Promotor:

Dr. ir. F.M. Rombouts

Hoogleraar in de Levensmiddelenhygiëne en -microbiologie, Landbouwuniversiteit Wageningen

Copromotor:

Dr. L.G.M. Gorris

Hoofd afdeling Voedselveiligheid en Toegepaste Microbiologie, Agrotechnologisch Onderzoeksinstituut (ATO-DLO)

Marjon H.J. Bennik

Biopreservation in modified atmosphere packaged vegetables

Proefschrift

ter verkrijging van de graad van doctor op gezag van de rector magnificus van de Landbouwuniversiteit te Wageningen, dr. C.M. Karssen, in het openbaar te verdedigen op maandag 22 december 1997 des namiddags te vier uur in de Aula.

The research described in this thesis has been performed at the Department of Food Safety and Applied Microbiology of the Agrotechnological Research Institute (ATO-DLO) and was funded by the Dutch Commodity Board of Fruits and Vegetables, the European Union (contract AIR1-CT92-0125) and the Agrotechnological Research Institute (ATO-DLO).

Bennik, M.H.J. - Biopreservation in modified atmosphere packaged vegetables - 1997.
Thesis Wageningen Agricultural University - With summary in Dutch - 96 p.
Subject headings: bacteriocins / biopreservation / lactic acid bacteria / modified atmosphere packaging / microflora / vegetables

ISBN: 90-5485-808-7

Cover photograph: Fotodienst Staringcentrum (SC-DLO), Wageningen
Inhibition of bacterial growth by bacteriocins/Remming van bacterie-groei door bacteriocines

Design: Max van Poorten

Stellingen behorend bij het proefschrift getiteld:
"Biopreservation in modified atmosphere packaged vegetables",
door Marjon H.J. Bennik.
Wageningen, maandag 22 december 1997

I De verlaagde activiteit van hybride classe IIa bacteriocines in de studie van Fimland et al. hangt samen met de aard van de α-helix in deze peptiden.

- Dit proefschrift,
- Fimland, G. et al. 1996. New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins: the C-terminal region is important for determining specificity. Appl. Environ. Microbiol. 62, 3313-3318.

II Bij de conclusie dat de werking van pediocine PA-1 geen eiwit-receptor vereist gaan Chen et al. voorbij aan de mogelijkheid dat de gebruikte lipiden-extracten in hun liposomenstudies nog hydrophobe eiwitten of peptiden bevatten.

- Chen, Y. et al. 1997. Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. Appl. Environ. Microbiol. 63, 524-531.

III Het grote aantal verschillende namen van bacteriocines suggereert ten onrechte een even groot aantal verschillende verbindingen.

- Onder meer dit proefschrift.

IV Bij de extrapolatie van resultaten verkregen d.m.v. modelsystemen naar de praktijksituatie dient de nodige zorgvuldigheid in acht te worden genomen.

- Dit proefschrift,
- Cai, Y. et al. 1997. Isolation and characterization of nisin-producing *Lactococcus lactis* subsp. *lactis* from bean sprouts. J. Appl. Microbiol. 83, 499-507.

V Om de microbiologische veiligheid van verse groenten te garanderen zijn hygiënische omstandigheden tijdens teelt, bewerking en distributie noodzakelijk, alhoewel ook de consument een deel van de verantwoordelijkheid moet nemen.

- Hyde, B. 1997. Consumers concerned about food contamination don't use safe practices at home, ASM News, 68: 352.

VI Bij een toenemende internationale handel in verse voedingsmiddelen zal niet alleen de omvang, maar ook de aard van voedselinfecties veranderen.

- Mahon, B.E. et al. 1997. An international outbreak of *Salmonella* infections caused by alfalfa sprouts grown from contamined seeds. J. Infect. Diseases 175(4): 876-882.
- Monge, R., et al. 1996. Occurrence of parasites and intestinal bacteria in vegetables that are consumed raw in Costa Rica. Rev. Biol. Trop. 44: 369-375.

VII Inburgeren in de VS is een kwestie van meer 'burgers'.

VIII Wetenschap brengt ons verder, ook van huis.

IX False facts are highly injurious to the progress of science, for they often endure long; but false views, if supported by some evidence, do little harm, for everyone takes a salutary pleasure in proving their falseness; and when this is done, one path toward error is closed and the road to truth is often at the same time opened.

- Charles Darwin

Contents

	Voorwoord	7
Chapter 1.	General introduction	9
	1. Modified atmosphere packaging of vegetables	9
	2. Biopreservation by the use of lactic acid bacteria	10
	3. Outline of this thesis	14
Chapter 2.	The influence of oxygen and carbon dioxide on the growth of prevalent *Enterobacteriaceae* and *Pseudomonas* species isolated from fresh and modified atmosphere stored vegetables	15
Chapter 3.	Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen	23
Chapter 4.	Vegetable-associated *Pediococcus parvulus* produces pediocin PA-1	32
Chapter 5.	A role for oblique peptides in pore formation by bacteriocins of lactic acid bacteria	37
Chapter 6.	Interactions of nisin and pediocin PA-1 with closely related lactic acid bacteria that manifest over 100-fold differences in bacteriocin sensitivity	49
Chapter 7.	Biopreservation for the control of *Listeria monocytogenes* on minimally processed, modified atmosphere stored vegetables	66
	References	75
	Summary and concluding remarks	85
	Samenvatting en slotopmerkingen	89
	Curriculum vitae	93
	List of publications	94

AAN MIJN OUDERS
AAN JAN ALBERT

Voorwoord

Het boekje is af! Bij de totstandkoming ervan is de hulp en betrokkenheid van een groot aantal personen erg belangrijk geweest.

Allereerst wil ik mijn copromotor Leon Gorris noemen. Leon, bedankt voor je steun en het vertrouwen dat je altijd in me hebt gesteld. Je deur stond altijd open en ondanks je volle agenda speelde je het toch steeds weer klaar om op vlotte wijze mijn teksten en manuscripten door te nemen en te stroomlijnen. Eddy Smid, zonder jouw enthousiasme en betrokkenheid had dit boekje er absoluut anders uit gezien. Bedankt voor de vele tips en nuttige discussies en, hoe zou ik het kunnen vergeten, je hulp achter de labtafel. Mijn promotor Frans Rombouts wil ik hartelijk bedanken voor zijn interesse in mijn onderzoek gedurende de afgelopen vier jaar en de snelle beoordeling van het uiteindelijke manuscript. Verder heb ik het erg gewaardeerd betrokken te worden bij aangelegenheden zoals werkbesprekingen en cursussen.

The research described in this thesis was part of an EU project, entitled "Improvement of the safety and quality of refrigerated, minimally processed ready-to-eat foods using novel mild preservation techniques". I would like to thank all of the participants in this multidisciplinary project for the productive collaboration and discussions, and the good atmosphere during the half-year meetings. Een aantal mensen wil ik hier noemen: Tjakko Abee, bedankt voor je interesse en voor het meedenken over bacteriocine-studies. De veelal informele besprekingen waren erg motiverend. Annette Verheul en Géke Naaktgeboren, jullie bedankt voor de hulp bij verschillende experimenten. Dichter bij huis, op het ATO, had ik de hulp van Herman Peppelenbos bij het gas doorstroom-systeem niet kunnen missen.

Dit brengt me terug bij de mensen waar ik de meeste tijd mee heb doorgebracht in het lab en die altijd gezorgd hebben voor een uitstekende sfeer. Yke, Patrick, Roy, beide Jannen en Athina, bedankt voor jullie hulp en betrokkenheid bij mijn onderzoek, en natuurlijk voor de gezelligheid in, maar ook buiten het lab. Van alle andere mensen die korte of langere tijd in het lab hebben gewerkt wil ik met name diegenen bedanken die zich hebben ingezet binnen het kader van het EG-projekt: Bastiënne, Christelle, Esther, Evelyn, Irene, Jeroen, Lianne, Marc, Marloes, Olga, Simone, Tonja, Trudy, Yvette, Wendy en Werner.

Het voert te ver om alle collega's op te noemen bij wie ik aan kon kloppen met vragen en die ervoor gezorgd hebben dat ik het ATO als een prettige werkomgeving heb ervaren. Hierbij denk ik vooral aan de mensen van de 'molbi'-labs, de 'eiwit'-labs, het 'hennep'-lab, het 'verpakkings'-lab en onze buurlabs op de begane grond.

Een aantal personen hebben een belangrijke rol hebben gespeeld in de afrondingsfase van dit proefschrift. Bedankt Max, voor het layouten van het boekje, en Edwin, voor het correctie- en 'koeriers'-werk. In het bijzonder wil ik Athina en mijn zus Angela bedanken voor het organiseren van het feest en het afhandelen van allerlei zaken die vanuit Boston niet een twee drie te regelen zouden zijn geweest.

Beste pap en mam, dit is 't dan! Wat me altijd erg geholpen heeft zijn een portie doorzettingsvermogen, nuchterheid en zelfstandigheid, zaken die jullie me hebben bijgebracht.

Tenslotte, lieve Jan Albert, de laatste loodjes waren ongetwijfeld zwaarder geweest zonder jouw hulp. Bedankt voor alles!

Marjon

CHAPTER 1
General introduction

Postharvest losses of both fresh and freshly processed agricultural produce have been estimated to be as high as 25 to 40% of the total production (132). These can be the result of inadequate control of physiological and microbiological deterioration processes that occur during storage, handling, transport and retailing (132). To counteract postharvest losses, the use of modified atmosphere (MA) storage techniques has increased for bulk storage, but also for consumer-sized packages. Moreover, MA packaging complies with recent trends toward the use of healthy convenient foods, that are minimally processed and less heavily preserved as compared with traditionally processed foods (85). This introduction focuses on the quality and safety aspects of fresh and minimally processed vegetables that are packaged under MA conditions, and introduces biopreservation, based on the use of lactic acid bacteria (LAB), as a potential safety measure.

1. Modified atmosphere packaging of vegetables

1.1 General characteristics

MA packaging in combination with refrigeration can effectively prolong the shelflife of fresh and minimally processed vegetables. Unlike other perishable foods that are packed under MA conditions, such as meats, fresh vegetables continue to respire. As a consequence, oxygen (O_2) is consumed and carbon dioxide (CO_2) is produced inside the package. Favorable gas concentrations for the storage of vegetables are in the range of 1 to 5% O_2 and 5 to 10% CO_2 (112,113). Under these reduced O_2 and elevated CO_2 conditions, the respiration rate of the product, which is positively associated with maturation and change of quality (*e.g.* softening, enzymic browning), is decreased. In addition, MA conditions can reduce microbial spoilage of the product (83,134). The antimicrobial effect of MA conditions is considered to result mainly from elevated concentrations of CO_2, which can reduce the intracellular pH and interfere with the cellular metabolism by the diffusion of H_2CO_3 across the bacterial membrane (56,195).

The equilibrium gas atmosphere inside the package depends on the gas diffusion properties of the packaging material, and the respiratory activity of the produce (50,83,113). The latter activity is affected by intrinsic factors such as the size/variety of produce and the stage of maturation, but also by external factors such as temperature, processing, relative humidity, fill weight, pack volume, film surface area, and the degree of illumination (49). Both high barrier package films and films with excessive permeability are known to have adverse effects. In the case of a high barrier film, undesirable situations may arise from the depletion of O_2, which leads to anaerobic respiration of the product and subsequently yields off-odors and off-flavors. Conversely, the use of a film of excessive permeability will result in little or no favorable change in the atmosphere inside the package. In general, optimal equilibrium MA conditions can be achieved with the use of a film with intermediate permeability and careful consideration of the above mentioned factors for each commodity.

1.2 Microbiological quality and safety of MA stored produce

Fresh or refrigerated minimally processed vegetables normally contain epiphytic microorganisms that can cause spoilage (*e.g., Enterobacteriaceae, Pseudomonas* species, lactic acid bacteria) but they are considered harmless to the consumer (reviewed in reference 148). In addition to spoilage bacteria, these products may incidently harbor pathogens. Especially the ability of cold tolerant pathogens to grow during storage is considered a potential safety hazard. Several studies have demonstrated the presence of *Listeria monocytogenes* on minimally processed vegetables (30,167,170). Other psychrotrophic pathogens, such as *Aeromonas hydrophila* and *Yersinia enterocolitica* are a concern as well (18). Furthermore, mesophylic pathogens, such as *Salmonella typhimurium* and *Staphylococcus aureus* can proliferate at abuse temperatures (reviewed in reference 148). Also the possible growth of strictly anaerobic pathogens, such as *Clostridium botulinum*, cannot be ignored when a MA package is used that allows the total depletion of O_2.

MA storage has been reported to suppress the growth of spoilage bacteria, especially *Pseudomonas* species, several of which can cause soft rot (134), but detailed studies on the effects of gas atmospheres on the prevailing vegetable-associated microorganisms are limited. High CO_2 concentrations have also been shown to suppress the growth of pathogens in a range of packed, nonrespiring products and in laboratory media, but CO_2 concentrations that were applied exceeded the levels that are generally tolerated by vegetable produce. On minimally processed MA-stored vegetables that were stored under refrigerated conditions, however, the growth of *L. monocytogenes* was not suppressed as compared with ambient conditions (12,35).

Although refrigerated MA packaging prolongs the initial fresh state and the shelf life of the product, it must be noted that it cannot overcome hygienic abuses in the production or handling of a product. Both a good physiological and microbiological quality of the product at the onset of storage is therefore essential. To avoid the potential hazard of pathogens in practice, adequate measures should be applied to prevent contamination during cultivation and processing. In addition, extra safety factors may be required for the safe use of MA stored vegetables.

2. Biopreservation by the use of lactic acid bacteria

2.1 The antimicrobial traits of lactic acid bacteria

Lactic acid bacteria can inhibit or eliminate the growth of a wide range of microorganisms, including bacteria, yeasts and fungi, by the production of a variety of antimicrobial compounds, such as organic acids, diacetyl, hydrogen peroxide, enzymes, defective phages, lytic agents and antimicrobial peptides (bacteriocins) (23,131,162).

Since the addition of chemical preservatives to foods has fallen in disfavor with consumers, there has been a large interest in the application of natural antimicrobial agents in foods (86). In this respect, LAB and their antimicrobial compounds could play an important role as biopreservation agents to extend the storage life and safety of products, including MA packaged vegetables. An additional favorable characteristic of LAB is their GRAS (GENERALLY RECOGNIZED AS SAFE) status, which is mainly based on the fact that they have been used for centuries to produce stable foods (*e.g.* dairy, yogurt, sausages, sauerkraut) without negative effects on the health of consumers (196). In particular, bacteriocins of LAB have provoked a great deal of interest for their potential as non-

toxic preservatives (53). Their characteristics and potential application in food preservation will be addressed below.

2.2 Bacteriocins produced by lactic acid bacteria

2.2.a Definition and classification

Bacteriocins are antimicrobial peptides and proteins which are ribosomally synthesized by bacteria (175). Although bacteriocin research was initially focused on proteins which are produced by Gram-negative bacteria (125), a large number of chemically diverse bacteriocins of Gram-positive bacteria have recently been identified and characterized, particularly those produced by LAB (184). Biochemical and genetic studies have enabled the classification of the latter bacteriocins into the following classes (121):

I Lantibiotics. These are defined as small heat-stable membrane active peptides which contain posttranslationally modified amino acids (*i.e.* lanthionine, β-methyl lanthionine and dehydrated amino acids [dehydroalanine and/or dehydrobutyrine]).

II Small (< 5 kDa), heat-stable membrane active bacteriocins that are characterized by the absence of unusual amino acids and the presence of a double glycine processing site in the leader sequence of the precursor. Most of the bacteriocins of LAB that have been characterized to date belong to this class in which three major subclasses are distinguished:
IIa Bacteriocins with activity towards *Listeria monocytogenes*, containing a consensus sequence at the N-terminus: Tyr-Gly-Asn-Gly-Val-Xaa-Cys (Xaa is His, Thr, Ser or Tyr). The bacteriocins pediocin PA-1 and mundticin, which are investigated in this thesis, are members of this class.
IIb Bacteriocins that form pores which require two peptides for activity.
IIc Thiol-activated peptides that require reduced cysteines for activity.

III Large (> 30 kDa) heat-labile proteins bacteriocins.

A fourth class of bacteriocins has been proposed, consisting of complex bacteriocins that are composed of a protein plus one or more nonproteinaceous moieties (lipid, carbohydrates). However, these bacteriocins have not yet been characterized adequately at a biochemical level and the recognition of this class seems therefore premature to date (147).

Members of class I and II have been isolated and characterized most frequently, and have been intensively studied because they are prominent candidates for industrial application. These compounds are synthesized as inactive prepeptides, which are activated upon cleavage of the leader sequence. For a detailed description of the biochemical properties and the characterization of the genes involved in production, immunity, secretion and regulation of production of lantibiotics and class II bacteriocins, the reader is referred to several extensive reviews (51,104,111,121-123,147,188).

2.2.b Mode of action

Of the class I bacteriocins that are produced by LAB, nisin A is a prominent member of which the mode of action has been studied in detail (57,111). Although over 15 class II bacteriocins have been characterized at the genetic level, the mode of action has only been studied in detail for lactococcins A and B (182,185), mesenterocin Y105 (136), pediocin PA-1 (39), lactacin F (3), and plantaricin C (81).

The class I and II bacteriocins have a number of characteristics in common: a molecular mass of 3 to 6 kDa, a high isoelectric point, and an amphiphilic, hydrophobic nature. In addition, they share the capacity to permeate the cytoplasmic membranes of target cells by the formation of oligomeric pores (57,151,187). The formation of pores subsequently causes an efflux of small cytoplasmic molecules and ions from the target cells and a dissipation of the proton motive force, which eventually may lead to cell death (39,72,164).

The mode of action of class I and II bacteriocins differs in some aspects: whereas nisin is believed to inhibit the growth of sensitive organisms in a voltage-dependent way in absence of a protein receptor (57,72), pediocin PA-1, lactococcin A and B and plantaricin C were found to act in a voltage-independent and protein receptor-mediated way (39,81,96,145). More detailed information on the mode of action of the lantibiotics and class II bacteriocins can be found in recent reviews and books (2,111,187).

2.3 Application of bacteriocins and bacteriocin-producing strains

Several factors have contributed to extensive research aimed at the application of bacteriocinogenic strains and their bacteriocins to preserve foods. These include the consumers' resistance to, and concern about the safety of chemical preservatives, the realization that LAB are naturally occurring on a wide variety of foods, and the approval of the bacteriocin nisin A for use in certain pasteurized cheese spreads by the FDA. Nisin A is currently permitted in at least 50 countries for the inhibition of sporeforming bacteria (clostridia, bacilli) in cheeses and canned foods, and is the only bacteriocin within the EU with a full legal status as a food additive to date (E234). Its broad spectrum of activity towards Gram-positive bacteria (including pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum*), its heat stability at low pH, and nontoxic nature have promoted its use in food processing and fermentation (91,183).

A number of bacteriocins of LAB other than nisin also exhibit fairly broad spectra of inhibition, which might make them suitable candidates for improvement of food safety (40,157,194,198). Of special interest are the heat stable class **IIa** bacteriocins, which generally show antimicrobial activity towards *L. monocytogenes*, but may also inhibit the growth of other Gram-positive pathogens (121). For industrial applications, it is noteworthy that these compounds are usually active over a wide pH range, whereas nisin has the highest antimicrobial activity at a low pH. In addition, the production of these compounds does not involve posttranslational modifications, and consequently does not require complex biosynthesis pathways. Class IIa bacteriocins have now been reported to be produced by a wide variety of LAB including *Pediococcus* (41,105,139,146), *Lactobacillus* (64,102,128,179), *Carnobacterium* (20,109,158), *Enterococcus* (8, AND THIS THESIS), and *Leuconostoc* (94,95).

The inhibitory spectra of most other LAB bacteriocins than those mentioned above are limited to bacteria that are closely related to the producer. Rather than for biopreservation, strains that produce such narrow spectrum bacteriocins are interesting from a food quality point of view, since they can be used as starter cultures in fermentations. Several of these narrow spectrum bacteriocinogenic LAB have been demonstrated to inhibit the growth of competing spoilage bacteria more effectively as compared with their nonproducing counterparts (53,93,188,190). Applications of bacteriocins in foods have been reviewed frequently (53,89,162,172).

It has become evident that the production of a certain bacteriocin is not necessarily linked to one species or restricted to organisms sharing the same environment. This is exemplified by the characterization of a number of identical bacteriocins that are produced by different strains which were isolated from a variety of food products: pediocin PA-1, AcH, JD and Bac by *Pediococcus acidilactici*, *Lactobacillus plantarum* and *P. parvulus* (41,64,105,139,146, AND THIS THESIS); sakacin 674, sakacin P and bavaricin A by *L. sake* and *L. bavaricus* (102,128,179); or curvacin A and sakacin A by *L. curvatus* and *L. sake* (102,178). In addition, the production of different bacteriocins by a single organism has been reported for several LAB (20,158,181). Evidently, the application of LAB bacteriocins as preservatives in foods requires a full characterization of the compounds that are produced.

2.3.a Modes of application

The application of bacteriocins in food systems can be achieved by three basic methods:

I **The use of a pure culture of the viable bacteriocin-producing LAB.** This offers an indirect way to incorporate bacteriocins in a food product. The success of this method depends on the ability of the LAB to grow and produce the bacteriocin to the desired extent in the food under the prevailing environmental conditions (*e.g.*, composition of the food, temperature, pH, gas atmosphere)

II **Application of a crude bacteriocin preparation obtained by growing the bacteriocin producing LAB on a complex, natural substrate (*e.g.* milk).** This method provides a bacteriocin preparation of known and constant activity and is currently employed for the production of nisin preparations on an industrial scale.

III **The use of a (semi-)purified preparation of the bacteriocin.** Here, the dosage of the bacteriocin is accurate and its effect is therefore more predictable. However, the application of this method is limited as a result of national regulations on food additives.

2.3.b Considerations for application

The success of the application of a bacteriocin or its producing strain to inhibit the growth of pathogenic or spoilage bacteria in foods depends on multiple factors. Firstly, as with all other preservative compounds, target bacteria may possess a certain degree of tolerance or resistance to bacteriocins or develop this upon prolonged exposure (47,142). Both the cell wall constitution and the membrane lipid composition have been demonstrated to be involved in acquired bacteriocin resistance (48,137,142). Secondly, the inhibitory action of these antimicrobial peptides is only di-

rected towards Gram-positive bacteria and can vary considerably between different genera, species of genera, identical species, and even for identical cultures under different environmental conditions (4,93,121). The exact factors that account for the naturally occurring variability in bacteriocin susceptibility have yet to be clarified. Thirdly, there are intrinsic factors in foods, such as pH and temperature, that can influence both the bacteriocin production and the activity of bacteriocins (115). The effectiveness of bacteriocins can furthermore be compromised by proteolytic cleavage, and by adsorption of these hydrophobic compounds to fats and phospholipids in the food matrix. Finally, when using viable cultures, the primary requirement is that optimal growth and bacteriocin production take place under the prevailing processing or storage conditions. The size of the inoculum applied may determine whether or not the bacteriocinogenic strain can outgrow the competing microflora, but may also be important in the view that bacteriocin production can be an autoregulated process (122).

Given the above concerns, adequate control of the growth of both pathogens and competing microflora requires extensive knowledge of factors that determine susceptibility and resistance of Gram-positive bacteria towards bacteriocins.

3. Outline of this thesis

This dissertation comprises studies that deal with the microbiological quality and safety of minimally processed vegetables which are stored under refrigerated modified atmosphere conditions, and the use of bacteriocinogenic LAB cultures and their bacteriocins as potential safety measures.

Chapter 1 gives a short introduction to MA packaging of vegetables and the general microbiological characteristics of this type of products. It furthermore describes the possibility to introduce LAB and their antimicrobial peptides as an additional barrier to the growth of pathogens.

Chapter 2 describes the composition of the spoilage microflora on produce during MA storage. Representatives of the predominant spoilage bacteria were evaluated for their growth under various constant gas atmospheres in an agar model system.

In **Chapter 3**, we investigated the influence of different oxygen and carbon dioxide concentrations on the growth of psychrotrophic pathogens that may be present on minimally processed vegetables.

Chapter 4 describes the isolation of vegetable-associated bacteriocinogenic LAB. The broad spectrum bacteriocins produced by two strains of *Pediococcus parvulus* were further characterized by both genetic and biochemical techniques.

The identification and characterization of a novel broad spectrum bacteriocin from *Enterococcus mundtii* is the subject of **Chapter 5**. Having established its membrane active bactericidal action, we performed further structural analysis on this bacteriocin, and related peptides.

To obtain better insight into the natural variability in the susceptibility of closely related Gram-positive bacteria towards bacteriocins, we selected two sets of strains with 100 to 1000 fold differences in sensitivity towards bacteriocins, and studied the effect of bacteriocins on physiological responses of whole cells in conjunction with their membrane lipid properties (**Chapter 6**).

In **Chapter 7**, we have investigated the use of *P. parvulus* and *E. mundtii* and their bacteriocins as biopreservation agents to control the growth of *Listeria monocytogenes* in a vegetable model system, and on minimally processed vegetables that were stored under MA conditions.

CHAPTER 2

The influence of oxygen and carbon dioxide on the growth of prevalent *Enterobacteriaceae* and *Pseudomonas* species isolated from fresh and modified atmosphere stored vegetables

Marjon H. J. Bennik, Werner Vorstman, Eddy J. Smid, Leon G.M. Gorris
Food Microbiology, submitted for publication

ABSTRACT

To obtain more insight into the specific impact of Modified Atmosphere (MA) packaging on the composition of the epiphytic microflora of minimally processed vegetables, we determined the prevalent bacteria on cut chicory endive and mungbean sprouts before and after storage under constant MA conditions at 8°C. On both fresh and MA stored mung bean sprouts, *Enterobacter cloacea, Pantoea agglomerans, Pseudomonas fluorescens, Ps. viridilivida* and *Ps. corrugata* constituted the main species. By contrast, we observed a shift from *Rahnella aquatilis* and several *Pseudomonas* species towards *Escherichia vulneris* and *Ps. fluorescens* on chicory endive before and after MA storage. For the prevalent epiphytes, an agar model system was used to quantify the growth in pure culture at 8°C under 1.5 or 21% O_2 in combination with 0, 5, 20, or 50% CO_2. In general, the various MA conditions tested had no strong influence on maximum population densities, while lag times were not detected. For each of the strains, however, maximum specific growth rates were reduced at increased CO_2 concentrations, independent of the O_2 concentration applied. This effect was more pronounced for *Pseudomonas* species than for *Enterobacteriaceae*. Notably, the agar model study showed that individual species of *Pseudomonas* or *Enterobacteriaceae* responded similarly to specific MA conditions applied, which did not correlate with the shift in the predominent species observed on chicory endive. The data obtained underline the complexity of the ecological conditions to which microorganisms on MA-packaged vegetables are subjected. Our results substantiate the merely partial suppression of the microflora under MA conditions optimal for minimally processed vegetable products. A possible implication for food safety is discussed.

INTRODUCTION

The shelf life of minimally processed vegetables can be prolonged by refrigerated storage under modified atmospheres (MA). These products harbor large and diverse populations of microorganisms, including potential spoilage organisms, that may rapidly multiply during storage (148).

While nonrespiring products can be exposed to very high CO_2 concentrations and/or anaerobic conditions to suppress the growth of undesirable organisms (67), fresh vegetables require the presence of O_2 and do not tolerate high levels of CO_2 to maintain their metabolic functions (153,154). Under typical MA storage conditions, *e.g.* 1 to 5% O_2 in combination with 5 to 10% CO_2 (112), the extended shelf life of vegetables is thought to result from a lowered product respiration and inhibition of the growth of spoilage microorganisms (46,67). The inhibitory effect of CO_2 on the growth of bacteria is believed to result from diffusion of H_2CO_3 across the bacterial membrane, thereby causing intracellular changes in pH which may affect enzymes that are involved in metabolic routes in the cell (56,195). This effect is stronger at low temperatures because of its better solubility at decreased temperature (34).

Generally, the microflora on vegetables primarily consists of epiphytic strains of *Enterobacteriaceae* and *Pseudomonas* species, whereas lactic acid bacteria (LAB) and fungi may be present at relatively lower numbers (148). Occasionally, pathogens can occur due to the use of contaminated irrigation water or organic fertilizer during cultivation or as a consequence of insufficient hygiene during processing (18,148). A suppression of the outgrowth of pathogens by isolates from the epiphytic microflora on minimally processed vegetables was previously observed and indicates that these epiphytes may play a role in assuring the safety of these nonsterile products (12,36).

Whereas MA packaging can cause specific changes in the composition of the microflora due to changes in the gas atmosphere, characteristics of this microflora on produce are generally monitored only for major groups of microorganisms. Information on the specific effect of MA conditions on the individual microorganisms residing on produce is insufficient to address a possible link to product safety. To gain more insight in this matter, we identified the prevailing epiphytic microflora before and after MA storage of minimally processed vegetables, using chicory endive and mungbean sprouts as representative products. Subsequently, representatives of the predominant *Enterobacteriaceae* and *Pseudomonas* strains were individually evaluated for their growth under various constant gas atmospheres in a previously described agar model system (14) using conditions relevant to MA packaged vegetables.

MATERIALS AND METHODS

Products and storage. Chicory endive (*Cichorium intybus* L., *var. foliosum*) was purchased from a local retailer and, after removal of the outer leaves and the kernel, aseptically cut in 1 cm pieces across the midrib. Mungbean sprouts (*Vigna radiata* (L.) Wilczek) were obtained directly from a local grower. The vegetables were incubated under controlled gas atmospheres at 8°C, thereby mimicking equilibrium gas conditions inside packages of vegetables (1.5% O_2, 20% CO_2, 78.5% N_2). Controls for storage under ambient conditions were taken along by storing both products under 21% O_2, 0% CO_2, 78% N_2. The gas concentrations were set and controlled as previously described (14).

Isolation and enumeration of micro-organisms. Duplicate samples (25 g) of each of the products were taken at day 0, 1, 3, 6, 9 and 13 and diluted with 0.85% NaCl supplemented with 225 ml of 0.1% (w/v) peptone (peptone salt solution). After blending in a Stomacher Lab-blender (Seward Laboratory, London) for 1 min, dilutions were plated in duplicate onto appropriate media for the enumeration of different groups of microorganisms. The media and culturing conditions used were as follows: mesophilic aerobes, Plate Count agar (PCA) (Oxoid, Basingstoke, England) incubated for 3 d at 30°C; *Pseudomonas* species, *Pseudomonas* agar base (Oxoid) containing cephaloridine-fucidin-cetrimide (CFC agar) (Oxoid), incubated for 2 d at 30°C; coliform bacteria, violet red bile glucose (VRBG) agar (Oxoid) incubated at 30°C for 1 d; lactic acid bacteria, Rogosa agar (Merck, Darmstadt, Germany) incubated under an O_2-

depleted/CO_2-enriched atmosphere in gas tight jars with Anaerocult C pouches (Merck) at 30°C for 3 to 5 d.

Selection and identification of micro-organisms. Twentyfive colonies were randomly isolated from the greatest dilution of VRBG and CFC agar plates after 0 and 7 days of storage of chicory and mungbean sprouts (under 1.5% O_2, 20% CO_2, 78.5% N_2). Subsequently, colonies were purified by repeated subculture on PCA. Samples from plates incubated for 24 h at 30°C were used for Gram stains, oxidase test by the use oxidase strips (Merck), catalase production, and oxidative or fermentative utilization of glucose (144). Members of the *Enterobacteriaceae*, which were isolated from VRBG, were further identified by using the Biolog GN MicroPlates system (Biolog Inc., Hayward, CA). *Pseudomonads* were grouped according to their capability of arginine hydrolysis, production of exopigment, and nitrate reduction (144), and subsequently identified by the Biolog GN system.

Culturing conditions of selected strains and inoculation of plates. Purified strains were stored at -80°C in brain heart infusion broth (BHI) (Oxoid) containing 20% glycerol. Prior to use, *Enterobacteriaceae* and *Pseudomonas* isolates were cultured in BHI broth at 30°C for 24 hours, and subsequently subcultured for another 16 hours, using 0.1% inocula. The agar plates to be incubated under modified atmospheres were inoculated with early-stationary-phase cultures, which were diluted in peptone salt to approximately 5.5 x 10^6 colony forming units (cfu) per ml. 50 µl samples of diluted culture were surface-spread onto 60 mm diameter Petri dishes, containing 9 ml of BHI agar to give an estimated initial populations of bacteria of 1 x 10^4 cfu.cm^{-2}. Unless stated otherwise, the medium was buffered at pH 7.2 with phosphate buffer (0.1M), using equimolar amounts of sodium and potassium phosphate (Na/K P_i). Control experiments for the acidifying effect of 50% CO_2 were carried out with buffered medium adjusted to pH 6.7 (14).

Storage and analysis of inoculated agar plates under modified gas phase atmospheres. Plates inoculated with pure cultures were stored at 8°C as described previously (14). In short, inoculated agar plates were incubated in flasks that were continuously flushed (flow rate = 200 ml.min^{-1}) with 1.5 or 21% O_2, combined with 0, 5, 20, or 50% CO_2 at 8°C. The remainder of each atmosphere was made up to 100% with N_2. At day 1, 2, 4, 7, 9 and 13 the last flask of a series was disconnected from the flow through system, which enabled plates to be removed individually, without disturbing the gas conditions of the remaining plates. Viable counts on the surface of the agar were determined in duplicate and expressed as cfu.cm^{-2}, using BHI medium for *Enterobacteriaceae* and *Pseudomonas* species.

Data handling. Bacterial growth data were fitted by the Dmodel (IFR inhouse model, provided by Józef Baranyi, Institute of Food Research, Reading, England) to render the lag time (λ), the maximum population density (MPD), the maximum specific growth rate (μ_m) and the standard errors (se) of the estimates (10).

RESULTS

Microbial populations during modified atmosphere storage. Fresh mungbean sprouts had a high initial microbial load of approximately 1 x 10^7 cfu.g^{-1} on PCA. These numbers increased to approximately 1 x 10^9 cfu.g^{-1} during MA storage for 9 days at 8°C. The microflora on this product primarily consisted of *Enterobacteriaceae* and *Pseudomonas* species, with LAB being present at an approximate 1000-fold lower number. A 3 day lag phase was apparent for the LAB, and despite a 50% higher maximum specific growth rate of this population as compared with the *Enterobacteriaceae* and *Pseudomonas*, their numbers remained 3 log units lower.

The initial microflora on fresh cut chicory endive was approximately 1 x 10^4 cfu.g^{-1} on PCA, and reached a maximum population density of approximately 1 x 10^8 cfu.g^{-1} after 9 days of storage. The microflora on chicory mainly consisted of *Enterobacteriaceae*, *Pseudomonas* species and

Table 1. Predominant *Enterobacteriaceae* and *Pseudomonas* species on chicory endive and mungbean sprouts before and after storage under 1.5% O_2 / 20% CO_2 / 78.5% N_2 at 8°C.

	Enterobacteriaceae				*Pseudomonas*			
	Day 0		Day 7[a]		Day 0		Day 7[a]	
Product	Organism	NR	Organism	NR	Organism	NR	Organism	NR
Chicory endive	*Rahnella aquatilis*	17	*Escherichia vulneris*	11	*Pseudomonas viridiflava*	5	*Pseudomonas fluorescens*	12
	Pantoea agglomerans	2	*Pantoea agglomerans*	5	*Pseudomonas corrugata*	4	*Pseudomonas corrugata*	3
	Serratia odorifera	2	*Rahnella aquatilis*	2	*Pseudomonas viridilivida*[c]	4	*Pseudomonas putida*	2
	Escherichia vulneris	1	*Enterobacter intermedius*	2	*Pseudomonas cichorii*	3		
	Klebsiella oxytoca	1	*Kluyvera cryocrescens*	2	*Pseudomonas marginalis*	2		
	Enterobacter cloacae	1	*Serratia proteamaculans*	1	*Pseudomonas fulva*	1		
			Buttiauxella agrestis	1				
	ni[b]	1	ni	1	ni	6	ni	8
Mungbean sprouts	*Enterobacter cloacae*	9	*Pantoea agglomerans*	9	*Pseudomonas fluorescens*	8	*Pseudomonas fluorescens*	7
	Pantoea agglomerans	6	*Enterobacter cloacae*	9	*Pseudomonas viridilivida*[c]	6	*Pseudomonas corrugata*	6
	Erwinia amylovora	4	*Rahnella aquatilis*	1	*Pseudomonas corrugata*	3	*Pseudomonas viridilivida*[c]	4
	Erwinia carotovora	1	*Erwinia amylovora*	1	*Pseudomonas synxantha*	1	*Pseudomonas cichorii*	1
			Buttiauxella agrestis	1	*Pseudomonas marginalis*	1		
					Pseudomonas putida	1		
	ni	5	ni	4	ni	5	ni	7

[a] Storage for 7 days at 8°C under 1.5% O_2, 20% CO_2, 78.5% N_2.

