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1 Synergistic interaction between pH and NaCl in the limits of germination and outgrowth of
2 *Clostridium sporogenes* and Group I *Clostridium botulinum* vegetative cells and spores after heat
3 treatment.

4

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23

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28 **Highlights**

- 29 • Individual isolates of *C. sporogenes* and *C. botulinum* grew at pH ≥ 4.5 and NaCl $\leq 11.5\%$
- 30 • No correlation between physiological and genetic characteristics of 42 isolates
- 31 • pH and NaCl growth limits differed between vegetative cells and spores
- 32 • Combined low heat treatment, pH 4.9 and NaCl 4% prevented growth

33 **Abstract**

34 Group I *Clostridium botulinum* and *Clostridium sporogenes* are physiologically and genetically closely
35 related. Both are widely distributed in the environment and can cause foodborne botulism. In this
36 work, a physiological study was conducted with 37 isolates from spoiled canned food and five
37 referenced strains of *C. sporogenes* (three isolates) and Group I *C. botulinum* (two isolates). Growth
38 limits of vegetative cells were established as a function of pH and NaCl concentration in PYG modified
39 medium (PYGm) at 30 °C for 48 days. The heat resistance of the spores was studied for 2 min and 10
40 min at 102 °C and 110 °C. This physiological study (pH, NaCl growth limits and heat resistance)
41 allowed the selection of 14 isolates of *C. sporogenes* (twelve isolates) and Group I *C. botulinum* (two
42 isolates) representative of the diversity found. This panel of 14 selected isolates (11 isolated from
43 spoiled canned food and three reference strains), were whole genome sequenced, but no association
44 of physiological and genetic characteristics could be detected. Finally, we studied the ability of spores
45 to germinate and grow from 5 isolates (four *C. sporogenes* and one Group I *C. botulinum*), under
46 stress conditions generated by pH and NaCl following a low intensity heat treatment. The
47 accumulation of these 3 stresses creates synergies that will strongly reduce the probability of spore
48 growth in pH and salt conditions where they usually proliferate. The effect is progressive as the
49 conditions become drastic: the number of decimal reduction observed increases translating a
50 probability of growth which decreases. This study provides a better understanding of the behaviour
51 of *C. sporogenes* and Group I *C. botulinum* isolates and shows how the combination of pH, NaCl and
52 heat treatment can help prevent or minimise foodborne botulism outbreaks.

53

54

55 1 Introduction

56 Strains of *Clostridium botulinum* Group I are widespread throughout the environment and can
57 enter the food chain, where they can cause foodborne botulism (due to ingestion of pre-formed
58 botulinum neurotoxin in contaminated food). Strains of Group I *C. botulinum* are able to form
59 strongly heat-resistant spores, which makes Group I *C. botulinum* the most concern pathogen in low-
60 acid canned food. Group I *C. botulinum* and *Clostridium sporogenes* are phylogenetically closely
61 related and share physiological characteristics including the high thermal resistance of their spores
62 and their mesophilic growth.

63 *C. botulinum* can produce highly potent neurotoxins (0.1–1 ng/kg for laboratory mice by injection but
64 100/1000 times less toxic by ingestion) with several different neurotoxin serotypes described (A, B, C,
65 D, E, F, G, HA and X). Foodborne botulism has been mainly described in home-prepared foods but can
66 also affect commercial products, and in the case of Group I *C. botulinum* with strains that produce
67 neurotoxin of serotypes A, B and/or F (Peck, 2009; Rasetti-Escargueil et al., 2020). For many years,
68 the difference between Group I *C. botulinum* and *C. sporogenes* was considered to be in the
69 production of botulinum toxin and *C. sporogenes* has often been used as a surrogate for Group I *C.*
70 *botulinum*. It was believed that Group I *C. botulinum* strains could produce botulinum neurotoxins,
71 whereas *C. sporogenes* strains could not. However, recent studies reveal a more complex picture.
72 Various approaches have been used to establish the diversity and the phylogenetic relationship
73 between strains of Group I *C. botulinum* and *C. sporogenes*. Comparisons of core genome single
74 nucleotide polymorphisms (SNP) following whole genome sequencing have shown that Group I
75 *C. botulinum* and *C. sporogenes* are genetically distinct (Peck, 2009; Peck and van Vliet, 2016;
76 Weigand et al., 2015). Brunt et al. (2020) showed the presence of type B neurotoxin genes in some *C.*
77 *sporogenes* strains, and botulinum neurotoxin genes were absent from some strains of Group I *C.*
78 *botulinum* (Brunt et al., 2020). The diversity and spread of botulinum neurotoxin genes is associated
79 with horizontal transfer via mobile elements, conjugative plasmids and various
80 recombination/insertion events (Nawrocki et al., 2018; Peck and van Vliet, 2016).

81 Group I *C. botulinum* strains can grow to a pH as low as pH 4.6 and above, making this value a
82 reference hazard target for low-acid food (Peck, 2009). Although occasional outbreaks of botulism
83 have been attributed to foods with a pH lower than 4.6, these foods were also contaminated with
84 molds, whose growth increased the pH of the food, enabling Group I *C. botulinum* to grow and
85 release toxin. Growth of Group I *C. botulinum* and *C. sporogenes* can be inhibited only at high
86 concentrations of sodium chloride ($\text{NaCl} \geq 10\%$, i.e. $a_w \geq 0.94$) or glycerol ($a_w > 0.93$) (Peck, 2009).

87 The decimal reduction value at 121.1 °C ($D_{121.1\text{ °C}}$) of Group I *C. botulinum* spores is commonly taken
88 to be 0.21 min (Esty and Meyer, 1922). This decimal reduction time corresponds to the time required
89 to inactivate 90% of the initial population in bacteria. A recent meta-analysis of 23 scientific papers
90 (394 D -values collected) found the mean value of $D_{121.1\text{ °C}}$ to be 0.2 min, with a standard deviation of
91 0.11 min, and reported values up to 0.48 min at 121.1 °C (Diao et al., 2014). Spores of *C. sporogenes*
92 strains are often reported to be more heat-resistant than those of Group I *C. botulinum* strains, with
93 $D_{121.1\text{ °C}}$ values ranging from 0.2 to 3.5 (Basaran-Akgul, 2013; Brown et al., 2012; Diao et al., 2014).

94 The difference in heat resistance is partially explained by the acquisition of a second *spoVA* operon,
95 which is responsible for the transport of dipicolinic acid into the spore core during the sporulation,
96 increasing the heat resistance of the spores (Brunt et al., 2020; Butler et al., 2017; den Besten et al.,
97 2018).

98 Previous work indicates a broad diversity in physiological and genetic characteristics within both
99 species. As consumer diet shifts toward the consumption of minimally processed foods, this
100 physiological and genetic diversity needs to be considered to reduce the risks of botulism outbreaks.

101 The purpose of the present study was to seek insights: (i) into the physiological and genetic diversity
102 of isolates of Group I *C. botulinum* and *C. sporogenes* and (ii) into the potential synergistic interaction
103 of food formulation (pH and sodium chloride content) and heat treatment on the probability of spore
104 germination and outgrowth. The growth limits of 37 isolates of *C. sporogenes* and Group I *C.*
105 *botulinum* isolated from spoiled canned food and five reference strains (three reference strains of *C.*
106 *sporogenes* : PA 3679 Campden collection, IFR 81/14/1 and PA 3679 ATCC 7955 and two reference

107 strains of Group I *C. botulinum* : Prevot 910 and 301-13 Institut Pasteur collection (Table 2) were
108 assessed in PYGm broth at sub-optimal temperature (30 °C) in anaerobic conditions, together with
109 the heat resistance of spores at 102 °C and 110 °C for 2 min and 10 min. Fourteen representative
110 isolates selected according to their physiological characteristics were also sequenced. This genetic
111 study allowed the selection of five diverse isolates (three isolates of *C. sporogenes* or Group I *C.*
112 *botulinum* and two reference strains) for further study. The ability of these isolates to grow from
113 vegetative cells or from heat-treated spores was assessed according to the pH and NaCl
114 concentration of the growth media.

115

116 2 Materials and Methods

117 2.1 Bacterial strains

118 The isolates of *Clostridium sporogenes* and *Clostridium botulinum* used in this study are listed in
119 Table 1. Isolates 1–40 were food contaminant isolates, isolated from spoiled cans by the Centre
120 Technique pour la Conservation des Produits Agricoles (CTCPA) and identified as *C. sporogenes* with
121 16S rRNA sequencing, negative *in vivo* test (mice) and absence of toxin genes by PCR. The reference
122 strains of Group I *C. botulinum* were Prevot 910 (isolate 41) and 301-13 (isolate 42) (Mazuet et al.,
123 2016). Each isolate was stored at –80 °C in beads (Biomérieux, AES, Chemunex, Bruz, France). Cell
124 recovery was carried out with three beads dropped into 10 mL of peptone-yeast-glucose modified
125 broth (PYGm). The 10 mL tube was incubated at 37 °C for 72 h. Two successive pre-cultures in 10 mL
126 tubes of PYGm broth inoculated at 1% were then incubated at 30 °C for 16 h and 22 h. Anaerobiosis
127 was maintained by pouring paraffin into the tubes after inoculation. PYGm medium preparation
128 followed DSMZ protocol (DSMZ, 2020). The PYGm modified broth composition was: yeast extract (10
129 g/L), beef extract (5 g/L), dextrose (5 g/L), peptone (5 g/L), tryptone (5 g/L) (Biokar Diagnostics,
130 Allonne, France), K₂HPO₄ (2 g/L), resazurin (1 mg/L), Tween® 80 (1 mL/L), and 40 mL/L of salt solution
131 (NaHCO₃, 10 g/L; NaCl, 2 g/L; K₂HPO₄, 1 g/L; KH₂PO₄, 1 g/L; MgSO₄ 7H₂O, 0.5 g/L; CaCl₂.2H₂O, 0.25 g/L)
132 (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The PYGm broth ingredients were combined in a
133 final solution volume of 1 L and mixed thoroughly, boiled for 5 min, and cooled to room temperature.
134 L-Cysteine HCl (0.5 g/L), vitamin K1 solution and haemin solution (Sigma-Aldrich, Saint-Quentin-
135 Fallavier, France) were then added to the PYGm broth. The vitamin K1 solution was 0.1 g of vitamin
136 K1 in 20.0 mL of 95% ethanol, mixed thoroughly, sterilised by filtration, and stored at –18 °C in a
137 brown glass bottle. The haemin solution was composed of 50.0 mg of haemin in 1.0 mL of 1N NaOH
138 made up to a final volume of 100.0 mL and stored at 4 °C. Resazurin (1 mg/L) (Sigma-Aldrich, Saint-
139 Quentin-Fallavier, France) added to the PYGm medium is a highly sensitive colour indicator. It turns
140 pink in the presence of oxygen and is colourless in its absence. Finally, the PYGm broth was
141 distributed in 10 mL tubes and autoclaved for 15 min at 121 °C.

