy-slime producing *L. sakei* strains (Björkroth & Korkeala, 1997) on frankfurters. Another interesting PC is *L. sakei* TH1; application of this strain allowed control of *L. monocytogenes* on cooked ham and frankfurters (Bredholt et al., 1999; 2001).

Mesophilic LAB acting as a biopreservative only under conditions of temperature abuse have been less studied. Degnan et al. (1992) observed a 2.7 log reduction of *L. monocytogenes* (compared to a bacteriocin-negative mutant) by the pediocin AcH producing *Pediococcus acidilactici* JBL1095 when applied at 10^5 cfu/g on vacuum packaged wieners at abuse temperature (25°C), while no inhibition occurred at refrigeration temperature (4°C). Elsser (1998) worked with *Lc. lactis* subsp. *lactis* L201 at levels of 10^5-10^7 cfu/cm^2 and this strain was able to inhibit growth of *Salmonella, St. aureus, B. cereus* and *Cl. perfringens* in vacuum packaged cooked sausages when temperature abused at 22°C.

Existing studies indicate that certain LAB may be used as protective cultures for CMP, provided that they inhibit *L. monocytogenes* and spoiling LAB, while they cause only a minimal change in the sensory properties of the treated product. However, according to Lücke (2000) addition of lactate/(di)acetate or glucono-δ-lactone (GDL) to the formulation of CMP is more effective than applying protective cultures.

Raw cured meat products

Few authors have investigated the use of PC on raw cured meat products since too high salt contents can limit the growth of the PC. The use of starter cultures and carbohydrates to produce a product which will safely spoil is, however, the basis of the Wisconsin Process for bacon production (Moore & Madden, 1997). Andersen (1995a) demonstrated that *L. sakei* BJ-33, when applied at 10^7 cfu/g, was able to control the endogenous LAB-flora and *L. monocytogenes* in MAP-packaged bacon cubes (3.5% salt) at 15°C.

Other food products

Rodgers (2001; 2003; 2004) reviewed the applications of bacteriocin-producing and acid-producing LAB in refrigerated ready-to-eat food products, e.g. soups, meals and salads, to

prevent them from growth of food born pathogens, in particular *Cl. botulinum* and/or *L. monocytogenes*. The effectiveness of a mixture of a nisin-producing *Lc. lactis* and a pediocin A-producing *P. pentosaceus* to prevent growth of *Cl. botulinum* and botulinal toxin formation after 10 days at 10°C varied with the type of cook-chill food product examined. Positive effects were noticed in seafood chowder, chicken casserole, vegetable curry, beef stroganoff, lamb hot pot, veal casserole and chicken satay (Rodgers, 2004).

3.3.2. Bacteriophages

Recently, there is an increased interest in the use of bacteriophages, viruses that infect - and usually kill - bacteria, as a means of inactivating food born pathogens and spoilage organisms in food products (Hudson et al., 2005). Endolysins or bacteriophage-encoded peptidoglycan hydrolases also have the potential to affect bacteria but their antibacterial efficacy in food products has not been reported (Greer, 2005; Loessner, 2005).

3.3.2.1. Life cycle

Phages are submicroscopic particles that typically consist of nucleic acid (DNA or RNA) surrounded by a protein coat (Hudson et al., 2005). They are abundant in the environment, in the human gastro-intestinal tract, in water and in food products, inclusive meat and meat products (Greer, 1983; Atterbury et al., 2003a).

Bacteriophages have been classified as lytic phages (virulent), those that employ only the lytic pathway, or lysogenic phages (temperate). Both types are only able to replicate after infecting a bacterial cell (obligate parasites). In the case of the lytic pathway, infection results in death of the bacterial cell by lysis to release new phage particles. In the case of lysogeny, infection does not result in cell death/cell lysis but results in integration of the phage genome into the bacterial chromosome. Being part of it, the phage genome is replicated during bacterial growth but no phage particles are formed. Some phages are strictly lytic while others can switch between the lytic cycle and a lysogenic status and vice versa following certain stimuli (Thiel, 2004; Hudson et al., 2005). Host lysis in the absence of phage replication is known as 'lysis from without'. This non-proliferative lytic mechanism can occur when a high number of phage particles adhere to the cell and lyse it through the activity of cell wall degrading enzymes (Hudson et al., 2005).

For preservation purposes, lytic phages are preferred. In this lytic pathway (Figure 1.5), (1) phages attach to cell surface receptor molecules of a specific host bacterium, (2) the phage

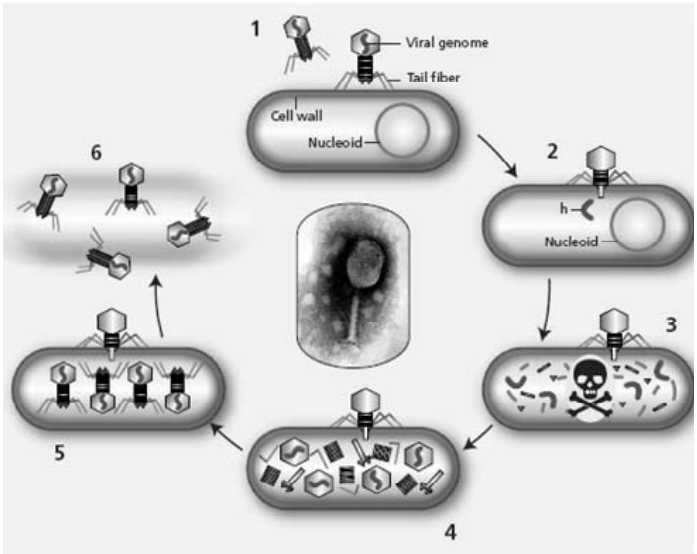

Figure 1.5. The lytic pathway of bacteriophages (Thiel, 2004)

genome is injected into the cell, (3) the bacterial genome is disrupted and the bacterium is killed as some of the bacteriophage genes are encoding for enzymes that turn off or even destroy the host's DNA, (4) replication of viral genome and transcription of genes that are translated to form the proteins of the phages' capsid, (5), assembly of new phages, up to several hundred per cell, and (6) their endolysins degrade the peptidoglycan of the bacterial cell wall which results in the release of the phages and death of the host by lysis. In this process, the bacterial cell is destroyed while the number of phages increases, amplifying the overall antibacterial effect. This cycle may last only 30-60 minutes (Thiel, 2004).

3.3.2.2. Effectiveness as food preservatives

Bacteriophages are attractive candidates for biopreservation of food products because they are self-perpetuating organisms designed to kill living bacterial cells. A valuable quality is their remarkable stability in refrigerated foods and their property to not impart any undesirable sensory changes. In what follows, the focus will be on the application of phages in meat and meat products.

Bacteriophages to control pathogens

The potential of phages for controlling food born pathogens is reflected in several recent studies on *Campylobacter* (Atterbury et al., 2003b; Goode et al., 2003), *Salmonella* (Goode et al., 2003; Whichard et al., 2003), *E. coli* O157:H7 (O'Flynn et al., 2004) and *L. monocytogenes* (Dykes & Moorhead, 2002; Carlton et al., 2005).

The application of the host-specific bacteriophage ϕ2 at approximately $5\times10^6/cm^2$ to the surface of chicken skin resulted in a significant 1 $\log_{10}$ reduction in the number of inoculated *Campylobacter jejuni* cells ($5\times10^5/cm^2$), when stored at 4°C (Atterbury et al., 2003b). O'Flynn et al. (2004) demonstrated the efficacy of a three-phage cocktail (multiplicity of infection (MOI) - the ratio of phages to host cells - was 10^6 pfu/cfu) in reducing *E. coli* O157:H7 on inoculated steak meat at 37°C but simultaneously reported that no lysis occurred in the absence of growth of the host at 12°C. Two phages, phage Felix O1 and a variant, were applied (MOI of 1.9 $\times10^4$ pfu/cfu) to frankfurters inoculated with *Salmonella* typhimurium and could reduce growth of the pathogen with ± 2 $\log_{10}$ after 24h at 22°C (Whichard et al., 2003). Goode et al. (2003) was able to inactivate partly or completely, depending on the MOI, *Salmonella* enterica and *C. jejuni* on chicken skin stored at 4°C for 48h.

Application of phage LH7 to two *L. monocytogenes* isolates inoculated onto vacuum packaged beef, which was stored at 4°C, had no effect compared to a control because of the MOI that had not been optimised (Dykes & Moorhead, 2002). When using phage P100, an antilisterial effect (3.5 $\log_{10}$ reduction) was obtained in surface-ripened red-smear soft cheese (Carlton et al., 2005). At present, the option of using phages to prevent proliferation of post-processing contaminating *L. monocytogenes* on anaerobically packaged CMP has not been reported.

Bacteriophages to prevent spoilage

Shelf-life extension through the action of phages on spoilage organisms has been considered (Hudson et al., 2005). Attempts have been made to increase the shelf-life of aerobically stored meat by using phages active against *Pseudomonas* spp. (Greer & Dilts, 1990) and *B. thermosphacta* (Greer & Dilts, 2002). In the study of Greer & Dilts (1990), a pool of seven bacteriophages was unable to control beef spoilage by *Pseudomonas* growth. The efficacy of the phage pool was limited by a narrow range of host specificity. Subsequent host range studies showed that only 57.2% of the 1023 tested *Pseudomonas* spp. were susceptible to phage lysis. Greer & Dilts (2002) tested phages to control spoilage by *B. thermosphacta* on pork adipose tissue. In the presence of the phages, counts were reduced over the first 2 days at both 2 and 6°C but then increased, presumably through the growth of a subpopulation of resistant cells. Phage treatment extended the shelf-life with four days compared to the control. In their study, the problem of insufficient host range was not addressed since the pork tissue was sterile and only one single *B. thermosphacta* isolate was used (Hudson et al., 2005).

3.3.2.3. Issues to consider in biopreservation with bacteriophages

Factors determining the efficacy of the phage application are the stability of the phage(s) under the physicochemical conditions of the food (pH, a_w) and under its storage conditions (temperature) and the ratio of phages to host cells (MOI) (Hudson et al., 2005). Furthermore, emergence of phage resistance and phage host range are two important issues to consider in the design of phage interventions (Whichard et al., 2003).

Phage stability

The phage stability depends on the pH with lower pH-values resulting in less phage stability. At low temperatures, phages are assumed to be stable at pH values between 4 and 10 (Hudson et al., 2005). Leverentz et al. (2003) found the phage LMP-102 to be active between pH-values of 5.5 and 8 and the greatest reduction of *L. monocytogenes* in broth by 10^7 pfu/ml of the LMP-102 phage was achieved within a pH range from 7 to 8. Experiments to control *L. monocytogenes* on fruit revealed that the titer of a phage mixture remained stable for 7 days on melon (pH 5.5-6.5) but declined to non-detectable levels within 30 min on apple slices (pH 3.8-4.2) (Leverentz et al., 2003).

It has been stated that phage replication does not occur at temperatures too low to permit growth of the host. Phage replication, however, is not necessary for inactivation through 'lysis from without' (Hudson et al., 2005). Activity of bacteriophages at refrigeration temperatures of 7 and 4°C has been demonstrated by Dykes & Moorhead (2002) and Atterbury et al. (2003b), respectively.

The effect of thermal stress on phages is variable, depending on the phage type, the matrix and the time/temperature combination. For two psychrophilic *Pseudomonas* phages, greater than 99% inactivation occurred after 1 min at 60°C for one of them and only 39% inactivation occurred after 30 min at 60°C for the other phage. In contrast, phage particles isolated from a hot spring (>80°C) had a wide thermal tolerance range with 18 to 30% surviving boiling (Hudson et al., 2005). In general, phages are more heat resistant than most vegetative bacteria and they may survive heat treatments routinely applied in the food industry. *Campylobacter* bacteriophages could survive commercial poultry processing procedures (Atterbury et al., 2003a), a necessary characteristic if phages are to retain their ability to control bacteria during the post processing storage of foods (Hudson et al., 2005).

Multiplicity of infection (MOI)

Phage adsorption to receptors on the bacterial cell wall requires an appropriate chance of collision of the phage with the bacterial cell. This implicates that the higher the MOI, the higher the phage's effectiveness. However, the MOI may also not be too high since phage replication has been reported to only proceed if also a minimum number of actively growing bacterial cells are present. A separate case is 'lysis from without', not requiring phage replication and thus occurring at very high MOI (Hudson et al., 2005, Greer, 2005).

Host specificity

The inherent host specificity of bacteriophages is based on the specific binding between elements of their capsid and specific molecules on the surface of their target bacteria and this ensures that they do not infect eukaryotic cells and even non-target bacterial cells. This means that pathogens can be removed and beneficial organisms (starter cultures, gastro-intestinal flora of the consumer or the background flora of the treated food product) stay. However, this narrow host range might also be a drawback, in particular when phage application is directed towards control of spoilage organisms (Greer, 2005). Even the use of mixtures of effective phages did not ensure adequate coverage of all target organisms and could therefore not prolong the shelf-life of aerobically stored beef (Greer & Dilts, 1990). Another disadvantage, however, of this specificity might be that resistance might develop when the specific cell surface receptors are lost (Carlton et al., 2005; Hudson et al., 2005; Greer, 2005).

Phage resistance

Host cells are not entirely defenceless against phage attack since phage resistance mechanisms have been identified and most of them are plasmid encoded (Emond et al., 1997; Hudson et al., 2005). The most common form of resistance is loss of a cell surface receptor resulting in the prevention of phage attachment (adsorption inhibition), but other forms are prevention of phage DNA-injection (Garcia & Molineux, 1995), restriction and modification of incoming phage DNA and abortive infection (Emond et al., 1997). In the latter case, the phage lytic cycle is terminated only after phage attachment, DNA-injection and early phage expression; further phage proliferation is prevented and lysis of the host fails to occur (Emond et al., 1997).

Some researchers have not been able to recover phage-resistant bacterial mutants during laboratory trials of phage biocontrol in foods (Atterbury et al., 2003b; Carlton et al., 2005),

while others did notice the emergence of phage-insensitive mutants (O'Flynn et al., 2004). In the latter study, the frequency of formation of bacteriophage-insensitive mutants of *E. coli* O157:H7 varied from 10^{-4} cfu for one particular phage to 10^{-6} cfu for two other phages and for the tested phage cocktails. Resistant mutants could revert to phage sensitivity. This led to the conclusion that phage-insensitive mutants should not hinder the use of phages as biocontrol agents. Moreover, most pathogens are typically encountered in the environment in low levels reducing therefore the chance of resistance development following phage applications in foods (O'Flynn et al., 2004). Furthermore, antagonistic co-evolution might occur: bacteria might evolve to resist phages but phages evolve too e.g. by altering their host-range (O'Flynn et al., 2004; Thiel, 2004). Despite the disparity in the published literature, phage-resistant mutants do emerge and are a concern. An option to resolve issues with resistance is the use of phage cocktails (Whichard et al., 2003; Carlton et al., 2005; Greer, 2005).

3.4. Effect of protective LAB on the sensory quality of anaerobically packaged cooked meat products

If LAB are used as biopreservatives for controlling growth of pathogens and extending storage life in CMP, careful consideration of their impact on the sensory properties is necessary. In CMP, the production of large amounts of organic acids and carbon dioxide by the applied LAB is highly undesirable (McMullen & Stiles, 1996).

A careful selection of the appropriate LAB is the first step to avoid these sensory effects. From a sensory point of view, especially homofermentative LAB are good candidates because they do not produce carbon dioxide and organic acids other than lactic acid. More recently, several *Leuconostoc* spp. were found to be candidate for the biopreservation of CMP (Table 1.5). Budde et al. (2003) found that the spoilage potential of several heterofermentative *Leuc. carnosum* strains differed depending on the strain and they selected *Leuc. carnosum* 4010 as a potential biopreservative because it did not produce any specific compounds that might affect the sensory quality of meat products. Furthermore, in CMP, which often contain added sucrose, the use of LAB-strains producing dextran from sucrose is not possible because this results in slime formation (McMullen & Stiles, 1996).

Secondly, a careful consideration of the formulation of a CMP might also overcome negative sensory effects. Cooked meat products with high levels of carbohydrates support sensory changes, while in CMP without added carbohydrate, little effect on the sensory quality of the product is to be expected (McMullen & Stiles, 1996). Jacobsen et al. (2003) suggested not

using *Leuc. carnosum* 4010 in frankfurter sausages or other products with high dextrose concentration (± 5%) since these products will be spoiled because of gas production.

Despite numerous reports investigating the effectiveness of PC, only few authors have included in their research their influence of the PC on the sensory characteristics of the treated products. Ignoring the effects on sensory quality of the food product may lead to a successful but misleading result.

The commercialised PC *L. sakei* BJ-33 (Table 1.6) is reported to be a weak acidifier with only limited proteolytic and lipolytic activities and no production of H_2O_2. These properties may, according to Jelle (1987) and Andersen (1995a), explain the cultures' low impact on the sensory quality of a meat product (Bactoferm^{TM} technical information, 2003). However, Jelle (1987) reported that vacuum packaged beef inoculated with several lactobacilli, including *L. sakei* BJ-33, had lower sensory scores than non-inoculated beef and that assessors preferred non-inoculated beef. However, inoculation with *L. sakei* BJ-33 did not turn the beef into an unacceptable product. The sensory effect of the lactobacilli was probably due to acid production since inoculation of the beef with lactobacilli resulted in a fast pH-drop (Jelle, 1987). Little information is available on the influence of *L. sakei* BJ-33 on the sensory quality of CMP. Andersen (2000) did not include pH-measurements and sensory evaluation in his publication. In the study on pariza of Kotzekidou & Bloukas (1998), the control treatment had an unacceptable sour taste after 4 weeks, whereas products treated with *L. sakei* BJ-33 had an acceptable odour and taste until 7-8 weeks of storage at 6-8°C. Despite the sour taste, the pH of the control treatment was the highest of all treatments and decreased only from 6.7 to 6.1 after 4 weeks. The authors assigned the negative sensory quality of the control to heterofermentative LAB producing organic acids of which particular acetic acid is unwanted. This hypothesis still does not agree with the pH-evolution of the product; it might be that other types of metabolites were causing the spoilage.

With regard to commercialised bacteriocinogenic *Leuc. carnosum* 4010 (Table 1.5), sensory evaluation of 38 commercial products biopreserved with the strain confirmed that the strain is suitable for almost all kinds of sliced meat products, only giving the products a slightly more acidic flavour and taste (Budde et al., 2003; Jacobsen et al., 2003). Similar results were found by Bredholt et al. (1999; 2001) (Table 1.7). They observed no statistically significant differences in consumer preferences between treated and untreated products and showed that addition of *L. sakei* TH1 to cooked ham or saveloy did not affect the acceptability of the meat products after 28 days of storage at 4°C although the flavour and taste was slightly more

acidic than the controls. Indeed, the pH of cervelat treated with *L. sakei* TH1 reached values of approximately 5.2-5.3, while CMP are most often considered as unacceptable from a sensory point of view when the pH is lowered below 5.3 (Korkeala et al., 1990).

Amezquita & Brashears (2002) (Table 1.6) did not observe any deviating sensory quality when applying the bacteriocin-producing *P. acidilactici* on frankfurters after 56 days at 5°C but did not evaluate the effect of the more acidifying non-bacteriocinogenic *L. casei* and *L. paracasei.*

3.5. Mechanism of inhibition of protective LAB in anaerobically packaged cooked meat products

Microbial interference is the antagonistic inhibition (antagonism) displayed by one micro-organism towards another micro-organism (Hugas, 1998). This type of microbial interaction is depicted in Figure 1.6.

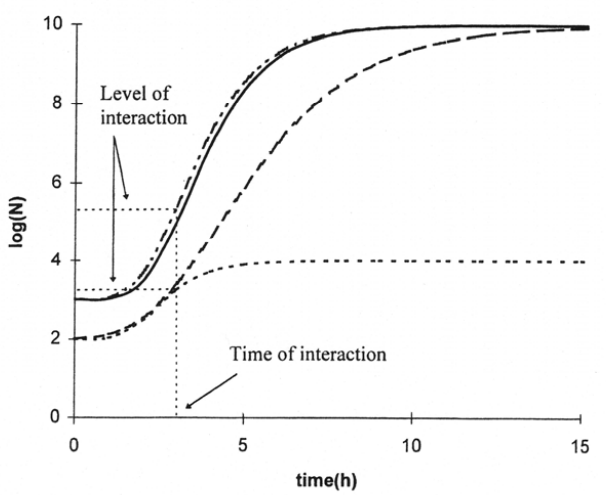

Figure 1.6. Simulation of the growth of pure cultures A (—) and B (— —) compared with mixed cultures of A (— — —) and B (---); interaction between A and B in the mixed culture decreases the growth of B (Malakar et al., 1999)

LAB-growth in meat and meat products can cause microbial interference to spoilage and pathogenic bacteria through one or a combination of several mechanisms like production of inhibitory substances, nutrient competition and competition for space (attachment/adhesion sites) (Holzapfel et al., 1995; Hugas, 1998). Whereas section 1.5 described the antimicrobial

activity of LAB in general, this section deals with only those mechanisms of inhibition that are of importance in the biopreservation of pre-packaged CMP with protective LAB.

3.5.1. Production of inhibitory substances

3.5.1.1. Lactic acid production and related pH-decrease

The in-situ production of organic acids, mainly lactic acid, in food products by LAB is an important mechanism of biopreservation and has been indicated as the cause of e.g. inhibition of *L. monocytogenes* by *L. sakei* BJ-33 in vacuum packaged ground beef (Juven et al., 1998). In the latter study, *L. sakei* BJ-33 produced ± 50 mM of lactic acid in the product, resulting in a final pH of 4.7. However, many factors determine the effectiveness of in-situ acidification: product's initial pH, its buffering capacity, the type and level of target organism, the nature and concentration of fermentable carbohydrate, viability and growth rate of LAB and target organism in the food matrix and the applied storage temperature (Montville & Winkowski, 1997). In anaerobically packaged CMP, LAB sometimes exert antilisterial effects that can not be explained in terms of lactic acid production and decrease of pH alone (Vermeulen et al., 2006) because of two main reasons:

(1) the produced amount of lactic acid, at the moment of inhibition, is negligible compared with that already present in CMP (Metaxopolous et al., 2002) due to the low carbohydrate content of most CMP;

(2) the high buffering capacity of most CMP limits the pH-reduction, following lactic acid production.

This means that another mechanism must be responsible for the biopreservative effect of protective LAB in anaerobically stored CMP.

3.5.1.2. Hydrogen peroxide production

Hydrogen peroxide production by LAB does not occur under anaerobic conditions (Kandler & Weiss, 1986). When using a high barrier packaging material for vacuum packaging or modified atmosphere packaging of CMP, low residual O_2-levels are obtained and H_2O_2 is playing a minor role in the antagonism of protective LAB.

3.5.1.3. Bacteriocin production

When using a bacteriocinogenic PC for biopreservation of CMP, production of the bacteriocin is automatically indicated as the main cause for observed inhibitions. To prevent that inhibition is wrongfully attributed to bacteriocin production but is rather caused by acid production, studies must include an isogenic bacteriocin-negative mutant as a control (Buchanan & Bagi, 1997). Such studies support the fact that bacteriocinogenic LAB inhibit *Listeria* spp. regardless of acid production (Skyttä et al., 1991; Buchanan & Klawitter, 1992; Mataragas et al., 2003a). However, acid production may have a potentiating effect by lowering the pH to a value closer to the optimal pH for bacteriocin production (Skyttä et al., 1991; Mataragas et al., 2003b).

3.5.1.4. Production of other antimicrobials

New antimicrobials are still being discovered, e.g. a mixture of low-molecular-mass molecules that act synergistically with lactic acid (Niku-Paavola et al., 1999), 3-hydroxy fatty acids (Sjögren et al., 2003), antifungal cyclic dipeptides (Ström et al., 2002), phenyllactic acid and 4-hydroxyphenyllactic acid (Ström et al., 2002; Valerio et al., 2004). Although most of these compounds are mainly having antifungal activity (Schnürer & Magnusson, 2005), some of them are also active towards Gram-positive bacteria such as *Listeria* (Leroy et al., 2006). Whether these compounds could play a role in the activity of protective LAB on anaerobically packaged CMP has not been studied yet.

3.5.2. Nutrient competition

Competition for nutrients between two micro-organisms occurs when a nutrient essential for growth of both micro-organisms, is limited. The limited availability of certain essential molecules needed for cell metabolism, including energetic compounds and building blocks for cell synthesis, like amino acids, vitamins, minerals and nucleotides, may slow down the growth. The micro-organism that can metabolise the nutrient most quickly is most competitive and will dominate the other micro-organism (Geisen et al., 1992).

It has been shown that the population of an endogenous microbial flora suppresses growth of pathogens in food products (Kleinlein & Untermann, 1990; Vold et al., 2000) and this probably due to a competitive advantage for nutrient uptake (Nilsson et al., 2004). This suppression of a particular organism by an overgrowing microflora is called the Jameson

effect (Gram et al., 2002). Several authors have proposed nutrient competition as an explanation for the inhibition of *L. monocytogenes* by non-bacteriocinogenic LAB in anaerobically packaged CMP (Buchanan & Bagi, 1997; Bredholt et al., 1999; Devlieghere et al., 2004) or in other food products (Nilsson et al., 1999; 2004). Up to now, it is not clear for which nutrient(s) competition occur(s). According to Chr. Hansen, the inhibitory effect of non-bacteriocinogenic *L. sakei* BJ-33 is due to competition for easy fermentable nutrients and rest oxygen (Juven et al., 1998; Andersen, 2000; Bactoferm™ technical information, 2003). The data of Buchanan & Bagi (1997) suggest that the suppression of the maximum population density of *L. monocytogenes*, when growing in co-culture with a non-bacteriocinogenic *C. piscicola*, is, at least in part, related to nutrient depletion since the extent of the suppression decreased when the two species were cultured in 3× or 6× brain heart infusion broth. Indeed, lactobacilli are extremely fastidious organisms, well adapted to complex organic substrates. They require not only carbohydrates as energy and carbon source, but also amino acids, vitamins and minerals (Kandler & Weiss, 1986).

3.5.2.1. Competition for carbohydrates

The carbohydrate content of meat products is relatively poor, except when carbohydrates are directly or indirectly added, and among the few sugars found in meat, glucose and ribose are the only sugars that *L. sakei* can utilise for its growth (Stentz et al., 2001). Glucose originates from glycogen and ribose from ATP hydrolysis (Champomier-Vergès et al., 2001). *L. monocytogenes* can utilise only a limited number of carbon sources for energy, glucose the preferred source (Premaratne et al., 1991). For this reason, glucose has often been proposed as the substrate for which competition could occur. In the study of Nilsson et al. (2005), the cell free supernatant of the non-bacteriocinogenic *C. piscicola* A9b caused a decrease in *L. monocytogenes* cell density, which was abolished by glucose addition suggesting that competition for glucose causes the inhibitory interactions. The results from Vermeulen et al. (2006) do not confirm this hypothesis since (1) in the presence of a higher glucose content compared to a lower glucose content, inhibition of *L. monocytogenes* by *L. sakei* was enhanced (Vermeulen et al., 2006) and (2) at the moment of inhibition glucose limitation could not be observed. Concerning ribose, its utilisation is not repressed by glucose and co-metabolism of glucose and ribose instead of sequential utilisation during growth of *L. sakei* on meat is suggested. Whether this metabolism would lead to an advantage of *L. sakei* towards other microbial flora on meat is not yet demonstrated. Other microbial flora might use only glucose whereas *L. sakei* might take an advantage in using both sugars (Stentz et al., 2001).

3.5.2.2. Competition for amino acids

The pattern of amino acid requirements of lactobacilli differs among species and even strains (Kandler & Weiss, 1986). According to Lauret et al. (1996) and Moretro et al. (1998), essential amino acids for growth of *L. sakei* are glycine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, valine, proline, threonine, tyrosine, asparagine and arginine. Leucine, isoleucine, arginine, methionine, valine and cysteine are essential growth factors for *L. monocytogenes* (Premaratne et al., 1991), while tryptophan, phenylalanine and histidine improve the growth of *L. monocytogenes* (Phan-Thanh & Gormon, 1997).

Vermeulen et al. (2006) investigated the possibility of competition for 18 different amino acids to explain antilisterial effects of a *L. sakei* strain in a chemically defined broth simulating CMP. They excluded competition for one of the amino acids as the cause of inhibition since only one amino acid, arginine, was exhausted during co-culture growth of *L. monocytogenes* and *L. sakei* and the decrease in concentration of arginine started later than the moment at which the inhibitory interaction occurred. Nilsson et al. (2005) found similar results since supplementation with amino acids did not eliminate the antilisterial activity of the cell free supernatant of non-bacteriocinogenic *C. piscicola* A9b.

3.5.2.3. Competition for vitamins

While pantothenic acid and nicotinic acid are required by almost all lactobacilli, thiamine is only necessary for the growth of heterofermentative lactobacilli. The requirement for folic acid, riboflavin, pyridoxal phosphate and *p*-aminobenzoic acid is scattered among the various species of lactobacilli, riboflavin being the most frequently required compound. Biotin and vitamin B_{12} are required by only a few strains. The pattern of vitamin heterotrophy is considered to be characteristic for a particular species (Kandler & Weiss, 1986; Moretro et al., 1998). In the study of Lauret et al. (1996), six vitamins were essential for growth of *L. sakei* being *p*-aminobenzoic acid, pyridoxal, nicotinic acid, folic acid, calcium panthotenate and riboflavin, while only riboflavin, nicotinic acid and calcium panthotenate were found to be essential in the study of Moretro et al. (1998). Vitamins essential for growth of *L. monocytogenes* are riboflavin, biotin, thiamine and thioctic acid according to Premaratne et al. (1991) and additionally nicotinamide, pyridoxal, *p*-aminobenzoic acid and calcium pantothenate (Phan-Thanh & Gormon, 1997).

CMP contain B-vitamins in concentrations ranging from 0 to 1 mg/100 g for thiamine, riboflavin and pyridoxal (Esteve et al., 2002). Therefore, it is not unthinkable that competition

for one or more of these vitamins occurs when LAB are growing together with *L. monocytogenes*. However, supplementation studies with yeast extract (as a source of vitamins and minerals) (Nilsson et al., 1999) or with eight different vitamins (Nilsson et al., 2005) did not eliminate the antilisterial effect of non-bacteriocinogenic *C. piscicola* A9b (Nilsson et al., 1999) and A10a (Nilsson et al., 2005).

3.5.2.4. Competition for minerals

Iron is an essential element for most micro-organisms, except for lactobacilli (Lauret et al., 1996; Imbert & Blondeau, 1998), and siderophores provide the cell with this element (Gram et al., 2002). Micro-organisms that produce siderophores and have a higher affinity for iron, have thus a competitive advantage compared to others lacking siderophores (Helander et al., 1997; Gram et al., 2002). Competition for iron as mediated by siderophore production explains the inhibition of *Shewanella putrefaciens* by *Pseudomonas* spp. in fish under iron-limited conditions (Gram et al., 2002). LAB have been recorded as capable of surviving without iron (Imbert & Blondeau, 1998) but *Lactobacillus* spp., particularly *L. johnsonii*, requires iron under particular environmental conditions with limited or specific nucleotide sources (Elli et al., 2000). *L. monocytogenes* requires iron and in particular when growing at refrigeration temperatures (Dykes & Dworaczek, 2002). Therefore, limiting iron at these temperatures might be an effective way to control this pathogen (Dykes & Dworaczek, 2002). Whether growth of LAB might lead to limitation of iron in CMP is yet to be determined.

Against this background, manganese and magnesium are also important minerals since these are known to stimulate LAB-growth (Raccach, 1985; Imbert & Blondeau, 1998; Moretro et al., 1998; Hugas et al., 2002), whereas potassium and magnesium are essential for growth of *L. sakei* (Moretro et al., 1998).

However, supplementation studies with yeast extract (as a source of vitamins and minerals) (Nilsson et al., 1999) or with individual minerals (Fe, Mg, K and Na) (Nilsson et al., 2005) did not eliminate the antilisterial effect of non-bacteriocinogenic *C. piscicola* A9b (Nilsson et al., 1999) and A10a (Nilsson et al., 2005).

3.5.3. Competition for space (attachment/adhesion sites)

An interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts could not be explained by nutrient depletion or production of inhibitory compounds but rather seemed to be mediated by a cell-to-cell contact mechanism at high cell densities and to a lesser ability

of the non-*Saccharomyces* yeasts to compete for space (Nissen & Arneborg, 2003). If this mechanism also occurs between LAB and target organisms such as *L. monocytogenes* is not yet clear. Nilsson et al. (2005) proved that cell-to-cell contact was not required for a non-bacteriocinogenic *C. piscicola* strain to cause inhibition of *L. monocytogenes*.

3.5.4. Quorum sensing

Another interesting theory to explain suppression of *L. monocytogenes* by LAB in CMP is the production and accumulation of 'signal molecules' at high population densities (by LAB) triggering the stationary phase (of *L. monocytogenes)* as a stress-adaptive response (Farkas et al., 2002). In fact, the idea of Farkas et al. (2002) can be seen as some type of quorum sensing. Quorum sensing is the regulation of gene expression as a function of cell-population density, providing a way of cell-to-cell communication. Quorum-sensing micro-organisms communicate through the production of chemical signals and in many Gram-positive bacteria, these signals are peptides (Kuipers et al., 1998; Gram et al., 2002). Quorum-sensing occurs widely in LAB. At present, all LAB-related cases of quorum sensing involve the biosynthesis of antimicrobial peptides, either lantibiotics such as nisin or linear peptides (Kuipers et al., 1998) and studies confirming the theory of Farkas et al. (2002) do not exist.

3.6. Practical implementation of protective cultures

3.6.1. Safety aspects

3.6.1.1. Lactic acid bacteria

Lactic acid bacteria are ubiquitous, they are natural components of the human microflora, occur in substantial numbers on food products and have been used in the production of a wide range of fermented foods since ancient times without adverse effects in humans. It is usually accepted that, with the exception of some streptococci, pathogenicity of LAB is rare (Kandler & Weiss, 1986; Aguirre & Collins, 1993; Pot et al., 1994; Adams & Marteau, 1995). However, there are reports and reviews on the involvement of LAB in human clinical infections (Aguirre & Collins, 1993; Adams & Marteau, 1995; Foulquié Moreno et al., 2005). These reports cite an increasing number of cases in which LAB have been implicated in human diseases, particularly bacterial endocarditis and bacteraemia. Attention is focused mainly on enterococci but some cases are also related to lactobacilli, pediococci and leuconostocs (Kandler & Weiss, 1986; Aguirre & Collins, 1993). In the vast majority of these

clinical cases, patients were immunocompromised and this lead to the conclusion that LAB fall into the category of opportunistic pathogens (Aguirre & Collins, 1993).

Of the various LAB, associated with clinical infection, enterococci probably represent the major cause of concern for the future because of (1) the increasing number of antibiotic-resistant strains, especially vancomycin-resistant strains, and (2) the description of several virulence factors (Aguirre & Collins, 1993; Adams & Marteau, 1995; Foulquié Moreno et al., 2005).
Lactobacilli, on the other hand, are sensitive towards most antibiotics active against Gram-positive bacteria (Kandler & Weiss, 1986), although antibiotic resistant *Lactobacillus* strains have been isolated from food products (Gevers et al., 2000; Mathur & Singh, 2005). Some potential virulence factors for some *Lactobacillus* strains are described, but these are present in the majority of oral strains (Oakey et al., 1995). The observed frequency of bacteraemias where lactobacilli are involved is only 0.1-0.24% (Wessels et al., 2004).

It is of importance to be cognisant of the association of LAB with clinical infections but there seems to be no evidence that fermented foods are a concern in the diet and, with the exception of enterococci, the overall risk of LAB infection is very low (Adams & Marteau, 1995; Stiles & Holzapfel, 1997). In the case of enterococci, Foulquié Moreno et al. (2005) suggested that the selection of new strains of interest for the food industry should be based on the absence of possible pathogenic properties or transferable antibiotic resistance genes.

3.6.1.2. Bacteriophages

Bacteriophages are the most numerous life forms on earth, occurring almost everywhere in our environment, in water, in foods of various origin, in the gastro-intestinal tract, etc. (Dabrowska et al., 2005). On fresh and processed meat and meat products, more than 10^8 viable phages per gram are often present (Carlton et al., 2005). This means that phages are routinely consumed in quite significant numbers and that mammalian organisms, including humans, are very frequently exposed to interactions with bacteriophages (Dabrowska et al., 2005). It is commonly believed that bacteriophages cannot infect cells of organisms more complex than bacteria, because of major differences in cell-surface molecules and in key intracellular machinery that is essential for phage replication. Therefore, bacteriophages are generally believed to have no intrinsic tropism towards eukaryotic cells. Nevertheless, there are some reports that show the ability of bacteriophages to interact with (but not infect)

mammalian cells. When exposed to bacteriophages, these virions penetrate to the blood and other tissues and they can multiply at sites of bacterial infections and anti-phage antibodies are produced (Dabrowska et al., 2005). However, other authors could not detect phage-specific antibodies in the serum of human volunteers consuming phage T4 (Carlton et al., 2005).

Some phages (temperate) are able to transfer virulence properties among bacteria. These phages integrate their genome into the host genome, forming a lysogen. This lysogenic status can potentially result in the expression of genes encoding properties which increase virulence of the host bacteria. This is never the case for lytic (virulent) phages as they lack the genetic factors required for integration of their phage DNA into the host genome and always enter the lytic cycle and eventually kill and lyse the infected cells. Since all infected cells are killed, this precludes the opportunity for increased virulence to occur (Whichard et al., 2003; Carlton et al., 2005). Therefore, phages intended for food applications should be strictly lytic and screened for virulence genes or genes that could increases virulence (Whichard et al., 2003).

In conclusion, there is little reason to assume that intake of phages with food may have negative effects on humans.

3.6.2. Regulatory aspects

3.6.2.1. Lactic acid bacteria

In the United States (US), a company can use a new LAB-strain for use in food without ever notifying the Food and Drug Administration (FDA). However, in practice, LAB for food are either classified as an additive or as a GRAS substance. Most food-associated LAB have acquired the 'generally regarded as safe' (GRAS) status (Wessels et al., 2004).

In the European Union (EU), there is no harmonised legislation that regulates the use of LAB in food products, whether as starter culture, protective culture or probiotic culture. Currently, seven EU laws are of importance when introducing a new LAB-strain for food applications. The most important one is the Novel Food Regulation (European Parliament and Council, 1997). If a given LAB has never been used before May 15, 1997, then it should theoretically be subject of this Novel Food Regulation. However, the regulation does not specify the level of novelty intended, whether it be genus, species or strain. In 2003, the approval process of this regulation had not yet been applied to any strain of LAB (Wessels, 2004). As there is no regulation at the moment, different EU member states regulate protective LAB to very

different degrees and classify them into different regulatory categories (additive, ingredient or processing aid). The latter categories are important for the labelling of food products containing these living bacteria.

The recent biosafety concerns about LAB in food, mainly related to the issue of transferable resistance genes, have resulted in the idea to propose criteria, standards, guidelines and regulations (Mathur & Singh, 2005). Also, the EU Commission and the European Food Safety Authority (EFSA) have perceived the need of a risk assessment of micro-organisms in food. This idea has also grown under influence of the severe legislation on LAB as feed additives since 1996 (Wessels et al., 2004). Up to now, there is no official announcement of an EU legislation covering LAB added to food products. In 2003, a working paper for public consultation, proposed the introduction of the 'qualified presumption of safety' or QPS system (European Commission, 2003). The QPS approach is similar in concept and purpose to the GRAS approach but takes into account the different regulatory practices in Europe (EFSA, 2005). It is a decision-tree approach leading to the QPS-status or not for certain groups of micro-organisms (Figure 1.7).

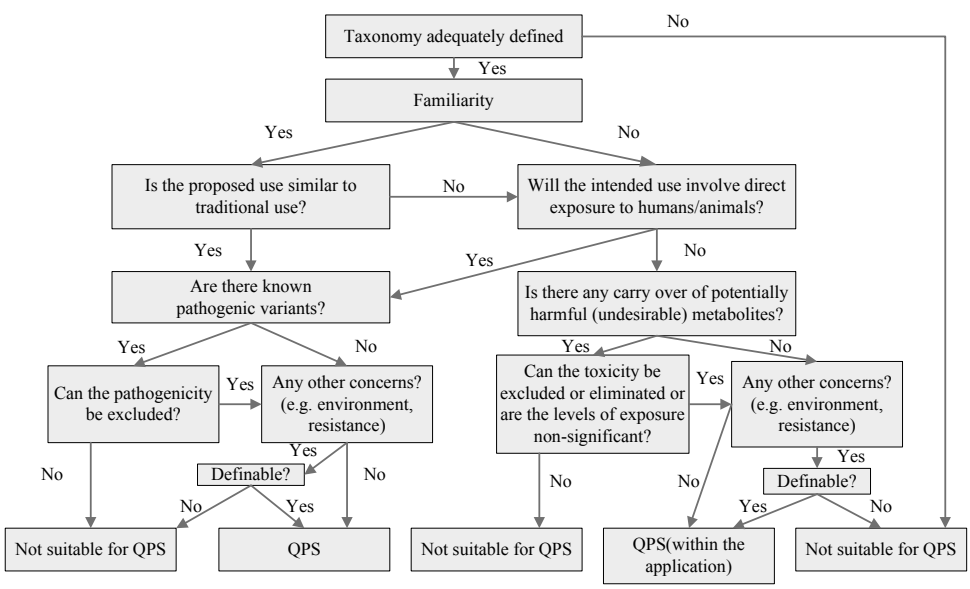

Figure 1.7. QPS-decision tree (modified from European Commission, 2003)

A micro-organism with the QPS-status would be freed from full safety assessment other than any specific requirements introduced as a qualification (European Commission, 2003). In

2005, the scientific committee of EFSA concluded that QPS could provide a system to be applied to all requests received by EFSA for the safety assessment of micro-organisms deliberately introduced into the food chain (Anon, 2005). This means that up to now, QPS is only suggested as an operating tool within EFSA for risk assessment (EFSA, 2005) and no steps have been taken to make the QPS concept part of an EU regulatory framework.

3.6.2.2. Bacteriophages

There is considerable commercial interest in phages but very few information is available on the regulatory status of bacteriophages to be used in food. Some phage companies are making large efforts to get an approval of the FDA for the use of specific phages in foods. What the FDA requires to approve a product is not clear, but discussions with regulators are going on (Thiel, 2004; Greer, 2005). In the European Union (EU), there is no harmonised legislation that regulates the use of bacteriophages in food products.

3.6.3. Technical aspects

In what follows, the focus is on the application of protective LAB-cultures but to a large extent the content also applies to the application of bacteriophages.

In general, high inocula (10^6-10^9 cfu/g) of a PC are needed. Besides the correct inoculum, the even distribution of the culture on the treated product surface is critical to prevent niches were the product is left unprotected (Rodgers, 2001; Jacobsen et al., 2003). The application of a PC does not require special equipment (Rodgers, 2003). The product can be added directly to the meat together with spices, e.g. in the case of minced meat. If the treated product is cured, e.g. in the case of cured raw meat, the PC can easily be added to the meat via the curing brine.

If the treated product is intended for cooking, as it is the case for CMP, the heat sensitivity of the culture should be taken into account. Because most LAB are heat sensitive, protective LAB-cultures are best applied after the pasteurisation step of the CMP in order to guarantee their viability and activity (Rodgers, 2001). The CMP can be immersed or dipped into a solution of the PC or the PC can be sprayed or dripped on the product during or after slicing or in the package immediately before sealing (Andersen, 1995b). In the case of spraying, an ordinary atomiser or, even better, an automatic dispensing and spraying device could be used to ensure the correct concentrations of the PC and its even distribution (Rodgers, 2003). Bredholt et al. (2001) applied *L. sakei* TH1 to cooked ham and cervelat sausages after cooking, immediately before slicing using a hand-operated spraying bottle and obtained in

this way an equal distribution over the whole surface. Jacobsen et al. (2003) evaluated different application techniques for the biopreservative *Leuc. carnosum* 4010 on pork saveloys. The technique giving the highest reduction in *L. monocytogenes* used a Disinfector 200® system equipped with two nozzles, one facing the slicing direction and the other perpendicular to the slicing direction, and mounted on the slicer for sprinkling the PC on all surfaces of each slice of the meat product. When spraying, dripping or dipping, the microbiological status of the PC and aseptic/hygienic handling is critical in order to prevent contamination of the final product (Rodgers, 2003). From this point of view, spraying or immersion may not be the most ideal application technique.

An alternative application technique, which excludes this problem of contamination, might be the addition of micro-encapsulated PC prior to pasteurisation of CMP (Rodgers, 2004). Micro-encapsulation is defined as the technology of packaging solid, liquid or gaseous materials in miniature sealed capsules that release their contents at controlled rates under the influence of certain stimuli (Pothakamuryn & Barbosa-Cánovas, 2004). The encapsulation material should be designed in such a way that it protects the PC from inactivation during heating but simultaneously the material needs to degrade during heating at a certain rate in order to release the PC into the product when the thermal processing step is completed. Appropriate encapsulation materials could be fats and hydrogenated oils, gelatine, carbohydrates, gums, hydrocolloids, sugars, proteins, starch and even polymers such as hydroxy propyl methyl cellulose (HPMC) (Domingues, 2000). Little information is available on the encapsulation of PC. Lemay et al. (2002a) tested encapsulation in lyophilised alginated beads supplemented with glycerol as a means to protect *L. sakei* BJ-33 (B-2 SafePro™) against heat treatment. Significantly enhanced cell protection was observed in broth but not in a meat model.

Chapter 2

Evaluation of meat born lactic acid bacteria as protective cultures for biopreservation of cooked meat products

Redrafted after

L. Vermeiren, F. Devlieghere & J. Debevere (2004)
International Journal of Food Microbiology, 96, 149-164

Chapter 2
Evaluation of meat born lactic acid bacteria as protective cultures for biopreservation of cooked meat products

Summary

In this study, 91 bacterial isolates, originating from meat products, were subjected to a step-by-step screening and characterisation procedure to select potential protective cultures for biopreservation of cured cooked meat products. Strains were first tested on their homofermentative and psychrotrophic character and salt tolerance. Secondly, the antibacterial capacities towards *Listeria monocytogenes*, *Leuconostoc mesenteroides*, *Leuconostoc carnosum* and *Brochothrix thermosphacta* were determined in an agar spot test. Of the tested strains, 38% was inhibitory towards all indicator strains and 91%, 88% and 74% of the strains could inhibit *L. monocytogenes*, *B. thermosphacta* and *Leuc. mesenteroides*, respectively. Finally, 12 strains - those with the highest antibacterial capacities - were evaluated on their competitive nature by comparing their growth rate, acidifying character and lactic acid production at 7°C under anaerobic conditions in a liquid broth. All 12 strains, except a bacteriocin producing *Lactobacillus plantarum* strain and the lactocin S producing *Lactobacillus sakei* 148, combined a fast growth rate with a deep and rapid acidification due to the production of high levels of lactic acid. The 12 selected strains were then further investigated for their growth capacity on a model cooked ham product to establish whether the presence of these cultures on the ham did not negatively influence the sensory properties of the ham. All strains grew in 6 days at 7°C from a level of 10^5-10^6 to 10^7-10^8 cfu/g and again the bacteriocin producing *L. plantarum* strain was the slowest growing strain. As the glucose level of the model cooked ham product was low (0.09 ± 0.03%), growth of the putative protective cultures, resulted in glucose depletion and a limited lactic acid production and accompanying pH-decrease. Cooked ham inoculated with isolates 13E, 10A, 14A (all 3 identified as *L. sakei* subsp. *carnosus*), strain LS5 (*L. sakei* 148) and LS8 (*L. sakei* subsp. *carnosus* SAGA 777) was not rejected by the sensory panel at the 34th day of vacuum packaged storage at 7°C. Therefore these strains could have potential for the use as protective culture in cooked meat products.

1. Introduction

Cured cooked meat products (CMP) are economically important refrigerated products with a high consumption in European countries. The spoilage flora of vacuum packaged (VP) or modified atmosphere packaged (MAP) CMP consists mainly of *Lactobacillus* spp., predominantly *L. sakei* and *L. curvatus*, followed by *Leuconostoc* spp., *Weisella* spp. and *Carnobacterium* spp. *Brochothrix thermosphacta* may also dominate the bacterial flora dependent on the film permeability and the residual oxygen obtained through the vacuum process (Borch et al., 1996; Samelis et al., 2000a). Also the psychrotrophic pathogen *Listeria monocytogenes* can be found as a result of post-contamination in CMP (Uyttendaele et al., 1999).

Biopreservation has gained increasing attention as a means of naturally controlling the shelf-life and safety of CMP. Studies on biopreservation in CMP have, until now, mainly focused on inhibition of food born pathogens such as *L. monocytogenes* (Andersen, 1995a; Hugas et al., 1998; Bredholt et al., 1999; Bredholt et al., 2001; Amezquita & Brashears, 2002; Budde et al., 2003; Mataragas et al., 2003a). Only a limited number of authors report on the activity of protective cultures against specific spoilage organisms of meat products (Kotzekidou & Bloukas, 1996; Björkroth & Korkeala, 1997; Kotzekidou & Bloukas, 1998; Metaxopoulos et al., 2002). To our knowledge, no study has ever investigated the effect of homofermentative lactic acid bacteria (LAB) on heterofermentative LAB on CMP. Furthermore, most studies investigate bacteriocin production, while other types of antagonism are less frequently studied. Despite the stream of promising information and laboratory studies (Budde et al., 2003; Mataragas et al., 2003a), bacteriocinogenic strains often suffer from a limited effectiveness in foods (Holzapfel et al., 1995; Rodriguez et al., 2002). An alternative to overcome these disadvantages is the use of non-bacteriocinogenic but nevertheless very competitive cultures e.g. *L. sakei* BJ-33 (Andersen, 1995a) and *L. sakei* TH1 (Bredholt et al., 2001).

In this chapter, a step-by-step isolation, screening and characterisation was performed to select potential protective cultures to be used in CMP. Especially LAB that are homofermentative, salt tolerant, psychrotrophic and adapted to meat substrates are good candidates for bioprotection of meat products. The screening was focused on cultures, showing inhibitory activity towards *L. monocytogenes* and spoilage organisms, typically associated with CMP. After studying their growth and acidifying character at low

temperatures, the characterisation of the potential cultures was continued by inoculating them onto a model cooked ham product to find out if they were not negatively influencing the sensory properties of the cooked ham, a prerequisite to use them as protective cultures.

2. Materials and methods

2.1. Collection of relevant strains

Based on a literature study, contacts with commercial suppliers of food starter cultures and earlier research experiments in our laboratory, 15 LAB were collected for this study. Seven of these 15 strains are known to produce bacteriocins and the other eight strains were considered to be bacteriocin negative and are known for their very competitive character. In this chapter, these cultures are further described as 'collected' strains.

2.2. Isolation and identification of isolates from meat products

Twenty-seven different types of commercial, cooked and/or fermented, vacuum packaged or MA-packaged, sliced meat products were obtained from different Belgian supermarkets and stored at 7°C up to the use by date. On the use by date, a 30 g sample of the product was taken aseptically and a decimal dilution serial in Peptone Physiologic Solution (PPS; 8.5 g/l NaCl (VWR, VWR International, Leuven, Belgium) and 1 g/l Peptone (Oxoid, Oxoid Limited, Basingstoke, Hampshire, UK)) was prepared to spreadplate the sample on de Man Rogosa Sharpe agar (MRS, Oxoid), supplemented with 1.4 g/l of sorbic acid (Sigma, Sigma-Aldrich Corporation, St. Louis, Missouri, USA) (pH 5.4) to inhibit yeast growth, and on modified CHALMERS medium (Vanos & Cox, 1986) to allow isolation of LAB. The modified CHALMERS medium gives easily distinguishable colonies for LAB due to the characteristic colony type (small pink-red colonies with a light halo) and allows to distinguish the high acid producing colonies among a large population of LAB as the halo around the colony, due to $CaCO_3$ dissolution by lactic acid, is larger for strongly acidifying LAB (Vanos and Cox, 1986). Composition of the modified CHALMERS medium according to Vanos and Cox (1986): 20 g/l lactose (VWR), 20 g/l D-(+)-glucose (Sigma), 3 g/l soy peptone (VWR), 3 g/l meat extract (VWR), 3 g/l yeast extract (Oxoid), 20 g/l $CaCO_3$ (Sigma), 15 g/l agar (Oxoid), 0.5 ml of 1% (w/v) neutral red (Sigma) solution, final pH of 6.0 before sterilisation and after sterilisation 3 vials/l (32.000 IU/vial) of the antibiotic polymyxin-B (International Medical Products - LabM, Brussels, Belgium) were added to reach a concentration of polymyxin-B-

sulphate of 100 IU/ml of medium. After micro-aerophilic incubation for 72h at 30°C of both media types, five colonies were picked from each of the two media. Attention was given to choose colonies with different macroscopic morphology and different halo-size on the modified CHALMERS medium. Isolates were reinoculated in MRS-broth, incubated at 30°C and checked for purity by streaking on MRS-agar. Plates with pure cultures were used to test for cell morphology by phase contrast microscopy, Gram reaction by the KOH method and catalase formation by dropping a 3% H_2O_2 (VWR) solution directly onto each plate. Gram-positive and catalase negative strains were further investigated for gas production from glucose and slimy appearance on MRS-agar and finally the carbohydrate fermentation profile was determined using the API50CH system (BioMERIEUX, Brussels, Belgium) to select for LAB not producing gas, not demonstrating a slimy or ropy appearance on MRS-agar and not identical to each other, as indicated by the fermentation profile.

2.3. Psychrotrophic character and salt tolerance

The collected and isolated strains were tested on their potential to grow in buffered modified BHI-broth at low temperatures (4°C and 7°C) combined with salt concentrations occurring in the water phase of CMP (3% and 6% of NaCl). The buffered modified BHI-broth consisted of Brain Heart Infusion broth (BHI, Oxoid) (37 g/l) supplemented with 18 g/l D-(+)-glucose (Sigma), 3 g/l yeast extract (Oxoid), 4.6 g/l Na_2HPO_4 (Sigma), 20 mg/l $NaNO_2$ (UCB, Leuven, Belgium) and the pH was adjusted to 6.2 before sterilisation. Devlieghere et al. (1998) demonstrated this medium to be suitable as simulation medium for cooked ham. Additional NaCl (VWR) was added to obtain a level of 3% or 6% of NaCl, being representative NaCl-concentrations for the broad spectrum of CMP. All strains were inoculated at a level of 10^6-10^7 cfu/ml in 5 ml of this broth containing either 3% or 6% of NaCl and stored at either 4°C or 7°C. During 5 and 8 weeks at 7°C and 4°C, respectively, growth was followed daily by visually examining the turbidity of the broth.

2.4. Antibacterial activity towards *L. monocytogenes*, *Leuc. mesenteroides, Leuc. carnosum* and *B. thermosphacta*

The strains, selected in the previous experiments, were investigated for their antibacterial properties towards *L. monocytogenes* and towards spoilage organisms, typically associated with CMP. Indicator organisms were *L. monocytogenes* LFMFP 235, isolated from cooked ham, *L. monocytogenes* LMG 10470, *L. monocytogenes* Scott A, *Leuc. mesenteroides* subsp.

dextranicum LMG 6908^T, *Leuc. carnosum* LMG 11498 and *B. thermosphacta* LFMFP 230, isolated from cooked ham. Antibacterial activity was assessed by an agar spot test (Juven et al., 1998). The putative protective culture was applied as a single spot of 10 µl on MRS-agar and incubated at 30°C for 24h in micro-aerophilic conditions. After incubation, plates were covered with 7 ml of semi-soft (0.7% agar) BHI-agar or MRS-agar inoculated with the indicator strain at a level of 1% (1 ml of an overnight culture in 100 ml of medium). Separate plates containing the test culture were overlaid with each of the six indicator strains and each test was performed in triplicate. After incubation for 24h at the optimal growth temperature of the indicator strain, lawns were examined for evidence of inhibition. Based on the results of this study and the tests on the potential of the strains to grow at refrigeration temperatures (4°C and 7°C) and at high salt concentrations (3% and 6% of NaCl), a selection was made of LAB for further study.

The selected LAB were further subjected to a bacteriocin assay according to Buncic et al. (1997) to find out if the antibacterial properties were the result of bacteriocin production. The putative producer strains were grown in MRS-broth for 24h at 30°C. Two 10 µl aliquots were spotted on MRS-agar containing 0.2% glucose to avoid acid production and these plates were incubated at 30°C anaerobically to avoid H_2O_2 production. After incubation, 10 µl spots of chymotrypsin (Sigma) and proteinase K (Sigma) (0.05g/100ml) were brought next to one of the lactobacilli spots to inactivate any bacteriocin produced. After adding the enzyme spots, plates were held for 2h at room temperature to allow diffusion of the enzyme before plates were overlaid with 7 ml semi-soft (0.7 % agar) BHI-agar, inoculated at a level of 1% with indicator strain *L. monocytogenes* LFMFP 235. Plates were incubated for 24h at 37°C. Bacteriocin production was indicated by a clear inhibition zone around the untreated spot and a half inhibition zone around the spot treated with enzymes.

2.5. Growth, acidification profile and lactic acid production of 12 selected putative protective cultures

Twelve meat born homofermentative, salt tolerant, psychrotrophic LAB with proven in-vitro antibacterial characteristics, as demonstrated in previous experiments, were selected for this experiment. The objective was to compare these putative protective cultures with regard to their growth characteristics and acidifying character at 7°C, under anaerobic conditions and at a pH and salt concentration representative for CMP. The strains were inoculated at 10^5 cfu/ml in an adapted BHI-broth (pH 6.2 and 3% NaCl). Growth of the cultures was followed during

storage under an atmosphere of 100% N_2 at 7°C to simulate refrigerated vacuum packaged conditions. Experiments were performed in jars provided with a Teflon valve and a central opening, which was closed with a silicone septum (Devlieghere et al., 1998). Methods for preparation, inoculation and flushing with 100% N_2 of these jars are described by Devlieghere et al. (1998). The adapted BHI-broth consisted of BHI (37 g/l) supplemented with 4 g/l yeast extract (Oxoid), 18 g/l D-(+)-glucose (Sigma), 1 ml/l Tween 80 (Sigma), 0.2 g/l $MgSO_4.7H_2O$ (Sigma) and 0.04 g/l $MnSO_4.H_2O$ (Sigma). Additional NaCl was added to obtain a level of 3% of NaCl. At several time intervals during storage, samples of 5 ml were taken by using a sterile needle to determine cell number and pH. Cell numbers were determined by spreadplating on MRS-agar by using a Spiral Plater (Spiral Systems, Model D, Led Techno, Eksel, Belgium) and pH-measurements were done by using a pH-electrode (Knick, type 763, Berlin, Germany). At the end of each growth experiment, when the pH was changing not more than 0.01 pH-units in 24h, a sample was taken for the determination of the concentration of lactic acid by using a high-performance liquid chromatograph. The analyses were performed isocratically with the cation exchange column Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA, USA) at a flow rate of 0.6 ml/min of 5 mM H_2SO_4 (VWR) at 35°C and a run time of 25 min. The HPLC-equipment consisted of a pump (Gilson, type 307, Villiers Le Bel, France), an injector (Rheodyne 9096, Bensheim, Germany) with a 20 µl-loop and a refractive index detector (Gilson, type 132). Growth curves were modelled by the model of Baranyi & Roberts (1994) and pH-curves by a modified Gompertz equation used by Linton et al. (1995) for modelling survival curves of *L. monocytogenes* and adjusted for this purpose into:

$$A \cdot e^{-e\left(\frac{(AR \cdot e \cdot (S-t))}{A}+1\right)} - A \cdot e^{-e\left(\frac{(AR \cdot e \cdot S)}{A}+1\right)}$$

(A= tail of the sigmoid curve or final pH; t= time (h); S= shoulder of the sigmoid curve (h), AR= acidification rate (h^{-1}) or slope of the linear part of the sigmoid curve).

In this way estimations for generation time (h), lagphase (h), acidification rate (AR) (h^{-1}), time to acidification starting from 10^6 cfu/ml (= t_{ac-6}) (h) and depth of acidification could be made.

2.6. Behaviour of 12 selected putative protective cultures on a model cooked ham

The major objective of this experiment was to establish if the 12 selected putative protective cultures were not negatively influencing the sensory properties when inoculated onto a cooked meat product. Furthermore, this experiment allows comparisons of the 12 LAB with regard to their growth and acidifying capacity on a cooked ham product.

To achieve this goal, a model cooked meat product was designed: model cooked ham (MCH). This model product was an imitation of cooked ham as it had a similar recipe. However, the production process was slightly modified. In contrast to industrially prepared cooked ham, the pork meat was cuttered and, after addition of nitrited salt and other ingredients, filled in a casing prior to pasteurisation. This modified production process was necessary to obtain a very homogeneous composition. On the surface of a slice of the MCH, the original muscle structure was not visible anymore. This was necessary to avoid differences in pH or differences in nutrient levels between different positions on the surface of a MCH-slice. Furthermore, the MCH was produced under very hygienic semi-industrial conditions in such a way that the contamination level after slicing was very low. This was an additional advantage as it allowed studying the inoculated protective culture without having to take into account the background flora. The MCH was manufactured at Dera Food Technology N.V. (Bornem, Belgium) with following recipe: 80% of pork meat, 20% of water, 18 g/kg nitrited salt (NaCl containing 0.6% of sodium nitrite), 5 g/kg Deraphos C107 (potassium and sodium- di-, tri- and polyphosphates) and 0.5 g/kg Na-ascorbate. After boning and defatting, hams were cut in pieces of ± 10 to 10 to 10 cm. These pieces were homogenised and further minced to 20 mm and finally cuttered in a vacuum bowl cutter (Kilia, Neumünster, Germany) together with the nitrited salt and other ingredients. The cutter mixture was filled in a cook-in-casing to a final diameter of 100 mm and tempered for minimum 2 hours at 4°C before pasteurisation occurred at 75°C to a core temperature of 70°C in a cooking chamber (Kerres, Sulzbach, Germany) during 2 hours and 45 min. After cooling at 4°C, the cooked ham sausages were sliced with a non-automatic slicer (Omas, S.Vittoria di Gualtieri, Italy) in slices of 2 mm thickness (± 20 g/slice). The product was quick-frozen in a blast freezer (Friginox-Le Froid Professionnel, Frispeed SR-range, Villevallier, France) at -40°C to a core temperature of -10°C to avoid formation of large ice crystals and finally further stored in a freezing room at -18°C. When an experiment started, the necessary amount of product was transferred from the freezer to a cooler at -3°C for 48h and later at 4°C for 24h.

