Improved applicability of nisin in novel combinations with other food preservation factors

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Irene E. Pol

Proefschrift

Ter verkrijging van de graad van doctor op gezag van de rector magnificus van Wageningen Universiteit, Prof. Dr. ir. L. Speelman, in het openbaar te verdedigen op 13 maart 2001 des namiddags te 16:00 uur in de Aula.

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Stellingen behorende bij het proefschrift:

Improved applicability of nisin in novel combinations with other food preservation factors
Door Irene Pol
Wageningen, dinsdag 13 maart, 2001, 16:00 uur

1. Carvacrol alsmede een behandeling met pulserende elektrische velden zijn beide in staat de bacteriocide werking van nisine te versterken.
   (Dit proefschrift).

2. Inactivatie van sporen door een behandeling met pulserende elektrische velden en nisine is alleen te bewerkstelligen wanneer deze behandelingen gecombineerd worden met ontkieming-stimulerende technieken.
   (Dit proefschrift; Knorr *et al.* 1994. Food application of high electric field pulses. *Trends in Food Science and Technology* 5:71-75).

3. Opgroeien in een schone omgeving kan aanleiding geven tot een vergrote kans op ontwikkeling van allergieën.
   (Steerenberg *et al.* 2000. Westerse leefstijl zet afweersysteem op verkeerde been. *Voeding Nu*, nr. 6, blz 23-26).

4. Het gebruik van veiligheidsindicatoren in verpakkingen om de aanwezigheid van pathogenen in voedsel aan te tonen, geeft de consument ten onrechte het idee dat het voedsel volkomen veilig is.
   (Spijkers, A. 2000. Nieuwe veelbelovende veiligheidsindicatoren. *VMT*, nr. 24, blz 47).

5. Tegenstanders van een donorcodicil zouden onder aan de wachtlijst voor organen geplaatst moeten worden.

6. Gemak dient de mens, maar veroorzaakt tevens een toename in het aantal welvaartsziekten en milieuproblemen.

CONTENTS

VOORWOORD

Op deze plaats wil ik graag iedereen bedanken die op welke wijze dan ook heeft bijgedragen bij de totstandkoming van dit proefschrift. Graag wil ik een aantal mensen bij naam noemen.
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Lieve Egon, jij weet hoe moeilijk ik het af en toe vond om te accepteren dat ik het allemaal misschien niet af kon maken en hoe blij ik was dat het uiteindelijk toch op zijn pootjes terecht is gekomen. Je hebt veel geduld met me gehad toen ik het zo druk had in de laatste fase van mijn proefschrift en jij hebt ontzettend veel gedaan zonder morren. Bedankt!

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"Als je doet wat je leuk vind, hoef je nooit te werken", Ghandi.

CHAPTER 1

General introduction

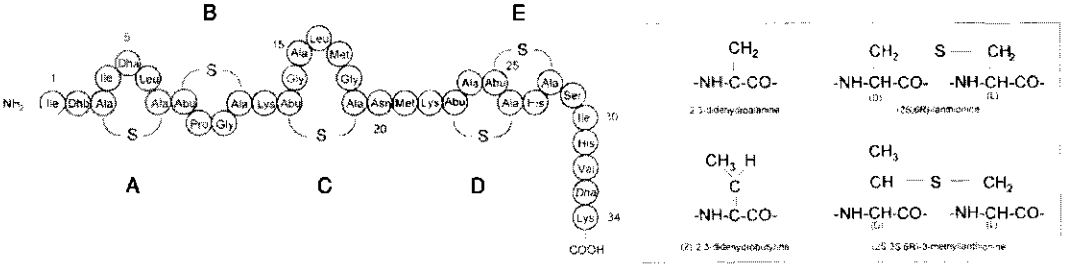
Foods stabilised by traditional preservation methods no longer meet with modern consumers' demand for more natural and fresh-like products. Food manufactures increasingly rely on mild preservation techniques in order to improve the organoleptic quality and nutritional properties of the products. However, mild preservation techniques applied singly are not sufficient to ensure complete safe products and combinations of preservation measures are needed to suppress microbial outgrowth (130). Bacteriocins, produced by lactic acid bacteria have been successfully used in food products to control the growth of pathogenic and spoilage organisms (7, 56). Nisin is the only bacteriocin which has been approved by the World Health Organization (WHO) to be used as a food preservative, however, its application is still limited (26). Limits to its use might be overcome by integrating more effectively various methods of food preservation in order to obtain a multi-target, yet mild preservation method, referred to as the hurdle concept (72). This thesis focuses on the development of novel combinations of nisin with other food preservation factors in order to expand the practical application with regard to mild food preservation.

NISIN

Structure

Lactic acid bacteria are capable of producing a wide variety of antimicrobial substances, including bactericidal peptides, known as bacteriocins (56). The most extensively studied bacteriocin is nisin, a lantibiotic produced by *Lactococcus lactis* ssp. *lactis* (26). Nisin is a small, 34 amino acid, peptide containing the unusual amino acids dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine and β-methyllanthionine. The latter two carry thioether bridges of which five occur in nisin, resulting in five so-called lanthionine ring structures (26). The structure of nisin is given in figure 1.

Figure 1



The primary structure of nisin A.
Ala-S-Ala: lanthionine, Abu-S-Ala: β-methyllanthionine, Dha: dehydroalanine, Dhb: dehydrobutyrine

The mature peptide consists of two domains, i.e. an N-terminal domain consisting of the three lanthionine rings A, B, and C and a C-terminal domain containing the intertwined rings D and E. Nisin is an amphiphilic molecule with the N-terminal domain being more hydrophobic and the C-terminal domain being more hydrophilic and has a net positive charge (56, 111). Two naturally occurring variants of nisin were discovered; Nisin A and Nisin Z. They differ in a single amino acid at position 27, being a histidine residue in nisin A and an asparagine residue in nisin Z (113, 149).

Mode of action

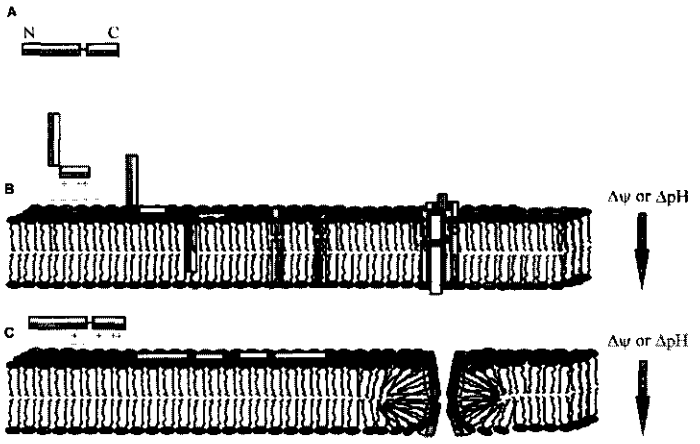
Nisin displays inhibitory activity towards a broad range of Gram-positive organisms, including *Listeria monocytogenes* and also inhibits the outgrowth of germinating spores of *Clostridium* and *Bacillus* species (47, 89, 111). The primary target is the cytoplasmic membrane. Intact cells of Gram-negative bacteria are generally resistant to nisin, since their cytoplasmic membrane is protected by the outer membrane (3, 50). The outer membrane is composed of phospholipids and lipopolysaccharides (LPS), and functions as a permeability barrier against hydrophilic compounds, but allows the entry of small hydrophobic compounds through porin proteins. Gram-negative bacteria can be sensitised to nisin by exposure to chelating agents, removing the stabilising cations resulting in release of LPS and appearance of phospholipids on the surface of the outer membrane. Now, hydrophilic compounds such as nisin can penetrate the outer membrane and reach their site of action at the cytoplasmic membrane (16, 49, 131). In Gram-positive bacteria nisin has to pass the cell wall to reach its target, however, the mechanism of passage through the cell wall is not known. Many non-lantibiotic bacteriocins, such as lactacin F, interact with membrane-associated receptor proteins, prior to interaction with the lipid bilayer (2). In contrast, a specific proteinaceous receptor for nisin or other lantibiotics on the outer surface of the bacterial membrane has not been found. Moreover, the fact that nisin is able to permeabilize lipid vesicles, implies that a specific receptor is not essential for activity (12, 38, 39, 152). However, recently, Breukink *et al.* (13) demonstrated that nisin combines its pore forming ability with a high affinity for Lipid II, a membrane-anchored cell wall precursor, thus causing the peptide to be highly active in the nanomolar range.

The primary biological target of nisin is the energy-transducing membrane of sensitive cells. Nisin permeabilizes the cytoplasmic membrane by forming pores in the membrane, resulting in a rapid efflux of small molecules (14, 38, 91). This pore formation leads to dissipation of the membrane potential and ionic gradients across the membrane and subsequently results in the destruction of energy metabolism and cell death (88, 151). Other mechanisms have been reported to be involved in the biological activity of nisin, like induction of autolytic enzyme activity and inhibition of cell wall biosynthesis. These were suggested to be secondairy effects, since these processes are rather slow and require relatively high concentrations of nisin (26, 47, 56). However, in recent view of Lipid II, we now understand that these effects are indeed primary effects related to the interaction of nisin with Lipid II (13).

The first step in the mechanism of action of nisin is considered to be the binding of the peptide to the cytoplasmic membrane of the target bacteria. Nisin was found to bind electrostatically to the negatively charged phospholipids in the membrane (3, 39, 41, 80). Subsequent pore formation involves the local perturbation of the bilayer structure and a membrane potential dependent or pH gradient dependent reorientation of these molecules from a surface bound into a membrane inserted configuration (29, 88). Two models have been proposed for pore formation; the barrel stave model and the wedge model (Fig. 2) (29). The models differ with regard to the insertion of the molecule. In the barrel stave model, nisin molecules insert into the membrane and subsequently switch into a membrane spanning orientation and form a cluster around a central water-filled pore. Martin *et al.* (80) provided evidence that the C-terminal part penetrates into the hydrophobic core of lipid bilayer. This view is supported by the physical characterisation of nisin (152). In addition, van Kraaij *et al.* (145) showed that the C-terminal part had the ability to translocate across the membrane. Alternatively, other studies suggest that primarily the N-terminal part of nisin penetrates into the lipid phase, while the C-terminal part is

responsible for the binding of nisin to the target membrane (12). The wedge model suggests that surface bound molecules of nisin insert together with phospholipids giving rise to wedge-like pores (29).
Nisin needs an energised membrane to exert its action (39). Both the membrane potential and the pH gradient across the membrane are equally effective in promoting insertion and pore formation of nisin in the membrane (38, 88). Several authors have demonstrated the dissipating effect of nisin on the proton motive force (3, 14, 38, 39, 88, 92, 100, 151, 152). Nisin increases the permeability of the membrane towards potassium ions and protons, resulting in a complete dissipation of the membrane potential and pH gradient across the cell membrane. In addition, depletion of the intracellular ATP Pool was observed as a result of phosphate efflux and exhaustion in an attempt to regenerate the proton motive force. Studies into the mode of action suggest that depletion of the proton motive force is a common mechanism amongst bacteriocins of lactic acid bacteria (15, 90).

Figure 2



Two proposed models for nisin pore formation (adapted from 91).

A. schematic representation of nisin with N-and C- terminal domain.
B. Barrel stave mdel for pore formation.
C. Wedge model for pore formation.

Nisin's action against spores is predominantly sporostatic rather than sporocidal. Its activity lies in preventing the swelling of the germinated spores (5, 76, 82). Nisin interacts with sulfhydryl groups in the membrane, interfering with spore growth by disrupting some vital functions (75, 93, 94).
An important drawback in the application of nisin is the appearance of nisin-resistant or tolerant organisms (81). Several researchers found resistance towards nisin to be correlated with both an altered fatty acid composition and an altered phospholipid composition. The changes were found to correspond with a decreased membrane fluidity, presumably preventing the insertion of nisin (82, 85, 86). Furthermore, changes in the phospholipid head groups, resulting in a decrease in net negative charge, were demonstrated to play a role in nisin resistance, thereby affecting the electrostatic interaction of nisin with the cytoplasmic membrane (21, 148). In addition to membrane compositional changes, the cell wall may also be involved in nisin resistance (27). In general, it is assumed that nisin reaches the membrane by diffusion through the peptidoglycan layer of Gram-positive bacteria. Differences in composition, thickness, charge or hydrophobicity of the cell wall could very well play a role in the differences in susceptibility towards nisin. The peptidoglycan layer might act as a sieve for nisin molecules, dictating the accessibility of the target membrane. This is supported by recent studies, demonstrating an altered cell wall composition in nisin-resistant strains (21, 23). In some Gram-positive bacteria, nisin resistance was found to be related to the presence of a nisin degrading enzyme, nisinase (58). Alltogether, the results point out that nisin resistance is probably more complex. Small changes in membrane composition can hardly explain the large variation in nisin resistance amongst different strains (12). An explanation could possibly be offered by the recent observation that the presence of the

peptidoglycan precursor molecules Lipid II substantially increased the susceptibility of liposomes to nisin. It was postulated that Lipid II, which is present at the outer surface of the membrane of Gram-positive bacteria, serves as a docking molecule for nisin, facilitating specific binding to the bacterial membrane. Therefore it was speculated that the amount and accessibility of Lipid II might contribute to nisin resistance (13).

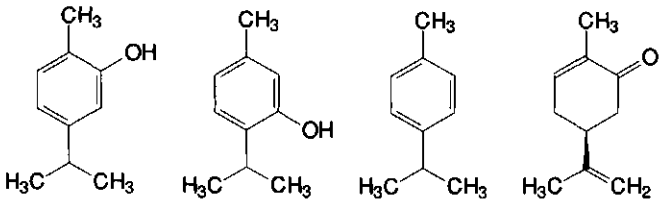
Application

Nisin is Generally Regarded As Safe (GRAS status) and is the only bacteriocin that has been approved by the World Health Organisation to be used as a food preservative. Nisin is currently permitted in over 50 countries (47, 97) and is used in processed cheese, canned foods, and other food products (25, 47, 60, 73, 97, 133). However, practical application of nisin is still limited because of its low solubility, and heat sensitivity at neutral pH. Furthermore nisin is ineffective against Gram-negative bacteria and has limited efficacy in certain food matrices due to binding to food ingredients as fat particles or degradation by proteolytic enzymes (7, 22, 117). Also emergence of nisin resistance or tolerance in certain bacteria like *L. monocytogenes* has been observed (21, 23, 81, 85, 86, 148). These restrictions might be overcome by combining nisin with other biopreservatives or mild processes. Several researchers have reported successful combinations of nisin with pH, temperature and/or NaCl (11, 57, 137, 141), potassium sorbate and sodium benzoate (32), fatty acids of sugars (138), fatty acids like monolaurin (78), carbon dioxide (98), other bacteriocins (46, 117) and mild processes such as High Hydrostatic Pressure (103, 136). Combining several hurdles might lead to additive or even synergistic inhibition allowing the individual hurdles to be set at lower intensities more suitable for mild preservation. This thesis describes the action of combinations of nisin with essential oils, like carvacrol, and Pulsed Electric Field treatment in more detail.

ESSENTIAL OILS

Herbs and spices have been used for centuries to provide distinctive flavours to food and beverages (10). However, many spices were also shown to exhibit antimicrobial activity towards a number of food borne pathogens and fungi (24, 63, 66, 67, 74, 83, 84, 87, 122, 128, 142). The compounds responsible for antimicrobial activity are often in the essential oil fraction, which consists of a mixture of esters, ethers, phenols, aldehydes, ketones and terpenes (99). Phenolic compounds appear to exhibit the strongest antimicrobial activity. The number of double bonds in terpenes or the introduction of a ketone radical does not play a large role (108). In general the presence of an aromatic ring containing a functional group is important for the antimicrobial activity (33). Introduction of a hydroxyl group directly bonded to a cyclohexane or benzene ring results in an increase of the antibacterial activity as illustrated by the different activities of carvacrol and thymol compared to cymene. The chemical structure of those compounds is essentially similar except that cymene does not carry a hydroxyl group and subsequently hardly exhibits antimicrobial activity (Fig. 3) (108).

Figure 3



The chemical structure of respectively carvacrol, thymol, cymene and S (+) carvone.

The hydroxyl group is quite reactive and can easily form hydrogen bonds with active sites of enzymes. But also the inductive effect of the isopropyl group and the aromaticity can contribute to the antimicrobial activity (33). Moleyar and Narasimham (87), suggested that the –CHO group conjugated to carbon-carbon double bond could be the site of antifungal activity.

Mode of action

The primary target of essential oils is the cytoplasmic membrane. Due to their lipophilic nature, these compounds can accumulate in the membrane according to their partition coefficient, specific for the component applied (126). The degree of accumulation depends on the hydrophobicity of the compound and the fluidity of the membrane (127). In general Gram-positive bacteria are more sensitive towards essential oils than Gram-negative bacteria (84, 122). In order to exert their action, essential oils have to pass the cell wall and partition in the cytoplasmic membrane. The presence of an outer membrane in Gram-negative bacteria might act as a molecular sieve through which molecules with a molecular mass greater than 600 to 1000 Da cannot penetrate (127). Despite the presence of porins with low specificity, the outer membrane shows a very low permeability towards hydrophobic compounds, explaining the lower sensitivity of Gram-negative bacteria (50, 99). However, Dean and Ritchie (24) and Tassou *et al.* (135), could not demonstrate a difference in susceptibility towards essential oils being reflected in the Gram reaction of the organism.

Accumulation of essential oils in the membrane leads to an increase in the permeability of the membrane, resulting in leakage of intracellular constituents and loss of the membrane barrier function (129). Exposure to cyclic hydrocarbons resulted in swelling of the membrane and an increase in membrane fluidity, leading to an increase in membrane permeability and subsequent loss of intracellular potassium ions (126, 127, 143). As a result, dissipation of the pH gradient and the membrane potential and depletion of internal ATP pool was observed (101, 126, 129, 143). With the loss of these ion gradients and the proton motive force the cell loses the driving force for a broad range of biological processes. The alteration in the membrane structure may lead to impairment of a variety of enzyme systems including those involved in energy production like ATPase and cytochrome C oxidase (64, 126). ATP synthesis might be inhibited and exhaustion of remaining ATP for maintenance purposes might explain the depletion of the internal ATP pool upon addition of lipophilic compounds (126, 143). The effects on the enzyme activity can be ascribed to the altered protein-lipid interactions, membrane thickness, fluidity and/or phospholipid headgroup hydration as a result of accumulation of essential oils (127). Carvacrol and thymol are amongst the most potent compounds (66, 69, 70, 128). Like vegetative cells, spores were also found to be sensitive towards essential oils to different extent (19, 142). The essential oils exhibited sporocidal or sporostatic mechanisms of action in inhibiting specifically one or more stages of the spore cycle or the commitment to germinate (19).

Application

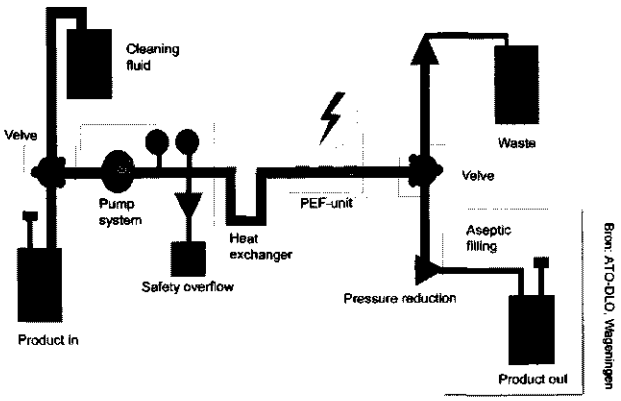
Herbs and spices as well as their antimicrobial constituents are considered to be GRAS and are already used as flavouring agents in certain food products. Since essential oils exhibit strong flavours, their application is limited to products in which these specific aromas are appreciated. Carvacrol, an important component of the essential oil fraction of oreganum (51-84.5%), thyme (45-60%) and savory (26-41%) (128, 142) is used as a flavouring agent in non-alcoholic beverages (28.5 ppm), soft candy (21.4 ppm), chewing gum (8.4 ppm), and baked goods (15.7 ppm) (34). Since essential oils posses antimicrobial activity it renders them interesting for the food industry as natural preservative agents. Herbs and spices containing the active compound can be added to products without any regulatory problems. However, to reach adequate levels of the active compound, large amount of herbs and spices will be needed. The purified compounds are more active, but their application is not permitted unless extensive toxicological data are provided. Purification will bring these chemicals into the same category as synthetic chemical compounds and thus require a non-toxicity report. Application of carvacrol, having the GRAS status, is currently permitted in the USA (Code of Federal Regulations (CFR)), however, if it is applied for other purposes than flavouring foods new toxicology data are necessary (34). Food ingredients have great influence on the activity of essential oils (135). Carbohydrates do not seem to affect the activity much, however, fat particles or proteins can provide protection against essential oils. For instance, the hydrophobic essential oils can bind to or dissolve in fat particles, thereby decreasing the effective concentration of the oils (61). However, other food ingredients like NaCl and ascorbic acid enhance the inactivation by essential oils (69, 133). The pH and the temperature of the food products are of great importance in determining the activity of the essential oils. According to Juven *et al.* (61) the essential oils become more hydrophobic at low pH and dissolve better in the lipid phase of the bacterial membrane leading to increased antibacterial activity. However, Ultee *et al.* (142) and Tassou and Nychas (134) observed a higher activity of carvacrol in either acidic as well as alkaline environment. A dip in activity was seen at neutral pH values. In general, they found carvacrol to be more active at higher pH values. The temperature affects the activity of essential oils, by decreasing the solubility at lower temperatures and hence the concentration in the cell membrane (99, 135).
Not much is known with respect to development of resistance against these essential oils. Bacteria adapt to toxic compounds by a broad range of adaptations ranging from altering their cell envelope composition to active transport systems. By changing their cytoplasmic membrane composition, like the phospholipid composition, the partition coefficient can be reduced significantly (55). Adaptation of *B. cereus* to carvacrol results in a decrease in the membrane fluidity, leading to a reduced sensitivity of the organism towards carvacrol since less carvacrol dissolves in a more rigid membrane (144).

PULSED ELECTRIC FIELD

Recent interest of industries in alternatives to conventional food preservation methods like heating has resulted in the revived attention towards technologies like Pulsed Electric Field (PEF). PEF treatment is a non-thermal pasteurisation technique, which inactivates microorganisms without adversely affecting the flavour, colour and nutrient content of products (8, 44). It involves the application of high voltage pulses to a food placed between two electrodes. Due to a treatment at an ambient or sub-ambient temperature for only a few microseconds, energy loss due to heating of the food is minimised (4). Bacterial cells are destroyed by mechanical effects rather than thermal effects (107, 115). There are two major waveforms in pulsed electric field technology; exponential decay pulses and square wave pulses (9). Exponential decay pulses rise rapidly to a maximum value and decay slowly to zero. The square wave pulses maintain peak voltage for a longer time. Square wave pulses are energy efficient and exhibit a more lethal effect than exponential decay pulses (4, 6, 153). A PEF treatment can be applied in a batch system as well as in a continuous system.

The advantages of a continuous system over a batch system are the minimised temperature rise, the higher field strengths and the higher conductivity ranges that can be processed. A continuous flow system as used in this study is illustrated in figure 4.

Figure 4

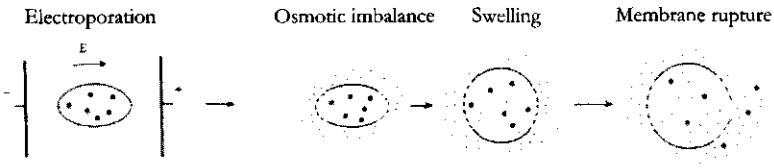


Continuous PEF system as used in this thesis.

Mode of action

Microbial inactivation is caused by structural changes in the membrane, resulting in pore formation and loss of the selective properties of the membrane (45, 102, 114, 116). When a cell suspension is exposed to an electric field, the ions inside the cell migrate according to the electric field across the electrodes until they are held back by the membrane. As a result, dissolved ions accumulate at both membrane surfaces leading to an increased transmembrane potential (6, 68, 156). The charges generated on the membrane surface are opposite of signs and attract each other. This attraction gives rise to a compression pressure that causes the membrane thickness to decrease (18, 156). The electric forces and compression pressure increase with a decrease in the membrane thickness (Fig. 5).

Figure 5

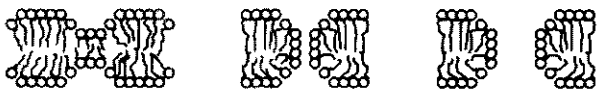


Electroporation of the cell membrane (adapted from 140).

Furthermore, the electric charges will cause the polar lipid molecules to reorient when subjected to an intense electric field strength resulting in formation of hydrophilic pores and impairment of the membrane barrier against ions (140) (Fig 6). Breakdown of the integrity of membrane is associated with a reversible permeability increase of the cell membrane. The extent of the permeability increase depends on the strength and duration of the electric field pulse (18, 68, 71, 116, 153, 156). Membrane disruption occurs when the induced transmembrane potential exceeds the threshold value of typically 1 Volt,

which for example corresponds to an external electric field of about 10 kV/cm for *Escherichia coli* (18, 20, 116, 156).

