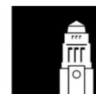

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1 **Investigating the transient and persistent effects of heat on Clostridium**
2 **difficile spores**

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11 **Keywords:** C. difficile, heat treatment, spores, broth enrichment, heat activation

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14 **Abbreviations**: TVC (total viable counts), DPA (dipicolinic acid), CDRN (Clostridium
15 difficile research network), CCEYL (cycloserine-cefoxitin egg yolk lysozyme agar),
16 BHI (brain heart infusion), CDI (Clostridium difficile infection).

Abstract

Purpose: Clostridium difficile spores are extremely resilient to high temperatures. Sub-lethal temperatures are associated with ‘reactivation’ of dormant spores, utilised to maximise C. difficile spore recovery. Spore eradication is of vital importance to the food industry. The current study seeks to elucidate transient and persisting effects of heating C. difficile spores at various temperatures.

Methods: Spores of five C. difficile strains of different ribotypes (001,015,020,027,078) were heated at 50,60,70 & 80℃ for 60 minutes in PBS and enumerated at 0,15,30,45 & 60 minutes. GInaFiT was used to model kinetics of spore inactivation. In subsequent experiments, spores were transferred to enriched BHI broths after 10 minutes of 80℃ heat treatment in PBS; samples were enumerated at 90 minutes and 24 hours.

Results: Spores of all strains demonstrated log-linear inactivation with tailing when heated for 60 minutes at 80℃ ($\bar{x}$=7.54±0.04log_{10}CFU/ml vs 4.72±0.09log_{10}CFU/ml; $P<0.001$). At 70℃, all strains except the 078 exhibited substantial decline in recovery over 60 minutes. Interestingly, 50℃ heat treatment had an inhibitory effect on 078 spore recovery at 0 vs 60 minutes (7.61±0.06log_{10}CFU/ml vs 6.13±0.05log_{10}CFU/ml; $P<0.001$). Heating at 70/80℃ inhibited the initial germination and outgrowth of both newly produced and aged spores in enriched broths. This inhibition appeared to be transient; after 24 hours vegetative counts were higher in heat treated vs non-heat treated spores ($\bar{x}$=7.65±0.04log_{10}CFU/ml vs 6.79±0.06log_{10}CFU/ml; $P<0.001$).

Conclusions: 078 spores were more resistant to the inhibitory effects of higher temperatures. Heat initially inhibits spore germination, but subsequent outgrowth of vegetative populations accelerates after the initial inhibitory period.

43 ## 1. Introduction

44 Clostridium difficile infection (CDI) is a major burden on healthcare services around
45 the world. A range of severity is observed in CDI, with most infections entailing a
46 mild to moderate episode of diarrhoea. Severe disease carries a risk of mortality and
47 the possibility of complications such as pseudomembranous colitis and ultimately
48 toxic megacolon, a surgical emergency (1). C. difficile spores are responsible for
49 transmission within the nosocomial environment, whether it be from environmental
50 contamination or recrudescent disease in the case of relapsing CDI (2).

51 Previous work has examined the effect of heat on spores of several species
52 including C. difficile, at several temperatures for a variety of time periods. Heat is
53 significant in the context of spores due to being reported as both inhibitory and
54 stimulatory to spore germination in different contexts. Studies have highlighted the
55 increased germination of some spore formers (B. subtilis, C. perfringens) in
56 response to sublethal heat treatment (3-5). Sublethal heat t reatment is usually
57 administered between 60-75 ℃ for ~30 minutes. A ~30% increase in germination
58 was noted when aged C. difficile spores were heat activated compared to fresh
59 spores (6). Other studies have also failed to observe an effect of sublethal heat
60 treatment on C. difficile spore germination in fresh spores (7, 8). Varying
61 temperatures and durations have been reported previously for C. difficile spore
62 germination optimisation (9-11). However, there remains no clear consensus on how
63 to optimise spore recovery in regards to heat.

64 Historically, the survival of clostridial spores at high temperatures has been a
65 concern for food processing, with Clostridium perfringens and Clostridium botulinum
66 being of particular importance. C. perfringens spores are particular resistant to heat
67 treatment, with spores being able to survive temperatures exceeding 100℃ for sh ort

68 periods of time (12). The effect of heat treatment on C. difficile spores was first
69 investigated in the 1980s (13). Historically, microbial heat inactivation has been
70 studied assuming log-linear kinetics with calculations of D and Z-values (14-16).
71 However, other models have increasingly been devised to describe thermal
72 inactivation, particularly in spores (17-20). Thermal inactivation of C. difficile spores
73 has been found to begin to occur at temperatures above 70 ℃ (6) (21) (22).

74 Although not fully appreciated, foodborne transmission of C. difficile is possible and
75 has been evaluated using thermal death models (21). Community acquired CDI
76 accounted for ~32% of cases in the United States in 2013 (23) and whole genome
77 sequencing has indicated a lack of a clear transmission pathway in a substantial
78 number of CDI cases in the UK (24). Food could serve as an environmental
79 reservoir; C. difficile has been isolated from a number of cooked meats and food
80 products (25), as well as from raw meat products (26).

81 A number of factors have been identified in influencing the heat resistance of spores;
82 the method of spore purification, spore concentration, the media used for spore
83 preparation (6, 13, 21, 27, 28) and the strains utilised for heat treatment assays.
84 Spores produced on different media have been found to exhibit altered resistance to
85 heat. Notably, the method of C. difficile spore production differs considerably with a
86 variety of solid and liquid media being utilised to produce C. difficile spores (6, 13,
87 21, 27, 28). Spores produced in liquid media in both Alicyclobacillus acidoterrestris
88 (29) and Bacillus subtilis (30) were observed to have lower thermal resistances than
89 their counterparts produced on solid agar. Similar differences in heat resistance are
90 conceivable for C. difficile spores. Different methodologies exist in regards to spore
91 purification. A number of laboratories now purify C. difficile spores using density
92 gradient centrifugation to remove cellular debris (9, 27, 31). In contrast, washing with

93 PBS is still routinely used (32). It is unclear what effect this has on the heat
94 resistance of spores, but comparable trends have been seen in studies using the
95 different methodologies (27, 32). However, it is plausible that increased levels of
96 cellular debris could ameliorate some of the effects of heat.