The MCH was inoculated with the 12 strains at 10^5-10^6 cfu/g in three separate experiments, spread in time. Each experiment consisted of five series: one blank series of non-inoculated ham and four series of ham inoculated with one of the 12 strains. Each series was performed in triplicate. The inoculum was subcultured twice (24h, 30°C) in 5 ml MRS-broth before use. To reach an inoculation level of 10^5-10^6 cfu/g, 200 µl of the inoculum was divided over and spread on the surface of 10 slices (125 g/10 slices) of MCH. After inoculation, slices were vacuum packaged (10 slices/package) and stored at 7 ± 1°C in a ventilated refrigerator. Packaging was performed using a Multivac A300/42 (Hagenmüller, Wolfertschwenden, Germany) gas packaging machine in a high barrier film (NX90, Euralpak, Wommelgem, Belgium) of 90 µm thickness with an oxygen transmission rate of 5.2 ml/m^2.24h.atm at 23°C and 85% of relative humidity. At day 0, 2, 6, 9, 13, 20, 27 and 34 of the storage period, cooked ham samples were analysed on growth of the inoculated strain, pH and concentration of metabolites. Furthermore, the sensory characteristics were evaluated.

For the microbial analyses, a 15 g sample of ham was taken aseptically and a decimal dilution series in PPS was prepared to plate the appropriate dilutions on MRS-agar (aerobic incubation at 22°C for 3-5 days) and M5-agar (anaerobic incubation at 30°C for 2 days) to determine the level of LAB. M5-agar differentiates between homo- and heterofermentative LAB (Zuniga et al., 1993). The blank samples were also plated on Plate Count Agar (PCA, Oxoid) (aerobic incubation at 22°C for 3-5 days), Reinforced Clostridial Agar (RCA, Oxoid) (anaerobic incubation at 37°C for 3-5 days) and Yeast Glucose Chloramphenicol Agar (YGC, Bio-Rad) (aerobic incubation at 22°C for 3-5 days) to determine total aerobic psychrotrophic count, total anaerobic count and number of yeasts and moulds, respectively.

The pH-measurements and HPLC-analyses were performed as described in section 2.5. Before HPLC-analysis, meat samples were subjected to an extraction procedure: a 10 g sample was homogenised with 50 ml of distilled water, 5 ml of Carrez I (0.407 M $K_4Fe^{II}(CN)_6$, Sigma) and 5 ml of Carrez II (0.814 M $ZnSO_4$, VWR) and finally filled up to 100 ml with distilled water. The deproteinised mixture was filtered (∅ 125 mm, Schleicher & Schuell Microscience, Dassel, Germany) and filtered once more (∅ 0.2 µm, Alltech Associates, Lokeren, Belgium) immediately before injection.

Cooked ham samples were evaluated by a nine-member trained sensory panel using a scoring method. Attributes were odour, acid odour, rot odour, taste, acid taste, general appearance, slimy appearance and colour. Attribute scales varied from one to nine with one being very weak, five being moderate and nine very strong. Samples with a score above five were

considered as unacceptable. Finally, the panel was asked to evaluate the fitness for human consumption. If five or more of nine persons indicated a sample as unfit, the sensorial quality was considered to be rejected. Samples for sensory analyses were offered to the panellists in plastic recipients that were closed and stored at 4°C up to the moment of sensory evaluation. Time between sampling and sensory evaluation was not longer than 1 hour. Sensory evaluation was performed under IR-light (except for the attributes general appearance, slimy appearance and colour) in a special room with individual booths.

Triplicate results of pH, glucose and lactic acid concentration were analysed for significant ($P<0.05$) differences between the 15 different series of the three experiments (three non-inoculated samples and 12 inoculated samples) on each day of analysis using analysis of variance (One-way ANOVA) and Post Hoc Multiple Comparison Tukey tests.

Data for pH, glucose and lactic acid concentration of each strain were further analysed for significant ($P<0.05$) differences between the different days of analysis using analysis of variance (One-way ANOVA) and Post Hoc Multiple Comparison Tukey tests.

For each of the three experiments, the score data from the sensory evaluation were analysed for significant differences ($P<0.05$) between the five different series (one non-inoculated sample and five inoculated samples) within one experiment by subjecting scores, obtained for each attribute and for each day of analysis, to analysis of variance (One-way ANOVA) and Post Hoc Multiple Comparison Tukey tests.

All statistical analyses were performed using the software SPSS 11.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Collection of relevant strains

The 'collected' LAB (Table 2.1) consisted of seven strains, known to produce bacteriocins, and eight strains, considered to be bacteriocin negative but known for their competitive nature.

3.2. Isolation and identification of isolates from meat products

From the 27 different meat products, 76 isolates were pure strains growing well in MRS-broth. All of these 76 isolates, except two, were Gram-positive and catalase negative confirming the selectivity of MRS supplemented with sorbic acid and the Chalmers medium.

Table 2.1. Overview of 'collected' lactic acid bacteria implicated in this chapter

Strain	Code	Origin (Reference)	Bacteriocin production	Obtained from
Lactobacillus plantarum ALC	LP1	Not reported	Pediocin AcH	Danisco
Pediococcus acidilactici PA-2	PA1	Not reported	Pediocin	Chr. Hansen
Lactococcus lactis BB24	LL3	Fermented sausage (Rodriguez et al., 1995)	Nisin	Dr. J.M. Rodriguez[a]
Lactococcus lactis G18	LL4	Fermented sausage (Rodriguez et al., 1995)	Nisin	Dr. J.M. Rodriguez
Lactobacillus sakei 148	LS5	Spanish dry sausage (Sobrino et al., 1991)	Lactocin S	Dr. J.M. Rodriguez
Lactobacillus sakei Lb 706	LS6	Vacuum packaged meat (Schillinger & Lücke, 1989)	Sakacin A	FRC[b] 195
Lactococcus lactis UW1	LL2	Frankfurter sausage	Nisin	LMG[c] 7930
Lactobacillus sakei BJ-33	LS1	MA-packaged fresh meat (Andersen, 1995a)	Not reported	Chr. Hansen
Lactococcus lactis subsp. *lactis* L201	LL1	Vacuum packaged cooked sausage (Elsser, 1998)	Not reported	Danisco
Lactobacillus plantarum	LP5	Fermented sausage	Not reported	LFMFP[d] 143
Pediococcus pentosaceus	PP1	Fermented sausage	Not reported	LFMFP 155
Lactobacillus curvatus	LC4	Fermented sausage	Not reported	LFMFP 540
Lactobacillus sakei subsp. *carnosus*	LS7	Cooked ham (Devlieghere et al., 1998)	Not reported	LFMFP 216
Lactobacillus sakei SAGA 777	LS8	Not reported	Not reported	Quest International
Lactobacillus plantarum	LP3	Not reported	Not reported	LMG 8155

[a], Departamento de Nutricion y Bromatologia III, Universidad Complutense de Madrid (Madrid, Spain)
[b], FRC, Federal Research Centre for Nutrition, Institute for Hygiene and Toxicology (Karlsruhe, Germany)
[c], LMG, Laboratory Microbiology Gent (Gent, Belgium)
[d], LFMFP, Laboratory of Food Microbiology and Food Preservation (Gent, Belgium)

From the 74 remaining strains, only three (4.0%) were heterofermentative and further identified as *Leuc. mesenteroides* subsp. *mesenteroides* using the API50CH system. Four (5.0%) strains demonstrated a slimy and/or ropy appearance on MRS-agar and were not further used. Based on their API50CH fermentation profile, 37 strains were selected for further study. These were Gram-positive, catalase negative and homofermentative, did not show a slimy appearance on MRS-agar and moreover were completely different from each other which means that either they were isolated from a different meat product or they were isolated from the same meat product but showed a different fermentation profile. This severe selection criterion explains why only 37 strains were further investigated. The 37 remaining strains are further described as 'isolated' strains.

The percentage of strains isolated from cooked or fermented meat products per species is presented in Table 2.2. Since *L. sakei* is not included in the database of the API50CH system and pure *L. sakei* strains are identified by the API50CH system as *Lactobacillus fermentum*, it was not surprising that the majority of the isolates (32.4% of total isolates and 47.6% of cooked meat isolates) was identified as *L. fermentum*, an organism that is not described as a typical spoilage organism of CMP.

Table 2.2. Distribution of homofermentative LAB isolated from cooked or fermented meat products per species as identified by the API50CH system

Species	**Total**	**Cooked meat products**	**Fermented meat products**
Number of isolates	37 (100%)	21 (100%)	16 (100%)
Lactobacillus fermentum[a]	12 (32.4%)	10 (47.6%)	2 (12.5%)
Lactococcus lactis subsp. *lactis*	9 (24.3%)	5 (23.8%)	4 (25.0%)
Lactobacillus curvatus	5 (13.5%)	2 (9.5%)	3 (18.8%)
Lactobacillus plantarum	2 (5.4%)	0 (0.0%)	2 (12.5%)
Carnobacterium divergens	3 (8.1%)	3 (14.3%)	0 (0.0%)
Pediococcus pentosaceus	1 (2.7%)	0 (0.0%)	1 (6.3%)
Leuconostoc lactis	1 (2.7%)	0 (0.0%)	1 (6.3%)
Lactobacillus acidophilus	1 (2.7%)	1 (4.8%)	0 (0.0%)
Lactobacillus brevis	1 (2.7%)	0 (0.0%)	1 (6.3%)
Leuconostoc mesenteroides subsp. *mesenteroides / dextranicum*	1 (2.7%)	0 (0.0%)	1 (6.3%)

[a], Since *L. sakei* is not included in the database of the API50CH system and pure *L. sakei* strains are identified by the API50CH system as *L. fermentum*, these isolates were presumably belonging to the *L. sakei/curvatus* group

Earlier results (not shown) indicate that such *L. fermentum* strains, in most cases, are identified through gelelectroforese (SDS-PAGE) and cluster analysis as members of the *L. sakei*/*curvatus* group (Devlieghere et al., 1998). Difficulties in correctly identifying strains of the *L. sakei*/*curvatus* group have been reported before (Champomier-Vergès et al., 2002).
Further, also *Lactococcus lactis* subsp. *lactis* (32.4%) and *L. curvatus* (13.5%) were frequently isolated from the meat products. Dominance of the *L. sakei/curvatus* group in the spoilage microbial association of CMP has been demonstrated by many authors (Devlieghere et al., 1998; Samelis et al., 2000a). *Lactococcus lactis* subsp. *lactis* is not typically associated with CMP but has already been isolated from CMP by Barakat et al. (2000) and Hamasaki et al. (2003) and from fermented products by Rodriguez et al. (1995). Other species such as *Carnobacterium divergens* are less frequently isolated but nevertheless typical for CMP (Samelis et al., 1998). *Lactobacillus sakei, L. curvatus, L. plantarum* and *Pediococcus pentosaceus*, commonly used as starter cultures (Montel, 1999), were isolated from the fermented meat products.

3.3. Psychrotrophic character and salt tolerance

This test was performed for the 15 collected strains (7 bacteriocinogenic and 8 non-bacteriocinogenic) and 37 isolated strains.
Within the group of the bacteriocinogenic strains only lactocin S producing *L. sakei* 148 (LS5) and sakacin A producing *L. sakei* Lb 706 (LS6) were able to grow at both, 4°C and 7°C combined with 3% and 6% of NaCl. It has been described before that only a limited number of bacteriocinogenic strains are able to grow at low temperatures (Hugas, 1998). None of the *Lc. lactis* strains could grow at 4°C or 7°C, while strains LP1 and PA1 could not grow anymore when the low temperature was combined with 6% of salt. From *Lc. lactis* it is known that they can grow at temperatures not lower then 10°C (Batt, 1999; Hamasaki et al., 2003). However, according to Sobrino et al. (1991), strains LL3 and LL4 are able to grow at 4°C but this was not confirmed in our study. Only LS5 was used for further study as not so much is known about the potential of this lactocin S producing strain in real meat applications.
Five out of eight strains within the group of the collected non-bacteriocinogenic strains were psychrotrophic and salt tolerant. All these 5 strains, *L. sakei* BJ33 (LS1), a *L. sakei* subsp. *carnosus* isolate from cooked ham (LS7), *L. sakei* SAGA777 (LS8), *L. plantarum* (LP5) and *L. curvatus* (LC4), were used for the next steps of the screening. Again, the *Lc. lactis* strain (LL1) could not grow at the low temperatures and LP3 and PP1 were sensitive for 6% of salt.

Among the isolates, 76% (28/37) was able to grow at low temperatures combined with higher salt concentrations, even though all of these cultures were isolated from refrigerated meat products. The group of seven isolates, that was not able to grow at 4°C or 7°C combined with 3% or 6% NaCl, consisted of three *C. divergens* isolates, one *L. plantarum* isolate, one *L. curvatus* isolate, the sole *P. pentosaceus* and the sole *Leuc. lactis* isolate. Most strains belonging to the *L. sakei/curvatus* group could grow at the low temperatures and high salt concentrations. *L. sakei* is known to be one of the most psychrotrophic species of lactobacilli since some strains grow at 2-4°C (Champomier-Vergès et al., 2002). In total, 34 strains were useful for further tests, consisting of 6 collected strains and 28 isolated strains.

3.4. Antibacterial activity towards *L. monocytogenes, Leuc. mesenteroides, Leuc. carnosum* and *B. thermosphacta*

The aim of this experiment was to examine the 34 selected LAB for their antibacterial activity towards *L. monocytogenes* and towards representative spoilage organisms. It should be noted that a positive result, this is the detection of an inhibition zone, may result from lactic acid, bacteriocin or hydrogen peroxide production. However, this test was meant to select for strains with the highest antibacterial activity, as it was not possible to continue working with all 34 strains. This test was not yet meant to reveal the mechanism of inhibition. All strains, except two, showed antibacterial activity towards at least one of the indicator strains. Only 13/34 (38.2%) strains were active towards all six indicator strains. Towards *L. monocytogenes* LFMFP 235, *L. monocytogenes* LMG 10470 and *L. monocytogenes* Scott A, 27/34 (79.4%), 31/34 (91.2%) and 26/34 (76.5%) LAB-strains, respectively, were demonstrating antibacterial activity. Towards *Leuc. mesenteroides*, *Leuc. carnosum* and *B. thermosphacta*, 25/34 (73.5%), 17/34 (50%) and 30/34 (88.2%) LAB-strains, respectively, were demonstrating antibacterial activity. The largest inhibition zones were observed towards *L. monocytogenes* LMG 10470 and *B. thermosphacta* LFMFP 230 while the *Leuc. carnosum* strain was only slightly inhibited. Within the group of collected LAB, LP5 showed the highest and LC4 the lowest antibacterial activity towards the six indicator strains, while the antibacterial activity of LS1, LS5, LS7 and LS8 was intermediate. LS5 did not show a larger inhibition zone in comparison to the other strains although this strain is known to produce lactocin S (Sobrino et al., 1991). Inhibition zones can be the result of different antimicrobial compounds e.g. for LS1, lactic acid production has been indicated as the causative agent for the antagonistic character (Juven et al., 1998).

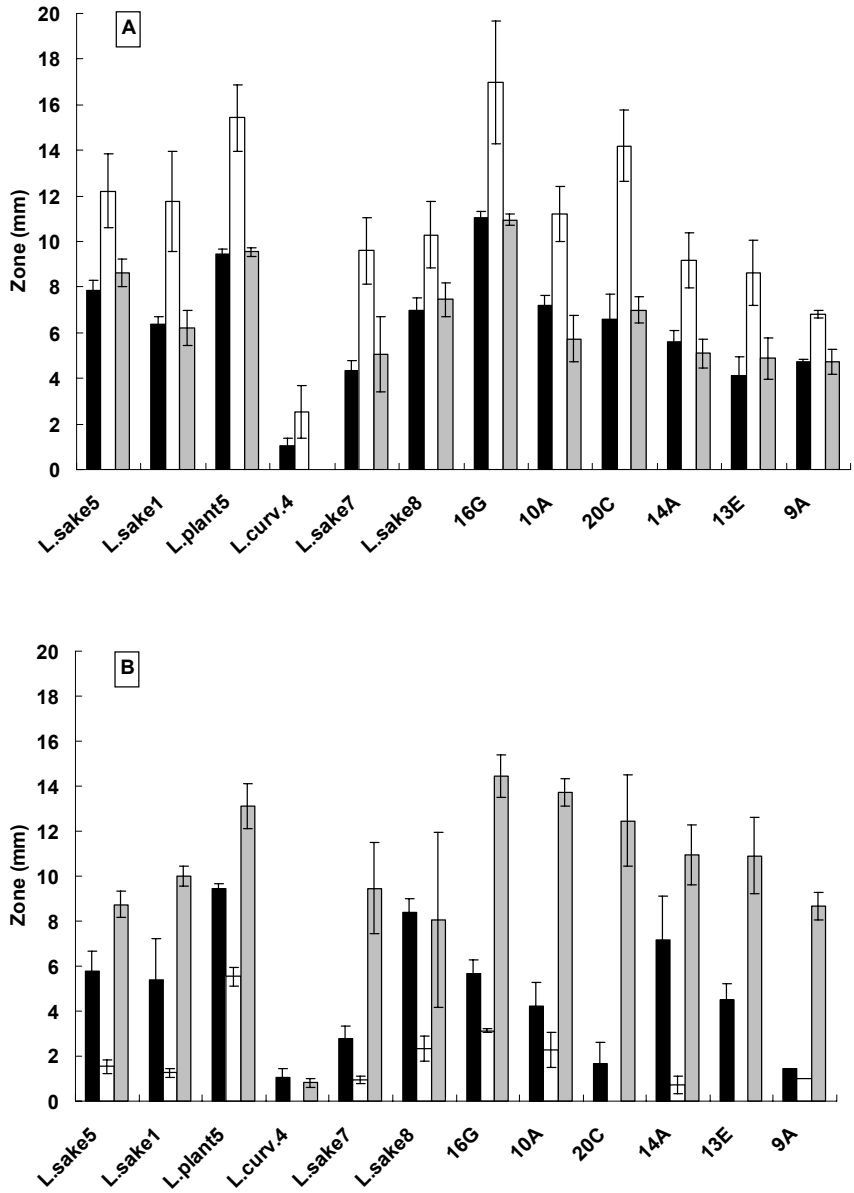

Figure 2.1. Antibacterial properties of 12 selected lactic acid bacteria towards (A) *L. monocytogenes* (black bars, *L. monocytogenes* LFMFP 235; white bars, *L. monocytogenes* LMG 10470; grey bars, *L. monocytogenes* Scott A) and (B) spoilage organisms (black bars, *Leuc. mesenteroides*; white bars, *Leuc. carnosum*; grey bars, *B. thermosphacta*); zone (mm) = radius of inhibition zone minus radius of the spot (error bars represent 95% confidence intervals, n=3)

Within the group of isolated strains, isolates 9A (vleeskoek, a typical Belgian cooked meat product), 20C (rolled pork), 10A (cooked turkey fillet), 13E (boulogne), 14A (fermented sausage) and 16G (chorizo) showed the highest antibacterial activities towards the indicator strains and were selected for further study. Especially strain 16G showed large inhibition zones. In Figure 2.1 the antibacterial activity of the six selected 'collected' LAB and six selected 'isolated' LAB towards the three *L. monocytogenes* indicator strains and the three spoilage indicator strains, respectively, is shown.

The 12 selected strains were subjected to a bacteriocin assay, confirming that LS5 produced a bacteriocin and revealing that LP5, previously thought to be bacteriocin negative, is also producing a bacteriocin. All other strains did not show inhibitory zones towards *L. monocytogenes* LFMFP 235 in repeated bacteriocin assays. To absolutely prove that they do not produce bacteriocins, specialised genetic techniques need to be used but this was beyond the scope of this study. Therefore, the ten other strains are further assumed to be non-bacteriocinogenic. Sobrino et al. (1991) investigated the inhibitory spectrum of lactocin S produced by *L. sakei* 148 (LS5). Activity was mainly observed towards indicator strains *L. curvatus*, *Leuc. mesenteroides* and *L. monocytogenes.*

The six selected isolates were further identified through SDS-PAGE of total soluble cell protein. Based on the API50CH system strains 9A, 20C and 10A were identified as *L. fermentum*, strains 13E and 14A as *Leuc. mesenteroides* subsp. *mesenteroides/dextranicum* and 16G as *L. curvatus*. However, through SDS-PAGE, isolates 9A, 20C, 10A, 13E and 14A were identified as *L. sakei* subsp. *carnosus* and 16G was confirmed to be a *L. curvatus* strain.

3.5. Growth, acidification profile and lactic acid production of 12 selected putative protective cultures

For the six collected strains (LS5, LS1, LS7, LS8, LP5 and LC4) and six isolated strains (9A, 20C, 10A, 13E, 14A and 16G), growth curves and pH-curves were determined. Using the model of Baranyi & Roberts (1994), an estimation of the growth rate (h^{-1}) and lagphase (h) was obtained. The pH-model resulted in a shoulder S (h), an acidification rate AR (h^{-1}) and a tail A or final pH, used to calculate the acidification depth, being the difference between the initial pH (6.2) and the estimated final pH. To obtain a parameter that indicates the time before acidification in the medium starts and that is independent on the initial experimental cell number (varying from 10^5 to 10^6 cfu/ml), the shoulder obtained from the pH-model was corrected for the time necessary to reach 10^6 cfu/ml starting from the initial count. In this

way, the time to acidification starting from 10^6 cfu/ml (= t_{ac-6}) was calculated. The most important model parameters and the amount of lactic acid produced during growth are summarised in Table 2.3. The majority of the strains (LS8, LS7, LS1, 10A, 13E, 9A, 20C and 14A) showed at 7°C a comparable growth and pH-evolution with short lagphases and short generation times. Within this group, all strains were *L. sakei* strains. Acidification depth for this dominant group ranged from 1.92 to 2.19 pH-units. The lactocin S producing LS5 demonstrated a different pattern: a longer generation time and accompanying longer t_{ac-6} was observed together with a limited acidification of 1.58 pH-units, which is less than for all other strains. Strains LC4 and 16G, both *L. curvatus*, had a comparable evolution with longer generation times and longer acidification rates than the *L. sakei* group.

Table 2.3. Model parameters of the growth and acidification experiment for the 12 selected lactic acid bacteria in adapted BHI-broth at 7°C under anaerobic conditions (mean ± standard deviation, n=3)

Strain	Lagphase (h)	Generation time (h)	Time to acidification t_{ac-6} (h)[a]	Acidification rate (h^{-1})	Depth of acidification[b]	Produced level of lactic acid (mM)
LS8	46.19 ± 4.40	3.88 ± 0.56	14.37 ± 2.00	0.047 ± 0.001	2.19 ± 0.01	131.98 ± 0.26
LS7	39.13 ± 2.20	3.00 ± 0.25	13.32 ± 3.31	0.030 ± 0.001	2.24 ± 0.01	149.86 ± 0.54
LS1	45.93 ± 5.29	3.78 ± 0.52	29.48 ± 8.50	0.039 ± 0.000	2.21 ± 0.01	139.76 ± 0.25
LS5	56.63 ± 6.08	7.07 ± 0.60	74.47 ± 6.72	0.018 ± 0.000	1.58 ± 0.01	82.28 ± 1.34
LP5	178.15 ± 1.96	10.39 ± 0.86	118.48 ± 4.13	0.015 ± 0.000	2.39 ± 0.01	121.91 ± 1.09
LC4	60.88 ± 4.66	4.50 ± 0.48	33.18 ± 0.72	0.040 ± 0.002	2.22 ± 0.00	128.25 ± 0.29
9A	91.17 ± 4.76	5.33 ± 0.42	23.70 ± 1.91	0.032 ± 0.001	2.17 ± 0.01	128.31 ± 0.31
10A	25.09 ± 0.74	3.67 ± 0.14	14.48 ± 1.24	0.023 ± 0.001	1.92 ± 0.01	128.37 ± 1.50
13E	54.17 ± 3.37	4.13 ± 0.40	27.17 ± 0.41	0.037 ± 0.001	2.13 ± 0.02	129.52 ± 0.65
14A	26.96 ± 7.31	4.37 ± 0.97	14.92 ± 1.34	0.033 ± 0.001	2.21 ± 0.01	153.59 ± 1.44
16G	17.61 ± 5.15	7.67 ± 0.54	32.32 ± 0.79	0.017 ± 0.000	1.86 ± 0.00	130.05 ± 2.09
20C	33.93 ± 2.97	3.10 ± 0.25	15.81 ± 1.62	0.045 ± 0.000	2.15 ± 0.01	128.49 ± 0.82

[a], starting from 10^6 cfu/ml; [b], difference between initial pH (6.2) and estimated final pH

The sole *L. plantarum* strain (LP5) grew and acidified the medium very slowly, but resulted in a deep acidification. Most strains produced levels of lactic acid of about 130 mM, whereas LS7 and 14A produced a higher level of approximately 150 mM. Lactocin S producing strain LS5 produced less (82 mM) lactic acid in comparison to the other strains. Especially strains

with fast growth rates at low temperatures have potential as protective cultures since fast growth rates are an indication for greater competitiveness for nutrients and give the LAB a selective advantage over slower growing competitors (Bredholt et al., 1999). However, a fast growth rate is for most LAB accompanied by a large acidification rate and acidification depth. On real CMP, this could result in a rapid and large pH-decrease, creating undesired sensory deviations. Therefore, strains were further investigated to determine to what extent they influenced the organoleptic properties of a cooked meat product.

3.6. Behaviour of 12 selected putative protective cultures on a model cooked ham

The chemical composition of the MCH was: 24.60 ± 0.63 % of dry matter, 2.68 ± 0.02 % of NaCl (on aqueous phase), pH of 6.09 ± 0.05 and a water activity of 0.983 ± 0.001.

The non-inoculated MCH had an initial contamination with LAB, enumerated on MRS-agar, of 1.3 $\log_{10}$(cfu/g). Aerobic count and LAB-count were of the same magnitude and no yeasts or moulds were detected. Near the end of the storage period (day 34), the level of endogenous LAB increased up to 10^4-10^6 cfu/g. The initial microbial load was very low compared to the obtained inoculation level of 5×10^5-5×10^6 cfu/g. Furthermore, dominance of inoculated strains over background flora was confirmed by identical API-profiles of inoculum and isolates (results not shown).

Figures 2.2 and 2.3 show the growth of the tested strains on the MCH, as enumerated on M5-agar. At day six, the majority of the strains reached a cell concentration of ± 10^8 cfu/g. Similar to the growth experiment in the adapted BHI-broth, strain LP5 grew slower than the other strains and reached a lower maximal population level, although the inoculation level was slightly higher than the mean inoculation level of the other LAB.

The initial level of glucose and lactic acid of the MCH was 0.09 ± 0.03% and 0.70 ± 0.12%, respectively. The latter level corresponds to the level of 0.76% lactate, reported to be a level naturally present in meat by Stekelenburg & Kant-Muermans (2001).

The pH of the MCH was about 6.09 ± 0.05 at the start of the experiment. Near the end of the storage period, the pH of the inoculated ham decreased significantly while the pH of the non-inoculated ham did not decrease (Figures 2.4 and 2.5). The pH-decrease at day 27 varied between 0.2-0.3 pH-units, dependent on the type of strain. In the cooked ham samples that were still sensory acceptable at day 34 (strains 13E, 10A, 14A, LS8 and LS5), the pH

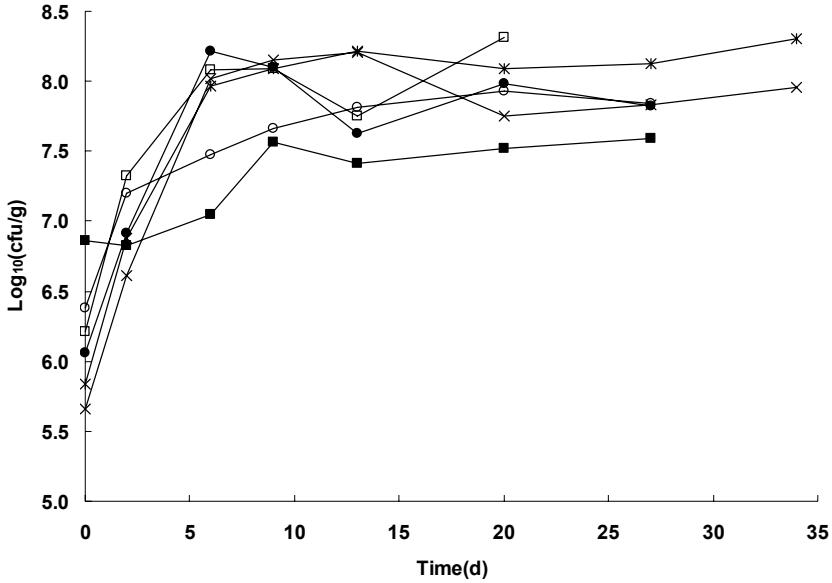

Figure 2.2. Growth of the six selected 'collected' LAB (●, LS7;○, LS1; □, LC4; ■, LP5; ×, LS5; ∗, LS8) on the vacuum packaged model cooked ham at 7°C (mean data of three replicates, error bars are not presented to avoid complexity of the figure)

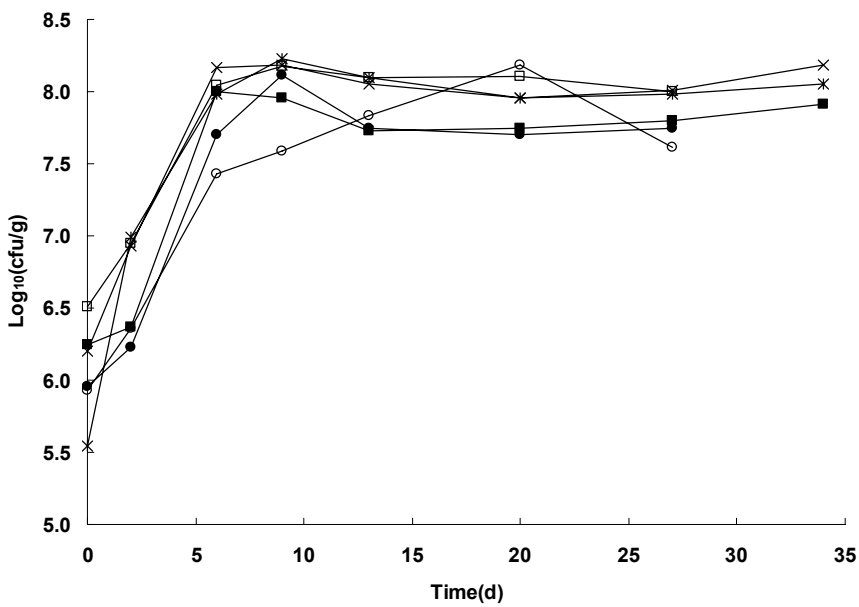

Figure 2.3. Growth of the six selected 'isolated' LAB (●, 20C;○, 13E; □, 9A; ■, 16G; ×, 10A; ∗, 14A) on the vacuum packaged model cooked ham at 7°C (mean data of three replicates, error bars are not presented to avoid complexity of the figure)

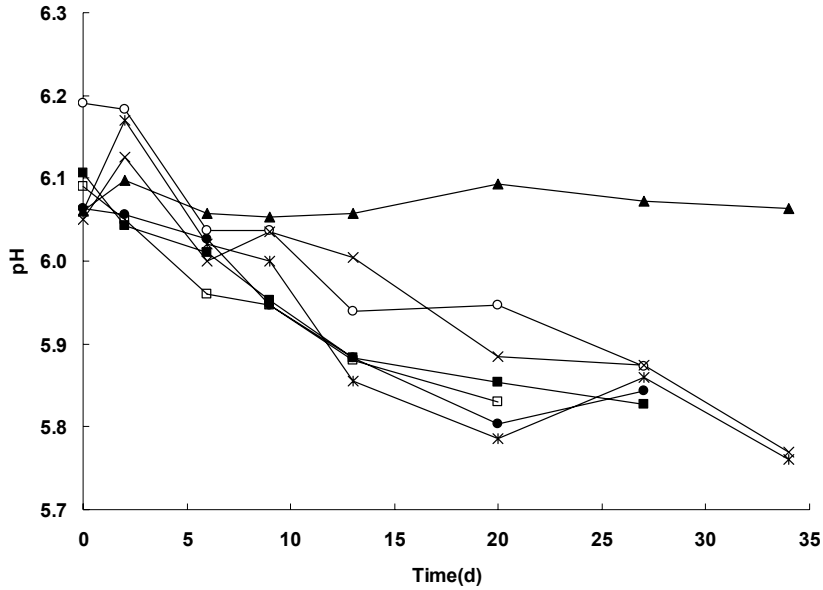

Figure 2.4. pH-evolution of the vacuum packaged model cooked ham, non-inoculated (▲) and inoculated with the six selected 'collected' LAB (●, LS7;○, LS1; □, LC4; ■, LP5; ×, LS5; ∗, LS8) at 7°C (mean data of three replicates, error bars are not presented to avoid complexity of the figure)

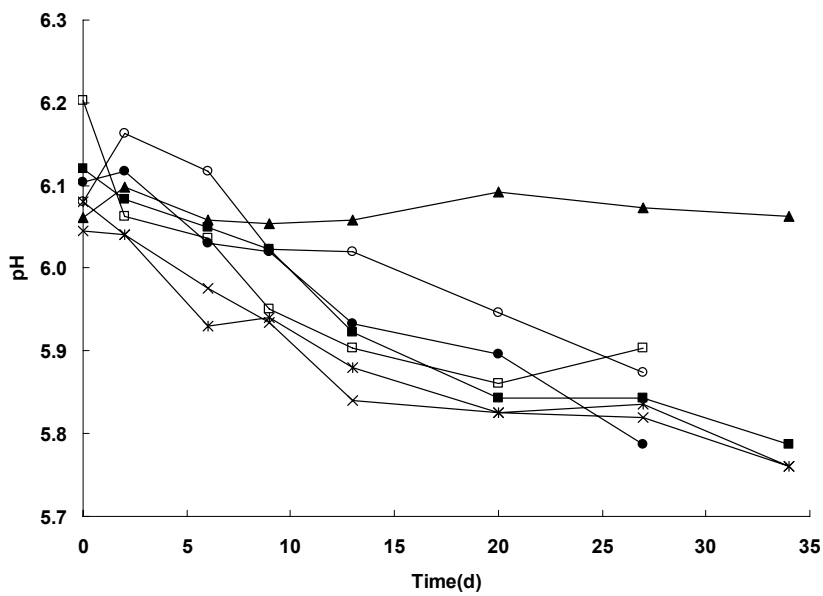

Figure 2.5. pH-evolution of the vacuum packaged model cooked ham, non-inoculated (▲) and inoculated with the six selected 'isolated LAB (●, 20C;○, 13E; □, 9A; ■, 16G; ×, 10A; ∗, 14A) at 7°C (mean data of three replicates, error bars are not presented to avoid complexity of the figure)

decreased to a value of about 5.80-5.75 on day 34. Significant differences in pH-values between inoculated and related non-inoculated samples were observed from day 9 on for LS7, 16G, LC4, 10A and 14A, from day 13 on for LP5, LS1 and LS8 and from day 20 on for 9A and LS5. This indicates that strains 10A, 14A, LC4, LS7 and 16G are more rapidly acidifying the MCH, while strains 9A and LS5 are less rapidly acidifying the MCH. LS5 had also a low acidification rate in the previous experiment when growing in the adapted BHI-broth.

However, the limited acidification by LS5 as it was occurring in the broth was not occurring in the cooked ham. The pH of the cooked ham, inoculated with LS5, decreased to 5.80 ± 0.05 only. In general, the pH-decrease in the MCH was very limited but this can easily be understood taking into account the small level of glucose (0.09 ± 0.03%), initially present in the MCH. Since LAB can form maximum 2 moles of lactic acid by conversion of 1 mole of glucose, the maximum expected amount of lactic acid produced out of ± 0.1% glucose is ± 0.1% of lactic acid, depending on the fermentative character of the strain. This level is very low compared to the initial lactic acid level of 0.70 ± 0.12% and explains why no significant lactic acid production was found in the MCH inoculated with the cultures (Table 2.5). Korkeala et al. (1990) concluded that a level of lactic acid above 0.4% is an indication of spoilage, while here levels of lactic acid varied between 0.4 and 1.2% during storage and high levels were not correlated with rejection of samples by our sensory panel. However, the study of Korkeala et al. (1990) was performed on cooked ring sausages, while this study was done on cooked ham. Juven et al. (1998) found a significant lactic acid production of 50 mM or 0.45% (from 90 mM or 0.81% lactic acid initially to 142 mM or 1.28% lactic acid after 9 weeks at 4°C) on ground beef, inoculated with *L. sakei* BJ-33 (LS1 in this study). Presumably, the glucose content of the ground beef was higher than the glucose content of the MCH used here. However, Juven et al. (1998) did not evaluate the sensory properties of this product after these 9 weeks. Near the end of storage, the glucose level of the MCH decreased significantly and finally no glucose was detected anymore from day 13 on for LS1, 16G, LC4, LS8 and 14A, from day 20 on for LS7, 13E and 10A and from day 27 on for LP5, 9A, LS5 and 20C. The glucose concentration at the last day of the storage period of the non-inoculated product did not differ significantly from the initial glucose level.

Table 2.4 gives the day of sensory rejection and the mean score of the sensory panel for the different sensory attributes at the day of sensory rejection or, in the case of no sensory rejection, at day 34. Results from the sensory analysis indicate that cooked ham inoculated with strains 13E, 10A, 14A, LS5 and LS8 was not rejected by the sensory panel even on the

34th day of the storage period, although the pH, at that moment, had already decreased to 5.75-5.80. Statistical analysis (analysis of variance, $P<0.05$) confirmed that there were no significant differences in all sensory attributes on day 34 between blank 1 and 13E and between blank 3 and 14A, LS5, 10A and LS8. Bredholt et al. (1999) also compared vacuum packaged reference cooked ham and cooked ham, inoculated with several homofermentative LAB, and found all of the inoculated packages after 21 days of storage at 8°C to be acceptable, although some strains resulted in a slightly more sour taste and smell. However, in the study of Bredholt et al. (1999) sensory properties were not followed as a function of time. Table 2.4 reveals that cooked ham inoculated with strains 20C, LP5 and LS7 was rejected due to a deviating taste, while cooked ham inoculated with strains 16G, 9A, LS1 and LC4 was unacceptable because of a deviating taste and a deviating odour. Furthermore, it is important to notice that scores for taste and odour on the day of rejection, in case of the rejected series, were never higher than 6.14 indicating that the deviating odour and taste were not very pronounced.

Table 2.4. Scores for the different sensory attributes at the day of rejection or in case of no rejection at the last day of the storage experiment

Strain	Day of rejection	Odour	Acid odour	Rot odour	Taste	Acid taste	Slime	Colour	General appearance	% yes[a]
Blank l	>34	3.38	2.50	2.50	3.13	3.13	1.50	2.00	2.00	100.0
20C	13	3.00	4.00	3.50	5.00	4.38	1.75	2.63	2.63	37.5
13E	>34	3.25	2.63	2.25	3.50	3.38	1.86	2.00	1.88	75.0
LP5	13	3.63	4.50	3.38	5.25	4.38	2.50	2.13	2.13	25.0
LS7	13	3.63	4.73	3.63	5.63	4.50	2.00	2.25	2.63	12.5
Blank 2	>27	3.71	2.50	2.50	3.13	2.29	1.00	1.71	1.57	85.7
16G	9	5.29	4.00	3.50	5.00	5.29	2.00	3.57	3.71	28.6
9A	20	5.25	4.25	4.38	5.50	5.13	1.25	2.00	2.00	42.9
LS1	9	5.14	4.50	3.38	5.25	4.29	2.14	3.43	3.57	28.6
LC4	6	6.14	4.14	3.14	5.57	4.00	1.43	1.86	2.57	28.6
Blank 3	>34	2.80	2.40	2.00	2.00	1.80	1.00	1.20	1.40	100.0
10A	>34	4.00	3.80	3.20	3.20	3.20	1.60	1.40	1.60	100.0
14A	>34	2.40	2.40	2.20	2.20	2.00	1.40	1.20	1.60	100.0
LS5	>34	3.60	2.40	3.00	3.00	2.80	1.00	1.40	1.40	100.0
LS8	>34	3.20	3.00	3.40	3.40	3.00	1.40	1.20	1.20	80.0

[a]; % of the panel members indicating the product as acceptable for consumption

Scores for general appearance, colour and slime production never reached a score higher than 3.5-4 and 2.5, respectively, indicating that inoculation with the LAB did not influence the colour and general appearance of the MCH in a negative way and that the strains did not produce slime on the surface of the product.

Table 2.5 summarises the number of LAB, pH and concentration of glucose and metabolites of the cooked ham at the day of sensory rejection or, in the case of no sensory rejection, at the last day of storage (day 34). The panellists considered the rejected samples unfit for human consumption after LAB reaching levels of 7.4 to 8.3 $\log_{10}$(cfu/g). This corresponds to the findings of Korkeala et al. (1987) on cooked ring sausages. However, samples still acceptable at day 34 did also reach these levels of LAB without causing sensory deviations in agreement with the findings of Bredholt et al. (1999). At the moment of sensory rejection, pH-values ranged from 5.79 to 5.96. This corresponds to the results of Korkeala et al. (1990), finding cooked ring sausages unfit when the pH decreases below 5.8-5.9. However, cooked ham samples, still sensory acceptable at day 34, demonstrated a similar pH-decrease without being judged unfit for consumption. Differences between our study and the study of Korkeala et al. (1990) might be explained by our primary selection towards homofermentative LAB while Korkeala et al. (1990) studied the behaviour of the natural spoilage flora.

Although the strains were previously selected on their homofermentative character, small levels of acetic acid (varying from 0.002 to 0.290%), ethanol (varying from 0.005 to 0.750%) or propionic acid (varying from 0.003 to 0.142%) were detected in all inoculated series and even in the non-inoculated series near the end of storage. This is in agreement with the results from Borch et al. (1991), demonstrating a metabolic switch from homolactic to heterolactic fermentation during anaerobic continuous growth of a homofermentative *Lactobacillus* due to glucose depletion. Production of acetate may be induced when the supply of glucose to each individual bacterial cell is insufficient to support a homofermentative metabolism, e.g. on a meat surface during storage (Borch & Agerhem, 1992). The study of Borch & Agerhem (1992) demonstrated that in the presence of the homofermentative *Lactobacillus* spp. on beef packaged in 5% CO_2 and 95% N_2, acetate reached a level of about 6 mmol/kg or 0.036% after 4 weeks at 4°C. Comparable concentrations were found in this study, but on a cooked ham product. However, for none of the inoculated series a persistent production of one of these metabolites from a certain moment on could be observed and 95% confidence intervals on the concentration values of these metabolites were considerably large. There was also no relation between formation of one of these metabolites and rejection by the sensory panel. Even in the cooked ham samples inoculated with 13E, 10A, 14A, LS5 and LS8 and not rejected at day 34

Table 2.5. Summary of the LAB-count on M5-agar, pH and concentration of glucose and metabolites of the cooked ham at the day of sensory rejection or in the case of no rejection at the last day of the storage experiment (mean ± standard deviation, n=3)

Strain	Day of sensory rejection	LAB-count (log_{10}(cfu/g))	pH	Glucose (g/kg)	Lactic acid (g/kg)	Acetic acid (g/kg)	Propionic acid (g/kg)	Ethanol (g/kg)
Blank l	>34	4.2 ± 0.7	6.04 ± 0.00	0.96 ± 0.38	6.98 ± 0.70	0.08 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
20C	13	7.8 ± 0.2	5.93 ± 0.02	0.72 ± 0.09	6.76 ± 0.78	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
13E	>34	7.9 ± 0.1	5.79 ± 0.01	0.00 ± 0.00	10.13 ± 0.98	0.27 ± 0.17	0.00 ± 0.00	0.96 ± 0.83
LP5	13	7.4 ± 0.3	5.88 ± 0.02	0.65 ± 0.23	9.91 ± 1.81	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LS7	13	7.6 ± 0.1	5.88 ± 0.02	0.15 ± 0.26	9.68 ± 1.90	0.08 ± 0.14	0.00 ± 0.00	0.00 ± 0.00
Blank 2	>27	5.2 ± 0.8	6.09 ± 0.07	1.15 ± 0.22	8.18 ± 0.37	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16G	9	8.2 ± 0.4	5.95 ± 0.03	0.13 ± 0.23	5.88 ± 0.96	0.02 ± 0.04	0.04 ± 0.07	0.00 ± 0.00
9A	20	8.2 ± 0.2	5.95 ± 0.02	0.26 ± 0.17	4.87 ± 0.98	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LS1	9	7.7 ± 0.3	6.04 ± 0.02	0.05 ± 0.04	3.89 ± 0.39	0.05 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
LC4	6	8.1 ± 0.2	5.96 ± 0.03	0.38 ± 0.18	6.28 ± 0.55	0.03 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
Blank 3	>34	6.3 ± 0.5	6.02 ± 0.04	0.90 ± 0.52	6.38 ± 3.72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10A	>34	8.2 ± 0.0	5.76 ± 0.01	0.00 ± 0.00	12.05 ± 5.24	0.00 ± 0.00	0.13 ± 0.00	0.10 ± 0.14
14A	>34	8.1 ± 0.0	5.76 ± 0.00	0.00 ± 0.00	7.84 ± 0.97	0.19 ± 0.02	0.97 ± 1.38	1.75 ± 2.47
LS5	>34	8.0 ± 0.0	5.77 ± 0.01	0.00 ± 0.00	11.81 ± 3.17	0.17± 0.24	0.00 ± 0.00	0.00 ± 0.00
LS8	>34	8.3 ± 0.1	5.76 ± 0.01	0.00 ± 0.00	6.35 ± 4.38	0.10 ± 0.15	0.26 ± 0.37	0.39 ± 0.55

of the storage experiment, on some days, a small amount of ethanol and/or acetic acid was detected, indicating that these metabolites could not be the reason for sensory rejection. In the experiment of Borch et al. (1991), anaerobic continuous growth of a homofermentative *Lactobacillus* during glucose depletion was accompanied by extensive utilisation of amino acids and also sulphide was produced. During our experiment, no typical sulphide odours were detected in the rejected products, although absence or presence of sulphide was not confirmed by chemical analysis. Amino acid catabolism produces a number of compounds including ammonia, amines, aldehydes, phenols, indole and alcohols, all being volatile flavours. Dainty (1996) confirmed that when glucose becomes depleted, other substrates begin to be metabolised. These include lactate, amino acids and creatine under aerobic storage and lactate and arginine during storage in vacuum or MAP.

4. Conclusion

Especially lactic acid bacteria that are homofermentative, salt tolerant, psychrotrophic and adapted to meat substrates have a good potential to be used for the biopreservation of cooked meat products. Screening of 91 meat born LAB resulted in the selection of 12 putative protective cultures, known for their psychrotrophic character, salt tolerance and antibacterial properties towards *L. monocytogenes* and towards spoilage organisms, associated with CMP. From these 12 strains, only LC4 could be excluded for further research because this strain had a very limited antibacterial activity and when inoculated on cooked ham, the product was sensory rejected on day 6 of vacuum packaged storage at 7°C. Strain LP5 was also less suitable for further investigations as this strain grew slowly on the MCH in comparison to the other LAB. Further, all strains resulting in sensory rejection of the cooked ham were considered to be not useful as protective culture. The remaining four non-bacteriocinogenic (13E, 10A, 14A and LS8, all four identified as *L. sakei* subsp. *carnosus*) and one bacteriocinogenic (LS5 or lactocin S producing *L. sakei* 148) LAB were found suitable for further research. Some selected strains from this chapter will be used in chapters 4, 5 and 6 to investigate the biopreservative effect in CMP due to an inhibitory effect towards heterofermentative LAB, *B. thermosphacta* and *L. monocytogenes*.

Chapter 3

In-vitro and *in-situ* growth characteristics and behaviour of spoilage organisms associated with anaerobically stored cooked meat products

Redrafted after

L. Vermeiren, F. Devlieghere, V. De Graef & J. Debevere (2005)
Journal of Applied Microbiology, 98, 33-42

Chapter 3
In-vitro and in-situ growth characteristics and behaviour of spoilage organisms associated with anaerobically stored cooked meat products

Summary

This chapter presents a systematic study on the behaviour of different types of spoilage organisms, relevant for vacuum packaged cooked meat products (CMP), to better understand the spoilage they cause and to establish the relationship between microbial growth, pH, metabolite formation and organoleptic deviations.

First, strains were characterised in a broth to compare their growth rate, acidifying character and metabolite production under conditions simulating refrigerated vacuum packaged conditions. *Brochothrix thermosphacta* grew faster than the lactic acid bacteria (LAB). All LAB-strains grew fast except *Leuconostoc mesenteroides* subsp. *dextranicum* and *Leuconostoc carnosum*. The acidification rate was related to the growth rate, while the acidification depth was more related to the acid end-products of fermenation.

Secondly, the growth of the organisms was studied on a model cooked ham (MCH). Strains spoiling the model product most rapidly belonged to the species *Leuc. mesenteroides* subsp. *mesenteroides* followed by the species *B. thermosphacta*, while *Lactobacillus sakei* grew more slowly on the MCH. *Leuconostoc citreum, Leuc. carnosum* and *Weissella viridiscens* demonstrated an intermediate spoilage capacity, whereas *Leuc. mesenteroides* subsp. *dextranicum* and *Leuc. carnosum* grew very slowly compared to the other LAB. Growth of the strains on the MCH resulted in a limited pH-decrease which was a function of the growth rate of the strains. Also the glucose consumption was a function of this growth rate. For none of the strains, a significant lactic acid production could be observed. Some small amounts of acetic acid, propionic acid and ethanol were detected for some strains near the end of the storage period. The time at which the MCH became unacceptable from a sensory point of view was linked to the growth rate of the strains, except for *Leuc. citreum* and *Leuc. mesenteroides* subsp. *dextranicum*, which were causing intensive spoilage despite their slow growth. Sensory rejection was mainly based on the attributes odour, taste and acid taste. No clear relation could be observed between metabolite production and the appearance of sensory changes.

1. Introduction

A considerable part of the cured cooked meat products (CMP), such as pâté, cooked ham, emulsion-style sausages and cooked poultry products, are sliced and pre-packaged (vacuum or modified atmosphere) commodities to be sold with sell-by-dates at 7°C varying from three to four weeks for e.g. cooked ham (Stekelenburg & Kant-Muermans, 2001) to six weeks for e.g. pâté. Since these products are heated to a temperature of 65-75°C, most vegetative cells are inactivated and post-heat treatment recontamination determines the shelf-life (Borch et al., 1996). Product handling after cooking plus slicing prior to packaging recontaminates the products with about 0.5 to 3 $\log_{10}$(cfu/g) of total bacteria (Samelis et al., 2000a). When stored anaerobically and under refrigeration, psychrotrophic LAB will dominate the spoilage flora because of their tolerance to micro-aerophilic or anaerobic atmospheres (Korkeala & Mäkelä, 1989; von Holy et al., 1991). The lactic acid flora of vacuum or MA-packaged CMP consists mainly of homofermentative *Lactobacillus* spp., predominantly *L. sakei* and *L. curvatus* (Korkeala & Mäkelä, 1989; von Holy et al., 1991; Devlieghere et al., 1998; Samelis et al., 2000a). In addition to these, obligate heterofermentative lactobacilli, e.g. *L. brevis* and *Leuconostoc* spp. (von Holy et al., 1991; Björkroth et al., 1998; Samelis et al., 1998), followed by other species such as *Weissella* spp. (Samelis et al., 2000a) and *Carnobacterium* spp. (Borch & Molin, 1989) have been found to cause spoilage. Homofermentative LAB ferment glucose exclusively to lactic acid, while heterofermentative LAB ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide (Stiles & Holzapfel, 1997). However, glucose limitation, e.g. during anaerobic growth of homofermentative *Lactobacillus* spp. on a meat surface, can induce a metabolic switch from homo- to heterolactic fermentation (Borch et al., 1991). *Brochothrix thermosphacta* may also form a dominant part of the spoilage flora depending on the amount of residual oxygen present in the headspace of the package (Borch et al., 1996; Samelis et al., 2000a). *B. thermosphacta* is reported not to be competitive under anaerobic conditions and to be rapidly outgrown by lactobacilli (especially *L. sakei* and *L. curvatus*) in refrigerated vacuum packaged meat products (Stiles & Holzapfel, 1997).

This chapter summarises the results of an in-depth study on the growth characteristics of and product formation by mainly heterofermentative LAB and *B. thermosphacta*, associated with spoilage of CMP. First, the strains were characterised in a liquid growth medium at 7°C under an atmosphere of 100% N_2 to compare the spoilage organisms with regard to their growth

rate, acidifying character and metabolite production under conditions simulating refrigerated vacuum packaged conditions and not causing glucose depletion. Further, the spoilage organisms were inoculated on a model cooked ham (MCH) product to (1) establish whether the MCH supported growth of the spoiling strains, (2) characterise the spoilage phenomena and (3) establish the relationship between microbial growth, pH, metabolite production and sensory changes.

2. Materials and methods

2.1. Bacterial strains and preparation of inoculum

Nine spoilage organisms, typically associated with vacuum packaged CMP, were chosen for this study and are presented in Table 3.1.

Table 3.1. Overview of strains used in this study

Strain	Code	Origin (Reference)	Obtained from
Leuconostoc mesenteroides subsp. *mesenteroides*	LM2	Fermented olives	LMG[a] 6893
Leuconostoc mesenteroides subsp. *dextranicum*	LM3	Not reported	LMG 6908
Leuconostoc mesenteroides subsp. *mesenteroides*	LM4	Vacuum packaged smoked turkey fillet	LFMFP[b] 666
Lactobacillus sakei subsp. *carnosus*	LS2	Cooked ham (Devlieghere et al., 1998)	LFMFP 217
Leuconostoc citreum	LC1	Not reported	LMG 9824
Leuconostoc carnosum	LC2	Vacuum packaged beef	LMG 11498
Weissella viridiscens	WV1	Frankfurters	LMG 13093
Brochothrix thermosphacta	BT1	Cooked ham	LFMFP 230
Brochothrix thermosphacta	BT2	Vacuum packaged turkey fillet	LFMFP 227

[a], LMG, Laboratory Microbiology Gent (Gent, Belgium); [b], LFMFP, Laboratory of Food Microbiology and Food Preservation (Gent, Belgium)

Stock cultures of the strains were maintained in de Man Rogosa Sharpe (MRS, Oxoid, Oxoid Limited, Basingstoke, Hampshire, UK) broth or Brain Heart Infusion (BHI, Oxoid) broth supplemented with 15% glycerol at -75°C. Working cultures of the strains were maintained on de Man Rogosa Sharpe (MRS, Oxoid) agar or Tryptone Soya Agar (TSA, Oxoid) slants at

7°C and revived by transferring a loop of inoculum into MRS-broth or BHI-broth followed by incubation at 30°C (22°C for BT1 and BT2) for 24h.

2.2. Growth, acidification profile and metabolite production in a liquid broth under anaerobic refrigerated conditions

The aim of this experiment was to characterise the nine spoilage organisms and to compare their growth characteristics, acidifying character and metabolite production profile in a broth at 7°C under anaerobic conditions at a pH and salt concentration relevant for CMP. Therefore, the strains were inoculated at 10^5 cfu/ml in an adapted BHI-broth (pH 6.2 and 3% NaCl). Growth of the cultures was followed during storage under an atmosphere of 100% N_2 at 7°C to simulate refrigerated vacuum packaged conditions. The composition of the adapted BHI-broth was described before in chapter 2. Additional NaCl was added to obtain a level of 3% of NaCl. The broth was not formulated to simulate cooked ham as the broth did not contain nitrite, contained a higher amount of glucose and had a different buffering capacity to that of cooked ham. This experiment should be seen as a pre-screening of the strains for the further ham model experiments. Twice a day (10 hours of difference), samples of 5 ml were taken to determine cell number and pH. Cell numbers were determined by plating on MRS-agar by using a Spiral Plater (Spiral Systems Inc., Model D, Led Techno, Eksel, Belgium) and pH-measurements were done by using a pH-electrode (Knick, type 763, Berlin, Germany). At the end of each growth experiment, when the pH was changing not more than 0.01 pH-units in 24h, a sample was taken for the determination of the concentration of glucose and metabolites (lactic acid, acetic acid, propionic acid and ethanol) by HPLC-analysis (Chapter 2). Growth curves were modelled by the model of Baranyi & Roberts (1994) and pH-curves by a modified Gompertz equation (Linton et al., 1995; Chapter 2).

2.3. Behaviour on a model cooked ham product

A model cooked ham (MCH) product was manufactured on a semi-industrial scale at Dera Food Technology N.V. (Bornem, Belgium) with the following recipe: 80% of pork meat, 20% of water, 18 g/kg nitrited salt (containing 0.6% of nitrite), 5 g/kg Deraphos C107 (potassium and sodium- di-, tri- and polyphosphates) and 0.5 g/kg Na-ascorbate. The production process of the MCH was similar to the process described before in chapter 2. In packages of 25 slices/package the product was quick-frozen in a blast freezer (Friginox-Le Froid Professionnel, Frispeed SR-range, France) at -40°C to a core temperature of -10°C to avoid

formation of large ice crystals and finally further stored in a freezing room at -18°C. When an experiment started, the necessary amount of product was transferred from the freezer to a cooler at -3°C for 48h and later at 4°C for 24h.

The MCH was inoculated with the nine spoilage organisms at a level of 10^4-10^5 cfu/g in three consecutive experiments. Each experiment consisted of four series: one blank series of non-inoculated ham and three series of ham inoculated with one of the nine strains. All series were tested in triplicate. The inoculum was subcultured twice (24h, 30°C) in 5 ml MRS-broth (for LAB) or BHI-broth (for *B. thermosphacta*). To reach an inoculation level of 10^4-10^5 cfu/g, 200 µl of the appropriate dilution of the 24h-culture was divided over and spread on the surface of 8 slices (110 g/8 slices) of MCH. After inoculation, slices were vacuum packaged (8 slices/package) and stored at 7 ± 1°C in a ventilated refrigerator. Packaging was performed using a Multivac A300/42 (Hagenmüller, Wolfertschwenden, Germany) gas packaging machine in a high barrier film (NX90, Euralpak, Wommelgem, Belgium) of 90 µm thickness with an oxygen transmission rate of 5.2 ml/m^2.24h.atm at 23°C and 85% of relative humidity.

At day 0, 2, 6, 9, 13, 20, 27, 34 and 41 of the storage period, cooked ham samples were analysed for growth of the inoculated strain, pH and concentration of metabolites. Furthermore, the sensory characteristics were evaluated.

For the microbial analyses, a 15g sample of ham was taken aseptically and a decimal dilution series in Peptone Physiologic Solution (PPS; 8.5 g/l NaCl (VWR, VWR International, Leuven, Belgium) and 1 g/l Peptone (Oxoid)) was prepared to plate the appropriate dilutions on MRS-agar (aerobic incubation at 22°C for 3-5 days) and M5-agar (anaerobic incubation at 30°C for 2 days) to determine the level of LAB. The M5-agar differentiates between homo- and heterofermentative LAB (Zuniga et al., 1993). The blank samples were also plated on Plate Count Agar (PCA, Oxoid) (aerobic incubation at 22°C for 3-5 days), Reinforced Clostridial Agar (RCA, Oxoid) (anaerobic incubation at 37°C for 3-5 d) and Yeast Glucose Chloramphenicol Agar (YGC, Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA) (aerobic incubation at 22°C for 3-5 d) to determine total aerobic psychrotrophic count, total anaerobic count and number of yeasts and moulds, respectively. In the case of inoculation with *B. thermosphacta*, STAA-agar (Oxoid) (aerobic incubation at 22°C for 2-3 days) supplemented with STAA (Streptomycin sulphate, Thallous acetate, Actidione, Agar) selective supplement (Oxoid) was used.

The pH-measurements and the determinations of the concentration of metabolites by extraction and subsequent HPLC-analysis were performed according to the methods described before in chapter 2.

The sensory quality of cooked ham samples was evaluated by a trained sensory panel (nine persons) using a scoring method, similar to the method described in chapter 2.
Statistical analysis of the results of pH, glucose and lactic acid concentration was done in a similar way as described before in chapter 2. All statistical analyses were performed using the software SPSS 11.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Growth, acidification profile and metabolite production in a liquid broth under anaerobic refrigerated conditions

For the nine spoilage organisms, growth curves and pH-curves at 7°C were obtained. By using the model of Baranyi & Roberts (1994) an estimation for the growth rate (h^{-1}) and lagphase (h) was calculated. The pH-model resulted in a shoulder S (h), an acidification rate AR (h^{-1}) and a tail A or final pH, used to calculate the acidification depth. The acidification depth was defined as the difference between the initial pH (6.2) and the estimated final pH. Furthermore, the time to acidification starting from 10^6 cfu/ml (= t_{ac-6}) was calculated in the same way as described in chapter 2. The most important model parameters are summarised in Table 3.2.

Table 3.2. Model parameters of the growth and acidification experiment for the nine spoilage organisms in adapted BHI broth at 7°C under anaerobic conditions (mean ± standard deviation, n=3)

Strain	Lagphase (h)	Generation time (h)	Time to acidification t_{ac-6} (h) [a]	Acidification rate (h^{-1})	Depth of acidification [b]
LS2	40.88 ± 4.11	3.70 ± 0.28	21.10 ± 2.87	0.033 ± 0.001	2.16 ± 0.02
LM2	33.21 ± 3.77	3.77 ± 0.37	46.39 ± 3.56	0.021 ± 0.000	1.77 ± 0.01
LM3	354.19 ± 32.55	10.90 ± 6.08	48.46 ± 4.29	0.011 ± 0.000	1.91 ± 0.04
LM4	40.44 ± 2.41	4.53 ± 0.33	49.52 ± 8.08	0.029 ± 0.001	2.10 ± 0.01
LC1	32.62 ± 4.75	5.82 ± 0.48	43.24 ± 1.61	0.024 ± 0.001	2.00 ± 0.01
LC2	52.29 ± 12.22	10.78 ± 1.05	76.93 ± 2.99	0.014 ± 0.000	1.90 ± 0.07
WV1	46.67 ± 1.09	5.61 ± 0.30	67.96 ± 1.35	0.022 ± 0.001	1.94 ± 0.01
BT1	11.98 ± 0.03	2.00 ± 0.16	25.53 ± 1.25	0.034 ± 0.001	1.63 ± 0.04
BT2	9.19 ± 1.15	2.39 ± 0.28	29.08 ± 0.52	0.037 ± 0.001	1.66 ± 0.01

[a], starting from 10^6 cfu/ml; [b], difference between initial pH (6.2) and estimated final pH

Figure 3.1 shows the levels of lactic acid, acetic acid, propionic acid and ethanol and the level of glucose that were produced and consumed, respectively, at the end of the growth experiment. The pattern of metabolite production reflects the homo- or heterofermentative character of the tested strains.

