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Impact of temperature and oxygen on the fate of *Bacillus weihenstephanensis* in a food-based  
medium

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23

24 **Abstract**

25 The capacity of the *Bacillus weihenstephanensis* KBAB4 strain, a psychrotolerant species of  
26 the *B. cereus sensu lato* group, to multiply in carrot broth at 8 °C and 30 °C, in presence or  
27 absence of oxygen was determined. In aerobic carrot broth tyndallized in presence of oxygen,  
28 at both temperatures, the population of vegetative cells of *B. weihenstephanensis* inoculated at  
29 a level of 10<sup>3</sup> or 10<sup>6</sup> CFU/ml dropped immediately. After 16h at 30 °C, *B. weihenstephanensis*  
30 reached around 10<sup>3</sup> CFU/ml, indicating that some vegetative cells had survived and  
31 multiplied, with lipid inclusions accumulated in cells, indicating possible stressing conditions.  
32 At 8 °C, no multiplication of *B. weihenstephanensis* was observed during 3 days to at least 12  
33 days, depending of carrot broth batches. In anaerobic carrot broth tyndallized without oxygen,  
34 the vegetative cells of *B. weihenstephanensis* were not killed upon inoculation and multiplied  
35 in the broth at both 30 °C and 8 °C. Comparison with results from previous studies shows that  
36 *B. weihenstephanensis* behaves differently in carrot broth and in laboratory media at 8 °C  
37 with regards to presence or absence of oxygen.

38

39 **Keywords** - *Bacillus cereus*; carrot; vegetable; polyhydroxybutyrate; bactericidal effect

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## 42 1 Introduction

43 *Bacillus cereus* is a foodborne pathogen and an endospore-forming bacteria causing diarrheal  
44 or emetic food poisoning (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008; Forghani  
45 et al., 2014). Because its spores survive a wide range of heat-treatments, (Luu-Thi et al.,  
46 2014), *B. cereus* represents a particular risk for cooked or pasteurised food products. It has  
47 been reported as the second leading cause of foodborne outbreaks in France  
48 (Santé\_publique\_France, 2019) since 2012. In EU the second leading cause of foodborne  
49 outbreaks corresponds to the “toxin producing bacteria” that includes *B. cereus*,  
50 *Staphylococcus aureus* and *Clostridium* spp other than *C. botulinum* (EFSA and ECDC,  
51 2018). *B. cereus* foodborne outbreaks are mostly associated to consumption of foods of non-  
52 animal origin, including vegetables and vegetables purées (Lund et al., 2000; Cadel Six et al.,  
53 2012; EFSA, 2012; Bennett et al., 2013; EFSA, 2013; Cadel Six et al., 2014; EFSA, 2014,  
54 2015). *B. cereus sensu lato* was divided into seven phylogenetic groups in function of their  
55 optimal growth temperature (Guinebretière et al., 2008). In particular, it includes groups of  
56 psychrotolerant strains which spores can germinate and grow in foods stored at refrigeration  
57 temperatures. Spores of *B. cereus* are particularly prevalent in vegetables and heat processed,  
58 non-sterilized, foods containing vegetables (Choma et al., 2000; Del Torre et al., 2001; Valero  
59 et al., 2002; Daelman et al., 2013). The capacity of psychrotolerant *B. cereus* strains to grow  
60 at temperatures of refrigeration in vegetables was reported (Valero et al., 2000; Valero et al.,  
61 2003; Valero et al., 2007; Samapundo et al., 2011; De Sarrau et al., 2013a). However, heat  
62 processed, non-sterilized, foods containing vegetables are stored under refrigeration and can  
63 be packaged without oxygen or under air. The aim of the present study was to determine the  
64 growth capacity of psychrotolerant *B. cereus* in a vegetable-based medium, at cold  
65 temperature, in presence or absence of oxygen. This study was performed with a  
66 psychrotolerant *B. weihenstephanensis* strain, belonging to the phylogenetic group VI of

67 *Bacillus cereus* sensu lato (Guinebretière et al., 2008; Guinebretiere et al., 2010). We used a  
68 buffered-carrot broth because this vegetable is widely consumed and because several studies  
69 previously showed that *B. cereus* can grow in products containing cooked carrots (Valero et  
70 al., 2000; Valero et al., 2002; Valero et al., 2003).

71

## 72 **2 Materials and methods**

### 73 *2.1 Strain and carrot broth preparation*

74 We used the psychrotolerant *Bacillus weihenstephanensis* KBAB4 strain isolated from a  
75 forest soil (Vilas-Boas et al., 2002; Sorokin et al., 2006).