142 The four reference strains of Group I *C. botulinum* were stored at $-80\text{ }^{\circ}\text{C}$ in 1 mL aliquots of trypticase
143 yeast extract glucose (TYG) mixed with 30% glycerol (v/v). A volume of 100 μL of aliquot added to
144 10 mL of TYG. The culture was incubated at $37\text{ }^{\circ}\text{C}$ for 24 h. A second pre-culture inoculated at 1% in
145 10 mL tubes of TYG broth was then incubated at $37\text{ }^{\circ}\text{C}$ for 22 h. The TYG broth composition was
146 trypticase (30 g/L) (Biomérieux, AES, Chemunex, Bruz, France), yeast extract (20 g/L) (Becton-
147 Dickinson, BD Difco, Grenoble, France), glucose (5 g/L) (Biokar Diagnostics, Allonne, France), DL-
148 cysteine (0.5 g/L) (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The TYG broth was then
149 distributed in 10 mL tubes and autoclaved for 20 min at $110\text{ }^{\circ}\text{C}$. The oxygen was removed from the
150 PYGm broth and TYG broth in a water bath (JULABO GmbH, Seelbach, Germany) at $95\text{ }^{\circ}\text{C}$ for 20 min
151 or by storage in an anaerobic chamber before use (90% N_2 , 5% H_2 , 5% CO_2 , Whitley A35 Workstation,
152 Don Whitley Scientific, United Kingdom).

153

154 **2.2 Spore suspension**

155 Spores were produced in modified meat liver sulphite reducer agar modified medium
156 (MLSRAm) supplemented with 2 g/L of yeast extract (Biokar Diagnostics, Allonne, France). An aliquot
157 (1 mL) of the pre-culture of vegetative cells (previously described) was inoculated on MLSRAM agar
158 plates. The plates were incubated under anaerobiosis in jars (Oxoïd™ AnaeroGen™, Sigma-Aldrich,
159 Saint-Quentin-Fallavier, France) at $30\text{ }^{\circ}\text{C}$ for 10 days. Sporulation was stopped when free spores
160 exceeded 95% of cells counted by phase-contrast microscopy at a magnification of 1,000 (Olympus
161 BX50; Olympus Optical Co., Ltd., Hamburg, Germany). Spores were harvested by pouring distilled
162 sterile water into the plates and scraping them in anaerobiosis conditions. Spore suspensions were
163 centrifuged at $6,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$, and the spores were resuspended in 5 mL of sterile
164 distilled water. Spore batches were divided into 1 mL aliquots and stored at $4\text{ }^{\circ}\text{C}$ until use. Final
165 concentration of the stock suspensions was 10^7 – 10^9 spores/mL.

166

167 **2.3 Measurement of the spore heat resistance**

168 To estimate the initial (t_0) spore concentration, the spore suspensions were firstly heated in a
169 water bath at 80 °C for 10 min. For physiology experiments, the spores were heated for 2 min and 10
170 min at 102 °C and at 110 °C. The spore suspensions (10^7 – 10^9 spores/mL) were diluted 1:10 (v/v) in
171 phosphate buffer pH 7.0 (Sigma-Aldrich, Saint-Quentin-Fallavier, France) before the heat treatment;
172 50 µL capillary tubes (Hirschmann Laborgeräte, Ringcaps®, Germany) were then filled with 50 µL of
173 spore suspension and heat-treated in a thermostatic oil bath (JULABO GmbH, Seelbach, Germany;
174 LAUDA Therm 180, Roissy-Charles-de-Gaulle, France). The capillary tubes were removed from the
175 bath after the set time and cooled in a water/ice bath. After cleaning/disinfection with ethanol, the
176 tubes were broken at both ends and their contents poured into a tube containing 3 mL of tryptone
177 salt broth (Biokar Diagnostics, Beauvais, France). The capillary tubes were rinsed with 50 µL of
178 tryptone salt broth. Viable cells were counted by plating in MLSRAm. The plates were incubated
179 under anaerobiosis in jars (Oxoid™ AnaeroGen™, Sigma-Aldrich, Saint-Quentin-Fallavier, France) at
180 30 °C for 48 hours at 30 °C.

181

182 **2.4 Study of the physiological diversity of *C. sporogenes* and Group I *C. botulinum* strains**

183 **2.4.1 Experimental design**

184 The isolates of *C. sporogenes* and Group I *C. botulinum* were incubated in PYGm broth at
185 37 °C for 72 h in anaerobiosis. Two successive cultures of 100 µL of revived culture inoculated in 10
186 mL tubes of PYGm broth were then incubated at 30 °C for 16 h or 22 h. The vegetative cell
187 suspensions (100 µL) at a concentration of 10^6 CFU/mL were inoculated in 10 mL Hungate tubes
188 (Dutscher, Issy-les-Moulineaux, France) of PYGm broth adjusted to different pH values and sodium
189 chloride concentrations. The initial pH values used were 7.0, 5.1, 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, and 4.4.
190 The pH of the broth was adjusted with 1N HCl. The broth was then autoclaved at 121 °C for 15 min.
191 The pH meter (Meterlab, Villeurbanne, France) and a Tuff-Tip™ electrode (Fisher Bioblock Scientific,
192 Illkirch-Graffenstaden, France) were calibrated using standard buffer solutions (Merck, Darmstadt,

193 Germany) at pH 4.0 and 7.0. The sodium chloride concentrations tested were 0, 5, 6, 7, 8, 9, 10, 11
194 and 12% (w/vol) of NaCl added to the PYGm broth before autoclaving at 121 °C for 15 min. The
195 calculated water activity were 0.997, 0.994, 0.988, 0.981, 0.975, 0.968, 0.962, 0.955, 0.949, 0.942,
196 0.935 and 0.928, for each sodium chloride concentration from 0 to 12% (w/vol) respectively. The
197 oxygen was removed from the PYGm broth in an anaerobic chamber (90% N₂, 5% H₂, 5% CO₂, Whitley
198 A35 Workstation, Don Whitley Scientific, United Kingdom). The Hungate tubes were incubated at 30
199 °C for 48 days. Growth of vegetative cells was estimated by visible broth turbidity in the Hungate
200 tubes. Visual observations were made weekly. Three biological replicates were acquired for each
201 condition.

202

203 **2.4.2 Statistical analysis**

204 The turbidity of the tubes was assessed by visual inspection for all pH and sodium chloride
205 concentration values. The observed growth limits of pH and sodium chloride concentration were
206 assessed by a median value calculated between the three replicates values of observed growth or no
207 growth.

208 The heat resistance was quantified by the time needed to inactivate 90% of the cells
209 population, and estimated from the ratio of surviving cells after one of the four led heat treatments.
210 Assuming a log-linear destruction curve, this time corresponds to a decimal reduction time and
211 usually named D-value, was calculated as:

$$212 \quad D_T = \frac{t_T}{\text{Log}_{10} \left(\frac{N_0}{N_t} \right)}$$

213 **Equation 1**

214 Where D_T is the decimal reduction time (min) at the heating temperature T (102 or 110°C), t_T is the
215 heating time (2 or 10 min) at the T temperature, N₀ and N_t are the CFU before and after heat
216 treatment.

217 In order to compare the heat resistance of the strains, D-values were calculated at the
218 reference temperature (121.1 °C) using the equation 2:

$$219 \quad D_{121.1^{\circ}\text{C}} = D_T \times 10^{\left(\frac{(T-121.1)}{z}\right)}$$

220 **Equation 2**

221 Where $D_{121.1^{\circ}\text{C}}$ is the decimal reduction time at the reference temperature 121.1 °C, D_T is the decimal
222 reduction time at the experimental temperature T (102 °C or 110 °C), and z is the temperature
223 difference required to change the D -value by a factor of 10 ($z = 10$ °C for Group I *C. botulinum*)
224 (Bigelow, 1921; Diao et al., 2014).

225 Finally, we performed a principal component analysis (PCA) on the growth limit as a function of pH
226 and sodium chloride concentration; the heat resistance of *C. sporogenes* and Group I *C. botulinum*
227 spores were taken as variables. The strain isolation matrix was used for illustrative qualitative
228 variables. We introduced two illustrative strains, namely Group I *C. botulinum* strain 41 (Prevot 910)
229 and Group I *C. botulinum* strain 42 (301-13). Statistical analysis was performed using the *FactoMineR*
230 package in R software (<https://cran.r-project.org/>) (Lê et al., 2008).

231

232 **2.5 Diversity and phylogeny of *C. sporogenes* and Group I *C. botulinum* strains and neurotoxin 233 production capacity**

234 **2.5.1 Genomic DNA preparation**

235 All genomic DNA preparations and whole-genome sequencing steps were performed by an
236 external partner (Genoscreen, Lille, France). Genomic DNA of *C. sporogenes* was extracted from
237 culture grown in PYGm broth as described previously. DNA extraction was performed by following
238 Gram-positive cell lysis with a Gentra Puregene kit (Qiagen, Valencia, USA). The DNA quality was
239 checked by fluorometric determination with SYBR Green and qualitatively controlled by agarose gel
240 electrophoresis according to the manufacturer's instructions.

241

242 **2.5.2 Whole genome sequencing**

243 Whole genome sequence data were generated with the Illumina sequencing technology.
244 Fragment libraries were constructed using a Nextera XT DNA Library Prep kit (Illumina, Cambridge,
245 UK) according to the manufacturer's instructions. Size selection was performed using an agarose gel
246 electrophoresis system (Life Technologies, Grand Island, USA) and quantitatively controlled by
247 fluorometric determination with SYBR Green. The pool of libraries was qualitatively and
248 quantitatively controlled using migration by fluorometric assay and by capillary electrophoresis on an
249 Agilent DNA high sensitivity chip (Agilent, Santa Clara, USA). Sequencing was generated with a 2 ×
250 150 bp read metric on an Illumina HiSeq sequencer.

