1 # Effects of Nisin and Reutericyclin on Resistance of Endospores of
2 # *Clostridium* spp. to Heat and High Pressure

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18

19 ## Abstract

20 The effects of high pressure, temperature, and antimicrobial compounds on
21 endospores of *Clostridium* spp. were examined. Minimal inhibitory concentrations (MIC)
22 of nisin and reutericyclin were determined for vegetative cells and endospores of *C.*
23 *sporogenes* ATCC 7955, *C. beijerinckii* ATCC 8260, and *C. difficile* 3195. Endospores
24 of *C. sporogenes* ATCC 7955 and *C. beijerinckii* ATCC 8260 were exposed to 90°C and
25 90°C/600 MPa in the presence of 16 mg L^{-1} nisin or 6.4 mg L^{-1} reutericyclin for 0 to 60
26 min in a 0.9% saline solution. Dipicolinic acid (DPA) release was measured using a
27 terbium-DPA fluorescence assay, and endospore permeability was assessed using 4',6-
28 diamidino-2-phenylindole (DAPI) fluorescence. Vegetative cells of *C. sporogenes* ATCC
29 7955 exhibited higher sensitivity to nisin relative to endospores, with MIC values 0.23 ±
30 0.084 mg L^{-1} and 1.11 ± 0.48 mg L^{-1}, respectively. Nisin increased DPA release when
31 endospores were treated at 90°C; however, only *C. sporogenes* ATCC 7955 exhibited
32 higher inactivation, suggesting strain or species specific effects. Reutericyclin did not
33 enhance spore inactivation or DPA release. Use of nisin in combination with high
34 pressure, thermal treatments enhanced inactivation of endospores of *Clostridium* spp. and
35 may have application in foods.

36

37 **Keywords:** *Clostridium*, dipicolinic acid, high-hydrostatic pressure, nisin, reutericyclin

38

39 ## 1. Introduction

40 Bacterial endospores are a dormant form of bacteria ubiquitous in the
41 environment. Endospores are composed of multiple layers that confer resistance to
42 adverse conditions, including UV radiation, heat, and chemicals (Beaman and Gerhard,
43 1986; Esty and Meyer, 1922; Mazotta et al., 1997; Setlow, 2006). Endospore dormancy
44 can last millions of years (Cano and Borucki, 1995). Germination and outgrowth of
45 endospores can be induced by exposure to nutrients, muropeptides, chemicals, high
46 temperature, and pressure (Moir, 2006; Setlow, 2003; Shah et al., 2008). Within the core
47 of an endospore, dipicolinic acid (DPA) forms a complex with divalent cations that
48 excludes water, which in turn contributes to thermal resistance (Gerhardt and Marquis,
49 1989; Setlow, 2006). As an endospore germinates, DPA is released from its core; DPA is
50 also released when an endospore's structural integrity is compromised by chemicals,
51 heat, or high pressure (Setlow, 2003). Measurement of DPA has been used to examine
52 the inactivation kinetics of endospores of *Bacillus* spp. following thermal and pressure
53 treatments. Loss of DPA contributes to the loss of endospore thermal resistance (Kort et
54 al., 2005; Margosch et al., 2004a).

55 The ubiquity and inherent resistance of endospores makes them a safety concern
56 for food processors. *Clostridium botulinum* is the most significant threat (Collins and
57 East, 1998; Esty and Meyer, 1922; Margosch et al., 2004b). The use of thermal
58 processing to eliminate endospores in foods compromises organoleptic properties. High-
59 hydrostatic pressure (HHP) has shown promise as an alternative for inactivating
60 endospores. Pressure-assisted thermal processing (PATP) makes use of pressure ranging
61 from 200 to 800 MPa, in combination with heat to enhance endospore inactivation (Ahn

62 et al., 2007; Gao and Yu, 2008; Reddy et al., 2003). However, a reduction of resistant
63 clostridial endospores by 5 log (cfu g^{-1}) is achieved only by processes that operate at high
64 pressure in combination with 120 °C (for review, see Black et al., 2007).

65 Application of antimicrobial compounds, such as nisin and the tetramic acid
66 reutericyclin (Gänzle et al., 2000; Lubelski et al., 2008), in combination with moderate
67 heat and pressure may be suitable to achieve minimal processing of foods and control of
68 endospore outgrowth and viability. Nisin, a pore-forming lantibiotic produced by
69 *Lactococcus lactis*, enhances inactivation of *Bacillus* endospores by heat and pressure
70 (Lopez-Pedemonte et al., 2003; Roberts and Hoover, 1996). Reutericyclin is a tetramic
71 acid that acts as a proton-ionophore with potent bactericidal activity against Gram-
72 positive organisms, including endospores of *Bacillus* spp. and *Clostridium* spp. (Gänzle,
73 2004; Hurdle et al., 2011). However, few data are available on the inactivation of
74 clostridial endospores by combined application of nisin, pressure, and temperature (Gao
75 and Ju, 2008) and effects of reutericyclin have not been evaluated.

