Sensitivities of Germinating Spores and Carvacrol-Adapted Vegetative Cells and Spores of Bacillus cereus to Nisin and Pulsed-Electric-Field Treatment

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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 sublethal damage. In contrast, germinating spores were found to be sensitive to PEF treatment. Nisin treatment was more efficient than PEF treatment for inactivating germinating spores. PEF resistance was lost after 50 min of germination, and not all germinated spores could be inactivated. Nisin, however, was able to inactivate the germinating spores to the same extent as heat treatment. Resistance to nisin was lost immediately when the germination process started. A decrease in the membrane fluidity of vegetative cells caused by incubation in the presence of carvacrol resulted in a dramatic increase in the sensitivity to nisin. On the other hand, inactivation by PEF treatment or by a combination of nisin and PEF treatments did not change after adaptation to carvacrol. Spores grown in the presence of carvacrol were not susceptible to nisin and/or PEF treatment in any way.

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 in order to combine stability and microbial safety with improved organoleptic quality. PEF treatment is a potential alternative to heat pasteurization. It is a nonthermal inactivation technique that results in minimal losses of flavor, color, and food quality (3, 42). PEF treatment inactivates microorganisms by causing irreversible structural changes in the membrane, resulting in pore formation and subsequent loss of cellular constituents (7, 8). Upon exposure of cells to electric fields, the ions inside and outside the cells migrate according to the electric field across the electrodes. Consequently, free charges accumulate on both sides of the membrane surface, which results in increased membrane potential and a reduction in the membrane thickness as a consequence of the increased attraction between the opposing charges. These charges force the polar lipid molecules in the membrane to reorient, which leads to formation of hydrophilic pores and impairment of the membrane barrier against ions (3, 42). The extent of the increase in permeability depends on the strength and duration of the electric pulse (19).

Recently, synergy between nisin and PEF treatments was demonstrated with vegetative cells of Bacillus cereus (30). Nisin, the only bacteriocin that is approved by the World Health Organization for use as a food preservative, increases the permeability of the membrane by pore formation, which results in a rapid efflux of small molecules. The efflux of cellular constituents results in complete collapse of the proton motive force and finally leads to cell death (10, 13, 41). It has been thought that a common primary target, the cytoplasmic membrane, provides an explanation for the observed synergy (30).

The synergy between nisin and PEF treatments makes this combination technology interesting for mild food preservation. However, thorough knowledge of the effects 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 heat 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, it is sporostatic and prevents the swelling of 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 cells 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 used. Some examples of such processes are heat shock, lysozyme, EDTA, pH, and high hydrostatic pressure treatments (2, 3). In contrast, Marquez et al. (23) observed inactivation of bacterial endospores by PEF treatment alone. In this study inactivation required a minimum field strength of 35 kV/cm and was enhanced by increasing the temperature, the number of pulses, and the pulse duration.

Factors that play major roles in overall spore resistance
include the low permeability of spores to toxic chemicals and the decreased spore core water content (33, 34). The latter, together with spore mineralization plays a major role in acquired heat and γ-radiation resistance and a less significant role in resistance to hydrogen peroxide (31, 35). The spore cortex is largely responsible for maintenance of the dehydrated state of the spore core (32, 34). Resistance to oxidizing agents and chemicals is largely due to the proteinaceous spore coat and cortex, which restrict access of potentially toxic molecules to the spore core (35). These characteristic resistance properties 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 nonnutrient (chemical, enzyme, etc.) germinants (12, 34). L-Alanine is the most common nutrient germinant and triggers a sequence of germination events, including uptake of water, loss of Ca²⁺ and dipicolinic acid, loss of refractility, and onset of core metabolism (12, 40).