[b] Not identified.

[c] The observed phenotype and metabolic patterns of these *Pseudomonas* isolates are characteristic for *Ps. viridilivida*, which is a strain with an uncertain taxonomic position (Dr. Janssens, Laboratorium voor Microbiologie (LGM), Gent, Belgium, pers. comm.; reference 118).

The reference *Ps. viridilivida* strain in the Biolog data base is known as a plant pathogen (Ms. Abraham, Biolog Inc. Hayward, CA., pers. comm.).

LAB, with the former two groups dominating after storage under the selected MA condition. The growth rates of the entire populations of *Enterobacteriaceae* and *Pseudomonas* species on this product were approximately 2.5 fold higher than on mung bean sprouts.

Both products had a good appearance after 9 to 13 days of storage at 8°C under 1.5% O_2/20% CO_2/78.5% N_2 and off-odors were absent. For the control samples, which were stored under ambient gas conditions, we observed 50% higher maximum specific growth rates of the *Enterobacteriaceae* and *Pseudomonas* populations. These products were not suitable for consumption after 6 days, as a result of brown discoloration and soft rot.

Identification of Gram-negative bacteria on fresh and MA stored vegetables. For mungbean sprouts, both before and after storage under MA, the predominant bacteria detected on VRBG agar were *Enterobacter cloacea* and *Pantoea agglomerans* while the bacteria prevailing on CFC agar were identified as *Ps. fluorescens*, *Ps. viridilivida* and *Ps. corrugata* **(Table 1)**. For fresh chicory endive, the prevalent bacterium detected on VRBG agar was *Rahnella aquatilis*. MA storage induced a shift in the *Enterobacteriaceae* population towards primarily *Escherichia vulneris*. Microorganisms isolated from this product by the use of CFC agar represented different species of *Pseudomonas* species, whereas *Ps. fluorescens* was found as the predominant strain after MA storage **(Table 1)**.

Overall, 20% of the isolates were not identified with the Biolog GN identification system, which is 10% higher than previously reported for clinical isolates and reference taxa (87,103,124). The unidentified *Enterobacteriaceae* had similarity scores <0.500 at 24 h of incubation, which were too low for characterization. Unidentified isolates from CFC agar were either atypical *Pseudomonas* species (based on preliminary testing of arginine hydrolysis, production of exopigment, and nitrate reduction) or had similarity scores <0.500 (24 h incubation) in the Biolog GN system.

Influence of gas atmospheres on vegetable-associated isolates. Isolates of the predominant *Enterobacteriaceae* and *Pseudomonas* species which were present on the two products before and after MA storage were individually evaluated for their growth on an agar surface under 1.5 or 21% O_2, combined with 0, 5, 20, or 50% CO_2. Lag times were not observed for any of the selected strains under the different test conditions. In addition, we found no significant differences for growth under 1.5% O_2 as compared with 21% O_2.

The predominant *Enterobacteriaceae* on cut chicory endive before and after MA storage, *i.e. Rahnella aquatilis* and *Escherichia vulneris*, showed similar growth patterns under the different CO_2 concentrations tested **(Figure 2A)**. On mungbean sprouts, we observed comparable growth patterns for the two strains of *P. agglomerans* which were isolated from fresh and MA stored product **(Figure 1A)**. Although *E. cloacea* was identified as one of the major species on the latter product both before and after MA storage, this isolate grew poorly at 8°C. Its μ_m was approximately fivefold lower under 0, 5 and 20% CO_2 as compared with the other *Enterobacteriaceae* under study, and growth was completely absent under 50% CO_2. In addition, its maximum population density on agar plates did not exceed 7.9×10^5 cfu.cm^{-2}. The other *E. cloacea* isolates from mungbean sprouts were found to have equal growth characteristics (data not shown).

For each of the selected psychrotrophic *Enterobacteriaceae*, a near linear relationship between the CO_2 concentration and the μ_m was found. In the absence of CO_2, the μ_m of these isolates ranged between 0.13 and 0.16 h^{-1} at 8°C whereas their rates were 50 to 60% reduced under 50% CO_2 **(Figure 1A and 2A)**. Starting from initial population densities of approximately 1×10^4

cfu.cm^{-2}, maximum population densities (MPDs) of 8 x 10^8, 2 x 10^9 and 2 x 10^9 cfu.cm^{-2} were reached by *Pantoea agglomerans*, *Rahnella aquatilis* and *Escherichia vulneris*, respectively, under 0% CO_2. These final levels were unaffected by CO_2 concentrations up to 20%, and approximately 0.5 log units reduced under 50% CO_2.

The inhibitory effect of CO_2 on the growth of *Pseudomonas* species was more pronounced than on the *Enterobacteriaceae*. The relationship between the μ_m and the CO_2 concentration appeared nonlinear for the *Pseudomonas* isolates: starting from μ_m's ranging from 0.13 to 0.17 h^{-1} in absence of CO_2, a 60 to 75% reduction was observed under 20% CO_2. The rates were reduced by 80 to 90% under 50% CO_2 **(Figure 1B and 2B)**. The MPDs of *Ps. corrugata*, *Ps. fluorescens* (both isolates) and *Ps. viridiflava* increased from initial levels of approximately 1 x 10^4 cfu.cm^{-2} to 1.6 x 10^9, 1 x

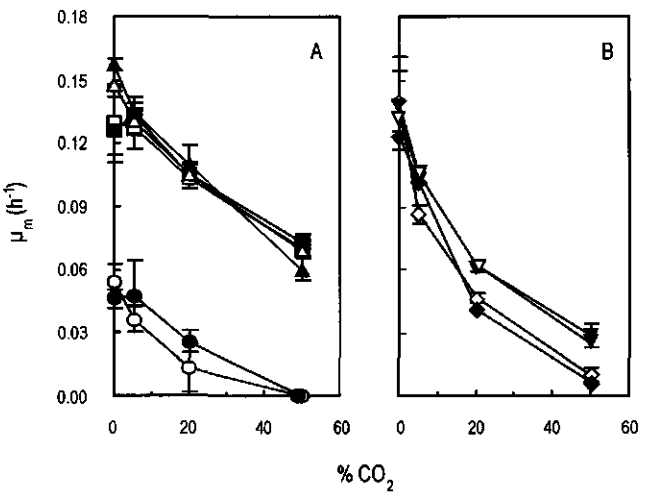

Figure 1.

Influence of CO_2 concentration on the maximum specific growth rates (μ_m) of isolates from mungbean sprouts as determined on BHIA (pH 7.2) under 1.5% O_2 (open symbols) and 21% O_2 (closed symbols).
(A) Predominant *Enterobacteriaceae* isolated from fresh product: (●○) *Enterobacter cloacea* and (▲△) *Pantoea agglomerans*; or from fresh product: (■□) *Pantoea agglomerans*.
(B) Predominant *Pseudomonas* isolated from fresh product: (◆◇) *Pseudomonas fluorescens*; or from MA stored product: (▼▽) *Pseudomonas corrugata*.
The error bars represent the standard errors of the estimates.

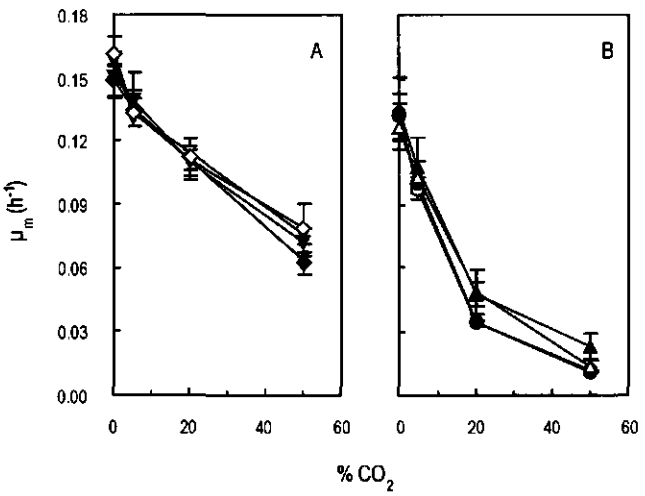

Figure 2.

Influence of CO_2 concentration on the maximum specific growth rates (μ_m) of isolates from chicory endive as determined on BHIA (pH 7.2) under 1.5% O_2 (open symbols) and 21% O_2 (closed symbols).
(A) Predominant *Enterobacteriaceae* isolated from fresh product: (▼▽) *Rahnella aquatilis*; or from fresh product: (◆◇) *Escherichia vulneris*.
(B) Predominant *Pseudomonas* isolated from fresh product: (●○) *Pseudomonas viridiflava*; or from MA stored product: (▲△) *Pseudomonas fluorescens*.
The error bars represent the standard errors of the estimates.

10^9 and 1.6 x 10^9 cfu.cm^{-2}, respectively, under 0% CO_2. Whereas the MPDs of the individual *Pseudomonas* species were essentially unaffected by CO_2 concentrations up to 20% (less than 0.5 log units lower under 20% CO_2 than under 0% CO_2), the MPDs were significantly lower under 50% CO_2, with maxima of 5 x 10^6, 1 x 10^6 and 5 x 10^4 cfu.cm^{-2} for *Ps. corrugata*, *Ps. fluorescens* (both isolates) and *Ps. viridiflava*, respectively.

The growth of the *Enterobacteriaceae* and *Pseudomonas* isolates was unaffected by the pH change of the medium from 7.2 to 6.7 (data not shown), indicating that the inhibitory effect of 50% CO_2 is not the result of the CO_2-induced reduction in the pH of the medium.

DISCUSSION

Although the presence of *Enterobacteriaceae* and *Pseudomonas* species on fresh and modified atmosphere (MA) stored vegetables is well established, only few studies on the composition of the prevalent microflora have detailed the specific species within these populations. In addition, information on the responses of the individual predominant strains to modified gas atmospheres is limited. Such information would be relevant to assess the impact of the growth of the individual species on the survival and growth of pathogens that occasionally occur on minimally processed vegetables. In this view, we identified *Enterobacteriaceae* and *Pseudomonas* species that prevailed on cut chicory endive and mungbean sprouts before and after storage under MA at a low temperature. Subsequently, the growth of the predominant isolates was evaluated in an agar model system under different O_2 and CO_2 concentrations at the same temperature (14). We demonstrated that the *Enterobacteriaceae* and *Pseudomonas* isolates had reduced maximum specific growth rates at increasing CO_2 concentrations, however, this effect was more pronounced for the *Pseudomonas* species. Of specific interest is our observation that the growth of the individual psychrotrophic species within each of the populations responded in a highly similar way to different CO_2 concentrations in the model system, whereas differences between the tested O_2 concentrations were absent. This accounted for isolates of both chicory endive and mungbean sprouts.

The vegetables investigated contained high numbers of *Enterobacteriaceae* and *Pseudomonas* species, including strains which are known as phytopathogenic bacteria that can cause rot in vegetables due to the production of pectate lyase (PL), such as *Ps. chicorii*, *Ps. viridiflava*, *Ps. marginalis*, *Ps. fluorescens*, *Erwinia carotovora* and *E. amylovora* (71,130,134). Despite the presence of these organisms, visible spoilage (*e.g.* maceration, soft-rot, discoloration) was absent after storage. This observation is in line with previous studies that suggested that the beneficial effect of MA storage of vegetables is for an important part the result of the physiological state of the product rather than the inhibition of soft-rot bacteria (134).

The observed high microbial load on mungbean sprouts is consistent with previously reported aerobic plate counts (1,6). These high numbers are generally not related to unhygienic conditions, but to the growth of the microflora during the germination of the beans, which typically takes place in a moist, warm (21 to 27°C) environment under 10% O_2/10% CO_2/80% N_2 (156,171). Whereas mungbean sprouts essentially harbored the same species of bacteria before and after MA storage, we observed a marked shift in both *Enterobacteriaceae* and *Pseudomonas* species on chicory endive.

On mungbean sprouts, the similar sensitivity of the various *Enterobacteriaceae* or *Pseudomonas* isolates towards CO_2 in our model system may explain the minor changes in the predominant strains before and after MA storage. For chicory endive, however, the observed shift from *R. aquatilis* to *E. vulneris* and from a mixed *Pseudomonas* population to *Ps. fluorescens* cannot be attributed to differences in CO_2 sensitivity as these strains were found to exhibit the same growth responses under the specified gas conditions. The outgrowth of these specific species is likely to be related to other factors than the gas concentrations that are suitable for vegetables. In this respect, one should consider the interactions between the different microorganisms (12,36), their specific nutritional requirements, the physiological state of the product and the nutrient availability (192). A nutritional limitation for the total bacterial populations on both products was indicated by the two to four-fold higher maximum specific growth rates of the individual organisms under identical gas conditions in the agar model system (data not shown). The twofold higher growth rates of the *Enterobacteriaceae* and *Pseudomonas* populations on cut chicory endive as compared with mungbean sprouts likely resulted from a higher nutrient availability on cut surfaces and from a lower competition on chicory endive that contained a 1000-fold lower initial bacterial load.

Whereas information on the effect of CO_2 on *Enterobacteriaceae* is limited, it is well known that *Pseudomonas* species are sensitive to CO_2 (61,63,79,90). The stronger inhibitory effect of CO_2 on pure cultures of *Pseudomonas* as compared with *Enterobacteriaceae* was mainly reflected by a more pronounced CO_2-induced decrease of their maximum specific growth rates. The observed 80 to 90% reduction in the maximum specific growth rates under 50% CO_2 at 8°C of the strains investigated, *i.e. Ps. viridiflava, Ps. corrugata* and *Ps. fluorescens*, agrees with a previously reported 90% relative inhibition of the growth rate of *Ps. fragi* under 50% CO_2 at 5°C (62). We furthermore observed a reduction of the maximum population densities for only the *Pseudomonas* species under 50% CO_2, while lag times were not detected. These findings are consistent with the effects of CO_2 on *Ps. fluorescens* as reported by Eyles et al. (65).

Under the MA conditions tested, the maximum specific growth rates of the the selected *Enterobacteriaceae* strains resembled those of a number of psychrotrophic pathogens as previously determined in our model system (14), whereas the maximum specific growth rates of *Pseudomonas* species were 20 to 60% lower. In conclusion, MA storage can cause a selective suppression of the outgrowth of different epiphytic populations. Since competition between epiphytes and pathogens may retard the outgrowth of pathogens on minimally processed vegetables (36), the inhibitory effect of MA storage on a subgroup of the epiphytes, namely the *Pseudomonas* species, and not on the psychrotrophic pathogens may bring this safety feature in unbalance. The complexity of the ecological conditions to which epiphytes and pathogens are subjected urges the need for more systematic studies to ensure the safety of minimally processed vegetables stored under MA conditions.

ACKNOWLEDGEMENTS

WE THANK IRENE POL, SIMONE OOLHORST AND MARC WALPOT FOR THEIR CAPABLE TECHNICAL ASSISTANCE, AND JÓSZEF BARANYI FOR INSTRUCTING THE USE OF THE DMODEL.

CHAPTER 3

Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen

Marjon H.J. Bennik, Eddy J. Smid, Frank M. Rombouts, and Leon G.M. Gorris
Food Microbiology (1995) 12: 509-519

ABSTRACT

A solid surface model system was developed to study the effect of gas atmosphere composition on the growth of *Aeromonas hydrophila, Yersinia enterocolitica, Listeria monocytogenes,* and a cold tolerant strain of *Bacillus cereus.* The organisms were incubated on an agar surface at 8°C under either 1.5 or 21% O_2, combined with 0, 5, 20 or 50% CO_2. The remainder of each atmosphere was made up to 100% with N_2. Growth was evaluated on the basis of three parameters, namely maximum specific growth rate, maximum population density and lag time. In all instances the maximum specific growth rate decreased significantly with increasing CO_2 concentration. Prolonged lag times were observed only for *Y. enterocolitica* under 50% CO_2 / 21% O_2 / 29% N_2, while a strong reduction in the maximum population density was noted only for *B. cereus* at the highest level of CO_2. The O_2 concentrations tested did not significantly affect maximum specific growth rates nor maximum population densities in any case. The results indicate that the model system may be a suitable means of estimating the growth of bacteria on minimally processed produce, packaged under modified atmospheres. Extrapolation of our results to modified atmosphere packaged fruits and vegetables using typical O_2 concentrations of 1 to 5% and CO_2 concentrations of 5 to 10%, suggest that growth of the above pathogens may occur at 8°C, thereby imposing a safety hazard for these products.

INTRODUCTION

Modified atmosphere (MA) packaging in combination with refrigeration is increasingly used to extend the storage life of minimally processed fresh (MPF) fruits and vegetables. The low oxygen (O_2) concentrations employed in MA packaging (1 to 5%) have been found to reduce oxidative processes as well as produce respiration and ripening (76,112,113). The presence of O_2 at a minimum concentration of 1 to 2% is thought to be required to prevent anoxic conditions that cause anaerobic metabolism of the product, thereby leading to the formation of off-odors (112). These minimum O_2 levels furthermore prevent the growth of obligatory anaerobes such as *Clostridium botulinum.* Typical carbon dioxide (CO_2) concentrations for MA packaging of fruits and vegetables are 5 to 10% (112). These relatively high CO_2 levels suppress the respiration of produce, and moreover seem to inhibit a number of common food spoilage microorganisms (46,67).

The microbiology of MPF fruits and vegetables has been reviewed extensively (148). Hazardous situations may arise with products that are organoleptically acceptable, *i.e.* harbor relatively low numbers of spoilage organisms, but contain cold tolerant pathogens (99,148). Although outbreaks of foodborne diseases with MA stored vegetables are scarce (170), the potential risk of such events may increase, due to increased consumption of fresh and minimally processed foods.

A number of studies have indicated that the storage of foods at refrigeration temperatures under MA conditions may select for the growth of psychrotrophic pathogens (25,99). In this respect, the proliferation and survival of bacteria such as *Aeromonas hydrophila, Yersinia enterocolitica* and *Listeria monocytogenes* are of particular concern (17,19,116,180). With regard to the prevailing gas concentrations during MA packaging of vegetables, it is known that especially elevated CO_2 concentrations can exert an antimicrobial action. In general, CO_2 causes a decrease in the pH of media, an effect that increases at lower temperatures when the solubility of the gas is enhanced (34). Its inhibitory effect on microorganisms is thought to result mainly from diffusion of H_2CO_3 across the bacterial membrane, causing intracellular changes in pH (195). Such intracellular pH changes may affect enzymes that are involved in metabolic routes in the cell. Furthermore, elevated CO_2 concentrations may inhibit decarboxylation reactions, in which CO_2 is released by feedback mechanisms (56). The direct inhibition of enzymatic processes in bacteria by CO_2 has been demonstrated previously (79,120).

The growth characteristics of pathogens under the influence of CO_2 have been found to depend on the microorganism, the presence of endogenous microorganisms, the age and load of the initial bacterial population, the incubation temperature, the type of food product, and the CO_2 concentration applied (56,107,150). Since many studies on the antimicrobial effect of CO_2 have been carried out with packaged products, CO_2 concentrations may not always be stable as a result of gas diffusion through the packaging material, the respiratory activity of the product inside the package and dissolving and binding of CO_2 in the food matrix (78,99,199). Many studies on the effect of CO_2 on the growth of pure bacterial cultures have been performed in liquid laboratory media to avoid the above mentioned variations (9,60,61,79,90,120,143). Only a few studies report the growth of pure cultures under modified atmospheres on solid surfaces of laboratory media (65,80). Considering that many foods are mainly contaminated at the surface, the latter approach is probably a more accurate reflection of the practical situation.

The aim of the present study was to develop a model test system to investigate the growth of pure cultures of psychrotrophic foodborne pathogens on a solid surface under constant gas phase conditions, and to design an objective assessment of their growth parameters.

MATERIALS AND METHODS

Microorganisms. Two Gram-negative and two Gram-positive psychrotrophic foodborne pathogens were studied. *Aeromonas hydrophila* DSM 30187 and *Yersinia enterocolitica* DSM 4780 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Listeria monocytogenes* Scott A was obtained from the Culture Collection of the Department of Food Science of the Agricultural University of Wageningen (The Netherlands). A psychrotrophic strain of *Bacillus cereus* F4626/90 (= IFR-NL94-25), isolated from milk by Dr. R. Gilbert (PHLS, Colindale, UK), was kindly provided by Dr. M. Peck (IFRN, Norwich, UK).

Media and cultural conditions. All cultures were stored at -80°C in brain heart infusion broth (BHI, Oxoid, UK) supplemented with 20% glycerol. Bacteria were cultivated at 30°C in BHI broth for 24 hours, and subsequently subcultured for another 16 hours, using 0.1% inocula. Cultures in stationary phase were diluted in 0.85% NaCl supplemented with 0.1% (w/v) peptone, to give approximately 5.5 x 10^6 colony forming units (cfu) per ml. Fifty µl samples of diluted culture were surface spread onto 60 mm diameter Petri dishes, containing 9 ml of brain heart infusion agar (BHIA). Initial populations of bacteria were about 10^4 cfu.cm^{-2}. Unless stated otherwise, the medium was buffered at pH 7.2 with phosphate buffer (0.1M), using equimolar amounts of sodium and potassium phosphate (Na/K P_i). A number of experiments were also performed using buffered BHIA adjusted to pH 6.7. Bacterial growth on the agar surface was examined under eight modified gas atmosphere compositions, *i.e.* 1.5 or 21% O_2, combined with 0, 5, 20, or 50% CO_2. The remainder of each atmosphere was made up to 100% with N_2.

Buffered media were used to minimize acidification caused by CO_2 dissolving in the medium. The effect of buffer strenght upon growth was examined at 30°C in BHI broth without additional phosphate and with final Na/K P_i concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25M (pH 7.2).

Assessment of pH reduction by dissolved CO_2. Acidification of buffered BHIA plates (pH 7.2) by CO_2 was examined in a gas flow-through system over 10 days incubation at 8°C. Uninoculated buffered plates (0.05, 0.10, 0.15 and 0.20M of Na/K P_i) were incubated in atmospheres containing 0, 1.0, 5.0, 10.0, 25.0, 50.0, and 100.0% CO_2 made up to 100% with N_2. On day 0, 1, 3, 6 and 10, duplicate samples were removed from the system and the pH was measured instantly at 8°C, using a flat surface pH electrode (Phoenix, USA).

Experimental design and storage of sample. Plates were incubated in a series of 1 l flasks, connected via silicon rubber tubings for each gasphase composition. Flasks were placed in a climatized room at 8°C and flushed continuously (flow rate = 200 ml.min^{-1}) with the desired gas atmosphere. Mass flow controllers (5850 TR series, Brooks Instrument b.v., The Netherlands) were used to mix the N_2, O_2 and CO_2. The incoming gas was humidified by passage through a 500 ml gas wash bottle. Concentrations of O_2 and CO_2 in the outcoming gas were measured and controlled at 2 h intervals by an O_2 and CO_2 analyzer (Servomex, analyzers series 1400). Agar plates were stored in the modified atmospheres at 8°C for up to 13 days. On day 2, 4, 7, 9 and 13 the last flask of a series was disconnected from the flow through system. This enabled plates to be removed individually, without disturbing the gas conditions of the remaining plates.

Microbial analysis. Viable bacterial counts were determined by analyzing samples of the agar medium in duplicate as follows. Agar samples were aseptically transferred from Petri dishes into stomacher bags and homogenized for one min with 41 ml of 0.85% NaCl using a stomacher (Seward, UK). Serial dilutions of each homogenized sample were made in 0.85% NaCl supplemented with 0.1% peptone and plated on BHIA in duplicate. Plates were incubated at 30°C for 24 h and the cfu.cm^{-2} values were determined. The pH of the homogenate from each agar sample was recorded.

Data handling. Bacterial growth curves were generated for each gas phase composition by fitting the data to the Gompertz equation, as modified by Zwietering et al. (200) to include microbiologically significant parameters (Equation 1). N is the number of microorganisms at time t, N_0 is the number of microorganisms at $t = 0$, A (= $\ln[N_\infty/N_0]$) is the asymptotic level of microorganisms, μ_m is the maximum specific growth rate achieved, $e = \exp(1)$, and λ is the lag time.

Equation 1

$$\ln\left(\frac{N}{N_0}\right) = A \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\}$$

Bacterial counts were fitted to equation 1 by a nonlinear regression program, using a Marquardt algorithm. This program estimates the maximum specific growth rate (μ_m), the final level of microorganisms ($A = \ln[N_\infty/N_0]$), the lag time (λ) and their 95% confidence intervals (200). Confidence intervals were based on the variance-covariance matrix of the parameters, calculated with the Jacobian matrix. Estimated μ_m values were subjected to linear regression using Equation 2.

Equation 2

$$y = b_0 + b_1 x$$

with regression coefficients b_0 (intercept) and b_1 (x coefficient), y representing the estimated growth parameter, and x the CO_2 concentration (%). 95% Confidence intervals of the linear regression parameters were also determined.

RESULTS

Acidification of the medium by CO_2 and gas analyses. The pH of the buffered BHIA (pH 7.2) was influenced by the concentration of CO_2 in the gas atmosphere. Equilibrium medium pH values of 7.2, 7.2, 7.0, 6.9, 6.8, 6.7 and 6.6 were observed in atmospheres containing 0, 1, 5, 10, 25, 50 or 100% CO_2 respectively, at 8°C. Due to permanent gas flushing of the incubators, the CO_2 concentrations in the gas phase were not influenced by CO_2 dissolving in the medium.

The maximum CO_2 concentration that was applied was 50%, and under these gas conditions, the medium pH decreased from 7.2 to 6.7. Therefore, growth was also monitored on BHIA buffered at pH 6.7 in the absence of CO_2, as a control for acidification of the medium.

General growth characteristics. The phosphate concentration employed (0.1 M) did not inhibit growth of the different pathogens in liquid medium at 30°C (data not shown). With incubation on agar surfaces, the growth of *A. hydrophila*, *Y. enterocolitica* and *B. cereus* resulted in a slight alkalinisation of the medium at CO_2 concentrations below 20% after 13 days at 8°C **(Table 1)**, with a maximum increase of 0.9 pH units. By contrast, growth of *L. monocytogenes* caused a slight acidification of the medium under each of the gas conditions tested, with a maximum pH drop of 0.5 units at maximum population density.

An example of growth of *Y. enterocolitica* on the surface of BHIA (pH 6.7 and 7.2) under 1.5% O_2 and 0, 5, 20, or 50% CO_2 is shown in **Figure 1**. The growth curves were obtained using the modified Gompertz equation **(Equation 1)**.

Lag times. Significant lag times were absent for the four pathogens grown on BHIA medium (pH 6.7 or 7.2) under the different gas phase compositions, notwithstanding the growth of *Y. enterocolitica* under 50% CO_2 and 21% O_2. Furthermore, viable counts of *B. cereus* gradually decreased under 50% CO_2 and 1.5% or 21% O_2 (data not shown).

Maximum specific growth rates. The maximum specific growth rates (μ_m) and their 95% confidence intervals, derived from the growth curves, are presented as a function of the CO_2 concentration in **Figure 2.** Linear regression analysis of these data **(Equation 2)** revealed a significant reduction of the maximum specific growth rates as a function of increasing CO_2 concentrations for all four pathogens, as indicated by negative values of both the estimates and the 95% confidence intervals of b_1 (x coefficient of regression) under 1.5% and 21% O_2 **(Table 2).** Linear regression ana-

Table 1. Medium pH (initial and final after 13 days of incubation) and estimated maximum population densities (PD) of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* on BHIA incubated at 8°C under 1.5% O_2 or 21% O_2. Measured initial population densities are also presented.

Microorganism	% CO_2	Medium pH			PD (log cfu.cm^{-2})		
		Initial	1.5% O_2 Final	21% O_2 Final	Initial	1.5% O_2 Maximum	21% O_2 Maximum
A. hydrophila	0	6.7	6.9	7.1	3.73	9.70	9.87
	0	7.2	7.4	7.5	3.73	9.56	9.75
	5	7.2	7.3	7.3	3.73	9.48	9.72
	20	7.2	7.1	7.1	3.73	9.31	9.44
	50	7.2	6.8	6.6	3.73	9.18	8.80
Y. enterocolitica	0	6.7	7.0	7.3	4.12	9.64	9.86
	0	7.2	7.8	7.8	4.12	9.51	9.82
	5	7.2	7.4	7.4	4.12	9.56	9.68
	20	7.2	7.2	7.3	4.12	9.31	9.56
	50	7.2	6.8	6.6	4.12	8.85	9.55[a]
L. monocytogenes	0	6.7	6.3	6.2	4.81	9.13	8.70
	0	7.2	6.8	6.8	4.81	9.21	9.14
	5	7.2	6.8	6.8	4.81	9.25	9.25
	20	7.2	6.8	6.8	4.81	9.04	9.11
	50	7.2	6.7	6.7	4.81	8.93	8.57
B. cereus	0	6.7	7.6	8.0	4.10	7.82	7.96
	0	7.2	7.8	8.1	4.10	8.22	8.12
	5	7.2	7.2	7.4	4.10	8.17	8.03
	20	7.2	6.8	6.9	4.10	7.28	7.54
	50	7.2	6.7	6.7	4.10	3.60[b]	3.88[b]

[a] Estimation based on growth under 20% CO_2

[b] Measured instead of estimated values

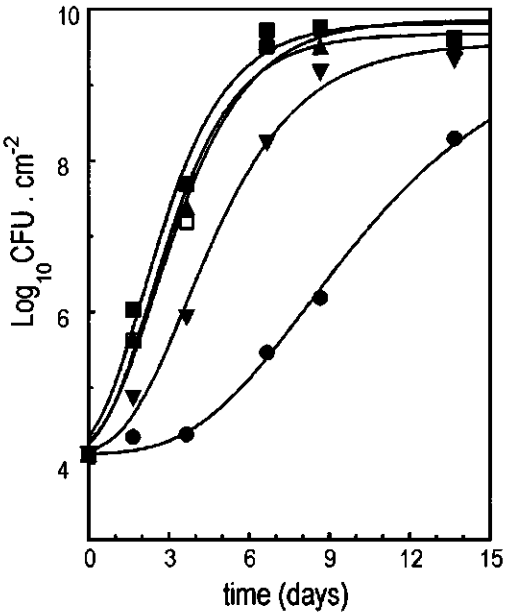

Figure 1.
Growth of *Y. enterocolitica* (Log_{10} cfu.cm^{-2}) on the surface of buffered BHIA (pH 7.2) stored under 1.5% O_2 and
(■) 0% CO_2,
(▲) 5% CO_2,
(▼) 20% CO_2 or
(●) 50% CO_2 and on buffered BHIA (pH 6.7) stored under 1.5% O_2 and 0% CO_2 (- -□- -).
Data are fitted by the modified Gompertz equation **(Equation 1)**.

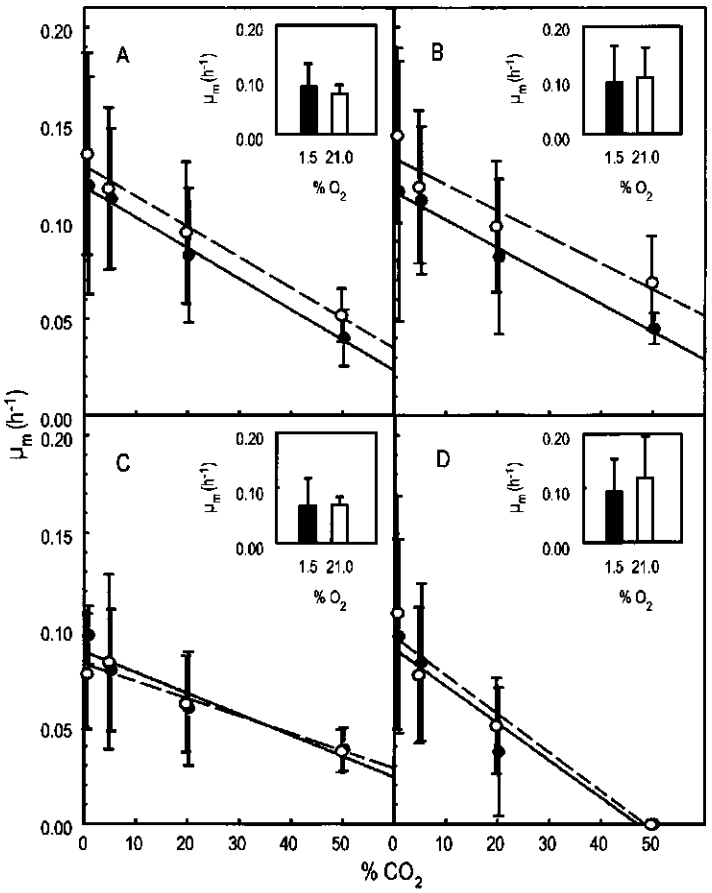

Figure 2.
Influence of CO_2 concentration on the maximum specific growth rates (μ_m) of
(A) *A. hydrophila*,
(B) *Y. enterocolitica*,
(C) *L. monocytogenes*, and
(D) *B. cereus* on BHIA (pH 7.2) stored under
(○) 1.5% O_2 and
(●) 21% O_2.
Lines represent linear regression under 1.5% O_2 (--) or 21% O_2 (—).
Inserts show μ_m on BHIA (pH 6.7) under 1.5% O_2 (white bar) or 21% O_2 (black bar) and 0% CO_2.
95% Confidence intervals of the estimated μ_m are indicated by error bars.

lysis furthermore substantiated the absence of significant differences in the maximum specific growth rates under 1.5% and 21% O_2, since estimated values of regression coefficients b_0 (intercept) and b_1 under 21% O_2 were situated within the 95% confidence intervals of the 1.5% O_2 regression coefficients in all cases **(Table 2)**.

For growth of *A. hydrophila, Y. enterocolitica* and *L. monocytogenes* under 50% CO_2, we observed maximum specific growth rates that were almost half of those under 0 and 5% CO_2 **(Figures 2A, 2B and 2C)**. In absence of CO_2, these three pathogens generally had lower maximum specific growth rates on medium with an initial pH of 6.7 as compared with pH 7.2. However, their maximum specific growth rates under 0% CO_2 at pH 6.7 were higher than the rates on medium with pH 7.2 during incubation under 50% CO_2 (the latter concentration was shown to cause a decrease in the pH of the medium to pH 6.7) **(Figures 2A, 2B and 2C plus inserts)**. The observed decrease in the maximum specific growth rates obtained under elevated CO_2 concentrations may therefore be attributed to both a direct inhibitory effect of CO_2 on the growing cells and to the acidifying effect of CO_2 on the medium.

In the case of *B. cereus*, we observed an approximate 50% reduction in the maximum specific growth rates during incubation under 20% CO_2 as compared with 0% CO_2. The antimicrobial effect of this gas on this organism was even more pronounced at higher CO_2 concentrations as indicated by a decline of viable counts during incubation under 50% CO_2 at 8°C for 13 days **(Table 1)**. The maximum specific growth rates of *B. cereus* were similar on pH 6.7 and pH 7.2 medium

Table 2. Linear regression parameter values (b_0 and b_1) and their 95% confidence intervals for the effect of CO_2 on the estimated maximum specific growth rate (μ_m) values of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* during growth on agar at 8°C under 1.5% O_2 or 21% O_2.

Microrganism	O_2 (%)	Parameter			
		b_0		b_1	
		Estimate	95% Conf. Int.[a]	Estimate	95% Conf. Int.
A. hydrophila	1.5	0.129	0.112 to 0.146	- 0.00158	- 0.00220 to - 0.00095
	21	0.118	0.109 to 0.127	- 0.00158	- 0.00192 to - 0.00125
Y. enterocolitica	1.5	0.133	0.097 to 0.168	- 0.00136	- 0.00265 to - 6.3E-05
	21	0.115	0.103 to 0.127	- 0.00144	- 0.00188 to - 0.00100
L. monocytogenes	1.5	0.082	0.067 to 0.097	- 0.00089	- 0.00146 to - 0.00034
	21	0.089	0.062 to 0.116	- 0.00108	- 0.00208 to - 8.5E-05
B. cereus	1.5	0.097	0.060 to 0.133	- 0.00200	- 0.00334 to - 0.00065
	21	0.090	0.054 to 0.127	- 0.00192	- 0.00327 to - 0.00057

[a] 95% Confidence interval.

under 0% CO_2, while growth was completely absent under 50% CO_2 **(Figure 2D plus insert)**. The observed reduction of the viable counts of this organism under 50% CO_2 is therefore likely to result from a direct inhibitory effect of CO_2.