251

252 **2.5.3 Genome assembly, phylogeny and bioinformatic analyses**

253 Genome sequences were assembled *de novo* using Shovill version 1.0.9
254 (<https://github.com/tseemann/shovill>), with default settings for the SPAdes assembler version
255 3.14.1, a minimum coverage of 5× and a minimum contig size of 200 bp (Bankevich et al., 2012). The
256 assembly metrics were determined using Quast version 4.6.3 (Gurevich et al., 2013). Illumina FASTQ
257 reads. Genome assemblies were uploaded in Genbank in BioProject PRJNA763108 (see
258 Supplementary Data Table S4). Assembly metrics and Genbank/SRA accession numbers of individual
259 samples are given in Supplementary Table S1.

260 Core genome single nucleotide polymorphisms (SNPs) were identified using ParSNP version 1.2 with
261 PhyPack enabled ("-x" switch). Phylogenetic trees were constructed using the built-in FastTree2
262 module (Treangen et al., 2014). The Group I *C. botulinum* and *C. sporogenes* genome sequences
263 included for comparison had been previously described (Brunt et al., 2020) (see Supplementary Data
264 Table S2). The genomes were searched for botulinum toxin genes using TBLASTN with the
265 representatives sequences of the A, B, C, D, E, F, G, HA, X and EBoNT/J toxins, with no toxin homologs
266 detected (Peck et al., 2017).

267

268 **2.6 Synergistic effect of pH and sodium chloride concentration and heat treatment on growth**
269 **limits of *C. sporogenes* and Group I *C. botulinum* strains**

270 **2.6.1 Experimental design**

271 Suspensions of cells (vegetative cells or spores) at different physiological states were used at
272 concentrations of 7.92 ± 0.52 log/mL (*C. sporogenes* isolate 1), 7.54 ± 0.85 log/mL (*C. sporogenes*
273 isolate 14), 7.76 ± 0.36 log/mL (*C. sporogenes* isolate 17), 8.09 ± 1.63 log/mL (*C. sporogenes* isolate
274 34) and 8.03 ± 0.87 log/mL (Group I *C. botulinum* isolate 41). We used three different cellular
275 physiological states: vegetative cells, spores heated at 80 °C for 10 min (“spores”), and spores heat-
276 treated with sublethal treatment (“heat-treated spores”). For the latter, the spores were heat-
277 treated to obtain approximately 1 log decimal reduction. The treatments were strain-dependent: (i)
278 strain 1 was heat-treated at 100 °C for 1.5 min, (ii) strain 14 at 100 °C for 1.8 min, (iii) strain 17 at
279 100 °C for 2.3 min, (iv) strain 34 at 110 °C for 1.7 min and (v) strain 41 at 100 °C for 4 min. These
280 growth limits refer to growth for vegetative cells, and to germination, outgrowth and growth for
281 spores.

282 Viable cell concentration was assessed using the most probable number (MPN) method in PYGm
283 broth in 96-well microplates (Nest, Wuxi, China). MPN was estimated by six successive 10-fold serial
284 dilutions in a 96-well microplate. For each dilution, eight wells were replicated. A reduced factorial
285 design, including 30 combinations of 11 pH levels and 13 sodium chloride concentrations, was used
286 (see in supplementary data Table S6). The PYGm broth pH values were 7.0, 6.0, 5.8, 5.6, 5.4, 5.2, 5.0,
287 4.8, 4.6, 4.5, and 4.4. The sodium chloride concentrations tested were 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11
288 and 12% (wt/vol) of NaCl added to PYGm broth before autoclaving at 121 °C for 15 min. The
289 microplates were inoculated in an anaerobic chamber (90 % N₂, 5 % H₂, 5 % CO₂, Whitley A35
290 Workstation, Don Whitley Scientific, United Kingdom). Then, the microplates were incubated in a bag
291 to obtain an anaerobic atmosphere (AnaeroGen™ Thermo Oxoid™, ThermoFisher Scientific, Dardilly,
292 France) at 30 °C for 48 days. Growth of vegetative cells or germination and outgrowth of spores were
293 determined based on visible broth turbidity in the wells. The growth was considered as positive when

294 1 out of 8 wells present growth. Observations were made weekly. The influence of pH and sodium
295 chloride concentrations was studied independently and in combination and replicated four times.

296

297 **2.6.2 Data processing and modelling**

298 For each combination of pH and sodium chloride concentration, the number of wells where
299 microbial growth was observed, was assessed in a set of serial dilutions. This allowed the
300 computation of the population density of cells able to grow (MPN function, Statistical Toolbox,
301 MATLAB R2013b, MathWorks, Natick, MA) (Fridman, 2020). Four experimental replicates were used
302 to calculate the mean of the observed number of decimal reductions (n_{obs}). Whenever one of the
303 experimental replicates was bounded by a censored value, the mean was given using the *fitdistrplus*
304 function available in the R package (<https://cran.r-project.org/>) (Pouillot and Delignette-Muller,
305 2010).

306 Based on this estimated density of cells able to grow, the number of decimal reductions observed
307 (n_{obs}) was computed with Equation 3, as follows:

$$308 \quad n_{\text{obs}} = \log(N_0) - \log(N_{\text{obs}})$$

309 **Equation 3**

310 where N_0 is the concentration of vegetative cells or spores able to grow when the pH is equal to 7
311 and sodium chloride concentration is 0% ($a_w = 0.997$), and N_{obs} is the concentration of vegetative cells
312 or spores able to grow at the given condition. The limit of quantification is reached when only one
313 well is positive. Beyond this limit of detection, the result is considered as censored data.

314 The decimal reduction number according to the environmental conditions was derived from the
315 model of Boix *et al.*, 2021 with Equation 4 (Boix *et al.*, 2021):

$$316 \quad \begin{matrix} n_{\text{pred}} \\ (0 \end{matrix} \quad , \text{if } \Pi \geq \Pi_0$$

317 **Equation 4**

318 where n_{pred} is the predicted decimal reduction number of vegetative cells or heat-treated spores at
 319 different combined pH values and sodium chloride concentrations, Π is the gamma product derived
 320 from the Gamma concept (Zwietering et al., 1992), and Π_0 is the Π value above which n_{pred} is not
 321 affected by pH and sodium chloride concentration. At the optimal condition, $\Pi = 1$ and $n_{\text{pred}} = 0$.
 322 When the conditions become adverse, $\Pi = 0$ and n_{pred} tends towards infinity as pH, sodium chloride
 323 concentration and their interaction approach the limit of growth for the vegetative cells and the limit
 324 of germination, outgrowth and growth for the spores.

325 The product Π is computed as followed for each condition of recovery:

$$326 \quad \Pi = \gamma_{\text{CM}_{\text{pH}}} \times \gamma_{\text{CM}_{a_w}}$$

327 **Equation 5**

328 Where γ_{CM_X} quantifies the impact of each environmental factor (pH, a_w) and γ_{ξ} quantifies their
 329 synergistic effect.

330 γ_{CM_X} is computed as follows:

$$331 \quad \gamma_{\text{CM}_X} = \frac{(X - X_{\text{max}}) \times (X - X_{\text{min}})^n}{(X_{\text{opt}} - X_{\text{min}})^{n-1} [(X_{\text{opt}} - X_{\text{min}})(X - X_{\text{opt}}) - (X_{\text{opt}} - X_{\text{max}})((n - 1) \times X_{\text{opt}} + X_{\text{min}} - nX)]}$$

332 **Equation 6**

333 Where X is the variable studied (a_w or pH), $a_{w_{\text{min}}}$, $a_{w_{\text{opt}}}$, $a_{w_{\text{max}}}$, pH_{min} , pH_{opt} and pH_{max} are
 334 minimum, optimum and maximum growth and germination and outgrowth values for the variables
 335 studied (a_w and pH), and n is a shape parameter which is 1 for a_w and pH (Rosso et al., 1995, 1993).
 336 The function describing the interaction between the studied factors was given by Le Marc *et al.*
 337 (2001) and later modified by Augustin *et al.* (2005) (Augustin et al., 2005; Le Marc, 2001). γ_{ξ} is
 338 computed as follows:

$$\gamma_{\xi} = \begin{cases} 1, & \psi \leq 0.5 \\ 0, & \psi \geq 1 \end{cases}$$

340 **Equation 7**

341 where

$$\psi = \sum \frac{\varphi(X_i)}{\dots}$$

343 **Equation 8**

344 and

$$\varphi(X) = \left(\frac{X_{\text{opt}} - X}{\dots} \right)^3$$

346 **Equation 9**

347 Where X_{min} and X_{opt} are minimum and optimum growth and germination and outgrowth values for
 348 the studied variables.

349

350 The parameters were estimated by minimizing the sum of square root error using a non-linear fitting
 351 function (*lsqcurvefit* function, Optimization Toolbox, MATLAB R2020a, MathWorks, Natick, MA). The
 352 95% confidence intervals of the estimated parameters were computed using the confidence intervals
 353 based on bootstrap percentiles (Efron and Tibshirani, 1993). Goodness of fit was evaluated by the
 354 coefficient of determination (R^2) and the root-mean-square error (RMSE). A value close to 1 indicates
 355 a best fit for R^2 . The smaller the RMSE, the closer the model fitted the data.