97 Spore concentrations utilised vary but concentrations of ~10^7 spores/ ml are widely
98 reported and accepted (27, 31, 32). Lower concentrations are also sometimes used
99 in spore germination assays (21, 33). A direct investigation comparing the effect of
100 spore concentrations on heat resistance has not been carried out. It is conceivable
101 that at higher spore concentrations spore aggregation and clumping could play a
102 larger role in protecting spores from heat. Differences in heat resistance between
103 strains have been observed even since the 1980s (28). This phenomenon has been
104 replicated in subsequent work (32). One study utilising four strains found heat
105 resistance did not correlate with hypervirulence (21). In contrast, Rodriguez-Palacios
106 et al suggested an increased thermal resistance of some 027 and 078 strains in
107 response to sub boiling temperatures (22).

108 C. difficile spores are routinely recovered on BHI (brain heart infusion) agar
109 supplemented with taurocholate (27, 32, 34, 35). This is due to previous studies
110 indicating the stimulatory nature of primary bile acids including taurocholate (9, 36,
111 37). Pickering et al recently demonstrated the comparability of CCEYL (cycloserine-
112 cefoxitin egg yolk lysozyme) agar to BHI supplemented with various concentrations
113 of taurocholate for recovery C. difficile spores (38). CCEYL agar inherently contains
114 the primary bile acid cholate, which is stimulatory to C. difficile spore germination (9,
115 39). Although CCEY and CCEYL were comparable for recovery of spores in previous
116 work, CCEYL was utilised in the current study due to evidence that lysozyme
117 increases recovery of heat-treated spores and clinical isolates (21, 40). The current

118 study seeks to provide a more comprehensive understanding of the effect of heat
119 treatment on C. difficile spore germination and outgrowth. This is achieved by
120 detailing both the transient and long term effects of heat on the transition of spores
121 from phase bright to phase dark and subsequent vegetative cell proliferation.

122 ## 2. Methods

123 ### 2.1 Spore Production

124 Spores of five PCR ribotypes (RT 001,015,020,027 & 078) of C. difficile were
125 prepared as previously described (41). All strains used were clinical isolates
126 obtained from the Clostridium difficile ribotyping network (CDRN). Spore stocks were
127 enumerated by serial dilution in phosphate-buffered saline (PBS) in a 96-well plate
128 and anaerobic growth of 20µl aliquots of each dilution on CCEYL agar.

129 All experiments were carried out in biological duplicate (different spore preparations)
130 and technical triplicate (each biological replicate processed in triplicate) and all
131 spores were <14 days old unless otherwise stated. In all experiments, agar plates
132 were incubated anaerobically at 37℃ and counts of colony forming units (CFU) were
133 carried out at 48 hours post-inoculation. Spore suspensions were vortexed
134 vigorously 20 seconds to achieve a homogenous suspension prior to use.

135 Spore viability was assessed by recovery on CCEYL agar. CCEYL agar is
136 comparable to BHI supplemented with taurocholate for recovery of C. difficile spores
137 (38). CCEYL inherently contains cholate, a stimulatory bile acid comparable to
138 taurocholate. Lysozyme was incorporated in to CCEY due to previous efficacy in
139 recovering heat-treated spores, environmentally aged spores and clinical isolates
140 (21, 40).

141 ### 2.2 Phase Contrast Microscopy

142 Slides were prepared by spreading 50µl of spore suspension uniformly over a
143 microscope slide and drying aerobically for 30 minutes at 50℃. Slides were overlaid
144 with 50µl of Wilkins-Chalgren agar and dried for a further hour. Phase bright spores,
145 phase dark spores and vegetative cells were visualised in ten fields of view and
146 counted on a phase contrast microscope at 1000X magnification. All entities were
147 counted in each field of view. In broth experiments one slide was prepared per
148 biological replicate (broth).

149 **2.3 Heat treatment in PBS**

150 Spore suspensions (50 µl) were aliquoted in to PBS (450 µl) in a 1.5ml Eppendorf
151 tube. The final concentration of spores for heating was ~ $2x10^7$ spores/ ml
152 Eppendorfs were transferred to a heat block and heated for 1 hour in aerobic
153 conditions under 1 atm of pressure..Heating at 50, 60, 70 and 80 ℃ were tested
154 independently. At time points 0, 15, 30, 45 and 60 minutes aliquots were serially
155 diluted (10-fold) in PBS in a 96-well plate. Twenty-microlitres of each dilution were
156 inoculated on to CCEYL agar plates.

157 **2.4 Heat treatment prior to broth inoculation**

158 Spore suspensions (50 µl) were aliquoted in PBS (950 µl) and heated at 50, 60, 70
159 or 80℃ aerobically for 10 minutes in a heat plate. The final concentration of spo res
160 for heating was ~10^7 spores/ ml. This concentration is in accordance with previous
161 work (31, 32). The heated suspensions were then transferred to a 4ml BHI broth
162 supplemented with 0.1% taurocholate and 0.4% glycine to produce a final volume of
163 5ml. Broths were incubated anaerobically for 90 minutes under 1atm of pressure. At
164 90 minutes, 20µl of broth was removed and serially diluted (10-fold) in PBS to 10^{-7}.
165 One-hundred microlitres of broth were aliquoted in to 100µl of 100% ethanol and
166 after an hour, serially diluted in PBS to 10^{-4}. Twenty-microlitres of each dilution were

167 inoculated on to CCEYL agar. In addition, 500µl of broth was removed and
168 centrifuged at 9500g for 1 minute. The supernatant was removed and spores were
169 resuspended in 50µl of PBS, which was spread on to a slide for phase contrast
170 microscopy. A zero time point aliquot was included for phase contrast microscopy.
171 Prior to commencement of the current study, a pilot study was undertaken to assess
172 spore germination over time; 90 minutes was the optimal time point for broth
173 enumeration.

174 2.5 Reversibility of heat treatment

175 Reversibility of heat treatment was tested for both fresh spores (<14 days old) and
176 aged spores (~12 weeks old). Spore suspensions (50 µl) were aliquoted into PBS
177 (950 µl) and heated at 80℃ for 10 minutes in a heat block. The final concentratio n of
178 spores for heating was ~10^7 spores/ ml Subsequently the contents were transferred
179 into supplemented Brain Heart Infusion (BHI) broth (4ml) to produce a final volume
180 of 5ml (0.1% taurocholate, 0.4% glycine). At 24 and 48 hours, broth (20 µl) was
181 removed and serially diluted in PBS to 10^{-7} in technical triplicate. Broth (100 µl) was
182 aliquoted in to 100µl of 100% ethanol and after an hour, serially diluted in PBS to 10^{-}
183 ^{4}. Twenty-microlitres of each dilution were aliquoted on to CCEYL agar.