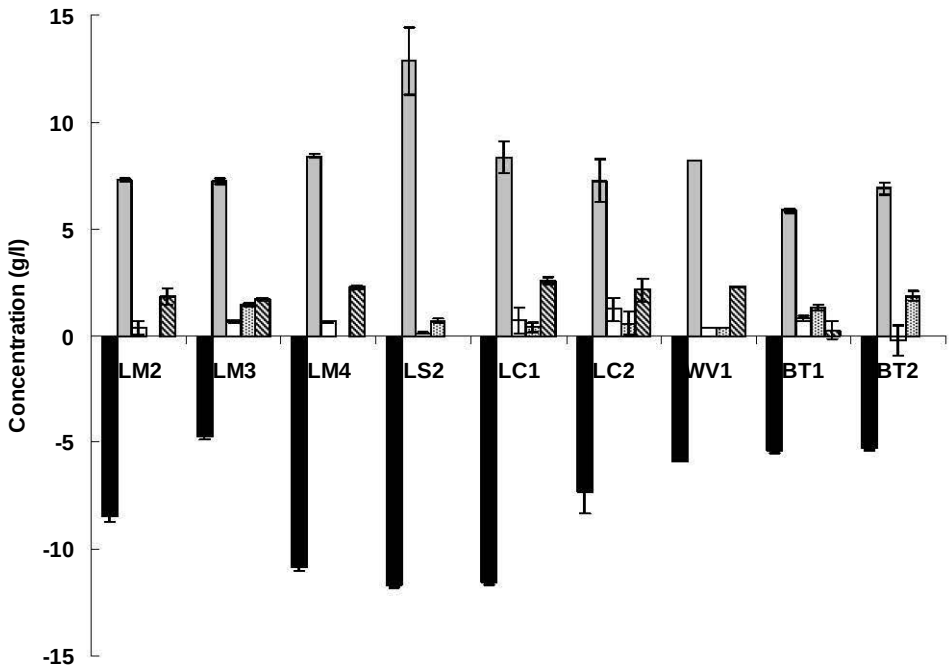

Figure 3.1. Production of metabolites and consumption of glucose at the end of the growth of the nine spoilage organisms in adapted BHI-broth at 7°C under anaerobic conditions (black bars, glucose; grey bars, lactic acid; white bars, acetic acid; dotted bars, propionic acid; striped bars, ethanol) (error bars represent 95% confidence intervals, n=3)

Each of the nine spoilage organisms was able to grow in the adapted BHI-broth at 7°C under anaerobic conditions, but large differences in growth characteristics could be observed. The most rapidly growing strains were the two *B. thermosphacta* strains followed by the two *Leuc. mesenteroides* subsp. *mesenteroides* strains. The *Leuc. carnosum* and *Leuc. mesenteroides* subsp. *dextranicum* strains grew slowly. The acidification rate was related to the growth rate since the fastest growing strains were acidifying the medium most rapidly. The acidification depth, on the other hand, was rather related to the pattern of metabolite production as the homofermentative LS2 acidified the medium to a greater extent than the heterofermentative LAB. The two *B. thermosphacta* strains acidified the medium to a

lesser extent compared to the LAB since these strains also produced lower levels of lactic acid than the LAB did. All strains converted glucose to lactic acid as major end-product, while the heterofermentative LAB and the *B. thermosphacta* strains also produced other metabolites, e.g. ethanol and acetic acid.

Few studies have investigated the behaviour of several spoilage causing LAB and *B. thermosphacta*, as has been done in this experiment. Blickstad & Molin (1984) investigated the growth and end-product formation in fermenter cultures of *B. thermosphacta*, *W. viridiscens* and a homofermentative *Lactobacillus* spp. in different gaseous atmospheres, including 100% N_2 and 5% CO_2 + 95% N_2. However, it is difficult to compare with their results since those experiments were performed at 25°C. In agreement with our results, all test strains produced under anaerobic conditions mainly lactic acid and *W. viridiscens* also produced ethanol while *B. thermosphacta* produced small amounts of ethanol. In 1983, Blickstad investigated the same three organisms as in the study of Blickstad & Molin (1984) but under anaerobic conditions at pH 6.3 and 8°C in a complex medium with 2% of glucose and no nitrite, thus very similar to the conditions of this experiment. The results for *W. viridiscens* correspond very well. Blickstad (1983) reported for *W. viridiscens* a growth rate of 0.06 h^{-1}, while in our study a very similar growth rate of 0.054 ± 0.003 h^{-1} was found and main metabolites were lactic acid and ethanol in both studies. Growth rates for *B. thermosphacta* did not correspond that well, since both *B. thermosphacta* strains grew very fast in our study, while Blickstad (1983) found a growth rate comparable to that of *W. viridiscens.* This might be attributed to inter-strain variations within the *Brochothrix* species.

3.2. Behaviour on a model cooked ham product

The chemical characteristics of the MCH were as follows: 24.60 ± 0.63 % of dry matter, 2.68 ± 0.02 % of NaCl (on aqueous phase), pH of 6.05 ± 0.02 and a water activity of 0.983 ± 0.001.

The non-inoculated cooked ham had an initial microbial contamination with LAB, enumerated on M5-agar, of 1×10^2 cfu/g to 4.2×10^2 cfu/g. Aerobic count and LAB-count were of the same magnitude and no yeasts or moulds were detected. Near the end of the storage period, the level of endogenous LAB increased up to 10^4-10^5 cfu/g. The initial microbial load was very low compared to the inoculation level of the LAB (10^4-10^5 cfu/g). Furthermore,

dominance of the inoculated strains over the background flora was confirmed by identical API-profiles of the inoculum and isolates (results not shown).

Figure 3.2 shows the growth of the tested strains on the MCH, as enumerated on M5-agar for LAB or STAA-agar for *B. thermosphacta*.

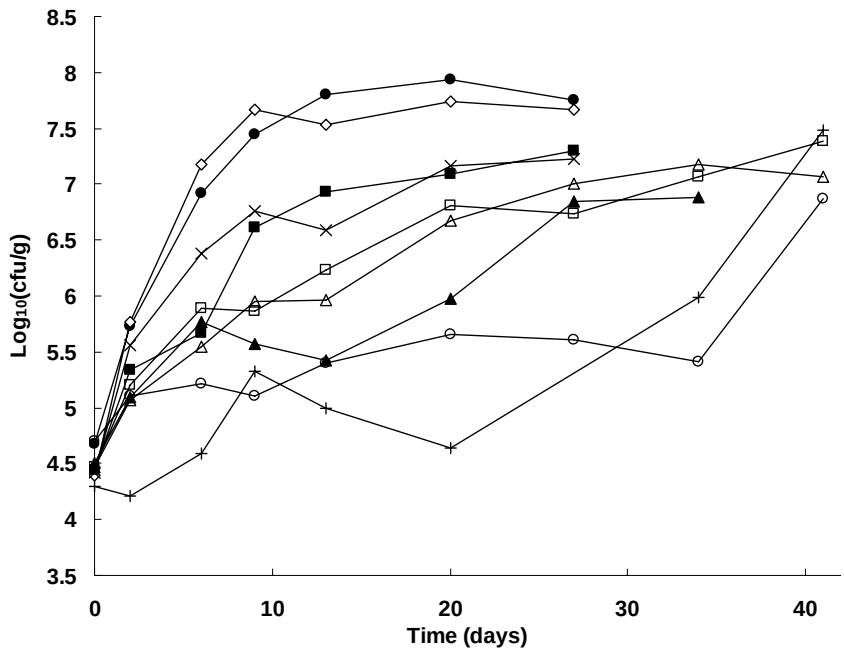

Figure 3.2. Growth of the nine spoilage organisms on the vacuum packaged model cooked ham during storage at 7°C. Strains: (□) LS2, (▲) LC1, (Δ) WV1, (●) LM2, (◊) LM4, (○) LC2, (+) LM3, (■) BT1 and (×) BT2 (mean data of three replicates, error bars are not presented to avoid complexity of the figure)

Most strains reached a maximal population of about 10^7-10^8 cfu/g on the cooked ham. The most rapidly growing organisms were *Leuc. mesenteroides* strains LM2 and LM4, followed by *B. thermosphacta* strains BT1 and BT2. Strains LS2 and WV1 had a comparable but slower growth than LM2, LM4, BT1 and BT2. Strains LC1, LC2 and LM3 grew very slowly on the cooked ham and reached a level of about 10^7 cfu/g only after 27 days for LC1 and after 41 days for LM3 and LC2. Strain LM3 was identified as *Leuc. mesenteroides* subsp. *dextranicum* whereas LM2 and LM4 were identified as *Leuc. mesenteroides* subsp. *mesenteroides.*

The pH of the cooked ham was about 6.05 ± 0.02 and decreased near the end of the storage period (Figure 3.3). Whereas the pH of the non-inoculated ham and ham inoculated with LC1,

LC2 and LM3 not significantly decreased compared to the initial pH-value, significant differences compared to the initial pH-value were observed for LS2 on day 41, for LM2 from day 13 on, for LM4 from day 20 on and for BT1 and BT2 on day 27. The overall pH-decrease was, however, very limited and varied between 0.1-0.2 pH-units. This can easily be understood taking into account the low level of glucose (0.10 ± 0.02%) initially present in the cooked ham.

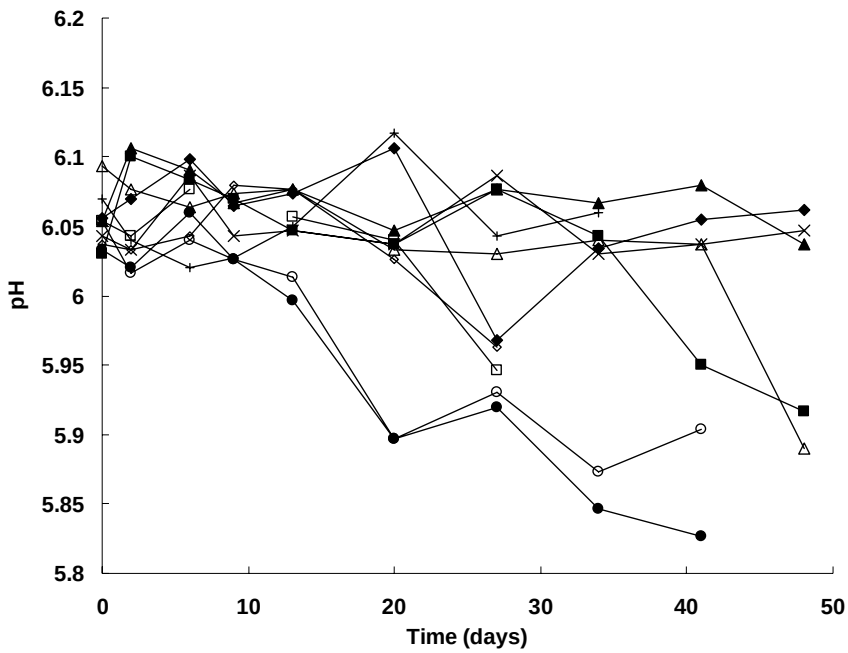

Figure 3.3. pH-evolution of the vacuum packaged model cooked ham inoculated with the nine spoilage organisms during storage at 7°C. Strains: (■) LS2, (▲) LC1, (△) WV1, (●) LM2, (○) LM4, (×) LC2, (◊) BT1, (□) BT2, (♦) blank and (+) LM3 (mean data of three replicates, error bars are not presented to avoid complexity of the figure)

Near the end of the storage period, the glucose level of the MCH decreased and this decrease occurred most rapidly for the fast growing strains. In the case of LM2 and LM4, no glucose was found anymore in the ham from the 20th day on. The non-inoculated ham and the ham with the slow growing strains LM3 and LC2 showed no significant decrease in glucose level compared to the initial glucose level. A significant decrease in glucose compared to the initial glucose concentration was observed for LM2, LM4 and BT1 from day 20 on and for LS2 from day 41 on.

For none of the strains, a significant lactic acid production could be observed. Some small amounts of acetic acid, propionic acid and ethanol were detected for some strains near the end of the storage period. However, these levels were accompanied by large 95% confidence intervals and consequently the production was not always found to be statistically significant ($P<0.05$). A slightly increasing but nevertheless not statistically significant trend in acetic acid concentration as a function of time was noticed for LM2 from day 13 on and for LM4 from day 9 on. Concerning ethanol production, a similar trend was observed for LM2, LM4 and BT2 from day 13 on and with regard to propionic acid production, for LS2 and LC1 from day 41 on and for LM4 and LC2 from day 13 on. Especially the growth of strains LM2 and LM4 resulted in the production of these heterofermentative end-products.

Table 3.3 gives the day of rejection and the mean score of the sensory panel for different sensory attributes at the day of rejection or, in the case of no rejection, on the last day of the storage period.

Table 3.3. Scores for the different sensory attributes at the day of rejection or in the case of no rejection at the 41th day of the storage experiment (mean, n=6)

Strain	Day of rejection	Odour	Acid odour	Rot odour	Taste	Acid taste	Slime	Colour	General appearance	% yes[a]
Blank l	34	6.00	5.11	4.51	6.11	5.11	2.78	2.78	3.22	22.2
LS2	34	5.11	3.67	3.29	6.00	5.11	1.67	2.67	2.89	22.2
LC1	34	7.22	5.33	4.71	7.67	6.22	2.33	2.78	3.44	0.0
WV1	34	5.89	4.78	4.37	5.67	5.11	2.78	4.22	4.78	22.2
Blank 2	41	2.38	1.75	1.63	3.50	3.00	1.75	2.38	2.38	62.5
LC2	>41	4.13	2.25	2.13	4.50	3.38	2.00	2.25	2.63	50.0
LM2	20	6.33	5.56	4.56	5.67	5.22	2.22	2.56	2.89	11.1
LM4	20	5.22	5.22	3.78	4.67	4.56	2.67	2.56	3.11	44.4
Blank 3	41	6.43	3.57	4.86	7.14	5.86	1.86	2.29	2.57	14.3
LM3	41	7.57	4.43	6.29	8.14	5.86	2.00	2.14	2.43	0.0
BT1	13	5.75	4.75	4.75	6.50	6.13	2.50	2.75	3.38	37.5
BT2	13	6.25	4.75	4.63	5.63	5.13	2.13	2.63	3.50	25.0

[a], % of the panel members indicating the product as acceptable for consumption

The day of rejection was linked to the growth rate of the strains. Ham with the fast growing strains BT1, BT2, LM2 and LM4 was rejected early in the storage period, while ham containing the slow growing strains was acceptable almost until the end of the storage period.

Sensory rejection was mainly based on the attributes odour, taste and acid taste. Sensory deviations could not always be described as acid but for some strains (LM3) a more fermented and rotten taste/odour was observed. Highest scores were given for LC1 and LM3 indicating that these organisms caused the most intensive spoilage, although they were growing rather slow. For all LAB, scores for general appearance, slime production and colour never reached a value higher than 3.50, 2.78 and 2.78, respectively, indicating that inoculation with the strains did not influence the colour and general appearance of the cooked ham in a negative way and that the strains did not produce slime on the surface of the product.

In this chapter, growth experiments on a MCH showed the most rapidly growing spoilage organisms to be *Leuc. mesenteroides* subsp. *mesenteroides* strains followed by *B. thermosphacta*. This result was in contradiction with the broth experiment, in which *B. thermosphacta* grew more rapidly than *Leuc. mesenteroides*. Since inoculum levels were similar, it might be that the MCH substrate is better supporting the growth of *Leuc. mesenteroides* than of *B. thermosphacta*. Another possible explanation might be the presence of residual nitrite in the cooked ham product, while this compound was not present in the adapted BHI-broth. It is known for some time that *B. thermosphacta* is more sensitive towards nitrite than LAB are (Gardner, 1981). Other differences between the broth and the MCH are the different level of glucose and the difference in buffering capacity. Furthermore, surface growth is difficult to compare with growth in a liquid broth. *L. sakei* has been identified as the specific spoilage organism of CMP but on this MCH it did not grow the fastest in comparison with other spoilage causing LAB. It has to be mentioned that this study was a pure culture study and is not showing which strain would grow the fastest when inoculating e.g. *L. sakei* and *Leuc. mesenteroides* together on the cooked ham. Similar as in the broth, *Leuc. carnosum* and *Leuc. mesenteroides* subsp. *dextranicum* grew slowly on the cooked ham.

Growth of the spoilage organisms on the MCH resulted in a limited pH-decrease and no significant lactic acid production could be observed throughout the storage period. This fact can be understood taking into account the initial levels of glucose ($0.10 \pm 0.02\%$) and lactic acid ($0.89 \pm 0.15\%$). The latter level corresponds to the level of 0.76% lactate, reported by Stekelenburg & Kant-Muermans (2001) to be a possible level naturally present in meat. Since LAB can form maximum two moles of lactic acid by conversion of one mole of glucose, the maximum expected amount of lactic acid produced out of $\pm 0.1\%$ glucose is $\pm 0.1\%$ of lactic

acid, depending on the heterofermentative character of the strain. This level is very low compared to the initial level of lactic acid of 0.89% and taking into account a mean 95% confidence interval of ± 0.2% on the lactic acid concentrations, no significant lactic acid production could be found. The small amount of lactic acid, which could be produced, is responsible for the limited decrease in pH. About 0.1% lactic acid is, from our experience, needed to create a pH-drop of about 0.1 pH-units in cooked ham.

Table 3.4 summarises the number of LAB or *Brochothrix* spp., as enumerated on M5-agar or STAA-agar, the pH and the concentration of some typical metabolites of the cooked ham at the day of rejection or in case of no rejection at the last day of the storage experiment.

Table 3.4. Summary of the cell number (log_{10}(cfu/g)), pH and concentration (g/kg of ham) of glucose and metabolites of the cooked ham at the day of rejection or in case of no rejection at the last day of the storage experiment (mean ± standard deviation, n = 3)

Strain	Rejected on day	Cell number	pH	Glucose	Lactic acid	Acetic acid	Propionic acid	Ethanol
BLl [a]	34	5.0 ± 1.0	6.07 ± 0.08	0.22 ± 0.38	12.3 ± 3.8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LS2	34	7.1 ± 0.1	6.04 ± 0.01	0.91 ± 0.11	9.5 ± 0.9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LC1	34	6.9 ± 0.2	6.07 ± 0.02	0.90 ± 0.07	8.7 ± 2.3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
WV1	34	7.2 ± 0.1	6.04 ± 0.02	0.55 ± 0.32	9.0 ± 2.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
BL2 [a]	41	3.2 ± 1.2	6.00 ± 0.01	0.82 ± 0.11	8.2 ± 1.7	1.76 ± 3.05	0.00 ± 0.00	0.00 ± 0.00
LC2	>41	6.9 ± 1.0	6.04 ± 0.06	0.67 ± 0.58	14.7 ± 6.2	0.00 ± 0.00	0.92 ± 1.59	0.00 ± 0.00
LM2	20	7.9 ± 0.4	5.90 ± 0.01	0.00 ± 0.00	9.9 ± 0.7	0.97 ± 0.90	0.00 ± 0.00	1.16 ± 1.09
LM4	20	8.2 ± 0.1	5.90 ± 0.03	0.00 ± 0.00	10.3 ± 0.6	0.42 ± 0.73	0.50 ± 0.86	1.63 ± 1.43
BL3 [a]	41	5.4 ± 0.7	6.01 ± 0.04	0.71 ± 0.23	8.3 ± 1.7	0.44 ± 0.76	0.00 ± 0.00	1.30 ± 2.25
LM3	41	7.5 ± 0.2	5.92 ± 0.09	0.00 ± 0.00	9.4 ± 7.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
BT1	13	6.9 ± 0.3	6.08 ± 0.01	1.14 ± 0.11	8.6 ± 0.8	0.05 ± 0.09	0.00 ± 0.00	0.00 ± 0.00
BT2	13	6.6 ± 0.3	6.06 ± 0.02	1.10 ± 0.21	12.4 ± 3.9	0.00 ± 0.00	0.00 ± 0.00	0.94 ± 0.87

[a], BL1 = blank 1; BL2 = blank 2; BL3 = blank 3

Samples were considered unfit for human consumption when the LAB-count reached levels of 6.9 to 8.2 log_{10}(cfu/g), corresponding to the findings of Korkeala et al. (1987) on cooked ring sausages. The highest maximum cell concentration was reached by LM2 and LM4. The samples inoculated with the *Brochothrix* strains were rejected when the cell number was lower, about 6.6 to 6.9 log_{10}(cfu/g). The non-inoculated samples were rejected at very low cell numbers, varying from 3.2 to 5.4 log_{10}(cfu/g). Also cooked ham samples inoculated with strains LS2, LC1 and WV1 were rejected at rather low cell numbers of 7.1, 6.9 and 7.2

$\log_{10}$(cfu/g), respectively. It might have been the case that other types of spoilage, e.g. chemical spoilage, were occurring after this prolonged storage explaining why these samples were rejected at a rather low level of LAB. At the moment of rejection, pH-values were for all strains, except for the three *Leuc. mesenteroides* strains, not significantly lower than the initial pH-value since glucose concentrations at that moment were also not significantly lowered. For the three *Leuc mesenteroides* strains, pH-values at the day of rejection were lowered to a value of about 5.90 and at that moment no glucose was present anymore in the ham. The main conclusion which can be drawn from the pattern of metabolite production at the day of rejection is that mainly *Leuc. mesenteroides* subsp. *mesenteroides* strains produced the typical heterofermentative end-products. Cooked ham containing *B. thermosphacta* was rejected on day 13 and from that day on, ethanol was produced for BT2 but not for BT1. It is possible that the sensory deviations caused by *B. thermosphacta* were caused by other metabolites or volatiles that were not analysed in this study.

Egan et al. (1980) performed a comparable study on vacuum packaged sliced cooked luncheon meat at 5°C. The tested LAB were a mix of four homofermentative and a mix of four heterofermentative LAB, but the identity was not further specified. Furthermore, the study of Egan et al. (1980) was less extensive than our study since pH and metabolite production were not examined. Egan et al. (1980) concluded that *B. thermosphacta* caused rapid spoilage, that homofermentative LAB caused spoilage much more slowly and that heterofermentative LAB were intermediate in their effect. Another study to compare with is the study of Borch & Agerhem (1992), investigating the chemical, microbial and sensory changes during anaerobic cold (4°C) storage of raw beef inoculated with a homofermentative *Lactobacillus* spp. or a *Leuconostoc* spp. However, care must be taken when comparing since the food products under study differ strongly. In their study, both species reached a maximal population of 10^7 cfu/cm^2 at the same moment i.e. after two weeks whereas in our study, two *Leuconostoc* strains grew faster and a third one grew slower than the tested *Lactobacillus* strain. Borch & Agerhem (1992) also observed a drastic decrease in glucose concentration on beef slices inoculated with *Leuconostoc*, while the change in glucose concentration was less drastic for *Lactobacillus*. Table 3.4 reveals that our results correspond to these findings.

Because of the low initial level of glucose in our test product and as a consequence limited pH-decrease and lactic acid production, a poor correlation between pH, lactic acid concentration and cell number was observed. CMP can contain higher concentrations of glucose and then the correlation between these parameters is more obvious as it was the case in the studies of Korkeala (Korkeala et al., 1987; 1989; 1990). In our study, rejection of

cooked ham samples inoculated with *Leuc. mesenteroides* subsp. *mesenteroides* was related to the production of heterofermentative end-products, mainly acetic acid and ethanol. However, for all other samples it was not possible to observe a relation between formation of one of the metabolites and rejection by the sensory panel. In the case of *Leuc. mesenteroides* subsp. *mesenteroides*, glucose was depleted. Dainty (1996) noticed that when glucose becomes depleted other substrates start to be metabolised. These include lactate, amino acids and creatine under aerobic storage and lactate and arginine during anaerobic storage. Amino acid catabolism produces a number of compounds including ammonia, aldehydes, phenols, indol and alcohols, all being volatile flavours (Borch et al., 1991).

4. Conclusion

The results of this pure culture study show that within a group of nine spoilage organisms, typically associated with anaerobically packaged sliced cooked meat products, *Brochothrix thermosphacta* and *Leuconostoc mesenteroides* subsp. *mesenteroides* seem to have the highest potential to cause rapid spoilage. This was demonstrated in broth experiments and in an inoculation study on a model cooked ham product. *Lactobacillus sakei*, identified as the specific spoilage organism of anaerobically packaged cooked meat products, was not the most rapidly growing organism on the model cooked ham. It has to be stressed that this study was a pure culture study and does not show which strain would grow the fastest in a real life situation when different types of spoiling LAB are present together on a cooked meat product. The next chapter (chapter 4) will investigate the interactions between two LAB, that were shown to be potential protective cultures in chapter 2, and the two spoilage organisms *Brochothrix thermosphacta* and *Leuconostoc mesenteroides* subsp. *mesenteroides*, that were found to be relevant spoilage organisms for CMP in this chapter.

Chapter 4

Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham

Redrafted after

L. Vermeiren, F. Devlieghere & J. Debevere (2006)
International Journal of Food Microbiology, 108, 68-77

Chapter 4
Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham

Summary

In this chapter, the usefulness of two lactic acid bacteria, a *Lactobacillus sakei* subsp. *carnosus* strain (10A) and the lactocin S producing *Lactobacillus sakei* 148 (LS5), to extend the shelf-life of cooked meat products (CMP) was investigated. The interactions between these potential protective cultures and the spoilage organisms, *Leuconostoc mesenteroides* (LM4) and *Brochothrix thermosphacta* (BT1), were examined in co-culture studies on a model cooked ham (MCH) product at 7°C under vacuum packaged conditions. The influence of the glucose content of the model cooked ham on the interaction phenomena was investigated by performing the co-culture studies on MCH with a low glucose content of about 0.2% (w/w) glucose and MCH with a high glucose content of about 1.3% (w/w) glucose. The difficulty in quantifying such an interaction was to individually follow the growth of the homofermentative LAB-strain on the one hand and the growth of the heterofermentative LAB-strain on the other hand when growing in co-culture on the MCH. To resolve this issue a new medium, TC8-MRS-agar, consisting of MRS-agar supplemented with tetracycline at 8 µg/ml, was developed. This agar medium allowed differentiation of LM4-colonies from 10A-colonies or LS5-colonies after incubation for three days at 30°C under anaerobic conditions.

When artificially contaminating the model cooked ham with BT1 at 10^2 cfu/g in combination with 10A at 10^5 cfu/g, the growth of BT1 was significantly slower compared to its simultaneous growth in mono-culture. In a similar experiment with LM4, this strain reached a level of 10^7 cfu/g approximately 14 days later if LM4 grew together with 10A compared to its growth in mono-culture. The lactocin S producing LS5 did not demonstrate an antagonistic action towards LM4 or BT1. On the MCH with low glucose content as well as on the MCH with high glucose content, antagonistic interactions of 10A towards LM4 and BT1 occurred; the antagonistic effect of 10A was not eliminated when glucose was abundant in the product.

The results of this chapter indicate that *Lactobacillus sakei* 10A has potential as protective culture for the shelf-life prolongation of CMP, while *Lactobacillus sakei* LS5 has not.

1. Introduction

Interest in the role of biopreservation in assuring food stability and food safety has increased as a variety of bacteria, mainly lactic acid bacteria (LAB), occurring commonly in foods have been evaluated for their potential to control spoilage organisms and food born pathogens while changing the sensory properties as little as possible (Lücke, 2000; Chapter 2). This natural way of preservation has gained increasing attention for controlling the shelf-life and safety of the new generation of minimally processed ready-to-eat food products of extended durability under refrigerated conditions (Rodgers, 2001). This product group includes the refrigerated, pre-packaged, cured, cooked meat products (CMP), which are either vacuum packaged (VP) or packaged in a modified atmosphere (MAP). Post-contamination after cooking determines the shelf-life of these meat products resulting in a spoilage flora mainly consisting of *Lactobacillus* spp., *Leuconostoc* spp. and in some cases *Brochothrix thermosphacta* (Borch et al., 1996; Samelis et al., 2000a, Chapter 3).

In CMP, protective cultures (PC) have mainly been evaluated for their potential to inhibit food born pathogens such as *Listeria monocytogenes* (Andersen, 1995a; Hugas et al., 1998; Bredholt et al., 1999; Bredholt et al., 2001, Amezquita & Brashears, 2002; Budde et al., 2003; Mataragas et al., 2003a). Less is known about the possibility to use PC for shelf-life prolongation of these products. Kotzekidou & Bloukas (1996) reported a one-week extension of the shelf-life of vacuum packaged, sliced, cooked ham at 4°C when inoculated with the bioprotective culture *Lactobacillus sakei* BJ-33 before cooking via the curing solution at a level of 10^{10} cfu/g. In 1998, Kotzekidou & Bloukas reported a shelf-life extension of 19 and 28 days at 6-8°C of vacuum packaged pariza, a frankfurter-type sausage, when inoculated with the same culture *L. sakei* BJ-33 at a level of 10^3 cfu/g and 10^5 cfu/g, respectively. In that study, the control sample was thought to be spoiled by heterofermentative LAB. However, the same culture failed to prevent growth of ropy slime producing *L. sakei* strains leading to spoilage on frankfurters (Björkroth & Korkeala, 1997). Metaxopoulos et al. (2002) inoculated sliced cooked cured pork shoulder with the bacteriocin producing *Leuc. mesenteroides* L124 and *L. curvatus* L422. Results under vacuum packaging showed that in the non-inoculated samples the spoilage microflora grew but in the inoculated ones the numbers of *B. thermosphacta* and enterococci reduced during storage. To our knowledge, no study has ever investigated the effect of homofermentative, protective LAB on heterofermentative, spoilage causing LAB in CMP.

Protective cultures can be divided in bacteriocinogenic cultures and non-bacteriocinogenic cultures. In most vacuum packaged CMP, the antagonistic character of non-bacteriocinogenic PC can not be explained by pH-reduction through an increasing lactic acid concentration. Probably, a more complex combined effect of production of antimicrobial compounds and competition for or depletion of specific nutrients might explain the protective effect of these cultures (Devlieghere et al., 2004; Chapter 1 & 2).

In chapter 2, four non-bacteriocinogenic LAB-strains (13E, 10A, 14A and LS8, all four identified as *L. sakei* subsp. *carnosus*) and one bacteriocinogenic LAB-strain (LS5 or lactocin S producing *L. sakei* 148) were found to have suitable properties to be candidate protective cultures for CMP. The results from chapter 3 showed that *B. thermosphacta* and *Leuc. mesenteroides* may cause rapid spoilage when growing as pure cultures on anaerobically packaged CMP. The objective of this chapter was to evaluate two of the selected LAB, the bacteriocinogenic strain LS5 and the non-bacteriocinogenic strain 10A, for their usefulness in prolonging the shelf-life of CMP. Therefore, co-culture experiments at 7°C with the two selected LAB (potential protective cultures), 10A and LS5, on the one hand and the spoilage organisms (target strains), *B. thermosphacta* BT1 and *Leuc. mesenteroides* LM4, on the other hand were set up. These experiments were performed on two types of model cooked ham (MCH) product: MCH with a low glucose content (0.2% (w/w) glucose) and MCH with a high glucose content (1.3% (w/w) glucose). The co-culture studies of this chapter aimed to (1) examine whether an inhibitory interaction between the potential protective cultures and the target strains occurred, (2) quantify this interaction and (3) investigate the role of the glucose content of the MCH in the interaction phenomena.

2. Materials and methods

2.1. Bacterial strains

Two lactic acid bacteria were investigated for their usefulness as protective culture. The first strain was the non-bacteriocin producing *Lactobacillus sakei* subsp. *carnosus* (coded as 10A), isolated from vacuum packaged, sliced, cooked and smoked turkey fillet in chapter 2. The second strain was the lactocin S producing *Lactobacillus sakei* 148 (coded as LS5) isolated from a Spanish dry sausage by Sobrino et al. (1991). Both strains were selected in chapter 2 out of a group of 91 meat born bacterial isolates based on a profound screening of their

growth characteristics, antibacterial properties and influence on the organoleptic properties of a MCH. In this chapter, these cultures are further described as 'potential protective cultures'.
The antagonistic effect of these two potential protective cultures was evaluated towards two spoilage organisms, representative for CMP and in this chapter further described as 'target strains'. The first target strain, a *Leuconostoc mesenteroides* subsp. *mesenteroides* strain (coded as LM4) had been isolated in chapter 2 from vacuum packaged, cooked turkey fillet. The second target strain was a *Brochothrix thermosphacta* strain (coded as BT1), isolated from cooked ham and present in the culture collection of the Laboratory of Food Microbiology and Food Preservation (LFMFP, Gent University, Belgium).

2.2. Model cooked ham (MCH)

Two types of model cooked ham (MCH) were manufactured on a semi-industrial scale at Dera Food Technology N.V. (Bornem, Belgium): a model cooked ham with a low glucose content (LG-product) and a model cooked ham with a high glucose content (HG-product). The recipe and production process of the LG-product were identical to what has been described before in chapters 2 and 3. The LG-product was expected to contain approximately 0.1% (w/w) glucose. The HG-product was prepared following the same recipe and procedure as for the LG-product but an additional amount of glucose (as dextrose monohydrate) was added to reach a final concentration of approximately 1% (w/w) glucose.

2.3. Co-culture experiments

In total, four co-culture experiments were conducted: two on the LG-product and two identical experiments on the HG-product (Table 4.1). Studies 1 and 3 aimed to test the effect of 10A or LS5 on *Leuc. mesenteroides* LM4 while studies 2 and 4 aimed to test the effect of 10A or LS5 on *B. thermosphacta* BT1. Each co-culture study consisted of four test series: (1) non-inoculated cooked ham (blank) used as a reference or control, (2) cooked ham inoculated with the target strain alone (BT1 or LM4), (3) cooked ham inoculated with the target strain and *L. sakei* 10A and (4) cooked ham inoculated with the target strain and *L. sakei* LS5. Each test series was performed in triplicate.
The inoculum was subcultured twice (24h, 30°C) in 5 ml de Man Rogosa Sharpe (MRS, Oxoid, Oxoid Limited, Basingstoke, Hampshire, UK) broth. To obtain an inoculation level of 10^5 cfu/g for the PC and an inoculation level of 10^2 cfu/g for the target strain, 100 µl of a proper dilution of each was divided over and spread on the surface of 6 slices (± 90 g/ 6

slices) of cooked ham. After inoculation, slices were vacuum packaged (6 slices/package) and stored at 7 ± 1°C in a ventilated refrigerator for 42 days. Vacuum packaging was performed as described before in chapter 2.

At regular time intervals of the storage period, cooked ham samples were analysed for microbial growth, pH and concentrations of lactic acid and glucose.

Table 4.1. Overview of the different co-culture experiments on the model cooked ham

	Study 1	Study 2	Study 3	Study 4
Model product	Low glucose	Low glucose	High glucose	High glucose
Target strain	*Leuc. mesenteroides*	*B. thermosphacta*	*Leuc. mesenteroides*	*B. thermosphacta*
Different series within a co-culture study	Blank[a]	Blank	Blank	Blank
	LM4[b]	BT1[e]	LM4	BT1
	LM4+10A[c]	BT1+10A[f]	LM4+10A	BT1+10A
	LM4+LS5[d]	BT1+LS5[g]	LM4+LS5	BT1+LS5

[a], Blank = non-inoculated MCH; [b], LM4 = MCH inoculated with *Leuc. mesenteroides* LM4; [c], LM4+10A = MCH inoculated with *Leuc. mesenteroides* LM4 and *L. sakei* 10A; [d], LM4+LS5 = MCH inoculated with *Leuc. mesenteroides* LM4 and *L. sakei* LS5; [e], BT1 = MCH inoculated with *B. thermosphacta* BT1; [f], BT1+10A = MCH inoculated with *B. thermosphacta* BT1 and *L. sakei* 10A; [g], BT1+LS5 = MCH inoculated with *B. thermosphacta* BT1 and *L. sakei* LS5

2.4. Chemical analyses

The pH of the cooked ham was measured in a mixed sample using a pH-electrode (Ingold, MGDX K57, Urdorf, Switzerland) connected to a pH-meter (Knick, type 763, Berlin, Germany). The concentrations of lactic acid and glucose were determined by using high-performance liquid chromatography (HPLC). Details of this HPLC-analysis have been described in chapter 2. Prior to HPLC-analysis, meat samples were subjected to a modified extraction procedure: a 25 g sample was homogenised with 75 ml of distilled water, filtered (∅ 125 mm, Schleicher & Schuell, Microscience, Dassel, Germany), heated for 15 min at 80°C in a hot water bath, centrifuged at 8000*g* for 10 min and filtered again (∅ 0.2 µm, Alltech Associates, Lokeren, Belgium) prior to injection.

2.5. Microbiological analyses

A 15 g sample of ham was taken aseptically and a decimal dilution series in Peptone Physiologic Solution (PPS; 8.5 g/l NaCl (VWR, VWR International, Leuven, Belgium) and

1 g/l Peptone (Oxoid)) was prepared to plate the appropriate dilutions on the appropriate agar media. The reference samples were plated on Plate Count Agar (PCA, Oxoid) (aerobic incubation at 22°C for 3-5 days), Reinforced Clostridial Agar (RCA, Oxoid) (anaerobic incubation at 37°C for 3-5 days), de Man Rogosa Sharpe (MRS, Oxoid) agar (aerobic incubation at 22°C for 3-5 days) and Yeast Glucose Chloramphenicol Agar (YGC, Bio-Rad Laboratories, Hercules, CA, USA) (aerobic incubation at 22°C for 3-5 days) to determine total aerobic psychrotrophic count, total anaerobic count, total lactic acid bacteria and number of yeasts and moulds, respectively.

To follow the growth of the PC, 10A and LS5, M5-agar (anaerobic incubation at 30°C for 2 days) was used. M5-agar differentiates between homo- and heterofermentative LAB (Zuniga et al., 1993) and gives very well recognisable colonies for 10A and LS5. In the case of co-inoculation with *B. thermosphacta*, STAA (Streptomycin sulphate, Thallous acetate, Actidione, Agar) (Oxoid) agar (aerobic incubation for 2-3 days at 22°C) supplemented with STAA selective supplement (Oxoid) was used to enumerate *B. thermosphacta*. In the case of co-inoculation with *Leuc. mesenteroides*, TC8-MRS-agar (anaerobic incubation at 30°C for 3 days) was used to enumerate *Leuc. mesenteroides*. TC8-MRS-agar consisted of MRS-agar supplemented with tetracycline (Sigma, Sigma-Aldrich Corporation, St. Louis, Missouri, USA) at a concentration of 8 µg/ml.

2.6. Development and optimisation of TC8-MRS-agar

In the co-culture experiments investigating the interaction between 10A or LS5 and LM4, the challenge was to follow the growth of a homofermentative LAB and a heterofermentative LAB on the same product. Although M5-agar differentiates between homofermentative (blue colonies) and heterofermentative LAB (white colonies) (Zuniga et al., 1993), this agar medium could not be used here. In the co-culture experiments of this chapter, the inoculum ratio of 10A/LM4 or LS5/LM4 was $10^5/10^2$ and this meant that, if M5-agar would be used, the few white colonies of the LM4-strain would be overgrown by the large number of blue colonies of 10A or LS5. For that reason, a new medium had to be developed.

The objective was to develop an agar medium that was supporting the growth of LM4 while inhibiting the growth of 10A and LS5 and this by supplementing MRS with an antibiotic; the idea was based on the work of Foegeding et al. (1992).

The first step in the development of the medium was testing the sensitivity of 10A, LS5 and LM4 towards 11 common antibiotics. Susceptibility testing was done according to a modified

disc diffusion method (Gevers et al., 2000) on MRS-agar by using Oxoid susceptibility test discs of bacitracin (10 µg), novobiocin (30 µg), clindamycin (10 µg), erythromycin (15 µg), kanamycin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), rifampicin (30 µg), gentamicin (10 µg), streptomycin (25 µg) and ampicillin (25 µg). Antibiotics that were inhibiting the growth of *L. sakei* 10A and *L. sakei* LS5 were selected for further tests.

In the second step, the breakpoint concentration of the selected antibiotics was determined (Gevers et al., 2000). The breakpoint concentration was defined as the minimal concentration of antibiotic that had to be supplemented to MRS-agar in order to inhibit microbial growth completely during three days of anaerobic incubation at 30°C. From overnight cultures of 10A, LS5 and LM4, a first decimal dilution in PPS was prepared and 0.1 ml of this dilution was spread on MRS-agar supplemented with the antibiotic in doubling concentrations ranging between 0 and 256 µg/ml. After incubation for three days at 30°C under anaerobic conditions, plates were investigated for growth/no growth and from these results the breakpoint concentration was deduced.

In a third step, the differentiating character of MRS-agar supplemented with the antibiotic(s), selected in step two, was tested. This was done by plating appropriate dilutions in PPS of mono-cultures of 10A, LM4 and LS5 and co-cultures of 10A/LM4 and LS5/LM4, with inoculum ratios of 1 and 100, on the supplemented MRS-agars. After anaerobic incubation at 30°C for three days, counts were performed and colonies were picked from the plates to determine the carbohydrate fermentation profile using the API50CH system (BioMerieux, Brussels, Belgium) to confirm whether the observed colonies were LM4 and not 10A or LS5.

2.7. Statistical analyses

Cell numbers, pH-values, glucose and lactic acid concentrations were analysed for significant ($P<0.05$) differences between mono-culture growth and co-culture growth or between growth on the LG-product and growth on the HG-product using independent samples *t*-tests in SPSS 11.0 (SPPS, Chicago, IL, USA).

3. Results and discussion

3.1. Development of TC8-MRS-agar

In general, no large differences in the antibiotic resistance/sensitivity patterns of 10A, LS5 and LM4 could be observed. LM4 was sensitive to all tested antibiotics. Strains 10A and LS5

were sensitive to 8/11 tested antibiotics. Strain 10A was resistant to kanamycin, gentamycin and streptomycin whereas LS5 was only resistant to streptomycin.

The antibiotics rifampicin, chloramphenicol, tetracycline and erythromycin were selected for the determination of their breakpoint concentrations for strain 10A, LS5 and LM4. Only in the case of tetracycline, the breakpoint concentration of *Leuc. mesenteroides* LM4 (256 µg/ml) was higher than the breakpoint concentration of the two *L. sakei* strains 10A (64 µg/ml) and LS5 (64 µg/ml). At a concentration between 4 and 64 µg/ml the growth of 10A and LS5 was strongly inhibited but only at concentrations >64 µg/ml their growth was completely inhibited. In the case of LM4, no growth inhibition occurred at concentrations ≤8 µg/ml; between 16 and 64 µg/ml, a very limited growth inhibition occurred; at 128 µg/ml growth was strongly inhibited and at 256 µg/ml no growth was observed anymore.

In a final experiment, the differentiating character of MRS, supplemented with tetracycline in doubling concentrations ranging between 4 and 64 µg/ml, was verified. Best results were obtained with MRS supplemented with tetracycline at a concentration of 8 µg/ml. This medium allowed differentiation between *Leuc. mesenteroides* LM4 and *L. sakei* 10A or LS5. During the three days incubation period, LM4 was able to grow on the medium to large colonies, while 10A and LS5 where not growing. When incubating the TC8-MRS-plates longer up to five days, very small colonies of 10A or LS5 became visible. The identity of all colony types was confirmed through determination of the carbohydrate fermentation profile.

3.2. Co-culture experiments

The mean chemical composition of the two types of model cooked ham was: 26.06 ± 0.95 % of dry matter, 37 ppm of residual nitrite, 2.56 ± 0.09 % of NaCl (on aqueous phase), pH of 6.16 ± 0.06 and a water activity of 0.982 ± 0.001. The mean glucose concentration of the LG-product and HG-product was 0.21 ± 0.06% (w/w) and 1.31 ± 0.24% (w/w), respectively.

The non-inoculated cooked ham had an initial total aerobic count and an initial LAB-count of <1.0 $\log_{10}$(cfu/g) and no yeasts or moulds were detected. In all experiments, the level of endogenous LAB exceeded the detection limit of 1.0 $\log_{10}$(cfu/g) between day 14 and day 21 of the experiment and at the end of the storage period (day 42) their level had increased up to 5-6 $\log_{10}$(cfu/g). The obtained inoculation level varied in the different inoculation studies from 5.13 to 5.35 $\log_{10}$(cfu/g) for 10A and from 5.08 to 5.52 $\log_{10}$(cfu/g) for LS5. This means that the initial level of lactic acid flora was very low compared to the inoculation level of the PC and could therefore not have been influencing the action of the PC.

Both potential PC grew well on the cooked ham. However, LS5 demonstrated a slower growth in the co-culture studies with LM4, where the strain did not reach a cell number of 7 $\log_{10}$(cfu/g) within the 42 days period of the experiments. In general, 10A reached a level of 7 $\log_{10}$(cfu/g) within 7-14 days. Independent of the tested target organism, 10A grew more rapidly than LS5, in all co-culture experiments but this effect was most obvious in the co-culture experiments with LM4. Significant (*t*-test, $P<0.05$) differences in growth between 10A and LS5, in co-culture with BT1, were found on day 3, 7 and 14 in the LG-product and on day 14 in the HG-product. Significant differences in growth between 10A and LS5, in co-culture with LM4, were found from day 3 on, in the LG-product and on day 14 and 21 in the HG-product. Therefore, it might be stated that LS5 grows slower than 10A, rather in the LG-product than in the HG-product. However, no significant differences were found for the growth of both strains, 10A and LS5, between the LG-product and the HG-product. This indicates that the glucose content of the cooked ham did not significantly influence the growth of the cultures 10A and LS5. A similar conclusion could be made for the mono-culture growth of the strains LM4 and BT1 since no significant ($P<0.05$) differences were observed for the growth of both strains between the LG-product and the HG-product.

3.2.1. Co-culture studies between the potential protective cultures and *Brochothrix thermosphacta*

In Figures 4.1 and 4.2 the interactions between 10A or LS5, respectively, and BT1 on both types of cooked ham are presented. Significant ($P<0.05$) differences were found in the LG-product and in the HG-product between the growth of BT1 in mono-culture and its growth in co-culture with *L. sakei* 10A. The slower growth of the *B. thermosphacta* strain in co-culture with *L. sakei* 10A was most obvious in the LG-product. A significantly slower growth was found in the LG-product on day 14, 21 and 28 and in the HG-product the inhibiting trend was clear but only statistically significant on day 42. In the LG-product, BT1-growth in co-culture with 10A, that was first inhibited, seemed to restart slightly from day 35 on with the result that growth differences between the mono-culture and the co-culture situation were not significant anymore on day 35 and 42. Each time, the inhibition of the BT1-growth by 10A started when *L. sakei* 10A reached a cell number of approximately 10^7 cfu/g and entered its stationary phase. Summarising, it may be stated that *L. sakei* 10A, when applied at a level of 10^5 cfu/g, can prolong the shelf-life of the MCH if this ham would be post-contaminated with *B. thermosphacta* at a level of 10^2 cfu/g or lower.

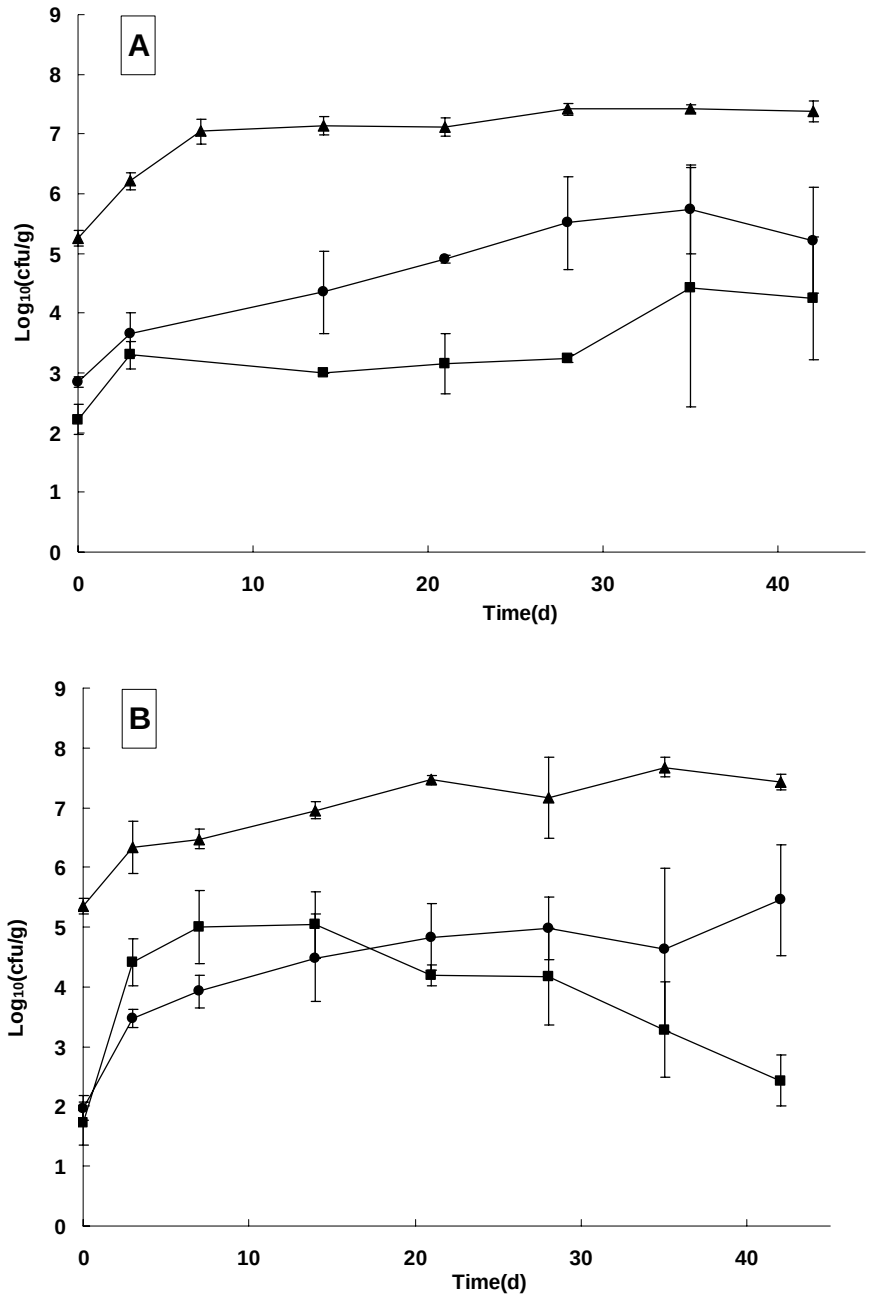

Figure 4.1. Interaction at 7°C between *L. sakei* 10A and *B. thermosphacta* BT1 on vacuum packaged model cooked ham with low (A) and high (B) glucose content. (▲, growth of 10A in co-culture with BT1; ■, growth of BT1 in co-culture with 10A; ●, growth of BT1 in mono-culture) (error bars represent 95% confidence intervals, n=3)

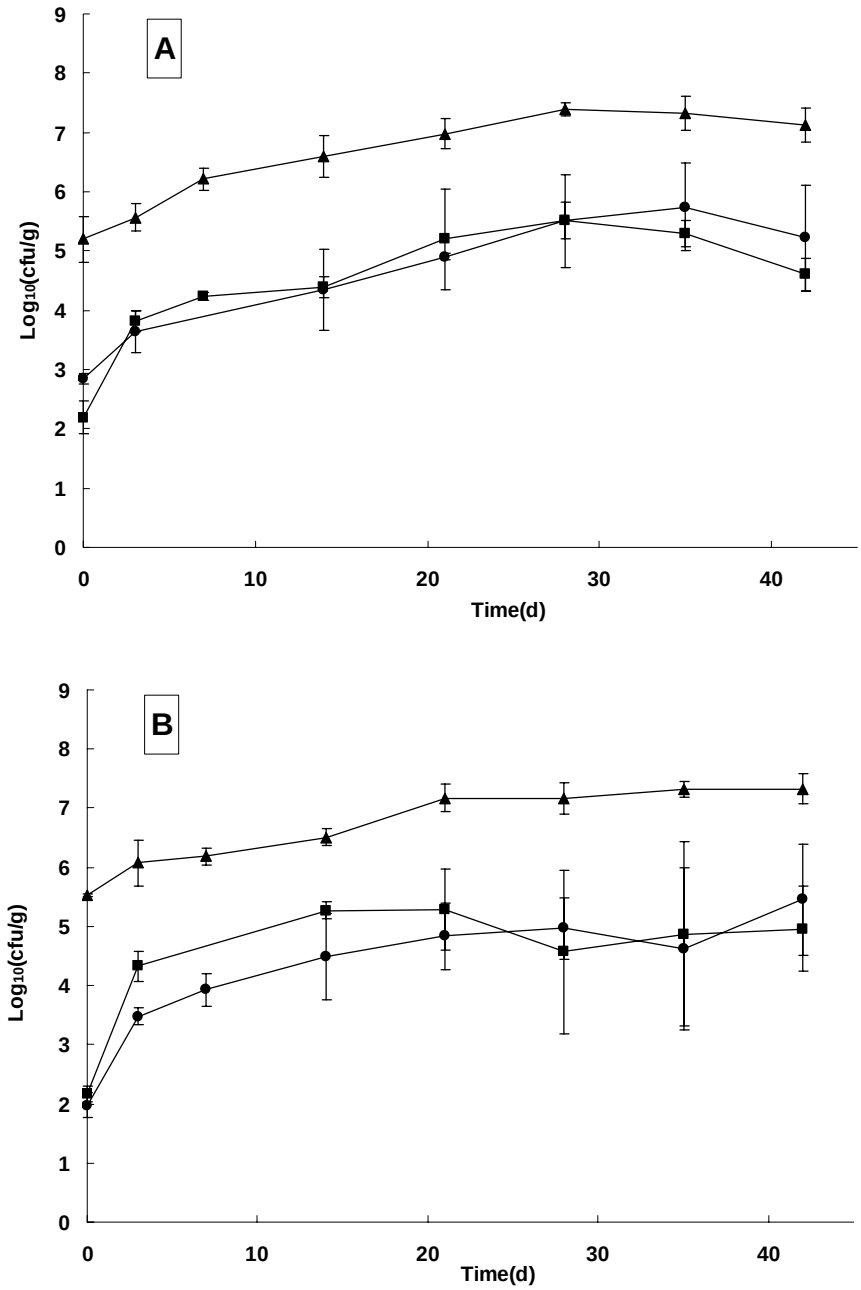

Figure 4.2. Interaction at 7°C between *L. sakei* LS5 and *B. thermosphacta* BT1 on vacuum packaged model cooked ham with low (A) and high (B) glucose content (▲, growth of LS5 in co-culture with BT1; ■, growth of BT1 in co-culture with LS5; ●, growth of BT1 in mono-culture) (error bars represent 95% confidence intervals, n=3)

On the other hand, no significant ($P<0.05$) differences were found in the growth of BT1 between its growth in mono-culture and its growth in co-culture with *L. sakei* LS5 and this in both types of MCH. Whereas strain LS5 clearly showed an in-vitro antibacterial activity towards *B. thermosphacta* in chapter 2 and was proven to produce a bacteriocin (Sobrino et al., 1991; Chapter 2), the strain was not able, at an inoculation level of 10^5 cfu/g, to prolong the shelf-life of a MCH that is contaminated with *B. thermosphacta* at 10^2 cfu/g.

The evolution of the pH during the co-culture studies is presented in Table 4.2. A significantly faster pH-decrease was found in the case of co-inoculation with *L. sakei* 10A in comparison to in the mono-culture experiment from day 35 in the LG-product and from day 21 in the HG-product. However, the differences in pH between day 0 and the end of the storage period were minimal; a decrease of 0.18 pH-units in the LG-product and a decrease of 0.20 in the HG-product. No significant different pH-patterns between mono-culture and co-culture growth of BT1 were obtained in the case of the co-culture experiments with LS5. These findings might be related to the slower growth of LS5 on the cooked ham compared to the growth of 10A.

The initial glucose content of the model cooked ham was 0.21 ± 0.06% (w/w) for the LG-product and 1.31 ± 0.24% (w/w) for the HG-product. The initial lactic acid concentration had a mean value of 1.10 ± 0.23% (w/w). The evolution of the glucose and lactic acid concentration as a function of time is presented in Tables 4.3 and 4.4. Findings correspond very well to the previous conclusions on microbial growth and pH.
In the interaction experiments between 10A and BT1, a significant lower glucose concentration was found in the co-culture situation compared to in the mono-culture situation from day 28 in the LG-product and on day 42 in the HG-product. In the case of the LG-product, growth of BT1 in co-culture with 10A resulted in a significantly lower pH and glucose level compared to the initial pH and the initial glucose content from day 14 on; the glucose was even completely depleted from day 35 on. In the case of the HG-product, a significant lower glucose level compared to the initial glucose level was observed on day 42 only. This result is rather logic as the lower the initial glucose concentration is, the sooner the glucose in the ham might be depleted.

Table 4.2. Evolution of the pH in the model cooked ham (MCH) with low and high glucose content for the four different co-culture studies (mean ± standard deviation, n= 3)

Time	MCH with low glucose content			MCH with high glucose content			MCH with low glucose content			MCH with high glucose content		
(days)	BT1	BT1+10A	BT1+LS5	BT1	BT1+10A	BT1+LS5	LM4	LM4+10A	LM4+LS5	LM4	LM4+10A	LM4+LS5
0	6.18 ± 0.01	6.16 ± 0.02	6.15 ± 0.00	6.18 ± 0.03	6.15 ± 0.01	6.16 ± 0.00	6.15 ± 0.02	6.16 ± 0.03	6.14 ± 0.04	6.16 ± 0.00	6.19 ± 0.01	6.19 ± 0.01
3	6.17 ± 0.02	6.18 ± 0.02	6.17 ± 0.01	6.16 ± 0.01	6.15 ± 0.02	6.15 ± 0.00	ND	ND	ND	ND	ND	ND
7	6.19 ± 0.01	6.15 ± 0.00	6.17 ± 0.01	6.18 ± 0.02	6.18 ± 0.01	6.18 ± 0.01	6.17 ± 0.01	6.16 ± 0.03	6.14 ± 0.01	6.19 ± 0.01	6.13 ± 0.00	6.16 ± 0.02
14	6.12 ± 0.02	6.10 ± 0.00	6.13 ± 0.01	6.17 ± 0.03	6.15 ± 0.02	6.14 ± 0.01	6.12 ± 0.00	6.14 ± 0.06	6.11 ± 0.00	6.05 ± 0.00	6.11 ± 0.02	6.06 ± 0.01
21	6.11± 0.03	6.12 ± 0.02	6.10 ± 0.06	6.18 ± 0.01	6.10 ± 0.02	6.12 ± 0.02	6.12 ± 0.01	6.06 ± 0.05	6.12 ± 0.01	6.10 ± 0.01	6.10 ± 0.03	6.06 ± 0.00
28	6.14 ± 0.06	6.07 ± 0.01	6.03 ± 0.02	6.16 ± 0.01	6.03 ± 0.03	6.11 ± 0.02	6.04 ± 0.04	6.05 ± 0.02	6.11 ± 0.02	6.14 ± 0.01	6.15 ± 0.03	5.99 ± 0.03
35	6.12 ± 0.02	6.02 ± 0.04	6.07 ± 0.07	6.13 ± 0.01	6.01 ± 0.02	6.13 ± 0.04	6.08 ± 0.04	6.02 ± 0.01	6.06 ± 0.04	6.18 ± 0.02	6.09 ± 0.05	5.99 ± 0.08
42	6.11 ± 0.06	5.98 ± 0.01	6.11 ± 0.03	6.19 ± 0.02	5.95 ± 0.02	6.06 ± 0.02	6.02 ± 0.02	5.98 ± 0.02	5.99 ± 0.01	6.03 ± 0.01	6.06 ± 0.01	5.83 ± 0.06

ND = no data available

Table 4.3. Evolution of the glucose concentration (%, w/w) in the model cooked ham (MCH) with low and high glucose content for the four different co-culture studies (ND = no data available; mean ± standard deviation, n=3)

Time	MCH with low glucose content			MCH with high glucose content			MCH with low glucose content			MCH with high glucose content		
(days)	BT1	BT1+10A	BT1+LS5	BT1	BT1+10A	BT1+LS5	LM4	LM4+10A	LM4+LS5	LM4	LM4+10A	LM4+LS5
0	0.17 ± 0.04	0.20 ± 0.01	0.22 ± 0.02	1.27 ± 0.24	1.14 ± 0.15	1.30 ± 0.25	0.25 ± 0.03	0.18 ± 0.03	0.30 ± 0.09	1.45 ± 0.38	1.15 ± 0.17	1.47 ± 0.18
3	0.19 ± 0.02	0.22 ± 0.02	0.24 ± 0.05	1.28 ± 0.33	1.15 ± 0.22	1.01 ± 0.01	ND	ND	ND	ND	ND	ND
7	0.17 ± 0.04	0.17 ± 0.03	0.22 ± 0.08	1.07 ± 0.04	1.17 ± 0.23	1.35 ± 0.55	0.22 ± 0.03	0.16 ± 0.03	0.15 ± 0.01	0.97 ± 0.05	1.10 ± 0.03	1.00 ± 0.02
14	0.17 ± 0.02	0.12 ± 0.04	0.16 ± 0.03	1.33 ± 0.35	1.21 ± 0.26	1.13 ± 0.27	0.29 ± 0.14	0.22 ± 0.05	0.23 ± 0.01	1.14 ± 0.36	1.13 ± 0.03	1.26 ± 0.36
21	0.17 ± 0.05	0.10 ± 0.03	0.17 ± 0.03	0.96 ± 0.03	0.91 ± 0.09	0.99 ± 0.02	0.29 ± 0.07	0.07 ± 0.13	0.21 ± 0.03	1.28 ± 0.06	1.29 ± 0.27	1.17 ± 0.23
28	0.13 ± 0.01	0.10 ± 0.01	0.04 ± 0.07	1.35 ± 0.44	1.02 ± 0.07	1.28 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.24 ± 0.14	1.18 ± 0.07	1.25 ± 0.14
35	0.17 ± 0.01	0.00 ± 0.00	0.09 ± 0.08	1.15 ± 0.15	1.05 ± 0.39	1.18 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.11 ± 0.06	1.02 ± 0.07	0.93 ± 0.29
42	0.19 ± 0.06	0.00 ± 0.00	0.19 ± 0.04	1.11 ± 0.07	0.82 ± 0.06	0.94 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.06 ± 0.11	0.95 ± 0.13	0.66 ± 0.05

Table 4.4. Evolution of the lactic acid concentration (%, w/w) in the model cooked ham (MCH) with low and high glucose content for the four different co-culture studies (ND = no data available; mean ± standard deviation, n=3)

Time	MCH with low glucose content			MCH with high glucose content			MCH with low glucose content			MCH with high glucose content		
(days)	BT1	BT1+10A	BT1+LS5	BT1	BT1+10A	BT1+LS5	LM4	LM4+10A	LM4+LS5	LM4	LM4+10A	LM4+LS5
0	0.83 ± 0.02	0.95 ± 0.04	0.93 ± 0.02	1.21 ± 0.23	1.10 ± 0.13	1.24 ± 0.26	1.11 ± 0.17	0.90 ± 0.10	1.25 ± 0.23	1.29 ± 0.33	1.04 ± 0.15	1.33 ± 0.16
3	0.97 ± 0.05	0.99 ± 0.03	0.93 ± 0.10	1.21 ± 0.38	1.06 ± 0.25	0.96 ± 0.01	ND	ND	ND	ND	ND	ND
7	1.14 ± 0.26	1.16 ± 0.18	0.97 ± 0.07	1.05 ± 0.15	1.08 ± 0.14	1.28 ± 0.50	1.10 ± 0.10	1.02 ± 0.19	0.98 ± 0.05	0.87 ± 0.05	1.01 ± 0.03	0.92 ± 0.01
14	1.13 ± 0.16	0.94 ± 0.14	0.97 ± 0.02	1.18 ± 0.25	1.10 ± 0.19	1.10 ± 0.33	1.33 ± 0.43	1.01 ± 0.02	1.05 ± 0.08	1.06 ± 0.34	1.00 ± 0.02	1.10 ± 0.33
21	1.07 ± 0.08	0.95 ± 0.22	1.13 ± 0.12	0.93 ± 0.02	0.91 ± 0.04	0.96 ± 0.01	1.29 ± 0.28	1.05 ± 0.16	1.11 ± 0.06	1.11 ± 0.14	1.29 ± 0.34	1.37 ± 0.29
28	0.98 ± 0.14	1.12 ± 0.01	1.07 ± 0.08	1.27 ± 0.42	1.19 ± 0.22	1.37 ± 0.41	1.15 ± 0.40	0.95 ± 0.09	0.98 ± 0.04	1.23 ± 0.33	1.20 ± 0.05	1.24 ± 0.02
35	0.99 ± 0.01	1.11 ± 0.07	1.12 ± 0.05	1.09 ± 0.15	1.25 ± 0.49	1.19 ± 0.25	0.89 ± 0.21	1.05 ± 0.02	1.20 ± 0.41	1.05 ± 0.25	1.13 ± 0.17	1.18 ± 0.04
42	1.00 ± 0.09	1.16 ± 0.02	1.11 ± 0.07	1.00 ± 0.08	1.11 ± 0.01	1.06 ± 0.06	0.99 ± 0.14	0.90 ± 0.24	1.28 ± 0.20	1.19 ± 0.17	0.99 ± 0.18	1.25 ± 0.19

In the interaction studies between 10A and BT1, co-culture growth resulted in a glucose consumption of 0.20 ± 0.01% in the LG-product, and of 0.32 ± 0.13% in the HG-product. In the interaction studies between LS5 and BT1, no significant differences could be observed in the glucose concentration between mono-culture and co-culture experiments. During growth of BT1 in co-culture with LS5, the glucose content of the cooked ham did not decrease significantly compared to the initial glucose level and this in both types of MCH.