76 Carrot broth was prepared with Nantaise carrots from France. Various types of carrots were  
77 used, two different batches of “winter carrots” purchased at the end of their storage period  
78 (beginning of spring), and three batches of “spring carrots” purchased at the beginning of  
79 summer. Carrots were washed, peeled, grated and vacuum -packed into plastic pouches  
80 (between 100 g and 150 g per plastic pouches) (Fig. 1). These pouches were heated 25 min at  
81 80 °C in a water bath. Grated carrots were then weighted, transferred into a stomacher bag  
82 with filter (< 100 g/stomacher bag), and stomached during 2 min after addition of phosphate  
83 buffer pH 7.0 (100 ml of buffer for 50 g of carrots) to achieve a pH of  $7.0 \pm 0.2$  for the  
84 tyndallized carrot broths. The filtered carrot broth was poured from the stomacher bag into a  
85 sterile flask and dispensed into KIMAX tubes, or into Hungate tubes (Dutscher) (10 ml/tube)  
86 or in Amsco flasks. Carrot broth was directly dispensed in KIMAX tubes under air (condition  
87 1 in Fig. 1), whereas oxygen was eliminated from carrot broth by boiling under a flow of  
88 nitrogen passed through a Hungate column (Guérin et al., 2016), before dispensing  
89 anaerobically into Amsco flasks and Hungate tubes (conditions 3 and 4 in Fig. 1,  
90 respectively). To assess the effect of boiling independently of anaerobic conditions, carrot  
91 broth was also boiled without nitrogen flow and dispensed in KIMAX tubes with air

92 (condition 2 in Fig. 1). All tubes and Amsco flasks were then sterilized following a  
93 tyndallization protocol, consisting in heating the carrot broth tubes and flasks three times at  
94 80 °C during 1 h in a water bath, with a 24 h-storage at room temperature in the dark between  
95 each heat treatment. The sterile tubes and flasks were finally stored at room temperature in the  
96 dark before use.

97

## 98 2.2 Growth conditions

99 Inocula of vegetative cells were prepared from a stock of *B. weihenstephanensis* KBAB4  
100 suspensions of exponential phase cells ( $OD_{600} = 0.5$ ), in a 30 % glycerol final concentration,  
101 stored at -80 °C (Guérin et al., 2016). Ten ml of Brain Heart Infusion (BHI; Biokar),  
102 pH  $7.4 \pm 0.2$  were inoculated with 100  $\mu$ l of the frozen KBAB4 culture in KIMAX tubes and  
103 incubated at 20 °C under shaking at 200 rpm until an  $OD_{600}$  equal to 0.5. Optical density of  
104 inocula was measured using a spectrophotometer (Helios Epsilon; Thermo Scientific,  
105 Rockford, IL). In parallel, spore suspensions were obtained after a 9 days-incubation at 30 °C  
106 on Fortified Nutrient Agar plates, as described previously (Bressuire-Isoard et al., 2016).  
107 Vegetative cells inocula or spore suspensions were diluted to inoculate 10 ml of carrot broth  
108 or BHI at around  $10^3$  CFU/ml (low inoculum) or  $10^6$  CFU/ml (high inoculum). For anaerobic  
109 cultures, we used carrot broth in Hungate tubes (condition 4 in Fig. 1) and for cultures with  
110 oxygen, we used carrot broth in KIMAX tubes tyndallized in aerobic conditions (conditions 1  
111 and 2 in Fig. 1), or carrot broth in Amsco flasks sterilized in anaerobic conditions, open to air  
112 and dispensed in sterile KIMAX tubes at time of inoculation (condition 3 in Fig. 1). Tubes  
113 were then incubated at 8 °C or 30 °C under shaking at 200 rpm. CFU were enumerated by  
114 sampling 100  $\mu$ l of each cultures and plating serial dilutions on Luria Bertani agar plates (LB;  
115 Biokar) then incubated at 30 °C overnight. One Hungate tube was inoculated and incubated  
116 for each sampling point and replicate because it was discarded once open, whereas the same

117 KIMAX tube was used for all the sampling points of one replicate growth curve. For each  
118 batch of carrot broth, three growth curves were performed with three independent inocula.  
119 Duration of incubation and intervals of sampling were adapted to the growth or decline  
120 observed.

121

### 122 *2.3 Microscopy and image analysis*

123 Samples were observed using a phase contrast microscope (Olympus BX 50 instrument,  
124 Rungis, France) with a BX-FLA reflected light fluorescence attachment. Pictures were taken  
125 with a black and white cool SNAP-EZ camera (Tucson, USA). Images were processed with  
126 Micromanager software, ImageJ and PMC Capture Pro software. Samples, stained with  
127 Live/Dead (Invitrogen) or Nile red (Sigma) as described below, were observed in  
128 epifluorescence microscopy, and pictures were taken separately with the corresponding filters  
129 and processed with PMC Capture Pro software (De Sarrau et al., 2013b).