Maximum population densities. The estimated maximum population densities of *A. hydrophila*, *Y. enterocolitica* and *L. monocytogenes* on BHIA medium (pH 7.2) were only slightly reduced under 50% CO_2 as compared to 0% CO_2 (maximum one log unit) under both 1.5% and 21% O_2 **(Table 1)**. Incubation of *B. cereus* under 20% CO_2 resulted in a reduction in the maximum population density of less than 1 log unit as compared to 0% CO_2, for both O_2 concentrations **(Table 1)**, but no growth was observed under 50% CO_2. The 95% confidence intervals of the estimated maximum population densities were less than one log unit (data not shown).

Since the estimated maximum population densities on pH 6.7 and pH 7.2 medium in absence of CO_2 were similar for each pathogen **(Table 1)**, the reduction of the maximum population densities under 50% CO_2 can be accounted for by a direct inhibitory effect of CO_2, rather than acidification of the medium.

DISCUSSION

In this study we evaluated the growth of the psychrotrophic pathogenic bacteria *A. hydrophila*, *Y. enterocolitica*, *L. monocytogenes*, and *B. cereus* under different O_2 and CO_2 concentrations. For this purpose, a model surface system was developed in which pure bacterial cultures can be incubated on an agar surface under controlled gas atmospheres at 8°C. The growth data were subsequently evaluated by using of the modified Gompertz equation (200), which rendered objective assessment of the maximum specific growth rates, maximum population densities and lag times.

The main effect of CO_2 on the growth parameters of each of the above pathogens was observed for the maximum specific growth rates, which were found to decrease significantly with increasing concentrations of CO_2. Within the range of 0 to 50% CO_2, we identified a linear relationship between the maximum specific growth rate and CO_2 concentration. The decreases in the maximum specific growth rates under elevated CO_2 concentrations which were observed for *A. hydrophila*, *Y. enterocolitica* and *L. monocytogenes* likely resulted from a combined effect of acidification of the medium and direct inhibition by CO_2. With *B. cereus*, however, the growth inhibition under 50% CO_2 probably resulted from a direct inhibitory effect of CO_2, since the maximum specific growth rates that were found on medium of pH 6.7 and pH 7.2 in the absence of CO_2 were similar. These results are in agreement with previous reports on the high sensitivity of *B. cereus* towards CO_2 (61,62,143).

With regard to the influence of CO_2 on the maximum population densities, we observed only small differences for *A. hydrophila*, *Y. enterocolitica*, *L. monocytogenes* under CO_2 concentrations up to 50%. For *B. cereus*, these values were slightly reduced under 20% CO_2 compared to 0% CO_2, while growth was completely absent under the highest CO_2 concentration tested (50%). The reduction of the maximum population densities of this pathogen under 50% CO_2 could not be attributed to the acidification of the medium. This is in line with the general thought that reduction of the medium pH by CO_2 is not the major cause of inhibition of bacterial growth (46,120).

In general, we did not observe significant lag times for *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* under the different gas conditions. Exceptions were *Y. enterocolitica* under 50% CO_2 combined with 21% O_2 and *B. cereus* under 50% CO_2. To our knowledge, there are no reports on the influence of CO_2 on the lag time for growth of *B. cereus*. Reports that CO_2 may influence lag times of the other pathogens are variable (62,65,67,100). Notably, extended lag times were observed in some of these studies at much higher CO_2 concentrations than applied in this study. The main variables that seem to determine lag times are the type of bacterium, the type of product, and the storage temperature. For *A. hydrophila*, prolonged lag phases have been reported during growth on BHI agar at 5°C in saturated CO_2 as compared to N_2 or air (80). Gill and Reichel (77) observed an extended lag phase for *Y. enterocolitica* on high pH beef packaged under saturated CO_2 at 5°C and 10°C, while the growth of *A. hydrophila* and *L. monocytogenes* was completely inhibited at 5°C. Their study, however, demonstrated the absence of a significant lag phase and growth of *A. hydrophila* and *L. monocytogenes* at 10°C. Furthermore, Hudson et al. (107) observed extended lag times for *A. hydrophila* and *L. monocytogenes*, but not for *Y. enterocolitica* on roast beef, packaged under saturated CO_2 at 3°C.

With regard to the O_2 concentrations tested, we did not observe significant differences in the growth patterns of *A. hydrophila, Y. enterocolitica, L. monocytogenes* and *B. cereus* under 1.5 and 21% O_2. Although the applied O_2 concentration of 1.5% approaches the minimal requirement for vegetables to maintain aerobic respiration (83,113,152), this concentration appears not to be inhibitory to the growth of aerobic respiratory bacteria. These results agree with the observation that strictly aerobic bacteria such as *Pseudomonas fragi* did not have decreased maximum specific growth rates at reduced O_2 concentrations, unless these levels were lower than 0.5 to 0.25% (43,61).

The extrapolation of our results to MA packaged fruits and vegetables suggest that growth of *A. hydrophila, Y. enterocolitica*, and *L. monocytogenes* and *B. cereus* may be possible at 8°C since the maximum specific growth rates were only slightly reduced and maximum population densities were not effectively reduced by typical MA gas concentrations of 1 to 5% O_2 combined with 5 to 10% CO_2. The surface model system presented together with the data analyses performed allowed an objective assessment of the effects of CO_2 on the growth of individual bacteria. This system can also be employed to investigate the effects of other gases as well as the interactions between epiphytic microflora and psychrotrophic pathogens under various gas phase compositions. It is noteworthy that *Pseudomonas* species are prominent members of the endogenous spoilage microflora on MA packaged vegetables (148). Since these organisms are sensitive to CO_2 (62), their suppression may influence the nutrient availability for other organisms. To elucidate the impact of CO_2 on vegetable-associated spoilage or other organisms, the evaluation of their growth in absence of an interfering microflora by the use of a systematic approach such as the model surface system may prove helpful.

ACKNOWLEDGEMENTS

We thank Marcel Zwietering, Claire Wilkinson and Maarten Hertog for valueable advice on data handling and Werner Vorstman and Marc Walpot for their capable technical assistance.

CHAPTER 4

Vegetable-associated *Pediococcus parvulus* produces pediocin PA-1

Marjon H.J. Bennik, Eddy J. Smid and Leon G.M. Gorris.
*Applied and Environmental Microbiology (1997) **63**: 2074-2076*

ABSTRACT

Two bacteriocin producing lactic acid bacteria, designated ATO34 and ATO77, were isolated from fresh vegetables and identified as *Pediococcus parvulus* on the basis of fermentation patterns and ribotyping. The strains inhibited the growth of a number of important foodborne pathogens, such as *Listeria monocytogenes* and nonproteolytic *Clostridium botulinum*. Recombinant DNA techniques revealed the presence of the pediocin PA-1 gene in both strains. Further biochemical analysis confirmed the production of pediocin PA-1 and excluded the presence of other bacteriocins. To our knowledge, this is the first case of bacteriocin production by *P. parvulus*.

INTRODUCTION

A recent trend towards the use of natural, healthy convenient foods has resulted in a new generation of chill-stored, minimally processed vegetables. Since these products rely heavily on refrigeration as the main preservation factor, psychrotrophic pathogenic bacteria that can be present as part of the microflora, such as *Listeria monocytogenes* and *Clostridium botulinum*, may pose a hazard (12,148). The application of bacteriocinogenic lactic acid bacteria (LAB) with activity against Gram-positive pathogens might therefore be effective to ensure the microbial safety of these products. We isolated a number of bacteriocinogenic LAB, including two strains of *Pediococcus parvulus*, from minimally processed vegetables. Although several bacteriocinogenic pediococci have been reported (reviewed in reference 52), only two pediocins produced by *Pediococcus acidilactici* of meat origin, have been described in more detail, namely, pediocin PA-1/AcH (139,145) and pediocin L50 (42). To our knowledge, this report describes the first case of bacteriocin production by *P. parvulus*.

METHODS, RESULTS AND DISCUSSION

Strains, growth conditions, and screening for bacteriocin production. In our search for bacteriocinogenic LAB, a total of 900 strains were randomly isolated from minimally processed vegetables by using MRS agar plates (Oxoid, Basingstoke, England) supplemented with delvocid (0.2 mg.liter^{-1}). All LAB isolates were cultured in MRS broth at 30°C. Cell-free supernatants of early-stationary-cultures, adjusted to pH 6.0, were tested for antimicrobial activity by a previously described well-diffusion assay (129). Supernatants of nine isolates produced clear zones against the indicator organism *Lactobacillus sake* DSM20017. The fermentation patterns of these isolates as determined with API Rapid CH fermentation strips in CHL medium (BioMerieux, Marcy, France) preliminary identified the strains as one *Lactobacillus plantarum* strain, one *Enterococcus* strain, five *Leuconostoc mesenteroides* strains, and two *Pediococcus* strains. The two pediococci, ATO34 and ATO77, which were isolated from separate batches of fresh chicory endive, were selected for further studies, since initial screening revealed activity against *L. monocytogenes*. Full characterization by the services of the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany) identified both strains as *P. parvulus* on the basis of fermentation patterns and 16S rRNA sequence similarity.

Characterization of the inhibitory compound. Evidence for bacteriocin production by the two *P. parvulus* strains was obtained by proteolytic treatment of culture supernatants. Incubation with trypsin, pepsin, α-chymotrypsin, papain, protease IX, and proteinase K (1 mg.ml^{-1}) (Boehringer, Mannheim, Germany), resulted in a complete loss of activity, as detemined by the well diffusion assay. The inhibitory compound of both strains was not affected by heating for 15 min at 100°C. Both bacteriocins were stable from pH 1 to pH 6 for 14 h at 4°C, whereas decreased activity was observed above pH 7.

Activity spectrum. Supernatants of *P. parvulus* ATO34 and ATO77 inhibited the growth of several types of LAB, *L. monocytogenes*, and *C. botulinum*. Gram-negative bacteria, yeasts and molds were not inhibited (**Table 1**). A broader range of microorganisms was sensitive to the inhibitory action of the culture supernatant of strain ATO77, as compared with ATO34. This can be explained by a difference in concentration, since the bacteriocin activity in culture supernatant of strain ATO77 was

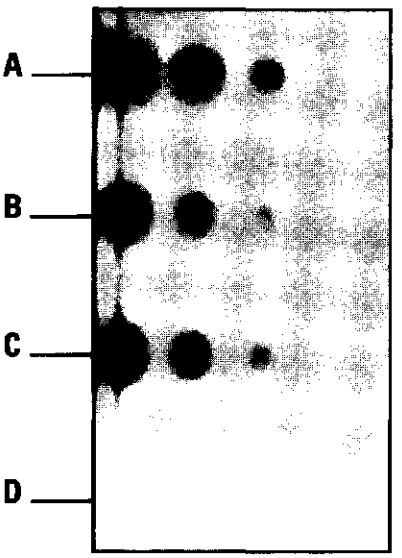

Figure 1.
Dot blot hybridization with 5' end-labeled *pedA* oligomer 1 and equal amounts (400, 40, 4, or 0.4 ng [left to right]) of plasmid DNA of *P. acidilactici* PAC1.0 (positive control) (**A**), *P. parvulus* ATO34 (**B**), *P. parvulus* ATO77 (**C**), and phage lambda DNA (**D**).

Table 1. Spectrum of bacteriocin activity of *P. parvulus* strains AT034 and AT077, as determined in duplicate in a well diffusion assay.

Indicator strain(s)[a]	Culture conditions[b]	Inhibition by: AT034	AT077
Lactobacillus brevis NCAIM B00509	MRS, 30°C, anaerobic	–	–
Lactobacillus casei WAU11	MRS, 30°C, anaerobic	–	–
Lactobacillus delbrueckii subsp. *bulgaricus* DSM20081	MRS, 30°C, anaerobic	+	+
Lactobacillus delbrueckii subsp. *lactis* DSM20072	MRS, 30°C, anaerobic	–	–
Lactobacillus plantarum ATCC8014	MRS, 30°C, anaerobic	–	–
Lactobacillus sake DSM20017, IFO12456	MRS, 30°C, anaerobic	+	+
Lactobacillus sake DSM20497, NCFB2812	MRS, 30°C, anaerobic	–	–
Lactobacillus salivarius subsp. *salicinius* DSM20555	MRS, 30°C, anaerobic	–	–
Lactobacillus xylosus WAU7	MRS, 30°C, anaerobic	–	–
Lactococcus lactis subsp. *lactis* NCDO495, NCDO497	MRS, 30°C, anaerobic	–	–
Leuconostoc mesenteroides subsp. *mes.* DSM20343	MRS, 30°C, anaerobic	–	+
Leuconostoc paramesenteroides DSM 20288	MRS, 30°C, anaerobic	–	–
Pediococcus dextrinicus DSM20335	MRS, 30°C, anaerobic	+	+
Pediococcus pentosaseus DSM20336	MRS, 30°C, anaerobic	–	–
Carnobacterium piscicola UI49	APT, 30°C, anaerobic	+	+
Enterococcus faecalis DSM20478	BHI, 30°C, aerobic	–	+
Enterococcus hirae ATCC9790	BHI, 30°C, aerobic	–	+
Micrococcus luteus DSM1790	NA, 30°C, aerobic	–	–
Streptococcus mutans DSM20523	NA, 30°C, aerobic	–	–
Listeria monocytogenes WAUI, WAUL4492, LDCD81-861, LCDC81-1081	BHI, 30°C, aerobic	+	+
Listeria monocytogenes WAU L028	BHI, 30°C, aerobic	–	+
Listeria monocytogenes Scott A WAU	BHI, 30°C, aerobic	–	–
Listeria innocua WAU II, WAU III	BHI, 30°C, aerobic	+	+
Listeria innocua DSM20649	BHI, 30°C, aerobic	–	+
Clostridium botulinum IFR81-23[c], 81-26[c], 86-32[c], 86-34[c]	VL, 30°C, 90% H_2/10% CO_2	–	+
Clostridium botulinum IFR81-1[c], 81-30[c], 81-31[c], 93-21[d], 83-42[d], 81-21[d], 93-25[d], 96-02[d], 93-23[d]	VL, 30°C, 90% H_2/10% CO_2	–	–
Clostridium beijerinkii NIZO B523	RC, 30°C, anaerobic	+	+
Clostridium sporogenes NIZO B545	RC, 30°C, anaerobic	+	+
Clostridium tyrobutyricum NIZO B570, B571, B599	RC, 30°C, anaerobic	–	–
Bacillus cereus DSM 31, IFR94-10, 94-15, 94-22, 94-23, 94-24, 94-25, 94-26	NA, 30°C, aerobic	–	–
Staphylococcus aureus ATCC6538	BHI, 30°C, aerobic	–	–
Aeromonas hydrophila subsp *hydrophila* DSM30187	BHI, 30°C, aerobic	–	–
Yersinia enterocolitica DSM4780	BHI, 30°C, aerobic	–	–
Salmonella typhimurium DSM554	BHI, 30°C, aerobic	–	–
Escherichia coli ATCC11775	BHI, 30°C, aerobic	–	–
Pseudomonas aeruginosa ATCC9027	BHI, 30°C, aerobic	–	–
Candida albicans ATCC10231	YNB, 25°C, aerobic	–	–
Aspergillus niger ATCC16404	YNB, 25°C, aerobic	–	–

[a] NCAIM, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary; WAU, Wageningen Agricultural University, Wageningen, The Netherlands; DSM, Deutsche Sammlung von Microorganismen, Braunschweig, Germany; ATCC, American Type Culture Collection, Rockville, USA; IFO, Institute for Fermentation, Osaka, Japan; NCFB, National Collection of Food Bacteria, Reading, UK; NCDO, National Collection of Dairy Organisms, Reading, UK; UI, University of Iceland, Reykjavik, Iceland; IFR, Institute of Food Research, Norwich, United Kingdom; NIZO, Institute for Dairy Research, Ede, The Netherlands.

[b] MRS medium, brain heart infusion (BHI) medium, and reinforced clostridium (RC) medium were obtained from Oxoid (UK); all-purpose tryptone (APT) medium and yeast nitrogen base (YNB) were obtained from Difco (Detroit, Mich.); VL agar base (11) was supplemented with 5% horse blood.

[c] Nonproteolytic strain.

[d] Proteolytic strain.

twofold higher as determined by critical dilution in a microtiter plate assay (75). In the case of *C. botulinum*, growth of the nonproteolytic strains was inhibited, whereas growth of the proteolytic strains was not. This is probably due to inactivation of the bacteriocins by secreted proteolytic enzymes. These enzymes may also be present in foods, originating from either the product or the endogenous microorganisms in it. This type of inactivation may reduce the effectiveness of bacteriocins in practical food applications.

Genetic characterization. Characteristics of the bacteriocins produced by *P. parvulus* ATO34 and ATO77, such as pH and thermal stability, were compared with those of all pediocins described to date. These characteristics coincided best with those of pediocin PA-1 (AcH) (21,82), which is encoded by the *pedA* gene (139).

Putative sequence homology of plasmid DNAs isolated from the two strains with the *pedA* gene was initially investigated by dot blot hybridization. Equal amounts of plasmid DNAs of strain ATO34, strain ATO77, and *P. acidilactici* PAC1.0, isolated by the method of Ahn and Stiles (5), and phage λ DNA (negative control) were blotted on a nylon membrane. A probe identical to the 3'-sequence of the *pedA* gene was obtained by 5' end labeling with [γ-^{32}P] ATP of oligomer 1 (5'- CATTTATGATTACCTTGATGTCCA-3'). Hybridization was performed in 6 x SSC (1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 45°C by established methods (166). Plasmid DNAs of strains ATO34 and ATO77 showed signals with the same intensity as those of plasmid DNA of *P. acidilactici* PAC1.0, in the absence of a signal for phage λ DNA (**Figure 1**).

The plasmid DNAs of strains ATO34 and ATO77 were subsequently screened for the presence of the *pedA* gene (139) by PCR with primers 2A (5'-TAAGGATAATTTAAGAAGAAGGAG-3') and 2B (5'-TAAAATCACCCCTTTATTGA-3'). Plasmid pSRQ220 (139), containing the *pedA* gene, was used as a positive control. PCRs were carried out in standard PCR buffer, with 200 μM deoxynucleoside triphosphates, 0.2 μM primers, and 0.5 U of *Taq* polymerase (Pharmacia, Uppsala, Sweden) in a total volume of 50 μl (30 cycles of 30 s at 95°C, 30 s at 52°C, 30 s at 72°C. We obtained DNA fragments of the expected size (260 bp). Purified PCR products were directly used for cloning, using the pGEM-T vector system (Promega, Madison, Wis.). Positive clones containing PCR products from ATO34 (n=5) and ATO77 (n=8) were pooled and sequenced. Sequence analysis revealed sequences fully identical to that of the *pedA* gene (139) for both ATO34 and ATO77.

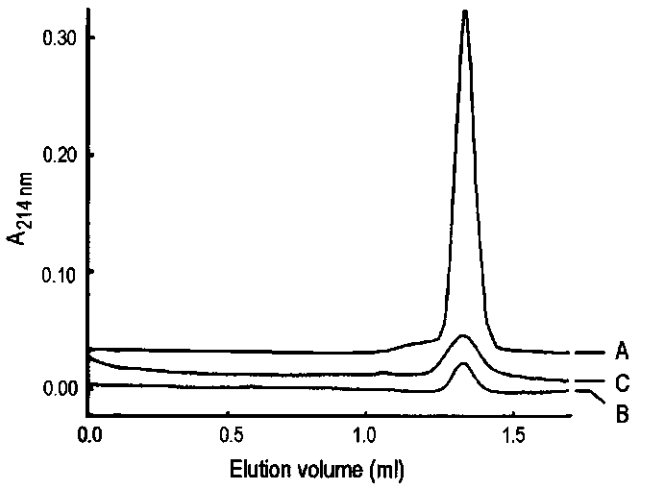

Figure 2.
Gelfiltration of bacteriocins of *P. acidilactici* PAC1.0 **(A)**, *P. parvulus* ATO34 **(B)**, and *P. parvulus* ATO77 **(C)**, after purification by hydrophobic-interaction chromatography and cation-exchange chromatography.

Biochemical characterization. To confirm that *P. parvulus* produces only pediocin PA-1, bacteriocins of strains ATO34 and ATO77 were purified to homogeneity from 500 ml of early-stationary-phase culture supernatant. *P. acidilactici* PAC1.0, producing pediocin PA-1 (139), was used as a reference. The maximum bacteriocin activities in culture supernatant of strains ATO34, ATO77, and PAC1.0 were 80, 160 or 2560 bacteriocin units per ml (75), respectively. Proteins were concentrated from the supernatants by a two-step ammonium sulfate precipitation, in which 94% of the total activity was recovered in the second step (25 to 70% saturation) for all three batches. This fraction was loaded on a 24-ml phenyl-Sepharose CL4B column (Pharmacia) which was equilibrated with 0.42 M ammonium sulfate in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5 (buffer 1). The column was eluted with a linear gradient (after 60 min in 100% buffer 1, in 140 min to 100% 50 mM MES buffer [pH 5.5], at 2 $ml.min^{-1}$) and subsequently washed with 40 ml of 70% ethanol (EtOH) in water. The EtOH fraction, containing all of the bacteriocin activity, was loaded on a 1-ml ResourceS cation-exchange column (Pharmacia) which was equilibrated with 10 mM formic acid-10% EtOH (A). Elution was performed with a linear gradient (in 10 min from [A] to 100% 1.0 M NaCl-10 mM formic acid-10% EtOH in water [B], at 2 $ml.min^{-1}$). Each batch showed the same elution profile, with bacteriocin activity in the 0.8 to 1.0 M NaCl fractions. These active fractions were pooled and concentrated by ultrafiltration (3000 molecular weight cutoff), and 50 µl (80% of the sample) was loaded on a Superdex Peptide PC3.2/30 gelfiltration column (SmartSystem, Pharmacia) equilibrated with 0.15 M NaCl-0.1% trifluoroacetic acid-20% EtOH. Bacteriocin was eluted by using a constant flow (80 $\mu l.min^{-1}$) with monitoring of A_{214}. An absorbance peak at 1.32 ml (**Figure 2**) corresponded to bacteriocin activity for all three batches. The peak areas reflected the initial bacteriocin activities, present in culture supernatants of the three different strains, which indicated similar yields. The homogeneity of each peak was confirmed by reversed-phase chromatography, using a C_2-C_{18} µRPC 3.2/30 column (SmartSystem: Pharmacia), and this confirmed the identity of the bacteriocin of *P. parvulus* as a single peptide, being pediocin PA-1. Interestingly, this bacteriocin has previously been reported to be produced by only meat- and dairy-associated strains of *P. acidilactici* (105,139,146).

In conclusion, this study shows that knowledge of physicochemical and biological properties in conjunction with the use of recombinant DNA technology were effective means of identifying bacteriocins of *P. parvulus*. The adaptation of this strain to the vegetable environment, its observed ability to grow at low temperatures, and its GENERALLY RECOGNIZED AS SAFE status make it a candidate for use as a biopreservation agent for minimally processed vegetables.

ACKNOWLEDGEMENTS

WE ACKNOWLEDGE THE TECHNICAL ASSISTANCE OF BASTIENNE PEELEN, IRENE POL AND MARC WALPOT. IN ADDITION, THE HELP OF SANDRA STRINGER (INSTITUTE OF FOOD RESEARCH, NORWICH, UNITED KINGDOM) IN TESTING BACTERIOCIN ACTIVITY AGAINST *C. botulinum* STRAINS IS HIGHLY APPRECIATED. *P. acidilactici* PAC1.0 AND PLASMID pSRQ220 WERE KINDLY PROVIDED BY A. M. LEDEBOER (UNILEVER RESEARCH LABORATORY, VLAARDINGEN, THE NETHERLANDS).

CHAPTER 5

A role for oblique peptides in pore formation by bacteriocins of lactic acid bacteria

Marjon H.J. Bennik, Berlinda Vanloo, Eddy J. Smid, Leon G.M. Gorris, Robert Brasseur
Biochimica et Biophysica Acta, submitted for publication

ABSTRACT

Bacteriocins are antimicrobial peptides and proteins which are produced by bacteria. The activity of those that are secreted by lactic acid bacteria is generally related to the formation of pores in the cytoplasmic membrane of their target organisms, eventually leading to cell death. This report addresses the initial steps involved in the mode of action of class IIa bacteriocins which share a highly conserved N-terminal 'YGNGV' motif. We report the complete primary amino acid sequence of mundticin, a novel class IIa bacteriocin of 43 amino acids (M_r 4287.2), produced by *Enterococcus mundtii*. This bacteriocin was demonstrated to permeate the cytoplasmic membrane of sensitive organisms. Furthermore, we have used mundticin as a prototype of class IIa bacteriocins to study the initial steps involved in the pore-forming activity of these compounds by computer modeling. A three-dimensional representation of the molecular hydrophobicity potential around lipid-associating helices revealed an asymmetric distribution of hydrophobic residues along the axis of an α-helix in the central region of mundticin, and the same was found for homologous class IIa bacteriocins. The predicted angle of insertion of these α-helical regions in a simulated hydrophobic-hydrophilic interface varied from 30 to 50°. Based on these predictions, we believe that the oblique orientation of these regions in bacteriocins results in the destabilization of the phospholipid bilayer and may facilitate the insertion and/or aggregation of monomers into functional pores in the cytoplasmic membrane.

INTRODUCTION

Bacteriocins are antimicrobial peptides and proteins that are ribosomally synthesized by bacteria (175). These compounds can inhibit or eliminate the growth of their target organisms by affecting the membrane permeability (111,197), or alternatively by interference with essential cell functions such as DNA replication (189) and translation (74). Traditionally, bacteriocin research has focused on proteins from Gram-negative bacteria (125). However, bacteriocins produced by Gram-positive organisms, particularly those from lactic acid bacteria (LAB), have recently provoked a great deal of interest for their potential as nontoxic preservatives in the food and feed industry (184). The action of bacteriocins that are produced by LAB seems to be confined to the formation of pores in the cytoplasmic membrane of target cells, explaining the leakage of small intracellular components and the dissipation of the proton motive force after exposure to these antimicrobial compounds (108,187).

Amongst LAB bacteriocins, the class IIa bacteriocins have received a lot of attention, especially because of their activity toward the pathogen *L. monocytogenes*. This class of bacteriocins consists of a group of small heat-stable cationic membrane active peptides that do not undergo substantial posttranslational modification and are predicted to form α-helices with varying amounts of hydrophobicity (108,121). The presence of the N-terminal consensus sequence 'Tyr-Gly-Asn-Gly-Val' (YGNGV) is typical for members of this class (121,158).

Pore formation by class II bacteriocins is thought to require membrane receptor proteins (39,186). However, the exact molecular events which precede pore formation remain to be elucidated to date. The action of these membrane-active peptides on lipid bilayers involves both hydrophobic interactions between the lipid acyl chains and the hydrophobic residues of the peptide, and electrostatic interactions between the polar residues and the phospholipid head groups (27,69). Although the presence of an α-helical domain in the class IIa bacteriocins is thought to play a key role in the interaction with the lipid bilayer (69), the structural and functional properties of such a lipid-protein complex are unknown. In the present study, we propose a possible mode of action.

A recent classification of lipid-associating helices based on the structural and functional characteristics of the lipid-protein complexes, distinguishes between lipid-associated peptides with a constant hydrophobicity, and peptides with a hydrophobic gradient along the axis of an α-helix (26,27). Peptides of the former category have been found to interact with lipid bilayers through helical structures which are positioned parallel or perpendicular to the hydrophobic-hydrophilic interface. Conversely, oblique peptides with a hydrophobicity gradient along their axis insert at an 30 to 60° angle at hydrophobic/hydrophilic interfaces. Such peptides have been identified in processes in which membrane perturbation underlies the action of the peptide by the partial penetration of the bilayer and destabilization of the phospholipid acyl chains (recently reviewed by 27). These processes include a wide range of biological phenomena, such as cell signaling events (176), fusion events that are induced by viral peptides (106,191) or the Alzheimer β-amyloid peptide (155), and processes in lipid metabolism (27).

The aim of this study was to identify the compound that is responsible for the antimicrobial activity of *Enterococcus mundtii* and to explain its mode of action on the cytoplasmic membrane of sensitive cells. Based on computer modeling studies, we furthermore propose the hypothesis that the presence of an oblique peptide in this protein plays a role in the initial steps of the pore-forming activity of mundticin and homologous class IIa bacteriocins.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *Enterococcus mundtii* ATO6 was isolated from minimally processed vegetables (13), and identified on the basis of fermentation patterns and 16S rRNA analysis by the services of the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany). *Lactobacillus sake* DSM 20017 was used as an indicator strain for the monitoring of bacteriocin activity. Both strains mentioned above were routinely grown in MRS medium (Oxoid, Basingstoke, England). *Listeria monocytogenes* LDCD81-861 (167) was grown in brain heart infusion (BHI) broth (Oxoid) supplemented with 0.5% (w/v) glucose. All cultivations were started with 1% inocula and performed at 30°C.

Bacteriocin assays, antimicrobial spectrum and stability. The antimicrobial activity of mundticin was determined in a microtiter assay as previously described (13). Hereby, the activity was expressed in bacteriocin units (BU) per ml,

which was calculated from the reciprocal of the highest dilution of a sample that reduced the OD_{660} of the indicator organism by 50% after 8 h of incubation at 30°C. This assay was also used to determine the minimally inhibitory concentration (MIC) of mundticin against *L. monocytogenes* LDCD81-861, starting from a purified bacteriocin solution with a known bacteriocin concentration.

To determine the nature of the inhibitory substance, the supernatant of *E. mundtii* ATO6 was treated with proteolytic enzymes, *i.e.* trypsin, α-chymotrypsin and proteinase K (1 mg/ml) (Boehringer, Mannheim, Germany) for 2 h at 37°C. The pH stability of the purified bacteriocin during storage at 4°C for 14 h was determined in the range of pH 1 to 12 with 1 pH unit intervals. Following incubation, the antimicrobial activity of the samples was determined by the use of the microtiter assay as described above. The heat resistance was assessed by boiling a bacteriocin solution which was adjusted to pH 6.0 for 1, 5, 10, 15, 30 or 60 minutes. Again, the remaining bacteriocin activity was measured by the use of the microtiter assay.

Growth inhibition of a panel of 65 bacteria and fungi in response to culture supernatant of *E. mundtii* was determined by the use of a well diffusion assay and a microtiter assay as described previously (13). To establish whether the action of mundticin was bacteriostatic or bactericidal, late- exponential-phase cells of *L. monocytogenes* LCDC81-681 in BHI broth were exposed to this bacteriocin (final concentrations 0.1 and 1.0 μg/ml) and after dilution of the cells, the viable counts were determined at regular time intervals during 5 h at 30°C.

Purification of mundticin. The bacteriocin produced by *E. mundtii* ATO6 was isolated from 2 liters of a 22 h static culture. The cells were removed by centrifugation, and proteins were subsequently concentrated from the supernatants by a two-step ammonium sulfate precipitation (0 to 25% and 25 to 70% saturation). The fraction obtained from the second step of this precipitation was dissolved in 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 5.5, containing 0.85 M ammonium sulfate. Aliquots (3 ml) were loaded on a 24-ml phenyl Sepharose CL4B column (Pharmacia, Uppsala, Sweden) which was equilibrated with 0.42 M ammonium sulfate in 50 mM MES buffer, pH 5.5. The elution was performed by a 140 min linear gradient from 0.42 to 0.0 M ammonium sulfate in 50 mM MES buffer, pH 5.5, at 2 ml/min. The most active fractions were pooled, diluted fivefold in 10 mM formic acid-10% EtOH (Solution A), and loaded on a 1 ml ResourceS cation-exchange column (Pharmacia) which was equilibrated with solution A. The column was eluted with a 30 min linear gradient from 0.0 to 1.0 M NaCl in 10 mM formic acid-10% EtOH at 5 ml/min. The active fractions were pooled, and further purification was accomplished by loading 50 μl samples on a Superdex Peptide PC3.2/30 gelfiltration column (Smartsystem, Pharmacia), followed by elution in 0.15 M NaCl-0.1% trifluoroacetic acid (TFA)-20% EtOH at 80 μl/min (monitoring at 214, 254, and 280 nm). The homogeneity of the peaks was confirmed by reversed phase chromatography with a C_2-C_{18} μRPC 3.2/30 column (Pharmacia) using a 30 min linear gradient from 20 to 95% EtOH in 0.1% TFA (100 μl/min, monitoring at 214 nm).

Enzymatic cleavage and alkylation of mundticin. Purified mundticin (40 μg/ml in 50 mM Tris- HCl, pH 7.4) was incubated with the sequence grade endoprotease Asp-N (Boehringer) at an enzyme to substrate ratio of 1:20 (w/w). After incubation for 7 h at 37°C, the reaction was stopped by the addition of trichloroacetic acid (TCA) (5% final concentration). The peptide fragments were immediately separated by injection of 50 μl samples on a Superdex Peptide PC3.2/30 gelfiltration column (Pharmacia), using the same conditions as described above. Reduction and alkylation of purified mundticin were performed by dissolving the peptide in 0.2M Tris-6M guanidine.HCl buffer, pH 8.4, containing 20mM DTT. After incubation of 1 h at 20°C, 1 μl of 4-vinylpyridine was added. This reaction mixture was further incubated for 1 h at 20°C, after which the sample was diluted (1:1 in water) and sequenced.

N-terminal amino acid sequence analysis and mass spectrometry. Purified peptides were analyzed by the Sequence Centre Utrecht (University of Utrecht, Utrecht, The Netherlands). The N-terminal sequences were obtained by Edman degradation on an automated gas phase sequencer (Applied Biosystems model 476A) with on-line phenylthiohydantoin derivate identification by reversed phase HPLC. Electronspray-mass spectrometry (ES-MS) was

performed at the Institute for Animal Science and Health (ID-DLO, The Netherlands). The mass spectrum of purified peptides was determined by direct injection (1.2 μg/μl in 0.1% TFA-50% EtOH) on a Quattro IISQ instrument (Micromass, Manchester, UK).

Measurement of the membrane potential with fluorescent probe. Cells of *L. monocytogenes* LDCD81-861 were harvested in the exponential phase of growth (OD_{660} 0.6) by centrifugation (5000 g, 15 min) at 4°C, washed twice in 50 mM potassium *N*-2-Hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (K-HEPES) buffer (pH 7.0), resuspended, concentrated approximately fifty-fold, and stored on ice. Membrane potential ($\Delta\psi$) measurements were performed by using the fluorescent probe 3,3-dipropylthiocarbocyanine ($DiSC_3[5]$). Cell suspensions were diluted in the K-HEPES buffer to a density of 0.06 mg bacterial protein/ml. Reactions were performed at 20°C in a Perkin Elmer LS 50B spectrofluorometer, and the $\Delta\psi$ was monitored with $DiSC_3(5)$ (excitation wavelength, 643 nm; emission wavelength, 666 nm) at a final concentration of 5 μM. The was generated after the addition of glucose in the presence of the H^+/K^+ exchanger nigericin (final concentrations of 0.2% wt/vol and 5 μM, respectively). After reaching a steady-state $\Delta\psi$, the purified mundticin was added to the cells. The K^+ ionophore valinomycin (final concentration 2 μM) was used as a control for the absence of a membrane potential. Protein concentrations of bacteriocin preparations were determined with the Nano-Orange Protein Quantitation kit (Molecular Probes Europe).

Measurement of ATP concentrations. To determine the influence of mundticin on the intracellular and extracellular ATP concentrations of *L. monocytogenes* LDCD81-681, cell suspensions were prepared essentially as described above for the $\Delta\psi$ measurements, however, by the use of 50 mM potassium phosphate (KP_i) buffer (pH 7.0). Cell suspensions were diluted in this KP_i buffer (0.10 mg bacterial protein/ml) and incubated for 6 min at 30°C with glucose (final concentration of 0.5% [wt/vol]), prior to the addition of purified mundticin (final concentrations of 0.14 μg/ml). At regular time intervals, 0.2 ml samples of the suspensions were removed, and the cells were immediately separated from the external medium by spinning them through a layer of silicon oil, which was placed on top of a layer of 50 μl 10% (w/v) TCA with 2 mM EDTA (177). 5 μl aliquots of both aqueous layers were used to determine the ATP content using the firefly luciferase assay as described previously (135). Luminescence was recorded using a BIO-Orbit 1250 luminometer (Turku, Finland). Protein concentrations of bacterial cell suspensions were determined and by the method of Lowry et al. (133).