In the case of the LG-product, a limited but significantly higher lactic acid concentration was found on day 35 and 42 when the cooked ham was inoculated with 10A and BT1 compared to the cooked ham inoculated with BT1 alone. In the case of the HG-product, no significant difference was observed in lactic acid concentration during the whole storage period between mono- and co-culture experiment. Furthermore, *t*-tests were performed for all mono- en co-culture experiments to test if a significant ($P<0.05$) lactic acid production occurred as a function of time. Only in the LG-product, when BT1 was grown in mono-culture or in co-culture with 10A and LS5, a significant but limited production of lactic acid was occurring. At day 42, 0.17 ± 0.07% of lactic acid was produced in the mono-culture situation; 0.20 ± 0.02% of lactic acid was produced for the interaction experiments between 10A and BT1, while 0.18 ± 0.09% of lactic acid was produced for the experiments between LS5 and BT1.

3.2.2. Co-culture studies between the potential protective cultures and *Leuconostoc mesenteroides*

Figures 4.3 and 4.4 present the interactions between 10A or LS5, respectively, and LM4. In the two types of cooked ham, LM4 demonstrated a significantly slower growth when growing in co-culture with 10A compared to its growth in mono-culture and this from day 21 on. This result confirms that *L. sakei* 10A, when added at 10^5 cfu/g, can prolong the shelf-life of the MCH and in particular if this ham would be contaminated with *Leuc. mesenteroides* at a level of 10^2 cfu/g or lower. Through the presence of 10A, the moment at which 10^7 cfu/g of *Leuc. mesenteroides* is reached, was postponed with approximately 14 days compared to the experiment in which LM4 grew in absence of 10A and this result was independent of the glucose level of the model product. Inhibition of LM4 was occurring from the moment that *L. sakei* 10A reached a cell number of 10^7 cfu/g.

The interaction studies between LS5 and LM4 resulted in some unexpected findings. Significant differences between the growth of LM4 in mono-culture and its growth in co-culture with LS5 were observed on day 21 and 28 in the LG-product and between day 3 and

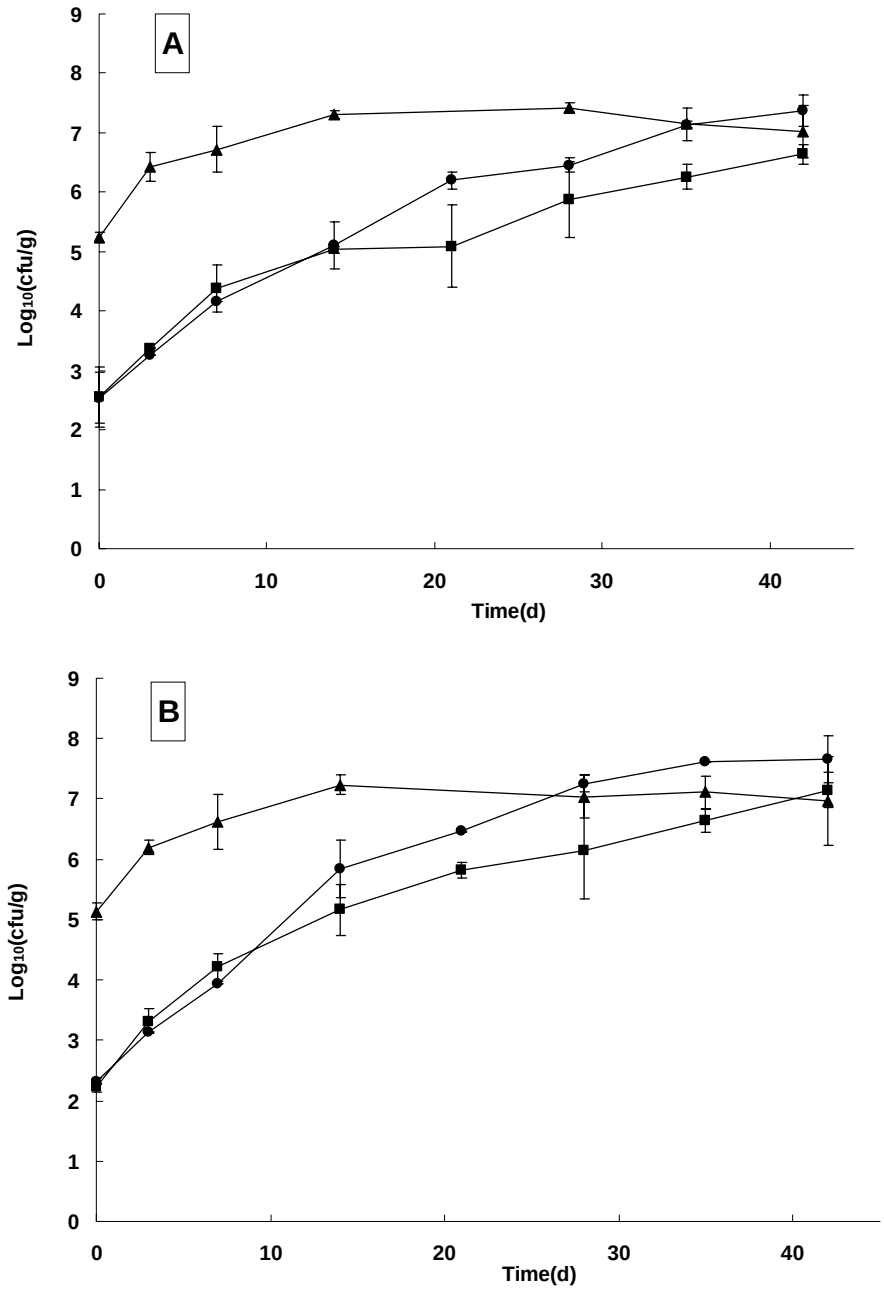

Figure 4.3. Interaction at 7°C between *L. sakei* 10A and *Leuc. mesenteroides* LM4 on vacuum packaged model cooked ham with low (A) and high (B) glucose content. (▲, growth of 10A in co-culture with LM4; ■, growth of LM4 in co-culture with 10A; ●, growth of LM4 in mono-culture) (error bars represent 95% confidence intervals, n=3)

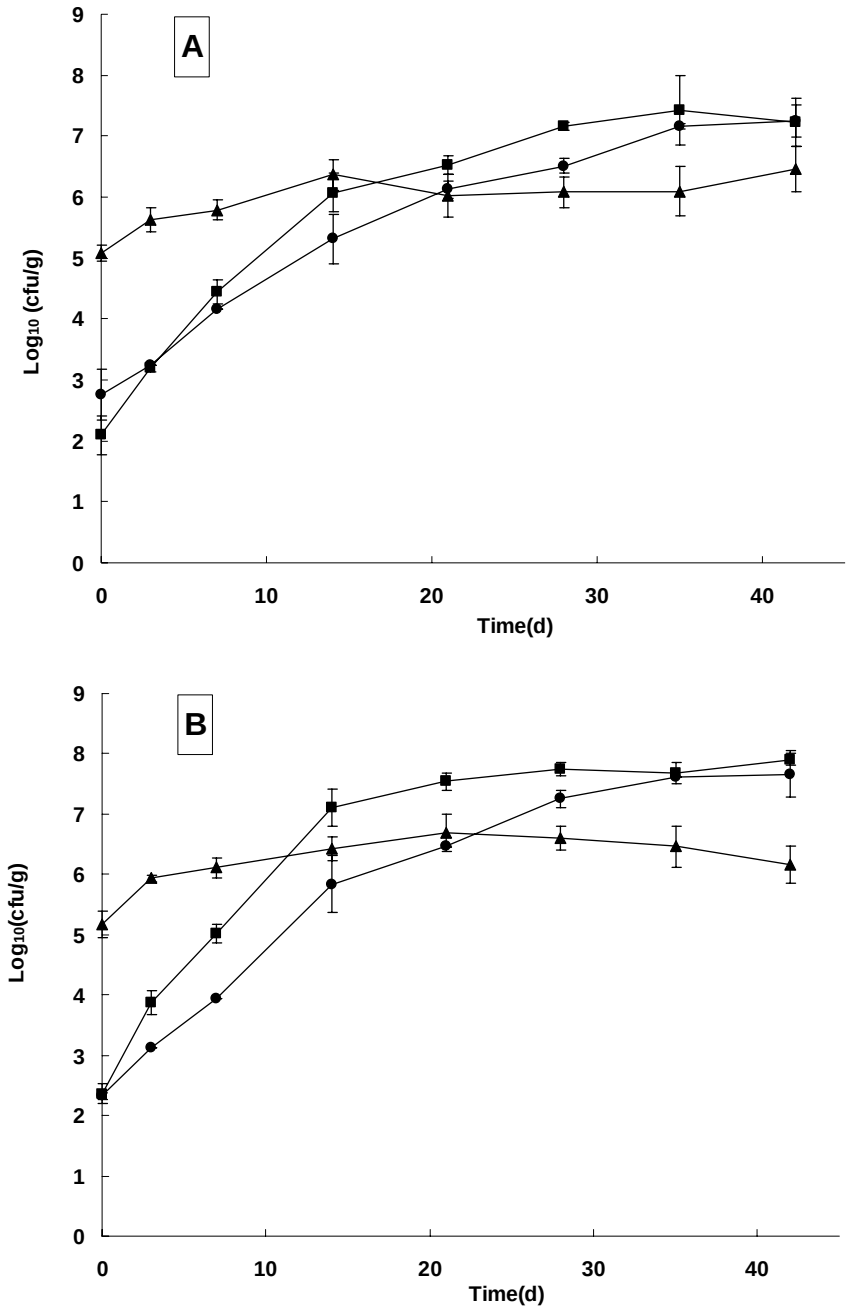

Figure 4.4. Interaction at 7°C between *L. sakei* LS5 and *Leuc. mesenteroides* LM4 on vacuum packaged model cooked ham with low (A) and high (B) glucose content. (▲, growth of LS5 in co-culture with LM4; ■, growth of LM4 in co-culture with LS5; ●, growth of LM4 in mono-culture) (error bars represent 95% confidence intervals, n=3)

day 28 in the HG-product. However, the interaction was not negative but positive as the growth of LM4 was faster in the presence of LS5. An explanation for this kind of reversed interaction phenomenon might be (1) competition for an essential compound that can be taken up by LM4 faster than by LS5 or (2) breakdown of meat compounds by LS5 to nutrients that are easier to metabolise by LM4 compared to in absence of LS5. However, these are only hypotheses. Nevertheless, this result indicates that LS5, at the tested application level of 10^5 cfu/g, is not able to inhibit the growth of *Leuc. mesenteroides* when contaminating the MCH at levels of 10^2 cfu/g or lower.

The evolution of the pH, glucose and lactic acid concentration of the model cooked ham in the different co-culture studies with LM4 is presented in Tables 4.2 to 4.4.

In the LG-product, no significant different evolution in pH, glucose and lactic acid concentration could be found between the growth of LM4 alone and the growth of LM4 in the presence of 10A or LS5. Nevertheless, a significant decrease in the glucose level of the LG-ham occurred from day 28 on in all performed experiments. At day 28 the glucose of the LG-product was completely depleted since the initial 0.2% (w/w) of glucose was completely consumed. This was, however, not reflected in the lactic acid profile since no significant lactic acid production was noticed. Only for the growth of LM4 in mono-culture and its growth in co-culture with 10A, a significant but limited pH-decrease compared to the initial pH was occurring.

In the HG-product, a significantly faster pH-decrease occurred when LM4 grew in the presence of LS5, as the pH decreased from a level of 6.20 to 5.83 at day 42. This finding is related to the fast outgrowth of LM4 in the presence of LS5 resulting in a significantly lower glucose concentration on day 42. However, it was not reflected in the lactic acid profile since no significant differences were found in the lactic acid content of the ham between the growth of LM4 alone and the growth of LM4 in the presence of 10A or LS5. Furthermore, *t*-tests demonstrated that no significant ($P<0.05$) glucose consumption and lactic acid production as a function of time (compared to day 0) occurred in all three cases (LM4 alone, LM4 with 10A and LM4 with LS5) with the exception of the LM4-LS5 experiment on day 35 and 42.

3.2.3. Discussion

In this chapter, *L. sakei* 10A was able to retard the growth of two spoilage organisms typically associated with CMP, being the heterofermentative *Leuc. mesenteroides* LM4 and strain

B. thermosphacta BT1. When artificially contaminating the LG-product and HG-product with BT1 at a level of 10^2 cfu/g in combination with 10A at a level of 10^5 cfu/g, the growth of BT1 was significantly slower compared to a simultaneous mono-culture experiment with BT1 alone. Although BT1, in the mono-culture experiment, did not reach a cell number that is linked to sensory unacceptability of the product (6.6-6.9 ± 0.03 $\log_{10}$(cfu/g), Chapter 3), it is clear from the results that such cell numbers will be reached later or even never in the presence of strain 10A. When repeating this experiment with the target organism LM4, a shelf-life prolongation of ± 14 days was observed if a LAB-count of 7 $\log_{10}$(cfu/g) was used as an end-point for the microbial shelf-life.

On both types of MCH, the timing of growth retardation coincided with *L. sakei* 10A entering its stationary phase. This suggests that either depletion of a critical nutrient or the production of an inhibitory extracellular agent, associated with *L. sakei* 10A reaching a specific population density, may be responsible for the observed inhibition (Buchanan & Bagi, 1997). Inhibition may be due to the effect of one or a synergism between several mechanisms: competition for nutrients, production of organic acids or other antimicrobial substances such as bacteriocins (Bredholt et al., 1999; Devlieghere et al., 2004; Chapter 1). In a bacteriocin assay, *L. sakei* 10A did not show inhibitory zones (Chapter 2) and was therefore assumed to be non-bacteriocinogenic. Further, a very limited pH-decrease was observed in the MCH in all experiments of this chapter. This finding might indicate that the interaction is not the result of a pH-drop through lactic acid production from glucose. With the exception of the co-culture experiment between BT1 and 10A on the LG-product, no significant lactic acid production was seen in the presence of 10A; this means that production of lactic acid/lactate is not a very probable mechanism to explain the observed antagonistic effects. Furthermore, no significant differences could be observed between the interaction phenomena on the LG-product and those on the HG-product. On the two products antagonistic interactions occurred indicating that the antagonistic effect of 10A was not eliminated when glucose was abundant in the product. Although the latter fact does not support the hypothesis of antagonism based on competition for glucose, this theory cannot be excluded based on the results of these experiments. Indeed, in the co-culture experiments between 10A and LM4 on the LG-product, growth retardation of LM4 started at the moment that glucose of the MCH was almost depleted (0.07%). In the experiments between BT1 and 10A, glucose depletion at the moment of inhibition could not be observed. Therefore, it might be possible that on the LG-product, glucose competition was, at least partly, responsible for the observed antagonism of 10A towards LM4. The inhibition might also be the effect of competition for nutrients other than

glucose. Buchanan & Bagi (1997) also suggested a mechanism involving nutrient depletion to explain the ability of *C. piscicola* to suppress *L. monocytogenes* in a broth experiment. It was thought to be possible that the depletion of nutrients like vitamins, minerals, trace elements, peptides causes the antagonistic interaction phenomena. In the HG-product, glucose depletion did not occur but still antagonism was observed. Besides, no significant lactic acid production occurred. Therefore, the mechanism behind the inhibition observed on the HG-products remains to be resolved. The experiments of chapter 7 aim to better understand this inhibition mechanism.

In contradiction to *L. sakei* 10A, the lactocin S producing *L. sakei* LS5 was not able to retard the growth of *Leuc. mesenteroides* LM4 or *B. thermosphacta* BT1. Although strain LS5 clearly demonstrated an in-vitro antibacterial activity towards *B. thermosphacta* and *Leuc. mesenteroides* in the agar spot tests of chapter 2 and was proven to produce the bacteriocin lactocin S (Sobrino et al., 1992; Chapter 2), the strain was not able, at an inoculation level of 10^5 cfu/g, to prolong the shelf-life of the MCH when artificially contaminated with 10^2 cfu/g of *B. thermosphacta* BT1 or *Leuc. mesenteroides* LM4. It is possible that the bacteriocin was not produced or produced at an insufficient level. The effectiveness of bacteriocin-producing strains in foods can be limited by a range of factors such as a limited diffusion in solid matrices, inactivation through proteolytic enzymes or binding to food ingredients such as lipids, a low production level, etc. (Holzapfel et al., 1995; Rodriguez et al., 2002).

4. Conclusion

Few authors have investigated the effect of protective cultures on the shelf-life of CMP. To our knowledge, the interaction between a single homofermentative LAB and a single heterofermentative LAB on a cooked meat product has not been studied before. The difficulty in quantifying such an interaction is to individually follow the growth of a homofermentative and a heterofermentative LAB-strain when growing together on a meat product. To resolve this issue, the medium TC8-MRS-agar, consisting of MRS-agar supplemented with tetracycline at 8 µg/ml, was developed. This agar medium allowed the differentiation of LM4-colonies from 10A-colonies or LS5-colonies after incubation for three days at 30°C under anaerobic conditions.

In this chapter, *Lactobacillus sakei* 10A, a lactic acid bacterium that had been isolated from cooked turkey fillet in chapter 2, showed to offer opportunities as protective culture for the

shelf-life prolongation of cooked meat products. Antagonistic interactions towards two main representatives of the spoilage flora of anaerobically packaged cooked meat products, *Leuconostoc mesenteroides* and *Brochothrix thermosphacta,* were demonstrated on a model cooked ham product at 7°C under vacuum packaging. The inhibitory effect of the biopreservative *L. sakei* 10A occurred when the strain entered its stationary phase and was independent of the glucose content of the model cooked ham.

In the next chapter (Chapter 5), similar co-culture experiments will be conducted but towards the pathogen *L. monocytogenes*. The objective of chapter 5 is to examine whether the shelf-life extending capacity of *L. sakei* 10A can be enlarged with the capacity to control the growth of *L. monocytogenes*. In chapter 5, all experiments are still performed on a model cooked ham product. Further validation studies are necessary to investigate whether the biopreservative effect of *L. sakei* 10A can be extended towards real and different types of cooked meat products and these studies will be the subject of chapter 6.

Chapter 5

The interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham

Redrafted after

L. Vermeiren, F. Devlieghere, I. Vandekinderen & J. Debevere (2006)
Food Microbiology, 23, 511-518

Chapter 5
The interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham

Summary

This chapter investigates the same potential protective cultures that have been studied in chapter 4, being the *Lactobacillus sakei* subsp. *carnosus* strain (10A) and the lactocin S producing *Lactobacillus sakei* 148 (LS5). Their capacity to increase the food safety and in particular to control the growth of *L. monocytogenes* on cooked meat products was investigated. The interaction between the potential protective cultures and a cocktail of three *Listeria monocytogenes* strains was examined in co-culture studies on a model cooked ham (MCH). Furthermore, the influence of the inoculum level (10^5 cfu/g versus 10^6 cfu/g), storage temperature (4°C versus 7°C) and packaging type (vacuum packaging versus modified atmosphere packaging) on the interaction phenomena was investigated. At 7°C, applying *L. sakei* 10A at 10^6 cfu/g limited the growth of *L. monocytogenes* to a level <1 $\log_{10}$(cfu/g) during 27 days, whilst an application level of 10^5 cfu/g failed to prevent growth to unacceptable levels. *L. sakei* LS5 did not demonstrate an antagonistic effect towards *L. monocytogenes*. Lowering the temperature to 4°C or switching from vacuum packaging to modified atmosphere packaging did not influence the ability of *L. sakei* 10A to grow on the MCH, as its dominance did not change. A combination of *L. sakei* 10A and 4°C or a combination of strain 10A and an atmosphere containing 50% of CO_2 completely inhibited the growth of *L. monocytogenes*. Sensory assessment and pH-measurements confirmed that *L. sakei* 10A, even when present at a high level (>7 $\log_{10}$(cfu/g)) for prolonged storage times (up to 42 days), did not acidify the cooked ham to a point of sensory rejection.

1. Introduction

Several authors have reported the occurrence of the psychrotrophic food born pathogen *L. monocytogenes* on anaerobically packaged sliced cooked meat products (CMP). Incidence rates vary from 2.6% in pâté samples (Rijpens et al., 1997) over 4.9% in a variety of CMP (Uyttendaele et al., 1999) to 8.8% in several cooked meats (Vitas & Garcia-Jalon, 2004). In general, the incidence rate is higher for minced CMP (e.g. pâté) than for whole CMP (e.g. cooked ham, cooked poultry), 6.14% and 3.96%, respectively in the study of Uyttendaele et al. (1999). Contamination of CMP with *L. monocytogenes* is often the result of cross contamination during slicing. Uyttendaele et al. (1999) found that incidence rates for CMP were higher after slicing (6.65%) than before slicing (1.65%). However, insufficient thermal processing and the presence of heat survivors might also be the cause of post-process contamination (Samelis & Metaxopoulos, 1999).

Further proliferation of the pathogen on CMP depends on several factors including pH, water activity, level of lactate and composition of the headspace atmosphere (Beumer et al., 1996; Blom et al., 1997; Barakat & Harris, 1999; Devlieghere et al., 2001; Uyttendaele et al., 2004). The competitive flora of a product also influences the growth of *L. monocytogenes*. Some studies have been published about successful use of bacteriocin producing LAB to control *L. monocytogenes* on CMP (Budde et al., 2003; Jacobsen et al., 2003; Mataragas et al., 2003a). However, antagonistic interactions between non-bacteriocin producing LAB and *L. monocytogenes* have also been observed (Andersen, 1995a; Buchanan & Bagi, 1997; Juven et al, 1998; Nilsson et al., 1999; Bredholt et al., 2001). In the study of Bredholt et al. (2001), a *L. sakei* strain was able to inhibit the growth of 10^3 cfu/g of *L. monocytogenes* at 8 and 4°C on cooked ham and cervelat sausage. Amezquita & Brashears (2002) found *L. casei* and *L. paracasei* to have antilisterial activities on cooked ham and frankfurters.

LAB that are homofermentative, salt tolerant, psychrotrophic and adapted to meat-based substrates have shown the greatest potential for use as protective cultures (PC) for the biopreservation of CMP. Furthermore, PC may not influence the sensory properties of the meat products on which they are applied. Based on these criteria, promising LAB were selected in chapter 2 for further study as protective culture. In chapter 4, two of the candidate PC, *L. sakei* 10A and LS5, were found to have an antagonistic effect on the spoilage organisms *Brochothrix thermosphacta* and *Leuconostoc mesenteroides* on a model CMP. In this chapter, their potential antagonistic activity towards *L. monocytogenes* was investigated

on the same model cooked ham (MCH) as well as how this antagonism might be influenced by inoculum level, storage temperature and atmosphere in the headspace of the packaging. Moreover, the effect of the PC on the sensory quality of the MCH was evaluated.

2. Materials and methods

2.1. Bacterial strains

The two lactic acid bacteria under study in this chapter were the non-bacteriocin producing *Lactobacillus sakei* subsp. *carnosus* (coded as 10A) and the lactocin S producing *Lactobacillus sakei* 148 (coded as LS5). Details on these strains are given in chapters 2 and 4. In the co-culture studies, a cocktail of three *Listeria monocytogenes* strains (coded as LIS) was used. *L. monocytogenes* Scott A and a *L. monocytogenes* strain (LFMFP 45), isolated from cooked pita meat, were obtained from the culture collection of the Laboratory of Food Microbiology and Food Preservation (LFMFP, Gent University, Belgium), while the third strain (*L. monocytogenes* LMG 13305), isolated from soft cheese, was obtained from the culture collection of the Laboratory Microbiology Gent (LMG, Gent University, Belgium). Working cultures of the LAB-strains and *L. monocytogenes* strains were maintained at 7°C on de Man Rogosa Sharpe (MRS, Oxoid, Oxoid Limited, Basingstoke, Hampshire, UK) slants or on Tryptone Soya Agar (TSA, Oxoid) slants, respectively, and revived by transferring a loop of inoculum into 5 ml MRS-broth (Oxoid) or Brain Heart Infusion (BHI, Oxoid) broth, respectively, followed by incubation at 30°C for 24h.

2.2. Model cooked ham

A model cooked ham (MCH) product was manufactured on a semi-industrial scale at Dera Food Technology N.V. (Bornem, Belgium). The recipe and production process are described before in chapter 2.

2.3. Co-culture experiments

In total, three different co-inoculation studies (Table 5.1) were performed on the MCH: (1) an interaction study at 7°C between 10A or LS5 and *L. monocytogenes* at an inoculum ratio of $10^5/10^2$ under vacuum packaging, (2) an interaction study at 7°C and 4°C between 10A or LS5 and *L. monocytogenes* at an inoculum ratio of $10^6/10^2$ under vacuum packaging (VP) and

(3) an interaction study at 7°C between 10A and *L. monocytogenes* at an inoculum ratio of $10^6/10^2$ under vacuum packaging and under modified atmosphere packaging (MAP). Each study was performed in triplicate.

Table 5.1. Overview of the different co-inoculation studies of this chapter

	Study 1	**Study 2**	**Study 3**
Target inoculation level of 10A or LS5	10^5	10^6	10^6
Temperature	7°C	4°C or 7°C	7°C
Packaging type	Vacuum	Vacuum	Vacuum or modified atmosphere
Different series within a co-inoculation study	Blank[a] LIS[b] 10A+LIS[c] LS5+LIS[d]	Blank LIS (7°C) LIS (4°C) 10A+LIS (7°C) 10A+LIS (4°C) LS5+LIS (7°C) 10A[e] (7°C) 10A (4°C)	Blank LIS (VP[f]) LIS (MAP[g]) 10A+LIS (VP) 10A+LIS (MAP) 10A (VP) 10A (MAP)

[a], Blank = non-inoculated MCH; [b], LIS = MCH inoculated with the cocktail of *L. monocytogenes*; [c], 10A+LIS = MCH inoculated with the cocktail of *L. monocytogenes* and *L. sakei* 10A; [d], LS5+LIS = MCH inoculated with the cocktail of *L. monocytogenes* and *L. sakei* LS5; [e], 10A = MCH inoculated with *L. sakei* 10A; [f], VP = vacuum packaging; [g], MAP = modified atmosphere packaging

The first co-inoculation study aimed to investigate whether both LAB, 10A and LS5, are useful to protect the MCH against growth of *L. monocytogenes.* The second co-inoculation study aimed to observe the effect of the application of a higher initial level of the protective cultures. Furthermore, also the influence of the storage temperature was studied here to assure that dominance of the protective culture remains when lowering the temperature from 7°C to 4°C. The third co-inoculation study was performed to find out whether a switch from VP to MAP influences the antagonistic activity of *L. sakei* 10A towards *L. monocytogenes.*

The inocula of 10A or LS5 and the *L. monocytogenes* strains were subcultured twice (24h, 30°C) in 5 ml MRS-broth or 5 ml BHI-broth, respectively. To reach the desired inoculation levels, appropriate dilutions in Peptone Physiologic Solution (PPS; 8.5 g/l NaCl (VWR, VWR International, Leuven, Belgium) and 1 g/l Peptone (Oxoid)) were prepared and 0.1 ml of each was divided over and spread on the surface of 5 slices (± 75 g/ 5 slices) of cooked ham. After

inoculation, slices were vacuum packaged or packaged in a modified atmosphere of 50% CO_2/ 50% N_2 with a gas/product ratio of 2/1 (5 slices/package) and stored at the appropriate temperature in a ventilated refrigerator for 35 or 42 days. Packaging was performed as described in chapter 2.

2.4. Microbiological analyses

At regular time intervals during storage, cooked ham samples were analysed for growth of the inoculated strains. Sampling and plating procedures were similar as in chapter 4. For the blank series, total aerobic psychrotrophic count, total anaerobic count, total lactic acid bacteria and number of yeasts and moulds were determined. To follow the protective cultures 10A and LS5 MRS-agar supplemented with sorbic acid (1.4 g/l) (Sigma, Sigma-Aldrich Corporation, St. Louis, Missouri, USA) (aerobic incubation for 48h at 30°C) was used. To determine the number of *L. monocytogenes* on the MCH, ALOA (Agar Listeria Ottaviani and Agosti) (Biolife, Biolife Italiana S.r.l, Milan, Italy) was used (aerobic incubation for 2 days at 37°C).

2.5. pH-measurements

For the MCH inoculated with *L. sakei* 10A alone, the pH was measured as a function of time in the same way as described in chapter 4.

2.6. Sensory analysis

On each day of analysis a sensory evaluation of the MCH, inoculated with *L. sakei* 10A, was performed by four panellists. The panel was asked to indicate if the samples had an acceptable or unacceptable (deviating) odour and taste. The sensory test was done under red lighting in a taste room with isolated booths.

2.7. Statistical analyses

Cell numbers were analysed for significant ($P<0.05$) differences between mono-culture growth and co-culture growth using independent samples *t*-tests in SPSS 11.0 (SPPS, Chicago, IL, USA).

3. Results and discussion

The chemical composition of the MCH was: 21.99 ± 0.25% of dry matter, 25.5 ± 0.7 ppm of residual nitrite, 2.48 ± 0.02 %of NaCl (on aqueous phase), a pH of 6.13 and a water activity of 0.984 ± 0.001.

The non-inoculated MCH had an initial total aerobic count and an initial LAB-count of <1.0 $\log_{10}$(cfu/g); no yeasts or moulds were detected. In all experiments, the number of endogenous LAB remained very low during storage and did not exceed 3.5 $\log_{10}$(cfu/g) after 42 days at 7°C. Consequently the initial LAB-count was much lower than the inoculation level of the protective cultures examined; this implies that the action of the protective cultures was not influenced by the native LAB-flora.

3.1. Influence of inoculum ratio

A comparison was made between the use of the potential protective cultures 10A and LS5 at levels of 10^5 or 10^6 cfu/g in the MCH and the influence this might have on the interaction phenomena between 10A or LS5 and *L. monocytogenes* and this at 7°C and under vacuum packaging. Figures 5.1 and 5.2 present the evolution of the counts of *L. monocytogenes* and LAB on the MCH during the experiments with the cultures 10A and LS5, respectively.

When 10A was applied to the MCH at a level of ± 10^5 cfu/g, the strain reached a level of 10^7 cfu/g between day 21 and day 28. From that moment on, a slightly but statistically significant ($P<0.05$) slower growth of the *L. monocytogenes* cocktail occurred when growing in co-culture with 10A compared to growth in absence of 10A. However, by this time the cocktail of *L. monocytogenes* had already increased with > 3 $\log_{10}$(cfu/g). Applying 10A at a higher level of ± 10^6 cfu/g limited the increase in counts of *L. monocytogenes* to <1 $\log_{10}$(cfu/g) over a 28 day period compared to an increase of >3 $\log_{10}$(cfu/g) when the cocktail was grown alone. After 27 days, the number of *L. monocytogenes* in co-culture with 10A seemed to increase slightly. However, after 42 days of storage a 2.5 $\log_{10}$(cfu/g) difference in growth was observed between the *L. monocytogenes* cocktail grown in co-culture with 10A and the *L. monocytogenes* cocktail grown alone.

When inoculated at approximately 10^6 cfu/g, 10A grew more rapidly on the MCH since the strain reached a cell number of about 10^8 cfu/g after 7 days. For a protective culture, a rapid growth to the number of cells that has an antagonistic action is very important.

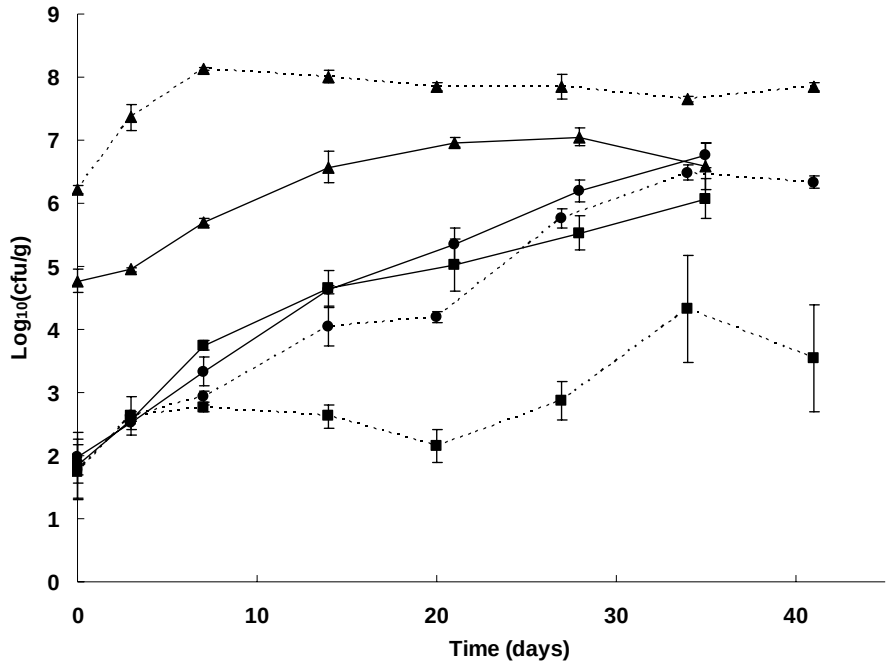

Figure 5.1. Interaction at 7°C between *L. sakei* 10A and *L. monocytogenes* on vacuum packaged MCH at an inoculum ratio of $10^5/10^2$ (full lines) or $10^6/10^2$ (broken lines) (•, growth of the *L. monocytogenes* cocktail; ■, growth of the *L. monocytogenes* cocktail in co-culture with 10A; ▲, growth of 10A in co-culture with the *L. monocytogenes* cocktail) (error bars represent 95% confidence intervals, n=3)

From this experiment and also from the interaction experiments of chapter 4 between *L. sakei* 10A and the spoilage organisms *Leuc. mesenteroides* and *B. thermosphacta*, it could be derived that inhibition by *L. sakei* 10A occurs when the strain reaches a level of ± 10^7 cfu/g. Whereas the strain has been selected for its fast growth at 7°C on the MCH (Chapter 2), growth up to the inhibitory level occurred more rapidly when starting at a level of 10^6 cfu/g. Bredholt et al. (1999) found, however, no difference in the inhibitory effect of a non-bacteriocinogenic strain of *L. sakei* on the growth of *L. monocytogenes* when two different inoculum levels, 10^4 and 10^6 cfu/g, were evaluated. In the study of Amezquita & Brashears (2002), the competitive inhibition of *L. monocytogenes* by a mix of three LAB was obtained when applying the cultures at a level of 10^7 cfu/g. In general, protective cultures are applied at levels varying between 10^5-10^7 cfu/g (Andersen, 1995a).

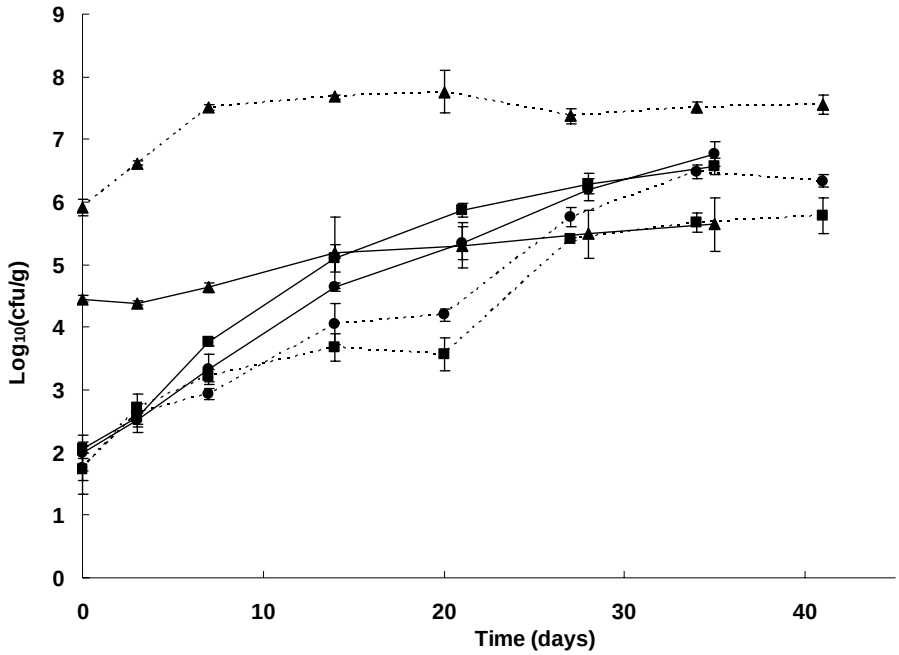

Figure 5.2. Interaction at 7°C between *L. sakei* LS5 and *L. monocytogenes* on vacuum packaged MCH at an inoculum ratio of $10^5/10^2$ (full lines) or $10^6/10^2$ (broken lines) (•, growth of the *L. monocytogenes* cocktail; ■, growth of the *L. monocytogenes* cocktail in co-culture with 10A; ▲, growth of 10A in co-culture with the *L. monocytogenes* cocktail) (error bars represent 95% confidence intervals, n=3)

In the case of co-inoculation with *L. sakei* LS5 a slightly lower (5×10^4 cfu/g) than the desired lower inoculation level (10^5 cfu/g) was reached. The desired higher inoculation level of 10^6 cfu/g was obtained. At each of the two investigated inoculation levels, the growth of *L. monocytogenes* was not inhibited by *L. sakei* LS5. At the lower inoculum ratio of $5\times10^4/10^2$ (LS5/*L. monocytogenes*) no significant differences in growth of *L. monocytogenes* between growth as a cocktail alone and growth as a co-culture of the cocktail with LS5 were observed. This was probably related to the application of a lower than desired inoculum which resulted in slow growth of LS5 on the MCH. During the 42 days storage period, LS5-levels did not exceed 10^6 cfu/g and the strain was consequently out grown by *L. monocytogenes*. At the higher inoculum ratio of $10^6/10^2$, growth of *L. monocytogenes* was significantly ($P<0.05$) slower when grown as a cocktail alone than when grown as a co-culture with LS5 from day 20 onwards. However, the difference in growth was very small and in both cases, *L. monocytogenes* grew to unacceptable counts. When starting from 10^6 cfu/g, growth of LS5 was, however, very rapid since LS5 reached a cell number of 5×10^7 cfu/g after

7 days and remained at that cell number during the subsequent storage period. These results confirm that *L. sakei* LS5 is not useful as a protective culture since the strain is not able to inhibit the growth of *L. monocytogenes*, even when applied at a higher level of 10^6 cfu/g. In the co-culture experiments of chapter 4, between LS5 and the spoilage organisms *Leuc. mesenteroides* and *B. thermosphacta*, similar results were obtained.

3.2. Influence of storage temperature

Rapid growth at refrigeration temperatures is an essential requirement for a protective culture that is meant for the biopreservation of refrigerated CMP. *L. sakei* 10A was selected because it is capable of growing at 4°C and 7°C in broth and for its ability to grow rapidly at 7°C on the MCH (Chapter 2). To examine whether strain 10A is still dominant on the MCH at 4°C, the interaction between 10A and *L. monocytogenes* was investigated at this lower temperature. A comparison of the interaction effects at 7°C and 4°C is presented in Figure 5.3.

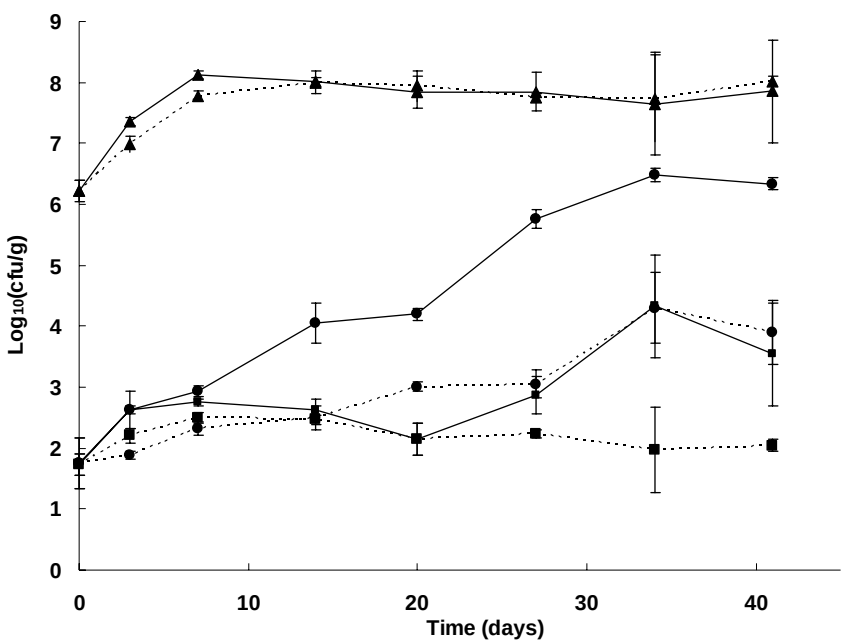

Figure 5.3. Interaction at 7°C (full lines) or 4°C (broken lines) between *L. sakei* 10A and *L. monocytogenes* on vacuum packaged MCH at an inoculum ratio of $10^6/10^2$ (●, growth of the *L. monocytogenes* cocktail; ■, growth of the *L. monocytogenes* cocktail in co-culture with 10A; ▲, growth of 10A in co-culture with the *L. monocytogenes* cocktail) (error bars represent 95% confidence intervals, n=3)

Clearly, the growth of *L. sakei* 10A was not influenced by lowering the temperature from 7°C to 4°C. At both temperatures, 10A grew rapidly (in 7 days) from the initial level of 6 $\log_{10}$(cfu/g) to a level of 7.5-8 $\log_{10}$(cfu/g), after which it remained at this level up to the end of storage. Most strains belonging to the *L. sakei/L. curvatus* group can grow at low temperatures and *L. sakei* is known to be one of the most psychrotrophic species of the lactobacilli since some of its strains can grow at 2-4°C (Champomier-Vergès et al., 2002). In contrast to the behaviour of *L. sakei* 10A, growth of the *L. monocytogenes* cocktail was strongly influenced by the storage temperature. At 4°C, its cell number increased with 2 $\log_{10}$(cfu/g) after 42 days. At 7°C, a level of 10^6 cfu/g was exceeded after approximately 30 days. *L. monocytogenes* is considered as a psychrotrophic pathogen and the minimum growth temperature varies from 0-5°C (Abee & Wouters, 1999). When *L. monocytogenes* was growing in co-culture with 10A, growth was strongly reduced at both temperatures. Whereas at 7°C limited growth of *L. monocytogenes* on the MCH still occurred, a complete inhibition of its growth by *L. sakei* 10A was observed at 4°C.

3.3. Influence of atmosphere in the headspace of the packaging

Although sliced CMP are often vacuum packaged, modified atmosphere packaging is nowadays in Europe a common packaging technique for this type of products. *L. sakei* 10A was selected on the basis of its growth characteristics on vacuum packaged MCH. To determine whether strain's dominance is maintained under MAP, the influence of CO_2 on the growth of *L. sakei* 10A was examined. For this experiment, a CO_2 level of 50% was chosen since CO_2 levels commonly used for CMP vary from 30-50%. The comparison between the interaction phenomena under VP and these under MAP is presented in Figure 5.4. The headspace CO_2 concentration of 50% did not influence the growth of *L. sakei* 10A. Under both packaging conditions (VP and MAP), 10A grew rapidly (in 7 days) from the initial level of 10^6 cfu/g to a level of nearly 10^8 cfu/g and this level was remained during the entire storage period. However, the growth of *L. monocytogenes* was strongly influenced by the packaging type. Under vacuum, its cell number increased with 4 $\log_{10}$(cfu/g) after 42 days, whilst under MAP no considerable growth was observed. It is known that *L. sakei* is less sensitive to modified atmospheres containing CO_2 than *L. monocytogenes* (Devlieghere, 2000). Under vacuum packaging, the antagonistic interaction of *L. sakei* 10A towards *L. monocytogenes* was once more confirmed. However, by analogy with the first co-inoculation study, a slight increase in the number of *L. monocytogenes* was observed after 30 days of storage resulting in

an increase in cell number of 2 $\log_{10}$(cfu/g) during the storage period. Combining the protective culture *L. sakei* 10A, applied at an initial level of 10^6 cfu/g, with MAP fully prevented the growth of *L. monocytogenes* on the MCH.

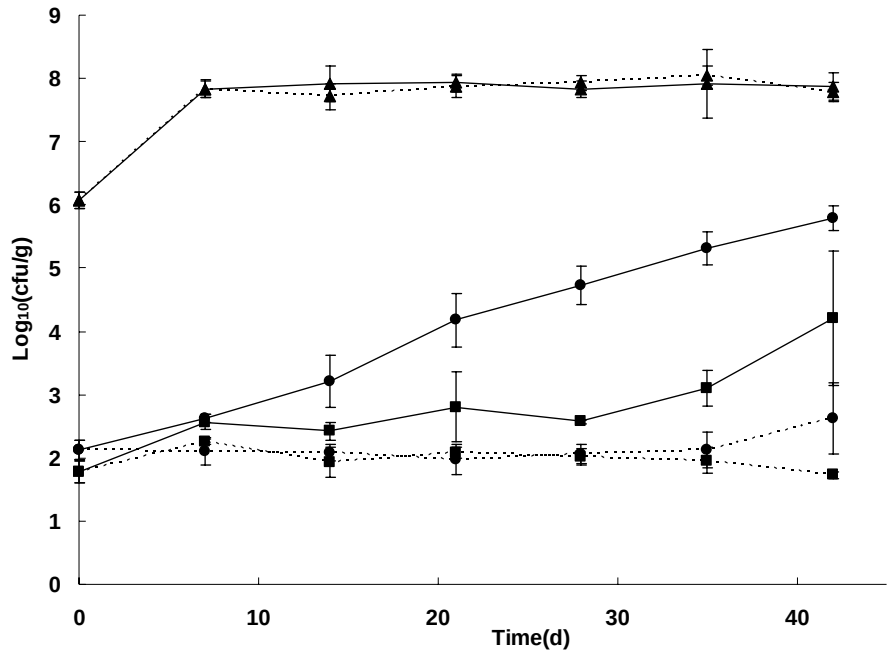

Figure 5.4. Interaction at 7°C between *L. sakei* 10A and *L. monocytogenes* on MCH under VP (full lines) or MAP (broken lines) at an inoculum ratio of $10^6/10^2$ (●, growth of the *L. monocytogenes* cocktail; ■, growth of the *L. monocytogenes* cocktail in co-culture with 10A; ▲, growth of 10A in co-culture with the *L. monocytogenes* cocktail) (error bars represent 95% confidence intervals, n=3)

3.4. Influence of 10A on the acidity of the MCH

pH-measurements and sensory evaluation of the MCH, inoculated with *L. sakei* 10A alone, were periodically done during the storage period. Table 5.2 shows the evolution of the pH of the MCH as a function of time during the growth of 10A in mono-culture at 4°C and 7°C (co-inoculation study 2) and under MAP and VP (co-inoculation study 3). The pH of the non-inoculated cooked ham (blank) did not change significantly (mean pH of 6.04 at day 35) compared to its initial pH-value. In all cases evaluated, the pH-change of the MCH containing *L. sakei* 10A was minimal, with a mean decrease of 0.35 pH-units over the entire storage period and this despite the fact that *L. sakei* 10A reached counts of 10^8 cfu/g after only seven days and remained at this level throughout the storage period.

Table 5.2. Evolution of the pH of the MCH during growth of *L. sakei* 10A in mono-culture at 4°C, 7°C, under VP and under MAP (mean ± standard deviation, n=3)

Study 2			Study 3		
Time (days)	**10A (7°C-VP)**	**10A (4°C-VP)**	**Time (days)**	**10A (7°C-VP)**	**10A (7°C-MAP)**
0	6.13 ± 0.00	6.13 ± 0.00	0	6.09 ± 0.01	6.09 ± 0.01
20	5.91 ± 0.05	5.91 ± 0.02	7	5.96 ± 0.01	5.94 ± 0.03
27	5.89 ± 0.04	5.85 ± 0.01	14	5.90 ± 0.02	5.84 ± 0.01
34	5.88 ± 0.01	5.83 ± 0.02	21	5.90 ± 0.01	5.88 ± 0.02
41	5.81 ± 0.02	5.82 ± 0.04	28	5.82 ± 0.03	5.83 ± 0.04
			35	5.77 ± 0.06	5.76 ± 0.03
			42	5.76 ± 0.01	5.72 ± 0.02

The small pH-drop was probably the result of the MCH having low glucose content (0.21 ± 0.06%) and large buffering capacity. The pH-measurements were reflected in the findings of the sensory analysis. Despite the extended storage period of 42 days, the panellists could not detect an unacceptable taste or odour in the MCH-samples containing *L. sakei* 10A. These findings confirm earlier results on the organoleptic evaluation of MCH containing high numbers of 10A (Chapter 2).

Since the presence of *L. sakei* 10A on the MCH, even at high numbers and for prolonged periods of time, did not result in significant acidification and sensory rejection of the MCH, the observed antagonistic activity towards *L. monocytogenes* cannot be caused by lactic acid production and the associated pH-decrease. According to the results of chapter 2, *L. sakei* 10A does not produce a bacteriocin. However, regrowth of the food born pathogen might suggest the diminution of an inhibitory substance (Amezquita & Brashears, 2002). The mechanism of the inhibition is therefore yet to be revealed. Probably, a more complex combined effect of production of antimicrobials and competition for or depletion of specific nutrients might explain the protective effect of this culture. In agreement with the results of this chapter, Buchanan & Klawitter (1992) demonstrated that the inhibition of *L. monocytogenes* Scott A was not a function of acid production by *Carnobacterium piscicola* LK5 in co-cultures of the two isolates grown in tryptose soya broth with and without glucose. It is possible that depletion of other nutrients like vitamins, minerals, trace elements or peptides caused the antagonistic interaction (Buchanan & Bagi, 1997). Recently, Nilsson et al. (2005) demonstrated that a non-bacteriocinogenic *C. piscicola* reduced growth of *L. monocytogenes*, at least partly, by depletion of glucose since they observed that the

inhibitory effect of glucose was abolished by the addition of glucose but not by the addition of amino acids, vitamins or minerals.

4. Conclusion

Lactobacillus sakei 10A offers opportunities as biopreservative since this protective culture can improve the safety of cooked meat products by inhibiting the growth of contaminating *Listeria monocytogenes* cells. The antagonistic interaction of *L. sakei* 10A towards *L. monocytogenes* was proven to occur on a model cooked ham product at 4°C, at 7°C, under vacuum packaging and under modified atmosphere packaging. The combination of the biopreservative *L. sakei* 10A and a storage temperature of 4°C or 10A and a modified atmosphere containing 50% of CO_2 fully prevented growth of *L. monocytogenes.* In addition, *L. sakei* 10A had no impact on the sensory quality of the model cooked ham, increasing its potential application for the control of *L. monocytogenes* growth in ready-to-eat meat products. Further validation studies, needed to explore the usefulness of *L. sakei* 10A in several types of real cooked meat products, will be the subject of chapter 6.

Chapter 6

The sensory acceptability of cooked meat products treated with a protective culture depends on glucose content and buffering capacity: a case study with *Lactobacillus sakei* 10A

Redrafted after

L. Vermeiren, F. Devlieghere, I. Vandekinderen, U. Rajtak and J. Debevere (2006)
Meat science, 74, 532-545

Chapter 6
The sensory acceptability of cooked meat products treated with a protective culture depends on glucose content and buffering capacity: a case study with *Lactobacillus sakei* 10A

Summary

Biopreservation has been proven to be a promising natural preservation technique, but the impact of protective cultures on the sensory properties of cooked meat products (CMP) is not well documented. This chapter presents a case study on the protective culture *Lactobacillus sakei* 10A to obtain a clear view on the real consequences of using protective cultures on the sensory quality of CMP. A preliminary screening study on 13 different CMP and more elaborate application trials at 7°C on vacuum packaged pâté, cooked ham, cooked sausage and two cooked poultry products demonstrated that *L. sakei* 10A inhibits the growth of endogenous lactic acid bacteria (LAB) and of artificially inoculated *Leuconostoc mesenteroides*, *Brochothrix thermosphacta* and *Listeria monocytogenes* cells. Despite these promising antagonistic effects, the application of *L. sakei* 10A to CMP was in some cases limited by a significant acidification resulting in an acid taste of the product. This was most obvious in pâté and cooked sausage and less obvious in cooked turkey fillet. From the results a hypothesis could be derived that high buffering capacity and low glucose content are key elements to avoid sensory deviations when applying protective cultures on CMP.

1. Introduction

During the last decades, research on preservation strategies of (micro)biological origin has flourished. In particular, the use of lactic acid bacteria (LAB) and/or their metabolites (e.g. lactic acid, bacteriocins, etc.) for use in biopreservation of foods has gained increasing attention (Lücke, 2000; Rodgers, 2001; Devlieghere et al., 2004). Although some successful studies were published (Aymerich et al., 2002; Jacobsen et al., 2003), the effectiveness of bacteriocin-producing strains in foods can be limited by a range of factors (Holzapfel et al., 1995; Rodriguez et al., 2002). Furthermore, concerns have been raised with respect to possible resistance development (Ennahar et al., 2000) as a consequence of widespread use of these peptides as food preservatives, although this is not generally accepted in literature (Cleveland et al., 2001). Recently, some studies have demonstrated that LAB, that do not produce bacteriocins, are capable of controlling microbial growth in food products (Nilsson et al., 1999) and more specifically in refrigerated, anaerobically packaged, sliced and cooked meat products (CMP) (Kotzekidou & Bloukas, 1996; Kotzekidou & Bloukas, 1998; Bredholt et al., 1999; Bredholt et al., 2001; Amezquita & Brashears, 2002; Chapters 4 and 5). In CMP, protective cultures have mainly been evaluated for their potential to inhibit food born pathogens such as *Listeria monocytogenes* and less is known about the possible use of protective cultures for controlling spoilage. It is important in the development of a protective culture that the assessment of its influence on the sensory characteristics of the treated products is reviewed; few authors have dealt with this subject. Bredholt et al. (2001) observed no statistically significant differences in consumer preferences after 11 days at 4°C between cooked ham and cervelat with and without *Lactobacillus sakei* TH1 and little sensory differences between treated and untreated products after 28 days at 4°C. Amezquita & Brashears (2002) found positive results for the bacteriocin-producing *Pediococcus acidilactici* on frankfurters after 56 days at 5°C but did not investigate the effect of the more acidifying non-bacteriocinogenic *Lactobacillus casei* and *Lactobacillus paracasei.* In the study on pariza of Kotzekidou & Bloukas (1998), the control treatment had an unacceptable sour taste after 4 weeks, whereas the treatments with *L. sakei* BJ-33 had an acceptable odour and taste until 7-8 weeks of storage at 6-8°C. Despite the sour taste, the pH of the control treatment was the highest of all treatments and decreased only from 6.7 to 6.1 after 4 weeks. The authors assigned the negative sensory quality of the control to heterofermentative LAB producing organic acids of which in particular acetic acid is unwanted (Kotzekidou & Bloukas, 1998).

This hypothesis still does not agree with the pH-evolution of the product; it might be that other types of metabolism were causing the spoilage.

The existing studies do not clearly demonstrate whether protective cultures have a positive or negative impact on the sensory properties of the treated product and how a negative impact can be explained and avoided. Therefore the objective of this chapter was to validate the biopreservative capacity of protective cultures on several industrially manufactured cooked meat products and to investigate the effect of protective cultures on the sensory quality of these CMP. The non-bacteriocinogenic *L. sakei* 10A, that has been found capable of controlling spoilage in chapter 4 and inhibiting growth of *L. monocytogenes* in chapter 5 on a vacuum packaged model cooked ham without altering the sensory properties of this product, was chosen as a case study. The first part of this work was a preliminary screening study of 13 different CMP for their suitability to be treated with culture 10A. Of most importance was the determination of the glucose content, the buffering capacity and the effect of 10A on the pH and the related acid taste of the product. In the second part of this work, more elaborate storage experiments at 7°C on vacuum packaged pâté, cooked ham, cooked sausage and two cooked poultry products were conducted. The effect of *L. sakei* 10A (applied at 10^6 cfu/g) on the growth of (1) the natural spoilage flora, (2) inoculated (100 cfu/g) *Leuc. mesenteroides*, (3) inoculated (100 cfu/g) *B. thermosphacta* and (4) inoculated (100 cfu/g) *L. monocytogenes* was investigated and the relationship between microbial growth, acidification, glucose consumption, lactic acid production and sensorial appreciation was assessed.

2. Materials and methods

2.1. Bacterial strains

The protective culture *Lactobacillus sakei* subsp. *carnosus* (coded here as 10A) was isolated from vacuum packaged, sliced, cooked and smoked turkey fillet in chapter 2. In the inoculation experiments with *Listeria monocytogenes*, a mix of three *L. monocytogenes* strains was used. Strains *L. monocytogenes* Scott A (coded here as LIS 1) and *L. monocytogenes* LFMFP 45 (coded here as LIS 3), isolated from cooked pita meat, were from the Laboratory of Food Microbiology and Food Preservation (LFMFP, Gent University, Belgium). Strain *L. monocytogenes* LMG 13305 (coded here as LIS 2), isolated from soft cheese, was obtained from the culture collection of the Laboratory Microbiology Gent (LMG, Gent University, Belgium). In the inoculation experiments with *Leuconostoc mesenteroides*, a

mixture of *Leuc. mesenteroides* LM2 (LMG 6893) and LM4 (LFMFP 666) was used. In the inoculation experiments with *Brochothrix thermosphacta*, a mixture of *B. thermosphacta* BT1 (LFMFP 230) and BT2 (LFMFP 227) was used. The latter four strains were previously described in chapter 4. Working cultures of the strains were maintained on de Man Rogosa Sharpe (MRS, Oxoid, Oxoid Limited, Basingstoke, Hampshire, UK) agar or Tryptone Soya Agar (TSA, Oxoid) slants at 7°C and revived by transferring a loop of inoculum into 5 ml MRS-broth (Oxoid) or into 10 ml Brain Heart Infusion broth (BHI, Oxoid) followed by incubation at 30°C (all strains except BT1 and BT2) or 22°C (BT1 and BT2) for 24h.

2.2. Chemical analyses

The pH was measured using a pH-electrode (Ingold, MGDX K57, Urdorf, Switzerland) connected to a pH-meter (Knick, type 763, Berlin, Germany). Salt content was determined according to the method of Mohr, a titrimetric determination of chloride ions (Skoog et al., 1996). Water activity was measured using an a_w-cryometer (Nagy AWK-20, Gäufelden, Germany). Fat, protein and phosphate levels were determined according to respectively ISO 1444, ISO 937 and ISO 1871, and ISO 13730. Moisture and ash were determined according to standard AOAC (1990) procedures.

Glucose was determined by a spectrophotometric method with an enzymatic reagent (glucose oxidase-peroxidase (GOP) reagent). This reagent contains two enzymes (glucose oxidase (Sigma, Sigma-Aldrich Corporation, St. Louis, Missouri, USA) and peroxidase reagent (Sigma)) (Karkalas, 1985). Glucose is first oxidized to gluconic acid and hydrogen peroxide. This peroxide reacts immediately with phenol (Acros Organics, Geel, Belgium) and 4-aminoantipyrine (Acros Organics) in the presence of peroxidase, so that quinoneimine is formed. This complex has an intense pink colour. Finally, the absorbance was measured at 505 nm against a reference (1 ml distilled water plus GOP-reagent) (Cary 50 UV-Vis, Varian, Sint-Katelijne-Waver, Belgium).