Figure 6



Visco elastic Model for electroporation of cell membrane (adapted from 6).
a. a growing number of membrane fluctuations.
b. molecular rearrangements leading to discontinuity.
c. expansion of the pore resulting mechanical breakdown of the membrane.

Chang and Reese (20) distinguished primary, secondary and tertiary effects on the structure of the membrane caused by PEF treatment. Dielectric breakdown due to an increased membrane potential and structural fatigue caused by mechanical stress were considered to be primary effects. Efflux of ions and molecules due to permeabilization of the membrane, local heating and membrane stress due to material flow were seen as secondary effects. Cell swelling, or shrinking and disruption of cytoskeletal structure were categorised as tertiary effects.

Inactivation of microorganisms by PEF treatment is affected by a number of factors including process parameters of the PEF treatment, the characteristics of the microorganims and the physical, chemical and electrical properties of the treatment media. Microbial inactivation is dependent on the electric field strength and the treatment time, defined as the product of the number of pulses and the pulse duration (4). Once the critical transmembrane potential is exceeded, inactivation increases with an increase in the applied electric field strength or treatment time (45, 53, 54, 107, 115, 116, 147, 154). Inactivation by PEF treatment is significantly enhanced at moderately high temperatures (~50-60°C) (44, 51, 71, 115, 116, 146, 154). This can be explained by the increased membrane fluidity resulting in a more efficient PEF treatment (8, 53, 59) or by the increase in electrical conductivity as a result of the higher temperature (79).

The physical and chemical characteristics of the treatment media like the conductivity, ionic strength and the presence of particles or gas bubbles are important determinants for the effectivity of the PEF treatment. Conductivity of the medium is related to the efficiency of the energy transferred and increases with ionic strength and temperature (6, 9). A low conductivity leads to a more effective PEF treatment (154). Lowering the conductivity of the liquid medium is thought to increase the difference between the conductivities of the medium and the microbial cytoplasm and weakens the membrane structure due to an increased flow of ionic substances across the membrane (6, 59). Additional factors that enhance PEF treatment are pH, High Hydrostatic Pressure and the presence of antimicrobials (62). Each of these factors imposes an additional stress to the microorganisms resulting in an increase in the total inactivation (62, 147). In general, a lower pH enhances inactivation by PEF treatment (154). The higher inactivation rate may be explained by transport of H^+ ions as a result of osmotic imbalance, leading to the inability of the cell to maintain the internal pH near neutral (147). The influence of PEF treatment on proteins, polysaccharide macromolecules, or lipids is not exactly known. Electric field can influence the conformational state of a protein through charge, dipole or induced dipole effects, causing protein unfolding and denaturation (9). Not all enzymes lose activity after PEF treatment. Ho *et al.* (52) found inactivation of lipase, glucose oxidase, alpha amylase, peroxidase and phenol oxidase upon PEF treatment, but increased activity of lysozyme and pepsin in a certain range of electric fields.

The efficiency of PEF treatment is also influenced by the intrinsic characteristics of the microorganisms (8). Yeast are more sensitive towards a PEF treatment than bacterial cells, as a result of their greater cell size (154). The transmembrane potential experienced by the cell is proportional to the cell size, thereby relating the critical field strength to the cell size. Subsequently, the critical field strength is much lower for bigger cells (44). Furthermore, Gram-positive bacteria are more sensitive towards a PEF treatment

than Gram-negative bacteria. Exponentially growing cells are also more sensitive than stationary cells. Microbial growth in the logarithmic phase is characterised by a high proportion of cells undergoing division, during which the cell is more susceptible to the applied electric field (6, 54, 154).
Compared to vegetative cells, microbial spores are resistant to extreme ambient conditions such as high temperatures and osmotic pressures, high and low pH's, and also towards PEF treatment (8). Their PEF resistance is associated not only with their small size, but also dehydration and mineralisation is thought to play an important role. Hamilton and Sale (45) found spores of *B. cereus* and *B. polymyxa* to be completely resistant to treatment with exponential decay pulses. During germination and outgrowth, however, the spores became sensitive at the point that the outer layers of the spore split open and the developing vegetative cell begins to emerge. It was suggested by several authors that inactivation might not be achieved by PEF treatment unless combination processes inducing germination are applied, such as High Hydrostatic Pressure, heat shock or lysozyme (6, 8, 44, 71, 155). In contrast, Marquez *et al.* (79) claimed direct inactivation of *B. subtilis* and *B. cereus* spores by PEF treatment only. A minimum field strength of 35 kV/cm was required and inactivation was enhanced at increasing temperatures, number of pulses and pulse duration. Structural damage was demonstrated by SEM revealing holes in the surface. Some spores were reported to be completely destroyed or enlarged.

Application

PEF treatment offers the advantage of limited heat production during treatment while still achieving sufficient microbial inactivation. The shelf life of foods can be significantly extended with minimum changes to physical, chemical and organoleptic properties of the product (44, 110). PEF treatment is an energy efficient process and is therefore an interesting alternative to heat pasteurisation. Examples of products processed by PEF are apple juice, orange juice, milk, eggs and green pea soup (109, 110, 146). PEF treated milk (40 pulses, over a 25 minute time interval) was thoroughly investigated by Barbosa *et al.* (6) and no chemical or physical changes in enzyme activity, fat or protein integrity, flavour, starter growth, rennet activity, clotting yield, cheese production, calcium distribution or casein structure were detected. Pure Pulse Technologies (Inc.) has patented the commercial process to preserve fluid foods (dairy products, fruit juices and liquid egg) both batch wise and continuously (6, 8). However, the application of PEF processing is limited to food products that can withstand high electric fields. Homogeneous fluids with low electrical conductivity are ideal fluids for continuous treatment with PEF. Non-fluid foods and foods containing particulates can also be processed, provided that dielectric breakdown is prevented by media pressurising and degassing or in case batch systems are used (4, 8). The presence of gas bubbles should be avoided since it leads to dielectric breakdown and arcing of sparks.
The food composition is very important in determining the efficiency of the PEF treatment. Factors like pH or the presence of antimicrobials, can enhance inactivation by PEF treatment. In addition, food ingredients such as proteins or fat particles and the presence of Ca^{2+} and Mg^{2+} ions can provide protection for microorganisms (44, 51, 53). At present, it is not known whether microorganisms can develop resistance towards PEF treatment. Further research is required to obtain sufficient information and gain a better understanding of PEF technology.

FOOD-BORNE PATHOGEN *BACILLUS CEREUS*

Vegetative cells

Pathogenic organisms can cause severe hazards in mild preserved foods especially when they are able to grow at low temperatures. This thesis will mainly focus on *B. cereus*, a pathogenic organism ubiquitously found in soil, water and foods. *B. cereus* is responsible for at least 6 - 50 outbreaks a year in the USA (28, 112). In the Netherlands, *B. cereus* has emerged as the most frequently identified bacterial pathogen in outbreaks reported at the IGB (Dutch Inspection of Health Protection) in 1995 - 1997 (28). The relatively mild symptoms and the short duration of the illness contribute to the underreporting of the food-borne pathogen (112). *B. cereus* is a Gram-positive, facultativly anaerobic rod and is characterised by its ability to form spores (118). Optimum growth temperature lies between 28 and 35 °C with a minimum of 4 - 5 °C and a maximum of 48°C. The organism can quickly grow (minimum generation time is 18 - 27 min) over wide pH range of 4.9 - 9.3 and at salt concentration of up to 7.5% (28, 118). *B. cereus* is easily transmitted from its natural soil habitat into vegetation and subsequently into foods. It can be present in a variety of foods, including dairy products, meats, spices and cereals. In general, foods that are processed by drying or are otherwise subjected to heating can still contain *B. cereus* since its spores can survive pasteurization and cooking (28, 112).

Two food-borne illnesses, associated with *B. cereus* are related to the production of two different toxins (95). The diarrhoeal illness is associated with abdominal pain, profuse watery diarrhoea, rectal tenesmus and on some occasions nausea that rarely produces vomiting. The illness reveals itself 6 - 18 h after consumption and lasts for 12 - 24 h. The infective dose is 10^5 - 10^7 and the delay is indicative of subsequent bacterial growth and toxin formation in the small intestine (infection). The diarrhoeal enterotoxin is a heat unstable toxin, rapidly losing activity at pH values outside the range of 5 - 10 (28, 95, 112). The exact mechanism is not yet clear, however, it is suggested that diarrhoeal toxin stimulates the adenylate cyclic AMP system, which synthesises AMP from ATP. Cyclic AMP acts inside human cells by affecting the storage of fluids in the cell, attributing to the accumulation of fluid in the intestines and consequently appearance of diarrhoea (28, 118). The cell permeabilizing activity of the enterotoxin is antagonized by Ca^{2+} ions (112).

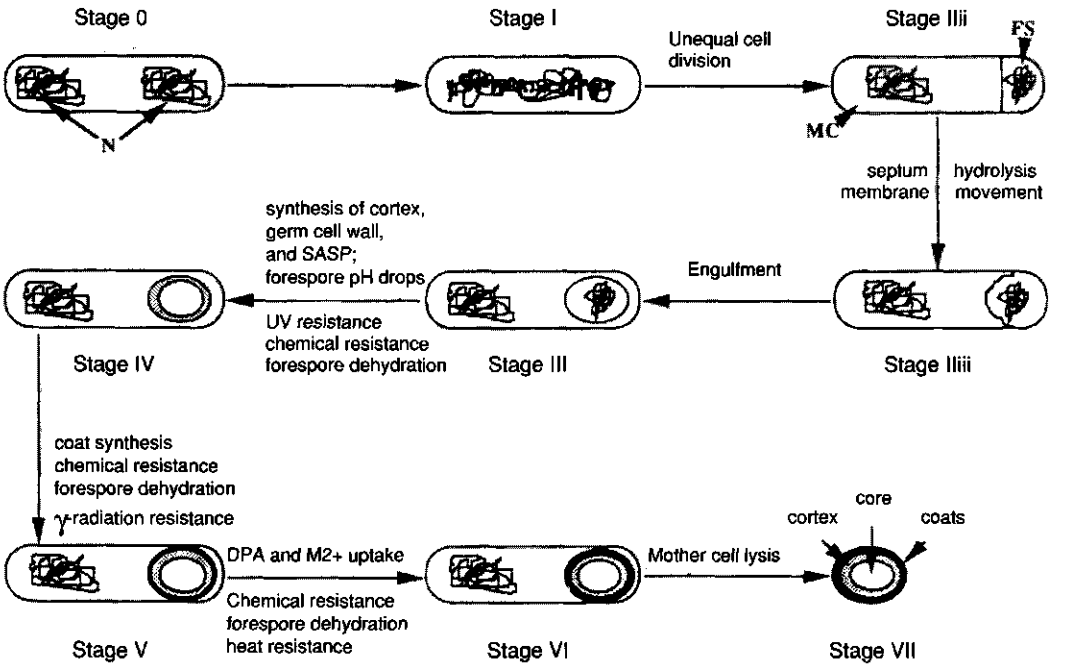
The emetic syndrome is caused by consumption of a preformed toxin as a consequence of the growth of toxinogenic strains of *B. cereus* in food (intoxication) and is most commonly associated with rice. The onset of the emetic syndrome occurs within 1 - 5 h of consumption and the symptoms (which include malaise, nausea, vomiting and occasionally diarrhoea) persist for 6 - 24 h. The diarrhoea is most probably caused by the concomitant synthesis of enterotoxin in some emetic strains. Infectious dose varies form 10^5- 10^8 cells or spores per gram (28, 95, 112). The emetic toxin is very stable to heat, extremes of pH and proteolysis with trypsin and pepsin.

Spores

Sporulation

Spore formation takes place when the cell culture reaches the stationary phase and is most commonly initiated by nutrient starvation (Fig. 7). The first notable morphological event in sporulation is the unequal cell division resulting in a larger mother cell compartment and a smaller forespore compartment (stage Iii). As the sporulation process proceeds, the mother cell engulfs the forespore (stage IIiii). As a result, the forespore is surrounded by two cytoplasmic membranes: the inner en outer forespore membranes (stage III) (35).

Figure 7



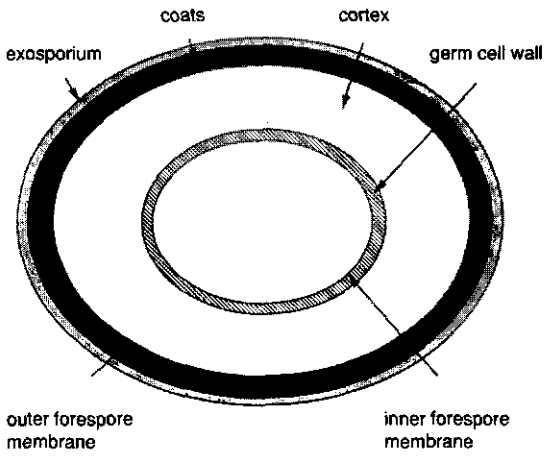
Sporulation process of rod-shaped *Bacillus* (adapted from 122).

In between these two layers, a large peptidoglycan layer, the cortex, is synthesised (stage IV). At this stage, the spore synthesises two biological markers, glucose dehydrogenase and Small Acid Soluble Proteins (SASP). The function of the enzyme is not known, the α/β-type SASP are involved in acquiring DNA protection (121). At this time, UV resistance and chemical resistance are completed and the pH drops by 1 - 1.3 units (77). Dehydration of the forespore is initiated and continues throughout stages III to V. A proteinaceous spore coat is laid down outside the outer forespore membrane and γ-radiation and further chemical resistance are acquired during this stage (stage V). Dipicoline acid (DPA) accumulation is accompanied by uptake of enormous amounts of divalent ions, predominantly Ca^{2+}, and some Mg^{2+} and Mn^{2+}, leading to further dehydration of the spore core and increased heat resistance (119) (stage VI). At this time the forespore is increasing in density and it becomes distinguishable by light microscopy as a refractile body within the mother cell. The spore than become metabolically dormant and the mother cell lyses and releases the fully formed spore (122).

Spore structure

The spore as released by the mother cell at the end of the sporulation is biochemically and physiologically different from a vegetative cell. The core is surrounded by 6 distinguishable envelope structures (Fig. 8) (35). These 6 structures are from outside to inside: the exosporium, the coat, the outer forespore membrane, the cortex, the germ cell wall, and the inner forespore membrane (121).

Figure 8



Structure of a dormant spore, not precisely drawn to scale (adapted from 122).

The outermost spore layer, the exosporium, varies significantly in size between species and its various components are not well characterised (96). It is thought to play a role in the hydrophobicity of the spores (150). Underlying the exosporium is the proteinaceous spore coat. The coat protects the spore cortex from attack by lytic enzymes, and may also provide an initial barrier to chemicals such as oxidising agents (96). The outer forespore membrane, underlying the spore coat, is largely composed of proteins and plays a large role in the extreme impermeability of the spore to small molecules. The cortex is synthesised in between the inner and outer forespore membrane and consists mainly of peptidoglycan (121, 139). The spore cortex is largely responsible for maintenance of the dehydrated state of the spore core and thus much of the spore resistance (106). The germ cell wall is one of the structures which exhibits great similarity with vegetative cells (139). Like the germ cell wall, the inner forespore membrane is a complete membrane with a phospholipid composition similar to that of vegetative cells (121). The inner forespore membrane is an extremely strong barrier to hydrophilic molecules, charged molecules and to most molecules with a molecular weight of $\geq$ 280 Da (40). The spore core contains the spore's DNA, ribosomes and most enzymes as well as the depots of DPA and divalent cations (96). The ions present in the core are electrostatically immobilised (17, 43). Protection of the DNA in the core is provided by α/β-type SASP, which binds to the DNA (121). The water content of the core is extremely low, in contrast to the normal water content of the other regions. The spore core contains only 0.3 - 0.7 g of water per g of dry weight, compared to 3 - 4 g of water per dry g weight of vegetative cells. The low water content is believed to play a major role in the spore dormancy and the acquired resistance to a variety of agents (121).

Spore Resistance

Spores are extremely resistant to a broad range of potentially lethal treatments such as heat, radiation, chemicals and desiccation. Factors important in overall spore resistance include the low permeability of spores to toxic chemicals and the decreased spore-core water content (121, 122). The spore cortex is largely responsible for the maintenance of the dehydrated state of the core by preventing the uptake of water (106, 122) and plays an important role in resistance to heat and oxidising agents (106, 123). Spore mineralisation is also implicated in heat resistance (103, 105, 125). Both the amount and type of mineral ions accumulated in the core, affect the heat resistance. The order of spore heat

resistance with different cations is given as: $H^+ < Na^+ < K^+ < Mg^{2+} < Mn^{2+} < Ca^{2+} <$ untreated. Alterations of spore mineralisation can alter spore core water content, which presumably has a significant effect on heat resistance. However, mineralisation may also affect spore heat resistance independently of effects on core water content (122). The mechanism of spore inactivation by heat is not by DNA damage but probably by denaturation of spore proteins or degradation of spore membranes (121). Large part of the resistance to oxidising agents and chemicals is achieved by the spore coat and the cortex, which play a role in restricting the access of potentially toxic molecules to the spore core by providing an impermeable barrier (119, 125). Resistance to UV-radiation is developed prior to acquisition of heat resistance and in parallel with synthesis of α/β-type SASP. Binding of α/β-type SASP to DNA results in a conformational change in DNA, leading to major alterations in DNA's properties like interactions with enzymes and also its UV photochemistry (31, 105, 119, 120, 121, 123). Upon UV-radiation, a spore photoproduct SP (5-thyminyl-5,6-dihydrothymine adduct) is formed instead of TT photoproduct (cyclobutane-type pyrimidine dimers), which is the major photoproduct in vegetative cells. Although SP is also a potentially lethal photoproduct, spores have a SP-specific repair system that rapidly and efficiently repairs lesions in the first minutes of spore germination and helps them overcome the DNA damage done by UV-radiation (121). α/β-type SASP also provide protection from hydrogen peroxide DNA damage (119, 120, 123).

Germination

Although spores are metabolically dormant and can remain in this state for many years, if given the proper stimulus they can return to active metabolism within minutes through the process of spore germination (122). Germination can be triggered by a wide variety of physical and chemical factors (42), which may be divided into nutrient (sugars, amino acids and ribonucleotides) and non-nutrient germinants (chemicals, enzymes, High Hydrostatic Pressure etc.) (30, 37, 43, 122, 155). A spore population will often initiate germination more rapidly and completely if activated prior to addition of a germinant by means of low pH, chemicals or sub-lethal heat treatment (65, 122). L-alanine is the most common nutrient germinant. It is thought to interact with its receptor (R), generating allosteric conformational changes (1, 36). The activated L-alanine receptor (R^*) has proteolytic activity, which converts a pro enzyme (L) to an active heat-sensitive cortex lytic enzyme (L^*) (36, 132). Cortex hydrolysis then allows the uptake of water and restores the activity of enzymes in the spore core (1, 48, 132). In general, the sequence of events in germination is as follows; 1 commitment to germinate, which involves an interaction of the germinant to the spore. Once commitment occurs, no further interaction is necessary for the spore to complete germination. 2 Loss of heat resistance and release of calcium and DPA. 3 Loss of spore cortex, resulting in complete hydration, mobilisation of ions, loss of refractility and UV resistance, release of hexosamine, further release of DPA and fall in extinction (17, 43). Heat resistance is lost early in germination and is therefore not associated with loss of DPA or spore hydration but most probably with the heat sensitivity of the activated enzyme (37, 121). Germination can be inhibited by factors like D-alanine, fatty acids or Hg^{2+}. D-alanine acts as an inhibitor of L-alanine-induced germination possibly by competing for the same receptor binding sites (37). Fatty acids inhibit germination by binding to the hydrophobic L-alanine receptor due to their lipophilic nature (1). Inhibition of germination by Hg^{2+} lies at the level of the L-alanine receptor and the active heat sensitive cortex lytic enzymes (37).

OUTLINE OF THIS THESIS

The aim of this research was to expand the range of application of nisin in food preservation by combinations with other biopreservatives or mild preservation techniques. Carvacrol and PEF technology were selected for their similarity in primary site of action, the cytoplasmic membrane. By

combining different techniques or preservatives with similar but not identical targets additive or even synergistic inactivation might be achieved. Combinations of nisin with essential oils against the food-borne pathogens *L. monocytogenes* and *B. cereus* are described in Chapter 2. The influence of three essential oils, namely carvacrol, thymol and carvone were examined at different temperatures. Furthermore, the possibility of increasing the amount of hurdles using lysozyme was tested against *L. monocytogenes*. In Chapter 3, the mechanism of synergy between nisin and carvacrol was studied at the level of energy transduction. The bioenergetic consequences of nisin and carvacrol were determined with regard to the membrane potential, the pH gradient and the ATP pools. In Chapter 4, a novel non-thermal, preservation technology, PEF treatment, was introduced as a suitable candidate to enhance the bactericidal activity of nisin. The kinetics of the combined action of nisin and PEF treatment were studied in more detail in order to determine the onset of synergy.
Chapter 5 describes the use of carvacrol as a third hurdle in combination with nisin and PEF treatment in order to enhance the synergy found between nisin and PEF treatment against *B. cereus*. Possible application of this combination in food products was evaluated using diluted milk as a food model matrix. Before these combination techniques can replace currently used pasteurisation or even sterilisation processes, inactivation of spores is crucial. In Chapter 6, inactivation of spores by combination of nisin with PEF treatment was investigated. Direct inactivation of spores could not be achieved, therefore the sensitivity of nutrient induced germinated spores was examined in different phases of their germination process. Furthermore, the influence of an altered membrane fluidity on the sensitivity of spores and vegetative cells towards nisin and/or PEF treatment was evaluated. Chapter 7 provides an overview of the potential of the combination techniques as mild food preservation with regard to microbial inactivation and mechanism of action.

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CHAPTER 2

Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*

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ABSTRACT

Nisin, a small antimicrobial protein, was tested for its bactericidal action against *Listeria monocytogenes* and *Bacillus cereus* and a typical biphasic reduction of the viable count was observed. The reduction was most fast during the first 10 minutes of exposure, while the viable count remained stable in the last part of the exposure period. *B. cereus* was more sensitive towards nisin than *L. monocytogenes* and the inhibitory effect of nisin was stronger towards cells cultivated and exposed at 8°C than towards cells cultivated and exposed at 20°C. Combining nisin with sublethal doses of carvacrol resulted in an increased reduction in the viable count of both organisms, indicating synergy between nisin and carvacrol. Addition of lysozyme as a third preservative factor increased the synergistic effect between nisin and carvone especially in the last part of the exposure period.

INTRODUCTION

Lactic acid bacteria produce a wide range of antimicrobial substances including bactericidal peptides known as bacteriocins. Nisin, produced by *Lactococcus lactis* ssp. *lactis*, is bactericidal against a broad range of Gram-positive bacteria including some lactic acid bacteria, *Listeria monocytogenes* and spore forming bacteria like *Clostridium* and *Bacillus* species (10, 12, 21). Nisin inhibits outgrowth of germinating spores and causes lysis of vegetative cells (7, 23). Its primary target is the cytoplasmic membrane of vegetative cells. Nisin permeabilizes the cytoplasmic membrane by forming pores in the membrane, resulting in a rapid efflux of small molecules (5, 7, 23). Pore formation caused by this cationic lantibiotic involves the local perturbation of the bilayer structure and a membrane potential dependent or pH gradient dependent reorientation of these molecules from a surface bound into a membrane inserted configuration (8, 9, 12). The efflux of cellular constituents results in a complete collapse of the proton motive force (2, 5, 10, 14).

Nisin is Generally Regarded As Safe (GRAS status) and is the only bacteriocin that has been approved by the WHO as a food preservative and is currently permitted in over 50 countries (1, 10). Nisin can be used in processed cheese, canned foods, and other food products (7, 15, 23) but the practical application is still limited because of its low stability and activity at high pH and its limited efficacy in certain food matrices. Also emergence of nisin resistance or tolerance in certain bacteria like *L. monocytogenes* has been observed (16, 24). By combining nisin with other biopreservatives, these restrictions might be overcome. In this study, nisin was combined with essential oils.

Many herbs and spices have been used for millennia to provide distinctive flavours, but they also exhibit antimicrobial activity (17, 27, 29). The compounds responsible for antimicrobial activity are often in the

essential oil fraction, which consists mainly of phenolic compounds (3, 4, 22, 27). The essential oils are hydrophobic and their primary site of toxicity is the membrane. They accumulate in the lipid bilayer according to a partition coefficient that is specific for the compound applied, leading to disruption of the membrane structure and function (11, 13). As a result, the permeability increases and the activity of enzyme systems, including those involved in energy production and structural component synthesis, will be affected (4, 22, 25). A critical concentration of the essential oils is needed to cause leakage of cellular constituents (18).

By combining nisin with plant essential oils, the restrictions in the use of nisin as a food preservative might be overcome and the range of application could be expanded. Since both compounds act on the cytoplasmic membrane an additive or synergistic effect could be expected and lower dosage of both compounds would be necessary to cause an inhibitory effect. In this research carvacrol was used at sublethal concentrations and the activity of nisin combined with carvacrol was determined on the food borne pathogens *L. monocytogenes* and *B. cereus*. Furthermore the influence of growth temperature on the sensitivity of both organisms towards nisin and carvacrol was determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Listeria monocytogenes Scott A (obtained from the Department of Food Science of the Agricultural University of Wageningen, The Netherlands) and *Bacillus cereus* IFR-NL94-25 (obtained from the Institute of Food Research, Norwich, UK) were used in all experiments. Cells were grown at 20 or 8°C in Brain Heart Infusion (BHI) medium (Oxoid, Hampshire, UK) containing 0.5 % glucose (wt/vol). Cultures were maintained in 30 % glycerol at – 80°C.