184 2.6 Data analysis

185 Statistical analysis was carried out in IBM SPSS Statistics 22. Data normality was
186 assessed using histograms and Kolmogorov-Smirnov tests. Homogeneity of
187 variance was assessed using Levene's test. In the case of a significant difference in
188 variance between groups, Welch's ANOVA was utilised with Games-Howell multiple
189 comparisons. The details of statistical analysis can be found with each experiment.
190 All means are reported with standard error of the mean (SEM). $\bar{x}$ represents the

191 combined average of two or more specified strains. P values <0.05 were considered
192 significant, <0.01 very significant and <0.001 highly significant.

193 For heat treatment in PBS experiments, appropriate curves were added to the data
194 using the GInaFiT software (18). Due to the non log-linear nature of spore
195 inactivation observed, the calculation of D and Z-values was not carried out.

196
3. Results

197
3.1 Heat treatment in PBS

198 The greatest decrease in spore recovery was observed in spores heated at 80℃
199 (Fig. 1). 80℃ heat treatment was inhibitory to all of the strains used ; after 15
200 minutes a highly significant decrease in spore recovery in all strains was observed
201 (P<0.001). Three of the strains showed an additional ~1log decrease after a further
202 15 minutes of heating, but the 078 strain appeared to drop only marginally at 15 vs
203 30 minutes (4.88 ± 0.06$\log_{10}$CFU/ml vs 4.70 ± 0.07$\log_{10}$CFU/ml; P<0.001). After 30
204 minutes counts began to stabilise in all but the 078 strain, and a small decrease was
205 observed in the 078 strain at 30 vs 45 minutes (4.70 ± 0.07$\log_{10}$CFU/ml vs 4.40 ±
206 0.05$\log_{10}$CFU/ml; P< 0.001). After 45 minutes, spore viability increased (4.40 ±
207 0.05$\log_{10}$CFU/ml vs 5.17 ± 0.04$\log_{10}$CFU/ml) in the 078 strain (P<0.001). All of the
208 strains at 80℃ exhibited a decrease in spore recovery consistent with a log-linear
209 with tailing model (Table 1).

210 The time-dependent decrease in spore recovery at 80℃ was also observed to a
211 lesser extent at 70℃; 3 of the strains (001, 015, 020) showed a drop of
212 ~2$\log_{10}$CFU/ml after 60 minutes of heat treatment. A highly significant decrease in
213 spore recovery took longer in the 027 strain, with a ~2$\log_{10}$CFU/ml drop at 0 vs the
214 60 minute time point (7.70 ± 0.03$\log_{10}$CFU/ml vs 5.74 ± 0.03$\log_{10}$CFU/ml) (P<

215 0.001). The rate at which the decline occurred differed between strains, after 15
216 minutes a ~2log_{10}CFU/ml decrease was observed in the 015 strain (7.55 ±
217 0.02log_{10}CFU/ml vs 5.65 ± 0.03log_{10}CFU/ml). In contrast, the 020 strain took 30
218 minutes for a substantial decrease to occur (7.34 ± 0.07log_{10}CFU/ml vs 5.40 ±
219 0.06log_{10}CFU/ml) and 45 minutes in the 027 strain (7.69 ± 0.03log_{10}CFU/ml vs 6.59
220 ± 0.03log_{10}CFU/ml). The 078 strain showed a modest but significant decrease in
221 spore recovery after 60 minutes (7.60 ± 0.04log_{10}CFU/ml vs 7.31 ± 0.01log_{10}CFU/ml;
222 $P< 0.05$) (data not shown). In contrast to heating at 80℃ , a variety of models were
223 used at 70℃ to fit the data depending on strain (Table 1). The models utilised to fit
224 the data were sigmoidal (001 & 020), log-linear with a shoulder (027) and biphasic
225 (015).

226 Spore counts remained stable across the 60 minute time period at 60℃ in fo ur of the
227 strains. The 078 strain showed sensitivity to heating at 60℃ with a similar drop in
228 spore recovery over 60 minutes compared to 70℃ (7.57 ± 0.03log_{10}CFU/ml vs 7.29
229 ± 0.06log_{10}CFU/ml). At 50℃, the 078 strain showed a highly statistically significant
230 decrease in spore recovery at the 60 minute time point (7.61 ± 0.06log_{10}CFU/ml vs
231 6.13 ± 0.05log_{10}CFU/ml; $P<0.001$) (data not shown).

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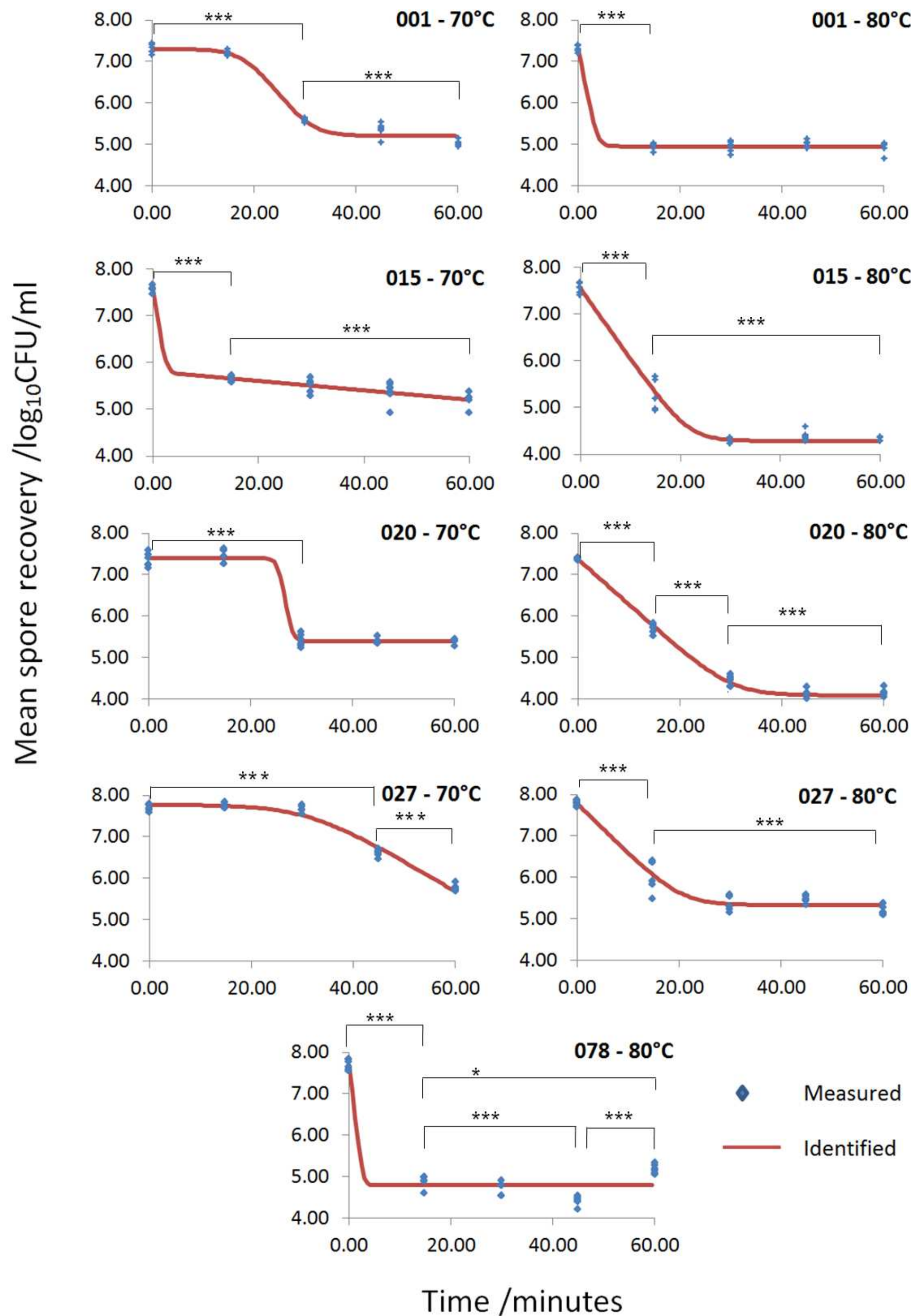