Lactic acid and sugars other than glucose were determined using a high-performance liquid chromatograph (HPLC). Details of this HPLC-analysis have been described in chapter 2. Prior to HPLC-analysis, meat samples were subjected to a ¼ extraction: a 25 g sample was homogenised with 75 ml of distilled water and filtered (∅ 125 mm, Schleicher & Schuell, Microscience, Dassel, Germany). In the case of sugar analysis, further dilutions in distilled water were prepared if necessary to ensure that the concentration was within the linear range (up to 160 mg/l). In the case of lactic acid analysis, the filtrate was heated for 15 min at 80°C

in a water bath, centrifuged at 8000g for 10 min and filtered (∅ 0.2 µm, Alltech Associates, Lokeren, Belgium) before injection.

Buffering capacity was determined by adding 8.5 % (w/w) of lactic acid (Sigma) in steps of 500 µl to a 90 g mixed and homogenised sample. After each addition, the meat sample was very well homogenised and the pH was measured on three positions in the sample. The procedure was continued until a pH value of 5 was obtained and repeated twice.

2.3. Microbiological analyses

A 15 g sample was taken aseptically and a decimal dilution series in Peptone Physiologic Solution (PPS; 8.5 g/l NaCl (VWR, VWR International, Leuven, Belgium) and 1 g/l Peptone (Oxoid)) was prepared to plate the appropriate dilutions on the appropriate agar media. Total aerobic psychrotrophic count, total anaerobic count and lactic acid bacteria were determined, according to the pour plate technique, on Plate Count Agar (PCA, Oxoid) (aerobic incubation at 22°C for 3-5 days), Reinforced Clostridial Agar (RCA, Oxoid) (anaerobic incubation at 37°C for 3-5 days) and de Man Rogosa Sharpe (MRS, Oxoid) agar (aerobic incubation at 22°C for 3-5 days), respectively. Yeasts and moulds were determined on Yeast Glucose Chloramphenicol Agar (YGC, Bio-Rad, Bio-Rad Laboratories Inc., Hercules, CA, USA) (aerobic incubation at 22°C for 3-5 days) according to the spread plate technique. The number of *L. monocytogenes* was determined on ALOA (Agar Listeria Ottaviani and Agosti) (Biolife, Biolife Italiana S.r.L., Milan, Italy) (aerobic incubation for 2 days at 37°C) supplemented with ALOA Enrichment selective supplement (Biolife). For the determination of the number of *B. thermosphacta* STAA-agar (Oxoid) (aerobic incubation for 2-3 days at 22°C) supplemented with STAA (Streptomycin sulphate, Thallous acetate, Actidione, Agar) selective supplement (Oxoid) was used.

2.4. Sensory analyses

Samples for sensory analyses were transferred in plastic recipients that were closed and stored at 4°C until sensory evaluation. Time between sampling and sensory evaluation was not longer than 1 hour. All sensory evaluations were performed under IR-light in a special room with individual booths.

Two types of sensory tests were used in this work. A paired comparison test (preference test) was used to investigate whether the CMP with the protective culture 10A tasted better or worse than the CMP without protective culture. The two samples were offered simultaneously

to a sensory panel of 18 non-trained persons. The order of the samples was changed systematically: orders A-B and B-A were occurring equally.

In the more elaborate shelf-life studies, this preference test was combined with a scoring test. Samples were evaluated by a 6-member sensory panel on the attributes odour and taste. The panel members were trained on the sensory characteristics of fresh and spoiled products and were experienced in tasting CMP (Chapters 2 and 3). Panel members were asked to first open the recipient and evaluate the odour. Thereafter, samples were taken out of the recipient and evaluated for taste. Attribute scales for odour and taste varied from 1-9 with 1 being fresh, 5 the limit of acceptability and 9 spoiled. A score above 5 indicated the sample being unacceptable. Finally, the 6-member panel was asked to evaluate the fitness for human consumption. If four of six persons considered a sample unfit, the sensory quality was considered to be rejected.

2.5. Screening of cooked meat products

Thirteen anaerobically packaged CMP (Table 6.1) were obtained from three different Belgian meat processing companies (B, G and S).

Table 6.1. Overview of the chemical parameters characterising the cooked meat products used in the screening study

Product	pH	Dry matter (%)	NaCl (% on water phase)	Water activity	Protein (%)	Phosphate (%)	Fat (%)
Cooked ham B1	6.36	27.49	2.95	0.9722	16.0	0.63	2.8
Cooked ham G1	6.29	26.82	2.67	0.9783	21.6	0.48	2.6
Cooked ham G2	6.27	25.74	2.37	0.9787	20.3	0.58	2.8
Meat Loaf S	6.30	40.31	4.07	0.9638	13.4	0.44	14.6
Saucisson de Strasbourg S	6.30	37.90	3.33	0.9787	11.8	0.39	21.0
Cooked chicken fillet B	6.56	27.18	2.55	0.9720	18.6	0.60	2.4
Cooked turkey fillet B	6.01	27.00	2.29	0.9772	20.2	0.60	1.8
Cervelat sausage S	6.03	40.95	4.31	0.9656	14.5	0.59	20.4
Veal sausage S	6.15	51.27	4.22	0.9753	7.7	0.16	36.6
Cooked sausage S1 (Parijzerworst)	6.29	33.62	3.57	0.9771	13.1	0.36	14.7
Cooked sausage S2 (Lunchworst)	6.06	48.19	3.69	0.9742	10.7	0.22	29.6
Cooked sausage S3 (Hespeworst S)	6.19	38.23	3.33	0.9803	10.4	0.22	19.0
Cooked sausage G1 (Hespeworst G)	6.07	39.91	3.97	0.9754	12.1	0.41	18.3

Immediately after production, products were transferred to the laboratory in cooled conditions and stored between 0-2°C up to the start of the screening experiment. Some of the products were whole products, whereas others were sliced. In a first step, products were analysed for glucose content, presence of other sugars than glucose and buffering capacity. Secondly, the products were inoculated with *L. sakei* 10A at 10^6 cfu/g and the influence of the strain on pH-evolution and off-flavour development was determined after storing them under vacuum for 11 days at 7°C. As a reference, a product without protective culture was used. On day 0 and on day 11, the pH and the microbial contamination (total aerobic psychrotrophic count and total number of LAB) of the products with and without protective culture were determined. Furthermore, on day 11, a taste panel was asked whether they preferred the reference product or the product containing *L. sakei* 10A according to the preference test described in section 2.4.

2.6. Storage experiments

Five CMP were selected for a more elaborate storage experiment: pâté, cooked ham, cooked sausage, cooked chicken fillet and cooked turkey fillet. Each storage experiment consisted of eight test series: (1) non-inoculated product or reference product (control), (2) product inoculated with *L. sakei* 10A (10^6 cfu/g) (10A), (3) product inoculated with 100 cfu/g of *Leuc. mesenteroides* LM2 and LM4 , (4) product inoculated with *L. sakei* 10A (10^6 cfu/g) and 100 cfu/g of *Leuc. mesenteroides* LM2 and LM4, (5) product inoculated with 100 cfu/g of *B. thermosphacta* BT1 and BT2, (6) product inoculated with *L. sakei* 10A (10^6 cfu/g) and 100 cfu/g of *B. thermosphacta* BT1 and BT2, (7) product inoculated with 100 cfu/g of the *L. monocytogenes* cocktail LIS1, LIS2 and LIS3 and (8) product inoculated with *L. sakei* 10A (10^6 cfu/g) and 100 cfu/g of the *L. monocytogenes* cocktail LIS1, LIS2 and LIS3. In this way the effect of 10A on the natural spoilage flora, on the artificially inoculated spoilage organisms *Leuc. mesenteroides* and *B. thermosphacta* and on the food born pathogen *L. monocytogenes* could be determined. Each test series was performed in duplicate.

The different inocula were subcultured twice (24h) in MRS-broth for the LAB (30°C) or BHI-broth for *L. monocytogenes* (30°C) and *B. thermosphacta* (22°C). Cells of 10A were harvested by centrifugation at 8000*g* for 10 min at 10°C, washed with a 0.85% salt solution and resuspended in fresh MRS-broth before further diluting to prepare the inoculum of 10A. The other strains were not washed before inoculation. The inoculum was divided over and spread on the surface of 200 g of product to reach the desired inoculation level. After

inoculation, 200 g portions of product were vacuum packaged and stored at 7 ± 1°C in a ventilated refrigerator. Packaging was performed using a Multivac A300/42 (Hagenmüller, Wolfertschwenden, Germany) gas packaging machine in a high barrier film (NX90, Euralpak, Wommelgem, Belgium) of 90 µm thickness with an oxygen transmission rate of 5.2 ml/m^2.24h.atm at 23°C and 85% relative humidity.

At regular time intervals, products were analysed for growth of the endogenous flora and/or the inoculated strains. All series were analysed for total aerobic psychrotrophic count, total anaerobic count, total LAB and number of yeasts and moulds. The series inoculated with *L. monocytogenes* or *B. thermosphacta* were additionally analysed for the number of *L. monocytogenes* or *B. thermosphacta.* At several points in time colonies were picked from MRS-plates to determine the carbohydrate fermentation profile using the API-system with API50CHL-medium for LAB (BioMERIEUX, Brussels, Belgium).

Simultaneously with the microbial analyses pH-measurements were performed. On the first and the final storage day, an additional sample was taken for determination of lactic acid and glucose. Furthermore, on each day of analysis, both types of sensory tests were performed on all test series except those inoculated with *L. monocytogenes*. For each product, analyses continued up to the moment that samples became unacceptable from a sensory point of view as described in section 2.4.

2.7. Statistical analyses

All statistical analyses were performed using the software SPSS 12.0 (SPSS, Chicago, IL, USA).

Data of the screening experiment were analysed for relationships between the parameters for the 13 different CMP. The existence of a linear relationship was investigated using the Pearson correlation between (1) buffering capacity and phosphate content and protein content and (2) pH-decrease in products containing 10A and glucose and buffering capacity. The relationship between the binomially distributed preference data of the sensory analysis and the normally distributed parameters glucose content, buffering capacity and pH-decrease in 10A-products was investigated using a logistic regression method (model expressing the dependent variable 'preference for product with 10A' as a function of the co-variates glucose, buffering capacity and pH-decrease on products with 10A).

In the storage experiment, duplicate data on microbial cell numbers, pH, glucose and lactic acid concentrations were analysed for significant ($P<0.05$) differences (1) between

biopreserved samples and control samples on each day of analysis using an independent samples *t*-test ($P<0.05$) and (2) between day 0 and the final storage day for both, biopreserved and control samples, using a paired-samples *t*-test ($P<0.05$). The results from the paired comparison test were analysed according to a two-sided binomial test. In the case of 18 assessors, the critical number of answers to obtain a statistically significant (level of significance = 0.95) preference for one of both samples is 14 (Brinkman, 2002). The results from the scoring test of each of the five meat products were analysed for significant differences ($P<0.05$) between biopreserved samples and control samples by subjecting scores obtained for each attribute at each day of analysis to a paired-samples *t*-test.

3. Results and discussion

3.1. Screening of cooked meat products

Evaluation of buffering capacity through acidification with lactic acid resulted in a titration curve for the 13 CMP. Van Slyke (1922) defined a ratio β to calculate buffering capacity in a defined pH-range. This ratio expresses the relationship between the increment of acid and the change in pH:

$$\beta = -\frac{d\mathrm{B}}{d\mathrm{pH}}$$

where *d*B is the number of moles of acid added and *d*pH is the pH-change (Salaün et al., 2005). This ratio can be calculated for each pH-value of the titration curve. Within the pH-interval 5 to 6, the titration curves were approximately linear (data not shown) and the ratio at each pH-value was more or less the same. The mean value of the different ratios within the range pH 5 to 6 was calculated and used as a measure to compare the buffering capacity of the different CMP. Figure 6.1 summarises the buffering capacity and glucose content of the 13 CMP and of the model cooked ham product used in chapters 2 to 5. This model product can be used as a reference product to compare with in the discussion of these two parameters.

The buffering capacity of all products was lower than that of the model cooked ham. Cooked turkey fillet, cooked chicken fillet, cooked ham G1 and cervelat sausage constituted a group with a relative high buffering capacity varying between 6 and 7 mmol lactic acid per pH-unit.

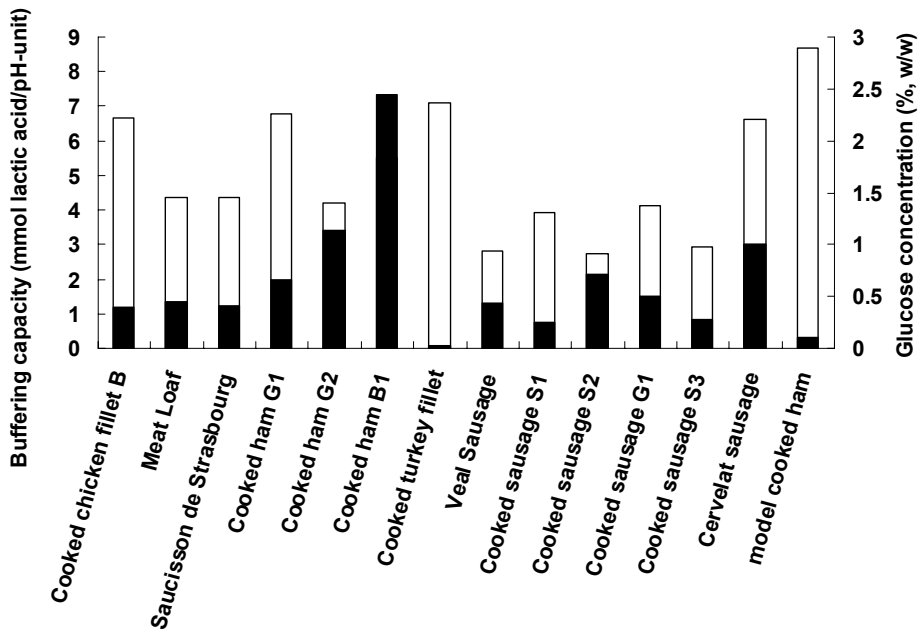

Figure 6.1. Buffering capacity (total bars) and glucose concentration (black part of the bars) of the cooked meat products implicated in the screening study

An intermediate group comprising meat loaf, saucisson de Strasbourg, cooked ham G2 and B1, cooked sausage S1 and G1 had buffering capacities of 4 to 5 mmol lactic acid per pH-unit. A last group consisting of veal sausage, cooked sausage S2 and cooked sausage G2 was characterised by a low buffering capacity varying between 2 and 3 mmol lactic acid per pH-unit. The buffering capacity of a product corresponds to the ability of a product to be acidified or alkalinised. Food products contain several constituents that are responsible for buffering capacity. These constituents are the small compounds and proteins containing one or several acid-base groups (Salaün et al., 2005). Positive significant Pearson correlations at the 0.01 level were found between buffering capacity (BC) and protein and phosphate contents (BC-protein: 0.794; BC-phosphate: 0.843); the higher the protein content or the phosphate content, the higher the buffering capacity of the meat product.

All products, except the cooked turkey fillet, contained more glucose than the model cooked ham product. Cooked ham B1 had a high glucose content of 2.44% while the cooked turkey fillet had a low glucose content of 0.02%. For the other products, glucose content ranged from 0.25 to 1.14%. Most products had glucose contents between 0.40 and 0.60%. Glucose that is

present in CMP can originate from either the raw meat ingredient(s) or the non-meat ingredients. In some recipes of CMP, glucose is added directly as dextrose or as dextrose syrup or indirectly as a carrier material for flavours, etc.

None of the 13 products contained detectable levels of sugars other than glucose. This was confirmed by the HPLC-chromatogram of the meat extracts (results not shown).

In the second part of the screening study, CMP were inoculated with *L. sakei* 10A and the influence of its presence on the pH and possible off-flavour development was investigated. The overall objective of this experiment was to find out if any relationship exists between potential sensory deviations caused by *L. sakei* 10A and the buffering capacity and glucose content determined in the first part of the study. The CMP had an initial count of LAB varying between 1.0 and 4.5 $\log_{10}$(cfu/g) with a mean contamination level of 2.5 ± 1.0 $\log_{10}$(cfu/g). In all cases, the total aerobic psychrotrophic count and LAB-count were of similar magnitude. All CMP were inoculated with *L. sakei* 10A to obtain the desired inoculated level of 10^6 cfu/g; LAB-counts of the inoculated products varied from 5.82 to 6.62 $\log_{10}$(cfu/g). On all types of CMP, *L. sakei* 10A grew very well. After 11 days of storage at 7°C, the products inoculated with 10A had LAB-counts varying between 7.88 and 8.90 $\log_{10}$(cfu/g) while LAB-counts of the reference products, not inoculated with 10A, had levels varying between 5.52 to 8.86 $\log_{10}$(cfu/g) (results not shown).

The pH of the reference and inoculated products decreased as a function of time. In all products, the pH-decrease from day 0 to day 11 was larger for the products inoculated with *L. sakei* 10A (Figure 6.2).

After 11 days of storage, 18 people were asked to indicate their sensory preference (taste): product with *L. sakei* 10A or reference product. For most CMP, except two, less then 14 out of 18 persons had a preference for one of the products indicating that there was no significant difference in taste on day 11 between the reference product and that containing *L. sakei* 10A. However, for two CMP, a significant preference for the reference product was observed. For veal sausage and cooked sausage S3, 17 and 14 panellists, respectively, preferred the reference product. For these two CMP, the product containing 10A had very low pH's of 4.79 and 5.05, respectively, after 11 days at 7°C. This low pH explains why most preferred the reference sample of these products. It should be noted that the results of this sensory evaluation represented a picture at a given moment (day 11). On day 11, cooked ham B1 and cervelat sausage had LAB-counts lower than 7 $\log_{10}$(cfu/g), 5.52 and 6.97 $\log_{10}$(cfu/g) respectively, indicating that for these two CMP the reference samples were not at the stage

where LAB-growth results in sensory changes. This might have influenced the results of the preference test for these two products.

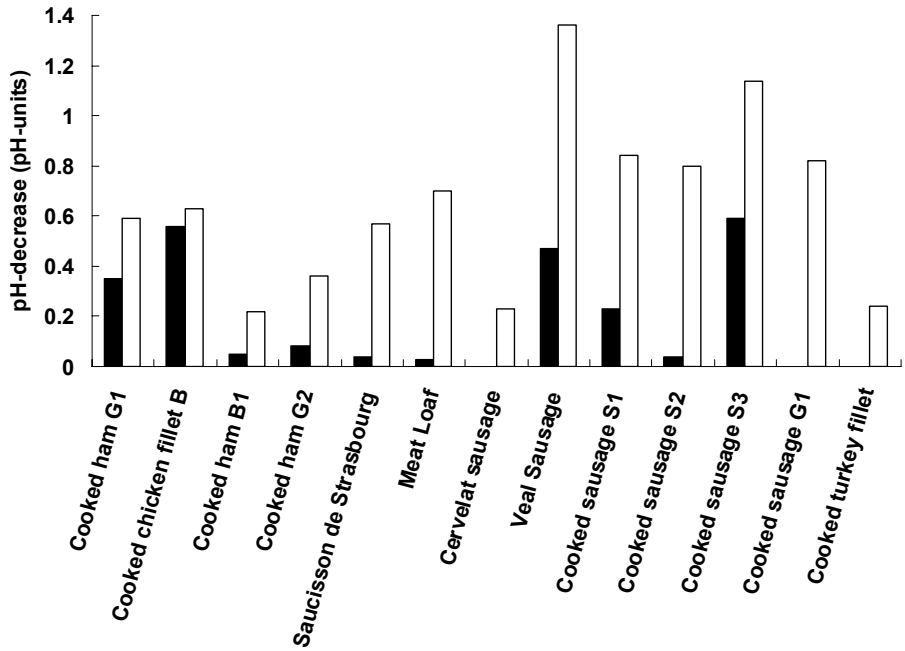

Figure 6.2. The pH-decrease from day 0 to day 11 of the cooked meat products in the screening study without (black bars) and with the protective culture *L. sakei* 10A (white bars)

The existence of a linear relationship between glucose content, buffering capacity and pH-drop on products with 10A after 11 days was investigated. A significant negative Pearson correlation at the 0.01 level (-0.739) was found between the pH-decrease from day 0 to day 11 in case of inoculation with 10A and the buffering capacity. The lower the buffering capacity of a product, the more the pH of the product decreases when the protective culture *L. sakei* 10A is applied. No clear linear relationship (Pearson correlation of –0.447) could be observed between glucose content and pH-decrease of the product inoculated with *L. sakei* 10A since this was dependent on the buffering capacity. In some products, e.g. cooked ham B1 and cervelat sausage, the glucose content was high, 2.44 % and 1.01% respectively, while the pH-decrease in 11 days through the presence of 10A was limited, 0.22 and 0.23 pH-units respectively. These two products were, incidentally, products having a high buffering capacity. In other products, e.g. cooked sausage G1 and S1, the glucose content was low, 0.28% and 0.25% respectively, while the pH-decrease through the presence of 10A was high,

0.82 and 0.84 pH-units respectively. These two products were products with a low buffering capacity.

Additional analysis, attempting to link preference data to the parameters glucose, buffering capacity and pH-drop on 10A-products, revealed that only 'pH-decrease on products with 10A' significantly influenced the preference for products containing 10A. This means that the preference for meat products treated with 10A is a function of the decrease in pH, caused by the presence of 10A, and there is no relationship between preference and glucose content or buffering capacity of the product.

From this study we could suggest that high buffering capacity and low glucose content are crucial to avoid sensory changes when applying protective cultures on CMP. To confirm our hypothesis and to further understand the role of these two parameters in the acidification of products treated with a protective culture more extended storage experiments were performed.

3.2. Storage experiments

For the more elaborate storage experiments, five products were chosen with different buffering capacities and glucose contents. An overview of the characteristics of these products is listed in Table 6.2. The effect of *L. sakei* 10A on the growth of the natural spoilage flora and on the growth of inoculated *Leuc. mesenteroides*, *B. thermosphacta* and *L. monocytogenes* strains was investigated and the relationship between microbial growth, acidification, glucose consumption, lactic acid production and sensorial appreciation was assessed.

Table 6.2. Chemical parameters characterising the cooked meat products investigated in the shelf-life studies

Product	pH	a_w	DM[a] (%)	NaCl[b] (%)	Fat (%)	Total phosphate (%, as P_20_5)	Lactic acid (%)	Glucose (%)	BC [c] (mmol lactic acid/pH-unit)
Pâté	6.43	0.9644	48.11	3.77	31.3	0.49	1.24	1.46	2.87
Cooked ham	6.30	0.9831	27.03	2.49	2.4	0.51	0.93	0.69	5.71
Cooked sausage	6.05	0.9778	39.91	3.97	19.6	0.36	0.59	0.31	4.12
Cooked chicken fillet	6.25	0.9797	27.41	3.11	4.8	0.70	1.01	0.40	6.65
Cooked turkey fillet	6.24	0.9701	24.89	2.81	1.1	0.61	2.87	0.09	7.09

[a], DM = dry matter; [b], in water phase; [c], BC = buffering capacity

3.2.1. Antagonistic activity of *L. sakei* 10A

Figure 6.3 presents for four out of five tested products (1) the growth of LAB on the reference samples and on the samples containing *L. sakei* 10A and (2) the growth of the *L. monocytogenes* cocktail on the samples inoculated with the *L. monocytogenes* cocktail alone and on the samples inoculated with the *L. monocytogenes* cocktail and *L. sakei* 10A .

In the reference samples of all five products, total aerobic psychrotrophic count and LAB-count were similar. In the reference samples, the rate of LAB-growth differed among the products depending on the initial contamination level and the intrinsic parameters of the products (Table 6.2). In the cooked turkey fillet, growth of endogenous LAB was retarded, probably due to the high concentration of lactic acid (2.87 ± 0.29%). High lactic acid contents may indicate that lactate is added during the preparation of the product. The inhibitory effect of salts of lactic acid on microbial growth has been reported and Na-lactate addition results in the shelf-life extension of anaerobically packaged CMP (Houtsma et al., 1993; Devlieghere et al., 2000; Stekelenburg & Kant-Muermans, 2001). The cooked ham was spoiled most rapidly as this product had the highest water activity and lowest salt content. Moreover, by identifying the endogenous LAB-flora on the final day of storage, it was seen that the LAB-flora of this cooked ham was dominated by a *Leuc. mesenteroides* strain and the results of chapter 3 demonstrate that this species has a high potential to cause rapid spoilage on CMP. On the final storage day, the LAB-flora of the other four products consisted of strains belonging to the *L. sakei/L. curvatus* group.

The LAB-count of the 10A-samples consisted of the endogenous LAB and the inoculated *L. sakei* 10A. The antagonistic effect of 10A on the endogenous LAB could not be assessed using traditional culture techniques. As long as the endogenous LAB were present in a lower number than the inoculated *L. sakei* 10A, dominance of 10A was expected and was shown by determination of API-profiles of isolated colonies. When the LAB-count of the reference samples reached the same level as the LAB-count of the 10A-samples, differentiation with *L. sakei* 10A using API-profiles was not always possible as the endogenous LAB consisted mainly of strains belonging to the *L. sakei/L. curvatus* group. Only in the study with cooked ham, the endogenous LAB mainly consisted of a *Leuc. mesenteroides* strain. On the final storage day on the cooked ham samples inoculated with 10A, the LAB-flora consisted completely of the protective culture 10A and any colony was identified as *Leuc. mesenteroides*.

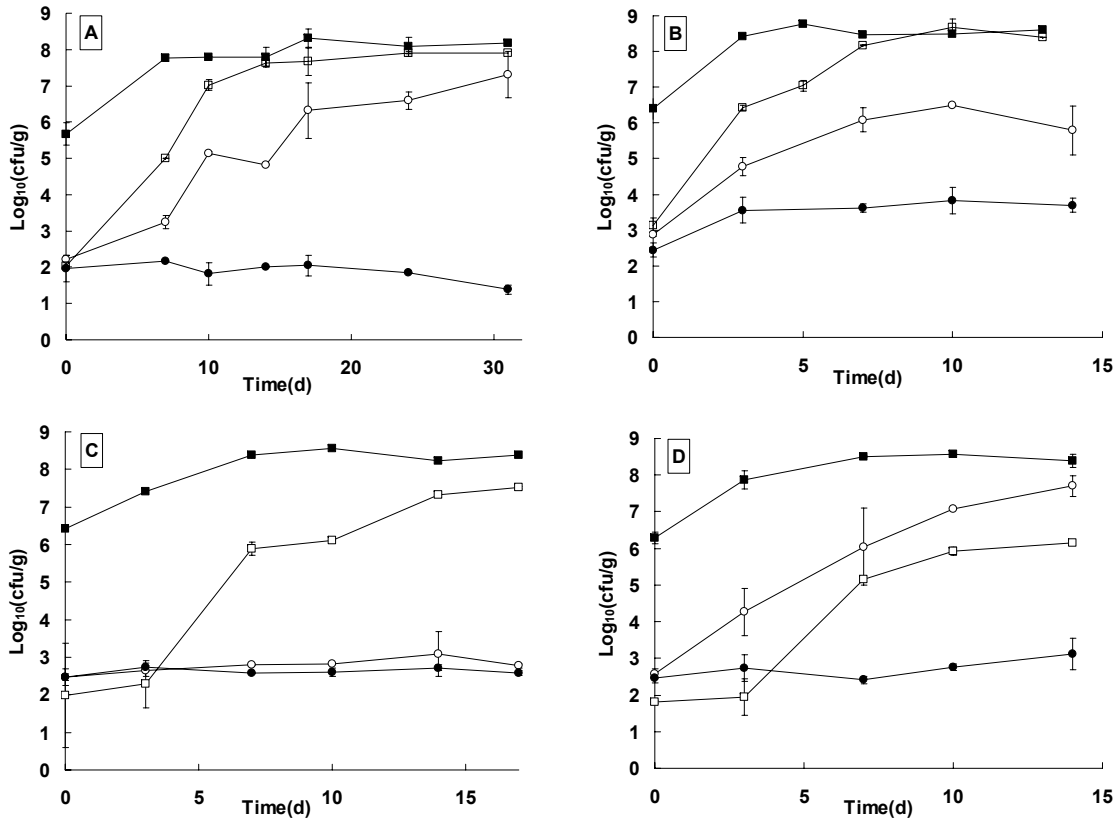

Figure 6.3. Growth of LAB (■,□) and *L. monocytogenes* (●,○) on different vacuum packaged meat products treated with the protective culture *L. sakei* 10A (full symbols) or untreated (empty symbols) and stored at 7°C: (A) pâté, (B) cooked ham, (C) cooked sausage and (D) cooked chicken fillet (mean data of two replicates; error bars represent standard deviations)

In all five products, *L. sakei* 10A exhibited rapid growth and became dominant in a very short time; starting from 10^6 cfu/g the culture reached $>10^8$ cfu/g on day 3 with the exception of its growth in the cooked sausage where 10A reached levels of $>10^8$ cfu/g on day 7. Even in the cooked turkey fillet containing a high level of lactic acid, dominance of 10A was still observed.

In all products that supported growth of the *L. monocytogenes* cocktail in the reference samples (pâté, cooked ham and cooked chicken fillet) there was significant inhibition of the growth of the *L. monocytogenes* cocktail when strain *L. sakei* 10A was present. In the reference samples of pâté, cooked ham and cooked chicken fillet where no *L. sakei* 10A was added, the pathogen exhibited a gradual growth of approximately 2.6, 2.8 and 5.3 $\log_{10}$(cfu/g) respectively after 14 days at 7°C. Applying *L. sakei* 10A prevented the growth of the *L. monocytogenes* cocktail on pâté and cooked chicken fillet and limited the growth to 1.3 $\log_{10}$(cfu/g) after 14 days on cooked ham. No growth of *L. monocytogenes* was observed on the reference and 10A-samples of cooked sausage and cooked turkey fillet. In cooked turkey fillet, this can be explained by the presence of lactate. In the case of the cooked sausage, the reason is not known.

In all of the tested products, except in the cooked turkey fillet, a significant inhibition of the *B. thermosphacta* cocktail was observed when the protective culture *L. sakei* 10A was added. On the reference samples of pâté, cooked ham, cooked sausage and cooked chicken fillet, the number of *B. thermosphacta* increased approximately 1.45, 2.55, 2.84 and 3.13 $\log_{10}$(cfu/g), respectively, during 14 days of storage. On the 10A-samples of pâté, cooked sausage and cooked chicken fillet, numbers of *B. thermosphacta* showed no significant changes during 14 days of storage. In the case of cooked ham, the number of *B. thermosphacta* increased approximately 2.1 $\log_{10}$(cfu/g) over 14 days in spite of the presence of *L. sakei* 10A. In the cooked turkey fillet, no growth of the *B. thermosphacta* cocktail was observed even in the reference samples, probably due to the presence of lactate.

The inhibitory effect of *L. sakei* 10A on the inoculated spoilage organism *Leuc. mesenteroides* was more difficult to assess due to the lack of an appropriate culture medium differentiating between endogenous lactic acid flora, protective culture *L. sakei* 10A and spoilage organism *Leuc. mesenteroides*. However, by identification (five colonies per plate) according to the API-system, dominance of *L. sakei* 10A towards the inoculated *Leuc. mesenteroides* was confirmed in all five CMP.

3.2.2. Glucose consumption and lactic acid production

In almost all products, an important part of the glucose was consumed near the end of the storage period and this was reflected in an increasing level of lactic acid (Table 6.3).

Table 6.3. Glucose consumption and lactic acid production (%, ± SD[a]) in different cooked meat products inoculated with *L. sakei* 10A (10A) in comparison to a non-inoculated product (control) during anaerobic storage at 7°C

		Glucose (%)		Lactic acid (%)	
Product	**Time**	**control**	**10A**	**control**	**10A**
Pâté	day 0	1.46±0.04[a]	1.46±0.04	1.25±0.01	0.95±0.10
	day 17	1.39±0.01	1.19±0.01	1.68±0.35	1.67±0.30
	consumption / production	0.07±0.05	0.27±0.05	0.44±0.36	0.72±0.21
Cooked ham	day 0	0.68±0.04	0.48±0.02	1.02±0.05	1.12±0.20
	day 14	0.12±0.04	0.02±0.00	1.56±0.34	1.50±0.18
	consumption / production	0.56±0.08	0.46±0.01	0.54±0.29	0.38±0.02
Cooked sausage	day 0	0.34±0.06	0.27±0.10	0.54±0.12	0.62±0.12
	day 17	0.21±0.00	0.00±0.00	0.65±0.13	1.19±0.21
	consumption / production	0.13±0.07	0.27±0.10	0.11±0.02	0.56±0.08
Cooked chicken fillet	day 0	0.47±0.01	0.50±0.00	0.98±0.07	1.04±0.00
	day 14	0.38±0.06	0.00±0.01	1.00±0.03	1.68±0.34
	consumption / production	0.09±0.06	0.50±0.00	0.02±0.10	0.64±0.34
Cooked turkey fillet	day 0	0.10±0.01	0.08±0.01	2.73±0.21	3.01±0.36
	day 25	0.04±0.00	0.00±0.00	2.68±0.00	3.38±0.47
	consumption / production	0.06±0.03	0.08±0.01	0.00±0.00	0.37±0.83

[a], SD = standard deviation (n=2)

Glucose consumption at the end of the storage period was more significant in the 10A-samples than in the reference samples; differences in glucose concentration between the first and final day of storage were not significant (*t*-test, $P<0.05$) in the reference samples of all products and in the 10A-samples of cooked sausage and pâté. With glucose, there was a trend towards more lactic acid production at the end of the storage period in the 10A-samples than in the reference samples. However, a *t*-test revealed that differences in lactic acid levels between the first and final day of storage were not significant ($P<0.05$).

In general, higher glucose consumption and higher lactic acid production occurred in products inoculated with *L. sakei* 10A. This trend was clear in all products apart from cooked ham, but

the glucose consumption in 10A-samples was only significantly ($P<0.05$) larger in the case of pâté and cooked chicken fillet and the lactic acid production was only significantly ($P<0.05$) larger in the case of cooked sausage and cooked chicken fillet. In the case of cooked ham, presence of 10A did not result in a higher glucose consumption or lactic acid production. This can be explained by the fast rate of spoilage by endogenous LAB in the reference sample of the cooked ham. As a result of that a high glucose consumption and lactic acid production occurred in the reference samples and no significant differences in glucose and lactic acid evolution could be observed compared to the 10A-samples.

In most products, the lactic acid production was of the same magnitude as the glucose consumption. In pâté, however, more lactic acid was produced than is possible from the amount of glucose that is present, indicating the presence of other fermentable substrates within the product formulation. By analysing the HPLC-chromatogram of an aqueous extract of the pâté, it became clear that the pâté contained also other carbohydrates, including sucrose. Moore & Madden (1997) also found additional fermentable substrates besides glucose in a pork liver pâté.

3.2.3. Influence of 10A on pH and sensory properties

Changes in pH over the storage period are summarised in Table 6.4. The pH was significantly higher in the control samples compared to the 10A-samples starting from day 10, day 3, day 3 and day 11 for pâté, cooked sausage, cooked chicken fillet and cooked turkey fillet respectively. In cooked ham, the pH-difference between the reference samples and the 10A-samples was not significantly different over the storage period. This can be explained by the fact that LAB were proliferating most rapidly on the reference cooked ham compared to the other products. The pH of the reference samples of cooked sausage, cooked chicken fillet and cooked turkey fillet did not decrease significantly between the beginning and the end of storage.

Conversely, in the case of pâté and cooked ham, the pH of the reference sample decreased significantly to values of 6.09 and 5.30, respectively. The rate of pH-decrease was related to and explained by the rate of LAB-growth. The pH of the samples inoculated with *L. sakei* 10A decreased significantly compared to their initial pH-values. On the time at which 10A-samples were unacceptable or on the final storage day, in the case of no sensory rejection, the pâté, cooked ham, cooked sausage, cooked chicken fillet and cooked turkey fillet reached low pH-values of 5.73, 5.55, 5.27, 5.61 and 5.83 respectively.

Table 6.4. Evolution of the pH ± SD[a] of different cooked meat products inoculated with *L. sakei* 10A (10A) in comparison to a non-inoculated product (control) during anaerobic storage at 7°C

Pâté			Cooked ham			Cooked sausage			Cooked chicken fillet			Cooked turkey fillet		
T(d)[b]	control	10A	T(d)	control	10A	T(d)	control	10A	T(d)	control	10A	T(d)	control	10A
0	6.43±0.00[a]	6.33±0.02	0	6.26±0.00	6.19±0.01	0	6.05±0.00	6.05±0.00	0	6.25±0.00	6.25±0.00	0	6.24±0.03	6.24±0.01
7	6.29±0.04	6.22±0.04	3	6.18±0.08	6.08±0.08	3	6.11±0.01	5.85±0.03	3	6.23±0.01	6.07±0.01	7	6.26±0.05	6.13±0.01
10	6.23±0.00	6.13±0.02	5	5.98±0.08	5.77±0.01	7	6.16±0.04	5.55±0.07	7	6.23±0.01	5.69±0.01	11	6.33±0.04	6.00±0.06
14	6.22±0.04	5.87±0.05	7	5.80±0.09	5.64±0.02	10	6.08±0.01	5.27±0.08	10	6.28±0.01	5.61±0.01	14	6.27±0.01	5.93±0.01
17	6.21±0.02	5.73±0.01	10	5.57±0.03	5.53±0.01	14	6.06±0.09	5.18±0.03	14	6.20±0.01	5.46±0.09	18	6.30±0.02	5.95±0.06
24	6.09±0.01	5.40±0.16	14	5.30±0.05	5.45±0.03							25	6.20±0.00	5.80±0.02

[a], SD = standard deviation (n=2); [b], T(d)= time in days

In general, CMP with such low pH-values are unacceptable from a sensory point of view (Korkeala et al., 1990; Chapter 3) and the effect of these low pH-values in the 10A-samples was reflected in the results of the sensory analyses. Figure 6.4 shows the mean scores for taste of the reference samples and the 10A-samples for all products except for the cooked chicken fillet.

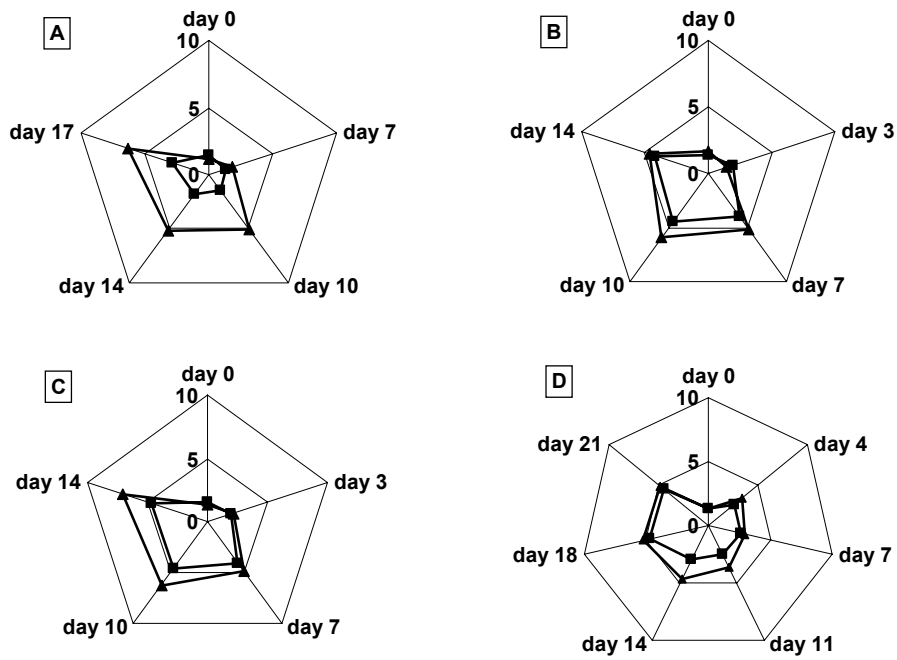

Figure 6.4. Evolution of the mean scores for taste (n=6) of different vacuum packaged meat products treated with the protective culture *L. sakei* 10A (▲) or untreated (■) and stored at 7°C: (A) pâté, (B) cooked ham, (C) cooked sausage and (D) cooked turkey fillet

The scores for odour are not shown but were all very similar. The results for the cooked chicken fillet are not shown since the results were very similar to those of the cooked sausage. In all products, taste scores were lower for the reference samples than for the 10A-samples but only in the case of pâté this difference was significant from day 10 on ($P<0.05$). Only the cooked turkey fillet scores for the 10A-samples remained under or near 5, the limit of acceptability, during the complete storage period, indicating that *L. sakei* 10A has no pronounced negative impact on the sensory quality of the cooked turkey fillet. Variations in the scores of the six different panellists were high and for this reason a second sensory test (preference test) was used. By combining the two sensory tests it was possible to determine

the time at which the products were not acceptable anymore from a sensory point of view (Table 6.5).

Table 6.5. Time at which the meat products were not acceptable for consumption from a sensory point of view based on two types of sensory tests

Product	Time (days) at which >3/6 persons consider the sample as unfit for human consumption		Time (days) at which there was a significant preference for the reference sample based on its taste
	Control	10A	
Pâté	>17	17	10
Cooked ham	>14	>14	>14
Cooked sausage	>14	10	10
Cooked chicken fillet	>10	10	10
Cooked turkey fillet	>21	>21	18

In all products, except in the cooked ham, a significant preference for the reference product was seen after a certain time. This could be explained by the rapid growth of *L. sakei* 10A on the 10A-samples resulting in more rapid acidification of the products. When the pH decreases below a certain value (5.3-5.7), the acidic conditions are noticed when eaten. In the case of cooked ham, there was no time at which the panellists had a significant preference for the reference product above the 10A-product. What's more, on day 14 a significant preference for the cooked ham with 10A was observed. As mentioned before, the specific spoilage organism on the reference cooked ham was a *Leuc. mesenteroides* strain while on the cooked ham treated with *L. sakei* 10A, the *L. sakei* 10A was dominant. At the beginning, more panellists preferred the reference cooked ham although the preference was not significant, in agreement with the results of the other products. However, near the end of storage, panellists preferred the 10A-sample above the reference sample spoiled by the *Leuc. mesenteroides* strain indicating that spoilage due to growth of *L. sakei* 10A is considered as less undesirable than spoilage due to growth of *Leuc. mesenteroides*. This result is not surprising since *Leuc. mesenteroides* is heterofermentative while *L. sakei* is homofermentative.

3.3. Discussion

3.3.1. Antagonistic activity of *L. sakei* 10A

To be useful as a protective culture in sliced CMP, a pronounced antilisterial activity at low temperature is highly desirable. The antilisterial action of *L. sakei* 10A was already described in chapter 5 in a model product. In the present chapter, *L. sakei* 10A showed inhibitory activity against a *L. monocytogenes* cocktail in different vacuum packaged CMP that supported growth of this pathogen during refrigerated storage. Antilisterial effects of non-bacteriocinogenic LAB in CMP have been reported before. In the study of Bredholt et al. (2001), *L. sakei* TH1 could prevent the growth of 10^3 cfu/g of *L. monocytogenes* at 8 and 4°C on cooked ham and cervelat sausage. Amezquita & Brashears (2002) observed effective but product-dependent antilisterial activities using a *L. casei* and a *L. paracasei* strain on cooked ham and frankfurters. Andersen (2000) demonstrated that *L. sakei* BJ-33 was able to inhibit the development of *L. monocytogenes* in cooked ham, an emulsion sausage and rolled cooked pork belly.

The results of this chapter also confirm that *L. sakei* 10A can suppress *B. thermosphacta* in several commercial CMP as shown before in chapter 4 by co-culture experiments on a model product. The antagonistic activity of non-bacteriocinogenic LAB against *B. thermosphacta* was also described by Kotzekidou & Bloukas (1996; 1998) in cooked ham and frankfurters, respectively. Also *L. sakei* BJ-33 has been reported to control the growth of *B. thermosphacta* (Bactoferm^{TM} technical information, 2003).

Few authors have investigated the effect of protective LAB on other LAB in CMP. In 1998, Kotzekidou & Bloukas reported a shelf-life extension of 19 and 28 days at 6-8°C on vacuum packaged pariza when inoculated with *L. sakei* BJ-33 at 10^3 and 10^5 cfu/g, respectively. In their study, the control sample was thought to be spoiled by heterofermentative LAB. However, the same culture failed to prevent growth of ropy slime producing *L. sakei* strains leading to spoilage on frankfurters (Björkroth & Korkeala, 1997). To our knowledge, only Vermeiren et al. (2006a) (Chapter 4) have studied the interaction between a single homofermentative, protective LAB and a single heterofermentative, spoilage causing LAB and clearly showed the inhibitory effect of *L. sakei* 10A on a *Leuc. mesenteroides* strain in a model product during vacuum packaged storage at 7°C. The present chapter confirms that *L. sakei* 10A, when applied at 10^6 cfu/g, is dominant towards *Leuc. mesenteroides*, initially present at 100 cfu/g in several industrially manufactured CMP.

3.3.2. Role of glucose and buffering capacity in sensory acceptability of protective cultures

Since LAB are acid producing bacteria, the development of protective LAB-cultures should include the assessment of their effect on the sensory quality of the treated products. In this chapter, the impact of protective cultures on the sensory properties of CMP was investigated using *L. sakei* 10A. In general, the application of the protective culture *L. sakei* 10A to CMP was promising unless limited by significant acidification resulting in an acid taste. This was most obvious for pâté and cooked sausage and less obvious for cooked turkey fillet. From the screening study the hypothesis was derived that both high buffering capacity and low glucose content are important to avoid sensory changes when applying *L. sakei* 10A. This hypothesis was confirmed in the storage experiments. The negative sensorial aspects on pâté and cooked sausage, when containing *L. sakei* 10A, were related to the low buffering capacity of these products. Furthermore, the pâté had a high glucose content and contained some other sugars and this acted in a synergistic way with the low buffering capacity in causing sensory changes. The buffering capacity and the glucose content of the cooked ham were intermediate; the buffering capacity of the cooked chicken fillet was high and the glucose content intermediate but still sensory changes occurred. Due to the high buffering capacity and the very low glucose content of turkey fillet, the presence of *L. sakei* 10A did not affect the sensory properties of this product up to day 17 (scores < 5). Nevertheless, the taste of the reference cooked turkey fillet was considered as better than the taste of the 10A-product. This was, however, not due to the acidifying effect of 10A as the pH on day 21 was only 5.80 but was due to the retarded spoilage in the reference sample through the presence of lactate. If no lactate had been present in the reference cooked turkey fillet, the sensory results might have been even more positive. Few authors have studied the effect of protective cultures on the sensory properties of the treated products. Andersen (2000) did not include pH-measurements and sensory evaluation. Bredholt et al. (2001) observed in a consumer preference trial after 11 days at 4°C no statistically significant differences in consumer preferences between cooked ham and cervelat with and without *L. sakei* TH1. However, after 11 days the *L. sakei* TH1 strain was only in the late exponential phase and sensory changes at that time are not expected. The same authors also observed little or no sensory differences between treated and untreated products since the investigated attributes were still satisfactory after 28 days at 4°C. This positive result was obtained despite the fact that after 28 days at 4°C, the pH of the cervelat treated with *L. sakei* TH1 was 5.2-5.3. In our study, CMP were unacceptable from a sensory point of view when pH-values reached levels of 5.3-5.7. Korkeala et al. (1990) found

that cooked ring sausages were deemed unfit for consumption when the pH was below 5.8-5.9 and similar results were also obtained in chapter 3. Amezquita & Brashears (2002) found positive sensory results for the bacteriocin-producing *P. acidilactici* on frankfurters after 56 days at 5°C but did not investigate the effect of the more acidifying non-bacteriocinogenic *L. casei* and *L. paracasei.* However, their co-inoculation studies also resulted in significant acidification of frankfurters and cooked ham.

In a study on pariza Kotzekidou & Bloukas (1998) found the control samples to have an unacceptable sour taste after 4 weeks, whereas those treated with *L. sakei* BJ-33 had an acceptable odour and taste up to 7-8 weeks of storage at 6-8°C. Despite the sour taste, the pH of the controls was the highest of all treatments and decreased only from 6.7 to 6.1 after 4 weeks. The authors assigned the negative sensory quality of the control to heterofermentative LAB producing organic acids, in particular acetic acid. This hypothesis does not agree with the pH-changes in the product; it might be that other types of metabolites caused spoilage.

The shelf-life studies of this chapter demonstrate that protective cultures might be a valuable natural preservation strategy to prolong the shelf-life and protect against the growth of *L. monocytogenes* for CMP. However, application of the culture without sensory loss is only possible in CMP with high buffering capacity and low glucose content.

A high buffering capacity is obtained when products contain a minimum level of phosphate but mainly when they are rich in meat-protein. Usually, phosphate levels of less than 0.5% phosphate are added and from this study it could be deduced that 0.5% of phosphate might be the minimal level necessary to have sufficient buffering. A high level of total meat-protein in a CMP is obtained when products are produced from lean meat pieces. Thus the protective culture is not useful in products with low protein levels, usually those with a high fat content such as pâté and cooked sausages. However, in lean products such as cooked ham of high quality and cooked poultry products, the culture can have potential as long as the glucose content is sufficiently low.

In theory, glucose that is present in cooked meat products can originate from either the raw meat ingredient(s) or the non-meat ingredients. However, in post-mortem meat muscle glucose content ranges from 0.05% or lower (Nubel, 1999) to 0.15% (Nychas et al., 1998) or even 0.2% (Borch et al., 1996). In this work, the glucose concentration of the raw poultry meat, used for the preparation of the cooked chicken fillet was 0.03%. This confirms that the glucose in CMP with glucose levels of approximately 0.5% originates from non-meat ingredients. In some recipes of CMP, glucose is added directly as dextrose or dextrose syrup or indirectly e.g as a carrier for flavours or as a dispersion agent for gums in the brine.

However, from a technological point of view it is possible to make CMP with glucose levels of 0.1% or lower. Low glucose contents can be obtained by avoiding direct addition of glucose or other sugars and by selecting ingredients for cooked meat manufacturing that do not contain glucose; the latter may require the development of e.g. flavours that use carrier material other than glucose.

4. Conclusion

The use of the protective culture *Lactobacillus sakei* 10A on anaerobically packaged cooked meat products maintains or, in some cases, prolong the shelf-life while protecting against growth of *Listeria monocytogenes*. The effect of protective cultures in general and of *Lactobacillus sakei* 10A in particular on the organoleptic characteristics of cooked meat products depends on the glucose content and the buffering capacity of these products. We developed the hypothesis that only protein rich cooked meat products containing low amounts of glucose benefit from the use of a protective culture. The next chapter (Chapter 7) studies the mechanism of action by which the protective culture *L. sakei* 10A inhibits the growth of *L. monocytogenes*.

Chapter 7

The contribution of lactic acid production versus nutrient competition to the growth inhibition of *Listeria monocytogenes* by the non-bacteriocinogenic *Lactobacillus sakei* 10A

Redrafted after

L. Vermeiren, F. Devlieghere, U. Rajtak, B. Kerkaert and J. Debevere

Journal of Applied Microbiology

Submitted for publication

Chapter 7
The contribution of lactic acid production versus nutrient competition to the growth inhibition of *Listeria monocytogenes* by the non-bacteriocinogenic *Lactobacillus sakei* 10A

Summary

The study presented in this chapter investigates possible mechanisms by which the non-bacteriocinogenic *Lactobacillus sakei* 10A inhibits *Listeria monocytogenes.*

First, the antagonistic character of *L. sakei* 10A was confirmed by the observation of growth inhibition of *L. monocytogenes* in the presence of *L. sakei* 10A in buffered BHI-broth (b-BHI) (7°C, anaerobic). When assessing the growth of *L. monocytogenes* in the cell free supernatants (CFS), prepared after centrifugation of a 10A-culture at different time points during its growth in b-BHI, it became clear that the older the culture was, the more inhibitory properties it had, meaning that either production of (an) antimicrobial compound(s) or nutrient competition caused the inhibition.

The precise role of lactic acid production and nutrient competition was more obvious during co-culture experiments in two types of broths, differing from each other in their glucose level and in the presence/absence of yeast extract, Mn^{2+} and Mg^{2+}. The presence of more nutrients did not prevent the growth inhibition of *L. monocytogenes* by *L. sakei* 10A. In the nutrient-poor broth, inhibition coincided with the moment of glucose depletion. In the nutrient-rich broth, an increased lactic acid production was thought to cause the inhibition. Subsequent challenge experiments with *L. monocytogenes* in the CFS, obtained from 10A's growth in the media with the two different nutrient levels, allowed distinction between the different antagonistic effects (pH-reduction, lactic acid production and nutrient competition). In the nutrient-poor broth, growth inhibition was exclusively caused by nutrient competition and competition for glucose was, at least partly, involved. In the nutrient-rich broth, growth inhibition was caused by a combination of several factors: the antimicrobial effect of the produced lactic acid/lactate, nutrient competition and pH-reduction as a consequence of lactic acid formation.

In a final experiment, no effect of supplementation with vitamins and minerals on the inhibition phenomena could be observed.

1. Introduction

The last two decades, biopreservation became an important field of research. The technology to control the food born pathogen *Listeria monocytogenes* in cooked meat products (CMP) by the use of protective lactic acid bacteria (LAB) has been demonstrated in several studies (Andersen, 1995a; Hugas et al., 1998; Bredholt et al., 1999; Bredholt et al., 2001; Amezquita & Brashears, 2002; Budde et al., 2003; Mataragas et al., 2003a) and also in the previous two chapters (Chapters 5 and 6). The search for protective cultures (PC) to be used in sliced CMP has mainly been directed towards LAB capable of producing bacteriocins. However, the sometimes limited effectiveness of these cultures in the meat substrate (Rodriguez et al., 2002) and the concern for resistance development (Ennahar et al., 2000) has driven the search to alternative non-bacteriocinogenic PC. Although the effectiveness of such PC to protect CMP against the proliferation of post-contaminating *L. monocytogenes* cells has been proven (Bredholt et al., 1999; 2001; Chapter 6), the mechanism behind the inhibitory activity is not well understood. Therefore, it was the objective of this chapter to study more in detail the mechanism behind the growth inhibition phenomena, mediated by *Lactobacillus sakei* 10A.

Hydrogen peroxide production by LAB does not occur under anaerobic conditions (Kandler & Weiss, 1986) and is therefore playing a minor role in the antagonism of protective LAB in CMP packaged under vacuum or under a modified atmosphere.

The in-situ production of organic acids, mainly lactic acid, and the subsequent in-situ acidification is another important mechanism of biopreservation (Juven et al., 1998). In anaerobically packaged CMP, LAB sometimes exert antilisterial effects that can not be explained by lactic acid production and pH-decrease alone (Chapters 5 and 6) and this because of mainly two reasons. First of all, the amount of lactic acid produced at the moment of inhibition by the PC is most often negligible compared with the amount already present in CMP (Metaxopolous et al., 2002; Chapter 2) due to the low carbohydrate content of most CMP. The high buffering capacity of most CMP further limits the pH-reduction, following lactic acid production as observed in chapter 6. This means that another mechanism must also be responsible for the biopreservative effect.

Several authors have proposed nutrient competition as an explanation for the inhibition of *L. monocytogenes* by non-bacteriocinogenic LAB in anaerobically packaged CMP (Buchanan & Bagi, 1997; Bredholt et al., 1999; Devlieghere et al., 2004; Vermeulen et al., 2006) or other

food products (Nilsson et al., 1999; 2004). Up to now it is, however, not clear for which nutrient(s) competition occurs.

In this chapter, to examine the mechanism(s) by which *L. sakei* 10A exerts its biopreservative effect, *L. monocytogenes* was chosen as target organism. Experiments were performed in liquid growth media with a pH of 6.2 at 7°C and under anaerobic conditions to simulate, at least partly, the conditions in vacuum packaged or modified atmosphere packaged sliced CMP. Interaction experiments between *L. sakei* 10A and *L. monocytogenes* were followed in time by challenge experiments with *L. monocytogenes* in the cell free supernatant obtained from the growth of *L. sakei* 10A. Furthermore, the effect of supplementing the growth medium with extra nutrients, glucose, vitamins and minerals on the inhibitory interaction was investigated. The work of this chapter aimed to determine the role of pH, lactic acid and nutrient competition in the inhibition of *L. monocytogenes* by *L. sakei* 10A.

2. Materials and methods

2.1. Bacterial strains

Lactobacillus sakei subsp. *carnosus* 10A and *Listeria monocytogenes* LMG 13305 were used throughout this study. The non-bacteriocinogenic *L. sakei* 10A was isolated from vacuum packaged, sliced, cooked turkey fillet in chapter 2. *L. monocytogenes* LMG 13305, isolated from soft cheese, was obtained from the culture collection of the Laboratory Microbiology Gent (LMG, Gent University, Belgium). Stock cultures of the strains were maintained in de Man Rogosa Sharpe (MRS, Oxoid, Oxoid Limited, Basingstoke, Hampshire, UK) broth or Brain Heart Infusion (BHI, Oxoid) broth supplemented with 15% glycerol at -75°C. Working cultures of the strains were maintained on MRS-slants or Tryptone Soya Agar (TSA, Oxoid) slants at 7°C and revived by transferring a loop of inoculum into MRS-broth or BHI-broth followed by incubation at 30°C for 24h.

2.2. Growth media

The different liquid growth media used in this study are summarised in Table 7.1. All media were adapted to pH 6.2 with 4N HCl before sterilisation (Fluka, Sigma-Aldrich Chemie GmbH, Bucks, Switzerland).

Table 7.1. Composition of the different growth media used in this chapter

	Concentration (g/l)					
Component	Buffered BHI-broth **(b-BHI)**	Buffered modified BHI-broth **(b-mBHI)**	Buffered adapted BHI-broth **(b-aBHI)**	Buffered modified glucose-supplemented BHI-broth **(b-mgBHI)**	Buffered modified BHI-broth supplemented with vitamins/minerals **(s-b-mBHI)**	Buffered modified glucose-supplemented BHI-broth supplemented with vitamins/minerals **(s-b-mgBHI)**
BHI[a]	37.0	37.0	37.0	37.0	37.0	37.0
KH_2PO_4[b]	15.9	15.9	15.9	15.9	15.9	15.9
K_2HPO_4[b]	21.6	21.6	21.6	21.6	21.6	21.6
Yeast extract[a]	-	-	4.0	-	-	-
D-(+)-glucose[c]	-	-	18.0	18.0	-	18.0
$MgSO_4.7H_2O$[c]	-	-	0.2	-	0.41	0.41
$MnSO_4.H_2O$[c]	-	-	0.04	-	0.02	0.02
NaCl[b]	-	25.0	25.0	25.0	25.0	25.0
Tween 80[c]	-	1 (ml/l)	1 (ml/l)	1 (ml/l)	1 (ml/l)	1 (ml/l)
Fe-citrate[c]	-	-	-	-	0.088	0.088
Biotin[d]	-	-	-	-	0.5	0.5
Riboflavin[c]	-	-	-	-	5	5
Thiamin[d]	-	-	-	-	1	1

[a], Oxoid; [b], Fluka; [c], Sigma (Sigma-Aldrich Corporation, St. Louis, Missouri, USA); [d], Acros Organics (Acros Organics, Geel, Belgium)

2.3. Experimental set-up

The outline of this chapter is schematically presented in Table 7.2.

Table 7.2. Overview of the different experiments of this chapter

First experiment: **Interaction experiment in b-BHI and subsequent challenge experiment in CFS[d] of *L. sakei* 10A**			
Part 1: co-culture experiment		**Part 2: challenge experiment in CFS**	
Medium	*Test series*	*Medium*	*Test series*
b-BHI	10A[a]	CFS[d] (d0), CFS (d3), CFS (d7), CFS (d10), CFS (d14)	LIS
	LIS[b]		
	10A+LIS[c]		
Second experiment: **Role of lactic acid production versus nutrient competition: co-culture experiment and challenge experiment in CFS**			
Part 1: co-culture experiment		**Part 2: challenge experiment in CFS**	
Medium	*Test series*	*Medium*	*Test series*
b-mBHI	10A	CFS, CFS-P[e], BHI-P[f], BHI-P-LA[g]	LIS
	LIS		
	10A+LIS		
b-aBHI	10A	CFS, CFS-P, BHI-P, BHI-P-LA	LIS
	LIS		
	10A+LIS		
Third experiment: **Interaction experiments – supplementation with vitamins and minerals**			
co-culture experiment			
Medium	*Test series*		
b-mBHI	10A		
	LIS		
	10A+LIS		
s-b-mBHI	10A		
	LIS		
	10A+LIS		
b-mgBHI	10A		
	LIS		
	10A+LIS		
s-b-mgBHI	10A		
	LIS		
	10A+LIS		

[a], 10A = broth inoculated with *L. sakei* 10A; [b], LIS = broth inoculated with *L. monocytogenes*; [c], 10A+LIS = broth inoculated with *L. sakei* 10A and *L. monocytogenes*; [d], CFS = cell free supernatant; [e], CFS-P = pH-adjusted CFS; [f], BHI-P = pH-adjusted BHI-broth; [g], BHI-P-LA = pH-adjusted and lactic acid supplemented BHI-broth

In all three experiments, growth of the cultures was followed in liquid broths in gastight jars (Chapters 2 and 3) filled with 100% N_2 during storage at 7°C to simulate the refrigerated vacuum packaged storage conditions of CMP. Inoculation levels were the same for all experiments throughout this study: 10^6 cfu/ml for *L. sakei* 10A (10A) and 10^2 cfu/ml for *L. monocytogenes* (LIS). At several time intervals during storage, samples were taken to determine cell number(s), pH and in some cases glucose and lactic acid content. Each growth experiment was performed in triplicate. Methods for preparation, flushing with 100% N_2, inoculation and sampling of the jars were similar to the ones applied in chapter 2 and 3 and are described in detail by Devlieghere et al. (1998).

During growth in mono-culture, cell numbers of *L. sakei* 10A and *L. monocytogenes* were determined on TSA-agar (aerobic incubation for 48h at 30°C). During growth in co-culture (10A+LIS), cell numbers of *L. sakei* 10A were determined on MRS-agar supplemented with sorbic acid (1.4 g/l) (pH=4.7) (aerobic incubation for 48h at 30°C) and cell numbers of *L. monocytogenes* were determined on ALOA (Agar Listeria Ottaviani and Agosti) (Biolife, Biolife Italiana S.r.l., Milan, Italy) (aerobic incubation for 2 days at 37°C) supplemented with ALOA Enrichment selective supplement (Biolife).