Antibacterial effect of nisin and carvacrol

To determine the effect of nisin (Aplin and Barrett Ltd., Trowbridge, UK) and carvacrol (Fluka Chemie AG, Buchs, Switzerland) separately or in combination on the growth of *L. monocytogenes* and *B. cereus*, cells were precultured at 20 and 8 °C for 16 hours, washed twice in BHI and diluted in BHI until an Optical Density (660 nm, at 9 mm) of 0.2 was reached. Of this cell suspension, 10 µl was added to microtiterplates in which a nisin gradient in the x-direction and an essential oil gradient in the y-direction were created in BHI containing 0.5 % glucose. Concentrations of nisin ranged from 0 to 10 µg/ml and concentrations of carvacrol ranged from 0 to 5 mmol/l. Carvacrol stock solution was held in 95 % ethanol at 4°C and a stock solution of nisin was made in 50 % ethanol, filter sterilized (Costar, 0.22 µm) and kept at – 20°C. During incubation at 20 and 8°C, the OD_{655} was monitored at different time intervals until a constant reading was observed. In all experiments the concentration of ethanol did not exceed 2 % (vol/vol).

Combined effect of nisin and carvacrol on the viable count

The effect of nisin in combination with carvacrol on the viable count of *L. monocytogenes* and vegetative cells of *B. cereus* was determined as described by Ultee *et al.* (29) using 50 mmol/l HEPES as buffer (pH 7.0) without the addition of $MgSO_4$. Washed cells in the exponential growth phase were exposed to nisin alone, carvacrol alone or a combination of both compounds (nisin was added directly after carvacrol). Samples were taken after 0, 0.3, 5, 10, 15, 23, 35 minutes of exposure and immediately diluted (10^2 - to 10^5- fold) in peptone-physiological salt solution (1g/l peptone and 8.5 gr/l NaCl). The tests were carried out at two different temperatures, namely 20 and 8°C.

Influence of lysozyme on the activity of nisin and carvone

To determine the influence of lysozyme (Chicken egg white, Sigma Chemicals Co, St Louis, USA) on the inhibiting capacity of nisin and/or carvone (Fluka Chemie, AG, Buchs, Switzerland) similar tests as described above were performed by combining nisin or carvone or the combination with lysozyme. A 2 mg/ml stock solution of lysozyme was prepared in demineralised water, filter sterilized (Costar, 0.22 μm) and kept at – 20°C until further use.

RESULTS

Antibacterial activity of nisin and carvacrol

The antibacterial effect of nisin in combination with carvacrol was determined by exposing *Listeria monocytogenes* and *Bacillus cereus* to 96 different combinations of both compounds and monitor the Optical Density during the incubation at test temperature. At low concentrations, nisin and carvacrol alone increased the lag phase of both organisms. At higher concentrations, both compounds inhibited the growth of the two strains completely. From these results the minimal inhibitory concentration (MIC value) could be determined. Table 1 shows the MIC values of nisin and carvacrol separately or in combination for growth of *L. monocytogenes* and *B. cereus* at 20 and 8°C. At 20°C, both *L. monocytogenes* and *B. cereus* are equally susceptible towards nisin.

Table 1

Minimum inhibitory concentrations (MIC value)* of nisin and carvacrol when used alone or in combination for *L. monocytogenes* Scott A and *B. cereus* F46.26.90 at given temperatures.

	T(°C)	MIC Nisin (μg/ml)	MIC Carvacrol (mmol/l)	MIC Combination	
				Nisin (μg/ml)	Carvacrol (mmol/l)
L monocytogenes	20	10	2.5	0.63	1.25
	8	10	2.5	2.5	0.63
B. cereus	20	10	1.25	1.25	0.63
	8	5	0.63	1.25	0.31

* = MIC value is defined as the minimum concentration of nisin or carvacrol at which no growth occurs.

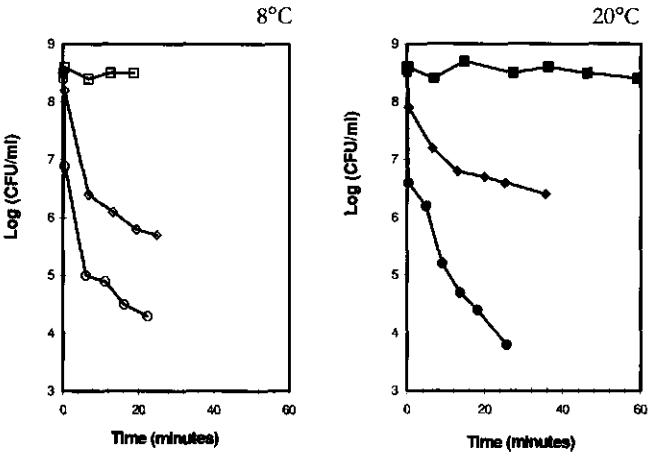
A concentration of 10 μg/ml completely suppressed the growth of both organisms. Lower temperatures did not influence the sensitivity of *L. monocytogenes* towards nisin. On the other hand, *B. cereus*, cultured and exposed at 8°C, exhibited an increased sensitivity towards nisin at this temperature. Like nisin, carvacrol was able to inhibit growth of both organisms completely. Full suppression of growth of *L. monocytogenes* was observed at both temperatures in the presence of 2.5 mmol/l carvacrol (Table 1). *B. cereus* was more sensitive, resulting in lower MIC values for carvacrol (Table 1). Again *B. cereus* showed increased sensitivity towards carvacrol at lower temperatures. The MIC values of the combination are based on the lowest nisin concentration. More combinations cause total inhibition, but this method was chosen since the aim of this study was to expand the use of nisin. In combination, less of both compounds was needed to achieve total inhibition. At 20°C, growth of *L. monocytogenes* was completely inhibited in the presence of a concentration of nisin which was 16 - fold lower than when nisin was applied as the sole preservative. Also lower concentrations of carvacrol were needed to achieve total inhibition indicating that carvacrol is able to enhance the inhibiting effect of nisin on the growth of *L. monocytogenes*. Similar results were found for *B. cereus*. In short, the MIC values of nisin and carvacrol used

together are lower than the MIC values of both compounds used separately, indicating an additive and possibly a synergistic effect of carvacrol with nisin.

Combined effect of nisin and carvacrol on the viable count

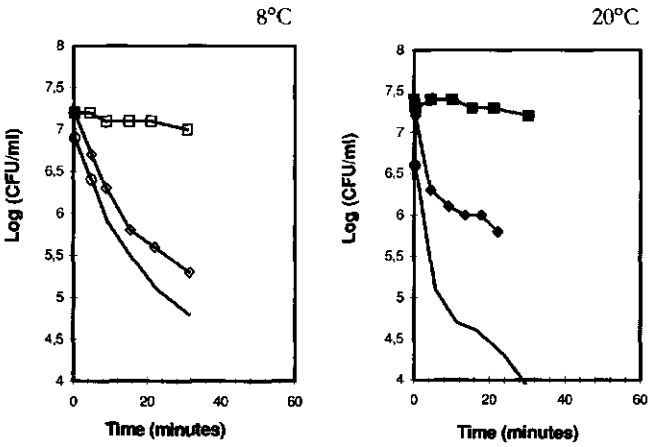
To determine the bacteriostatic or bactericidal action of nisin and carvacrol, the effect of these compounds on the viable count of both organisms was studied. The organisms were exposed to one of the preservatives or to both simultaneously and the viable count was monitored during 35 minutes (Fig. 1 and 2).

Figure 1



Exposure of *L. monocytogenes* to nisin and/or carvacrol during 35 minutes and the effect of both compounds on the viable count. Closed symbols represent precultivation and exposure at 20°C and open symbols represent precultivation and exposure at 8°C: nisin 5.3 µg/ml (◆), carvacrol 1.3 mmol/l (■), nisin 5.3 µg/ml + carvacrol 1.3 mmol/l (●), nisin 5.3 µg/ml (◇), carvacrol 0.7 mmol/l (□), nisin 5.3 µg/ml + carvacrol 0.7mmol/l (○). Cells were exposed in HEPES buffer (50 mmol/l, pH 7.0). All data represent mean values of triplicate measurements and detection limit lies at log N of 3.5.

Figure 2



Exposure of *B. cereus* to nisin and/or carvacrol during 30 minutes and the effect of both compounds on the viable count. Closed symbols represent precultivation and exposure at 20°C and open symbols represent precultivation and exposure at 8°C: nisin 0.3 µg/ml (◆), carvacrol 0.7 mmol/l (■), nisin 0.3 µg/ml + carvacrol 0.3 mmol/l (●), nisin 0.16 µg/ml (◇), carvacrol 0.7 mmol/l (□), nisin 0.16 µg/ml + carvacrol 0.7 mmol/l (○). Cells were exposed in HEPES buffer (50 mmol/l, pH 7.0). All data represent mean values of triplicate measurements. Detection limit lies at log N of 3.5.

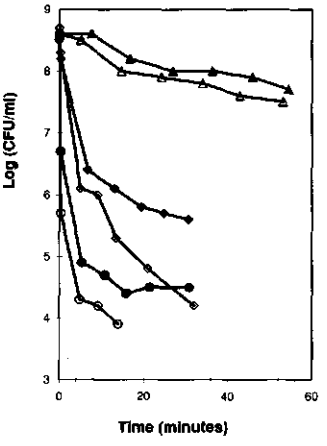
Control experiments with no additions showed a stable viable count over the 40 minutes of incubation in HEPES buffer (data not shown). Carvacrol at a concentration of 1.3 mmol/l did not reduce the viable count. However, nisin (5.3 µg/ml) caused a biphasic reduction of the viable count of

L. monocytogenes. In the first 5 - 7 minutes a rapid decline in viable count was observed while at the end of the exposure time the viable count remained stable. A total reduction of 2 log units was achieved. When nisin was combined with carvacrol a similar trend was observed. A large decline in viable count was achieved in the first 5 - 7 minutes of exposure and the decline became less strong when the exposure time increased. Interestingly, the combined exposure resulted in an extra reduction of almost 3 log units. This clearly indicates that carvacrol acts synergistically with nisin. This synergy was also observed at lower temperatures. Under these conditions, *L. monocytogenes* was more sensitive towards nisin. The reduction at 8°C was approximately 1 log unit more than at 20°C. Again, carvacrol potentated the antibacterial activity of nisin. Compared to *L. monocytogenes*, *B. cereus* was found to be much more sensitive towards nisin (Fig. 2). In addition, these experiments have been performed with other *B. cereus* strains as well and all strains showed greater sensitive towards nisin in comparison with *L. monocytogenes* (data not shown). *B. cereus* is more sensitive towards nisin at lower temperatures, but the synergism between nisin and carvacrol is greater at 20°C. At 20°C nisin was combined with 0.3 mmol/l carvacrol to achieve an extra reduction of 2.5 log units, while at 8°C this concentration did not lead to an extra reduction. A concentration of at least 0.7 mmol/l was needed to cause an extra reduction of 1 log unit. Similar results were obtained with other essential oils like thymol and carvone (data not shown).

Influence of lysozyme on the activity of nisin and carvone

Exposure to lysozyme as a third preservative factor resulted in a further decrease in viable count of *L. monocytogenes* (Fig. 3). *L. monocytogenes* was not very sensitive towards lysozyme (313 units/l), only a minor reduction in viable count was observed during 60 minutes of exposure to lysozyme.

Figure 3



Effect of lysozyme on the inhibitory effect of nisin and/or carvone on *L. monocytogenes* at 8°C. Nisin 5.3 µg/ml (◆), nisin 5.3 µg/ml + lysozyme 313 units (◇), nisin 5.3 µg/ml + carvone 10 mmol/l (●), nisin 5.3 µg/ml + carvone 10 mmol/l + lysozyme 313 units (○), lysozyme 313 units (△), carvone 10 mmol/l + lysozyme 313 units (▲).
Detection limit lies at log N of 3.5.

Carvone (10 mmol/l) on its own was not effective in decreasing the viable count of *L. monocytogenes* (data not shown) and it was not able to enhance the effect of lysozyme when used simultaneously. Combining lysozyme with nisin (5.3 µg/ml) resulted in an extra reduction in the last part of the exposure period. This reduction was comparable with the reduction obtained when nisin was used simultaneously with carvone. All three components together caused an even further reduction of the viable count below the detection limit.

DISCUSSION

This paper describes the combined effect of nisin and carvacrol towards *Listeria monocytogenes* and *Bacillus cereus*. Both compounds were found to show antibacterial activity towards the food pathogens used. At low concentrations the lag phase was extended, while at higher concentrations the growth was completely suppressed. MIC values of carvacrol found for *B. cereus* are in agreement with results described by Ultee *et al.* (29). They tested the bactericidal activity of carvacrol on *B. cereus* grown at 8°C and found MIC values of 0.75 mmol/l. Combining nisin with carvacrol increased the inhibitory effect of nisin as indicated by the lower MIC values for both compounds at both temperatures. A sublethal concentration of carvacrol which in it self did not influence the viable count resulted in an extra reduction of the viable count when combined with nisin.

The increased sensitivity of both pathogens for nisin at lower temperatures could be explained by the altered fluidity of the membrane, which is the primary target for nisin and essential oils. Ultee *et al.* (29) have shown that *B. cereus* cultured at 8°C has a lower phase transition temperature compared to cells grown at 30°C. Cells adapt to lower temperatures by changing their membrane composition. In general, the proportion of unsaturated fatty acyl chains of the lipids is increased to maintain an optimum fluidity (2, 26). At lower temperatures, cells have a higher membrane fluidity, which could facilitate the incorporation of lipophilic compounds as nisin or carvacrol. Ueckert *et al.* (28) found an improved activity of nisin towards heat stressed cells of *L. monocytogenes* and concluded that insertion of nisin in the membrane was facilitated because of temperature induced increased membrane fluidity. On the contrary, Abee *et al.* (2) found a decreased action of nisin Z (a variant of nisin A) at lower temperatures. This can be explained by the fact that these cells were cultured at higher temperatures and react to a lowering of temperature by ordering their lipid hydrocarbon chain which leads to a less fluid membrane and therefore to a decrease in the efficiency of nisin Z. Cells cultured at 30°C and subsequently put at 8°C have a lower membrane fluidity than cells cultured and exposed at 8°C (29). Ming and Daeschel (19) found that insertion of nisin was prevented by an increased rigidity of the membrane. Their nisin resistant mutants had a higher phase transition temperature, a higher percentage of straight-chain fatty acids, and a lower percentage of branched-chain fatty acids consistent with a more rigid membrane. This was confirmed in later studies in which they found an increased resistance with alteration of membrane composition (20). However, Davies *et al.* (6) could not detect differences in total phospholipid between *L. monocytogenes* wild type and its resistant mutant, but found a correlation between cell surface hydrophobicity and nisin sensitivity, indicating that the nisin resistant mutant adsorbs smaller amounts of nisin (20).

These findings can not entirely explain the increased sensitivity at lower temperatures found in this study. Although cells grown at 8°C have in general a lower phase transition temperature than cells grown at 30°C, cells grown at 30°C and exposed at 30°C have the same phase transition temperature as cells grown and exposed at 8°C (29). An explanation could be offered by Crandall and Montville (5) who believe that an altered membrane composition could lead to a decrease in negative charges of the phospholipids in the bilayer and therefore might hinder nisin's ability to bind and interact with the membrane (20). This is supported by the fact that adsorption of nisin Z to the membrane is decreased by the presence of cations in a concentration dependent manor. Di- and trivalent cations might inhibit the electrostatic interactions between the positive charges in the nisin molecule and negatively charged phospholipid head groups. Alternatively or additionally, the neutralisation of the negative head group charges may induce a condensation of these phospholipids resulting in a more rigid membrane (2). Crandall and Montville (5) did detect alterations in the cytoplasmic membrane and cell wall of their nisin resistant mutant, consistent with a more rigid membrane, but found that cations did not prevent nisin from binding and subsequent killing of wild type cells. Therefore they concluded that cations do not only interfere with the electrostatic binding of nisin with the anionic phospholipid head groups, but they might also be required to sufficiently stabilise the altered nisin resistant cells against disruption by nisin. The exact mechanism of nisin action remains unclear. Several models have been described but non of these offer sufficient explanation for the observed results. In conclusion, nisin and carvacrol are

bactericidal against *L. monocytogenes* and *B. cereus,* and act synergistically when used in combination. Addition of lysozyme increased the synergistic effect between nisin and carvone in the last part of the exposure time, suggesting an increased lifetime of the created pores. At lower temperatures, *L. monocytogenes* and *B. cereus* are more sensitive towards nisin and carvacrol which could be due to an altered membrane composition and subsequently an altered membrane fluidity or decreased electrostatic interaction of nisin with phospholipids due to a decrease in negative charges. Synergy between nisin and carvacrol enables us to use lower amounts of both compounds for effective food preservation. Low concentrations of carvacrol in combination with nisin are sufficient to reduce the viable count with 4-log cycles. The actual mechanism of synergy is not known. Carvacrol might enhance nisin's action by increasing the life time of pores created by nisin or by increasing the number or size of the pores formed both leading to an increased reduction of viable numbers. This needs to be elucidated. Our results further prove that increasing the number of preservative factors by combining compounds is effective in decreasing the number of colony forming units of food borne pathogens. It might be an effective and promising approach for preservation of food products in the future.

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CHAPTER 3

Bioenergetic consequences of nisin combined with carvacrol towards *Bacillus cereus*

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ABSTRACT

This paper describes the influence of the combination of nisin and carvacrol on the membrane potential, the pH gradient and the intracellular ATP pools of vegetative cells of *Bacillus cereus*. Both the membrane potential and the pH gradient were dissipated by nisin and carvacrol. The intracellular ATP pool was rapidly depleted. In combination, carvacrol enhanced the membrane potential dissipating activity of nisin, and acted synergistically in dissipating the pH gradient and depleting the intracellular ATP pool. The synergistic depletion of ATP could not be explained by an increased ATP efflux, indicating that carvacrol does not enlarge pores formed by nisin or increase the lifetime or the number of pores.

INTRODUCTION

Lactic acid bacteria are capable of producing a wide variety of antimicrobial substances, including bactericidal peptides, known as bacteriocins (2, 15). The most extensively studied bacteriocin is nisin, a lantibiotic produced by *Lactococcus lactis* ssp. *lactis* (13). Nisin displays inhibitory activity towards a broad range of Gram-positive organisms, including the food pathogens *Listeria monocytogenes* and *Bacillus cereus* (3, 16). Nisin is the only bacteriocin which has been approved by the World Health Organisation (WHO) to be used as a preservative in the food industry. It inhibits the outgrowth of germinating spores and causes lysis of the vegetative cell (10). The primary biological target is the energy-transducing cytoplasmic membrane of Gram-positive bacteria. Nisin binds electrostatically to the negatively charged phospholipids and increases the permeability of the membrane by pore formation, resulting in rapid efflux of essential intracellular small molecules (1, 5, 9, 24). The efflux of cellular constituents results in a complete collapse of the proton motive force and subsequently in cell death (33). Nisin needs an energised membrane to exert its action (12). Both the membrane potential as well as the pH gradient across the membrane are equally effective in promoting insertion and pore formation of nisin in the membrane (11, 21). Breukink *et al.* (6) demonstrated that nisin combines its pore forming ability with high affinity binding to Lipid II, a membrane-anchored cell wall precursor.

Recently, synergy between nisin and carvacrol against vegetative cells of *B. cereus* was observed (27). Carvacrol, present in the essential oil fraction of oregano and thyme, was found to exhibit antimicrobial activity towards a number of foodborne pathogens, including *B. cereus* (17, 18, 25, 30). Due to its hydrophobic nature, carvacrol accumulates into the cytoplasmic membrane resulting in leakage of potassium ions (28, 31). Different studies have shown that efflux of potassium ions is a first indication of membrane damage (28). Consequently a decrease of the intracellular pH, collapse of the membrane potential and inhibition of the ATP synthesis was observed (28, 31). Dissipation of the proton motive

force is a shared mechanism of action of nisin and carvacrol, offering a possible explanation for the observed synergy between the two compounds. In this paper, we have studied the influence of carvacrol on the dissipating effect of nisin on the membrane potential and the intercellular pH. Furthermore, the combined effect of nisin and carvacrol on both the intra- and extracellular ATP pools was determined.

MATERIALS AND METHODS

Growth of bacteria

Bacillus cereus IFR-NL94-25, obtained from the Institute of Food Research (Norwich, UK) was grown at 20°C in Brain Heart Infusion (BHI) broth (Oxoid), containing 0.5 % (wt/vol) glucose. Cell cultures were maintained at – 20°C in 30 % glycerol as a cryoprotectant.

Chemicals

Nisaplin, containing 2 % nisin, was obtained from Aplin and Barrett Ltd. (Wilts, UK). A stock solution of nisin was made in 50 % ethanol, centrifuged, filter sterilised (Costar, 0.22 µm) and stored at – 20°C before use. Purified carvacrol was obtained from Fluka Chemie AG (Buchs, Switzerland). A stock solution was made in 95 % ethanol and stored at 4°C. Final concentration of ethanol in the experiments was always kept below 2 % (vol/vol).

Influence of nisin and/or carvacrol on the membrane potential ($\Delta\Psi$)

Cells of *B. cereus* were harvested in the exponential growth phase and washed twice in 50 mM potassium-N-2-hydroxy-ethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer (pH 7.0). The cell pellet was resuspended in HEPES buffer until an OD_{660} of 10 was reached (light path 1cm). An aliquot of 30 µl of the cell suspension was diluted in 2 ml buffer containing 5 µM 3,3-dipropylthiacarbocyanine (DiSC3(5), Molecular Probes, Leiden, The Netherlands). A Perkin elmer LS 50B spectrophotometer was used to monitor the fluorescence of the probe as a qualitative measure of the membrane potential ($\Delta\Psi$) (excitation wavelength 643 nm, emission wavelength 666 nm). Glucose was added to energise the cells (final concentration 15 mM). The influence of nisin and/or carvacrol on the membrane potential was measured in the presence of nigericin (final concentration 1 nM) to prevent generation of a transmembrane pH gradient. Valinomycin (final concentration 1 nM) was used as a control. Measurements were performed twice.

Influence of nisin and/or carvacrol on the intracellular pH

The intracellular pH was measured using the fluorescent probe 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (cFDASE), as described by Breewer *et al.* (4) and modified by Ultee *et al.* (31). *B. cereus* cells were harvested in the exponential phase, washed three times with 50 mM HEPES buffer (pH 7.0) and resuspended until OD_{666} of 0.1 was reached (light path 1 cm). The cells were incubated with 1.5 µM cFDASE (10 min, 30°C) to allow hydrolysation of cFDASE to cFSE (carboxyfluorescein succinimidyl ester) and subsequent conjugation to aliphatic amines in the cell. Cells were washed with 50 mM potassium phosphate buffer (pH 5.8) and non-conjugated cFSE was removed by incubating the cells in the presence of 10 mM glucose for 30 min at 30°C. Again, the cells were washed twice and resuspended in potassium phosphate buffer (50 mM, pH 5.8) and kept on ice until further use. Fluorescence were measured using a Perkin elmer LS 50B spectrophotometer at excitation wavelengths of 490 nm (pH sensitive) and 440 nm (pH insensitive) by rapidly altering the monochromator between both wavelengths. The emission was determined at 525 nm and the excitation and emission slit widths were set on 5 and 10 nm respectively. The intracellular pH was calculated from the ratio of the emission at 490 and 440 nm excitation. The analysis was started by diluting 30 µl of the

cell suspension in a quartz cuvette containing 3 ml of potassium phosphate buffer (50 mM, pH 5.8) and stirred continuously. The measurements were performed in the presence of valinomycin (1 μM) to dissipate the membrane potential, and nigericin (1 μM) was used as a control. A calibration curve was determined in buffers with pH-values ranging from pH 3 to 10, adjusted with NaOH or HCl. Buffers contained 50 mM glycerine, 50 mM citric acid, 50 mM $Na_2HPO_4.2H_2O$ and 50 mM KCl. Measurements were performed twice.

Determination of intra- and extracellular ATP

Cells of *B. cereus* were harvested in the exponential growth phase (OD_{660} = 0.8), washed three times in 50 mM HEPES buffer (pH 7.0) and resuspended in HEPES buffer to an OD_{660} of 0.1 (light path 1 cm). Glucose was added at time zero to a final concentration of 0.5 % (wt/vol), and after 2 min of incubation, nisin and/or carvacrol were added in different concentrations. In the control experiments no nisin or carvacrol was added. The intracellular and extracellular ATP concentrations were determined at regular time intervals by separating the cells from the external medium by silicon oil centrifugation. Samples (200 μl) from the cell suspension were transferred to microcentrifugation tubes containing 200 μl of 2:1 mixture of silicon oil AR200 (ϱ = 1.05 g/ml) and silicon oil AR20 (ϱ = 0.96 g/ml) on the top a layer of 100 μl 10 % (w/vol) trichloracetic acid (TCA) with 2 mM ethylenediamineteracetic acid (EDTA) buffer. The mixture was centrifuged at 12000 g for 5 minutes, and samples (5 μl) of both aqueous layers were taken to determine the ATP content using 1243-107 ATP assay Kit (Bio-orbit, Turku Finland). Luminescence was recorded with a model 1250 luminometer (Bio-orbit, Turku, Finland). Measurements were done in duplicate.