Figure 1. Spore recovery of five ribotypes (001, 015, 020, 027 & 078) of C. difficile heated for 60 minutes at 70/80℃. Spores were enumerated at 0, 15, 30, 45 & 60 minutes. Experiments were carried out in biological duplicate and processing in technical triplicate. Spore recovery was compared between time points using RM-ANOVA with Tukey's multiple comparisons. Statistically significant results (P< 0.05) are highlighted by *, very significant (P < 0.01) by ** and highly significant (P< 0.001) by *. Curves of best fit were fitted using the GInaFiT Excel add-in (18). The models fitted included linear with shoulder, sigmoidal and biphasic (iii) (Table 1). The lower limit of detection for this experiment was 1.52log$_{10}$CFU/ml.**

	Temperature/ ℃			
	70		80	
Ribotype	Model	r^2	Model	r^2
001	Sigmoidal(42)	0.98	Linear with tailing(42)	0.99
015	Biphasic(43)	0.97	Linear with tailing	0.98
020	Sigmoidal	0.98	Linear with tailing	0.99
027	Linear with shoulder(42)	0.98	Linear with tailing	0.96
078	N/A	N/A	Linear with tailing	0.94

233

234 **Table 1. The model used to fit the data shown in Figure 1 with the**
235 **corresponding r^2 correlation coefficient value (2 decimal places).**

236 **3.2 Heat treatment prior to broth inoculation**

237 After aerobic heat treatment, spores were aliquoted in to BHI containing taurocholate
238 to induce germination. The sensitivity of vegetative cells to ethanol allowed the
239 differentiation between non-germinating spores and germinating/vegetative cells.
240 The difference between total viable counts and spores indicates the number of
241 spores that have germinated. Heat treatment at 70℃ and 80℃ appeared to inhibit
242 spore recovery to differing extents (Fig. 2a). TVC (total viable counts) and spore

243 counts at 50℃ and 60℃ were comparable to the non-heated control (TVC; $\bar{x}$ = 6.63
244 ± 0.08log_{10}CFU/ml vs 6.63 ± 0.06log_{10}CFU/ml vs 6.68 ± 0.06log_{10}CFU/ml). Spore
245 counts were on average ~3log_{10}CFU/ml lower than total viable counts (TVC) in the
246 non-heated control and spores heated at 50℃ and 60℃. In three strains (001, 015,
247 020) heating at 70℃ exhibited comparable TVC and spore counts to 50℃/6 0℃,
248 however spore counts were higher in the 027 and 078 strain (5.48 ±
249 0.04log_{10}CFU/ml & 4.78 ± 0.05log_{10}CFU/ml) compared to the other three strains ($\bar{x}$ =
250 3.76 ± 0.05log_{10}CFU/ml). At 80℃, the 027 and 078 strains exhibited lower TVC ($\bar{x}$
251 =4.05 ± 0.05log_{10}CFU/ml vs 4.80 ± 0.12log_{10}CFU/ml; $P< 0.001$) and spore counts
252 (3.56 ± 0.13log_{10}CFU/ml vs 4.69 ± 0.14log_{10}CFU/ml; $P<0.001$) compared with the
253 other strains.

254 Phase contrast microscopy revealed a highly significant increase in visualised phase
255 bright spores in 80℃ heat treated spores vs the control ($\bar{x}$ =82.7 ± 1.1% vs 0.8 ±
256 0.3%; $P< 0.001$) (Fig. 2b). Results were similar for the control and 50/60℃ treated
257 spores in all strains; the majority of entities identified were phase dark (germinated)
258 spores ($\bar{x}$ = 81.8 ± 1.3%, 83.2 ± 1.3%, and 84.9 ± 1.1%). Strain variation was
259 observed at 70℃, the 027 and 078 strains showed similar levels of phase b right and
260 phase dark spores ($\bar{x}$ = 50.6 ± 2.1% vs 39.9 ± 1.8%), the other strains (001, 015 &
261 020) showed results consistent with the control ($\bar{x}$ = 0.3 ± 0.2% vs 85.4 ± 1.1%).