Identification of an oblique-orientated peptide and molecular modeling of peptide insertion into the lipid bilayer environment. The search for an oblique-orientated peptide was carried out by scanning the entire bacteriocin sequences according to the procedure of Rahman et al. (161). The hydrophobicity gradient for a 12-13 residue peptide window along the different bacteriocin sequences was calculated according to the method of Jähnig (110), using the Eisenberg's hydrophobicity scale (59). Any helical peptide with a minimal mean hydrophobicity of 0.2 and a hy-drophobicity gradient along the axis of the helical peptide between the C-terminal and N-terminal end of the peptide was considered as an acceptable candidate. Modeling of all peptides was carried out as described previously (26) and the method used is that applied to the study of amphiphilic molecule conformation (28). The method used for the prediction of the conformational structure of the peptides accounted for the contribution of classic energy (Van der Waals, electrostatic and hydrophobic binding energy) as well as the lipid-water interface properties, including the concomitant variation of the dielectric constant and the transfer energy of atoms from a hydrophobic to a hydrophilic environment (29). The structure, mode of insertion, and orientation of the peptides were predicted as in a hydrophobic-hydrophilic interface. In this model, the interaction energy was calculated and minimized until the lowest energy state of the entire peptide-lipid aggregate was reached. All calculations were performed on a Pentium Pro processor station, by using PC-TAMMO+ (Theoretical Analysis of Molecular Membrane Organization) and PC-PROT+ (Protein Plus Analysis) software. Graphs were drawn with the WINMGM program.

Accession number. The amino acid sequence of mundticin determined in this study has been deposited in the SWISS-PROT database under accession number P80925.

RESULTS

Characterization and purification of mundticin. A lactic acid bacterium with a broad spectrum of antimicrobial activity was isolated from minimally processed vegetables and identified as *Enterococcus mundtii* ATO6. The supernatant of *E. mundtii* ATO6 cultures exhibited strong antimicrobial activity against the indicator strain *Lactobacillus sake* DSM 20017, which reached a maximum after approximately 22 h of growth, yielding 2.6 x 10^6 BU/l culture fluid. Proteolytic treatment of the supernatant resulted in total loss of this activity, indicating the proteinaceous nature of the antimicrobial compound. After a two-step ammonium sulfate precipitation of the supernatant, with near total recovery of bacteriocin activity (99%) in the second step, the antimicrobial compound was purified to homogeneity by chromatographic techniques. Hydrophobic interaction chromatography and subsequent cationic exchange chromatography gave 60% and 55% recovery of the initial activity, respectively. A single peak eluted from the gelfiltration column (95% yield of activity) **(Peak i, Figure 1)**, which homogeneity was confirmed by reversed phase chromatography. The specific activity of the purified peptide against *L. sake* was 1500 BU/μg, and the final yield of

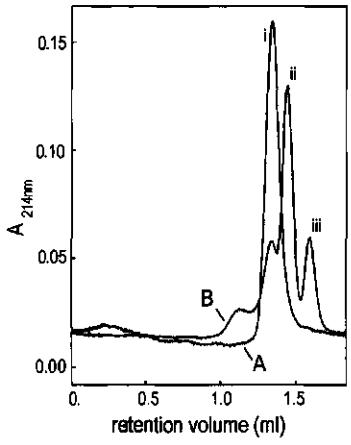

Figure 1.
Gelfiltration elution profile of **(A)** purified mundticin and **(B)** mundticin cleaved with Asp-N on a Superdex Peptide PC3.2/30 column.
Peak i, amino acids 1-43;
Peak ii, amino acids 17-43;
Peak iii, amino acids 1-16.

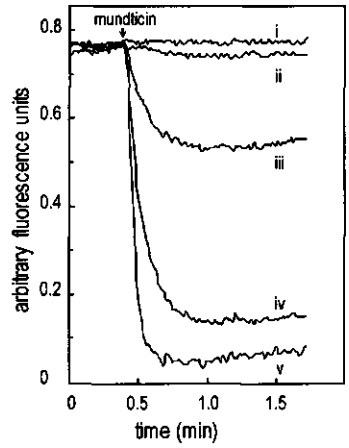

Figure 2.
Effect of mundticin on the membrane potential ($\Delta\psi$) of energized, nigericin-treated whole cells of *Listeria monocytogenes* LDCD81-861 in absence or presence of purified mundtcin: **i**, 0; **ii**, 1.4.10^{-3}; **iii**, 2.8.10^{-3}; **iv**, 1.4.10^{-2}; **v**, 0.14 μg mundticin/ml. *The $\Delta\psi$ was measured with the* fluorescent probe $DiSC_3(5)$. The absence of the $\Delta\psi$ was established at 0.05 arbitrary fluorescence units by the addition of valinomycin (2 μM).

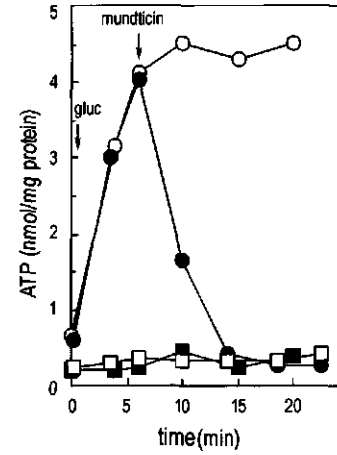

Figure 3.
Intracellular ATP concentrations (dots) or extracellular ATP concentrations (squares) of *L. monocytogenes* LDCD81-861 cells after the addition of glucose at t = 0 (gluc; final concentration 0.5% wt/vol), in absence (○□, control) or presence of (●■) *mundticin* (0.14 μg/ml). The values represent the average of duplicate determinations.

the peptide from the culture supernatant was 0.5 mg/liter. The antimicrobial activity of the peptide was not affected by heating for 15 min at 100°C, but prolonged heating for 1 h at 100°C resulted in a 50% loss of activity. The peptide was stable from pH 1 to 10 for 14 h at 4°C.

N-terminal amino acid sequence analysis of the purified bacteriocin revealed a 43 amino acid (a.a.) residue peptide, which was designated mundticin. Initial sequence analysis did not identify the residues at positions 9 and 14 and the residues at positions 37 and 40 to 43 could not unequivocally be appointed. To fully elucidate the primary structure of the bacteriocin, the peptide was cleaved with Asp-N specific endoprotease. The two resulting fragments were separated by gel-filtration chromatography, yielding two clear absorbance peaks as shown in **Figure 1 (Peak ii and iii)**. The full sequence of the C-terminal region was obtained after N-terminal amino acid sequencing of the larger fragment (a.a.17 to 43). Following reduction and alkylation with 4-vinyl pyridine of the uncleaved peptide, the residues at position 9 and 14 were identified as pyridethylated cysteinyl residues, rendering the complete primary structure of a novel bacteriocin, which was designated mundticin: KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK. The presence of a YGNGV motif in the N-terminus of this bacteriocin characterized it as a class IIa bacteriocin of LAB (121). The homology to other members of this class is described below. The average mass of mundticin as determined by electronspray mass spectrometry was 4287.21 ± 0.59 Da, which fully agrees with a calculated mass of 4287.8 in the presence of one disulfide bridge, linking the cysteine residues at position 9 and 14.

Mundticin targets the cytoplasmic membrane and dissipates the $\Delta\psi$. Mundticin was found to inhibit the growth of a wide range of Gram-positive bacteria at nanomolar concentrations. Specifically, this bacteriocin caused a 10,000-fold reduction of viable counts of the pathogen *L. monocytogenes* within 25 or 150 min at a concentration of 1.0 μg/ml or 0.1 μg/ml, respectively, which demonstrated its vigorous bactericidal action. Furthermore, mundticin prevented the outgrowth of spores and vegetative cells of toxin-producing strains of *Clostridium botulinum* and inhibited the growth of different species of LAB *(Lactobacillus salivarius, L. sake, Leuconostoc paramesenteroides, Leuc. mesenteroides, Carnobacterium piscicola, Pediococcus dextrinicus, P. pentosaseus, Enterococcus faecalis, E. hirae,* and *Listeria innocua).* We did not observe activity against Gram-negative bacteria and fungi.

Table 1. Sequence and properties of the tilted peptides identified in class **IIa** bacteriocins.

Bacteriocin	Sequence[a]	% homol	% ident	Ho	μH	Angle of insertion (°)	SwissProt accession nr
Mundticin	SVDWGKAIGIIGN	100	100	0.37	0.37	40	P80925
Piscicolin 126	TVDWSKAIGIIGN	100	85	0.33	0.33	45	P80569
Sakacin P	TVDWGTAIGNIG	83	75	0.42	0.41	35	P35618
Pediocin PA-1	SVDWGKATTCIIN	77	69	0.2	0.2	30	P29430
Leucosin A	SVNWGEAFSAGVH	69	37	0.32	0.26	30	P34034
Mesentericin Y105[37]	SVNWGEAASAGIH	69	37	0.3	0.23	35	P38577

[a] Sequence of residue 15 to 26 for sakacin P; sequence of residue 15 to 27 for the other bacteriocins.

The mean hydrophobicity (Ho) and the hydrophobic moment (μH) of the peptide was computed using the Eisenberg consensus scale (59). The angle of insertion in a lipid bilayer was calculated according to Brasseur (26).

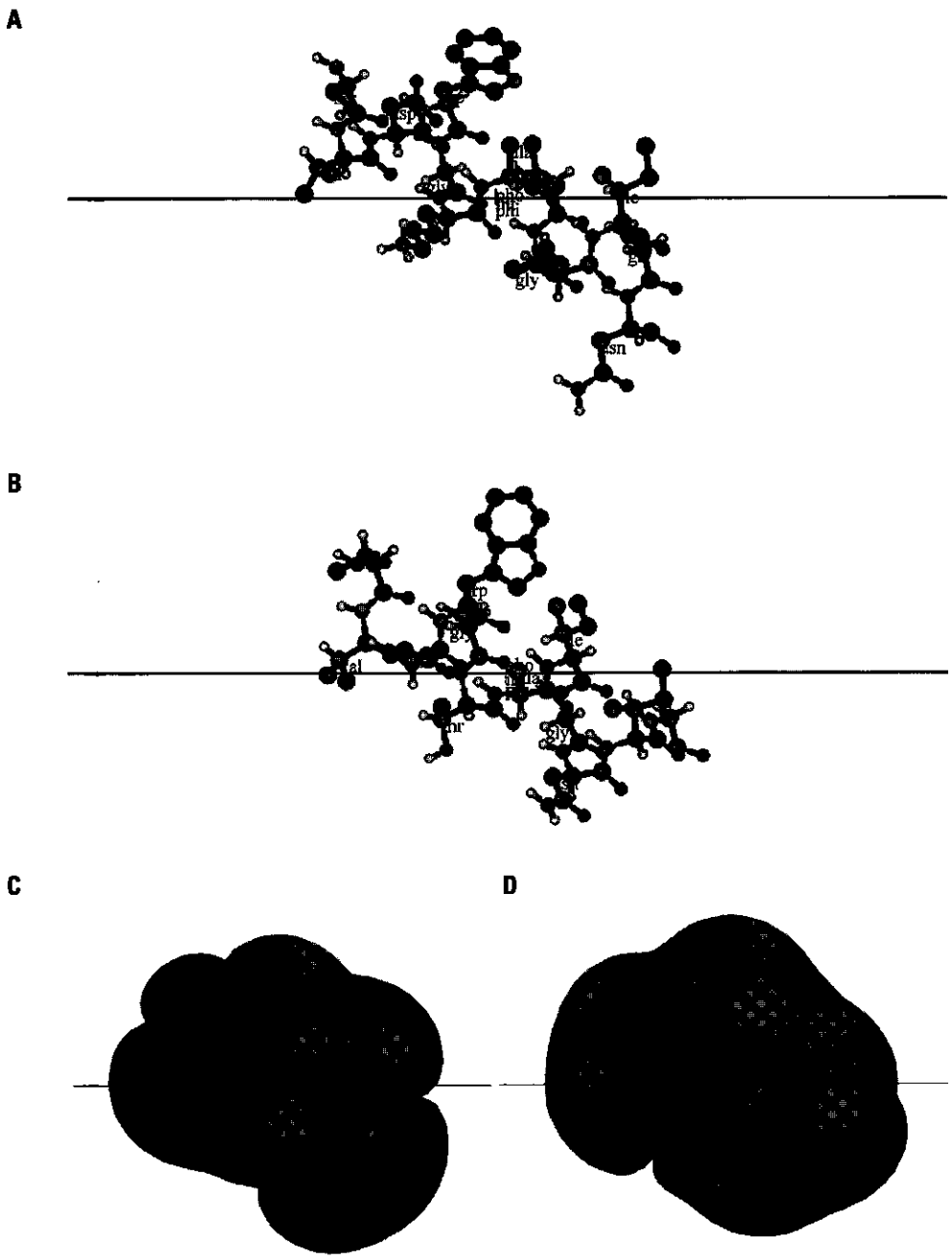

Figure 4.

Steric representation of the tilted peptide in the bacteriocins **(A)** mundticin (residue 15-27) and **(B)** sakacin P (residue 15-26) at a lipid (upper)/ water (lower) interface. The peptides are orientated at an angle of 40 and 45°, respectively.

The molecular hydrophobicity potential around these α-helical regions, which indicates both the hydrophobicity gradient along the peptide sequence and the magnitude of the hydrophobic envelope around the helix, is presented in panel **C** (mundticin) and **D** (sakacin P). The hydrophobic and hydrophilic envelopes are shown in light and dark grey, respectively.

To investigate the effect of mundticin on the cytoplasmic membrane, the membrane potential ($\Delta\psi$) of whole cells of *L. monocytogenes* LDCD81-861 was measured after exposure to the purified bacteriocin. At increasing bacteriocin concentrations, a more rapid $\Delta\psi$ dissipation was observed in conjunction with a lower final membrane potential **(Figure 2)**. The addition of mundticin to energized cells of the same strain at a concentration that caused the complete dissipation of the $\Delta\psi$ resulted in an almost complete loss of intracellular ATP within 10 min, in absence of ATP leakage **(Figure 3)**. The elimination of the growth of *L. monocytogenes* was achieved at 7 ng/ml (= MIC), which is a lower concentration than required for full dissipation of the $\Delta\psi$.

Identification of the oblique-orientated peptides in class IIa bacteriocins. A homology search for the entire sequence of mundticin through the SwissProt database rendered eight other class IIa bacteriocins of lactic acid bacteria with the characteristic YGNGV motif near the N-terminus: piscicolin 126 (P80569), sakacin P (P35618), pediocin PA-1 (P29430), leucocin A (P34034), mesentericin Y105 (P38577), carnobacteriocin B2 (P38580), sakacin A (P80097), and carnobacteriocin BM1 (P38579). The homology of these respective sequences to mundticin decreased from 82 to 47% and the identity decreased from 74 to 26%, as determined by multiple sequence alignment with the Clustal program (98). Overall, the sequence similarities were high at the N-terminal region (varying between 62 and 100% homology for the first 21 residues), but low for the remaining 22 residues, apart from residues 34 to 36.

The sequence of mundticin was entirely scanned to identify a peptide meeting the criteria of an oblique-orientated peptide (as defined in the materials and methods section). This analysis identified the Ser-15 to Asn-27 domain of mundticin as the best candidate, with a mean hydrophobicity of 0.37. Due to the hydrophobicity gradient between the more hydrophobic N-terminal end of the peptide and the more hydrophilic C-terminal residues, the peptide was calculated to insert in the lipid bilayer at an angle of 40° **(Table 1)**. Using the same criteria, oblique-orientated peptides

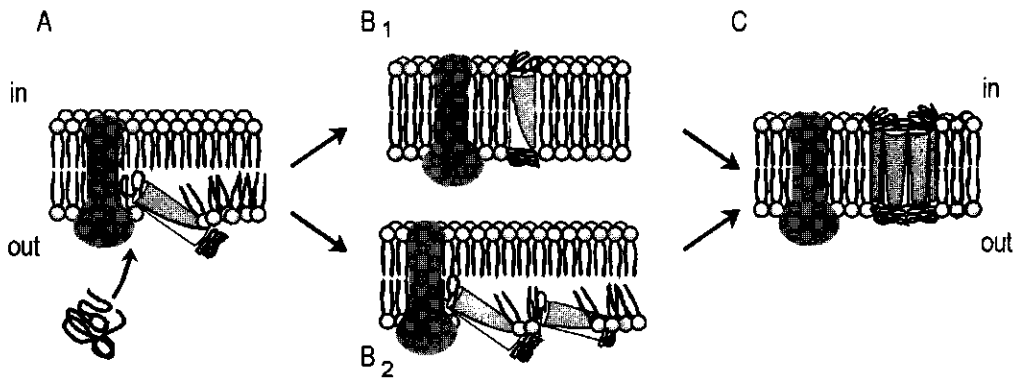

Figure 5.

Suggested initial steps of pore-formation by the YGNGV bacteriocins. Following interaction of the water soluble bacteriocin with a membrane protein receptor **(A)**, the α-helical region of the bacteriocin inserts at an approximate angle of 40° in the lipid bilayer of the target strain, thereby disrupting the regular parallel orientation of the phospholipids.
Subsequently, bacteriocin monomers insert into the membrane **(B1)**, followed by pore formation through aggregration of the monomers into oligomeric structures **(C)**; or alternatively, bacteriocin monomers aggregate at the surface **(B2)** and then form a water filled pore **(C)**.

were also identified in the other bacteriocins, and predicted to insert into the hydrophobic/hydrophilic interface with their N-termini at an approximate angle of 40° relative to a lipid/water interface (**Table 1**). The homology of the oblique orientated fragments of mundticin and the other bacteriocins varied between 69 and 100% (**Table 1**). Carnobacteriocin B2, carnobacteriocin BM1 and sakacin A were not included in this study, because of their low similarity of both the entire sequence, and the oblique peptide region (<34% identical; <50% homologous). In addition to mundticin and homologous class IIa bacteriocins, we also analyzed hybrid class IIa bacteriocins, that have been described by Fimland et al. (68) (SEE DISCUSSION).

The most probable conformation of the bacteriocin peptides at a hydrophobic/hydrophilic interface is illustrated for mundticin (residue 15 to 27) and sakacin P (residue 15 to 26) in **Figures 4A** and **4B**, respectively. Including the Asn at position 27 in the oblique segment of sakacin P (residue 15 to 27) rendered a slightly less hydrophobic peptide (Ho 0.32 and μH 0.32), with an angle of insertion of 55°, that thereby also met the criteria for a tilted peptide as defined in the materials and methods section. The conformations of the oblique segments of piscicolin 126, pediocin PA-1, leucocin A and mesenterocin Y105[37] were similar to that shown in **Figures 4A** and **4B** (data not shown). For each of these peptides, the hydrophobic Trp-18 was similarly positioned at the hydrophobic face of the simulated lipid/water interface as shown in **Figure 4**.

The molecular hydrophobicity potential (MHP) of the α-helix, which indicates both the hydrophobicity gradient along the peptide sequence and the magnitude of the hydrophobic envelope around the helix (26), is sterically represented for the oblique peptide of mundticin (residue 15 to 27) and the sakacin P segment (residue 15 to 26) in **Figure 4C** and **4D**, respectively. This illustrates the hydrophobicity gradient that underlies the oblique insertion of these peptides, which in turn perturbs the lipid acyl chains and disrupts the regular parallel orientation of the phospholipids.

DISCUSSION

We have identified the novel antimicrobial peptide, designated mundticin, that was found to belong to the class IIa bacteriocins of lactic acid bacteria (121). This study describes its isolation, characterization and antimicrobial action, and focuses on the initial steps involved in the pore-forming activity of mundticin and homologous bacteriocins. Molecular modeling revealed an oblique hydrophobic α-helical region in mundticin, that was calculated to insert at an angle of 40° at a hydrophobic/hydrophilic interface. Similarly, other class IIa bacteriocins were found to contain a oblique peptides, which were calculated to insert at angles varying between 30 and 45°. We hypothesize that the presence of this oblique peptide, due to the hydrophobicity gradient along the axis of the helical peptide, constitutes an important factor for bacteriocin permeation of the cytoplasmic membrane of target cells, similar to the destabilizing effects of tilted peptides in a variety of other processes in which membrane perturbation underlies the mode of action of the peptide in question (27).

Mundticin is a positively charged, hydrophobic, 43 amino acid peptide, which is produced and secreted by the Gram-positive bacterium *Enterococcus mundtii*. Our data indicate the presence of one disulfide bridge between Cys-9 and Cys-14, and the absence of further posttranslational modifications. In addition to the conserved N-terminal Tyr-Gly-Asn-Gly-Val (YGNGV) motif at posi-

tion 3 to 7, these two cysteines were also present in each of the homologous class IIa bacteriocins. Although this disulfide bridge was required for the activity of pediocin PA-1 and mesenterocin Y105[37] (39,69), it was reported to be not essential for activity of leucocin A and carnobacteriocin B2 (94,158).

A common feature of the YGNGV bacteriocins is their activity towards *Listeria monocytogenes*, a Gram-positive bacterium that can cause severe infections of the central nervous system (167). Mundticin was shown to inhibit the growth of a broad spectrum of bacteria, including the toxin producing *Clostridium botulinum*. Its good solubility in water, the absence of posttranslational modifications, and its stability over a wide pH and temperature range are favorable characteristics for a possible application as a food preservative. The bactericidal effect of mundticin on cells of *L. monocytogenes* was shown to be related to a rapid loss of the membrane potential, which indicated the dissipation of ionic gradients. In absence of ATP leakage, the observed reduction of the pool size of ATP can be explained by the accelerated consumption of ATP to regenerate the decreased PMF and/or by a shift in the ATP hydrolysis equilibrium resulting from phosphate efflux (88). The absence of ATP efflux furthermore indicates that the size exclusion limit of mundticin-induced pores is smaller than 500 Da. The action of mundticin hereby resembles that of the structurally related class IIa bacteriocins pediocin PA-1 (75% homologous) (37) and of mesenterocin Y105[37] (71% homologous) (136).

The class IIa bacteriocins are believed to interact with a membrane bound protein receptor prior to insertion into the cytoplasmic membrane in a membrane potential independent way (39). This mode of action resembles that of lactococcin A (class II) (187), but is different from a number of extensively posttranslationally modified lanthionine-containing class I bacteriocins that act in absence of a protein receptor in a voltage-dependent way (111). From recent studies on bacteriocins with a N-terminal YGNGV motif it became evident that mutations or deletions in both the N-terminus and the C-terminus of these peptide can cause complete loss of activity (69,160). The highly conserved N-terminal region has been suggested to form part of a recognition sequence for a putative membrane-bound protein receptor, and the more variable C-terminal region was put forward to determine the specificity for target organisms (68,160). Following interaction with the protein receptor, the actual pore formation by bacteriocins has been proposed to take effect through a 'barrel-stave' mechanism involving subsequent binding of monomers to the membrane, insertion into the membrane, and the aggregation of monomers in a 'barrel-staves' manner, surrounding a central core, analogous to a variety of pore-forming toxins (151,187).

The interactions of the YGNGV bacteriocins with the cytoplasmic membrane involves the electrostatical interaction of these cationic compounds with the phospholipid head groups, and hydrophobic interactions of the α-helix with the lipid acyl chains, but the exact interactions remain to be elucidated. Our results indicate that the bacteriocin mundticin and the highly homologous bacteriocins piscicolin 126, sakacin P, pediocin PA-1, leucocin A, and mesenterocin Y105[37] contain α-helical hydrophobic peptide regions that insert at an angle of 30 to 45° in the lipid/ water interface. This oblique orientation most likely destabilizes the phospholipid bilayers, and is thereby thought to facilitate the insertion and/or aggregation of bacteriocin molecules prior to forming pores in the cytoplasmic membrane of their target organisms. Interestingly, each of these oblique peptides contained a tryptophane residue at position 18, which was similarly positioned in the hydrophobic region of the hydrophobic/hydrophilic interface. The presence of this conserved hydrophobic residue is likely to constitute an important determinant in the action of these peptides.

The proposed involvement of an oblique peptide in the initial steps of pore formation by class IIa bacteriocins is schematically represented in **Figure 5**. Following interaction of the water-soluble bacteriocin with a putative membrane protein receptor, the α-helical region of the bacteriocin inserts at an angle of approximately 40° in the lipid bilayer of the target strain, thereby disrupting the regular parallel orientation of the phospholipids. Subsequently, bacteriocin monomers insert into the membrane, and pore formation occurs by aggregation of the monomers into oligomeric structures. Alternatively, bacteriocin monomers aggregate at the surface, where they destabilize the phospholipid bilayer, and then form a water filled pore. Our hypothesis was strengthened by analyzing data of other investigators. Fimland et al. (68) constructed hybrid bacteriocins from pediocin PA-1, sakacin P and curvacin A (sakacin A) by combining the N-terminal parts (residue 1 to 21) with the C-terminal parts (position 22 to end) of these peptides which involved their α-helical region. Each of the hybrid bacteriocins had an antimicrobial spectrum similar to the parent C-terminal bacteriocin. Interestingly though, Ped-Sak was as potent as the parent bacteriocin whereas the other hybrids exhibited 10 to 10000 times reduced activities (68). Our analysis of the constructed hybrids Ped-Sak, Sak-Ped, Sak-Cur and Cur-Sak revealed that only Ped-Sak had a hydrophobic oblique peptide with an angle of insertion of 45°. The hydrophobic α-helical regions in Sak-Ped and Sak-Cur had angles of insertion of 25 and 0°, respectively, indicating a near parallel or full parallel orientation to the hydrophobic-hydrophilic interface. In addition, Cur-Sak contained a less hydrophobic peptide with an angle of insertion of 70° that resembles a perpendicular orientation. Thus, the reduced activity of the latter three hybrids is likely related to the unfavorable angles of insertion of the α-helical region as compared to the parent bacteriocin (calculated to have angles of insertion of ca. 40°). This observation agrees with the proposed relevance of the oblique peptide in the initial steps of pore formation. The involvement of a tilted α-helix in the action of the YGNGV bacteriocins is further substantiated by data on leucocin A and mesentericin $Y105^{37}$, that have the same activity against various indicator strains (69). Although these bacteriocins differ at two residues, which are both situated in the oblique peptide region, their calculated angles of insertion in a bilayer are similar (ca. 30°), which agrees with their comparable action.

The present study identified the novel pore-forming bacteriocin mundticin and demonstrated its action on the cytoplasmic membrane of target cells. This bacteriocin was used as a prototype of class IIa bacteriocins of LAB to study the initial steps that are involved in pore formation. Our study indicates that not only mundticin, but also other members of the class IIa bacteriocins of LAB contain oblique α-helical regions. The variety of biological phenomena in which such domains have been identified to date (106,155,176,191) underlines that the perturbing effect of tilted peptides at hydrophobic/hydrophilic interfaces is a wide spread phenomenon in nature. The present analysis of oblique peptides may enable the rational design of molecules with enhanced or altered activities, and renders valuable information for the construction of bacteriocins with better preservative properties.

ACKNOWLEDGEMENTS

THE AUTHORS THANK WIM SCHAAPER AND TRUUS POSTMA FOR PROVIDING THE MASS SPECTRA; FRÉDOLIN VAN DER LECQ FOR PERFORMING THE N-TERMINAL AMINO ACID SEQUENCE ANALYSES; SANDRA STRINGER FOR THE TESTS WITH *C. botulinum*; AND JAN ALBERT KUIVENHOVEN FOR CRITICAL READING OF THE MANUSCRIPT.

CHAPTER 6

Interactions of nisin and pediocin PA-1 with closely related lactic acid bacteria that manifest over 100-fold differences in bacteriocin sensitivity

Marjon H.J. Bennik, Annette Verheul, Tjakko Abee, Geesje Naaktgeboren-Stoffels, Leon G.M. Gorris, and Eddy J. Smid

Applied and Environmental Microbiology (1997), volume 63, 3628-3636

ABSTRACT

The natural variation in the susceptibilities of Gram-positive bacteria towards the bacteriocins nisin and pediocin PA-1 is considerable. This study addresses the factors associated with this variability for closely related lactic acid bacteria. We compared two sets of nonbacteriocinogenic strains for which the MICs of nisin and pediocin PA-1 differed 100 to 1000-fold: *Lactobacillus sake* DSM20017 and *L. sake* DSM20497; and *Pediococcus dextrinicus* and *P. pentosaceus*. Strikingly, the bacteriocin-sensitive and -insensitive strains showed a similar concentration-dependent dissipation of their membrane potential ($\Delta\psi$) after exposure to these bacteriocins. The bacteriocin-induced dissipation of $\Delta\psi$ below their MICs for the insensitive strains did not coincide with a reduction of intracellular ATP pools and glycolysis rates. This was not observed with the sensitive strains. Analysis of membrane lipid properties revealed minor differences in the phospho- and glycolipid compositions of both sets of strains. The interactions of the bacteriocins with strain-specific lipids were not significantly different in a lipid monolayer assay. Further lipid analysis revealed a higher *in situ* membrane fluidity of the bacteriocin-sensitive *Pediococcus* strain compared with that for the insensitive strain, but the opposite was found for the *L. sake* strains. Our results provide evidence that the association of bacteriocins with the cell membrane and their subsequent insertion takes place in a similar way whether cells have a high or a low natural tolerance towards bacteriocins. For insensitive strains, overall membrane constitution rather than mere membrane fluidity may preclude the formation of pores with sufficient diameters and lifetimes to ultimately cause cell death.

INTRODUCTION

Bacteriocins from lactic acid bacteria (LAB) are ribosomally synthesized antimicrobial peptides of a cationic, amphiphilic nature. Their antimicrobial activity is directed towards Gram-positive bacteria while the cells that produce bacteriocins are insensitive (33,127,159). The potential of these peptides for ensuring the microbial safety and quality of various food products has provoked great interest (reviewed in reference 172). Three major classes of bacteriocins have been distinguished: lantibiotics (class I), small heat-stable nonlantibiotics (class II) and large heat labile proteins (class III) (121,147). Nisin is the best-characterized class I bacteriocin of LAB (111) and to date the only bacteriocin that has been accepted by the WORLD HEALTH ORGANIZATION as a preservative

in foods. The action of pediocin PA-1, a class II bacteriocin which is identical to pediocin AcH (96,145), has been described in detail by several investigators (33,39). Class I and II bacteriocins are believed to form pores in the membranes of target cells through a "barrel-stave" mechanism, which involves the binding of monomers to the membrane, their insertion into the membrane, and finally their aggregation, leading to the formation of pores surrounding a central core (151,187). Alternatively, a "wedge" model has been proposed as a mode of action for nisin, whereby monomers associate with the membrane surface and cause destabilization of the bilayer structure, thereby promoting the formation of a wedge-state pore (57). Pore formation by bacteriocins causes a rapid efflux of small cytoplasmic molecules and ions from the target cells and the collapse of the proton motive force (PMF), which may lead to cell death (39,72,164). Yet, the molecular mechanism by which nisin and pediocin PA-1 act, appears to differ considerably. Whereas nisin is believed to inhibit the growth of sensitive organisms in a voltage-dependent way in the absence of a protein receptor (57,72), pediocin PA-1 has been suggested to act in a voltage-independent protein receptor-mediated way (33,39). However, it was demonstrated recently that pediocin PA-1 can function in absence of a protein receptor (38).

The success of the application of bacteriocins or bacteriocinogenic LAB to eliminate or inhibit the growth of pathogenic or spoilage bacteria in complex food systems depends on multiple factors. Firstly, the effectiveness of the excreted bacteriocins can be compromised by proteolytic cleavage through the action of enzymes originating from the product or from endogenous microflora. In addition, the prolonged exposure of bacteria to bacteriocins may induce resistance to these compounds (47,142). Moreover, bacteriocinogenic LAB used for the suppression of pathogens need a competitive advantage over phylogenetic-related genera (or species) that may be present in the same ecological niche. The excretion of a bacteriocin may provide such an advantage, but the inhibitory action of these antimicrobial peptides against Gram-positive bacteria can vary considerably between different genera, species of genera, identical species, and even identical cultures under different environmental conditions (4,93,121). Adequate control of the growth of the pathogens and the competing microflora requires extensive knowledge of factors that determine bacteriocin susceptibility.

Thus far, the majority of studies on bacteriocin susceptibility have been performed with bacteriocin-sensitive strains and their variants which gained bacteriocin resistance through serial passages in bacteriocin-containing media. This has been shown to yield cultures of trained variants for which MICs are 2 to 10 times higher than those for the original cultures (47,92,137,142). Both cell wall constitution and membrane lipid composition have been demonstrated to be involved in such acquired bacteriocin resistance (48,137,142). However, observations that the stability of this acquired resistance can be variable and may be lost upon subculturing in medium without the bacteriocin (142,163) indicate that trained bacteriocin resistance might be related to a more general adaptive response of the cells. The factors that account for the naturally occurring variability in bacteriocin susceptibility have yet to be clarified.

The present study was conducted to obtain better insight into the natural variation in bacteriocin sensitivity of closely related LAB that can be present in the same ecological niche. Therefore, two sets of strains with 100- to 1000-fold differences in sensitivity were selected. Having studied the effect of nisin and pediocin PA-1 on the glycolysis, intracellular ATP (ATP_{in}) pools, and the membrane potential ($\Delta\psi$) of whole cells in conjunction with membrane lipid properties, we suggest that the differences in bacteriocin susceptibility of LAB are associated not only with variation in membrane lipid composition but also with the overall membrane constitution.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The LAB strains used throughout this study were *Pediococcus dextrinicus* DSM20335 which is bacteriocin sensitive (*P. dextr.*^{SENS}), *Pediococcus pentosaceus* DSM20336 which is bacteriocin insensitive (*P. pent.*^{INSENS}), *Lactobacillus sake* DSM20017 (*L. sake*^{SENS}) (all type strains), and *L. sake* DSM20497 (*L. sake*^{INSENS}). The identity of the latter strain has previously been confirmed by DNA-DNA homology analysis (114). Strains that were used in the initial screening for bacteriocin sensitivity are listed in **Table 1**. All *Lactobacillus*, *Leuconostoc*, and *Pediococcus* strains were grown in MRS broth (Oxoid, Basingstoke, England). Strains of *Carnobacterium*, *Enterococcus*, *Lactococcus* and *Streptococcus* were grown in M17 broth (Oxoid) supplemented with 0.5% (wt/ vol) glucose (GM17). The following bacteriocin producing strains were used: *Lactococcus lactis* subsp. *lactis* NCDO 497, which produces nisin A; *Pediococcus acidilactici* PAC1.0, which produces pediocin PA-1 (139) (provided by Dr. A. M. Ledeboer, Unilever Research Laboratory, Vlaardingen, The Netherlands); *Carnobacterium piscicola* UI49, which produces carnobacteriocin UI49 (173); *L. sake* DSM6333, which produces sakacin A (101); *Enterococcus mundtii* ATO6, which produces mundticin (15). Cultivations were performed statically at 30°C unless stated otherwise.

Bacteriocin preparations. Strains producing nisin A or pediocin PA-1 were grown to the late exponential phase. After the removal of the cells by centrifugation, the supernatant was adjusted to pH 6.0 and filter sterilized, with 0.2 μm pore-size filters (Costar, Cambridge, Mass.). The supernatant containing pediocin PA-1 was concentrated 10-fold by freeze drying and resuspension in water, while the supernatant containing nisin was used directly to determine the sensitivities of various LAB **(see Table 1)** towards these bacteriocins.

For further experiments, pediocin PA-1 was purified from culture supernatant of *P. acidilactici* PAC1.0 as described by Bennik et al. (13); after ammonium sulfate precipitation, pediocin PA-1 was purified to homogeneity by hydrophobic interaction-, cation-exchange-, and gelfiltration chromatography. Nisin A was purified from a commercially available preparation containing 2.5% nisin (Sigma Chemical Co., St. Louis, Mo.), which was dissolved in water. After precipitation of the whey proteins from this solution by centrifugation, nisin was purified to homogeneity by using the hydrophobic-interaction, cation-exchange and gelfiltration columns as described previously (13).

The purified nisin and pediocin PA-1 preparations were desalted by ultrafiltration (molecular weight cutoff 3000, Costar) with 10 mM potassium 2-(*N*-morpholino) ethanesulfonic acid (K-MES) buffer (pH 5.5) containing 20% ethanol. The homogeneity of the final samples was confirmed by reversed phase chromatography on a C_2-C_{18} μRPC 3.2/30 column (Smart System) with a linear gradient (20% to 95% ethylalcohol-0.1% trifluoroacetic acid in 30 min, 100 $\mu l.min^{-1}$). All purification steps were performed at room temperature. All chromatographic equipment was obtained from Pharmacia Biotechnology, Uppsala, Sweden.

Determination of bacteriocin sensitivity and MICs. Bacteriocin sensitivities of indicator organisms were determined in duplicate by a critical dilution assay. Twofold diluted bacteriocin solutions (50 μl) were prepared in MRS or GM17 broth by using 96-well microtiter plates (Costar). The wells were filled with a mixture of the indicator strain (grown to optical density at 660 nm [OD_{660}] of 0.9) and the appropriate culture medium at a ratio of 1:9 to a final volume of 200 μl. After 6 h of incubation at 30°C, growth was evaluated by measuring the OD_{655} in a microplate reader (Model 3550-UV, Bio-Rad, Richmond, Calif.). The bacteriocin sensitivities of the tested strains were calculated from the reciprocal of the highest dilution of the bacteriocin preparation that inhibited growth of the indicator strain by 50%, and expressed in bacteriocin units per milliliter ($BU.ml^{-1}$). Furthermore, the MIC of nisin or pediocin PA-1 was calculated from the lowest concentration at which the growth of the indicator was inhibited.