Determination of protein content

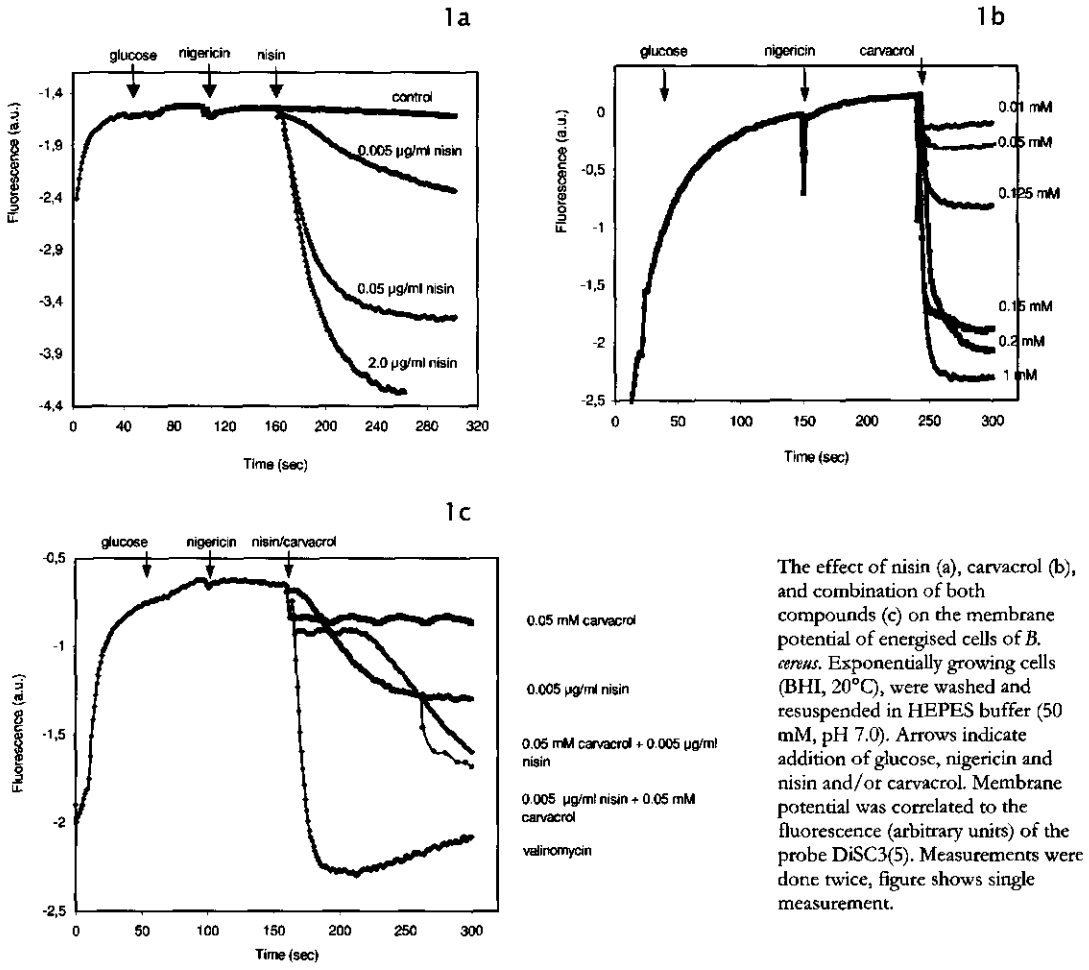
The internal and external ATP content was measured per mg protein. The amount of protein in the cells was determined according to Lowry *et al.* (20) using bovine serum albumin as a standard.

RESULTS

Influence of nisin and/or carvacrol on the membrane potential ($\Delta\Psi$)

Nisin is known for its pore forming ability in the cytoplasmic membrane resulting in dissipation of the proton motive force. The observed synergy between nisin and carvacrol might be caused by an increased membrane permeability resulting in collapse of the membrane potential. Therefore, the effect of both nisin and carvacrol on the membrane potential of *Bacillus cereus* was investigated. Cells of *B. cereus* were incubated in the presence of the fluorescent probe DiSC3(5). Changes in the fluorescence of the potentiometric dye as a result of addition of nisin and/or carvacrol correlates with changes in the membrane potential. As expected, nisin dissipated the membrane potential (Fig. 1a). Already at 0.005 μg/ml, nisin gradually decreased the membrane potential. An increase in nisin concentration resulted in an increased rate of reduction and a lower steady state membrane potential was reached. Carvacrol decreased the membrane potential, when present at concentrations of 0.01 mM or higher. Like nisin, higher concentrations of carvacrol resulted in a higher rate of reduction and a lower steady state membrane potential (Fig. 1b). Interestingly, both nisin and carvacrol enhanced each other membrane dissipating capacity (Fig. 1c). When carvacrol was added 1.5 minutes after nisin, the decreased in membrane potential first showed the gradual reduction curve of nisin followed by the typical more immediate reduction curve of carvacrol. Moreover, when carvacrol was added first, the reduction in membrane potential first followed the typical reduction caused by carvacrol and continued with the typical gradual reduction curve caused by nisin. Note that either way the steady state membrane potential was similar.

Figure 1



The effect of nisin (a), carvacrol (b), and combination of both compounds (c) on the membrane potential of energised cells of *B. cereus*. Exponentially growing cells (BHI, 20°C), were washed and resuspended in HEPES buffer (50 mM, pH 7.0). Arrows indicate addition of glucose, nigericin and nisin and/or carvacrol. Membrane potential was correlated to the fluorescence (arbitrary units) of the probe DiSC3(5). Measurements were done twice, figure shows single measurement.

Influence of nisin and/or carvacrol on the intracellular pH

Nisin and carvacrol both dissipate the membrane potential, however, the combination effects found could not explain the synergistic effect observed on the viable count of *B. cereus* (27). Therefore the explanation might be found in the influence of nisin and/or carvacrol on the pH gradient. The intracellular pH was measured using the fluorescent probe carboxyfluorescein diacetate succinimidyl ester (cFDASE). Measurements were performed in the presence of valinomycin to diminish the membrane potential. Nigericin was used as a control. As expected, both nisin and carvacrol were able to lower the pH_{in} of cells of *B. cereus* (Table 1). More nisin was needed to completely dissipate the pH gradient than to diminish the membrane potential. A concentration of 2 µg/ml hardly affected the pH gradient, however, at 2.5 µg/ml a complete collapse of the pH gradient was observed. Carvacrol dissipated the pH gradient completely when added at concentrations of 2 mM and higher. Lower amount of carvacrol still caused a reduction in the internal pH, however when added in concentrations

below 1 mM, no effect was observed anymore. Interestingly, carvacrol was able to enhance the reducing effect of nisin when used simultaneously. When 2 μg/ml of nisin was combined with 0.5 mM of carvacrol, a complete dissipation of the pH gradient was observed, while both concentrations of nisin as well as carvacrol were ineffective when used as a single treatment.

Table 1

		pH_{in}
Untreated		7.8
Nisin	2 μg/ml	7.7
	2.5 μg/ml	5.95
Carvacrol	1 mM	7.5
	1.5 mM	6.65
	2 mM	5.95
Nisin + Carvacrol	2 μg/ml+ 0.5 mM	5.95
Nigericin		5.95

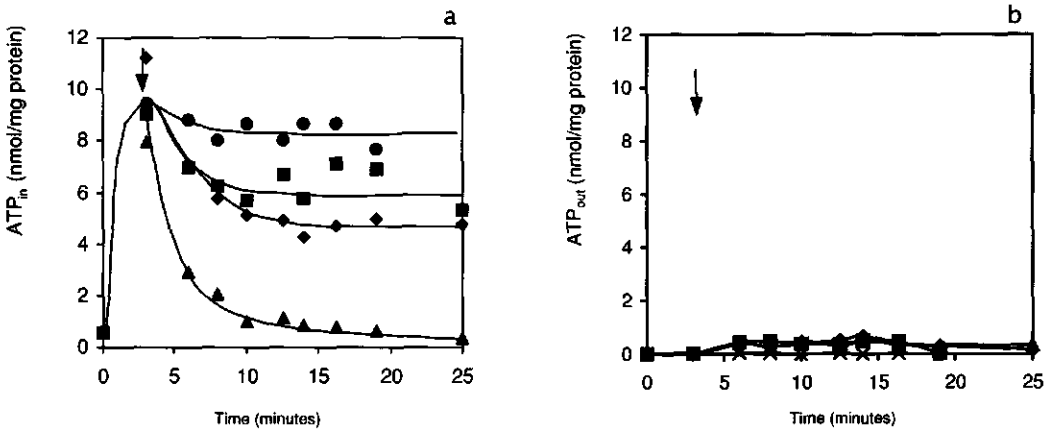
Effect of nisin and/or carvacrol on the intracellular pH of energised cells of *B. cereus*. Exponentially growing cells were washed and resuspended in potassium phosphate buffer (50 mM, pH 5.8) according to materials and method. Measurements were done twice, table shows single measurement.

Influence of nisin and/or carvacrol on the intra- and extracellular ATP pools

Nisin and carvacrol both interfered with the two components of the proton motive force. The proton motive force is the driving force for ATP synthesis and interference with the proton motive force will influence the energy requirements. The influence of nisin and/or carvacrol on the intra- and extracellular ATP pools was studied in more detail. Both carvacrol and nisin were able to reduce the intracellular amount of ATP (Fig. 2). Addition of 0.16 μg/ml of nisin to the energised cells resulted in an immediate decrease in internal ATP pool from 9.4 to 4.7 nmol/mg of protein. At the same time a small increase in external ATP pool to a level of approximately 0.5 nmol ATP/mg of protein was observed. Carvacrol (1 mM) reduced the internal ATP pool levels almost to the same extent as nisin. However, the simultaneous addition of nisin and carvacrol resulted in a huge reduction in internal ATP pool to almost zero. The carvacrol concentration used in the combination treatment was only half that of the single treatment, indicating synergistic effects between nisin and carvacrol in depleting the intracellular ATP pool. The increase in extracellular ATP was not proportional to the observed decrease in intracellular ATP pool. Moreover, the extent of the increase in external ATP was similar for all three treatments, excluding ATP leakage as the sole explanation for the depletion of the intracellular ATP pools.

Figure 2

The effect of nisin and/or carvacrol on the intracellular (a) and extracellular (b) ATP pools of energised cells of *B. cereus*. Arrows indicated addition of nisin and/or carvacrol at t = 2 min. Control (●), carvacrol 1 mM (■), nisin 0.16 μg/ml (◆), nisin 0.16 μg/ml and carvacrol 0.5 mM (▲). Data are means of duplicate measurements.



DISCUSSION

This paper describes the combined effect of nisin and carvacrol on a number of bioenergetic parameters of vegetative cells of *Bacillus cereus*. To our knowledge, the effect of simultaneous addition of two components on the proton motive force has not been described in literature before. Both nisin and carvacrol alone were able to dissipate the membrane potential as well as the pH gradient across the membrane. The membrane potential was found to be affected at low concentrations of nisin (0.005 μg/ml) and carvacrol (0.01 mM), while the pH gradient was affected only at higher concentrations (nisin> 2 μg/ml, carvacrol >1 mM). In addition, depletion of the internal ATP pool was observed upon exposure to both compounds. These results are in agreement with studies reported by other authors (7, 8, 22, 26, 29, 31).

Carvacrol is able to enhance the bactericidal activity of nisin towards vegetative cells of *B. cereus* at concentrations, which in it self do not reduce the viable count. The mode of action of this synergy is not yet understood, but the similarity in dissipation of the proton motive force and depletion of the internal ATP pool might offer an explanation. The membrane potential is already influenced by low concentrations of nisin and carvacrol that do not cause any reduction in the viable count (27, 30). Combining these two components resulted in an enhanced dissipation of the membrane potential. In addition, a synergistic reduction of the pH gradient and the intracellular ATP pool was observed. The observed reduction in internal ATP was not proportional to the increase in external ATP. Several authors have demonstrated that the ATP efflux was only partially responsible for the observed decrease in intracellular ATP (1, 14, 24, 31, 33). Abee *et al.* (1) suggested that hydrolysis was caused by phosphate efflux and a subsequent shift in the ATP hydrolysis equilibrium. In addition, cells exhaust the intracellular ATP in an attempt to regenerate the proton motive force (23). Depletion of ATP pool by carvacrol was suggested to result from a reduced rate of ATP synthesis or increased ATP hydrolysis (31). The increase in extracellular ATP caused by the combination of nisin and carvacrol was identical to the increase caused by the single treatments, indicating that carvacrol does not increase ATP efflux caused by nisin as a result of increased pore formation or size or lifetime of the pores. Presumably, the

rate of ATP hydrolysis is increased upon simultaneous addition or the internal ATP pool is exhausted in an attempt to reenergize the membrane. Alternatively, the disturbance of the membrane permeability by carvacrol and nisin might lead to malfunction of membrane bound enzymes like ATPase, resulting in a decreased ATP synthesis (19, 26, 31). Measurements of phosphate efflux, indicating ATP hydrolysis could elucidate this.

Clearly cells of *B. cereus* are able to cope with low concentrations of both nisin and carvacrol since the decrease in membrane potential is not related to the reduction in viable count. When concentrations increase, cells are no longer able to compensate for loss of membrane integrity and their intracellular ATP pools are depleted in a futile attempt to regenerate their proton motive force. No increase in extracellular ATP was detected upon combining nisin and carvacrol, indicating that carvacrol does not increase nisin's pore forming ability by enlarging the pore size, the life time or the number of pores or by increasing nisin's affinity for Lipid II. Therefore, our results can only be explained by an increased hydrolyses of ATP to regenerate the proton motive force resulting in increased ATP depletion. Or alternatively, the extra ATP depletion observed might result from a disturbed membrane integrity. The latter phenomenon could be directly caused by both carvacrol and nisin. Carvacrol is known to accumulate in the lipid bilayer, causing an increase in the membrane fluidity (32) and nisin was demonstrated to bind to the phospholipids in the membrane, thereby disturbing the ordering of the membrane. Disturbance of the integrity could lead to malfunction of the membrane bound enzymes, including ATPase, leading to a decreased ATP synthesis.

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CHAPTER 4

Pulsed Electric Field treatment enhances the bactericidal action of nisin against *Bacillus cereus.*

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ABSTRACT

Nisin was combined with Pulsed Electric Field (PEF) treatment in order to expand the range of application of nisin as a mild preservative agent. Vegetative cells of *Bacillus cereus* were subjected to low doses of nisin (0.06 µg/ml) and mild PEF treatment (16.7 kV/cm, 50 pulses each of 2 µs duration) separately and in combination. Both treatments, when applied separately, reduced the viable count of *B. cereus* by 0.8 and 1.2 log units respectively, but the combined treatment resulted in a remarkable increase in the reduction of the viable count of 3.8 log units. The observed reduction obtained by the combined treatment was 1.8 log units more than the sum of the reductions obtained with the single treatments. These results clearly demonstrate that PEF treatment acts synergistically with nisin in reducing the viable count of vegetative cells of *B. cereus*.

INTRODUCTION

Nisin, an antimicrobial protein produced by *Lactococcus lactis* ssp. *lactis*, is the only bacteriocin that is approved by the WHO to be used as a food preservative today (1, 9). Nisin is bactericidal against a broad range of Gram-positive bacteria including some lactic acid bacteria, *Listeria monocytogenes* and spore forming bacteria like *Clostridium* and *Bacillus* species (9, 12, 20). Yeast, molds and Gram-negative bacteria are normally resistant, but Gram-negative bacteria can be sensitized by exposure to chelating agents which change the permeability properties of the outer membrane (1, 4, 30). The primary target of nisin is the cytoplasmic membrane of vegetative cells. Nisin interacts via electrostatic interactions with the phospholipids in the membrane and increases the permeability of the membrane by pore formation, resulting in a rapid efflux of small molecules (3, 26). Pore formation is a membrane potential or pH gradient dependent process and involves reorientation of nisin molecules from a surface bound into a membrane bound configuration (4, 5, 6, 12). The efflux of cellular constituents results in a complete collapse of the proton motive force and cellular death.

Nisin is used as a natural food preservative in products like processed cheese and canned foods (19, 26), but its practical application is limited due to its low stability and activity at high pH and its limited efficacy in certain food matrices (29). Essential oils were studied previously and have been shown to act synergistically with nisin (22). In this study, a Pulsed Electric Field (PEF) treatment was chosen to combine with nisin. PEF treatment is a non-thermal pasteurization technique, which inactivates microorganisms without adversely affecting the flavour, color and nutrient content of products (2, 24,

25, 34). Lysis of microorganisms is caused by irreversible structural changes in the membrane resulting in pore formation and loss of the selective permeability properties of the membrane (2, 8, 21, 33). An imposed electric field causes polarization and subsequently accumulation of free charges at both sides of the cell surface leading to an increased transmembrane potential difference and a reduction of the membrane thickness. Furthermore, the electric charges will cause the polar lipid molecules to reorient when subjected to an intense electric field strength resulting in formation of hydrophilic pores and impairment of the membrane barrier against ions (31). Breakdown of the integrity of membrane is associated with a reversible permeability increase of the cell membrane. The extent of the permeability increase depends on the strength and duration of the electric field pulse (2, 8, 17, 33, 34). Exceeding a transmembrane potential of typically 1 Volt leads to irreversible membrane damage (28), the pores created in the membrane remain open after removing the applied potential (2, 23, 33). Electroporation does not only occur in the lipid bilayer but also affects protein channels in the membrane. The opening and closing of many of these channels is controlled by the membrane potential in the 50 mV range, which is smaller than the dielectric strength of a lipid bilayer. Thus, when a pulsed electric field is applied, many voltage sensitive protein channels can open before the transmembrane electric potential reaches the breakdown potential of the lipid bilayer. Once open, protein channels may experience currents by ion transport which are much larger than these channels can endure. As a result, protein channels may be irreversibly denatured by Joule heating or electric modifications of functional groups and contribute to impairment of the cell membrane (2, 31).
In the present study nisin was combined with PEF treatment in order to obtain increased lysis of vegetative cells of *Bacillus cereus*. Both treatments were used at mild doses. This study provides evidence that PEF treatment acts synergistically with nisin in reducing the viability of *B. cereus*. In addition, the kinetics of the combined action of nisin and PEF treatment has been studied in more detail.

MATERIALS AND METHODS

Growth of bacteria

Bacillus cereus IFR-NL94-25, obtained from the Institute of Food Research (Norwich, UK) was grown at 20°C in Brain Heart Infusion (BHI) broth (Oxoid), containing 0.5 % (wt/vol) glucose. Cells were harvested after 16 hours of growth, washed and resuspended in 5 mM potassium-N-2-hydroxy-ethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer (pH 7.0) to an OD_{660} of 0.2 (light path of 9 mm) and stored on ice until further use. Cell suspensions were checked for spores microscopically before harvesting and subsequently by analysis of surviving spores after a standard heat treatment (80°C, 5 min).

Pulsed Electric Field treatment

The inactivation of vegetative cells of *B. cereus* by PEF treatment has been studied using a custom built batch system. The system consisted of an electronic high voltage pulser connected to a treatment chamber with a volume of 800 µl. The chamber was formed by two stainless steel cylinders with a diameter of 12.6 mm which was tightly fitted into a plexiglass tube with the same inner diameter. The plexiglass tube separated the electrodes and provided electrical isolation. In this work the electrode distance was fixed to 6 mm. The space between the electrodes contained the organisms under investigation. Two ports in the middle of the plexiglass tube provided access for filling and rinsing. The pulser was a custom built device and produced high voltage pulses up to 25 kV with a duration in the range of 0.5 - 2 µs. The electronic output circuit of the pulser has been configured to match the impedance of the chamber. In this way single rectangular shaped pulses have been obtained. The rise time of a pulse was less than 100 ns and the repetition rate could be varied in the range of 1 Hz to 1 kHz. During experiments, the pulses were monitored using an oscilloscope connected to the terminals

of the treatment chamber through a Tektronix high voltage probe. In all cases the temperature during treatment was kept below 30°C in order to discriminate for thermal effects.

Filling and cleaning procedure

The bacterial suspensions were pipetted into the chamber through the filling ports at the side. The second port acted as an air relief such that the chamber could be filled completely with liquid in absence of air bubbles. At this point, the chamber was inserted into an electrically isolated box where the electrodes were connected to the high voltage terminals. After treatment the chamber was released from the box and the content was analyzed in a sterile environment. As a cleaning procedure the chamber was first rinsed five times with 70 % ethanol, followed by rinsing fives times with demineralised water and finally rinsed once with sterile demineralised water.

Determination of critical electric field strength and treatment time

Cells of *B. cereus* were harvested and washed as described above. To determine the critical electric field strength for this strain, 800 µl of the washed cell suspension was subjected to increasing electric field strengths in a range of 0 to 25 kV/cm at room temperature (25°C) and serial diluted after homogenizing in peptone physiological salt (8.5 g sodium salt and 1 g of peptone per liter demineralised water). The total treatment time was 1 ms and was realized by applying 500 pulses of each 2 µs duration. To determine the characteristic inactivation time, the total treatment time was varied in the range 0 - 800 µs at a fixed field strength of 16.7 kV/cm.

Effect of nisin on viability of *B. cereus*

The effect of nisin on the viability of *B. cereus* cells was determined by exposing 2 ml of the cell suspension to different concentrations of nisin. Nisaplin, containing 2 % nisin, was obtained from Aplin and Barrett Ltd. (Wilts, UK) and stock solution of nisin was made in 50 % ethanol and filter sterilized (Costar, 0.22 µm) before use. The inactivating action of nisin was quenched after 11.5 minutes by a 100-fold dilution in peptone physiological salt solution (see above) and the number of surviving cells was determined on BHI agar.

Combined effects of nisin and Pulse Electric Field on viability

The effect of nisin and/or PEF treatment on the viability of *B. cereus* was determined by exposing the cells to nisin alone (0.06 µg/ml, 11.5 min), PEField treatment alone (16.7 kV/cm, 50 pulses) or to a combination of both treatments. The combined treatment was carried out by subjecting *B. cereus* cells to PEF treatment 1.5 minutes after addition of nisin to the cell suspension. The PEF treatment was spread over 10 minutes and the number of surviving cells was determined by dilution in peptone physiological salt solution and plating on BHI agar.

Kinetics of the combined treatment

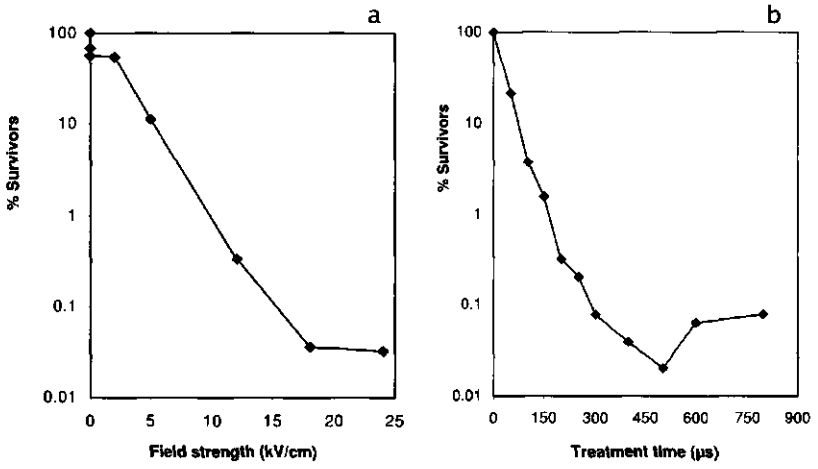
B. cereus cell suspensions were subjected to nisin alone, PEF treatment alone or a combination of both according to the above-mentioned method. Each minute, the first time 1.5 minutes after nisin was added to the cell suspension, a treatment of 5 pulses was applied, such that after 11.5 minutes a total number of 50 pulses was reached. The treatments were stopped after 0, 2.5, 3.5, 5.5, 7.5, 9.5, 11.5 minutes to determine the number of surviving cells. Conditions used here were identical to the conditions in the previous experiment.

RESULTS

Determination of critical electric field strength and treatment time

The critical field strength for *Bacillus cereus* was determined by exposing the organisms to different field strengths ranging from 0 to 25 kV/cm (Fig. 1a). The temperature increase of the suspension by Ohmic heating has been measured directly after treatment and under circumstances of steady state operation. A temperature increase of maximum 5°C was observed at the highest field strength applied. This can be explained by the large contact surface of the liquid with the electrodes, which provides substantial cooling. The influence of an altered pH on the viability of *B. cereus* could be excluded while no pH changes were detected between the PEF treated and non-treated HEPES buffer (data not shown). Thus, inactivation of vegetative cells of *B. cereus* could solely be attributed to the PEF treatment itself.

Figure 1



Determination of critical field strength (kV/cm) (a) and critical treatment time (b) for *Bacillus cereus*. (a) The total treatment time was 1000 µs (500 pulses of 2 µs duration). Data points represent means of 2 independent measurements. (b) PEF treatment applied was 16.7 kV/cm, with pulses of 2 µs duration. Data points represent the average of 3 measurements.