76 The aim of this study was to examine the effects of high-pressure, temperature,
77 and nisin or reutericyclin on survival of endospores of selected *Clostridium* spp. *C.*
78 *sporogenes* ATCC 7955 was chosen for its heat-resistant endospores and genetic
79 similarity to *C. botulinum* Group I (Collins and East, 1998); *C. beijerinckii* ATCC 8260
80 was chosen due to its ability to grow at refrigeration temperature (4 °C) (Broda et al.,
81 1996); *C. difficile* 3195 is a gastrointestinal human pathogen. *B. amyloliquefaciens* FAD
82 11/2 was used as a pressure-resistant reference strain (Margosch et al., 2006). Nisin and
83 reutericyclin were selected as antimicrobials that target cell membranes but have different
84 modes of action. Nisin is not inactivated by heat or pressure; reutericyclin is a heat- and

85 pressure stable tetramic acid derivative (Gänzle et al., 2000). Loss of DPA from
86 endospore populations was quantified by fluorescence spectroscopy in the presence of
87 terbium (Tb) ions, which form stable fluorescent complexes (Kort et al., 2005). 4’6-
88 Diamidino-2-phenylindole (DAPI) was used to determine the accessibility of endospore
89 DNA to chemical compounds (Den Blaauwen et al., 1999).
90

91 **2. Materials and Methods**

92

93 *2.1. Strains, growth and sporulation conditions*

94

95 *Clostridium beijerinckii* ATCC 8260, *Clostridium difficile* 3195 (CanBiocin Inc.,
96 Edmonton, AB), *Clostridium sporogenes* ATCC 7955, and *Bacillus amyloliquefaciens*
97 FAD 11/2 were inoculated from endospore stocks, maintained at -80 °C in 60% glycerol
98 in sterile water, into Reinforced Clostridial Medium (RCM; Difco, Becton, Dickinson
99 and Company, Sparks, USA) broth and incubated anaerobically (*Clostridium* spp.) or
100 aerobically (*B. amyloliquefaciens* FAD 11/2) at 37 °C. Weihenstephan Südhang (WSH)
101 medium was prepared as described by Margosch et al. (2004a). Anaerobic handling of
102 cultures was performed in an anaerobic chamber. Cultures were sporulated by plating
103 onto the surface of WSH agar that was incubated anaerobically or aerobically at 37 °C.
104 Following 14 d of incubation, sporulation was confirmed by phase-contrast microscopy.
105 Endospores were harvested from the surface of WSH agar plates by washing the surface
106 of the plates with sterile 0.9% saline. Harvested endospores were washed five times by

107 centrifugation at 2,700 x *g* and re-suspended in sterile 0.9% saline. Endospore
108 suspensions were standardized to OD_{600} values of 0.5, 1.5, and 2.0 in sterile 0.9% saline.
109 Endospore suspensions were plated onto RCM agar and incubated anaerobically or
110 aerobically at 37 °C for 6 d to verify that increasing OD_{600} values corresponded to an
111 increase in the density of endospore populations. An OD_{600} of 2.0 equated to
112 approximately 10^8 endospores/ml for each *Clostridium* spp. and *B. amyloliquefaciens*
113 FAD 11/2. Endospore stocks were stored at -20 °C. Sterile 0.9% saline was used as the
114 endospore suspension medium throughout subsequent experiments.

115

116 *2.2. Minimum inhibitory concentration (MIC)*

117

118 Nisin (Chrisaplin, Chr. Hansen HS, Hørsholm, Denmark) was diluted in aqueous
119 0.05% acetic acid and stored at -20 °C. Reutericyclin was purified by solvent extraction
120 from the culture supernatant of *Lactobacillus reuteri* LTH2584 as described by Gänzle et
121 al. (2000), and stored in 80:20 isopropanol:water solution at -20 °C. The concentration of
122 reutericyclin was determined by a critical dilution assay using *Lactobacillus*
123 *sanfranciscensis* as an indicator strain (Gänzle et al., 2000). Critical dilution assays
124 (Gänzle et al., 2000) were used to determine the MIC of nisin and reutericyclin against
125 germination of endospores or growth vegetative cells of *Clostridium* spp. MIC
126 procedures were carried out in an anaerobic hood. In brief, 100 µl of RCM broth was
127 added to each well of a microtitre plate. Nisin or reutericyclin stock solutions (100 µl)
128 were added to separate wells and serially diluted. Stationary phase cultures of
129 *Clostridium* spp. or endospore suspensions with an OD_{600} 2.0 were diluted to one-tenth of

130 the initial concentration in RCM broth, and microtitre plates were inoculated with 50 µl
131 of the diluted culture. Plates were incubated anaerobically at 37 °C for 24 h (vegetative
132 cells) or 6 d (endospores).

133

134 *2.3. Thermal treatment of endospores*

135

136 Nisin and reutericyclin were mixed with endospore suspensions (OD_{600} 2.0) at
137 16X the endospore MIC values (16 mg L^{-1} and 6.4 mg L^{-1}, respectively). Endospore
138 suspensions with antimicrobials were heat-sealed in borosilicate glass capillary tubes
139 (Disposable Micropipets, Fisher Scientific, Toronto, Canada) and kept on ice overnight
140 until treatment. Thermal treatments were performed in an oil bath. Samples were placed
141 into pre-heated canola oil at 90˚C, 100˚C, and 120 ˚C for up to 60 min. Initiation of
142 thermal treatment of samples was taken as the moment samples were placed into pre-
143 heated oil, and designated as 0 min treatment time. Samples were stored on ice for <60
144 min following treatment until extracted from glass capillaries. Samples were plated onto
145 RCM agar, and incubated anaerobically or aerobically at 37 °C for 6 d.