Bacteriocin degradation by extracellular protease. Extracellular proteases were extracted from cells of bacteriocin-sensitive and -insensitive strains by the method of Mills and Thomas (141). Cells were grown in 500 ml of MRS broth

containing 10 mM $CaCl_2$ at 30°C, harvested at an OD_{660} of 0.7, and washed twice in 50 mM K-MES - 10 mM $CaCl_2$ buffer (pH 6.3). Cells were resuspended in 1 ml of 20 mM Tris HCl (pH 7.8) in the absence of $CaCl_2$ and incubated without stirring for 30 min at 30°C. After the removal of cells by centrifugation, the supernatant containing extracellular proteases was kept on ice. After the protein concentration was determined, the extracts were diluted in 50 mM KMES (pH 6.0) to a final concentration of 0.2 $mg.ml^{-1}$ of protein. Directly afterwards, purified nisin (130 $\mu g.ml^{-1}$) was incubated with these solutions, with final concentrations of 13 μg of nisin per ml and 0.1 mg of protein per ml of the enzyme extracts. Following incubation for 2 h at 30°C, solutions of nisin incubated with the protein extracts, the enzyme extracts alone, or nisin without the protein extracts were analyzed on a Superdex Peptide PC 3.2/30 gelfiltration column as described previously (13), and antimicrobial activity was monitored.

Determination of glycolysis rate. Exponential-phase cultures (OD_{660} of 0.8) of selected LAB were harvested by centrifugation and washed twice in a 0.5 mM potassium-piperazine-*N*-*N*'-(2-ethanesulfonic acid) (K-PIPES), 0.5 mM K-HEPES, 0.5 mM K-MES buffer (polybuffer pH 6.0 or 7.6) containing 50 mM KCl. (Cultures were grown to an OD_{660} of 0.8 instead of 0.7. The viability of these cells was better on ice; thereby, the reproducibility of the assay was increased. Freshly harvested cells at OD_{660} of 0.7 and 0.8 had the same responses). The cells were concentrated (≈ 50-fold) and kept on ice until use. Washed cells were diluted in the appropriate buffer (pH 6.0 or 7.6) in a temperature-regulated vessel (5 ml at 30°C) to a density of 0.2 mg of bacterial protein per ml. Following equilibration, the cells were energized with the fermentable substrate glucose (final concentration of 0.2% [wt/vol]). The recorded pH changes were converted into micromoles of H^+ released by calibration of the buffer with a standardized HCl solution.

Glycolysis rates were determined in the presence or absence of purified nisin, purified pediocin PA-1, or the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Fivefold serial dilutions of these substances were added to the above mentioned vessel, with the highest final concentrations being 5 and 0.80 $\mu g.ml^{-1}$ and 100 μM, respectively. For the experiments performed in polybuffer with a pH of 6.0, additions were made at a pH of 5.7; when the polybuffer had a pH of 7.6, additions were made at a pH of 7.3.

Acid analysis. Acids produced by LAB during glycolysis were analyzed by high pressure liquid chromatography by the method of Gosselink et al. (84) with an Ionpak KC-811 (300 by 8 mm [inside diameter]; Shodex, Tokyo, Japan) packed with strong cation-exchange resin gels. Acids produced by LAB during glycolysis were determined under standard glycolysis conditions in the presence or absence of CCCP (final concentrations of 10 or 100 μM, respectively), with polybuffer of pH 6.0. Samples (0.5 ml) were taken after 15 and 45 min of incubation. After removal of the cells by centrifugation, the acid composition of 50 μl of filtered (0.2 μm) supernatant was analyzed.

Intracellular pH measurements. The intracellular pH (pH_{in}) of the LAB strains in the absence or presence of CCCP (maximum, 10 μM; 5fold serial diluted) was determined based on the method of Breeuwer et al. (31). Harvested cells (OD_{660} of 0.7) were washed and resuspended in 50 mM K- HEPES buffer (pH 8.0). Subsequently, the cells were incubated for 10 min at 30°C in the presence of 1 μM carboxyfluorescein diacetate succinimidyl ester (cFDASE) (Molecular Probes Europe, Leiden, The Netherlands), washed, and resuspended in 50 mM potassium phosphate buffer (pH 6.5). In the cytoplasm, cFDASE is hydrolyzed to carboxyfluorescein succinimidyl ester (cFSE) and subsequently conjugated to aliphatic amines. To eliminate nonconjugated cFSE, the cells were incubated in the presence of glucose (0.2% wt/vol). After being washed, the cells were diluted to a concentration of 0.013 mg of bacterial protein per ml in a 3 ml glass cuvette and placed in the cuvette holder of the spectrofluorometer (equipped with a stirrer and a thermostate) (Perkin-Elmer LS 50B). Fluorescence intensities at the emission wavelength of 530 nm were measured at excitation wavelengths of 500 and 440 nm by rapidly altering the monochromator between the two wavelengths. The intracellular pH was calculated from the 500 to-440 ratio's as described previously (31).

Determination of ATP_{in} and ATP_{out}. Exponential-growth-phase cells of bacteriocin-sensitive and -insensitive LAB (OD_{660} of 0.8) were harvested, washed twice in 50 mM potassium phosphate buffer (pH 5.7 or 7.3), and concentrated

(≈ 50 fold). Washed cells were subsequently diluted in phosphate buffer (pH 5.7 or 7.3) to a concentration of 0.2 mg of protein per ml in a temperature-controlled vessel at 30°C. Glucose was added to a final concentration of 0.5% (wt/vol), and after 5 min of incubation, purified nisin (final concentrations of 0.2 or 5 $\mu g.ml^{-1}$), purified pediocin (final concentrations of 0.8 or 0.08 $\mu g.ml^{-1}$), or CCCP (final concentration of 20 μM) was added. A control experiment was run. ATP_{in} and ATP_{out} concentrations were determined at regular time intervals by separating the cells from the external medium by silicon oil centrifugation (177). Specifically, samples (500 μl) from a cell suspension were transferred to microcentrifugation tubes containing 750 μl of a 2:1 mixture of silicon oil AR200 (ρ = 1.05 $g.ml^{-1}$) and silicon oil AR20 (ρ = 0.96 $g.ml^{-1}$) (Wacker Chemicals, Munich, Germany) on top of a layer of 100 μl of 10% (wt/vol) trichloroacetic acid with 2 mM EDTA. The cells were spun through the silicon oil for 5 min at 12000 x *g*, and samples (5 μl) of both aqueous layers were taken to determine the ATP content by using the firefly luciferase assay as described by Lundin and Thore (135). Luminescence was recorded using a model 1250 luminometer (BIO-Orbit, Turku, Finland).

Monitoring the $\Delta\psi$ with a fluorescent probe. Cells of selected LAB (OD_{660} of 0.8) were washed twice in 50 mM K-HEPES buffer (pH 7.0), concentrated (≈ 50-fold), and stored on ice until use. Washed cells were diluted (≈ 200-fold) in this buffer, which contained the fluorescent dye 3,3 dipropylthiacarbocyanine ($DiSC_3[5]$) (5 μM) (Molecular Probes Europe), to a density of 0.05 mg of bacterial protein per ml.$\Delta\psi$ was monitored with $DiSC_3(5)$ (excitation wavelength, 643 nm; emission wavelength, 666 nm) with a Perkin Elmer LS 50B spectrofluorometer at 25°C. Following equilibration, the $\Delta\psi$ was built up by the addition of glucose (final concentration 0.2% wt/vol) in the presence of the K^+/H^+ exchanger nigericin (5 μM). At the maximum $\Delta\psi$, purified nisin (final concentrations of 7.8×10^{-1}, 7.8×10^{-2}, or 7.8×10^{-3} $\mu g.ml^{-1}$) or pediocin PA-1 (final concentration of 9.3×10^{-2}, 4.7×10^{-2}, or 2.3×10^{-2} $\mu g.ml^{-1}$) was added to the cells. Valinomycin (2 μM) was used as a control.

Lipid extraction and analysis. Lipids extractions of LAB were performed by the method of Bligh and Dyer (22) as detailed by Kates (117). Exponential-growth-phase cells (OD_{660} of 0.7) of 2 liter cultures were harvested by centrifugation, washed twice in 50 mM potassium phosphate buffer (pH 6.9) containing 5 mM $MgSO_4$, and concentrated 250-fold. Prior to lipid extraction, concentrated cell suspensions were incubated with lysozyme (3 mg) and mutanolysin (150 U) for 45 min at 37°C to degrade glycosidic bonds of the cell wall glycan layer.

The lipid composition of the extracts was determined by thin layer chromatography (TLC) (silica 60, Fertigplatten, Merck, Germany) with chloroform-methanol-acetic acid-water (85:15:10:3.5 by volume). General lipid staining was performed with I_2 vapor, glycolipids were indicated by spraying with α-naphtol (0.5 g in 100 ml of methanol-water = 1:1), and phospholipids were visualized with sulfuric acid in water (95% [vol/vol]). Plates were heated at 90°C until maximal staining intensity of the glycolipids (blue-purple) and the polar lipids (yellow) was observed (117). The R_f values of individual spots were compared to the reference phospholipids diphosphatidylglyceride (DPG or cardiolipin), phosphatidylglyceride (PG), phosphatidyl choline, phosphatidylethanolamine, phosphatidyl serine, and lysophosphatidylglyceride (LPG) and the glycolipids monogalactosyldiacylglycerol and digalactosyldiacylglycerol (Sigma). These reference lipids included phospholipids previously reported to be present in lactobacilli and pediococci, *i.e.*, PG, DPG, LPG, and phosphatidic acid (58,149).

Analysis of the fatty acyl chains of lipid extracts was performed in duplicate by gas-liquid chromatography (GLC). Aliquots of the total lipid extracts were used for the preparation of fatty acid methyl esters by transmethylation with concentrated 2.5% H_2SO_4 (vol/vol) in anhydrous methanol. The fatty acid methyl esters were extracted with petroleum ether (boiling point, 60 to 80°C) and subsequently separated isothermally at 165°C by GLC with a Perkin-Elmer F33 gas chromatograph equipped with a glass column (1 m by 4 mm [inside diameter]) packed with 5% (wt/wt) SP2100 (Supelco, Bellefonte, Pa.) as the stationary phase. Fatty acid methyl ester compositions were determined by measuring peak areas after identification with authentic standards and comparison of retention times.

Monolayer experiments. The change in surface pressure of bacterial lipid monolayers after the addition of bacterio-

cin was considered as a measure of penetration of the peptide into the surface lipid phase by using the Wilhelmy plate method at 30°C (70). Monolayers with initial surface pressures of 18 to 40 $mN.m^{-1}$ were formed by spreading purified bacterial total lipid extracts (dissolved in chloroform) on a subphase of 10 mM Tris buffer (pH 7.4). These purified lipid extracts were obtained by the conversion of the anionic lipids to their sodium salts and the removal of proteins from the lipid extract by silica column chromatography (119). A saturating concentration of purified nisin or pediocin PA-1 (1 $\mu g.ml^{-1}$) was added to the continuously stirred subphase through a small injection channel. The increase in surface pressure as a result of the added bacteriocin was monitored until the surface pressure became stable.

Whole cell membrane lipid phase transition temperature. For each strain of LAB, two separate batches of exponential-growth-phase cells (OD_{660} of 0.7) were harvested and washed twice with 50 mM potassium buffer (pH 6.0). The wet bacterial pellet was placed between two CaF_2 windows and subsequently, Fourier transform infrared (FTIR) spectra were recorded on a Perkin-Elmer 1725 spectrometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector and a Perkin-Elmer microscope interfaced to a personal computer. The FTIR spectra to study the phase-transition temperature of lipids in whole cells were recorded in a temperature-controlled cell. After the samples were cooled to -50°C, they were slowly heated (1°C/min), and a spectrum was recorded every 2-3°C. Each spectrum was the average of 32 scans at each temperature. Membrane fluidity was monitored by observing the band position of the CH_2 symmetric stretch band around 2850 cm^{-1}. The spectral region between 3000 and 2800 cm^{-1} was selected, and second derivative spectra were calculated. The second derivative spectrum was normalized, and the band position was calculated as the average of the spectral positions at 80% of the total peak height. Plotting of the wave number (expressed per cm) against temperature resulted in a curve from which the phase transition temperature was determined.

Protein determination. Protein concentrations of bacterial cells were determined by the method of Lowry et al. (133). Protein concentrations of purified bacteriocin preparations were assayed by using the NanoOrange Protein Quantitation kit (Molecular Probes Europe) according to the manufacturer's instructions. In both assays, bovine serum albumin was used as a standard.

RESULTS

Sensitivity of LAB to bacteriocins. To select strains with high and low bacteriocin sensitivities, twenty strains of LAB were examined for their sensitivities towards nisin and pediocin PA-1 in a critical dilution assay **(Table 1)**. Seven strains were relatively insensitive towards the two bacteriocins, three strains showed high sensitivity, and the remaining ten strains were moderately sensitive. Two strains of identical species with the highest difference in bacteriocin sensitivity were selected for further studies: the bacteriocin sensitive *L. sake* DSM20017 (*L. sake*^{SENS}) and the relatively bacteriocin-insensitive strain *L. sake* DSM20497 (*L. sake*^{INSENS}). In addition, two different species of the same genus were selected, namely the bacteriocin-sensitive *P. dextrinicus* DSM20335 (*P. dextr.*^{SENS}) and the bacteriocin-insensitive *P. pentosaceus* (*P. pent.*^{INSENS}), based on the same criterion. The difference in the bacteriocin sensitivities of the strains is reflected by the different MICs of the bacteriocins for the strains **(Table 2)**. Similar nisin and pediocin PA-1 concentrations were needed to inhibit the growth of the two selected sensitive strains, whereas 100- to 1000-fold higher concentrations were required to inhibit the growth of the insensitive strains. Full inhibition of the growth of the insensitive strains by the protonophore CCCP was observed at concentrations only twofold higher than those required for the sensitive strains **(Table 2)**. Production of bacteriocins by the four selected strains could not be detected with the indicator strains listed in **Table 1.** We tested the sensitivities of the four selected strains towards the class I bacteriocin carnobacteriocin UI49 (173) and two

Table 1. Sensitivity of indicator organisms towards bacteriocins as determined by a critical dilution assay.

Indicator organism[a]	Sensitivity (BU.ml^{-1})[b] towards	
	Nisin	Pediocin PA-1
Carnobacterium gallinarum NCDO2766	20	10240
Enterococcus faecalis DSM20478[t]	< 20	< 20
Lactococcus lactis subsp. *lactis* NCDO497	< 20	< 20
Lactococcus lactis subsp. *lactis* WAU IL1403 pMB580	640	20
Lactobacillus acidophilus UOT705-R-1	160	< 20
Lactobacillus fermentum UOT22-0-6	160	< 20
Lactobacillus plantarum ATCC8014[t]	320	1280
Lactobacillus sake DSM20017[t, c]	40960	81920
Lactobacillus sake DSM6333	640	640
Lactobacillus sake DSM20497[c]	40	80
Lactobacillus sake DSM20498	320	320
Lactobacillus sake IFO3541	640	640
Lactobacillus sake IFO12456	6240	20480
Lactobacillus sake NCFB2812	1280	160
Lactobacillus sake NCFB2813	160	1280
Lactobacillus sake NCFB2814	1280	160
Leuconostoc mesenteroides subsp. *mes.* DSM20343[t]	640	10240
Pediococcus dextrinicus DSM20335[t, c]	20480	40960
Pediococcus pentosaceus DSM20336[t, c]	80	160
Streptococcus mutans DSM20523[t]	< 20	< 20

[a] Sources: NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; WAU, Wageningen Agricultural University, Wageningen, The Netherlands; UOT, University of Otaga, Otaga, Iceland; ATCC, American Type Culture Collection, Rockville, Md.; IFO, Institute for Fermentation, Osaka, Japan; NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

[b] BU.ml^{-1}, bacteriocin units per milliliter (FURTHER DEFINED IN MATERIALS AND METHODS).

[c] Strain used throughout the study, and designated as follows: *L. sake* DSM20017 = *L. sake*^{SENS}, *L. sake* DSM20497 = *L. sake*^{INSENS}, *P. dextrinicus* DSM20335 = *P. dextr.*^{SENS}, *P. pentosaceus* DSM20336 = *P. pent.*^{INSENS}.

[t] Type strain.

Table 2. MICs of nisin, pediocin PA-1 and CCCP for selected strains.

Strain	MIC[a] of:		
	Nisin (mg.liter^{-1})	Pediocin PA-1 (mg.liter^{-1})	CCCP (μM)
L. sake^{SENS}	3.0×10^{-3}	2.3×10^{-3}	39
L. sake^{INSENS}	3.06	1.19	78
P. dextr.^{SENS}	6.0×10^{-3}	4.6×10^{-3}	39
P. pent.^{INSENS}	1.53	0.59	78

[a] Mean values of duplicate determinations are presented.

class IIa bacteriocins: sakacin A (101) and mundticin (a novel bacteriocin) (15) in addition to nisin and pediocin PA-1. This indicated similar differences in the sensitivities of the sensitive and insensitive strains (data not shown).

Bacteriocin degradation. Since the inactivation of bacteriocins by extracellular proteolytic activity could explain differences in the bacteriocin sensitivities, peptide cleavage was tested by the coincubation of nisin with extracellular proteins extracted from each of the four selected strains. No degradation of nisin or decrease in antimicrobial activity was observed after 2 h of incubation at 30°C for any of the strains.

Effect of bacteriocins on glycolysis. The insertion of bacteriocins into the cell membrane can result in the dissipation of the PMF and the efflux of cytoplasmic components (32,39). To evaluate differences in the bacteriocin sensitivities of the selected strains, the effect of bacteriocins on the glycolytic activities of cells was analyzed. Exponential-growth-phase, glycolyzing cells which were

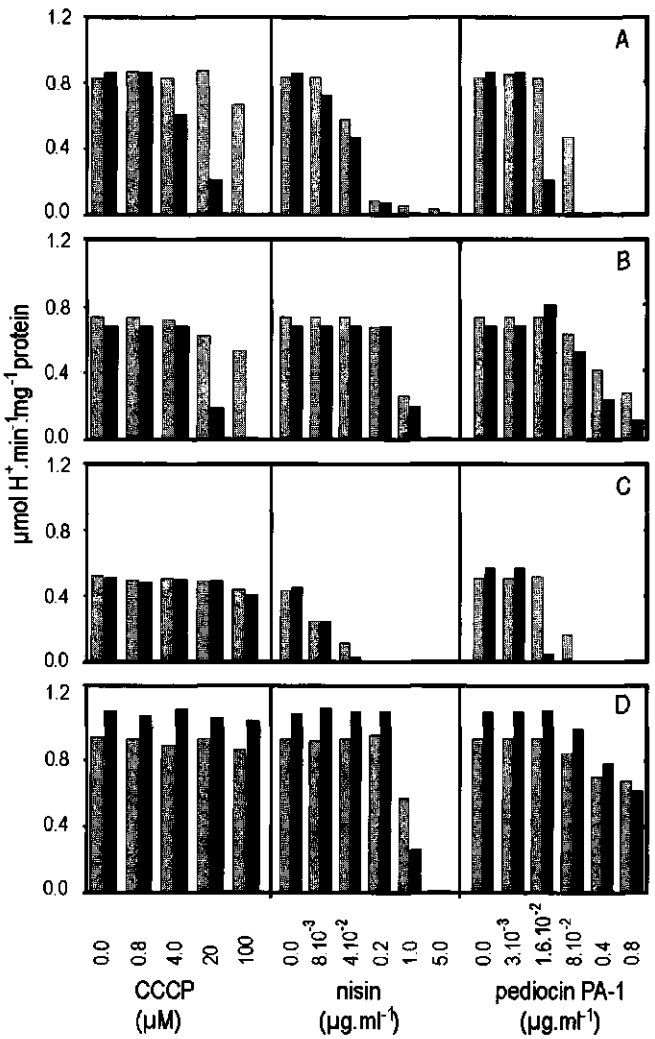

Figure 1.
Effects of CCCP, nisin, and pediocin PA-1 on the glycolysis of *L. sake*^{SENS} **(A)**, *L. sake*^{INSENS} **(B)**, *P. dextr.*^{SENS} **(C)**, and *P. pent.*^{INSENS} **(D)**.
Different concentrations of CCCP, nisin, and pediocin PA-1 were added to exponential-growth-phase cells of the selected strains, which were resuspended in a 0.5 mM polybuffer of pH 7.3 (grey bars) or pH 5.7 (black bars) to a final concentration of 0.2 mg of bacterial protein.ml^{-1}.
Bars represent the average results of duplicate experiments.

energized with the fermentable substrate glucose (referred to hereafter as glucose-energized cells) were therefore exposed to different concentrations of nisin and pediocin PA-1 at extracellular pHs (pH_{out}) of 5.7 and 7.3, respectively. The direct effect of the PMF dissipation on the glycolysis of cells was determined by the addition of the protonophore CCCP.

First, the actual insertion of CCCP into the cytoplasmic membrane was assessed by the measurement of the intracellular pH (pH_{in}) of the glucose-energized cells with the fluorescent probe cFSE. The rapid dissipation of the pH gradient confirmed the action of CCCP against each of the selected strains at concentrations exceeding 10 μM (data not shown). The influence of CCCP on the glycolytic activities of these strains is represented in **Figure 1 (left panels)**. Exposure of the two *L. sake* strains to the highest CCCP concentration at a pH_{out} of 7.3 resulted in only slightly inhibited glycolysis, whereas CCCP added at a pH_{out} of 5.7 caused a significant reduction in the glycolysis rates at concentrations of 4 to 20 μM. By contrast, the glycolysis of *P. dextr.*^{SENS} and *P. pent.*^{INSENS} was not inhibited by CCCP at either of the pH values tested.

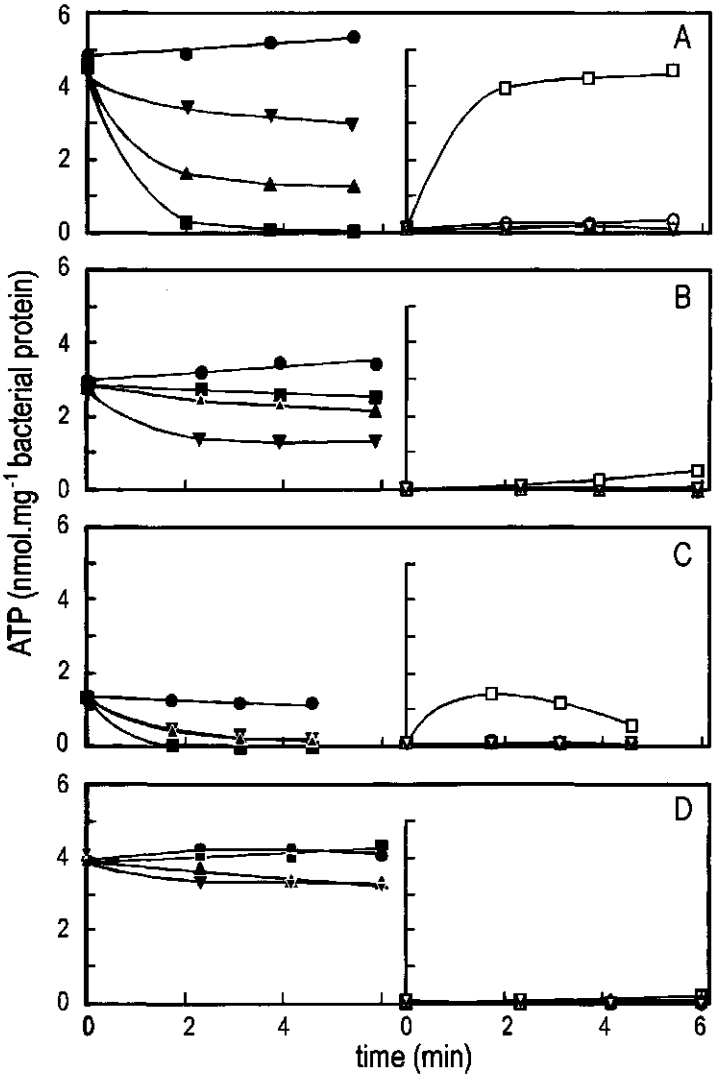

Figure 2.
ATP_{in} concentrations (closed symbols) and ATP_{out} concentrations (open symbols) of glucose-energized, exponential-growth-phase cells of *L. sake*^{SENS} **(A)**, *L. sake*^{INSENS} **(B)**, *P. dextr.*^{SENS} **(C)**, and *P. pent.*^{INSENS} **(D)** in the absence (○●, control) and presence of CCCP (20 μM) (▽▼), nisin (0.2 μg.ml^{-1}) (□■), and pediocin PA-1 (8×10^{-2} μg.ml^{-1}) (△▲). A cell density of 0.2 mg of bacterial protein.ml^{-1} in 50 mM potassium-phosphate buffer (pH 5.7) was used, and inhibitory substances were added at 0 min. Values represent the average of duplicate experiments.

The addition of nisin at a pH of 5.7 resulted in reduced glycolysis rates for all selected strains, but a concentration more than 100-fold higher was needed to inhibit the glycolysis of *L. sake*^{INSENS} and *P. pent.*^{INSENS} **(Figures 1B and 1D, middle panels)** compared with that of *L. sake*^{SENS} and *P. dextr.*^{SENS} **(Figures 1A and 1C, middle panels)**. For each strain, the reduction of the glycolysis in the presence of equal nisin concentrations was less pronounced at pH 7.3 than at pH 5.7 **(Figure 1, middle panels)**. The addition of pediocin PA-1 at pH 5.7 also caused a reduction in the glycolysis rates for the selected strains, and again, higher concentrations were needed (≈ 50-fold) for the inhibition of the insensitive strains than for the sensitive strains **(Figure 1, right panels)**. The glycolysis rates of *L. sake*^{INSENS} and *P. pent.*^{INSENS} gradually decreased at pediocin PA-1 concentrations exceeding $8x10^{-2}$

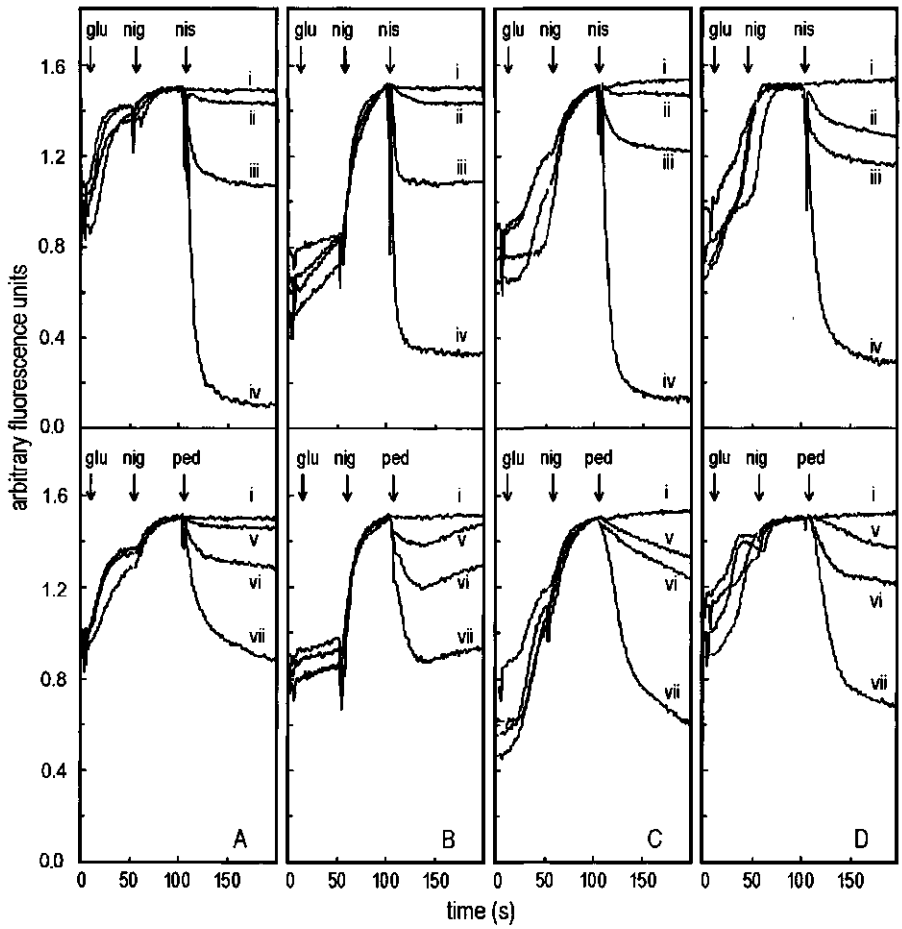

Figure 3.

Effects of nisin and pediocin PA-1 (upper and lower panels, respectively) on the $\Delta\psi$ of exponential-growth-phase cells of *L. sake*^{SENS} **(A)**, *L. sake*^{INSENS} **(B)**, *P. dextr.*^{SENS} **(C)**, and *P. pent.*^{INSENS} **(D)** with a cell density of 0.05 mg of bacterial protein.ml^{-1} in 50 mM K-HEPES buffer (pH 7.0). At the indicated times, glucose (glc; 0.2% [wt/vol]), nigericin (nig; 5 μM) or bacteriocin was added.

Bands: **i** no additions; **ii, iii,** and **iv** = 7.8×10^{-3}, 7.8×10^{-2}, and 7.8×10^{-1} μg of nisin (nis) per ml, respectively; **v, vi, vii** = 2.3×10^{-2}, 4.7×10^{-2}, and 9.3×10^{-2} μg of pediocin PA-1 (ped) per ml, respectively. $\Delta\psi$ was measured with the fluorescent probe $DiSC_3(5)$ as described in the MATERIALS AND METHODS. Valinomycin (2 μM) was used as a control (absence of $\Delta\psi$), resulting in 0.1 arbitrary fluorescence units for the different strains.

$\mu g.ml^{-1}$, but even at the highest concentration, 15 and 55% of the initial glycolysis rates were maintained, respectively. When pediocin PA-1 was added at pH 7.3, the same trends were observed; however, the pediocin PA-1 concentrations were fivefold higher **(Figure 1, right panels)**.

In the absence of bacteriocins or CCCP, the maximum glycolysis rates of the two *L. sake* strains were comparable **(Figure 1)**. By contrast, the glycolysis rate of *P. dextr.*^{SENS} was only 40% of the rate of *P. pent.*^{INSENS} **(Figure 1)**. Acid analysis of cell suspensions confirmed homolactic fermentation under standard glycolysis assay conditions (initial pH 7.3) for the four different organisms with and without CCCP (data not shown).

Effect of bacteriocins on the ATP_{in} pools. The steady state ATP_{in} concentrations of energized cells of *L. sake*^{SENS}, *L. sake*^{INSENS}, *P. dextr.*^{SENS} and *P. pent.*^{INSENS} at pH 5.7 were approximately 5.0, 3.5, 1.3 and 4.2 $nmol.mg^{-1}$ bacterial protein, respectively **(Figure 2)**. Comparable values were found at a pH of 7.3 (data not shown). ATP_{out} was not observed at either pH value. The protonophore CCCP (final concentration, 20 μM) was added at pHs 7.3 and 5.7 to determine the effect of PMF dissipation on the ATP_{in} and ATP_{out} concentrations. CCCP reduced ATP_{in} to approximately 3.0, 1.3, 0.2, and 3.2 nmol per mg of bacterial protein at a pH of 5.7 **(Figure 2)** and 4.4, 2.2, 0.9, and 3.3 nmol per mg of bacterial protein at a pH of 7.3 (data not shown) for *L. sake*^{SENS}, *L. sake*^{INSENS}, *P. dextr.*^{SENS} and *P. pent.*^{INSENS}, respectively. Again, extracellular ATP was not detectable.

The addition of nisin at a higher concentration than required for the inhibition of the growth of all selected strains (5 $\mu g.ml^{-1}$) caused a total loss of ATP_{in} from *L. sake*^{SENS} and *P. dextr.*^{SENS} and an increase of ATP_{out} within 2 min. The sum of the ATP_{in} and ATP_{out} remained constant, which indicated that the reduction in ATP_{in} resulted directly from leakage (data not shown).

Exposure of *L. sake*^{INSENS} and *P. pent.*^{INSENS} to this high nisin concentration resulted in a more gradual reduction of the ATP_{in} to approximately 10% of the initial concentration within 5 min. The sum of ATP_{in} and ATP_{out} slightly decreased with time, suggesting that the reduction in ATP_{in} was a result of ATP leakage, with a minor contribution of ATP hydrolysis (data not shown). Nisin was also added to the selected strains at pH 5.7 at a concentration that was inhibitory to the growth of the sensitive strains but not to that of insensitive strains (0.2 $\mu g.ml^{-1}$) **(Table 2)**. Again, this resulted in a rapid total loss of ATP_{in} and an increase of ATP_{out} for the two sensitive strains **(Figures 2A and 2C)**. However, *L. sake*^{INSENS} showed only a small decrease in ATP_{in} and a slight increase in ATP_{out} under these conditions. Moreover, *P. pent.*^{INSENS} showed neither a reduction in ATP_{in} nor an increase in ATP_{out}. When nisin (0.2 $\mu g.ml^{-1}$) was added at pH 7.3, similar results were obtained (data not shown).

The selected strains were also exposed to a concentration of pediocin PA-1 that was inhibitory to the growth of all strains (1.5 $\mu g.ml^{-1}$). This resulted in a gradual decrease of ATP_{in} to approximately 30% of the initial value within 5 min for both sensitive strains, whereas a reduction of approximately 50% was found for the insensitive strains. ATP leakage was not observed (data not shown). Furthermore, pediocin PA-1 was added at a concentration ($8x10^{-2}$ $\mu g.ml^{-1}$) inhibitory to the growth of *L. sake*^{SENS} and *P. dextr.*^{SENS}, but not to that of *L. sake*^{INSENS} or *P. pent.*^{INSENS}. This caused ATP_{in} reductions of 75% for *L. sake*^{SENS} and 90% for *P. dextr.*^{SENS} at pH 5.7, but no increase in ATP_{out} **(Figures 2A and 2C)**. Exposure of the insensitive strains to this pediocin PA-1 concentration led to a minor decrease in the ATP_{in} of *L. sake*^{INSENS} in the absence of ATP leakage **(Figure 2B)**, whereas the ATP_{in} and ATP_{out} concentrations of *P. pent.*^{INSENS} were unaffected **(Figure 2D)**. When pediocin PA-1 ($8x10^{-2}$ $\mu g.ml^{-1}$) was added at pH 7.3, similar results were obtained (data not shown).

$\Delta\psi$ of whole cells. The influence of bacteriocins on the $\Delta\psi$ of whole cells was investigated for each of the selected strains with different final concentrations of nisin or pediocin PA-1. At increasing bacteriocin concentrations, both a higher rate of $\Delta\psi$ reduction and a lower final potential were observed for the four different strains with the $\Delta\psi$ indicator $DiSC_3(5)$ **(Figure 3).** *L. sake*^{SENS} and *L. sake*^{INSENS} $\Delta\psi$ responses after the addition of nisin or pediocin PA-1 were similar, even though the former strain is 500- to 1000-fold more sensitive towards these bacteriocins **(Figures 3A and 3B).** Comparable $\Delta\psi$ responses at different nisin and pediocin PA-1 concentrations were also observed for *P. dextr.*^{SENS} and *P. pent.*^{INSENS}, whose sensitivities towards the two bacteriocins differ more than 100-fold **(Figures 3C and 3D).** Whereas the addition of nisin at the highest concentration caused the complete dissipation of the $\Delta\psi$ (compared to the addition of valinomycin [control]), the full dissipation of the $\Delta\psi$ by pediocin PA-1 was not observed for the different strains at the maximum concentration applied.

Membrane glyco- and phospholipid composition and the interaction between bacteriocins and lipid monolayers. The initial association of bacteriocins with the phospholipid bilayer may be influenced by the (di)acylglycerol head group composition of the membrane lipids. To determine possible differences between the phospho- and glycolipid compositions of strains sensitive and insensitive towards bacteriocins, extracted lipids of the four selected strains were analyzed by TLC. As shown in **Table 3**, each strain contained the major phospholipid DPG in addition to a major glycolipid (R_f = 0.47) which was not further characterized. The migration profile of the latter compound was similar to the reference digalactosyldiacylglycerol (R_f = 0.44) and probably indicated the presence of a dihexosyldiacylglycerol containing stereo isomers of galactose, since the most abundant glyco-

Table 3. Membrane lipid characteristic of *Lactobacillus* and *Pediococcus* species, grown at 30°C[a].