130

### 131 *2.4 Cell viability*

132 Live/Dead staining was done according to manufacturer instructions. Stained cells were  
133 observed in fluorescence microscopy with a blue filter (B excitation cube Wide band; U-  
134 MWB; exciter filter BP450-480), to observe both green and red cells, and with a green filter  
135 (G excitation cube Wide band; U-MWB; exciter filter BP510-550) to observe red cells.  
136 Pictures were taken alternatively in phase contrast and in fluorescence microscopy with the  
137 green filter. The proportion of viable (green) and dead (red) cells was determined on one  
138 sample, grown 3 days at 30 °C in aerobiosis in a carrot broth tyndallized without oxygen, by  
139 cells enumeration on five different microscopic fields (n=632 cells).

140

### 141 *2.5 Presence of cell lipid inclusions*

142 Nile red coloration (Garton et al., 2002) was used to observe the presence of lipid inclusions  
143 in *B. cereus* cells. Five  $\mu$ l of a Nile red solution prepared by dissolving 0.5 mg/ml Nile red  
144 (Sigma) in absolute ethanol, were added to 100  $\mu$ l of cultures, vortexed and incubated 10 min  
145 at 37 °C in the dark. This suspension was centrifuged 10 min at 4, 000 g, the supernatant was  
146 discarded and the cells pellet was then vortexed in 100  $\mu$ l of phosphate buffer saline (PBS)  
147 with Tween 80. This step was performed twice and cells were finally suspended in 100  $\mu$ l of  
148 PBS for fluorescence microscopy using the green filter. Pictures of each sample were taken  
149 alternatively in phase contrast and in fluorescence microscopy.

150

## 151 2.6 Statistical analysis

152 The results are expressed as means of three independent biological replicates. A Student's T-  
153 test was used to compare mean values with the null hypothesis was rejected for  $p < 0.05$ .

154

## 155 3 Results

### 156 3.1 Growth of *B. weihenstephanensis* in carrot broths

157 In broth prepared with the two batches of winter carrots, an immediate death of  
158 *B. weihenstephanensis* cells upon inoculation was observed in the broth tyndallized in  
159 presence of oxygen (condition 1 in Fig. 1). At time zero, we did not recover the  $10^3$  CFU/ml  
160 inoculated in the broth, when incubated with oxygen and whatever the temperature of growth  
161 subsequently applied (Fig. 2A). No CFU were recovered upon inoculation of a higher  
162 inoculum of  $10^6$  CFU/ml (Table 1). No growth was subsequently observed at 8 °C and in  
163 aerobiosis (condition 1 in Fig. 1) during the 12 d of incubation (Fig. 2A). With 2 batches of  
164 spring carrots, an initial reduction of 2 log cycles was observed while no reduction in counts  
165 was observed for a third batch. In these three batches, no increase in numbers of cells was  
166 subsequently observed during 3-4 days at 8 °C (result not shown). At 30 °C, after the initial



167 decline in number observed for 4 batches of carrots, cells grew in carrot broth (Fig. 2A) and  
168 *B. weihenstephanensis* reached a maximal population ( $N_{\max}$ ) of approximately  
169  $8.2 \log_{10}$  CFU/ml, followed by a rapid decline (Fig. 2A).  
170 *B. weihenstephanensis* behaved similarly in carrot broth boiled in air before tyndallisation in  
171 air (condition 2 in Fig. 1) and in carrot broth directly tyndallized in air, without boiling  
172 (condition 1 in Fig. 1), with an initial reduction in counts upon inoculation and no growth (or  
173 delayed growth depending on carrot batches) at 8 °C (data not shown).  
174 When carrot broth was prepared and tyndallized anaerobically (conditions 3 and 4 in Fig. 1)  
175 we enumerated the expected  $10^3$  CFU/ml inoculated at time zero (Fig. 2A and 2B), showing  
176 that the lethal effect did not occurred or was abolished in this condition. This result was  
177 observed in both anaerobic broths kept anaerobic (condition 4 in Fig. 1, Fig. 2B) and aerated  
178 (condition 3 in Fig. 1, Fig. 2A), after tyndallization. In these carrot broths prepared according  
179 to conditions 3 and 4, *B. weihenstephanensis* multiplied at 30 °C and at 8 °C, and reached a  
180  $N_{\max}$  from 7.5 to 8  $\log_{10}$  CFU/ml (Fig. 2A and B).  
181 Because *B. weihenstephanensis* can be present in foods as spores, we then tested inoculations  
182 with suspension of spores at  $10^3$  CFU/ml and  $10^6$  CFU/ml in carrot broth in tubes tyndallized  
183 with oxygen (condition 1 in Fig. 1) (Table 1). We recovered the expected populations at time  
184 zero, suggesting that unlike vegetative cells spores were not killed shortly after inoculation.  
185 When tubes inoculated with spores were incubated at 30 °C, numbers of CFU remained stable  
186 for 2 h before starting to increase and reached the same  $N_{\max}$  as with a vegetative cells  
187 inoculum (data not shown).