356

357 **3 Results and Discussion**

358 **3.1 Study of physiological diversity of *C. sporogenes* and Group I *C. botulinum* strains**

359 **3.1.1 Minimum growth pH for *C. sporogenes* and Group I *C. botulinum* strains**

360 The lower pH limits for the growth of vegetative cells of the 42 isolates (37 *C. sporogenes*/Group I
361 *C. botulinum* isolates isolated from spoiled canned food, three reference *C. sporogenes* strains and
362 two reference Group I *C. botulinum* strains) are presented in Table 2 and Figure 1a. The growth limits
363 of the 42 studied isolates ranged from pH 4.49 (*C. sporogenes* strain 17) to pH 5.05 (*C. sporogenes*
364 strain 32). Fifty percent of the isolates studied grew at pH 4.6. The median pH limit for growth was
365 pH 4.61. Among these isolates, 18 isolates (43%) grew at a pH below 4.6. A pH of 4.6 is normally
366 defined as the minimum pH needed for Group I *C. botulinum* to grow (Peck, 2009). Several studies
367 have shown that the minimum pH for growth of Group I *C. botulinum* is between pH 4.6 and pH 4.8,
368 although for some strains it may far exceed pH 4.9–5.0 (Hauschild, 1993; Odlaug and Pflug, 1978;
369 Townsend et al., 1954). The determination of the minimum pH for growth is dependent on several
370 factors (growth medium, size of the inoculum...), so it is difficult to have the absolute limit of the
371 species but rather a range of growth and no growth. The occurrence of strains of *C. sporogenes*
372 growing at pH 4.5 raises the possibility that certain strains of *C. sporogenes* might form botulinum
373 toxin at this pH. This needs further investigation, given that in canning, the risk presented by Group I
374 *C. botulinum* is currently only considered for products with a pH above 4.6 (CTCPA and DGAL/SDSSA,
375 2015; FDA and GMA, 2010).

376

377 **3.1.2 Maximum sodium chloride concentration for growth of *C. sporogenes* and Group I**
378 ***C. botulinum* strains**

379 The sodium chloride (NaCl) growth limits are presented in Table 2 and Figure 1b. Most of the isolates
380 (98%) of this study did not grow at an NaCl concentration above 10%. The median concentration was
381 7.5% NaCl. No growth was observed over 5% NaCl concentration for *C. sporogenes* strain 32. For this
382 strain, the growth limit as a function of NaCl concentration could not be determined more precisely

383 because only concentrations of NaCl between 5% and 12% were tested. By contrast, the
384 *C. sporogenes* strain 14 grew at 11.5% NaCl. No growth was observed at or above 12% NaCl, as
385 previously reported in the literature. The growth limit NaCl concentration for Group I *C. botulinum*
386 species is 10% (ANSES, 2019; Peck, 2009). The heterogeneity of growth limits as a function of NaCl
387 concentration observed here was consistent with the literature (Roberts and Ingram, 1973; Russell
388 and Gould, 2003).

389

390 **3.1.3 Heat resistance of *C. sporogenes* and Group I *C. botulinum* spores**

391 The heat resistance of 40 isolates of *C. sporogenes* and Group I *C. botulinum* spores was studied at
392 102 °C and 110 °C for 2 min and 10 min. To compare the heat resistance of all isolates, an
393 extrapolated *D*-value was calculated at 121.1 °C and *z*-value of 10 °C. The results are presented in
394 Table 2, and Figure 1c. *C. sporogenes* isolates 2 and 32 were not tested. Group I *C. botulinum* strains
395 used in this study had a heat resistance $D_{121.1\text{ °C}}$ of 0.03 min (strain 41) and 0.066 min (strain 42). The
396 maximum *D*-value at 121.1 °C was 0.50 min (*C. sporogenes* strain 16) with the median heat resistance
397 value of 0.02 min at 121.1 °C. The reference value for heat resistance of Group I *C. botulinum* species
398 is $D_{121.1\text{ °C}} = 0.21$ min (ANSES, 2019; Esty and Meyer, 1922; FDA and GMA, 2010; Peck, 2009). None of
399 the Group I *C. botulinum* strains tested in our study reached this reference heat resistance. It is
400 recognised that the heat resistance of *C. sporogenes* spores is higher than that of Group I *C.*
401 *botulinum* spores (Brown et al., 2012; Diao et al., 2014). Diao et al. (2014) in a meta-analysis showed
402 that *C. sporogenes* PA 3679 strain spores had an average heat resistance $D_{121.1\text{ °C}}$ of 1.3 min in
403 contrast to Group I *C. botulinum*, whose average heat resistance $D_{121.1\text{ °C}}$ was 0.2 min (Diao et al.,
404 2014). Spores of isolates labelled *C. sporogenes* strain PA 3679 (isolates 1 and 40) examined in the
405 present study had a lower heat resistance ($D_{121.1\text{ °C}} = 0.02$ and 0.16 min) than measured elsewhere
406 (Diao et al., 2014).

407

408 **3.1.4 Correlation between physiological characteristics of *C. sporogenes* and Group I *C.***
409 ***botulinum* strains**

410 Principal component analysis (PCA) was performed on growth limit values as a function of pH, NaCl
411 concentration and spore heat-resistance at 121.1 °C for the *C. sporogenes* and Group I *C. botulinum*
412 isolates (37 isolates from spoiled canned food and five reference strains). This included two
413 reference Group I *C. botulinum* strains (strains 41 and 42, Figure 2b).

414 The three axes in the covariance circle represented pH, NaCl concentration and spore heat resistance
415 (TT) (Figure 2a). The three axes were not correlated with each other. PCA statistics showed that
416 dimension 1 was positively correlated with pH growth limit, and negatively correlated with the NaCl
417 concentration growth limit. Dimension 1 explained 46.57% of the variance of the individual profiles.
418 Dimension 2 was positively correlated to the spore heat resistance, and explained 36.27% of the
419 variance of the individual profiles.

420

421 The PCA map shows the 42 isolates of *C. sporogenes* and Group I *C. botulinum* (37 isolates isolated
422 from spoiled canned food and five reference strains) as a function of growth limit pH and sodium
423 chloride concentration (NaCl) and spore heat resistance (TT) (Figure 2b). The population fell into
424 three groups: (i) a heat resistant group (top), (ii) a group resistant to acid pH and high sodium
425 chloride concentration (bottom left) and (iii) a group that grew only at high pH and low NaCl
426 concentration and had low spore heat resistance (bottom right). Three isolates in particular had
427 opposite pH and NaCl characteristics (*C. sporogenes* isolates 14, 16 and 32). *C. sporogenes* strain 14
428 was acid- and NaCl-resistant; it could grow at pH 4.51 or 11.5% NaCl and had a $D_{121.1\text{ }^{\circ}\text{C}}$ value of 0.139
429 min. *C. sporogenes* strain 32 was sensitive to acid and NaCl, and could grow at pH 5.05 or 5% NaCl. *C.*
430 *sporogenes* strain 16 was intermediate for pH and NaCl growth limits, growing at pH 4.50 and 7.5%
431 NaCl but was the most heat-resistant strain with an estimated $D_{121.1\text{ }^{\circ}\text{C}}$ value of 0.50 min. There was
432 no link or correlation between the origin (culture collection, fish, meat, milk-based dessert, prepared

433 meat meal and vegetables) and the physiological characteristics of the isolates as observed on the
434 PCA map.

435

436 **3.2 Genetic diversity a of *C. sporogenes* and Group I *C. botulinum* strains and their neurotoxin** 437 **production ability**

438 Eleven isolates of *C. sporogenes* (nine isolates isolated from spoiled canned food and two
439 reference strains), and three isolates of non-toxigenic Group I *C. botulinum* (isolated from spoiled
440 canned food) and one reference Group I *C. botulinum* strain (strain 41) were selected for a genetic
441 diversity study (Table 2). In this study, the production of botulinum toxin was not quantified, only the
442 presence of the genes allowing the production of botulinum toxin were identified by comparison of
443 the sequences and the reference sequences by Blast. The selection was made according to their
444 growth limits for pH and/or NaCl concentration and/or the heat resistance of their spores. For
445 example, *C. sporogenes* strain 17 grew at an acid pH of 4.49, *C. sporogenes* strain 14 grew up to a
446 sodium chloride concentration of 11.5%, and Group I *C. botulinum* strain 34 showed a high spore
447 heat resistance, $D_{121,1\text{ }^{\circ}\text{C}} = 0.25$ min.

448

449 Two major lineages were identified: Group I *C. botulinum* and *C. sporogenes*. Strain 41 (Prevot 910) is
450 currently recognised as a Group I *C. botulinum* producer of type A neurotoxin (Figure 3). Among our
451 newly isolated isolates, three (isolates 5, 23 and 34) were initially identified as *C. sporogenes* strains
452 (based on 16S ribosomal DNA sequencing, *in vivo* tests on mice and toxin gene detection by PCR) and
453 redefined by WGS as Group I *C. botulinum* strains but lacking a botulinum toxin gene (using SNP
454 analysis and BLAST). Toxin production was not experimentally determined for the *C. sporogenes*
455 strains belonging to the Group I *C. botulinum* strains. Whole genome sequencing analysis provides a
456 powerful approach to show the phylogenetic relatedness of neurotoxin-producing and non-
457 neurotoxin-producing strains, and the genetic modifications and exchanges between closely related
458 strains. Core genome SNP typing is currently the standard method for building large phylogenetic

459 trees to establish relations between strains. ParSNP performs microbial genome alignments to
460 identify both structural and point variations (Pightling et al., 2018; Treangen et al., 2014). The non-
461 toxigenic Group I *C. botulinum* isolates (5, 23 and 34) are closely genetically related, clustered
462 together and were isolated from meat. These three isolates showed high heat resistance at 121.1 °C
463 up to 0.25 min for strain 34, 0.21 min for strain 5 and 0.20 min for strain 23. These three isolates had
464 a median tolerance to sodium chloride concentration (6.5–7.5%) and a median tolerance to acid (pH
465 4.50–4.73).

466 The remaining 11 isolates were classified as belonging to the *C. sporogenes* species. The *C.*
467 *sporogenes* group was divided into two subgroups, and our *C. sporogenes* isolates (11 isolates) were
468 located in both. The phylogenetic tree showed that isolates 8 and 40 were closely related but
469 differed in their physiological characteristics. The strain 40 was more acidophilic and more resistant
470 to NaCl concentration and heat treatment (pH: 4.51, 9.50% and $D_{121.1\text{ }^\circ\text{C}} = 0.02$ min) than strain 8 (pH
471 4.83, 7.50%, and $D_{121.1\text{ }^\circ\text{C}} = 0.01$ min). These results showed a genomic similarity but a physiological
472 diversity, although both isolates were isolated from plant material. By contrast, strain 1 (pH 4.50 and
473 8.50%) and strain 40 (pH 4.51 and 9.50%) had close physiological characteristics, although the heat
474 resistance of the spores of strain 1 ($D_{121.1\text{ }^\circ\text{C}} = 0.16$ min) was higher than that of strain 40 ($D_{121.1\text{ }^\circ\text{C}} =$
475 0.02 min). Isolates 1 and 40 are described as versions of strain PA 3679 and are in the same group of
476 *C. sporogenes* in the phylogenetic tree, but distantly related. Originally, these isolates came from two
477 different collections, one from the Campden collection (strain 1) and the other from the ATCC
478 collection (strain 40, ATCC 7955). This divergence between these isolates may also be due to
479 mutations accumulated over time by successive subcultures (Treangen et al., 2014). Other studies
480 have reported significant heterogeneity among isolates described as PA 3679. Some isolates with a
481 lower spore heat resistance genetically resembled *C. sporogenes*, whereas other isolates with a
482 higher spore heat resistance genetically resembled Group I *C. botulinum* (Bradbury et al., 2012;
483 Bradshaw et al., 2010; Schill et al., 2016; Weigand et al., 2015). The two isolates described as strain

484 PA 3679 (isolates 1 and 40) tested in the present study align with isolates in the *C. sporogenes*
485 grouping (Figure 3).