Strain	Diacyl head group composition[b]					% Fatty acid composition (acyl chains)		Phase-transition temp (°C)		
	Phospholipid			Glycolipid[c] with R_f of:		Short	Saturated[e]	T_m	T_b	T_e
	DPG	PG	LPG	0.23	0.47					
L. sake^{SENS}	+++	+−	+−	−	+++	41.8	49.0	10.0	-4.4	22.4
L. sake^{INSENS}	+++	+−	+−	−	+++	41.9	39.8	0.4	-3.1	3.9
P. dextr.^{SENS}	+++	+−	−	++	+++	32.0	20.2	-6.4	-34.7	21.1
P. pent.^{INSENS}	+++	+−	+−	−	+++	31.9	26.3	5.1	-5.7	16.0

[a] Mean values of duplicate determinations are presented.

[b] Diameters of spots as determined by TLC analysis: −, 0 mm; +−, 0 to 2 mm; ++, 2 to 5 mm; +++, 5 to 8 mm. Equal amounts of total lipids were applied for the different strains.

[c] Unidentified with R_fs determined by TLC analysis.

[d] Percentage of short fatty acids ($[C_{14:0} + C_{16:0} + C_{16:1}]$/total).

[e] Percentage of saturated fatty acids ($[C_{14:0} + C_{16:0} + C_{18:0}]$/total).

lipid of lactobacilli has been identified as galactosylglucosyl-diacylglycerol (58,149). Only *P. dextr.*^{SENS} contained an additional major glycolipid (R_f = 0.23), that was not further studied (**Table 3**). In addition, one minor phospholipid (R_f = 0.94) and two minor glycolipids (R_fs = 0.06 and 0.90) were observed for each strain (data not shown). Lysozyme treatment did not result in a higher yield of crude lipid extract from the cell pellets, whereas the lipid head group composition was similar to that for extraction without lysozyme.

The impact of the differences in the diacyl head group composition on the interactions between bacteriocins and lipids was evaluated by determining the interaction of the lipid extracts of the four selected strains with either nisin or pediocin PA-1 by using monomolecular lipid layers. Peptide-induced pressure changes of the lipid film were interpreted as partial penetration of the peptide into the lipid phase. The nisin-induced pressure changes in the lipid monolayers of the bacteriocin-sensitive or -insensitive strains were not significantly different from each other (**Figure 4**). The surface pressure changes induced by pediocin PA-1 were much lower than those for nisin: a pressure increase of only 3 $mN.m^{-1}$ at an initial surface pressure of 18 $mN.m^{-1}$ was observed for the lipid monolayers of both the sensitive and insensitive strains, whereas a pressure increase did not occur in the initial pressure range of 30 to 35 $mN.m^{-1}$ (data not shown).

Fatty acid composition and phase transition temperatures of the cytoplasmic membrane. The actual insertion of bacteriocins into the lipid membrane of LAB might be influenced by membrane fluidity, which depends on the length of the acyl chains and the degree of saturation (174). Therefore, the fatty acid composition of membrane lipids from sensitive and insensitive strains

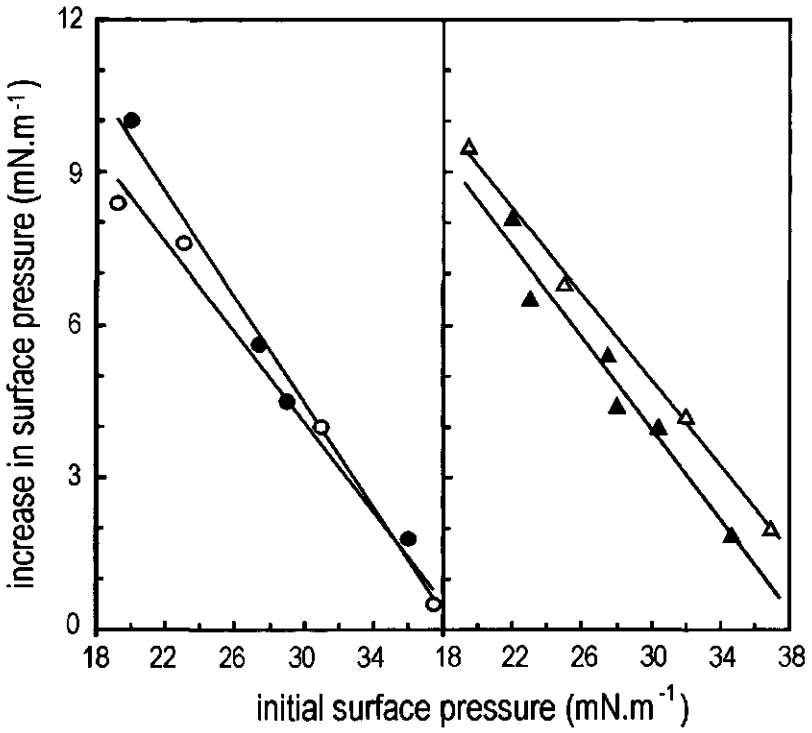

Figure 4.
Increases in surface pressure after the injection of nisin (final concentration, 1 $\mu g.ml^{-1}$) underneath monolayers of purified lipids isolated from *L. sake*^{SENS} (●), *L. sake*^{INSENS} (○), *P. dextr.*^{SENS} (▲), and *P. pent.*^{INSENS} (△) at different initial surface pressures.

was determined. Each strain predominantly contained $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$, with the latter fatty acid being the major component of each strain, and minor amounts of $C_{14:0}$ and $C_{18:0}$. In addition, only the two *L. sake* strains contained one extra fatty acid with a chain length of >18 (data not shown). This unidentified acid most likely indicates the presence of 11,12 methylene octadecenoic acid ($\Delta C_{19:0}$, lactobacillic acid), which has previously been identified in *L. sake* ATCC 15521 (DSM20017, *L. sake*^{SENS}) as the only fatty acid with a chain length of >18 (45).

As an indication of membrane fluidity, we calculated the percentages of short acyl chains and saturated fatty acids for all four strains **(Table 3)**. Compared with the *Pediococcus* strains, the two *L. sake* strains contained a substantially higher percentage of short acyl chains and saturated fatty acids. Comparison of the two *L. sake* strains showed similar percentages of short acyl chains but a higher degree of saturation of the fatty acids for *L. sake*^{SENS}. For the two *Pediococcus* strains, similar percentages of short acyl chains were found, with *P. pent.*^{INSENS} containing a higher percentage of saturated fatty acids **(Table 3)**.

The *in situ* membrane fluidity of whole cells was studied by FTIR spectroscopy. The membrane phase transition temperature, T_m, and the beginning (T_b) and ending (T_e) temperatures of the melting curve were determined by plotting the symmetric CH_2 stretching band position against the temperature. The T_m of the sensitive *Pediococcus* strain was considerably lower than the T_m of the insensitive strain (ΔT_m, 11.5°C). By contrast, the T_m of the sensitive *L. sake* strain was considerably higher than that of the insensitive strain (ΔT_m, 9.6°C) **(Table 3)**.

In short, both the fatty acid analysis and the *in situ* membrane fluidity measurements indicate that the membrane of the sensitive *Pediococcus* strain is more fluid than that of the insensitive strain. By contrast, the sensitive *L. sake* strain was found to have a membrane that was less fluid than that of the insensitive strain. Notably, a comparison of the two sets of organisms shows that the range over which the membrane phase transition occurs is broader for the sensitive strains than for the the insensitive strains, as can be deduced from the increase between T_e and T_b **(Table 3)**.

DISCUSSION

We have investigated the susceptibilities of two sets of closely related LAB with large differences in sensitivity towards the bacteriocins nisin and pediocin PA-1. Strikingly, the bacteriocin-induced dissipation of the $\Delta\psi$ of whole cells as well as the interaction of the bacteriocins with strain-specific lipids were similar for the sensitive and insensitive strains. Despite the dissipation of the $\Delta\psi$, the insensitive strains were able to maintain high ATP_{in} pools and high glycolytic rates below the MICs of these compounds. Furthermore, it became clear that the differences in bacteriocin sensitivity of the selected LAB could not be explained only by the variation in their membrane lipid composition. In this respect, our findings suggest an important role for the overall membrane constitution in the determination of LAB sensitivities towards nisin and pediocin PA-1.

Both nisin and pediocin PA-1 are known to induce the dissipation of the $\Delta\psi$ and chemical gradients of ions, which directly disturb membrane-associated energy transducing processes in target cells. In addition, pore formation by bacteriocins may affect energy generation via the glycolysis by the efflux of pathway intermediates and/or reduced enzyme activities after intracellular acidification **(32,37,39,57,164)**. The bacteriocins were shown to cause a depletion of the ATP_{in} pools and a reduction of the glycolysis rates at concentrations similar to the MICs for the selected LAB.

Consistent with the results of previous studies (4,193), nisin concentrations above the MIC for each strain induced a rapid, near total efflux of ATP (M_r, 507), indicating that the energy depletion and reduced glycolysis are primarily determined by the efflux of essential metabolites. Furthermore, our results agree with predictions that nisin induces the formation of pores with a maximum diameter of 0.9 nm, allowing the passage of molecules with molecular masses up to 500 Da (165).

In contrast to nisin, pediocin PA-1 did not induce ATP leakage above concentrations that caused complete growth inhibition. Evidently, the size exclusion limit of pediocin PA-1-induced pores is smaller than 500 Da. The stronger depletion of ATP_{in} pools by pediocin PA-1 compared with that by the protonophore CCCP implies that this bacteriocin also affects ion gradients other than protons, possibly coinciding with the efflux of essential metabolites (<500 Da). This suggestion agrees with the findings of Chen and Montville (37). The observed reduction of the pool size of ATP can be explained by the accelerated consumption of ATP to regenerate the decreased PMF and/or by a shift in the equilibrium of ATP hydrolysis resulting from an efflux of phosphate (4,88).

The dissipation of the proton gradient was studied by the use of the protonophore CCCP, which acts as a monomeric proton shuttle (97). At a low pH, this compound caused a reduction of the glycolysis rates of both *L. sake* strains, probably because of an inhibition of the glycolytic enzymes at the observed reduced intracellular pH. By contrast, the glycolysis of the *Pediococcus* strains was not affected by this protonophore, indicating that these organisms are less sensitive to internal pH variation. At a neutral pH, the CCCP-induced reduction of the ATP_{in} pools indicated an enhanced use of ATP to regenerate the PMF. The effect of this compound on the glycolysis and ATP_{in} pools was comparable for the sensitive and insensitive cells for both sets of strains, and only twofold higher concentrations of CCCP were required to kill insensitive strains as compared with those for their sensitive counterparts. The insertion of this protonophore into the cytoplasmic membrane is therefore thought to take place in a similar way for each of the selected strains.

Importantly, the exposure of the sensitive and insensitive strains to either nisin or pediocin PA-1 resulted in a similar concentration-dependent dissipation of the $\Delta\psi$ in these cells that manifest 100- to 1000-fold differences in bacteriocin sensitivity. Whereas the perturbation of the cytoplasmic membrane upon exposure to low concentrations of bacteriocins caused rapid energy depletion in the sensitive strains, the glycolysis rates and ATP_{in} pools of the insensitive cells appeared to be unaffected. As opposed to the results for CCCP, these results suggest that nisin and pediocin PA-1 molecules interact differently with the cytoplasmic membranes of sensitive and insensitive strains. The $\Delta\psi$ measurements indicate that the accessability of the cytoplasmic membrane to the bacteriocins was not impaired for the insensitive strains. This finding excludes the possibility of a role for the cell wall in the high bacteriocin tolerance of these strains, as has been proposed for the acquired nisin resistance of *Listeria monocytogenes* (48,137). Our findings further indicate that the inactivation of bacteriocins by proteases does not underlie the mechanism of the bacteriocin resistance of *L. sake*^{INSENS} and *P. pent.*^{INSENS}.

It is unlikely that the observed differences in sensitivity are related to the presence of putative immunity genes in the insensitive strains. To date, strains which express immunity towards a specific bacteriocin have been reported not to exhibit a loss of $\Delta\psi$ when they are exposed to the respective bacteriocin (39,81,182). The insensitive strains in this study, however, showed a dissipation of $\Delta\psi$ when they were exposed to bacteriocins, even at low concentrations. In addition, immunity to a specific bacteriocin has been reported only for strains that produce the cognate bacteriocin (7,127,159,186). In this respect, we have shown that the strains we studied are nonbacteriocinogenic.

The first step in bacteriocin pore formation involves the hydrophobic and electrostatic interactions with the phospholipid bilayer, which may account for the local disorganization of membrane phospholipids (55,57). In our monolayer studies, we identified substantial nisin-induced changes in the pressure of strain-specific lipid films with initial surface pressures of 30 to 35 mN/m, which are believed to be relevant for biological membranes (54). On the other hand, pediocin PA-1 did not induce an increase in pressure under these conditions, whereas a slight increase was observed at lower initial surface pressures. Considering that the above experiments were conducted in the absence of membrane proteins, these results agree with previous findings that nisin acts in a receptor-independent way (57,72), whereas pediocin PA-1 is believed to require membrane-associated protein receptor (39). Despite differences in the diacyl head group compositions of the LAB investigated, no significant differences were observed between the interactions of nisin or pediocin PA-1 with the respective lipid monolayers. In a similar monolayer study, Demel et al. (55) demonstrated that nisin had a high affinity for anionic phospholipids, while it exhibited low interaction with neutral or Zwitterionic phospholipids or glycolipids. Since the sensitive and insensitive strains in the current study varied mainly in their neutral and glycolipid compositions, this might explain our findings of the similar interactions of the bacteriocins with strain-specific lipids.

The actual pore formation by bacteriocins may be influenced by their specific biochemical characteristics, concentration, and $\Delta\psi$ (37-39,57,73,165). To explain differences in bacteriocin sensitivities among bacteria, Ming and Daeschel (142) have recently postulated a role of membrane fluidity, wherein a more compact membrane, as determined by its membrane acyl chain composition, was suggested to affect the action of nisin. Our data indicate higher membrane fluidity for *P. dextr.*^{SENS} than for *P. pent.*^{INSENS}. In line with the above hypothesis, a less compact membrane of the sensitive *Pediococcus* strain might facilitate pore formation by bacteriocins. However, a similar explanation does not hold true for the sensitivity of the *L. sake* strain, since the insensitive *L. sake* strain had a more fluid membrane. Interestingly, though, the temperature range for the membrane phase transition was considerably broader for the sensitive strains than for their insensitive counterparts. This result might indicate that the membrane of the sensitive *L. sake* is less homogeneous, which in turn might enhance the pore-forming action of bacteriocin molecules.

It is likely that the association of bacteriocins with the cell membrane, followed by the insertion of these compounds, takes place in a similar way for sensitive and insensitive cells. This speculation was supported by the similar interactions of nisin or pediocin PA-1 with strain-specific lipid monolayers, their comparable effects on the $\Delta\psi$ of whole cells, and by the comparable action of CCCP on the LAB strains, which was independent of the aggregation of molecules (97). However, the aggregation of bacteriocin monomers in oligomeric structures appeared to occur more efficiently in the membranes of sensitive cells than in those of insensitive cells. This assumption is strengthened by the observation that the perturbation of the cytoplasmic membrane by low bacteriocin concentrations caused rapid ATP depletion only in the sensitive strains. We suggest that not only membrane fluidity but also overall membrane constitution, in which membrane proteins can affect lipid ordering (66), might influence the pore-forming activity of bacteriocins. In this respect, the formation of transient, multistate pores with varying diameters, rather than uniform pores, has been demonstrated in planar lipid bilayers for various lanthionine-containing bacteriocins (45,126,165). Whereas our data indicate that the selected LAB strains with a natural high tolerance towards bacteriocins cannot hinder the insertion of bacteriocin monomers, the overall constitution of their membranes may preclude the formation of pores with sufficient diameters and lifetimes to ultimately cause cell death. Clearly, the *in vivo* formation of pores by bacteriocins in the cytoplasmic membranes of intact cells is a multifaceted and complex phenomenon. Having

provided evidence that naturally occurring differences in bacteriocin susceptibilities cannot be explained only by variation in membrane lipid composition, we suggest that further in-depth analysis of this phenomenon requires focusing on the role of membrane proteins in the constitution of biological membranes.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Claudia van den Boom, Yvette Feenstra and Bastienne Peelen. We thank Wim Wolkers and Folkert A. Hoekstra (Department of Plant Physiology, Wageningen Agricultural University, Wageningen, The Netherlands) for the determination of the phase transition temperatures.

CHAPTER 7

Biopreservation for the control of *Listeria monocytogenes* on minimally processed, modified atmosphere stored vegetables

Marjon H.J. Bennik, Wendy van Overbeek, Eddy J. Smid and Leon G.M. Gorris
Letters in Applied Microbiology, submitted for publication

ABSTRACT

Two *Pediococcus parvulus* strains and one *Enterococcus mundtii* strain, isolated from minimally processed vegetables, were evaluated for their potential to control the growth of *Listeria monocytogenes* on refrigerated, modified atmosphere (MA) stored mungbean sprouts. In culture broth, only *E. mundtii* was capable of growth and bacteriocin production at low temperatures (4 to 8°C). This strain inhibited the growth of *L. monocytogenes* on sterile vegetable-medium but not on fresh produce during storage at 8°C under MA-conditions. However, mundticin, the bacteriocin produced by *E. mundtii*, was found to have potential as a biopreservative agent for minimally processed vegetables when used in a washing step or a coating procedure.

INTRODUCTION

Minimally processed, modified atmosphere (MA) packaged vegetables generally contain few or no antimicrobial additives and rely on refrigeration as the main preservation factor. MA-conditions that are commonly used (1 to 5% O_2 and 5 to 10% CO_2) can extend the shelflife of produce by five to seven days at 4 to 8°C. The major safety concern with these non-sterile, ready-to-eat products is the possible outgrowth of psychrotrophic pathogens, such as *Listeria monocytogenes*, in the absence of sensory defects (24). *L. monocytogenes* has been isolated from a wide variety of raw or processed vegetables, and several cases of listeriosis have been related to the consumption of vegetable products (148,167). Thus, there may be a genuine need to introduce additional safety measures for minimally processed vegetables.

Fresh vegetables generally harbor low numbers of lactic acid bacteria (LAB) (148). The relatively high resistance of LAB towards CO_2 (143), their possibility to grow at low temperatures, and their ability to produce several different antimicrobial substances, including bacteriocins (121), make them suitable candidates for a natural preservation of vegetables under rerigerated MA-conditions. Bacteriocin producing LAB exhibit activity against Gram-positive bacteria, which may include *L. monocytogenes*. The application of these LAB as protective cultures or their bacteriocins as biopreservation agents on minimally processed vegetables could inhibit or prevent the growth of this pathogen.

The use of LAB and/or their antimicrobial products has already been demonstrated to extend the storage life and safety of chilled, MA-stored meats (140). In ready-to-eat salads, favorable

shifts in microbial populations after inoculation with several bacteriocin producing LAB have been reported by Vescovo et al. (188). However, the strains used in their study were isolated from environments which are ecologically different from minimally processed vegetables, *i.e.* tomato pulp, cheese, cucumber brines or silage. Since efficient inhibition of the growth of pathogens by bacteriocin producing LAB on minimally processed vegetables may be achieved by strains that grow and secrete these compounds under refrigerated MA-conditions, ecological adaptation may be an important factor. We have previously isolated a number of bacteriocinogenic LAB from minimally processed vegetables with activity towards *e.g. L. monocytogenes* and nonproteolytic *Clostridium botulinum*. These included two strains of *Pediococcus parvulus* (13), producing the bacteriocin pediocin PA-1 (96), and one strain of *Enterococcus mundtii*, producing the novel class IIa bacteriocin mundticin (13,15). These isolates are well adapted to the vegetable ecosystem, and have now been studied for their ability to control the growth of *L. monocytogenes* on mungbean sprouts under refrigerated MA-conditions.

The aim of this study was to investigate the potential use of *P. parvulus* and *E. mundtii* and their bacteriocins as biopreservative agents for MA-stored vegetables. Therefore, the strains were evaluated for their growth, bacteriocin and acid production at different temperatures. In addition, the influence of CO_2 on their growth was studied in a model agar system. The ability of *E. mundtii* to suppress *L. monocytogenes* under MA-storage conditions at 8°C was assessed on a sterile vegetable medium, and on fresh mung bean sprouts. Additionally, we determined whether it would be possible to use mundticin, the bacteriocin produced by *E. mundtii*, as a biopreservation factor in a washing step and in a coating procedure for minimally processed vegetables.

MATERIALS AND METHODS

Strains, media and culturing conditions. The bacteriocin producing (bact$^+$) strains *Enterococcus mundtii* AT06 as well as *Pediococcus parvulus* AT034 and AT077 have been isolated from minimally processed vegetables (13,15). The non-producing (bact$^-$) strains *E. mundtii* DSM3848 and *P. parvulus* DSM20332 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Listeria monocytogenes* LDCD861 and LDCD1087 were isolated during an outbreak of listeriosis related to the consumption of raw cabbage (167). LAB were cultivated in all purpose tryptone (APT) medium (Difco, Detroit, Mich.), and *Listeria monocytogenes* in brain heart infusion broth (BHI) (Oxoid, Basingstoke, England). Media were supplemented with 0.5% glucose, and incubations were performed at 30°C, unless stated otherwise.

Vegetable agar medium was prepared by submerging 1 kg of fresh mungbean sprouts, obtained from a local grower, in 1 liter of water of 90°C. After 3.5 minutes, the mungbean sprouts were removed from the exudate and blended in a foodprocessor. The exudate and the blended vegetables were centrifuged (30 min, 5000 g) at 4°C. The pooled supernatants were adjusted to pH 6.5 with NaOH, filter sterilized (0.2 μm, Costar, Cambridge, MA), and stored at -20°C until use.

To prepare vegetable agar medium, the above sterile vegetable supernatant was warmed to 50°C, and mixed at a ratio of 2:1 with a 3% agar-containing sterile phosphate buffer (0.3 mol.l^{-1} of equimolar amounts of sodium and potassium phosphate (Na/K P_i) at pH 7.2), to give a final concentration of 0.1 mol.l^{-1} phosphate and 1% agar.

Temperature-dependent growth, bacteriocin production and medium acidification. 100 ml of APT broth was inoculated with the bacteriocin producing LAB, using initial levels of approximately 10^4 cfu.ml^{-1}. Incubations were perfor-

med at 4, 8, 15 and 30°C. At regular time intervals, duplicate aliquots of the culture fluid were used to determine viable counts (colony forming units (cfu) per ml), pH, and bacteriocin activity (arbitrary bacteriocin units (BU) per ml). The growth and final pH of the medium were also monitored for the nonbacteriocinogenic strains *E. mundtii* DSM3848 and *P. parvulus* DSM20332.

Influence of CO_2 on bacteriocinogenic strains. The bacteriocinogenic LAB were evaluated for their growth on an agar surface under 1.5% O_2 combined with either 0, 5, 20 or 50% CO_2 at 8°C, employing a model system described previously (14). The strains were individually inoculated onto APT agar plates supplemented with 0.5% glucose and 0.1 mol.l^{-1} Na/K P_i buffer **(see above)** at pH 7.2. Viable counts on the surface of the agar were determined in duplicate and expressed as cfu.cm^{-2}.

Bacterial growth in a vegetable model system. Early-stationary-phase cultures of *L. monocytogenes* LDCD861 and LDCD1087 (OD_{660} 0.9) were mixed (ratio 1:1) and washed in a 4°C physiological salt solution (0.85 w/v % NaCl and 0.1 w/v % peptone). A dilution of this mix of *L. monocytogenes* was surface spread onto Petridishes (diameter 6 cm) containing 9 ml of vegetable agar medium to render an initial population density of approximately 1 x 10^4 cfu.cm^{-2}. Following the same procedure, plates were coinoculated with washed cells of bact$^+$ *E. mundtii* AT06 or bact$^-$ *E.mundtii* DSM3848 cultures to a population density of 1 x 10^4 cfu.ml^{-1} (low inoculum) or 1 x 10^6 cfu.ml^{-1} (high inoculum). Pure cultures of each of the above LAB strains were used as controls.

Inoculation of vegetables. Fresh mungbean sprouts which were either dipped in a mundticin- containing solution, or coated with an alginate film **(see below)**, were inoculated with approximately 1 x 10^4 cfu.ml^{-1} of the *L. monocytogenes* mix by spraying 10 ml of approximately 1 x 10^6 cfu.ml^{-1} per kg of produce. *E. mundtii* was inoculated on the product at initial levels of approximately 1 x 10^4 or 1 x 10^6 cfu.ml^{-1} by spraying 10 ml of a cell suspension of 1 x 10^6 or 1 x 10^8 cfu.ml^{-1}, respectively, per kg of produce.

Treatment of mungbean sprouts with bacteriocin-containing solution or coating. Prior to inoculation of a mix of *L. monocytogenes* LDCD681 and 1087, fresh mungbean sprouts were dipped in sterile water containing 200 BU.ml^{-1} of purified mundticin (15) and subsequently dried on a grid at 4°C for 30 minutes. Mungbean sprouts were coated with an alginate film by dipping them in a 10% solution of Saltiagine SG 300 (Systems Bio-Industries, Lannilis, France) containing 200 BU.ml^{-1} of mundticin, and subsequently in a sterile, 200 BU.ml^{-1} of mundticin containing 3% (w/v) $CaCl_2$ solution. The spouts were dried on a grid at 4°C for 30 to 40 min before inoculation with *L. monocytogenes* and storage under appropriate conditions. With both treatments, appropriate controls were examined that did not contain mundticin.

Vegetable model system and mungbean sprouts: MA storage conditions and enumeration. Mungbean sprouts and inoculated vegetable-medium plates were incubated under a constant flow (200 ml.min^{-1}) of 1.5% O_2 /20% CO_2/78.5% N_2 at 8°C. At regular time intervals, duplicate viable counts were determined by plating dilutions of either the agar medium or the mungbean sprouts onto Palcam agar medium (Oxoid) for *L. monocytogenes* and onto MRS agar (Oxoid) for LAB, including *E. mundtii*. Plates were incubated at 30°C for 2 to 3 days.

Bacteriocin activity. The bacteriocin activity in filter-sterilized (0.2 μm filters, Costar) culture supernatant of the various bacteriocinogenic strains was determined a previously described microtiter plate system (13). Bacteriocin activities were expressed as Bacteriocin Units per ml (BU.ml^{-1}). *L. monocytogenes* LDCD861 was used as an indicator strain.

Data handling. Bacterial growth data were fitted by the Dmodel (IFR inhouse model, provided by József Baranyi, Institute of Food Research, Reading, England) to determine the lag time (λ), the maximum population density (MPD), the maximum specific growth rate (μ_m) and the standard errors(se) of the estimates (10).

RESULTS AND DISCUSSION

We evaluated the potential of different bacteriocinogenic LAB, that were isolated from minimally processed vegetables, to control the growth of *Listeria monocytogenes* on vegetable agar medium, and fresh mungbean sprouts under refrigerated MA conditions. The LAB studied, *i.e.* two strains of *P. parvulus* (13) and one strain of *Enterococcus mundtii* (15), are homofermentative and typically associated with plants (44,169). The experiments were conducted in systems of increasing complexity, *i.e.* in culture broth, on sterile vegetable (mungbean sprouts) medium, and on fresh produce.

For growth in culture broth at 4, 8, 15 or 30°C, respectively, both pediocin PA-1 producing *P. parvulus* strains showed similar maximum specific growth rates (μ_m), maximum population densities (MPDs), and final pH values of their growth medium (**Table 1**). These growth characteristics matched those of the nonbacteriocinogenic *P. parvulus* DSM20332, which was used as a control (data not shown). Neither *P. parvulus* ATO34 nor ATO77 exhibited bacteriocin activity at 4 or 8°C, but both strains produced pediocin PA-1 when cultured at 15 and 30°C, with strain ATO77 displaying a twofold higher activity than ATO34. Further examination of the growth potential of *P. parvulus* ATO77 under different CO_2 concentrations indicated an increase of the μ_m under elevated CO_2 concentrations (**Figure 1**). This is in line with previous reports on the high resistance of LAB towards CO_2 (143). Due to the lack of bacteriocin production at 4 and 8°C, the bact$^+$ *P. parvulus* strains were considered unsuitable as biopreservation agents on refrigerated, MA-stored vegeta-

Table 1. Growth and bacteriocin production of the bacteriocinogenic strains of *Pediococcus parvulus* and *Enterococcus mundtii* at 4, 8, 15 and 30°C, respectively. Cultivations were performed in APT broth, supplemented with 0.5% glucose. Values represent the average of duplicate experiments.

Strain	Incubation temperature	Growth characteristics				Final pH medium	Maximum bacteriocin activity
	(°C)	μ_m (h^{-1})[a]		MPD[a] (log cfu.ml^{-1})			(BU.ml^{-1})[b]
P. parvulus ATO34	4	0.018	± 0.001	8.2	± 0.1	3.8	0
	8	0.030	± 0.002	8.0	± 0.1	3.8	0
	15	0.122	± 0.005	8.3	± 0.1	3.9	40
	30	0.403	± 0.036	8.0	± 0.1	3.9	1280
P. parvulus ATO77	4	0.019	± 0.001	7.9	± 0.1	3.9	0
	8	0.045	± 0.006	8.5	± 0.3	3.8	0
	15	0.141	± 0.017	8.1	± 0.1	3.9	80
	30	0.389	± 0.016	8.1	± 0.1	3.9	2560
E. mundtii ATO6	4	0.053	± 0.008	8.9	± 0.2	4.7	1280
	8	0.059	± 0.008	9.1	± 0.2	4.6	5120
	15	0.256	± 0.039	9.2	± 0.1	4.4	5120
	30	1.490	± 0.106	9.0	± 0.1	4.4	5120

[a] Growth of LABs was determined in APT broth, supplemented with 0.5% glucose.

[b] Bacteriocin activity was expressed as Bacteriocin Units per ml, measured against *L. monocytogenes* LDCD861.

bles. However, these LAB may find use as biopreservatives in food products that are processed or stored at higher temperatures in which *L. monocytogenes* poses a safety hazard (*e.g.* soft cheese, paté).

The μ_m, MPDs and the final pH values of the media that were found for *E. mundtii* in culture broth during incubation at 4, 8, 15 and 30°C were higher than those of both *P. parvulus* strains **(Table 1)**. The growth characteristics of the control bact$^-$ strain *E. mundtii* DSM3848 were similar to its bact$^+$ counterparts (data not shown). On agar medium, the μ_m of bact$^+$ *E. mundtii* was found to decrease with increasing CO_2 concentrations, whereas the MPDs were unaffected **(Figure 1)**. Especially at CO_2 concentrations below 20%, which are relevant for MA-storage of vegetables (113), we observed significantly higher growth rates for *E. mundtii* than for *P. parvulus*. Importantly, supernatants of *E. mundtii* that were obtained from cultures grown at 8, 15 and 30°C, all exhibited high (*i.e.* 5120 BU.ml^{-1}) bacteriocin activities against *L. monocytogenes*. A more moderate antilisterial activity was observed in the supernatants from cultures grown at 4°C **(Table 1)**.

Although *E. mundtii* has been reported to be associated with plants (44), it does not have a GENERALLY RECOGNISED AS SAFE status. However, the above favorable characteristics of this strain prompted us to further evaluate its potential as a model biopreservation agent. Therefore, coino-

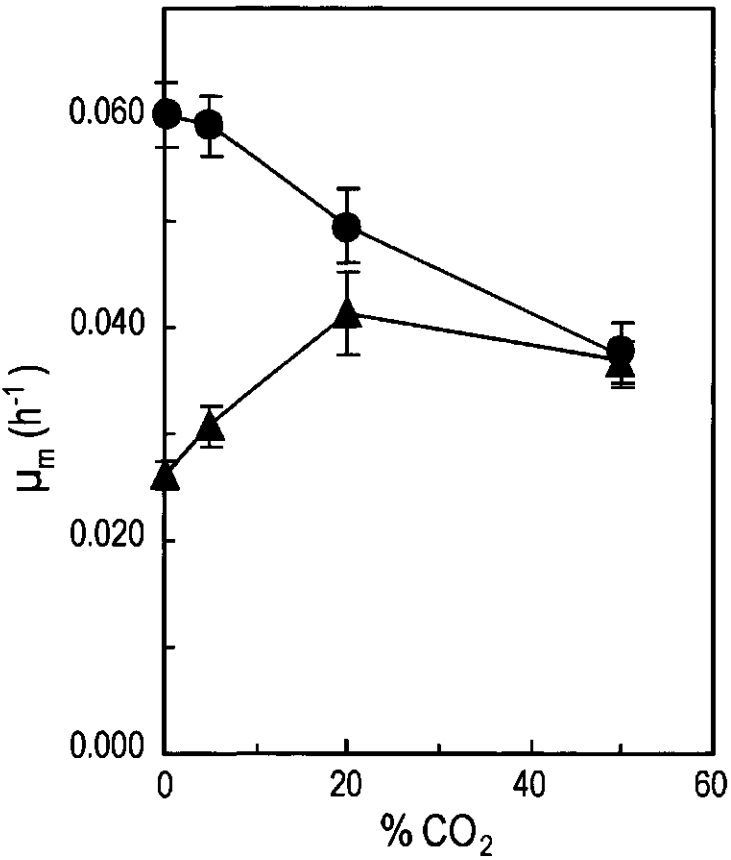

Figure 1.
The influence of CO_2 on the maximum specific growth rates of (▲) *P. parvulus* AT077 and (●) *E. mundtii* AT06 in a model agar surface system at 8°C.
APT agar plates supplemented with 0.5% glucose were inoculated at initial levels of approximately 1 x 10^4 cfu.ml^{-1} and incubated under a constant flow of 1.5% O_2 combined with 0,5, 20 or 50% CO_2, the remainder being N_2.

culation with *L. monocytogenes* was performed on the surface of sterile agar medium that was prepared from mungbean sprouts. *E. mundtii* was applied at both a low and a high inoculation density, since bacteriocin activity in its culture broth was specifically observed at the end of the exponential growth phase (data not shown). Both inoculum levels prevented the outgrowth of *L. monocytogenes* for 5 days during storage at 8°C under 1.5% O_2/20% CO_2/78.5% N_2. The biopreservative effect was most pronounced with the highest inoculum, as evidenced by a one log reduction in the initial number of the pathogen **(Figure 2)**. In a control experiment, the growth of the pathogen was not affected when the bact$^-$ strain *E. mundtii* DSM3848 was used as the coinoculant **(Figure 2)**.

The inhibition of *L. monocytogenes* by the bact$^+$ *E. mundtii* is likely to result from the production of mundticin on the vegetable medium, since both the bact$^+$ and the bact$^-$ *E. mundtii* strains showed similar growth characteristics on vegetable agar medium, reaching MPDs of $5x10^8$ cfu.cm^{-2} after 5 or 8 days while using the high or low initial inoculum, respectively. In addition, the growth of both the bact$^+$ and the bact$^-$ *E. mundtii* strains resulted in an identical final pH of the medium (pH 6.3).

An increase in the viable counts of *L. monocytogenes* after 5 days of cocultivation with bact$^+$ *E. mundtii* in the vegetable model system **(Figure 2)**, may indicate that part of the *L. monocytogenes*

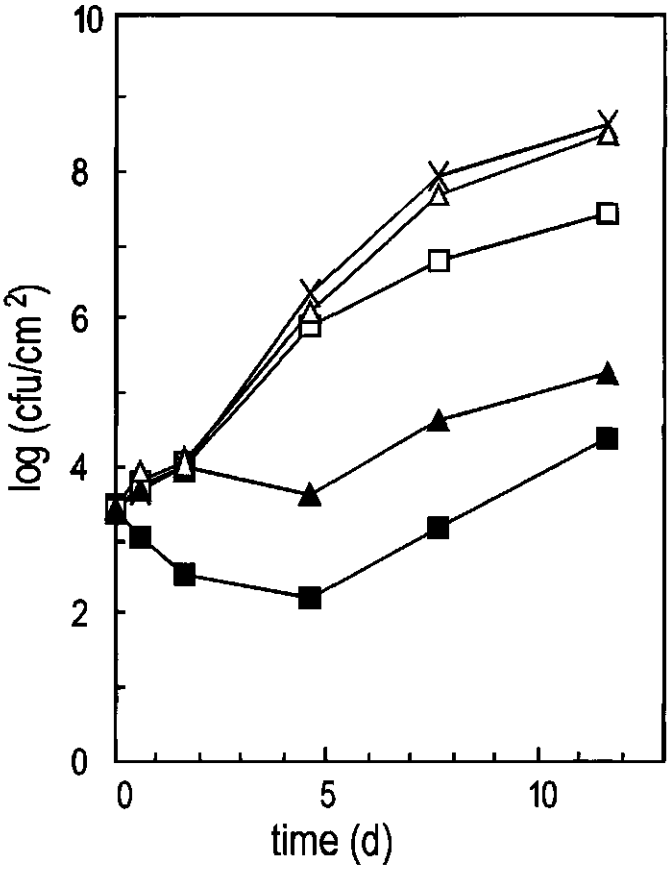

Figure 2.
Growth of *L. monocytogenes* (1:1 mix of LDCD681 : LDCD1087) on vegetable agar medium in absence of *E. mundtii* (X); cocultured with (▲) bact$^+$ *E. mundtii* or (△) bact$^-$ *E. mundtii* DSM3848 using initial levels of 1 x 10^4 cfu.cm^{-2}; or cocultured with (■) bact$^+$ *E. mundtii* or (□) bact$^-$ *E. mundtii* DSM3848 using initial levels of 1 x 10^6 cfu.cm^{-2}. All strains were washed prior to inoculation. Incubations were performed under a constant flow of 1.5% O_2/20% CO_2/78.5% N_2 at 8°C.

population gained resistance against mundticin. Similar findings were previously reported with respect to other bacteriocins (163). Alternatively, the loss of mundticin activity may be due to adsorption or proteolytic degradation.

