Influence of Food Matrix on Inactivation of Bacillus cereus by Combinations of Nisin, Pulsed Electric Field Treatment, and Carvacrol

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ABSTRACT

Carvacrol was used as a third preservative factor to enhance further 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 potassium-N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid (HEPES) buffer. The influence of food ingredients on bactericidal activity was tested using skimmed milk that was diluted to 20% with sterile demineralized 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, as 5% milk still allows synergy between carvacrol and PEF treatment to the same extent as 20% milk.

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 (18). Pulsed electric field (PEF) is a nonthermal inactivation method that 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 nonthermal technology allows the process to be energy efficient (25, 37, 38). Microbial inactivation is caused by structural changes in the membrane, resulting in pore formation and loss of the selective properties of the membrane (10, 12, 22, 27). Ions in- and outside the cell migrate according to the electric field across the electrodes, resulting in accumulation of free charges at both membrane surfaces leading to an increased transmembrane potential and a decreased membrane thickness (5, 39). In addition, the electric charges 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 (31).

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 (14). Researchers have shown that inactivation increases with an increase in the applied electric field strength and treatment time and that higher temperatures act synergistically with PEF treatment (9, 17, 23, 27, 28, 34, 36). Other factors that enhance PEF treatment are pH and the presence of antimicrobials that 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 (35).

In general, preservation by a single preservative factor is not sufficient to ensure completely safe products, and multiple hurdles are advised (18). Recently, synergy between the bacteriocin nisin and PEF treatment was demonstrated in our laboratory (23). 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, 8, 26). 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, 6, 19, 20). The fact that nisin and PEF treatment share a common primary target might offer an explanation for the synergy found between the two treatments. Another example of mild preservation by multiple hurdles is based on the synergy between nisin and essential oils like thymol and carvacrol (24). Due to their lipophilic nature, components like carvacrol can accumulate in the lipid bilayer, thereby disturbing its function, leading to cell death (32). Again, combining two compounds with similar primary targets re-
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 broth (Oxoid, Basingstoke, UK), 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-ethanesulfonic acid (HEPES; Sigma-Aldrich, Zwijndrecht, The Netherlands) buffer (pH 7.0) until an OD₆₆₀ 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). No spores could be found in the used cell suspensions.

**Influence of carvacrol on the synergy between nisin and PEF treatment.** To determine the effect of the combination of nisin (Aplin and Barrett Ltd., Wilts, England) 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 to 0.5 mM). The action of nisin and carvacrol was quenched 11.5 min after addition to the cell suspension by a 100-fold dilution in peptone physiological salt solution and the number of survivors was determined on brain heart infusion 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, Leeuwarden, The Netherlands; skimmed [0% fat], ultrahigh temperature sterilized). 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 to 20% with sterile water. The cells were grown and harvested as described above. The pellet was resuspended in 20% skimmed milk (G = 1.4 mS/cm at 20°C) to a concentration of 10⁷ cells/ml and subjected to nisin (0.13 μg/ml), carvacrol (concentration range: 0.4 to 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 colinear 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 min at high flow rate before treatment to get a homogenous distribution of the cells. During experiments a total volume of 400 ml of inoculated medium was circulated through the system for 12 min at a 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 was analyzed on brain heart infusion 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 *B. cereus* (23, 24). Because the primary target of all 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 (23). For this study, the nisin concentration was lowered to 0.04 μg/ml and complemented with carvacrol until a reduction of 1 log unit was achieved (Fig. 1). A concentration of 0.5 mM of

**FIGURE 1. Log reduction of vegetative cells of *B. cereus* F46.26.90 caused by nisin (0.04 μg/ml) combined with different concentrations of carvacrol. Error bars are indicated and data points represent means of an average of four measurements.**
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 min 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 the 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.

**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 (σ = 1.4 mS/cm at 20°C) and recirculated for 5 min 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 min 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 (23).

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 the food model matrix, nisin was complemented with 0.4 mM of carvacrol and combined with PEF treatment. 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 that 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 (22); 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 (Table 1).