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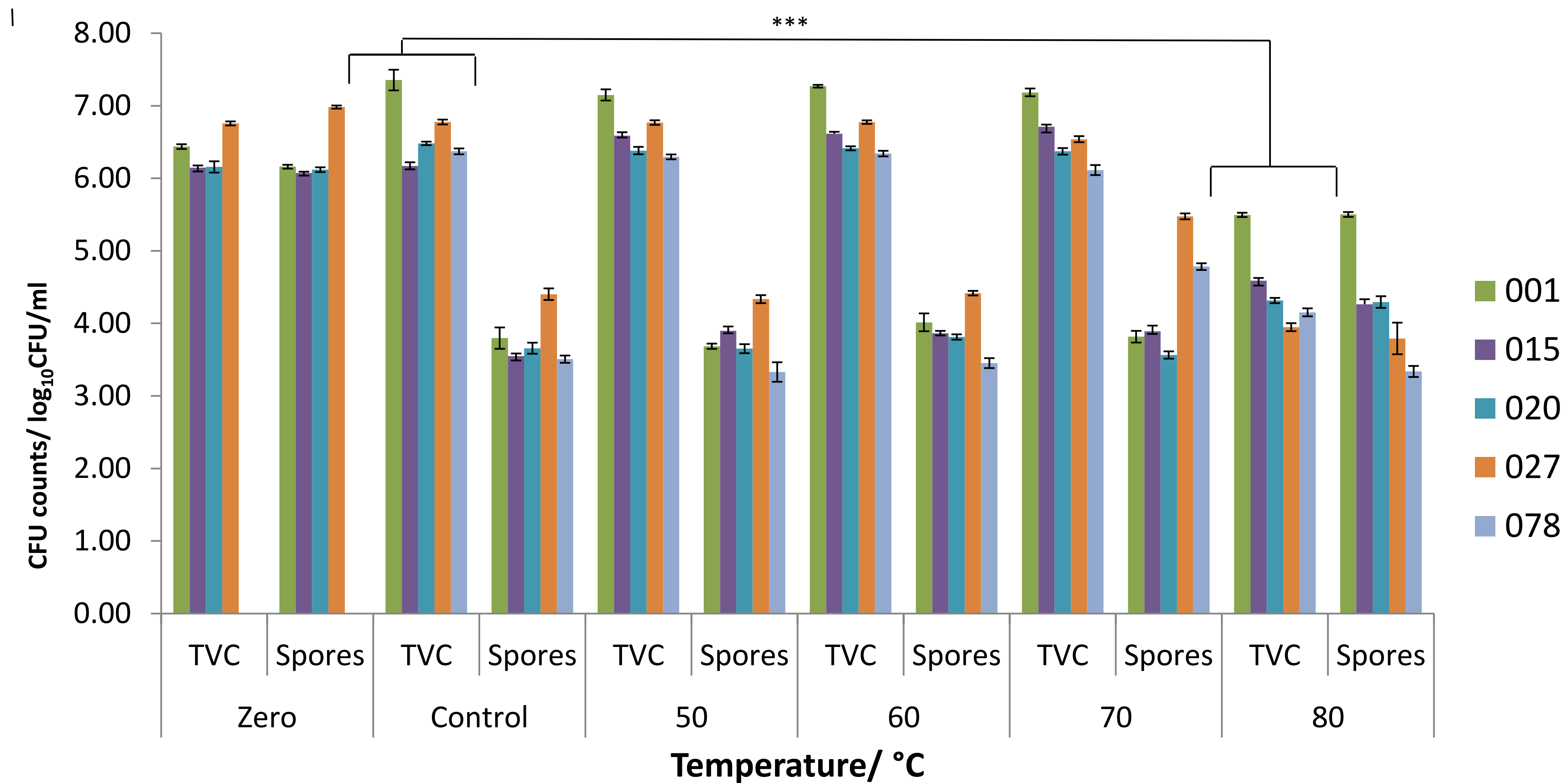

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Figure 1a. Mean ± SE TVC and spore counts of five ribotypes of C. difficile incubated for 90 minutes in BHI supplemented with 0.1% taurocholate/ 0.4% glycine. Spores were heat treated at 50, 60, 70 or 80℃ fo r 10 minutes prior to broth inoculation. TVC/spore counts are also included from the zero time point and from a control broth. Broths were carried out in biological duplicate and technical triplicate. TVC counts were compared using Welch's ANOVA with post-hoc Games-Howell multiple comparisons. Highly significant (P< 0.001) differences are highlighted with *. The lower limit of detection for this experiment was 1.52log$_{10}$CFU/ml.**