To obtain more information on the putative application of bact$^+$ *E. mundtii*, this strain was tested for its activity against *L. monocytogenes* on mungbean sprouts stored at 8°C under MA conditions. The bact$^+$ *E. mundtii* did not suppress the growth of the pathogen after the application of either a low or high inoculum (**Figure 3**). This inability is probably not due to lack of *in situ* bacteriocin production, as suggested by the secretion of mundticin in the model vegetable agar system under identical storage conditions. However, since the addition of the protective culture was performed on non-sterile produce, it is likely that the secreted bacteriocin was degraded by proteolytic enzymes. These can originate from the produce or from its endogenous microflora, which consisted of high numbers of *Enterobacteriaceae* and *Pseudomonas* species, including several proteolytic strains (16,138).

Although the direct application of *E. mundtii* on mungbean sprouts was not effective in reducing the initial viable count of *L. monocytogenes* nor its growth potential, a decline of two log units in the initial numbers was achieved when the produce was dipped in a solution of mundti-

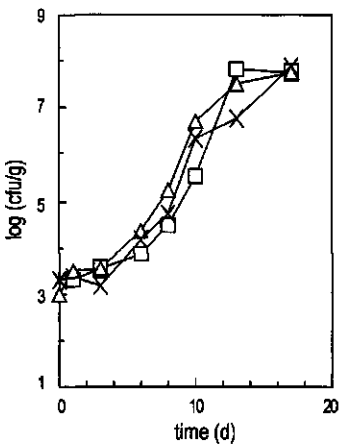

Figure 3.
The influence of bacteriocinogenic strains on the growth of *L. monocytogenes* (1:1 mix of strain LDCD681 and LDCD1087) on mungbean sprouts, incubated under 1.5% O_2/20% CO_2/78.5% N_2 at 8°C.
Growth of (X) *L. monocytogenes* in absence of *E. mundtii*;
L. monocytogenes coinoculated with (△) low (approximately 10^4 cfu.g^{-1}) or (□) high (approximately 10^6 cfu.g^{-1}) initial inocula of bact$^+$ *E. mundtii*.

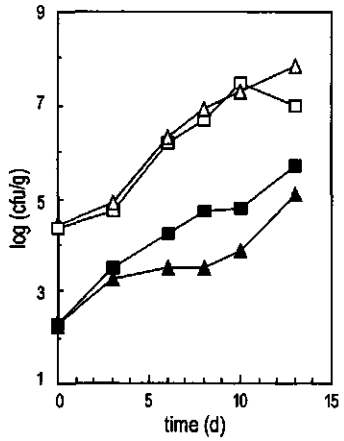

Figure 4.
Growth of *L. monocytogenes* on mungbean sprouts after treatment with purified bacteriocin of *E. mundtii*.
The product was (△) dipped in sterile water;
(□) coated with alginate film without mundticin;
(▲) dipped in sterile water containing 200 BU.ml^{-1} of mundticin;
or (■) coated with an alginate film containing 200 BU.ml^{-1} mundticin.
Incubations were performed under 1.5% O_2/20% CO_2/78.5% N_2 at 8°C.

cin prior to contamination with the pathogen (**Figure 4**). Identical results were obtained when the product was treated with a mundticin containing alginate film. The increase of the viable count of the pathogen after five days may, again, be attributed to proteolytic degradation. Noteworthy is that the counts of the pathogen did not exceed the initial inoculation level for approximately eight days.

In conclusion, two vegetable-associated bact$^+$ *P. parvulus* strains were considered unsuitable as biopreservation agents for refrigerated, MA-stored vegetables due to a lack of bacteriocin production at low temperatures (4 to 8°C). By contrast, the bact$^+$ *E. mundtii* strain displayed a number of characteristics that favor its putative use as a biopreservation agent, such as limited product acidification, production of a bacteriocin with anti-listeria activity at refrigeration temperatures, and high growth rates on vegetable medium under MA-conditions. Despite these properties, this strain failed to inhibit the growth of *L. monocytogenes* on fresh mungbean sprouts and was therefore not likely to be feasible as a protective culture against *L. monocytogenes* on MA-stored vegetables. However, our first successful trials of the applications of mundticin in a washing solution or as a component of a coating warrant further research on the potential for this compound as a natural preservation agent for minimally processed vegetables.

ACKNOWLEDGEMENTS

THE AUTHORS THANK LIANNE VAN IERSEL, CHRISTELLE LEGOFF AND TRUDY ZWEDEN FOR THEIR CAPABLE TECHNICAL ASSISTANCE.

REFERENCES

1. Abdullah, A., R. E. Baldwin, M. Fields, and A. L. Karr. 1984. Sensory attributes and safety aspects of germinated small-seeded soybeans and mungbeans. J. Food Prot. **47**:434-437.

2. Abee, T. 1995. Pore-forming bacteriocins of Gram-positive bacteria and self-protection mechanisms of producer organisms. FEMS Microbiol. Lett. **129**:1-9.

3. Abee, T., T. R. Klaenhammer, and L. Letellier. 1994. Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. Appl. Environ. Microbiol. **60**:1006-1013.

4. Abee, T., F. M. Rombouts, J. Hugenholtz, G. Guihard, and L. Letellier. 1994. Mode of action of nisin Z against *Listeria monocytogenes* Scott A grown at high and low temperatures. Appl. Environ. Microbiol. **60**:1962-1968.

5. Ahn, C. and M. E. Stiles. 1990. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl. Environ. Microbiol. **56**:2503-2510.

6. Andrews, W. H., P. B. Mislivec, C. R. Wilson, V. R. Bruce, P. L. Poelma, R. Gibson, M. W. Trucksess, and K. Young. 1982. Microbiological methods: Microbial hazards associated with bean sprouting.
J. Assoc. Off. Anal. Chem. **65**:241-248.

7. Axelsson, L., A. Holck, S.-E. Birkeland, T. Aukrust, and H. Blom. 1993. Cloning and nucleotide sequence of a gene from *Lactobacillus sake* Lb706 necessary for sakacin A production and immunity.
Appl. Environ. Microbiol. **50**:2868-2875.

8. Aymerich, T., H. Holo, L. S. Havarstein, M. Hugas, M. Garriga, and I. F. Nes. 1996. Biochemical and genetic characterisation of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. **62**:1676-1682.

9. Baker, R. C., R. A. Qureshi, and J. H. Hotchkiss. 1986. Effect of an elevated level of carbon dioxide containing atmosphere on the growth of spoilage and pathogenic bacteria at 2, 7, and 13°C. Poultry Sci. **65**:729-737.

10. Baranyi, J., T. A. Roberts, and P. McClure. 1993. A non-autonomous differential equation to model bacterial growth. Food Microbiol. **10**:43-59.

11. Barnes, E. M. and C. S. Impey. 1968. Anaerobic Gram-negative non-sporing bacteria from the caeca of poultry. J. Appl. Bacteriol. **31**:530-541.

12. Bennik, M. H. J., H. W. Peppelenbos, C. Nguyen-The, F. Carlin, E. J. Smid, and L. G. M. Gorris. 1996. Microbiology of minimally processed, modified-atmosphere packaged chicory endive.
Posth. Biol. Technol. **9**:209-221.

13. Bennik, M. H. J., E. J. Smid, and L. G. M. Gorris. 1997. Vegetable-associated *Pediococcus parvulus* produces pediocin PA-1. Appl. Environ. Microbiol. **63**:2074-2076.
14. Bennik, M. H. J., E. J. Smid, F. M. Rombouts, and L. G. M. Gorris. 1995. Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen. Food Microbiol. **12**:509-519.
15. Bennik, M. H. J., B. Vanloo, E. J. Smid, L. G. M. Gorris, and R. Brasseur. 1997. A role for oblique peptides in the pore-formation by bacteriocins of lactic acid bacteria. Biochim. Biophys. Acta, submitted.
16. Bennik, M. H. J., W. Vorstman, E. J. Smid, and L. G. M. Gorris. 1997. The influence of oxygen and carbon dioxide on the growth of prevalent *Enterobacteriaceae* and *Pseudomonas* species isolated from fresh and modified atmosphere stored vegetables. Food Microbiol., submitted.
17. Berrang, M. E., R. E. Brackett, and L. R. Beuchat. 1989. Growth of *Aeromonas hydrophila* on fresh vegetables stored under a controlled atmosphere. Appl. Environ. Microbiol. **55**:2167-2171.
18. Beuchat, L. R. 1995. Pathogenic microorganisms associated with fresh produce. J. Food Prot. **59**:204-216.
19. Beuchat, L. R. and R. E. Brackett. 1990. Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. J. Food Sci. **55**:755-758.
20. Bhugaloo-Vial, P., X. Dousset, A. Metivier, O. Sorokine, P. Anglade, P. Boyaval, and D. Marion. 1996. Purification and amino acid sequences of Piscicocins V1a and V1b, two class **IIa** bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. Appl. Environ. Microbiol. **62**:4410-4416.
21. Bhunia, A. K., M. C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. **65**:261-268.
22. Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911-917.
23. Blom, H. and C. Morvedt. 1991. Anti-microbial substances produced by food associated micro-organisms. Biochem. Soc. Trans. **19**:694-698.
24. Brackett, R. E. 1992. Microbial safety of chilled foods: current issues. Trends Food Sci. Technol. **3**:81-85.
25. Brackett, R. E. 1994. Microbiological spoilage and pathogens in minimally processed refrigerated fruits and vegetables, p. 269-312. In R. C. Wiley (ed.), Minimally processed refrigerated fruits and vegetables. Chapman and Hall, New York.
26. Brasseur, R. 1991. Differentiation of lipid-associating helices by the use of three-dimensional molecular hydrophobicity potential calculations. J. Biol. Chem. **266**:16120-16127.
27. Brasseur, R., T. Pillot, L. Lins, J. Vandekerckhove, and M. Rosseneu. 1997. Peptides in membranes: tipping the balance of membrane stability. Trends Biochem. Sci. **22**:167-171.
28. Brasseur, R. and J. M. Ruysschaert. 1986. Conformation and mode of organization of amphiphilic membrane components: a conformational analysis. Biochem. J. **238**:1-11.
29. Brasseur, R., B. Vanloo, R. Deleys, L. Lins, C. Labeur, J. Taveirne, J. M. Ruysschaert, and M. Rosseneu. 1993. Synthetic model peptides for apolipoproteins. I. Design and properties of synthetic model peptides for the amphipathic helices of the plasma apolipoproteins. Biochim. Biophys. Acta **1170**:1-7.
30. Breer, C. and A. Baumgartner. 1992. Occurrence and behaviour of *Listeria monocytogenes* on salads, vegetables, and in fresh vegetable juices. Arch. Lebensmittelhyg. **43**:108-110.
31. Breeuwer, P., J. L. Drocourt, F. M. Rombouts, and T. Abee. 1996. A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. Appl. Environ. Microbiol. **62**:178-183.
32. Bruno, M. E. C., A. Kaiser, and T. J. Montville. 1992. Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. Appl. Environ. Microbiol. **58**:2255-2259.
33. Bruno, M. E. C. and T. J. Montville. 1993. Common mechanistic action of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. **59**:3003-3010.
34. Butler, J. N. 1982. Carbon dioxide equilibria and their applications. p.1-259. Addison-Wesley Publishing Company, Inc. Reading, Massachusetts.
35. Carlin, F., C. Nguyen-The, A. A. Da Silva, and C. Cochet. 1996. Effects of carbon dioxide on the fate of *Listeria monocytogenes*, of aerobic bacteria and on the development of spoilage in minimally processed fresh endive. Int. J. Food Microbiol. **32**:159-172.
36. Carlin, F., C. Nguyen-The, and C. E. Morris. 1996. The influence of the background microflora on the fate of *Listeria monocytogenes* on minimally processed fresh broad leaved endive. J. Food Prot. **59**:698-703.

37. Chen, Y. and T. J. Montville. 1995. Efflux of ions and ATP depletion induced by pediocin PA-1 are concomitant with cell death in *Listeria monocytogenes* Scott A. J. Appl. Bacteriol. **79**:684-690.
38. Chen, Y., R. Shapira, M. Eisenstein, and T. J. Montville. 1997. Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. Appl. Environ. *Microbiol.* **63**:524-531.
39. Chikindas, M. L., M. J. Garcia Garcera, A. J. M. Driessen, A. M. Ledeboer, J. Nissen-Meyer, I. F. Nes, T. Abee, W. N. Konings, and G. Venema. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Appl. Environ. Microbiol. **59**:3577-3584.
40. Choi, S. Y. and L. R. Beuchat. 1994. Growth inhibition of *Listeria monocytogenes* by a bacteriocin of *Pediococcus acidilactici* M during fermentation of kimchi. Food Microbiol. **11**:301-307.
41. Christensen, D. P. and R. W. Hutkins. 1992. Collapse of the proton motive force in *Listeria monocytogenes* caused by a bacteriocin produced by *Pediococcus acidilactici*. Appl. Environ. Microbiol. **58**:3312-3315.
42. Cintas, L. M., J. M. Rodriguez, M. F. Fernandez, K. Sletten, I. F. Nes, P. E. Hernandez, and H. Holo. 1995. Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. Appl. Environ. Microbiol. **61**:2643-2648.
43. Clark, D. S. and T. Burki. 1972. Oxygen requirements of strains of *Pseudomonas* and *Achromobacter*. Can. J. Microbiol. **18**:321-326.
44. Collins, M. D., J. A. E. Farrow, and D. Jones. 1986. *Enterococcus mundtii* sp. nov. Int. J. Syst. Bact. **36**:8-12.
45. Dainty, R. H., C. M. Hibbard, and R. A. Edwards. 1984. Cellular fatty acids of *Streptobacteria* isolated from vacuum packaged meats. Syst. Appl. Microbiol. **5**:233-240.
46. Daniels, J. A., R. Krishnamurthi, and S. S. H. Rizvi. 1985. A review of the effect of carbon dioxide on microbial growth and food quality. J. Food Prot. **48**:532-537.
47. Davies, E. A. and M. R. Adams. 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. Int. J. Food Microbiol. **21**:341-347.
48. Davies, E. A., M. B. Falahee, and M. R. Adams. 1996. Involvement of the cell envelope of *Listeria monocytogenes* in the acquisition of nisin resistance. J. Appl. Bacteriol. **81**:139-146.
49. Day, B. P. F. 1992. Applications-fruits and vegetables, R. T. Parry (ed.), Modified atmosphere packaging of foods. Blackie and Son Ltd. Glasgow, United Kingdom.
50. Day, B. P. F. and L. G. M. Gorris. 1993. An update on the perspective of modified atmosphere packaging of fresh produce on the West-European market. ZFL **44**:32-37.
51. De Vos, W. M., O. P. Kuipers, and J. R. Van der Meer. 1995. Maturation pathway of nisin and other lantibiotics: post-translationally modified antimicrobial peptides exported by Gram-positive bacteria. Mol. Microbiol. **17**:427-437.
52. De Vuyst, L. 1994. Bacteriocins of *Pediococcus*, p. 461-464. In L. Devuyst and E. J. Vandamme (eds.), Bacteriocins of lactic acid bacteria. Blackie Academic & Professional, London, United Kingdom.
53. De Vuyst, L. and E. J. Vandamme. 1994. Bacteriocins of lactic acid bacteria. Blackie Academics and Professionals, London, UK.
54. Demel, R. A., W. S. M. Geurt van Kessel, R. F. A. Zwaal, B. Roelofsen, and L. L. M. van Deenen. 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. Biochim. Biophys. Acta **406**:97-107.
55. Demel, R. A., T. Peelen, R. J. Siezen, B. DeKruijff, and O. P. Kuipers. 1996. Nisin Z, mutant nisin Z and lactacin 481 interactions with anionic lipids correlate with antimicrobial activity. A monolayer study. Eur. J. Biochem. **235**:267-274.
56. Dixon, N. M. and D. B. Kell. 1989. A review: the inhibition by CO_2 of the growth and metabolism of micro-organisms. J. Appl. Bacteriol. **67**:109-136.
57. Driessen, A. J. M., H. W. Vandenhooven, W. Kuiper, M. Vandekamp, H. G. Sahl, R. N. H. Konings, and W. N. Konings. 1995. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry **34**:1606-1614.
58. Drucker, D. B., G. Megson, D. W. S. Harty, I. Riba, and S. J. Gaskell. 1995. Phospholipids of *Lactobacillus* spp. J. Bacteriol. **177**:6304-6308.
59. Eisenberg, D., R. M. Weiss, and T. C. Terwilliger. 1982. The helical hydrophobic moment: a measure of the amphiphilicity of a helix. Nature **299**:371-374.
60. Eklund, T. and T. Jarmund. 1983. Microculture studies on the effect of various gas atmospheres on microbial growth at different temperatures. J. Appl. Bacteriol. **55**:119-125.

61. Enfors, S. O. and G. Molin. 1980. Effect of high concentrations of carbon dioxide on growth rate of *Pseudomonas fragi, Bacillus cereus* and *Streptococcus cremoris.* J. Appl. Bacteriol. **48**:409-416.
62. Enfors, S. O. and G. Molin. 1981. The influence of temperature on the growth inhibitory effect of carbon dioxide on *Pseudomonas fragi* and *Bacillus cereus.* Can. J. Microbiol. **27**:15-19.
63. Enfors, S. O., G. Molin, and A. Ternström. 1979. Effect of packaging under carbon dioxide, nitrogen or air on the microbial flora of pork stored at 4°C. J. Appl. Bacteriol. **47**:197-208.
64. Ennahar, S., D. Aoude-Werner, O. Sorokine, A. Van Dorsselaer, F. Bringel, J.-C. Hubert, and C. Hasselmann. 1996. Production of Pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. Appl. Environ. Microbiol. **62**:4381-4387.
65. Eyles, M. J., C. J. Moir, and J. A. Davey. 1993. The effects of modified atmospheres on the growth of psychrotrophic pseudomonads on a surface in a model system. Int. J. Food Microbiol. **20**:97-107.
66. Fabrie, C. H., J. M. Smeets, B. de Kruijff, and J. de Gier. 1994. The cryoprotectant trehalose destabilises the bilayer organisation of *Escherichia coli*-derived membrane systems at elevated temperatures as determined by ^{2}H and ^{31}P-NMR. Chem. Phys. Lipids **70**:133-145.
67. Farber, J. M. 1991. Microbiological aspects of modified atmosphere packaging technology - a review. J. Food Prot. **54**:58-70.
68. Fimland, G., O. R. Blingsmo, K. Sletten, G. Jung, I. F. Nes, and J. Nissenmeyer. 1996. New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins - the C-terminal region is important for determining specificity. Appl. Environ. Microbiol. **62**:3313-3318.
69. Fleury, Y., M. A. Dayem, J. J. Montagne, E. Chaboisseau, J. P. Le Caer, P. Nicolas, and A. Delfour. 1996. Covalent structure, synthesis, and structure-function studies of Mesentericin Y 105^{37}, a defence peptide from Gram-positive bacteria *Leuconostoc mesenteroides.* J. Biol. Chem. **271**:14421-14429.
70. Gaines, G. L. 1966. Insoluble monolayers at liquid-gas interfaces. Interscience Publishers, Inc. New York, N.Y.
71. Gallois, A., R. Samson, E. Ageron, and P. A. D. Grimont. 1992. *Erwinia carotovora* subsp. nov. associated with odorous soft rot of chicory *(Cichorium intybus L.).* Int. J. Syst. Bact. **42**:582-588.
72. Gao, F. H., T. Abee, and W. N. Konings. 1991. Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome C oxidase-containing proteoliposomes. Appl. Environ. Microbiol. **57**:2164-2170.
73. Garcia Garcera, M. J., M. G. L. Elferink, A. J. M. Driessen, and W. N. Konings. 1993. *In vitro* pore-forming activity of the lantibiotic nisin. Role of proton motive force and lipid composition. Eur. J. Biochem. **212**:417-422.
74. García-Bustos, J. F., N. Pezzi, and E. Mendez. 1985. Structure and mode of action of microcin C7, an antibacterial peptide produced by *Escherichia coli.* Antimicrob. Agents Chemother. **27**:791-797.
75. Geis, A., J. Singh, and M. Teuber. 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. **45**:205-211.
76. Gill, C. D. and G. Molin. 1993. Modified atmospheres and vacuum packaging, p. 172-199. In N. J. Russell and G. W. Gould (eds.), Food preservatives. Blackie, Glasgow.
77. Gill, C. D. and M. P. Reichel. 1989. Growth of the cold-tolerant pathogens *Yersinia enterocolitica, Aeromonas hydrophila* and *Listeria monocytogenes* on high-pH beef packaged under vacuum or carbon dioxide. Food Microbiol. **6**:223-230.
78. Gill, C. O. 1988. The solubility of carbon dioxide in meat. Meat Sci. **23**:65-71.
79. Gill, C. O. and K. H. Tan. 1979. Effect of carbon dioxide on growth of *Pseudomonas fluorescens.* Appl. Environ. Microbiol. **38**:237-240.
80. Golden, D. A., M. J. Eyles, and L. R. Beuchat. 1989. Influence of modified atmosphere storage on the growth of uninjured and heat-injured *Aeromonas hydrophila.* Appl. Environ. Microbiol. **55**:3012-3015.
81. Gonzalez, B., E. Glaasker, E. R. S. Kunji, A. J. M. Driessen, J. E. Suarez, and W. N. Konings. 1996. Bactericidal mode of action of plantaricin C. Appl. Environ. Microbiol. **62**:2701-2709.
82. Gonzalez, C. F. and B. S. Kunka. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici.* Appl. Environ. Microbiol. **53**:2534-2538.
83. Gorris, L. G. M. and H. W. Peppelenbos. 1992. Modified atmosphere and vacuum packaging to extend the shelf life of respiring produce. HortTechnology **2**:303-309.
84. Gosselink, R. J. A., J. E. G. Van Dam, and F. H. A. Zomers. 1995. Combined HPLC analysis of organic acids and furans formed during organosolv pulping of fiber hemp. J. Wood Chem. Technol. **15**:1-25.
85. Gould, G. W. 1992. Ecosystem approach to food preservation. J. Appl. Bacteriol. **73** Supplement: 58S-68S.
86. Gould, G. W. 1996. Industry perspective on the use of natural antimicrobials and inhibitors for food applications. J. Food Prot. Supplement: 82-86.

87. Grimont, P. A. D., M. Vancanney, M. Lefevre, P. Vandemeulebroecke, L. Vauterin, R. Brosch, K. Kersters, and F. Grimont. 1996. Ability of Biolog and Biotype-100 systems to reveal the taxonomic diversity of the pseudomonads. Syst. Appl. Microbiol. **19:**510-527.
88. Guihard, G., H. Benedetti, M. Besnard, and L. Letellier. 1993. Phosphate efflux through the channels formed by colicins and phage T5 in *Escherichia coli* cells is responsible for the fall in cytoplasmic ATP.
J. Biol. Chem. **268:**17775-17780.
89. Hammes, W. P. and P. S. Tichaczek. 1994. The potential of lactic acid bacteria for the production of safe and wholesome food. Zeitschrift fur Lebensmittel-Untersuchung und -Forschung **198:**193-201.
90. Hao, Y. Y. and R. E. Brackett. 1993. Influence of modified atmosphere on growth of vegetable spoilage bacteria in media. J. Food Prot. **56:**223-228.
91. Harris, L. J., H. P. Fleming, and T. R. Klaenhammer. 1992. Developments in nisin research.
Food Res. Int. **25:**57-66.
92. Harris, L. J., H. P. Fleming, and T. R. Klaenhammer. 1992. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A and UAL 500 to nisin. J. Food Prot. **54:**836-840.
93. Harris, L. J., H. P. Fleming, and T. R. Klaenhammer. 1992. Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant *Leuconostoc mesenteroides* strain and a nisin-producing *Lactococcus lactis* strain. Appl. Environ. Microbiol. **58:**1484-1489.
94. Hastings, J. W., M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1991. Characterization of leucocin A-UAL187 and cloning of the bacteriocin gene from *Leuconostoc gelidum.* J. Bacteriol. **173:**7491-7500.
95. Hechard, Y., B. Derijard, F. Letellier, and Y. Cenatiempo. 1992. Characterization and purification of mesentericin Y105, an anti-listeria bacteriocin from *Leuconostoc mesenteroides.* J. Gen. Microbiol. **138:**2725-2731.
96. Henderson, J. T., A. L. Chopko, and P. D. Vanwassenaar. 1992. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. Arch. Biochem. Biophys. **295:**5-12.
97. Heytler, P. G. 1979. Uncouplers of the oxidative phosphorylation. Methods Enzymol. **55:**562-572.
98. Higgins, D. G. and P. M. Sharp. 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73:**237-244.
99. Hintlian, C. B. and J. H. Hotchkiss. 1986. The safety of modified atmosphere packaging: a review. Food Technol. **12:**70-76.
100. Hintlian, C. B. and J. H. Hotchkiss. 1987. Comparative growth of spoilage and pathogenic organisms on modified atmosphere packaged cooked beef. J. Food Prot. **50:**218-223.
101. Holck, A., L. Axelsson, S.-E. Birkeland, T. Aukrust, and H. Blom. 1992. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J. Gen. Microbiol. **138:**2715-2720.
102. Holck, A. L., L. Axelsson, K. Huhne, and L. Krockel. 1994. Purification and cloning of sakacin 674, a bacteriocin from *Lactobacillus sake* Lb674. FEMS Microbiol. Lett. **115:**143-149.
103. Holmes, B., M. Costas, M. Ganner, S. L. W. On, and M. Stevens. 1994. Evaluation of the Biolog system for identification of some Gram-negative bacteria of clinical importance. J. Clin. Microbiol. **32:**1970-1975.
104. Hoover, D. and L. Steenson. 1993. Bacteriocins of lactic acid bacteria. Academic Press, New York, N.Y.
105. Hoover, D. G., P. M. Walsh, K. M. Kolaetis, and M. M. Daly. 1988. A bacteriocin produced by *Pediococcus* species associated with a 5.5-megadalton plasmid. J. Food Prot. **51:**29-31.
106. Horth, M., B. Lambrecht, M. C. L. Khim, F. Bex, C. Thiriart, J. M. Ruysschaert, A. Burny, and R. Brasseur. 1991. Theoretical and functional analysis of the SIV fusion peptide. EMBO J. **10:**2747-2755.
107. Hudson, J. A., S. J. Mott, and N. Penney. 1994. Growth of *Listeria monocytogenes, Aeromonas hydrophila,* and *Yersinia enterocolitica* on vacuum and saturated carbon dioxide controlled atmosphere packaged sliced roast beef. J. Food Prot. **57:**204-208.
108. Jack, R. W., J. R. Tagg, and B. Ray. 1995. Bacteriocins of Gram-positive bacteria. Microbiol. Rev. **59:**171-200.
109. Jack, R. W., J. Wan, J. Gordon, K. Harmark, B. E. Davidson, A. J. Hillier, R. E. H. Wettenhall, M. W. Hickey, and M. J. Coventry. 1996. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl. Environ. Microbiol. **62:**2897-2903.
110. Jähnig, F. 1990. Structure predictions of membrane proteins that are not that bad.
Trends Biochem. Sci. **15:**93-95.
111. Jung, G. and H.-G. Sahl. 1991. Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
112. Kader, A. A. 1980. Prevention of ripening in fruits by use of controlled atmospheres. Food Technol. **34:**50-54.
113. Kader, A. A., D. Zagory, and E. L. Kerbel. 1989. Modified atmosphere packaging of fruits and vegetables. Crit. Rev. Food Sci. Nutr. **28:**1-30.

114. Kagermeier-Callaway, A. S. and E. Lauer. 1995. *Lactobacillus sake* Katagiri, Kitahara, and Fukami 1934 is the senior synonym for *Lactobacillus bavaricus* Stetter and Stetter 1980. Int. J. Syst. Bact. **45**:398-399.
115. Kaiser, A. L. and T. J. Montville. 1993. The influence of pH and growth rate on production of the bacteriocin, bavaricin MN, in batch and continuous fermentations. J. Appl. Bacteriol. **75**:536-540.
116. Kallander, K. D., A. D. Hitchins, G. A. Lancette, J. A. Schmieg, G. R. Garcia, H. M. Solomon, and J. N. Sofos. 1991. Fate of *Listeria monocytogenes* in shredded cabbage stored at 5 and 25°C under a modified atmosphere. J. Food Prot. **54**:302-304.
117. Kates, M. 1986. Laboratory techniques in biochemistry and molecular biology. p.1-464. Elsevier, Amsterdam, The Netherlands.
118. Kersters, K., W. Ludwig, M. Vancanneyt, P. De Vos, M. Gillis, and K.-H. Schleifer. 1996. Recent changes in the classification of the pseudomonads: an overview. Syst. Appl. Microbiol. **19**:465-477.
119. Killian, J. A., M. C. Koorengevel, J. A. Bouwstra, G. Gooris, W. Dowhan, and B. de Kruijff. 1994. Effect of divalent cations on lipid organization of cardiolipin isolated from *Escherichia coli* strain AH930. Biochim. Biophys. Acta **1189**:225-232.
120. King, A. D. and C. W. Nagel. 1975. Influence of carbon dioxide upon the metabolism of *Pseudomonas aeruginosa*. J. Food Sci. **40**:362-366.
121. Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39-86.
122. Kleerebezem, M., L. E. N. Quadri, O. P. Kuipers, and W. M. De Vos. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol. Microbiol. **24**:895-904.
123. Kleinkauf, H. and H. von Döhren. 1987. Biosynthesis of peptide antibiotics. Annu. Rev. Microbiol. **41**:259-289.
124. Klingler, J. M., R. P. Stowe, D. C. Obenhuber, T. O. Groves, S. K. Mishra, and D. L. Pierson. 1992. Evaluation of the Biolog automated microbial identification system. Appl. Environ. Microbiol. **58**:2089-2092.
125. Kolter, R. and F. Moreno. 1992. Genetics of ribosomally synthesized peptide antibiotics. Annu. Rev. Microbiol. **46**:141-163.
126. Kordel, M., R. Benz, and H.-G. Sahl. 1988. Mode of action of the staphylococcin-like peptide Pep 5: voltage-dependent depolarization of bacterial and artificial membranes. J. Bacteriol. **170**:84-88.
127. Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. De Vos. 1993. Characterization of the gene cluster nisABTCIPR of *Lactococcus lactis*; requirement of expression of the nisA and nisI genes for development of immunity. Eur. J. Biochem. **216**:281-291.
128. Larsen, A. G., F. K. Vogensen, and J. Josephsen. 1993. Antimicrobial activity of lactic acid bacteria isolated from sour doughs - purification and characterization of bavaricin-A, a bacteriocin produced by *Lactobacillus bavaricus* MI401. J. Appl. Bacteriol. **75**:113-122.
129. Lewus, C. B. and T. J. Montville. 1991. Detection of bacteriocins produced by lactic acid bacteria. J. Microbiol. Methods **13**:145-150.
130. Liao, C. H., D. E. McCallus, and J. M. Wells. 1993. Calcium-dependent pectate lyase production in the soft-rotting bacterium *Pseudomonas fluorescens*. Phytopathol. **83**:813-818.
131. Lindgren, S. E. and W. J. Dobrogosz. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. **87**:149-164.
132. Lioutas, T. S. 1988. Challenges of controlled and modified atmosphere packaging: a food company's perspective. Food Technol. **42**:78-86.
133. Lowry, O. H., N. J. Rosebourg, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265-275.
134. Lund, B. M. 1983. Bacterial spoilage, p. 219-257. In C. Dennis (ed.), Postharvest pathology of fruits and vegetables. Academic Press, London.
135. Lundin, A. and A. Thore. 1975. Analytical information obtainable by evaluation of the time course of firefly bioluminescence in the assay of ATP. Anal. Biochem. **66**:47-63.
136. Maftah, A., D. Renault, C. Vignoles, Y. Hechard, P. Bressolier, M. H. Ratinaud, Y. Cenatiempo, and R. Julien. 1993. Membrane permeabilization of *Listeria monocytogenes* and mitochondria by the bacteriocin mesenterocin Y105. J. Bacteriol. **175**:3232-3235.
137. Maisnier-Patin, S. and J. Richard. 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations. FEMS Microbiol. Lett. **140**:29-35.
138. Margesin, R. and F. Schinner. 1994. Psychrotrophic proteolytic bacteria of dairy origin. Biodeterioration Abstr. **8**:1-7.

139. Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenbergh. 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0.
Appl. Environ. Microbiol. **58**:2360-2367.
140. McMullen, L. M. and M. E. Stiles. 1996. Potential for use of bacteriocin-producing lactic acid bacteria in the preservation of meats. J. Food Prot. **Suppl**:S:64-71.
141. Mills, O. E. and T. D. Thomas. 1978. Release of cell wall-associated proteinases from lactic streptococci. N. Z. J. Dairy Sci. Technol. **13**:209-215.
142. Ming, X. and M. A. Daeschel. 1993. Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* Scott-A. J. Food Prot. **56**:944-948.
143. Molin, G. 1983. The resistance to carbon dioxide of some food related bacteria. Eur. J. Appl. Microbiol. Biotechnol. **18**:214-217.
144. Mossel, D. A. A. and W. F. Jacobs-Reitsma. 1990. Microbiological research of foods: strategy, principles and methods (Dutch: Mikrobiologisch onderzoek van levensmiddelen: strategie, principes en methoden). P.C. Noordervliet B.V. Zeist, The Netherlands.
145. Motlagh, A., M. Bukhtiyarova, and B. Ray. 1994. Complete nucleotide sequence of psmb-74, a plasmid encoding the production of pediocin AcH in *Pediococcus acidilactici*. Lett. Appl. Microbiol. **18**:305-312.
146. Motlagh, A. M., A. K. Bhunia, F. Szostek, T. R. Hansen, M. C. Johnson, and B. Ray. 1992. Nucleotide and amino acid sequence of pap-gene (pediocin AcH production) in *Pediococcus acidilactici*. H. Lett. Appl. Microbiol. **15**:45-48.
147. Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek **70**:113-128.
148. Nguyen-The, C. and F. Carlin. 1994. The microbiology of minimally processed fresh fruits and vegetables. Crit. Rev. Food Sci. Nutr. **34**:371-401.
149. O'Leary, W. M. and S. G. Wilkinson. 1988. Gram-positive bacteria, p. 117-201. In C. Rutledge and S. G. Wilkonson (eds.), Microbial lipids. Acadamic Press, London, England.
150. Ogrydziak, D. M. and W. M. Brown. 1982. Temperature effects in modified atmosphere storage of seafoods. Food Technol. **35**:86-96.
151. Ojcius, D. M. and J. D.-E. Young. 1991. Cytolytic pore-forming proteins and peptides: is there a common structural motif ? Trends Biochem. Sci. **16**:225-229.
152. Peppelenbos, H. W. 1995. A systematic approach to research on modified atmosphere packaging of produce. Proceedings European Concerted Action COST94, 1-2 October 1992, Istanbul, Turkey, pp 51-63.
153. Peppelenbos, H. W., L. Brien, and L. G. M. Gorris. 1997. The influence of carbon dioxide on gas exchange rates of mungbean sprouts under aerobic and anaerobic conditions. J. Sci. Food Agric. in press.
154. Peppelenbos, H. W., L. M. M. Tijskens, J. Van 't Leven, and E. C. Wilkinson. 1996. Modelling oxidative and fermentative carbon dioxide production of fruits and vegetables. Posth. Biol. Technol. **9**:283-295.
155. Pillot, T., M. Goethals, B. Vanloo, C. Talussot, R. Brasseur, J. Vandekerckhove, M. Rosseneu, and L. Lins. 1996. Fusogenic properties of the C-terminal domain of the Alzheimer β-amyloid peptide.
J. Biol. Chem. **271**:28757-28765.
156. Price, T. V. 1988. Seed sprout production for human consumption - a review.
Can. Inst. Food Sci. Technol. J. **21**:57-65.
157. Pucci, M. J., E. R. Vedamuthu, B. S. Kunka, and P. A. Vandenbergh. 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0.
Appl. Environ. Microbiol. **54**:2349-2353.
158. Quadri, L. E. N., M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. J. Biol. Chem. **269**:12204-12211.
159. Quadri, L. E. N., M. Sailer, M. R. Terebiznik, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. J. Bacteriol. **177**:1144-1151.
160. Quadri, L. E. N., L. Z. Yan, M. E. Stiles, and J. C. Vederas. 1997. Effect of amino acid substitutions on the activity of carnobacteriocin B2: overproduction of the antimicrobial peptide, its engineered variants and its precursor in *Escherichia coli*. J. Biol. Chem. **272**:3384-3388.
161. Rahman, M., L. Lins, A. Soumarmon, and R. Brasseur. 1997. Are amphipathic asymmetric peptides ubiquitous structures for membrane destabilisation. J. Mol. Model. **3**:203-215.