486

487 **3.3 Synergistic effect of pH, sodium chloride concentration and heat treatment on growth limits** 488 **of *C. sporogenes* and Group I *C. botulinum* strains**

489 Physiological and genomic studies of *C. sporogenes* (34 isolates from spoiled canned food and three
490 reference strains), non-toxigenic Group I *C. botulinum* (three isolates isolated from spoiled canned
491 food) and Group I *C. botulinum* (two reference strains) showed heterogeneity in growth limits as a
492 function of pH and NaCl concentration and as a function of spore heat resistance (although no link
493 was established between physiological characteristics and genetic diversity). These observations
494 prompted us to determine how the growth limits of vegetative cells, spores and heat-treated spores
495 varied as a function of pH and NaCl concentration.

496

497 Tests were carried out with five isolates of *C. sporogenes* or Group I *C. botulinum*: isolates 14 and 17
498 isolated from spoiled canned food, reference strain 1, strain 34 isolated from spoiled canned food,
499 and reference strain 41. Strain 14 (pH 4.51, 11% NaCl and $D_{121.1\text{ }^{\circ}\text{C}} = 0.14$ min) was the most salt-
500 tolerant and strain 17 (pH 4.49, 9.5% NaCl and $D_{121.1\text{ }^{\circ}\text{C}} = 0.02$ min) was the most acid-tolerant. These
501 two isolates were classified in the two subgroups of the species *C. sporogenes*. Strain 34 was included
502 based on the results of the genetic analysis (identified as non-toxigenic Group I *C. botulinum*) and the
503 physiological study (growth limit: pH 4.50, 7.5% NaCl and $D_{121.1\text{ }^{\circ}\text{C}} = 0.25$ min). The reference Group I
504 *C. botulinum* strain 41 had a medium growth limit and heat resistance (pH 4.68, 6.75% NaCl and $D_{121.1\text{ }^{\circ}\text{C}}$
505 $= 0.03$ min). Figure 4a shows the observed germination, outgrowth and growth limit as a function
506 of pH of *C. sporogenes* (isolates 1, 14 and 17) and Group I *C. botulinum* (isolates 34 and 41). The
507 status of the inoculum (vegetative cells, spores or heat-treated spores) greatly impacted the limits
508 for growth. These limits correspond to growth for vegetative cells and germination, outgrowth and

509 growth for the spores. For vegetative cells, the growth limits were between pH 4.5 and pH 4.7
510 depending on the isolates, in line with the literature (pH limit of 4.6) (Peck, 2009).

511 The pH germination/outgrowth limits for spores that were heat-activated (80 °C, 10 min) were higher
512 than the growth limit of vegetative cells by 0.24 pH units (mean value). When a stronger heat
513 treatment was applied to *C. sporogenes* and Group I *C. botulinum* spores, the observed values of the
514 pH growth limits increased. Indeed, the pH increased by 0.20 units between spores and heat-treated
515 spores, and the pH increased by 0.40 between vegetative cells and spores heated at 100/110 °C. The
516 growth limits as a function of pH thus differed between species and within the same species (0.3 pH
517 units increase).

518 The limit of a_w (controlled by NaCl) for growth followed the same pattern as the pH growth limit
519 (Figure 4b): (i) spores (activated at 80 °C for 10 min) showed a smaller interval of a_w limit for
520 germination and outgrowth compared with the growth limits of vegetative cells (increasing by 0.007
521 on average), and (ii) the a_w -dependent germination/outgrowth limits for heat-treated spores were
522 higher than for spores or vegetative cells (increasing by 0.013 on average) (see Figure 4b). Strain 17
523 seems to be the strain least affected by the pH or NaCl concentration on the germination and
524 outgrowth limits of spores. This result illustrated the variability of growth limits among isolates.

525

526 **3.3.1 Assessment of the recovery probability of *C. sporogenes* and Group I *C. botulinum***

527 The synergistic effect of environmental factors on growth was then further investigated for
528 the five isolates of *C. sporogenes* and Group I *C. botulinum* (presence or absence of toxin gene). The
529 growth range of vegetative cells and germination and outgrowth of spores and heat-treated spores
530 was studied using a factorial design combining different pH values and NaCl concentrations. A total
531 of 30 combinations of pH (pH 4.4–6.5) and sodium chloride concentration (0–12% NaCl) were
532 studied. Decimal reduction number as a function of pH and NaCl concentration was modelled with
533 Equation 3. The lowest observed probability of growth or of germination and outgrowth was 8.22
534 log, which corresponds to less than one cell in a hundred million. Estimated coefficients of regression

535 modelled for the probability of growth of vegetative cells and spores of *C. sporogenes* and Group I *C.*
536 *botulinum* isolates and the outcomes of variance analysis of the results are shown in Table 3 and
537 Figure 5. The developed model displayed a good fit with the observed data (root-mean-square error
538 (RMSE) values in Table 3). The parameters of the model were distant from the actual limits observed,
539 because pH_{min} and a_{wmin} values would only be determined from an infinite inoculation concentration.
540 It was not possible to experimentally determine these absolute values of pH and sodium chloride
541 concentration for high inoculation concentrations, whence insufficient data to make accurate
542 estimations for the low probabilities.

543

544 The growth probability of vegetative cells and the germination, outgrowth and growth probability of
545 heat-treated spores was established. Contour plot for the non-toxigenic Group I *C. botulinum* strain
546 34 is shown in Figure 6 as an example. The interactions between pH and sodium chloride
547 concentration, and so the probability of growth, are affected by these three physiological states. The
548 growth range of the vegetative cells was greater than that of the spores, with a boundary at pH 4.5
549 and 6% sodium chloride for a probability of $-8 \log$ (Figure 6a). By contrast, for spores (heat-activated
550 at 80 °C for 10 min), germination and outgrowth range decreased to pH 4.8 and 5% sodium chloride
551 (Figure 6b). Spore germination and outgrowth range decreased even more as the intensity of the
552 heat treatment increased (pH 5 and 3% of NaCl) (Figure 6c).

553 Similar results were observed for *C. sporogenes* strain 1 (PA 3679) and Group I *C. botulinum* strain 41
554 (Prevot 910) (see Supplementary Data Figure S1 and Figure S4). For Group I *C. botulinum* strain 41,
555 only few data were acquired for spores (activated at 80 °C) so it was not possible to fit the model.
556 However, the observed data also show an effect of heat treatment on the germination limits of the
557 spores (see Supplementary Data, Table S6). For *C. sporogenes* strain 14, the effect of heat treatment
558 was less marked than for the other isolates (Table 3). The probability of germination and outgrowth
559 for spores was slightly different between vegetative cells and spores (see Supplementary Data Figure
560 S2). For the *C. sporogenes* strain 17, the effect of heat treatment between spores and heat-treated

561 spores was less pronounced than for the other isolates. The probability of germination and
562 outgrowth for the spores was not affected by the more intense heat treatment. However, the
563 probability of growth was different between vegetative cells and spores (see Supplementary Data
564 Figure S3). The effect of heat treatment on pH and sodium chloride concentration interactions and
565 growth probabilities depended on isolates, but the same impacts of physiological state were
566 observed.

567 Although the present dataset and derived models are extremely useful in helping to prevent the
568 spoilage of ambient products by *C. sporogenes*, we note that food safety with respect to Group I
569 *C. botulinum* will also require a demonstration that no botulinum neurotoxin is formed, as foodborne
570 botulism is an intoxication.

571

572 **4 Conclusion**

573 Physiological and genetic parameters of *C. sporogenes* isolates (34 isolates isolated from spoiled food
574 and three reference strains), non-toxigenic Group I *C. botulinum* (three isolates isolated from spoiled
575 food) and reference strains of Group I *C. botulinum* (two reference strains) were studied. The growth
576 limit as a function of pH and sodium chloride concentration and the spore heat resistance revealed a
577 strong diversity within the two species. No relationship was observed between the physiological
578 characteristics and the isolation matrix of the isolates. The genetic study revealed that three isolates
579 isolated from spoiled canned food, initially identified as *C. sporogenes* isolates, belonged to Group I
580 *C. botulinum*. No link was established between physiological characteristics and phylogeny. The
581 ability of selected isolates to grow as vegetative cells or spores was assessed according to pH and
582 NaCl concentration of the growth media. The process of germination of spores (germination,
583 outgrowth and cell division into vegetative cells) was impacted by the pH, sodium chloride
584 concentration and heat treatment.

585 One main result is the impact of pH and sodium chloride concentration on growth range, stronger for
586 vegetative cells than for heat-treated spores. The growth/germination limits were strain-dependent

587 and acted synergistically. In addition, the accumulated data on growth limits provide a better
588 understanding of the behaviour of isolates, including isolates with extreme characteristics. The study
589 of the heterogeneity of physiological and genetic characteristics allows a better assessment of the
590 risks and helps prevent and/or minimise foodborne botulism outbreaks and canned food spoilage. In
591 addition, the pH limit of industrial processes for acid or acidified pasteurised products stored at room
592 temperature is currently 4.6. This pH limit is safe, based on the vegetative cell growth limits. If the
593 recovery limits of heat-treated spores are taken into account, the pH limit for food formulation could
594 be increased (e.g. 4.8–4.9). If the synergistic effect between food formulation and heat treatment on
595 spore recovery is taken into account, acid canned food can be formulated up to pH 5 with 4% of salt.
596 It is important to recognise that to ensure food safety with regard to Group I *C. botulinum*,
597 establishing the synergistic factors also helps to avert the formation of botulinum neurotoxin. This
598 type of synergistic action is already considered for the control of *Listeria monocytogenes*
599 (“Règlement (CE) N°2073/2005 de la Commission,” 2005).