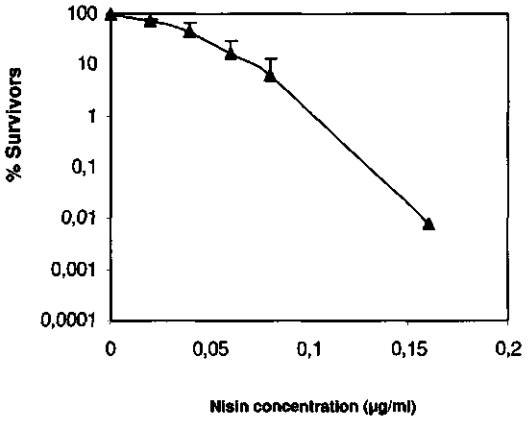
From figure 1a, a critical field strength of 3.9 kV/cm has been determined for inactivation of vegetative cells of *B. cereus* by PEF treatment. The critical field strength has been defined by extrapolating the straight section of the survival curve to 100 % (7, 17). Applying low electric field strength did not cause a reduction in viable count. Only after passing a critical field strength a reduction in viable count was observed which was log-linear with the applied field strength. At field strengths of 20 kV/cm and higher the inactivation saturated. This effect has been analyzed and has been ascribed to fading of the electrical field near the metal-liquid-plexiglass interface. Due to edge effects near the interface of the electrode and the test fluid, locally the critical field strength was not reached (data not shown). This limited the resolution of our experiments to a reduction of 4 log cycles.

The critical treatment time was determined by subjecting *B. cereus* to an increasing treatment time in the range of 0 to 800 µs with constant electric field strength (16.7 kV/cm). Treatment time was defined as the product of the pulse duration and the number of pulses (17). Figure 1b shows that the relation between the number of survivors and the treatment time is log-linear for a certain range of pulses. When subjecting *B. cereus* to a treatment longer than 300 µs, a plateau value for the reduction was reached caused by edge effects described above. The shape of the inactivation curves are in agreement with results reported by Sale and Hamilton (27), Jayaram *et al.* (13, 14) and Knorr *et al.* (17). A mild treatment, causing only 1 log unit reduction, was obtained using a treatment time of 100 µs.

Effect of nisin on viability of *B. cereus*

The effect of nisin was determined by adding different concentrations of nisin to the cell suspension. Note that nisin was active throughout the whole volume of the reaction chamber and therefore its activity was not influenced by edge effects. The reaction was quenched 11.5 minutes after the addition of nisin by a 100-fold dilution of the cell suspension.

Figure 2



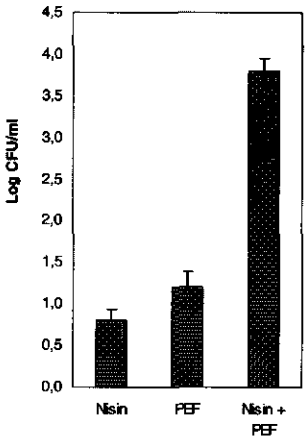
Influence of nisin concentration on the viability of *Bacillus cereus*. Treatment time was 11.5 minutes. Data points represent means of an average of 4 measurements.

Figure 2 shows that nisin was already active at very low concentrations. A concentration of 0.04 µg/ml nisin caused a minor reduction in the percentage of survivors. Higher concentrations resulted in an increased reduction in viable count. In order to obtain a mild reduction of only 1 log unit, a concentration of 0.06 µg/ml nisin was chosen for further experiments. Since PEF treatment did not alter the pH of the buffer, no differences in activity of PEF treated nisin and non-treated nisin are expected.

Combined effects of nisin and Pulsed Electric Field treatment on viability

Since nisin shows the highest activity in the first 10 minutes after addition (22), the combination treatment was also given in this time interval. To study possible synergism between nisin and PEF treatment, mild doses of both nisin and PEF treatment have been chosen. Nisin was used at a concentration of 0.06 µg/ml and a PEF treatment of 16.7 kV/cm (50 pulses of each 2 µs duration) was applied 1.5 minutes after addition of nisin. Treatment of vegetative cells of *B. cereus* with either nisin or PEF treatment alone, caused a minor decrease in the viable count of 0.8 and 1.2 log units, respectively (Fig. 3). However, when cells of *B. cereus* were subjected to the combined treatment, the viable count is reduced with 3.8 log units. This is close to the resolution of the experiment as explained. Combining the two treatments resulted in a decrease of 1.8 log units more than the sum of both treatments alone, indicating synergy between the PEF treatment and nisin.

Figure 3

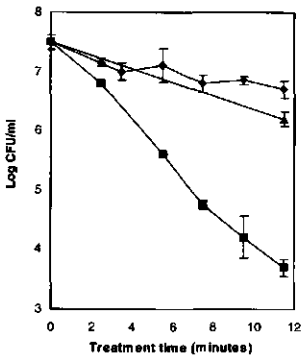


The reducing effect of PEF treatment alone, nisin alone and the combined treatment on the viability of *Bacillus cereus*. PEF treatment given was 16.7 kV/cm (100 µs) spread over 10 minutes. Data points represent means of duplicate measurements.

Kinetics of the combined treatment

The inactivation of *B. cereus* by the combined treatment and by both treatments alone has been determined as a function of time. The inactivation of *B. cereus* by a PEF treatment was found to be log-linear in time (Fig. 1b). In this particular experiment only a control sample at t = 11.5 minutes was taken (Fig. 4). In the presence of 0.06 µg/ml nisin, only a marginal reduction of the viable count of *B. cereus* cells was observed. At this low concentration, the typical biphasic inactivation curve (22) is not observed. In the combined treatment, the viable count decreased more rapidly than the sum of the effect of the two treatments alone, again indicating towards the existence of synergy between nisin and PEF treatment.

Figure 4



Kinetics of combined action of nisin and PEF treatment on the viability of *Bacillus cereus* monitored in time. PEF treatment, 16.7 kV/cm, 100 µs treatment time (▲), nisin 0.06 µg/ml (◆) and nisin (0.06 µg/ml) + PEF treatment, 16.7 kV/cm, 100 µs treatment time (■). Data points represent means of duplicate measurements.

Note that viable counts after 11.5 minutes of the three treatments were consistent with results found in Figure 3, again demonstrating the reproducibility of the procedures applied. In sharp contrast to what is observed with high doses of nisin alone (22), the inactivation kinetics associated with the combined treatment follows a log-linear pattern.

DISCUSSION

To increase the range of application of nisin, this antimicrobial peptide was combined with a Pulsed Electric Field treatment and the possible occurrence of synergy with nisin was investigated. Both treatments were tested at mild doses and applied together to determine the effect on the viability of vegetative cells of *Bacillus cereus*. The PEF treatment was spread over a period of 10 minutes to match the relevant time scale of nisin's action. Furthermore, this method minimizes the temperature rise so that heat inactivation is excluded and the inhibitory effect can be solely ascribed to the PEF treatment (27). The results found here clearly show that a PEF treatment enhances the inhibitory effect of nisin. Almost 2 log units extra reduction is achieved when nisin and PEF treatment are applied in combination compared to the sum of the reduction achieved by the single treatments.

Kalchayanand *et al.* (15) combined hydrostatic pressure (UHP) and electroporation (EP, single pulse treatment) with nisin, pediocin AcH or a combination of both and found greater antibacterial activity when UHP and EP were combined with bacteriocins than when UHP and EP were used alone. The bacteriocins were able to increase the effectiveness of UHP and EP towards *Escherichia coli, Listeria monocytogenes* and *Salmonella typhimurium*. However, from these data no conclusions with regard to synergy between the bacteriocins and UHP or EP could be drawn. Although *S. typhimurium* and *E. coli* are both Gram-negative bacteria and therefore rather insensitive towards nisin, nisin is able to inactivate *L. monocytogenes* and therefore the observed effect of EP treatment in the presence of nisin could be solely attributed to nisin alone. In contrast, data presented here clearly demonstrate synergy between nisin and PEF treatment since both treatments were applied in mild doses.

Kalchayanand *et al.* (15) reported that electroporation and ultra high pressure can induce sublethal injury and these cells become more sensitive to different physical and chemical environments to which the normal cells are resistant. This is supported by Ho *et al.* (10) who found that the critical field strength required for cell lysis was reduced by inducing additional stress to the cell membrane like sodium chloride or osmotic pressure. This could apply for the combined action described in this paper as well. The PEF treatment can be regarded as an additional stress to nisin and possibly facilitates the incorporation of nisin into the cytoplasmic membrane resulting in more or larger pores or pores with a longer lifetime. Larger pores are obtained either by using a higher field strength, by increasing the pulse duration or by reducing the ionic strength of the buffer medium (16, 18). The process of pore formation is relatively insensitive to temperature in contrast to the marked temperature dependence of the repair process which is slow at low temperatures and high at elevated temperatures (16, 31).

The ionic strength of the media plays an important role in the effectiveness of the inactivation. Fluids with low ionic strength favor bacterial inactivation (14, 32). This is explained by the reduced stability of the cell membrane when exposed to a medium of low ionic strength. Divalent cations such as Ca^{2+} and Mg^{2+} play a role in the stability of biological membranes. They induce a protective mechanism against electrical treatments in bacterial cell membranes (11, 31, 33). It is also known that certain components of food, like proteins, can offer protection to bacteria against different stresses. Ho *et al.* (10) found that the critical electric field strength required for cell lysis was increased by formation of a protective layer for the cells by xanthan gum.

In conclusion, our results clearly demonstrate that PEF treatment potentiates the antimicrobial action of nisin against vegetative cells of *B. cereus*. The synergism found between nisin and PEF treatment opens new perspectives to apply the hurdle concept as a preservation method.

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CHAPTER 5

Influence of food matrix on inactivation of *Bacillus cereus* by combinations of nisin, Pulsed Electric Field treatment and carvacrol

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Journal of Food Protection

ABSTRACT

Carvacrol was used as a third preservative factor to further enhance the synergy between nisin and Pulsed Electric Field (PEF) treatment against vegetative cells of *Bacillus cereus*. When applied simultaneously with nisin (0.04 µg/ml), carvacrol (0.5 mM) enhanced the synergy found between nisin and PEF treatment (16.7 kV/cm, 30 pulses) in HEPES buffer. The influence of food ingredients on the bactericidal activity was tested using skimmed milk, which was diluted to 20 % with sterile demineralised water. The efficacy of PEF treatment was not affected by the presence of proteins and results found in HEPES buffer correlated well with results in milk (20 %). Nisin showed less activity against *B. cereus* in milk. Carvacrol was not able to enhance the synergy between nisin and PEF treatment in milk, unless used in high concentrations (1.2 mM). This concentration in itself did not influence the viable count. Carvacrol did act synergistically with PEF treatment in milk, however not in HEPES buffer. This synergy was not influenced by proteins in milk, since 5 % milk still allow synergy between carvacrol and PEF treatment to the same extent as 20 % milk.

INTRODUCTION

Modern consumers nowadays demand more natural food products with a long shelf life and preferentially mildly preserved. To guarantee the microbial safety of these products, several mild preservative factors need to be combined (20). Pulsed Electric Field (PEF) is a non-thermal inactivation method, which has potential as an alternative to thermal processing. PEF treatment has been proven to inactivate microorganisms with minimal losses of flavor and food quality and the low processing temperatures used in this non-thermal technology allows the process to be energy efficient (40, 42). Microbial inactivation is caused by structural changes in the membrane, resulting in pore formation and loss of the selective properties of the membrane (10, 24, 30). When a cell suspension is exposed to an electric field, the ions in- and outside the cell migrate according to the electric field across the electrodes. As a result, free charges accumulate at both membrane surfaces leading to an increased transmembrane potential. In addition, the attraction between the opposing charges leads to a compression pressure that causes the membrane thickness to decrease (6, 43). These electric forces and compression pressure

increase with a decrease in the membrane thickness. Furthermore, the electric charges will cause the polar lipid molecules to reorient when subjected to an intense electric field strength resulting in formation of hydrophilic pores and impairment of the membrane barrier function against ions (34). Breakdown of the integrity of membrane is associated with a reversible permeability increase of the cell membrane. The extent of the permeability increase depends on the strength and duration of the electric field pulse (6, 10, 19, 31, 40, 42). Exceeding the threshold transmembrane potential of typically 1 Volt leads to irreversible membrane damage (31), the pores created in the membrane remain open after removing the applied electric field (6, 27, 40). In general, pore formation involves a two step mechanism of initial perforation followed by pore expansion; the entire process depends on electric field intensity and pulse duration. The area of pore formation increases with an increase in electric field, whereas the pore radius increases with an increase in pulse duration (2).

Inactivation of microorganisms by PEF treatment is affected by a number of factors including process parameters of the PEF treatment, the characteristics of the microorganisms and the physical, chemical and electrical properties of the treatment media. Researchers have shown that inactivation increases with an increase in the applied electric field strength and treatment time, and that higer temperatures act synergistically with PEF treatment (9, 19, 26, 30, 31, 37, 41).

The growth phase and size of the target microorganisms can influence the efficiency of the PEF treatment. Cell and membrane properties are different at different stages of growth and logarithmic phase cells are more sensitive than lag and stationary phase cells (14, 27, 40, 41). The characteristics of the treatment medium, like the conductivity, pH, ionic strength, the presence of particles or gas bubbles and the dielectric properties of a medium are strongly influencing the efficiency of the PEF treatment. Electrical conductivity is related to the efficiency of the energy transferred, and a low conductivity leads to a more effective PEF treatment. Electrical conductivity increases with ionic strength (13, 15, 38, 41). Other factors that enhance PEF treatment are pH and the presence of antimicrobials, which act as additional preservative factors (hurdles): each factor imposes an additional stress to the microorganisms and the result is an increase in the total antimicrobial action of the combined treatment.

In general, preservation by a single preservative factor is not sufficient to ensure complete safe products and multiple hurdles are advised (20). Recently, synergy between the bacteriocin nisin and PEF treatment was demonstrated in our laboratory (26). The primary target of nisin is the cytoplasmic membrane of vegetative cells. Nisin binds to the bacterial membrane via electrostatic interactions with the phospholipids and increases the permeability of the membrane by pore formation, resulting in a rapid efflux of small molecules (4, 7, 28). This pore formation leads to dissipation of the membrane potential and ionic gradients across the membrane and subsequently results in the destruction of energy metabolism and cell death (1, 5, 21, 22). The fact that nisin and PEF treatment share a common primary target might offer an explanation for the synergy found between the two treatments. An other example of mild preservation by multiple hurdles is based on the synergy between nisin and essential oils like thymol and carvacrol (25). Due to their lipophilic nature, components like carvacrol can accumulate in the lipid bilayer, thereby disturbing its function, leading to cell death (35). Again, combining two compounds with similar primary targets resulted in improved bactericidal activity compared to the sum of the reduction of the single treatment. In this research carvacrol was added as a third hurdle in combination with nisin and PEF treatment in order to enhance the synergy found between nisin and PEF treatment. In addition, the possible application of these combinations in food products was evaluated using diluted milk as a food model matrix.

MATERIALS AND METHODS

Growth of bacteria

Bacillus cereus IFR-NL94-25, obtained from the Institute of Food Research (Norwich, UK) was grown at 20°C in Brain Heart Infusion (BHI) broth (Oxoid), supplemented with 0.5 % (wt/vol) glucose. Cells were harvested in the exponential growth phase, washed and resuspended in 5 mM

potassium-N-2-hydroxy-ethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer (pH 7.0) until an OD_{660} of 0.2 (10 mm light pathway) and stored on ice until further use. Cell suspensions were checked for spores microscopically before harvesting and subsequently by analysis of surviving spores after a standard heat treatment (80°C, 5 min).

Influence of nisin combined with carvacrol on viability of *B. cereus*

To determine the effect of the combination of nisin (Aplin and Barrett Ltd., Wilts, UK) and carvacrol (Fluka Chemie AG, Buchs, Switzerland) on the viability of *B. cereus*, 2 ml of the cell suspension was exposed to different combinations of nisin (0.04 µg/ml) and carvacrol (0.3 - 0.5 mM). The action of nisin and carvacrol was quenched, 11.5 minutes after addition to the cell suspension, by a 100-fold dilution in peptone physiological salt solution (8.5 g sodium salt and 1 g of peptone per liter demi water) and the number of surviving cells was determined on BHI agar. A stock solution of carvacrol (0.5 mM)was held in 95 % ethanol and kept at 4°C. Nisin solution (2 µg/ml) was made in 50 % ethanol, filter sterilized (Costar, 0.22 µm) and kept at - 20°C until further use.

The influence of carvacrol on the synergy between nisin and PEF treatment

The batch PEF system, described in Pol *et al.* (26), consisted of an electronic high voltage pulser connected to a treatment chamber with a volume of 800 µl. The pulser produced rectangular shaped, high voltage pulses up to 25 kV with a duration in the range of 0.5 - 2 µs. The effect of carvacrol on the synergy between nisin and PEF treatment against *B. cereus* was determined by exposing the cells to nisin alone (0.04 µg/ml), carvacrol alone (0.5 mM), PEF treatment alone (16.7 kV/cm, 20 pulses) or a combination of the three treatments. The combined treatments were carried out by applying the PEF treatment 1.5 minutes after addition of nisin and/or carvacrol to the cell suspension. The PEF treatment was spread over a 10 minutes time interval, to maximize interaction with nisin and/or carvacrol. The reaction was quenched by a 100-fold dilution in peptone physiological salt solution and the number of survivors was determined on BHI agar. In all cases the temperature during treatment was kept below 30°C in order to discriminate for thermal effects.

Combined effect of nisin, carvacrol and PEF treatment in food products using a continuous PEF system

The application of a combination of nisin, PEF treatment and carvacrol in food products was tested using diluted milk as a food matrix (DOMO LANG LEKKER®, FCDF, skimmed (0 % fat), ultra high temperature sterilized (UHT)). The conductivity of the milk was 5.09 mS/cm. In order to lower the conductivity of the milk within the application range of the PEF treatment chamber, the milk was diluted till 20 % with sterile water. The cells were grown and harvested as described above. The pellet was resuspended in 20 % skimmed milk (σ = 1.4 mS/cm at 20 °C) till a concentration of 10^7 cells/ml and subjected to nisin (0.13 µg/ml), carvacrol (concentration range: 0.4 - 1.2 mM), PEF treatment (20 kV/cm, 30 pulses of 2 µs duration) or a combination of all three treatments. PEF treatment was applied using a continuous flow system instead of a batch system. The continuous PEF system consists of an electronic high voltage pulser and a treatment device as part of an aseptic fluid handling system. The treatment device is of co-linear design as reported by Yin *et al.* and discussed by Barbosa *et al.* (2) and the gap distance is 4 mm in length and 2 mm in diameter. By regulating the flow rate of the fluid and the repetition frequency of the pulser, the average number of pulses that fluid elements received was set to 2.5 pulses per minute. *B. cereus* was added to the diluted milk and recirculated for 5 minutes at high flow rate before treatment to get a homogeneous distribution of the

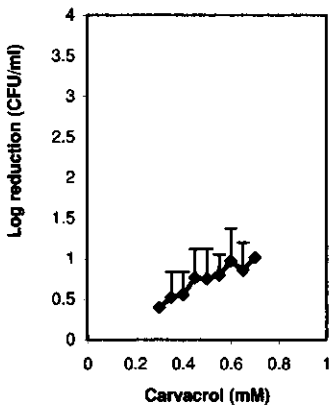
cells. During experiments a total volume of 400 ml of inoculated medium was circulated through the system for 12 minutes at 660 Hz pulse repetition rate and a flow rate of 100 ml/min. Under these conditions the temperature was kept below 25°C. Samples were drawn at appropriate time intervals and the number of survivors were analyzed on BHI agar.

RESULTS

The influence of carvacrol on the synergy between nisin and PEF treatment

Carvacrol as well as PEF treatment has previously been proven to act synergistically with nisin against *Bacillus cereus* (25, 26). Since the primary target of all these three treatments is the cytoplasmic membrane, combining them might show additive or even synergistic effects. Therefore carvacrol was added as a third hurdle simultaneously with nisin and the effect of this combination on the viable count of vegetative cells of *B. cereus* was determined. The reduction caused by a nisin treatment of 0.06 µg/ml was approximately 1 log unit (26). For this study, the nisin concentration was lowered to 0.04 µg/ml and complemented with carvacrol till a reduction of 1 log unit was achieved (Fig. 1).

Figure 1



Log reduction of vegetative cells of *Bacillus cereus*. caused by nisin (0.04 µg/ml) combined with different concentrations of carvacrol. Data points represent means of an average of 4 measurements. Error bars are indicated.

A concentration of 0.5 mM of carvacrol, which by itself did not lead to a reduction in viable count (Fig. 2a), was combined with nisin and PEF treatment. The PEF treatment was started 1.5 minutes after simultaneous addition of carvacrol and nisin according to the above-described method. Addition of nisin (0.04 g/ml) only, caused a small reduction in the viable count of approximately 1 log unit (Fig. 3). Carvacrol in a concentration of 0.5 mM did not show any bactericidal effect but a simultaneous addition of these two components led to a reduction of 2.5 log units. This indicates synergy between nisin and carvacrol, proven by the fact that the sum of the reduction of the single treatments is smaller than the reduction found for the combined treatment. A single PEF treatment was not able to reduce the viable count with more than 1 log unit, however, when all three treatments were combined, a reduction of more than 4 log units was observed. This reduction is much more than can be expected on basis of the sum of the reductions of the three single treatments, even when the synergy between nisin and carvacrol is taken into account, indicating synergy between the three components. Although, synergy was detected between nisin and PEF treatment, carvacrol did not act synergistically with PEF treatment.

Figure 2

Influence of carvacrol on the viable count of *Bacillus cereus* in HEPES buffer (50 mM, pH 7.0) (a), and in milk (20 %) (b). Treatment time was 11.5 minutes. Error bars are indicated.

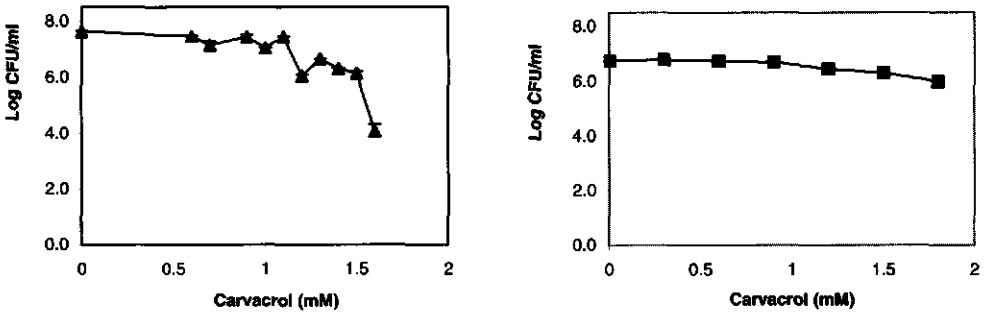
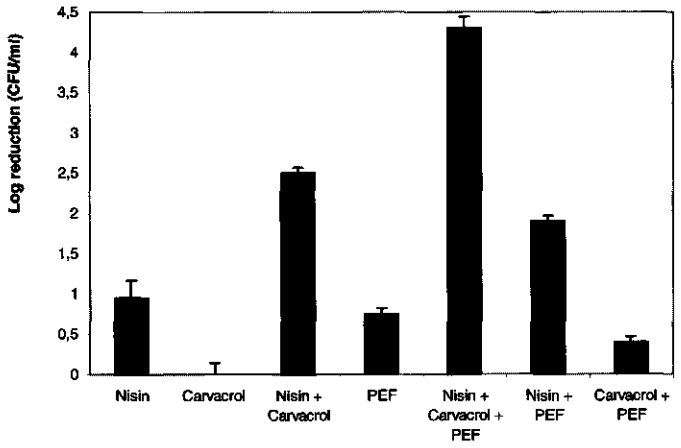


Figure 3

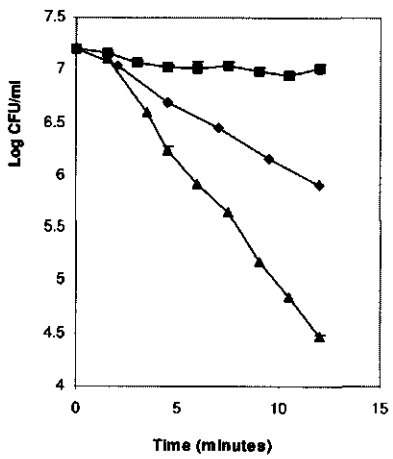


The reducing effect of PEF treatment alone, nisin alone, carvacrol alone and the combination treatments on the viable count of *Bacillus cereus* suspended in HEPES buffer (5 mM, pH 7.0). PEF treatment given was 16.7 kV/cm (20 pulses of 2 µs duration) spread over 10 minutes. Nisin was used at 0.04 µg/ml and carvacrol was used at 0.5 mM. Data points represent means of duplicate measurements. Error bars are indicated.