146

147 *2.4. High hydrostatic pressure (HHP) treatment of endospores*

148

149 Nisin and reutericyclin were mixed with endospore suspensions (OD_{600} 2.0) at
150 16X the endospore MIC values (16 mg L^{-1} and 6.4 mg L^{-1}, respectively). Mixed solutions
151 were heat-sealed in Tygon tubing (Application Specific Tubing, Saint-Gobain

152 Performance Plastics, Pittsburg, USA) using a hair-straightener (TONI&GUY®, Model
153 TGST2976F, London, UK). Samples were kept on ice overnight until treatment. To
154 achieve isothermal conditions during compression and pressure holding times, adiabatic
155 heating during compression was compensated for in accordance with heating curves of
156 pressurized water. Sealed samples were preheated in a water bath to a temperature
157 corresponding to (target temperature – adiabatic heating). For treatments at 90 °C,
158 samples were conditioned at 74 °C for 5 min prior to pressurization. Samples were placed
159 in vessels of a U111 Unipress (Warsaw, Poland) conditioned at 90 °C, compressed to 600
160 MPa in 1 min, held at this pressure and temperature for up to 60 min, and decompressed
161 in 30 sec or less. Initiation of high-pressure thermal treatment of samples was taken as the
162 moment samples were fully compressed. Time at which compression was achieved was
163 designated as 0 min treatment time. Pressure vessels had dimensions of 12 x 58 mm.
164 Temperature control of the pressure vessels was achieved using an external circulating
165 propylene glycol system (LAUDA Proline, Delran, USA). Samples were stored on ice for
166 < 1 h following treatment, plated onto RCM agar, and incubated 37 °C for 6 d.

167

168 *2.5. Terbium-DPA fluorescence assay*

169

170 The protocol described by Kort et al. (2005) was adapted to a 96-well microtitre
171 plate. Control samples to determine maximum DPA release were obtained for each
172 endospore crop using samples heated at 120 °C for 60 min. Standardized endospore
173 suspensions were subjected to HHP or thermal treatments as described above. Samples
174 were centrifuged (10,600 x *g* for 4 min.) to remove insoluble material. Following

175 centrifugation, supernatants were transferred into a 96-well microtitre plate and mixed
176 with an equal volume of 20 mM terbium chloride in Tris buffer (pH 7.5). Fluorescence
177 measurements were done in a spectrofluorometer (Varioskan Flash, Thermo Electron
178 Corporation, Nepean, Canada) with excitation and emission wavelengths of 270 and 545
179 nm, respectively. Results were calculated as % DPA release relative to control samples
180 prepared from the same endospore suspension. Fluorescence was recorded as relative
181 fluorescence units (RFU).

182 Terbium-DPA fluorescence assays were done to establish a relationship between
183 endospore density and % DPA release following lethal thermal treatments of endospores.
184 Suspensions of *C. beijerinckii* endospores, standardized to OD_{600} values of 0.25, 0.5, 1.0,
185 and 2.0, were heated at 120 °C for 60 min. Increasing OD_{600} values resulted in a
186 corresponding increase of the amount of DPA released from the endospores (data not
187 shown). Treatment of endospores at 120 ºC caused a near complete DPA release in under
188 a minute for all three *Clostridium* spp. (data not shown).

189

190 *2.6. 4',6-Diamidino-2-phenylindole (DAPI) fluorescence assay*

191

192 Endospores were dyed with DAPI as outlined by Den Blaauwen et al (1999).
193 Samples were immobilized on glass slides using 2% agarose (Sigma-Aldrich, St Louis,
194 MO). Visualization was done using a fluorescence microscope (Zeiss AXIOVision
195 Imager, Toronto, Canada) with a Hoechst filter set (Ex. 365 nm; Em. 445 ± 50 nm). The
196 fluorescence intensity of individual endospores was measured and integrated in

197 quintuplicate per treatment (data not shown). All sample measurements were calibrated
198 against background intensities. Vegetative cells dosed with DAPI were used as controls
199 for maximal fluorescence intensity.

200

201 **3. Results**

202

203 *3.1. MICs of nisin and reutericyclin for vegetative cells and endospores of* Clostridium
204 *spp.*

205 The MIC of nisin and reutericyclin against *Clostridium* spp. vegetative cells and
206 endospores was determined. The concentration of nisin required for inhibition of
207 vegetative cells was similar to that required for inhibition of endospores of all
208 *Clostridium* spp., except *C. sporogenes* (Table 1). There was no significant difference in
209 the MIC of reutericyclin between vegetative cells and endospores of *Clostridium* spp.
210 The MIC of reutericyclin against *Clostridium* spp. endospores and vegetative cells were
211 lower than those of nisin, except for vegetative cells of *C. sporogenes* (Table 1). In
212 subsequent experiments with high temperature / high pressure treatments, reutericyclin
213 and nisin were used at a concentration exceeding their MIC towards endospores 16-fold,
214 corresponding to about 5 μM nisin and 18 μM reutericyclin, respectively.