600

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607

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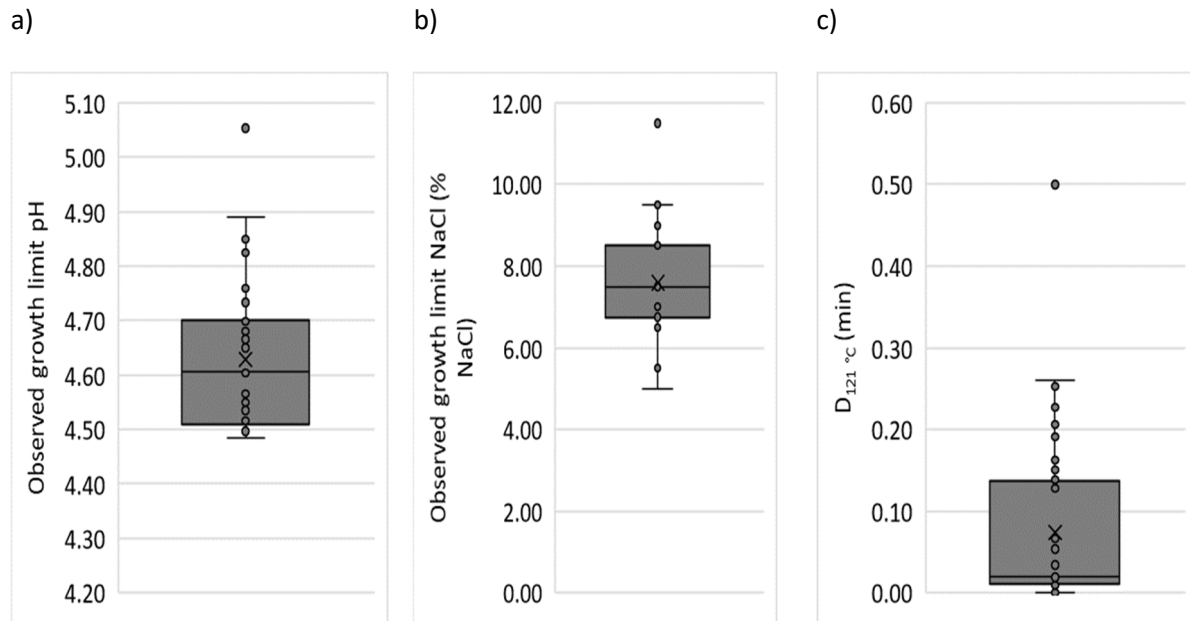
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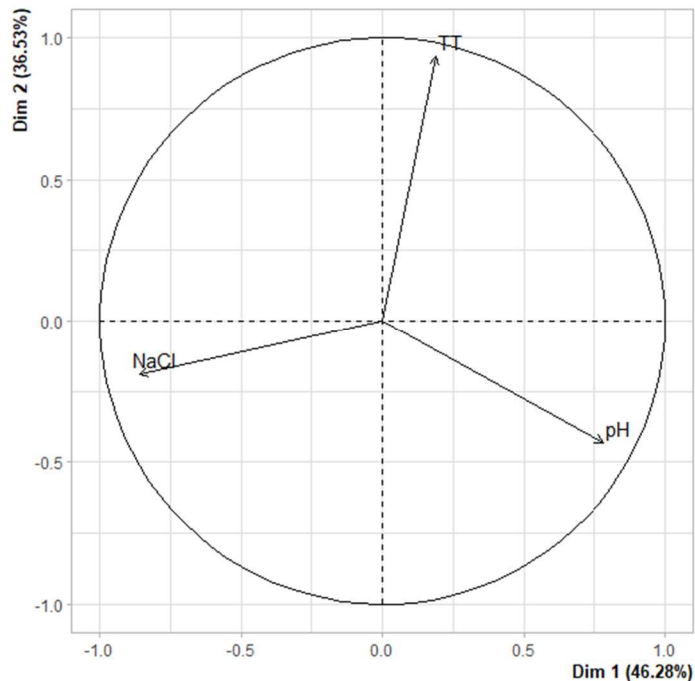
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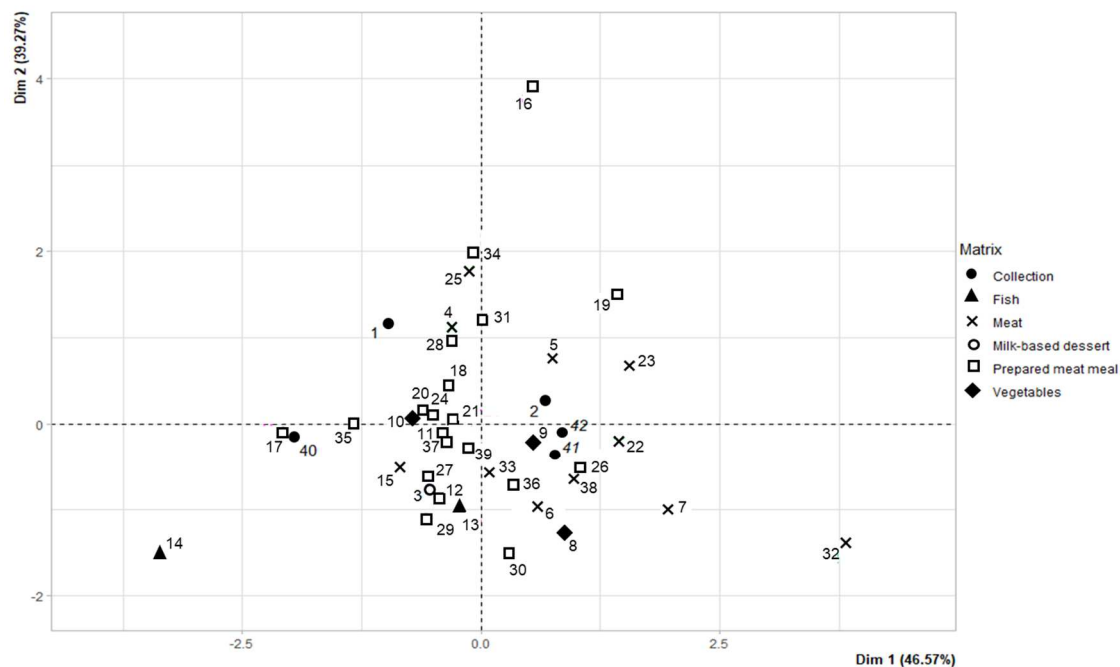


1 **Figure 1. Boxplots of (a) observed growth limit pH, (b) observed growth limit sodium chloride**
 2 **concentration and (c) D -value calculated at 121 °C of 42 strains. The median observed growth limit**
 3 **and D -value were pH 4.61, 7.5% NaCl and 0.021 min at 121.1 °C.**

a)



b)



5 **Figure 2. (a) Principal component analysis (PCA) correlation circle for growth limit as a function of pH and sodium chloride concentration (NaCl) and heat**
 6 **resistance (TT) of spores of 42 *C. sporogenes* and Group I *C. botulinum* isolates (at 121.1 °C). The correlation circle between the growth limits according to**
 7 **the three physiological characteristics represented by the arrows. The variability of the strains is explained by 47% according to the growth limits in NaCl,**
 8 **35% according to the heat resistance of the spores and 18% according to the growth limits in pH. The arrangement of each arrow then indicates that the**
 9 **factors pH, NaCl concentration and heat treatment are independent. (b) PCA map for *C. sporogenes* isolates and Group I *C. botulinum* strains. Each**

- 10 number corresponds to the strain number. Each shape corresponds to the source of isolation of each strain (see legend). The individuals on the bottom
- 11 left are halophilic strains, the individuals on the top right have high heat resistance and the individuals on the bottom right are acid sensitive strains.