The pH was measured by using a pH-electrode (Ingold, MGDX K57, Urdorf, Switzerland) connected to a pH-meter (Knick, type 763, Berlin, Germany).

Glucose and lactic acid were determined by a spectrophotometric method with an enzymatic reagent (glucose-oxidase-peroxidase (GOP) reagent) and a HPLC-method, respectively, as previously described in chapters 6 and 2, respectively.

2.4. Co-culture experiment in b-BHI and subsequent challenge experiment in CFS of 10A

For this first experiment, buffered BHI-broth (b-BHI), containing 0.2% of glucose, was used (Table 7.1). The goal was (1) to determine whether *L. sakei* 10A can inhibit *L. monocytogenes* when growing in co-culture in b-BHI, (2) to determine whether *L. monocytogenes* is inhibited in its growth when cultured in the cell free supernatant (CFS) obtained from the growth of *L. sakei* 10A in b-BHI and (3) to determine whether the extent of the latter inhibition differs according to the time during 10A's growth at which the CFS is obtained.

In the first part of this experiment (Table 7.2), the growth of *L. monocytogenes* in mono-culture and in co-culture with *L. sakei* 10A was determined. Simultaneously, the growth of *L. sakei* 10A in mono-culture was determined and at day 0, 3, 7, 10 and 14 of 10A's growth,

three jars were opened to prepare from the growth medium the cell free supernatant (CFS) by centrifugation at 8000*g* for 10 min followed by filter sterilisation through a bottle top filter (ϕ 0.2 µm, Nalgene, Rochester, US) and finally freezing at -75°C until further use. The CFS taken at time x was designated as CFS(dx).
In the second part of this experiment (Table 7.2), the growth of *L. monocytogenes* was studied in the different cell free supernatants.

2.5. Role of lactic acid production versus nutrient competition

The objective of this experiment was to further elucidate the mechanism behind the growth inhibition of *L. monocytogenes* by *L. sakei* 10A and this by investigating two potential antagonistic systems: either lactic acid/lactate production or nutrient competition. To investigate which role each of these two mechanisms might play, the inhibition of *L. monocytogenes* by *L. sakei* 10A was studied in two growth media with a different nutrient level. It was the aim to find out if supplementation of the growth medium with a mixture of different nutrients (carbohydrate, amino-nitrogen, vitamins and minerals) could result in elimination of the growth inhibition of *L. monocytogenes* by *L. sakei* 10A. Therefore, two different growth media, b-mBHI and b-aBHI (Table 7.1), were used. The two growth media differed in their nutrient level and more specifically in the glucose level and in the presence/absence of yeast extract, Mn^{2+} and Mg^{2+}. Therefore, b-mBHI can be seen as a nutrient-poor broth, while b-aBHI can be considered as a nutrient-rich broth.
Similar as in section 2.4., co-culture experiments between *L. sakei* 10A and *L. monocytogenes* were followed by challenge experiments with *L. monocytogenes* in the cell free supernatant obtained from the growth of *L. sakei* 10A.

2.5.1. Co-culture experiment

In the first part of this experiment (Table 7.2), the growth of *L. monocytogenes* in mono-culture and in co-culture with *L. sakei* 10A was determined in the two types of broths. The main question was whether the inhibition of *Listeria* by 10A would be abolished in the broth with the higher nutrient level or not. During the growth experiment, also the evolution in pH, glucose and lactic acid concentration of the media was determined.

2.5.2. Challenge experiment in CFS

To distinguish the different antagonistic effects (pH-reduction, lactic acid production and nutrient depletion) that occured simultaneously in the co-culture experiments (2.5.1), the growth of *L. monocytogenes* was further studied in the CFS obtained from the mono-culture growth of *L. sakei* 10A after 12 days at 7°C under anaerobic conditions. From chapter 2 it was known that strain 10A reaches its stationary phase after 12 days. This was done for both types of growth media (b-mBHI and b-aBHI) and always in triplicate. The cell free supernatant (CFS) was prepared by centrifugation at 8000*g* for 10 min followed by filter sterilisation through a bottle top filter (ϕ 0.2 µm, Nalgene). To investigate the role of the pH-decrease, the CFSs of both media were split in two different parts (Table 7.3). One part was pH-adjusted to pH 6.2 (designated as CFS-P) and the other was not pH-adjusted (designated as CFS). Adaptations of the pH were done using 4M HCl or 8M NaOH (Fluka). The role of lactic acid was investigated by including a medium (designated as BHI-P-LA), that was either b-mBHI or b-aBHI that was adjusted to pH 6.2 and supplemented with lactic acid to contain finally the same amount of lactic acid as produced by *L. sakei* 10A after 12 days of growth.

Table 7.3. Overview of the different media used in the challenge experiment meant to separately investigate the role of pH-decrease, lactic acid production and nutrient competition

Designation of the medium	Description	Potential antagonistic properties
CFS	CFS obtained after 12 days of 10A-growth in either b-mBHI or b-aBHI	Lowered pH, lowered nutrient level and produced lactate/lactic
CFS-P	CFS obtained after 12 days of 10A-growth in either b-mBHI or b-aBHI and adjusted to pH 6.2	Lowered nutrient level and produced lactate/lactic acid
BHI-P	Either b-mBHI or b-aBHI, adjusted to pH 6.2	None
BHI-P-LA	Either b-mBHI or b-aBHI, adjusted to pH 6.2 and supplemented with lactic acid to have the same concentration of lactic acid as in CFS or CFS-P	Added lactate/lactic acid

To obtain the same lactic acid content as in CFS(d12), small volumes of 0.85% (w/w) lactic acid (Sigma-Aldrich) were added in repeating steps, each time followed by a verifying measurement of the lactic acid concentration through HPLC-analysis until the desired concentration was reached. Finally, a fourth medium serving as a positive control was included in the test: b-mBHI or b-aBHI, adjusted to pH 6.2 (BHI-P). Media BHI-P-LA and

BHI-P differed from CFS-P and CFS in the absence of nutrient depletion through 10A's growth, allowing the evaluation of the role of nutrient depletion. The media BHI-P-LA and BHI-P were also centrifuged and filter sterilised in the same way as was done for the preparation of the CFS and this to prevent differences in nutrient composition compared to CFS and CFS-P due to these treatments.

2.6. Co-culture experiments – supplementation with vitamins and minerals

The objective of this experiment was to investigate whether supplementation of the growth medium with some specific vitamins and minerals results in elimination of the antagonistic effect of *L. monocytogenes* LMG 13305 by *L. sakei* 10A. The minerals under study were Fe^{3+}, Mn^{2+} and Mg^{2} while the vitamins under study were biotin (vitamin B8), riboflavin (vitamin B2) and thiamine (vitamin B1). These vitamins and minerals were chosen because it is reported that they are essential for the growth of *L. monocytogenes* as well as for *L. sakei* (Premaratne et al., 1991; Phan-Thanh & Gormon, 1997; Moretro et al., 1998). The growth of *L. sakei* 10A in mono-culture and the growth of *L. monocytogenes* in mono-culture and in co-culture with *L. sakei* 10A was determined in four different media (Tables 7.1 and 7.2): (1) b-mBHI, (2) s-b-mBHI or b-mBHI supplemented with the vitamins and minerals, (3) b-mgBHI and (4) s-b-mgBHI or b-mgBHI supplemented with the vitamins and minerals. To supplement the media with these six micro-nutrients, stock solutions in distilled water were prepared of Iron(III)-citrate (Sigma), $MgSO_4.7H_2O$ (Sigma), $MnSO_4.H_2O$ (Sigma), biotin (Acros Organics), riboflavin (Sigma) and thiamine (Acros Organics) at concentrations of 3.77, 175.48, 8.56, 0.04, 0.09 and 0.43 g/l, respectively. These stock solutions were filter sterilised through a bottle top filter (ϕ 0.2 µm, Nalgene) and an appropriate volume of each of the sterile stock solutions was added to the sterile b-mBHI or b-mgBHI medium in order to reach final concentrations of 0.088 g/l Fe-citrate, 0.41 g/l $MgSO_4.7H_2O$, 0.02 g/l $MnSO_4.H_2O$, 0.5 mg/l biotin, 5 mg/l riboflavin and 1 mg/l thiamine. These concentrations were reported to be minimal concentrations for growth of *L. monocytogenes* in several chemically defined minimal media (Premaratne et al., 1991; Phan-Thanh & Gormon, 1997; Moretro et al., 1998). During the experiment not only the evolution of the cell numbers but also the evolutions of the pH and the glucose concentration were determined.

3. Results and discussion

3.1. Co-culture experiment in b-BHI and subsequent challenge experiment in CFS of 10A

The growth of *L. monocytogenes* in mono-culture and in co-culture with *L. sakei* 10A in b-BHI at 7°C under anaerobic conditions is presented in Figure 7.1.

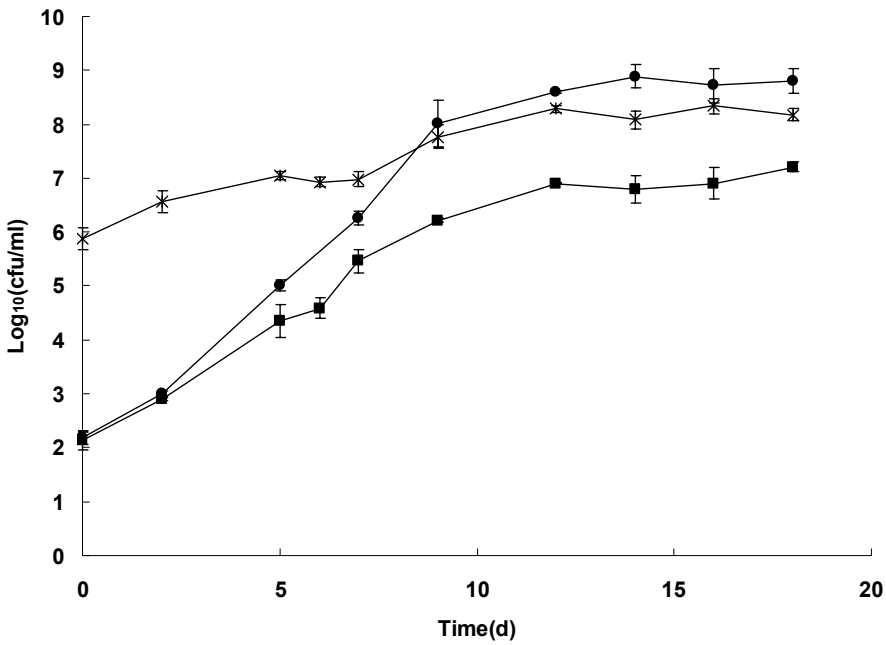

Figure 7.1. Interaction at 7°C between *L. sakei* 10A and *L. monocytogenes* LMG 13305 in b-BHI under anaerobic conditions (■, growth of *L. monocytogenes* in co-culture with 10A; ×, growth of *L. sakei* 10A in co-culture with *L. monocytogenes*; ●, growth of *L. monocytogenes* in mono-culture) (error bars represent 95% confidence intervals, n=3)

The results confirm once more the antilisterial activity of *L. sakei* 10A, which was already observed before on CMP in chapters 5 and 6. The presence of *L. sakei* 10A resulted in a lower growth rate and a difference in the maximal population density of ± 2 $\log_{10}$(cfu/ml) compared to the growth of *L. monocytogenes* in mono-culture. However, in the presence of *L. sakei* 10A, *L. monocytogenes* still reached very high levels of ± 7 $\log_{10}$(cfu/ml). The growth inhibition of *L. monocytogenes* in b-BHI was less pronounced than these observed in chapters 5 and 6 on CMP. This might be linked to the fact that *L. sakei* 10A grew slower in b-BHI-

broth than on CMP. Furthermore, the growth of *L. sakei* 10A in b-BHI was characterised by an intermediate lagphase phenomenon (Figure 7.1), as described before by Vereecken (2002). The reason for the occurrence of this intermediate lagphase is not clear but Vereecken (2002) considered two main hypotheses: (1) diauxic growth due to exhaustion of a substrate or (2) absence of agitation of the medium during incubation. Different to what was observed in CMP, the growth inhibition started not when 10A entered the stationary phase but earlier when 10A was still in the exponential growth phase. This corresponds, however, to the findings of Vermeulen et al. (2006) in a chemically defined medium, simulating CMP. In general, surface growth on solid media is difficult to compare with growth in liquid broths. Furthermore, CMP have a more complex composition than liquid broths.

In the second part of this trial, the growth of *L. monocytogenes* was studied in several cell free supernatants, obtained at different points in time during 10A's growth in b-BHI (7°C, anaerobic). Results are presented in Figure 7.2. Error bars, indicating the 95% confidence interval (n=3), are not presented to avoid complexity of the figure.

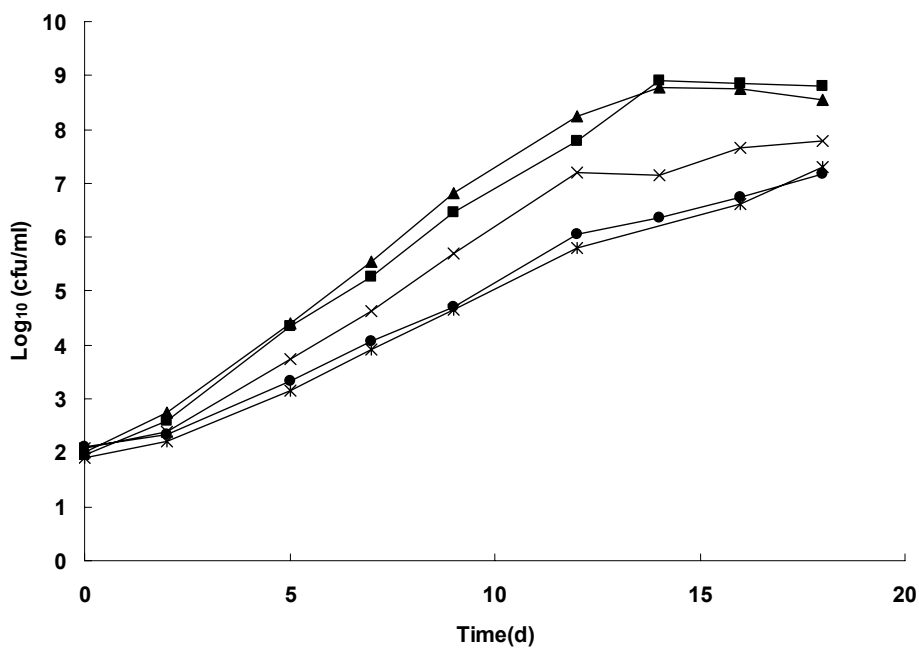

Figure 7.2. Growth of *L. monocytogenes* LMG 13305 in cell free supernatant obtained from the growth of *L. sakei* 10A at 7°C under anaerobic conditions at different points in time (■, CFS(d0); ▲, CFS(d3); ×, CFS(d7); ✳, CFS(d10); ●, CFS(d14)) (mean data of three replicates, error bars are not presented to avoid complexity of the figure)

However, cell numbers were analysed for significant ($P<0.05$) differences between the different CFS's by analysis of variance ($P<0.05$). In general, *L. monocytogenes* grew slower as the culture, from which the CFS was prepared, became older. This effect was most obvious when comparing the growth of *L. monocytogenes* in CFS(d0) and in CFS(d14).

The result could indicate that the later the CFS was obtained during 10A's growth, either the more extracellular, antimicrobial metabolite(s) were present or the less nutrients were present. Since the experiment was performed in a buffered growth medium, the pH-reduction due to lactic acid production was very limited (0.25 pH-units) and could be excluded as the cause of the antagonism. With regard to the production of antimicrobial(s), hydrogen peroxide was excluded because it can only be produced in the presence of oxygen and not in the anaerobic conditions that were used here to mimic vacuum packaging or modified atmosphere packaging. Furthermore, bacteriocin production was excluded since strain 10A did not produce bacteriocin(s) in repeating agar assays (Chapter 2). Therefore, lactic acid/lactate was thought to be the most probable antimicrobial that had interfered in the growth inhibition of *L. monocytogenes* by *L. sakei* 10A in b-BHI. The pH of the medium in this experiment was 6.2 to 6.3 and this implies the action of lactic acid in its dissociated form. It has been shown that the effects of lactic acid on microbial growth at different pH can only be explained if both the dissociated and undissociated form is taken into account (Gonçalves et al., 1997).

In conclusion, two main hypotheses to explain the antagonism were left: (1) inhibition through the antimicrobial action of lactic acid/lactate and (2) inhibition through nutrient competition. The latter hypothesis was thought to be of prime importance in this b-BHI-medium since (1) glucose measurements indicated that CFS(d14) contained no glucose anymore (0.008 ± 0.001 %) after the 14-days period of 10A-growth and (2) the low initial glucose level of BHI (0.2 % before sterilisation) probably had limited the amount of lactic acid that was produced. However, in this first experiment, no lactic acid measurements were done to confirm our hypotheses. Therefore, it was the objective of the next experiment to further investigate these two hypotheses and to find out the role of either lactic acid/lactate production or nutrient competition in the observed growth inhibition of *L. monocytogenes* by *L. sakei* 10A.

3.2. Role of lactic acid production versus nutrient competition

3.2.1. Co-culture experiment

The objective of this experiment was to evaluate the influence of the nutrient level of the growth medium on the growth inhibition of *L. monocytogenes* by *L. sakei* 10A and to find out whether the presence of a mix of different nutrients (carbohydrate, peptides, amino acids, vitamins and minerals) can abolish the growth inhibition. Therefore, the growth of *L. sakei* 10A and *L. monocytogenes* in mono-culture and in co-culture was determined in b-mBHI, a nutrient-poor broth and in b-aBHI, a nutrient-rich broth. b-mBHI-broth was not supplemented with extra glucose and contained only the 0.2% of glucose that is present in the recipe of BHI (Oxoid). b-aBHI-broth was supplemented with 1.8% of glucose in order to reach a final glucose content of 2%. The media further differed in the presence/absence of yeast extract (as a source of amino-nitrogen, vitamins and minerals), Mn^{2+} and Mg^{2+}. During sterilisation of the media, glucose concentrations decreased due to the Maillard reaction and other degradation reactions of glucose. The glucose concentrations that were measured immediately after sterilisation were more or less halved compared to the initial glucose concentrations before sterilisation: b-mBHI-broth had a mean glucose content of 0.09 ± 0.01 % while b-aBHI-broth had a mean glucose content of 0.89 ± 0.07%.

The growth of *L. sakei* 10A in mono-culture and the corresponding pH-decrease, evolution in glucose and lactic acid concentration in b-mBHI and in b-aBHI are presented in Figures 7.3a and 7.3b, respectively.

In b-mBHI, strain 10A grew well and reached ± 7 $\log_{10}$(cfu/ml) after 5 days and its level further increased up to 8 $\log_{10}$(cfu/ml) after 12 days. During this time, the glucose of the broth was completely consumed. The glucose consumption was 0.076 ± 0.002% and the glucose depletion occurred already after 7 days. The glucose consumption resulted in the production of lactic acid. After 12 days, lactic acid production amounted to 0.24 ± 0.06%. This level exceeded the level of lactic acid that could have been produced from the present glucose. This indicates that, after the glucose was depleted, other nutrients were probably also converted to lactic acid. The produced lactic acid resulted in a slight pH-drop of the buffered broth from 6.62 ± 0.01 to 6.38 ± 0.02.

In b-aBHI, strain 10A grew remarkably faster and to a higher maximal cell number due to the higher nutrient level of this broth. Glucose depletion still occurred but now later in time (day 12). The total glucose consumption after 12 days was 0.80 ± 0.04% and the resulting lactic

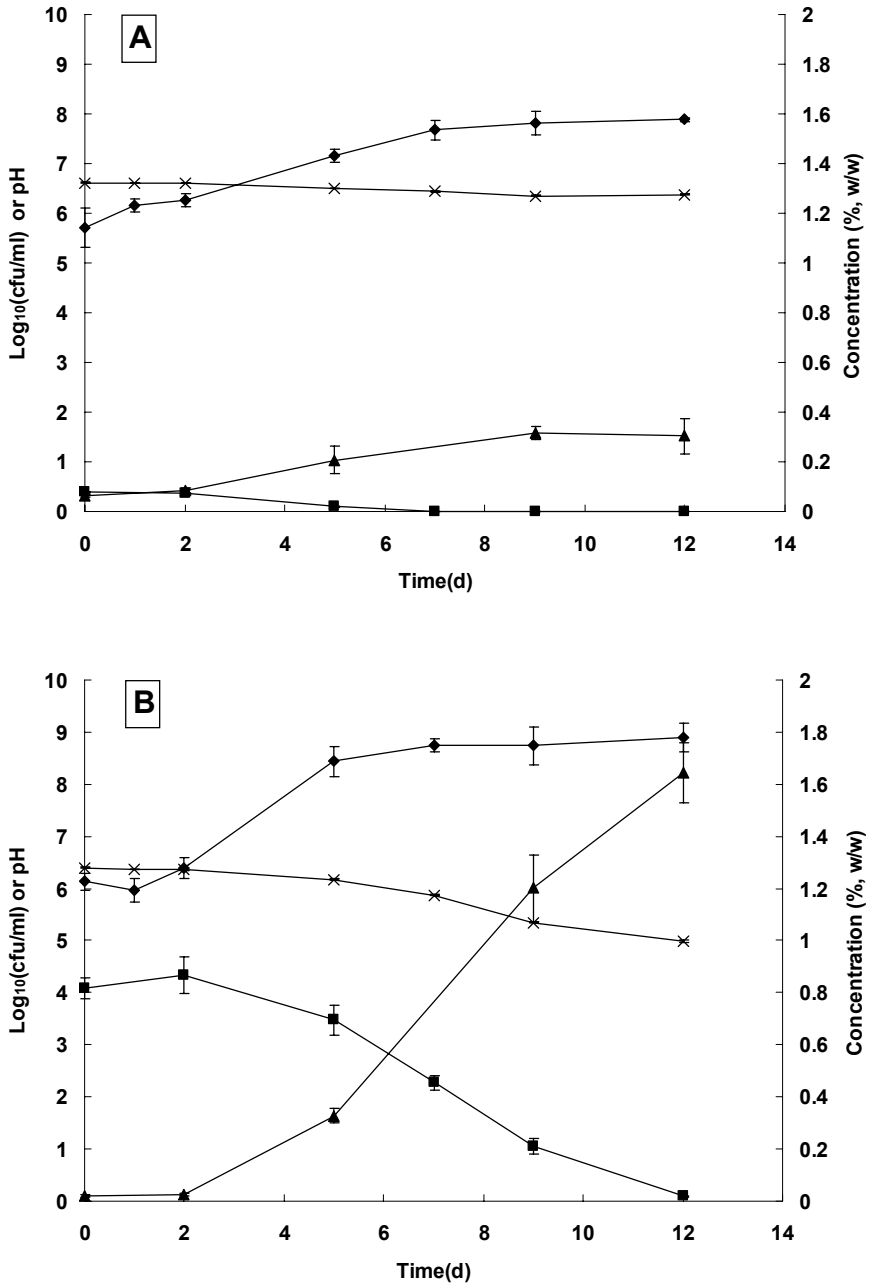

Figure 7.3. The mono-culture growth (♦) of *L. sakei* 10A at 7°C in b-mBHI-broth (A) and in b-aBHI-broth (B) under anaerobic conditions and the corresponding changes in pH (×), glucose (■) and lactic acid (▲) concentration (error bars represent 95% confidence intervals, n=3)

acid production amounted to 1.62 ± 0.10 %. This level again exceeded the level of lactic acid that could have been produced from the consumed glucose, indicating that other nutrients might have been converted to lactic acid. Although the medium was buffered, the strong production of lactic acid resulted in an important pH-drop from 6.40 ± 0.01 to 4.99 ± 0.03.

The growth of *L. monocytogenes* in mono-culture and in co-culture with *L. sakei* 10A and the corresponding changes in glucose and lactic acid concentration in b-mBHI and in b-aBHI are presented in Figures 7.4a and 7.4b, respectively.

When comparing the mono-culture growth of *L. monocytogenes* in the two types of broth, it can be seen that *L. monocytogenes* grew faster in b-mBHI, the broth with less nutrients, than in b-aBHI, the nutrient-rich broth. This was unexpected and in contradiction to what was observed for the mono-culture growth of *L. sakei* 10A. This difference was probably attributed to the difference in water activity between both media (a_w(b-mBHI) = 0.975 and a_w(b-aBHI) = 0.972). Furthermore, there was a small pH-difference of 0.2 pH-units between both media after sterilisation ((pH(b-mBHI) = 6.6 and pH(b-aBHI) = 6.4). Using the predictive model 'pathogen modeling program' (PMP version 7.0, Eastern Regional Research Centrum, Wyndmoor, Pennsylvania, US) it was confirmed that these pH-differences and a_w-differences could explain the slower growth of *L. monocytogenes* in the nutrient-rich broth.

After having discussed the mono-culture growth of *L. sakei* 10A and *L. monocytogenes* in the two different broths, the interaction between the two bacteria in the two broths will now be discussed.

In b-mBHI, *L. sakei* 10A resulted in growth inhibition of *L. monocytogenes* since between day 5 and day 7, the growth rate of *L. monocytogenes* in co-culture with 10A started to decrease compared to the growth rate of *L. monocytogenes* in mono-culture. After a period of 14 days this resulted in a difference of the maximal population density with ± 2 $\log_{10}$(cfu/ml). The moment, at which the inhibition occurred (between day 5 and day 7), corresponded to the moment at which the glucose of the broth was depleted. At day 7, also a higher amount of lactic acid (0.36 ± 0.02%) was present compared to the amount of lactic acid (0.13 ± 0.01%) produced during mono-culture growth of *L. monocytogenes.* However, this level was too low to result in an antilisterial effect.

In b-aBHI, *L. sakei* 10A resulted again in growth inhibition of *L. monocytogenes* but inhibition started between day 7 and day 9, being slightly later than in b-mBHI.

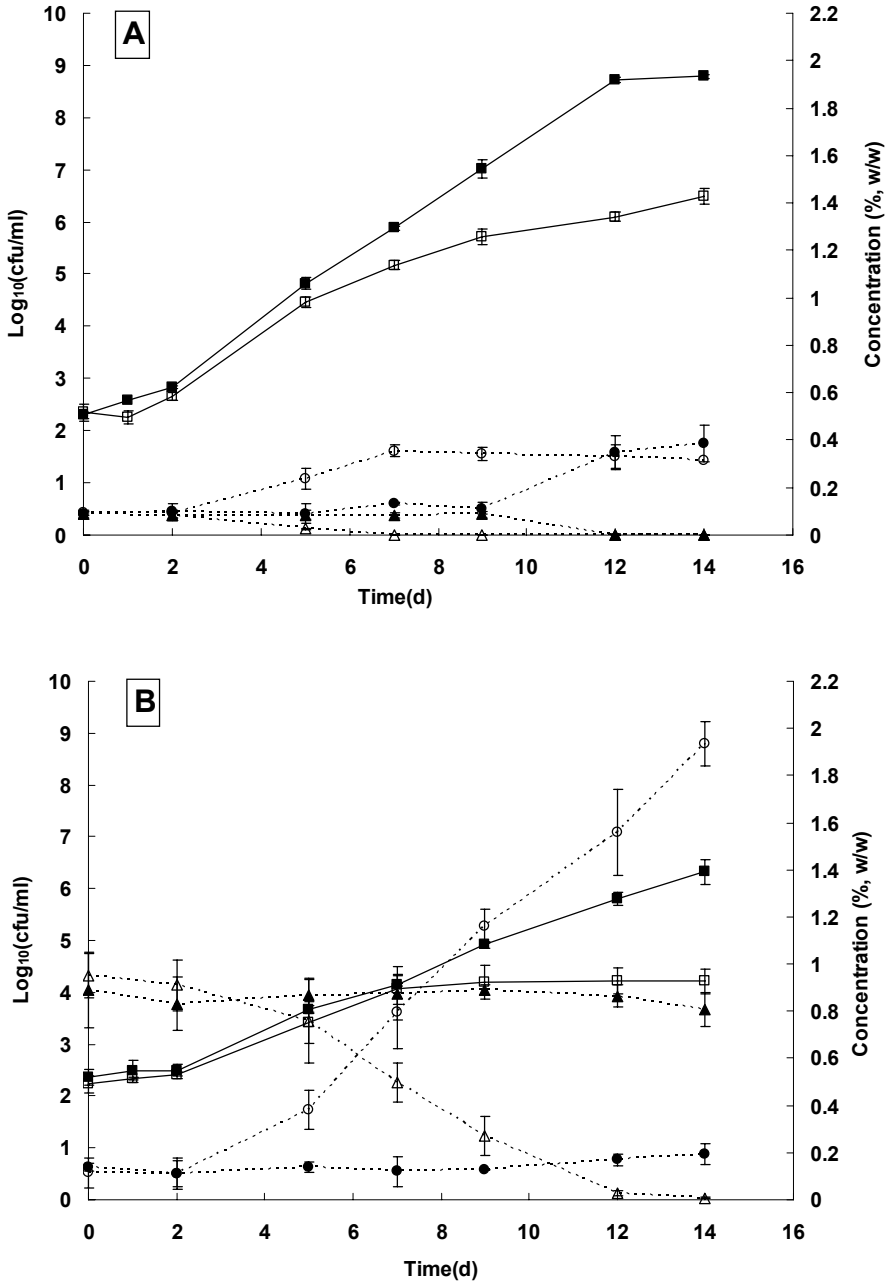

Figure 7.4. The growth (full lines; ■,□) of *L. monocytogenes* in mono-culture (closed symbols) and in co-culture (open symbols) with *L. sakei* 10A and the corresponding changes in glucose (▲, Δ) and lactic acid (●, ○) concentrations (broken lines) in b-mBHI (A) and in b-aBHI (B) (error bars represent 95% confidence intervals, n=3)

This time, growth inhibition was not characterised by a lower growth rate, as it was in b-mBHI, but from day 7 on an early stationary phase was induced and this resulted in a difference in cell number of ± 2 $\log_{10}$(cfu/ml) after the 14-days period. Between day 7 and day 9, the moment at which the *Listeria* number started to stagnate, still sufficient glucose was present but the lactic acid content of the medium had already increased up to a level of 0.79 ± 0.14% (day 7) and 1.16 ± 0.06% (day 9).

In conclusion, the higher nutrient level of the nutrient-rich broth did not counteract the antagonism of *L. sakei* 10A towards *L. monocytogenes*. On the one hand, the higher glucose level of the nutrient-rich broth delayed the glucose depletion phenomenon that was observed in b-mBHI but on the other hand the higher glucose level resulted in a larger lactic acid production, causing another type of inhibition. However, conclusions about the real contribution of competition for glucose and/or other nutrients and lactic acid production were still difficult to make since the concentrations of the nutrients, including glucose, as well as of lactic acid changed simultaneously in the media. To examine the individual contribution of (1) nutrient competition, (2) lactic acid production and (3) pH-reduction to the observed inhibition phenomena, this co-culture experiment was followed by a challenge experiment with *L. monocytogenes* in the CFS from 10A's growth in both, the nutrient-poor and the nutrient-rich broth.

3.2.2. Challenge experiment in CFS

To distinguish the different antagonistic effects (pH-reduction, lactic acid production and nutrient competition) that occurred simultaneously in the co-culture experiments of section 3.2.1, the growth of *L. monocytogenes* was further studied in the CFS from 10A's growth in the nutrient-poor and the nutrient-rich broth. As explained in section 2.5.2. and schematically presented in Tables 7.2 and 7.3, the growth of *L. monocytogenes* was followed in pH-adjusted (CFS-P) and non pH-adjusted CFS (CFS) compared to pH-adjusted b-BHI-broth (either b-mBHI or b-aBHI) (BHI-P) and pH-adjusted b-BHI-broth (BHI-P-LA) that was supplemented with lactic acid to have the same lactic acid concentration as produced by *L. sakei* 10A. The results from these growth experiments are presented in Figure 7.5.

L. monocytogenes grew the best in BHI-P since this medium had no lowered pH, did not contain lactic acid, either supplemented or produced by *L. sakei* 10A and still contained all its nutrients compared to CFS-P or CFS (Table 7.3).

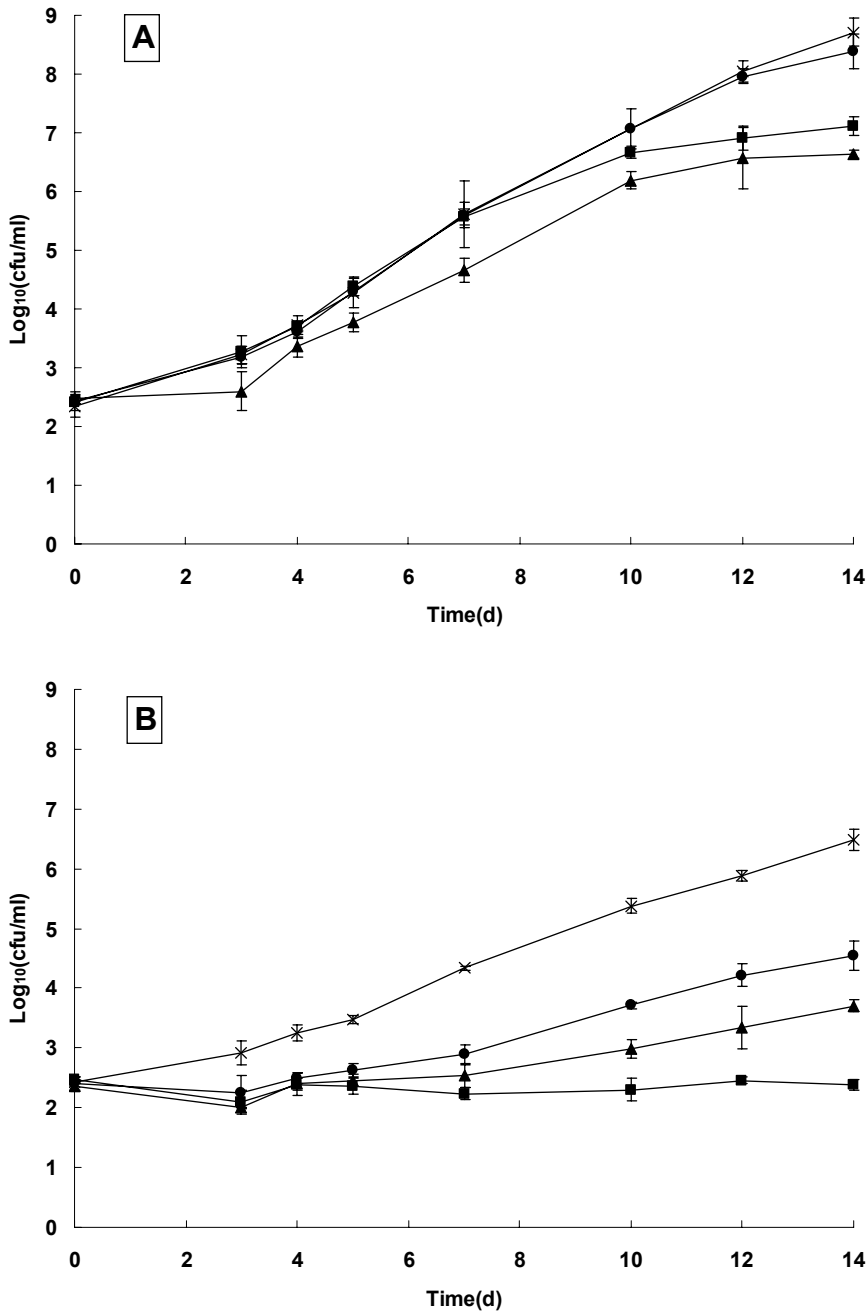

Figure 7.5. Growth of *L. monocytogenes* in CFS obtained from the growth of *L. sakei* 10A (7°C, 100% N_2) in b-mBHI-broth (A) and in b-aBHI-broth (B) (×, pH-adjusted broth (BHI-P); ●, pH-adjusted and lactic acid supplemented broth (BHI-P-LA); ■, CFS; ▲, pH-adjusted CFS (CFS-P)) (error bars represent 95% confidence intervals, n=3)

By comparing the growth of *Listeria* in BHI-P with its growth in BHI-P-LA, the contribution of the produced amount of lactic acid/lactate to the inhibition, which was observed in the first part of the experiment (section 3.2.1), could be revealed. In the nutrient-poor broth (Figure 7.5a) lactic acid/lactate did not contribute to the inhibition since there was no significantly different growth of *L. monocytogenes* between BHI-P and BHI-P-LA. This indicates that the limited lactic acid production (0.24 ± 0.06%) by *L. sakei* 10A after 12 days of growth in b-mBHI is not sufficient to have an antilisterial effect. However, in the nutrient-rich broth (Figure 7.5b), *L. monocytogenes* grew much slower in BHI-P-LA than in BHI-P indicating that the amount of lactic acid produced (1.62 ± 0.10 %) by *L. sakei* 10A after 12 days of growth in b-aBHI ìs sufficient to result in growth inhibition of *L. monocytogenes.*

By comparing the growth of the *L. monocytogenes* strain in BHI-P-LA and CFS-P, the role of nutrient competition could be elucidated, since the only difference between these two media was that CFS-P was depleted in glucose and maybe in other nutrients because of the prior growth of *L. sakei* 10A (Table 7.3). Further, these two media had the same pH and the same lactic acid content. In both, the nutrient-poor and the nutrient-rich broth (Figure 7.5), nutrient competition seemed to play a role since *L. monocytogenes* grew significantly slower in the nutrient-depleted CFS-P compared to in BHI-P-LA. It is highly probable that the absence of glucose in the CFS-P of both media (b-mBHI and b-aBHI) (see Figure 7.4) caused this difference in growth rate. However, the CFS-P contained possibly also lower concentrations of other nutrients or might even have been depleted in other nutrients.

By comparing the growth of *L. monocytogenes* in CFS-P and in CFS, the additional effect of the pH-reduction, occurring during *L. sakei* 10A's growth, could be determined since these media only differed in their pH.
The difference in pH between CFS and CFS-P, both obtained from the growth of *L. sakei* 10A in the nutrient-poor b-mBHI, was limited (CFS; pH = 6.38 ± 0.02 and CFS-P; pH = 6.20 ± 0.01). Still, there was a small but significant difference in growth of *Listeria* between CFS and CFS-P. Because the b-mBHI had initially (after sterilisation) a pH of 6.6 and we had chosen to adapt the pH of the CFS to pH 6.2 (pH relevant for CMP) and the total pH-decrease in b-mBHI was limited to 0.2 pH-units, the pH of the CFS (6.4) was still higher than the one of CFS-P (6.2). It had been better if the initial pH of the two broths, b-mBHI and b-aBHI, before the start of the co-culture experiment (after sterilisation), had been adapted to 6.2. In

that case the pH of the CFS would have been lower than the one of the CFS-P and the role of pH-reduction under these nutrient-limited conditions would have been easier to evaluate.
The difference in pH between CFS and CFS-P, both obtained from the growth of *L. sakei* 10A in the nutrient-rich b-aBHI, was much larger (CFS; pH = 4.99 ± 0.03 and CFS-P; pH = 6.20 ± 0.01) and consequently there was a significant slower growth of *L. monocytogenes* in CFS compared to in CFS-P.

Further, the challenge study confirmed the earlier observations of the co-culture experiments of section 3.2.1. In the nutrient-poor broth, the growth inhibition of *L. monocytogenes* by *L. sakei* 10A was almost exclusively caused by nutrient competition. There is a high chance that glucose competition was at least partly involved in this nutrient competition phenomenon since the CFS from both media were depleted in glucose due to 10A's growth. However, also other nutrients might have been depleted during 10A's growth. Further, in the nutrient-rich broth, the growth inhibition of *L. monocytogenes* by *L. sakei* 10A was caused by a combination of several factors. Part of the observed inhibition could be attributed to the antimicrobial effect of the produced lactic acid/lactate, while the other part of the inhibition was attributed to nutrient depletion and pH-reduction as a consequence of lactic acid formation.
The results indicate that the inhibition of *L. monocytogenes* in CMP by the protective culture *L. sakei* 10A is not based on one mechanism only. However, the inhibition mechanism probably differs according to the type of CMP in which the culture is used and is based on the combination of nutrient competition on the one hand and lactic acid/lactate production together with pH-reduction on the other hand. The results strongly indicate that glucose plays a major role in this nutrient competition phenomenon. The contribution of either nutrient competition or lactic acid production/pH-reduction probably depends on the nutrient content of the product. In CMP with a low glucose content, the observed inhibition will be caused by glucose depletion and almost no lactic acid will be produced, while in CMP with a high glucose content, the contribution of lactic acid's antimicrobial effect will become dominant. The results from this section indicated that competition for glucose plays a role in the observed nutrient competition phenomenon. However, they did not exclude that also other nutrients are involved. Therefore, the next experiment tried to find out if competition for minerals/vitamins could play a further role.

3.3. Co-culture experiments - supplementation with vitamins and minerals

This experiment aimed to examine the effect of supplementation of the growth medium with vitamins and minerals on the inhibitory interaction of *L. monocytogenes* by *L. sakei* 10A. The growth of *L. sakei* 10A in mono-culture and the growth of *L. monocytogenes* in mono-culture and in co-culture with *L. sakei* 10A was determined in four different media: b-mBHI, s-b-mBHI, b-mgBHI and s-b-mgBHI (see section 2.6), differing either in the presence/absence of supplemented vitamins and minerals or in their glucose level.

The mono-culture growth of *L. sakei* 10A was strongly influenced by the presence/absence of the vitamins and minerals. In the media supplemented with these vitamins/minerals, 10A immediately started to grow at a higher growth rate compared to its growth in the non-supplemented media and this was independent on the glucose level. In both media, supplemented and non-supplemented with vitamins/minerals, the growth of *L. sakei* 10A was at first not significantly different as the glucose level was different. However, from a certain moment during growth (day 7 in the absence of supplementation and day 4 in the case of supplementation), the growth of *L. sakei* 10A started to slow down or even stagnate in the media that contained the lower glucose level. Glucose measurements confirmed that the moment, at which this stagnation in growth occurred, corresponded to the moment at which the glucose of the medium was depleted. This is illustrated in Figure 7.6.

Similar results and conclusions were obtained for the co-culture growth of *L. sakei* 10A with *L. monocytogenes* since no significant differences were observed in the growth of *L. sakei* 10A between mono-culture and co-culture (data not shown).

Supplementation with vitamins/minerals, however, did not influence the mono-culture growth of *L. monocytogenes*, neither in the medium with little glucose nor in the medium with a lot of glucose. The influence of the glucose level on the growth of *L. monocytogenes* was similar as observed in the experiment of section 3.2.: a faster growth at a lower glucose level independent on the presence of vitamins/minerals. Again, small pH- and a_w-differences between both media were thought to be responsible for these differences in growth.

By analogy with the mono-culture growth, the presence of extra vitamins/minerals did not influence the growth of *L. monocytogenes* in co-culture with *L. sakei* 10A. No statistically significant differences were obtained in the co-culture growth of *L. monocytogenes* between s-BHI or BHI (either b-mBHI or b-mgBHI). This means that the presence of the investigated vitamins and minerals did not eliminate the inhibitory effect of 10A on *L. monocytogenes.*

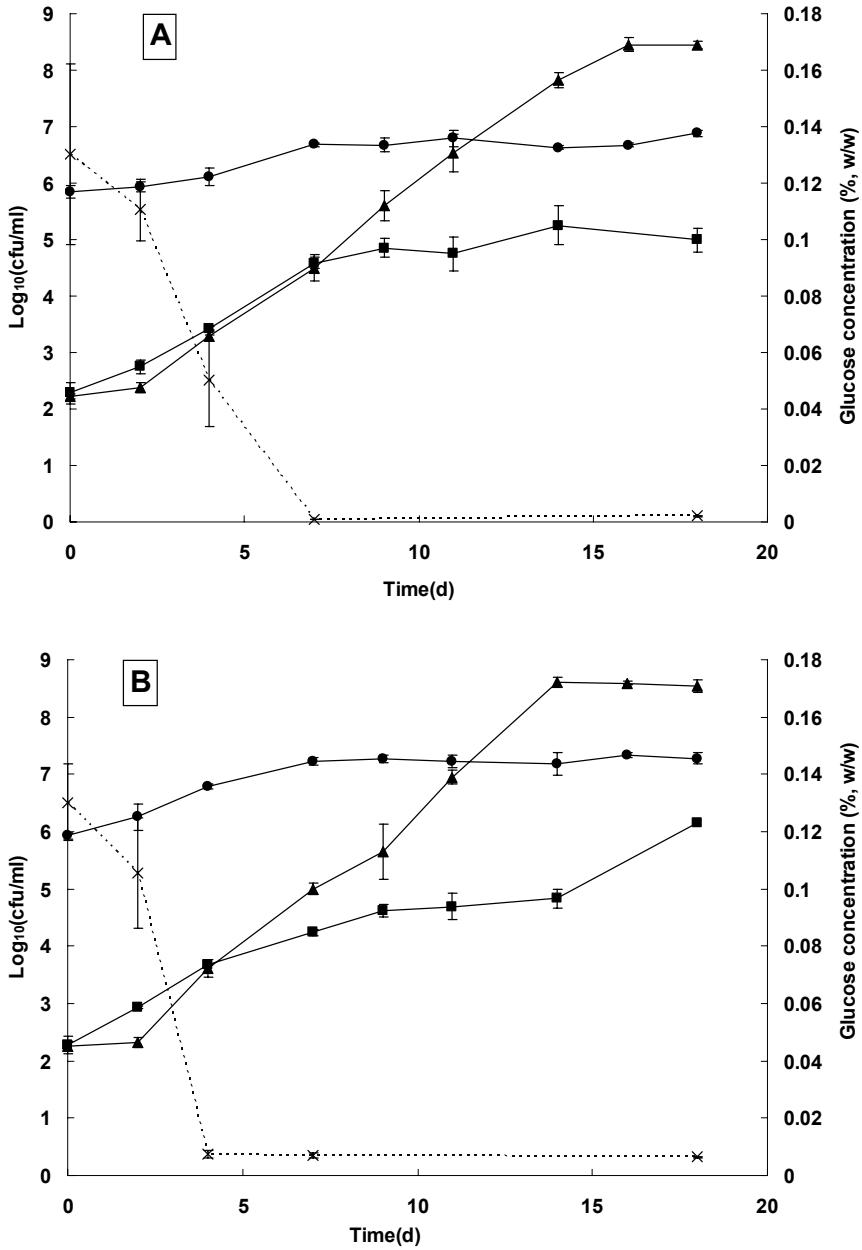

Figure 7.6. Interaction (7°, vacuum) between *L. sakei* 10A and *L. monocytogenes* in b-mBHI (A) and in s-b-mBHI (B) (full lines; ●, growth of *L. sakei* 10A in co-culture with *L. monocytogenes*; ▲, mono-culture growth of *L. monocytogenes*; ■, growth of *L. monocytogenes* in co-culture with *L. sakei* 10A) and the evolution of the glucose concentration during co-culture growth of *L. monocytogenes* and *L. sakei* 10A (broken lines, ×) (error bars represent 95% confidence intervals, n=3)

This result might indicate that the inhibition phenomena that were observed in the BHI-broths are not caused by competition for biotin, riboflavin, thiamine, Fe^{3+}, Mn^{2+} or Mg^{2+}. However, this hypothesis is not fully proven by our results since in both media, with and without additional glucose, inhibition occurred also due to other reasons than potential competition for vitamins and minerals. In b-mBHI (Figure 7.6a) and in s-b-mBHI-broth (Figure 7.6b), the moment of inhibition corresponded again exactly to the moment at which the glucose supply of the medium was depleted. This indicates that glucose depletion, at least partly, contributed to the inhibition phenomenon in this low-glucose broth. In b-mgBHI and in s-b-mgBHI-broth, the inhibition was probably, by analogy with section 3.2., mainly caused by the production of inhibitory levels of lactic acid/lactate from the higher level of glucose. The mean lactic acid production at day 18 in b-mgBHI and in s-b-mgBHI was 1.29 ± 0.17% and 1.83 ± 0.17%, respectively. The higher lactic acid production in s-b-mgBHI compared to in b-mgBHI is caused by the faster growth of *L. sakei* 10A in the presence of the supplemented vitamins/minerals. In addition, it can not be excluded that the concentrations of the supplemented vitamins/minerals were maybe not high enough to avoid nutrient depletion. However, we strongly think this was not the case since the chosen concentrations of the supplemented nutrients are quite high concentrations that are used in chemically defined minimal media for the optimal growth of *L. monocytogenes* (Phan-Thanh & Gormon, 1997; Moretro et al., 1998).

3.4. Discussion

The results of this chapter prove that the mechanism behind the inhibition of *L. monocytogenes* by *L. sakei* 10A is based on a combination of nutrient competition and lactic acid production/pH-reduction. The higher the glucose content, the more lactic acid is produced which leads to a more extended inhibition. With regard to that part of the inhibition that is caused by nutrient competition, it was found that, at least in the BHI-broths used here, inhibition starts when glucose is depleted. Also in co-culture experiments on a model cooked ham (Chapter 4) growth inhibition of *Leuc. mesenteroides* LM4 by *L. sakei* 10A occurred approximately at the moment that glucose was almost depleted. However, the same conclusion could not be derived from co-culture experiments between *B. thermosphacta* BT1 and 10A (Chapter 4) and also not from co-culture studies between *L. monocytogenes* and *L. sakei* 10A in several real CMP (Chapter 6). In the latter application tests, *L. monocytogenes* did not start to grow in the presence of 10A; the moment of inhibition was in fact the start of

these experiments when there was still sufficient glucose present. So, the translation of the results of the broth experiments of this chapter towards what really occurs in a CMP is a difficult issue. The complexity of the substrate and the presence of the background flora makes it more difficult to analyse the inhibition mechanism in meat products compared to in artificial growth media.

Nutrient competition as an explanation for the inhibition of *L. monocytogenes* by non-bacteriocinogenic LAB has been proposed by several other authors (Buchanan & Bagi, 1997; Bredholt et al., 1999; Nilsson et al., 1999; 2004; Vermeulen et al., 2006). The data of Buchanan & Bagi (1997) suggest that the suppression of the maximum population density of *L. monocytogenes*, when growing in co-culture with a non-bacteriocinogenic *C. piscicola*, is related to nutrient depletion since the extent of the suppression decreased when the two species were cultured in 3× or 6× BHI-broth. In the study of Nilsson et al. (2005), the cell free supernatant of the non-bacteriocinogenic *C. piscicola* A9b caused a decrease in *L. monocytogenes* cell density, which was abolished by glucose addition, suggesting that competition for glucose, at least partly, causes the inhibitory interaction. The results of this work are somewhat different since in the presence of a higher glucose content compared to in the presence of a lower glucose content, inhibition by *L. sakei* 10A was still occurring. However, the fact that at the moment of inhibition, glucose limitation could be observed, confirms the results of Nilsson et al. (2004) and supports the hypothesis that glucose depletion, at least partly, causes the inhibition.

Up to now, there are no studies demonstrating that competition for other nutrients than glucose occurs. Vermeulen et al. (2006) could exclude competition for one of 18 different amino acids as the cause of inhibition of *L. monocytogenes* by a *L. sakei* strain in a chemically defined broth simulating CMP. Nilsson et al. (1999; 2005) found comparable results since supplementation with amino acids and also with individual vitamins and minerals did not eliminate the antilisterial activity of the cell free supernatant of a non-bacteriocinogenic *C. piscicola* A9b. Also this study could not identify any role of vitamins and minerals in the inhibition phenomenon.

4. Conclusion

In this chapter, the non-bacteriocinogenic protective culture *Lactobacillus sakei* 10A was observed to inhibit the food born pathogen *Listeria monocytogenes* in different types of liquid broths and depending on the composition of the broth, either nutrient competition or lactic

acid production/pH-reduction was indicated as the most probable mechanism behind the inhibition. The results strongly indicate that glucose is the main component for which competition occurs.

This chapter was the last chapter dealing with biopreservation by means of lactic acid bacteria. In the next chapter (Chapter 8), another type of biopreservation will be investigated: bacteriophages to prevent growth of *L. monocytogenes* on vacuum packaged sliced CMP.

Chapter 8

Treatment with bacteriophage P100 as an effective biopreservation method to control *Listeria monocytogenes* on sliced and vacuum packaged cooked meat products

Redrafted from

L. Vermeiren, F. Devlieghere, D. De Meester, M. Schellekens and J. Debevere

International Journal of Food Microbiology

In preparation for submission

Chapter 8
Treatment with bacteriophage P100 as an effective biopreservation method to control *Listeria monocytogenes* on sliced and vacuum packaged cooked meat products

Summary

This chapter reports on the use of bacteriophage P100 to prevent proliferation of post-processing contaminating *L. monocytogenes* cells on vacuum packaged, sliced, cooked meat products.

First, broth experiments revealed that the three *L. monocytogenes* strains, used in chapters 5 and 6 of this PhD-work, were each susceptible to the action of bacteriophage P100 and this at 30°C as well as at 7°C. However, at 30°C the susceptibility towards P100 was strain-dependent since the time, at which the OD(600nm) of the growth medium containing *L. monocytogenes* and P100 started to increase, differed among the three strains. Therefore subsequent application trials on cooked meat products were making use of a cocktail of the three *L. monocytogenes* strains.

In a preliminary application test on a cooked poultry product the presence of phage P100 resulted in a reduction of the *L. monocytogenes* count with 3.32 $\log_{10}$(cfu/g) compared to the untreated control after 21 days of storage (7°C, vacuum). A more elaborate application test on cooked ham confirmed the antilisterial effect of P100 on CMP during storage at 7°C under vacuum packaged conditions. In the latter experiment, treatment with P100 at a level of 1×10^7 pfu/cm^2 or 5×10^6 pfu/cm^2 reduced the population of *L. monocytogenes* after 10 days of storage (7°C, vacuum) with 0.97 and 0.61 $\log_{10}$(cfu/g), respectively, compared to the untreated control. However, the difference in antilisterial effect between the two different phage doses was shown to be not significant.

In conclusion, this chapter provides evidence on the usefulness of bacteriophage P100 to control the growth of *L. monocytogenes* on sliced, cooked meat products during anaerobic storage at 7°C.

1. Introduction

Listeria monocytogenes is a widely distributed opportunistic food born pathogen and ingestion of food contaminated with this pathogen may cause listeriosis (Bell & Kyriakides, 2005). *L. monocytogenes* is of great concern due to its high mortality rate, wide distribution on raw products, psychrotrophic character and its ability to establish itself in various food processing environments (Muriana, 1996). Several food items have been associated with major outbreaks of listeriosis including vegetables, dairy products (pasteurised milk and cheese), fish and fish products (smoked and marinated fish) and also meat products such as cured cooked meat products (CMP) (pâté, frankfurters, etc.) (Jemmi et al., 2002; Vitas & Garcia-Jalon, 2004; Roberts et al., 2005). Details on prevalence of *L. monocytogenes* in CMP have been described before in chapters 1 and 5.

Cooked meat products are not only prone to contamination with *L. monocytogenes* but the substrate properties and storage conditions are adequate for the development of this pathogen (Bredholt et al., 2001; Devlieghere et al., 2001; Uyttendaele et al., 2004; Chapter 6). Outbreaks of listeriosis due to consumption of CMP show the possible risks associated with the presence of this pathogen in CMP (Vitas & Garcia-Jalon, 2004; Bell & Kyriakides, 2005). The search for innovative strategies to control *L. monocytogenes* is a major research topic within the field of food microbiology. In view of the consumer trends towards natural and healthy food products, preservation by means of micro-organisms has been proposed. In general, the applied micro-organisms are bacteria and in particular lactic acid bacteria (LAB) (Holzapfel et al., 1995; Lücke, 2000). Recently, there is an increased interest in the use of bacteriophages as a means of inactivating food born pathogens (Hudson et al., 2005). Several recent studies investigated the potential of phages for controlling *Campylobacter* (Atterbury et al., 2003b; Goode et al., 2003), *Salmonella* (Goode et al., 2003; Whichard et al., 2003), *E. coli* (O'Flynn et al., 2004) and *L. monocytogenes* (Dykes & Moorhead, 2002; Carlton et al., 2005).

Dykes & Moorhead (2002) are, to our knowledge, the sole authors that report on phage control of *L. monocytogenes* in meat. They investigated the effect of listeriophage LH7 on the growth and survival of two strains of *L. monocytogenes* on vacuum packaged beef, which was stored at 4°C. The listeriophage had little effect on either of the *L. monocytogenes* strains probably because of the less than optimal bacteria-phage ratio. Carlton et al. (2005) selected

for its work the strictly lytic phage P100 and demonstrated its successful application on artificially contamined soft cheese to control *L. monocytogenes*.

At present, the option of using phages to prevent proliferation of post-contaminating *L. monocytogenes* cells on anaerobically packaged CMP has not been reported. Therefore, the objective of this chapter was to investigate the feasibility of bacteriophage P100 to control *L. monocytogenes* in CMP. A first experiment aimed at testing the sensitivity of the three *L. monocytogenes* strains, used in chapters 5 and 6 of this PhD-work, towards P100 at 30°C and at 7°C in broth. Further, application trials on cooked chicken fillet and on cooked ham were performed to prove the antilisterial effect of P100 during storage at 7°C under vacuum packaged conditions.

2. Materials and methods

2.1. Bacterial strains and bacteriophage P100

Three *L. monocytogenes* strains were used in this study. Strain *L. monocytogenes* Scott A and strain *L. monocytogenes* LFMFP 45, isolated from cooked pita meat, were present at the Laboratory of Food Microbiology and Food Preservation (LFMFP, Gent University, Belgium). Strain *L. monocytogenes* LMG 13305, isolated from soft cheese, was obtained from the culture collection of the Laboratory Microbiology Gent (LMG, Gent University, Belgium). The indicator strain used for the phage titer determination was *Listeria innocua* B1488, obtained from EBI Food Safety (Den Haag, The Netherlands). Stock cultures of the strains were maintained in Brain Heart Infusion (BHI, Oxoid, Oxoid Limited, Basingstoke, Hampshire, UK) broth supplemented with 15% glycerol at -75°C. Working cultures of the strains were maintained on Tryptone Soya Agar (TSA, Oxoid) slants at 7°C and revived by transferring a loop of inoculum into 10 ml BHI-broth followed by incubation at 30°C for 24h.

Bacteriophage P100, isolated from a sewage effluent sample from a dairy plant in southern Germany, was provided by EBI Food Safety (Den Haag, The Netherlands) (Carlton et al., 2005). The Listex^{TM} P100 solution contained a high concentration (varying from 2.3×10^{10} to 5.3×10^{10} pfu/ml) of P100-phages and was stored at 4°C.

2.2. Effect of P100 on *L. monocytogenes* in broth

The effect of listeriophage P100 on the growth of three *L. monocytogenes* strains (Scott A, LFMFP 45 and LMG 13305) was determined in broth at two different growth temperatures

(7°C and 30°C). Experiments were performed in microtiter plates and in triplicate for each *L. monocytogenes* strain and for each incubation temperature. Part of the microtiter plate wells contained BHI with *L. monocytogenes* alone (5×10^5 cfu/ml) while another part contained BHI with *L. monocytogenes* (5×10^5 cfu/ml) and P100 (1×10^8 pfu/ml). Wells containing *L. monocytogenes* alone were prepared by filling them with 100 µl of BHI-broth and 100 µl of the appropriate dilution (in BHI-broth) of a 24h culture of the respective *L. monocytogenes* strain. Wells containing *L. monocytogenes* and P100 were prepared by filling them with 100 µl of the appropriate dilution (in BHI-broth) of a 24h culture of the respective *L. monocytogenes* strain and 100 µl of the appropriate dilution (in BHI-broth) of the Listex™ P100 solution. Microtiter plates were incubated at 30°C for 72h or at 7°C for 7 days. Growth was followed by measuring the optical density at 600 nm using a Versamax microplate reader (Molecular devices, Sunnyvale, CA, USA). OD-measurements of the broth inoculated with *L. monocytogenes* alone were corrected by the OD of a control consisting of only pure BHI, while measurements of the broth inoculated with *L. monocytogenes* and P100 were corrected by the OD of a control consisting of BHI and P100.

2.3. Effect of P100 on *L. monocytogenes* in cooked chicken fillet

This experiment on cooked chicken fillet was a preliminary experiment to test the feasibility of bacteriophage P100 to control the growth of *L. monocytogenes* on cooked meat products. Further, this experiment allowed evaluation of the practical procedure for studying the interaction between phages and bacteria on CMP.

The test product was industrially prepared, sliced, vacuum packaged, cooked chicken fillet. The product was obtained from a Belgian meat company and immediately after production transferred to the laboratory under cooled conditions. An 'industrially prepared' product was chosen to test the antilisterial effect of P100 in the presence of a realistic initial level of background flora.

The product was treated in three different ways: (1) non-inoculated product (control), (2) product inoculated with a cocktail of three *L. monocytogenes* strains (Scott A, LFMFP 45 and LMG 13305) at 10 cfu/g, (3) product inoculated with the same three-strain cocktail of *L. monocytogenes* and subsequently treated with P100 (treatment level of $1x10^7$ pfu/cm^2) (MOI of $4x10^6$). In the case of inoculation with *L. monocytogenes*, a 24h culture (BHI-broth, 30°C) of each *L. monocytogenes* strain was diluted in Peptone Physiologic Solution (PPS; 8.5 g/l NaCl (VWR, VWR International, Leuven, Belgium) and 1 g/l peptone (Oxoid)) to prepare

the inoculum of the three-strain *L. monocytogenes* cocktail. From the appropriate dilution of the *L. monocytogenes* cocktail, 500 µl was divided over and spread on the surface of ± 150 g of meat product with a spatula to reach the desired inoculation level of *L. monocytogenes* (± 10 cfu/g). In the case of treatment with P100, the product was inoculated with the Listex^{TM} P100 solution more or less 60 seconds after inoculation with *L. monocytogenes*. From the appropriate dilution of the phage solution containing 2×10^{10} pfu/ml, 500 µl was divided over and spread on the surface of ± 150 g of product with a spatula to reach the desired inoculation level of $1x10^{7}$ pfu/cm^{2}.

After inoculation, the 150 g portions of product were vacuum packaged and stored at 7 ± 1°C in a ventilated refrigerator. Vacuum packaging was performed as described before in Chapter 2. At regular time intervals during storage, samples were analysed to determine the number of LAB, the presence of *L. monocytogenes* in 25 g of sample and/or the number of *L. monocytogenes*.