188

### 189 3.2 Microscopic observations of *B. weihenstephanensis* cells in carrot broths

190 Morphology of *B. weihenstephanensis* KBAB4 cells changed with the different culture  
191 conditions in carrot-broth (Fig. 3): vegetative cells were opaque, slightly swollen, with some

192 inclusions when grown in aerobiosis with or without oxygen during tyndallization (conditions  
193 1 and 3 in Fig. 1) (white arrows in Fig. 3A and B). In contrast, cells exhibited classical shape,  
194 and no inclusion was observed when grown in anaerobiosis at 30 °C (condition 4 in Fig. 1)  
195 (Fig. 3C, white arrow) for both young cells (1 day) and cells from declining population (3  
196 days). Cells grown at 8 °C in anaerobiosis (condition 4 in Fig. 1) were longer with no  
197 inclusion (Fig 3D). As we hypothesised that these inclusions could be lipid granules, we  
198 performed a Nile red coloration of cells cultivated 3 days at 30 °C in aerobiosis in a carrot  
199 broth tyndallized without oxygen (condition 3 in Fig. 1) (Fig. 4A and B). In fluorescence  
200 microscopy, several inclusions appeared in each Nile red-stained cells (white arrow in  
201 enlarged Fig. 4B and same cells are shown under phase contrast microscopy in Fig. 4A). A  
202 Live/Dead coloration of the same cultures as in Fig 4A and Fig. 4B showed that dead cells  
203 represented 49 %  $\pm$  15 % of total cells (n=632 cells), irrespective of the presence of  
204 inclusions. When grown at 30 °C in aerobiosis in BHI medium, cells also contained lipid  
205 inclusions but less than in cells cultivated in carrot broth (Fig. 4C and D).

206

#### 207 **4 Discussion**

208 The presence of oxygen during tyndallization of carrot-broth (conditions 1 and 2 in Fig. 1)  
209 seemed to create lethal compounds for *B. weihenstephanensis* cells because immediately (e.g.  
210 within 5 min) upon inoculation, the vegetative cells were no longer recovered on medium  
211 plates. In contrast, spores were resistant to this lethal effect as the expected concentration of  
212 spores was recovered after their inoculation in tyndallized carrot broth, as observed in  
213 previous studies (Valero et al., 2000; Valero et al., 2003; Valero et al., 2007). These authors  
214 did not test inoculation with vegetative cells and logically did not report any lethal effect from  
215 processed carrot.

216 No vegetative cells were detected upon contact of the highest inoculum with carrot broth  
217 suggesting a reduction of cells of at least  $10^5$ -fold. However, growth of *B. weihenstephanensis*  
218 resumed at 30 °C after inoculation of 10 ml of carrot-broth with  $10^4$  CFU, indicating that  
219 some cells survived in the vegetable-broth and that the lethal compounds caused a reduction  
220 of less than  $10^4$ -fold. To explain this contradiction between the impacts of carrot broth on the  
221 two inoculum levels, we may assume that part of the inoculated cells, not recovered on the  
222 plate count medium, was not killed but rather in a viable but not cultivable state and could  
223 resume growth after some time. Another possibility is that few spores were present in the  
224 inoculum and resist the lethal compounds from the carrot broth and then multiplied.

225 When vegetative cells were inoculated into carrot-broth tyndallized without oxygen  
226 (conditions 3 and 4 in Fig. 1), all the inoculated vegetative cells were recovered, whatever the  
227 presence or absence of oxygen during inoculation and incubation. This indicates that the  
228 lethal compound(s) was (were) not generated during inoculation or incubation of the carrot  
229 broth in air. Boiling before tyndallization under air did not prevent the production of the lethal  
230 compound(s) (condition 2 in Fig. 1) indicating that the boiling phase requested to create strict  
231 anaerobiosis in conditions 3 and 4 (Fig. 1) was not the cause of the absence of the lethal  
232 compound(s) in these conditions. Overall our results indicate that the lethal compound(s) are  
233 likely produced during exposure of the carrot broth to oxygen during heat treatment.

