In Situ Determination of *Clostridium* Endospore Membrane Fluidity during Pressure-Assisted Thermal Processing in Combination with Nisin or Reutericyclin

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This study determined the membrane fluidity of clostridial endospores during treatment with heat and pressure with nisin or reutericyclin. Heating (90°C) reduced laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) general polarization, corresponding to membrane fluidization. Pressure (200 MPa) stabilized membrane order. Reutericyclin and nisin exhibit divergent effects on heat- and pressure-induced spore inactivation and membrane fluidity.

Pressure-assisted thermal sterilization (PATs) of food is an alternative to thermal processing (1). PATs releases dipic-

olinic acid (DPA) from endospores, rehydrates the core of endospores, and allows for spore inactivation (2, 3, 4). A combination of 200 to 800 MPa with 120°C eliminates resistant spores of *Clostridium botulinum* (2, 5). At or above 120°C, however, pressure may fail to accelerate thermal inactivation or even exerts protective effects (2, 5). Antimicrobials acting in concert with PATs may enhance the inactivation of endospores, thus ensuring product safety at reduced treatment intensity. Nisin and reutericyclin exhibit antimicrobial activity against endospores (6, 7). Nisin is a pore-forming lantibiotic (6), reutericyclin is a proton ionophore (8). Remarkably, nisin enhances pressure-induced spore inactivation (9, 10), whereas reutericyclin had no effect or even attenuated pressure-induced inactivation of bacterial endospores (11).

Endospores have multiple, distinct layers that contribute to resistance and metabolic dormancy (2, 12, 13). Dehydration of the spore core contributes to endospore resistance (12). Endospores possess an outer membrane, a remnant of sporulation, and an inner membrane separating the dehydrated core from the hydrated exterior (14, 15). Lipids of the inner membrane are compressed, and the surface area expands during germination without lipid synthesis (16). Disruption of the inner membrane of endospores rehydrates the core and allows inactivation of endospores by antimicrobials (17, 18). An improved understanding of the effect of pressure on endospore membranes will improve the control of endospores by pressure and facilitate the selection of antimicrobials to support pressure-assisted sterilization; however, little is known about the

FIG 1 Second derivative FT-IR spectra of laurdan-labeled endospores of *C. sporogenes* heated to 90°C. Samples were heated in the absence of antimicrobials (A), in the presence of 16 mg liter⁻¹ nisin (B), or in the presence of 6.4 mg liter⁻¹ reutericyclin (C). Measurements were taken every 10 min as samples were heated to 90°C at a rate of 5°C/10 min. The graph highlights the area corresponding to the CH₂ asymmetrical stretching absorbance; i and ii denote the wavenumbers corresponding to gel state and liquid-crystalline membranes, respectively. Comparable results were obtained with *C. beijerinckii* (data not shown).

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FIG 2 Generalized polarization of laurdan-labeled endospores of *C. sporogenes* (A and B) and *C. beijerinckii* (C and D) standardized to an optical density at 600 nm of 0.5 and treated at 90°C (A and C) or 90°C and 200 MPa (B and D). Samples were treated in the presence of 6.4 mg liter⁻¹ reutericyclin (red lines) or 16 mg liter⁻¹ nisin (blue lines) or in the absence of antimicrobials (black lines). Measurements were taken every 12 s during treatments. Each value is the average of 10 measurements. Dotted lines indicate treatment conditions as follows: (A and C) i, heating to 90°C; ii, hold at 90°C; iii, cooling to 4°C and refrigerated storage; iv, rescan after overnight storage at -20°C; (B and D) i, compression to 200 MPa; ii, heating to 90°C; iii, hold at 200 MPa, 90°C; iv, cooling to 4°C and hold at 4°C and 200 MPa; v, measurements following decompression and overnight storage at -20°C.

behavior of membranes of endospores during pressure processing.

This study examined the effects of pressure and temperature in combination with nisin or reutericyclin on the membrane fluidity of endospores of *Clostridium* spp. Nisin and reutericyclin were selected because they are both membrane active but differ in their modes of action and their effects on spore survival after heat or pressure treatment (7, 8, 11). Nisin and reutericyclin were applied at 16 and 6.4 mg liter⁻¹, respectively, concentrations that exceed their MICs 16-fold. Experiments employed *Clostridium sporogenes* ATCC 7955 and *Clostridium beijerinckii* ATCC 8260, which were previously characterized with respect to spore inactivation and DPA release by heat and pressure (11). Membrane fluidity changes and phase transitions were assessed with the fluorescent dye laurdan (6-dodecanoyl-2-dimethylaminonaphthalene). The laurdan general polarization (GP) values indicate membrane fluidity of bacterial cells and endospores (19, 20). Laurdan was previously used to characterize the response of bacterial membranes to high pressure, nisin, and reutericyclin (21, 22). Fourier transform infrared (FT-IR) spectroscopy scans of cells complemented information on membranes (22, 23). For details of the experimental protocol used for the *in situ* assessment of membrane fluidity, see the supplemental material.

Analysis of spore membranes with FT-IR spectroscopy of endospores. FT-IR spectroscopy of clostridial endospores examined changes in the hydrocarbon chain region of membranes during

heating to 90°C. The symmetrical CH₂-stretching mode at ~2,850 cm⁻¹ was analyzed (23, 24). FT-IR spectra were recorded and analyzed after drying of spores on a CaF₂ window as described previously (25). During treatment, spores of both *Clostridium* spp. exhibited a shift at wavenumber 2,849 cm⁻¹ to 2,853 to 2,854 cm⁻¹ (Fig. 1 and data not shown), indicating a membrane phase transition from a gel phase to the liquid-crystalline phase.