215

216 *3.2. Heat resistance of endospores*

217

218 At 100 ºC *C. beijerinckii* and *C. difficile* endospores were inactivated within 25
219 min of treatment (Figure 1). Near-maximal DPA release was achieved for *C. difficile* and
220 *C. beijerinckii* after 60 min. The heat inactivation profile for *C. sporogenes* exhibited a
221 tailing effect. In comparison to the other strains, *C. sporogenes* exhibited a higher
222 resistance and a higher level of DPA release at 100 ºC with a near-maximal DPA release
223 occurring at 30 min. *C. sporogenes* and *C. beijerinckii* were selected for subsequent
224 experiments to determine the combined sporicidal effects of high temperature, high
225 pressure, and antimicrobial compounds. *C. difficile* was omitted from further
226 investigation as its response to heat was similar to other *Clostridium* spp.

227

228 *3.3. Inactivation of endospores by high temperature and antimicrobials*

229

230 The effects of combined thermal and antimicrobial treatments on endospore
231 inactivation and DPA release were determined and compared to the effects of thermal
232 treatments alone. No increased inactivation of *C. beijerinckii* endospores was observed
233 when heated in combination with nisin and 90 ºC relative to 90 ºC alone, but an increase
234 in % DPA released was observed (Figure 2A). The application of nisin and heat at 90 ºC
235 to endospores of *C. sporogenes* resulted in a more rapid inactivation in comparison to
236 that observed for endospores of *C. sporogenes* at 90 ºC without nisin, and in comparison
237 to *C. beijerinckii* (Figure 2). Percentage of DPA release was higher when endospores
238 were treated with a combination of nisin and 90 ºC relative to 90 ºC alone. Addition of
239 reutericyclin to endospores of *C. sporogenes* and *C. beijerinckii* had no effect on DPA
240 release or endospore inactivation (Figure 2).

241

242 *3.4. Inactivation of endospores by high-pressure, high temperature, and antimicrobials*

243

244 Combined treatments of high-pressure, thermal, and antimicrobials were done to
245 determine the effects on endospore inactivation and DPA release for comparison to
246 thermal treatments. In the absence of antimicrobials, both *C. sporogenes* and *C.*
247 *beijerinckii* endospores treated at 600 MPa and 90 ºC were inactivated more rapidly
248 compared to endospores heated at 100 ºC (Figure 1) or 90 ºC (Figure 2). Both
249 *Clostridium* spp. exhibited a complete release of DPA in the time it took the high-
250 pressure equipment to ramp up to 600 MPa (1 min) (Figure 3).

251 Nisin and reutericyclin initially accelerated inactivation of *C. beijerinckii*
252 endospores at 600 MPa and 90 ºC, but both compounds induced a tailing effect (Figure
253 3A). Addition of nisin resulted in a more rapid inactivation of *C. sporogenes* at 600 MPa
254 and 90 °C relative to 90 ºC alone (Figure 3B). The presence of reutericyclin produced a
255 tailing effect in the inactivation of *C. sporogenes* endospores (Figure 3B).

256 Because the effects of nisin and reutericyclin on endospore survival appeared to
257 be species- or strain-specific, the effect of these antimicrobials on *B. amyloliquefaciens*
258 FAD 11/2 was assessed (Figure 4). *B. amyloliquefaciens* was more resistant to heat or
259 pressure than any of the *Clostridium* spp. investigated in this study. Reutericyclin had no
260 effect on endospore survival at 90 °C, or at 600 MPa and 90 °C. Nisin accelerated
261 endospore inactivation at 90 °C, and at 600 MPa and 90 °C when compared to treatments
262 with reutericyclin or without antimicrobials. In comparison to treatments at 90 °C,

263 pressure accelerated endospore inactivation in the absence of antimicrobials or in the
264 presence of reutericyclin. However, relative to 90 °C, pressure application did not
265 accelerate endospore inactivation when nisin was present.

266

267 *3.5. DAPI fluorescence in treated endospores*

268

269 To determine whether the divergent effects of nisin and reutericyclin on heat- and
270 pressure-mediated spore inactivation relate to membrane permeabilization, endospore
271 permeability was assayed by determination of the accessibility of DAPI to endospore
272 DNA. Untreated endospores of *C. sporogenes* and *C. beijerinckii* did not stain with
273 DAPI, indicating that endospores were impermeable to DAPI (Figure 6 and data not
274 shown). *C. sporogenes* and *C. beijerinckii* endospores fluoresced after all treatments at 90
275 ºC and 600 MPa (Figure 5A). An average value of 76 ± 19% and 70 ± 23% fluorescence
276 intensity was recorded for endospores relative to vegetative cells of *C. sporogenes* and *C.*
277 *beijerinckii*, respectively. The observation of 100% fluorescence intensity in vegetative
278 cells was not expected, as endospores are markedly smaller than vegetative cells.
279 Following treatment at 90 ºC for 0 to 60 min, endospores of both *C. sporogenes* and *C.*
280 *beijerinckii* did not exhibit increased fluorescence after being exposed to DAPI (Figure
281 5B and data not shown). No fluorescence was observed among samples heated at 90 ºC in
282 the presence of reutericyclin, or in the control samples. However, increased fluorescence
283 was observed in endospores treated at 90 ºC in the presence of nisin (Figure 5B),
284 indicating that nisin increased the permeability of the endospore to DAPI.