2.4. Effect of P100 on *L. monocytogenes* in cooked ham

An application trial on cooked ham was performed in order to investigate the efficiency of bacteriophage P100 to control the growth of *L. monocytogenes* in sliced and vacuum packaged cooked ham during refrigerated storage.

The test product was an industrially prepared, sliced, vacuum packaged, cured cooked ham with the Belgian quality label 'Meesterlyck'. The product was obtained from a Belgian meat company and immediately after production transferred to the laboratory under cooled conditions. The company estimated the product's shelf-life at 7°C for ± 2 weeks under vacuum packaging.

The cooked ham was treated in four different ways: (1) non-inoculated product (control), (2) product inoculated with a cocktail of three *L. monocytogenes* strains at 10 cfu/g (Scott A, LFMFP 45 and LMG 13305), (3) product inoculated with the same three-strain cocktail of *L. monocytogenes* and subsequently treated with P100 at $1x10^{7}$ pfu/cm^{2} (MOI of $5x10^{6}$) and (4) product inoculated with the three-strain cocktail of *L. monocytogenes* and treated with P100 at $5x10^{6}$ pfu/cm^{2} (MOI of $2.6x10^{6}$). Two treatment levels were tested to optimise the treatment level and allow selection of the most economical phage dose. Each treatment was tested in triplicate.

Inoculation with *L. monocytogenes* and treatment with P100 was done in the same way as described before in section 2.3 of this chapter. From the appropriate dilution of the

L. monocytogenes cocktail, 100 µl was spread on the surface of ± 150 g product to obtain approximately 10 cfu/g of *L. monocytogenes*. From the appropriate dilution of the phage solution (5.3×10^{10} pfu/ml), 150 µl was spread on the surface of ± 150 g of product to obtain $1x10^7$ pfu/cm^2 or $5x10^6$ pfu/cm^2 of P100.

After inoculation, the 150 g portions of product were vacuum packaged and stored at 7 ± 1°C in a ventilated refrigerator. Vacuum packaging was performed as described in section 2.3. At regular time intervals during storage, cooked ham samples were analysed to determine the total aerobic psychrotrophic count, the number of LAB, the presence of *L. monocytogenes* in 25 g of sample and/or the number of *L. monocytogenes*. Samples treated with P100 were also subjected to phage number enumeration.

2.5. Chemical analyses

The cooked meat products were characterised by determining the pH, water activity, NaCl level, dry matter, lactate level and nitrite level. The pH was measured by using a pH-electrode (Ingold, MGDX K57, Urdorf, Switzerland) connected to a pH-meter (Knick, type 763, Berlin, Germany). NaCl-content was determined according to the method of Mohr, a titrimetric determination of chloride ions (Skoog et al., 1996). The water activity was measured by using an a_w cryometer (Nagy AWK-20, Gäufelden, Germany). Lactate was determined following an enzymatic method (method Boehringer). The level of nitrite was determined according to ISO 2918. Moisture and ash were determined according to standard AOAC (1990) procedures.

2.6. Microbiological analyses

A 15 g sample was taken aseptically and a decimal dilution series in PPS was prepared to plate the appropriate dilutions on the appropriate agar media. Total aerobic psychrotrophic count and number of LAB were determined according to the pour plate technique on Plate Count Agar (PCA, Oxoid) (aerobic incubation at 22°C for 3-5 days) and de Man Rogosa Sharpe (MRS, Oxoid) agar (aerobic incubation at 30°C for 3-5 days), respectively.

Quantitative enumeration of *L. monocytogenes* was done by spreadplating onto ALOA (Agar Listeria Ottaviani and Agosti) (Biolife, Biolife Italiana S.r.L., Milan, Italy) (aerobic incubation for 2 days at 37°C) supplemented with ALOA Enrichment selective supplement (Biolife). The presence of *L. monocytogenes* in 25 g was determined in three steps: (1) a primary enrichment (24h, 30°C) of a 10-fold diluted homogenised 25 g sample in demi-Fraser broth (Biomérieux, Brussels, Belgium), (2) subsequently, 0.1 ml of incubated demi-Fraser

broth was transferred to 10 ml Fraser broth (Biomérieux) for secondary enrichment (24h, 30°C) and (3) identification using MiniVidasLMOII (Biomérieux).

2.7. Phage titer determination

For phage number enumeration of phage-treated products, a soft agar overlay technique was used based on the method described by Carlton et al. (2005). As a positive control, the Listex^{TM} P100 stock solution was included in the test.

Samples (10 g) from phage-treated products were first diluted tenfold in sterile 0.1 M phosphate buffer of pH 7.4 (Sigma, Sigma-Aldrich Corporation, St. Louis, Missouri, USA) and then filter sterilised through a membrane filter (∅ 0.45 µm, Schleicher & Schuell Microscience, Dassel, Germany) to avoid bacterial contamination of the soft agar double layer plates in the subsequent plaque assay.

The phage titers of the filtrates of the phage-treated products and of the Listex^{TM} P100 stock solution were determined by counting plaques from their serial 10-fold dilutions. Volumes of 20 µl of each phage dilution were mixed with 200 µl (10^6 cfu) of cells of the indicator strain *L. innocua.* After incubation for 30 min at 30°C, the cell-phage mixture was mixed with 3 ml of preheated 4YT (yeast trypton) semi-soft agar (32 g/l trypton (Oxoid), 20 g/l yeast extract (Oxoid), 5 g/l NaCl (VWR) and 7.5 g/l agar (Oxoid)) and this final mixture was quickly poured onto pre-heated 4YT-agar plates (32 g/l trypton, 20 g/l yeast extract, 5 g/l NaCl and 15 g/l agar). Following incubation for 24h at 30°C, plaques were counted.

2.8. Statistical analyses

The application test on cooked ham was conducted in triplicate. All statistical analyses were performed by using the software SPSS 12.0 (SPSS, Chicago, IL, USA). *t*-tests were applied to determine the significance ($P<0.05$) of differences in phage titers between the two different treatment levels and between the beginning and the end of storage. Analysis of variance (One-way ANOVA) was applied to determine the significance ($P<0.05$) of differences in bacterial counts between the three different treatments.

3. Results and discussion

3.1. Effect of P100 on *L. monocytogenes* in broth

The effect of phage P100 on *L. monocytogenes* Scott A, LFMFP 45 and LMG 13305 in BHI-broth at 30°C and 7°C is presented in Figures 8.1 and 8.2, respectively.

At 30°C, in absence of phage P100, the three *L. monocytogenes* strains displayed a very similar pattern since all reached a maximal OD-value of approximately 0.6 already after about 8h and the three strains grew with a comparable growth rate as indicated by the slope of the OD-curves.

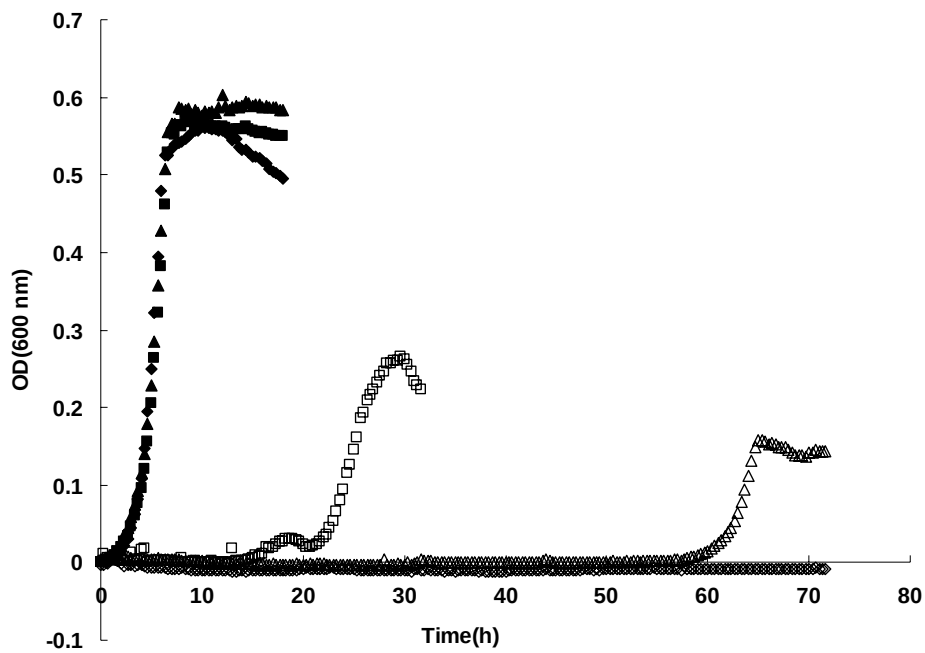

Figure 8.1. The growth of *L. monocytogenes* in BHI-broth at 30°C without (full symbols) and with (hollow symbols) listeriophage P100 (◊,♦LFMFP 45; ■,□ Scott A;▲,△ LMG 13305) (symbols represent mean OD-value of three replicates)

In the presence of phage P100, the growth of the *L. monocytogenes* strains was inhibited. The extent of inhibition varied among the strains (Figure 8.1). In the case of strain LFMFP 45, no increase in OD was observed during 72h. The detection limit or the time at which the OD(600nm) starts to increase was known to be $\pm$ 1×10^7 cfu/ml for *L. monocytogenes* (Francois et al., 2005). Therefore, no increase in OD can mean that the strain either has not

grown due to the action of P100 or that the strain had grown to a level lower than 7 $\log_{10}$(cfu/ml) due to the action of P100. In the case of strain Scott A and LMG 13305, the OD increased but only after a prolonged time: at ± 15 hours for strain Scott A and at ± 58 hours for strain LMG 13305 compared to ± 1 hour in the absence of P100. The extension of the detection time (the time at which the OD(600nm) starts to increase) in the presence of P100 means that the phage is active. However, a part of the cells was probably still intact and these cells were doubling in time since the OD finally started to increase. When finally growing, *L. monocytogenes* Scott A and LMG 13305 grew at a lower growth rate compared to in the absence of P100. Furthermore, the maximal OD-value of the strains was reduced by the presence of P100 and after having reached a maximal OD-value, the OD started to decrease slightly proving the lytic action of the phage. According to Carlton et al. (2005) P100 is strictly lytic. The action of lytic phages results in the disruption of the microbial cell wall (Hudson et al., 2005) and in clearing of the growth medium (Whichard et al., 2003).

The results indicate that the Listex™ P100 solution has an antilisterial effect at 30°C in broth towards all three *L. monocytogenes* strains. Similar experiments at 30°C were performed by Dykes & Moorhead (2002) in Tryptone Soya Broth. However, these authors observed a more limited effect of the investigated listeriophage LH7. They reported only a slight extension of ± 2 hours in the moment of OD-increase for two different *L. monocytogenes* strains by adding listeriophage LH7 at the start of the incubation period.

Since the final goal of this experiment was to find out if P100 could control the growth of *L. monocytogenes* under refrigerated conditions, broth experiments were repeated at 7°C. The latter experiment lasted 7 days and during this period no increase in OD occurred when P100 was added while in absence of P100, the strains reached approximately their maximal OD-value after this 7-days period (Figure 8.2).

Dykes & Moorhead (2002) observed at 7°C only for one of two investigated *L. monocytogenes* strains an extension (of 9 days) of the time at which the OD started to increase. Although we cannot exclude that growth of the *L. monocytogenes* strains could have started later than the 7th day, our results clearly demonstrate the in-vitro effectiveness at 7°C of the P100 solution towards *L. monocytogenes.*

In broth, Listex™ P100 was effective towards all three investigated *L. monocytogenes* strains but at 30°C the efficiency of the activity of P100 was strain dependent and the highest effectiveness was observed towards *L. monocytogenes* LFMFP 45. Strain variability in the

susceptibility of the genus *Listeria* towards phages is known (Loessner & Busse, 1990) and may be due to strain variation in the cell wall composition and more specifically in the number and type of receptor molecules (Deutsch et al., 2004; O'Flynn et al., 2004), differences in restriction/modification systems in the host (O'Flynn et al., 2004) or differences in burst rate and burst size or the rate at which phages escape and the number of phages that escape from the bacterial host. According to Carlton et al. (2005) almost all of the phages infecting organisms of the genus *Listeria* feature a very narrow host range, while P100 features an unusually broad host range within the genus *Listeria*, similar to phage A511 (Loessner & Busse, 1990). The lytic reaction of P100 is not related to one species or serovar within the genus *Listeria* (Carlton et al., 2005).

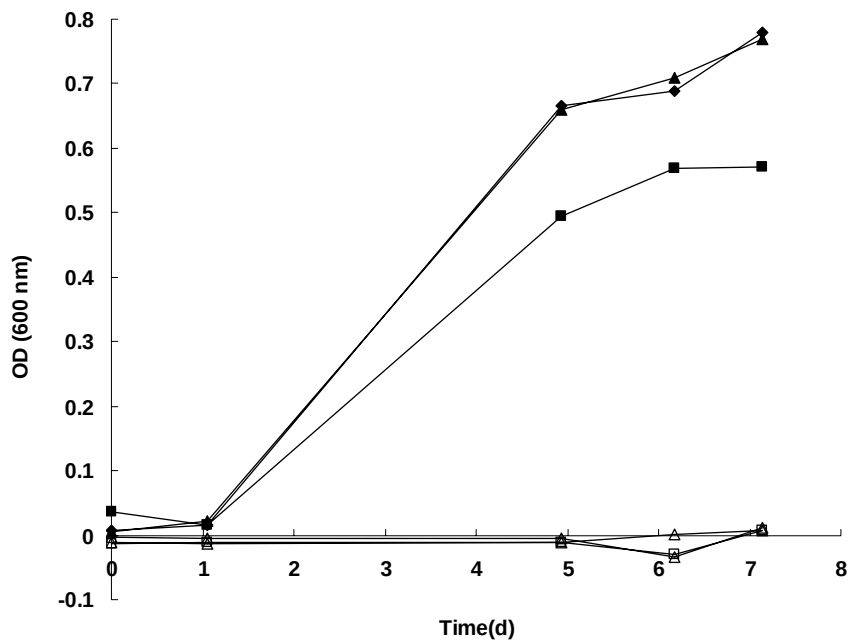

Figure 8.2. The growth of *L. monocytogenes* in BHI-broth at 7°C without (full symbols) and with (hollow symbols) listeriophage P100 (◊,♦LFMFP45; ■,□ Scott A;▲,Δ LMG 13305) (symbols represent mean OD-value of three replicates)

3.2. Effect of P100 on *L. monocytogenes* in cooked chicken fillet

The chemical composition of the cooked chicken fillet was: 27.41% of dry matter, 3.11% NaCl (in water phase), pH of 6.25, water activity of 0.9797, residual nitrite level <5 mg/kg (as $NaNO_2$) and (D+L)-lactic acid level of 1.01%.

Table 8.1 shows the effect of phage P100 on the growth of *L. monocytogenes* on the surface of cooked chicken fillet during vacuum packaged storage at 7°C.

Table 8.1. The growth (log_{10}(cfu/g)) of the artificially inoculated three-strain *L. monocytogenes* cocktail on vacuum packaged cooked chicken fillet at 7°C without listeriophage P100 (LIS) and with listeriophage P100 (LIS+P100) at a dose of $1x10^7$ pfu/cm^2

Time (days)	LIS	LIS + P100
0	1.00	1.00
7	2.46	2.04
14	4.62	1.85
21	4.32	1.00

This experiment was performed only once and not in triplicate since it was only a preliminary experiment. Therefore, statistical analysis of the data was not possible. However, the effect of P100 could be undoubtedly determined. In the non-inoculated cooked poultry product (= blank), *L. monocytogenes* was absent (in 25 g of sample) during the whole storage period, indicating that the test product was not contaminated with this pathogen. In the product inoculated with the cocktail of the three *L. monocytogenes* strains, *L. monocytogenes* grew approximately 3.5 log_{10}(cfu/g) within two weeks. After these two weeks, the level of *L. monocytogenes* stagnated probably due to a growth-inhibiting effect of the endogenous lactic acid flora. Indeed, the number of lactic acid bacteria reached a mean value of 7.7 log_{10}(cfu/g) at day 14. It is known that LAB can affect the growth of *L. monocytogenes* when they reach levels of 10^7 cfu/g or more (Chapters 5 & 6).

In the product inoculated with *L. monocytogenes* and phage P100, *L. monocytogenes* could only grow ± 1 log_{10}(cfu/g) after 7 days but during further storage its level decreased and after three weeks no *L. monocytogenes* cells could be detected anymore. Although the endogenous lactic acid flora might have contributed partly to the observed antilisterial effect from day 14 on, it is clear that the presence of the phage solution retards the growth of *L. monocytogenes.* In this experiment, no effect of the phage solution on the endogenous LAB-flora could be observed. Initially, 2.2 log_{10}(cfu/g) of LAB were present. This corresponds to the levels that normally are present on CMP: 0.5-3 log_{10}(cfu/g) (Samelis et al., 2000a; Chapter 6). After one and two weeks of storage, the LAB-count of the control sample was increased to a level of 5.5 and 7.4 log_{10}(cfu/g), respectively. A cell number of 7 log_{10}(cfu/g) is often considered as the

end-point for the microbial shelf-life of cooked meat products (Korkeala et al., 1987; Chapter 3).

3.3. Effect of P100 on *L. monocytogenes* in cooked ham

The chemical composition of the cooked ham was: 28.45 ± 2.05 % of dry matter, 2.38 ± 0.00 % of NaCl (in water phase), pH of 6.11 ± 0.02, water activity of 0.9827 ± 0.0001, residual nitrite level of 5.50 ± 0.71 mg/kg (as $NaNO_2$) and (D+L)-lactic acid level of 0.67 ± 0.06 %.
The control cooked ham did not contain *L. monocytogenes* (in 25 g) and was also not artificially contaminated with the pathogen. Figure 8.3 shows the effect of P100 on the proliferation of *L. monocytogenes* on the surface of cooked ham that was stored under vacuum packaging at 7°C.

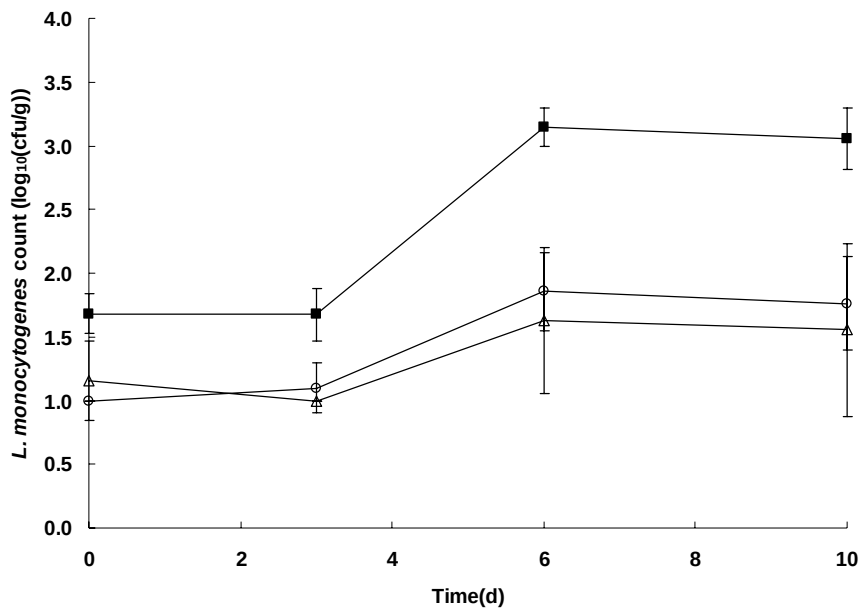

Figure 8.3. The growth of the three-strain *L. monocytogenes* cocktail in vacuum packaged cooked ham at 7°C without (full symbols, ■) and with (hollow symbols) listeriophage P100 (Δ, higher treatment level of $1x10^7$ pfu/cm^2; ○, lower treatment level of $5x10^6$ pfu/cm^2)(symbols represent mean of three replicates, error bars represent 95% confidence intervals)

Analysis of variance (P<0.05) showed that there was a significantly lower *L. monocytogenes* number on the P100-treated samples compared to on the untreated samples and this throughout the storage period and independent of the phage treatment level. This means that a

treatment of cooked ham with P100 significantly retarded the growth of *L. monocytogenes*. On the product inoculated with the cocktail of *L. monocytogenes* and not treated with P100, the number of *L. monocytogenes* increased with 1.37 ± 0.34 $\log_{10}$(cfu/g) after 10 days. On the cooked ham inoculated with the cocktail of *L. monocytogenes* but treated with P100 at a level of $1x10^7$ pfu/cm^2 or $5x10^6$ pfu/cm^2 the number of *L. monocytogenes* increased only with 0.40 ± 0.36 and 0.76 ± 0.32 $\log_{10}$(cfu/g), respectively (after 10 days). The limited growth of *L. monocytogenes* on the cooked ham product in absence of P100 was related to the high LAB-count of the product. After 7 days the product reached already a LAB-number of 7 $\log_{10}$(cfu/g) resulting in growth inhibition of *L. monocytogenes*.

Already at the start of the experiment (day 0), the *L. monocytogenes* number was significantly lower in the P100-containing samples compared to in the untreated sample (Figure 8.3). This could mean that the lower and the higher phage treatment level resulted in an immediate reduction with 0.52 and 0.68 $\log_{10}$(cfu/g), respectively, of the number of *L. monocytogenes*. Additional experiments were performed to explain this initial difference in cell number of *L. monocytogenes*. These experiments revealed that the initial difference in *Listeria*-number was not due to phage activity on the surface of the ALOA-agar plates during incubation since no significantly different *L. monocytogenes* counts could be observed when enumerating *L. monocytogenes* in BHI-broths inoculated with either *L. monocytogenes* alone or with *L. monocytogenes* and P100 (plating was done immediately after inoculation). A small part of the initial difference might, however, be attributed to the action of the phages in PPS in the period between diluting the sample and plating onto ALOA. A small (0.1 log) but statistically significant (*t*-test, $P<0.05$) decrease in *Listeria*-number was observed when *L. monocytogenes* cells were subjected to a phage treatment (same multiplicity of infection (MOI) as on cooked ham trial) in PPS and then stored for 3 hours at room temperature compared to untreated *L. monocytogenes* cells. Although these observations not fully explain the initial lower cell number in the presence of P100, it is possible that the phages, when inoculated onto the product, immediately start to act on the *Listeria* cells that are present on the surface of the cooked ham and that in the time during inoculation, sampling and plating (estimated at ± 2 hours) the phage's activity leads to a slightly reduced number of *L. monocytogenes* cells.
Using analysis of variance ($P<0.05$), it could be demonstrated that there was no significant difference in antilisterial effect between the two investigated phage treatment levels. Treatment of cooked ham with the lower, more economical P100-dose (5×10^6 pfu/cm^2) might,

therefore, be sufficient to control *L. monocytogenes*. It might be interesting to examine in future experiments the influence of even lower P100-doses.

The evolution of the phage titers on the phage-treated cooked ham samples during storage under vacuum at 7°C is presented in Figure 8.4.

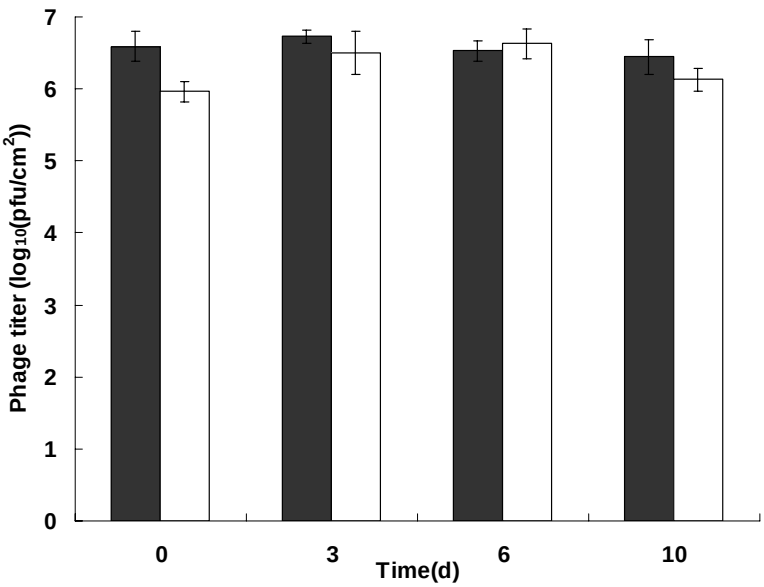

Figure 8.4. The evolution of the phage titer on the vacuum packaged cooked ham during storage at 7°C (black bars, phage treatment level of $1x10^7$ pfu/cm^2; white bars, phage treatment level of $5x10^6$ pfu/cm^2) (error bars represent 95% confidence intervals, n=3)

On day 0, the obtained phage titers were about 0.5 log-cycle lower than the desired phage titers and this was the case for the two treatment levels. Furthermore, the lower treatment level was about 0.5 log-cycle lower than the higher treatment level but this difference could not be observed anymore on the other days of analysis. On day 3, 6 and 10, there was no significant (*t*-test; $P<0.05$) difference anymore in phage titer between the two different treatment levels.

Phage titers did not change significantly (*t*-test; $P<0.05$) between day 0 and day 10 of the experiment, demonstrating the stability of phage P100 on the cooked ham product. Initially, 2.3 $\log_{10}$(cfu/g) of LAB were present. The total aerobic psychrotrophic count and the LAB-count of the non-inoculated cooked ham were of the same magnitude. After 1 week of storage, the LAB-count of the control sample had evolved already to a level of 7.4 $\log_{10}$(cfu/g), thereby exceeding the limit of 7 $\log_{10}$(cfu/g) that is often used for the microbial

shelf-life of cooked meat products (Korkeala et al., 1987; Chapter 3). Treatment of the cooked ham with P100 did not influence the presence of bacteria other than *L. monocytogenes.* The evolution of the total aerobic psychrotrophic count and of the LAB-count as a function of time did not differ significantly between cooked ham samples treated with P100 and untreated cooked ham samples.

To our knowledge, Dykes & Moorhead (2002) are the only authors that have reported before on the effect of a bacteriophage on *L. monocytogenes* on meat. In their study, the exposure of beef to listeriophage LH7, by immersion in a bath containing 3×10^3 pfu/ml, had no significant effect on numbers of *L. monocytogenes* (10^6 cfu/cm^2) compared to the control during refrigerated storage under vacuum. No phage titer determinations were performed but the authors appointed the less than optimal bacteria-phages ratio for this difference. Our results compare well to the reports of Leverentz et al. (2003) and Carlton et al. (2005). Leverentz et al. (2003) examined the effect of a mixture of lytic listeriophages on *L. monocytogenes* in artificially contaminated fresh-cut melons (pH 5.5 to 6.5). The phage mixture reduced *L. monocytogenes* populations by 2.0 to 4.6 log-units over the control. Carlton et al. (2005) have also studied the application of bacteriophage P100 but on cheese. When applying P100 to surface-ripened red-smear soft cheese through the washing/smearing solution a dose-dependent inhibitory effect of P100 was recorded. A lower concentration of 1.5×10^8 pfu per ml of smearing solution resulted in an approximately 2-3 log decrease of *Listeria* viable counts. When a higher concentration of 3×10^9 pfu per ml of smearing solution was used, complete eradication of viable *L. monocytogenes* was observed. In contrast, the untreated control cheeses supported growth of *L. monocytogenes* to titers of generally more than 7 $\log_{10}$(cfu/g).

Phage resistance and host specificity are two important issues to consider when using bacteriophages as biopreservatives. The frequency of formation of bacteriophage-insensitive mutants of *E. coli* O157:H7 has been reported to vary from 10^{-4} to 10^{-6} (O'Flynn et al., 2004). To our knowledge, frequency of resistance formation has not been reported for P100. Carlton et al. (2005) found no evidence for phage resistance in the *Listeria* isolates recovered from P100-treated cheese samples and concluded that the development of insensitivity of *Listeria* cells against strictly virulent phages such as P100, if occurring at all, is a rare event. Indeed, the fact that on the surface of cooked meat products 'lysis from without' occurs in stead of the classic lytic pathway and that *L. monocytogenes* typically occurs in low levels on

contaminated cooked meat products, reduces the risk for resistance development following phage applications in food products.

With regard to host specificity, P100 has been reported to be one of the few known virulent phages for this genus, which are strictly lytic and which have an unusually broad host range within the genus *Listeria* (Carlton et al., 2005).

4 Conclusion

Public and regulatory concern related to *L. monocytogenes* has led to the implementation of microbiological standards, aiming at regulating the levels of *L. monocytogenes* in food products. Since 1st of January 2006, a new EU regulation on microbiological criteria for foodstuffs has come into force (Commission Regulation (EC) No 2073/2005) (European Commission, 2005). This regulation sets a maximum level of 100 cfu/g for *L. monocytogenes* at the end of the shelf-life of ready-to-eat foods (including cooked meat products) when absence of *L. monocytogenes* in 25 g of the product can not be guaranteed. Driven by this new EU-regulation, research on treatment of food products with bacteriophages to prevent growth of *L. monocytogenes* has gained interest. This chapter provides evidence on the usefulness of the lytic *Listeria*-specific phage P100 to control the growth of *L. monocytogenes* on sliced cooked meat products. Activity of phage P100 was observed at a MOI of 10^6 pfu/cfu, at 7°C and under anaerobic storage conditions. The applied phages did not replicate during storage but their titer remained stable, suggesting that P100 is active through 'lysis from without', a mechanism that occurs at very high MOI's. Important issues to consider in the design of phage interventions for food products are the emergence of phage resistance and the host specificity of the phage. These topics could be the subject of future research.

General discussion, conclusions and perspectives

General discussion, conclusions and perspectives

1. General Discussion

When studying literature on biopreservation of meat and meat products, it became clear that most studies of the past have mainly focused on the use of bacteriocinogenic protective cultures to control food born pathogens such as *Listeria monocytogenes* and this in particular on fermented meat products. Recently, the use of protective cultures for preservation of non-fermented meat products such as cured cooked meat products gained more interest. However, little information was available on the influence of non-bacteriocinogenic micro-organisms on the spoilage organisms relevant for these cooked meat products. Therefore, the primary objective of this work was to investigate the possibility of preserving sliced cooked meat products (CMP), which are packaged either under vacuum or under a modified atmosphere, by means of non-bacteriocinogenic micro-organisms and to elucidate possible mechanisms of their biopreservation activity. Besides improvement of food stability, also food safety and in particular control of the growth of *L. monocytogenes* on these CMP was an important research topic of this PhD-work. Two types of non-bacteriocinogenic micro-organisms were investigated: lactic acid bacteria (LAB) on the one hand and bacteriophages on the other hand. The main part of this research dealt with the application of non-bacteriocinogenic LAB. Only in the final chapter, preservation of vacuum packaged CMP by means of bacteriophages was investigated.

1.1. Relevant spoilage organisms for anaerobically packaged cooked meat products

Prior to development of a biopreservation technology for CMP it was necessary to collect more information on the food product under study: sliced, cured, cooked meat products packaged under vacuum or under a modified atmosphere. To study these CMP, a model cooked ham (MCH) product was designed. From literature it was known that spoilage of anaerobically packaged CMP is mainly caused by LAB, in particular *Lactobacillus* spp. and *Leuconostoc* spp., and in some cases also by *Brochothrix thermosphacta*. It was the objective of chapter 3 to study the behaviour of several relevant spoilage organisms on the MCH-product that would be used throughout this work. The results of broth experiments and of an inoculation study on MCH showed that within a group of nine spoilage organisms, typically

associated with anaerobically packaged sliced CMP, *B. thermosphacta* and *Leuc. mesenteroides* subsp. *mesenteroides* seemed to have the highest potential to cause rapid spoilage. MCH-samples were considered unfit for consumption when the LAB-count reached values of 6.9 to 8.2 $\log_{10}$(cfu/g). Sensory rejection of MCH-samples containing high levels of *Leuc. mesenteroides* subsp. *mesenteroides* was related to the production of heterofermentative end-products, mainly acetic acid and ethanol. Sensory spoilage of MCH containing *B. thermosphacta* occurred at a lower cell number of 6.6 to 6.9 $\log_{10}$(cfu/g). *Lactobacillus sakei*, a strain that is often reported in literature to be the specific spoilage organism of CMP, was not the most rapidly growing organism on the MCH. It has to be stressed that this study was a pure culture study excluding in this way interactions between the different spoilage organisms that normally occur on an industrially manufactured cooked meat product.

1.2. Shelf-life extending capacity of protective LAB-cultures

Is it possible to find lactic acid bacteria that can extend the shelf-life of anaerobically packaged, sliced CMP? To answer this question, the PhD-study started with the selection of appropriate lactic acid bacteria (LAB) that could serve as potential protective culture(s) throughout this work (Chapter 2). Starting from 91 meat born strains, either collected or isolated in this work, 12 putative protective cultures were selected. These cultures were homofermentative, salt tolerant and psychrotrophic LAB with interesting antibacterial properties towards *L. monocytogenes*, *Leuc. mesenteroides*, *Leuc. carnosum* and *B. thermosphacta*. From these 12 strains, only one non-bacteriocinogenic (*Lactobacillus sakei* subsp. *carnosus* 10A) and one bacteriocinogenic (LS5 or lactocin S producing *L. sakei* 148) strain were the subject of further research in chapters 4 and 5.

Chapter 4 investigated whether *L. sakei* 10A and *L. sakei* LS5 were able to inhibit growth of the spoilage organisms *Leuc. mesenteroides* and *B. thermosphacta* on the MCH. To resolve the difficulty of individually following the growth of a homofermentative LAB-strain and a heterofermentative LAB-strain when growing in co-culture on the MCH, TC8-MRS-agar was developed. This medium allowed differentiation between the colonies of strain 10A or strain LS5 and the colonies of the *Leuc. mesenteroides* strain.

The lactocin S producing strain *L. sakei* LS5 was found to be not effective since its presence did not affect the growth of both spoilage organisms. However, LS5 clearly showed an in-vitro antibacterial activity towards the same bacteria in the agar spot tests of chapter 2 and was proven to produce the bacteriocin lactocin S (Sobrino et al., 1991; Chapter 2). It is

possible that the bacteriocin was not produced or produced at an insufficient level on the MCH or the effectiveness of the strain may have been limited by a range of factors (Chapter 1) such as a limited diffusion in solid matrices, inactivation through binding to food ingredients such as lipids, etc.

In contradiction to strain LS5, *L. sakei* 10A demonstrated to offer opportunities for the prolongation of the shelf-life of cooked meat products since antagonistic effects towards *Leuc. mesenteroides* and *B. thermosphacta* were observed on the MCH at 7°C under vacuum packaging. The inhibitory effect of the biopreservative 10A occurred when the strain entered its stationary phase. When inoculated with *Leuc. mesenteroides*, the time necessary to reach 7 $\log_{10}$(cfu/g) – a value that is often considered as the end-point of the microbial shelf-life – was prolonged with approximately 14 days. To our knowledge, this is the first time that an antagonistic interaction between a non-bacteriocinogenic, homofermentative LAB-strain and a heterofermentative, spoilage causing LAB-strain is reported on a cooked meat product.

Up to that moment, the action of the culture was only tested on the MCH. However, elaborate application trials (Chapter 6) at 7°C under vacuum packaged conditions demonstrated that *L. sakei* 10A also acts on the endogenous LAB-flora and on artificially inoculated *Leuc. mesenteroides* and *B. thermosphacta* cells on pâté, cooked ham, cooked sausage and two cooked poultry products.

In conclusion, the application of *L. sakei* 10A on anaerobically packaged CMP can at least maintain and in some cases prolong the shelf-life. This conclusion will be further refined when taking into account the sensory aspects (section 1.4).

1.3. Capacity of protective LAB-cultures to prevent proliferation of *L. monocytogenes*

The ability of a protective LAB-culture to prevent spoilage in the case of post-contamination with *Leuc. mesenteroides* and *B. thermosphacta* is interesting, but the capacity of the culture to improve the food safety of CMP by hindering the growth of *L. monocytogenes* would be considered as an important added value. Since CMP are susceptible for post-contamination with this food born pathogen and in view of the new regulation on microbial criteria in food (the current Commission Regulation (EC) No 2073/2005) (European Commission, 2005) this was certainly a relevant research purpose.

In chapter 2 the in-vitro antilisterial activity of 12 selected LAB was investigated. The two LAB that were selected in that chapter for further research in chapters 4 and 5, the

bacteriocinogenic *L. sakei* 10A and non-bacteriocinogenic *L. sakei* LS5, were shown to produce growth inhibition zones in agar spot assays and this for all three tested *L. monocytogenes* strains. Co-culture experiments (Chapter 5) showed that *L. sakei* LS5 could not prevent that *L. monocytogenes* grew to unacceptable levels on the MCH (7°C, vacuum). Possible explanations have already been discussed in part 1.2. of this discussion. However, co-culture experiments with *L. sakei* 10A clearly proved the antilisterial action of this strain on the MCH and this at 4°C and at 7°C, under vacuum packaging and under modified atmosphere packaging. The combination of the biopreservative *L. sakei* 10A and a storage temperature of 4°C or strain 10A and a modified atmosphere containing 50% of CO_2 fully prevented growth of *L. monocytogenes* on the MCH during 42 days. Another important conclusion of chapter 5 was that the application level of *L. sakei* 10A had to be at least 6 $\log_{10}$(cfu/g) in order to protect against proliferation of *L. monocytogenes* to unacceptable levels.

The antilisterial activity was further verified by challenge tests on five different industrially manufactured cooked meat products: pâté, cooked ham, cooked sausage and two cooked poultry products. In all products that supported growth of *L. monocytogenes* in the reference samples (pâté, cooked ham and cooked chicken fillet), there was a significant inhibition of the growth of *L. monocytogenes* when strain *L. sakei* 10A was added. Applying *L. sakei* 10A prevented growth of the *L. monocytogenes* cocktail on pâté and on cooked chicken fillet and limited its growth to 1.3 $\log_{10}$(cfu/g) after 14 days on cooked ham.

1.4. Sensory acceptability of CMP treated with *L. sakei* 10A

Since LAB are acid producing bacteria, the development of protective LAB-cultures for biopreservation purposes should include the assessment of their effect on the sensory quality of the treated products. Already at the first stage of this work (Chapter 2) this aspect was part of the investigations and seen as a prerequisite for a suitable protective culture. In chapter 2, *L. sakei* 10A was already selected as one of the LAB strains that did not significantly influence the sensory properties of the MCH even when present for a long time at high cell numbers. In chapter 5 this was once more confirmed since panellists were unable to detect an unacceptable taste or odour in MCH-samples inoculated with *L. sakei* 10A. These positive sensory results were all obtained on the MCH-product, a product that contained a low level of glucose, limiting in this way the amount of lactic acid that could be produced by *L. sakei* 10A. When evaluating the biopreservative capacity of *L. sakei* 10A on five industrially

manufactured cooked meat products (pâté, cooked ham, cooked sausage and two cooked poultry products), it became clear that despite the promising antagonistic effects, the application of *L. sakei* 10A to CMP was in some cases limited by a significant acidification resulting in an acid taste of the product. The effect of protective cultures in general and of *L. sakei* 10A in particular on the organoleptic characteristics of cooked meat products depends on the glucose content and the buffering capacity of these products. From the results of chapter 6, a hypothesis could be derived that high buffering capacity and low glucose content are key elements to avoid sensory deviations when applying protective cultures on CMP. A high buffering capacity is obtained when products contain a minimum level of phosphate but mainly when they are rich in meat-protein. A high level of total meat-protein in a CMP is obtained when products are produced from lean meat pieces. Thus protective cultures are not useful in products with low protein levels, usually those with a high fat content such as pâté and cooked sausages. In lean products such as cooked ham of high quality and cooked poultry products, the culture can have potential as long as the glucose content is sufficiently low. Most CMP contain approximately 0.5% glucose originating from non-meat ingredients. In some recipes of CMP, glucose is added directly as dextrose or as dextrose syrup or indirectly e.g. as a carrier for flavours or as a dispersion agent for gums in the brine. However, from a technological point of view it is possible to make CMP with glucose levels of 0.1% and lower. Low glucose contents can be obtained by avoiding direct addition of glucose or other sugars and by selecting ingredients for cooked meat manufacturing that do not contain glucose; the latter may require the development of e.g. flavours that use carrier materials other than glucose.

1.5. Inhibition mechanism of *L. sakei* 10A

To complete the work on the biopreservative culture *L. sakei* 10A, its mode of action was studied in chapter 7. The mechanism by which the non-bacteriocinogenic *L. sakei* 10A inhibits *L. monocytogenes* was an issue that had remained unsolved up to that stage of the PhD-work. Also in the existing literature, no clear answer for this question could be found. The results indicated that growth inhibition of *L. monocytogenes* by the non-bacteriocinogenic *L. sakei* 10A is not based on one mechanism only. The strain was observed to retard the growth of *L. monocytogenes* in different types of liquid broths and depending on the composition of the broth either nutrient competition or lactic acid production/pH-reduction was indicated as the most probable mechanism behind the inhibition. The results strongly

indicate that glucose is the main component for which competition occurs. Also in the co-culture experiments on the MCH (Chapter 4) growth inhibition of *Leuc. mesenteroides* LM4 by 10A occurred approximately at the moment that glucose was almost depleted. The same conclusion could not be derived from the co-culture experiments between *B. thermosphacta* BT1 and 10A (Chapter 4) and also not from the co-culture studies between *L. monocytogenes* and 10A in several real CMP (Chapter 6). In the latter application tests, *L. monocytogenes* did not start to grow in the presence of 10A; the moment of inhibition was in fact the start of these experiments when there was still sufficient glucose present. So, the translation of the results of the broth experiments of this chapter towards what really occurs in a CMP is a difficult issue. The complexity of the substrate and the presence of the background flora makes it more difficult to analyse the inhibition mechanism in meat products compared to in artificial growth media.

1.6. Bacteriophage P100 to control growth of *L. monocytogenes*

In chapter 8, an innovative type of biopreservation using bacteriophage P100 was examined. Challenge testing with *L. monocytogenes* on vacuum packaged cooked chicken fillet and cooked ham in the presence and absence of the lytic *Listeria*-specific phage P100 provided evidence for its effectiveness in controlling growth of *L. monocytogenes*. Activity of phage P100 was observed at a multiplicity of infection (MOI) of 10^6 pfu/cfu, at 7°C and under anaerobic storage conditions. The applied phages did not replicate during storage but their titer remained stable, suggesting that P100 is active through 'lysis from without', a mechanism that occurs at very high MOI's.

2. Conclusions

The main realisations from this work with regard to biopreservation using protective LAB-cultures are summarised below.

- A new and promising non-bacteriocinogenic protective culture *L. sakei* 10A has been isolated and characterised.
- This culture was found to offer a solution for both spoilage and safety problems associated with anaerobically packaged cooked meat products.
- An important drawback of the biopreservative culture was its influence on the sensory quality of some CMP; its presence sometimes resulted in acidification of the treated product. This work revealed, however, the conditions at which these sensory

deviations can be prevented and led to the conclusion that application of *L. sakei* 10A without sensory loss is only possible in CMP with high buffering capacity and low glucose content. Implementation of non-bacteriocinogenic protective cultures in general and of *L. sakei* 10A in particular may therefore require adaptations of the recipe of the CMP.

- A better insight was gained into the mechanism by which the non-bacteriocinogenic culture *L. sakei* 10A inhibits *L. monocytogenes.*

Opposed to other published studies, this work evaluated biopreservation by means of LAB for applications on CMP using an integrated approach. Not only the effectiveness of the culture was investigated but the mechanism of inhibition was studied in detail and the culture was critically evaluated with regard to its effect on the sensory quality of CMP. In particular the latter aspect is crucial for the development of a protective culture and was not always sufficiently investigated and/or discussed in existing studies.

With regard to biopreservation using bacteriophages, it can be concluded that treatment with bacteriophage P100 as a non-bacterial type of biopreservation was demonstrated to be promising for the control of *L. monocytogenes* in vacuum packaged CMP.

From this work, it is evident that there might be a role for non-bacteriocinogenic micro-organisms such as *L. sakei* 10A or bacteriophage P100 in the preservation of anaerobically packaged cooked meat products. Biopreservation may offer a valuable alternative for chemical preservatives such as lactate or CO_2, especially for the control of *L. monocytogenes*. In terms of shelf-life prolongation, effects of chemical preservatives will be more difficult to rival by protective cultures.

Provided that the recipe of the cooked meat products is adjusted towards low glucose content and high buffering capacity, protective cultures are a biological alternative way of preserving cooked meat products. The choice for a protective culture instead of a chemical preservative can be made in view of the consumer concerns about chemicals in foods. In particular, meat companies producing bio-products might consider using protective cultures. Whether meat producing companies will make this choice depends on the technical, regulatory and economical aspects related to the use of these cultures. Technically seen, there are no limitations for treating food products with protective LAB-cultures or bacteriophages. The question is whether this technology is sufficiently cost-effective for the producer compared to the classical chemical preservatives and whether the cost is in proportion to the added value.

From a scientific point of view, lactic acid bacteria and bacteriophages can be considered safe for consumption but in the European Union there is no harmonised legislation that regulates (neither forbids nor approves) the use of either LAB or bacteriophages in food products. Further, implementation of these technologies will require careful communication and marketing in order to get it accepted by the general public.

3. Perspectives

Subjects for further research are:

- The uniqueness or generality of the antagonistic character of *L. sakei* 10A: do other non-bacteriocinogenic *L. sakei* strains have similar biopreservative properties indicating that this capacity is typical for the species *L. sakei* or even typical for the genus *Lactobacillus*.
- Is there a difference in inhibition mechanism when *L. sakei* 10A inhibits *L. monocytogenes* or when it acts towards *B. thermosphacta* or *Leuc. mesenteroides*?
- Development of an industrial application method for *L. sakei* 10A and/or bacteriophage P100. Options to investigate are spraying, immersion and encapsulation techniques. Heat sensitivity is an important drawback for biopreservative cultures and research directed towards a solution of this problem might be very useful in order to simplify the application method.
- The technology of using bacteriophages for combating pathogenic organisms in food products requires more fundamental research. Host range, resistance development, mode of action are important issues that need to be addressed.

List of abbreviations

List of abbreviations

AR	Acidification rate
a_w	Water activity
ALOA	Agar Listeria Ottaviani and Agosti
BC	Buffering capacity
BHI	Brain Heart Infusion
cfu	Colony forming unit
CMP	Cooked meat products
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EMP	Embden-Meyerhof-Parnas
EPS	Extracellular polysaccharides
EU	European Union
FDA	Food and Drug Administration
FRC	Federal Research Center
GRAS	Generally recognised as safe
HG-product	MCH-product with high glucose content
HPLC	High performance liquid chromatography
HPMC	Hydroxy propyl methyl cellulose
LAB	Lactic acid bacteria
LG-product	MCH-product with low glucose content
LFMFP	Laboratory of Food Microbiology and Food Preservation
LMG	Laboratory of Microbiology Gent
MA	Modified atmosphere
MAP	Modified atmosphere packaging
MCH	Model cooked ham
MOI	Multiplicity of infection
MRS	de Man Rogosa Sharpe
ND	Not determined
OD	Optical density
PC	Protective culture(s)
PCA	Plate Count Agar

pfu	plaque forming unit
PPS	Pepton Physiologic Solution
QPS	Qualified presumption of safety
rRNA	Ribosomal ribonucleic acid
RCA	Reinforced Clostridial Agar
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
STAA	Streptomycin sulphate, Thallous acetate, Actidione Agar
TSA	Trypton Soya Broth
VP	Vacuum packaging
YGC	Yeast Glucose Chloramphenicol
YT	Yeast Trypton

References

References

1. **Aasen, I.M., Markussen, S., Moretro, T., Katla, T., Axelsson, L. & Naterstad, K.** (2003). Interactions of the bacteriocins sakacin P and nisin with food constituents. International Journal of Food Microbiology, 87, 35-43.

2. **Abee, T., Krockel, L. & Hill, C.** (1995). Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. International Journal of Food Microbiology, 28, 169-185.

3. **Abee, T. & Wouters, J.A.** (1999). Microbial stress response in minimal processing. International Journal of Food Microbiology, 50, 65-91.

4. **Adams, M.R. & Marteau, P.** (1995). On the safety of lactic acid bacteria from food. International Journal of Food Microbiology, 27, 263-264.

5. **Aguirre, M. & Collins, M.D.** (1993). Lactic acid bacteria and human clinical infection. Journal of Applied Bacteriology, 75, 95-107.

6. **Amezquita, A. & Brashears, M.M.** (2002). Competitive inhibition of *Listeria monocytogenes* in ready-to-eat meat products by lactic acid bacteria. Journal of Food Protection, 65(2), 316-325.

7. **Andersen, L.** (1995a). Biopreservation with FloraCarn L-2. Fleischwirtschaft, 75(11), 1327-1329.

8. **Andersen, L.** (1995b). Preservation of meat products with a lactic acid bacteria culture - Flora Carn L-2. Proceedings of the 41th ICoMST meeting, San Antonio (Texas, US), 20-25 August, p. 303-304.

9. **Andersen, L.** (2000). Bioprotection: an additional safety hurdle. Proceedings of the 46th ICoMST meeting, Buenos Aires (Argentina), 27 August - 1 September, p.646-647.

10. **Anon** (2005). Opinion of the Scientific Committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of micro-organisms used in food/feed and the production of food/feed additives. The EFSA Journal, 226, 1-12.

11. **AOAC** (1990). Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia.

12. **Arena, M.E., Saguir, F.M. & Manca de Nadra, M.C.** (1999). Arginine dihydrolase pathway in *Lactobacillus plantarum* from orange. International Journal of Food Microbiology, 47, 203-209.

13. **Atterbury, R.J., Connerton, P.L., Dodd, C.E.R., Rees, C.E.D. & Connerton, I.F.** (2003a). Isolation and characterization of *Campylobacter* bacteriophages from retail poultry. Applied and Environmental Microbiology, 69(8), 4511-4518.

14. **Atterbury, R.J., Connerton, P.L., Dodd, C.E.R., Rees, C.E.D. & Connerton, I.F.** (2003b). Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. Applied and Environmental Microbiology, 69(10), 6302-6306.

15. **Aymerich, M.T., Garriga, M., Costa, S., Monfort, J.M. & Hugas, M.** (2002). Prevention of ropiness in cooked pork by bacteriocinogenic cultures. International Dairy Journal, 12, 239-246.

16. **Bactoferm™ technical information** (2003). Innovative solutions for the meat and poultry industry. Chr. Hansen, Denmark.

17. **Barakat, R.K. & Harris, L.J.** (1999). Growth of *Listeria monocytogenes* and *Yersinia enterocolitica* on cooked modified-atmosphere-packaged poultry in the presence and absence of a naturally occurring microbiota. Applied and Environmental Microbiology, 65, 342-345.

18. **Barakat, R.K., Griffiths, M.W. & Harris, L.J.** (2000). Isolation and characterization of *Carnobacterium*, *Lactococcus* and *Enterococcus* spp. from cooked, modified atmosphere packaged, refrigerated, poultry meat. International Journal of Food Microbiology, 62, 83-94.

19. **Baranyi, J. & Roberts, T.A.** (1994). A dynamic approach to predict bacterial growth in food. International Journal of Food Microbiology, 23, 277-294.

20. **Batt, C.A.** (1999). *Lactococcus*. In: Robinson, R.K., Batt, C.A., Patel, P.D. (Eds.), Encyclopedia of Food Microbiology. Academic Press, San Diego, p. 1164-1166.

21. **Bedie, G.K., Samelis, J., Sofos, J.N., Belk, K.E., Scanga, J.A. & Smith, G.C.** (2001). Antimicrobials in the formulation to control *Listeria monocytogenes* postprocessing contamination on frankfurters stored at 4°C in vacuum packages. Journal of Food Protection, 64(12), 1949-1955.

22. **Bell, C. & Kyriakides, A.** (2005). *Listeria*: a practical approach to the organism and its control. 2nd edition. Blackwell Publishing Ltd, Oxford, 288p.

23. **Benkerroum, N., Daoudi, A. & Kamal, M.** (2003). Behaviour of *Listeria monocytogenes* in raw sausage in presence of a bacteriocin-producing lactococcal strain as a protective culture. Meat Science, 63, 479-484.

24. **Benkerroum, N., Daoudi, A., Hamraoui, T., Ghalfi, H., Thiry, C., Duroy, M., Evrart, P., Roblain, D. & Thonart, P.** (2005). Lyophilized preparations of bacteriocinogenic *Lactobacillus curvatus* and *Lactococcus lactis* subsp. *lactis* as potential protective adjuncts to control *Listeria monocytogenes* in dry-fermented sausages. Journal of Applied Microbiology, 98, 56-63.

25. **Bennik, M.H.J., Smid, E.J. & Gorris, L.** (2000). Use of biopreservation in minimal processing of vegetables. In: Alzamora, S.M., Tapia, M.S., López-Malo, A. (Eds.), Minimally Processed Fruits and Vegetables: fundamental aspects and applications. Aspen Publishers Inc., Gaithersburg, Maryland, p. 265-276.

26. **Berry, E.D., Liewen, M.B., Mandigo, R.W. & Hutkins, R.W.** (1990). Inhibition of *Listeria monocytogenes* by bacteriocin-producing *Pediococcus* during the manufacture of fermented semidry sausage. Journal of Food Protection, 53(3), 194-197.

27. **Berry, E.D., Hutkins, R.W. & Mandigo, R.W.** (1991). The use of bacteriocin-producing *Pediococcus acidilactici* to control postprocessing of *Listeria monocytogenes* contamination of frankfurters. Journal of Food Protection, 54(9), 681-686.

28. **Beumer, R.R., te Giffel, M.C., de Boer, E. & Rombouts, F.M.** (1996). Growth of *Listeria monocytogenes* on sliced cooked meat products. Food Microbiology, 13, 333-340.

29. **Björkroth, J. & Korkeala, H.** (1997). Ropy slime-producing *Lactobacillus sake* strains possess a strong competitive ability against a commercial biopreservative. International Journal of Food Microbiology, 38, 117-123.

30. **Björkroth, K.J., Vandamme, P. & Korkeala, H.J.** (1998). Identification and characterization of *Leuconostoc carnosum*, associated with production and spoilage of vacuum-packaged, sliced, cooked ham. Applied and Environmental Microbiology, 64(9), 3313-3319.

31. **Blickstad, E.** (1983). Growth and end-product formation of two psychrotrophic *Lactobacillus* spp. and *Brochothrix thermosphacta* ATCC 11509^T at different pH values and temperatures. Applied and Environmental Microbiology, 46(6), 1345-1350.

32. **Blickstad, E. & Molin, G.** (1984). Growth and end-product formation in fermenter cultures of *Brochothrix thermosphacta* ATCC 11509^T and two psychrotrophic *Lactobacillus* spp. in different gaseous atmospheres. Journal of Applied Bacteriology, 57, 213-220.

33. **Blom, H., Nerbrink, E., Dainty, R., Hagtvedt, T., Borch, E., Nissen, H. & Nesbakken, T.** (1997). Addition of 2.5% lactate and 0.25% acetate controls growth of *Listeria monocytogenes* in vacuum-packed sensory-acceptable servelat sausage and cooked ham stored at 4°C. International Journal of Food Microbiology, 38, 71-76.

34. **Bloukas, J.G., Paneras, E.D. & Fournitzis, G.C**. (1997). Sodium lactate and protective culture effects on quality characteristics and shelf-life of low-fat frankfurters produced with olive oil. Meat Science, 45(2), 223-238.

35. **Bogaert, J-C. & Naidu, A.S.** (2000). Lactic acid. In: Naidu, A.S (Ed.), Natural food antimicrobial systems. CRC Press LLC, Florida, p. 613-636.

36. **Borch, E., Nerbrink, E. & Svensson, P.** (1988). Identification of major contamination sources during processing of emulsion sausage. International Journal of Food Microbiology, 7, 317-330.

37. **Borch, E. & Molin, G.** (1989). The aerobic growth and product formation of *Lactobacillus*, *Leuconostoc*, *Brochothrix* and *Carnobacterium* in batch cultures. Applied Microbiology and Biotechnology, 30, 81-88.

38. **Borch, E. & Agerhem, H.** (1992). Chemical, microbial and sensory changes during the anaerobic cold storage of beef inoculated with a homofermentative *Lactobacillus* spp. or a *Leuconostoc* spp. International Journal of Food Microbiology, 15, 99-108.

39. **Borch, E., Berg, H. & Holst**, **O.** (1991). Heterolactic fermentation by a homofermentative *Lactobacillus* spp. during glucose limitation in anaerobic continuous culture with complete cell recycle. Journal of Applied Bacteriology, 71, 265-269.

40. **Borch, E., Kant-Muermans, M. & Blixt, Y**. (1996). Bacterial spoilage of meat and cured meat products. International Journal of Food Microbiology, 33, 103-120.

41. **Brashears, M.M., Reilly, S.S. & Gilliland, S.E.** (1998). Antagonistic action of cells of *Lactobacillus lactis* toward *Escherichia coli* on refrigerated raw chicken meat. Journal of Food Protection, 61(2), 166-170.

42. **Bredholt, S., Nesbakken, T. & Holck, A.** (1999). Protective cultures inhibit growth of *Listeria monocytogenes* and *Escherichia coli O157:H7* in cooked, sliced, vacuum- and gaspackaged meat. International Journal of Food Microbiology, 53, 43-52.

43. **Bredholt, S., Nesbakken, T. & Holck, A.** (2001). Industrial application of an antilisterial strain of *Lactobacillus sakei* as a protective culture and its effect on the sensory acceptability of cooked, sliced, vacuum-packaged meats. International Journal of Food Microbiology, 66, 191-196.

44. **Breidt, F., Crowley, K.A. & Fleming, H.P.** (1995). Controlling cabbage fermentations with nisin and nisin-resistant *Leuconostoc mesenteroides*. Food Microbiology, 12, 109-116.

45. **Brillet, A., Pilet, M.-F., Prevost, H., Cardinal, M. & Leroi, F.** (2005). Effect of inoculation of *Carnobacterium divergens* V41, a biopreservative strain against *Listeria monocytogenes* risk, on the microbiological, chemical and sensory quality of cold-smoked salmon. International Journal of Food Microbiology, 104, 309-324.

46. **Brinkman, J.** (2002). Proeven van succes. Sensorisch onderzoek: techniek, toepassingen en procedures. Keesing Noordervliet BV, Houten, 280p.

47. **Buchanan, R.L. & Klawitter, L.A.** (1992). Characterization of a lactic acid bacterium, *Carnobacterium piscicola* LK5, with activity against *Listeria monocytogenes* at refrigeration temperatures. Journal of Food Safety, 12, 199-217.

48. **Buchanan, R.L. & Bagi, L.K.** (1997). Microbial competition: effect of culture conditions on the supression of *Listeria monocytogenes* Scott A by *Carnobacterium piscicola*. Journal of Food Protection, 60(3), 254-261.

49. **Budde, B.B., Hornbaek T., Jacobsen, T., Barkholt, V. & Koch, A.G.** (2003). *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification and meat application experiments. International Journal of Food Microbiology, 83, 171-184.

50. **Buncic, S., Avery, S.M. & Moorhead, S.M.** (1997). Insufficient antilisterial capacity of low inoculum *Lactobacillus* cultures on long-term stored meats at 4°C. International Journal of Food Microbiology, 34, 157-170.

51. **Buyong, N., Kok, J. & Luchansky, J.B.** (1998). Use of a genetically enhanced pediocin-producing starter culture, *Lactococcus lactis* subsp. *lactis* MM217, to control *Listeria monocytogenes* in cheddar cheese. Applied and Environmental Microbiology, 64(12), 4842-4845.

52. **Cai, Y., Ng, L.-K. & Farber, J.M.** (1997). Isolation and characterization of nisin-producing *Lactococcus lactis* subsp. *lactis* from bean-sprouts. Journal of Applied Microbiology, 83, 499-507.

53. **Callewaert, R., Hugas, M. & De Vuyst, L.** (2000). Competitiveness and bacteriocin production of *Enterococci* in the production of Spanish style dry fermented sausages. International Journal of Food Microbiology, 57, 33-42.

54. **Campanini, M., Pedrazzoni, I., Barbuti, S. & Baldani, P.** (1993). Behaviour of *Listeria monocytogenes* during the maturation of naturally and artificially contaminated salami: effect of lactic-acid bacteria starter cultures. International Journal of Food Microbiology, 20, 169-175.

55. **Caplice, E. & Fitzgerald, G.F.** (1999). Food fermentations: role of micro-organisms in food production and preservation. International Journal of Food Microbiology, 50, 131-149.

56. **Carlton, R.M., Noordman, W.H., Biswas, B., de Meester, E.D. & Loessner, M.J.** (2005). Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study and application. Regulatory Toxicology and Pharmacology, 43(3), 301-312.

57. **Carr, F.J., Chill, D. & Maida, N.** (2002). The lactic acid bacteria: a literature survey. Critical Reviews in Microbiology, 28(4), 281-370.

58. **Castellano, P. & Vignolo, G.** (2004). Protective effect of *Lactobacillus casei* CRL705 compared with the bacteriocins it produces during storage of refrigerated fresh meat under vacuum. Proceedings of the 19th International ICFMH symposium FOOD MICRO 2004, Portoroz (Slovenia), 12-16 September, p. 287.

59. **Cayré, M.E., Garro, O. & Vignolo, G.** (2005). Effect of storage temperature and gas permeability of packaging film and *Brochothrix thermosphacta* in cooked meat emulsions. Food Microbiology, 22, 505-512.

60. **Champomier-Vergès, M.-C., Zuniga, M., Morel-Deville, F., Pérez-Martinez, G. & Ehrlich, S.D.** (1999). Relationships between arginine degradation, pH and survival in *Lactobacillus sakei*. FEMS Microbiology Letters, 180, 297-304.

61. **Champomier-Vergès, M.-C., Chaillou, S., Cornet, M. & Zagorec, M.** (2001). *Lactobacillus sakei*: recent developments and future prospects. Research in Microbiology, 152, 839-848.

62. **Champomier-Vergès, M.-C., Chaillou, S., Cornet, M. & Zagorec, M.** (2002). Erratum to "*Lactobacillus sakei*: recent developments and future prospects" [Research in Microbiology, 152 (2001) 839]. Research in Microbiology, 153, 115-123.

63. **Chung, K.T., Dickson, J.S. & Crouse, J.D.** (1989). Effects of nisin on growth of bacteria attached to meat. Applied and Environmental Microbiology, 55(6), 1329-1333.

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

65. **Coventry, M.J., Muirhead, K. & Hickey, M.W.** (1995). Partial characterization of pediocin PO_2 and comparison with nisin for biopreservation of meat products. International Journal of Food Microbiology, 26, 133-145.