Determination of the combined effect of nisin, carvacrol and PEF treatment in food products using a continuous PEF system

In a buffer system, the combination of nisin and PEF treatment acted synergistically against *B. cereus*. To evaluate the potential of this preservation method in real food products, diluted milk was used as a food model matrix to test the bactericidal activity of this combination. *B. cereus* cells were added to the diluted milk ($\sigma = 1.4$ mS/cm at 20°C) and recirculated for 5 minutes at high flow rate to get a homogeneous distribution of the cells. The effect of nisin and/or PEF treatment on the viable count of *B. cereus* was determined by exposing the cells to nisin alone (0.13 µg/ml, 11.5 min), PEF treatment alone (20 kV/cm, 30 pulses) or to a combination of both treatments. Control experiments showed that the viable count remained constant for 12 minutes in the diluted milk (data not shown). Simultaneous treatment of the cells with nisin and PEF resulted in an additional reduction of 1.5 log units when compared to inactivation achieved with a single PEF treatment (Fig. 4). A single nisin treatment did not cause a substantial reduction in the viable count of *B. cereus* in 20 % milk, indicating true synergy between nisin and PEF treatment. Nisin lost some of its bactericidal activity in diluted milk compared to HEPES buffer (26).
Carvacrol was able to enhance the synergy found between nisin and PEF treatment in buffer systems and to test whether this combination was suitable for application in food model matrix, nisin was complemented with 0.4 mM of carvacrol and combined with PEF treatment.

Figure 4

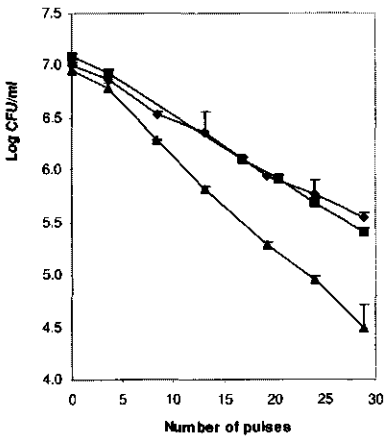


Effect of nisin 0.13 µg/ml (■), PEF treatment (20 kV/cm, 30 pulses of 2 µs duration (◆) and the combination of the two treatments (▲) on the viable count of *Bacillus cereus* in diluted milk (20 %). Data points represent means of duplicate measurements. Error bars are indicated.

The combination of nisin and carvacrol did not exhibit much bactericidal activity in milk (20 %) and in contrast to results found for HEPES buffer, no extra reduction was obtained upon addition of carvacrol when compared with the reduction caused by nisin combined with PEF treatment (Fig. 5). The reductions found for both combinations were 3 log units. Higher concentrations of carvacrol, up to 1.2 mM, were tested, however, no increased reduction was found upon addition of carvacrol to the combination of nisin and PEF treatment. Except for 1.2 mM of carvacrol, which was able to enhance the reduction found with 1 log unit (Fig. 5). This high concentration of carvacrol in itself, was proven not to be bactericidal for *B. cereus* in 20 % milk (Fig. 2b), therefore, the increased reduction could be ascribed to the synergy between the three components. In contrast to results found for HEPES buffer, synergy was found between PEF treatment and carvacrol in milk (Table 1). Low concentrations of

carvacrol (0.4 mM), not able to enlarge synergy between nisin and PEF treatment, did show synergy when combined with PEF treatment to a similar extent as synergy found for nisin and PEF treatment, as indicated by the identical inactivation rates.

Figure 5



Effect of nisin 0.08 µg/ml + PEF treatment (20 kV/cm, 30 pulses of 2 µs duration (◆), nisin 0.08 µg/ml + PEF treatment (20 kV/cm, 30 pulses of 2 µs duration) combined with 0.8 mM carvacrol (■) or 1.2 mM carvacrol (▲) on the viable count of *Bacillus cereus* in diluted milk (20 %). Data points represent means of duplicate measurements. Error bars ae indicated.

Components found in milk could be responsible for this improved bactericidal effect. Possibly, carvacrol interacts with proteins in milk leading to improved bactericidal activity when combined with PEF treatment. Alternatively, PEF treatment might change the structure of proteins leading to interactions with carvacrol resulting in improved activity. By varying the protein content in the diluted milk this theory was validated. Lowering of the protein content by further dilution of the milk did not result in a significant decreased synergy between carvacrol and PEF treatment against *B. cereus* (Fig. 6). Even a 4 times-dilution of the milk till 5 % did not lead to a reduced bactericidal effect of carvacrol combined with PEF treatment, suggesting that the protein content of 5 % milk is still sufficient to enhance bactericidal activity between carvacrol and PEF treatment.

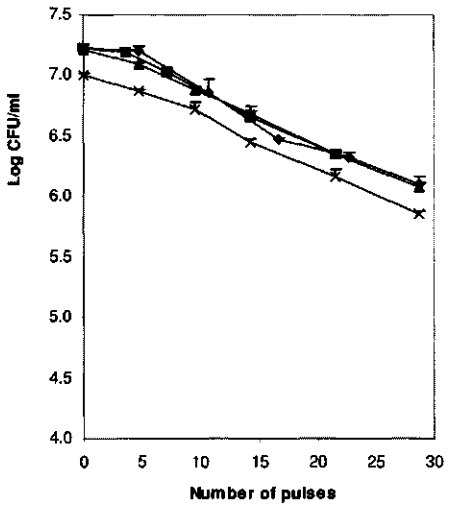
Table 1

Inactivation rates (^{10}log per pulse) for *Bacillus cereus* in HEPES buffer (5 mM, pH 7.0) and in milk (20 %). In HEPES buffer the nisin concentration was 0.06 µg/ml, the PEF treatment was 16.7 kV/cm (50 pulses of 2 µs duration). In milk (20 %) the PEF treatment was 20 kV/cm (30 pulses of 2 µs duration). The nisin concentration was 0.13 µg/ml, when combined with carvacrol (0.4 mM) and PEF treatment, the concentration was lowered to 0.08 µg/ml)

	PEF	Nisin + PEF	Nisin + PEF + carvacrol	PEF + carvacrol
HEPES buffer	-0.0474 ± 0	-0.1451 ± 0.0060	n.d.	n.d.
Milk (20 %)	-0.0461 ± 0.0012	-0.1025 ± 0.0025	-0.1066 ± 0.0035	-0.0982 ± 0.0050

n.d. is not determined

Figure 6



Influence of protein content on *Bacillus cereus* suspended in milk upon treatment with carvacrol (0.8 mM) and PEF (20 kV/cm, 30 pulses of 2 µs duration). 5 % milk (◆), 10 % milk (■), 15 % milk (▲), and 20 % milk (X). Error bars are indicated.

DISCUSSION

Previously, synergy was detected between nisin and PEF treatment (26) and between nisin and carvacrol (25). In this work, carvacrol was used as a third hurdle to further increase synergy found between nisin and PEF treatment. All three treatments have similar primary targets and therefore an additive or even synergistic effect could be expected when these treatments were combined against *B. cereus*. The PEF treatment was spread over a 10 minutes time interval to maximize interaction with nisin and/or carvacrol. Furthermore, this method minimizes the temperature rise during the treatment so that heat inactivation is excluded and the inhibitory effect can be solely ascribed to the PEF treatment (30). When applied simultaneously with nisin, carvacrol is able to enhance the synergy found between nisin and PEF treatment in HEPES buffer. This might be explained by the facilitated incorporation of nisin into the membrane by carvacrol and/or PEF treatment so that more pores or pores with a longer lifetime are formed. Another possible explanation for the observed synergy could be the increased size of the pores. Whether both carvacrol and PEF treatment stimulate the bactericidal effect of nisin via similar ways remains unknown.

The fact that synergy was found between the three treatments renders the combination very interesting for mild food preservation. However, extrapolation of the results from labscale experiments in buffer systems to food model matrices is usually difficult and the newly discovered compounds or techniques don't always work in food model matrices. In order to be able to use food preservatives, the influence of food ingredients on the bactericidal activity must be established. The influence of proteins was tested using milk as a food model matrix. Skimmed milk was chosen to exclude the influence of fat particles. Both nisin and PEF treatment inactivated vegetative cells of *B. cereus* in diluted milk (20 %) according to first order inactivation kinetics. The proteins in milk did not interfere with the inhibiting effect of PEF treatment against *B. cereus*. Moreover, results obtained with the batch PEF system in buffer show a similar trend as results found using the continuous PEF system as indicated by the similar inactivation rates in Table 1. The protein content did influence the nisin activity negatively. More nisin was needed to achieve 1 log unit reduction in diluted milk compared to HEPES buffer either because the bioavailability of nisin is decreased due to binding to proteins or the microorganisms are protected by

the proteins (26). This might also explain the reduced extent of the synergy observed between nisin and PEF treatment. The slope of the inactivation curve of nisin combined with PEF treatment is significantly lower in milk although a higher concentration of nisin was used (Table 1).

In sharp contrast to the improved bactericidal activity found in HEPES buffer, carvacrol was not able to enhance the synergy between nisin and PEF treatment in diluted milk (Table 1). Different concentrations of carvacrol were tested but all of them exhibited the same reductions when combined with nisin and PEF treatment. Carvacrol concentrations in the range of 0.3 – 1.2 mM were used and only the highest concentration (1.2 mM) was able to enlarge the reduction with 1 log unit. Reactions between carvacrol and the proteins might explain the reduced antimicrobial effect. Tassou and Nychas (32) found a reduced inhibitory effect of phenolic extracts in milk and attributed this to the binding of the phenolics by the milk proteins (16, 23). Also the bacteria might be protected by the proteins against carvacrol. Although carvacrol was not able to improve the bactericidal activity of nisin and PEF treatment, it was however able to increase the inhibiting activity of the PEF treatment (Table 1). This observation rules out the possibility of the protecting effect of the proteins. On the contrary, since carvacrol did not improve the bactericidal effect of PEF treatment in HEPES buffer but only in milk, these proteins probably play an important role in the increased activity observed. Possibly, carvacrol interacts with proteins in milk leading to improved bactericidal activity when combined with PEF treatment. Alternatively, PEF treatment might change the structure of proteins leading to interactions with carvacrol resulting in improved activity. The influence of PEF treatment on the behavior of proteins, but also on polysaccharides macromolecules, or lipids subjected to an intense electric field is not exactly known. Proteins and some polysaccharides can carry electric charges and might behave as dipoles when subjected to PEF treatment, which cause the macromolecules to reorient or deform (such as protein unfolding and denaturation), and possibly some breakdown of covalent bonds may occur (3, 34).

Alkaline phosphatase (ALP) is an example of a globular protein with activity depending on a specific internal structure. PEF treatment alters the intramolecular linkage of active center and the entire globular configuration of ALP, inducing conformational changes and subsequently loss of activity (2). Not all enzymes lose activity after PEF treatment. Ho *et al.* (12) found inactivation of lipase, glucose oxidase, alpha amylase peroxidase and phenol oxidase upon PEF treatment, but increased activity of lysozyme and pepsin in a certain range of electric fields. Although contradicting results have been published on the effect of a PEF treatment on proteins, probably no significant changes have occurred in the milk upon PEF treatment in this work. This is supported by Barbosa *et al.* (2), who found no chemical or physical changes in enzyme activity, fat or protein integrity, starter growth rennet, clotting yield, cheese production, calcium distribution, casein structure or flavor degradation in milk treated with 40 pulses over a 25 minute time interval. The PEF treatment applied in this study was less severe (20 kV/cm, 30 pulses for a 12 minutes time interval), so that no changes are to be expected in the milk as a consequence of PEF treatment and therefore an altered protein conformation could not have attributed to the profound inactivation found upon treatment with carvacrol and PEF. This is supported by the fact that lowering of the protein content did not lead to a significant decrease in activity of carvacrol combined with PEF treatment. Even a low concentration of 5 % milk was still sufficient for carvacrol to increase the inhibitory activity of the PEF treatment (Fig. 6). Therefore it was suggested that the essential oil-protein complex is sensitive towards modification brought about by PEF treatment and could have caused the improved bactericidal effect. Further research is needed to elucidate the exact mechanism.

Other factors which might have influence on the inhibitory spectrum of the treatments are the temperature during the treatment, the characteristics of the treatment medium like the pH, the conductivity, ionic strength and the presence of cations (41).

The pH of the treatment medium could alter the inhibitory spectrum of nisin and carvacrol since the activity of both compounds is depended on the pH. At neutral pH carvacrol shows a dip in the inactivation rate, which increases again in acidic and alkaline environment. In general carvacrol seemed to work a bit better at higher pH values (32, 35). The efficacy of nisin and other bacteriocins is more

profound at acidic pH values. Nisin is an acidic molecule which is more stable and easier to dissolve at lower pH values (8, 29). In order to be effective it is believed that nisin must first adsorb to the cytoplasmic membrane. This adsorption has previously been shown to be reduced at acid pH values and that the amount of nisin adsorbed correlated with the sensitivity of *Listeria monocytogenes* to nisin. This was suggested to explain the reduced activity of nisin at lower pH values (22, 33, 39). However, the PEF treatment did not alter the pH of the milk or the buffer and no changes in activity of nisin or carvacrol were detected upon PEF treatment.

Factors like the temperature, the conductivity and ionic strength could also not be responsible for the improved inactivation. The temperature increase of the suspension by PEF treatment did not exceed the 5°C with a maximum end temperature of 25°C and this is not sufficient enough to observe additional inactivation in combination with a PEF treatment. The conductivity of the two different treatment media did differ (conductivity of HEPES 5 mM = 85 μS/cm and milk 20 % = 1.4 mS/cm) and could have resulted in lower inactivation in milk. However, previously reported results found in HEPES buffer (26) could directly be extrapolated to milk (20 %). Therefore the protecting effect of ions like Ca^{2+} (13) can be excluded.

PEF treatment has been shown to be a very good alternative preservation method to currently used techniques. Several researchers have conducted extensive research to the quality of PEF treated products and found significant shelf-life extensions with minimum changes in the physical and chemical properties of foods or the sensory properties (2, 6, 19, 27, 37). Safety becomes a concern when cells that are injured but not inactivated by electric field treatment have the ability to recover and reproduce during the storage of food. Kalchayanand *et al.* (17) have proven sub-lethal injury in Gram-positive and Gram-negative cells after Ultra High Pressure (UHP) and Electroporation (EP) treatment. This can be circumvented by applying the multiple hurdles concept by combining different preservative factors. Kalchayanand *et al.* (17) proved the success of a combination of UHP or EP with antimicrobials as pediocin or nisin against *L. monocytogenes*, *Escherichia coli O157:H7* and *Salmonella typhimurium*. A bottleneck for application of these combinations is the reduced efficacy in foods. However, recent publications show application examples in foods. Ultee *et al.* (36) found that carvacrol is able to reduce the growth of *B. cereus* in rice. At concentrations of 0.45 mg/ml the growth is completely inhibited. Combining carvacrol with other components as cymene or soya sauce further increased the inhibitory effect against *B. cereus*. Recent findings by Karatzas *et al.* (18) shows that *L. monocytogenes* was inactivated by High Hydrostatic Pressure (HHP) in undiluted semi skimmed milk to the same extent as in ACES buffer. Combining HHP with carvacrol increased the reduction of viable count of *L. monocytogenes* both in buffer and in milk. However, the extra reduction found in milk was somewhat less, which can be explained by the presence of fat to which the hydrophobic essential oil components bind.

The presented data demonstrate that the evaluated treatments have potential to be used as a mild food preservative in the near future. Evidently, more research needs to be done to verify the influence of other food ingredients like fat etc. By combining different preservation techniques many of the currently known restrictions might be overcome leading to practical mild preservation processes.

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CHAPTER 6

Sensitivity of germinating spores and carvacrol adapted vegetative cells and spores of *Bacillus cereus* towards nisin and Pulsed Electric Field

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ABSTRACT

Treatment of *Bacillus cereus* spores with nisin and/or Pulsed Electric Field (PEF) treatment did not lead to direct inactivation of the spores or increased heat sensitivity as a result of sub-lethal damage. On the contrary, germinating spores were found to be sensitive towards nisin and PEF treatment. Nisin is more efficient than PEF treatment to inactivate germinating spores. PEF resistance was lost after 50 minutes of germination and not all the germinated spores could be inactivated. Nisin, however, was able to inactivate the germinating spores to the same extent as heat treatment. The resistance to nisin was lost immediately upon initiation of the germination process. A decrease in the membrane fluidity of vegetative cells by incubation in the presence of carvacrol resulted in a dramatic increase in the sensitivity towards nisin. On the other hand, inactivation by PEF treatment or by a combination of nisin and PEF treatment did not change upon adaptation to carvacrol. Spores grown in the presence of carvacrol were not susceptible to nisin and/or PEF treatment in any way.

INTRODUCTION

Mild preservation techniques are becoming increasingly popular in modern food industries, since consumers ask for more natural and mildly preserved food products. Novel preservation techniques, including Pulsed Electric Field (PEF) treatment and High Hydrostatic Pressure, are being developed aiming to combine stability and microbial safety with improved organoleptic quality. PEF treatment is a potential alternative to heat pasteurization. It is a non-thermal inactivation technique yielding minimal losses of flavor, color and food quality (3, 42). PEF treatment inactivates microorganisms by irreversible structural changes in the membrane, resulting in pore formation and subsequent loss of cellular constituents (7). Upon exposure of cells to electric fields, the ions in- and outside the cell migrate according to the electric field across the electrodes. Consequently, free charges accumulate at both sides of the membrane surface resulting in an increased membrane potential and a reduction in the

membrane thickness as a result of the increased attraction between the opposing charges. These charges force the polar lipid molecules in the membrane to reorient leading to the formation of hydrophilic pores and impairment of the membrane barrier against ions (3, 42). The extent of the permeability increase depends on the strength and duration of the electric pulse (19).

Recently, synergy between nisin and PEF treatment was demonstrated against vegetative cells of *B. cereus* (30). Nisin, the only bacteriocin that is approved by the WHO to be used as a food preservative, increases the permeability of the membrane by pore formation, resulting in a rapid efflux of small molecules. The efflux of cellular constituents results in a complete collapse of the proton motive force, finally leading to cell death (10, 13, 41). A common primary target, the cytoplasmic membrane, was thought to offer an explanation for the observed synergy (30).

The synergy found between nisin and PEF treatment renders this combination technology interesting for mild food preservation. However, thorough knowledge of the effect of these novel techniques on inactivation of spores is needed before these processes can be used in food industries as alternatives to heat-pasteurization or even -sterilization treatments. Spores are considerably more resistant than vegetative cells and can cause spoilage or even health risks after germination and subsequent outgrowth. Nisin is not able to directly inactivate spores, however, its sporostatic activity lies in preventing the swelling of the germinated spores. Nisin interacts with sulfhydryl groups in the membrane, interfering with spore growth by disrupting some vital functions (22, 27, 28). Bacterial endospores are resistant to PEF treatment (3). Hamilton and Sale (15) could not detect alterations in the cortex and coat structure of spores after a PEF treatment and no inactivation of the spores was found. Spores became sensitive to PEF treatment only late in the germination process when the vegetative cell began to emerge. Likewise, Knorr *et al.* (20) did not detect inactivation of spores by PEF treatment and suggested that inactivation might not be achieved by PEF treatment unless combination processes inducing germination are applied. Examples of such hurdles are heat shock, lysozyme, EDTA, pH and HHP (2, 3). In contrast, Marquez *et al.* (23) claimed inactivation of bacterial endospores by PEF treatment only. The inactivation required a minimum field strength of 35 kV/cm and was enhanced by increasing temperature, the number of pulses and the pulse duration.

Factors playing a major role in overall spore resistance include the low permeability of spores to toxic chemicals and the decreased spore-core water content (33, 34). This dehydration together with spore mineralization plays a major role in the acquired heat and γ-radiation resistance and partly in resistance against hydrogen peroxide (31, 35). The spore cortex is largely responsible for the maintenance of the dehydrated state of the spore core (32, 34). The resistance to oxidizing agents and chemicals is largely achieved by the proteinaceous spore coat and the cortex, restricting the access of potentially toxic molecules to the spore core (35). These characteristic resistances are lost upon germination and could allow inactivation of spores by nisin and/or PEF treatment. Germination of spores is triggered by a number of factors, which may be divided into nutrient and non-nutrient (chemical, enzymes etc.) germinants (12, 34). L-alanine is the most common nutrient germinant triggering a sequence of germination events including uptake of water, loss of Ca^{2+} and DPA release, loss of refractility and onset of core metabolism (12, 40).

The aim of this work was to inactivate germinated spores by combinations of nisin with other preservative factors like PEF treatment (21). The sensitivity of nutrient-induced germinated spores towards nisin and/or PEF treatment was examined in different phases of germination. Furthermore, a possible increase in sensitivity of *B. cereus* spores by altering the membrane fluidity using plant derived antimicrobials like carvacrol was investigated.

MATERIALS AND METHOD

Growth of bacteria

Bacillus cereus IFR-NL94-25, obtained from the Institute of Food Research (Norwich, UK) was grown at 20°C in Brain Heart Infusion (BHI) broth (Oxoid), containing 0.5 % (wt/vol) glucose. Cell cultures were maintained at –20°C in 30 % glycerol as a cryoprotectant. Spores of *B. cereus* were produced on SPO 8 medium (per liter: 8 g Nutrient broth, 0.51 g $MgSO_4.7H_2O$, 0.97 g KCl, 0.2 g $CaCl_2.2H_2O$, $3*10^{-3}$ g $MnCl_2.4H_2O$, $0.55*10^{-3}$ g $FeSO_4.7H_2O$, 1.5 % agar) (11) by spreading 1 ml of a fully grown culture on the plate and incubate at 20°C for 4 days. The spores were harvested by scraping the agar surface, washed twice in sterile demineralized water and stored at –20°C until further use. A heat treatment of 10 minutes at 70°C showing no decrease in total count indicated that the spore suspension contained no vegetative cells.

Influence of nisin and/or PEF treatment on spores of *B. cereus*

Spores of *B. cereus* were resuspended in 50 mM potassium-N-2-hydroxy-ethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer (pH 7.0) till a concentration of 10^7 spores/ml and subjected to a single nisin treatment (3.0 µg/ml), a PEF treatment (53 kV/cm, 43 pulses of 2 µs duration, square wave pulses) or a combination of both treatments for 12 minutes. PEF treatment was applied using a continuous flow system. The treatment device is of co-linear design as reported by Yin *et al.* and discussed by Barbosa *et al.* (2), with a gap distance of 2 mm and a inner diameter of 1 mm. Spores of B. cereus were added to the buffer and recirculated for 5 minutes at high flow rate before treatment in order to get a homogeneous distribution of the spores throughout the system. During the experiments a total volume of 400 ml of inoculated medium was circulated through the system for 12 minutes at 980 Hz pulse frequency and a flow rate of 100 ml/min, to adjust the average number of received pulses per minute to 4. The combined treatments were carried out by applying the PEF treatment 1.5 minutes after addition of nisin to the spore suspension. The PEF treatment was spread over a 10 minutes time interval (recirculation experiment), to maximize interaction with nisin. The inactivation by nisin was quenched by a 100-fold dilution in peptone physiological salt solution (1g/l peptone and 8.5 g/l NaCl) and the number of survivors was determined on BHI agar before and after an additional heat treatment of 10 minutes at 70°C to distinguish between germinated spores and spores. In all cases the temperature during treatment was kept below 30°C in order to discriminate for thermal effects (Inlet temperature = 20°C).

Nutrient induced spore germination and heat activation

The germination of the *B. cereus* spores in liquid media was triggered using L-alanine or BHI broth as germinants. Spores of *B. cereus* were added to HEPES buffer (50 mM, pH 7.0) supplemented with 10 mM L-alanine (Merck) or to BHI broth, and incubated at 30°C. Germination on solid media was determined by spreading spores of *B. cereus* on BHI agar and incubating the plates at 30°C. At appropriate time intervals, samples were taken and analyzed for germinated spores by plating on BHI agar before and after an additional heat treatment of 10 minutes at 70°C.

In case of germination on solid media, the agar was diluted in 100 ml of peptone physiological salt solution, stomachered for 1 minute, and serially diluted before analyzing the number of spores by plate counting. The percentage of germination was calculated as follows: [{Spores/ml (count before heating – count after heating)} / {Viable cells/ml (count before heating)}]* 100 % (1).

To determine the influence of a heat activation step on the germination rate, the spores were subjected to a sub-lethal heat treatment of 10 minutes at 70°C prior to inoculation in BHI broth at 30°C. At appropriate time intervals samples were taken and analyzed for germination as described above.

Sensitivity of germinating spores of *B. cereus* to nisin and/or PEF treatment

Germinating spores were subjected to nisin, a single pass PEF treatment or a combination of both treatments in different stages of their germination to test which particular phase in the germination process renders them sensitive towards the used treatments. Therefore spores of *B. cereus*, suspended in BHI broth (10-fold diluted, σ = 4 mS/cm) supplemented with 0.5 % glucose till OD_{660} = 0.1 (light path 1 cm), were treated with either nisin, PEF treatment or a combination of both at regular time intervals during the germination period of 5 hours. In case of nisin treatment only, the spores were subjected to nisin for 12 minutes before the reaction was quenched by a 100-fold dilution in peptone physiological salt solution. The PEF treatment (27 kV/cm, 30 pulses of 2 µs duration, flow 10 ml/min) was given at once instead of spreading the treatment over a 10 minutes time interval (single pass experiment). The temperature rise during the single pass PEF treatment did not exceed 20°C (Inlet temperature = 20°C). The number of spores was determined before and after an additional heat treatment of 10 minutes at 70°C.