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

MATERIALS AND METHODS

Growth of bacteria. B. cereus IFR-NL94-25, obtained from the Institute of Food Research (Norwich, United Kingdom), 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 the presence of 30% glycerol as a cryoprotectant. Spores of B. cereus were produced on SPO 8 medium (8 g of nutrient broth per liter, 0.51 g of MgSO₄·7H₂O per liter, 0.97 g of KCl per liter, 0.2 g of CaCl₂·2H₂O per liter, 3 × 10⁻² g of FeSO₄·7H₂O per liter, 1.5% agar (11) by spreading 1 ml of a fully grown culture on a plate and incubating the culture 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 they were used. A heat treatment consisting of 10 min at 70°C resulted in no decrease in the total counts, and this 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-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (pH 7.0) at a concentration of 10⁷ spores/ml and subjected to a single nisin treatment (3.0 µg/ml), a PEF treatment (53 kV/cm), or a combination of the two treatments for 12 min. The PEF treatment was applied by using a continuous flow system. The treatment device was a colinear device as described by Yin et al. and discussed by Barbosa-Cánovas et al. (2); it had a gap diameter of 2 mm and an inner diameter of 1 mm. Spores of B. cereus were added to the buffer and recirculated for 5 min at a high flow rate before treatment in order to obtain homogeneous distribution of the spores throughout the system. During the experiments 400 ml (total volume) of inoculated medium was circulated through the system for 12 min at a 980-Hz pulse frequency and a flow rate of 100 ml/min in order 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 min after nisin was added to the spore suspension. The PEF treatment was spread over a 10-min interval (recirculation experiment) in order to maximize the interaction with nisin. Inactivation by nisin was quenched by 100-fold dilution in a peptone physiological salt solution (1 g of peptone per liter, 8.5 g of NaCl per liter), and the number of survivors was determined on BHI agar before and after an additional heat treatment consisting of 10 min 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 thermal effects (inlet temperature, 20°C).

Nutrient-induced spore germination and heat activation. Germination of the B. cereus spores in liquid media was triggered by using L-alanine or BHI broth as the germinant. 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 plates and incubating the plates at 30°C. At appropriate times samples were taken and analyzed for germinated spores on BHI agar before and after an additional heat treatment consisting of 10 min at 70°C. In the case of germination on solid media, the agar was diluted in 100 ml of the peptone physiological salt solution, treated with a stomacher for 1 min, and serially diluted before the number of spores was analyzed by plate counting. The percentage of germination was calculated as follows: [(number of spores per milliliter before heating-number of spores per milliliter after heating)] × 10⁶ (1).

To determine the influence of a heat activation step on the germination rate, spores were subjected to a sublethal heat treatment consisting of 10 min at 70°C prior to inoculation into BHI broth at 30°C. At appropriate times 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 treatment, a single-pass PEF treatment, or a combination of the two treatments at different stages of germination to test which phase in the germination process is sensitive to the used treatments. Spores of B. cereus suspended in BHI broth (10-fold diluted; conductivity = 4 mS/cm) supplemented with 0.5% glucose at an optical density at 660 nm (OD₆₆₀) of 0.1 (light path, 1 cm) were treated with either nisin, PEF, or a combination of the two at regular intervals during the germination period (5 h). When nisin treatment was used alone, the spores were exposed to nisin for 12 min before the reaction was quenched by 100-fold dilution in the peptone physiological salt solution. The PEF treatment (27 kV/cm; 302-μs pulses; flow rate, 10 ml/min) was applied once instead of being spread over a 10-min period (single-pass experiment). The temperature increase during the single-pass PEF treatment did not exceed 20°C (inlet temperature, 30°C). The number of spores was determined before and after an additional heat treatment consisting of 10 min at 70°C.

Influence of preincubation with carvacrol or nisin on sensitivity to nisin, carvacrol, and/or PEF treatment. To test the sensitivity of B. cereus to nisin and/or carvacrol, cells were grown in BHI broth containing 0.5% (wt/vol) glucose in the presence and absence of carvacrol (0.3 mM) or nisin (0.3 µg/ml) overnight at 20°C. The cells were harvested in the exponential growth phase, washed, and resuspended in 50 mM HEPES buffer (pH 7.0) at an OD₆₆₀ of 0.1 (light path, 1 cm). The adapted cells were exposed to nisin, carvacrol, or a combination of the two compounds at 20°C. Samples were taken at regular intervals during the 30-min exposure period and immediately diluted (10⁻² to 10⁻³-fold) in the peptone physiological salt solution to quench the inactivation reaction. The numbers of survivors were determined on BHI agar. To determine the sensitivity to 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 diluted 1:100 in fresh BHI broth containing 0.5% (wt/vol) glucose and 0.4 mM carvacrol and incubated at 30°C for approximately 4 h. Cells were harvested at an OD₆₆₀ of 0.1 (light path, 1 cm), washed and resuspended in HEPES buffer (50 mM, pH 7.0), and kept on ice until they were used. Spores of B. cereus were preincubated in SpO 8 agar in a concentration of 10⁶ spores/ml and subjected to a single nisin treatment (3.0 µg/ml), a PEF treatment (53 kV/cm; 43 2-µs pulses; square wave pulses), or a combination of the two treatments for 12 min. The PEF treatment was applied by using a continuous flow system. The treatment device was a colinear device as described by Yin et al. and discussed by Barbosa-Cánovas et al. (2); it had a gap diameter of 2 mm and an inner diameter of 1 mm. Spores of B. cereus were added to the buffer and recirculated for 5 min at a high flow rate before treatment in order to obtain homogeneous distribution of the spores throughout the system. During the experiments 400 ml (total volume) of inoculated medium was circulated through the system for 12 min at a 980-Hz pulse frequency and a flow rate of 100 ml/min in order 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 min after nisin was added to the spore suspension. The PEF treatment was spread over a 10-min interval (recirculation experiment) in order to maximize the interaction with nisin. Inactivation by nisin was quenched by 100-fold dilution in a peptone physiological salt solution (1 g of peptone per liter, 8.5 g of NaCl per liter), and the number of survivors was determined on BHI agar before and after an additional heat treatment consisting of 10 min 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 thermal effects (inlet temperature, 20°C).