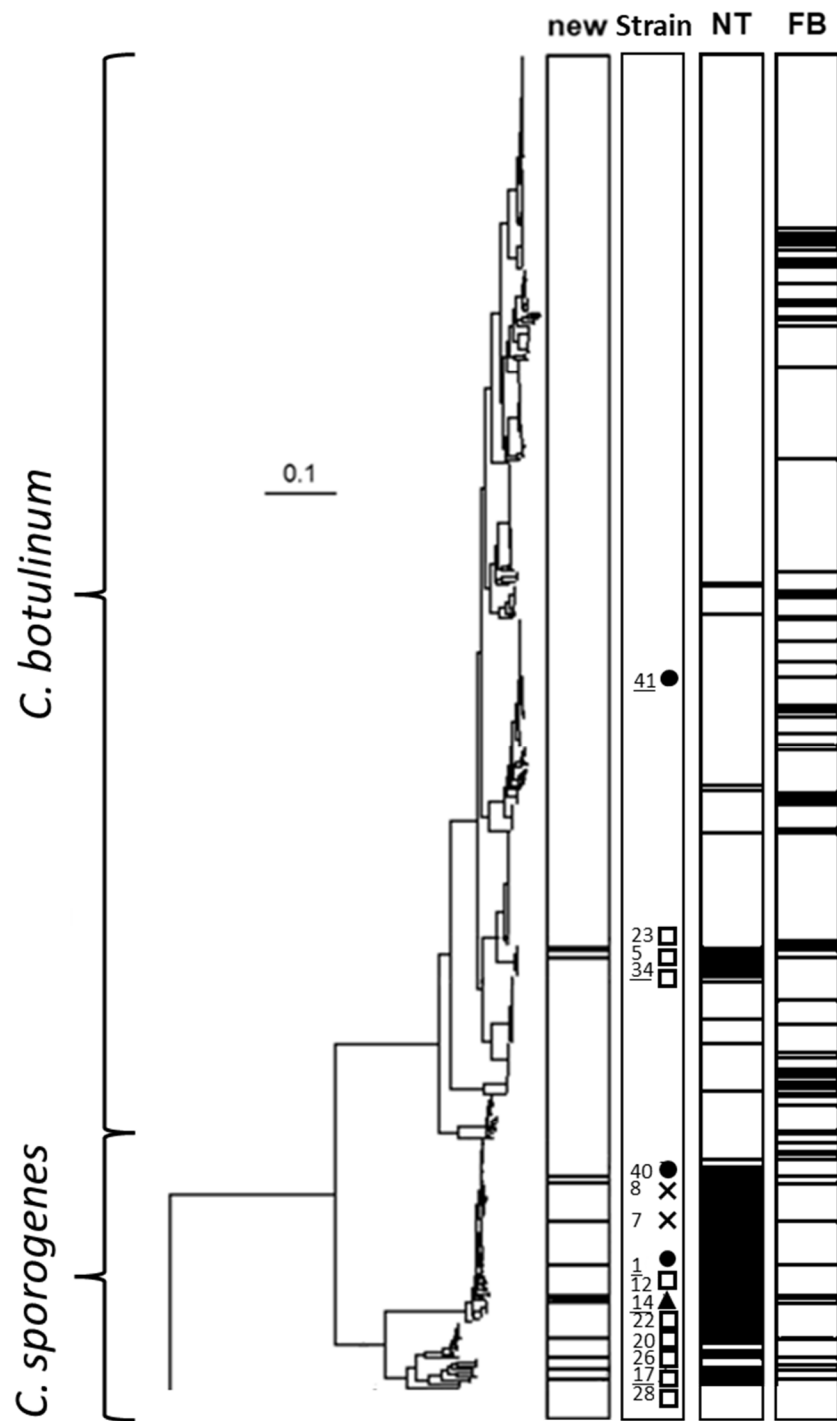
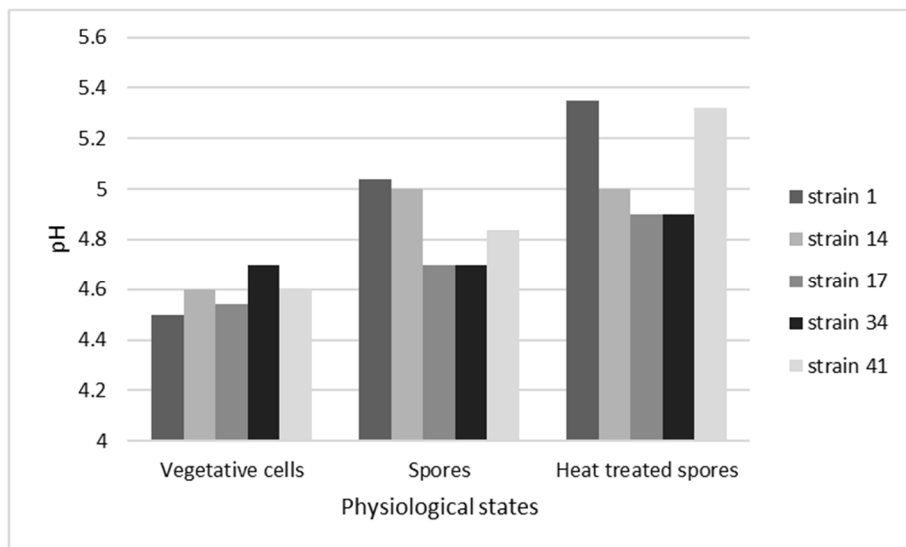


Figure 3. Phylogeny of genomes of Group I *C. botulinum* and *C. sporogenes* based on the phylogeny tree of Brunt (Brunt et al., 2020). The phylogenetic tree was created by comparing core single nucleotide polymorphisms (SNP) identified using the ParSNP program. Treegraph v2, MEGA7 and Figtree were used to annotate and visualise the phylogenetic tree. The distance 0.10 represents

the nucleotide substitution number per site for a given branch, based on the number of SNPs found in the core genome. Isolates 5, 23 and 34 are in the Group I *C. botulinum* clade and the other isolates are in the *C. sporogenes* clade (new frame). The underlined isolates (41, 34, 1, 14 and 17) were selected to assess their growth potential in conditions combining suboptimal levels of environmental factors. Black circles indicate collection strains, cross isolates isolated in meat, diamonds isolates isolated in vegetables, triangles isolates isolated in fish and empty squares isolates isolated in prepared meat meals (Table 1). NT: toxin genes. FB: isolates isolated from foodborne botulism incidents.

a)



b)

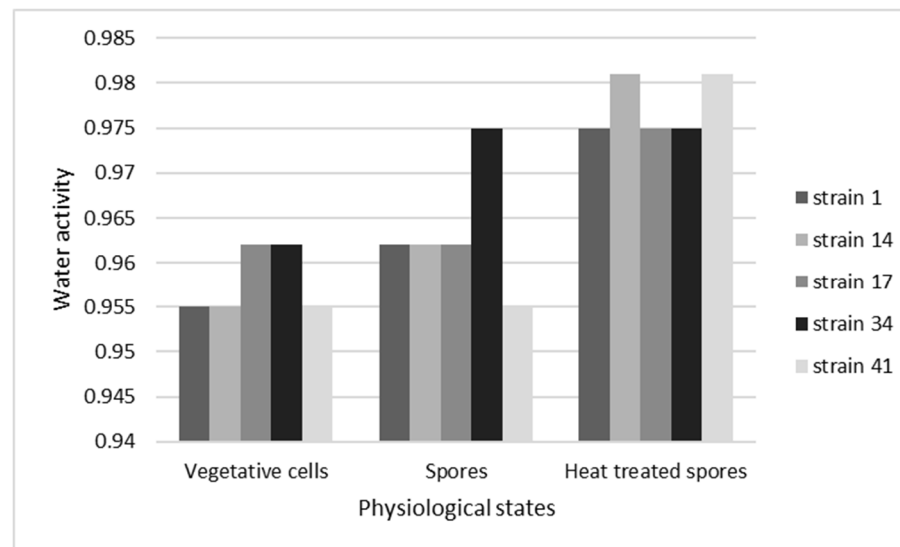


Figure 4. Effect of physiological state on (a) observed minimal pH for growth and (b) minimal water activity for growth (controlled by NaCl) for *C. sporogenes* isolates 1, 14 and 17, and Group I *C. botulinum* isolates 34 and 41. Three physiological states were used, vegetative cells, spores (heat treated at 80 °C for 10 min) and heat treated spores (the targeted heat treatment corresponds to the decimal reduction of one log).

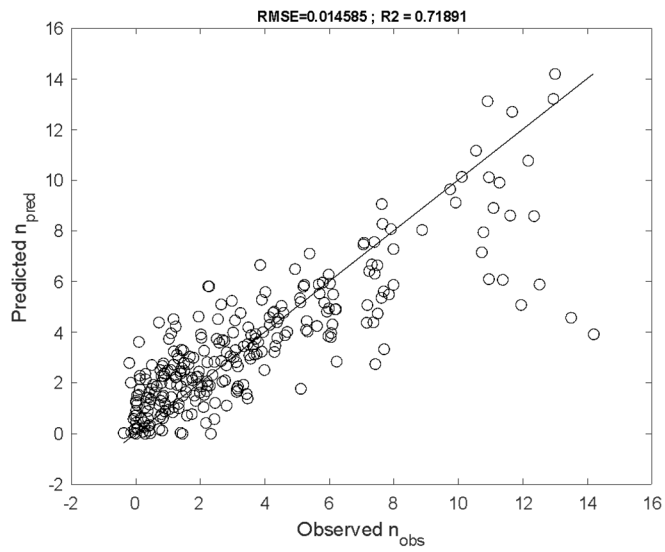
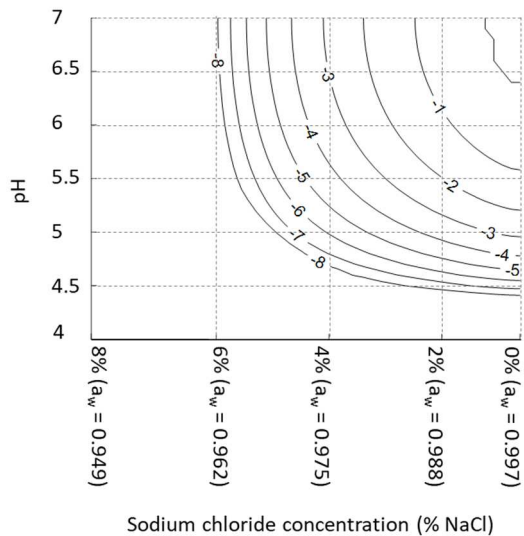
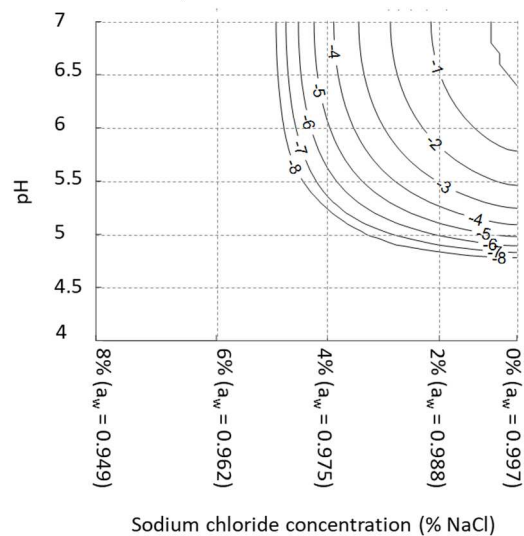


Figure 5. Comparison between observed (n_{obs}) and predicted (n_{pred}) decimal reduction numbers of *C. sporogenes* (isolates 1, 14 and 17) and non-toxigenic Group I *C. botulinum* (strain 34) and Group I *C. botulinum* (strain 41)

a)



b)



c)

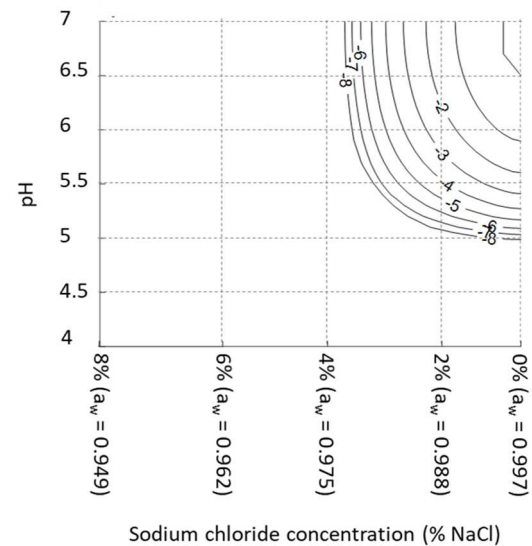


Figure 6. Contour plots (solid lines) showing the growth or recovery (including germination and outgrowth) probabilities of Group I *C. botulinum* strain 34

(a) vegetative cells, (b) spores, (c) heat-treated spores. Each solid line represents the decimal reduction number obtained by stress during recovery.