Influence of preincubation on carvacrol or nisin on sensitivity towards nisin, carvacrol and/or PEF treatment

To test the sensitivity of *B. cereus* towards nisin and/or carvacrol, cells were grown in BHI broth containing 0.5 % glucose (wt/vol) in the presence and absence of carvacrol (0.3 mM) or nisin (0.3 µg/ml) for approximately 16 hours at 20°C. The cells were harvested in the exponential growth phase, washed and resuspended in 50 mM HEPES buffer (pH 7.0) till OD_{660} = 0.1 (light path 1 cm). The adapted cells were exposed to nisin, carvacrol or a combination of both compounds at 20°C. Samples were taken at regular time intervals during 30 minutes exposure period and immediately diluted (10^2 - to 10^5- fold) in peptone physiological salt solution to quench the inactivation reaction. Numbers of survivors were determined on BHI agar.

To determine the sensitivity towards nisin and/or PEF treatment, vegetative cells and spores were cultivated in the presence and absence of carvacrol in order to change the cell membrane fluidity without changing the growth temperature (37). An overnight culture of *B. cereus* was 100 times diluted in fresh BHI containing 0.5 % of glucose (wt/vol) and 0.4 mM of carvacrol and incubated at 30°C for approximately 4 hours. Cells were harvested at OD_{660} = 0.1 (light path 1 cm) and washed and resuspended in HEPES buffer (50 mM, pH 7.0) and kept on ice until further use. Spores of *B. cereus* were produced on Spo 8 agar in the presence or absence of carvacrol (0.4 mM) at 20°C. Carvacrol was added to Spo 8 liquid agar (50°C) just before pouring the plates. Spo 8 medium containing carvacrol was poured in glass Petri-dishes, which were sealed with Viscose self-shrinking cellulose bands (Viscose closure Ltd. Fleming way, Crawley RH10 2NX, W-Sussex, UK) to minimize volatile carvacrol to evaporate. Control cells sporulated within 5 days, while in the presence of carvacrol the sporulation took 12 days to complete. The carvacrol concentration remained stable over the 12 days incubation period (data not shown). The spores were harvested by scraping the agar surface, washed twice in sterile demineralized water and stored at –20°C until further use. A heat treatment of 10 minutes at 70°C showing no decrease in total count of the control spores indicated that the spore suspension contained no vegetative cells. In the presence of carvacrol, the sporulation was not 100 % and the spore suspension was heat-treated (10 minutes 70°C) before starting the experiments to inactivate any vegetative cells present. Control cells and spores and carvacrol-adapted cells and spores were treated with nisin, PEF or a combination of both treatments like described above. The PEF treatment was spread over a 10 minutes time interval (recirculation experiment) and was started 1.5 minute after the addition of nisin to the cell- or spore suspension. In case of vegetative cells, nisin was used at a concentration of 0.08 µg/ml and PEF treatment was given at 27 kV/cm (30 pulses of 2 µs duration). In case of spores, nisin was used at a concentration of 3 µg/ml and PEF treatment was given at 53 kV/cm (43 pulses of 2 µs duration). The reaction was quenched by a 100-fold dilution in peptone physiological salt solution and the number of survivors was determined on BHI agar, before and after an additional

heat treatment of 10 minutes at 70°C in case of spores. In all cases the temperature during treatment was kept below 30°C in order to discriminate for thermal effects (Inlet temperature = 20°C).

Chemicals

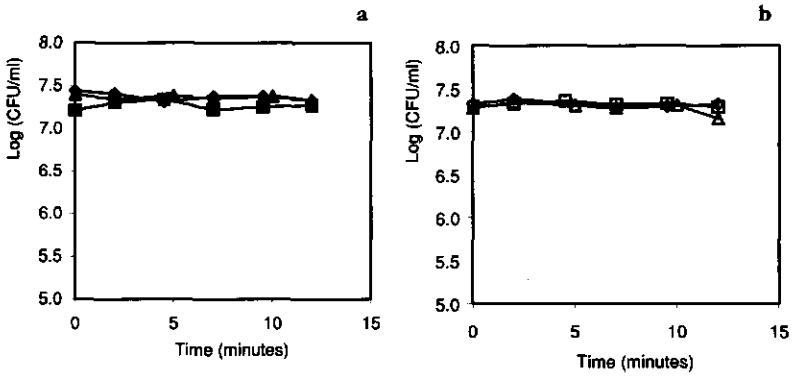
Carvacrol stock solution was held in 95 % ethanol at 4°C and stock solution of nisin (Nisaplin, containing 2 % nisin, Aplin and Barrett Ltd., Wilts, UK) was made in 50 % ethanol, filter sterilized (Costar, 0.22 µm) and kept at -20°C. The nisin concentration was not influenced by filter sterilization (data not shown).

RESULTS

Susceptibility of spores of *B. cereus* towards nisin and/or PEF treatment

Spores of *B. cereus*, developed at 20°C, were subjected to nisin, PEF treatment or a combination of both treatments and the effect of these treatments on the germination and viable count of the spores was determined. Although vegetative cells of *B. cereus* are very sensitive towards these treatments (30), spores were able to resist both the nisin treatment as well as the high intensity PEF treatment (Fig. 1a). An additional heat treatment did not cause any reduction in the viable count of the spores leading to the conclusion that nisin and PEF treatment did not initiate germination of the spores (Fig. 1b).

Figure 1



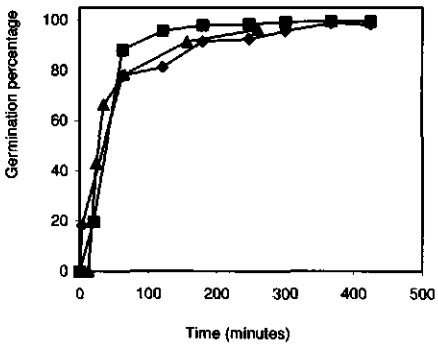
Effect of nisin treatment (3.0 µg/ml (■), PEF treatment (53 kV/cm, 43 pulses of 2 µs duration, recirculation) (◆) and the combination of the two treatments (▲) on spores of *Bacillus cereus* in HEPES buffer (50 mM, pH 7.0). Samples were plated on BHI agar before (a, closed symbols) and after (b, open symbols) receiving a heat treatment (70 °C, 10 minutes) to distinguish between spores and germinated spores. Data points represent means of duplicate measurements.

Nutrient induced spore germination and heat activation

The characteristics of the germination process was monitored in BHI broth and HEPES buffer containing L-alanine (10 mM) as a germinant. In both media, germination was initiated, however, L-alanine was not able to induce germination to the same extent as BHI broth (data not shown). Only 35 % of the spores were able to germinate in the presence of L-alanine (corresponding to 0.5 log units), while in BHI broth more than 95 % of the spores germinated (corresponding to 1 - 2 log units). Either way, complete germination was never observed. Maximum germination was reached after 3 hours of incubation in BHI broth or HEPES containing L-alanine. In order to improve the extent of

germination, BHI agar was used as an alternative germination medium. However, germination on BHI plates was not accelerated, indicated by similar germination percentages in figure 2.
After receiving a heat activation treatment, the spores germinated slightly faster than untreated spores, however the differences are very small (Fig. 2). Heat activation was therefore not used in further experiments.

Figure 2

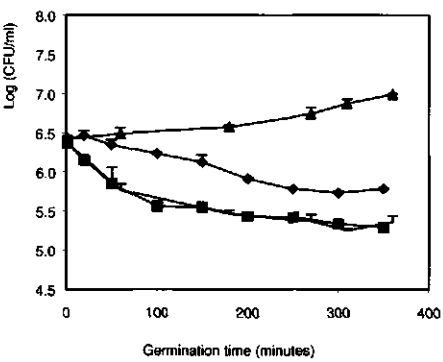


Germination of *Bacillus cereus* spores in BHI broth, with (■) and without (◆) a prior heat treatment (10 minutes 70°C), and on BHI plates (▲) at 30°C.

Sensitivity of germinating spores of *B. cereus* to nisin and/or PEF treatment

Upon germination, spores lose their resistance to several agents or treatments, like UV and oxidizing agents and become metabolically active. By exposing spores in different phases of their germination process to nisin, PEF treatment or a combination of both treatments, the onset of the loss of PEF and nisin resistance could be determined. After about 100 minutes of germination, the spore numbers declined slightly, possibly indicating the onset of loss of PEF resistance (data not shown). Experiments were repeated with an increased number of pulses (30 instead of 15 pulses) to verify the loss of PEF resistance. In the 6 hours germination period, growth was observed after 2.5 - 3 hours and loss of heat resistance indicated the immediate onset of germination (Fig. 3).

Figure 3

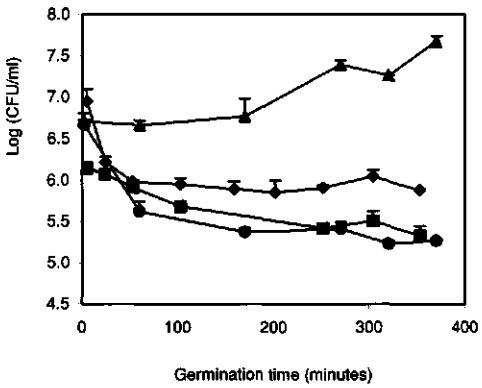


Inactivation of germinating spores (BHI broth, 10 x diluted, 20°C) by PEF treatment (27 kV/cm, 60 pulses of 2 µs duration, single pass) ▲: control, ●: control with additional heat treatment (10 minutes 70°C), ◆: PEF treatment, ■: PEF treatment with additional heat treatment (10 minutes 70°C). Standard deviation is indicated as error bars.

The germinating spores were subjected to the PEF treatment and loss of PEF resistance occurred 50 minutes after the onset of germination. A clear reduction caused by PEF treatment of 0.8 log unit was found. However, not all the germinated spores were inactivated since the reduction by PEF treatment

did not reach the same level as the reduction caused by heat treatment. Similar experiments were conducted to test the sensitivity of germinating spores to nisin. Figure 4 shows that germinating spores are very sensitive towards a nisin treatment. Immediately from the start of germination, spores became sensitive towards nisin almost to the same extent as to a heat treatment.

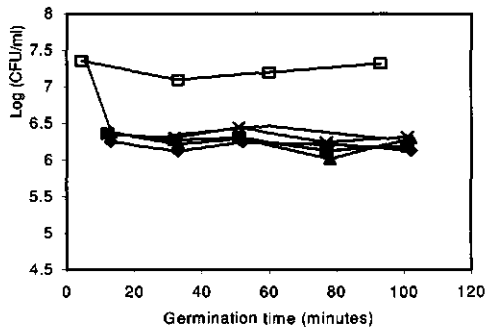
Figure 4



Inactivation of germinated spores (BHI broth, 10 x diluted, 20°C) by nisin (1.25 µg/ml). ▲: control (untreated), ●: control with additional heat treatment (10 minutes 70°C), ◆: nisin treatment, ■: nisin treatment with additional heat treatment (10 minutes 70°C). Standard deviation is indicated as error bars.

An additional heat treatment did not result in more reduction than the heated control samples. This was confirmed by determining these reductions during the first 30 minutes of germination (data not shown). Lower amounts of nisin, 0.6 µg/ml (Fig. 5) and 0.3 µg/ml (data not shown), were tested and similar results were found. Reductions obtained by nisin, nisin followed by an additional heat treatment and control with additional heat treatment were identical over the whole incubation period. Combining nisin and PEF treatment resulted in synergistic action against vegetative cells (30). This synergy was not seen or not clear when used against germinating spores. When PEF treatment was combined with nisin, the reduction obtained was similar to the reduction obtained with nisin only. The nisin concentration used already caused the maximum obtainable reduction and enhanced inactivation by PEF treatment would be hard to distinguish.

Figure 5



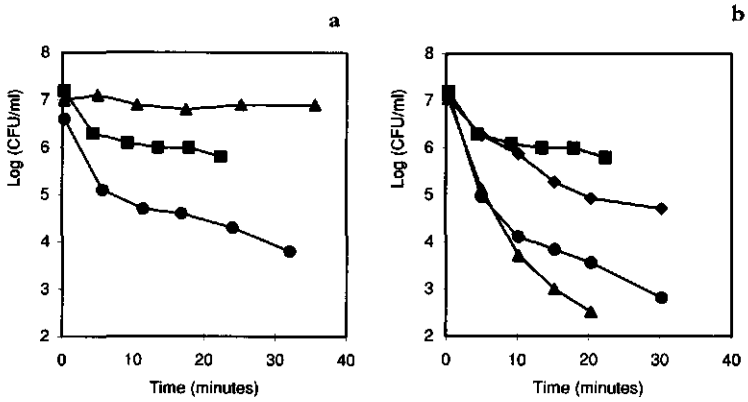
Inactivation of germinated spores (BHI broth, 10 x diluted, 20°C) by a combination of nisin (0.6 µg/ml) and PEF treatment (27 kV/cm, 60 pulses of 2 µs duration, single pass). □: control (untreated), ●: control with additional heat treatment (10 minutes 70°C), ▲: nisin treatment, ◆: nisin treatment with additional heat treatment (10 minutes 70°C), x : nisin combined with PEF treatment, ■: PEF treatment with additional heat treatment (10 minutes 70°C).

Lowering the nisin concentration to 0.3 μg/ml still resulted in the maximum reduction achievable. The combination of nisin and PEF treatment was also not able to damage the spores in such a way that increased susceptibility to a heat treatment could be observed.

Influence of preincubation on carvacrol or nisin on sensitivity towards nisin, carvacrol and/or PEF treatment

Carvacrol is a lipophilic, plant derived, antimicrobial compound that accumulates into the lipid bilayer, thereby disturbing its functions. Ultee *et al.* (37) have demonstrated that cells of *B. cereus* which were adapted to sub-lethal concentrations of carvacrol are less sensitive to this compound upon subsequent exposure. Since the primary target of nisin is the cytoplasmic membrane, changes in the membrane by other compounds could change the sensitivity of those cells to nisin. Cells of *B. cereus* were grown in the presence of 0.3 mM of carvacrol at 20°C, harvested in the exponential phase and subsequently exposed to nisin (0.3 μg/ml) for 30 minutes. The concentration of carvacrol used, was shown to be non-lethal, however, growth was inhibited to a certain extent (29, 37). The viability of control cells exposed to nisin decreased with 1 log unit within 30 minutes. When nisin was applied simultaneously with carvacrol (0.3 mM) a synergistic reduction of 3 log units was observed (Fig. 6a). Interestingly, when carvacrol-adapted cells were exposed to the same concentration of nisin, a reduction below the detection limit within 15 minutes was observed (Fig. 6b).

Figure 6

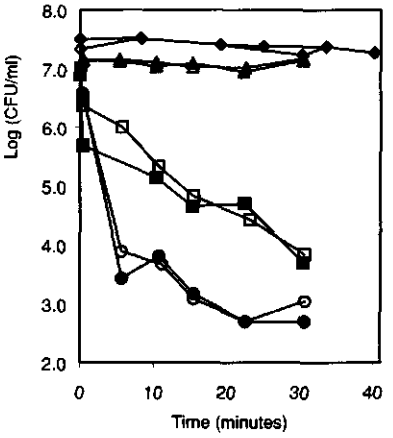


Influence of pre-cultivation on carvacrol on the sensitivity of *Bacillus cereus* towards nisin. A) Effect of nisin (■, 0.3 μg/ml), carvacrol (▲, 0.3 mM) and the combination of both treatments (●) on the viability of control cells of *B. cereus*. B) Effect of nisin on the viability of *B. cereus* grown in the presence of carvacrol. ■: nisin 0.3 μg/ml (control cells); ◆: nisin 0.16 μg/ml (pre-cultivation on 0.2 mM carvacrol); ●: nisin 0.16 μg/ml (pre-cultivation on 0.3 mM carvacrol); ▲: nisin 0.3 μg/ml (pre-cultivation on 0.3 mM carvacrol).

This reduction is even larger than the reduction obtained with control cells combining nisin and carvacrol, indicating the extreme sensitivity increase upon adaptation to carvacrol. Even with lower amounts of nisin (0.16 μg/ml) the reduction found was larger than for nisin combined with carvacrol against control cells. Lowering the adaptation concentration of carvacrol to 0.2 mM still allowed nisin (0.16 μg/ml) to cause larger reductions than nisin (0.3 μg/ml) on control cells. These results clearly indicate that adaptation towards carvacrol increases the sensitivity of *B. cereus* towards nisin dramatically. Nisin does not accumulate in the cytoplasmic membrane, but cells grown in the presence of nisin might change their lipid composition in such a way that the sensitivity towards carvacrol or nisin is altered. In order to test this, cells of *B. cereus* were grown in the presence of 0.3 μg/ml of nisin at 20°C and harvested in the exponential growth phase. This concentration of nisin is bactericidal to a certain extent but survivors do grow and possibly adjust their membrane composition to the presence of nisin.

However, no changes in the sensitivity towards nisin, carvacrol or the combination of both compounds could be detected in adapted cells (Fig. 7).

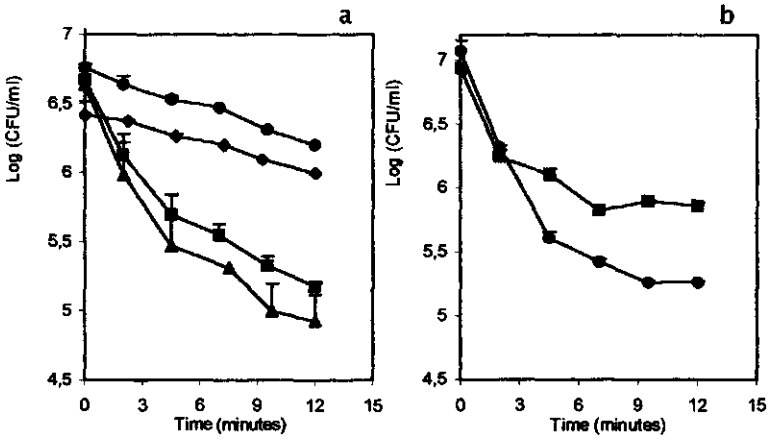
Figure 7



Influence of pre-cultivation on nisin on the sensitivity of *Bacillus cereus*. ◆: control; ▲: 0.3 mM carvacrol; ■: 0.3 µg/ml nisin; ●: 0.3 µg/ml nisin combined with 0.3 mM carvacrol. Open symbols represent control cells, closed symbols represent cells adapted to 0.3 µg/ml nisin.

Changes in the composition of the membrane might alter the sensitivity of *B. cereus* to either nisin or PEF treatment. Carvacrol proved to be a helpful tool to change the membrane fluidity of the cells. Cells were grown in the presence of carvacrol (0.4 mM) and subsequently exposed to nisin, PEF treatment or a combination of both. The number of survivors was monitored during the course of the treatment (Fig. 8).

Figure 8



Influence of incubation in the presence of carvacrol on activity of nisin and/or PEF treatment on cells of *Bacillus cereus* (30°C). A) Effect of PEF treatment (20 kV/cm, 30 pulses of 2 µs duration, recirculation) on control cells (◆) and adapted cells (●) and the effect of the combination of PEF treatment and nisin (0.04 µg/ml) on control cells (■) and adapted cells (▲). B) Effect of nisin (0.08 µg/ml) on control (■) and adapted (●) cells of *B. cereus*. Standard deviation is indicated as error bars.

The sensitivity of adapted cells towards PEF treatment is similar to that of non-adapted cells. When nisin was combined with PEF treatment, again the adapted cells seemed to be slightly more sensitive

than control cells. Interestingly, the difference is not as big as could be expected on basis of the increased sensitivity towards nisin. Clearly, the observed synergy between nisin and PEF is not increased in adapted cells and the slightly enhanced reduction found can be totally attributed to the increased sensitivity of adapted cells to nisin.
Spores are very resistant to a PEF treatment and nisin is only able to inhibit outgrowth of spores. Perhaps by interfering with the membrane composition of the mother cell by adding carvacrol to the sporulation medium, the emerging spore might exhibit different characteristics regarding sensitivity towards nisin and PEF treatment. As expected, the control spores did not show any reduction in the viable count upon treatment with either nisin or PEF or a combination of both (Fig. 1a and b) nor did the treatments initiate the germination process. Unfortunately, the used treatments were not able to induce any reduction in the viable count of adapted spores (data not shown). Even the most extreme possible PEF treatment did not result in a reduction in the viable count or initiated the germination process rendering the spores more susceptible to heat. Thus carvacrol is able to increase the sensitivity of vegetative cells towards nisin but not towards PEF or a combination of the two treatments, nor can it sensitize spores of *B. cereus* in any way to nisin and/or PEF treatment.

DISCUSSION

Previous work has demonstrated that PEF treatment is able to enhance the bactericidal activity of nisin against vegetative cells of *Bacillus cereus* (30), thereby opening new possibilities for applying this combination as a mild preservation method for foods. Before such techniques can replace currently used thermal processes, more research into spore inactivation is needed. Spores are highly resistant to a spectrum of stress factors such as heat, oxidizing agents, and UV radiation (33, 34) and indeed could not be inactivated directly or sensitized to heat by nisin or PEF treatment. Even the combination of these two treatments did not result in any damage to the spores. Several authors have found spores to be extremely resistant to PEF treatment and have suggested that spores could not be inactivated by PEF treatment unless germination inducing processes are used in combination (2, 3, 15, 20). PEF treatment itself is not able to induce germination (3). In contrast, Marquez *et al.* (23) claimed direct inactivation of spores by PEF treatment only. Treated samples at 13,000x magnification showed spores with holes, enlarged or completely damaged. However, these results could not be confirmed in this study.
Germinated spores were inactivated by nisin or PEF treatment to a certain extent. They lost their PEF resistance 50 minutes after the onset of germination, however not all the germinated spores could be inactivated. Conclusions have to be drawn with care since germination in all cases was incomplete. The germination media always contained both dormant spores as well as germinated spores and in a later stage also vegetative cells. The effects of the treatments on all of these populations could not be separated. Ideally, complete and synchronized germination is needed to quantify the inactivation by PEF treatment and determine precisely the onset of loss of PEF resistance. This incomplete germination is generally ascribed to the natural biovariability in the spore suspension (4). An alternative explanation could be the presumed accumulation of an inhibiting compound preventing germination of the remaining spores. However, Wuytack (40), was not able to demonstrate the existence of such compounds.
The late loss of PEF resistance can be explained by the dependence on the degradation of the spore coat. In dormant spores, the charges within the core are not free to migrate according to the electric field, but are immobilized by other molecules like proteins (7, 14). Furthermore, the cortex and the coat are more rigid structures than the cytoplasmic membrane of vegetative cells, which makes it more difficult to compress the membrane under influence of an imposed electric field and create pores. PEF treatment is expected to act in a later stage of the germination since it requires free movement of charges for its action (2). Full hydration of the core is dependent on the spore coat degradation, which is consistent with the late loss of PEF treatment resistance.

Germinating spores are immediately inactivated upon exposure to nisin. Even low concentrations of 0.3 μg/ml inactivated germinated spores to the same extent as heat treatment, suggesting that like heat resistance, loss of nisin resistance is one of the first events of spore germination. Apparently, nisin has gained access to the membrane by penetrating the coat, which was made more permeable upon germination or alternatively, the protective coat was degraded by spore lytic enzymes, allowing nisin to reach the cytoplasmic membrane. Inactivation of germinated spores by nisin was also found by Morris *et al.* (27), who suggested that sulfhydryl groups in the membrane, not available in ungerminated spores, are the natural target for nisin and therefore access to the membrane is a prerequisite. Combining nisin and PEF treatment did not result in additional inactivation or injury rendering the germinated spores more sensitive to heat. This is attributed to the high inactivation already caused by nisin itself and the small margin to observed synergy at all caused by the incomplete germination. In addition, loss of nisin resistance seems to be an early event in spore germination while loss of PEF resistance only occurs after 50 minutes of germination. Synergy would therefore be less likely due to different time scales of action.

One of the main problems associated with the use of antimicrobial compounds is the development of tolerance or resistance (25). One example is the increased tolerance of *Listeria monocytogenes* against nisin upon repeated exposure to increasing concentrations of nisin (26, 38). In addition, Ultee *et al.* (37) reported an increased resistance of *B. cereus* towards carvacrol upon adaptation. Both phenomena were explained by the influence of nisin or carvacrol on the membrane composition (24, 26, 37, 38). The disturbance of the lipid-lipid or lipid-protein interactions by accumulation of carvacrol in the membrane induces a change in the membrane composition to counteract this effect (18, 39). The change in fatty acid composition was consistent with a decreased membrane fluidity (16, 36, 37), resulting in a limited accumulation of carvacrol in the membrane, thereby decreasing the susceptibility of the cells towards carvacrol. In this study, however, cells grown in the presence of carvacrol (0.3 mM) became more sensitive towards nisin compared to control cells. A decrease in the membrane fluidity is not expected to increase nisin's action, but a change in the head group composition, with an increase in negatively charged lipids, might stimulate the electrostatic binding of nisin and this way enhance nisin's action (5, 9, 38). Ultee *et al.* (37) detected some additional phospholipids in adapted cells and one lacking phospholipid compared to control cells. However, these phospholipids were not further identified and no differences in the relative amounts of phosphatidyl ethanolamine, diphosphatidyl glycerol and phosphatidyl glycerol could be detected explaining the increased activity of nisin. Recently, Breukink *et al.* (6) demonstrated that nisin combines pore forming ability with high-affinity binding to peptidoglycan precursor, Lipid II. An increase in the Lipid II content of the cell markedly increased the activity of nisin. Therefore, an alternative explanation for the increased nisin activity towards adapted cells might be the increased Lipid II content, as a result of changes in membrane composition induced by carvacrol.