234 *B. weihenstephanensis* KBAB4 cells were able to grow aerobically in carrot broth at 30 °C  
235 with a  $N_{max}$  around  $8 \log_{10}$  CFU/ml. Apart of the initial decline, no difference was observed in  
236 growth kinetic and in cell morphology in carrot-broth tyndallized with or without oxygen,  
237 suggesting that the effect of the lethal compound(s) present in carrot-broth tyndallized  
238 aerobically was rather short in these conditions. It is possible that the compounds were  
239 inactivated while reacting with the bacterial cells after inoculation. In contrast, no growth  
240 (winter carrots) or delayed growth (spring carrots) was observed at 8 °C in aerobic conditions,

241 in carrot broth tyndallized in presence of oxygen, suggesting that the cells may not recover  
242 (winter carrots) or needed 3-4 days to recover (spring carrots) at 8 °C from the effect of the  
243 lethal compound(s) of the aerobically tyndallized carrot broth. Lethal effect of fresh carrots on  
244 various bacterial species has previously been described (Beuchat and Brackett, 1990; Nguyen-  
245 the and Lund, 1991, 1992; Babic et al., 1994; Noriega et al., 2010; Degirmenci et al., 2012),  
246 although not on species of the *B. cereus* group. As in the present study, an antimicrobial effect  
247 of fresh carrot was previously observed on *Listeria monocytogenes*, generated upon de-  
248 structuration of carrot tissue, short-lived, and active only in presence of oxygen (Nguyen-the  
249 and Lund, 1991). However, this antimicrobial effect on *L. monocytogenes* was shown to be  
250 heat labile (Beuchat and Brackett, 1990; Nguyen-the and Lund, 1991) and is therefore  
251 presumably different from the one observed in the present study.

252 *B. weihenstephanensis* vegetative cells grown aerobically at 30 °C in carrot broth were  
253 slightly swollen and contained several lipid inclusions, as shown after the Nile red staining.  
254 More inclusions were observed in these cells than in those grown in BHI in the same  
255 conditions. The lipid inclusions might be polyhydroxybutyrate granules (PHB), which have  
256 been described in cells of *B. thuringiensis* (Chen et al., 2010), another species also belonging  
257 to of the *B. cereus sensu lato* group. PHB frequently accumulates in bacterial cells upon stress  
258 including nutrient limitation and may help bacteria to cope with stress (Zhao et al., 2007;  
259 Wang et al., 2009; Wu et al., 2011; Lopez et al., 2012; Obruca et al., 2016). This may  
260 indicates some stressing conditions for *B. weihenstephanensis* in aerobic carrot-broth at  
261 30 °C. However, the proportion of dead cells in carrot- broth grown cultures (49 %) were  
262 similar to those reported previously for *B. cereus* grown in Luria broth medium in non-  
263 stressing conditions (Pandiani et al., 2010)

264 We observed that when *B. weihenstephanensis* cells grew without oxygen at 8 °C in carrot-  
265 broth, they reached a  $N_{max}$  of 7.5 log<sub>10</sub> CFU/ml after 6 days and had regular shapes with no

266 lipid inclusions, suggesting that they were less stressed than those grown at 30 °C with  
267 oxygen. However, it was previously shown that anaerobiosis strongly reduced sporulation by  
268 *B. cereus* (Abbas et al., 2014), and that lipid (PHB) accumulation depended of Spo0A, a  
269 master regulator of sporulation (Chen et al., 2010). Therefore, absence of lipid inclusions  
270 might not only be explained by absence of stress, but also by an action of anaerobiosis on the  
271 sporulation regulation cascade. We previously observed that the same strain as the one used in  
272 the present study (KBAB4) did not grow at 8 °C in BHI medium under anaerobiosis (Guérin  
273 et al., 2016), suggesting that carrot-broth was a better growth medium than BHI medium in  
274 the tested conditions.

275 In conclusion, compounds lethal for *B. weihenstephanensis* are presumably produced in  
276 carrot-broth heat treated in presence of oxygen. Whenever *B. weihenstephanensis* grew in  
277 carrot broth in presence of oxygen, cells were swollen and contained many lipid inclusions. In  
278 absence of oxygen, *B. weihenstephanensis* grew in carrot broth as typical bacilli without  
279 inclusions and at 8°C it grew better than in BHI. This study highlights the interactions  
280 between the food matrix and environmental conditions, such as temperature and oxygen,  
281 during preparation and storage of food products, on the fate of *B. weihenstephanensis* cells.