1 **Table 1. List of *C. sporogenes* isolates and Group I *C. botulinum* strains used in this study.**

Strain number	Species	Toxin genes (toxin type)	Isolation year	Origin
1	<i>C. sporogenes</i> ¹	No	1927	Collection
2	<i>C. sporogenes</i> ¹	No	Unknown	Collection
3	<i>C. sporogenes</i>	No	2016	Milk-based dessert
4	<i>C. sporogenes</i>	No	2010	Meat
5	<i>C. botulinum</i> ²	No	2010	Meat
6	<i>C. sporogenes</i>	No	2010	Meat
7	<i>C. sporogenes</i>	No	2010	Meat
8	<i>C. sporogenes</i>	No	2001	Vegetables
9	<i>C. sporogenes</i>	No	2002	Vegetables
10	<i>C. sporogenes</i>	No	2005	Vegetables
11	<i>C. sporogenes</i>	No	2015	Prepared meat meal
12	<i>C. sporogenes</i>	No	2006	Prepared meat meal
13	<i>C. sporogenes</i>	No	2007	Fish product
14	<i>C. sporogenes</i>	No	2006	Fish product
15	<i>C. sporogenes</i>	No	2001	Meat
16	<i>C. sporogenes</i>	No	2006	Prepared meat meal
17	<i>C. sporogenes</i>	No	2008	Prepared meat meal
18	<i>C. sporogenes</i>	No	2009	Prepared meat meal
19	<i>C. sporogenes</i>	No	2010	Prepared meat meal
20	<i>C. sporogenes</i>	No	2010	Prepared meat meal
21	<i>C. sporogenes</i>	No	2011	Prepared meat meal
22	<i>C. sporogenes</i>	No	2011	Meat
23	<i>C. botulinum</i> ²	No	2011	Meat
24	<i>C. sporogenes</i>	No	2012	Prepared meat meal
25	<i>C. sporogenes</i>	No	2012	Meat
26	<i>C. sporogenes</i>	No	2013	Prepared meat meal
27	<i>C. sporogenes</i>	No	2013	Prepared meat meal
28	<i>C. sporogenes</i>	No	2013	Prepared meat meal

29	<i>C. sporogenes</i>	No	2013	Prepared meat meal
30	<i>C. sporogenes</i>	No	2013	Prepared meat meal
31	<i>C. sporogenes</i>	No	2014	Prepared meat meal
32	<i>C. sporogenes</i>	No	2014	Meat
33	<i>C. sporogenes</i>	No	2015	Prepared meat meal
34	<i>C. botulinum</i> ²	No	2011	Prepared meat meal
35	<i>C. sporogenes</i>	No	2006	Prepared meat meal
36	<i>C. sporogenes</i>	No	2006	Prepared meat meal
37	<i>C. sporogenes</i>	No	2008	Prepared meat meal
38	<i>C. sporogenes</i>	No	2006	Meat
39	<i>C. sporogenes</i>	No	2005	Prepared meat meal
40	<i>C. sporogenes</i> ¹	No	1927	Collection
41	<i>C. botulinum</i> ¹	Yes (A1)	1953	Collection
42	<i>C. botulinum</i> ¹	Yes (A2)	2013	Collection

2 ¹ Strain 1: PA 3679 Campden collection; strain 2: IFR BL 81/14/1; strain 40: PA 3679 ATCC 7955; strain 41: Prevot 910 Institute Pasteur collection; strain 42:
3 301-13 Institute Pasteur collection (Mazuet et al., 2016); ² Renamed non-toxigenic Group I *C. botulinum* instead of *C. sporogenes* after whole genome
4 sequencing analysis (section 3.2).

5

Table 2. Growth limits as a function of pH and sodium chloride concentration and *D*-value at 121.1 °C of 42 strains of *C. sporogenes* and Group I *C.*

botulinum

Strain number	Species	Observed growth limit pH	Observed growth limit sodium chloride concentration (% NaCl)	Calculated <i>D</i> -value at 121.1 °C (min)
1	<i>C. sporogenes</i> ^{1,3,4}	4.50	8.5	0.16
2	<i>C. sporogenes</i> ¹	4.60	6.5	ND*
3	<i>C. sporogenes</i>	4.67	8.5	0.01
4	<i>C. sporogenes</i>	4.50	7.5	0.15
5	<i>C. botulinum</i> ^{2,3}	4.70	7.5	0.21
6	<i>C. sporogenes</i>	4.76	7.5	0.02
7	<i>C. sporogenes</i> ³	4.89	6.5	0.05
8	<i>C. sporogenes</i> ³	4.83	7.5	0.01
9	<i>C. sporogenes</i>	4.60	6.5	0.01
10	<i>C. sporogenes</i>	4.50	7.5	0.01
11	<i>C. sporogenes</i>	4.55	7.5	0.02
12	<i>C. sporogenes</i> ³	4.69	8.5	0.01
13	<i>C. sporogenes</i>	4.73	8.5	0.02
14	<i>C. sporogenes</i> ^{3,4}	4.51	11.5	0.14
15	<i>C. sporogenes</i>	4.61	8.5	0.01
16	<i>C. sporogenes</i>	4.50	7.5	0.50
17	<i>C. sporogenes</i> ^{3,4}	4.49	9.5	0.02
18	<i>C. sporogenes</i>	4.54	7.5	0.07
19	<i>C. sporogenes</i>	4.66	6.5	0.26
20	<i>C. sporogenes</i> ³	4.51	7.5	0.02
21	<i>C. sporogenes</i>	4.56	7.5	0.04
22	<i>C. sporogenes</i> ⁴	4.65	5.5	0.01
23	<i>C. botulinum</i> ^{2,3}	4.73	6.5	0.19

24	<i>C. sporogenes</i>	4.52	7.5	0.02
25	<i>C. sporogenes</i>	4.51	7.5	0.23
26	<i>C. sporogenes</i> ³	4.71	6.5	< 0.01
27	<i>C. sporogenes</i>	4.66	8.5	< 0.01
28	<i>C. sporogenes</i> ³	4.51	7.5	0.13
29	<i>C. sporogenes</i>	4.73	9.0	0.02
30	<i>C. sporogenes</i>	4.85	8.5	0.01
31	<i>C. sporogenes</i>	4.50	7.0	0.15
32	<i>C. sporogenes</i>	5.05	5.0	ND*
33	<i>C. sporogenes</i>	4.66	7.5	0.01
34	<i>C. botulinum</i> ^{2,3,4}	4.50	7.5	0.25
35	<i>C. sporogenes</i>	4.50	8.5	0.02
36	<i>C. sporogenes</i>	4.71	7.5	0.02
37	<i>C. sporogenes</i>	4.57	7.5	0.01
38	<i>C. sporogenes</i>	4.70	6.5	0.01
39	<i>C. sporogenes</i>	4.60	7.5	0.02
40	<i>C. sporogenes</i> ^{1,3}	4.51	9.5	0.02
41	<i>C. botulinum</i> ^{1,3,4}	4.68	6.75	0.03
42	<i>C. botulinum</i> ¹	4.68	6.75	0.07

¹ Strain 1: PA 3679 Campden collection; strain 2: IFR BL 81/14/1; strain 40: PA 3679 ATCC 7955; strain 41: Prevot 910 Institut Pasteur collection; strain 42: 301-13 Institut Pasteur collection; ²Renamed *C. sporogenes* as non-toxigenic Group I *C. botulinum* after whole genome sequencing analysis (this study, Section 3.2); ³ Isolates selected for the genetic study (results in Section 3.2); ⁴ Isolates selected to assess their growth potential in conditions combining suboptimal levels of environmental factors (results in Section 3.3); *ND: not determined

Table 3. Estimation of the parameters of the probability model (Equation 3) for *C. sporogenes* (Isolates 1, 14 and 17) and Group I *C. botulinum* (Isolates 34 and 41)

Species	<i>C. sporogenes</i>						Non-toxigenic Group I <i>C. botulinum</i>			Group I <i>C. botulinum</i>					
Strain	Strain 1 ²		Strain 14 ³		Strain 17 ³		Strain 34 ³			Strain 41 ²					
Physiological state	Vegetative cells	Spores		Vegetative cells	Spores		Vegetative cells	Spores		Vegetative cells	Spores		Vegetative cells	Spores	
		80 °C 10 min	100 °C 1.5 min		80 °C 10 min	100 °C 1.8 min		80 °C 10 min	100 °C 1.7 min		80 °C 10 min	110 °C 1.46 min		80 °C 10 min	100 °C 4 min
pH_{min}	4.01	4.79	5.13	4.46	4.67	4.96	4.36	4.58	4.76	4.19	4.58	4.81	4.40	ND	5.25
CI lower¹	3.77	4.70	4.96	4.37	4.33	4.91	4.27	4.44	4.64	3.75	4.43	4.73	4.26	ND	5.20
CI upper¹	4.14	4.89	5.25	4.52	4.80	4.98	4.41	4.61	4.82	4.41	4.64	4.85	4.50	ND	5.29
α_{wmin}	0.944	0.951	0.966	0.950	0.960	0.968	0.958	0.957	0.967	0.956	0.964	0.973	0.947	ND	0.962
CI lower¹	0.936	0.932	0.961	0.945	0.959	0.706	0.955	0.952	0.945	0.951	0.936	0.972	0.932	ND	0.576
CI upper¹	0.948	0.957	0.969	0.952	0.961	0.976	0.959	0.959	0.972	0.960	0.971	0.974	0.949	ND	0.973
Number of data	50	24	12	20	15	10	21	17	14	18	14	13	20	18	11
α										10					
Π_0										0.937					
R^2										0.71					
RMSE										0.014					

¹CI corresponding to 95% confidence intervals; ² Strain 1 is *C. sporogenes* reference strain (PA 3679) and Strain 41 is Group I *C. botulinum* reference strain

(Prevot 910); ³ Isolates 14 and 17 are *C. sporogenes* isolates and strain 34 is non-toxigenic Group I *C. botulinum* isolated from spoiled food.