285

286 4. Discussion

287

288 This study established that the membrane-active antimicrobial agents nisin and
289 reutericyclin have divergent effects on the heat or heat and pressure mediated inactivation
290 of clostridial endospores. Release of DPA from clostridial endospores, as well as their
291 permeability to DAPI, indicated that this divergent activity of nisin and reutericyclin was
292 related to their mode of action.

293 Sensitivity of clostridial cells and endospores to nisin falls within the previously
294 observed MIC range for *Clostridium* spp. endospores, ranging from approximately 0.17
295 mg L^{-1} to 59 mg L^{-1} (Bartoloni et al., 2004; Mazotta et al., 1997; Megrous et al., 1999;
296 Rayman et al., 1981). The MICs of reutericyclin are comparable to MICs of *Bacillus*
297 spp., *C. difficile,* and other Gram-positive bacteria (Gänzle, 2004; Hurdle et al., 2011). In
298 keeping with previous data, reutericyclin sensitivity of endospores was comparable to
299 vegetative cells (Gänzle et al., 2000). Remarkably, the MIC of reutericyclin towards *C.*
300 *difficile*, 1 µM or less, was substantially lower when compared to the MIC of synthetic
301 tetramic acids, 30 µM, probably reflecting the higher hydrophobicity of reutericyclin
302 (Gänzle et al., 2000; Ueda et al., 2010).

303 Endospores of *Bacillus* spp. release DPA during thermal treatments or high
304 temperature / high pressure treatments at 98 °C to 140 °C, and measurement of DPA
305 release was proposed as a rapid screening tool for the heat resistance of *Bacillus*
306 endospores (Koet et al., 2005; Margosch et al., 2004a). However, pressure-induced

307 release of DPA from endospores of *C. botulinum* was observed only after inactivation of
308 more than 90% of the endospore population (Margosch et al., 2004b). Release of DPA
309 from *C. beijerinckii* and *C. sporogenes* endospores at 90 °C in this study was quite
310 comparable (30 and 50% loss of DPA, respectively, after 60 min treatments). However,
311 sporicidal effects of the same treatments differed by 4 log (cfu g^{-1}). The current results do
312 not support the use of DPA release profiles as a measure of endospore inactivation for
313 comparison between strains. However, they do allow for intra-strain comparison of the
314 effects of high temperature or high temperature / high pressure on endospore inactivation.
315 *C. sporogenes* and *C. beijerinckii* endospores released DPA after treatment at 90 °C and
316 600 MPa with or without antimicrobials; these treatments also permeabilised endospore
317 walls to DAPI. Treatments at 90 °C without nisin did not allow access of DAPI to the
318 endospore cytoplasm, and did not result in an appreciable DPA release. The effect of
319 nisin on endospore permeability to DAPI corresponded to its effect on DPA release and
320 spore inactivation, and indicates pore formation in membranes of resting spores.

321 Addition of nisin enhanced inactivation of clostridial endospores in both thermal,
322 and high temperature / and high-pressure treatments, in keeping with literature data (Gao
323 and Yu, 2008; Lopez-Pedemonte et al., 2003; Roberts and Hoover, 1996), and the
324 expectation that a combination of several antagonistic principles (nisin, heat, pressure)
325 leads to additive or synergistic effects. Nisin activity against endospores at high
326 temperature conditions was reflected by accelerated release of DPA, and an increased
327 DAPI uptake (Figures 3 and 6). The magnitude of the combined effect of nisin was
328 strongly dependent on the species and decreased in the order *B. amyloliquefaciens* > *C.*
329 *sporogenes* > *C. beijerinckii*. For endospores of *C. beijerinckii*, the initial synergistic

330 inactivation effect of nisin with high temperature / high pressure was followed by
331 antagonistic effects and tailing during later stages of the pressure-death time curves.