Microorganisms are often found to be more sensitive to electric pulses at higher temperatures (17, 43), probably because membrane phospholipids are more fluid and the cytoplasmic membrane correspondingly more fragile (3). Surprisingly, cells adapted to carvacrol, consistent with a more rigid membrane, did not exhibit a decreased susceptibility towards PEF treatment. The more rigid membrane is less easily compressed by accumulating charges as a result of applied field strength and the ordered state of the phospholipids in the membrane decreases the chance of reorientation, which would reasonably lead to a decreased inactivation by PEF treatment. Obviously, other factors also play a role in the PEF sensitivity. The increase in membrane fluidity, described by the authors mentioned above, is caused by temperature induced shift (physical process) and not by a change in membrane composition (chemical process) as described in this research. Surprisingly, the observed synergy between nisin and PEF treatment was not influenced by a change in membrane fluidity and membrane composition. The mechanism of synergy between nisin and PEF treatment is not yet understood, apparently other factors concerning the cytoplasmic membrane might influence the observed synergy.

In conclusion, spores of *B. cereus* are rather resistant to nisin and/or PEF treatment and can only be inactivated after initiation of germination. Nisin resistance of spores is lost very early in germination, suggesting the importance of permeabilisation of the spore coat. Resistance to PEF treatment was lost in a later stage of the germination. These different time scales might explain the absence of synergy

between nisin and PEF treatment against germinated spores. Changing the membrane composition, and subsequently the membrane fluidity by growing the cells in the presence of carvacrol, resulted in a dramatic increase of the nisin sensitivity, however, the efficiency of PEF treatment was not changed. The influence of the membrane composition either by adaptation to carvacrol or other components or by temperature on the PEF treatment efficiency is not clear and should receive more attention, since microorganisms in food generally adapt to their environment. These combination techniques are a welcome alternative to currently used pasteurization methods, especially when synergy is detected between the techniques allowing reductions of the used intensities. Nisin and PEF treatment could be integrated as key elements in newly designed preservation strategies provided effective measures to activate dormant spores are implemented.

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CHAPTER 7

General discussion

Modern consumers nowadays, have a preference for more natural, mildly preserved food products with a fresh appearance over traditionally preserved products. Mild preservation techniques applied singly are usually not sufficient to control microbial outgrowth and combinations of measures are needed to ensure complete safe products (16). Bacteriocins, produced by lactic acid bacteria have been successfully used as biopreservatives in a number of food products to inhibit the growth of pathogenic and spoilage organisms (27). Up till now, nisin is the only bacteriocin that has been approved by the WHO to be used as a food preservative. Due to its restricted inhibition spectrum and the decreased solubility and heat sensitivity at neutral pH, application is still limited (10). The study described in this thesis aimed to increase the practical application of nisin by combinations with other biopreservatives or mild preservation techniques.

Nisin and essential oils

Essential oils, derived from plants, are known for their flavor characteristics. Many of the compounds found in essential oils possess antimicrobial activity (4, 9, 14, 22), and therefore are suitable candidates for mild food preservation in combination with nisin. The essential oils dramatically enhance the bactericidal activity of nisin at concentrations, which alone do not affect the bacterial cell counts of the foodborne pathogens *Listeria monocytogenes* and *Bacillus cereus* (chapter 2). Adaptation of these cells to lower temperatures resulted in an increased sensitivity towards nisin, possibly due to an altered membrane composition leading to a change in membrane fluidity or to an increased electrostatic interaction of nisin with phospholipids in the membrane caused by an increase in negative charges (8, 18 - 21, 31). Alternatively a decrease in lipid II content as a result of changes in the membrane composition might explain the decreased activity of nisin (5). Lowering the temperature had a negative influence on the synergy between nisin and the essential oils, which might result from the lower sensitivity of the cells towards essential oils at lower temperatures (28).
The exact mechanism underlying this synergy is not exactly known. Both nisin and carvacrol cause a dissipation of the proton motive force as well as depletion of the internal ATP pool (6, 12, 23, 26, 30, 32, chapter 3). In combination, carvacrol enhances the membrane potential dissipating effect of nisin, at concentrations which do not affect the viable count of *B. cereus*. Apparently cells are able to cope with low concentrations of nisin and carvacrol. When concentrations increase, cells are no longer able to compensate for loss of membrane integrity and a synergistic reduction of the pH gradient and depletion of the intracellular ATP pool were observed. The reduction in internal ATP is not proportional to the increase in external ATP and no additional increase in external ATP was observed upon simultaneous exposure to nisin and carvacrol. This observation excludes increased leakage of ATP as an explanation for the synergistic depletion of the intracellular ATP pool. Consequently, the underlying mechanism of the synergistic inactivation of *B. cereus* is most likely not the increased poreforming ability of nisin by carvacrol. Presumably, the rate of ATP hydrolysis is increased upon simultaneous addition of nisin and carvacrol or the internal ATP pool is exhausted in an attempt to reenergize the membrane (1, 23, 29). Alternatively, the disturbance of the membrane permeability by carvacrol and nisin might lead to impairment of membrane bound enzymes like ATPase, resulting in a decreased ATP synthesis (15, 26).

Nisin and PEF treatment

In addition to essential oils, Pulsed Electric Field treatment was also found to improve the antimicrobial action of nisin against *B. cereus*. Synergy was only found when PEF treatment was spread over a period of 10 minutes to match the relevant inactivation time scale of nisin's action. The additional stress imposed by PEF treatment possibly facilitates the incorporation of nisin into the cytoplasmic membrane resulting in more or larger pores or pores with a longer lifetime (chapter 4). Further reduction of the intensities of the treatments was achieved by adding carvacrol as a third hurdle to the combination of nisin and PEF treatment (chapter 5).

The fact that synergy was found between the three treatments renders the combination very interesting for mild food preservation. However, extrapolation of the results from labscale experiments in buffer systems to food model matrices is usually difficult and the influence of food ingredients on the efficiency of preservation techniques are not fully understood. The efficiency of PEF treatment against vegetative cells of *B. cereus* is not affected by proteins in skimmed milk (20 %). However, the proteins do have a negative influence on the nisin activity, either as a result of a decreased bioavailability of nisin due to binding of the molecule to proteins or because of protection of the microorganisms by the proteins. As a consequence, the synergy between nisin and PEF treatment is less pronounced in skimmed milk (20 %).

In sharp contrast to the improved bactericidal activity found in HEPES buffer, carvacrol is not able to enhance the synergy between nisin and PEF treatment in diluted milk (only in high concentrations (1.2 mM)). Possibly, carvacrol binds to the proteins, reducing the availability of the molecule. However, this is not consistent with the fact that carvacrol increases the antimicrobial activity of PEF treatment in milk. Therefore, the absence of synergy between nisin, PEF treatment and carvacrol is more likely explained by the decreased bioavailability of nisin, thereby decreasing the extent of synergy between nisin and carvacrol and consequently between all three treatments. The influence of PEF treatment on the behavior of proteins is not exactly known. Proteins can carry electric charges and might behave as dipoles when subjected to PEF treatment, which cause the macromolecules to reorient or deform (such as protein unfolding and denaturation), and possibly some breakdown of covalent bonds or casein micelles may occur (3). These PEF induced changes in the structure of proteins may play a role in the existence of synergy between carvacrol and PEF. Dilution of the milk to 5 % still provides enough proteins to stimulate synergy between carvacrol and PEF treatment (chapter 5).

Before such novel techniques can replace currently used thermal processes, more insight into spore inactivation is needed (chapter 6). Nisin and PEF treatment do not directly inactivate or damage spores of *B. cereus*, however germinated spores can be inactivated by nisin or PEF treatment to a certain extent. The PEF resistance of the germinated spores is lost 50 minutes after the onset of germination. Nisin resistance was lost immediately in parallel to heat resistance, suggesting that loss of nisin resistance might be ascribed to changes in the dehydrated state of the core. Sulfhydryl groups in the membrane, not available in ungerminated spores, were suggested to be the natural target for nisin and therefore access to the membrane is a prerequisite for inactivation (17, 24, 25). In addition, the increase in availability of the membrane-anchored cell wall precursor Lipid II upon germination could also play a role in the loss of nisin resistance (5). Apparently, nisin has gained access to the membrane by penetrating the coat, which was made more permeable upon germination or alternatively, the protective coat was degraded by spore lytic enzymes, allowing nisin to reach the cytoplasmic membrane. The late loss of PEF resistance can be explained by its dependence on the degradation of the spore coat. To exert antimicrobial inactivation by PEF treatment, free migration of ions is needed to increase the transmembrane potential of the spores. Formation of pores occurs after compression of the membrane and reorientation of the phospholipids in the membrane. In spores the ions are immobilized by proteins or DPA, restricting their mobility (7, 13) and subsequently the build up of an increased transmembrane potential is prevented. Secondly, the spore core is surrounded by several rigid protecting layers limiting the compression and reorientation of the phospholipids (2).

Combining nisin and PEF treatment did not result in additional inactivation of the germinating spores. Since loss of PEF resistance occurs only after 50 minutes of germination and loss of nisin resistance

seems to be an early event in spore germination, synergy would therefore be less likely due to different time scales of action. Furthermore, the incomplete germination of the spores reduces the margins to observe synergy. Ideally, complete and synchronized germination is needed to quantify the inactivation by nisin or PEF treatment and determine precisely the onset of loss of nisin or PEF resistance.
One of the main problems associated with the use of antimicrobial compounds is the development of tolerance or resistance to certain compounds. Adaptation of cells to carvacrol was correlated to a decrease in membrane fluidity as demonstrated by Ultee *et al.* (30). In addition, they observed a change in phospholipid composition of the membrane. Cells adapted to carvacrol exhibited an increased sensitivity towards nisin compared to control cells (chapter 6). A decrease in the membrane fluidity is not expected to increase nisin's action, but a change in the head group composition, with an increase in negatively charged lipids, might stimulate the electrostatic binding of nisin and in this way enhance nisin's action (8, 18 - 21, 31). Alternatively an increase in lipid II content in carvacrol-adapted cells as a result of changes in the membrane composition might explain the increased activity of nisin (5). A decrease in the membrane fluidity did not change the susceptibility towards a PEF treatment. A more rigid membrane is less likely to be compressed by accumulating charges as a result of applied field strength and the ordered state of the phospholipids in the membrane decreases the chance of reorientation, which would reasonably lead to a decreased inactivation by PEF treatment. Although the bactericidal activity of nisin was increased by adaptation to carvacrol, the synergy between nisin and PEF treatment was not influenced by a change in membrane fluidity and membrane composition. Attemps to change the membrane composition of spores by adaptation of vegetative cells to carvacrol prior to and during sporulation did not lead to inactivation of spores by either nisin or PEF treatment.

Application

Combinations of nisin with essential oils or PEF treatment have been successful in overcoming the restrictions in practical application of nisin. For instance, the inhibition spectrum of nisin can be widened by combination with other preservation technologies like PEF treatment. In addition, the limited activity of nisin at higher temperatures can be complemented by the increased synergy between nisin and essential oils.
The application of multiple hurdles has great potential to be used as a mild food preservation technology. The occurrence of synergy between nisin and essential oils or PEF technology allows for a reduction in the intensities of the treatments demonstrating the suitability for mild preservation. Increasing the number of hurdles (lysozyme) improves the observed synergy and further increases the mildness of the preservation technology (chapter 1).
Consumer's acceptation of these combination techniques in case of the essential oils is not expected to meet difficulties. This combination meets with present preference for more natural and mild preservation methods. Herbs and spices, of which essential oils are the active components, are already used for centuries as flavoring agents and in homeopathic products and medicines. Currently, carvacrol is Generally Recognized As Safe (GRAS) and has been approved by the Code of Federal Regulation (CFR) to be used as a flavoring agent (11). However, when the essential oils are used for their antimicrobial activity, they will be regarded as new food additives and subsequently require a non-toxicity report (27). To circumvent these problems, the original herbs and spices can be used as food flavoring agents, while at the same time advantage can be taken of their antimicrobial activity. However, the producer has to take into account the low concentration of the active compound in herbs and spices. Furthermore, the essential oils have a strong and specific flavor and can only be applied in products where this aroma is appreciated.
Acceptance of PEF technology is expected to give more problems and introduction of this technology has to be handled carefully. Consumers might associate PEF treated foods with residual electromagnetic raditation, just like radiated foods are associated with radioactivity. Only when PEF technology is

introduced carefully and the consumers are supplied with the right information, they will accept this technology as mild preservation.

At the moment, not enough information is known about PEF technology and its mechanism of action. Evidently, more research needs to be done to verify the influence of other food ingredients including fat particles on the antimicrobial activity. Furthermore the influence of PEF treatment on the product quality needs to be investigated. The fresh-like appearance, color and the vitamin content are seemingly unaffected however, the influence of PEF treatment on proteins, polysaccharides macromolecules, or lipids is not exactly known.

The development of tolerance or resistance to the PEF treatment or the combination treatments is not clear and should receive more attention, since microorganisms generally adapt to environmental stress factors. Increased tolerance towards nisin and carvacrol has been studied in more detail (8, 18 - 21, 31) however, no such research has been conducted concerning PEF technology. Combining preservation technologies in which the microorganism is attacked from different sides should reduce the development of tolerance to a minimum. Inactivation of spores is another challenge to be overcome before such combination technologies can be implemented in current preservation strategies.

In conclusion, these combination techniques are a welcome alternative to currently used pasteurization methods. The current limitations in the application of nisin can be complemented by the inhibition spectrum of the combination treatment. In addition, the synergy observed between the different preservation techniques allows for a reduction of the used intensities increasing the suitability for mild preservation.

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SUMMARY

In order to meet consumer's demand for more natural and fresh-like products, food manufactures increasingly rely on mild preservation techniques to improve the organoleptic quality and nutritional properties of their products. Nisin, the only bacteriocin which has been approved by the WHO to be used as a food preservative, has been successfully used in suppressing the growth of pathogenic and spoilage organisms. However, its practical application is limited due to its restricted inhibition spectrum and its low stability and solubility in certain food matrices. By combining nisin with other biopreservatives or mild processes these restrictions might be overcome.

Combining nisin with essential oils, like carvacrol, thymol and carvone, resulted in a synergistic reduction in viable count of both *L. monocytogenes* and *B. cereus*. Exposure to carvacrol resulted in a first order inactivation while exposure to nisin resulted in a typical biphasic reduction, being most fast during the first 10 minutes. In concentrations which did not affect the viable count, the essential oils dramatically enhanced the bactericidal activity of nisin, indicating the existence of synergy between the two components. The inhibitory effect of nisin was stronger towards cells cultivated and exposed at 8°C, while the synergy observed between nisin and the essential oils was larger towards cells cultivated and exposed at 20°C. Addition of lysozyme as a third preservative factor further increased the synergistic effect between nisin and carvacrol.

Both nisin and carvacrol, as single treatments, are able to affect the membrane integrity by increasing the permeability of the membrane for cations. Subsequently, dissipation of the membrane potential and the pH gradient across the membrane is observed as well as depletion of the internal ATP pool. In combination, carvacrol enhanced the membrane potential dissipating activity of nisin, and acted synergistically in dissipating the pH gradient and depleting the intracellular ATP pool. The synergistic depletion of ATP could not be explained by an increased ATP efflux, indicating that carvacrol does not enlarge pores formed by nisin or increase the lifetime or the number of pores.

In addition to essential oils, nisin (0.06 µg/ml) was combined with PEF treatment (16.7 kV/cm, 50 pulses each of 2 µs duration) and synergy was observed between the two treatments. Each of the two treatments, when applied separately, were able to reduced the viable count of *B. cereus* with 0.8 (nisin) and 1.2 log units (PEF treatment), while the combined treatment resulted in a remarkable increase in the reduction of the viable count of 3.8 log units. The observed reduction obtained by the combined treatment is 1.8 log units more than the sum of the reductions obtained with the single treatments. The bactericidal action of nisin was enhanced by PEF treatment throughout the whole exposure period. Increasing the number of hurdles by addition of carvacrol further enhanced the synergy between nisin and Pulsed Electric Field (PEF) treatment. The influence of food ingredients on the bactericidal activity was tested using skimmed milk in water (1:4 (vol/vol)). The efficacy of PEF treatment was not affected by the presence of proteins and results found in HEPES buffer correlated well with results in milk (20 %). Nisin showed less activity against *B. cereus* in milk. Carvacrol was not able to enhance the synergy between nisin and PEF treatment in milk, only if used in high concentrations (1.2 mM). This concentration in itself did not influence the viable count. Carvacrol was found to act synergistically with PEF treatment in milk, however not in HEPES buffer. This synergy was not influenced by proteins in milk, since 5 % milk (vol/vol) still allowed synergy between carvacrol and PEF treatment to the same extent as 20 % (vol/vol) milk.

Spores of *B. cereus* were not inactivated or sub-lethally damaged by treatment with nisin and/or PEF and could only be inactivated after initiation of germination. The resistance to nisin was lost immediately upon initiation of the germination process. Resistance to PEF treatment was lost after 50 minutes of germination. Synergy between nisin and PEF treatment could not be demonstrated against germinating spores. Attemps to decrease the membrane fluidity of vegetative cells by incubation in the presence of carvacrol resulted in a dramatic increase in the sensitivity towards nisin. On the other hand, inactivation

by PEF treatment or by a combination of nisin and PEF treatment did not change upon adaptation to carvacrol. Spores grown in the presence of carvacrol were not susceptible to nisin and/or PEF treatment in any way.

In conclusion, these results demonstrate that essential oils and PEF treatment can profitably be combinated with nisin in food preservation. Restrictions in the application of nisin caused by limitations in inhibition spectrum or instabilities in food matrices can be complemented by the combination treatment. In addition, the observed synergy enables us to further reduce the intensities of the treatment, improving the mildness of the technology.

SAMENVATTING

Het gebruik van milde conserveringstechnieken met een minimale invloed op de organoleptische eigenschappen en de voedingswaarde van producten is de laatste jaren sterk toegenomen door de groeiende vraag van de consument naar producten met een meer natuurlijk en vers karakter. Nisine is een goed voorbeeld van een natuurlijk conserveringsmiddel dat de groei van voedselpathogenen en bederforganismen remt en is tot op heden het enige bacteriocine dat door de Wereld Gezondheid Organisatie (WHO) is toegelaten om gebruikt te worden als conserveringsmiddel. Echter, het beperkte werkingsspectrum en de geringe stabiliteit en oplosbaarheid in bepaalde voedingsmatrices beperken de toepassingsmogelijkheden. Door nisine te combineren met andere bio-conserveringsmiddelen of met milde conserveringstechnieken kan voor deze beperkingen worden gecompenseerd.
Het combineren van etherische oliën als carvacrol, thymol en carvon met nisine resulteerde in een synergetische reductie van het aantal levensvatbare cellen van zowel *Listeria monocytogenes* als van *Bacillus cereus*. Cellen blootgesteld aan carvacrol alleen werden geïnactiveerd volgens een eerste orde kinetiek. Inactivatie door nisine daarentegen vertoonde twee fasen: een sterke afname in levensvatbaarheid gedurende de eerste 10 minuten gevolgd door een afvlakkende inactivatie. De bactericide werking van nisine werd al versterkt door lage concentraties etherische oliën welke zelf geen invloed hadden op levensvatbaarheid van de cellen. Dit duidt op de aanwezigheid van synergie tussen de beide componenten. De anti-microbiële werking van nisine was groter bij een kweek- en blootstellingstemperatuur van 8°C, daarentegen was de synergie tussen nisine en de etherische oliën groter bij een kweek- en blootstellingstemperatuur van 20°C. Het toevoegen van lysozym als derde conserveringsfactor leidde tot een verdere vergroting van de synergie tussen nisine en carvon.
Zowel nisine als carvacrol bleken de membraanintegriteit te beïnvloeden door de permeabiliteit van de membraan voor kationen als K^+ en H^+ te vergroten. Als gevolg hiervan daalde de pH gradiënt alsook de intracellulaire ATP concentratie. De membraanpotentiaal verlagende werking van nisine werd versterkt door simultane toevoeging van carvacrol. Tevens werd een synergetische verlaging van de pH gradiënt en de intracellulaire ATP pool door een combinatie van beide componenten waargenomen. De geringe toename in de externe ATP concentratie correleerde niet met de waargenomen afname in intracellulaire ATP concentratie. Bovendien werd er geen extra ATP lekkage waargenomen na simultane toediening van nisine en carvacrol. Hieruit volgt dat carvacrol niet in staat is de porie-vormende werking van nisine te versterken alsmede de levensduur of het aantal poriën te vergroten.
Nisine werd ook gecombineerd met een Pulsed Electric Field (PEF) behandeling. Synergie tussen de twee behandelingen werd alleen waargenomen indien de PEF behandeling werd gespreid over een periode van 10 minuten. Zowel nisine als de PEF behandeling, veroorzaakten een afname in levensvatbaarheid van *B. cereus* met respectievelijk 0.8 en 1.2 log eenheden. De gecombineerde behandeling echter, resulteerde in een opmerkelijke reductie van 3.8 log eenheden. Deze reductie was 1.8 log eenheden hoger dan de som van de aparte behandelingen. De bactericide werking van nisine werd versterkt door de PEF behandeling gedurende de gehele blootstellingsperiode. Een toename in het aantal horden door toevoeging van carvacrol resulteerde in een verdere versterking van de waargenomen synergie tussen nisine en de PEF behandeling.
Ook in magere melk verdund met water (1:4 (v/v)) versterkte de PEF behandeling de bactericide werking van nisine, doch in geringere mate vergeleken met HEPES buffer. De effectiviteit van de PEF behandeling werd niet beïnvloed door de eiwitten en resultaten gevonden in HEPES buffer bleken goed te correleren met de resultaten gevonden in verdunde melk (20 %). Dit in tegenstelling tot nisine, dat in verdunde melk (20 %) een verminderde activiteit tegen *B. cereus* vertoonde. Deze verlaagde bactericide werking veroorzaakte de geringere intensiteit van de waargenomen synergie tussen nisine en PEF behandeling in melk. In tegenstelling tot de situatie in HEPES buffer, was carvacrol niet in staat om de

synergie tussen nisine en PEF behandeling in melk te vergroten, tenzij toegevoegd in hoge concentratie (1.2 mM). Deze hoge concentratie had geen invloed op de levensvatbaarheid van *B. cereus*. Carvacrol vertoonde wel synergie met de PEF behandeling in melk. Een verlaging van de concentratie eiwitten door verdere verdunning van de melk van 20 % naar 5 % gaf geen vermindering van de intensiteit van de waargenomen synergie.

Sub-letale beschadiging of inactivatie van sporen van *B. cereus* kon niet worden bewerkstelligd door een behandeling met nisine en/of PEF. Ontkiemde sporen daarentegen waren wel gevoelig voor deze behandeling. Onmiddellijk na aanvang van het ontkiemingsproces verloren de sporen hun nisine resistentie. De PEF-resistentie ging pas verloren na 50 minuten ontkieming. Synergie tussen de twee behandeling kon niet worden aangetoond. Door middel van incubatie van de vegetatieve cellen in aanwezigheid van carvacrol kon de membraanvloeibaarheid van de cellen worden verlaagd zonder de groeitemperatuur te veranderen. Cellen met een verlaagde membraanvloeibaarheid vertoonden een sterk verhoogde gevoeligheid voor nisine. De gevoeligheid voor PEF behandeling of een combinatie van nisine en PEF behandeling werd niet beïnvloed door adaptatie van de cellen aan carvacrol. Het opkweken van sporen in de aanwezigheid van carvacrol leidde niet tot een verandering in de gevoeligheid van de sporen voor nisine of PEF behandeling.

Samenvattend demonstreren deze resultaten de potentie van het hordentechnologie-concept bij milde voedselconservering. Voor de geringe toepassingsmogelijkheden van nisine als gevolg van het beperkte remmingsspectrum alsmede de instabiliteit in voedselmatrices kan worden gecompenseerd door combinaties met andere behandelingen als etherische oliën of PEF technologie. Daarnaast stelt de waargenomen synergie ons in staat de intensiteit van de behandelingen te verlagen, waardoor de combinatietechnologie milder wordt.

CURRICULUM VITAE

Irene (Elisabeth) Pol werd geboren op 17 januari 1973 te Deventer. In 1991 behaalde zij haar VWO diploma aan het Isala college te Silvolde. In dat zelfde jaar begon zij de studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit in Wageningen (nu deel van Wageningen Universiteit en Researchcentrum). Met de afstudeervakken Levensmiddelenmicrobiologie, uitgevoerd op het ATO-DLO in Wageningen, Levensmiddelenchemie, uitgevoerd aan de sectie Levensmiddelen chemie van de Landbouwuniversiteit en haar stage Levensmiddelen microbiologie, uitgevoerd in Engeland bij het Institute for Food Research (IFR) in Norwich, werd deze studie in september 1996 afgerond. Vanaf december 1996 was ze werkzaam als wetenschappelijk onderzoeker bij het ATO-DLO in Wageningen (thans deel van Wageningen Universiteit en Research centrum), waar het in dit proefschrift beschreven onderzoek werd verricht. Het onderzoek werd financieel ondersteund door de Commission of the European Union (FAIR CT 96-1148).

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