66. **Dabrowska, K., Switala-Jelen, K., Opolski, A., Weber-Dabrowska, B. & Gorski, A.** (2005). Bacteriophage penetration in vertebrates. Journal of Applied Microbiology, 98, 7-13.

67. **Dainty, R.H.** (1996). Chemical/biochemical detection of spoilage. International Journal of Food Microbiology, 33, 19-33.

68. **Davies, E.A., Bevis, H.E. & Delves-Broughton, J.** (1997). The use of the bacteriocin nisin as a preservative in ricotta-type cheeses to control the food-borne pathogen *Listeria monocytogenes*. Letters in Applied Microbiology, 24, 343-346.

69. **Debevere, J.** (1989). The effect of sodium lactate on the shelf life of vacuum-packed coarse liver pate. Fleischwirtschaft, 69, 223-224.

70. **De Boer, E.** (1990). *Listeria monocytogenes* in kip(produkten) en vlees(waren). De Warenchemicus, 20, 101-108.

71. **De Boer, E. & van Netten, P**. (1990). De aanwezigheid en groei van *Listeria monocytogenes* in pâté. Voedingsmiddelentechnologie, 13, 15-17.

72. **Degnan, A.J., Yousef, A.E. & Luchansky, J.B.** (1992). Use of *Pediococcus acidilactici* to control *Listeria monocytogenes* in temperature-abused vacuum-packaged wieners. Journal of Food Protection, 55(2), 98-103.

73. **De Muynck, C., Leroy, A.I.J., De Maeseneire, S., Arnaut, F., Soetaert, W. & Vandamme, E.J.** (2004). Potential of selected lactic acid bacteria to produce food compatible antifungal metabolites. Microbiological Research, 159, 339-346.

74. **Deutsch, S.-M., Guezenec, S., Piot, M., Foster, S. & Lortal, S.** (2004). Mur-LH, the broad-spectrum endolysin of *Lactobacillus helveticus* temperate bacteriophage ϕ-0303. Applied and Environmental Microbiology, 70(1), 96-103.

75. **Devlieghere, F., Debevere, J. & Van Impe, J.** (1998). Effect of dissolved carbon dioxide and temperature on the growth of *Lactobacillus sake* in modified atmospheres. International Journal of Food Microbiology, 41, 231-238.

76. **Devlieghere, F**. (2000). Predictive modelling of the spoilage and the microbial safety of modified atmosphere packaged cooked meat products. Ph.D. Thesis, Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium, 207 p.

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

78. **Devlieghere, F., Geeraerd, A.H., Versyck, K.J., Vandewaetere, B., Van Impe, J. & Debevere, J.** (2001). Growth of *Listeria monocytogenes* in modified atmosphere packed cooked meat products: a predictive model. Food Microbiology, 18, 53-66.

79. **Devlieghere, F., Vermeiren, L. & Debevere, J.** (2004). New preservation technologies: possibilities and limitations. International Dairy Journal, 14(4), 273-285.

80. **De Vuyst, L. & Vandamme, E.J.** (1994a). Antimicrobial potential of lactic acid bacteria. In: De Vuyst, L. & Vandamme, E.J. (Eds.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic and Professional, London, p. 91-129.

81. **De Vuyst, L. & Vandamme, E.J.** (1994b). Nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and applications. In: De Vuyst, L. & Vandamme, E.J. (Eds.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic and Professional, London, p. 151-199.

82. **Domingues, D.** (2000). Food products with biocontrol preservation. Patent WO 00/57712.

83. **Dykes, G.A. & Dworaczek, M**. (2002). Influence of interactions between temperature, ferric ammonium citrate and glycine betaine on the growth of *L. monocytogenes* in a defined medium. Letters in Applied Microbiology, 35, 538-542.

84. **Dykes, G.A. & Moorhead, S.M**. (2002). Combined antimicrobial effect of nisin and a listeriophage against *Listeria monocytogenes* in broth but not in buffer or on raw beef. International Journal of Food Microbiology, 73, 71-81.

85. **EFSA** (2005). Qualified presumption of safety of micro-organisms in food and feed. Summary report EFSA Scientific colloquium 2, 13-14 December 2004, Brussels, Belgium, 142 p.

86. **Egan, A.F., Ford, A.L. & Shay, B.J.** (1980). A comparison of *Microbacterium thermosphactum* and lactobacilli as spoilage organisms of vacuum-packaged sliced luncheon meats. Journal of Food Science, 45, 1745-1748.

87. **Einarsson, H. & Lauzon, H.L.** (1995). Biopreservation of brined shrimp (*Pandalus borealis*) by bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology, 61(2), 669-676.

88. **Eklund, T.** (1989). Organic acids and esters. In: Gould, G.W. (Ed.), Mechanisms of action of food preservation procedures. Elsevier Science Publishers Ltd., London, p. 161-200.

89. **Elli, M., Zink, R., Rytz, A., Reniero, R. & Morelli, L.** (2000). Iron requirement of *Lactobacillus* spp. in completely chemically defined growth media. Journal of Applied Microbiology, 88, 695-703.

90. **Elsser, D**. (1998). Minimierung des hygienerisikos durch toxiinfektiöse und toxinogene bakterien bei nitritverminderten brühwursterzeugnissen durch den einsatz von schutzkulturen. Dissertation, 160 p.

91. **El-Ziney, M.G. & Debevere, J.M.** (1998). The effect of reuterin on *Listeria monocytogenes* and *Escherichia coli* 0157:H7 in milk and cottage cheese. Journal of Food Protection, 61, 1275-1280.

92. **Emond, E., Holler, B.J., Boucher, I., Vandenbergh, P.A., Vedamuthu, E.R., Kondo, J.K. & Moineau, S.** (1997). Phenotypic and genetic characterization of the bacteriophage abortive infection mechanism AbiK from *Lactococcus lactis*. Applied and Environmental Microbiologyg, 63(4), 1274-1283.

93. **Ennahar, S., Sonomoto, K. & Ishizaki, A.** (1999). Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. Journal of Bioscience and Bioengineering, 87(6), 705-716.

94. **Ennahar, S., Deschamps, N. & Richard, J.** (2000). Natural variation in susceptibility of *Listeria* strains to class IIa bacteriocins. Current Microbiology, 41, 1-4.

95. **Esteve, M.J., Farré, R., Frígola, A. & Pilamunga, C.** (2002). Contents of vitamins B1, B2, B6 and B12 in pork and meat products. Meat Science, 62, 73-78.

96. **European Commission** (2003). On a generic approach to the safety assessment of micro-organisms used in feed/food and feed/food production. A working paper open for discussion. http://europa.eu.int/comm/food/fs/sc/scf/out178_en.pdf.

97. **European Commission** (2005). Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, L338/1-26.

98. **Farkas, J., Andrássy, E., Beczner, J., Vidács, I. & Mészáros, L.** (2002). Utilizing luminometry for monitoring growth of *L. monocytogenes* in its liquid or gelified monocultures and cocultures with "acid-only" *Lactococcus lactis*. International Journal of Food Microbiology, 73, 159-170.

99. **Foegeding, P.M., Thomas, A.B., Pilkington, D.H. & Klaenhammer, T.R.** (1992). Enhanced control of *Listeria monocytogenes* by in-situ produced pediocin during dry-fermented sausage production. Applied and Environmental Microbiology, 58(3), 884-890.

100. **Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E. & De Vuyst, L.** (2005). The role and application of enterococci in food and health. International Journal of Food Microbiology, 106(1), 1-24.

101. **Francois, K., Devlieghere, F., Smet, K., Standaert, A.H., Geeraerd, J.F., Van Impe, J. & Debevere, J.** (2005). Effect of temperature and pH on the individual cell lag distribution of *Listeria monocytogenes*. International Journal of Food Microbiology, 100, 41-53.

102. **Gänzle, M.G.** (2004). Reutericylin: biological activity, mode of action and potential applications. Applied Microbiology and Biotechnology, 64, 326-332.

103. **Garcia, L.R. & Molineux, I.J.** (1995). Incomplete entry of bacteriophage T7 DNA into F plasmid-containing *Escherichia coli*. Journal of Bacteriology, 177(14), 4077-4083.

104. **Gardner, G.A.** (1981). *Brochothrix thermosphacta* (*Microbacterium thermosphactum*) in the spoilage of meats: a review. In: Roberts, T.A., Hobbs, G., Christian, J.H.B. & Skovgaard, N. (Eds.), Psychrotrophic micro-organisms in spoilage and pathogenicity. Academic Press, London, p.140-173.

105. **Garneau, S., Martin, N.I. & Vederas, J.C.** (2002). Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie, 84, 577-592.

106. **Geisen, R., Lücke, F.K. & Kröckel, L.** (1992). Starter and protective cultures for meat and meat products. Fleischwirtschaft, 72(6), 894-898.

107. **Gevers, D., Huys, G., Devlieghere, F., Uyttendaele, M., Debevere, J. & Swings, J**. (2000). Isolation and identification of tetracycline resistant lactic acid bacteria from prepacked sliced meat products. Systematic and Applied Microbiology, 23, 279-284.

108. **Giraffa, G., Neviani, E. & Tarelli, G.T.** (1994). Antilisterial activity by enterococci in a model predicting the temperature evolution of taleggio, an Italian soft cheese. Journal of Dairy Science, 77, 1176-1182.

109. **Gonçalves, L.M.D., Ramos, A., Almeida, J.S., Xavier, A.M.R.B. & Carrondo, M.J.T.** (1997). Elucidation of the mechanism of lactic acid growth inhibition and production in batch cultures of *Lactobacillus rhamnosus*. Applied Microbiology and Biotechnology, 48, 346-350.

110. **Goode, D., Allen, V.M. & Barrow, P.A.** (2003). Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. Applied and Environmental Microbiology, 69(8), 5032-5036.

111. **Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B. & Givskov, M**. (2002). Food spoilage – interactions between food spoilage bacteria. International Journal of Food Microbiology, 78, 79-97.

112. **Greer, G.G.** (1983). Psychrotrophic *Brochothrix thermosphacta* bacteriophages isolated from beef. Applied and Environmental Microbiology, 46(1), 245-251.

113. **Greer, G.G. & Dilts, B.D.** (1990). Inability of a bacteriophage pool to control beef spoilage. International Journal of Food Microbiology, 10, 331-342.

114. **Greer, G.G. & Dilts, B.D.** (1995). Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens on pork. International Journal of Food Microbiology, 25, 141-151.

115. **Greer, G.G. & Dilts, B.D.** (2002). Control of *Brochothrix thermosphacta* spoilage of pork adipose tissue using bacteriophages. Journal of Food Protection, 65(5), 861-863.

116. **Greer, G.G**. (2005). Bacteriophage control of foodborne bacteria. Journal of Food Protection, 68(5), 1102-1111.

117. **Guinane, C.M., Cotter, P.D., Hill, C. & Ross, R.P**. (2005). Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. Journal of Applied Microbiology, 98, 1316-1325.

118. **Hamasaki, Y., Ayaki, M., Fuchu, H., Sugiyama, M. & Morita, H.** (2003). Behaviour of psychrotrophic lactic acid bacteria isolated from spoiling cooked meat products. Applied and Environmental Microbiology, 69(6), 3668-3671.

119. **Harris, L.J., Daeschel, M.A., Stiles, M.E. & Klaenhammer, T.R.** (1989). Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. Journal of Food Protection, 52(6), 384-387.

120. **Helander, I.M., von Wright, A. & Mattila-Sandholm, T.-M.** (1997). Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. Trends in Food Science and Technology, 8(5), 146-150.

121. **Hertel, C., Schmidt, G., Fischer, M., Oellers, K. & Hammes, W.P.** (1998). Oxygen-dependent regulation of the expression of the catalase gene katA of *Lactobacillus sakei* LTH677. Applied and Environmental Microbiology, 64, 1359-1365.

122. **Holzapfel, W.H., Geisen, R. & Schillinger, U.** (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International Journal of Food Microbiology, 24, 343-362.

123. **Holzapfel, W.H.** (1998). The Gram-positive bacteria associated with meat and meat products. In: Davies, A. & Board, E. (Eds.), The microbiology of meat and poultry. Blackie Academic & Professional, London, p. 35-84.

124. **Houtsma, P.C., de Wit, J.C. & Rombouts, F.M.** (1993). Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. International Journal of Food Microbiology, 20, 247-257.

125. **Hudson, J.A., Billington, C., Carey-Smith, G. & Greening, G.** (2005). Bacteriophages as biocontrol agents in food. Journal of Food Protection, 68(2), 426-437.

126. **Hugas, M., Garriga, M., Aymerich, M.T. & Monfort, J.M.** (1995). Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lactobacillus sakei* CTC 494. Journal of Applied Bacteriology, 79, 322-330.

127. **Hugas, M., Neumeyer, B., Pagés, F., Garriga, M. & Hammes, P.** (1996). Die antimikrobielle wirkung von bakteriozin bildenden kulturen in fleischwaren 2. Vergleich des effektes unterschiedlicher bakteriozin bildender laktobazillen auf listerien in rohwurst. Fleischwirtschaft, 76(6), 649-652.

128. **Hugas, M.** (1998). Bacteriocinogenic lactic acid bacteria for the biopreservation of meat and meat products. Meat Science, 49 (S1), 139-150.

129. **Hugas, M., Pagés, F., Garriga, M. & Monfort, J.M.** (1998). Application of the bacteriocinogenic *Lactobacillus sakei* CTC494 to prevent growth of *Listeria* in fresh and cooked meat products packaged with different atmospheres. Food Microbiology, 15, 639-650.

130. **Hugas, M., Garriga, M., Pascual, M., Aymerich, M.T. & Monfort, J.M.** (2002). Enhancement of sakacin K activity against *Listeria monocytogenes* in fermented sausages with pepper or manganese as ingredients. Food Microbiology, 19, 519-528.

131. **Hugenholtz, J.** (1993). Citrate metabolism in lactic acid bacteria. FEMS Microbiology Reviews, 12, 165-178.

132. **Ito, A., Sato, Y., Kudo, S., Sato, S., Nakajima, H. & Toba, T.** (2003). The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. Current Microbiology, 47, 231-236.

133. **Imbert, M. & Blondeau, R.** (1998). On the iron requirement of lactobacilli grown in chemically defined medium. Current Microbiology, 37, 64-66.

134. **Jacobsen, T., Budde, B.B. & Koch, A.G.** (2003). Application of *Leuconostoc carnosum* for biopreservation of cooked meat products. Journal of Applied Microbiology, 95, 242-249.

135. **Jack, R.W., Tagg, J.R. & Ray, B.** (1995). Bacteriocins of gram-positive bacteria. Microbiology Reviews, 59(2), 171-200.

136. **Jelle, B.** (1987). The preserving effect of lactobacilli on vacuum packed beef. Master thesis, Department of Dairy and Food Science, Royal Danish Veterinary & Agricultural university.

137. **Jemmi, T., Pak, S.-I., Salman, M.D.** (2002)**.** Prevalence and risk factors for contamination with *Listeria monocytogenes* of imported and exported meat and fish products in Switzerland, 1992-2000. Preventive Veterinary Medicine, 54(1), 25-36.

138. **Juven, B.J., Barefoot, S.F., Pierson, M.D., McCaskill, L.H. & Smith, B**. (1998). Growth and survival of *Listeria monocytogenes* in vacuum-packaged ground beef inoculated with *Lactobacillus alimentarius* FloraCarn L-2. Journal of Food Protection, 61, 551-556.

139. **Kalalou, I., Faid, M. & Ahami, A.T.** (2004). Extending shelf life of fresh minced camel meat at ambient temperature by *Lactobacillus delbrueckii* subsp. *delbrueckii*. Electronic Journal of Biotechnology, 7(3), 243-248.

140. **Kandler, O. & Weiss, N.** (1986). Genus *Lactobacillus*. In: Sneath, P.H.A., Mair, N.S., Sharpe M.E. & Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins, Baltimore MD, p. 1209-1234.

141. **Karkalas, J.** (1985). An improved enzymatic method for the determination of native and modified starch. Journal of the Science of Food and Agriculture, 36, 1019-1027.

142. **Katla, T., Moretro, T., Sveen, I., Aasen, I.M., Axelsson, L., Rorvik, L.M. & Naterstad, K.** (2002). Inhibition of *Listeria monocytogenes* in chicken cold cuts by addition of sakacin P and sakacin P-producing *Lactobacillus sakei.* Journal of Applied Microbiology, 93, 191-196.

143. **Katikou, P., Ambrosiadis, D., Georgantelis, D., Koidis, P. & Georgakis, S.A.** (2005). Effect of *Lactobacillus*-protective cultures with bacteriocin-like inhibitory substances' producing ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. Journal of Applied Microbiology, 99, 1303-1313.

144. **Klaenhammer, T.R.** (1988). Bacteriocins of lactic acid bacteria. Biochimie, 70, 337-349.

145. **Klaenhammer, T.R.** (1993). Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Reviews, 12, 39-86.

146. **Kleinlein, N. & Untermann, F.** (1990). Growth of pathogenic *Y. enterocolitica* strains in minced meat with and without protective gas with consideration of the competitive background flora. International Journal of Food Microbiology, 10, 65-72.

147. **Korkeala, H., Lindroth, S., Ahvenainen, R. & Alanko, T.** (1987). Interrelationship between microbial numbers and other parameters in the spoilage of vacuum-packed cooked ring sausages. International Journal of Food Microbiology, 5, 311-321.

148. **Korkeala, H., Alanko, T., Mäkelä, P. & Lindroth, S**. (1989). Shelf life of vacuum-packed cooked ring sausages at different chill temperatures. International Journal of Food Microbiology, 9, 237-24.

149. **Korkeala, H. & Mäkelä, P**. (1989). Characterization of lactic acid bacteria isolated from vacuum-packed cooked ring sausages. International Journal of Food Microbiology, 9, 33-43.

150. **Korkeala, H., Alanko, T., Mäkelä, P. & Lindroth, S**. (1990). Lactic acid and pH as indicators of spoilage for vacuum-packed cooked ring sausages. International Journal of Food Microbiology, 10, 245-254.

151. **Korkeala, H.J. & Björkroth, K.J.** (1997). Microbiological spoilage and contamination of vacuum-packaged cooked sausages. Journal of Food Protection, 60(6), 724-731.

152. **Kotzekidou, P. & Bloukas, J.G.** (1996). Effect of protective cultures and packaging permeability on shelf life of sliced vacuum-packed cooked ham. Meat Science, 42 (3), 333-345.

153. **Kotzekidou, P. & Bloukas, J.G**. (1998). Microbial and sensory changes in vacuum-packed frankfurter-type sausage by *Lactobacillus alimentarius* and fate of inoculated *Salmonella enteritidis*. Food Microbiology, 15 (1), 101-111.

154. **Kuipers, O.P., de Ruyter, P.G.G.A., Kleerebezem, M. & de Vos, W.M.** (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. Journal of Biotechnology, 64, 15-21.

155. **Lauková, A., Czikková, S., Laczková, S. & Turek, P.** (1999). Use of enterocin CCM 4231 to control *Listeria monocytogenes* in experimentally contaminated dry fermented Hornad salami. International Journal of Food Microbiology, 52, 115-119.

156. **Lauret, R., Morel-Deville, F., Berthier, F., Champomier-Vergès, M.-C., Postma, P., Ehrlich, S.D. & Zagorec, M.** (1996). Carbohydrate utilization in *Lactobacillus sakei*. Applied and Environmental Microbiology, 62(6), 1922-1927.

157. **Leisner, J.J., Greer, G.G., Dilts, B.D. & Stiles, M.E.** (1995). Effect of growth of selected lactic acid bacteria on storage life of beef stored under vacuum and in air. International Journal of Food Microbiology, 26, 231-243.

158. **Leisner, J.J., Greer, G.G. & Stiles, M.E.** (1996). Control of beef spoilage by a sulphide-producing *Lactobacillus sake* strain with bacteriocinogenic *Leuconostoc gelidum* UAL187 during anaerobic storage. Applied and Environmental Microbiology, 62(7), 2610-2614.

159. **Lemay, M.J., Champagne, C.P., Gariépy, C & Saucier, L.** (2002a). A comparison of the effect of the heat resistance of free or encapsulated cultures of *Lactobacillus sakei*. Journal of Food Science, 67(9), 3428-3434.

160. **Lemay, M.J., Choquette, J., Delaquis, P.J., Gariépy, C., Rodrigue, N. & Saucier, L.** (2002b). Antimicrobial effect of natural preservatives in a cooked and acidified chicken meat model. International Journal of Food Microbiology, 78, 217-226.

161. **Leroy, F. & Devuyst, L.** (1999a). Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. Applied and Environmental Microbiology, 65(3), 974-981.

162. **Leroy, F. & Devuyst, L.** (1999b). The presence of salt and a curing agent reduces bacteriocin production by a *Lactobacillus sakei* CTC 494, a potential starter culture for sausage fermentation. Applied and Environmental Microbiology, 65(12), 5350-5356.

163. **Leroy, F., Verluyten, J. & De Vuyst, L.** (2006). Functional meat starter cultures for improved sausage fermentation. International Journal of Food Microbiology, 106(3), 270-285.

164. **Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M., Saftner, R. & Sulakvelidze, A.** (2003). Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. Applied and Environmental Microbiology, 69(8), 4519-4526.

165. **Lind, H., Jonsson, H. & Schnürer, J.** (2005). Antifungal effect of dairy propionibacteria – contribution of organic acids. International Journal of Food Microbiology, 98, 157-165.

166. **Linton, R.H., Carter, W.H., Pierson, M.D. & Hackney, C.R.** (1995). Use of a modified Gompertz equation to model nonlinear survival curves for *L. monocytogenes* Scott A. Journal of Food Protection, 58, 946-954.

167. **Liu, S.-Q.** (2003). Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. International Journal of Food Microbiology, 83, 115-131.

168. **Loessner, M.J. & Busse, M.** (1990). Bacteriophage typing of *Listeria* species. Applied and Environmental Microbiology, 56 (6), 1912-1918.

169. **Loessner, M.J.** (2005). Bacteriophage endolysins – current state of research and applications. Current Opinion in Microbiology, 8, 480-487.

170. **Luchansky, J.B., Glass, K.A., Harsono, K.D., Degnan, A.J., Faith, N.G., Cauvin, B., Baccus-Taylor, G., Arihara, K., Bater, B., Maurer, A.J. & Cassens, R.G**. (1992). Genomic analysis of *Pediococcus* starter cultures used to control *Listeria monocytogenes* in Turkey summer sausage. Applied and Environmental Microbiology, 58(9), 3053-3059.

171. **Lücke, F.K.** (2000). Utilization of microbes to process and preserve meat. Meat Science, 56, 105-115.

172. **Mäkelä, P. & Korkeala, H.** (1987). *Lactobacillus* contamination of cooked ring sausages at sausage processing plants. International Journal of Food Microbiology, 5, 323-330.

173. **Malakar, P.K., Martens, D.E., Zwietering, M.H., Béal, C. & van 't Riet, K.** (1999). Modelling the interactions between *Lactobacillus curvatus* and *Enterobacter cloacae*. II. Mixed cultures and shelf life predictions. International Journal of Food Microbiology, 51, 67-79.

174. **Marceau, A., Zagorec, M. & Champomier-Vergès, M.-C.** (2003). Positive effects of growth at suboptimal temperature and high salt concentration on long-term survival of *Lactobacillus sakei*. Research in Microbiology, 154 (1), 37-42.

175. **Marceau, A., Zagorec, M., Chaillou, S., Méra, T. & Champomier-Vergès, M.-C.** (2004). Evidence for involvement of at least six proteins in adaptation of *Lactobacillus sakei* to cold temperatures and addition of NaCl. Applied and Environmental Microbiology, 70(12), 7260-7268.

176. **Marshall, D.L., Cotton, L.N. & Bala, F.A.** (2000). Acetic acid. In: Naidu, A.S. (Ed.), Natural food antimicrobial systems. CRC Press LLC, Florida, p. 661-688.

177. **Mataragas, M., Drosinos, E.H. & Metaxopoulos, J.** (2003a). Antagonistic activity of lactic acid bacteria against *Listeria monocytogenes* in sliced, cooked cured pork shoulder stored under vacuum or modified atmosphere at 4±2°C. Food Microbiology, 20, 259-265.

178. **Mataragas, M., Metaxopoulos, J., Galiotou, M. & Drosinos, E.H.** (2003b). Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. Meat Science, 64, 265-271.

179. **Mathur, S. & Singh, R.** (2005). Antibiotic resistance in food lactic acid bacteria – a review. International Journal of Food Microbiology, 105, 281-295.

180. **Mattila, K., Saris, P. & Työppönen, S.** (2003). Survival of *Listeria monocytogenes* on sliced, cooked sausage after treatment with pediocin AcH. International Journal of Food Microbiology, 89, 281-286.

181. **McAuliffe, O., Ross, R.P. & Hill, C.** (2001). Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiology Reviews, 25, 285-308.

182. **McDonald, K. & Sun, D** (1999). Predictive food microbiology for the meat industry: a review. International Journal of Food Microbiology, 52, 1-27.

183. **McMullen, L.M. & Stiles, M.E.** (1996). Potential for use of bacteriocin-producing lactic acid bacteria in the preservation of meats. Journal of Food Protection, S, 64-71.

184. **Metaxopoulos, J., Mataragas, M. & Drosinos, E.H.** (2002). Microbial interaction in cooked cured meat products under vacuum or modified atmosphere at 4°C. Journal of Applied Microbiology, 93, 363-373.

185. **Minor-Pérez, H., Ponce-Alquicira, E., Macías-Bravo, S. & Guerrero-Legarreta, I.** (2004). Changes in fatty acids and microbial population of pork inoculated with two biopreservative strains. Meat Science, 66, 793-800.

186. **Montel, M.C.** (1999). Fermented meat products. In: Robinson, R.K., Batt, C.A., Patel, P.D. (Eds.), Encyclopedia of Food Microbiology. Academic Press, San Diego, p. 744-753.

187. **Montville, T.J. & Bruno, M.E.C.** (1994). Evidence that dissipation of proton motive force is a common mechanism of action for bacteriocins and other antimicrobial proteins. International Journal of Food Microbiology, 24, 53-74.

188. **Montville T.J. & Winkowski, K.** (1997). Biologically based preservation systems and probiotic bacteria. In: Doyle, M.P., Beuchat, L.R. & Montville, T.J. (Eds.), Food Microbiology: Fundamentals & Frontiers. ASM Press, Washington, p. 557-577.

189. **Moore, J.E. & Madden, R.H.** (1997). Preservation of vacuum-packaged pork liver pâté by fermentation. Journal of Food Protection, 60(7), 791-794.

190. **Motlagh, A.M., Holla, S., Johnson, M.C., Ray, B. & Field, R.A.** (1992). Inhibition of *Listeria* spp. in sterile food systems by pediocin AcH, a bacteriocin produced by *Pediococcus acidilactici* H. Journal of Food Protection, 55, 337-343.

191. **Moretro, T., Hagen, B.F. & Axelsson, L.** (1998). A new, completely defined medium for meat lactobacilli. Journal of Applied Microbiology, 85, 715-722.

192. **Muriana, P.M.** (1996). Bacteriocins for control of *Listeria* spp. in food. Journal of Food Protection, S, 54-63.

193. **Nes, I.F., Mørtvedt, C.I., Nissen-Meyer, J. & Skaugen, M.** (1994). Lactocin S, a lanthionine-containing bacteriocin isolated from *Lactobacillus sakei* L45. In: De Vuyst, L. & Vandamme, E.J. (Eds.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic and Professional, London, p. 435-449.

194. **Nettles, C.G. & Barefoot, S.F.** (1993). Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. Journal of Food Protection, 56(4), 338-356.

195. **Nielsen, J.W., Dickson, J.S. & Crouse, J.D.** (1990). Use of bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. Applied and Environmental Microbiology, 56(7), 2142-2145.

196. **Niku-Paavola, M.L., Laitila, A., Mattila-Sandholm, T. & Haikara, A.** (1999). New types of antimicrobial compounds produced by *Lactobacillus plantarum*. Journal of Applied Microbiology, 86, 29-35.

197. **Nilsson, L., Gram, L. & Huss, H.H.** (1999). Growth control of *Listeria monocytogenes* on cold-smoked salmon using a competitive lactic acid bacteria flora. Journal of Food Protection, 62(4), 336-342.

198. **Nilsson, L., Ng, Y.Y., Christiansen, J.N., Jorgensen, B.L., Grotinum, D. & Gram, L.** (2004). The contribution of bacteriocin to inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola* strains in cold-smoked salmon systems. Journal of Applied Microbiology, 96, 133-143.

199. **Nilsson, L., Hansen, T.B., Garrido, P., Buchrieser, C., Glaser, P., Knochel, S. Gram, L. & Gravesen, A.** (2005). Growth inhibition of *Listeria monocytogenes* by a

nonbacteriocinogenic *Carnobacterium piscicola*. Journal of Applied Microbiology, 98, 172-183.

200. **Nissen, P. & Arneborg, N.** (2003). Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae.* Archives of Microbiology, 180, 257-263.

201. **Nubel** (1999). Belgische voedingsmiddelentabel. 3th edition. Nubel, Brussel, 80 p.

202. **Nychas, G.-J.E., Drosinos, E.H. & Board, R.G.** (1998). Chemical changes in stored meat. In: Davies, A. and Board, E. (Eds.), Microbiology of meat and poultry. Blackie Academic & Professional, London, p. 289-326.

203. **Oakey H.J., Harty, D.W.S. & Knox, K.W.** (1995). Enzyme production by lactobacilli and the potential link with endocarditis. Journal of Applied Bacteriology, 78, 142-148.

204. **O'Flynn, G.O., Ross, R.P., Fitzgerald, G.F. & Coffey, A.** (2004). Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Applied and Environmental Microbiology, 70(6), 3417-3424.

205. **O'Sullivan, L., Ross, R.P. & Hill, C.** (2002). Potential of bacteriocin producing LAB for improvements in food safety and quality. Biochimie, 84, 593-604.

206. **Peirson, M.D., Guan, T.Y. & Holley, R.A.** (2003). Thermal resistances and lactate and diacetate sensitivities of bacteria causing bologna discolouration. International Journal of Food Microbiology, 86, 223-230.

207. **Phan-Thanh, L. & Gormon, T.** (1997). A chemically defined minimal medium for the optimal culture of *Listeria*. International Journal of Food Microbiology, 35, 91-95.

208. **Piard, J.C. & Desmazeaud, M.** (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end products. Lait, 71, 525-541.

209. **Pin, C., García de Fernando, G.D. & Ordóñez, J.A.** (2002). Effect of modified atmosphere composition on the metabolism of glucose by *Brochothrix thermosphacta*. Applied and Environmental Microbiology, 68(9), 4441-4447.

210. **Pot, B., Ludwig, W., Kersters, K. & Schleifer, K.H**. (1994). Taxonomy of lactic acid bacteria. In: De Vuyst, L. & Vandamme, E.J. (Eds.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic and Professional, London, p. 13-90.

211. **Pothakamury, U.R. & Barbosa-Cánovas, G.V.** (1995). Fundamental aspects of controlled release in foods. Trends in Food Science and Technology, 6, 397-406.

212. **Premaratne, R.J., Lin, W.-J. & Johnson, E.A.** (1991). Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Applied and Environmental Microbiology, 57(10), 3046-3048.

213. **Pucci, M.J., Vedamuthu, E.R., Kunka, B.S. & Vandenbergh, P.A.** (1988). Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. Applied and Environmental Microbiology, 54(10), 2349-2353.

214. **Qvist, S., Sehested, K. & Zeuthen, P.** (1994). Growth suppression of *Listeria monocytogenes* in a meat product. International Journal of Food Microbiology, 24, 283-293.

215. **Raccach, M.** (1985). Manganese and lactic acid bacteria. Journal of Food Protection, 48(10), 895-898.

216. **Rijpens, N.P., Jannes, G. & Herman, L.M.F.** (1997). Incidence of *Listeria* spp. and *Listeria monocytogenes* in ready-to-eat chicken and turkey products by polymerase chain reaction and line probe assay hybridisation. Journal of Food Protection, 60, 548-550.

217. **Roberts, T.A., Pitt, J.I., Cordier, J.-L., Gorris, L.G.M., Gram, L., Swanson, KM.J. & Tompkin, R.B.** (2005). Micro-organisms in foods 6: Microbial ecology of food commodities (2nd edition). ICMFS, Kluwer Academic/ Plenum Publishers, New York, 615 p.

218. **Rodgers, S.** (2001). Preserving non-fermented refrigerated foods with microbial cultures: a review. Trends in Food Science and Technology, 12, 276-284.

219. **Rodgers, S.** (2003). Potential applications of protective cultures in cook-chill catering. Food Control, 14, 35-42.

220. **Rodgers, S.** (2004). Novel approaches in controlling safety of cook-chill meals. Trends in Food Science and Technology, 15, 366-372.

221. **Rodriguez, J.M., Sobrino, O.J., Moreira, W.L., Fernandez, M.F., Cintas, L.M., Casaus, P., Sanz, B. & Hernandez, P.E.** (1994). Inhibition of *Listeria monocytogenes* by *Lactobacillus sake* strains of meat origin. Meat Science, 38, 17-26.

222. **Rodriguez, J.M., Cintas, L.M., Casaus, P., Horn, N., Dodd, H.M., Hernandez, P.E. & Gasson, M.J.** (1995). Isolation of nisin-producing *Lactococcus lactis* strains from dry-fermented sausages. Journal of Applied Bacteriology, 78, 109-115.

223. **Rodriguez, J.M., Martinez, M.I., Horn, N., & Dodd, H.M.** (2002). Heterologous production of bacteriocins by lactic acid bacteria. International Journal of Food Microbiology, 80, 101-116.

224. **Ross, R.P., Morgan, S. & Hill, C.** (2002). Preservation and fermentation: past, present and future. International Journal of Food Microbiology, 79, 3-16.

225. **Rossland, E., Andersen Borge, G.I., Langsrud, T. & Sorhaug, T.** (2003). Inhibition of *Bacillus cereus* by strains of *Lactobacillus* and *Lactococcus* in milk. International Journal of Food Microbiology, 89 (2-3), 205-212.

226. **Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J. & Jiménez-Díaz, R.** (1994). Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in spanish-

style green olive fermentations. Applied and Environmental Microbiology, 60(6), 2059-2064.

227. **Russell, J.B.** (1992). Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. Journal of Applied Bacteriology, 73, 363-370.

228. **Salaün, F., Mietton, B. & Gaucheron, F.** (2005). Buffering capacity of dairy products. International Dairy Journal, 15, 95-109.

229. **Samelis, J., Kakouri, A., Georgiadou, K.G. & Metaxopoulos, J.** (1998). Evaluation of the extent and type of bacterial contamination at different stages of processing of cooked ham. Journal of Applied Microbiology, 84, 649-660.

230. **Samelis, J. & Metaxopoulos, J.** (1999). Incidence and principle sources of *Listeria* spp. and *Listeria monocytogenes* contamination in processed meats and a meat processing plant. Food Microbiology, 16, 465-477.

231. **Samelis, J. & Georgiadou, K.G.** (2000). The microbial association of Greek taverna sausage stored at 4 and 10°C in air, vacuum or 100% carbon dioxide, and its spoilage potential. Journal of Applied Microbiology, 88, 58-68.

232. **Samelis, J., Kakouri, A. & Rementzis, J.** (2000a). Selective effect of the product type and the packaging conditions on the species of lactic acid bacteria dominating the spoilage microbial association of cooked meats at 4°C. Food Microbiology, 17, 329-340.

233. **Samelis, J., Kakouri, A. & Rementzis, J.** (2000b). The spoilage microflora of cured, cooked turkey breasts prepared commercially with or without smoking. International Journal of Food Microbiology, 56, 133-143.

234. **Samelis, J., Bedie, G.K., Sofos, J.N., Belk, K.E., Scanga, J.A. & Smith, G.C.** (2002). Control of *Listeria monocytogenes* with combined antimicrobials after postprocess contamination and extended storage of frankfurters at 4°C in vacuum packages. Journal of Food Protection, 65(2), 299-307.

235. **Schillinger, U. & Lücke, F.K.** (1987). Lactic acid bacteria on vacuum-packaged meat and their influence on shelf life. Fleischwirtschaft, 67(10), 1244-1248.

236. **Schillinger, U. & Lücke, F.K.** (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. Applied and Environmental Microbiology, 55(8), 1901-1906.

237. **Schillinger, U., Kaya, M. & Lücke, F.K.** (1991). Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sakei*. Journal of Applied Bacteriology, 70, 473-478.

238. **Schillinger, U., Geisen, R. & Holzapfel, W.H.** (1996). Potential of antagonistic micro-organisms and bacteriocins for the biological preservation of foods. Trends in Food Science and Technology, 7, 58-64.

239. **Schmidt, U. & Leistner, L.** (1993). Unpackaged sliced frankfurter-type sausage - behavior of *Listeria monocytogenes*. Fleischwirtschaft, 73(7), 733-740.

240. **Schnürer, J. & Magnusson, J.** (2005). Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science and Technology, 16, 70-78.

241. **Senne, M.M. & Gilliland, S.E.** (2003). Antagonistic action of cells of *Lactobacillus delbrueckki* subsp. *lactis* against pathogenic and spoilage micro-organisms in fresh meat systems. Journal of Food Protection, 66(3), 418-425.

242. **Shelef, L.A.** (1994). Antimicrobial effects of lactates: a review. Journal of Food Protection, 57(5), 445-450.

243. **Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J. & Kenne, L.** (2003). Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MILAB 14. Applied and Environmental Microbiology, 69(12), 7554-7557.

244. **Skoog, D.A., West, D.M. & Holler, F.J.** (1996). Fundamentals of Analytical Chemistry. 7th edition. Saunders College Publishing, Fort Worth, p. 870.

245. **Skyttä, E., Hereijgers, W. & Mattila-Sandholm, T.** (1991). Broad spectrum antibacterial activity of *Pediococcus damnosus* and *Pediococcus pentosaceus* in minced meat. Food Microbiology, 8, 231-237.

246. **Sobrino, O.J., Rodriguez, J.M., Moreira, W.L., Fernandez, M.F., Sanz, B. & Hernandez, P.E.** (1991). Antibacterial activity of *Lactobacillus sake* isolated from dry fermented sausages. International Journal of Food Microbiology, 13, 1-10.

247. **Sobrino, O.J., Rodriguez, J.M., Moreira, W.L., Cintas, L.M., Fernandez, M.F., Sanz, B. & Hernandez, P.E.** (1992). Sakacin M, a bacteriocin-like substance from *Lactobacillus sake* 148. International Journal of Food Microbiology, 16, 215-225.

248. **Stekelenburg, F. & Kant-Muermans, M.** (2001). Effects of sodium lactate and other additives in a cooked ham product on sensory quality and development of a strain of *Lactobacillus curvatus* and *Listeria monocytogenes*. International Journal of Food Microbiology, 66, 197-203.

249. **Stekelenburg, F.** (2003). Enhanced inhibition of *Listeria monocytogenes* in frankfurter sausage by the addition of potassium lactate and sodium diacetate mixtures. Food Microbiology, 20, 133-137.

250. **Stentz, R., Cornet, M., Chaillou, S. & Zagorec, M.** (2001). Adaptation of *Lactobacillus sakei* to meat: a new regulatory mechanism of ribose utilization? Lait, 81, 131-138.

251. **Stiles, M.E. & Holzapfel, W.H.** (1997). Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology, 36, 1-29.

252. **Stolle, A., Wawerla, M., Krauβe, G., Sperner, B., Schalch, B., Marx, H., Stock, K. & Schroeter, G**. (2001). Untersuchungen von SB-Fleisch-Erzeugnissen im Hinblick auf deren Verkehrsfähigkeit. Fleischwirtschaft, 4, 152-154.

253. **Stratford, M.** (2000). Traditional preservatives – organic acids. In: Robinson, R.K., Batt, C.A., Patel, P.D. (Eds.). Encyclopedia of Food Microbiology, Vol 3. Academic Press, London, p. 1729-1737.

254. **Ström, K., Sjögren, J., Broberg, A. & Schnürer, J.** (2002). *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Applied and Environmental Microbiology, 68(9), 4322-4327.

255. **Tavaria, F.K., Dahl, S., Carballo, F.J. & Malcata, F.X.** (2002). Amino acid catabolism and generation of volatiles by lactic acid bacteria. Journal of Dairy Science, 85, 2462-2470.

256. **Teixeira de Carvalho, A.A., Aparecida de Paula, R., Mantovani, H.C. & Alencar de Moraes, C.** (2006). Inhibition of *L. monocytogenes* by a lactic acid bacterium isolated from Italian sausage. Food Microbiology, 23, 213-219.

257. **Temmerman, R., Huys, G. & Swings, J.** (2004). Identification of lactic acid bacteria: culture-dependent and culture-independent methods. Trends in Food Science and Technology, 15, 348-359.

258. **Thiel, K.** (2004). Old dogma, new tricks – 21st Century phage therapy. Nature Biotechnology, 22(1), 31-36.

259. **Torriani, S., Van Reenen, C.A., Klein, G., Reuter, G., Dellaglio, F. & Dicks, L.M.T.** (1996). *Lactobacillus curvatus* subsp. *curvatus* subsp. nov. and *Lactobacillus curvatus* subsp. *melibiosus* subsp. nov. and *Lactobacillus sake* subsp. *sake* subsp. nov. and *Lactobacillus sake* subsp. *carnosus* subsp. nov., new subspecies of *Lactobacillus curvatus* Abo-Elnaga and Kandler 1965 and *Lactobacillus sake* Katagiri, Kitahara, and Fukami 1934 (Klein et al. 1996, emended descriptions), respectively. International Journal of Systematic Bacteriology, 46(4), 1158-1163.

260. **Työppönen, S., Markkula, A., Petäjä, E., Suihko, M.-L. & Mattila-Sandholm, T.** (2003a). Survival of *Listeria monocytogenes* in North European type dry sausages fermented by bioprotective meat starter cultures. Food Control, 14, 181-185.

261. **Työppönen, S., Petäjä, E. & Mattila-Sandholm, T.** (2003b). Bioprotectives and probiotics for dry sausages. International Journal of Food Microbiology, 83, 233-244.

262. **Urbach, G.** (1995). Contribution of lactic acid bacteria to flavour compound formation in dairy products. International Dairy Journal, 5, 877-903.

263. **Uyttendaele, M., De Troy, P. & Debevere, J.** (1999). Incidence of *Listeria monocytogenes* in different types of meat products on the Belgian retail market. International Journal of Food Microbiology, 53(1), 75-80.

264. **Uyttendaele, M., Rajkovic, A., Benos, G., Francois, K., Devlieghere, F. & Debevere, J.** (2004). Evaluation of a challenge testing protocol to assess the stability of ready-to-eat cooked meat products against growth of *Listeria monocytogenes*. International Journal of Food Microbiology, 90, 219-236.

265. **Valerio, F., Lavermicocca, P., Pascale, M. & Visconti, A.** (2004). Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. FEMS Microbiology Letters, 233, 289-295.

266. **Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J.** (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews, 60(2), 407-438.

267. **Vanos, V. & Cox, L.** (1986). Rapid routine method for the detection of lactic acid bacteria among competitive flora. Food Microbiology, 3, 223-234.

268. **Van Slyke, D.** (1922). On the measurement of buffer values and on the relationship of buffer value to the dissociation constant of the buffer and the concentration and reaction of the buffer solution. Journal of Biological Chemistry, 52, 525-571.

269. **Vereecken, K.** (2002). Predictive microbiology for multiple systems: design and validation of a model for lactic acid induced interaction in complex media. Ph.D. Thesis, Faculty of Applied Sciences, K.U. Leuven, Belgium, 221 p.

270. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2004). Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. International Journal of Food Microbiology, 96, 149-164.

271. **Vermeiren, L., Devlieghere, F., De Graef, V. & Debevere, J.** (2005). In-vitro and in-situ growth characteristics and behaviour of spoilage organisms associated with anaerobically stored cooked meat products. Journal of Applied Microbiology, 98, 33-42.

272. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2006a) Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham. International Journal of Food Microbiology, 108, 68-77.

273. **Vermeiren, L., Devlieghere, F., Vandekinderen, I. & Debevere, J.** (2006b). The antagonistic interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham. Food Microbiology, 23, 511-518

274. **Vermeiren, L., Devlieghere, F., Vandekinderen, I., Rajtak U. & Debevere, J.** (2006c). The sensory acceptability of cooked meat products treated with a protective culture depends on glucose content and buffering capacity: a case-study with *Lactobacillus sakei* 10A. Meat Science, in press.

275. **Vermeulen, A., Devlieghere, F., Ooghe, W. & Debevere, J.** (2006). Interactions between *Listeria monocytogenes* and *Lactobacillus sakei* in a chemically defined broth simulating cooked ham. International Journal of Food Microbiology, under revision.

276. **Vescovo, M., Orsi, C., Scolari, G. & Torriani, S**. (1995). Inhibitory effect of selected lactic acid bacteria on microflora associated with ready-to-use vegetables. Letters in Applied Microbiology, 21, 121-125.

277. **Vitas, A.I. & Garcia-Jalon, V.A. e I.** (2004). Occurrence of *L. monocytogenes* in fresh and processed foods in Navarra (Spain). International Journal of Food Microbiology, 90, 349-356.

278. **Vold, L., Holck, A., Wasteson, Y. & Nissen, H.** (2000). High levels of background flora inhibits growth of *Escherichia coli* O157:H7 in ground beef. International Journal of Food Microbiology, 56, 219-225.

279. **von Holy, A., Cloete, T.E. & Holzapfel, W.H.** (1991). Quantification and characterization of microbial populations associated with spoiled vacuum-packed Vienna sausages. Food Microbiology, 8, 95-104.

280. **Wessels, S., Axelsson, L., Hansen, E.B., De Vuyst, L., Laulund, S., Lähteenmäki, L., Lindgren, S., Mollet, B., Salminen, S. & von Wright, A.** (2004). The lactic acid bacteria, the food chain, and their regulation. Trends in Food Science and Technology, 15, 498-505.

281. **Whichard, J.M., Sriranganathan, N. & Pierson, F.W.** (2003). Suppression of *Salmonella* growth by wild-type and large-plaque variants of bacteriophage Felix O1 in liquid culture and on chicken frankfurters. Journal of Food Protection, 66(2), 220-225.

282. **Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O.P., Bierbaum, G., de Kruijff, B. & Sahl, H.-G.** (2001). Specific binding of nisin to the peptidoglycan precursor Lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. The Journal of Biological Chemistry, 276 (1), 1172-1779.

283. **Winkowski, K., Crandall, A.D. & Montville, T.J.** (1993). Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus* MN in beef systems at refrigeration temperatures. Applied and Environmental Microbiology, 59(8), 2552-2557.

284. **Yang, R. & Ray, B.** (1994). Prevalence and biological control of bacteriocin-producing psychrotrophic leuconostocs associated with spoilage of vacuum-packaged processed meats. Journal of Food Protection, 57(3), 209-217.

285. **Zhang, G. & Holley, R.A.** (1999). Development and PFGE monitoring of dominance among spoilage LAB from cured meats. Food Microbiology, 16, 633-644.

286. **Zottola, E.A., Yezzi, T.L., Ajao, D.B. & Roberts, R.F.** (1994). Utilization of cheddar cheese containing nisin as an antimicrobial agent in other foods. International Journal of Food Microbiology, 24, 227-238.

287. **Zuniga, M., Pardo, I. & Ferrer, S.** (1993). An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. International Journal of Food Microbiology, 18, 37-42.

Curriculum vitae

Curriculum vitae

Lieve Vermeiren werd geboren op 19 augustus 1975 te Gent en groeide op in Ouwegem. In 1993 beëindigde ze het secundair onderwijs, richting Latijn-Wiskunde, aan het Sint-Pieters Instituut te Gent. Vijf jaar later studeerde ze af als bio-ingenieur scheikunde aan de Universiteit Gent.

Na een korte opdracht als assistente ‘ad interim’ aan de Vakgroep Toegepaste Analytische en Fysische chemie’ (Faculteit Bio-ingenieurswetenschappen, Universiteit Gent), startte ze in februari 1999 als wetenschappelijk personeelslid op het Laboratorium voor Levensmiddelenmicrobiologie en -conservering (Faculteit Bio-ingenieurswetenschappen, Universiteit Gent). Daar was zij onder leiding van Prof. dr. ir. F. Devlieghere verantwoordelijk voor de coördinatie van de werkgroep ‘actieve verpakkingen’ in het kader van het Europese FAIR-project ‘Actipak’ CT98-4170 ‘Evaluating safety, effectiveness, economic-environmental impact and consumer acceptance of active and intelligent packagings’.
Ongeveer twee jaar later, in oktober 2000, werd zij aangesteld als assistente in het vakgebied ‘Technologie van vlees en vis’ aan de Vakgroep Voedselveiligheid en Voedselkwaliteit opnieuw aan het Laboratorium voor Levensmiddelenmicrobiologie en -conservering en dit voor drie opeenvolgende perioden van twee jaar.
Tijdens deze zes jaar werkte zij aan dit doctoraat onder leiding van de promotoren Prof. dr. ir. J. Debevere en Prof. dr. ir. F. Devlieghere. In juni 2005 behaalde ze het getuigschrift van de doctoraatsopleiding in de toegepaste biologische wetenschappen aan de Universiteit Gent.
Naast haar onderzoeksopdracht was zij verantwoordelijk voor het uitwerken, organiseren en doceren van de practica van de opleidingsonderdelen ‘Technologie van vis’, ‘Vleeskennis en vleestechnologie’ in het kader van de opleiding ‘Bio-ingenieur’ alsook van de opleidingsonderdelen ‘Fisheries and Fishery Products’ and 'Meat and Meat Products’ in het kader van de specialisatieopleiding ‘Master in Food Science and Technology’ en ‘Technology of Fishery Products’ in het kader van de opleiding ‘Master in Aquaculture’.
Met grote interesse nam zij ook deel aan interne dienstverlening binnen de faculteit en binnen de vakgroep alsook aan externe dienstverlening door middel van samenwerkingsverbanden met industriële partners van het Laboratorium voor Levensmiddelenmicrobiologie en -conservering.

List of publications

Papers in internationally distributed journals with peer-review

1. **Vermeiren, L., Devlieghere, F., Van Beest, M., De Kruijf, N. & Debevere, J.** (1999). Developments in the active packaging of foods. Trends in Food Science and Technology, 10(3), 77-86.

2. **Devlieghere, F., Vermeiren, L. & Debevere, J.** (2000). Study on antimicrobial activity of potassium sorbate in active food packaging. Acta Alimentaria, 29(2), 137-146.

3. **Devlieghere, F., Vermeiren, L., Jacobs, M. & Debevere, J.** (2000). The effectiveness of hexamethylenetetramine-incorporated plastic for the active packaging of foods. Packaging Technology and Science, 13(3), 117-121.

4. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2002). Effectiveness of some recent antimicrobial packaging concepts. Food additives and contaminants, 19, Supplement, 163-171.

5. **Devlieghere, F., Vermeiren, L. & Debevere, J.** (2004). New preservation technologies: possibilities and limitations. International Dairy Journal, 14(4), 273-285.

6. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2004). Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. International Journal of Food Microbiology, 96(2), 149-164.

7. **Vermeiren, L., Devlieghere, F., De Graef, V. & Debevere, J.** (2005). In-vitro and in-situ growth characteristics and behaviour of spoilage organisms associated with anaerobically stored cooked meat products. Journal of Applied Microbiology, 98, 33-42.

8. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2006) Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham. International Journal of Food Microbiology, 108, 68-77.

9. **Vermeiren, L., Devlieghere, F., Vandekinderen, I. & Debevere, J.** (2006). The antagonistic interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham. Food Microbiology, 23, 511-518.

10. **Vermeiren, L., Devlieghere, F., Vandekinderen, I., Rajtak U. & Debevere, J.** (2006).The sensory acceptability of cooked meat products treated with a protective culture depends on glucose content and buffering capacity: a case study with *Lactobacillus sakei* 10A. Meat Science, 74, 532-545.

11. **Vermeiren, L., Devlieghere, F., Rajtak, U., Kerkaert, B. & Debevere, J.** (2006). The contribution of lactic acid production versus nutrient competition to the growth inhibition

of *Listeria monocytogenes* by the non-bacteriocinogenic *Lactobacillus sakei* 10A. Submitted for publication in Journal of Applied Microbiology.

Papers in national journals and journals without peer-review

1. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2000). Actief verpakken in Europa: de stand van zaken. Voedingsmiddelentechnologie, 9, 28 april 2000, 11-14.

2. **Vermeiren, L., Devlieghere, F., Schepens, N. & Debevere, J.** (2000). Study on the feasibility of a triclosan-containing film as antimicrobial food packaging material. Communications in Agricultural and Applied Biological Sciences, 65/3b, 511-517.

3. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2003). Evaluation of the potential of meat born lactic acid bacteria for use as protective cultures in the biopreservation of cooked meat products. Communications in Agricultural and Applied Biological Sciences, 68/2(b), 441-448.

4. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2003). Characterization of spoilage through heterofermentative lactic acid bacteria and *Brochothrix* spp. on vacuum packaged cooked ham. Communications in Agricultural and Applied Biological Sciences, 68/3, 23-26.

5. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2004). Co-culture experiments between protective cultures and spoilage organisms on vacuum packaged ham. Communications in Agricultural and Applied Biological Sciences, 69/2, 329-331.

Short papers and abstracts published in conference proceedings

1. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (1999). Evaluating safety, effectiveness, economic-environmental impact and consumer acceptance of active and intelligent packagings, FAIR-project PL 98-1470 'Actipak'. 4th Conference on Food Microbiology, Luik (Belgium), June 16-17, p.189.

2. **Vermeiren, L., Devlieghere, F., Schepens, N. & Debevere, J.** (2000). Study on the effectiveness of a triclosan containing film as a food packaging material for vacuum packaged meat products. 5th Conference on Food microbiology, Luik (Belgium), June 22-23, p.171.

3. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2000). Potential applications of antimicrobial films for food packaging. Proceedings of the International Conference on active and intelligent packaging, Campden (UK), September 7-8.

4. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2000). Effectiveness of some recent antimicrobial packaging concepts. Proceedings of the 2nd International Symposium on food packaging: ensuring the safety and quality of foods, Vienna (Austria), November 8-10, p. 33.

5. **Devlieghere, F., Vermeiren, L. & Debevere, J.** (2000). Estimation of the safety of gas packaged cooked meat products: a predictive modelling approach. Proceedings of the

International Symposium on food packaging: ensuring the safety and quality of foods, Vienna (Austria), 8- November 10, p. 50bis.

6. **Vermeiren, L., Devlieghere, F., Assi, A. & Debevere, J.** (2002). Inhibitory spectrum of bacteriocin producing lactic acid bacteria as a primary screening of their potential use as protective cultures for meat preservation. 7th Conference on Food Microbiology, Luik (Belgium), June 20-21, p 115-116.

7. **Vermeiren, L., Devlieghere, F., Assi, A. & Debevere, J.** (2002). Inhibitory spectrum of bacteriocin producing lactic acid bacteria as a primary screening of their potential use as protective cultures for meat preservation. Food Micro 2002. 18th international ICFMH Symposium, Lillehammer (Norway), August 18-23, p. 304.

8. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2003). Growth and acidification profile of potential protective cultures for meat preservation. 8th conference on Food Microbiology, Luik (Belgium), June 19-20, p. 76.

9. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2004). Protectieve culturen ter verlenging van de houdbaarheid. 9th Conference on Food Microbiology, Merelbeke (Belgium), September 16-17, p. 26-34.

10. **Vermeiren, L., Devlieghere, F. & Debevere, J.** (2005). The use of *Lactobacillus sakei* 10A for the biopreservation of cooked meat products'. 8th Symposium on Lactic Acid Bacteria, Egmond aan Zee (The Netherlands), August 28 to September 1, A 007.

Books, chapters in books and dissertations

1. **Vermeiren, L.** (1998). Groeikarakterisitieken en productie van extracellulaire virulentiefactoren door *Aeromonas* spp. geïsoleerd uit levensmiddelen. Afstudeerwerk Landbouwkundige en Toegepaste Biologische Wetenschappen - Universiteit Gent.

2. **Vermeiren, L., Devlieghere, F. & Debevere, J., Smolander, M. Hurme, E., Ahvenainen, R., Van Beest, M.D. & Rijk, M.A.H.** (1999). An in-depth review of technologies, legislation, market and consumer needs and trends in active and intelligent packaging in relation to current European food packaging regulations. Literature review of FAIR-project Actipak (CT 98-4170), October, 55p.

3. **Vermeiren, L., Heirlings, L., Devlieghere, F. & Debevere, J.** (2003) Oxygen, ethylene and other scavengers. In: Ahvenainen, R. (Ed.) Novel Food packaging techniques, Woodhead Publishing Limited, Cambridge, UK, p. 22-49.

Conferences

Conferences with oral presentation

1. Symposium Voeding en kunststofverpakkingen - Beurs 'plastics for the industry', April 5, 2000, Kortrijk (Belgium): 'Trends in het verpakken van levensmiddelen'.

2. ILSI Europe 2nd International Symposium on Food Packaging - Ensuring the safety and quality of foods, November 8-10, 2000, Vienna (Austria): 'Effectiveness of some recent antimicrobial packaging concepts'.

3. International Conference on active and intelligent packaging, September 7-8 , 2000, Campden (UK): 'Potential applications of antimicrobial films for food packaging'.

4. The 14th Forum for Applied Biotechnology, September 27-28, 2000, Brugge (Belgium): 'Study on the feasibility of a triclosan-containing film as antimicrobial food packaging material'.

5. The 17th Forum for Applied Biotechnology, September 18-19, 2003, Gent (Belgium): 'Evaluation of the potential of meat born lactic acid bacteria for use as protective cultures in the biopreservation of cooked meat products'.

6. The 9th PhD-symposium on Applied Biological Sciences, October 16, 2003, Leuven (Belgium): 'Characterization of spoilage through heterofermentative lactic acid bacteria and *Brochothrix* spp. on vacuum packaged cooked ham'.

7. The 9th Conference in Food Microbiology, September 16-17, 2004, Merelbeke (Belgium): 'Protectieve culturen ter verlenging van de houdbaarheid'.

8. Seminarie 'Actuele verpakkingstrends met bijzondere aandacht voor actieve verpakking', Februari 17, 2005, Diepenbeek (Belgium): 'Antimicrobiële verpakkingsconcepten'.

9. BAMST Studiedag 'Microbiologie en vlees: welke toekomst?', April 5, 2006, Gembloux (Belgium): 'Verpakken van vlees en vleeswaren: microbiologische aspecten'.

10. NVvM-symposium ' Consumentengedrag en microbiële risico's / microbiologisch bederf, June 13, 2006, Wageningen (The Netherlands): 'Microbiologisch bederf van verpakte gekookte vleesproducten: karakterisering en het gebruik van beschermende culturen ter preventie'.

Conferences with poster presentation

1. 4th Conference on Food Microbiology, June 16-17, 1999, Luik (Belgium): 'Evaluating safety, effectiveness, economic-environmental impact and consumer acceptance of active and intelligent packagings'.

2. 5th Conference on Food Microbiology, June 22-23, 2000, Luik (Belgium): 'Study on the effectiveness of a triclosan containing film as a food packaging material for vacuum packaged meat products'.

3. 7th Conference on Food Microbiology, June 20-21, 2002, Luik (Belgium): 'Inhibitory spectrum of bacteriocin producing lactic acid bacteria as a primary screening of their potential use as protective cultures for meat preservation'.

4. FOOD MICRO 2002 - 18th International Symposium of the International Committee on Food Microbiology and Hygiene, 18-23 Augustus 2002, Lillehammer (Norway):

'Inhibitory spectrum of bacteriocin producing lactic acid bacteria as a primary screening of their potential use as protective cultures for meat preservation'. (not attended)

5. 8th Conference on Food Microbiology, June 19-20, 2003, Luik (Belgium): 'Growth and acidification profile of potential protective cultures for meat preservation'.

6. 10th PhD-symposium on Applied Biological Sciences, September 29, 2004, Gent (Belgium): 'Co-culture experiments between protective cultures and spoilage organisms on vacuum packaged ham'.

7. 8th Symposium on Lactic Acid Bacteria, August 28 to September 1, 2005, Egmond aan Zee (The Netherlands): 'The use of *Lactobacillus sakei* 10A for the biopreservation of cooked meat products'.

Conferences attended without poster or oral presentation

1. Bijeenkomst studiegroep VTEC O157 - *E. coli* 0157:H7, een gevaar voor u en mij? September 9, 1999, Gent, Belgium.

2. FOOD MICRO 99 - 17th International Symposium of the International Committee on Food Microbiology and Hygiene. September 13-17, 1999, Veldhoven, The Netherlands. (one day attended)

3. The 13th Forum for Applied Biotechnology. September 22-23, 1999, Gent, Belgium.

4. Studiedag TNO - Vers verpakken: systemen en materialen. November 17, 1999, Zeist, The Netherlands.

5. Studiedag KVIV-Genootschap Voeding - Omgaan met risico's in de voedingsindustrie, microbiologische risico's Deel II. March 23, 2000, Antwerp, Belgium.

6. The 6th Conference in Food Microbiology. June 21-22, 2001, Luik, Belgium.

7. Studiedag KaHo Sint-Lieven - Zouten en drogen in relatie tot de microbiologische veiligheid van gedroogde zouterijwaren. March 16, 2001, Gent, Belgium.

8. Studiedag KaHo Sint-Lieven - Nitriet in vleeswaren. May16, 2003, Gent Belgium.

9. Symposium 'Microbiologische, technologische en functionele aspecten van melkzuurbacteriën, probiotica en prebiotica'. October 24, 2003, Gent, Belgium.

10. BAMST studiedag - Oxidaties bij de productie van vlees en vleeswaren: (on)gewenst? November 26, 2003, Melle, Belgium.

11. BAMST avondseminarie - Gefermenteerde worst in Europa: Onderzoek en degustatie. March 25, 2004, Gent, Belgium.

12. Flemisch-Romanian Workshop on lactic acid bacteria. November 23, 2004, Brussels, Belgium.

13. EFSA Colloquium On Microorganisms in Food and Feed: Qualified presumption of Safety – QPS. December 13-14, 2004, Brussels, Belgium.

14. International Symposium Safety and shelf-life assessment of meat products in Europe. February 18, 2005, Ede, The Netherlands.

15. BAMST avondseminarie - Kookham in Europa: Grondstof, technologie, kwaliteit en degustatie. March 16, 2006, Gent, Belgium.

16. Food2Know ontbijtmeeting - Thema Levensmiddelentechnologie. April 11, 2006, Gent, Belgium.