332 In contrast to nisin, and in contrast to the expectation that the combination of
333 several antagonistic principles enhance sporicidal or bactericidal effects, reutericyclin did
334 not enhance endospore inactivation, DPA release, or permeability to DAPI by high
335 temperature or high temperature / high pressure treatments. Reutericyclin protected
336 endospores of *C. sporogenes* during high temperature or high temperature / high pressure
337 treatments, confirming interaction of the antimicrobial compound with the endospore
338 envelope previously shown using the fluorescent membrane-prove LAURDAN
339 (Hofstetter et al., 2012). Nisin and reutericyclin target cell membranes, but exert
340 antimicrobial activity by different mechanisms. Nisin forms pores of a size expected to
341 accommodate entry of water or DAPI, and the release of DPA (Lubelski et al., 2008),
342 whereas reutericyclin acts as a proton ionophore (Gänzle, 2004). Synergistic effects of
343 nisin, pressure, and temperature on spore inactivation indicate that nisin interacts with
344 membranes of resting endospores. Ionophore activity does not directly facilitate DPA
345 release and DAPI uptake by endospores. Moreover, the assay systems used here and
346 elsewhere (Gänzle et al., 2000) to determine reutericyclin activity against germinating
347 endospores does not determine whether reutericyclin is active against resting endospores.
348 The mode of action of ionophores necessitates translocation of ions from the outer leaflet
349 of the cytoplasmic membrane to the inner leaflet. Translocation may be inhibited by
350 biophysical properties of the endospores' inner membrane. Lipids of endospore inner
351 membranes are known to be in a highly compressed and immobile state (Cowan et al.,
352 2004).

In conclusion, endospore inactivation by high temperature or high temperature / high pressure processes may be accelerated by membrane-active antimicrobials. However, the effect of antimicrobial compounds present during high temperature / high-pressure treatments on endospore inactivation appears to depend on their mode of action. Nisin, an antimicrobial that facilitates the release of DPA via pores, accelerated endospore inactivation but reutericyclin, a proton ionophore, has no effect or antagonistic effects. Remarkably, the effect of nisin was most pronounced for pressure and heat resistant *B. amyloliquefaciens* endospores. The development of high pressure / high pressure processes for food preservation will benefit from insights into mechanisms responsible for endospore resistance. With a more definitive understanding of endospore physiology, particularly the properties of endospore membranes and responses to processing, highly specific and effective intervention strategies can be designed.

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464 **Figure Legends.**

465 **Figure 1.** Survival and DPA release from endospores of *C. difficile* (●, ○), *C. sporogenes*
466 (▲, Δ), and *C. beijerinckii* (■, □). Endospore suspensions were standardized to OD_{600}
467 2.0 and subjected to treatment at 100 ºC. Solid symbols, log CFU ml^{-1}; Open symbols, %
468 max RFU. Lines dropping below the x-axis indicate values below the detection limit of 2
469 log CFU ml^{-1}. Symbols represent means ± standard deviations of three independent
470 replicates.

471 **Figure 2.** Survival and DPA release from endospores of *C. beijerinckii* (A) and *C.*
472 *sporogenes* (B) standardized to OD_{600} 2.0 and heated to 90 ºC in the presence of 6.4 mg
473 L^{-1} reutericyclin (▲, Δ), 16 mg L^{-1} nisin (■, □), or in the absence of antimicrobials (●,
474 ○). Solid symbols, log CFU ml^{-1}; open symbols, % max RFU. Symbols represent means
475 ± standard deviations of three independent replicates.

476 **Figure 3.** Survival and DPA release from endospores of *C. beijerinckii* (A) and *C.*
477 *sporogenes* (B) standardized to OD_{600} 2.0 and treated at 600 MPa and 90 ºC in the
478 presence of 6.4 mg L^{-1} reutericyclin (▲, Δ), 16 mg L^{-1} nisin (■, □), or in the absence of
479 antimicrobials (●, ○). Solid symbols, log CFU ml^{-1}; open symbols, % max RFU. Lines
480 dropping below the x-axis indicate values below the detection limit of 2 log CFU ml^{-1}.
481 Symbols represent means ± standard deviations of three independent replicates.

482 **Figure 4.** Survival of endospores of *B. amyloliquefaciens* FAD 11/2 standardized to
483 OD_{600} 2.0 and treated at 90 ºC (A) or 90 ºC and 600 MPa (B). Endospores were treated in
484 presence of 6.4 mg L^{-1} reutericyclin (▲), 16 mg L^{-1} nisin (■), or in the absence of
485 antimicrobials (●). Symbols represent means ± standard deviations of three independent
486 replicates.

487 **Figure 5.** Photos of *C. sporogenes* endospores dyed with DAPI following 0 and 60 min
488 of thermal and high-pressure treatment. (A) 90°C and 600 MPa; (B) 90°C. (i), phase
489 contrast microscopy; (ii) fluorescence microscopy to detect DAPI fluorescence.
490 Photographs shown are representative of results observed in multiple photographs taken
491 for each treatment, with each photograph showing several dozen of endospores exhibiting
492 the same behaviour as those shown. The fluorescence intensities of the five endospores
493 per treatment were integrated and standardized against background interference and
494 vegetative cell controls for each photo. Comparable results were obtained with
495 endospores of *C. beijerinckii* (data not shown).
496

497 **Table 1. Hofstetter et al.**

498 **Table 1.** Minimum inhibitory concentration of nisin and reutericyclin against vegetative