In the case of vegetative cells, nisin was used at a concentration of 0.08 µg/ml and subjected to nisin and/or PEF treatment. 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 plates and incubating the plates at 30°C. At appropriate times samples were taken and analyzed for the number of survivors was determined by spreading spores of B. cereus on BHI agar plates and incubating the plates at 30°C.
heat treatment consisting of 10 min at 70°C in the case of spores. In all cases the temperature during treatment was kept below 30°C in order to discriminate thermal effects (inlet temperature, 20°C).

Chemicals. The carvacrol stock solution in 95% ethanol was kept at 4°C, and the stock solution of nisin (Nisaplin, containing 2% nisin; Aplin and Barrett Ltd., Wilts, United Kingdom) in 50% ethanol was filter sterilized (pore size, 0.22 μm; Costar) and kept at ~20°C. The nisin concentration was not influenced by filter sterilization (data not shown).

RESULTS

Susceptibility of spores of B. cereus to nisin and/or PEF treatment. Spores of B. cereus, which developed at 20°C, were subjected to nisin treatment, PEF treatment, or a combination of the two treatments, and the effects of these treatments on germination and the viable counts of the spores were determined. Although vegetative cells of B. cereus are very sensitive to these treatments (30), spores were able to resist both the nisin treatment and the high-intensity PEF treatment (data not shown). An additional heat treatment did not result in any reduction in the viable counts of the spores, leading to the conclusion that nisin and PEF treatment did not initiate germination of the spores (data not shown).

Nutrient-induced spore germination and heat activation. The characteristics of the germination process were 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 germinated in the presence of L-alanine (corresponding to 0.5 log unit), while in BHI broth more than 95% of the spores germinated (corresponding to 1 to 2 log units). In either case, complete germination was never observed. The maximum germination value was reached after 3 h 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 agar plates was not accelerated. After a heat activation treatment, the spores germinated slightly faster than untreated spores; however, the differences were very small (data not shown). Heat activation was therefore not used in further experiments.

Sensitivity of germinating spores of B. cereus to nisin and/or PEF treatment. Upon germination, spores lose their resistance to several agents or treatments, such as UV and oxidizing agents, and become metabolically active. By exposing spores in different phases of the germination process to nisin treatment, PEF treatment, or a combination of the two treatments, when loss of PEF resistance and nisin resistance began could be determined. After about 100 min of germination, the numbers of spores declined slightly, possibly indicating that loss of PEF resistance was beginning (data not shown). The experiments were repeated with more pulses (30 instead of 15 pulses) to verify that PEF resistance was lost. In the 6-h germination period, growth was observed after 2.5 to 3 h, and loss of heat resistance indicated that germination was beginning (Fig. 1).

The germinating spores were subjected to PEF treatment, and loss of PEF resistance occurred 50 min after germination began. A clear 0.8-log unit reduction caused by PEF treatment was found. However, not all the germinated spores were inactivated since the reduction caused 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 2 shows that germinating spores were very sensitive to nisin treatment. Immediately at the start of germination, spores became sensitive to nisin, and they were almost as sensitive to nisin as they were to heat treatment. An additional heat treatment did not result in more reduction than the reduction in the heated control samples. This was confirmed by determining the reduction during the first 10 min of germination (data not shown). Smaller amounts of nisin, 0.6 μg/ml (Fig. 3) and 0.3 μg/ml (data not shown), were tested, and similar results were obtained. Combining nisin and PEF treatments resulted in synergistic activity against vegetative cells (30). This synergy was not seen or was not clear when germinating spores were used. When PEF treatment was combined with nisin treatment, the reduction obtained was similar to the reduction obtained with nisin alone. The nisin concentration used resulted in the maximum obtainable reduction, and enhanced inactivation by PEF treatment would have been hard to distinguish. A nisin concentration of 0.3 μg/ml...
still resulted in the maximum reduction achievable. The combination of nisin and PEF treatments also did not damage the spores in such a way that increased susceptibility to heat treatment was observed.