282

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442  
443

444 Table 1. Counts of *B. weihenstephanensis* KBAB4 vegetative cells 5 min after inoculation in  
 445 carrot broth.  
 446

		Initial inoculum concentration (CFU/ml)			
		Vegetative cells		Spores	
Tyndallization	Condition of inoculation <sup>a</sup>	10 <sup>3</sup>	10 <sup>6</sup>	10 <sup>3</sup>	10 <sup>6</sup>
With oxygen	Aerobiosis	< 10 <sup>b</sup>	< 10	10 <sup>3</sup>	10 <sup>6</sup>
Without oxygen	Aerobiosis	10 <sup>3</sup>	NT <sup>c</sup>	NT	NT
Without oxygen	Anaerobiosis	10 <sup>3</sup>	NT	NT	NT

447

448 <sup>a</sup> Inoculation was done at room temperature with cells or spores

449 <sup>b</sup> Limit of detection was 10 CFU/ml.

450 <sup>c</sup>NT: not tested.

451

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458 **Legend of figures**

459

460 Fig. 1 - Conditions for preparation and inoculation in carrot broth.

461 Tyndallization was done by three successive incubations at 80 °C for 1 h at 24 h intervals.

462 *Italic fonts indicate steps done in strict anaerobic conditions, under a flow or a head space of*  
463 *nitrogen passed through a Hungate column to remove any trace of oxygen.*

464 Numbers referred to conditions cited in the text.

465

466 Fig. 2 - Growth of *B. weihenstephanensis* KBAB4 strain in carrot broth (winter carrots)  
467 incubated in air (panel A) (conditions 1 and 3 in Fig 1), or in anaerobic conditions (panel B)  
468 (condition 4 in Fig 1). Before inoculation, carrot broth was sterilized by tyndallization in  
469 presence (open symbols) (condition 1 in Fig 1) or in absence (closed symbols) of oxygen  
470 (conditions 3 and 4 in Fig 1). Carrot broths, inoculated with  $10^3$  and  $10^{3.5}$  CFU/ml, were  
471 incubated at 8 °C (triangles) or at 30 °C (squares).

472

473 Fig. 3. Microscopy observations under phase contrast (X 1, 000) of *B. weihenstephanensis*  
474 KBAB4 cells, at  $N_{max}$  in carrot -broth (winter carrots). Cells grown 3 days with oxygen at  
475 30 °C, in aerobically tyndallized carrot-broth (condition 1 in Fig. 1) (A); or in anaerobically  
476 tyndallized carrot-broth (condition 3 in Fig. 1) (B). White arrows show cells with inclusions.  
477 Cells grown under anaerobiosis, in anaerobically tyndallized carrot broth (condition 4 in  
478 Fig. 1), for 1 and 3 days at 30 °C (C), or 13 days at 8 °C (D). Bars represent 10  $\mu$ m.

479

480 Fig. 4. Microscopic observations of *B. weihenstephanensis* KBAB4 under phase contrast (A  
481 and C) and epifluorescence (B and D) (X 1,000). KBAB4 cells were cultivated aerobically for  
482 4 days at 30 °C in carrot broth (winter carrots) tyndallized without oxygen (condition 3 in