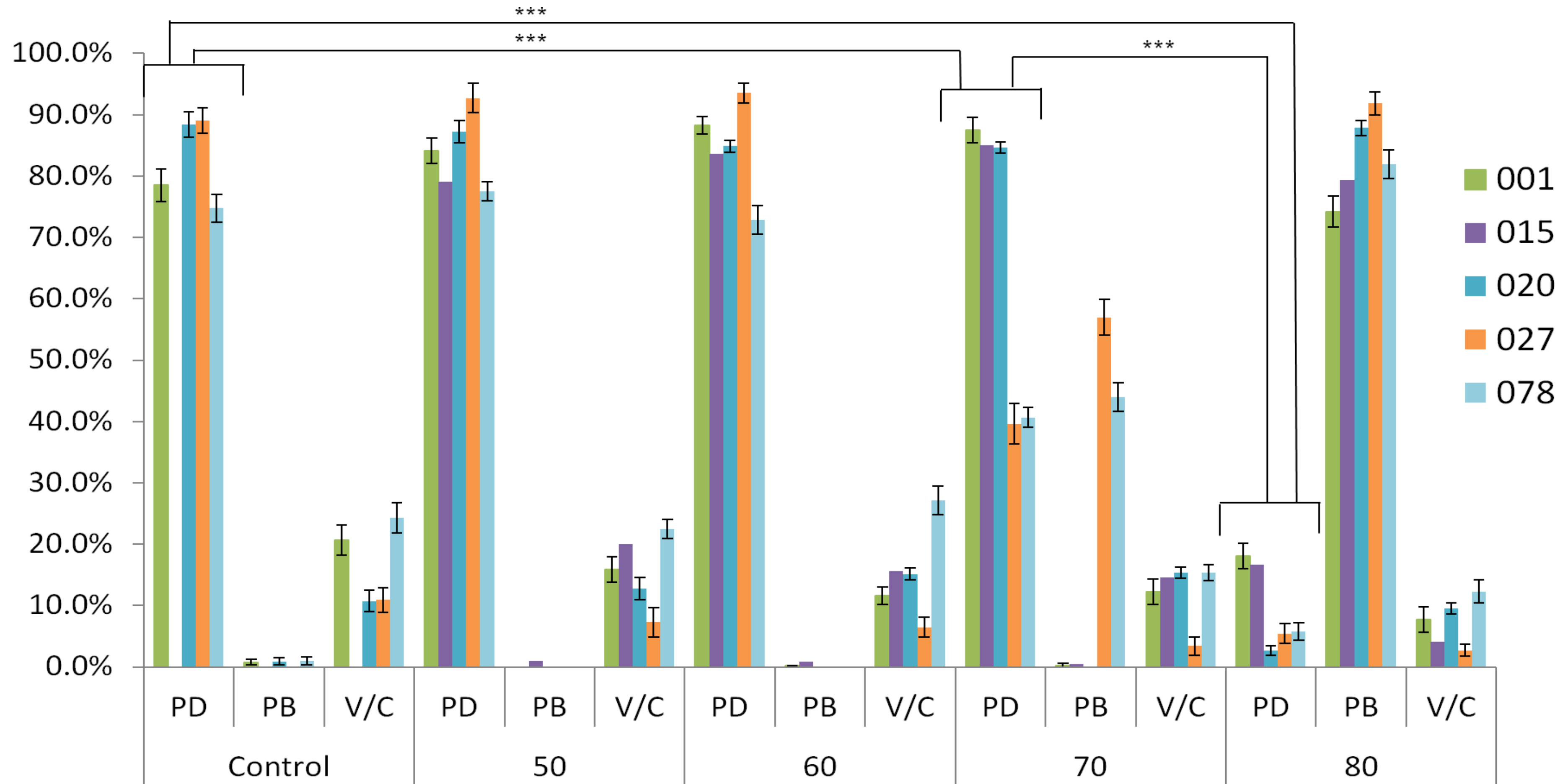

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Figure 2b. Percentage ± SE of phase dark spores (PD), phase bright spores (PB) and vegetative cells (VC) of five ribotypes (001, 015, 020, 027 & 078) incubated for 90 minutes in BHI supplemented with 0.1% taurocholate/ 0.4% glycine. Spores were heat treated at 50, 60, 70 or 80℃ for 10 minutes prior to broth inoculation. Data r epresents 10 different fields of view and one slide was prepared per broth. Broths were carried out in biological duplicate and technical triplicate. PD counts were assessed using Welch's ANOVA with post-hoc Games-Howell multiple comparisons. Highly significant (P< 0.001) differences are indicated using *.**

266 ### 3.3 Reversibility of heat treatment

267 In newly produced (<14 days old) spores, heat treatment prior to broth inoculation
268 caused an increase in vegetative populations at 24 hours vs the non-heat treated
269 control (Fig. 3). In three strains (001, 027, 078) this difference was highly significant
270 ($\bar{x}$ =7.40 ± 0.03log_{10}CFU/ml vs 6.45 ± 0.04log_{10}CFU/ml; $P< 0.001$). The 015 and 020
271 strains showed an increase in TVC in response to heat, but to a lesser extent than
272 the other 3 strains ($\bar{x}$ = 7.62 ± 0.03log_{10}CFU.ml vs 7.45 ± 0.04log_{10}CFU/ml; $P< 0.05$).

273 In old (>12 week old) spores the same phenomenon of increased vegetative growth
274 in heat-treated samples vs non-heat treated samples was observed (Fig. 3). All of
275 the strains showed an extremely significant increase in TVCs in heat treated vs non-
276 heat treated spores (7.81 ± 0.06log_{10}CFU/ml vs 6.76 ± 0.08log_{10}CFU/ml; $P< 0.001$).

277 The trend exists in both new and old spores, but differences between time points in
278 individual ribotypes existed. Vegetative populations only increased marginally in the
279 015 and 020 strains in response to heat in new spores ($\bar{x}$ = 7.61 ± 0.03log_{10}CFU/ml
280 vs 7.45 ± 0.05log_{10}CFU/ml). However, a greater increase occurred in old heat
281 treated spores ($\bar{x}$ =7.60 ± 0.03 log_{10}CFU/ml vs 6.33 ± 0.04log_{10}CFU/ml). The old
282 non-heat treated spores of ribotypes 015 and 020 produced a smaller vegetative
283 population in contrast to new spores. Spores of the other three strains (001, 027 &
284 078) exhibited similar behaviour independent of age.

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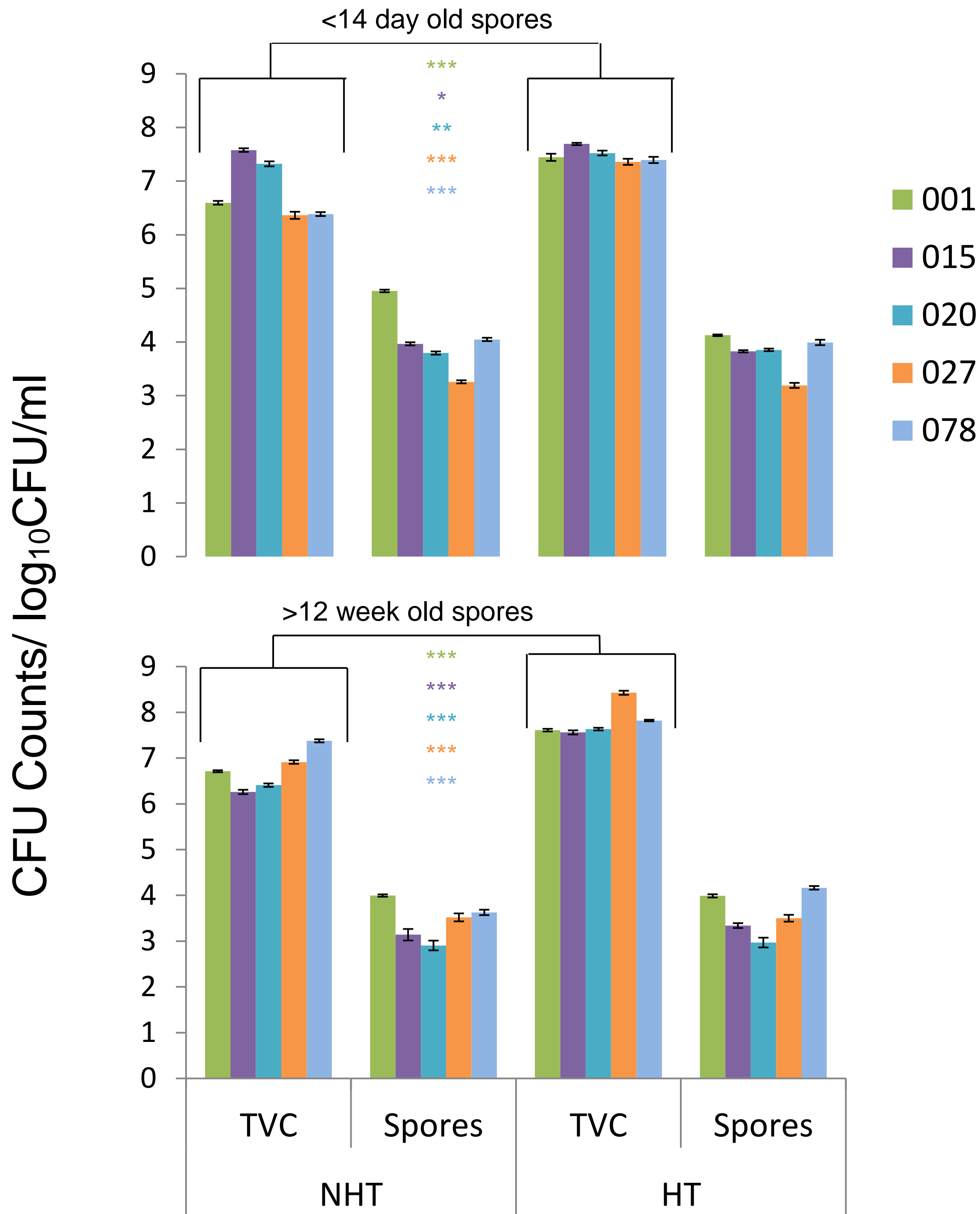