**Influence of preincubation with carvacrol or nisin on sensitivity to nisin, carvacrol, and/or PEF treatment.** Carvacrol is a lipophilic, plant-derived, antimicrobial compound that accumulates in the lipid bilayer and disturbs its functions. Ultee et al. (37) demonstrated that cells of *B. cereus* which were adapted to sublethal concentrations of carvacrol were less sensitive to this compound upon subsequent exposure. Since the primary target of nisin is the cytoplasmic membrane, changes in the membrane caused by other compounds could change the sensitivity of cells to nisin. Cells of *B. cereus* were grown in the presence of 0.3 mM carvacrol at 20°C, harvested in the exponential phase, and subsequently exposed to nisin (0.3 μg/ml) for 30 min. The concentration of carvacrol used was shown to be nonlethal; however, growth was inhibited to a certain extent (29, 37). The viability of control cells exposed to nisin decreased 1 log unit within 30 min. When nisin was added simultaneously with carvacrol (0.3 mM), a synergistic 3-log unit reduction was observed (Fig. 4A). Interestingly, when carvacrol-adapted cells were exposed to the same concentration of nisin, reduction to a value below the detection limit within 15 min was observed (Fig. 4B). This reduction was even larger than the reduction obtained with control cells when both nisin and carvacrol were used, indicating that the increase in sensitivity upon adaptation to carvacrol was great. Even with lower amounts of nisin (0.16 μg/ml) the reduction was larger than that for nisin combined with carvacrol when control cells were used. Decreasing the adaptation concentration of carvacrol to 0.2 mM still allowed 0.16 μg of nisin per ml to cause larger reductions than 0.3 μg of nisin per ml with control cells. These results clearly indicate that adaptation to carvacrol increases the sensitivity of *B. cereus* to nisin dramatically.

Nisin does not accumulate in the cytoplasmic membrane, but the lipid composition of cells grown in the presence of nisin might change in such a way that the sensitivity to carvacrol or nisin is altered. In order to test this hypothesis, cells of *B. cereus* were grown in the presence of 0.3 μg of nisin per ml 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 may adjust their membrane composition to the presence of nisin. However, no changes in the sensitivity to nisin, carvacrol, or a combination of the two compounds could be detected in adapted cells (Fig. 5).

Changes in the composition of the membrane might alter the sensitivity of *B. cereus* to PEF treatment. Carvacrol proved to be a helpful tool for changing 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 the two. The number of survivors was monitored during the treatment (Fig. 6). The sensitivity of adapted cells to PEF treatment was similar to that of nonadapted cells. When nisin treatment was combined with PEF treatment, the adapted cells seemed to be slightly more sensitive than the control cells. Interestingly, the difference was not as great as
activated by PEF treatment unless germination-inducing processes are also used (2, 3, 15, 20). PEF treatment itself is not able to induce germination (3). In contrast, Marquez et al. (23) claimed that they observed direct inactivation of spores by PEF treatment alone. Treated samples at were examined at a magnification of $\times13,000$, and spores had holes, were enlarged, or were completely destroyed. However, these results could not be confirmed in this study.

Germinated spores were inactivated by nisin or PEF treatment to some extent. They lost their PEF resistance 50 min after the onset of germination; however, not all the germinated spores could be inactivated. Conclusions should be drawn with care since germination in all cases was incomplete. The germination media always contained both dormant spores and germinated spores, and at a later stage they also contained 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 inactivation by PEF treatment and determine precisely when loss of PEF resistance begins. Incomplete germination is generally ascribed to the natural biovariability in spore suspensions (4). An alternative explanation could be accumulation of an inhibiting compound that prevents germination of the remaining spores. However, Wuytack (40) was not able to demonstrate the existence of such a compound.

The late loss of PEF resistance can be explained by dependence on degradation of the spore coat. In dormant spores, the changes in the core are not free to migrate according to the electric field but are immobilized by other molecules, such as proteins (7, 14). Furthermore, the cortex and the coat are more rigid than the cytoplasmic membrane of vegetative cells, which makes it more difficult to compress the membrane under the influence of an imposed electric field and create pores. It is thought that PEF treatment acts at a later stage of germination since it requires free movement of charges in order to act (2). Full hydration of the core is dependent on spore coat degradation, which is consistent with the late loss of resistance to PEF treatment.