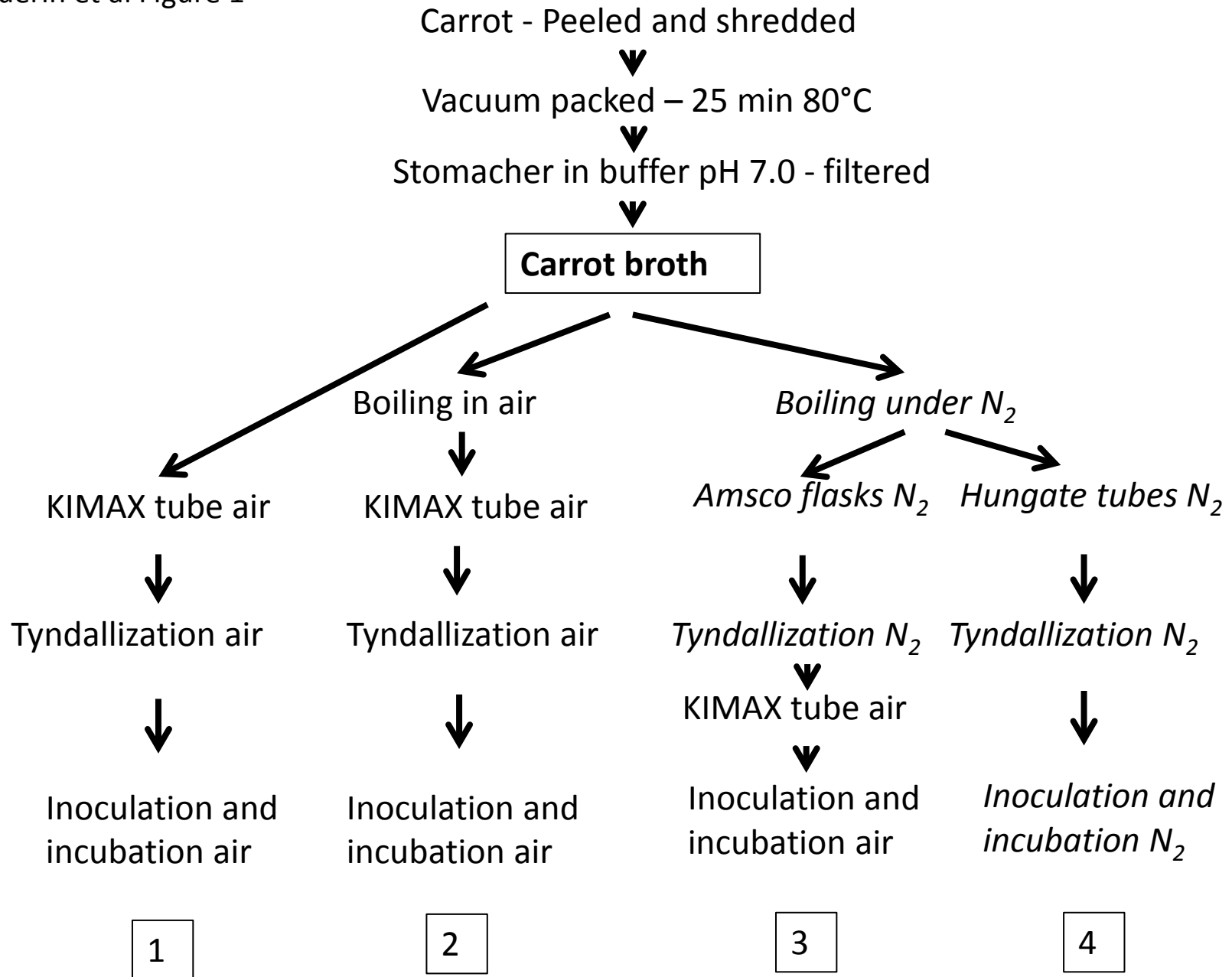
483 Fig. 1) (A and C) or in BHI during 2 days (B and D) and stained with Nile red. White (in  
484 panels B and D) and black (in panels A and C) arrows show lipid inclusions, fluorescing in  
485 panel B and D.

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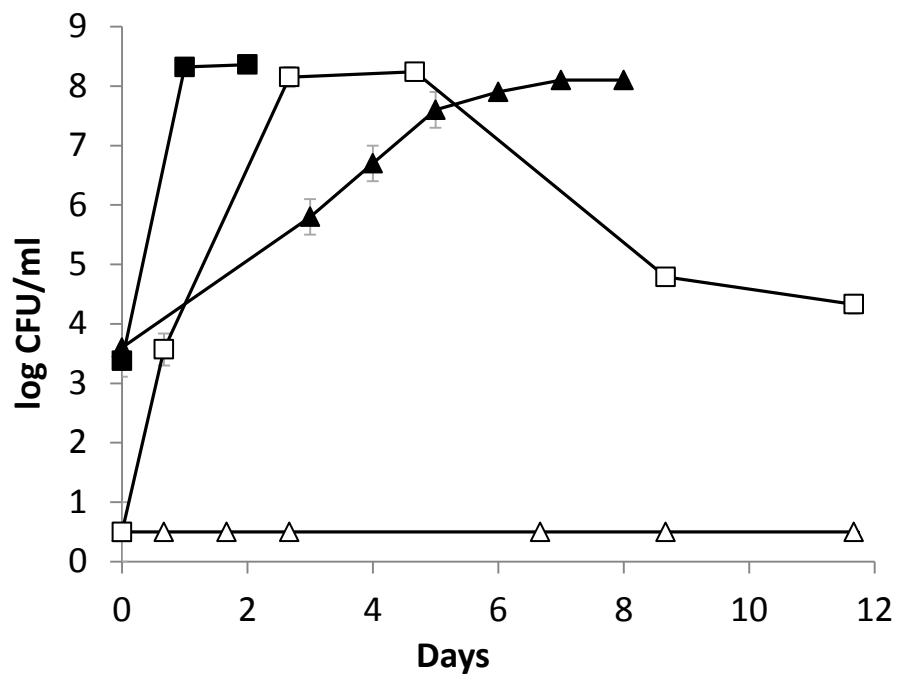
Guerin et al Figure 1