Figure 2. Mean ± SE TVC and spore counts of five ribotypes (001, 015, 020, 027 & 078) 24 hours post-broth inoculation. Both new spores (< 14 days old) and old spores (> 12 weeks old) were utilised. Heat treated spores (HT) were heated for 10 minutes at 80℃, non-heat treated (NHT) received no treatment. A trend towards increased TVCs in heat treated samples is present. Broths were carried out in biological duplicate and technical triplicate. TVC means were compared using two-tailed paired T tests. Very significant ($P < 0.01$) findings are highlighted by ** and highly significant ($P < 0.001$) by *. The lower limit of detection for this experiment was 1.52log$_{10}$CFU/ml.**

287 # 4. Discussion

288 **C. difficile spores display non log-linear inactivation kinetics**

289 Traditionally, thermal inactivation of microorganisms has been illustrated using log-
290 linear kinetic models, based on the assumption of a homogeneous population which
291 share the same intrinsic heat resistance (44). This approach is particularly prevalent
292 in food microbiology and food safety, where it is possible to quickly calculate D-
293 values and Z-values. The D-value is defined as the time taken for a 90% (or 1log)
294 reduction in microbial numbers from the starting population, the Z-value is the
295 temperature increase required to decrease the D-value by a magnitude of ten. This
296 method has been used contemporaneously to evaluate thermal resistance in C.
297 difficile spores (6, 21). However, in this study 70℃/80℃ heat data were not
298 adequately modelled using log-linear kinetics. In order to accurately assess and
299 model the thermal inactivation of C. difficile spores, a number of different survival
300 curves were used to describe the data (18).

301 When spores were heated in PBS for 60 minutes, substantial variation was
302 observed. At 70℃, heat inactivation data was fitted using a variety of models;
303 sigmoidal (001, 020), biphasic (015) and linear with tailing (027). One possible
304 explanation of the ‘shoulder’ observed in the sigmoidal curve is that bacterial spores
305 are surrounded by a protective matrix that buffers heat (18). Once this protective
306 matrix is destroyed/inactivated by sufficient heat, log-linear heat inactivation of
307 spores resumes. The spores used in this experiment were not purified; it is plausible
308 that proteinaceous cellular debris (in the form of dead vegetative cells) acts as a
309 protective matrix. Indeed, Permpoonpattana et al demonstrated by transmission
310 electron microscopy the presence of detached exosporium surrounding C. difficile

311 spores in one strain (45). One study found that extracellular matrix began to be
312 incorporated in to the exosporium of biofilm produced spores after 7-14 days of
313 incubation (46). In addition, cellular debris has been identified in 'pure' spores
314 prepared using the density centrifugation methodology (31).. In any event,
315 purification appears to have little effect on C. difficile spore heat resistance when
316 contrasting similar experiments (27, 32). The conditions encountered in the current
317 study are potentially more reflective of conditions encountered by spores in the food
318 processing industry.

319 It is unclear why some strains have a prolonged buffering period, for example the
320 027 strain. Differences in the composition and structure of the spore coat may exist
321 between different strains; one recent study found 70% of C. difficile spoVM mutants
322 of an 012 PCR ribotype strain (CD630) exhibited an increased sensitivity to heat
323 compared to the wild-type. However, only ~30% of spoVM mutants were subject to
324 cortex abnormalities.SpoVM is a basement membrane protein present in the spore
325 coat, and is suggested to be involved in the construction of the spore cortex (47).
326 Other reasons for the presence of a shoulder in thermal inactivation curves have
327 been proposed; clumping, the inability of bacteria in vegetative populations to
328 continually synthesise protective proteins over time and the cumulative damaging
329 effect of heating over a sustained period (19, 48). As spores are metabolically
330 dormant, the synthesis of protective proteins during heating is irrelevant.
331 Interestingly, shouldering was not observed when lower C. difficile spore loads
332 ($5\log_{10}$CFU/ml) were used in heat treatment assays (21, 22). This suggests some of
333 the proposed above mechanisms explaining shouldering may only operate with
334 higher spore loads, consistent with data from the current study.

335 Interestingly, the 027 and 078 spores appear to possess a more heat-resistant
336 subpopulation at 70℃. Spore populations exhibited on average a smaller decrease
337 in recovery than the other strains over 60 minutes. These data support the findings
338 of Rodriguez-Palacios et al who have previously suggested the increased thermal
339 resistance of 027 and 078 strains (22).In addition, 'tailing' was observed at 70°C in
340 some strains. Tailing has been discussed since the 1970s (43). Intra-strain
341 variability is a possible mechanism by which tailing occurs; a subpopulation of
342 spores are maintained due to their higher intrinsic heat resistance. A number of
343 potential mechanisms have been proposed to explain differences in heat resistance
344 between spores, including core DPA content, enzymes responsible for DPA
345 transport, and enzymatic degradation at high temperatures (27, 49).