Germinating spores are immediately inactivated upon exposure to nisin. Even a low concentration (0.3 $\mu$g/ml) inactivated germinated spores to the same extent as heat treatment, suggesting that like loss of heat resistance, loss of nisin resistance is one of the first events in spore germination. Apparently, nisin gains access to the membrane by penetrating the coat, which is more permeable during germination, or alternatively, the protective coat is degraded by spore lytic enzymes, which allows nisin to reach the cytoplasmic membrane. Inactivation of germinated spores by nisin was also observed by Morris et al. (27), who suggested that sulfhydryl groups in the membrane, which are not available in ungerminated spores, are the natural target for nisin and therefore access to the membrane is a prerequisite.

Combining nisin and PEF treatments did not result in additional inactivation or injury which made the germinated spores more sensitive to heat. This finding was attributed to the high level of inactivation caused by nisin itself and the small margin for the observed synergy caused by incomplete germination. In addition, loss of nisin resistance seems to be an early event in spore germination, while loss of PEF resistance occurs only after 50 min of germination. Synergy would therefore be less likely due to the different time scales for the different activities.
One of the main problems associated with the use of antimicrobial compounds is the development of tolerance or resistance (25). One example of this is the increased tolerance of *Listeria monocytogenes* to nisin after repeated exposure to increasing concentrations of nisin (26, 38). In addition, Uldue et al. (37) reported increased resistance of *B. cereus* to carvacrol after adaptation. Both phenomena were explained by the influence of nisin or carvacrol on membrane composition (24, 26, 37, 38). Disturbance of lipid-lipid or lipid-protein interactions by an accumulation of carvacrol in the membrane induces a change in the membrane composition which counteracts this effect (18, 39). The change in fatty acid composition was consistent with decreased membrane fluidity (16, 36, 37), which resulted in limited accumulation of carvacrol in the membrane and thereby decreased the susceptibility of the cells to carvacrol. In this study, however, cells grown in the presence of carvacrol (0.3 mM) became more sensitive to nisin than control cells. A decrease in membrane fluidity is not expected to increase nisin’s activity, 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 activity (5, 9, 38). Uldue et al. (37) detected some additional phospholipids in adapted cells and one missing phospholipid compared to control cells. However, these phospholipids were not identified, and no differences in the relative amounts of phosphatidylethanolamine, diphasphatidylglycerol, and phosphatidylglycerol were detected to explain the increased activity of nisin. Recently, Breukink et al. (6) demonstrated that nisin combines pore-forming activity with high-affinity binding to a peptidoglycan precursor, lipid II. An increase in the lipid II content of cells markedly increased the activity of nisin. Therefore, an alternative explanation for the increased nisin activity against adapted cells might be an 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 is more fragile (3). Surprisingly, cells adapted to carvacrol, which is consistent with a more rigid membrane, did not exhibit decreased susceptibility to PEF treatment. A 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 lead to decreased inactivation by PEF treatment. Obviously, other factors also play a role in PEF sensitivity. The increase in membrane fluidity described by the authors mentioned above is caused by a temperature-induced shift (physical process) and not by a change in membrane composition (chemical process) as described in this paper. Surprisingly, the observed synergy between nisin and PEF treatments was not influenced by a change in membrane fluidity and membrane composition. The mechanism of synergy between nisin and PEF treatments is not understood yet; apparently other cytoplasmic membrane factors may influence the observed synergy.

In conclusion, spores of *B. cereus* are rather resistant to nisin and/or PEF treatment and can be inactivated only after germination begins. The nisin resistance of spores is lost very early in germination, suggesting that access to the membrane early in germination is important. Resistance to PEF treatment was lost at a later stage of germination. The different time scales might explain the absence of synergy between nisin and PEF treatments when they are used against germinated spores. Changing the membrane composition and, subsequently, the membrane fluidity by growing cells in the presence of carvacrol resulted in a dramatic increase in nisin sensitivity; however, the efficiency of PEF treatment was not increased. The effect of a change in membrane composition caused either by adaptation to carvacrol or other components or by temperature on PEF treatment efficiency is not clear and should receive more attention, since microorganisms in foods generally adapt to their environments. Combination techniques are a welcome alternative to currently used pasteurization methods, especially when there is synergy between techniques that allows reductions in the intensities used. Nisin and PEF treatments could be integrated and become key elements in newly designed preservation strategies provided that effective measures to activate dormant spores are used.

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