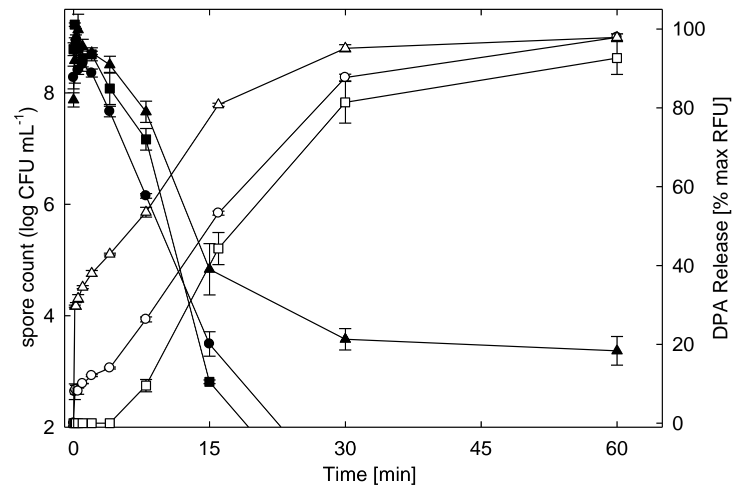
499 cells and endospores of *Clostridium* spp.[a]

	Nisin (mg L^{-1})		Reutericyclin (mg L^{-1})	
	Vegetative Cells	Endospores	Vegetative Cells	Endospores
C. sporogenes	0.23 ± 0.084	1.11 ± 0.48	0.33 ± 0.12	0.40 ± 0.00
C. beijerinckii	1.30 ± 0.00	1.09 ± 0.38	0.33 ± 0.12	0.27 ± 0.12
C. difficile	5.20 ± 0.00	3.47 ± 1.50	0.67 ± 0.23	0.53 ± 0.23

500 [a] Values represent mean ± standard deviation of three replicates.

501

502 **Figure 1. Hofstetter et al.**



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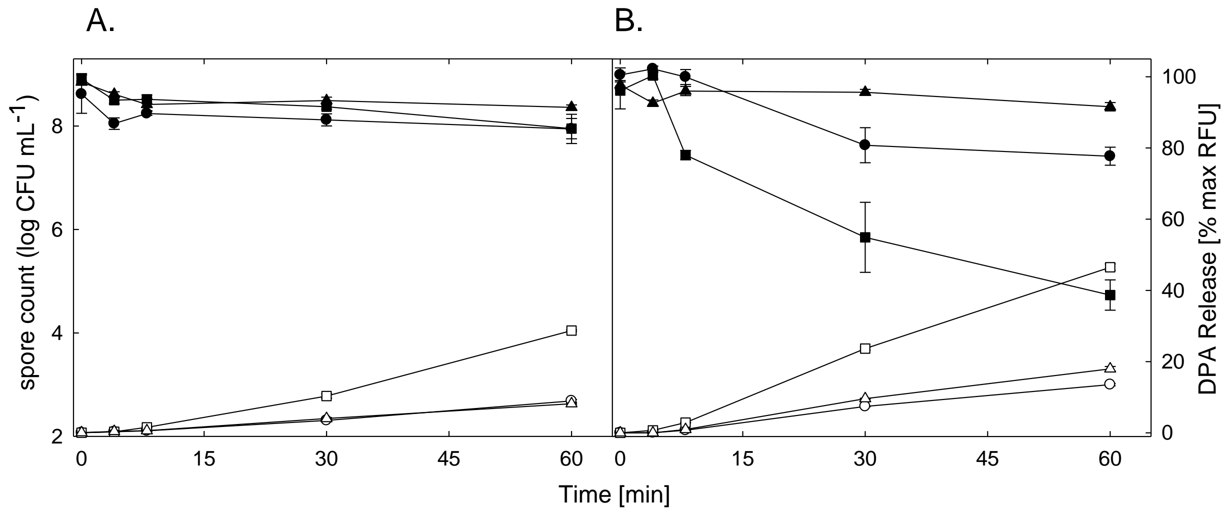
506 **Figure 2. Hofstetter et al.**



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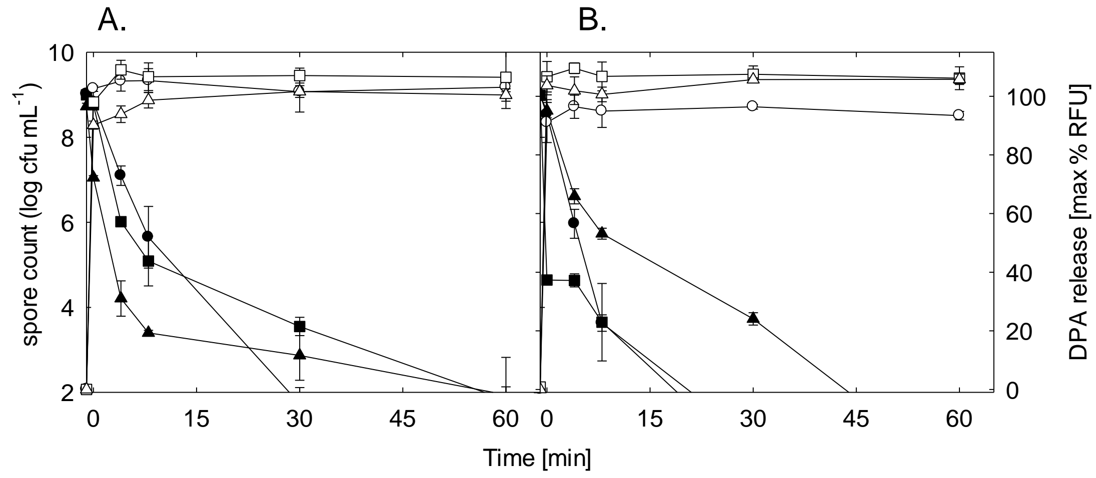
510 **Figure 3. Hofstetter et al.**