162. Ray, B. and M. Daeschel. 1992. Food biopreservatives of microbial origin. p.1-386. CRC Press, Boca Raton, Ann Arbor, London, Tokyo.
163. Rekhif, N., A. Atrih, and G. Lefebvre. 1994. Selection and properties of spontaneous mutants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. Curr. Microbiol. **28**:237-241.
164. Ruhr, E. and H.-G. Sahl. 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob.
Agents Chemother. **27**:841-845.
165. Sahl, H.-G., M. Kordel, and R. Benz. 1987. Voltage-dependent depolarization of bacterial membranes and artificial lipid bilayers by the peptide antibiotic nisin. Arch. Microbiol. **149**:120-124.
166. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.
167. Schlech, W. F., P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome. 1983. Epidemic Listeriosis - evidence for transmission by food. N. Engl. J. Med. **308**:203-206.
168. Siezen, R. J., O. P. Kuipers, and W. M. De Vos. 1996. Comparison of lantibiotic gene clusters and encoded proteins. Antonie van Leeuwenhoek **69**:171-184.
169. Simpson, W. J. and H. Taguchi. 1995. The genus *Pediococcus*, with notes on the genera *Tetratogenococcus* and *Aerococcus*, p. 125-172. In B. J. B. Wood and W. H. Holzapfel (eds.), The lactic acid bacteria. Blackie Academic & Professionals, London.
170. Sizmur, K. and C. W. Walker. 1988. Listeria in prepacked salads. Lancet **i**:1167
171. Splittstoesser, D. F., D. T. Queale, and B. W. Andaloro. 1983. The microbiology of vegetable sprouts during commercial production. J. Food Saf. **5**:79-86.
172. Stiles, M. E. 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek **70**:331-345.
173. Stoffels, G., J. Nissen-Meyer, A. Gudmundsdottir, K. Sletten, H. Holo, and I. F. Nes. 1992. Purification and characterization of a new bacteriocin isolated from a *Carnobacterium* sp. Appl. Environ. Microbiol. 58:1417-1422.
174. Suutari, M. and S. Laakso. 1994. Microbial fatty acids and thermal adaptation.
Crit. Rev. Microbiol. **20**:285-328.
175. Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. 1976. Bacteriocins of Gram-positive bacteria.
Bacteriol. Rev. **40**:722-756.
176. Talmud, P., L. Lins, and R. Brasseur. 1996. Prediction of signal peptide functional properties: a study of the orientation and angle of insertion of yeast invertase mutants and human apolipoprotein B signal peptide variants. Protein Eng. **9**:317-321.
177. Ten Brink, B., R. Otto, U.-P. Hansen, and W. N. Konings. 1985. Energy recycling by lactate efflux in growing and nongrowing cells of *Streptococcus cremoris*. J. Bacteriol. **162**:383-390.
178. Tichaczek, P. S., R. F. Vogel, and W. P. Hammes. 1993. Cloning and sequencing of *curA* encoding curvacin-A, the bacteriocin produced by *Lactobacillus curvatus* LTH1174. Arch. Microbiol. 160:279-283.
179. Tichaczek, P. S., R. F. Vogel, and W. P. Hammes. 1994. Cloning and sequencing of *sak P* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. Microbiology **140**:361-367.
180. Toan, P. V. and D. Beutling. 1992. Studies on the behaviour of *Yersinia enterocolitica* in artificially contaminated vietnamesian and german type vegetable salads. Arch. Lebensmittelhyg. **43**:8-11.
181. Van Belkum, M. J., B. J. Hayema, R. J. Jeeninga, J. Kok, and G. Venema. 1991. Organization and nucleotide sequences of two lactococcal bacteriocin operons. Appl. Environ. Microbiol. **57**:492-498.
182. Van Belkum, M. J., J. Kok, G. Venema, H. Holo, I. F. Nes, W. N. Konings, and T. Abee. 1991. The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-dependent, protein-mediated manner. J. Bacteriol. **173**:7934-7941.
183. Vandenberg, D. J. C., A. Smits, B. Pot, A. M. Ledeboer, K. Kersters, J. M. A. Verbakel, and C. T. Verrips. 1993. Isolation, screening and identification of lactic acid bacteria from traditional food fermentation processes and culture collections. Food Biotechnology **7**:189-205.
184. Vandenbergh, P. A. 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiol. Rev. **12**:221-238.
185. Venema, K., T. Abee, A. J. Haandrikman, K. J. Leenhouts, J. Kok, W. N. Konings, and G. Venema. 1993. Mode of action of lactococcin-B, a thiol-activated bacteriocin from *Lactococcus lactis*.
Appl. Environ. Microbiol. 59:1041-1048.
186. Venema, K., R. E. Haverkort, T. Abee, A. J. Haandrikman, K. J. Leenhouts, L. Deleij, G. Venema, and J. Kok.

1994. Mode of action of LciA, the lactococcin A immunity protein. Mol. Microbiol. **14**:521-532.
187. Venema, K., G. Venema, and J. Kok. 1995. Lactococcal bacteriocins: mode of action and immunity. Trends Microbiol. **3**:299-304.
188. Vescovo, M., C. Orsi, G. Scolari, and S. Torriani. 1995. Inhibitory effect of selected lactic acid bacteria on microflora associated with ready-to-use vegetables. Lett. Appl. Microbiol. **21**:121-125.
189. Vizán, J. L., C. Hernández-Chico, I. del Castillo, and F. Moreno. 1991. The peptide antibiotic microcin B17 induces double-strand cleavage of DNA mediated by *E. coli* DNA gyrase. EMBO J. **10**:467-476.
190. Vogel, R. F., B. S. Pohle, P. S. Tichaczek, and W. P. Hammes. 1993. The competitive advantage of *Lactobacillus curvatus* LTH 1174 in sausage fermentations is caused by formation of curvacin A. Syst. Appl. Microbiol. **16**:457-462.
191. Voneche, V., D. Portelle, R. Kettmann, L. Willems, K. Limbach, E. Paoletti, J. M. Ruysschaert, A. Burny, and R. Brasseur. 1992. Fusogenic segments of bovine leukemia virus and simian immunodeficiency virus are interchangeable and mediate fusion by means of oblique insertion in the lipid bilayer of their target cells. Proc. Natl. Acad. Sci. U. S. A. **89**:3810-3814.
192. Wilson, M. and S. E. Lindow. 1994. Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Appl. Environ. Microbiol. **60**:4468-4477.
193. Winkowski, K., M. E. C. Bruno, and T. J. Montville. 1994. Correlation of bioenergetic parameters with cell death in *Listeria monocytogenes* cells exposed to nisin. Appl. Environ. Microbiol. **60**:4186-4188.
194. Winkowski, K., A. D. Crandall, and T. J. Montville. 1993. Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus* MN in beef systems at refrigeration temperatures.
Appl. Environ. Microbiol. **59**:2552-2557.
195. Wolfe, S. K. 1980. Use of CO- and CO_2-enriched atmospheres for meats, fish, and produce. Food Technol. **34**:54-63.
196. Wood, B. J. B. and W. H. Holzapfel. 1995. The genera of lactic acid bacteria. p.1-398. Blackie Academics and Professionals, Glasgow, UK.
197. Yang, C. C. and J. Konisky. 1984. Colicin V-treated *Escherichia coli* does not generate membrane potential.
J. Bacteriol. **158**:757-759.
198. Yousef, A. E., J. B. Luchansky, A. J. Degnan, and M. P. Doyle. 1991. Behavior of *Listeria monocytogenes* in Wiener exudates in the presence of *Pediococcus acidilactici* H or pediocin AcH during storage at 4 or 25°C. Appl. Environ. Microbiol. **57**:1461-1467.
199. Zagory, D. and A. A. Kader. 1988. Modified atmosphere packaging of fresh produce. Food Technol. **42**:70-77.
200. Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. Van 't Riet. 1990. Modeling of the bacterial growth curve. Appl. Environ. Microbiol. **56**:1875-1881.

SUMMARY AND CONCLUDING REMARKS

Recent trends in food preservation are the use of mild preservation techniques, such as modified atmosphere (MA) packaging and refrigeration, to prolong the shelflife of foods without affecting the fresh character of the product. This has resulted in the development of a new generation of chill stored, minimally processed foods, such as vegetables that are packaged under reduced oxygen (O_2) and elevated carbon dioxide (CO_2) concentrations. These gas atmospheres in combination with refrigerated storage conditions reduce the respiration rates of these products, and can limit the growth of endogenous spoilage bacteria, thereby rendering prolonged shelflives as compared with storage under ambient conditions. Although MA packaged vegetables have a fair safety record, they may incidently harbor foodborne pathogens. Especially the outgrowth of psychrotrophic (cold tolerant) pathogens can be considered a safety concern, but quantitative data on their growth under MA conditions that are suitable for produce are essentially lacking. Detailed information on the outgrowth of both pathogens and vegetable-associated spoilage bacteria under these conditions may give better insight into possible antagonistic action of competing microflora on the growth of pathogens, and the development of adequate countermeasures toward their growth to hazardous levels.

One of the objectives of the studies that are described in this thesis was to obtain information on the impact of refrigerated MA storage conditions on the growth of microorganisms on minimally processed, MA packaged produce. Since our initial studies substantiated the possible hazard which can be posed by psychrotrophic pathogens, the use of biopreservation for adequate control of these microorganisms was investigated. In this respect, we have focused on lactic acid bacteria (LAB) because they occur naturally on fresh and minimally processed vegetables, and are able to produce a variety of antimicrobial substances, amongst which bacteriocins.

Chapter 1 gives a short introduction on the technology of MA packaging of minimally processed vegetables and their current microbiological safety and quality status. Furthermore, the possible role of LAB as biopreservation agents in foods is addressed with special emphasis on those strains that are able to produce bacteriocins. The characteristics and mode of action of different bacteriocins that are produced by LAB are described, as well as aspects which are relevant to the application of these antimicrobial compounds or their producing organisms as food preservatives.

Detailed information on the impact of refrigerated MA storage on the population dynamics of the endogenous microflora on vegetables is limited. Therefore, the prevailing epiphytic microorganisms on model produce (*i.e.* chicory endive, and mungbean sprouts) were identified before and after MA storage. Subsequently, the growth characteristics of the predominant spoilage bacteria were determined under MA conditions **(Chapter 2)**. It was found that the major strains on both products were *Enterobacteriaceae* and *Pseudomonas* species. There were changes in the prevalent species on chicory endive before and after MA storage, but this was not observed for mungbean sprouts. To quantify the influence of CO_2 and O_2 on the growth of the individual spoilage bacteria, a model agar system was developed that mimicked the *in situ* growth of bacteria on the surface of vegetables. The analysis of the growth of the predominant strains under controlled O_2 and CO_2 concentrations at 8°C indicated that, in general, lag times were not present under the various conditions tested, and that the maximum population sizes were not affected. However, the maximum specific growth rates generally decreased with increasing CO_2 concentrations, which was independent of the O_2 concentrations applied. This effect was more pronounced for *Pseudomonas* species than for *Enterobacteriaceae*. Representatives of the former subpopulation already showed significantly reduced maximum specific growth rates at CO_2 concentrations that are suitable for MA packaged vegetables.

In **Chapter 3**, the effects of different O_2 and CO_2 concentrations on the growth of *Aeromonas hydrophila, Yersinia enterocolitica, L. monocytogenes,* and a cold tolerant strain of *Bacillus cereus* were quantified in the above mentioned model agar system at 8°C. Again, the maximum specific growth rates of the various microorganisms decreased significantly with increasing CO_2 concentration, while the maximum population densities were not affected and lag times were not observed. Extrapolation of the results from the model system to MA packaged vegetables suggested that growth of the above pathogens may occur at 8°C to the same maximum population densities as compared to ambient conditions, but at 10 to 20% lower maximum specific growth rates. Because we have shown that MA packaging can only moderately retard the growth of psychrotrophic pathogens, these bacteria can be considered a safety hazard to which countermeasures need to be developed.

L. monocytogenes is a relevant psychrotrophic pathogen on minimally processed vegetables that requires adequate control measures. This Gram-positive bacterium can be inhibited by a number of bacteriocins that are produced by LAB. Thus, biopreservation using bacteriocin producing LAB strains may effectively prevent the growth of this bacterium, and possibly other psychrotrophic pathogens. With respect to practical application, an effort was made to obtain bacteriocinogenic LAB from minimally processed vegetables since these strains might be best adapted to the specific condi-

tions under which their antimicrobial activity is required. Out of a total of 890 LAB isolates from mungbean sprouts and chicory endive, only nine strains were found to produce bacteriocins **(Chapter 4)**. Three of these strains exhibited antimicrobial activity towards a wide variety of Gram-positive bacteria, including the foodborne pathogens *L. monocytogenes*, and nonproteolytic *Clostridium botulinum*. Two of these bacteriocinogenic isolates were identified as *Pediococcus parvulus* on the basis of fermentation patterns and ribotyping. Recombinant DNA experiments revealed the presence of the pediocin PA-1 gene in both LAB strains, and further biochemical analysis confirmed the production of pediocin PA-1 in absence of other bacteriocins.

The third broad spectrum bacteriocin-producing strain was identified as *Enterococcus mundtii* **(Chapter 5)**. Its bacteriocin was purified to homogeneity and characterized. Elucidation of the complete primary amino acid sequence revealed a novel bacteriocin of 43 amino acids (M_r 4287.2), designated as mundticin, that belongs to the class **IIa** bacteriocins of LAB. Other LAB that produce this class of antimicrobial peptides have previously been isolated from a wide variety of meat and dairy products, and their apparent wide-spread occurrence in foods may allow for the selection of strains that are ecologically adapted to specific food environments.

As further described in **Chapter 5**, mundticin exhibited bactericidal activity towards *L. monocytogenes*, and was shown to dissipate the membrane potential of whole cells, and to deplete their intracellular ATP pools. Mundticin was selected as a prototype of class **IIa** bacteriocins to investigate the biophysical properties of this peptide by molecular computer analysis. It was found that mundticin and related bacteriocins, contain a central α-helical region that was predicted to insert at an angle of 30 to 50° in a simulated hydrophobic/hydrophilic interface. This oblique insertion is proposed to cause a destabilisation of the phospholipid bilayer and facilitate the insertion and/or aggregation of monomers into functional pores in the cytoplasmic membrane. This concept is of interest for the rational design of bacteriocins with, for example, better biopreservation properties.

For the effective application of bacteriocins as biopreservation agents, it is essential to gain more insight into the variation in the susceptibility of Gram-positive bacteriocins towards bacteriocins. The comprehensive study that is described in **Chapter 6** addresses several factors which are associated with the natural variability in the bacteriocin-sensitivity of closely related non-bacteriocinogenic LAB. Two sets of strains for which the minimal inhibitory concentrations for nisin and pediocin PA-1 differed 100 to 1000-fold were compared. Our data provide evidence that the association of bacteriocins with the cell membrane and their subsequent insertion into the membrane take place in a similar way for cells that have a high or a low natural bacteriocin tolerance. For insensitive strains, the overall constitution of the membrane, rather than the mere membrane fluidity, may preclude the formation of pores with sufficient diameters and lifetimes to ultimately cause cell death. Further analysis of this phenomena may require more attention for the structural role of proteins in biological membranes.

The last part of this dissertation comprises a study on the potential of the vegetable-associated bacteriocin-producing bacteria and mundticin to act as biopreserva-

tion agents on refrigerated, MA-stored vegetables **(Chapter 7)**. Both *P. parvulus* strains were found to be unsuitable for this purpose, due to the lack of bacteriocin production at 4 and 8°C. By contrast, *E. mundtii* displayed a number of characteristics that favor an application as a biopreservative agent, such as limited acid production, production of a bacteriocin with anti-listeria activity at low temperatures, and high growth rates under MA conditions. Despite these properties, this strain failed to effectively inhibit the growth of *L. monocytogenes* on fresh mungbean sprouts. This is probably not due to a lack of *in situ* bacteriocin production, but rather the result of proteolytic degradation of mundticin or the growth of *L. monocytogenes* cells that are resistant to mundticin. Experiments with mundticin in a washing step and as a component of a coating were successful and warrant further research on the potential of this compound as a natural preservative agent for minimally processed vegetables.

In conclusion, this dissertation has contributed to a better understanding of the microbiological characteristics of minimally processed vegetables that are stored under MA conditions. A general concern for the possible outgrowth of psychrotrophic pathogens on MA stored vegetables was substantiated by our investigations, which stresses that hygienic cultivation and processing conditions of this type of products remain indispensable. Although bacteriocins cannot be used as the sole preservatives to enhance the safety of refrigerated MA-stored minimally processed vegetables, they can contribute to tackle safety problems that may arise from certain cold tolerant, Gram-positive pathogens.

SAMENVATTING EN SLOTOPMERKINGEN

Een recente trend in de voedselconservering is de toepassing van milde technieken, zoals natuurlijke anti-microbiële verbindingen en het verpakken onder gewijzigde gascondities. Dit heeft geleid tot de ontwikkeling van gekoelde, minimaal bewerkte levensmiddelen, waaronder groenten die zijn verpakt onder verlaagde zuurstof (O_2) en verhoogde kooldioxide (CO_2) concentraties. De combinatie van koeling en dergelijke gascondities leidt tot een verlengde houdbaarheid als gevolg van een verlaging van de respiratie snelheden van deze produkten en een remming van de groei van de endogene microbiële bederf-flora. Hoewel gasverpakte groenten als veilig te boek staan, kunnen er incidenteel pathogene bacteriën op voorkomen. Met name de uitgroei van koude-tolerante (psychrotrofe) stammen kan de veiligheid van deze produkten in gevaar brengen. Er is relatief weinig bekend over de groei van deze organismen onder gascondities die geschikt zijn voor groenten. Om de uitgroei van pathogenen doeltreffend tegen te kunnen gaan, is het niet alleen van belang een beter beeld te krijgen van de invloed van gewijzigde gascondities op deze micro-organismen, maar ook van de invloed ervan op mogelijk antagonistische/competerende microflora.

Een van de doelstellingen van deze dissertatie vormde het verdiepen van het inzicht in de invloed van gewijzigde gascondities op de groei van micro-organismen op minimaal verwerkte, gasverpakte groente tijdens gekoelde bewaring. Omdat uit initiële studies **(Hoofdstuk 3)** naar voren kwam dat psychrotrofe pathogene bacteriën een mogelijk veiligheidsrisico met zich mee kunnen brengen, is nagegaan of de groei van deze micro-organismen kan worden tegengegaan d.m.v. bioconservering. Hierbij was de aandacht met name gericht op melkzuurbacteriën, omdat deze micro-organismen anti-microbiële verbindingen kunnen produceren (waaronder bacteriocines) en ze bovendien van nature op de betreffende produkten voorkomen.

In de introduktie (**Hoofdstuk 1**) wordt kort ingegaan op technologische aspekten van het verpakken van verse en minimaal bewerkte groenten onder gewijzigde gascondities en de microbiologische kwaliteit en veiligheid van deze produkten. Hierna wordt de rol van melkzuurbacteriën in bioconservering behandeld, waarbij het accent is gelegd op bacteriocine-producerende stammen. Vervolgens worden de eigenschappen en werkingsmechanismen van bacteriocines beschreven, evenals een aantal aspekten die van belang zijn voor de toepassing van deze verbindingen of hun producerende stammen als mogelijke bioconserveringsmiddelen.

Om een beter inzicht te krijgen in de invloed van gewijzigde gascondities op de microbiële populatie-dynamica op groente tijdens gekoelde bewaring is, zowel voor als na bewaring, de samenstelling van de microflora op twee model-produkten (witlof en taugé) bepaald (**Hoofdstuk 2**). De belangrijkste micro-organismen op beide produkten waren *Enterobacteriaceae* en *Pseudomonaden*. Op witlof werd een verschuiving van de belangrijkste soorten waargenomen tijdens bewaring, terwijl dit niet het geval was voor taugé. Om de invloed van CO_2 en O_2 op de groei van de afzonderlijke bederf-veroorzakende bacteriën te kwantificeren, is een model systeem ontwikkeld om de *in situ* groei van bacteriën op een oppervlak na te bootsen. In de groeicurven van de groei van de meest voorkomende *Enterobacteriaceae* en *Pseudomonas* isolaten opgenomen onder constante O_2 en CO_2 concentraties bij 8°C, bleken lag tijden afwezig te zijn, terwijl de maximum populatie dichtheden niet of nauwelijks van elkaar verschilden. Echter, alle geteste isolaten hadden verlaagde maximale groeisnelheden onder verhoogde CO_2 concentraties. De groei van *Pseudomonaden* werd duidelijk sterker geremd dan de groei van *Entero-bacteriaceae* en er werd een significante verlaging van hun maximale specifieke groeisnelheden waargenomen onder CO_2 concentraties die geschikt zijn voor gasverpakte groenten.

In **Hoofdstuk 3** is het effect van verschillende O_2 en CO_2 concentraties op de groei van *Aeromonas hydrophila, Yersinia enterocolitica, L. monocytogenes,* en een koude-tolerante stam van *Bacillus cereus* gekwantificeerd in het boven beschreven model systeem bij 8°C. Deze bacteriën vertoonden een reduktie van de maximale specifieke groeisnelheid als funktie van toenemende CO_2 concentratie onder verschillende O_2 concentraties. De maximale populatie dichtheden werden echter niet of nauwelijks beïnvloed door verhoogde CO_2 concentratie, en lag tijden waren afwezig. Een extrapolatie van deze resultaten naar gasverpakte groente geeft aan dat de bovengenoemde pathogenen bij 8°C dezelfde maximale populatie-dichtheden kunnen bereiken onder optimale gascondities voor groente als onder atmosferische condities, met slechts 10 tot 20% verlaagde groeisnelheden. De geringe vertraging van de groei van deze pathogenen kan een probleem voor de veiligheid van deze produkten opleveren, waartegen maatregelen dienen te worden getroffen.

L. monocytogenes is een belangrijke psychrotrofe pathogeen die kan voorkomen op minimaal verwerkte groenten. Aangezien de groei van deze Gram-positieve bacterie, en mogelijk andere ongewenste soorten, onderdrukt kan worden door bacteriocine-producerende melkzuurbacteriën, zou de toepassing van zulke cultures als bioconserveringsmiddel uitkomst kunnen bieden. Een efficiënt gebruik van levende cultures vereist echter een goede aanpassing van de betreffende stammen aan condities waaronder hun activiteit gewenst is. Dit gaf aanleiding tot de isolatie van melkzuurbac-

teriën van witlof en taugé, die vervolgens werden getest op bacteriocine-produktie. Uit totaal van 890 melkzuurbacteriën bleken slecht negen isolaten bacteriocines te produceren (**Hoofdstuk 4**). Drie hiervan vertoonden antimicrobiële activiteit tegen een breed scala aan Gram-positieve bacteriën, waaronder *Listeria monocytogenes* en *Clostridium botulinum*. Twee van deze isolaten zijn vervolgens op basis van hun fermentatie profielen en de typering van hun ribosomale DNA geïdentificeerd als *Pediococcus parvulus*. Met behulp van recombinant DNA technieken werd het gen dat codeert voor pediocine PA-1 in beide stammen aangetoond en middels biochemische analyse werd tenslotte de produktie van pediocine PA-1 bevestigd.

De derde bacteriocine-producent met een breed antimicrobiëel werkingsspectrum werd geïdentificeerd als *Enterococcus mundtii* (**Hoofdstuk 5**). Het door deze stam geproduceerde bacteriocine is uitgebreid gekarakteriseerd. Op basis van zowel de primaire aminozuursequentie als de molekulaire massa bleek het een nieuw bacteriocine te betreffen (mundticin), welke behoort tot de klasse **IIa** bacteriocines van melkzuurbacteriën. Stammen die deze klasse van verbindingen produceren zijn door andere onderzoekers geïsoleerd van vlees- en zuivel-produkten en hun brede verspreiding in voedsel kan nuttig zijn voor de selectie van stammen met een goede ecologische aanpassing voor een specifiek voedingsmiddel.

Zoals beschreven in **Hoofdstuk 5**, veroorzaakte de blootstelling van *L. monocytogenes* aan mundticin een dissipatie van de membraan potentiaal en een depletie van de intracellulaire ATP pools, hetgeen uiteindelijk resulteerde in celdood. M.b.v. computer analyse werd aangetoond dat mundticin en sterk homologe bacteriocines (allen behorend tot klasse **IIa**) een α-helix bevatten welke met een hoek van 30 tot 50° inserteert in een gesimuleerd hydrofoob-hydrofiel scheidingsvlak. Vermoedelijk veroorzaakt deze oblique insertie een destabilisatie van de fosfolipiden bilaag, wat de insertie en/of aggregatie van bacteriocine-monomeren in de cytoplasmatische membraan vergemakkelijkt. Dit concept is van belang voor de mogelijke ontwikkeling van bacteriocines met verbeterde antimicrobiële eigenschappen.

Voor de effectieve toepassing van bacteriocines als bioconserverende verbindingen is een beter inzicht in de variatie in gevoeligheid van Gram-positieve bacteriën voor bacteriocines essentiëel. De uitgebreide studie in **Hoofdstuk 6** is gericht op factoren die de verschillen in gevoeligheid tussen nauw verwante bacteriën kunnen verklaren. Twee paar stammen met een 100- tot 1000-voud verschil in hun gevoeligheid voor nisine en pediocine PA-1 zijn met elkaar vergeleken. De resultaten suggereren dat de associatie van bacteriocines met de celmembraan en de daaropvolgende insertie in de membraan op een vergelijkbare manier plaatsvindt voor cellen met een hoge en een lage natuurlijke tolerantie voor bacteriocines. Voor de ongevoelige stammen lijkt de vorming van poriën met voldoende diameter en levensduur (wat leidt tot celdood) bepaald te worden door de gehele membraansamenstelling, en niet alleen de membraanvloeibaarheid. Verdere analyse van dit fenomeen vraagt meer aandacht voor de structurele rol van eiwitten in biologische membranen.

In **Hoofdstuk 7** is de toepassing van mundticin en bacteriocine-producerende groente-isolaten als bioconserveringsmiddelen op gekoelde gasverpakte groente onderzocht. Twee *P. parvulus* stammen bleken ongeschikte kandidaten te zijn door de

afwezigheid van bacteriocine-produktie bij 4 en 8°C. *E. mundtii* had echter een aantal gunstige eigenschappen voor gebruik als bioconserveringsmiddel, zoals een gematigde zuurproduktie, de produktie van mundticin met activiteit tegen *L. monocytogenes* bij lage temperaturen en hoge groeisnelheden onder gascondities die geschikt zijn voor de bewaring van groente. Desondanks werd de groei van *L. monocytogenes* op verse taugé niet onderdrukt door *E. mundtii.* Dit is waarschijnlijk niet toe te schrijven aan een gebrek aan *in situ* bacteriocine-produktie, maar aan proteolytische inactivatie van het bacteriocine, of aan de groei van *L. monocytogenes* cellen met resistentie tegen mundticin. Het gebruik van mundticin in een wasstap of als component in een coating was wel echter wel succesvol. Dit laatste vormt de basis voor mogelijk verder onderzoek naar de potentie van dit peptide als een natuurlijke antimicrobiële verbinding voor minimaal bewerkte groente.

Samenvattend heeft deze dissertatie bijgedragen aan een beter begrip van de microbiologische karakteristieken van minimaal verwerkte groenten die onder gewijzigde gascondities worden bewaard. De algemene zorg m.b.t. de uitgroei van koude tolerante pathogenen op deze produkten werd bevestigd door onze studies, hetgeen onderstreept dat hygiënische omstandigheden tijdens teelt en verwerking onontbeerlijk zijn. Hoewel bacteriocines niet geschikt zijn als enige barrière tegen de groei van ongewenste bacteriën om de veiligheid van minimaal bewerkte, gasverpakte groenten te garanderen, kunnen zij wel bijdragen tot de oplossing van specifieke veiligheidsproblemen die worden veroorzaakt door een aantal koude-tolerante Gram-positieve pathogenen.

CURRICULUM VITAE

Marjon (Maria Henrica Jacoba) Bennik werd geboren op 19 juni 1969 te Elst (Gld). Na het behalen van het VWO-B diploma aan het Canisius College-Mater Deï te Nijmegen in 1987, begon zij in datzelfde jaar aan een studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. Met de hoofdvakken Levensmiddelenmicrobiologie en Toxicologie en een stage aan de University of Alberta (Department of Food Science, Edmonton, Canada), werd deze studie in januari 1993 *cum laude* afgerond. Eerder die maand was zij reeds in dienst getreden bij het Agrotechnologisch Onder-zoeksinstituut (ATO-DLO) te Wageningen, waar het onderzoek zoals beschreven in dit proefschrift werd verricht. Sinds mei 1997 is zij door het ATO-DLO gedetacheerd in Boston (Verenigde Staten) aan de Harvard School of Public Health, Department of Molecular and Cellular Toxicology.

1

LIST OF PUBLICATIONS

Peer reviewed articles

Denbesten, C., M. H. J. Bennik, I. Bruggeman, P. Schielen, F. Kuper, A. Brouwer, J. H. Koeman, J. G. Vos, and P. J. van Bladeren. 1993. The role of oxidative metabolism in hexachlorobenzene-induced porphyria and thyroid hormone homeostasis: A comparison with pentachlorobenzene in a 13-week feeding study. Toxicol. Appl. Pharmacol. **119**:181-194.

Denbesten, C., M. H. J. Bennik, M. Van Iersel, M. A. W. Peters, C. Teunis, and P. J. van Bladeren. 1994. Comparison of the urinary metabolite profiles of hexachlorobenzene and pentachlorobenzene in the rat. Chem. Biol. Interact. **90**:121-137.

Bennik, M. H. J., E. J. Smid, F. M. Rombouts, and L. G. M. Gorris. 1995. Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen. Food Microbiol. **12**:509-519.

Essers, A. J. A., M. H. J. Bennik, and M. J. R. Nout. 1995. Mechanisms of increased linamarin degradation during solid-substrate fermentation of cassava. World J. Microbiol. Biotechnol. 11:226-270.

Bennik, M. H. J., H. W. Peppelenbos, C. Nguyen-The, F. Carlin, E. J. Smid, and L. G. M. Gorris. 1996. Microbiology of minimally processed, modified-atmosphere packaged chicory endive. Posth. Biol. Technol. **9**:209-221.

Bennik, M. H. J., E. J. Smid, and L. G. M. Gorris. 1997. Vegetable-associated *Pediococcus parvulus* produces pediocin PA-1. Appl. Environ. Microbiol. **63**:2074-2076.

Bennik, M. H. J., A. Verheul, T. Abee, G. Naaktgeboren-Stoffels, L. G. M. Gorris, and E. J. Smid. 1997. Interactions of nisin and pediocin PA-1 with closely related lactic acid bacteria that manifest over hundred-fold differences in bacteriocin sensitivity. Appl. Environ. Microbiol. **63**:3628-3636.

Bennik, M. H. J., B. Vanloo, E. J. Smid, L. G. M. Gorris, and R. Brasseur. A role for oblique peptides in the pore-formation by bacteriocins of lactic acid bacteria. Biochim. Biophys. Acta, submitted.

Bennik, M. H. J., W. van Overbeek, E. J. Smid, and L. G. M. Gorris. Biopreservation to control the growth of *Listeria monocytogenes* on minimally processed, modified atmosphere stored vegetables. Lett. Appl. Microbiol., submitted.

Bennik, M. H. J., W. Vorstman, E. J. Smid, and L. G. M. Gorris. The influence of oxygen and carbon dioxide on the growth of prevalent *Enterobacteriaceae* and *Pseudomonas* species isolated from fresh and modified atmosphere stored vegetables. Food Microbiol., submitted.

Bennik, M. H. J. , T. van Dommelen, H. Peppelenbos, L. G. M. Gorris, E. J. Smid. Contribution of spoilage microorganisms to the overall respiration of stored mung bean sprouts *(in preparation).*

Abstracts and other publications

Gorris, L. G. M., M. H. J. Bennik, and T. Abee. 1993. The contribution of biopreservation to the quality and safety of respiring produce kept under moderate vacuum with refrigeration. Abstract at the Food Micro '93 Symposium, p66. Bingen, Germany, August 31-September 3, 1993.

Bennik, M. H. J. and L. G. M. Gorris. Storage of fresh produce under modified atmospheres, introducing bioconservation as a safety hurdle. Proceedings of the Sixth International Symposium of the European Concerted Action Program COST94. Oosterbeek, The Netherlands, October 19-22, 1994.

Gorris, L. G. M., Y. de Witte, and M. H. J. Bennik. 1994. Refrigerated storage under moderate vacuum. Int. J. of Food Technol., Marketing, Packaging and Analysis 45(6):63-66.

Bennik, M. H. J., E. J. Smid, and L. G. M.Gorris. Bacteriocins of *Pediococcus parvulus* and *Enterococcus mundtii*. Abstract at of the INSERC Canada Advanced Research Workshop "Bacteriocins of lactic acid bacteria: fundamentals and applications". Banff, Canada, April 17-22, 1995.

Gorris, L. G. M. and M. H. J. Bennik. 1995. Improvement of the safety and quality of refrigerated ready-to-eat foods using novel mild preservation techniques: bioconservation as a safety hurdle. Proceedings of the 19th Congress IIR/IIF 1995, Volume I, pp. 392-398. The Hague, The Netherlands, August 20-26, 1995.

Bennik, M. H. J., W. van Overbeek, E. J. Smid, and L. G. M. Gorris. Biopreservation for ready-to-use vegetables. Abstract C23 of the "Fifth Symposium on lactic acid bacteria: genetics, metabolism, and applications". Veldhoven, The Netherlands, September 8-12, 1996.

Bennik, M. H. J., E. J. Smid, and L. G. M. Gorris. *Pediococcus parvulus* produces pediocin PA-1. Abstract C24 of the "Fifth Symposium on lactic acid bacteria: genetics, metabolism, and applications". Veldhoven, The Netherlands, September 8-12, 1996.

Gorris, L. G. M. and M. H. J. Bennik. 1996. Bacteriocins for food preservation. Int. J. of Food Technol., Marketing, Packaging and Analysis 45(11):65-71.

Bennik, M. H. J., E. J. Smid, and L. G. M. Gorris. 1997. Mild food preservation using biopreservation as an additional safety hurdle. Proceedings of the COPERNICUS workshop "Process Optimization and Minimally Processing of Foods". Vol. 5, pp. 44-48, J.C. Oliveira (ed.), Escola Superior de Biotecnologia, Porto, Portugal.

Bennik, M. H. J., A. Verheul, T. Abee, G. Naaktgeboren-stoffels, L. G. M. Gorris and E. J. Smid. Variation of bacteriocin susceptibility of phylogenetic closely related lactic acid bacteria. Abstract at "Lactic 97". Caen, France, September 10-12, 1997.

Bennik, M. H. J., W. van Overbeek, E. J. Smid and L. G. M. Gorris. Biopreservation for ready-to-use vegetables. Proceedings of COPERNICUS workshop "Process Optimization and Minimally Processing of Foods". Warsaw, Poland, December 1996 *(in press)*.

Gorris, L. G. M. and M. H. J. Bennik. Modelling interaction between micro-organisms on vegetables. COST 914 Workshop "Microbial Interaction Modelling". Wageningen, The Netherlands, December 9-10, 1996 *(in press)*.

Gorris, L. G. M., M. H. J. Bennik, and E. J. Smid. Modelling bacterial growth on prepared vegetables. Proceedings International Symposium "Predictive Modelling applied to chilled food preservation", IIR-COST. Quimper, France, June 16-18, 1997 *(in press)*.