346 Dipicolinate (DPA) is responsible for maintaining a dehydrated core and is important
347 in wet-heat resistance in B. subtilis (49). High DPA levels and low water content in
348 the spore core have long been known to facilitate high wet heat resistance (50, 51).
349 SpoVA is an ion transporter responsible for the transport of DPA in to the core in C.
350 perfringens; spoVA mutants have a loss of wet-heat resistance as well as impaired
351 germination (52). Additionally, foodborne B. subtilis strains harbouring the
352 $spoVA^{2mob}$ operon were found to have increased heat activation requirements than
353 non-foodborne strains (53); high heat resistance has been attributed to this operon
354 (54). More recently, the importance of DPA has been highlighted in C. difficile
355 spoVAC and dpaAB mutants. DpaAB is the gene from which an enzyme responsible
356 for DPA synthesis is synthesised. Both sets of mutants showed statistically
357 significant decreases in wet-heat resistance at temperatures above 50℃ versus the
358 wild-type (27). Cortex degradation was unaffected; heating affected a downstream
359 process.

360 The cortex has previously been implicated in heat resistance by its osmoregulatory
361 capacity in reducing the water content of the spore core (55) . The expanded
362 peptidoglycan cortex was hypothesised to facilitate the dehydration of the core.

363 On the contrary, none of these factors address the slight but significant spore
364 inactivation seen at 50/60℃ in the 078 strain. The 078 ribotype has be en found to be
365 phylogenetically dissimilar and highly divergent from other C. difficile strains based
366 on lineage (56). Based on the data, it is feasible that as well as the described
367 mechanisms, the 078 strain possesses an additional mechanism of heat resistance,
368 interrupted by 50 degree heat.

369 Inter-strain variability is something that has been observed previously in the literature
370 as early as 1985 (28). The reason for these observed differences is not clear. It is
371 possible that natural heterogeneity exists in different C. difficile strains of the levels
372 of proteins responsible for DPA synthesis and transport, and therefore DPA levels.
373 Indeed, intra-strain spore heterogeneity must be present due to the survival of a
374 small subpopulation of spores at higher temperatures. In addition, Rose et al. 2007
375 (30) showed that in B. subtilis wet-heat resistance is probably dependent on factors
376 other than just spore DPA content. This is likely true for C. difficile. Of note, pH has
377 been shown to be an important factor in thermal inactivation of microorganisms (20),
378 but clearly does not account for the variation observed in the current study.

379 At 80℃ the thermal death curve of all the strains was more consistent; all of the
380 strains showed a drop in recovery consistent with log-linear kinetics with tailing. The
381 increased heat is likely to be detrimental to any impurities in the spore suspensions,
382 eliminating the buffering properties observed at 70℃. Nevertheless, a small (~0.1%
383 of the original population) subpopulation of heat resistant spores persisted after 60
384 minutes for all strains.

385 **80℃ heat treatment is initially inhibitory to C. difficile spores but promotes**
386 **later outgrowth**

387 Both freshly produced (<7 days old) and environmentally aged (>3 months old)
388 spores were heat treated prior to broth inoculation. Initially this experiment was
389 performed to assess the immediate effect of heat on spore recovery and outgrowth
390 (90 minute incubation times) but the longer term effects of heat on spore recovery
391 were also documented (24). In both fresh and aged spores the same overall trends
392 were observed. When spores were heat treated and left for 90 minutes, spore
393 outgrowth was inhibited compared to prior heat treatment at lower temperatures and
394 the control. Both TVCs and spore counts decreased, indicating a global decrease in
395 spore recovery. On the other hand, when spores were left for a longer time period, at
396 24 hours the heat treated samples contained higher levels of vegetative cells and
397 comparable levels of spores to non-heat treated samples.

398 Initially these results appear contradictory, if heat is inhibiting spore germination at
399 an early stage it is unclear why a more rapidly growing vegetative population is
400 present at a later time point. One study also observed that heat treatment at 85℃
401 decreased recovery, but this was due to an impairment of vegetative growth and not
402 germination (6). These results are complementary to the current study, as spores
403 were able to progress to vegetative growth. It could be hypothesised that the heat
404 treatment causes a greater number of spores to germinate, but also an initial lag in
405 outgrowth.

406 Additionally, although the overall trend of increased cell proliferation in response to
407 heat was true for both newly produced and aged spores, there were differences
408 between strains. Heat treatment of newly produced spores of the 015 and 020
409 strains caused only marginal increases in vegetative proliferation at 24 hours

410 compared to the 001, 027 and 078 strains. In contrast, when 015 and 020 spores
411 were aged, heat treatment increased TVC counts at 24 hours substantially versus
412 the control. These results highlight that the ageing 'process' in spores may vary
413 between different strains.

414 Heat activation has commonly been used as a strategy to increase spore
415 germination in B. subtilis (3) and more recently C. difficile in older spores (6). The
416 previously cited study also found that aged spores (>20 weeks old) exhibited
417 increased recovery (30% increase) in response to heat treatment at 63℃ vs freshly
418 produced spores (6). The results of that study are somewhat congruent with the
419 current study; on the contrary newly produced spores of some strains exhibited heat
420 activation, albeit in producing vegetative populations. Heat activation has also not
421 been observed in newly produced C. difficile spores in other work (8). Heat has been
422 suggested to mediate its stimulatory effects by inducing germinant receptor
423 conformational changes. C. difficile spores are not prone to heat activation due to an
424 absence of inner membrane bound germinant receptors that are sensitive to heat
425 activation (36, 57). This is consistent with work demonstrating heat has no activation
426 effect on germinant-receptor independent germination in B. subtilis (58, 59)..

427 The different methodologies utilised should also be acknowledged, in the previous
428 study spores were heated in microtubes and directly plated on to a germinant free
429 solid agar (blood agar) (6), in contrast to the broth incubation used in this study. On
430 the contrary, the variations could be due to inherent differences in the ageing
431 processes between C. difficile strains.

432 In conclusion, the current study offers novel insights in to the effect of high
433 temperature on C. difficile spore germination. The differing heat inactivation kinetics
434 displayed by strains of different ribotypes is demonstrated. This is of particular

435 interest to the food industry for the eradication of contaminating spores. Secondly,
436 the transient and long-term effects of high temperatures on spore germination and
437 outgrowth are documented. After an initial inhibitory period, both newly produced and
438 aged spores are found to produce more rapidly proliferating vegetative populations in
439 response to heat treatment. These findings build on and elucidate previous findings
440 discussing heat activation in C. difficile spores.

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444

445 Conflicts of Interest

446 The authors have no conflict of interest to declare.

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