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 ac-

#### Table 1. Inactivation rates (log [CFU/ml] reduction per pulse) for *B. cereus* in HEPES buffer (5 mM, pH 7.0) and in milk (20%)<sup>a</sup>

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<tr>
<th></th>
<th>PEF</th>
<th>Nisin + PEF</th>
<th>Nisin + PEF + carvacrol</th>
<th>PEF + carvacrol</th>
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<tr>
<td>HEPES buffer</td>
<td>-0.0474 ± 0</td>
<td>-0.1451 ± 0.0060</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Milk (20%)</td>
<td>-0.0461 ± 0.0012</td>
<td>-0.1025 ± 0.0025</td>
<td>-0.1066 ± 0.0035</td>
<td>-0.0982 ± 0.0050</td>
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<sup>a</sup> 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. Standard deviation is indicated.

<sup>b</sup> ND, not determined.
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 significantly decreased synergy between carvacrol and PEF treatment against \textit{B. cereus} (Fig. 6). Even a 4\times dilution of the milk to 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.

**DISCUSSION**

Previously, synergy was detected between nisin and PEF treatment (23) and between nisin and carvacrol (24). In this work, carvacrol was used as a third hurdle to increase further the synergy found between nisin and PEF treatment. The PEF treatment was spread over a 10-min 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 (27). 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 an increased lifetime or pore size are formed. Whether both carvacrol and PEF treatment stimulate the bactericidal effect of nisin via similar modes of action 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 do not always work in food model matrices. The influence of proteins was tested using skimmed milk as a food model matrix. Both nisin and PEF treatment inactivated vegetative cells of \textit{B. cereus} in diluted milk (20%) according to first-order inactivation kinetics. The proteins in milk did not seem to interfere with the inhibiting effect of PEF treatment against \textit{B. cereus}. Results obtained in buffer with the batch PEF system show similar trends in inactivation as results found in diluted milk (20%) using the continuous PEF system (Table 1). This is supported by the fact that lowering the protein content did not change the inactivation rate of PEF and carvacrol (Fig. 6). The protein content did influence the nisin activity negatively. More nisin was needed to achieve a 1-log unit reduction in diluted milk compared to HEPES buffer (23) either because the bioavailability of nisin is decreased due to binding to proteins or the microorganisms are protected by the proteins. 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 (0 to 1.2 mM) were tested, but all of them exhibited the same reductions when combined with nisin and PEF treatment. 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 (29) found a reduced inhibitory effect of phenolic extracts in milk and attributed this to the binding of the phenolics by the milk proteins (15, 21). Although carvacrol was not able to improve the bactericidal activity of nisin and PEF treatment, it was however able to increase the inhibitory activity of the PEF treatment (Table 1). This observation rules out the possibility of the protective effect of the proteins. On the contrary, because 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 that cause the macromolecules to reorient or deform (such as protein unfolding and denaturation), and possibly some breakdown of covalent bonds may occur (3, 31). Alkaline phosphatase is an example of a globular protein with an internal structure that is lost upon PEF treatment resulting in loss of activity (2). Not all enzymes lose activity after
PEF treatment. Ho et al. (11) found inactivation of lipase, glucose oxidase, α-amylase peroxidase, and phenol oxidase upon PEF treatment, but increased activity of lysozyme and pepsin in a certain range of electric fields. Although contradictory results have been published on the effect of a PEF treatment on proteins, the authors believe that no radical changes have occurred in the milk upon PEF treatment in this work. 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 were found in milk treated with 40 pulses of 36.7 kV/cm over a 25-min time interval (2), which is a more severe PEF treatment than used in this study (20 kV/cm, 30 pulses for a 12-min time interval). Therefore an altered protein conformation as a consequence of PEF treatment 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 toward modification brought about by PEF treatment and could have caused the improved bactericidal effect and might explain the absence of synergy when all three treatments are combined.

The characteristics of the treatment media such as the pH, conductivity, and presence of cations can influence the efficacy of the treatments (30). However, the PEF treatment did not alter the pH of the milk or the buffer and therefore could not have played a role in the increased activity found (36). Furthermore, no changes in activity of nisin and carvacrol were detected upon PEF treatment. The temperature and the conductivity of the treatment media are also important factors determining the inactivation efficiency. However, 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 (23) showed similar trends with results found in milk (20%). Therefore the protecting effect of ions like Ca²⁺ (13) can be excluded.

By combining different preservation techniques, many of the currently known restrictions might be overcome, leading to practical mild preservation processes (16, 32). A bottleneck for application of these combinations is the reduced efficacy in foods (33). 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 determine the exact mechanism of inactivation and to verify the influence of other food ingredients.

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