***In situ* GP measurements of endospores.** GP values of laurdan-labeled endospores (20) exposed to nisin or reutericyclin at 90°C or 90°C and 200 MPa were measured *in situ* to assess membrane fluidity during treatments (26). Samples were sealed in quartz vials, placed in a custom pressure vessel, and heated at ambient pressure or after compression to 200 MPa. Heating to 90°C decreased GP values, corresponding to a phase transition from the gel phase to the liquid-crystalline phase (Fig. 2A and C). The GP values of the samples treated at 90°C did not return to the initial levels upon cooling (Fig. 2A and C). Higher GP values were maintained in the presence of nisin than in control samples and samples with reutericyclin. Heating to 90°C at 200 MPa also decreased the GP values of both *Clostridium* spp., but GP values remained high and indicative of a gel phase membrane (Fig. 2B and D).

Effects of heat, pressure, and antimicrobials on spore membrane properties. FT-IR and laurdan measurements confirm a gel state of inner membranes of endospores (16, 20). A phase shift toward a liquid-crystalline phase was observed after heating, in keeping with the effects of heat and pressure on model membranes

FIG 3 GP of laurdan-labeled endospores of *C. sporogenes* (triangles) and *C. beijerinckii* (circles) standardized to an optical density at 600 nm of 0.5 and treated at 90°C (A) or 90°C and 600 MPa (B). Samples were treated in the presence of 6.4 mg liter⁻¹ reutericyclin (gray) or 16 mg liter⁻¹ nisin (white) or in the absence of antimicrobials (black). A treatment time of 0 min corresponds to the placement of samples into the 90°C bath and immediate withdrawal (A) or compression to 600 MPa, followed by immediate decompression to 0.1 MPa and cooling (B). Measurements were taken after the samples had been held overnight at 4°C. Each value is the average $\pm$ standard deviation of three measurements. Viable-spore counts were obtained by using the same strains and identical treatment conditions (11).

(27). Changes in the GP during the heating of laurdan-labeled endospores persisted after decompression and overnight storage, suggesting that treatment at 90°C altered the ordered state of the inner membrane of endospores. This effect may relate to the heat activation of spore germination (28). *In situ* GP measurements of endospores heated to 90°C at 200 MPa highlight the antagonistic effect of pressure on fluidization of membranes. A high degree of order, consistent with gel-state membranes, was maintained for all treatments of both *Clostridium* spp. Nisin and reutericyclin exerted divergent effects on inner membrane fluidity. Nisin forms tetrameric pores (7) and increased resistance to membrane fluidization by treatment at 90°C, thereby introducing order to a membrane. Reutericyclin preferentially interacts with the hydrophobic interior of lipid bilayers and increases the membrane fluidity of *Lactobacillus reuteri* (21). Reutericyclin counteracted the ordering effect of pressure but not to an extent that caused a deviation from a gel state membrane during treatment at 200 MPa.

Correlation of membrane properties to spore inactivation.

GP values of laurdan-labeled endospores exposed to nisin or reutericyclin at 90°C or 90°C and 600 MPa were measured *ex situ* to relate membrane properties to spore inactivation. Spore preparation, treatments, and GP measurements were performed as described previously (11, 20). Treatment of spores at 90°C for 8 min reduced viable spore counts by less than 1 log CFU ml⁻¹, and the spores released less than 5% DPA (11) but GP values of clostridial endospores were lowered (Fig. 3). Reutericyclin produced GP values lower than those of controls, but high GP values were maintained in the presence of nisin. Treatment of clostridial endospores at 600 MPa and 90°C for 8 min reduced viable spore counts by more than 3 log CFU ml⁻¹ and resulted in the release of more than 95% DPA for all treatments (11) but did not affect GP values (Fig. 3).

Spores thus retained DPA and remained viable after heating, indicating that the core remained dehydrated despite the heat-induced membrane phase transition to the liquid-crystalline phase. Heating in the presence of nisin reduced spore counts by 90% (11) but also mitigated thermal effects on membrane fluidity (this study). Conversely, 90°C and 600 MPa treatments released DPA from clostridial endospores and inactivated a majority of the spore population (11) but did not severely alter membrane rigid-

ity (this study). The presence of nisin during treatment at 90°C and 600 MPa did not affect spore membranes (this study) but accelerated endospore inactivation (11). Addition of reutericyclin resulted in more-fluid membranes during and after high-pressure thermal processing (this study) but did not consistently enhance spore inactivation (11). Taken together, these findings suggest that pressure-mediated spore inactivation and release of DPA do not require disturbance of the highly ordered state of endospore membranes.

In conclusion, high pressure counteracts the fluidizing effects of heat on the inner membrane of endospores. The antimicrobials nisin and reutericyclin exert opposite effects on the membrane fluidity of endospores. Reutericyclin increased disorder within membranes during thermal and combined thermal and high-pressure treatments. Nisin facilitates a return to a highly ordered membrane state following high-pressure thermal processing. These findings demonstrate that endospores can be inactivated by heat and pressure without altering the highly ordered state of the membrane. Moreover, the divergent effects of reutericyclin and nisin on spore membrane fluidity provide a rationale for their divergent effects on heat- and pressure-induced spore inactivation and DPA release.

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