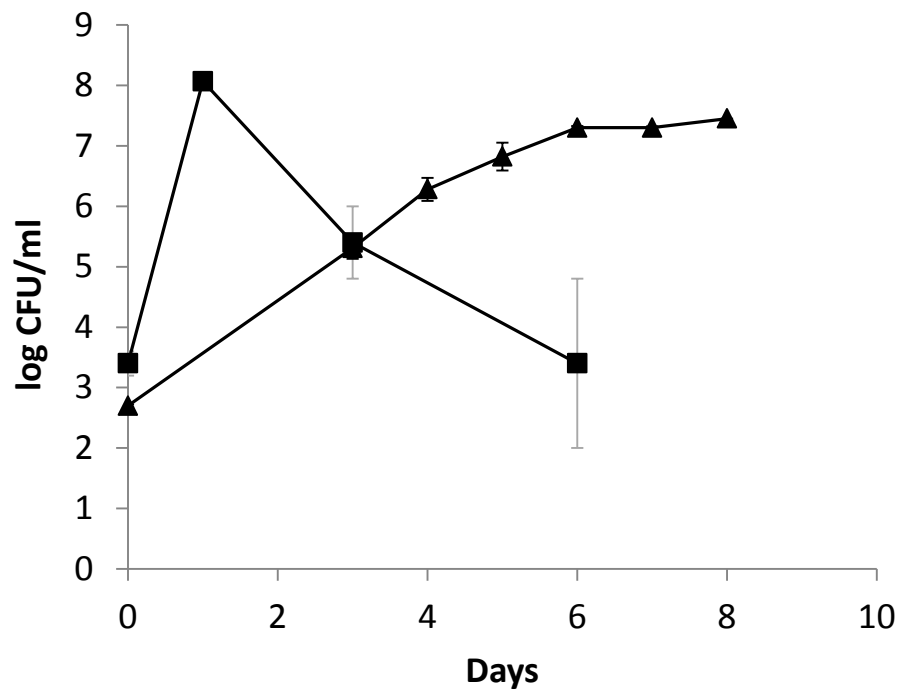


Guerin et al Figure 2

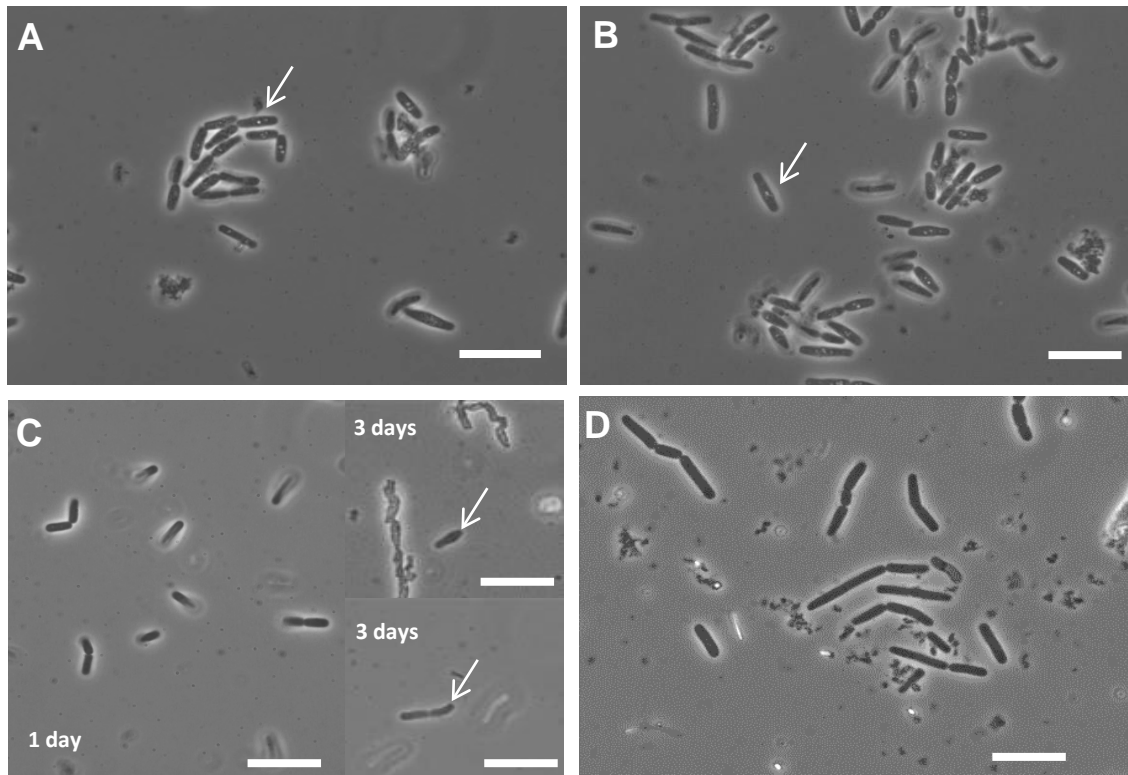
**A**



**B**



Guerin et al Figure 3



Guerin et al Figure 4