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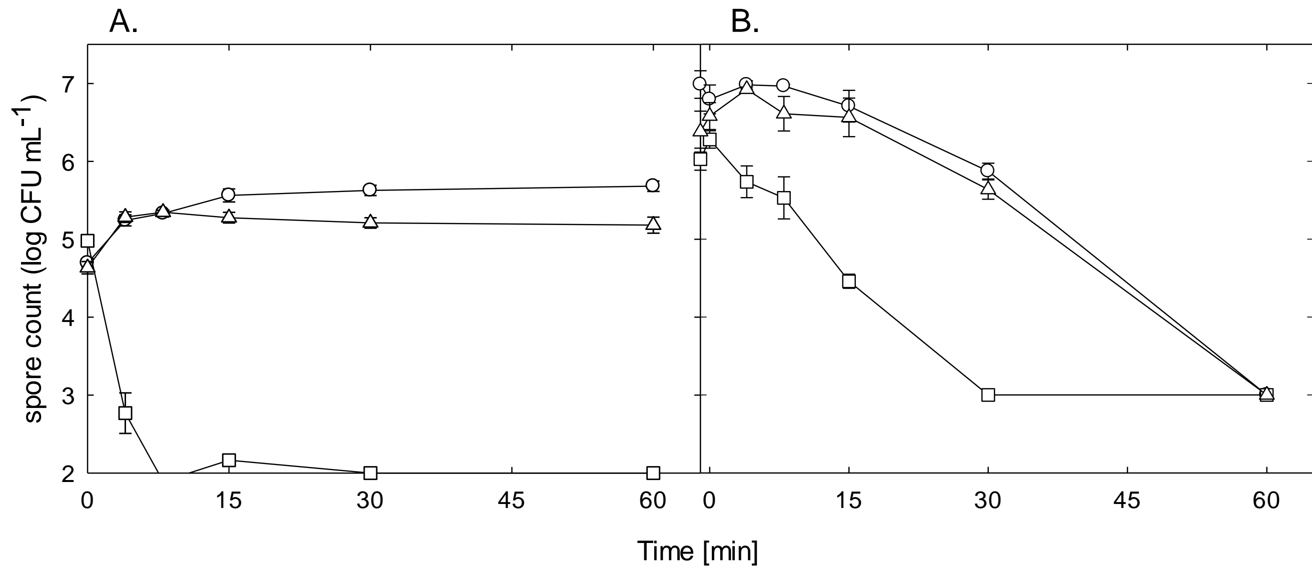
513 **Figure 4. Hofstetter et al.**



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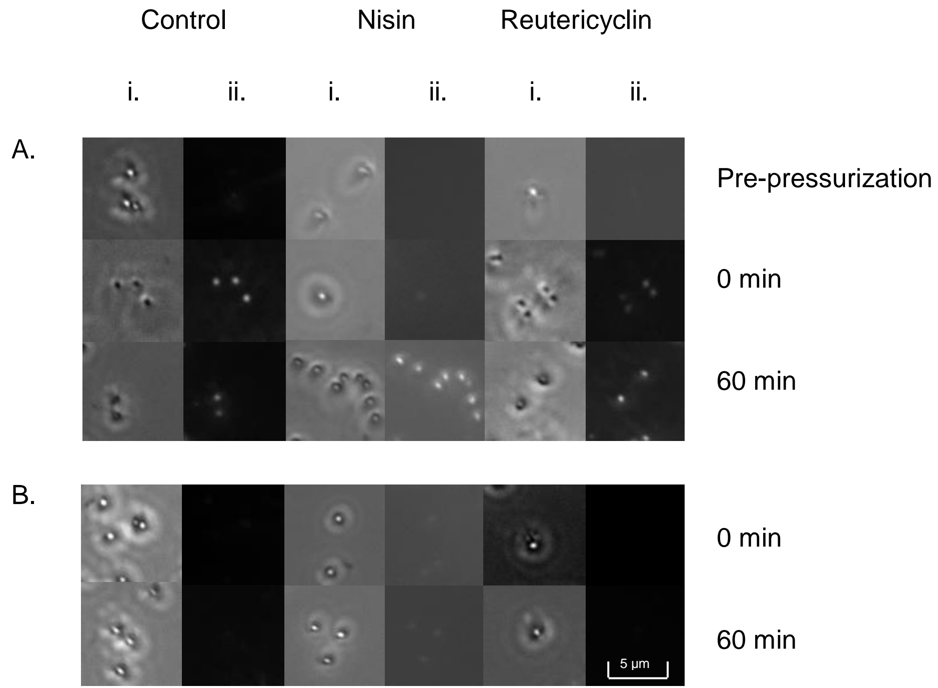
516 **Figure 5. Hofstetter et al.**



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